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Editorial

Diabetic pregnant women and perinatal outcomes

Diabetes mellitus (DM), one of the metabolic syndromes, accompanied with co-existence or subsequent development of comorbidities, contributing to the main cause of disability and mortality of diseased subjects, has become one of the biggest global health problems and also results in a heavy socio-economic burden in the world [1–4]. Its high global prevalence and progressively and continuously increased incidence is in the further burnout of the health-provider and socio-economic systems. All aware the urgent need to dissolve the DM-related problems. Therefore, it is important to conduct the effective strategy for prevention, early identification, and prompt management of pre-DM or DM subjects. The main goal attempts to avoid the occurrence of development of DM and subsequent DM-related morbidity and mortality [5,6]. In theory, DM can be grouped based on their genetic background, and simply, be classified by Type 1 DM (T1DM) and Type 2 DM (T2DM), based on the different underlying pathophysiology of diseases and subsequent different therapeutic strategies [7,8]. T1DM is frequently diagnosed in younger age and often needed insulin replacement therapy and T2DM is often associated with ageing process needing various kinds of anti-diabetes agents (ADAs) or insulin treatment [7,8]. Sometimes, it is difficult to simply distinguish T1DM from T2DM, because overlapping phenomenon is found [7,8]. With a continuously increased incidence of DM and a trend to attacks to the younger-age population, it is not surprising to find more and more reproductive-age women to have a diagnosis of DM. Similar to DM to elder population, DM in pregnancy also results in a big challenge to modern obstetric practice, partly because DM not only apparently deteriorates global health of these reproductive age women during pregnancy (exacerbating pre-existence of retinopathy and nephropathy and concomittent with hypertensive disorders of pregnancy [HDP]), but also worsens pregnancy and results in poor perinatal outcomes, such as an increased incidence of congenital anomalies, stillbirth, growth problems, such as macrosomia and intrauterine fetal growth retardation as well as neonatal hypoglycemia, birth trauma, and childhood obesity either in utero or in the lactational-neonatal period and childhood [9–13]. The real cause of the above-mentioned deteriorating global health of both mother and fetus is still uncertain, but it is believed that complex physiological and hormonal changes of mother leads to a progressive decrease of insulin sensitivity (a resultant insulin-resistant [IR] status) and a tendency to elevated blood glucose level to offer the optimal fetal development, physiology, metabolism and growth [9]. It is well-known that pregnant women have a vulnerability to disturbing their glucose homeostasis and enhancing the fluctuation of blood glucose levels and subsequently developing gestational diabetes mellitus (GDM), and in addition, women with pre-existed DM often have a great chance to exacerbate their diseases during pregnancy, resulting in an occurrence of hyperglycemia, which is a main cause of producing congenital fetal malformations in fetus and to be associated with adverse events (AEs) of both mother and offspring during and after pregnancy. To ameliorate the severe hyperglycemic status of pregnant women, some interventions, either by physiology (healthy life styles, such as exercise, limited calorie intake, adequate nutrition support, mineral supplement, and adequate and appropriate gestational weight gain) or by medication are often recommended. However, a long-term concern of the side effects and teratogenic effects of ADAs always bothers both health-providers and pregnant women. Therefore, the use of insulin, particularly performed by continuous subcutaneous insulin infusion (CSII) assisted by continuous glucose monitor (CGM) in the management of pregnant DM women, regardless of type 1 or type 2 is considered one of the best approaches based on its stringent glycemic control. However, the outcome of aforementioned strategies is not consistent, resulting in controversies [14].

A recent article [14] published in the 2023 September issue of the *Taiwanese Journal of Obstetrics and Gynecology* (*TJOG*) attempted to compare the difference of perinatal outcomes between T1DM and T2DM pregnant women treated by insulin. Additionally, some had received the stringent glycemic control and some did not, offering a better chance to clarify the role of using CSII and CGM for the therapy of T1DM pregnant women.

The authors retrospectively enrolled 37 women with T1DM ($n = 22$) and T2DM ($n = 15$) to compare the characteristics and pregnancy outcomes between two groups [14]. The results showed that T2DM pregnant women had a significantly shorter disease period (reflective by lower glycosylated hemoglobin level [HbA1c]) and advanced age than T1DM pregnant women did (3.0 years vs. 8.5 years and 6.7% vs. 7.2% before pregnancy and 6.2% vs. 6.7% in the third trimester; 36 years of age vs. 31 years of age, for T2DM and T1DM, respectively) [14]. Additionally, T2DM pregnant women had a higher body mass index (BMI) and also were associated with a high risk of HDP than T1DM [14]. The present study is interesting and worthy of further discussion.

First, although case number is only one for the occurrence of congenital fetal anomaly in the authors' study [14], we can find

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that this case was born from T1DM mother [14]. It is not surprising to find that T1DM pregnant women had a high HbA1c% than T2DM pregnant women, not only reflective by higher median (7.2%) HbA1C but also by a worse control of blood sugar (am upper limit as HbA1c 10.0%). It is well-known that the high possibility of severe damage to the early embryonic development and significant increases of hazards to fetus and pregnancy outcomes [9]. The complicated and high HbA1c secondary to a long-term hyperglycemia contributes to the importance of preconception counseling to avoid unintended pregnancy with abnormal glucose levels (high HbA1c levels) during the early pregnancy [9].

Second, it is interesting to find that the risk of preterm delivery either before 37 or before 34 gestational weeks was higher in T2DM pregnant women compared to T1DM pregnant women (33.3% vs. 9.1% and 13.3% vs. 0%, respectively); however, the admission rate of neonatal intensive care was not comparable with higher rates of preterm delivery in T2DM pregnancy. By contrast, we find that the newborns born from the T1DM pregnant women had a higher risk to need an intensive neonatal care than T2DM (45.5% vs. 40.0%), although all of the above-mentioned parameters did not reach the statistical significance. Additionally, Apgar score at 1 min of newborns was lower in T2DM group but pH levels of newborns were higher in T2DM group and pH levels <7.1 were more frequently noted in T1DM group, although both were not statistically significant. The aforementioned data seemed to be conflicted by each other. The authors failed to explain their findings, but the main cause we believe is secondary to very small case number of the present study. According to so limited case number in their study, any statistical significance may be meaningless and absence of any statistically significant difference is also nothing requiring to interpretate. All claims based on their study should be interpretated in caution. For example, the authors concluded that a well-controlled BMI is not important to decrease rate of large for gestation age (LGA) newborns in T2DM pregnant women. We do not think that based on so limited data, the authors can recommend the above claim. In fact, a well-controlled BMI and adequate/appropriate gestational weight gain is the critical components for minimizing the risk of delivering LGA newborn and macrosomia newborns, and this concept is supported by much evidence, particularly for T2DM concomittent with obesity, high BMI, and increased gestational weight gain [12,15]. Additionally, the authors found that T1DM pregnant women with stringent control of blood sugar and maintenance of lower HbA1c by the combination of CSII and CGM strategy had a higher risk of delivering heavier neonates than T1DM without CSII and CGM [14]. By contrast, the authors mentioned that controlling maternal diabetes in the third trimester of pregnancy is a key factor in the prevention of perinatal outcomes associated with LGA [14]. All may make the audience confused. In fact, we do not understand what the authors meant.

Although we have raised many questions about the authors' publication, we respect the authors' findings and interpretation. However, the discrepancy between the each evaluated item in their article needs further clarification. Originally, the authors attempted to establish the difference between T1DM and T2DM in pregnancy, but as shown above, more and more confusions are found. We highlight the controversy of their study. To offer a better care of T1DM and T2DM pregnant women, we are looking forward to seeing more and more researchers to focus on this hot spot.

Conflicts of interest

All authors declare no conflict of interest.

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Editorial

Integrating molecular pathology to endometrial cancer

Since the consensus of the most recent FIGO (the International Federation of Gynecology and Obstetrics) for endometrial cancer (EC) by Berek JS and members of the FIGO Women's Cancer Committee and Endometrial Cancer Staging subcommittee, 2021–2023 in 2023 has been published, the new staging system of EC offers a better chance to define the prognostic groups based on integration of various histological types, tumor patterns and molecular classification [1]. The 2023 FIGO staging of EC provides a more evidence-based context for treatment recommendations and for the more refined future collection of outcome and survival data [1]. Previously, the staging system of EC is mainly dependent on the surgical-pathological findings (clinico-pathological features), such as histological type (endometrioid carcinoma [EEC], serous carcinoma [SC], clear cell carcinoma [CCC], mixed carcinoma [MC], undifferentiated carcinoma [UC], carcinosarcoma [CS], other unusual types, such as mesonephric-like and gastrointestinal mucinous type carcinomas); tumor grade (1 [up to 5% solid non-glandular growth], 2 [6%–50% solid non-glandular growth], and 3 [>50% solid non-glandular growth]); degree of myometrial invasion (<50% or≥50%, also called as deep myometrial invasion [DMI]); lymphovascular space invasion (LVSI, absence [no], focal [<5 vessels] and substantial or extensive [≥5 vessels]); cervical stroma invasion; adnexal involvement (ovary or fallopian tubes); uterine serosal involvement; lymph node (LN) metastases (macro-metastases [>2 mm], micro-metastases [0.2–2 mm and or > 200 cancer cells] and isolated tumor cells); and distant metastases [1–3]. Conventionally, the decision of postoperative adjuvant therapy is often accorded to the risk classification, including low risk group, intermediate group, high-intermediate group and high group as well as advanced and metastatic group, which is made by the conventional identifiable risk factors, including final clinico-surgical pathological findings [2]. With an assistance of the aforementioned identifiable risk factors, the better recommendation of postoperative adjuvant therapy has been made [2]. The old system has offered a prediction of prognosis and outcome and all of them are often judged by recurrence of disease (progression-free survival [PFS]) and mortality of patients (overall survival [OS]) [2]. However, advanced FIGO stage (stage III and IV) plays a main and decisive role for prognosis and outcome, regardless of other risk factors are absent or present [2], supporting the critical importance of early diagnosis of EC patients who have an early-stage disease (stage I and II), who have the excellent prognosis, particularly for those patients with FIGO IA. Majority of them are cured by simple and comprehensive surgical staging surgery alone, either by minimally invasive procedure (the preference recommended by recent guideline [1,2]) or by conventional exploratory laparotomy, reaching up to more than 95% of PFS rates and >95% of OS rates, without the need of any postoperative adjuvant therapy [2–4]. However, the outcome is not always predictable using the conventional and old FIGO stating system, since a few of them have a recurrence and finally die of diseases [5–7]. By contrast, some of advanced-stage EC patients can be cured by adequate multi-modality therapy [2,8]. All suggest that conventional and old FIGO staging system can't fit all clinical situations.

A recently published article in the 2023 September issue of the *Taiwanese Journal of Obstetrics and Gynecology* (*TJOG*) tried to add molecular pathology into the conventional clinico-pathological findings to evaluate the outcome of patients with EEC [9]. The authors retrospectively enrolled 163 patients with EEC to compare the outcome between deficient mismatch repair gene (dMMR, n = 44, 27%) and proficient mismatch repair gen (pMMR, n = 119, 73%) [9]. The results showed that EEC patients with dMMR had a more frequent grade 3 ECC (25% vs. 12%), and a positive LVSI rate (55% vs. 25%), as well as a higher risk of LN metastasis (23% vs. 8%), especially para-aortic LN metastasis (18% vs. 4%), and a higher rate of negative estrogen receptor (21% vs. 6%) than EEC patients with pMMR did in the univariate analysis [9]. Consistent with the aforementioned findings, multivariate analysis showed that the EEC patients with dMMR had a higher risk of the positive LVSI with a risk ratio (RR) of 3.4 (95% confidence interval [CI] 1.4–8.2) and a trend of a higher risk to be associated with LN metastases (RR 2.8, 95% CI 0.9–8.6), contributing to worse PFS and a tendency to the resultant worse OS than EEC patients with pMMR did [9]. Among the dMMR EEC patients, the authors further found that loss of MLH1/PMS2 was most common type of dMMR status (n = 30, 68%), which was apparently associated with worse PFS compared to other dMMR subgroups, such as loss of MSH2/MSH6 (n = 8, 18%), MSH6 alone (n = 4, 9%) and PMS2 alone (n = 2, 5%) [9]. Based on the above findings, the authors concluded that ECC patients with dMMR had a statistically and apparently higher risk of associated with conventionally clinico-pathological risk factors, such as LVSI and LN metastases [9]. Additionally, loss of MLH1/PMS2 is particularly critical, since it was associated with more aggressive tumor behavior and contributed to the worse prognosis, not only for reducing PFS but also for tendency to reducing OS [9]. The current study is interesting and worthy of discussion.

First, the prognostic implication of MMR in EC patients may be still in uncertainty, because the agreement is not consistent. Another group from Korea by Dr. Kim showed the prevalence rate of dMMR in EC patients was 21% (38/177) in ECC type and 17% (5/29) in non-ECC type [7] compared to 27% in pure ECC type of the current study [9], consistent with 16%–31% of all EC patients with dMMR in the literature review [10]. Additionally, both studies found that the most common dMMR was for MLH1 (63%, 27/43

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vs. 68%, 30/44), followed by MSH6 (37%, 16/43 vs. 27%, 12/44) [7,9]. Furthermore, both studies showed patients with dMMR EC were significantly associated with unfavorable prognostic factors [7,9]. All are in agreement. However, it is interesting to find that patients with the aforementioned unfavorable prognostic factors seemed to have a favorable outcome, which is relatively confusing. Unlike the current study showing that EEC patients with dMMR had worse prognosis with a reduced OS [9], a previous Korea's study showed a favorable trend for OS in dMMR group [7], and this favoring was more apparent in dMMR patients who were advanced stage EC status or who underwent postoperative adjuvant therapy, despite that these patients had more unfavorable prognostic factors (LVSI and DMI) that EC patients with pMMR status [7]. All suggest the prognostic value of MMR status seemed to be worthy of further validation. We found that the characteristics of enrolled subjects in both studies seemed to be different (pure EEC patients [9], and all EC patients, including EEC or non-EEC [7]), although this proposal is still uncertain.

Although the value of MMR status in predicting prognosis of EC patients is unknown, MMR status-guided therapeutic benefits are consistent in EC patients, regardless of which history type is [10–13]. The biomarker value of MMR status for therapy may be more important than for outcome prediction.

It is well-known that advanced-stage or recurrent diseases of EC patients have extremely poor outcomes [10]. The conventional targeted therapies and platinum-based chemotherapy have had relatively limited efficacy, substantial toxic effects or both [10]. Moreover, these patients are not rare, and in fact, appropriately 10–20% of EC patients belong to metastatic and recurrent EC group [2]. Fortunately, immune checkpoint inhibitors (ICIs) [14], including pembrolizumab (a programmed cell death 1 [PD-1] inhibitor), had compelling antitumor activity, as assessed on the basis of objective response and duration of response, in patients with microsatellite instability-high (MSI-H) or dMMR advanced or recurrent EC [10,12]. Evidence supports pembrolizumab monotherapy has shown less activity in patients with microsatellite-stable or pMMR EC than in those EC patients with MSI-H or dMMR [10]. All hint MMR status of tumors is one of the most determinant factors to respond to ICIs, supporting that biomarker-guiding therapeutic choice may be much more critical than biomarker-related prognosis is. Since the authors did not mention this part, their application of MMR status to predict the outcome may be a risk of mis-interpretation of their data, particularly for those patients with dMMR treated with ICIs. The results have confirmed that ICIs therapy has a more favorable outcome of EC patients with dMMR compared to those EC patients with dMMR treated by conventional platinum-based chemotherapy [10,12].

Finally, the detection of the MMR status for patients with EC is highly and strongly recommended in the College of American Pathologists (CAP) and the American Society of Clinical Oncology (ASCO) if the patients are considered for ICIs therapy, suggesting that the role of MMR status in prediction outcome may be less important, if ICIs therapy is not planned. This concept is not only limited to EC patients, and for fighting multiple cancer types (colorectal cancer [CRC], gastroesophageal and small bowel cancer), biomarkers-guided therapy is very popular and effective [13]. In fact, all cancer patients being considered for ICIs therapy should test their MMR status of tumors [13]. At least three tests (MMR status and MSI status) are available to fulfill the indication of using ICIs in the management of cancer patients, including immunohistochemistry (IHC) for MMR and polymerase chain reaction (PCR) or next generation sequencing (NGS) for MSI [13]. Finally, ASCO endorses CAP guideline to recommend strongly that for patients with EC, being considered for ICIs therapy, pathologists should use MMR-IHC over MSI by PCR or NGS for the detection of DNA MMR defects [13]. As shown here, both Korea's studies had used MMR-IHC to detect DNA MMR defects in EC patients [7,9]. It is worthy of applause, although their purpose did not comply to the "consideration of using ICIs therapy for their patients". Besides of very promising advance in application of MMR-IHC for EEC patients, the aggressive (conventional grade 3) and non-aggressive histological types (conventional grade 1 and 2) had better be integrated by molecular classification for improved prognostication and for treatment decision-making [1]. The FIGO Women's Cancer Committee and Endometrial Cancer Staging subcommittee, 2021–2023 in 2023 recommends that grade 3 EEC are a prognostically, clinically, and molecularly heterogenous disease and the tumor type that benefits most from applying molecular classification to precisely predict outcome and offer a better choice for treatment plan [1]. Without molecular classification, grade 3 EEC cannot appropriately be allocated to a risk group and thus molecular profiling is particularly recommended in these patients with grade 3 EEC [1]. Therefore, if the molecular classification is unknown, grade 3 EEC were grouped together with the aggressive histological types in the actual FIGO classification in our clinical routine practice [1].

Taken together, immuno-oncology has become one of the leading therapeutic advanced therapies for cancer patients, not only due to successful transformation of the treatment landscape but also clear prediction of outcome [15]. Adding molecular classification into the conventional clinico-pathological staging system of the EC patients has become more and more important and critical. All of us should update our knowledge about the management of EC patients. The promising bullet agents are available for the treatment of EC patients, particularly for those patients with advance stage or recurrence status, which is conventionally considered a lethal disease. We welcome more and more experts' contribution to share their real-world clinical data to offer a better care of patients.

Conflicts of interest

All authors declare no conflict of interest.

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Editorial

Good outcome of endometrial cancer patients coexisting with adenomyosis is a result of their associated favorable clinico-pathological parameters

Endometrial cancer (EC) has become one of rapidly increasing women's cancers, contributing to the most common cancer of the female genital tract in Taiwan [1]. EC is believed its favorable outcome (both progression free survival [PFS] and overall survival [OS]) compared to other gynecologic organ-related cancers [2], partly because of early symptoms and signs presented by the patients, contributing to the early diagnosis and early stage of EC [3], and partly because of preceding risk factors hinting patients to look for help by health-care providers, such as metabolic syndrome, polycystic ovary syndrome, endometriosis and others [4–6]. Among these, endometriosis and its variance such as adenomyosis are one of the major bothersome problems occurred in women during reproductive age, particularly for those patients with adenomyosis, who present dysmenorrhea, menorrhagia, heavy menstrual bleeding, and chronic pelvic pain typically [6]. Additionally, pathogenesis favored the close correlation between endometriosis and EC, because of sharing a common pathogenic mechanism, including hormone factors, genetic predisposition, growth factors, inflammation, immune system dysregulation, environmental factors and oxidative stress [7]. Moreover, epidemiologic studies support that women with endometriosis and/or adenomyosis may increase the risk of development of EC in the whole follow-up period, with a crude odd ratio (OR) of 2.44 (95% confidence interval [CI] 2.24–2.66) and age-adjusted OR of 2.58 (95% CI 2.37–2.81) compared to those women without endometriosis and/or adenomyosis, although most women with endometriosis/adenomyosis do not develop EC [8,9]. Another study in Taiwan also confirmed women with endometriosis had significantly higher risk of EC (adjusted hazard ratio, aHR 2.92, 95% CI 2.12–4.03) [9].

Besides the risk of EC being increased in women with endometriosis/adenomyosis, the outcomes have also been compared between women with and without endometriosis/adenomyosis. Although many studies have shown the potential better prognosis in EC women coexisting with endometriosis/adenomyosis compared to those EC women without endometriosis/adenomyosis, some studies did not support the aforementioned results, particularly for those EC patients after adjusting the other worse prognostic factors, such as age, stage, and pathologic findings and molecular classification [10–14]. Therefore, it is happy to learn the recent publication has attempted to compare clinicopathological features and survival outcomes in patients with endometrial cancer, with and without associated adenomyosis [15].

The authors conducted a systematic review and meta-analysis, including 21 studies (n = 46,420) to explore the clinicopathological features and outcomes in EC patients with and without adenomyosis [15]. EC women coexisting with adenomyosis had a better 5-year OS (HR 0.62, 95% CI 0.50–0.79) and PFS (HR 0.60, 95% CI 0.44–0.82). The aforementioned better outcomes of EC patients with adenomyosis could be well reflective by these patients with favorable tumor grade (decreased risk of the International Federation of Gynecology and Obstetrics [FIGO] grade 2 or 3, OR 0.51, 95% CI 0.42–0.62) and early FIGO stage (higher percentage of stage I or II, OR 2.23, 95% CI 1.65,~3.01) [15]. Additionally, EC patients coexisting with adenomyosis had lower risk of tumor invasion of adnexa, cervical stromal invasion, deep myometrial involvement (DMI), lympho-vascular space invasion (LVSI) and peritoneal invasion [15]. However, it is interesting to find that disease-specific survival (DSS) was similar between two groups (HR 0.60, 95% CI 0.35–1.05) [15]. The present study is interesting and worthy of further discussion.

First, in term of survival-related outcomes (PFS, OS, and DSS), the authors clearly and apparently demonstrated that EC women with adenomyosis have better survival-related outcomes (with a 40% reduction of disease recurrence [PFS], death [OS] and disease-related mortality [DSS]) than those without, and the former two reach to the statistical significance but the latter does not have a statistical significance [15]. However, compared with the two formers based on analyzing 9 studies, including PFS and OS, the results of DDS were only based on analysis of 3 studies. Additionally, although DSS between EC women with and without adenomyosis did not reach the “statistically significant difference”, the HR was 0.60, which is relatively consistent with 0.62 of HR for OS and 0.60 of HR for PFS, suggesting that EC women with adenomyosis may really have a better prognosis than EC women without adenomyosis.

Second, as shown by authors [15], the better survival-related outcomes in EC women with adenomyosis are mainly dependent on presence of “favorable clinico-pathological parameters”, including absence of LVSI, absence of DMI, absence of cervix and/or adnexal involvement and low histological FIGO grade (grade 1) and early detection (early-stage, such as FIGO I or II, EC) [15]. The aforementioned correlation can be easily explained by patients' awareness, since women with adenomyosis may be complicated with dysmenorrhea, heavy menstrual bleeding, and chronic pelvic pain and all trigger them to look for the care of health-providers earlier or frequently, and seek periodical follow-up [15]. In fact, adenomyosis women have a higher chance to receive “relatively suppressed endometrium” like medication, since the use of oral contraceptive or long-term prescription of “progestins”-like

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hormones may have a significantly protective effect to reduce the development of EC [8]. Additionally, in the good responders (for adenomyosis), the long-term use of these "protective hormones" may further decrease the risk of developing EC in these patients. By contrast, for those without response to "protective hormones" treatment, the surgical intervention (hysterectomy) may be applied earlier. All result in the early diagnosis and early stage of EC in women with adenomyosis. In clinical practice, compared to other types of gynecological organ-related malignancy [16–18], EC is often diagnosed at the early stage [1].

Third, good outcomes of the EC women with adenomyosis may be partly explained by "accidental finding" or "background data" of the general population. Hermens and colleagues found an age-aOR for EC of 2.63 (95% CI 2.40–2.87) and an age-aOR of 1.11 (95% CI 0.82–1.5) after excluding the first year of follow-up [8]. In fact, the prevalence of adenomyosis in women with diagnosed EC was similar to the prevalence reported in hysterectomy for other gynecological conditions [8,12], and this observation may be reflective as early-stage EC (particularly for stage I) in nearly majority of the patients with presumed benign uterine diseases undergoing hysterectomy procedure. Compared with all women with diagnosed EC, at least 10% of diseased women were advanced-stage patients. All suggest that if excluding the advanced-stage EC women, the outcomes of EC patients with adenomyosis may be similar to those without adenomyosis.

Forth, although epidemiology supports the possibility of increased risk of EC in patients with adenomyosis compared to that without adenomyosis, the favorable clinico-pathological parameters, and early diagnosis of EC in these women with adenomyosis can be successfully compensated by higher prevalence of EC in patients of endometriosis and/or adenomyosis.

Taken together, similar to good compensation (better response to platinum-based chemotherapy, and successful prolongation of both PFS and OS after maintenance therapy by poly(ADP-ribose) polymerase [PARP] inhibitors [PARPi]) of BRCA-mutated epithelial ovarian cancer (EOC) women who have an absolute increased risk of the development of EOC in their life-spans, women with adenomyosis are also demonstrated to have a higher risk of developing EC in their lives. However, the favorable outcomes of these patients may be alleviated by anxiety of women with these troublesome diseases (adenomyosis and/or endometriosis).

Conflicts of interest

All authors declare no conflict of interest.

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Editorial

Ultrasonography for menopausal endometrium

Menopausal endometrium is a plethora of various kinds of disease processes secondary to significantly decreased female sex hormones entering inactive and free of cyclic changes of endometrium [1]. Among disorders of menopausal endometrium, the most sinister is an endometrial cancer (EC), which is secondary to the combination of genetic, environmental, and lifestyle risk factors-related development and has become one of the rapidly increasing women's cancer worldwide and the most common female genital tract cancer in the middle- and high-economic countries [2–5]. Fortunately, most patients with EC are symptomatic and the most presenting symptom of which is postmenopausal bleeding (PMB), which always alert patients looking for help, contributing to an early diagnosis and the following prompt treatment for the majority of the diseased patients [6,7]. The excellent outcomes of these early-stage EC patients can be achieved by either minimally invasive surgery (MIS) or conventional exploratory laparotomy [6,7], and the former is a preferred procedure based on its immediate postoperative advantages [6,8]. To approach symptomatic postmenopausal women (PMW), ultrasonography, particularly transvaginal ultrasonography (TVS) is always considered as the first priority and most effective, minimally invasive and convenient tool. Although the consensus of using TVS to detect underlying pathology of endometrium for PMW complicated with PMB is well-established [9,10], there is no consensus about the routine use to screen the asymptomatic PMW and it is also uncertain that the cut off value of the thickness of the endometrium in asymptomatic PMW, while a measurement of >5 mm and PMB is suspect and requires further examination [10]. By contrast, one study recommended that observation of asymptomatic PMW without risk factors and with an endometrial thickness of less than 10 mm may be reasonable [9]. Furthermore, some clinical situations, including tamoxifen-induced endometrial change of women with breast cancer may cause the controversies about the clinical practice using TVS in the evaluation of underlying endometrial pathologies [11]. Therefore, the relationship between atrophic endometrium and ultrasound findings is an important issue about our routine clinical practice for PMW, particularly for those PMW accompanied with PMB. A recently published article in the 2023 September issue of the *Taiwanese Journal of Obstetrics and Gynecology* (*TJOG*) by Drs. Lin and Chang trying to evaluate the endometrial thickness under sonogram in the women with atrophic endometrium, with or without PMB provides us useful and relevant information about this issue [12].

The authors conducted a retrospective study enrolling 202 PMW with pathological evidence of atrophic endometrium. They used the conventionally accepted cut off value of endometrial thickness to separate them into three groups, including 42 (20.8%), 109 (54%) and 51 (25.2%) women with endometrial thickness ≤4 mm, >4 mm–10 mm and >10 mm respectively [12]. The authors found tamoxifen use dramatically increased the thickness of endometrium and near two-thirds of patients (61.5%, 16/26) had a >10 mm endometrial thickness compared to 19.9% (35/176) in patients who did not receive tamoxifen treatment before [12]; however, it is interesting to find that women with a >10 mm endometrial thickness had a less frequency to present PMB (62.8% vs. 84.4% as well as 83.3% in 4 mm–10 mm and ≤4 mm, respectively) [12]. No women undergoing hormone replacement therapy (HRT) had an endometrial thickness >10 mm [12]. By the way, the authors found the typical endometrial change as cystic change, called as a “Swiss cheese” pattern in the women treated with tamoxifen, and the aforementioned pattern appears in 44% (7/16) of women with a >10 mm endometrial thickness [12]. The authors finally concluded that endometrial thickness may not be the only factor for indicating an invasive procedure, such as endometrial sampling, diagnostic dilation and curettage and others, because certain proportion of these patients with thickened endometrium (>10 mm) had a pathological diagnosis of atrophic endometrium [12]. The current study is interesting and worthy of discussion.

First, many studies suggested that endometrial thickness is of the critical determining factor for women with/without PMB [9–11,13,14]. Vitale et al. performed a systematic review and meta-analysis to demonstrate that the risk of EC and atypical endometrial hyperplasia (AEH) in asymptomatic PMW with an incidental ultrasonographic finding of ≥3.0 mm endometrial thickness is 3-fold higher than asymptomatic PMW with an ultrasound endometrial thickness under the 3.0 mm threshold to perform endometrial sampling; however, it is interesting to find that no substantial risk difference was found compared with higher ultrasonographic thresholds for endometrial thickness [15]. However, considering a significant increase of endometrial biopsy by threshold of 3 mm, which is associated with a meaningful emotion burden of women, secondary screening can be implemented for a cutoff of 4 or 5 mm, which could dramatically decrease the need of endometrial biopsy from 44.8% to 12.7% or 9.5%, respectively [15]. By contrast, if a cutoff of ≥14 mm is recommended, it can reach the highest specificity although the sensitivity may be significantly impaired [16]. All hint that the endometrial thickness alone may not be a good indication to perform endometrial biopsy, which is also supported by ACOG (The American College of Obstetrics and Gynecology) that using TVS as screening tool to evaluate asymptomatic PMW is not recommended for asymptomatic PMW [13]. Furthermore. an evidence-based guideline for clinical practice suggests an incidental finding of a thickened endometrium in a PMW

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without PMB is not an immediate indication for further more invasive testing, also supported the concept that endometrial thickness is not an indication for immediate invasive procedure [15,16].

Second, besides patients with thickened endometrium, there are many other different clinical scenarios possibly requiring endometrial biopsy, such as patients presenting with abnormal uterine bleeding (AUB) or PMB [16], suggesting that endometrial biopsy had better be accorded by presence of many clinical scenarios in these patients. In clinical practice, a careful evaluation of the other ultrasound findings, taking the patient's characteristics into account, including age, body mass index, parity, comorbidity and sharing the decision making with patient can minimize the need of invasive procedures [15]. Under the comprehensive review of history and clinical data, the better cost-effectiveness of invasive procedures can offer to the patients with a satisfactory sensitivity and specificity [15].

Third, the argument about the choice of invasive procedures to evaluate the underlying endometrial pathology is continuous, based on a possibility of presence of a false-negative result, missing the diagnosis due to biopsy technique, non-representative sampling, underlying endometrial pathology (e.g., polyp) and variable pathological interpretation [16]. Compared to conventional blind diagnostic dilation and curettage (D & C) performed in the operating room under general anesthesia to evaluate the PMW at risk of EC, office hysteroscopy may be a better alternative, based on its reducing cost, reducing complexity, enhancing accuracy inherent to directly visualized biopsy of pathology, facilitating identification of cornual pathology, which would be frequently and easily to be missed when blind procedure disproportionately assesses the posterior midline of an anteflexed uterus [15].

Taken together, TVS following invasive procedures to diagnose endometrial pathology (e.g., AEH or EC) is a well-accepted tool for evaluate PMW with PMB. Due to absence of consensus about the threshold of endometrium thickness, and front-line invasive procedure, we welcome more and more researches focusing on this topic, which not only buffers emotional stress of patients but also minimize the risk of missing diagnosis of EC.

Conflicts of interest

All authors declare no conflict of interest.

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Editorial

Endometrial thickness in tamoxifen-treated women

Endometrium is a plethora of various kinds of disease processes secondary to significantly altered various kinds of hormones or hormone-like agents entering its cyclic change, which is initiated in the proliferative phase and transforms into the secretory phase or becomes an inactive endometrium and ceased cyclic change [1]. Endometrial changes are conventionally and strictly controlled by the orchestration of hormones [2,3]. The disruption of axis of hormone system and over-production or depletion of hormone secretion, either mediated through endogenous or exogenous pathways have been implicated in the pathogenesis of endometrial disorders [2,3]. Among these hormone agents, the most popular and well-known agent available in the modern medicine is tamoxifen, one of selective estrogen receptor modulators (SERMs) mediated through different types of estrogen receptor (ER) to act either anti-estrogenic or estrogen-mimic effects, which has become the "gold standard", and established the principles of breast cancer (BC) targeting and identifying the appropriate treatment strategy to aid survival in BC patients, with enhancement of disease-free survival (DFS), and a 50% decrease in recurrence observed in ER-positive patients 15 years after diagnosis [4–7].

The use of tamoxifen is not without adverse events (AEs), and therefore, there are many other SERMS attempting to replace tamoxifen in the management of BC women with the same purpose. However, so far, no one succeeds, and tamoxifen is still a preferred agent for treating ER-positive BC patients [7]. The benefits of tamoxifen in lives saved from BC far outweigh concerns about an increased incidence of EC in post–or pre-menopausal women, although some AEs have contributed to slightly but significantly increased morbidity and mortality, such as the development of EC, change of coagulation system and the subsequent occurrence of acquired resistance [7]. Among the aforementioned AEs, EC is always in concern and worthy of further discussion.

Compared to BC, an easy and frequent diagnosis at the early stage of EC can be made, regardless whether women have a diagnosis of BC not. Early-stage EC contributes to a favorable outcome [8–10]. This is particularly apparent in tamoxifen-treated BC patients, partly because of well awareness of an increased risk of patients themselves or partly because of frequent and routinely using transvaginal ultrasonography (TVS) as screening tool to evaluate the tamoxifen-related endometrial abnormality or endometrial pathology by physicians. Although it is popular, it is still highly debated about the routine use of TVS to screening the asymptomatic BC women treated with tamoxifen and it is also uncertain that the cut off value of endometrial thickness in asymptomatic BC women undergoing tamoxifen or post-tamoxifen therapy [11–14]. The different definition of cutoff of endometrial thickness will result in psychological and physical stress and embarrassment of BC patients, because the following endometrial biopsy, either through the sampling procedure or through more invasive and cost diagnostic dilation and curettage (D&C) under general anesthesia in the operative room will be scheduled [12–14]. This dilemma and challenge is also found in general population, particularly for those asymptomatic postmenopausal women (PMW) [12]. The agreement of using TVS to screening underlying endometrial pathology for PMW accompanied with or without postmenopausal bleeding (PMB) is still in argument [12]. While a measurement of >5 mm (or > 3 mm in recent systemic review and meta-analysis) of endometrium in general population with or without presenting symptoms, such as PMB is suspect and requires further examination [11–14]; therefore, an issue about the surveillance of endometrial lesion in BC patients, particularly for those patients undergoing tamoxifen treatment is important. A recently published article in the 2023 July issue of the *Taiwanese Journal of Obstetrics and Gynecology* (*TJOG*) trying to evaluate the endometrial thickness of BC patients with tamoxifen treatment [15].

The authors conduced a retrospective study and enrolled 98 women receiving tamoxifen as postoperative BC treatment to compare endometrial thickness between two-dimensional ultrasonography (2-D TVS) and elastosonography (ultrasound elastography) [15]. The results showed the mean value of 5.81 mm (standard deviation [SD] 3.09 mm) in the conventional 2-D TVS and 3.07 mm (SD 1.62 mm) in the elastography, respectively and difference between two groups was statistically significant [15]. Additionally, based on the median value of the delta of 2.2 mm endometrial thickness, the difference was increased with age, presence of vaginal bleeding (presenting symptom), presence of ductal carcinoma in site (DCIS), and duration of tamoxifen treatment, and furthermore, after adjustment (multivariate analysis), only age and tamoxifen duration were still playing important variables that exerted a statistically significant effect [15]. All suggest that the results of endometrial thickness of BC patients will influence by many factors. However, how to evaluate the endometrial thickness may be another big issue, since reproducibility and accuracy may be significantly affected by technology and technicians.

Although the use of endometrial thickness alone screened by TVS for indicating the need to perform invasive procedures to detect atypical endometrial hyperplasia (AEH) or EC is long-term considered as convenient and cheap screening choice based on the fact that the increased risk of AEH or EC is dependent on endometrial thickness [11–14]. However, there is absence of consensus to clearly demonstrate the best cutoff of endometrial thickness. Over the cutoff of endometrial thickness, the following invasive procedures are needed to exclude the possibility of AEH or EC, resulting in a main critique-the gap between cost and effectiveness

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of screening tools. A ≥ 3.0 mm endometrial thickness is 3-fold higher than asymptomatic PMW with an ultrasound endometrial thickness under the 3.0 mm threshold to perform endometrial sampling [14]. If screening threshold was increased to a cutoff of 4 or 5 mm, a dramatically decreased rate of endometrial biopsy from 44.8% to 12.7% or 9.5%, respectively can be obtained [14]. Therefore, it is easy to expect that higher cutoff may minimize the need of subsequent invasive endometrial biopsy. For example, a threshold increases to a cutoff of ≥14 mm, the highest specificity can be achieved although the risk of missing diagnosis of AEH and EC may also be significantly increased [14]. How to balance the false-positive inducing unnecessary invasive procedures from the false-negative inducing delayed diagnosis of life-threatened EC needs our efforts.

Conventionally, 2-D TVS is an easy, cheap, and convenient tool fulfilling the requirement for the first line screening or evaluation purpose; however, the diagnostic accuracy and reproducibility of inter-observers or intra-observers are often in doubt, although some of them can be corrected by well-training process and typical landmark guideline for examination. To overcome the limitation of conventional 2-D ultrasound, many advanced technologies, such as 3-D TVS, saline-infusion TVS, and elastosonography, reported in the current article has been developed with various degree of success, because these strategies not only provide the reliable results comparable with much expensive and time-consuming diagnostic tools, such as magnetic resonance image (MRI) but also take advantages over 2-D TVS without compromising time-saving, convenience, cost-effectiveness, and compliance of both physicians and patients [15,16]. Compared to conventional 2-D TVS, the authors have shown elastosonography is a more powerful and more valuable screening tool to evaluate the endometrial thickness and morphologic characteristics, because of its real-time, objective, and repeatable characteristics [15]. The absolutely measured thickness of the endometrium by elastosonography was dramatically and significantly thinner than that by conventional 2-D TVS [15], suggesting the possibility that endometrial thickness may be over-estimated by 2-D TVS and by contrast, endometrial thickness measured by elastosonography may be more reliable to search for cutoff of endometrial thickness or to guide the indication for endometrial biopsy. The latter is important, since false-positive screening results not only cause the patients heavy emotional burden but also increase the expense of medical fare. Additionally, endometrial biopsy is not totally free of risk, particularly for those patients who receive diagnostic dilation and curettage (D&C) with or without assistance of hysteroscopy which needs general anesthesia and is held in the operating room [17]. Based on the recent most popular useful reference by measuring endometrial thickness as well as cutoff of endometrial thickness as 4 mm, although still debated, conventional 2-D TVS may be at higher risk of overestimated endometrium thickness resulting in false positive indication to request further invasive procedures to evaluate the underlying endometrial pathology than elastosonography. However, the sensitivity rate of elastosonography was not evaluated by Dr. Jo's group [15], since it is well known when specificity rate is increased, sensitivity rate is expected to be decreased. One study showed ≥15.5 mm endometrial thickness in premenopausal patients treated with tamoxifen can be used as the most accurate ultrasound diagnostic threshold for the diagnosis of abnormal endometrial hyperplasia with an area under the curve of 0.888 (95% confidence interval [CI] 0.716–1.000), a sensitivity of 100% and a specificity of 75%, but for PMW treated with tamoxifen, ≥5 mm endometrial thickness was suggested as cutoff for routine TVS diagnosis of abnormal postmenopausal endometrial hyperplasia with a sensitivity of 100%, specificity of 5.0%, positive predictive value of 9.5%, and negative predictive value of 100% [11]. All suggest it is still uncertain whether the cutoff of endometrial thickness should be defined, because when sensitivity rate is increased, the specificity rate is decreased in following, which results in a big gap between overtreatment and missing diagnosis and this controversial issue needs further more studies. That is why Dr. Jo attempted to use elastosonography in place of original 2-D TVS, to overcome the high false-positive rate and the low frequency of significant findings of 2-D TVS. Elastonography is a new technology that uses sound waves to estimate the physical properties of tissues in response to mechanical pressure on the target anatomy, which in theory, is used to detect differences in tissue properties such as stiffness by Youngs's modulus with the larger value, the greater the elastic hardness of the tissue and elasticity by the elastic hardness map with red indicating the softest and blue the hardest, allowing for the identification of various pathologies [15].

The frequent findings or features of ultrasonography for endometrium in BC patients undergoing tamoxifen therapy are not mentioned in detail in Dr. Jo's study [14]. Common ultrasound features include thickened endometrial with a typical “Gruyere cheese” appearance or “Swiss cheese” pattern in up to three quarters of tamoxifen users, which is reflective of pseudopolypoid glandulocystic endometrium and glandulocystic polyps as well as endometrial cysts (57%) and subendometrial cyst (15%) [5,19], contributing to no surprising to find that BC patients treated with tamoxifen had statistically significantly increased endometrial thickness than those treated either with or without other endocrine drugs and had a higher percentage of cystic change of endometrium [11].

Taken together, the limitation of high false-positive endometrial thickness for screening BC patients treated with tamoxifen still bother both physicians and patients. Due to absence of consensus about the threshold of endometrium thickness, and following front-line invasive procedure, we welcome more and more researches focusing on this topic.

Declaration of competing interest

All authors declare no conflict of interest.

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Review Article

Front-line chemo-immunotherapy for treating epithelial ovarian cancer: Part I CA125 and anti-CA125

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ABSTRACT

The current standard therapy of epithelial ovarian cancer (EOC) is the combination of surgery (primary cytoreductive surgery or interval cytoreductive surgery) and platinum-based chemotherapy (mainly using paclitaxel and carboplatin either by neoadjuvant chemotherapy and/or by postoperative adjuvant chemotherapy) with/without adding targeted therapy (mainly using anti-angiogenesis agent- bevacizumab). After front-line chemotherapy, the advanced-stage EOC can be successfully controlled and three-quarters of patients can achieve a complete clinical remission. Unfortunately, nearly all patients will recur and progression-free survival (PFS) of these patients is seldom more than 3 years with a dismal median PFS of 12–18 months. With each recurrence, patients finally develop resistance to standard chemotherapy regimen, contributing to fewer than half of women who survive for more than 5 years after diagnosis with a median overall survival (OS) of 40.7 months. Due to the lower PFS and OS, particularly for those advanced-stage patients, novel therapeutic options during the front-line therapy are desperately needed to decrease the occurrence of recurrence, and the majority of them are still under investigation. It is well-known that overexpression of CA125 has been associated with attenuated cellular apoptosis, platinum chemotherapy resistance, tumor proliferation and disease progression, suggesting that anti-CA125 may play a role in the management of patients with EOC. The current review is a Part I which will focus on development of anti-CA125 monoclonal antibody, hoping that alternation of the front-line therapy by chemo-immunotherapy will be beneficial for prolonged survival of patients with EOC.

Introduction

Epithelial ovarian cancer (EOC) is the seventh most commonly associated with the seventh deadliest cancer amongst women in Taiwan [1]. An age-standardized rate of EOC in 2020 is 10.66 cases (n = 1824) per 100,000 women, contributing to 3.61 deaths (n = 724) per 100,000 women in Taiwan [1]. The current standard-of-care for EOC is mainly dependent on a thorough and complete cytoreduction and following adjuvant platinum-based chemotherapy plus paclitaxel regimen with or without an angiogenesis inhibitor, such as bevacizumab [2–7]. Prognosis can be predicted by many clinical and biological parameters, such as tumor stage/grade/histology at diagnosis, extensive spreading pattern and invasion of tumor, and residual disease after cytoreductive surgery (complete cytoreduction without any residual visible tumor [R0], or minimal residual tumor [R1], or suboptimal debulking surgery [R2]) [4–7].

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Among these, the success of cytoreductive surgery, meaning complete cytoreductive surgery reaching the R0 status remains the most critically and independently prognostic factor to predict the patients' outcome, which can be directly measured by either progression-free survival (PFS) or overall survival (OS), and the latter is the gold standard endpoint in cancer clinical trials, as it is an objective measure, can be measured accurately by documenting the date of death, and has a minimal risk of bias in reporting [3,7]. However, primary cytoreductive surgery (PCS) is not always achieved, partly because of aggressive tumor behaviors (such as, the Ovarian Cancer Comorbidity Index [OCC]) and partly because of the characteristics of diseased subjects (such as, Charlson Comorbidity Index [CCI]), contributing to the need of some modified methods (neoadjuvant therapy [NAT]) to attempt to reduce the severity of the disease, downstage of the disease and compromise the diseased subjects not fitting to undergo the PCS [8–11].

After NAT treatment, interval cytoreductive surgery (ICS) may be applied [12–15]. This approach is reported to have the lower risk of immediate operation-related morbidity and mortality; therefore, it is more and more popular for the treatment of women with far-advanced staged EOC recently [5]. Furthermore, the success rate of ICS is significantly higher than that of PCS, resulting in the higher chance to achieve the R0 status, although it is uncertain whether the outcome of the patients treated by ICS is better or worse compared to that of PCS [12–15].

After cytoreductive surgery (PCS or ICS), the following platinum-based chemotherapy, called as front-line chemotherapy is an essential component for treating all patients with EOC, particularly for those patients with advanced-stage EOC [15]. After front-line chemotherapy, the advanced-stage EOC can be successfully controlled and three-quarters of patients can achieve a complete clinical remission [3]. Unfortunately, nearly all patients will recur and the PFS of these patients is seldom more than 3 years with a dismal median PFS of 12–18 months [16]. With each recurrence, patients finally develop resistance to standard chemotherapy regimen, contributing to fewer than half of women who survive for more than 5 years after diagnosis with a median OS of 40.7 months [2,3,14–17]. All result in EOC as most highly lethal cancer for women [2,14–17].

To overcome this limitation, the treatment landscape for EOC has changed in recent years with the introduction of maintenance therapy to treat patients with a certain genetic background, particularly for those patients with advanced disease [16–24]. All efforts attempt to have a better chance to prolong PFS and possibly cure the diseases and maintain OS of patients [15–24]. However, it relies on the advance and better understanding of pathophysiology of cancers as well as continuous improvement of technology in the cancer research [9,24–41], resulting in the availability to develop new agents (magic bullets), including small molecules, monoclonal antibody (MAb), antibody drug conjugates (ADCs), based on the following mechanisms, such as (a) targeting the specific cancer-specific antigens, (b) attacking the underlying repair system of cancer cells, (c) blocking the nutrition or oxygen supply (for example, antiangiogenic drugs), (d) changing the interaction between cancer cells and surrounding cells, (e) altering or modifying behaviors of cancer cells, (f) enhancing immune clearance ability (for example, immune checkpoint inhibitors [ICIs], immune system modulators), (g) enhancing the therapeutic effect of the original chemotherapy with complex engineered molecules that consist of MAb directed toward or targeted tumor-associated or tumor-specific antigens (Ag), conjugated via a stable linker to a potent cytotoxic agent, contributing to an ADC's biodistribution, tumor specificity and cytotoxic effects and many others in the management of women with advanced-stage EOC [4–6,16–24,40–44]. In fact, the aforementioned strategies based on recent guidance on maintenance therapy has evolved to include poly(ADP-ribose) polymerase (PARP) inhibitors (PARPi) and/or antiangiogenic agent, bevacizumab, which are a rapidly growing class of oncologic therapeutics, particularly for those patients in the platinum-sensitive setting [17,22–25]. It has likely positively affected survival outcomes for patients with platinum-sensitive EOC, and possibly contributed to the steady decline in the annual death rate.

Unfortunately, these increases in prevalence and survival cannot be totally reflective as patients free or absence of diseases, and they should require adjustments to the treatment for long-term care, especially when the patients become platinum-resistant EOC (PROC) [17]. Due to the lower PFS and OS, particularly for those patients with PROC, novel therapeutic options during the front-line therapy are desperately needed to decrease the occurrence of PROC, and majority of them are still on investigation. Additionally, many subsequent novel therapies, have not been associated with improved overall outcomes in patients with PROC [17]. Despite the success of bevacizumab, alternative antiangiogenic therapies, such as ofranergene obadenovec (ofra-vec), have either failed to improve outcomes or have been withdrawn from development [17]. All contribute to the reconsideration of modification of the front-line therapy. The current review will update the recent advances in new front-line strategies for the patients with advanced-stage EOC.

The standard front-line therapy for epithelial ovarian cancer

The backbone of front-line treatment has changed little in the past three decades, with the use of platinum-based chemotherapy, typically including a carboplatin/paclitaxel chemotherapy regimen [2,11,16,17]. In the past few decades, the emergence of bevacizumab into the standard platinum-based chemotherapy has revolutionized the treatment of advanced-stage EOC [18,19,22], since NCCN Guidelines Version 2.2023 Ovarian cancer/fallopian tube cancer/primary peritoneal cancer has recommended both paclitaxel/carboplatin with/without bevacizumab regimen as primary systemic therapy regimens [10,45–47]. Bevacizumab, a mAb binding vascular endothelial growth factor (VEGF), given with chemotherapy and continued as maintenance, shows a relatively promising result.

In ICON 7, PFS was 20.3 months with standard therapy, in comparison to 21.8 months with standard therapy plus bevacizumab (hazard ratio [HR] 0.81, 95% confidence interval [CI] 0.70–0.94), suggesting the benefit of adding bevacizumab to standard chemotherapy [47]. Additionally, the benefit was the greatest for patients with a high risk of progression, such as patients belonging to International Federation of Gynecology and Obstetrics (FIGO) stage III >1 cm or IV or suboptimally debulked surgery, where the estimated median PFS was 10.5 months with standard therapy, as compared with 15.9 months with bevacizumab (HR 0.68, 95% CI 0.55–0.85) [45,47], deriving a benefit on OS with the addition of bevacizumab with a median OS of 39.3 months versus 34.5 months, an absolute increase of 4.8 months [45,47].

In the GOG 218 trial, the benefits of adding bevacizumab in the front-line and maintenance therapy setting are also shown with prolonging 10.3 months–14.1 months (PFS, HR 0.72, 95% CI 0.63–0.82) in all corners but with prolonging 32.6 months–42.8 months (OS, HR 0.75, 95% CI 0.59–0.95) in stage IV EOC patients [45,46]. The above results make many gynecologic oncologists favor prescribing paclitaxel/carboplatin/bevacizumab regimen for EOC patients as a standard front-line therapy for patients with advanced EOC [45–50].

However, the 2022 Cochrane systemic review makes a relatively different conclusion, showing bevacizumab unlikely results in apparent difference in either PFS or OS compared to chemotherapy

alone (HR 0.82, 95% CI 0.64–1.05; two studies, 2746 participants; very low-certainty evidence and HR 0.97, 95% CI 0.88–1.07; two studies, 2776 participants; moderate-certainty evidence, respectively) [22]. Additionally, the combination of bevacizumab and chemotherapy likely increases any adverse events (grade ≥3) (risk ratio [RR] 1.16, 95% CI 1.07–1.26, one study, 1485 participants, moderate-certainty evidence) and may result in a significant increase in hypertension (grade ≥2) (RR 4.27, 95% CI 3.25–5.60, two studies, 2707 participants, low-certainty evidence), contributing to a slight reduction in global quality of life (QoL) (mean difference (MD) −6.4, 95% CI −8.86 to −3.94, one study, 890 participants, high-certainty evidence) [22].

Because of the uncertainty of absolute benefits of paclitaxel/carboplatin/bevacizumab regimen for EOC patients and bevacizumab considering as maintenance therapy (in fact, a bevacizumab treatment duration of 15 months remains the standard of care following completion of primary treatment) [48], the front-line therapy seemed to be no change in the past three decades in the management of women with EOC.

Additionally, triplet combinations of agents have been tested to improve outcomes, but a large phase III intergroup trial failed to demonstrate its efficacy [51]. Compared to the standard paclitaxel and carboplatin, addition of a third cytotoxic agent provided no benefit in PFS or OS in patients with EOC, regardless of optimal or suboptimal cytoreduction [51]. Other efforts to enhance the response of EOC treatment, such as (a) altered delivery method of chemotherapeutic agents (the use of intraperitoneal route in place of intravenous route); (b) changed dose and interval of chemotherapy administration (dose-dense chemotherapy); (c) intraperitoneal hyperthermia treatment [5]; however, all have yielded inconsistent benefit and failed to change the standard of care [52]. All suggest that primary front-line therapy needs further optimization (Fig. 1).

In recent years, immuno-oncology has become one of the leading therapeutic advanced therapies for cancer patients, not only due to successful transformation of the treatment landscape but also clear prediction of outcomes [5,32,40,52–59]. In fact, all we know is that modification of host immune systems has been demonstrated to improve outcomes in multiple solid tumors using ICIs as monotherapy and in scheduled combinations with chemotherapy and other targeted agents but has proven elusive thus far in ovarian cancer [52]. Therefore, strategy switching from ICIs to other potential candidates may be an alternative. The following will explore the recent development of oregovomab (MAb B43.13) acting as a front-line chemoimmunotherapy in patients with advanced-stage EOC with elevated CA125 (carbohydrate antigen 125 or cancer antigen 125).

Carbohydrate antigen 125 (cancer antigen 125, CA125)

Before evaluating the role of oregovomab (MAb B43.13) for treating EOC, the target should be explored. A recent article focusing on CA125 has been extensively reviewed by Dr. Gandhi or Dr. Giamougiannis [60,61]. CA125, also known as mucin 16 (MUC16) [62,63], a cell-surface, glycoprotein antigen normally expressed in tissues derived from coelomic epithelia, such as ovary, fallopian tube, peritoneum, pleura, pericardium, colon, kidney, and stomach was detected using the MAb OC 125 [64,65], followed by developing three Ab groups to identify the CA125, including OC 125-like Abs, M 11-like Abs, and OV197-like Abs [59,60,65]. The first attempt to evaluate the tumor marker of CA for diagnosing epithelial ovarian cancers occurred in 1983 by Dr. Bast [60,61,67,68]. CA125 has been extensively investigated as a serum biomarker in four separate clinical scenarios: (a) as part of screening algorithms for the early detection of ovarian cancer; (b) as part of diagnostic algorithms to distinguish between benign and malignant disease in women presenting with a pelvic mass; (c) to monitor response to treatment and (d) to detect recurrent disease [61]. Since this review is limited to discuss the role of anti-CA125 MAb in the management of women with EOC, we will focus on the association of CA125 and EOC. It is well-known that overexpression of CA125 has been associated with attenuated cellular apoptosis, platinum chemotherapy resistance, tumor proliferation and disease progression [61], suggesting that anti-CA125 may play a role in the management of patients with EOC.

Development of oregovomab (anti-CA125 MAb)

The key and active component of oregovomab is B43.13, which was first identified in 1990 and first clinical evaluation of a

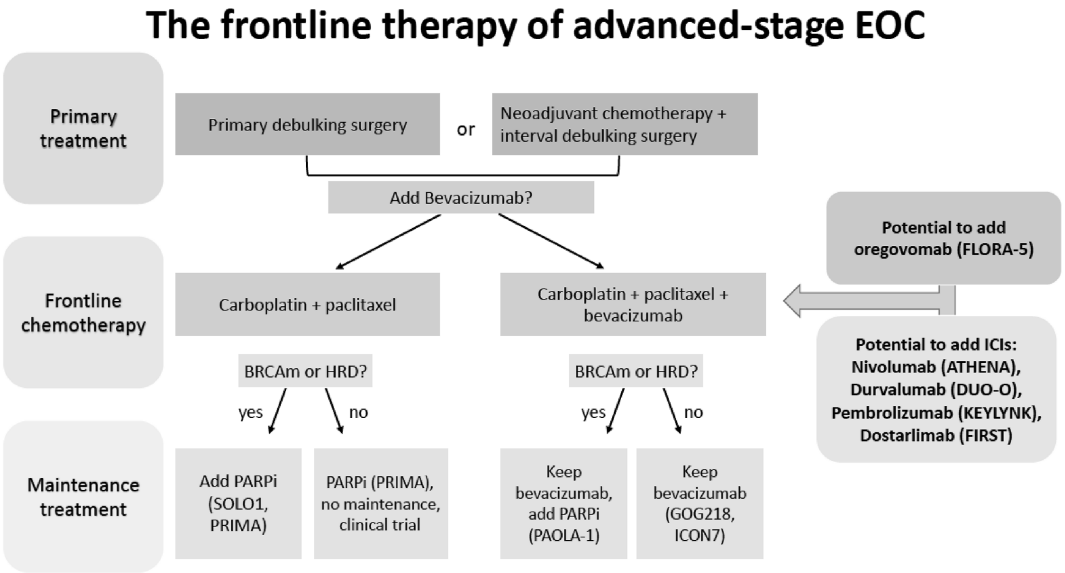

Fig. 1. Frontline therapy of advanced-stage epithelial ovarian cancer. BRCAm: breast cancer gene mutation; HRD: homologous recombination deficiency; PARPi: Poly (ADP-ribose) polymerase inhibitors; ICI: immune checkpoint inhibitors.

radioimmunoassay to monitor CA125 in assessing response to chemotherapy and determining those likely to benefit from chemotherapy (sero-diagnostic assay or sero-monitoring assay) [69,70]. After modification of murine MAb-B43.13, an IgG1k subclass immunoglobulin, oregovomab (OvaRex monoclonal antibody [MAb]-B43.13; Unither Pharmaceuticals, Wellesley Hills, MA) has been developed with a very high affinity (1.16×10^{10}/M) to CA125 [66]. The following unique mechanism makes oregovomab acting as an immunotherapeutic agent in the management of patients with EOC expressing CA125 [66].

The first clinical use of oregovomab in the management of patients with EOC was in 1993, and the authors utilized the immune response to the Ab (including human anti-mouse Ab [HAMA]) to investigate the therapeutic effects of oregovomab in the management of patients of EOC [71]. As expected, the tumor regression (better clinical courses) was closely correlated with stronger HAMA reaction in patients under repeated simulation of HAMA using MAb OC125 and B43.13 [71], suggesting the possible therapeutic role of oregovomab for ovarian cancer. The same group by Dr. Buam studied 32 patients with EOC and found a HAMA frequency of 34% (11/32: 3/7 after the first administration, 6/13 after the second, and 2/2 after the third), and this anti-idiotypic HAMA may trigger an antitumor effect either by suppressing the growth of CA125-expressing cancer cells directly, or by producing anti-anti-MAb B43.13 Abs (anti-anti-idiotype Abs), named as Ab3, which recognized the original MUC16 antigen, resulting in immune cell-mediated killing of MUC16-expressing tumor cells hoping that induction of anti-idiotypic HAMA will be beneficial for prolonged survival of patients with ovarian carcinoma [72]. Madiyalakan et al. studied 50 EOC patients who were treated with anti-CA125 murine MAb B43.13 (murine monoclonal anti-CA125 antibody B43.13 [Ovarex: Ab1]), and found 26 patients had elevated levels of anti-(mAb B43.13) Abs (Ab2), and eleven of these 26 patients also had high titer of Ab3 as well as 8 of the 22 patients had increased interferon-gamma (IFN-γ) levels [73]. Of the most importance, a tentative correlation was found between survival of these patients' anti-idiotype induction [73]. Additionally, Madiyalakan et al. further tested 100 EOC patients treated with Ovarex, and found those patients with prolonged survival after treatment had CA125-specific humoral and cellular response, and induced IFN-γ production [74]. Further in vitro studies indicated that the expression of major histocompatibility complex (MHC) I, MHC II (also called as HLA I and II, human leucocyte antigen I and II), and intercellular adhesion molecule (ICAM) I in EOC cells were upregulated in response to IFN-γ [74]. Such tumor cells were also found to be more sensitive to CA125-specific cytotoxic T cells compared to cells that were not incubated with IFN-γ [74]. Reinsberg and Krebs proposed that MAb B43.13 and MAb OC 125 may have different immune response after Ab infusion [75], suggesting that the immune response after MAb B43.13 may be more complicated. This above hypothesis was confirmed immediately, since the Ab-dependent cell-mediated cytotoxicity in EOC patients who received one to ten injection of a 2-mg dose of Ovarex can be successfully induced [76]. Seventy-five EOC patients who received one to ten injection of a 2-mg dose of oregovomab and 48 women developed Ab2; 18 of these patients also had elevated levels of Ab3 compared to pre-injection values, and additionally, these anti-CA125 Abs were able to conduct Fc-mediated tumor cell killing (Ab-dependent cell-mediated cytotoxicity) [76]. *In vivo* study using a human-PBL-SCID/BG mouse model demonstrated the successful engraftment of a human immune system in those mice, suggesting that OvaRex MAb-B43.13 treatment could (a) delay or prevent development of tumors; (b) reduce the size of small established tumors (SC tumor injection) or suppress ascites formation; (c) delay tumor growth when injected prior to tumor implantation; and (d) prolong the survival of the mice (i.p. tumor injection) [77]. Noujaim et al. found that the EOC patients generated anti-CA125 Abs that were directed against various epitopes on the Ag and were not restricted to the specific epitope recognized by MAb-B43.13, and the generation of CA125-specific B and T cell responses after MAb-B43.13 injection correlated with improved survival [78]. The influence of circulating CA125 for the induction of CA125-specific immune responses and the multi-epitopic nature of the human anti-CA125 Abs suggest that the majority of these Abs were not induced via the idiotypic

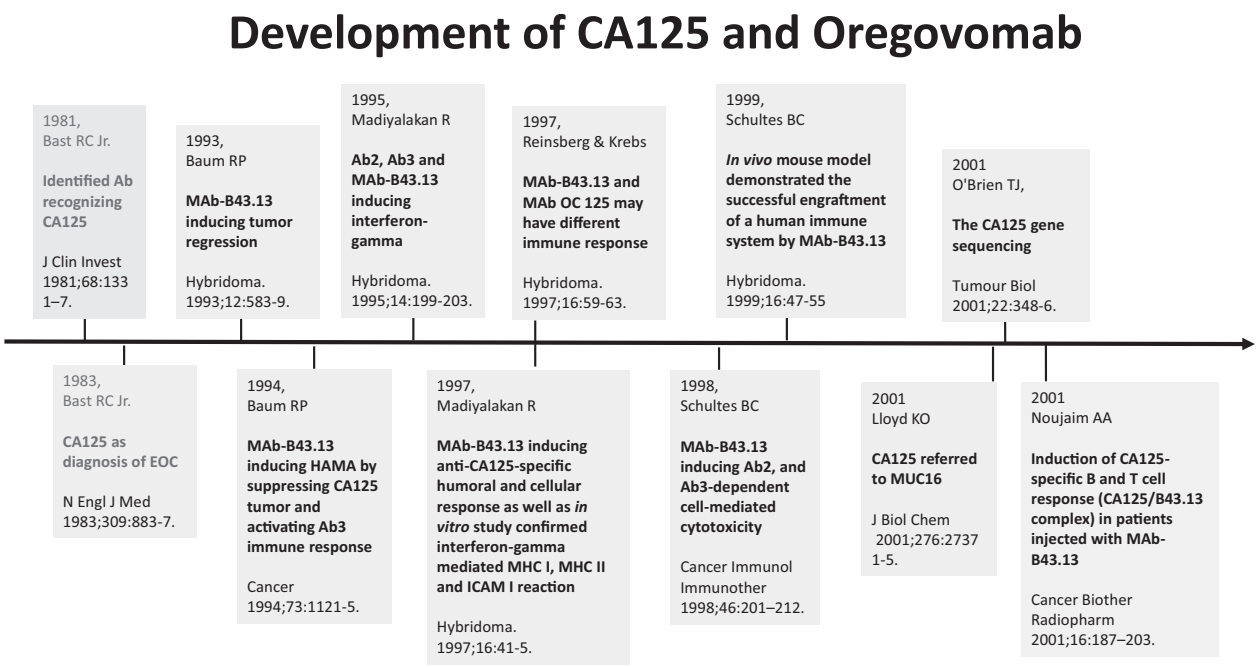

Fig. 2. Development of CA125 and Oregovomab. Ab: antibody; CA125: carbohydrate antigen 125 or cancer antigen 125; MAb: monoclonal antibody; HAMA: human anti-mouse antibody; OC: ovarian cancer; MHC: major histocompatibility complex; ICAM: intercellular adhesion molecule; MUC: mucin.

network but by the autologous antigen itself, hypothesizing that complex formation of MAb-B43.13 with circulating antigen triggers the induction of CA125-specific immune responses [66,78–81]. Gordon et al. tried to further evaluate immune responses and clinical outcomes for combined oregovomab and chemotherapy treatment of patients with advanced recurrent EOC and during the median follow-up of 15.8 months, oregovomab was well tolerated and induced multiple Ag-specific immune responses, maintained during concomitant chemotherapy [79]. Additionally, Gordon et al. found a significant survival benefit in patients mounting a T-cell response to CA125 and/or autologous tumor, and of the most important, this concomitant combination of oregovomab and chemotherapy did not produce any serious adverse events [79], confirming the safety profile of oregovomab.

Conclusion

The summary of the aforementioned hard works [71–81] is shown as Fig. 2, which clearly demonstrates the development of anti-CA125 MAb (OvaRex MAb-B43.13 or oregovomab). Viewing the oregovomab an effective agent in the management of patients with EOC can be mediated by (a) forming more efficient immune complexes with serum soluble CA125 and the CA125/B43.13 complex (Ab/Ag complex) binds to antigen-presenting cells (APCs), such as macrophages and dendritic cells; and (b) being cross-presented in the context of both HLA class I and HLA class II which triggers induction of CA125-specific immune responses, including anti-CD125 antibodies against various epitopes and CA125-specific B and T cell responses, but also CD4 and CD8 T-cell responses specific for B43.13 [66,80,81].

Taken together, there is good evidence to suggest that CA125 is a relevant target for antigen-mediated immunotherapy of ovarian cancer. Additionally, Berek's group announced the possibility that OvaRex exerts its therapeutic effects via stimulation of specific and non-specific immune response based on their observation in patients with EOC [80,81]. They found patients treated with OvaRex were 2.7 times less likely to die from EOC than were control patients managed with chemotherapy alone with a doubling in median survival time compared with the control group (59 vs 30 months, adjusted for FIGO stage, residual tumor, and response to chemotherapy), contributing to 40.7% of the 5-year OS rate in the OvaRex group compared with 11.4% in the control group [80,81]. Furthermore, survival was correlated with changes in three humoral immune parameters including nonspecific HAMA responses and Ab2 and anti-CA125 antibody development. Therefore, the following clinical trials were conducted to investigate the effectiveness of oregovomab in the management of women with advanced-stage EOC.

Declaration of competing interest

Dr. Peng-Hui Wang, an editorial board member at Taiwanese Journal of Obstetrics and Gynecology, had no role in the peer review process of or decision to publish this article. The other authors declare that they have no conflicts of interest related to the subject matter or materials discussed in this article.

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Original Article

Pregnancy outcomes following antenatal screening for intrahepatic cholestasis of pregnancy (ICP)

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ABSTRACT

Objective: To evaluate the maternal and perinatal outcomes following antenatal screening for ICP using a retrospective approach.
Materials and methods: A retrospective study was conducted at the second affiliated hospital of Chongqing Medical University, Chongqing, China, from 2012 to 2017. Pregnant women registered for antenatal in our hospital were screened for ICP. The pregnant women with detailed delivery record and presenting with the diagnosis of ICP based on TBA level ≥10 mmol/L and abnormal liver enzymes were included in the study.
Method: The pregnant women with detailed delivery records presenting with the diagnosis of ICP based on TBA level ≥10 mmol/L and abnormal liver enzymes were included in the study. 1410 pregnant women were enrolled in this study. We selected 940 pregnant women without the diagnosis of ICP as our control and 470 pregnant women diagnosed with ICP as our case study. Data collection and sampling in the control group was done using microsoft excel (version 16.61) random number generator.
Results: The mean age of the pregnant women and the gestational age at the time of diagnosis of ICP were 29.01 ± 4.3 years and 31.90 ± 8.83 weeks, respectively. It was found that a significant number of patients with ICP had a preterm birth and low birth weight (LBW), n = 151 (32.5%) $P < 0.001$ and n = 70 (14.9%) $P < 0.001$, respectively. A significant number of patients in the case group had a history of liver disease and gall bladder disease, $p < 0.001$ and $p = 0.005$, respectively, and a higher rate of GDM $p < 0.001$. Despite treatment, high TBA titer among ICP patients was associated with preterm delivery.
Conclusion: ICP in pregnancy leads to complications and poor perinatal outcomes. Fetal outcomes depend on the TBA levels; therefore, early diagnosis of ICP through routine screening followed by treatment is recommended in high-risk persons/areas.

Introduction

Intrahepatic cholestasis of pregnancy (ICP), also known as cholestasis gravidarum, is a common liver disorder associated with pregnancy, mainly in the late second and early third trimesters. However, sometimes it is found in the first trimester. The entity was first described as a "jaundice in pregnancy" by Ahlfeld in 1883 [1]. The prevalence of ICP varies based on ethnicity or geographical location. A total of 0.5–1% of the population around the globe is affected, 0.6% in South Australia and 5% in Araucanian-Indians [2,3]. The incidence rate can be as high as 6.06% in China, 9.2%–15.6% in South America [4], 1.5% in Scandinavian countries, and 0.1–0.2% in Europe [5].

The exact pathophysiology of ICP leading to the accumulation of bile acids and elevated levels of liver enzymes is not understood entirely. The knowledge about the disease has advanced over the years, especially for the risk factors associated with the disease, such as genetic disorders in ABCB4, ABCB11, fXR genes, mutations of the hepatobiliary transport system (the bile salt export protein (BSEP) or multidrug resistance protein 3 (MDR3)), reproductive hormones such as high circulating levels of estrogen during pregnancy, progesterone sulfated metabolites, anticardiolipin antibodies, hepatitis C infection, drugs, in addition to the seasonal and environmental factors [6,7].

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ICP is characterized by increased levels of serum bile acids and other liver enzymes or liver enzymes alone, along with pruritis. Sometimes, the patients are asymptomatic and have elevated total bile acids (TBA) levels above 10 mmol/L and abnormal liver enzymes. The maternal repercussions of ICP are limited, but it is very harmful to the fetus. Previous studies have found an association of ICP with increased fetal risk compared to normal pregnancy, which includes preterm birth, neonatal respiratory distress syndrome, meconium stained amniotic fluid (MSAF), neonatal intensive care unit admission, a low Apgar score in 5min, intrauterine growth restriction (IUGR), and stillbirth [2,8–11]. Increased serum bile acid levels above 40 μmol/L increase the risk of poor fetal outcomes [12]. On the other hand, pregnant women with ICP are at increased risk of gestational diabetes mellitus (GDM), preeclampsia, lipidemia, and cesarean section [9,13,14]. Maternal risk factors include increased maternal age above 35yrs, history of ICP in a previous pregnancy, family history of ICP, multiparity, multiple pregnancies, and pregnancy inadequate weight gain [4,15].

The Royal College of Obstetrics and Gynecology does not recommend early delivery of women with mild and moderate ICP nor routine screening in areas with a high incidence of ICP or high-risk patients [16]. On the other hand, the American College of Obstetricians and Gynecologists recommends active management of pregnant women with ICP but does not recommend routine screening for ICP in pregnancy in high-incidence areas and high-risk patients [17]. However, in China, the guideline recommends screening in areas with a high incidence of ICP and high-risk patients (such as people with chronic liver and gallbladder diseases, which include hepatitis C, gallbladder stones, history of oral contraceptives, family history of ICP or previous personal history of ICP, twin/multifetal pregnancy, and pregnant women with artificial insemination/IVF [29]. Due to controversy in the management protocol, we aimed to evaluate the maternal and perinatal outcomes in women screened for ICP using a retrospective approach.

Objectives

- To assess the clinical characteristics and outcome of asymptomatic and symptomatic presentation in ICP.
- To determine the pregnancy outcome of early (<28 weeks) and late (≥28 weeks) onset of ICP.
- To evaluate the relationship between TBA levels and pregnancy outcomes.

Materials and method

Ethical approval

The Second Affiliated Hospital of Chongqing Medical University approved the study and waived the need for informed consent due to the study's retrospective nature (ratification number: 162/2022).

Data collection

A retrospective case–control study was conducted at the Second Affiliated Hospital of Chongqing Medical University, Chongqing, China. We registered and screened 22,060 pregnant women for ICP in our hospital from 2012 to 2017. This study was carried out under the Declaration of Helsinki Ethical Principles for Medical Research involving human subjects protocol.

The diagnosis, management, and antenatal screening was based on the Chinese Medical Association of Obstetrics and Gynecology guideline (2011, 2015) [45,29]. The pregnant women were routinely screened for ICP in their first, second, and third trimesters to rule out ICP diagnosis by checking their liver function and TBA levels, as the region has a high incidence of ICP. In addition to this, they were also tested for routine urine analysis, thyroid function, renal function, routine blood analysis, and systolic and diastolic blood pressure.

Data collection and sampling in the control group was done using microsoft excel (version 16.61) random number generator. The inclusion criteria for the case group were the diagnosis of ICP based on TBA level ≥10 mmol/L and abnormal liver enzymes (alanine aminotransferase (ALT) > 40U/L and aspartate aminotransferase (AST) > 35U/L). A total of 865 pregnant women were diagnosed with ICP. Exclusion criteria included all pregnant women with ICP who did not have a complete antenatal and delivery history record in the hospital database. Based on this, 395 pregnant women were excluded from the study. In the control group, pregnant women without the diagnosis of ICP, n = 1300, were selected within the same population, and year as the case group from the hospital database, of which 360 pregnant women were excluded, the remaining 940 pregnant women in the control group were matched according to age, gravidity and parity at the ratio of 1:2 with the 470 ICP patients in the case group. The exclusion criteria for the control group were incomplete antenatal and delivery records in the hospital's electronic database. A total of 1410 pregnant women (control n = 940 and case n = 470) were included in the final analysis, as seen in Fig. 1.

The following data was collected from the hospital electronic database, which includes demographic and pregnancy characteristics such as maternal age, gravidity, parity, gestational age at delivery, gestational age at diagnosis, gestational age at itching or symptom onset, mode of delivery, past maternal history of ICP, invitro fertilization (IVF), abnormal liver enzymes (ALT >40U/L and AST >35U/L), TBA level, neonatal weight at birth, 1-, 5-, and 10-min APGAR scores. Maternal adverse outcomes include gestational diabetes mellitus (GDM), preeclampsia, pregnancy-induced hypertension (PIH), and postpartum hemorrhage (PPH). Neonatal outcomes include fetal distress, low birth weight (LBW) (birth weight of less than 2500 g) [18], small for gestational age (SGA), Meconium stained amniotic fluid (MSAF), preterm birth (gestational age less than 37 weeks), stillbirth/intrauterine fetal demise (IUFD) (defined as fetal death at or after 20 weeks of gestation), intrauterine growth restriction (IUGR), and neonatal asphyxia (Fig. 2).

In our hospital, the patient can decide the mode of delivery, even though we encourage vaginal delivery when there is no indication for a c-section. However, the doctor decides the delivery time based on the peculiarity of the patient's condition or health status.

The criteria for delivering an ICP pregnant woman in our hospital include if the patient presents with mild ICP (TBA≥10–39 mmol/L), delivery is considered at about 38–39 weeks. While in patients with severe ICP (TBA≥40), delivery is recommended at 34–37 weeks gestation based on treatment outcome, and maternal and fetal complications. For the mode of delivery, a patient with mild ICP can opt for labor induction and have a vaginal delivery if there is no indication for a cesarean section. While for a patient with severe ICP, fetal distress, and other contraindication for vaginal delivery, a cesarean section was performed.

Statistical analysis

Data analysis was performed using SPSS (26.0) Version. The results were expressed in descriptive statistics (percentages, frequencies, and statistics of location and dispersion) and inferential statistics (correlation analysis). For a relationship involving two categorical variables, the chi-square test was used. When assumptions of the chi-square test were not met, Fishers exact test was used. A p-value <0.05 was used to determine statistical

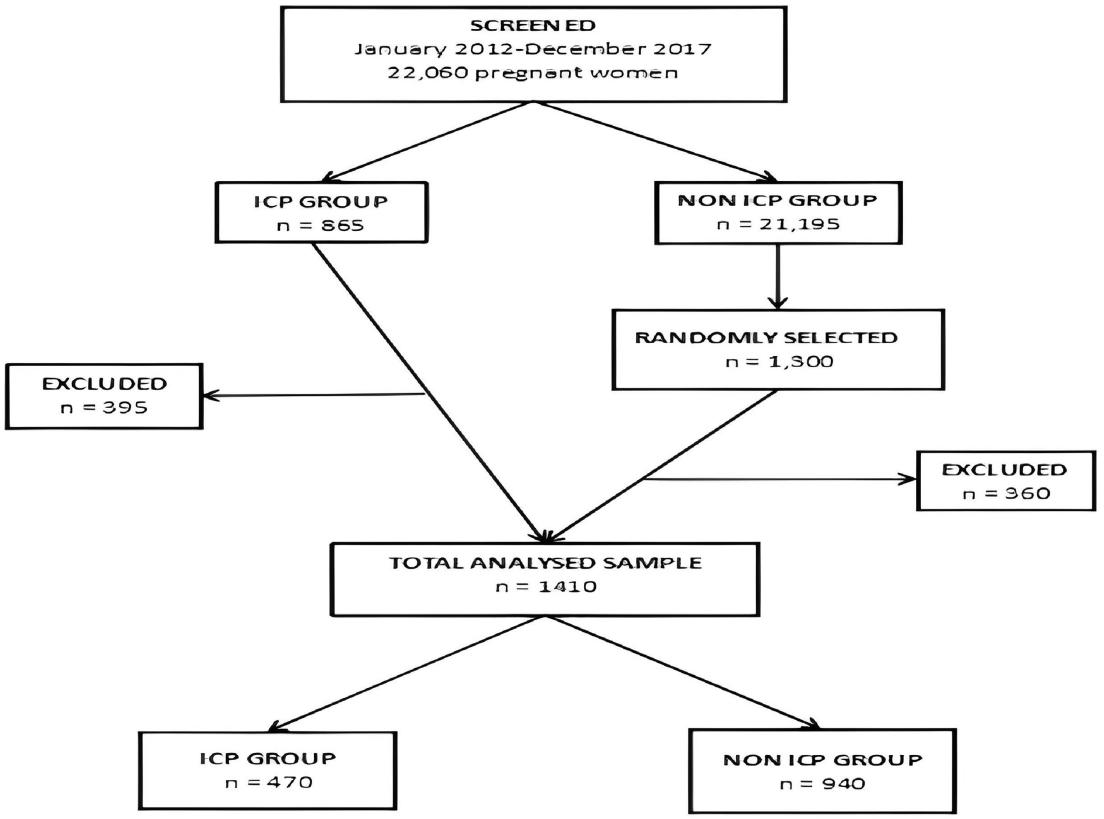

Fig. 1. A flowchart of enrolled and analyzed samples in this study.

significance. A power analysis depicted the sample size as 387 in the case group (ICP), which will provide a power of 95%. Since the sample size for the case and control group was above 387, we considered the power of this study to be more than 95%.

Results

Twenty-two thousand and sixty (22,060) pregnant women were registered for antenatal checkups and delivered at the university hospital during the study period. Among them, 470 pregnant women with ICP and 940 without ICP (n = 1410) were included in the case and control groups, respectively. The yearly incidence of ICP was 39 new cases per 1000 pregnant women.

The mean age of the patients was 29.01 ± 4.37 years, while the mean gestational age at delivery was 38.00 ± 3.10 weeks, indicating that most of the pregnancies were term delivery. The mean time of diagnosis was 31.90 ± 8.83 weeks, and the mean period for early-onset and late-onset ICP diagnosis were 18.07 ± 7.59 weeks and 34.02 ± 2.99 weeks, respectively. The cut-off for early-onset ICP was before 28 weeks, and late-onset ICP was after 28 weeks. The 1-min, 5-min, and 10-min APGAR scores were 9.69 ± 1.35, 9.86 ± 1.12, and 9.87 ± 1.02, respectively. The mean presentation time with itching was 31.97 ± 5.47 weeks, which could have contributed to the late-onset diagnosis in most patients. The mean TBA level was 18.05 ± 24.09 mg/dl. See Table 1 for the demographics and pregnancy attributes of the patients included in the study.

Most patients were symptomatic (57.6%), and (43.4%) had an absence of itching, with 10% having GDM. All patients (100%) presenting with ICP received treatment with UDCA. The incidences of other clinical outcomes, such as PIH, preeclampsia, and pregnancy-induced hypertension, ranged between 2% and 4%. Other feto-maternal outcomes such as IUGR, PPH, stillbirth/IUFD, thrombocytopenia, and premature rupture of membrane (PROM) were low (0%–6%), as presented in Table 2. However, LFTs were compromised in most patients, as evident in high aspartate aminotransferase (AST) > 35U/L and alanine transaminase (ALT) levels>40U/L, 43.4%, and 43.8%, respectively, indicating chronic liver disease. Most of the patients (60.4%) underwent cesarean delivery. In this study, we did not consider the high incidence of cesarean section as a potential outcome of ICP complications because some women who do not meet the criteria for cesarean section opted for elective C-sections. Perinatal outcomes were more or less uncomplicated because low APGAR scores at 1, 5, and 10 min were observed in less than 6% of the babies (a score lower than 7 signifies that the baby needs medical attention). A substantial number of newborns (208; 20.1%) were born prematurely, and 9.1% of the babies were LBW.

We examined the relationships between health history, demographics, and reproductive health factors in the case (n = 470) and control group (n = 940). History of liver disease (49 vs. 5, $p < 0.001$, OR 21.76, CI 8.61–55.02) and gall bladder disease (13 vs. 2, $p = 0.005$, OR 55.50, 95%CI 3.29–935.8) were significantly more in the cases compared to the controls. The odds ratio supported the findings, which showed that a history of liver disease and gallbladder disease carried the highest risks for ICP. The significant increase in the incidences of multifetal pregnancies ($p < 0.001$, OR 3.94, 95%CI 2.12–7.12) in the case group compared to the control group implies that the odds of having ICP in twin pregnancy is 3.94 times higher than the control. There was no significant difference in the number of participants between cases and control for both age groups (<35 and ≥ 35) (P = 0.95 for both). This is summarized in Table 3.

Since the p-values for most maternal and neonatal parameters (PIH, GDM, thrombocytopenia, preterm delivery, neonatal asphyxia, APGAR scores) between case and controls were less than

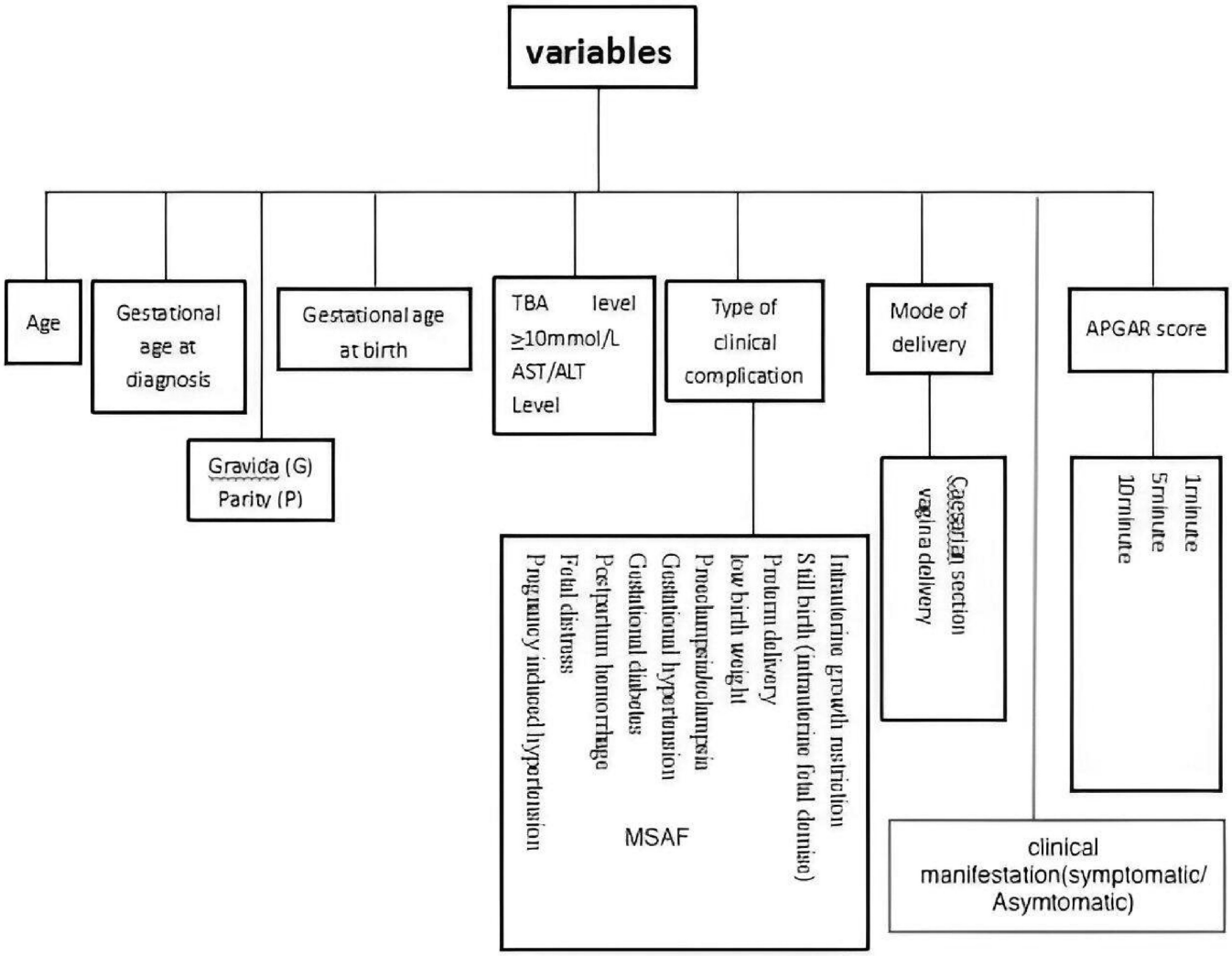

Fig. 2. Parameters needed for the study.

0.05, it was concluded that ICP deteriorates pregnancy outcomes. ICP significantly increases the risk of preterm delivery ($p < 0.001$) compared to controls despite treatment. Preterm delivery (32.5% vs. 10%; $p < 0.001$) and LBW (15% vs. 6.1%; <0.001) were significantly higher in the case group as compared to the control groups, as shown in Table 4. Babies having lower APGAR scores at 1-, 5-, and 10-min were significantly more in the case group (5.5%, 3.2%, 3% vs. 1.3%, 0.5%, and 0.2%) respectively; $p < 0.001$). The calculation of the odds ratios for the risk of poor maternal and neonatal outcomes between ICP and controls, even after treatment of the former, were PIH, GDM, thrombocytopenia, Low 10-Minute APGAR score, and preterm delivery, OR 3.24, 2.68, 11.72, 14.4, and 7.33, respectively. These findings further emphasize the need for appropriate perinatal care and treatment of hypertension in mothers awaiting labor.

Table 1
Demographic and pregnancy characteristics (n = 1410, ICP = 470 and controls = 940) M (sd).

Variable	Case (n = 470)	Control (n = 940)	Combined (n = 1410)
Age	28.90 (4.63)	29.07 (4.24)	29.01 (4.37)
Gravidity	2.33 (1.45)	2.24 (1.41)	2.27 (1.42)
Parity	1.21 (0.57)	1.26 (0.48)	1.24 (0.51)
Gestational weeks at birth	37.12 (3.14)	38.72 (2.87)	38.00 (3.10)
Weight gain in pregnancy (kg)	9.58 (7.05)	13.90 (5.01)	12.46 (6.12)
Baby weight at birth (g)	2953.8 (576.0)	3226.4 (484.5)	3135.4 (532.4)
APGAR score (1 min)	9.41 (1.97)	9.83 (0.86)	9.69 (1.35)
APGAR score (5 min)	9.68 (1.70)	9.94 (0.65)	9.86 (1.12)
APGAR score (10 min)	9.70 (1.61)	9.96 (0.50)	9.87 (1.02)
Time of diagnosis	31.90 (8.83)		
Diagnosis before 28 weeks	18.07 (7.59)		
Diagnosis after 28 weeks	34.02 (2.99)		
Time of itching onset (weeks)	31.97 (5.47)		
Total bile acids (TBA)	18.05 (24.09)		

No significant difference was observed between the percentages of preterm birth in early and late-onset ICP (30.9% versus 32.4%, $p = 0.61$) when the treatment is received early (see Table 5). However, the analysis showed that the preterm birth percentage is marginally higher in late-onset ICP (32.4%). The percentages of preeclampsia, PIH, GDM, PPH, SGA, IUGR, neonatal mortality, and stillbirth/IUFD did not significantly differ between early and late-onset ICP. However, fetal distress is more significant in early-onset ICP cases than in counterparts (6.2% vs. 0.5%; $p = 0.03$).

There was no significant difference in maternal and fetal parameters such as MSAF, PIH, DIC, PPH, gravidity, parity, stillbirth, preterm birth, LBW, and the APGAR scores between symptomatic

Table 2
Detailed clinical outcomes in patients n (%).

Variable	Case (n = 470)	Control (n = 940)	Combined (n = 1410)
Vaginal delivery	43 (9.1)	513 (54.6)	556 (39.5)
Cesarean section	427 (90.9)	424 (45.2)	851 (60.4)
Low APGAR Score (1 min)	26 (5.5)	12 (1.3)	38 (2.7)
Low APGAR Score (5 min)	15 (3.2)	5 (0.5)	20 (1.4)
Low APGAR Score (10 min)	14 (3.0)	2 (0.2)	16 (1.1)
Twin birth	30 (6.4)	16 (1.7)	46 (3.3)
Preterm birth	151 (32.5)	57 (10.0)	208 (20.1)
Low birth weight	70 (15.0)	57 (6.1)	127 (9.1)
Small for gestational age (SGA)	1 (0.2)	2 (0.2)	3 (0.2)
Gestational diabetes (GDM)	77 (16.4)	64 (6.8)	141 (10.0)
Preeclampsia	10 (2.1)	12 (1.3)	22 (1.6)
Pregnancy-induced hypertension (PIH)	13 (2.8)	9 (1.0)	22 (1.6)
Fetal growth restriction (FGR)	9 (1.9)	4 (0.4)	13 (0.9)
Postpartum hemorrhage (PPH)	8 (1.7)	5 (0.5)	13 (1.0)
Stillbirth or intrauterine death	2 (0.4)	0 (0)	2 (0.1)
Thrombocytopenia	17 (3.6)	3 (0.3)	20 (1.4)
Premature rupture of membrane (PROM)	35 (7.5)	49 (5.2)	84 (6.0)
In vitro fertilization (IVF)	8 (1.7)	8 (0.9)	16 (1.1)
History of liver disease	49 (10.5)	5 (0.5)	54 (3.8)
History of gallbladder disease	13 (2.8)	2 (0.21)	15 (1.1)
History of ICP	4 (0.9)	0 (0)	4 (0.3)
Fetal distress	7 (1.5)	4 (0.4)	11 (0.8)
Neonatal asphyxia	6 (1.3)	1 (0.1)	7 (0.5)
Meconium staining of amniotic fluid (MSAF)	4 (0.9)	0 (0)	4 (0.2)
Asymptomatic disease	204 (43.4)		
Symptomatic disease	266 (57.6)		
Time of Diagnosis	31.90 (8.83)		
Early onset ICP diagnosis (<28 weeks)	81 (17.2)		
Late onset ICP diagnosis (≥28 weeks)	389 (82.8)		
Elevated alanine transaminase (ALT) levels	206 (43.8)		
Elevated aspartate aminotransferase (AST)	204 (43.4)		
Abnormal cholic acid levels	134 (28.5)		

Table 3
Relationships between health history, demographics, and reproductive health factors and ICP (n = 940).

Variable	Case(470)	Control (940)	P-value	OR [95%CI]
History of liver disease, n (%)	49 (10.5)	5 (0.5)	<0.001	21.76 [8.61–55.02]
History of gallbladder disease, n (%)	13 (2.8)	2 (0.2)	0.005	55.50 [3.29–935.8]
History of ICP, n (%)	4 (0.9)	0 (0)	0.052	18.14 [0.97–337.7]
Age, n (row %)				
<35 years	418 (88.9)	835 (88.8)	0.95	1.01 [0.71–1.43]
≥35 years	52 (11.1)	105 (11.2)	0.95	0.99 [0.70–1.41]
Twin or multiple pregnancy, n (%)	30 (6.4)	16 (1.7)	<0.001	3.94 [2.12–7.30]

and asymptomatic patients (see Table 6). When the relationships between pregnancy outcomes and TBA levels among those with ICP (n = 470) were analyzed, the findings suggested that the mean TBA levels were significantly higher for mothers with preterm delivery (see Table 7).

Discussion

This retrospective study assessed pregnant women's maternal and perinatal outcomes with ICP. It was found that ICP affects about 0.1–2% of pregnant women and is characterized by increased

Table 4
Relationships between pregnancy outcomes and ICP diagnosis (n = 940).

	Case (n = 470) n (%)	Control (n = 940) n (%)	P-value	OR [95% CI]
Preeclampsia	10 (2.1)	12 (1.3)	0.229	1.68 [0.72–3.92]
PIH	13 (2.8)	9 (1.0)	0.014	2.94 [1.24–6.93]
GDM	77 (16.4)	64 (6.8)	<0.001	2.68 [1.89–3.81]
Preterm delivery	151 (32.5)	57 (10.0)	<0.001	7.33 [5.27–10.20]
PPH	8 (1.7)	5 (0.5)	0.040	3.24 [1.05–9.95]
SGA	1 (0.2)	2 (0.2)	0.999	1.00 [0.09–11.10]
Fetal growth restriction	9 (1.9)	4 (0.4)	0.012	4.57 [1.40–14.91]
Stillbirth	2 (0.4)	0 (0)	0.137	10.04 [0.48–209.5]
APGAR <7 (1 min)	26 (5.5)	12 (1.3)	<0.001	4.52 [2.26–9.06]
APGAR <7 (5 min)	15 (3.2)	5 (0.5)	<0.001	6.16 [2.23–17.07]
APGAR<7 (10 min)	14 (3.0)	2 (0.2)	<0.001	14.40 [3.26–63.63]
Low birth weight	70 (15.0)	57 (6.1)	<0.001	2.71 [1.87–3.92]
Fetal distress	7 (1.5)	4 (0.4)	0.045	3.54 [1.03–12.15]
Neonatal asphyxia	6 (1.3)	1 (0.1)	0.021	12.14 [1.46–101.2]
MSAF	4 (0.9)	0 (0)	0.052	18.14 [0.97–337.7]
Thrombocytopenia	17 (3.6)	3 (0.3)	<0.001	11.72 [3.42–40.20]
Cesarean Section	427 (90.9)	424 (45.2)	<0.001	12.08 [8.61–16.96]

Table 5
Relationships between pregnancy outcomes and time of diagnosis for participants with ICP (n = 470) n (%).

Variable	Diagnosed <28 weeks (n = 81) n (column %)	Diagnosed ≥28 weeks (n = 389) n (column %)	P-Value
Preeclampsia	0 (0)	10 (2.6)	0.22[a]
PIH	3 (3.7)	10 (2.6)	0.71[a]
Gestational Diabetes	17 (20.98)	60 (15.4)	0.61
Preterm delivery	25 (30.9)	126 (32.4)	0.79
Postpartum hemorrhage	0 (0)	8 (2.1)	0.12[a]
Small for gestational age	0 (0)	1 (0.26)	1.00[a]
Fetal growth restriction	2 (2.1)	7 (1.8)	0.6[a]
Stillbirth/IUFD	1 (1.23)	1 (0.26)	1.00[a]
Low APGAR (1 min)	9 (11.1)	17 (4.4)	0.07[a]
Low APGAR (5 min)	5 (6.2)	10 (2.6)	0.71[a]
Low APGAR (10 min)	4 (4.9)	10 (2.6)	0.70[a]
Fetal distress	5 (6.2)	2 (0.5)	0.03[a]
Neonatal asphyxia	2 (2.5)	4 (1.0)	0.09[a]

[a] Fisher's Exact test was used. n = number IUFD=Intrauterine fetal demise APGAR score<7 PIH=Pregnancy induced hypertension.

Table 6
Pregnancy outcomes for symptomatic and asymptomatic patients.

Pregnancy Outcomes	Symptomatic (n = 266)	Asymptomatic (n = 204)	p-value
MSAF n (%)	2 (0.75)	2 (0.98)	>0.05
Fetal distress n (%)	4 (1.5)	3 (1.5)	0.09
Postpartum Hemorrhage n (%)	2 (0.75)	6 (2.94)	0.08
DIC n (%)	1 (0.38)	0 (0)	>0.05
Birth weight (M, SD)	2967.25 ± 577.75	2971 ± 687.47	0.55
Gravidity (M, SD)	2.37 ± 1.48	2.22 ± 1.39	>0.05
Parity (M, SD)	1.21 ± 0.55	1.19 ± 0.47	>0.05
Stillbirth n (%)	1 (0.38)	1 (0.49)	>0.05
PIH n (%)	8 (3)	5 (2.5)	>0.05
Preterm birth (M, SD)	77 (34.24)	49 (29.7)	0.27
Low birth weight n (%)	42 (15.78)	28 (13.7)	>0.05
Weeks of diagnosis (M, SD)	33.94 ± 5.75	34.10 ± 6.67	>0.05
APGAR 1 (M, SD)	9.78 ± 1.78	9.7 ± 2.22	0.19
APGAR 5 (M, SD)	9.92 ± 1.69	9.9 ± 1.76	>0.05
APGAR 10 (M, SD)	9.94 ± 1.56	9.98 ± 1.72	>0.05

M = Mean; SD= Standard deviation; MSAF = meconium stained amniotic fluid; APGAR score(1,5 and 10 min) = mean APGAR score at birth; DIC = Disseminated intravascular coagulation.

Table 7
Relationships between pregnancy outcomes and TBA levels among those with ICP (n = 470).

Variable	Mean TBA Level (SD)	P-value
Preeclampsia		0.26
Preeclampsia (n = 10)	22.79 (24.13)	
No preeclampsia (n = 460)	17.09 (24.32)	
Pregnancy-induced hypertension (PIH)		0.66
PIH (n = 13)	18.49 (19.31)	
No PIH (n = 457)	18.08 (23.85)	
Gestational Diabetes (GDM)		0.85
GDM (n = 77)	20.22 (27.58)	
No GDM (n = 393)	17.76 (23.53)	
Preterm delivery		0.02
Preterm delivery (n = 151)	23.06 (30.92)	
No preterm delivery (n = 319)	14.84 (18.32)	
Postpartum hemorrhage (PPH)		0.70
PPH (n = 9)	16.81 (25.22)	
No PPH (n = 461)	18.20 (24.25)	
Small for gestational age (SGA)		0.24
SGA (n = 1)	5.50 (0)	
No SGA (n = 469)	18.20 (24.26)	
Intrauterine growth restriction (IUGR)		0.37
IUGR (n = 9)	8.47 (2.66)	
No IUGR (n = 461)	18.37 (24.4)	
Stillbirth or intrauterine fetal demise (IUFD)		0.14
Stillbirth or IUFD (n = 2)	5.55 (1.06)	
No stillbirth/IUFD (n = 468)	18.23 (24.28)	
Low APGAR Score (1 min)		0.32
Low APGAR Score (n = 25)	18.70 (23.63)	
No low APGAR Score (n = 445)	18.54 (30.1)	
Low APGAR Score (5 min)		0.23
Low APGAR Score (n = 16)	22.38 (39.34)	
No low APGAR Score (n = 454)	18.32 (23.6)	
Low APGAR Score (10 min)		0.49
Low APGAR Score (n = 14)	23.57 (40.59)	
No low APGAR score (n = 456)	18 (23.59)	
Low birth weight		0.15
low birth weight (n = 70)	24.57 (38.16)	
No low birth weight (n = 400)	16.73 (19.62)	
Fetal distress		>0.05
Fetal distress (n = 7)	13.29 (10.71)	
No fetal distress (n = 463)	18.01 (25.33)	
Neonatal asphyxia		0.7
Neonatal asphyxia (n = 6)	11.15 (5.67)	
Neonatal asphyxia (n = 464)	16.26 (24.38)	

n = sample M = mean SD = standard deviation.

serum bile acids and gestational pruritis [6]. ICP was also included in the list of medical disorders that can cause stillbirth in pregnancies by the 2007 stillbirth workshop [19]. In this five-year study, out of 22,060 pregnant women who registered in our hospital for antenatal checkups and delivery, only 865 (3.9%) were diagnosed with ICP, which is slightly lower compared to another domestic study by Gao X.X [4], whereas, in comparison to other studies conducted in Nepal (1.1%) [20] and Sweden (0.5%) [10,21], the incidence of ICP as found in our study was much higher. Regarding the optimal delivery time of such cases, the mean gestation in our study was 37.12 ± 3.14 weeks, indicating that most of the pregnancies were term delivery. Past studies calculated the composite mortality risk using data registries for 5545 California women with ICP and their matched controls; hence, they suggested that the optimal delivery time should be 36 weeks [22,23]. Our study's cut-off for early and late-onset ICP was before and after 28 weeks, respectively, whereas Jing L et al. used the 34-week mark as a cut-off [6]. There is no consensus regarding a cut-off for the diagnosis of early or late ICP, as some researchers consider the cut-off to be at 28th week of gestation [24,25]. Like our study, others used 32 or 34 weeks as their cut-off. Most babies in the case group had significantly lower APGAR scores at 1-, 5-, and 10-min, which means that children born to ICP mothers (despite receiving treatment or being diagnosed early) would require special perinatal care. We found that the incidence of ICP was insignificant regardless of maternal age. These findings indicate that ICP is associated with poor outcomes in gestational health, which could have implications for the mother and fetus.

This retrospective study indicated that a history of liver and gall bladder disease increases the probability of ICP, and it is consistent with a recent meta-analysis suggesting that HBV infection causing liver damage is a high-risk factor for ICP [26]. Another meta-analysis demonstrated a higher risk of ICP among pregnant women with HCV infection than non-HCV-infected women [27]. Pruritus and liver dysfunction symptoms are reversible after delivery, and the maternal prognosis is good [6]. Pruritis usually subsides within 24–48 h after delivery, but the liver enzymes and TBA levels may take up to 2–3 weeks postpartum to normalize [28,29].

Our study shows a significant relationship between ICP and maternal and neonatal outcomes (PIH, GDM, preterm delivery, and low APGAR scores), similar to previous studies [30,38,39], indicating perinatal complications in 33% of births [30]. Although preeclampsia was insignificant in our study; however, the analysis showed that the percentage of pregnant women with preeclampsia was marginally more in the case group compared to the control group, 2.1% vs. 1.3%, respectively. In China, the prevalence rate of pre-eclampsia and GDM in the general population is about 2.6% and 14.6%, respectively [40,41]. In a recent study in China, the prevalence rate of GDM and preeclampsia in ICP pregnancy were about 17.1% and 5.5%, respectively, compared to the control 12.4% and 2.4%, respectively [42]. However, our study showed the prevalence rate of GDM and preeclampsia in ICP to be 16.4% and 2.1%, compared to the control group, at 6.8% and 1.3%, respectively. A study in Sweden reported an increased risk of preterm delivery, GDM, and pre-eclampsia but not of stillbirth associated with ICP [31], while an Australian study reported favorable outcomes with ICP [32].

Although the incidence of feto-maternal outcomes such as FGR, MSAF, PPH, PROM, neonatal asphyxia, and fetal distress were very low, we could find a significant difference in maternal and fetal parameters, indicating that ICP does influence most feto-maternal outcomes despite proper treatment received. As a result, special postpartum care might suffice if diagnosed with ICP. In our study, the risk of stillbirth in the case group was insignificant 2 (0.4%) $P < 0.137$. These could also be due to the effectiveness of the treatment options that mitigated adverse outcomes of ICP, contrary to other studies that showed the risk of fetal complications such as MSAF, fetal distress, and IUFD increase in pregnancies affected by ICP. Another study reported meconium staining and IUFD among 0.4% and 24% of ICP cases [33].

A recent survey on ICP reported that 1.8% of patients were diagnosed without itching, and 5.6% of pregnancies with ICP were diagnosed in the first trimester [42]. Although the results of symptomatic and asymptomatic ICP were insignificant in our study, the analysis showed that preterm birth and low birth weight in symptomatic ICP mothers was marginally higher than those who presented without symptoms. A recent study conducted in Chongqing, a municipality with a high prevalence of ICP in China, compared the clinical features and fetal outcome of asymptomatic hypercholanemia of pregnancy (AHP), normal pregnancy, and classic ICP. The result indicated that patients with asymptomatic hypercholanemia of pregnancy had adverse pregnancy outcomes such as fetal distress, MSAF, APGAR score $\leq$7, and NICU admissions compared to normal pregnancy. However, MSAF and NICU admission risks were higher in classic ICP patients than in AHP patients [43]. A similar study on ICP reported no significant difference in pregnancy outcomes between asymptomatic and symptomatic patients [44].

Current pharmacologic treatment includes the use of ursodeoxycholic acid (UDCA) (orally 3–4 times at a dose of 15 mg/kg per day) [29], which has been shown to have greater efficacy than other treatment modalities along with improving the TBA and serum transaminase levels. Although there was treatment with UDCA, PIH, preterm delivery, and LBW were significant in pregnancies complicated by ICP in the present study. Similarly, Rook et al. [30] and Glantz et al. [33] found no clinically significant effect of UDCA on fetal complications, with the drug improving the serum biochemical markers of ICP.

We found that high TBA titer in ICP patients would predispose the risk of preterm delivery and associated complications, which is in line with a Swedish cohort study showing that serum BA of 40 μmol/L or more was three times more likely to be associated with several complications such as spontaneous preterm labor, MASF, and fetal asphyxia [33]. The guidelines for ICP state that "higher TBA levels might cause severe contraction of placental villus vessels, resulting in acute hypoxia and sudden fetal death" [29]. A higher incidence of TBA level was also found in twin pregnancies (2.1%) and pregnancies conceived by IVF (2.7%) [34]. Jing L et al. found that early-onset ICP would lead to higher serum bile acid concentrations and a more severe course of ICP [6]. A recent meta-analysis of 23 studies found that high serum BA levels (>100 μmol/L) were significantly affected by the increased risk of IUFD [35]. Although there is regular screening for liver function and serum TBA level for the early diagnosis of ICP in the referred population, the prevalence of ICP is still high in such a population, possibly due to the asymptomatic nature of the disease in some patients and late-onset of the disease.

In our study, most patients exhibited late-onset ICP because the mean time of diagnosis was more than 28 weeks. The percentage of preterm birth is marginally more in late-onset ICP, indicating that the treatment in pregnant women could prevent the risk of preterm delivery despite late onset, whereas Jing L et al. found that the preterm birth rate of early-onset ICP patients was 49.73%, which was significantly higher, $P < 0.001$, than that of late-onset ICP patients (20.28%) which is in contrast with our findings [6]. A substantial number of patients (32.5%%) in our study had a preterm birth, which could be due to complications or delayed diagnosis of ICP, and it is in line with the findings that 82.8% of the patients were diagnosed after 28 weeks. Glantz et al. concluded that pregnancies in women with TBA>40 μmol/L increased the fetal risk of preterm delivery [33]. Other studies in Turkey and China also reported an increased preterm birth rate among women with ICP [24,36]. The exact mechanism by which ICP leads to a higher incidence of premature delivery remains unclear. Some researchers suggest that the high concentration of TBA level may affect fetal hormone metabolism, which can cause changes in cell membrane permeability, leading to increased uterine fiber reactivity and stimulation of the prostaglandin release pathway, which altogether induces spontaneous premature delivery [37]. However, a high TBA level may influence myocardial contraction, which is suggested by a study conducted in rodents and humans that shows the myometrial cells of women with ICP respond efficiently to oxytocin compared to control and maybe a possible reason for the high rate of preterm delivery in women with ICP [9,37].

Our study also suggests that ICP could increase the risk of LBW. Similarly, the early-onset ICP group neonates had significant LBW in a study by Jing L et al. [6]. We found that the diagnosis of late-onset ICP was insignificantly related to the Apgar scores, showing that the treatment eliminates the difference in Apgar scores. Such findings could be attributed to the lesser complications for late-onset ICP, especially if they are close to term delivery. It could also be possible that treatment could lower the complications of ICP even if the diagnosis is late.

Limitation

Our study has a high study power of more than 95%, a large sample size for both case and control groups, and the use of an accurate statistical analytic tool. Nevertheless, our study had a few limitations. As a population-level retrospective analysis, our study can only provide evidence of an association, not causation. Due to the retrospective nature of our study, we could not ascertain if patients who were asymptomatic at the time of screening and diagnosis later presented with any symptoms related to classic ICP cases. This study included only the Chinese population; hence, it does not represent other ethnic populations. With the use of a single study center, a potential selection/sample bias could occur.

Conclusion

We found that the case group had a significant history of liver disease and a higher rate of GDM and preterm deliveries than the control group. Fetal outcomes depend on the TBA levels; therefore, early diagnosis of ICP through routine screening followed by treatment is recommended in ICP high-risk persons/areas. These findings could be considered while planning future diagnostic or therapeutic interventions for ICP patients, especially for pregnant women in healthcare settings where pregnancy management is poor.

Ethical approval

The medical and ethics committee of The Second Affiliated Hospital of Chongqing Medical University approved this study.

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No funding was received for conducting this study.

Conflict of interest

The authors have no competing interests relevant to this article to declare.

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Original Article

Association of serum progesterone levels on the transfer day with pregnancy outcomes in hormone replacement frozen-thawed cycles with oral dydrogesterone for strengthened luteal phase support

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ABSTRACT

Objective: To investigate the relationship between serum progesterone (P) levels on the day of blastocyst transfer and pregnancy outcomes in frozen-thawed embryo transfer (FET) cycles using hormone replacement therapy (HRT) with oral dydrogesterone for strengthened luteal phase support (LPS).

Materials and methods: This was a retrospective study including 1176 FET cycles. All patients received 40 mg of intramuscular (IM) P daily for endometrium transformation plus oral dydrogesterone 10 mg BID from transfer day for strengthened LPS. Pregnancy outcomes were compared between serum P levels on the transfer day $\geq$10 ng/ml and <10 ng/ml. Furthermore, cycles were divided into 10 groups by deciles of P and ongoing pregnancy rate (OPR) was calculated in each group. Analyses using deciles of serum P were completed to see if these could create further prognostic power.

Results: No differences were observed in clinical pregnancy rates (CPRs), OPRs and live birth rates (LBRs) between serum P levels $\geq$10 ng/ml and <10 ng/ml. Patients with serum P levels <5.65 ng/ml (10th percentile) had a significantly lower OPR (48.31% vs. 58.98%, $p = 0.03$) and LBR (43.22% vs. 57.75%, $p = 0.003$) than the rest of the patients. Multivariate logistic regression analysis showed serum P levels on the transfer day were not associated with pregnancy outcomes.

Conclusion: Measuring serum P levels on the day of HRT-FET is of clinical importance. Lower serum P levels impact the success of HRT-FET cycles, suggesting that there may be a threshold below which it is difficult to improve pregnancy outcomes via oral dydrogesterone to strengthen LPS.

Introduction

Over the last decade, the application of frozen-thawed embryo transfer (FET) procedure has increased dramatically worldwide. The main reasons are the increased use of selective single embryo transfer, "freeze-all" policies for avoiding the risk of ovarian hyperstimulation syndrome, and negative effects of supraphysiologic estradiol levels and premature progesterone (P) elevation on embryo implantation in fresh embryo transfer cycle [1]. At present, there is no consensual recommendation for endometrial preparation protocol [2]. Hormone replacement therapy (HRT) is a common endometrial preparation regimen because it makes it possible to schedule the day of embryo transfer and reducing monitoring requirements. However, there is still room for improvement in the administration route of estradiol and P, the ideal dose, duration and schedule. With the wide application of HRT-FET cycles, the association of serum P levels around the day of embryo transfer with pregnancy outcomes is sparking a hot debate because defining an optimal level may allow the individualization of FET in HRT [3–6]. Currently, the optimal timing for serum P measurement and further adaptive management for patients with low serum P remain to be determined [7]. And most existing data were based on vaginal P administration. Data regarding the optimal range of serum P values on the day of embryo transfer in cycles using intramuscular (IM) administration of P for endometrium transformation are limited and conflicting [8].

Therefore, in order to evaluate the effectiveness of P supplementation from the day of frozen blastocyst transfer, the association of serum P levels on the transfer day with pregnancy outcomes was analyzed in these HRT-FET cycles using IM administration of P for endometrium transformation with the addition of oral dydrogesterone for strengthened LPS.

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Materials and Methods

Patients

Data of 1176 frozen-thawed blastocyst transfer cycles with HRT for endometrial preparation at the Reproductive Medicine Center of the 901th Hospital from January 2017 to December 2020 were analyzed in this retrospective study. Inclusion criteria were: 1) women age less than 38 years old, 2) single blastocyst transfer, 3) blastocoele re-expansion before transfer > 30%, 4) endometrial thickness ≥7 mm. Exclusion criteria were: 1) having a history of recurrent abortion or repeated implant failure, 2) diagnosed with conditions including uterus duplex, uterus unicornis, uterus septus, intrauterine adhesions, adenomyosis or other uterine pathological factors. This study was approved by the Institutional Review Board of the 901th Hospital (approval number: 901YY-2021-07).

Endometrial preparation for FET

All patients underwent endometrial preparation with HRT. Briefly, each woman was administered oral Estradiol valerate tablets (Progynova; Bayer Schering Guangzhou, China) 4–8 mg per day from day 3 of menstrual cycle. And the dose was modified according to the endometrial thickness and morphology. After 12–14 days, transvaginal ultrasonography was performed to measure endometrial thickness. When endometrial thickness reached ≥7 mm and serum P < 1.0 ng/ml, IM administration of 40 mg P (Progesterone Injection, Jinyao Tianjin, China) daily was used to initiate secretory transformation, and original dosage of oral estradiol supplementation was continued. Blastocyst transfer was performed on the 6th day of IM P administration. Meanwhile, oral dydrogesterone (Dydrogesterone Tablets, Abbott Biologicals B.V., Netherlands) 10 mg BID was added to all patients.

Blastocyst vitrification/warming and transfer

The procedure of blastocyst vitrification/warming was described in our previous study [9]. Post-warming blastocyst was cultured for 1–2 h to assess the blastocyst survival and blastocoele re-expansion, then transferred into the uterine cavity under abdominal ultrasound guidance. A good-quality blastocyst was defined as having a well-expanded blastocoele (grades 4–6) on day 5 or day 6, a well-defined ICM (subgrades A or B) and a single layer of TE cells surrounding the cavity (subgrades A or B) in oocyte retrieval cycle according to Gardner's criteria [10].

Pregnancy diagnosis

A human chorionic gonadotrophin (β-hCG) test was conducted around 12 days after blastocyst transfer. Biochemical pregnancy was considered positive if the β-hCG level was >20 IU/l. Clinical pregnancy was defined as detection of an intrauterine gestational sac under ultrasound image on the day 30–35 after embryo transfer. Ongoing pregnancy was defined as a viable pregnancy detected by ultrasound examination at 12 weeks of gestation.

Serum P measurement

Blood samples were obtained prior to embryo transfer and the sixth day of P administration. Serum P concentration was measured by an electrochemiluminescence immunoassay (Beckman unicel DXI 800, the U.S). The intra-assay coefficient of variation was 2.8–3.2% and the inter-assay coefficient of variation was 5.0–5.5%. The sensitivity of the assay was 0.03 ng/ml.

Statistical analysis

Statistical analysis was performed using SPSS Statistics 21.0 (IBM Corp., Armonk, NY, USA). Continuous variables were expressed as mean ± SD, whereas categorical variables were expressed as percentage. For all dichotomous parameters, chi-squared or Fisher's Exact tests were adopted as appropriate. Continuous outcomes were analyzed using either independent T-test or Mann–Whitney U-test based on the normality of the distribution. To define the predictive capability of serum P on the OPR, the receiving operating characteristic (ROC) curve was described and the area under the curve (AUC) was calculated. A multivariate logistic regression, controlling for factors including female age at the time of oocyte retrieval, BMI, day 5 or day 6 blastocyst, blastocyst quality and serum P concentrations on the transfer day, was evaluated for the influence of pregnancy outcomes. A p-value <0.05 was considered as statistically significant.

Results

Descriptive analysis

During the study period, a total of 1176 HRT-FET cycles were performed in 1049 patients. The mean female age and body mass index (BMI) were 29.87 ± 3.77 years and 23.32 ± 3.57 kg/m^2, respectively. The mean serum P level on the day of blastocyst transfer was 12.31 ± 7.57 ng/ml with a large range (0.28–44 ng/ml), as shown in Fig. 1. The total CPR was 65.6% (95% CI: 62.8–68.2), OPR was 57.9% (95% CI: 55.1–60.7), and LBR was 56.3% (95% CI: 53.4–59.1). ROC curve showed that P levels on the day of blastocyst transfer had no predictive value for the OPR owing to the AUC = 0.5139 (95% CI: 0.4802–0.5476) ($p = 0.4155$).

Pregnancy outcomes of patients with serum P < 10 ng/ml and ≥10 ng/ml

Referring to the defined value (10 ng/ml) of luteal phase defect (LPD), the subjects were divided into two groups based on the serum P levels on the transfer day: P < 10 ng/ml group and P ≥ 10 ng/ml group. The CPRs, OPRs and LBRs were not significantly different between the two groups. Likewise, no differences were observed in the cycle characteristics such as age, the ratio of good-quality blastocyst and endometrial thickness between the two groups, to the exclusion of BMI. The average BMI in P < 10 ng/ml group was higher compared with that in P ≥ 10 ng/ml group ($p < 0.05$) (Table 1).

Pregnancy outcomes of patients with serum P < 5.65 ng/ml and ≥5.65 ng/ml

The subjects were divided into 10 groups by deciles of serum P levels, and OPR was calculated in each group (Fig. 2). Considering the lower limit of the 95% CI of the overall OPR (55.1%) as a fair rate, a critical P cutoff value of 5.65 ng/ml was observed. This cutoff value corresponded to the 10th percentile. As shown in Table 2, there were no significant differences in mean age, BMI, endometrial thickness and the ratio of good-quality blastocyst between P levels ≥ 5.65 ng/ml group and P levels <5.65 group. However, patients with serum P levels <5.65 ng/ml yielded a significantly lower OPR of 48.31% vs 58.98% ($p = 0.03$) and lower LBR of 43.22% vs 57.75% ($p = 0.003$).

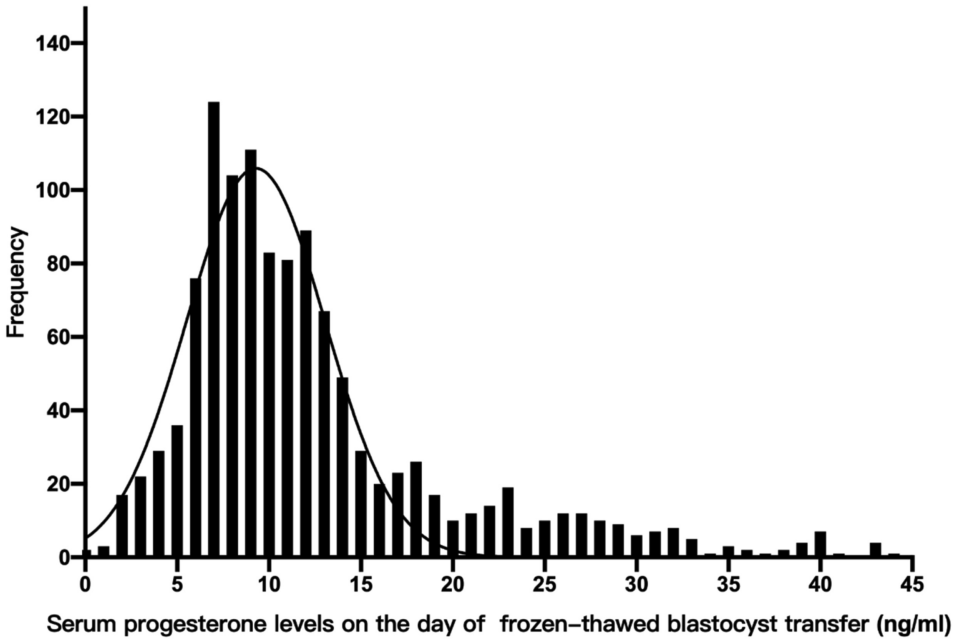


Fig. 1. Frequency distribution histogram of serum progesterone levels on the day of frozen blastocyst transfer.

Multivariate logistic regression analysis of factors related to pregnancy outcomes

Additionally, a multivariate logistic regression was performed in 1049 patients with their first FET cycles, controlling for variables including female age, serum P concentration on the transfer day, BMI, D5 vs. D6 blastocyst, and good-quality vs. non-good-quality blastocyst. The results showed age, D5 vs. D6 blastocyst and blastocyst quality was significantly associated with clinical and ongoing pregnancies. BMI, D5 vs. D6 blastocyst and blastocyst quality was significantly associated with live births. However, multivariate analysis failed to demonstrate serum P level on the transfer day was associated with pregnancy outcomes (Table 3).

Discussion

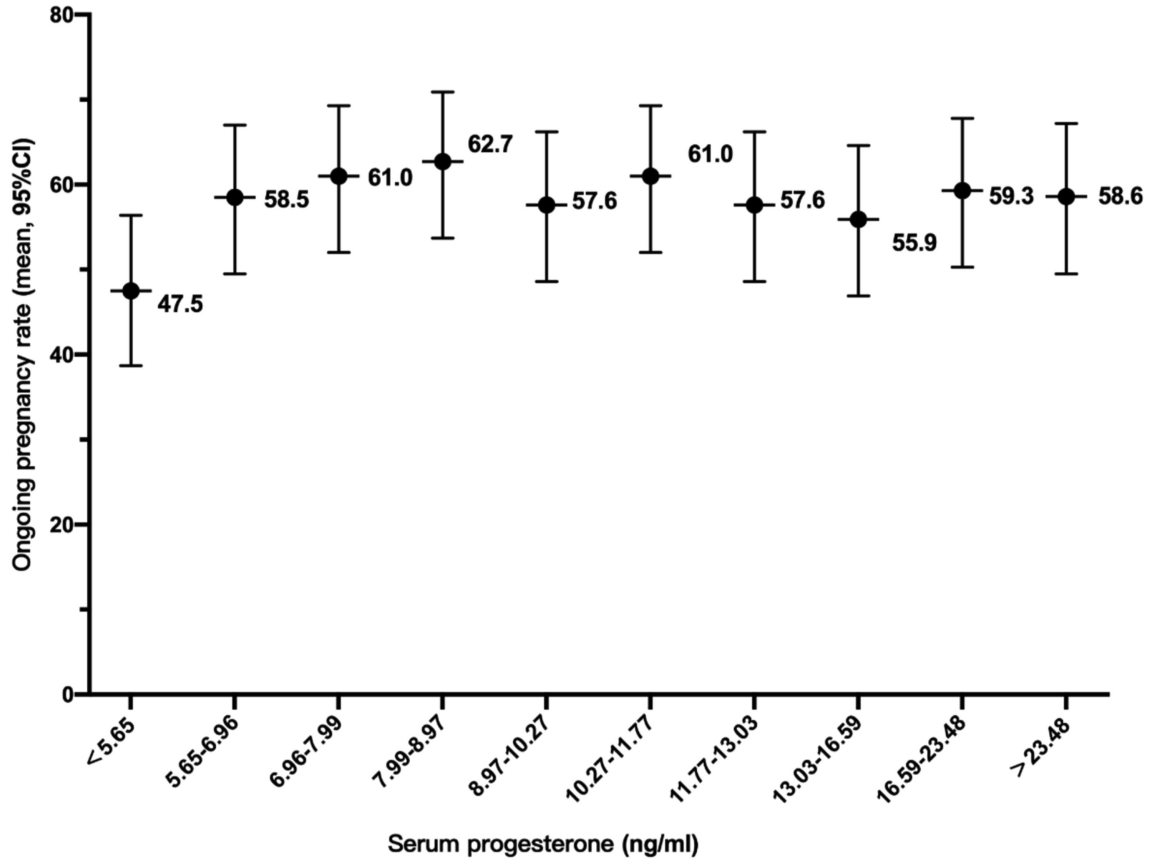
The aim of this retrospective study was to analyze the correlation between serum P levels on the day of blastocyst transfer and pregnancy outcomes in HRT-FET cycles with oral dydrogesterone for strengthened LPS. The results showed only patients with serum P levels below 5.65 ng/ml yielded a significantly lower OPR and LBR.

Table 1
Comparison of cycle characteristics and pregnancy outcomes of patients with serum P levels <10 ng/ml and ≥10 ng/ml.

	Serum P level		P-value
	<10 ng/ml	≥10 ng/ml	
Cycles (n)	565	611	
Age (years)	29.80 ± 3.81	29.93 ± 3.73	0.5546
BMI (kg/m^2)	23.98 ± 3.78	23.15 ± 3.77	0.0002
Endometrial thickness (mm)	8.9 ± 2.5	8.8 ± 2.4	0.3225
Good-quality blastocyst rate (%)	84.60 (478/565)	85.27 (521/611)	0.75
Biochemical pregnancy rate (%)	70.44 (398/565)	69.56 (425/611)	0.74
Clinical pregnancy rate (%)	65.84 (372/565)	64.88 (399/611)	0.73
Ongoing pregnancy rate (%)	57.52 (325/565)	58.27 (356/611)	0.80
Live birth rate (%)	55.4 (313/565)	57.12 (349/611)	0.55

Note: BMI, body mass index.

The interaction between embryo and receptive endometrium is a complex molecular process, in which P is essential to trigger secretory transformation and allow embryo implantation, pregnancy achievement and maintenance. In an HRT-FET cycle, all of available P derives from exogenous administration owing to the lack of a functional corpus luteum [11]. To date, there has been no consensus on the best P administration route. Even if using the same dose and route, significant individual differences still exist in the serum P levels on the day of embryo transfer. Thus, it is essential to consider the P administration route (vaginal, IM, subcutaneous or oral, etc.), dose and time of P measure when evaluating serum P values in the luteal phase of artificial cycles. Recently, several prospective studies showed that low serum P levels on the day of blastocyst transfer were associated with diminished OPRs in both own and donated oocyte cycles when patients received a dose of 400mg/12h micronized vaginal progesterone (MVP) for endometrial preparation. The threshold of serum P levels was identified as 8.8, 9.2 or 9.8 ng/ml. Serum P levels below the threshold were observed in approximately 30% of cycles [5,12,13]. In report of Boynukalin et al. [14], patients with serum P levels <13.6 ng/ml on the transfer day had a significantly lower likelihood of an ongoing pregnancy in single euploid frozen blastocyst transfer cycles when endometrial transformation was performed by IM of 100 mg P. It is well-known that vaginal P administration has a first-pass uterine effect. High concentrations of P in the myometrium and endometrium have been demonstrated after 4h vaginal P administration. However, the systemic absorption of P by vaginal administration is very limited. On the contrary, the serum P level increases rapidly after 2 h, and reached a peak value after 8 h IM administration [15]. Consequently, serum P levels do not represent the P levels in the endometrium. In addition, some factors maybe affect P levels after vaginal administration, such as sexual intercourse, patient compliance and differences in vaginal absorption, distribution, and metabolism. Similarly, some variations were also observed in IM administration [16]. In this study, we also observed serum P levels on the transfer day exhibited a wide range. Therefore, the differences of pharmacokinetic probably lead to significant differences in the timing of serum P reaching the peak value, which may in part explain the varying efficacy of different serum P levels.



Percentiles	P10	P20	P30	P40	P50	P60	P70	P80	P90
Progesterone	5.65	6.96	7.99	8.97	10.27	11.77	13.03	16.59	23.48

Fig. 2. Ongoing pregnancy rate according to the deciles of serum progesterone on the day of frozen blastocyst transfer. Data are expressed as mean.

Although low serum P level on the day of FET is associated with diminished pregnancy outcomes in HRT cycles, there is no consensus on whether women with low serum P levels may benefit from additional P supplementation. Recently, several studies demonstrated that patients with low serum P levels who received a daily subcutaneous injection of 25 mg P additionally on the day or prior to blastocyst transfer produced similar LBRs to those with adequate serum P levels and standard LPS by MVP [17–19]. Nevertheless, Cedrin-Durnerin et al. [7] found that increasing MVP supplementation from 600 mg to 1200 mg daily after FET, pregnancy outcome of the patients whose low P levels were corrected (≥10 ng/ml) on 2 days post-embryo transfer was no different to those whose P levels remained <10 ng/ml. And serum P levels

Table 2
Comparison of cycle characteristics and pregnancy outcomes of patients with serum P levels <5.65 ng/ml and ≥5.65 ng/ml.

	Serum P level		P-value
	<5.65 ng/ml	≥5.65 ng/ml	
Cycles (n)	118	1058	
Age (years)	29.97 ± 3.82	29.85 ± 3.77	0.7433
BMI (kg/m^2)	23.90 ± 4.04	23.51 ± 3.77	0.2902
Endometrial thickness (mm)	8.9 ± 2.5	8.8 ± 2.3	0.6571
Good-quality blastocyst rate (%)	81.36 (96/118)	85.35 (903/1058)	0.25
Biochemical pregnancy rate (%)	65.25 (77/118)	68.57 (746/1058)	0.46
Clinical pregnancy rate (%)	57.63 (68/118)	66.45 (703/1058)	0.06
Ongoing pregnancy rate (%)	48.31 (57/118)	58.98 (624/1058)	0.03
Live birth rate (%)	43.22 (51/118)	57.75 (611/1058)	0.003

Note: BMI, body mass index.

Table 3
Multivariate logistic regression analysis of factors related to pregnancy outcomes.

	Adjusted OR (95% CI)	P-value
Clinical pregnancy		
Age (years)	0.97 (0.93–1.00)	0.044
BMI (kg/m^2)	0.98 (0.94–1.01)	0.199
D5 blastocyst	1.75 (1.21–2.55)	0.003
Good-quality blastocyst	2.56 (1.79–3.66)	<0.001
Serum P level on the transfer day	1.01 (0.99–1.03)	0.451
Ongoing pregnancy		
Age (years)	0.96 (0.93–0.99)	0.019
BMI (kg/m^2)	0.98 (0.95–1.01)	0.196
D5 blastocyst	1.68 (1.15–2.45)	0.007
Good-quality blastocyst	2.65 (1.84–3.82)	<0.001
Serum P level on the transfer day	1.01 (0.99–1.03)	0.217
Live birth		
Age (years)	0.97 (0.94–1.01)	0.104
BMI (kg/m^2)	0.96 (0.93–1.00)	0.029
D5 blastocyst	1.60 (1.09–2.33)	0.015
Good-quality blastocyst	2.77 (1.91–4.01)	<0.001
Serum P level on the transfer day	1.01 (1.00–1.03)	0.135

Note: BMI, body mass index.

below 10 ng/ml remained in about 10% patients regardless of P dose. Thus, some authors suggested the addition of exogenous P, and combination of two or three routes rather than increasing the dose of a single route, may be a reasonable choice to ameliorate pregnancy outcomes in patients with low P levels on the day of FET [7,20]. In this study, comparable pregnancy outcomes were observed between the patients with P $\geq$ 10 ng/ml and P < 10 ng/ml. And multivariate regression analysis also demonstrated serum P level on the transfer day was not associated with pregnancy outcomes. The possible reason may be related to our LPS protocol. Namely, oral dydrogesterone was added daily to all of patients from the transfer day for strengthened LPS. Dydrogesterone is a synthetic oral P which is a stereoisomer of the natural P. On account of its chemical structure, Dydrogesterone binds almost exclusively to P receptors and has a higher oral bioavailability and progestogenic activity. Nevertheless, it is difficult for dydrogesterone or its metabolites to compare P measurements due to structural differences with P. It could induce endometrial transformation at a dose 10 to 20 times lower than that of oral micronized P [21,22]. And dydrogesterone supplementation has been shown to improve endometrial blood flow in women with threatened or recurrent miscarriage, suggesting dydrogesterone maybe facilitate normal embryo implantation [23]. Moreover, dydrogesterone was effective and well tolerated when used to provide LPS after fresh embryo transfer [24]. As far as FET cycle was concerned, Vuong et al. [25] found LPS with MVP plus oral dydrogesterone had a higher LBR and lower miscarriage rate compared with MVP alone. These results suggested strengthened LPS by addition of dydrogesterone for patients with low serum P on the day of FET could contribute to improve pregnancy outcomes.

In addition, our results showed lower OPR and LBR was observed in patients with serum P levels below 5.65 ng/ml on the transfer day, which accounted for 10% of transfer cycles. It was similar with Volovsky's study that serum P $\geq$ 10 ng/ml on the day of FET did not confer a statistically significant improvement in pregnancy outcomes compared with serum P < 10 ng/ml with the administration of MVP 200 mg TID for endometrium preparation, and only serum P < 5 ng/ml was associated with lower LBR [16]. This paper also mentioned if serum P levels were less than 8 ng/ml, P replacement was increased at the discretion of the treating clinician, although it was not standardized. In report of Gao et al. [26], CPR and LBR was still lower in patients with low serum P (<10 ng/ml) and strengthened LPS, compared with normal serum P ($\geq$10 ng/ml) and routine LPS. As such, it may be that the mechanism behind implantation failure in the case of insufficient serum P in HRT is probably not a simple endometrial receptivity defect and additional P administration cannot mitigate the effects of an initially very low serum P level [27,28].

It is noteworthy that too high P levels in the uterine cavity could induce a premature opening of the "implantation window" theoretically and thus a disruption of the physiological synchronicity between endometrial maturation and embryonic development. There were some studies showed serum P levels greater than 20 ng/ml or 32.5 ng/ml on the day of FET were associated with lower LBR when the patients received IM of 50–70 mg P daily or a combination of both vaginal P and IM of P with dose escalation method, respectively [3,29]. Yovich et al. [4] found concentrations of serum P below 50 nmol/l (16 ng/ml) and above 99 nmol/l (31.68 ng/ml) were associated with decreased implantation rates in HRT-FET cycles. However, in our study, decreased OPR was not observed in patients with serum P levels greater than 23.48 ng/ml which corresponded to the 90th percentile. Similar results were also observed in Boynukalin's study that OPR in patients with serum P levels greater than 53.2 ng/ml did not decrease, even reached 82.9% in single euploid frozen blastocyst transfer cycles by IM of 100 mg P for endometrium transformation [14]. These results suggested that the association between serum P levels and the probability of achieving a live birth varied depending on the route and dose of P administration [20].

In addition, recent data suggested a negative relationship between BMI and serum P levels [13,19,30]. In this study, there was no correlation between BMI and serum P level (data not shown), although the patients with P < 10 ng/ml showed a significantly higher BMI.

The main limitation of the study was that there isn't a group of patients who were not augmented with dydrogesterone after FET. The effects of dydrogesterone supplement on the clinical outcomes of patients with lower serum P levels should be evaluated in further studies via prospective control trial. Moreover, only young women with single blastocyst transfer and appropriate endometrial thickness were included. Extrapolation to different populations or to other administration routes of P will require further validation.

In conclusion, we demonstrated that measuring serum P levels on the day of HRT-FET is of clinical importance. As far as the majority of patients are concerned, IM administration of 40 mg P daily plus oral dydrogesterone 10 mg BID from the transfer day might be sufficient for endometrial transformation and LPS in HRT cycles. Lower serum P levels impact the success of HRT-FET cycles, suggesting that there may be a threshold below which it is difficult to improve pregnancy outcomes via oral dydrogesterone to strengthen LPS.

Conflicts of interest

None declared.

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Original Article

Setting up a specialized maternity unit in a tertiary hospital: An oasis for pregnant women with COVID-19 during the pandemic

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ABSTRACT

Objective: The COVID-19 pandemic has had an enormous impact on society and the medical environment in Taiwan in 2022. As pregnant women with COVID-19 are at higher risk for multiple complications, Taiwan needs a COVID-19 specialized maternity unit to improve the quality of maternal and neonatal care.
Materials and methods: We share our experience with specialized maternity unit for pregnant women with COVID-19 at the National Cheng Kung University Hospital, where we can have careful evaluation, safe birth, and comprehensive postpartum care.
Results: Our COVID-19 specialized maternity unit enrolled 253 pregnant women with COVID-19, 90 (35.6%) pregnant women were admitted to the specialized maternity unit, and 71 (28.1%) pregnant women gave birth during hospitalization in two months. All pregnant women recovery well and real-time polymerase chain reaction tests on all infants were negative for COVID-19.
Conclusion: A specialized maternity unit can provide pregnant women with a safe birth environment, immediate maternity care, and high medical quality. It can also help health workers in non-specialized maternity units deal with COVID-19-related psychological stress. Therefore, setting up one specialized maternity unit in the city during the pandemic should be guardedly considered.

Introduction

Since acute respiratory tract infection due to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was first reported in December 2019 [1], SARS-CoV-2 has spread rapidly worldwide. Until June, 2022, coronavirus disease 2019 (COVID-19) had been reported in more than 500 million patients and led to 6 million deaths [2]. Undoubtedly, it has had a major impact on health systems and societies. There were only about 14,000 confirmed cases of COVID-19 and 800 deaths in Taiwan before 2022 [3]. However, the number of confirmed cases amplified as 3 million, and more than 4000 deaths related to COVID-19 were reported from January, 2022 to June, 2022 [3]. According to data from the Taiwan Centers for Disease Control (CDC) and Bureau of Civil Affairs Tainan City Government [3,4], the estimated cumulative infection numbers in Tainan City may reach 200,000 COVID-19 cases per month, which may account for approximately 10% of the population of Tainan.

Immunologic changes in pregnant women with COVID-19 may alter the susceptibility and severity of infectious diseases [5]. Pregnant women are at higher risk of adverse outcomes, including mortality, intubation, intensive care unit (ICU) admission, preterm birth, preeclampsia, venous thromboembolism, and other severe comorbidities [6–8]. As for newborns, COVID-19 infection in pregnancy is also reported to be significantly associated with an increased risk of neonatal complications [9,10]. Therefore, COVID-19 is an important issue for pregnant women during the pandemic. Due to the serious effects of the COVID-19 pandemic on obstetric care, we established a specialized COVID-19 maternity unit to provide safe and holistic care for pregnant women with COVID-19.

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Here, we share the experience of instituting a COVID-19 specialized maternity unit in National Cheng Kung University Hospital (NCKUH), a tertiary hospital in southern Taiwan. Our clinical assessment and management for pregnant women with COVID-19, from the special isolation unit in the emergency department, quarantine maternity unit, dedicated birthing room and operation room, to postnatal care team, were organized and operated rapidly.

Materials and methods

Obstetric isolation emergency room

An isolation room or ward with traffic control is an infection control effort to avoid the spread of infectious microbes in healthcare facilities [11]. Therefore, NCKUH built a separate quarantine station with negative-pressure ventilation, which has been referred to as the "QurE" since 2020. It was designed to assemble rapidly into an isolation station [12]. In response to the COVID-19 outbreak and the privacy of pregnant women, an obstetric isolation room was established for each pregnant woman. Medical equipment for fetal and labor assessments, such as ultrasound (Medison Accuvix V20) and cardiotocography (Philips Avalon FM 20), were prepared at this quarantine station. It was also equipped with telemedicine for history taking and consultation.

Initial assessment at the emergency department

All the confirmed pregnant women are diagnosed with PCR. If they are referred to our hospital due to a positive rapid antigen test, PCR will also be performed for confirmation. When a pregnant woman with COVID-19 arrived at the emergency department, she was transferred to the obstetric isolation room for primary assessment of vital signs. Obstetricians identified the risk factors for severe COVID-19, such as cerebrovascular disease, chronic kidney disease, diabetes mellitus, and obesity, so on [13]. Disease severity was evaluated using the classification of the COVID-19 Treatment Guidelines of the National Institutes of Health (NIH) [14]. For example, upper respiratory symptoms such as sore throat and cough without dyspnea, acute lung injury, or abnormal chest imaging are classified as mild illnesses. If the pregnant women presented with moderate or severe COVID-19, the emergency physicians were consulted for further evaluation and multidisciplinary care was coordinated accordingly. After the initial assessment of maternal status, obstetricians evaluated maternal and fetal conditions by pelvic examination, cardiotocography, and ultrasound. In addition to medications for respiratory symptoms and tocolytic agents, the prescription of antiviral drugs (i.e., nirmatrelvir and ritonavir or remdesivir) were discussed and depended on the result of communication between the doctor and the pregnant women. If it was necessary, pregnant women with COVID-19 were taken to the COVID-19 specialized maternity unit, which was set up in the obstetric ward. The criteria of hospitalization followed general obstetric guidelines.

Establishment of the COVID-19 specialized maternity unit

COVID-19 infection during pregnancy is a complicated event and requires intimate collaboration with a multidisciplinary team, including obstetricians, infectious disease specialists, centers for infection control, and engineering and maintenance offices. We constructed different pathways for pregnant women and healthcare personnel (i.e., traffic control). However, we modified the outflow of the ventilation at the aisle and station to achieve a pressure gradient. After the departure of pregnant women with COVID-19, the environment was also sterilized with ultraviolet light. Cardiotocography (Philips Avalon FM 20/30) was available in all isolation rooms and was connected to the central dashboard at the nursing station. Ultrasound (Aloka Prosound Alpha 6) was available at the specialized maternity unit. In addition, the station was equipped with a new self-developed telemedicine system designed by our obstetric team. This telemedicine system connects to every space in specialized maternity units and provides effective and convenient communication. Furthermore, this system thrifts a lot of time and energy for the staff. After the integral facility was set up, 15 beds for pregnant women with COVID-19 were put in isolation.

This specialized maternal unit provides a multidisciplinary team of obstetricians, neonatologists, anesthetists, infectious disease specialists, and obstetric nurses. All physicians and nurses were attired in standard personal protective equipment for dealing with pregnant women with COVID-19. Once admitted to the specialized maternal unit, pregnant women were primarily cared for by obstetricians, with close communication and coordination with other medical staff.

Management in the COVID-19 specialized maternity unit

Every pregnant woman admitted to this maternity unit underwent standard laboratory tests, including routine blood examinations and biochemical tests. Rectovaginal culture of group B streptococci (GBS) was performed if it was not performed within the previous five weeks. Cardiotocography was used for antepartum fetal assessment every eight hours and obstetric ultrasound was performed, if required. If the gestational age of pregnant women were less than 37 weeks, the management of preterm labor follows the guidelines of the American College of Obstetricians and Gynecologists (ACOG), such as administering tocolytic agents, antenatal corticosteroids, and antibiotics [15]. The timing and mode of birth were individualized based on maternal status, concurrent disorders, gestational age, and shared decision-making with the mother. The anesthesia during delivery followed general medical practices and the epidural anesthesia is allowed for vaginal delivery. After a thorough evaluation and explanation, all pregnant women who were admitted to the COVID-19 specialized maternal unit discussed the birth plan with obstetricians.

Preparing for vaginal birth in the COVID-19 specialized maternity unit

The COVID-19 birthing room was separated from the other rooms and equipped with a birthing table, an infant warmer, and cardiotocography (Philips Avalon FM 20). The birth trolley contained a disposable vaginal birth set and sterile instruments for vaginal examination or repair of perineal wounds. When the pregnant woman was transferred to the birthing room, the birth team consisted of at least two obstetricians and two obstetric nurses. Neonatologists are on standby outside the birthing room with full personal protective equipment, prepared for intervention as needed. We did not permit the mother and newborn skin-to-skin contact in the birthing room because of vertical transmission concerns. Once the obstetric nurse finished preliminary management, newborns were handed over to neonatologists in a transport incubator and sent to the neonatal isolation room for further care. For mothers, proper postpartum care, such as wound repair, uterine massage, and uterotonic agents, was provided. The detailed traffic flow for vaginal birth in the COVID-19 birthing room is shown in Fig. 1.

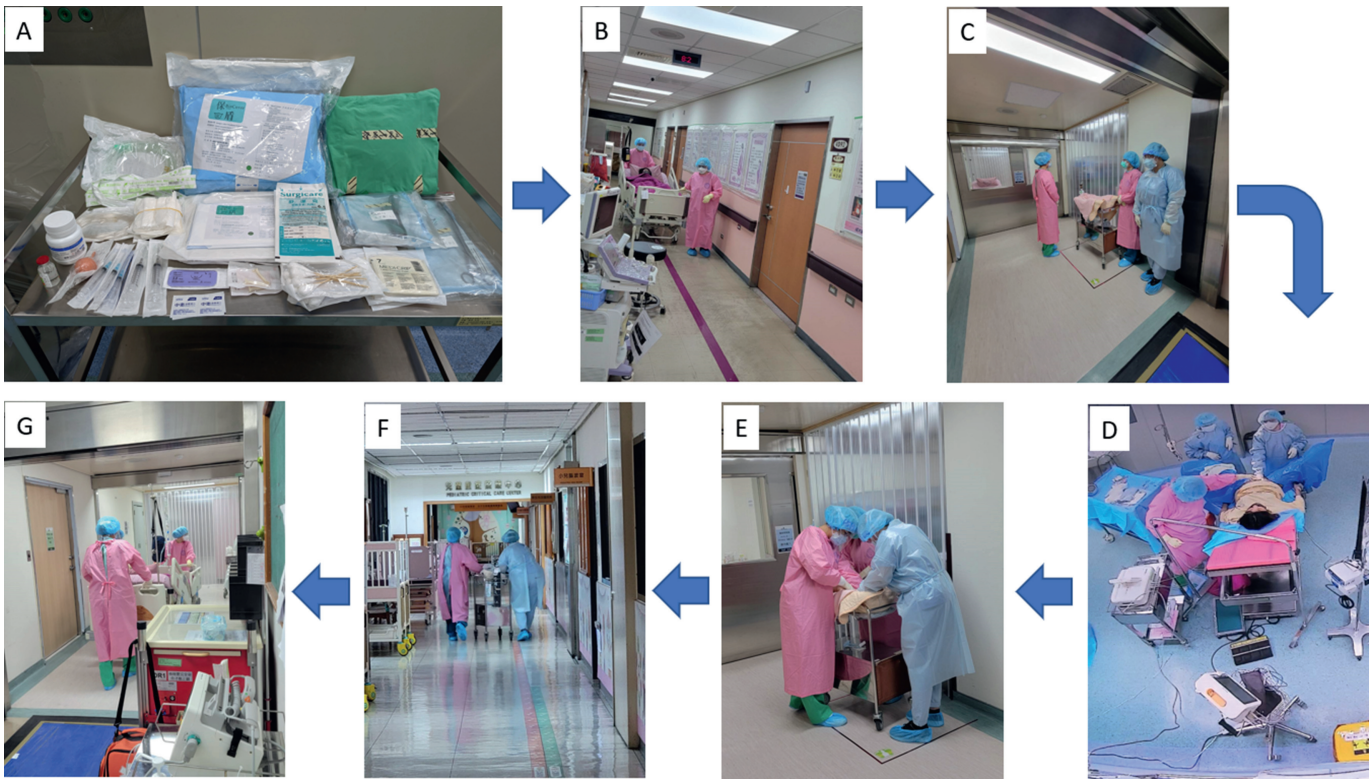

Fig. 1. Traffic flow for vaginal birth in the COVID-19 birthing room A. Preparing the disposable vaginal birth set and sterile instruments; B. Transferring the mother to the birthing room; C. Neonatologists on standby and prepared for intervention as needed; D. Obstetricians performs vaginal birth; E. Obstetric nurse hands the baby over to neonatologists; F. Transferring the baby to neonatal isolation ward; G. Transferring the mother back to the postpartum room after management.

Preparing for cesarean section for pregnant women with COVID-19

When pregnant women with COVID-19 required an emergency cesarean section, a multidisciplinary team consisting of obstetricians, neonatologists, anesthetists, obstetric nurses, and operating nurses was activated immediately. An operating room with an independent air-conditioning system was prepared as soon as possible. To minimize delays and achieve infection control, the hospital security team secured the transfer route and cleared the path ahead for the pregnant women. The type of anesthesia used for cesarean section depends on clinical condition. The management of newborns was the same as that of vaginal birth. After the initial assessment, if the condition of the newborn was found to be unstable, neonatologists entered the operating room and performed resuscitation.

Postpartum care of women with COVID-19 and neonates

All women with COVID-19 mandatorily underwent comprehensive postpartum assessment and care for physical, social, and psychological well-being. Adequate nutritional support, medication for pain control, and uterotonic agents were administered. At least every eight hours, healthcare workers evaluated vital signs, fundal height, uterine contraction, lochia, and breastfeeding. To avoid uncertain transmission, we encourage breastfeeding with appropriate infection control precautions. In addition, venous thromboembolism (VTE) risk assessment was performed [16], and thromboprophylaxis with low-molecular-weight heparin (LMWH) was considered based on individual risk factors.

The specialized maternity unit of NCKUH was established on May 20, 2022 for pregnant women with COVID-19, and a complete workflow was implemented (Fig. 2). We conducted a review of all cases admitted to the specialized maternity unit until July 21, 2022 to evaluate its effectiveness and safety. This study was approved by the Institutional Review Board of NCKUH (B-ER-111-164).

Results

In the first two months, the obstetric team of the NCKUH enrolled 253 pregnant women with COVID-19. Ninety (35.6%) pregnant women were telemedicine cared for at home with case managers closely following up. One hundred and sixty-three (64.4%) pregnant women visited our obstetric emergency department and 73 (28.9%) pregnant women were discharged from the emergency department after initial assessment and appropriate management. Another 90 (35.6%) pregnant women were admitted to the specialized maternity unit, and 71 (28.1%) pregnant women gave birth during hospitalization. The cumulative infection numbers in Tainan and pregnant women with COVID-19 in the NCKUH since establishment are presented in Fig. 3.

The characteristics and clinical outcomes of 71 pregnant women and 76 infants in the COVID-19 specialized maternity unit are shown in Table 1. The mean maternal age was 32.8 years old (range: 22–44 years), and the mean maternal body mass index was 26.7 kg/m^2 (range: 19.5–51.1 kg/m^2). Among the mothers, thirty-one (43.7%) were primiparous and 40 (56.3%) were multiparous. In terms of the mode of birth, there were 39 (54.9%) cases of normal spontaneous birth and 32 (45.1%) cases of cesarean section. Forty-six (64.8%) had completed primary series of vaccination and 23 (32.4%) received booster dose. All the pregnant women were classified into mild illness based on the classification of NIH and the mean cycle threshold (Ct) value of the real-time polymerase chain

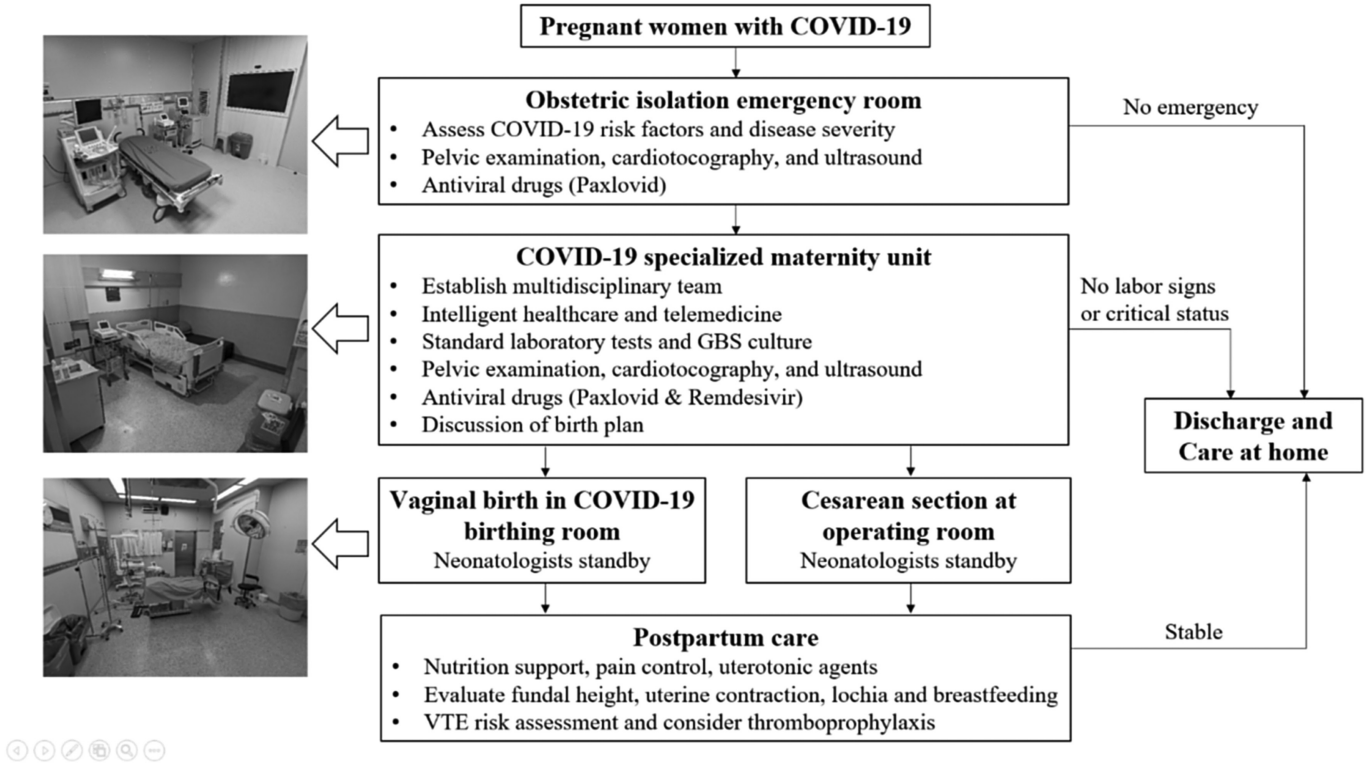

Fig. 2. Workflow for pregnant women with COVID-19 in National Cheng Kung University Hospital.

reaction (PCR) of SARS-CoV-2 was 21.3 (range: 11.5–33.8). Fifteen (21.1%) mothers received antiviral drugs, including intravenous remdesivir (five mothers) and oral Paxlovid (nirmatrelvir and ritonavir) (ten mothers). There were 12 mothers diagnosed with postpartum hemorrhage, and most were stabilized with conservative treatment. In addition, thromboprophylaxis with enoxaparin was administered to two mothers during hospitalization.

A total of 76 infants were gave birth with a mean birth weight of 2840.7 g (range: 1635–3790 g). The mean gestational age at birth was 37.4 weeks (range: 31–40 weeks). All the infants' Apgar scores were good, and SARS-CoV-2 was not found in their nasopharyngeal samples using real-time PCR.

Discussion

The COVID-19 pandemic has had a serious impact on human society in terms of health, economy, and lifestyle and has brought extreme stress and strain to general hospitals. According to the data presented in Fig. 3, the number of confirmed cases of COVID-19 is rapidly increasing in Tainan, and the medical needs of pregnant women are also increasing. National Cheng Kung University Hospital is the first hospital to completely transform its delivery rooms into COVID-19 specialized maternity unit and receive all the pregnant women with COVID-19 in Tainan city. We prepared a COVID-19 specialized maternity unit before the outbreak in the city and

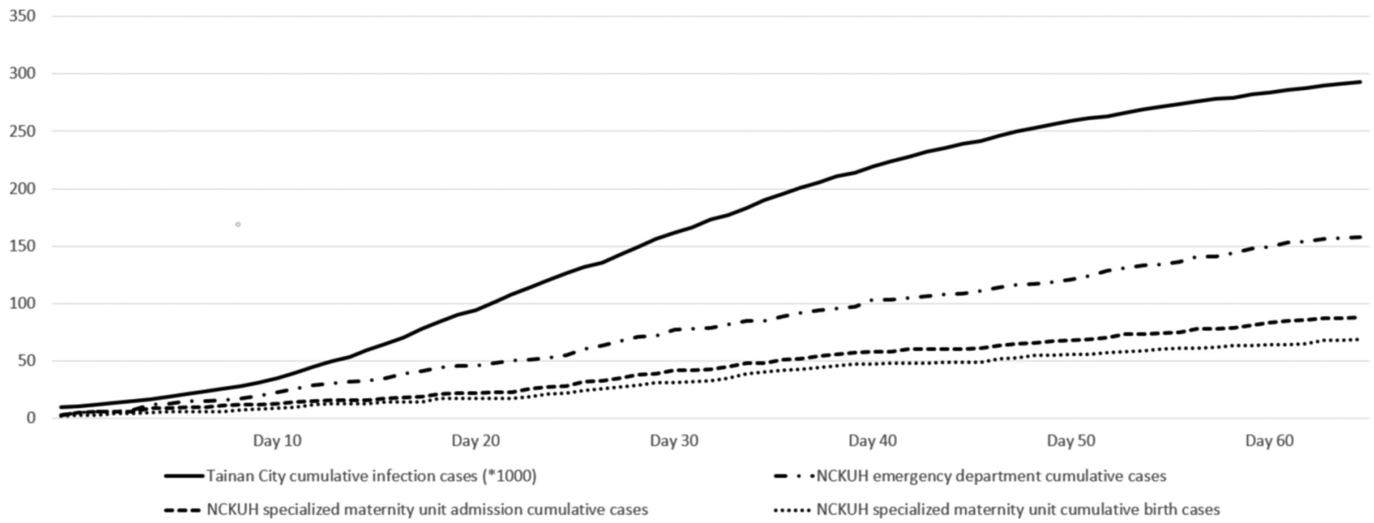

Fig. 3. The cumulative numbers of Tainan and the COVID-19 specialized maternity unit of National Cheng Kung University Hospital since establishment.

Table 1
The characteristics and clinical outcomes of 71 gave birth women and 76 infants in the COVID-19 specialized maternity unit of National Cheng Kung University Hospital.

Maternal age (year)	32.8 ± 4.6
Parity, n (%)	
Primiparous	31 (43.7%)
Multiparous	40 (56.3%)
Race, n (%)	
Asian	71 (100%)
Other	0 (0%)
Maternal BMI (kg/m^2)	26.7 ± 4.8
Vaccination status	
Not vaccinated	12 (16.9%)
Primary series	46 (64.8%)
Primary series and 1 booster	23 (32.4%)
COVID-19 RT-PCR Ct value	21.3 ± 6.1
GA at birth (week)	37.4 ± 2.6
Preterm birth, n (%)	18 (25.3%)
Mode of birth, n (%)	
Vaginal birth	39 (54.9%)
Epidural anesthesia	11 (28.2%)
Cesarean section	32 (45.1%)
Spinal anesthesia	29 (90.6%)
General anesthesia	3 (9.4%)
Maternal comorbidity, n (%)	
Previous cesarean section	13 (18.3%)
Hypertensive disorders	10 (14.1%)
Gestational diabetes mellitus	11 (15.5%)
Twin pregnancy	5 (7.0%)
Precipitate labor	11 (15.5%)
Malpresentation	8 (11.3%)
PTL	18 (25.3%)
PPROM	3 (4.2%)
Placenta abruption	5 (7.0%)
PPH	12 (16.9%)
Antiviral drug, n (%)	
Paxlovid	10 (14.1%)
Remdesivir	5 (7.0%)
Thromboprophylaxis, n (%)	2 (2.8%)
Maternal death, n (%)	0 (0%)
Birth weight (g)	2840.7 ± 622.1
Apgar score	
1 min	8.4 ± 1.3
5 min	9.4 ± 0.9
Neonatal COVID-19 infection, n (%)	0 (0%)
Neonatal death, n (%)	0 (0%)

Data are presented as mean ± standard deviation or number (percentage).
BMI, body mass index; RT-PCR, reverse transcription polymerase chain reaction; Ct value, cycle threshold value; GA, gestational age; PTL, preterm labor; PPROM, preterm premature rupture of membrane; PPH, postpartum hemorrhage.

this approach greatly alleviated the pressure on other hospitals and clinics in the same city because of the convenient transfer system. We have presented relevant data to demonstrate the effectiveness and safety of this approach. In our opinion, setting up a specialized maternity unit during the pandemic has great benefit to the city in terms of public health and maternal care, and it should be guardedly considered.

Establishing such a unit and its complete workflow from the point of arrival of the pregnant women at the emergency room to the point of birth greatly benefits women with this infection. The use of telemedicine in specialized maternity units can improve the quality of health services. In addition, an experienced multidisciplinary team is important for providing safe and comprehensive care for hospitalized women. The clinical outcomes of all pregnant women and infants in the COVID-19 maternity unit were satisfactory. The establishment of COVID-19 as a maternity unit has multiple features and benefits, as shown in Table 2. An experienced multidisciplinary team, complete workflow, and rapid traffic flow can ensure an efficient and safe medical environment. Moreover, a specialized maternity unit can provide immediate maternity care, high medical quality, and a comfortable birth experience for pregnant women with COVID-19. Further, healthcare workers in non-specialized maternity units can diminish their stress of facing COVID-19 and provide optimal health care services for pregnant women without COVID-19.

Decisions regarding the use of COVID-19 antiviral drugs should involve maternal disease severity, underlying risk factors, gestational age, and uncertain risk to the fetus and newborn. In Taiwan, two common antiviral drugs are administered to pregnant women. The Taiwan CDC has authorized the use of Paxlovid (nirmatrelvir and ritonavir) for pregnant women since May, 2022. It has been shown to reduce hospitalization and death in patients with mild to moderate COVID-19 [17]. We could offer Paxlovid in the emergency room and share decision-making with the mother. Notably, ergot derivatives, which are common uterotonic agents, should be avoided in pregnant women receiving Paxlovid during the postpartum period because of drug–drug interactions [18]. However, if the pregnant women are contraindicated, intravenous remdesivir is advised during hospitalization. Remdesivir has been proven to prevent disease progression with a three-day course of use in COVID-19 infection [19], and showed no fetal toxicity in a previous study on Ebola and Marburg virus diseases [20]. In our maternity unit, most pregnant women are unwilling to use antiviral drugs due to the limited human data and there are only 15 pregnant women received antiviral drugs. All these pregnant women and their infants revealed no adverse reaction or complication.

The timing and mode of birth should be modified based on multiple factors, including individual pregnancy risk factors, obstetric and surgical history, and the severity of COVID-19 infection. Although COVID-19 may result in an increased rate of cesarean section for pregnant women [21], the rates of neonatal COVID-19 infection, neonatal deaths, and maternal deaths are no greater than when the mother has vaginal birth [22,23]. According to our presented data, more than half mothers gave birth via vaginal route which proved that COVID-19 specialized maternity unit can provide a safe and reliable birth environment. Nonetheless, our data revealed a higher proportion of placental abruption and postpartum hemorrhage. We attribute this to the higher percentage of high-risk pregnancies, including multiple pregnancies, precipitate labor, preterm labor, and so forth. With the presence of a rapid and complete medical team, all cases of postpartum hemorrhage were effectively controlled with conservative treatment. In addition, the relatively low proportion of epidural anesthesia during vaginal birth may be due to our higher percentage of multiparous women and precipitate labor.

Skin-to-skin contact is not allowed in the birthing room because we believe that the sooner the transfer to neonatologists, the higher the quality of care for newborns. However, breastfeeding is still allowed after delivery with appropriate infection control precautions, such as proper hand cleaning and mask wearing [24]. There is no evidence that breastmilk can contain an infectious virus or that breastfeeding presents as a risk factor for the transmission of infection to infants [25]. Furthermore, antibodies against COVID-19 have been identified in breastmilk among mothers who have received the vaccine, which suggests a potential protective effect against infection in infants [26].

Thromboprophylaxis is also an important issue in pregnant women with COVID-19. Pregnant women are at an increased risk of VTE owing to hypercoagulability. Compared to women who are not pregnant, the risk of VTE during pregnancy is increased four-fold and even higher in the postpartum period [27]. The intravascular inflammation of pregnant women can be exaggerated in the context of COVID-19 infection [28], and the Royal College of Obstetricians and Gynecologists has recommended that all pregnant women with COVID-19 should undergo VTE risk assessment [29].

Table 2
Features and benefits of the COVID-19 specialized maternity unit.

Features and benefits of the COVID-19 specialized maternity unit	
Pregnant women with COVID-19	Safe and reliable birth environment • Immediate and effective maternity care • Equitable and of high medical quality • Appropriate and individual birth plan • Comfort and confidence in their birth experiences
Manpower	A complete and experienced multidisciplinary team • Obstetricians and nurses • Neonatologists and nurses • Anesthetists and nurses • Infectious disease physicians • Case manager
Workflow	Set up a complete, convenient, and efficient workflow • Fast assessment and management at the Emergency Department • Professional care for high-risk pregnancy with COVID-19 • Appropriate care in the COVID-19 specialized maternity unit • Intelligent healthcare and telemedicine • Secure vaginal birth and cesarean section • Comprehensive postpartum assessment and care
Traffic flow	Established a fast, safe, and smooth traffic flow • Entrance of reception with clear view and enough space • Unidirectional traffic flow with clearly marked signages • Establish pressure gradient between spaces with clearly demarcated • Close to the intensive care unit and operating room for easy transfer
Non-specialized maternity unit	Diminished the stress of caring for pregnant women with COVID-19 • Ensure an effective referral system and triage of high-risk pregnancy • Optimal and cost-effective health care services • Focus on providing care of non-COVID pregnancy

Thromboprophylaxis with LMWH is considered based on individual risk factors, such as personal history, body mass index, and maternal age. There were only two gave birth women received thromboprophylaxis, who had multiple risk factors, including severe obesity and advanced maternal age. None of the 71 gave birth women experienced thrombotic events before discharge.

Although many medical centers in Taiwan have similar ward setups, the specialized maternity unit of NCKUH possesses three distinct features apart from other hospitals. Firstly, we have established a dedicated ward within Tainan city instead of having dispersed ward setups in the same city that can result in resource fragmentation, inefficient manpower usage and reduced work efficiency. Secondly, we have completely transformed the ward into a specialized maternity unit, exclusively admitting pregnant women with COVID-19 without any other pregnant women. This approach effectively mitigates the risks of cross-infection within the hospital, facilitates patient transfer, and prevents unnecessary wastage of hospital space. Lastly, our postpartum care continues within the specialized maternity unit rather than being transferred to other wards and this measure can enhances the quality of postpartum due to the same professional obstetric team.

Establishing a COVID-19 specialized maternity unit and a complete workflow has multiple benefits, such as a safe birth environment, immediate maternity care, and high medical quality. Although we did not conduct questionnaire survey specifically targeting frontline healthcare workers at regional hospitals or local clinics, we believe that the specialized maternity unit primarily alleviate the pressure of frontline healthcare workers. Such a public health policy can accelerate referral efficiency, improve pregnant women safety, and ease the burden of frontline healthcare workers. In addition, the use of telemedicine in specialized maternity units provides a satisfactory experience for medical care. The strengths of this study are that we provide detailed description of the approach to establishing specialized maternity unit and relevant data to demonstrate the significant benefits of this public healthcare policy. However, the disadvantages are that the specialized maternity unit requires more property and resources, and pregnant women without COVID-19 must be transferred to the non-COVID-19 maternity unit of cooperating hospitals.

Conclusion

During the COVID-19 pandemic, establishing a COVID-19 specialized maternity unit and a complete workflow with telemedicine from the arrival of the emergency room to birth had remarkable benefits for pregnant women with COVID-19. A specialized maternity unit can provide pregnant women with a safe birth environment, immediate maternity care, and high-quality medical care. It should be guardedly considered about setting up one specialized maternity unit in the city during the pandemic.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

We would like to thank all the team members involved in the COVID-19 specialized maternity unit, including obstetricians, obstetric nurses, infectious disease physicians, and the Center for Infection Control of NCKUH. We would also like to express our gratitude to all obstetricians and obstetric nurses for their hard work and dedication to these pregnant women during the pandemic.

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Original Article

A novel missense variant in *CDK5RAP2* associated with non-obstructive azoospermia

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ABSTRACT

Objective: The most severe type of male infertility is non-obstructive azoospermia (NOA), where there is no sperm in the ejaculate due to failure of spermatogenesis, affecting 10%–20% of infertile men with azoospermia. Genetic studies have identified dozens of NOA genes. The main aim of the present study is to identify a novel monogenic mutation that may cause NOA.
Materials and methods: We studied the pedigree of a consanguineous family with three NOA and one fertile brother by a family-based exome-sequencing, segregation analysis, insilico protein modeling and single-cell RNA sequencing data analysis.
Results: Bioinformatics analysis followed by sanger sequencing revealed that three NOA brothers were homozygous for a rare missense variant in Cyclin Dependent Kinase Regulatory Subunit Associated Protein 2 (Centrosomin) *CDK5RAP2* (NM_018249:exon26:c.A4003T:p.R1335W, rs761196443). Protein modeling demonstrated that CDK5RAP2, Arg1335Trp resided nearby the Microtubule Associated Protein RP/EB Family Member 1 (EB1/MAPRE1) interaction site. As a consequence of the R1335W mutation, the positively charged Arginine was replaced by to the hydrophobic tryptophan residue, possibly leading to local instability in the structure and perturbation in the CDK5RAP2-MAPRE1 interaction.
Conclusion: Our study reports a novel missense variant of *CDK5RAP2* that segregates in homozygosity with male infertility and NOA in a consanguineous family. In silico structural predictions and gene expression data indicate a potential role of the *CDK5RAP2* variant in causing defective centrosomic maturation during spermatogenesis.

Introduction

One percent of worldwide men and 10%–20% of infertile men with azoospermia suffer of non-obstructive azoospermia (NOA). NOA is described as the absence of sperm in the ejaculate because of spermatogenesis arresting. Main types of testicular pathological phenotypes in NOA are sertoli cell-only syndrome [SCOS], maturation arrest [MA], hypo spermatogenesis, and mixed forms [1]. In NOA men, follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels, testis volume, and degree of androgenization can differ. About 30–50% of male infertility cases are idiopathic. Genetic abnormalities related to NOA affect about 30% of cases and consist of aberrations of sex chromosomes and monogenic defects related to spermatogenesis. About 40 genes have been involved in NOA so far [2]. For example, mutations in *STAG3, MSH4, Tex11 and PNLDC1* have been associated with NOA [3–6]. Therefore, searching for

Abbreviations: NOA, Non-obstructive azoospermia; NGS, Next Generation Sequencing; WES, Whole-Exome Sequencing; γTuRC, γ-Tubulin ring complex; TOC, Microtubule organising centre; MTs, Microtubules; PCM, Pericentriolar material; EB1/MAPRE1, Microtubule Associated Protein RP/EB Family Member 1; CDK5RAP2, Cyclin Dependent Kinase Regulatory Subunit Associated Protein 2 (Centrosomine).

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unknown pathogenic causes of defected spermatogenesis can be a valued work. Next generation sequencing (NGS) technologies, such as whole exome sequencing (WES) is a precise technique in the identification of novel candidate genes associated with NOA [5].

In animal cells, the centrosome is a compound organelle that functions as a microtubule organizing center (MTOC) and its role is a cell cycle regulator [7]. In S-phase, duplication of the centrosome supports the formation of two MTOCs in the M-phase that define the poles of the spindle fibers and ensure equal distribution of chromosomes and centrosomes to the two genetically identical daughter nuclei [8]. Centrosomes contain a pair of centrioles bounded by layers of pericentriolar material (PCM) [9]. A centriolar MTOC assembly can be driven by self-organization of PCM and microtubules (MTs) minus ends and needs γ-tubulin, pericentrin, CDK5RAP2 and ninein [10,11]. CDK5RAP2 with EB1/MAPRE1, stimulate MTs polymerization from the centrosome, bundle formation, growth and dynamics at the plus ends [12]. *Cdk5rap2* mt/mt male mice have reduced germ cell pool and are infertile. Germ cell reduction happens because of early development through a mitotic delay, long cell cycle, and apoptosis [13,14]. Akap9 interacts with Cdk5rap2 which is essential for spermatogenesis and sertoli cell maturation in mice [15]. LGALS3BP also interacts with CDK5RAP2, which is known as one of the biomarkers of azoospermia in humans [16].

Here, we report a missense variant in *CDK5RAP2* (NM_018249: exon26:c.A4003T: p.R1335W, rs761196443) nearby the EB1/MAPRE1 interaction site segregating in homozygosity in three brothers born to consanguineous parents and affected by NOA. A role of CDK5RAP2 in NOA is proposed for the first time.

Materials and methods

Patient selection

An infertile 28-year-old male (IV-3) was enrolled at Royan Institute Infertility Clinic in Tehran, Iran. He has been married for 7 years and has suffered from infertility for eight years. Genetic counseling shows that he was born to first-cousin parents with a history of infertility in three brothers, IV-3, IV-4, IV-5 and 2 uncles, III-1 and III-4 (Fig. 1). Because of the consanguinity of his parents and the frequency of infertility in his brothers, we chose IV-3, IV-4, IV-5 as a NOA and the healthy brother IV-6 as a control candidate for WES. The routine diagnostic ways failed to determine the reason of infertility, too. Each participant signed an informed written agreement form. This study was approved by the Royan Institute Ethics Committee (IR.ACECR.ROYAN.REC.1400.019).

Clinical investigations

We analyzed the semen of the infertile males (IV-3, IV-4, IV-5) in accordance with the World Health Organization (WHO) 2010 manual and recorded their reproductive history. The man with no clinical evidence of obstruction is the NOA patient. All infertile brothers did complete andrological examinations, investigation of their medical histories, physical assessments, and gonadal ultrasonography. Serum concentrations of FSH, LH, and testosterone were analyzed by electrochemiluminescence immunoassay (ECLIA) using the COBAS E-601 and E-411 devices (Roche, Germany). We performed karyotyping of lymphocytes collected from the patients' peripheral blood samples and AZF microdeletions analysis tests by multiplex PCR assay for the standard genetic testing [17] (Fig. 2).

DNA isolation and whole exome sequencing

We extracted genomic DNA from peripheral blood samples of the available family members (IV-3, IV-4, IV-5, IV-6), by using the salting-out procedure (Fig. 1) and high-quality DNA samples from individuals were selected for WES. DNA integrity, purity and quality were verified through gel electrophoresis and a260/280 and a260/a230 ratios by NanoDrop (Thermo Fisher Scientific, USA). SureSelect Human All Exon V5 kit was performed for library construction and capturing target regions (Agilent, USA), tracked by paired-end 150-bp sequencing on the Illumina HiSeq 2500 platform (Illumina, USA). DNA fragments were barcoded and sequenced on lanes of a fl2500 p. Reads were mapped to the UCSC reference human genome assembly GRCh37 (hg19) using the Burrows-Wheeler Aligner (BWA) package version 0.7.17-r1188 [18]. Mapped files in SAM format were converted to the BAM format and sorted by SAM tools version 0.1.18. Marking PCR duplicates was performed by Picard tools (https://broadinstitute.github.io/picard/) version 1.107. GATK 4.1.1.1 was used for the processing steps, including base quality score recalibration [19]. These steps built appropriate BAM files that underwent variant calling by HaplotypeCaller [20]. We used ANNOVAR for annotating and filtering VCF files and shortlisted candidates which were arranged according to the American College of Medical Genetics and Genomics (ACMG) guideline [21,22]. Thus, rare or novel variants, excluding synonymous variants, stayed for further analysis. *In silico* assessment of missense variants was directed with BayesDel_addAF, DANN, EIGEN, FATHMM-MKL, LIST-S2, M-CAP, Mutation assessor, MutationTaster, SIFT, SIFT4G, CADD, PolyPhen-2, LRT, PROVEAN, DEOGEN2 [23,24].

Segregation analysis (Sanger sequencing)

Sanger sequencing is the standard test for genotyping. Oligonucleotide primers nearby the candidate variant were designed by PerlPrimer-1.1.21. They used to amplify the region of interest for the pedigree participants by polymerase chain reaction (PCR): 5' -AACAAGTGGAATAGTGCTTCTG - 3' (forward primer) and 5' - GTTTATCTTGCAGGTATCTGGG - 3' (reverse primer). The amplicons were sequenced through Sanger sequencing with Macrogen (Seoul, Korea). The produced sequence was aligned with the human reference genome (hg19/b37) and analyzed by Chromas software.

In silico protein modeling of CDK5RAP2 and single-cell RNA sequencing data analysis

The 3D structure of CDK5RAP2 was modeled due to the lack of an experimental 3D structure of this protein in the protein data bank (PDB) [25]. The protein modeling was carried out with the I-TASSER web server [26]. This tool only models proteins with a sequence length between 10 and 1500, whereas the protein sequence length of the CDK5RAP2 is 1893 amino acids. So, The FASTA sequence of CDK5RAP2 (420-1893) was extracted from the UniProtKB database (UniProtKB ID: Q96SN8) [27] and submitted as input to the I-TASSER server. The I-TASSER uses threading alignments and pair-wise structural similarity to build models from the most significant templates and cluster them, respectively. Finally, the best structural model with the highest TM-score and C-score was selected for further analysis. The TM-score and C-score measure structural similarity and structural quality, respectively. The psfgen plugin in VMD1.9.3 was used to implement the R1335W mutation in the best model of CDK5RAP2, and the structural model was shown by VMD 1.9.3 [28].

The expression level of CDK5RAP2 and MAPRE1in the spermatogenesis cells was extracted from our previous research on the integration of scRNA-seq data of spermatogenesis [29]. That integrated scRNAseq data was generated based on GEO datasets of GSE109037 [30] and GSE106487 [31]. The scRNA-seq data analysis was performed by the Seurat3.2 R package [32] (see Table 1).

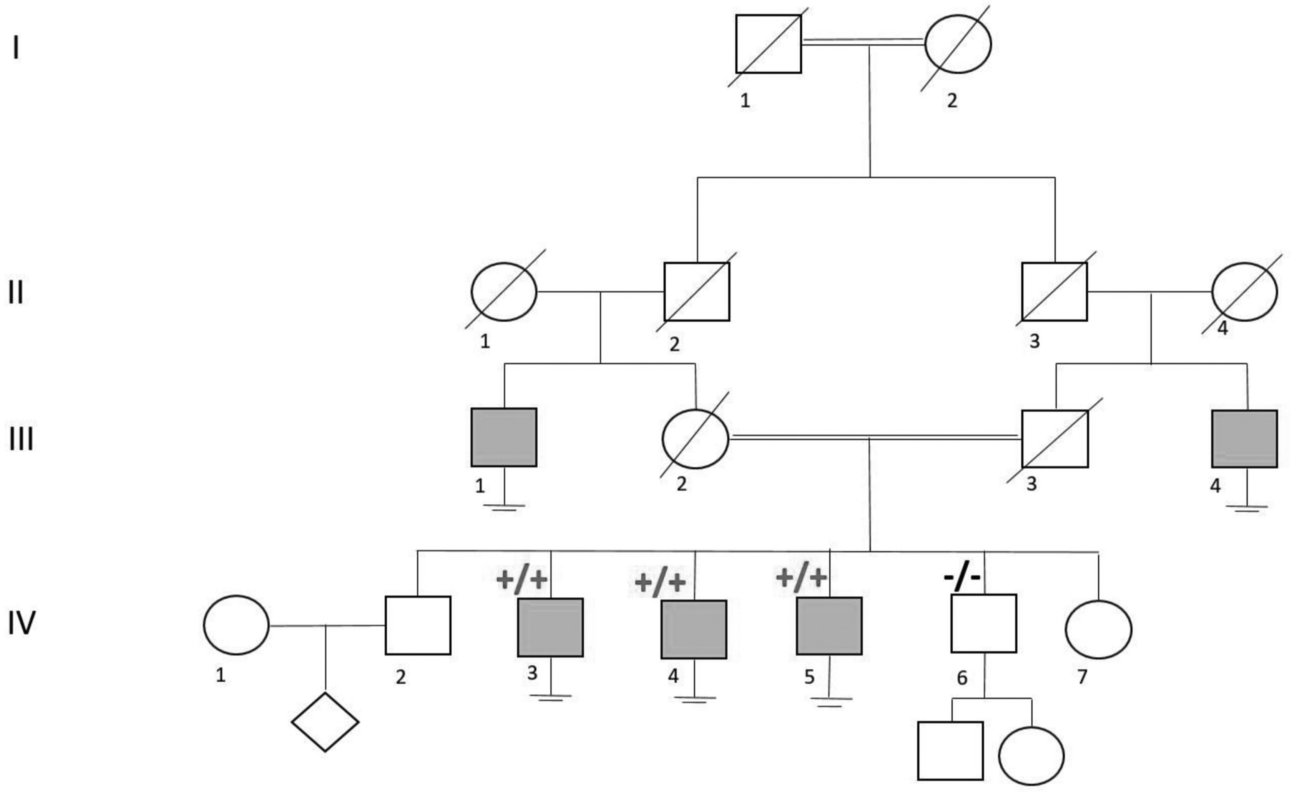

Fig. 1. Pedigree of a consanguineous family with non-obstructive azoospermia patients. A consanguineous marriage between first cousins has produced three infertile offsprings. The NOA patients are shown in orange squares. IV-3, IV-4, IV-5 were exome sequenced. All NOA patients were homozygous for a rare missense variant in *CDK5RAP2* (NM_018249:exon26:c.A4003T:p.R1335W). The "-" marks the wild-type allele. The proband is marked with an arrow. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Result

Clinical findings

Pedigree's analysis revealed that the three brothers were infertile, and genetic counseling demonstrated they were born to first-cousin parents and they have two infertile uncles (Fig. 1). Physical examination of the patients showed normal external male genitalia, palpable vas deferens and normal testicular volume. Further clinical investigations revealed no history of infection, chronic disease, vasectomy and no obstructions were informed by transrectal and scrotal ultrasonography. They had normal karyotype 46, XY and Y chromosome microdeletion genetic tests that included azoospermia factors a, b, and c (AZFa, AZFb, and AZFc) were negative (Table 1) (Fig. 2). Semen analysis of all affected cases indicated NOA in cases IV-3, IV-4, and IV-5. The serum levels of FSH, LH, and testosterone were within the normal range in these individuals (Table 2) (see Table 2).

Cdk5rap2 variant detection and impact

We performed WES on proband (IV-3) and his brothers (IV-4, IV-5, IV-6). A minimum of 94% of the total reads passed the initial quality filter ($Q \geq 30$), and 24.6 gigabases of sequence data were generated. The patients were born after two generations of consanguineous marriage between first cousins. Our estimated family inheritance pattern was autosomal recessive. We reached a shortlist *(RP1L1, CDK5RAP2)* after the removal of intronic, intergenic, and synonymous single nucleotide variants (SNVs) that shared between the affected subjects (Supplementary Table 1). Because *RP1L1* had a autosomal dominant inheritance pattern and didn't involve in spermatogenesis and didn't have any damaging report by pathogenic computational verdict, we prioritized a deleterious variant in *CDK5RAP2* that shared between the affected subjects for NOA, expressed in the testis and involved in cell cycle, and spermatogenesis. Also, we dindn't see This variant in healthy brother. This rare homozygous nonsynonymous SNV was in the *CDK5RAP2* gene located on chromosome 9 (NM_018249:exon26:c.A4003T:p.R1335W, rs761196443) in the proband (IV-3) and his brothers (IV-4, IV-5) in homozygous form with NOA. The candidate mutation was termed damaging by pathogenic computational verdict based on 7 predictions from DEOGEN, LIST-S2, PROVAN, Mutation Assessor, SIFT, SIFT 4G, Polyphen2_HDIV [23,24]. GnomAD exomes homozygous allele count was 0 with the good gnomAD exomes coverage (69.5). GERP++ was used which ranked this variant as highly conserved with a score of 4.87 out of a maximum score of 6.17, for conservation assessment [33]. PhyloP100way was 3.71, which is less than 6.8. The variant is quite rare, with a MAF of 0.00002, and according to gnomAD, it had never been reported in homozygous states. Furthermore, the variant is absent in the Iranome database consisting of 800 healthy Iranian individuals. The identified mutation was located in exon 26 of the *CDK5RAP2* gene, which participates in encoding of the CDK5RAP2 protein (Fig. 3A). Multiple alignments of the residues encoded by exon 26 of the human *CDK5RAP2* with several species from different kingdoms confirmed conservation as the Arginine residue (Fig. 3B). Subsequent Sanger sequencing confirmed the homozygous status (+/+) of the c.A4003T mutation in the azoospermic proband (IV-3) and in his azoospermic brothers (IV-4, IV-5). In contrast, the unaffected brother (IV-6) was homozygous (−/−)

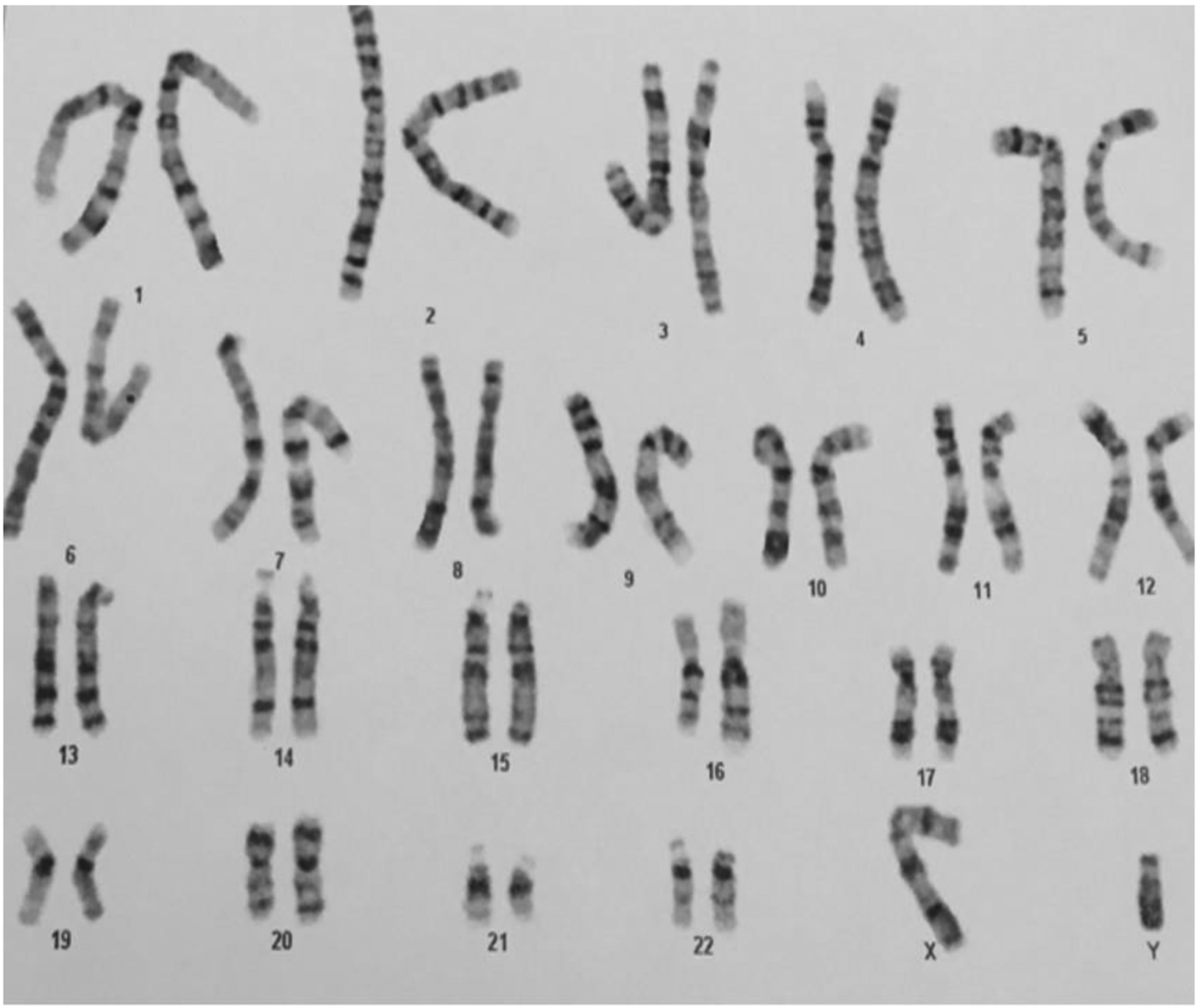

Fig. 2. Karyotype result of IV-3. 15 metaphase spreads were studied on the basis of GTG teqnicque at 420-450 band resolution revealing 46 chromosomes. No chromosomal aberration detected. 46, XY, compatible with apparently normal male.

Table 1
Clinical findings.

Subject (see Fig. 1)	IV-3	IV-4	IV-5
Age (y)	28	25	22
History of infertility (y)	7	5	3
Attempted microTESE	No	No	No
External genitalia	Normal	Normal	Normal
Secondary traits	Normal	Normal	Normal
Testis size (ml)	Reduction	Reduction	Reduction
vas deferens	Palpable	Palpable	Palpable
Semen analysis	Azoospermic	Azoospermic	Azoospermic
Ultrasonography	No obstructions	No obstructions	No obstructions
Histopathology	–	–	–
AZF microdeletions testing	No	No	No
Karyotype	46,XY	46,XY	46,XY
Past Hospital Admission	No	No	No

Table 2
Hormone concentrations.

Patient	IV-3	IV-4	IV-5	Reference Range
FSH (IU)	4.68	11.40	6.30	Male: 1.5–12.4 Female: 3.5–12.5
LH (IU)	8.86	4.90	4.50	Male: 1.7–9.1 Female: 2.4–12.6
Testosterone (ng/ml)	6.25	5.90	5.60	Male: 2.49–8-36

wild-type (Fig. 3C). These findings were consistent with the autosomal recessive pattern of inheritance. Genotyping results of the full pedigree are available in Fig. 1. This mutation causes substituting an Arginine residue with Tryptophan near residues 938-9 which is as important residues are in an interaction site with EB1/MAPRE1 (Fig. 4A).

Population study

CDK5RAP2 mutation (c.A4003T) was studied in 30 Iranian men with idiopathic azoospermia who had normal physical examinations, normal karyotype, and no Y chromosome microdeletions to evaluate whether this mutation can explain the failure in sperm counts. No homozygous patients with *CDK5RAP2* (c.A4003T) were found.

In silico protein modeling of CDK5RAP2 and single-cell RNA sequencing data analysis

The 3D structural model of wild-type of CDK5RAP2 was constructed which showed the closest structural similarity to the chromosome partition protein MukB (PDB ID: 7NYX: A) with a 1.78 Å RMSD value. The chromosome partition protein MukB in the threading alignments showed 89% coverage with a 5.69 normalized Z-score while a normalized Z-score greater than 1 means a high confidence alignment in the I-TASSER results. The R1335W mutation was implemented in the model and shown in Fig. 4A. The interaction of CDK5RAP2 protein with the NCKAP5L was reported to regulate MTs stability [12]. However, the binding site of NCKAP5L in CDK5RAP2 (residues 58-196) is far from the R1335W mutation point. Also, interactions of CDK5RAP2 with PCNT and AKAP9 were detected and residues of 1726–1893 were indicated as the binding site in CDK5RAP2 [34]. On the other hand, residues 926 to 1208 in CDK5RAP2 were reported as the binding region for MAPRE1 binding and residues 938-9 as important residues in this binding region whose changes can cause loss of interaction with MAPRE1 [35]. The binding regions of CDK5RAP2 for PCNT, AKAP9, and MAPRE1 were indicated in our model (Fig. 4A), which also shows that the important residues of the MAPRE1 binding site are closer to the R1335W mutation point.

We looked at the expression levels of CDK5RAP2 and MAPRE1 during spermatogenesis by interrogating our previously described dataset [29]. We found that the highest expression levels for CDK5RAP2 and MAPRE1 were in Zygotene spermatocytes and differentiated spermatogonia cells, respectively (Fig. 4B).

Discussion

Spermatogenesis is an evolutionarily conserved progression including mitotic cell division, meiosis and spermiogenesis to

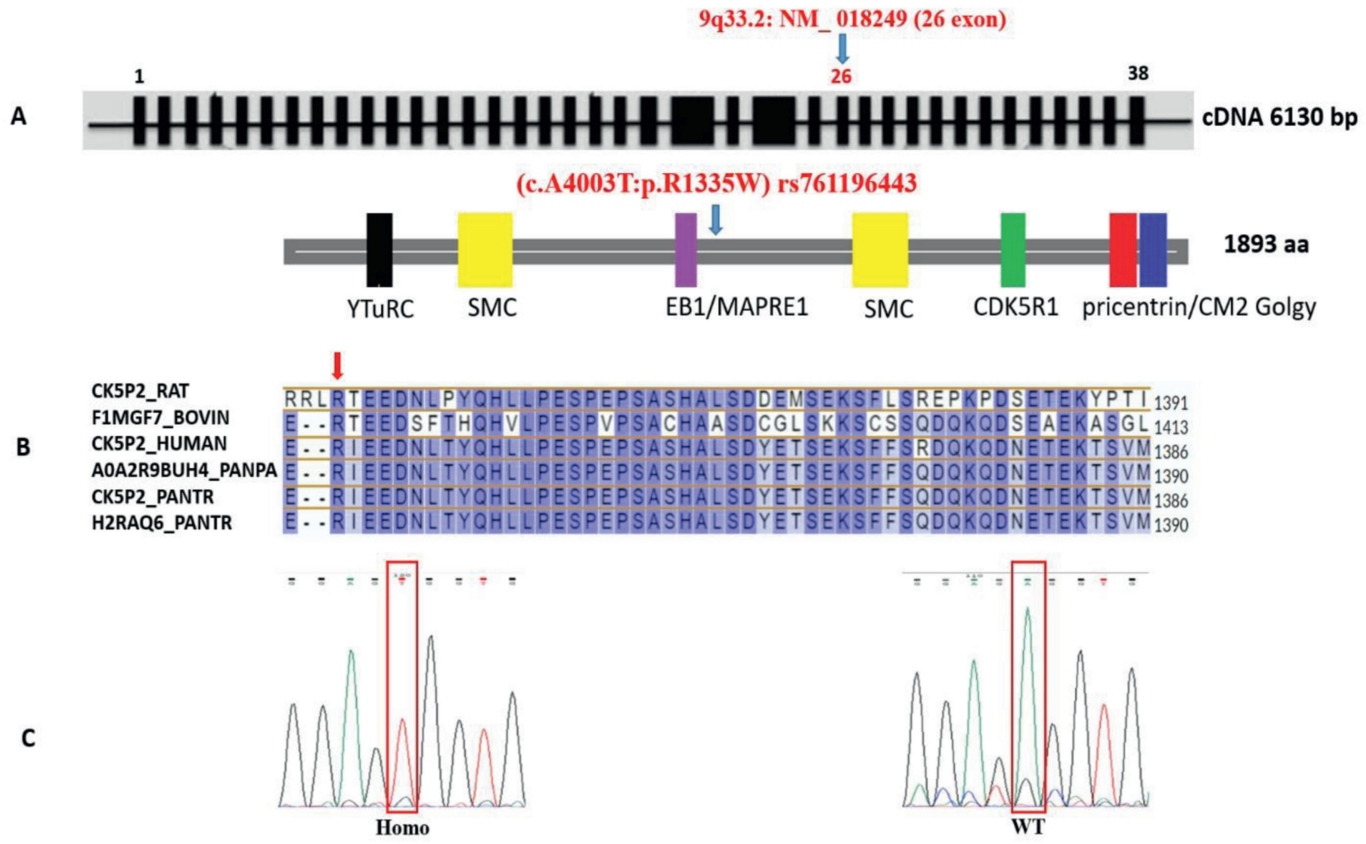

Fig. 3. Identification of *CDK5RAP2* variant in the homozygous state leading to non-obstructive azoospermia. A) *CDK5RAP2* is composed of 38 exons encoding a protein with 1893 amino acid residues. The discovered variants (R1335W) is close to EB1/MAPRE1 binding site in the protein. In the R1335W mutation, the arginine as a positively charged amino acid was changed to the tryptophan as a hydrophobic amino acid. B) Orthologous alignment showing that the amino acid residues is conserved, https://www.uniprot.org. C) Sanger sequencing validated WES results. Homo: homozygous for the variant; WT: homozygous for the wild type allele.

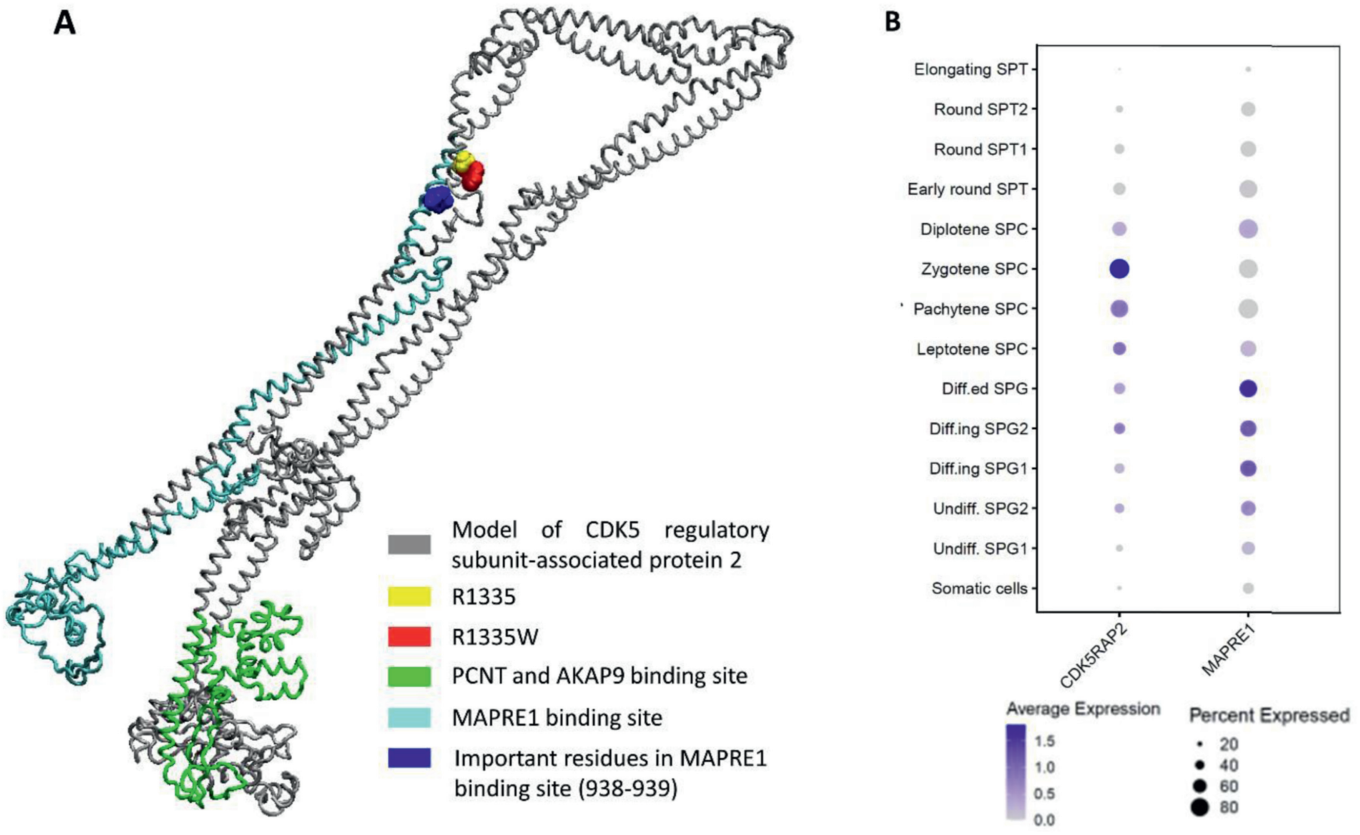

Fig. 4. *In silico* protein modeling and scRNA-seq expression of CDK5RAP2 (A) The 3D structural model of CDK5RAP2. The R1335, R1335W, PCNT, and AKAP9 binding site, MAPRE1 binding site, and important residues in the MAPRE1 binding site (residues of 938 & 939) are highlighted. (B) The expression pattern of CDK5RAP2 and MAPRE1 in spermatogenesis scRNAseq data.

produce mature sperms from stem cells [36]. A number of mutant genes were identified in previous studies which were associated with meiotic arrest and male infertility; namely, *MSH4, STAG3*, *SYCP1 and PNLDC1* [3–5,37]. Centrosomes are elaborated in these processes as MTOC. So, centrosomes and MTs perform critical roles during cell division and differentiation [7]. Centrosomin (CDK5RAP2) is a joint section of the mitotic and meiotic centrosomes and is essential for suitable formation, anchoring, and orientation of MTs from the centrosome (Fong et al., 2008). Also, CDK5RAP2 participates in centriolar MTOC assembly which is driven by self-organization of PCM in interphase mammalian cells [10].

The rare nonsynonymous SNV discovered here for NOA is described as *CDK5RAP2*: (NM_018249:exon26:c.A4003T:p.R1335W). The *CDK5RAP2* gene contains 38 exons, maps to chromosome 9q33.2, and encodes a protein with 1870 amino acids (Fig. 3A). CDK5RAP2 regulates mitotic spindle checkpoint activation by performing as a transcriptional regulator of BUBR1 and MAD2 promoters [12]. Moreover, the interaction of CDK5RAP2 and EB1/MAPRE1 stimulates MTs polymerization, bundle formation, and growth at the plus ends by connecting the γ-tubulin ring complex (γTuRC) to the centrosome [12]. EB1/MAPRE1 is involved in DNA damage and separation of sister chromatids by associating with the centrosomes and spindle MTs in interphase cells during mitosis [35]. We hypothesize that this variation severely disrupts the interaction of CDK5RAP2 and MAPRE1 and increases germ cell centrosome deficiency.

MTs are highly dynamic and polarized. They are controlled by a group of various proteins that binds to the plus ends, such as EB1 and CDK5RAP2. They are named plus-end tracking proteins (+TIPs). The structure of EB1 involves a calponin homology domain, a coiled-coil region, and a tail region. The first domain is responsible for MTs binding, the second one controls its homodimerization and the last one is for binding to +TIPs. Also, the association between CDK5RAP2 and EB tracks MTs plus ends. Structurally, CDK5RAP2 contains a basic and Ser-rich motif similar to the other +TIPs. This motif is responsible for EB1 interaction and is conserved in the chimpanzee, bovine, and dog but not in the rat and mouse [35]. CDK5RAP2 has 2 isoforms containing 1893 and 1814 amino acids. Both isoforms have an N-terminal microtubule-association domain and 10 coiled-coil domains. CDK5RAP2 staining indicates its presence on centrosomes during cell cycle [38]. Consistent with previous studies [29], our single-cell RNA sequencing data indicate that the highest expression levels for CDK5RAP2 and MAPRE1 during human spermatogenesis are in Zygotene spermatocytes and differentiated spermatogonia cells, respectively (Fig. 4B).

CDK5RAP2 has been associated with autosomal recessive primary microcephaly and its functional role has been studied mostly in brain development. However, its function in other organs has been poorly investigated. Concerning male infertility, *Cdk5rap2* mutant mice results in lack of spermatogenic cells in adults because of mitotic delay, prolonged cell cycle, and apoptosis [13,14].

CDK5RAP2 has a multifunctional role at centrosomes, including a MTs organizing function and an indirect control of centrosome cohesion. To achieve the first role, CDK5RAP2 binds to γTuRC to assemble centrosomal γ-tubulins. In this way, it interacts with EB1 through the basic and Ser-rich motif [35]. Centrosome deficiency is related to the DNA damage checkpoint response because mutations of several PCM components (PCNT, MCPH1 and CDK5RAP2) are associated with a damage checkpoint-mediated cell cycle arrest at

the G2/M boundary, also activation of the DNA damage response pathway initiates p53 signaling and causes cell cycle arrest or cellular death [7]. Moreover, CDK5RAP2 interacts with AKAP9 and LGALS3BP which are critical for spermatogenesis and sertoli cell maturation in mice and considered biomarkers of azoospermia in humans [15].

Our study demonstrated a rare homozygous nonsynonymous SNV in the *CDK5RAP2* gene in three siblings of a consanguineous family, which segregated with NOA. The homozygous status (+/+) of the c. R1335W mutation of the three azoospermic brothers and the homozygous wild-type status (−/−) of the healthy brother (Fig. 3C). These findings were consistent with the autosomal recessive pattern of inheritance (Fig. 1).

Construction of the 3D structural model of wild-type CDK5RAP2 allowed to map the binding region for MAPRE1 and demonstrate that the R1335W mutation point is close to important residues for MAPRE1 binding. The R1335W variant changes a positively charged amino acid into a hydrophobic one, possibly leading to local instability in the structure and perturbation of the CDK5RAP2-MAPRE1 interaction. We expect that this alteration may disturb the binding of the complex to centrosomes and plus ends of spindle MTs in interphase cells during mitosis. Then, dysfunction of centrosome MTs organization would activate the DNA damage checkpoint response, p53 signaling, and apoptosis. Finally, raising of cell cycle arresting would lead to spermatogenic cell death.

Altogether, our findings indicate a novel function for the *CDK5RAP2* gene within male infertility and NOA and linked with alteration of CDK5RAP2-MAPRE1 interaction and germ cell centrosome deficiency.

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Statement of ethics

This study was approved by the Royan Institute Ethics Committee (IR.ACECR.ROYAN.REC.1400.019). Each participant signed an informed written agreement form.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgment

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tjog.2023.03.015.

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Original Article

The effect of various air pollution and participants' age on semen quality in southern Taiwan

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ABSTRACT

Objective: This study aimed to investigate the association between semen quality and air pollution in southern Taiwan.
Materials and methods: In this retrospective study, 4338 males aged 21–70 years were recruited between 2001 and 2018 from a reproductive medical center. Semen quality was assessed according to standardized methods outlined in the World Health Organization (WHO) Laboratory Manual 1999 and 2010, including total sperm count, progressive sperm motility (%), rapid progressive sperm motility (%), and sperm with normal morphology (%). All designated national air quality automatic continuous monitoring stations measured the levels of air pollution [particulate matter (PM_{10} and $PM_{2.5}$), sulfur dioxide (SO_2), nitrogen dioxide (NO_2), carbon monoxide (CO), ozone (O_3)], and was documented by Environmental Protection Administration in Taiwan. We collected data on the levels of air pollution based on the participants' residential addresses.
Results: In our study, we found that progressive and rapid progressive sperm motility significantly decreased annually ($p < 0.05$). In addition, increasing age influenced total sperm count, progressive sperm motility, rapid progressive sperm motility, and sperm with normal morphology ($p < 0.05$). Among different air pollution, we observed SO_2 was associated with lower rapid progressive sperm motility and lower sperm with normal morphology ($\beta = -0.103$, $p = 0.043$; $\beta = 0.118$, $p = 0.001$, respectively). However, NO_2 was associated with higher rapid progressive sperm motility and a high number of sperm with normal morphology ($\beta = 0.129$, $p = 0.002$; $\beta = 0.127$, $p < 0.001$, respectively).
Conclusions: The semen quality in southern Taiwan appears to have declined in recent years. The participant's age for semen analysis was most strongly associated with semen parameters, Moreover, a significant association between SO_2 and NO_2 levels and semen motility was observed, even after adjusting for multiple comparisons. Further study is required to analyze the dose-dependent effect of SO_2 and NO_2 on semen parameters.

Introduction

Over the past 50 years, decreasing sperm counts has been a subject of much analysis but the results of these studies have been controversial [1]. Certain semen parameters play a significant role in male infertility (concentration) while others (morphology, apart from globozoospermia) do not. DeVilbiss et al. reviewed semen parameters were critical for achieving live birth in couples using ovulation induction only or no treatment [2]. Therefore, the decline in semen quality is a serious issue in human infertility and may be one of the causes of fertility rates decline. In 1992, evidence of a decline in sperm concentration over half a century in the USA and Europe was reported [3]. In 2017, a systematic review and meta-regression analysis on trends in human sperm count was published, which showed a significant decline in sperm concentrations between 1973 and 2011 among "unselected" (−1.38 million per mL per year) and fertile men (−0.68 million per mL per year) [4]. In

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China, several studies have reported a remarkable decline in semen quality among sperm donors [5,6]. However, inconsistencies have been reported in other Asian countries [7–9].

Climate change effects on reproductive health have attracted attention [1].

Air pollution is the world's largest environmental health risk. There has been a large volume of epidemiological studies examining air pollution and its various health outcomes, including birth defects. However, only a few studies with small samples have investigated the health effects of air pollution on semen quality in humans, and the results have been inconsistent [10–12]. Furthermore, most of these studies have focused on acute or short-term influences, despite the prolonged exposure people generally suffer. Chronic and low-dose exposure may contribute to significant impairment of spermatogenesis [13]. This study aimed to investigate semen quality by analyzing semen parameters between 2001 and 2018, and evaluate the health effects of exposure to different air pollutants on semen quality in Taiwanese men.

Materials and methods

Patients

Data were collected between 2001 and 2018 from a reproductive medical center at Kaohsiung Chang Gung Memorial Hospital in this retrospective study. All patients came to our reproductive center for fertility counseling, and their spouses received routine semen analysis. Participant with a history of testicular surgery or injury, history of cryptorchidism and hypogonadal men were excluded from the study. Four-thousand three-hundred and thirty-eight male participants were included in this study. It was approved by the Institutional Review Board (IRB) of Chang Gung Memorial Hospital (CGMH 201901110B0; approved August 12, 2019). It is not a research study that involve human participation. Therefore, the need of the written informed consent was waived by the Institutional Review Board (IRB) of Chang Gung Memorial Hospital. All methods were performed in accordance with the relevant guidelines and regulations of the Institution.

Semen analysis

All participants abstained for 3–7 days, and semen samples were obtained by masturbation at home or in the laboratory. The semen sample was placed in a sterile container and sent to the College of American Pathologists (CAP)-accredited laboratory; a qualified laboratory technician must complete routine semen analysis within 3 h after ejaculation. Semen quality parameters were assessed according to standardized methods outlined in the WHO Laboratory Manual 1999 and 2010, including total sperm count, progressive sperm motility (%), rapid progressive sperm motility (%), and sperm with normal morphology (%). For internal quality control, all routine semen analyses were conducted by the same laboratory director to remove technician bias. Sperm concentration (count) was estimated using a microscope (400× magnification) and the grid of the counting chamber. Scanning the slide and calculating the number of spermatozoa per field provides an approximate sperm concentration in 10^6/mL, then, multiplied by semen volume amounts to the total sperm count. Sperm motility was divided into four grades: (a) rapid progressive motility, (b) slow or sluggish progressive motility, (c) nonprogressive motility, and (d) motility. Progressive sperm motility was graded as (a) and (b). To classify sperm morphology, samples were analyzed using stained smear slides. Two-hundred sperm cells were examined, and the percentage of morphologically normal sperm was calculated.

Air pollution

The designated national air quality automatic continuous monitoring stations measured the levels of air pollution [particulate matter (PM_{10} and $PM_{2.5}$), sulfur dioxide (SO_2), nitrogen dioxide (NO_2), carbon monoxide (CO), ozone (O_3)] in different residential area, and was documented by Environmental Protection Administration in Taiwan (Supplemental Fig. 1, Supplemental Table 1). The annual average levels of air pollution were collected based on the participants' residential areas and the year of semen collection. The $PM_{2.5}$ level was available after 2008.

Statistical analysis

Participants were categorized the year of examination (2001–2018). The reproductive medical center was closed for one year in 2006 for maintenance. Before linear regression analysis, log-transformation of semen parameters was performed when the semen parameter data were not normally distributed. A simple linear correlation was used to evaluate the effect of age and year of examination on the semen parameters. Multiple linear regression was used to predict the effect of age or year of examination on semen parameters after adjusted for confounders such as BMI, and abstinence time. Multiple linear regression was used to predict the effect of air pollution on semen parameters after adjusted for confounders such as age, year of examination, BMI and abstinence time. All data were analyzed using SPSS version 22 (SPSS, Chicago, Illinois, USA) for Windows.

Age-period-cohort (APC) analyses were performed to determine the effect of age on semen parameters. All the participants were categorized into birth cohorts in accordance with their age in 2001, and the change in their semen parameters was observed between 2001 and 2018 with an age of 59 years as the follow-up endpoint. For example, a participant aged 25 years old in 2006 and the other aged 29 years old in 2010, these two participants were both at same age-period-cohort (20 in 2001).

Semen parameters for different birth cohorts were plotted to demonstrate the APC effect. The APC analysis was performed using Microsoft Office Excel 2019 (Microsoft Corporation, USA). The results were considered significant when two-sided $p < 0.05$.

Results

Total of 4338 participants were recruited for our study. The age of the participants ranged from 21 to 70 years, with a mean age of 36.9 ± 5.65 years. Most of participants sought infertility treatment in 2016 (10.7%) and 2017 (10.2%) (Table 1).

In all participants, the mean total sperm count was 73.0 (IQR: 30.0–107.0) × 10^6, progressive sperm motility (%) was 58.5% (IQR: 36.1–72.0%), rapid progressive sperm motility was 14.1% (IQR: 4.1–25.4%), and sperm with normal morphology was 42.8% (IQR: 25.3–53.1%).

After adjusted the confounders such as the year of examination, BMI, and abstinence time of participant, the rudimentary results of the distribution of semen parameters by age are shown in Fig. 1. To understand the relationship between semen parameters and age, linear regression was used to calculate the changes in semen parameters with increasing age. In Table 2, increasing age (per year) was significantly and negatively associated with progressive sperm motility, rapid progressive sperm motility, and sperm with normal morphology (−1.309%, −1.315%, and −1.429%, respectively; all $p < 0.0001$). However, the total sperm count was not associated with age.

After adjusted the confounders such as age, BMI, and abstinence time of participant, the rudimentary results of the distribution of semen parameters by year are shown in Fig. 2. Linear regression

Table 1
Summary of different semen parameters by year of data collection.

			Total sperm count (10^6)	Progressive motility (%)	Rapid progressive motility (%)	Normal morphology (%)
Year	N	%	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)
2001	239	5.5%	68.0 (32.0–90.0)	66.7 (40.5–80.6)	17.6 (4.0–30.0)	50.0 (41.2–57.1)
2002	229	5.3%	77.0 (42.0–98.0)	71.9 (43.8–82.7)	22.5 (10.0–30.8)	51.0 (41.7–57.1)
2003	135	3.1%	71.0 (33.5–92.0)	54.6 (32.1–75.4)	15.4 (3.9–23.4)	47.2 (39.3–52.3)
2004	175	4.0%	72.5 (29.5–94.0)	56.3 (37.2–77.5)	14.7 (2.7–25.2)	46.9 (38.1–52.6)
2005	88	2.0%	70.5 (35.0–94.0)	61.0 (39.3–75.6)	13.4 (6.1–23.0)	48.7 (41.7–53.3)
2007	74	1.7%	59.5 (6.5–82.3)	43.7 (20.3–63.1)	10.7 (0–19.3)	47.1 (30.4–53.0)
2008	177	4.1%	71.0 (18.0–101.0)	52.7 (32.9–70.6)	11.5 (0–25.0)	48.9 (31.0–55.0)
2009	214	4.9%	60.0 (12.5–102.8)	45.4 (19.4–71.6)	9.8 (0–25.4)	47.4 (22.2–57.1)
2010	216	5.0%	75.0 (19.8–117.3)	52.4 (26.9–69.5)	11.8 (0–26.1)	45.3 (23.0–55.4)
2011	278	6.4%	84.0 (40.3–119.8)	56.8 (33.5–70.6)	15.0 (3.2–26.6)	45.4 (31.7–53.9)
2012	287	6.6%	72.0 (30.0–114.0)	57.4 (32.5–69.0)	13.6 (3.9–25.5)	42.9 (32.0–52.8)
2013	320	7.4%	76.0 (33.8–115.0)	58.1 (35.9–69.9)	17.2 (4.8–26.2)	47.1 (33.3–55.6)
2014	360	8.3%	82.0 (24.5–119.0)	56.1 (33.2–69.3)	14.3 (3.6–25.7)	42.7 (23.3–54.7)
2015	426	9.8%	81.0 (35.0–114.8)	57.4 (36.6–68.1)	13.9 (4.5–25.2)	35.1 (21.1–48.4)
2016	465	10.7%	78.0 (35.0–115.0)	60.4 (43.1–71.8)	14.0 (6.0–23.6)	33.3 (20.2–47.6)
2017	441	10.2%	66.0 (35.0–102.0)	61.7 (44.8–72.9)	12.3 (5.6–21.4)	34.2 (20.7–45.9)
2018	214	4.9%	60.0 (28.5–102.8)	61.8 (43.5–73.0)	13.1 (4.9–21.5)	32.0 (18.2–43.2)
Total	4338	100.0%	73.0 (30.0–107.0)	58.5 (36.1–72.0)	14.1 (4.1–25.4)	42.8 (25.3–53.1)
p for trend			0.676	0.031*	<0.001*	0.228

*$p < 0.05$.

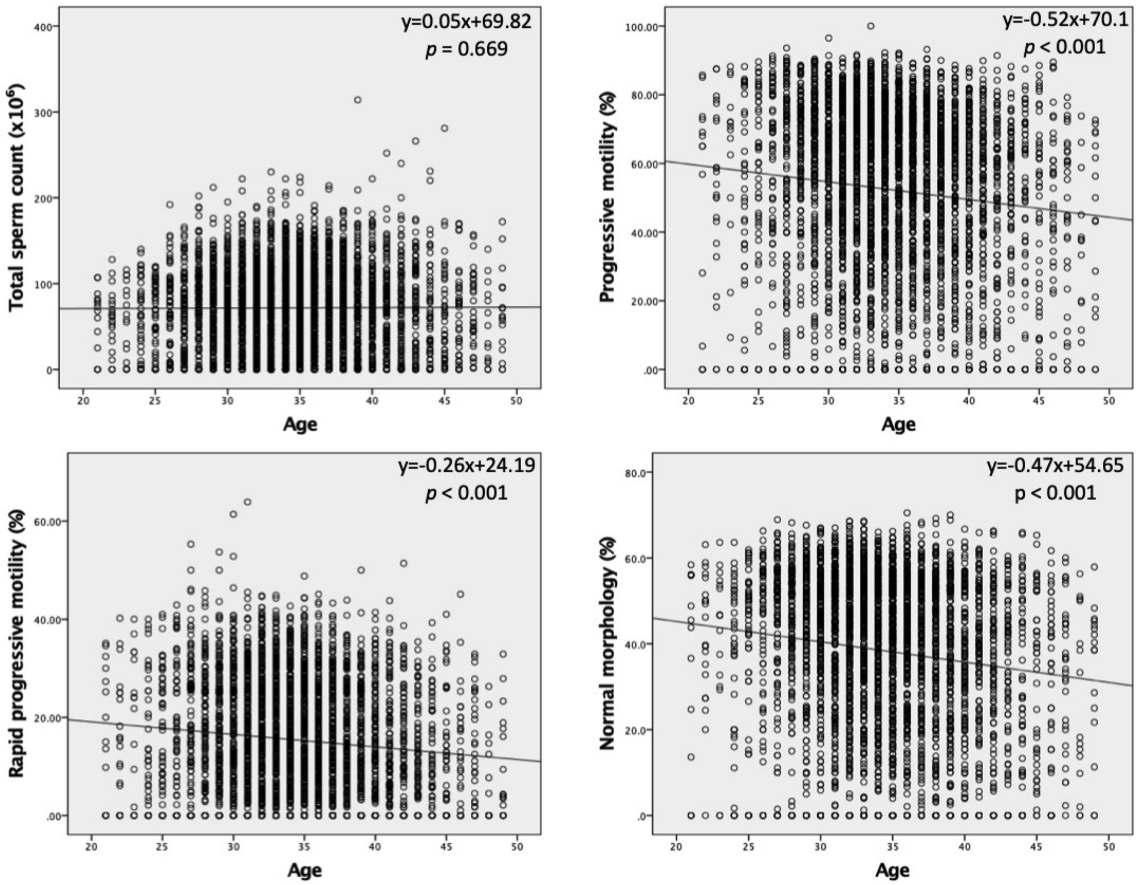

Fig. 1. Different semen parameters change with age.

was used to calculate the annual changes in semen parameters. In Table 2, rapid progressive sperm motility and sperm with normal morphology significantly reduced annually (−1.309%, and −1.991%, respectively; both $p < 0.0001$).

The mutual adjustments between the effects of age and year on the semen parameters are shown in Table 2. The effect of age on semen parameters was similar for both adjusted and unadjusted data (all semen parameters) after adjusting for the year of examination. Nevertheless, the effects of year on semen parameters (both adjusted and unadjusted data) were similar, except for progressive sperm motility (antilog-transformation $\beta = -0.001$, $p = 0.125$, unadjusted; antilog-transformation $\beta = 0.001$, $p = 0.489$, adjusted).

Table 2
Effect of age and year of data collection on different semen parameters.

	Age		Year	
Semen parameters	Coefficient[a] 95% CI	Coefficient[b] 95% CI	Coefficient[a] 95% CI	Coefficient[b] 95% CI
Number of sperm (10^6)	−0.003 (−0.006 to 0.001)	−1.007 (−1.014 to 1.002)	0.002 (−0.001 to 0.006)	1.005 (−1.002 to 1.014)
Progressive motility sperm (%)	−0.006* (−0.007 to 0.004)	−1.014* (−1.016 to 1.009)	−0.001 (−0.003 to 0)	−1.002 (−1.007 to 1)
Rapid progressive motility sperm (%)	−0.007* (−0.009 to 0.005)	−1.016* (−1.020 to 1.012)	−0.007* (−0.009 to 0.005)	−1.016* (−1.020 to 1.012)
Normal morphology sperm (%)	−0.007* (−0.009 to 0.006)	−1.016* (−1.020 to 1.014)	−0.013* (−0.015 to 0.012)	−1.030* (−1.035 to 1.028)
	Adjust-age[c]		Adjust-year[d]	
Semen parameters	Coefficient[a] 95% CI	Coefficient[b] 95% CI	Coefficient[a] 95% CI	Coefficient[b] 95% CI
Number of sperm (10^6)	−0.004 (−0.007 to 0)	−1.009 (−1.016 to 1)	0.003 (0 to 0.007)	1.007 (1 to 1.016)
Progressive motility sperm (%)	−0.006* (−0.007 to 0.004)	−1.014* (−1.016 to 1.009)	0.001 (−0.001 to 0.002)	1.002 (−1.002 to 1.005)
Rapid progressive motility sperm (%)	−0.006* (−0.008 to 0.004)	−1.014* (−1.018 to 1.009)	−0.005* (−0.007 to 0.003)	−1.012* (−1.016 to 1.007)
Normal morphology sperm (%)	−0.003* (−0.005 to 0.002)	−1.007* (−1.012 to 1.005)	−0.012* (−0.014 to 0.011)	−1.028* (−1.033 to 1.026)

*$p < 0.05$.
[a] Semen parameters were log-transformed.
[b] Coefficients were anti log-transformed.
[c] Adjusted by year.
[d] Adjusted by age.

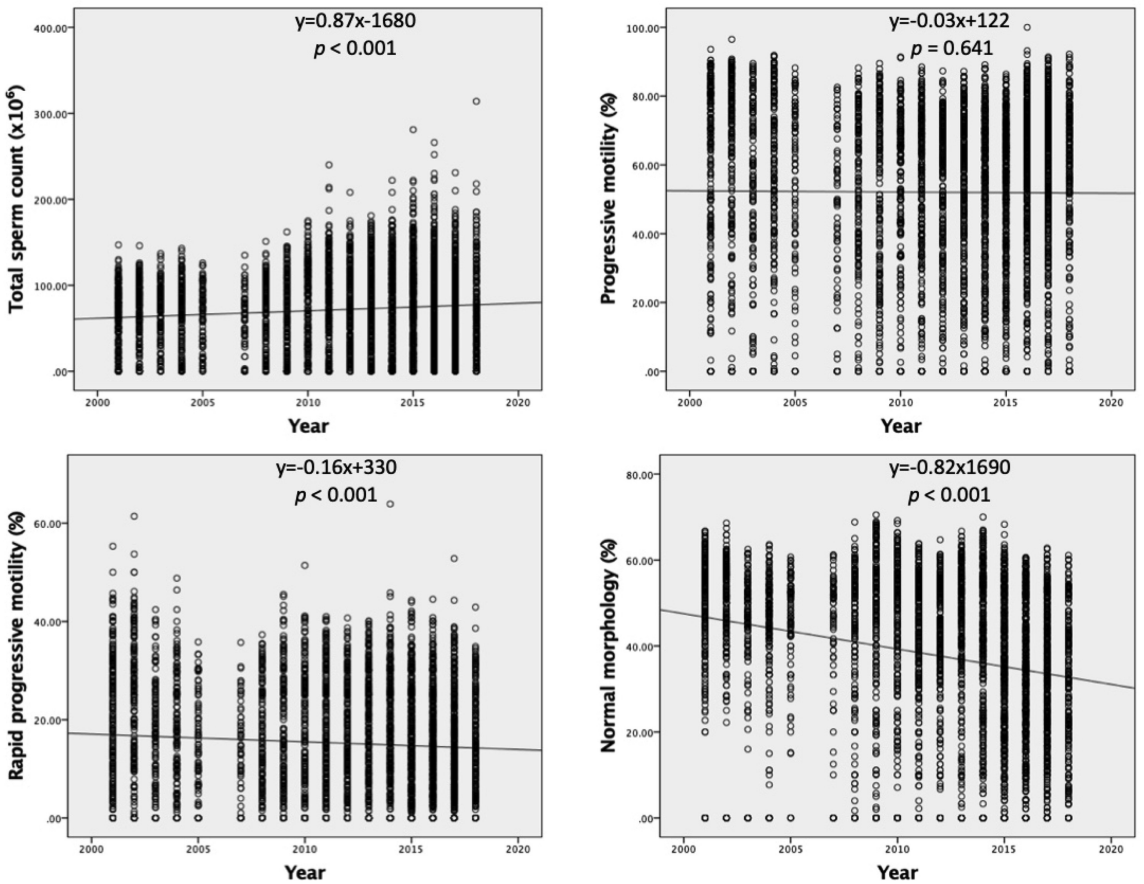

Fig. 2. Changes in semen parameters by year of data collection.

An age-period-cohort (APC) curve was used to demonstrate the cohort effect in Fig. 3. The decline effect was observed with increasing age among several birth cohorts with progressive, rapidly progressive sperm motility and sperm with normal morphology.

Among different air pollution (Table 3), after adjusted the confounders such as age, the year of examination, BMI, and abstinence time of participant, we observed SO_2 was associated with lower rapid progressive sperm motility and lower sperm with normal morphology ($\beta = -0.103$, $p = 0.043$; $\beta = -0.118$, $p = 0.001$,

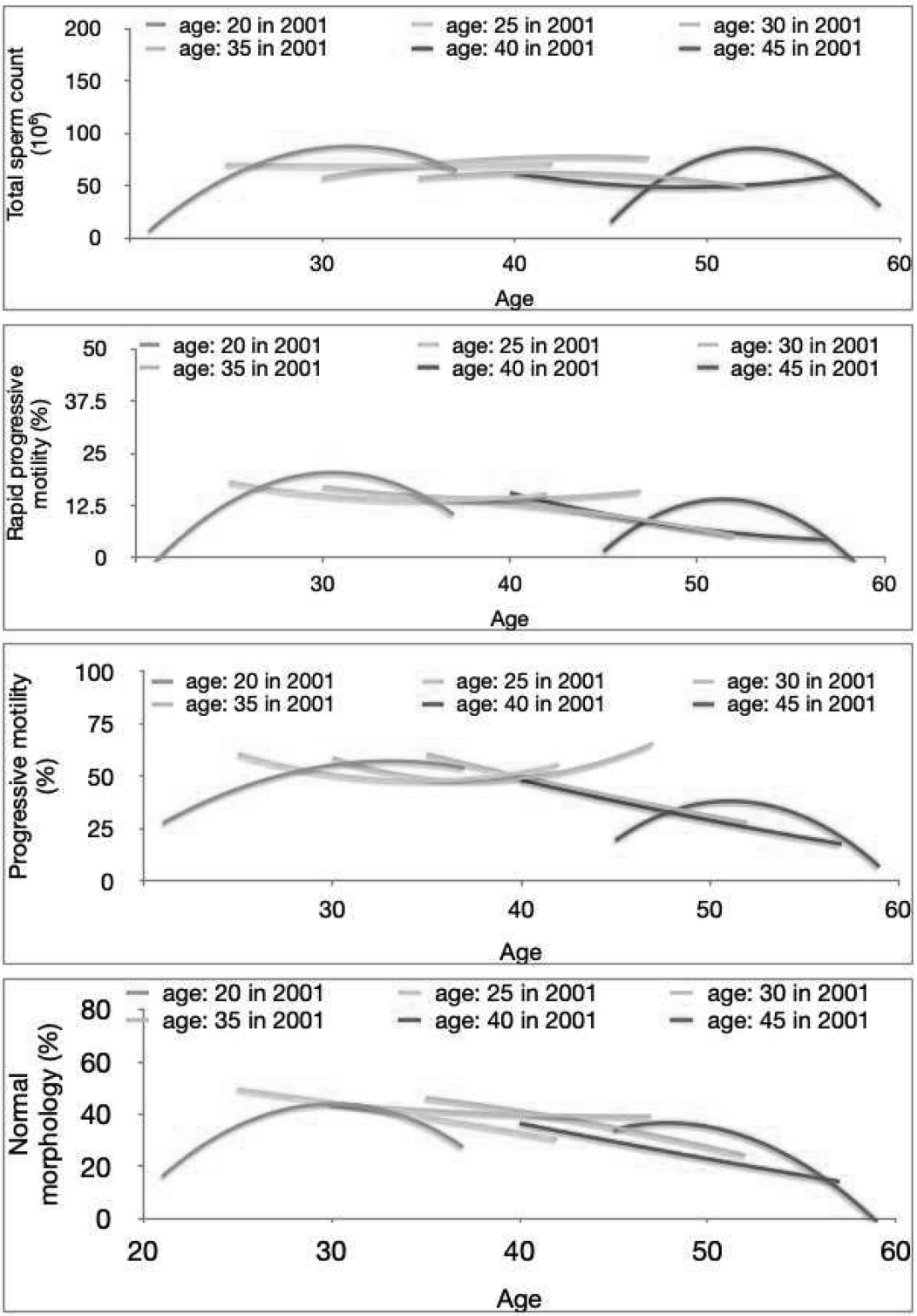

Fig. 3. Age-period-cohort (APC) model of total sperm count, progressive, rapid progressive sperm motility and sperm with normal morphology.

Table 3
Association between semen parameters and air pollutants.

Semen parameters	Air pollutant	β (95% CI)	*p*
Number of sperm (10^6)	PM_{10}	0.018 (−0.011, 0.046)	0.218
	$PM_{2.5}$	−0.010 (−0.027, 0.008)	0.268
	SO_2	−0.140 (−0.319, 0.039)	0.125
	NO_2	0.102 (−0.047, 0.251)	0.180
	CO	0.720 (−3.521, 4.960)	0.739
	O_3	−0.032 (−0.078, 0.014)	0.172
Progressive motility sperm (%)	PM_{10}	−0.001 (−0.013, 0.010)	0.842
	$PM_{2.5}$	0.001 (−0.006, 0.008)	0.708
	SO_2	0.052 (−0.021, 0.124)	0.163
	NO_2	0.006 (−0.055, 0.066)	0.853
	CO	0.315 (−1.405, 2.035)	0.720
	O_3	−0.005 (−0.024, 0.014)	0.598
Rapid progressive motility sperm (%)	PM_{10}	0.003 (−0.013, 0.018)	0.740
	$PM_{2.5}$	<0.001 (−0.009, 0.010)	0.930
	SO_2	−0.103 (−0.202, −0.003)	0.043*
	NO_2	0.129 (0.046, 0.212)	0.002*
	CO	1.301 (−1.071, 3.674)	0.282
	O_3	0.011 (−0.015, 0.036)	0.420
Normal morphology sperm (%)	PM_{10}	0.003 (−0.008, 0.015)	0.606
	$PM_{2.5}$	0.003 (−0.004, 0.010)	0.443
	SO_2	−0.118 (−0.191, −0.046)	0.001*
	NO_2	0.127 (0.067, 0.187)	<0.001*
	CO	0.571 (−1.141, 2.284)	0.513
	O_3	0.011 (−0.007, 0.030)	0.232

*$p < 0.05$.

respectively). However, NO_2 was associated with higher rapid progressive sperm motility and higher sperm with normal morphology ($\beta = 0.129$, $p = 0.002$ and $\beta = 0.127$, $p < 0.001$, respectively). The other air pollution (PM_{10}, $PM_{2.5}$, CO, O_3) did not have significant association with semen parameters.

Discussion

Emerging studies have documented that semen quality declines annually, which has become an important issue in male fertility. Taiwan is an urbanized and industrialized island where air pollution is an imminent problem. However, research on semen quality in the Taiwanese population is scarce. A reproductive medical center in northern Taiwan that recruited 7187 male partners of women undergoing assisted reproduction found sperm concentration, semen volume, number of sperm, progressive sperm motility, rapid progressive sperm motility, and sperm with normal morphology were significantly reduced annually by 1.013×10^6/mL, 1.015 mL, 1.028×10^6, 1.021%, 1.017%, and 1.016%, respectively [14]. After adjustment for multiple comparisons, age-reduced progressive, and rapidly progressive sperm motility which is comparable to the results of our study. A cross-sectional study was conducted among 6475 male participants in Taiwan, and estimated 3-month and 2-year average $PM_{2.5}$ concentrations using a spatio-temporal model [15]. Exposure to $PM_{2.5}$ is associated with a lower level of sperm normal morphology (increment of 5 $\mu g/m^3$ in 2-year average $PM_{2.5}$ was significantly associated with a decrease of 1.29% in sperm normal morphology), and a higher level of sperm concentration (increment of 5 $\mu g/m^3$ in 2-year average $PM_{2.5}$ was significantly associated with an increase of 1.03×10^6/mL in sperm concentration). However, $PM_{2.5}$ did not have significant association with semen parameters in our study.

There were two studies conducted in China, levels of SO_2 were several times higher than our studies median value of 11.8 $\mu g/m^3$ (IQR 10.2, 15.7), with observed median values of SO_2 ranging from 20.5 (5–95% 7.0–48.9) to 66.0 (5–95% 25.0–127.9) $\mu g/m^3$ in rural and urban areas in Chongqing [16] and 23.0 $\mu g/m^3$ (5–95% 14.5, 35.2) in Wuhan [17]. Compared to our findings, a study from Chongqing enrolled 1346 men with no history of infertility, and found that PM_{10}, SO_2 and NO_2 were associated with lower normal morphology ($\beta = -0.212$, $p < 0.001$; $\beta = -0.378$, $p < 0.001$; and $\beta = -0.381$, $p < 0.001$, respectively) [16]. The other study in Wuhan, which enrolled 1759 male partners of women undergoing assisted reproduction, found exposure to SO_2 for 0–90 days was significantly associated with decreased sperm concentration (β: −0.14; 95% CI: −0.23, −0.05), sperm count (β: −0.21; −0.30, −0.12), and total motile sperm count (β: −0.16; −0.25, −0.08) [17]. These findings were similar to those of our study. Moreover, exposure to SO_2 during spermatocytogenesis is a critical exposure window for impaired semen quality [18]. Testicular volume was also negatively associated with SO_2 exposure [19]. Further investigation of the effects of SO_2 exposure on semen quality is warranted.

Among other air pollutants (PM_{10}, $PM_{2.5}$, CO, O_3), was not significantly associated with semen parameters in our study. NO_2 was associated with higher rapid progressive sperm motility and higher sperm with normal morphology ($\beta = 0.129$, $p = 0.002$; $\beta = 0.127$, $p < 0.001$, respectively). $PM_{2.5}$, PM_{10}, CO, and NO_2 were found to have a detrimental impact on sperm morphology [20], whereas Michniewicz et al. found no correlation between exposure to NO_2 and the normal sperm percentage. Exposure to NO_2 and PM_{10} in the air reduced sperm motility in Wuhan, whereas Santi et al. found no correlation between PM_{10} exposure and the percentage of progressive sperm [21], which is in line with the present findings. However, these associations were severely attenuated after adjusting for site and may reflect site-level differences in air pollution or other unmeasured factors.

Deng et al. reviewed ten studies to obtain qualitative evidence of the influence of ambient air pollution on sperm quality, and collected data from six of the ten studies to conduct a meta-analysis [22]. Participants were classified into high- and low-exposure groups based on their exposure levels from the original study. The overall trends and evidence from this review indicate that chronic exposure to high levels of ambient pollutants can alter male sperm quality. Another meta-analysis enrolled 11 studies and classified them into high- and low-exposure groups [23]. Higher air pollution levels were associated with significant decreases in

semen volume (WMD: −0.16, 95% CI: −0.27 to −0.05), sperm concentration (WMD: −5.52, 95% CI: −9.88 to −1.16), progressive motility (WMD: −6.23, 95% CI: −11.64 to −0.81), total motility (WMD: −7.65, 95% CI: −14.09 to −1.20), and normal sperm morphology rate (WMD: −3.71, 95% CI: −5.59 to −1.82). Given these previous studies, there are implications for the association between air pollution and semen quality.

The annual decline in semen quality in Taiwan might also be caused by exposure to toxicants, as well as endocrine disruptors such as polychlorinated biphenyl (PCB), nonylphenol (NP), di-(2-ethylhexyl) phthalate (DEHP), and lead [14]. Lifestyle-related factors such as dietary patterns and obesity are also risk factors for semen quality. Diets rich in fruits and vegetables reportedly improve sperm motility [24]. However, Taiwanese men aged 19–30 years have insufficient intake of fruits and vegetables [25]. About obesity, the Nutrition and Health Survey in Taiwan (NAHSIT) revealed the prevalence of obesity (BMI > 27) in men aged 19–44 years increased from 15.1% in 1993–1996 to 29.6% in 2017–2020. A significant negative correlation was observed between the total number of sperm and body mass index (BMI) among men aged 20–30 years [26].

Our study has two limitations. First, we did directly not measure individual air pollution exposure but relied on outdoor ambient air pollution concentrations estimated at the residential addresses as a proxy for individual exposures. This approach may introduce some level of exposure misclassification. However, we expect our results tend to toward of null association between air pollution and semen quality as supported by previous research [27, 28]. Second, our study population was men who attended an infertility clinic for semen examination. The proportion of men with poor semen quality is higher than the general population. Thus, the findings of our study generalized to the general population need to be cautious. On a positive note, our study is one of the largest sample sizes to systemically study the exposure-lag-response relationship between air pollution and semen quality.

Conclusions

In summary, we found that semen quality in southern Taiwan appears to have declined annually, suggesting that increasing age for semen analysis mainly influences sperm with normal morphology, total sperm count, and progressive and rapid progressive sperm motility. Furthermore, we observed associations between SO_2 and NO_2 may be related with semen motility, which was robust to adjustment for multiple comparisons. However, among our study population with relatively low exposure, there is insufficient evidence to suggest an association between ambient air pollution exposure and adverse changes in semen quality. Further evidence of semen quality among populations exposed to higher levels of ambient air pollution is required.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tjog.2023.08.002.

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Original Article

Significantly shortened telomere length and altered androgen receptor level in cumulus cells from women with polycystic ovary syndrome

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ABSTRACT

Objective: The aim of this study was to investigate the correlation between hormone receptor levels and telomere length (TL) in infertile women with and without polycystic ovary syndrome (PCOS).
Materials and methods: This prospective cohort study recruited a total of 431 cumulus oocyte complex (COC) from 88 infertile women between July 2012 and June 2014. The participants were divided into three groups: young age (<38 years, n = 42 and 227 COC), advanced age (≥38 years, n = 33 and 107 COC) and PCOS patients (n = 13 and 97 COC). Cumulus cells were collected from individual follicle during oocyte pick-up, and the mRNA levels of hormone receptors and TL were measured using real-time PCR.
Results: The cumulus cells of PCOS patients demonstrated lower mRNA levels of LH receptor (75.57 ± 138.10 vs. 171.07 ± 317.68; $p < 0.01$) and androgen receptor (1.13 ± 1.52 vs. 4.08 ± 9.57; $p < 0.01$), as well as a shorter TL (2.39 ± 2.58 vs. 3.96 ± 4.72; $p < 0.01$) compared to those of the young age group. In the young age group, only androgen receptor mRNA level showed a significant association with TL (rho = 0.148, $p = 0.026$), while FSH receptor mRNA level was the only factor associated with TL (rho = 0.247, $p = 0.015$) in PCOS patients. For advanced-aged patients, no significant relationship was observed between hormone receptor mRNA levels and TL. Alternative splicing of androgen receptors was identified in some PCOS patients but not in young age controls.
Conclusion: The findings suggest that the androgen receptor level and function may be altered in the cumulus cells of PCOS patients, leading to a shorter TL in cumulus cells in PCOS patients.

Introduction

Polycystic ovary syndrome (PCOS) is a common cause of infertility [1,2], characterized by symptoms and signs of ovarian dysfunction [3]. The prevalence of PCOS ranges from 4% to 21% [4]. Patients with PCOS often exhibit hyperandrogenism, insulin resistance with hyperinsulinemia, low-grade chronic inflammation, and metabolic disorders, all of which contribute to an elevated level of reactive oxygen species (ROS) [5].

Telomeres are complex structures located at the ends of linear chromosomes. They are composed of repetitive DNA sequences (TTAGGG repeats) and a protein called shelterin [6]. The primary function of telomeres is to protect chromosome ends from end-to-end fusions and maintain chromosomal stability [7]. Additionally, telomeres play an important role in chromosome alignment and spindle integrity during mitosis and meiosis [5]. The length of telomeres, known as telomere length (TL), undergoes shortening with each cell division [8]. In peripheral leukocytes, the rate of telomere shortening is approximately 20–40 Kb per year [9]. When telomeres become critically short, a p53-dependent pathway is activated, leading to apoptosis or cell senescence [6].

Telomerase is an enzyme with reverse transcriptase activity and an internal RNA template. It adds TTAGGG repeats to the 3' end of the chromosome, thereby maintaining telomere length [10]. However, in most somatic cells of adults, telomerase activity is absent, except in germ line cells, embryonic stem cells, and immune

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cells [7]. Therefore, TL is a well-known biological marker of aging and age-related disorders. Moreover, the G-rich telomere repeat sequence is highly susceptible to direct damage caused by ROS [7]. Consequently, oxidative stress has a detrimental effect on telomeres, leading to direct telomere shortening [5,9].

Androgens have been found to increase the expression and activity of telomerase, leading to an increase in TL [9]. Several studies have been conducted to investigate TL in patients with PCOS using peripheral leukocytes or granulosa cells due to their phenomenon of hyperandrogenism, but these studies have produced mixed or even conflicting results [5,9,11–18]. Therefore, further investigation is needed to determine whether TL is altered in PCOS patients.

The objective of this study was to measure the gene expression levels of hormone receptors, including FSH, LH, progesterone, and androgen receptors, as well as TL in cumulus cells from patients with and without PCOS. Additionally, the correlation between TL and these receptors was analyzed.

Materials and methods

Patient selection

This prospective case-control study was conducted at Lee Women's Hospital in Taichung, Taiwan, from July 2012 to June 2014. This study recruited infertile patients who were undergoing assisted reproductive technologies (ART) during this period of time. The exclusion criteria for participation in the study were as follows: previous prolonged usage of oral contraceptive pills or hormone replacement therapy, stage III or IV endometriosis, primary ovarian insufficiency, and participation in oocyte donation programs.

The diagnosis of PCOS was based on the Rotterdam criteria [2], which required meeting at least two out of three criteria: oligo- or anovulation, clinical and/or biochemical signs of hyperandrogenism, and the presence of polycystic ovaries. Prior to participation, written informed consent was obtained from each participant, and the study received approval from the Institutional Review Board of Chung Shan Medical University Hospital (CS11264).

Controlled ovarian stimulation (COH) protocol

All patients recruited for this study underwent a standard GnRH agonist long protocol, consistent with our previous report [19,20]. In brief, starting from the mid-luteal phase of the previous menstrual cycle, a subcutaneous injection of 0.5 mg leuprolide acetate (Lupron®; Takeda Chemical Industries, Japan) was administered daily. Follitropin alfa (Gonal-f®; Merck Sereno) and/or hMG (Menopur®; Ferring) were given daily for COH starting from cycle day 3, with the dosage adjusted based on the ovarian response. Transvaginal ultrasound and serum estradiol levels were used to monitor the ovarian response. Once at least two dominant follicles reached a threshold diameter of 17 mm or greater, recombinant human chorionic gonadotropin (hCG) (Ovidrel®; Merck Sereno) was injected to trigger final oocyte maturation. Ovum pick-up was performed approximately 34–36 h after the trigger.

Collection of cumulus cells

The cumulus cells surrounding the oocyte in individual follicles were collected immediately after oocyte recovery, and sufficient cumulus cells were still left around the oocyte to allow normal fertilization. To separate the cumulus cells from blood cells and follicular fluid, aggregated cumulus cells were aspirated by Pasteur pipette and transferred to a dish containing phosphate-buffered saline (PBS; Invitrogen Corp, Carlsbad, CA, USA), washed three times, and subsequently used for DNA and RNA extraction.

Measurement of mRNA levels of hormone receptors in cumulus cells

Messenger RNA (mRNA) was extracted using the Dynabeads mRNA DIRECT Kit (Dynal Biotech ASA, Oslo, Norway) following the manufacturer's protocol. In brief, 120 μL of Lysis/Binding Buffer was added to the cumulus cells, and the tube was gently inverted to ensure complete lysis. The lysate was then transferred to another tube containing 10 μL of Dynabeads Oligo (dT) 25. The beads were mixed with the lysate and incubated at room temperature with continuous mixing for 3–5 min, allowing the poly (A) tail of the mRNA to anneal to the Oligo-dT on the beads. The tube was placed on a magnet for 2 min, and then the supernatant was subsequently removed. The bead-mRNA complexes were washed twice with 240 μL of Washing Buffer A and once with 120 μL of Washing Buffer B at room temperature. The magnet was used to separate the beads from the solution after each washing step. The beads containing mRNA were then prepared for reverse transcription into cDNA.

The following reagents were added to a tube containing the bead-mRNA complex, resulting in a final reaction volume of 30 μL: 17 μL of diethyl pyrocarbonate (DEPC)-treated water, 1 μL of RNaseOUT recombinant RNase inhibitor (40 U/μL, Invitrogen), 4 μL of dNTPs (10 mM each dATP, dGTP, dCTP, and dTTP), 1 μL of Oligo (dT) 20 primer (50 μM, Invitrogen), 4 μL of 5X First-Strand Buffer (Invitrogen), 2 μL of DTT (0.1 M), and 1 μL of SuperScript III Reverse Transcriptase (200 U/μL). The reaction was performed as our previous publication [20]. The resulting single-stranded cDNA served as the template for quantitative real-time polymerase chain reaction (qPCR).

The qPCR reaction volume consisted of 1 μL of cDNA, 12.5 μL of 2x HotSybr PCR Reaction Mix (NuStar Laboratory, USA), 5 μL of each gene-specific primer (0.2–0.5 μM, depending on the gene), and 6.5 μL of sterile distilled water. The specific primers used in this study are listed in Table 1. These primers were selected based on their specificity for FSH receptor, LH receptor, androgen receptor (including the primers used for the confirmation of the wild type and deletion isoform), and progesterone receptor, as confirmed using the BLAST program (http://www.ncbi.nlm.nih.gov). The thermal cycling was carried out using the 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) [21]. Each gene expression was tested in triplicate for each sample in this study. β-actin cDNA was used as a positive control, while a reaction mixture without template cDNA served as the negative control for each gene. Threshold cycle (CT) values were determined using Applied Biosystems software and used for relative quantitation with the $2^{-\Delta\Delta Ct}$ method [22]. Two pairs of primers were designed to check the alternative splicing of androgen receptor. The primers, wild type-2, were designed to amplify the PCR product spanning from exon 2 to 4, resulting in a DNA segment of 360 bp. In the case of alternative splicing with the deletion isoform, exon 3 is omitted. The forward primer binds to the junction of exon 2 and exon 4, resulting in a PCR product of 206 bp, and the sequence of the deletion isoform was identified using the GenBank sequence database.

Measurement of telomere length

The DNA extraction from the cumulus cells was performed using the DNeasy Tissue Kit (Qiagen, Inc., Mississauga, Ontario, Canada) following the provided instructions. The relative telomere length of granulose cells was measured using a modified real-time quantitative polymerase chain reaction (qPCR) protocol [23], similar to our previous report [20]. The relative telomere length was calculated as the ratio of telomere repeat copy number to 36B4 single-gene copy number (T/S ratio) between an unknown sample and a reference DNA sample. The T/S ratio is proportional to the average TL.

Table 1
The sequences of primers.

Gene name	Primers	Length (bp)
FSH receptor	F: 5′ GAGATCTCTCAGAATGATGTC 3′ R: 5′ TTGATGTAGAGCAGGTTGTTG 3′	110
LH receptor	F: 5′ GATGTGCTCCTGAACCAGAT 3′ R: 5′ GTTTGTAACGACTTGTCAGGA 3′	147
Androgen receptor (wild type-1)	F: 5′ GAAGCTGCAAGGTCTTCTTC 3′ R: 5′ TCCGAAGACGACAAGATGGA 3′	123
Androgen receptor (wild type-2)	F: 5′ AAGCTGCAAGGTCTTCTTCAAAA 3′ R: 5′ AAGGAGTCGGGCTGGTTGTT 3′	360
Androgen receptor (deletion isoform)	F: 5′ CGCTGAAGCCCGGAAGCTGA 3′ R: 5′ GCTGGTTGTTGTCGTGTCC 3′	206
Progesterone receptor	F: 5′ CTTGCATGATCTTGTCAAAC 3′ R: 5′ CTTCAGACAGCATTTCTGG 3′	98
β-actin	F: 5′ CTGGCACCCAGCACAATG 3′ R: 5′ GCCGATCCACACGGAGTACT 3′	181
Telomere	tel1: 5′ GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT 3′ tel2: 5′ TCCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA 3′	
36B4	F: 5′ CAGCAAGTGGGAAGGTGTAATCC 3′ R: 5′ CCCATTCTATCATCAACGGGTACAA 3′	

Table 2
Basic characteristics.

	Young age (<38 years)	Advanced-aged (≥38 years)	PCOS
Patient number (n)	42	33	13
Age (year)[a,b]	29.88 ± 3.75	41.61 ± 2.29	28.46 ± 4.58
BMI	22.10 ± 4.82	22.01 ± 5.09	19.96 ± 1.61
AMH (ng/mL)[a,b,c]	4.64 ± 2.38	1.86 ± 1.59	9.83 ± 2.97
Basal FSH (IU/L)	6.57 ± 2.50	8.00 ± 4.61	5.03 ± 2.10
Basal LH (IU/L)	5.15 ± 1.88	3.75 ± 3.05	4.59 ± 3.69
Basal estrogen (pg/mL)	22.15 ± 24.53	27.24 ± 17.89	22.68 ± 13.48
Gonadotropin dosage (IU)[a,b]	2908.33 ± 504.47	4195.16 ± 957.33	2273.08 ± 696.45
Peak estrogen (pg/mL)[a,b,c]	2776.30 ± 1369.53	1040.97 ± 805.74	4004.23 ± 1752.65
Peak Progesterone[a,b]	1.90 ± 0.87	1.23 ± 0.63	2.24 ± 1.09

Abbreviation: AMH: anti-Mullerian hormone; BMI: body mass index.
Note: Data are presented as mean ± SD. *P* value through ANOVA test.
[a] $p < 0.05$ between young patients and advanced-aged patients.
[b] $p < 0.05$ between advanced-aged patients and PCOS patients.
[c] $p < 0.05$ between young patients and PCOS patients.

Statistical analysis

The baseline hormone characteristics, mRNA levels and TL in cumulus cells were compared using ANOVA test with Bonferroni post-hoc comparisons. The correlation between mRNA levels of FSH receptor, LH receptor, androgen receptor, progesterone receptor and telomere length in cumulus cells was determined using Spearman correlation tests. A p level <0.05 was considered statistically significant.

Results

A total of 100 patients undergoing COH participated in the study initially, and 12 patients were excluded due to missing data. The patients were divided into three groups: the young age group (<38 years, n = 42), the advanced age group (≥38 years, n = 33), and the PCOS group (n = 13). The demographic characteristics of the participants are presented in Table 2. The advanced age group had a significantly higher age compared to the other two groups (41.61 ± 2.29, vs. 29.88 ± 3.75 and 28.46 ± 4.58; $p < 0.01$), while there was no difference in age between the young age group and the PCOS group. Moreover, serum anti-Mullerian hormone (AMH) level was significantly higher in PCOS patients compared to both the young age group and the advanced age group (9.83 ± 2.97 vs. 4.64 ± 2.38, vs. 1.86 ± 1.59, respectively; $p < 0.01$). There were no differences in body mass index (BMI), basal FSH, basal LH and basal estrogen levels in these three groups. The total dosage of gonadotropin used in the COH cycle was higher in the advanced age group

Table 3
The mRNA level of hormone receptors and telomere length.

	Young age (<38 years)	Advanced-aged (≥38 years)	PCOS
Follicles (n)	227	107	97
FSH receptor	1.75 ± 6.29	2.86 ± 6.83	1.40 ± 3.56
LH receptor[b]	171.07 ± 317.68	126.06 ± 200.99	75.57 ± 138.10
Progesterone receptor[a]	6.09 ± 13.75	12.81 ± 31.11	4.97 ± 12.67
Androgen receptor[b]	4.08 ± 9.57	2.40 ± 4.80	1.13 ± 1.52
Telomere length[b]	3.96 ± 4.72	2.83 ± 3.54	2.39 ± 2.58

Note: Data are presented as mean ± SD. *P* value through AVNOVA test.
[a] $p < 0.05$ between young patients and advanced-aged patients.
[b] $p < 0.05$ between young patients and PCOS patients.

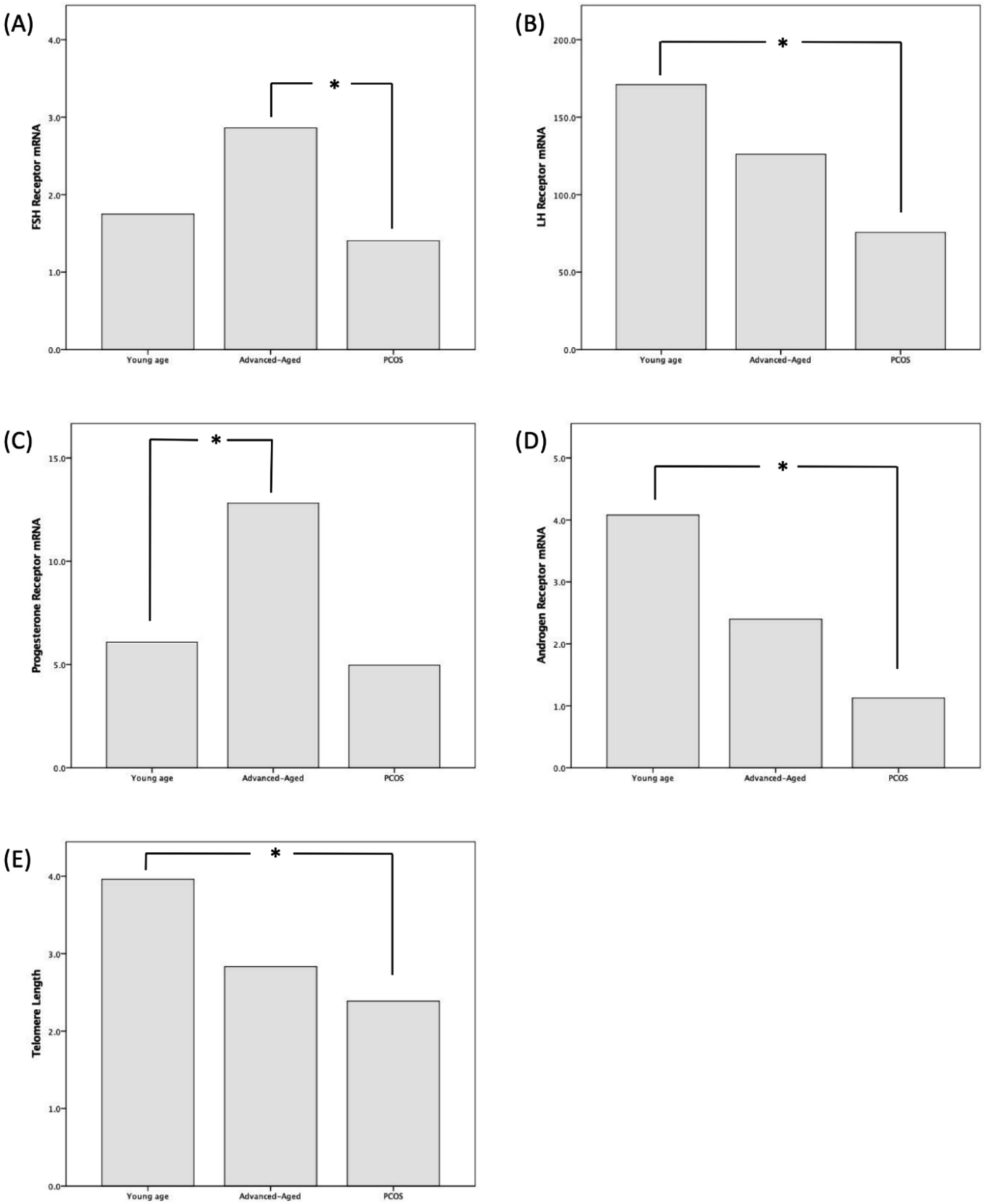

Fig. 1. The mRNA levels of hormone receptors and telomere length in the cumulus cells from 431 follicles of young age group (n = 227 follicles), advanced aged group (n = 107 follicles) and PCOS patients (n = 97 follicles). (A) The mRNA levels of FSH receptors. (B) The mRNA levels of LH receptors. (C) The mRNA levels of progesterone receptors. (D) The mRNA levels of androgen receptors. (E) The mean relative telomere length (T/S ratio). *P < 0.05.

compared to the other two groups (4195.2 ± 957.3 vs. 2908.3 ± 504.5 and 2273.1 ± 696.5; $p < 0.01$).

The mRNA levels of hormone receptors and telomere length in the cumulus cells from 431 follicles are shown in Table 3 and Fig. 1. The PCOS patients had lower mRNA levels of LH receptor (75.57 ± 138.10 vs. 171.07 ± 317.68; $p < 0.01$) and androgen receptor (1.13 ± 1.52 vs. 4.08 ± 9.57; $p < 0.01$), as well as shorter TL (2.39 ± 2.58 vs. 3.96 ± 4.72; $p < 0.01$) compared with the young age group.

Table 4 represents the correlation between TL and mRNA levels of hormone receptors. In the young age group, the mRNA level of androgen receptor was the only factor associated with TL (rho = 0.148, $p = 0.026$), while in PCOS patients, the mRNA level of FSH receptor was the only factor associated with TL (rho = 0.247, $p = 0.015$). There was no significant association between mRNA levels of hormone receptors and telomere length in the advanced age patients.

In 2015, Wang et al. demonstrated the phenomenon of alternative splicing of the androgen receptor in PCOS patients [24]. The deletion isoform of alternative splicing was found in some of our PCOS patients, as shown in Fig. 2A. The sequence of the alternatively-spliced cDNA of the androgen receptor was confirmed using GenBank, presenting in Fig. 2B.

Discussion

The telomere length shortens with cell aging as age advances, making it an excellent marker for cellular senescence. However, this study revealed that PCOS patients exhibit short telomeres in cumulus cells, even at a young age, similar to those of advanced age patients.

PCOS is characterized by proinflammatory conditions, and PCOS patients have been found to have increased levels of inflammatory biomarkers such as C-reactive protein (CRP) [5]. A case-control study published in 2015 showed that PCOS patients had higher CRP levels compared to the control group, and these levels were negatively correlated with TL in peripheral leukocytes [5]. The chronic inflammation status and the high incidence of metabolic syndrome in PCOS patients may directly contribute to oxidative stress, leading to telomere shortening [9].

Androgen has been shown to increase the amount and activity of telomerase in ovarian cancer cells through the PI3K/Akt pathway [25]. Additionally, Kim et al. discovered that the androgen receptor directly interacts with telomeric proteins in prostate cancer cells, thereby maintaining the stability of telomeric structure [26]. In a phase 1-2 study conducted by Townsley et al., the synthetic androgen, danazol, demonstrated a telomere elongation effect in patients with telomere-related diseases [27]. Consequently, the phenomenon of hyperandrogenism in PCOS may potentially play a role in the regulation of TL.

Li et al. demonstrated that PCOS patients have a short TL in granulosa cells, but no relationship was found between serum testosterone level and TL [13]. Other studies have also reported a shorter TL in peripheral leukocytes of PCOS patients compared to the control group [14,15]. Conversely, some research has shown a longer TL in granulosa cells [11] and peripheral leukocytes [9,12] of PCOS patients compared to the control group. However, other studies have indicated no difference in TL in granulosa cells [14] and peripheral leukocytes [5,11,16–18] between PCOS patients and the control group. These findings suggest a possible compensatory effect of chronic inflammation and hyperandrogenism in PCOS patients. Paradoxical findings have been observed in the female reproductive system [28]. The contradictory results from previous studies may be attributed to the differences in the cell types analyzed, the severity of hyperandrogenism among patients, and the age ranges of the patients [9]. It has also been hypothesized that racial, genetic, and environmental differences may contribute to variable TL in terminal differentiated cells, such as granulosa cells or leukocytes, in PCOS patients.

In our study, we observed lower mRNA levels of LH receptor and androgen receptor in cumulus cells of PCOS patients compared to those of the control group at the same age. Cumulus cells, which originate from undifferentiated granulosa cells, play a crucial role in connecting with the oocyte through gap junctions and directly influencing each other's gene expression and protein synthesis [29]. Therefore, cumulus cells are considered to be more representative of the oocytes than granulosa cells. Our findings in this study are consistent with a previous study [30] that also reported a disruption in the interaction between hormones and their receptors in PCOS patients [31]. The decreased levels of LH receptor and androgen receptor may diminish the positive effect of androgens on telomere maintenance, leading to shorter TL in PCOS patients.

Regarding the relationship between TL and mRNA levels of hormone receptors, we found a positive correlation between androgen receptor level and TL in the young age group. However, in PCOS patients, the FSH receptor level was positively correlated with TL. Devillers et al. demonstrated that the androgen receptor is involved in maintaining FSH receptor levels, contributing to the stimulation effect of FSH and promoting folliculogenesis in mini-puberty mice [32]. Fujibe et al. also found that androgens increase the mRNA level of FSH receptors and support follicle development in a mouse model [33]. These findings suggest that androgens, in conjunction with gonadotropins, play a vital role in ovarian function. In our study, although not statistically significant, the FSH receptor level in PCOS patients tended to be lower than that in control group. This is likely the result of the decreased androgen receptor level in PCOS patients, and the decreased levels of both FSH receptor and androgen receptor may contribute to the short TL.

The human mRNA of the androgen receptor is 10.6 Kb in length and consists of 8 exons and 7 introns, encoding the 910 amino-acid androgen receptor protein [34,35]. Alternative splicing of androgen receptor mRNA was first observed in the prostate as early as 1988 [36]. This alternative splicing can result in mRNA with altered lengths and sequences, leading to products with different amino acid sequences and, subsequently, different structures and functions of the androgen receptor [34]. While most studies have focused on prostate cancer, Wang et al. identified two alternative splice variants of the androgen receptor in granulosa cells of PCOS patients: the insertion and deletion isoforms [24]. These variants were associated with hyperandrogenism and abnormal folliculogenesis. The alternative splicing process alters the sequence at both the mRNA and protein levels, affecting the three-dimensional structure and subsequent function of the androgen receptor [24]. In our current study, we also found the presence of the deletion isoform of alternative splicing in the androgen receptor mRNA in some of PCOS patients. This finding could potentially explain the decreased androgen receptor levels and shortened TL observed in PCOS patients. Further

Table 4
The relationship between telomere length and mRNA level of hormone receptors in the cumulus cells from the individual follicle.

	Young age (n = 227)		Advanced-aged (n = 107)		PCOS (n = 97)	
Telomere length	Rho	P value	rho	P value	rho	P value
Androgen Receptor	0.148	0.026[a]	−0.056	0.567	−0.029	0.776
FSH receptor	−0.080	0.235	−0.159	0.102	0.247	0.015[a]
LH receptor	−0.103	0.123	0.039	0.688	0.052	0.612
P4 receptor	−0.128	0.054	−0.024	0.808	0.007	0.948

[a] Spearman rank correlation coefficients.

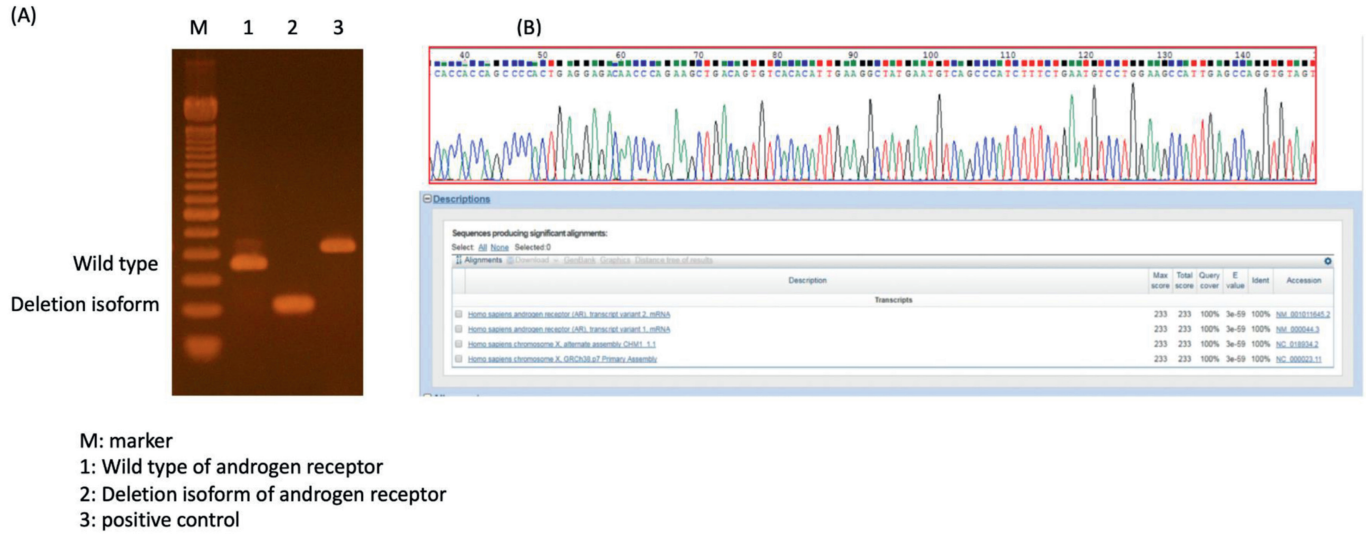

Fig. 2. Identification of the deletion isoform of androgen receptor in cumulus cells of PCOS patients. (A) Simultaneous presence of wild type and deletion isoform of androgen receptors in a PCOS patient. (B) Sequencing results confirming the presence of the deletion isoform of androgen receptor, as documented in GenBank.

investigation is warranted to explore the relationship between alternative splicing of the androgen receptor and TL in PCOS patients.

When comparing PCOS patients to advanced age patients, there were no significant differences in the mRNA levels of LH receptor, progesterone receptor, and androgen receptor. Additionally, although not statistically significant, the TL was found to be shorter in PCOS patients compared to advanced age patients. TL serves as one of the markers of cell aging [37], suggesting a potential oocyte aging and poor embryo quality in PCOS patients. However, further studies are needed to confirm the underlying mechanism.

One limitation of our study is that we did not assess the protein levels of hormone receptors. It should be noted that the relative expression levels of DNA transcription and mRNA translation may not always be consistent [31]. However, the measurement of protein levels is prohibited by the small number of cumulus cells in a single follicle. Furthermore, the prevalence of alternative splicing in PCOS patients was assessed, and we did not determine the percentage of deletion isoform compared to the wild type in each individual patient.

In conclusion, PCOS patients exhibited lower levels of LH receptor and androgen receptor, and shorter TL in the cumulus cells compared to non-PCOS patients of the same age. The decreased mRNA level of the androgen receptor may be associated with alternative splicing in some PCOS patients, potentially impeding the positive effect of androgen on telomere maintenance. In young non-PCOS patients, TL showed a positive correlation with the androgen receptor level, whereas in PCOS patients, TL was solely associated with the FSH receptor level. These findings suggest the involvement of alternative splicing of androgen receptors and short TL in PCOS patients. Further investigation is still required to elucidate the pathophysiology of PCOS.

Authorship statement

The author contributions to this manuscript were: T.H.L. and M.S.L. designed the research; E.H.C, Y.P.L., Y.C.C, and C.C.H. conducted the collection of cumulus cells and laboratory analyses; T.N.Y., E.H.C., and Y.P.L. contributed to data analysis; T.N.Y. and T.H.L. wrote the manuscript. All authors contributed to editing the manuscript and agreed the submission.

Declaration of Generative AI and AI-assisted technologies in the writing process

The authors declare nothing to disclose.

Conflict of interest

The authors have no conflicts of interest relevant to this article.

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Original Article

Risk factors of heavy uterine bleeding in patients with endometriosis and adenomyosis treated with dienogest

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ABSTRACT

Objective: Dienogest (DNG), a fourth-generation progestin, reduces pain associated with endometriosis and uterine adenomyosis; however, it is associated with irregular uterine bleeding that can cause anemia and poor quality of life. We investigated risk factors for heavy bleeding following DNG administration.
Materials and methods: We retrospectively investigated patients who received DNG for risk factors of heavy uterine bleeding, including clinical diagnosis, use of pretreatment gonadotropin-releasing hormone agonist, smoking, cancer antigen 125, and blood hormone levels. We additionally assessed the uterine area in patients with uterine adenomyosis, the major axis of the uterine body, the major axis of myometrial thickness, the site of tumor development, and the site of myoma development in patients with uterine fibroids.
Results: Eighty Japanese patients were administered DNG. The median age was 41 (range: 24–51) years. The odds ratio (OR) for moderate-to-severe bleeding according to clinical diagnosis were 0.33 ($P = 0.011$) for endometrioma and 9.00 ($P = 0.049$) for uterine adenomyosis. Receiver operating characteristic curve analysis of the uterine area associated with uterine adenomyosis showed an area under the curve (AUC) of 0.909 between those with major and minor bleeding, with an optimal cut-off value of 7388.2 mm^2. The uterine body major axis had an AUC of 0.946, with an optimal cut-off value of 78.3 mm. The major axis of myometrial thickness had an AUC of 0.855, with an optimal cut-off value of 46.8 mm.
Conclusion: Patients with endometrioma treated with DNG were less likely to experience heavy uterine bleeding. Uterine bleeding in patients with uterine adenomyosis and adenomyosis associated with uterine fibroids should be closely monitored while administering DNG.

Introduction

Dienogest (DNG) is a fourth-generation progestin used to lessen pain associated with endometriosis and adenomyosis. DNG produces excellent therapeutic effects with few side effects [1–3]; the only side effect is frequent, irregular uterine bleeding peculiar to progesterone. Prior investigations reported irregular uterine bleeding caused by long-term DNG administration in 70%–90% patients [2,4–6] secondary to progesterone-related endometrium thinning and entry into a secretory state [7].

Breakthrough bleeding due to pseudodecidua secondary to progesterone is presumed to be the main cause of irregular uterine bleeding. A Japanese study of long-term DNG administration in patients with adenomyosis found that irregular uterine bleeding peaked 2–3 months after treatment initiation and tended to decrease after that [8]. Unfortunately, this bleeding may occur unexpectedly, even in patients administered long-term treatment. Heavy uterine bleeding caused by DNG can lead to anemia and poor quality of life. Therefore, identifying factors that increase the risk of heavy bleeding may help avoid serious adverse events. We therefore investigated risk factors associated with heavy uterine bleeding.

Materials and Methods

A retrospective study was conducted between January 2008 and December 2021 at Kanazawa Medical University Hospital. We investigated risk factors associated with heavy uterine bleeding in patients administered DNG (2 mg/day). Patients with insufficient primary endpoints and those who discontinued DNG within 6 months were excluded.

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The study was approved by the Kanazawa Medical University Ethics Committee. Data were extracted from electronic patient files and included clinical diagnosis (endometriosis, uterine adenomyosis, and uterine fibroid with endometriosis and/or adenomyosis), presence or absence of pretreatment gonadotropin-releasing hormone (Gn-RH) agonist, smoking status, cancer antigen 125 (CA125), and blood hormone levels (estradiol [E2] at the end of the third month of treatment). We additionally assessed the uterine area in patients with uterine adenomyosis, the major axis of the uterine body, the major axis of myometrial thickness, the site of tumor development, and the site of myoma development in patients with uterine fibroids. The uterine area was calculated by measuring the length from the cervix to the fundus as the long axis diameter of the uterus, and the maximum diameter perpendicular to the long axis diameter as the short axis diameter of the uterus.

Uterine bleeding severity was classified as no bleeding, minimal bleeding (spotting or a few drops of blood), mild bleeding (soaking fewer than 1 pad or tampon in more than 3 h), moderate bleeding (soaking more than 1 pad or tampon in 3 h), and severe bleeding (soaking 1–2 pads or tampons in 1–2 h). Based on the above categories, Uterine bleeding was then categorized as no-to-mild or moderate-to-severe bleeding.

Clinical diagnosis was performed using ultrasonography (SONOVISTA FX, Mochida Siemens, Tokyo, Japan) and magnetic resonance imaging (MAGNETOM Avanto 1.5T, Siemens, Erlangen, Germany). The clinical diagnosis was based on magnetic resonance imaging, and ultrasonographic images were analyzed by multiple radiologists.

Fisher's exact test, Mann–Whitney U test, and Receiver operating characteristic (ROC) curves were used for statistical analyses using GraphPad Prism 9 version 9.2.0 (GraphPad Software, San Diego, CA, USA). The significance level was set at 5%.

Results

There were 181 Japanese females who consented during the study period. Of these, 71 patients lacked data, 30 patients were excluded because DNG was discontinued within 6 months, and 80 remained in the study group. Adverse events that occurred within 6 months of DNG treatment were uterine bleeding (n = 9), palpitations (n = 1), weight gain (n = 1), allergies (n = 1), and hair loss (n = 1) (Fig. 1).

The patients' characteristics are summarized in Table 1. The median age was 41.0 years, with a range of 24–51 years. The median body mass index (BMI) was 22.0 kg/m^2, with a range of 15.1–33.1 kg/m^2. The median and range of E2 were 47.0, 5.1–231.0 pg/mL, and CA125 was 23.3, 5.2–275.8 U/L. Among the included 80 patients, no blood hormones were detected in 5 patients (E2 at 3 months of treatment).

We calculated the risk of moderate-to-severe bleeding according to the characteristics of patients with endometriosis and adenomyosis and examined significant differences between the groups. There were 58 patients with endometriosis, with a median age of 40.0 years (range 24–50 years) and median BMI of 22.5 kg/m^2 (range 15.1–33.1 kg/m^2). There were 22 patients with uterine adenomyosis, with a median age of 43.0 years (range 35–51 years) and median BMI of 20.2 kg/m^2 (range 16.2–28.0 kg/m^2). There was no significant difference in moderate-to-severe bleeding in characteristics, excluding for clinical diagnosis (Tables 2 and 3).

According to the clinical diagnosis, the odds ratios for moderate-to-severe bleeding were OR = 0.33 (P = 0.011) for endometrioma and OR = 9.00 (P = 0.049) for adenomyosis. There was a significant difference in moderate-to-severe bleeding in patients with endometrioma and adenomyosis who were administered with DNG (Fig. 2).

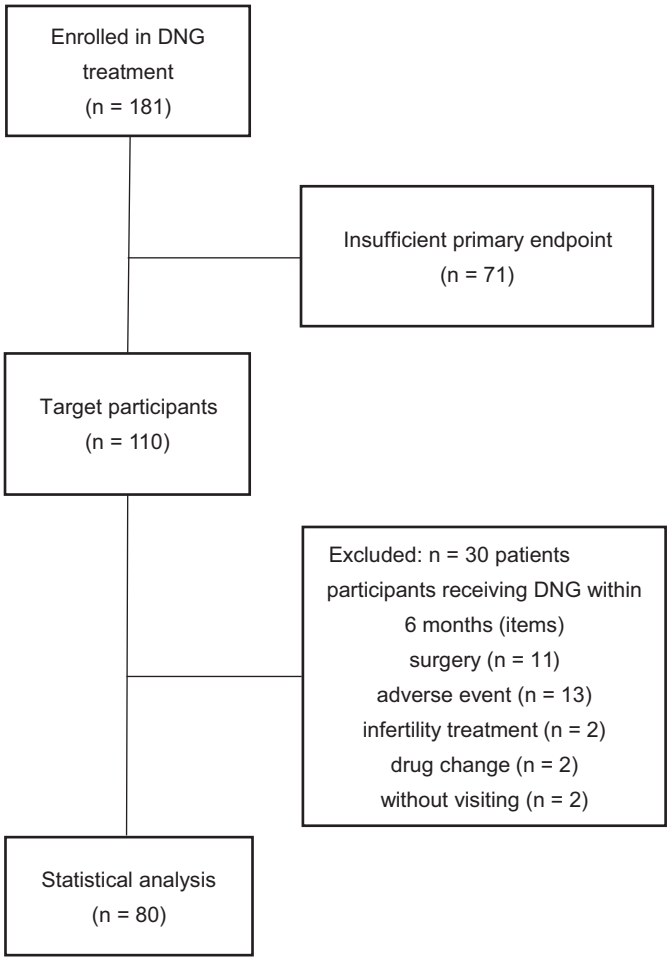


Fig. 1. Flowchart for patients administered dienogest.

Fig. 3 shows the measurement sites for uterine adenomyosis.

Between-group analysis of patients with uterine adenomyosis with no-to-mild and moderate-to-severe bleeding using ROC curves is shown in Fig. 4. The between-group area under the curve (AUC) of the uterine area was 0.909 (95% CI 0.761–1.000; P = 0.011), with an optimal cut-off value of 7388.2 mm^2 (sensitivity: 0.73; specificity: 1.00) (Fig. 3A). The major axis of the uterine body showed an AUC of 0.946 (95% CI 0.838–1.000; P = 0.006), with an optimal cut-off value of 78.3 mm (sensitivity: 0.82; specificity: 1.00) (Fig. 3B). The major axis of the myometrial thickness showed an AUC of 0.855 (95% CI 0.639–1.000; P = 0.027), with an optimal cut-off value of 46.8 mm (sensitivity: 0.82; specificity: 0.80) (Fig. 3C). Measured AUCs were considered outstanding discrimination or excellent discrimination. These cut-off values suggest an increased likelihood of heavy uterine bleeding when the reference values are exceeded.

Of the 16 cases of adenomyosis with uterine adenomyosis and endometriomas, 12 had moderate-to-severe bleeding. Of the 12 cases associated with uterine adenomyosis, 5 were intrinsic-type, and 5 were extrinsic-type; there was no significant between-group difference.

Among patients with uterine fibroids who were administered DNG, there was no significant difference in uterine submucosal fibrosis for moderate-to-severe bleeding; however, these patients were prone to uterine bleeding (Fig. 5).

Table 1
Characteristics of patients administered dienogest.

Characteristics	n, (%)
Age (year)	80, (100)
20–29	5, (6.3)
30–39	28, (35.0)
≥40	47, (58.7)
BMI (kg/m^2)	
≤19	21, (26.2)
20–29	55, (68.8)
≥30	4, (5.0)
Clinical diagnosis	
Uterine adenomyosis	10, (12.5)
Uterine adenomyosis with endometrioma	6, (7.5)
Uterine Adenomyosis with uterine fibroma	6, (7.5)
Endometriosis (endometrioma)	40, (50.0)
Endometrioma with uterine fibroma	18, (22.5)
Uterine bleeding	
No bleeding	14, (17.5)
Minimal bleeding	4, (5.0)
Mild bleeding	28, (35.0)
Moderate bleeding	27, (33.8)
Severe bleeding	7, (8.7)
Parity	
Nulliparous	41, (51.3)
Multiparous	39, (48.7)
Pretreatment for Gn-RH agonist	
No	67, (83.8)
Yes	13, (16.2)
Current smoker	
No	66, (82.5)
Yes	14, (17.5)
E2 (pg/mL)	
<47	37, (49.3)
≥47	38, (50.7)
CA125 (U/L)	
<35	55, (68.8)
≥35	25, (31.2)

BMI: Body Mass Index.

Table 2
Risk Factors and P-values for uterine bleeding in endometriosis-related patients administered dienogest.

Characteristics	Moderate to severe bleeding risk %	Odds ratio 95%CI	P value
Age (year)			
20 - 29	(1/5), 20	0.68 (0.06–5.84)	>0.99
30 - 39	(6/23), 26.1	0.89 (0.31–2.48)	0.78
≥ 40	(10/30), 33.4	1.14 (0.45–2.82)	
BMI (body mass index)			
≤ 19	(4/11), 36.4	1.24 (0.39–4.47)	0.49
20 - 29	(10/43), 23.3	0.79 (0.33–1.88)	0.17
≥ 30	(3/4), 75.0	2.56 (0.59–10.17)	
Parity			
nulliparous	(9/30), 30.0	1.02 (0.43–2.63)	>0.99
multiparous	(8/28), 28.6	0.98 (0.38–2.41)	
Pretreatment for GnRH agonist			
no	(16/53), 30.2	1.03 (0.46–2.30)	>0.99
yes	(1/5), 20.0	0.68 (0.06–5.84)	
Current smoker			
no	(15/50), 30.0	1.02 (0.48–2.33)	>0.99
yes	(2/8), 25.0	0.85 (0.17–4.27)	
E2 (pg/mL)			
< 47	(4/26), 15.4	0.53 (0.18–1.77)	0.15
≥ 47	(12/29), 41.4	1.42 (0.62–3.46)	
CA125 (U/L)			
< 35	(13/42), 30.2	1.06 (0.45–2.32)	>0.99
≥ 35	(4/16), 25.0	0.85 (0.28–2.70)	

Table 3
Risk Factors and P-values for uterine bleeding in uterine adenomyosis-related patients administered dienogest.

Characteristics	Moderate to severe bleeding risk %	Odds ratio 95%CI	P value
Age (year)			
20 - 29	–	–	–
30 - 39	(3/5), 60.0	0.83 (0.20–4.01)	>0.99
≥ 40	(13/17), 76.5	1.05 (0.39–2.80)	
BMI (body mass index)			
≤ 19	(8/10), 80.0	1.10 (0.39–3.57)	>0.99
20 - 29	(8/12), 66.7	0.92 (0.33–2.72)	
≥ 30	–	–	–
Parity			
nulliparous	(7/11), 63.7	0.88 (0.29–2.82)	0.75
multiparous	(9/11), 81.8	1.13 (0.36–3.35)	
Pretreatment for GnRH agonist			
no	(10/14), 71.4	0.98 (0.36–2.59)	>0.99
yes	(6/8), 75.0	1.03 (0.31–3.40)	
Current smoker			
no	(11/16), 68.8	0.95 (0.369–2.681)	>0.99
yes	(5/6), 83.3	1.146 (0.31–3.92)	
E2 (pg/mL)			
< 47	(7/11), 63.7	0.91 (0.29–3.09)	>0.99
≥ 47	(7/9), 77.8	1.11 (0.33–3.41)	
CA125 (U/L)			
< 35	(8/13), 61.5	0.85 (0.30–2.44)	0.74
≥ 35	(8/9), 88.9	1.22 (0.42–3.63)	

Discussion

In the present study, we used statistical analytic methods to investigate risk factors for heavy uterine bleeding in 80 Japanese females who were administered DNG.

In these patients, moderate-to-severe bleeding in patients administered DNG was not associated with pretreatment Gn-RH agonist administration. Miyawaki et al. reported that premedication with Gn-RH agonists reduced uterine bleeding, frequently occurring in the early stages of DNG therapy [9]. Pretreatment Gn-RH agonists effectively suppress the irregular uterine bleeding that tends to occur 2–3 months following DNG therapy initiation by thinning the endometrium. However, long-term administration of DNG (6 months or longer) may reduce this effect.

In a study analyzing clinical diagnoses, patients with only endometriomas were less likely to experience moderate-to-severe bleeding when treated with DNG; those with uterine adenomyosis associated with uterine adenomyosis and uterine fibroids were more likely to experience moderate-to-severe bleeding. The bleeding pattern in patients with endometriosis on DNG 2 mg was generally well tolerated, with only 2 women (0.6%) reporting a bleeding event as the main reason for early discontinuation [3]. Patients with endometriosis alone are less likely to develop heavy, uterine bleeding and may be treated aggressively using DNG. Also, 12 of 16 patients with uterine adenomyosis showed moderate-to-severe bleeding; however, treatment was not discontinued. Two patients with uterine adenomyosis developed heavy bleeding, and DNG was stopped 3 months after the start of treatment, leading to their exclusion from the final analysis. Neriishi et al. reported that 4 of 18 patients with uterine adenomyosis (22.2%) discontinued DNG treatment due to severe metrorrhagia [10]. Osuga et al. reported that the metrorrhagia was tolerable, and no clinically significant differences in bleeding severity were observed during the 52-week DNG treatment period [8]. According to post-marketing surveillance of DNG in Japan, 82.6% of patients who developed heavy

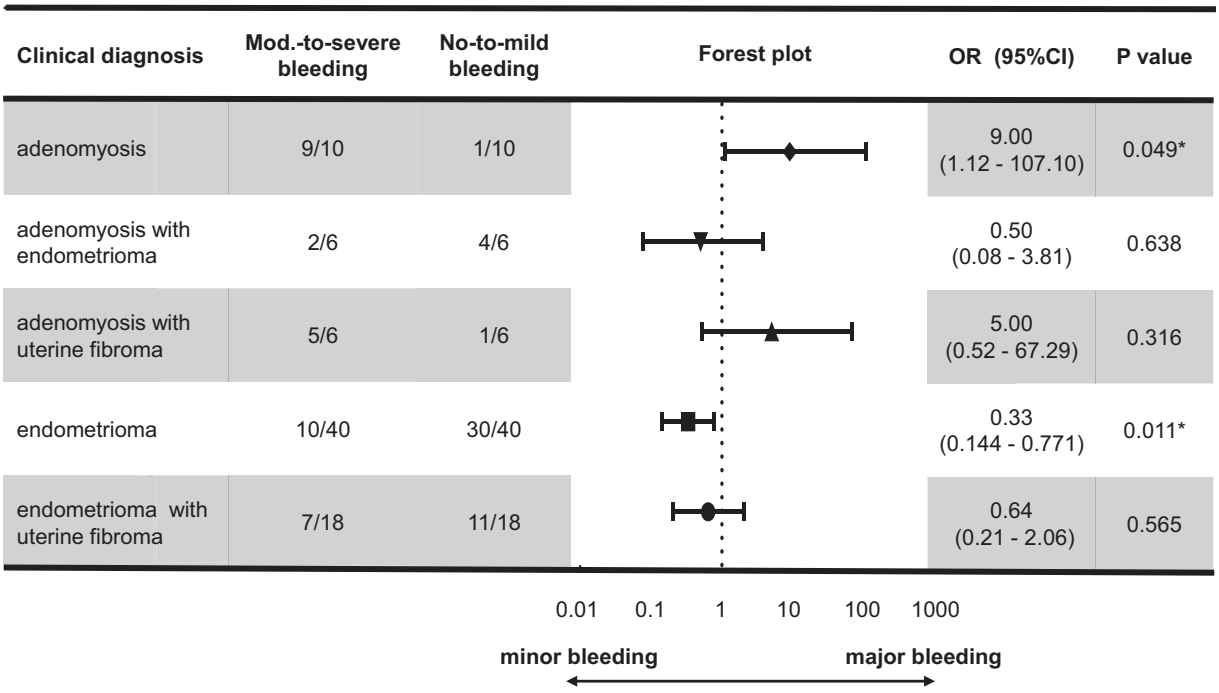


Fig. 2. The odds ratio of uterine bleeding by clinical diagnosis.
*p < 0.05.

uterine bleeding had uterine adenomyosis. In addition, heavy uterine bleeding was observed in 47.2% of patients with maximum uterine diameters of 10 cm or more and in those where the maximum myometrial thickness was 4 cm or more. These reports reaffirmed that DNG-treated patients with uterine adenomyosis are more prone to heavy uterine bleeding. According to our research data, patients with uterine adenomyosis with a major axis of the uterine body of 78.3 mm or more and a major axis of the myometrial thickness of 46.8 mm or more should be closely monitored for heavy, uterine bleeding.

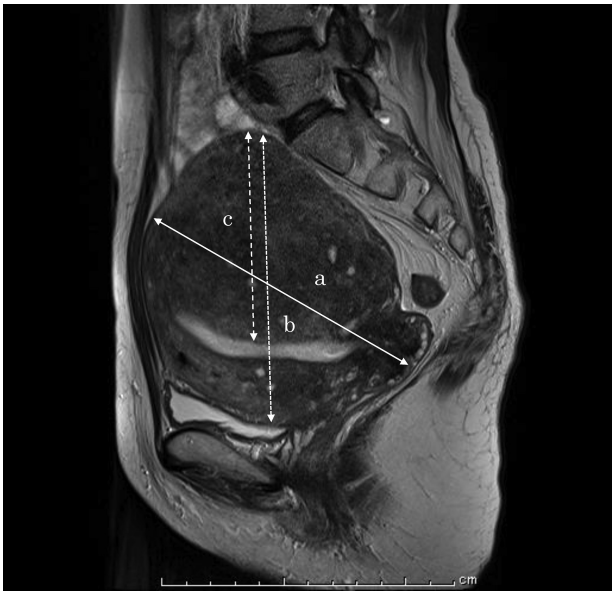


Fig. 3. Measurement site of the uterus.
Representative MRI T2-weighted image (WI) from this study.
We used the sagittal T2-WI of the uterus to measure (a) the long axis diameter of the uterus; (b) the major axis of the uterine body; and (c) the major axis of myometrial thickness.

Patients with intrinsic- and extrinsic-type uterine adenomyosis showed no significant differences in moderate-to-severe bleeding. However, Matsubara et al. reported that DNG-related severe, unpredictable bleeding was associated with uterine adenomyosis structural type (subtype I) in patients with symptomatic adenomyosis [11].

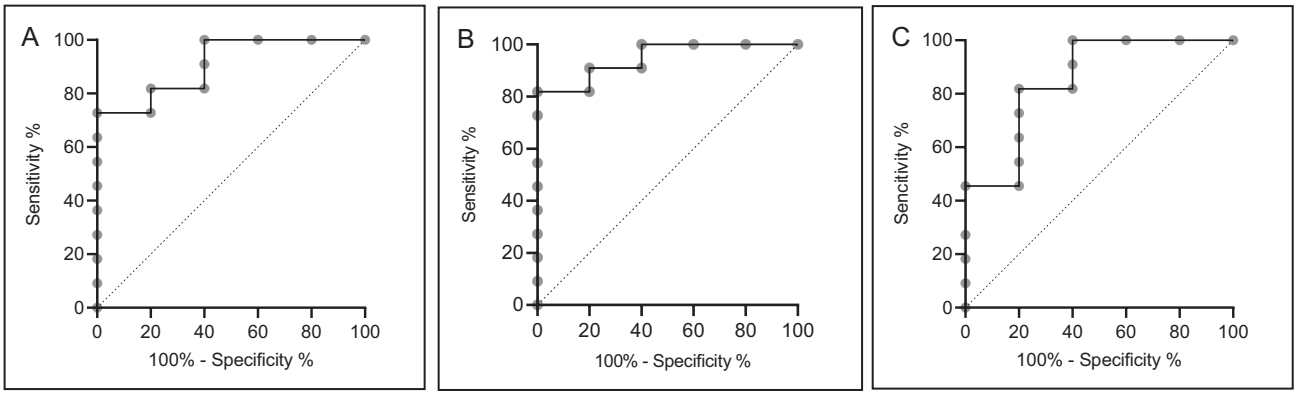
Submucosal fibromas are prone to uterine bleeding; however, patients with uterine fibroids were not significantly different from others with heavy uterine bleeding. No paper reports explain heavy uterine bleeding in patients with uterine fibroma treated with DNG.

Moderate-to-severe bleeding in patients with uterine adenomyosis who were administered DNG was not associated with a 47 pg/mL cut-off value for E2. However, Nagata et al. reported that patients with uterine adenomyosis treated with DNG had increased risk of treatment discontinuation due to uterine bleeding when estradiol is mildly or unsuppressed [12].

We must examine more patients with uterine adenomyosis and uterine fibromas who are administered DNG, with particular attention to types that are prone to uterine bleeding and the relationship between uterine bleeding and E2.

As a limitation, Uterine bleeding data obtained solely from interviews may not provide the most accurate measurement of blood volume and may have potentially biased our statistical data. Furthermore, our statistical analysis was based on pooling patients with moderate and severe bleeding. Therefore, our results do not reflect severe bleeding alone. Additionally, this was an observational study and the level of evidence was not high.

In conclusion, patients with endometrioma who are treated with DNG are less likely to experience heavy uterine bleeding. Additionally, physicians treating patients with uterine adenomyosis and uterine fibromas should be aware of the major axis cut-off values for the uterine body and myometrial thickness when



Measurement site of the uterus	Area under the curve (95%CI)	P value	Cut-off point (mm^2 or mm)	Sensitivity (%)	Specificity (%)
Area of the uterus	0.909 (0.761 ～ 1.000)	0.011*	7388.2	0.73	1.00
Major axis of the uterine body	0.946 (0.838 ～ 1.000)	0.006**	78.3	0.82	1.00
Major axis of myometrial thickness	0.855 (0.639 ～ 1.000)	0.027*	46.8	0.82	0.80

Fig. 4. Analysis between no-to-mild and moderate-to-severe groups of bleeding in uterine adenomyosis using ROC curves.
Subjects: Ten patients with uterine adenomyosis, six patients with uterine adenomyosis with endometrioma
The ROC curves A, B, and C represent the area of the uterus, the major axis of the uterine body, and the major axis of myometrial thickness)
*p < 0.05, **p < 0.01.

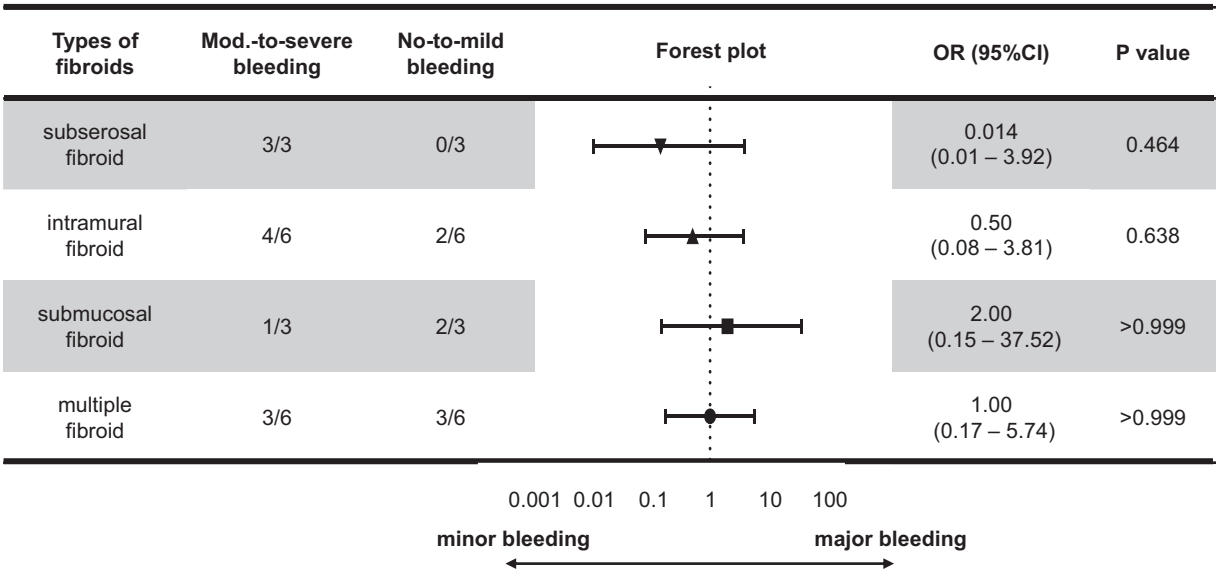


Fig. 5. The odds ratio for uterine bleeding due to uterine fibroids.

administering DNG to minimize the risk of heavy, uterine bleeding in these patients.

Conflict of interest

Authors declare no conflict of interests for this article.

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Original Article

Dinoprostone tablet versus continuous vaginal insert (Propess®) for elective induction in low-risk nulliparous women at term

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ABSTRACT

Objective: To evaluate the efficacy and safety of dinoprostone tablet and continuous vaginal insert (Propess®) in low-risk nulliparous women at term with insufficient cervical ripening receiving elective induction.

Materials and methods: A retrospective study was conducted between March 2020 and February 2022 and included 230 women who underwent elective induction with dinoprostone tablet or vaginal insert. The primary endpoint was failure of induction. Secondary endpoints included time to vaginal delivery, vaginal delivery rate, as well as maternal and neonatal complications and adverse outcomes.

Results: No statistically significant differences were found between the two groups regarding the main outcome measures; however, the high responders had a significant higher proportion of hyperstimulation and non-reassuring fetal status. The high responder in the Propess group was statistically significant younger (31.68 ± 4.73 vs. 33.82 ± 4.39, $p = 0.027$), while they had a significantly lower BMI at delivery time of the tablet group (24.49 ± 2.24 vs. 27.42 ± 4.32, $p = 0.024$). Factors associated with success of vaginal delivery within 24 h ($p = 0.015$, OR = 0.9, 95%CI = 0.82–0.98) and the Cesarean section ($p < 0.001$, OR = 1.17, 95%CI = 1.08–1.27) was BMI at delivery time.

Conclusion: Slow-release vaginal insert and dinoprostone tablet had similar efficacy and safety for elective induction in low risk nulliparous women at term. Women with younger maternal age or lower BMI at delivery time may have a better response to dinoprostone and had a significantly higher proportion of hyperstimulation and non-reassuring fetal status.

Introduction

Induction of labor (IOL), as a common obstetric intervention, may be recommended for medical indication or nonmedically concern. Although elective induction is generally not recommended before 39 0/7 weeks of gestation due to worse neonatal outcomes [1], the actual condition, wishes, preferences and cervical status of each woman should be taken into account [2]. In Taiwan, since the COVID-19 first outbroke in May, 2021, all patients who were admitted to our hospital first needed to check the PCR. Therefore, we suggested elective induction in most low-risk nulliparous women at term for disaster preparedness.

To improve the success rate of IOL, sufficient cervical ripening by pharmacological or mechanical methods is a key factor. There are many methods available to induce labor, but none has been internationally agreed to be the safest or most effective [3,4]. There are two dinoprostone formulations used in our institution. One is a 3 mg dinoprostone vaginal tablet and the other is a continuous vaginal insert (Propess®) that slowly releases a dose of dinoprostone at a rate of 0.3 mg/h up to 24 h and is retrievable when uterine hyperstimulation develops.

Three previous studies have compared these two agents and reported different results. Hunter and Parveen included 55 nulliparous women and reported a higher normal vaginal delivery rate in the dinoprostone insert group (81% vs. 52%), but two groups had a high degree of cross-over [5]. Rabl et al. revealed no differences in vaginal delivery in 24 h between the two groups [6]. Ahmed Abdelaziz et al. reported the contrary result (79% normal delivery in Propess group vs. 85% in tablet group) but included nulliparous and multiparous women [7]. Multiparous women were an independent predictor factor for successful induction with a slow-release dinoprostone insert (Propess), but the effect was not confirmed in nulliparous women [8].

Therefore, this retrospective study aimed to compare the efficacy and safety of Propess and dinoprostone tablet for elective

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induction in low-risk nulliparous women between 37 0/7 weeks of gestation of gestation and 41 0/7 weeks with insufficient cervical ripening.

Patients and methods

This retrospective study analyzed data collected from a single institution, VGHTC, between March 2020 and February 2022. The inclusion criteria were nulliparous, singleton gestation, cephalic presentation, gestation age from 37 0/7 weeks to 41 0/7 weeks, unfavorable cervix evaluated as Bishop score ≤6, and intact membranes or no labor progress 6 h after prelabor rupture of membranes. We excluded pregnant women who met the following criteria: multiparous women, multiple gestations, in labor, previous uterine surgery, malpresentation, fetal anomaly, nonassuring fetal heart rate, any antenatal complication, or any contraindication to dinoprostone or vaginal delivery.

Before induction, all women received pelvic examination to assess the Bishop score. The maternal uterine contractions and the fetal heart rate were monitored by cardiotocography for at least 30 min. The same assessment will be performed every 4 h in the dinoprostone tablet group. Half of 3 mg dinoprostone tablet (1.5 mg) was repeated unless the Bishop score improved to be > 7, or there is the presence of regular uterine contraction every 5 min or non-reassuring fetal heart rate pattern.

In the Propess group, the insert was placed in the posterior vaginal fornix. It was removed regardless of Bishop score after 24 h and may be immediately removed if the Bishop score improved to be > 7 or when there was rupture of membrane, uterine tachysystole or non-reassuring fetal heart rate pattern. Uterine tachysystole was defined as more than five contractions in 10 min for at least 30 min.

After the cervical ripening procedure, to achieve adequate uterine contraction, oxytocin augmentation with a starting dose of 1 mU/minute and titrated with 1 mU/minute every 15–30 min can be used in both groups. The women who had adequate uterine contraction without oxytocin augmentation was defined as a high responder to dinoprostone.

Because Propess was a self-pay choice and the dinoprostone tablet was provided by national health insurance, the choice of method depended on patient's will taking two drugs.

All demographic data including age, parity, Bishop score, body mass index (BMI), maternal and neonatal outcome were obtained from the medical record. The primary outcome was IOL failure, which was defined as Cesarean section. Indications for Cesarean section included fetal distress and failure of ripening or progression of labor. Failure of labor progression was defined to as stopping of cervix dilation or fetal descending after cervix ripening, oxytocin augmentation and rupture of membranes. Secondary outcomes included the time interval from induction to full dilation and vaginal delivery, vaginal delivery in 12 and 24 h, fetal distress, hyperstimulation, postpartum hemorrhage, intrapartum fever, placenta abruption, 4th degree perineal laceration, admission to the NICU, the Apgar scores at 1 min and 5 min, support of ventilator.

IBM® SPSS® Statistics version 21 (IBM® Corporation, Armonk, NY, USA) was used for statistical analysis. Continuous variables were presented as mean (SD) and categorical data as number (%). The two groups were compared with the Chi-square test, Mann–Whitney U test or Kruskal–Wallis test. A P value < 0.05 was considered statistically significant. Logistic regression was used to evaluate the impact of age, gestational age, BMI at delivery time, and birth weight on the probability of cesarean section and successful vaginal delivery in 24 h.

Table 1
Demographic and clinical data of woman in the two study groups (N = 230).

	Propess (n = 89)		Tablet (n = 141)		*P* value
Age	33.0	(30.0–36.5)	33.0	(29.0–36.0)	0.390
Gestational Age	39.0	(38.0–39.0)	39.0	(38.5–40.0)	0.107
PROM status	4	(4.5%)	33	(23.4%)	<0.001**
BMI at delivery time	26.9	(24.3–29.7)	26.4	(24.2–29.3)	0.638

Chi-square test or Mann–Whitney U test, Median (IQR). *$P < 0.05$, **$P < 0.01$.

The research ethics clearance approval letter was obtained from the Institutional Review Board I &II of Taichung Veterans General Hospital, No. CE22197B, on Jun 10, 2022.

Results

A total of 230 women included in this study were initially divided into two groups. 89 cases received Propess insertion and 61 of them needed to add intravenous oxytocin to achieve adequate uterine contraction. Among 141 cases in dinoprostone tablet group, 132 of them also received intravenous oxytocin.

The baseline characteristics are presented in Table 1. There were no statistically significant differences between the groups with respect to maternal age, gestational age and body mass index (BMI) at delivery time. However, more women in the tablet group (4.5% vs 23.4%, $p < 0.001$) had premature rupture before intervention.

Table 2 showed that the rate and indications of cesarean sections were not statistically significantly different between both groups. Additionally, there were no significant differences in induction time or vaginal delivery in 12 and 24 h. However, the Propess group had a significantly higher rate of spontaneous membrane rupture (95.5% vs. 76.4%, $p < 0.001$) and a lower rate of oxytocin augmentation (68.5% vs. 93.6%, $p < 0.001$).

Intrapartum, maternal, and neonatal outcomes were not statistically significant between both groups, except the rate of neonatal Apgar score at 5 min (7.9% vs 2.1%, $p = 0.049$).

Although most of the results were not significantly different, during the review of the data, we noticed that some women had a better response to the dinoprostone, with a shorter time of cervical ripening and adequate uterine contraction, regardless of the formulation. Therefore, we reevaluated the result after divided each group into two groups for induction with or without oxytocin augmentation.

In Table 3, the high responder in the Propess group was statistically significant younger (31.68 ± 4.73 vs. 33.82 ± 4.39, $p = 0.027$), while they had a significantly lower BMI at delivery time of the tablet group (24.49 ± 2.24 vs. 27.42 ± 4.32, $p = 0.024$).

The rate of cesarean section was not statistically significantly different in Table 4 but all the women only received dinoprostone received a cesarean section due to non-reassuring fetal status. They also had higher rates of normal vaginal delivery and statistically significant more cases delivered in 12 and 24 h. Also, results showed statistically significant outcomes of the median time from induction to full and to delivery, and the proportion of hyperstimulation and 4th degree laceration, but no significant difference in neonatal outcomes in each four groups (see Table 5, Table 6).

Maternal age, gestational age, membrane status, BMI at delivery time and birth weight were included in a univariate logistic regression analysis. Univariate study showed that BMI at delivery time was significantly correlated with vaginal delivery within 24 h ($p = 0.015$, OR = 0.9, 95%CI = 0.82–0.98) and cesarean section ($p < 0.001$, OR = 1.17, 95%CI = 1.08–1.27). Furthermore, multivariate regression analysis showed that BMI at delivery time was an independent predictor of both.

Table 2
Delivery outcomes in the two study groups (N = 230).

	Propess (n = 89)		Tablet (n = 141)		*P* value
Outcome during labor induction					
Induction to full dilatation time, median (mins)	1230.0	(840.0–1890.0)	1362.5	(1021.3–1732.5)	0.633
Induction to delivery, median (mins)	1437.0	(1023.0–2021.0)	1487.0	(1090.5–1894.0)	0.598
Total doses of dinoprostone	–		1.0	(1.0–2.0)	–
Need for repeat	–		54	(38.3%)	–
How long did propess remain (hrs)	12.0	(7.0–22.0)	–		–
Cx when remove propess (cms)	2.0	(1.8–3.0)	–		–
Spontaneous rupture of membranes	84	(95.5%)	107	(76.4%)	<0.001**
Oxytocin augmentation	61	(68.5%)	132	(93.6%)	<0.001**
Intrapartum outcomes					
Fetal distress	28	(31.5%)	33	(23.4%)	0.232
hyperstimulation	18	(20.2%)	15	(10.6%)	0.068
IOL failure (Cesarean section)	12	(13.5%)	28	(19.9%)	0.287
Mode of delivery					0.158
Vaginal delivery	59	(66.3%)	96	(68.1%)	
Vacuum/forcep	18	(20.2%)	17	(12.1%)	
Cesarean section	12	(13.5%)	28	(19.9%)	
Indications for C/S					
failure ripening	2	(16.7%)	9	(32.1%)	0.451
failure to progress	7	(58.3%)	15	(53.6%)	1.000
Non-reassuring fetal status	3	(25.0%)	4	(14.3%)	0.410
Delivery outcomes					
NSD within 12 h	9	(11.7%)	10	(8.8%)	0.694
NSD within 24 h	39	(50.6%)	49	(43.4%)	0.401
Maternal outcomes					
Post-partum hemorrhage	19	(21.3%)	27	(19.1%)	0.813
4th laceration	5	(5.6%)	7	(5.0%)	1.000
Placenta abruption	2	(2.2%)	0	(0.0%)	0.149
Intra-partum fever	9	(10.1%)	14	(9.9%)	1.000
Neonatal outcomes					
Birth weight (g)	3081.90	±325.86	3159.10	±367.61	0.065
A/P (1min)	6.84	±1.32	7.20	±0.87	0.088
A/P (5min)	8.84	±0.89	8.94	±0.66	0.883
A/P < 7 at 5 min	7	(7.9%)	3	(2.1%)	0.049*
NICU admission	12	(13.5%)	12	(8.5%)	0.327
Ventilator support (nasal prong/intubation)	12	(13.5%)	9	(6.4%)	0.113

Chi-square test or Mann–Whitney U test, Median (IQR). *$P < 0.05$, **$P < 0.01$.

Table 3
Demographic and clinical data of woman in the subgroups (N = 230).

	Propess only		Propess + oxytocin		*P* value	Tablet only		Tablet + oxytocin		*P* value
N	28		61			9		132		
Age	31.68	±4.73	33.82	±4.39	0.027*	32.22	±3.07	32.56	±5.13	0.748
Age group					0.031*					0.509
20-25	1	(3.6%)	2	(3.3%)		0	(0.0%)	9	(6.8%)	
25-30	12	(42.9%)	9	(14.8%)		3	(33.3%)	39	(29.5%)	
30-35	10	(35.7%)	29	(47.5%)		5	(55.6%)	48	(36.4%)	
>35	5	(17.9%)	21	(34.4%)		1	(11.1%)	36	(27.3%)	
Gestational Age	39.13	±0.89	39.37	±0.70	0.148	39.52	±0.99	39.41	±0.87	0.586
Gestational Age group					0.119					0.952
37- 37 + 6	3	(10.7%)	3	(4.9%)		1	(11.1%)	10	(7.6%)	
38- 38 + 6	9	(32.1%)	11	(18.0%)		1	(11.1%)	23	(17.4%)	
39- 39 + 6	9	(32.1%)	36	(59.0%)		4	(44.4%)	56	(42.4%)	
> 40	7	(25.0%)	11	(18.0%)		3	(33.3%)	43	(32.6%)	
PROM status	3	(10.7%)	1	(1.6%)	0.090	2	(22.2%)	31	(23.5%)	1.000
BMI at delivery time	26.28	±3.04	27.90	±4.15	0.150	24.49	±2.24	27.42	±4.32	0.024*
BMI group					0.207					0.205
20-27	17	(60.7%)	29	(47.5%)		8	(88.9%)	70	(53.0%)	
27-30	8	(28.6%)	14	(23.0%)		1	(11.1%)	35	(26.5%)	
30-35	3	(10.7%)	12	(19.7%)		0	(0.0%)	20	(15.2%)	
>35	0	(0.0%)	6	(9.8%)		0	(0.0%)	7	(5.3%)	

Chi-square test or Mann–Whitney U test. *$P < 0.05$, **$P < 0.01$.

Discussion

This retrospective study was designed to compare the efficacy and safety between the slow-release dinoprostone insert (Propess) and the 3 mg dinoprostone tablet. The cesarean section rates between two groups were not statistically different (13.5 vs 19.9%), neither the most secondary outcomes. However, the women in the Propess group had a lower rate of oxytocin augmentation, a higher rate of spontaneous rupture of membranes and Apgar score <7 at 5 min. Furthermore, the high responders of the Propess group were statistically significantly younger (31.68 ± 4.73 vs. 33.82 ± 4.39, $p = 0.027$), while they had a significantly lower BMI at delivery time

Table 4
Delivery outcomes in the subgroups (N = 230).

	Propess only		Propess + oxytocin		Tablet only		Tablet + oxytocin		*P* value
N	28		61		9		132		
Outcome during labor induction									
Induction to full dilatation time, median (mins)	695.0	(482.5–1085.0)	1617.5	(1182.5–2160.0)	672.5	(388.8–1092.5)	1387.5	(1140.0–1777.5)	<0.001**
Induction to delivery, median (mins)	869.0	(588.5–1177.5)	1718.5	(1347.0–2280.5)	767.5	(519.0–1156.8)	1540.0	(1263.0–1941.5)	<0.001**
Total doses of dinoprostone	–		–		1.0	(1–2)	1.0	(1–2)	0.800
Need for repeat	–		–		3	(33.3%)	51	(38.6%)	–
How long did propess remain (hrs)	7.5	(5.3–15.0)	13.0	(9.0–24.0)	–		–		–
Cx when remove propess (cms)	3.0	(3.0–8.0)	2.0	(1.0–3.0)	–		–		–
Spontaneous rupture of membranes	24	(88.9%)	60	(98.4%)	6	(75.0%)	101	(76.5%)	0.001**
Intrapartum outcomes									
Fetal distress	12	(42.9%)	16	(26.2%)	4	(44.4%)	29	(22.0%)	0.081
hyperstimulation	10	(35.7%)	8	(13.1%)	3	(33.3%)	12	(9.1%)	0.001**
IOL failure (Cesaream section)	3	(10.7%)	9	(14.8%)	1	(11.1%)	27	(20.5%)	0.518
Mode of delivery									0.299
Vaginal delivery	21	(75.0%)	38	(62.3%)	8	(88.9%)	88	(66.7%)	
Vacuum/forcep	4	(14.3%)	14	(23.0%)	0	(0.0%)	17	(12.9%)	
Cesarean section	3	(10.7%)	9	(14.8%)	1	(11.1%)	27	(20.5%)	
Indications for C/S									
failure ripening	0	(0.0%)	2	(22.2%)	0	(0.0%)	9	(33.3%)	0.551
failure to progress	0	(0.0%)	7	(77.8%)	0	(0.0%)	15	(55.6%)	0.079
Non-reassuring fetal status	3	(100.0%)	0	(0.0%)	1	(100.0%)	3	(11.1%)	<0.001**
Delivery outcomes									
NSD within 12 h	8	(32.0%)	1	(1.9%)	3	(37.5%)	7	(6.7%)	<0.001**
NSD within 24 h	23	(92.0%)	16	(30.8%)	7	(87.5%)	42	(40.0%)	<0.001**
Post-partum hemorrhage	4	(14.3%)	15	(24.6%)	3	(33.3%)	24	(18.2%)	0.449
4th laceration	3	(10.7%)	2	(3.3%)	2	(22.2%)	5	(3.8%)	0.046*
Placenta abruption	1	(3.6%)	1	(1.6%)	0	(0.0%)	0	(0.0%)	0.259
Intra-partum fever	1	(3.6%)	8	(13.1%)	1	(11.1%)	13	(9.8%)	0.581
Neonatal outcomes									
Birth weight (g)	2984.14	±304.32	3126.77	±327.96	3113.67	±219.67	3162.20	±375.93	0.077
A/P (1min)	6.61	±1.81	6.95	±1.02	7.11	±0.78	7.20	±0.88	0.387
A/P (5min)	8.79	±1.07	8.87	±0.81	9.11	±0.33	8.92	±0.67	0.889
A/P < 7 at 5 min	3	(10.7%)	4	(6.6%)	0	(0.0%)	3	(2.3%)	0.156
NICU admission	5	(17.9%)	7	(11.5%)	0	(0.0%)	12	(9.1%)	0.388
Ventilator support (nasal prong/intubation)	5	(17.9%)	7	(11.5%)	0	(0.0%)	9	(6.8%)	0.193

Chi-square test or Kruskal–Wallis test, Median (IQR). *$P < 0.05$, **$P < 0.01$.

Table 5
Outcome: NSD within 24 h.

	Univariate				Multivariable			
	OR	95%CI		*p* value	OR	95%CI		*p* value
Age	0.98	(0.93-	1.04)	0.604				
Gestational Age	0.94	(0.66-	1.33)	0.720				
PROM status	1.98	(0.87-	4.49)	0.102	1.84	(0.80-	4.22)	0.151
BMI at delivery time	0.90	(0.82-	0.98)	0.015*	0.90	(0.82-	0.98)	0.017*
Birth weight	1.00	(1.00-	1.00)	0.107	1.00	(1.00-	1.00)	0.121

Logistic regression. *$p < 0.05$, **$p < 0.01$.

Table 6
Outcome: Cesarean section.

	Univariate				Multivariable			
	OR	95%CI		*p* value	OR	95%CI		*p* value
Age	1.06	(0.99-	1.14)	0.108	1.08	(1.00-	1.16)	0.059
Gestational Age	1.16	(0.76-	1.76)	0.487				
PROM status	1.68	(0.72-	3.90)	0.228				
BMI at delivery time	1.17	(1.08-	1.27)	<0.001**	1.18	(1.09-	1.28)	<0.001**
Birth weight	1.00	(1.00-	1.00)	0.065	1.00	(1.00-	1.00)	0.093

Logistic regression. *$p < 0.05$, **$p < 0.01$.

of tablet group (24.49 ± 2.24 vs. 27.42 ± 4.32, p = 0.024). These women had significantly shorter time from induction to cervix full dilation and delivery, a higher proportion of vaginal delivery in 12 and 24 h, and a higher risk of hyperstimulation, non-reassuring fetal status and 4th perineal laceration. But the rates for cesarean sections were not different.

In our study, the overall success rate of IOL was found to be 86.5% vs. 80.1%, including spontaneous vaginal delivery (66.3% vs. 68.1%) and operative vaginal delivery (20.2% vs. 12.1%). There is no statistical difference between two groups. The result was different from previous studies [5,7]. Two groups in the Hunter et al. study had a high degree of cross-over, while Ahmed Abdelaziz et al.

included nulliparous and multiparous women in each group [5,7]. Higher parity is one of the predictive factors for the success of cervical ripening and labor induction with slow-release dinoprostone inserts (Propess) [8,9]. Therefore, the inclusion of only nulliparous women in our study may explain the different result.

The results of the safety evaluation included the intrapartum, maternal, and neonatal outcomes were not statistically significant between both groups, except the rate of spontaneous membrane rupture, oxytocin augmentation and neonatal Apgar score <7 at 5 min (7.9% vs. 2.1%, $p = 0.049$). The previous one result may contribute to a higher proportion of premature rupture of membranes before intervention in the tablet group. The last two results may contribute to the high proportion of women in the vaginal insert group having a higher risk of hyperstimulation and non-reassuring fetal status. The phenomenon was more distinct in the subgroup. Intrapartum stress may explain the lower Apgar score observed in the vaginal insert group.

Although efficacy and safety were proved in previous results, some women had better response to dinoprostone and may have potentially dangerous consequences. The high responders were defined as women who had adequate uterine contraction without oxytocin augmentation and this can explain why they had a statistically significant higher proportion of hyperstimulation and the consequence of non-reassuring fetal status [10]. This adverse effect did not result in longer delivery time, cesarean section or worse neonatal outcomes [11].

The high responders in the Propess group were statistically significant younger (31.68 ± 4.73 vs. 33.82 ± 4.39, $p = 0.027$), while they had a significantly lower BMI at delivery time of the tablet group (24.49 ± 2.24 vs. 27.42 ± 4.32, $p = 0.024$). Obesity was shown to have statistically significant impact on cervical ripening, achieving active phase labor, labor induction and cesarean birth in previous studies [9,12–15]. It was unexpectedly that our study found that a higher BMI was a risk factor for poor response to dinoprostone, successful vaginal delivery in 24 h and cesarean section.

Additionally, Yair Daykan et al. revealed that maternal age showed a trend towards induction failure, although it didn't have a statistically significant impact on cervical ripening [9]. Moreover, Lei Zhao et al. proved that maternal age was a risk factor for failure of vaginal delivery in dinoprostone induced labor. Our finding is thought to be incidental given the small sample size in comparison [15]. The impact of maternal age on cervical ripening or labor induction should be further studied.

The strength of our study was that we only included nulliparous women who receive induction of labor without medical indication. Confounders such as parity, maternal and neonatal medical condition were excluded. Furthermore, the same obstetric protocols were used in two groups since this study was conducted in a single center. The main limitation was that this study was a retrospective study with a small sample size. Furthermore, half of the tablet was administered in our protocol and it was a lower dose compared to previous studies.

In conclusion, slow-release dinoprostone insert and dinoprostone tablet had similar efficacy for elective induction in low risk nulliparous women at term. Women with younger maternal age or lower BMI at delivery time may have a better response to dinoprostone and had a significantly higher proportion of hyperstimulation and non-reassuring fetal status. Since our study was retrospective and had the limitation of small sample size, larger prospective studies are worthy of further investigation.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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Original Article

The correlation with abnormal fetal outcome and a high level of amniotic fluid alpha-fetoprotein in mid-trimester

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ABSTRACT

Objective: To evaluate the correlation of high levels [>2.0 multiples of median (MoM)] of amniotic fluid alpha-fetoprotein (AFAFP) in midtrimester with abnormal fetal outcome.
Materials and methods: We retrospectively studied 6245 pregnant women with singleton pregnancy who had undergone amniocentesis between 15 and 27 weeks' gestation at Mackay Memorial Hospital between January 2014 and June 2020. Fifty-five cases had high AFAFP levels (>2.0 MoM). We investigated the abnormal fetal outcomes.
Results: Among the fifty-five cases with high AFAFP levels (>2.0 MoM), thirty (54.5%) had fetal chromosomal abnormalities, major structural abnormalities, and/or adverse obstetric events. Eight cases (14.5%) had chromosomal abnormalities including trisomy 21 (3 cases), trisomy 18 (3 cases), mosaic trisomy 18 (1 cases), and mosaic ring 13 (1 case). Seventeen cases (30.9%) had major structural abnormalities including abdominal wall defect (6 cases) and central nervous system (5 cases), gastrointestinal tract (3 cases), cardiovascular (2 cases), and genitourinary tract (2 cases) abnormalities. Fifteen cases (27%) had adverse obstetric events, including preterm delivery (5 cases), intrauterine fetal demise (4 cases), small for gestational age (4 cases), preeclampsia (4 cases), gestational diabetes mellitus (2 cases), gestational hypertension (1 case), preterm prelabor rupture of membrane (1 case), prolonged labor (1 case), and preterm uterine contraction (1 case).
Conclusion: A high AFAFP level (>2.0 MoM) in midtrimester can be associated with abnormal fetal outcome, including chromosomal abnormalities, major structural abnormalities, and adverse obstetric events. Women with a prenatal diagnosis of high AFAFP levels (>2.0 MoM) should be alerted of the possibility of abnormal fetal outcomes, and further detailed genetic studies and serial sonographic examinations are recommended.

Introduction

Alpha-fetoprotein (AFP) was first discovered in 1956 in the fetal serum, which is synthesized from the liver, yolk sac, and gastrointestinal tract of the fetus [1–3]. It is the dominant serum protein early in fetal life, reaching its peak level at approximately 12–13 weeks' gestation and decreasing abruptly after 32 weeks until birth [4].

Due to the fetal renal system's immaturity, fetal serum AFP is filtered through the glomeruli and then can be found in fetal urine. Since the amniotic fluid mainly consists of fetal urine, amniotic fluid alpha-fetoprotein (AFAFP) would be expected to be measurable. In maternal serum, maternal serum AFP(MSAFP) is also detectable due to AFAFP transposition from the amniotic fluid through the placenta, while AFP level in the circulation of healthy non-pregnant women is essentially undetectable [5].

The AFP level may increase in fetuses with (1) neural tube defect (NTD)or ventral wall defect; (2) glomerular diseases causing expedited protein filtration and passage into the urea; and (3)

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impaired fetal swallowing or digestion causing AFP to accumulate in the amniotic fluid [1]. Conversely, if the lesions are covered by healthy skin, maternal and amniotic fluid AFP concentrations are generally normal [6].

Since the early 1970s, the relationship between high AFAFP levels and open NTD has been established [7]. In the 1980s, AFP was used for predicting Down syndrome [8,9]. Later, AFP has been used as a gestational age-dependent fetal biomarker to screen for fetal malformations or chromosomal abnormalities [7,10,11], and became widely used in prenatal screening programs after 2000s [12]. The cut-off AFAFP value of >2.0 multiples of median (MoM) can detect anencephaly, open NTDs, and encephalocele with rates of 100%, 95%, and 78%, respectively [13,14].

This study aimed to evaluate the correlation of high AFAFP levels (>2.0 MoM) in midtrimester with abnormal fetal outcome.

Materials and methods

This retrospective study was reviewed and approved by the institutional review board of Mackay Memorial Hospital (MMH) (IRB No. 21MMHISO14e).

Altogether, 7213 pregnant women who had undergone amniocentesis at MMH between January 2014 and June 2020 were enrolled in this study. Of these, 6675 cases were singleton births with gestational age between 15 and 27 weeks. Altogether, 430 cases had incomplete data; thus, we finally included 6245 cases (Fig. 1). Maternal age, obstetric history, indications for amniocentesis, fetal karyotype, and perinatal outcome of the included cases were recorded. Results from screening ultrasound performed by obstetric sonography specialists were also recorded. All amniotic fluid samples were obtained by obstetrical specialists under direct sonographic guidance and were processed at the cytogenetic laboratory of MMH. All amniotic fluid samples were clear without blood contamination. The AFAFP's MoM values were established based on the 6245 cases included in our study.

We identified 55 cases with high AFAFP levels (>2.0 MoM) of and investigated the abnormal fetal outcomes in these cases.

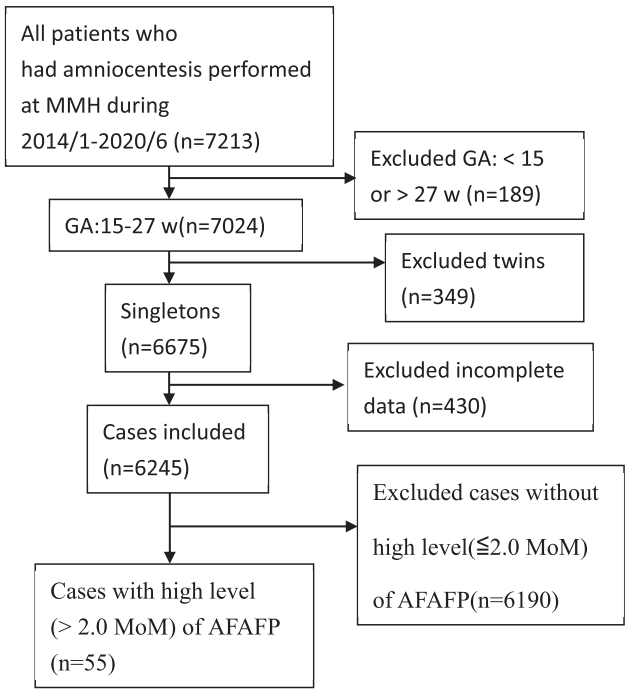

Fig. 1. Participants selection flowchart.

Results

Maternal age, indications for amniocentesis, gestational age for amniocentesis, MoM of AFAFP, fetal karyotype, fetal ultrasound report, and perinatal outcome of the 55 cases with high AFAFP levels (>2.0 MoM) were listed in Table 1. The lists were sorted in descending order of the MoM values of AFAFP.

Amniocentesis was performed most frequently at 17 (18 cases) and 18 (12 cases) weeks' gestation. Advanced maternal age was the most common indication for amniocentesis (38 cases), followed by abnormal fetal ultrasound (7 cases), elective reasons (4 cases), abnormal maternal serum screening (4 cases), and previous/family history of fetal anomaly (2 cases).

The highest risk for adverse perinatal outcomes, including fetal chromosomal abnormalities, major structural abnormalities, or adverse obstetric events, were noted in cases with AFAFP MoM >5 (2/2, 100%), followed by 11 of 19 cases (57.8%) with AFAFP MoM >2.5 and 30 (54.5%) of 55 cases with AFAFP MoM >2. Eight cases (14.5%) had chromosomal abnormalities, including trisomy 21 (3 cases), trisomy 18 (3 cases), mosaic trisomy 18 (1 cases), and mosaic ring 13 (1 case) (Table 2). Seventeen cases (30.9%) had major structural abnormalities including abdominal wall defect (6 cases) (Table 3) and central nervous system (5 cases) (Table 4), gastrointestinal tract (3 cases) (Table 5), cardiovascular (2 cases) (Table 6), and genitourinary tract (2 cases) (Table 7) abnormalities. Fifteen cases (27%) with adverse obstetric events were recorded, including preterm delivery (5 cases), intrauterine fetal demise (IUFD, four cases), small for gestational age (SGA, four cases), preeclampsia (four cases), gestational diabetes mellitus (GDM, two cases), gestational hypertension (one case), preterm prelabor rupture of membrane (PPROM, one case), prolonged labor (one case), and preterm uterine contraction (one case).

Discussion

Clinically, there were many indications for amniocentesis; in our study, we divided them into five items: advanced maternal age (>34 years), abnormal maternal serum screening, abnormal fetal ultrasound, previous/family history of fetal anomaly, and elective amniocentesis. Our data showed that advanced maternal age and abnormal fetal ultrasound were the most common and secondary indications for amniocentesis.

The AFAFP data were routinely analyzed when amniocentesis was performed at MMH. If the AFAFP level was high (>2.0 MoM), further sonographic examination, such as level II ultrasound, is suggested to rule out fetal structural abnormalities not detectable by general ultrasound. Moreover, further genetic studies are advised for possible genetic abnormalities.

AFAFP data were widely used in prenatal screening since the late 1970s, because elevated AFAFP levels improved the diagnostic accuracy of NTD or ventral wall defect, compared with ultrasound alone [15]. Therefore, AFAFP measurements would be valuable in antenatal diagnosis and would offer more information on fetal outcomes for the parents. AFAFP measurement, followed by detailed sonography, was highly successful for detecting fetal anomaly in approximately 99% of cases [16,17].

In our study, the risk for adverse perinatal outcomes was increased with higher AFAFP MoM values. The most common chromosomal abnormalities were trisomy 21 and 18, comparable to the findings of previous studies reporting trisomy 21 and trisomy 18 as the first and second most common chromosomal abnormalities among live births [18,19]. The AFAFP MoM tends to decrease in trisomy 13, 18, or 21 cases [20]. However, our trisomy cases had elevated AFAFP MoM, which might be complicated by the major structural defect, such as omphalocele.

Table 1
Cases with high level (>2 MoM) of amniotic fluid alpha-fetoprotein.

Case ID	Age	Indication	GA	AFAFP MoM	Karyotype	Abnormal fetal ultrasound	Perinatal outcome
#1	30y	Abnormal fetal ultrasound	17w	12.23	46,XX	Gastroschisis	Loss of follow-up
#2	40y	AMA	16w	5.00	47,XY+18	Omphalocele, radius dysplasia	IUFD, TOP
#3	34y	AMA	17w	3.72	46,XY	Oligohydroamnios	Delivery at 38 weeks of gestation, right subependymal cyst, spontaneously resolved at age 6 months
#4	36y	AMA	18w	3.63	46,XY	Omphalocele	TOP
#5	36y	AMA	19w	3.48	46,XY	Nil	Normal
#6	34y	AMA	21w	3.40	46,XX	Nil	Normal
#7	34y	AMA	18w	3.36	46,XY	Nil	Normal
#8	30y	Abnormal maternal serum screening	20w	3.26	47,XY,+21	Nil	TOP
#9	35y	AMA	17w	3.26	46,XY	Nil	Normal
#10	36y	AMA	17w	3.23	46,XY,inv(9) (p12q13)	Nil	Normal
#11	35y	AMA	23	3.10	46,XY	Nil	Normal
#12	31y	Abnormal fetal ultrasound	18w	2.86	46,XX	Omphalocele	TOP
#13	34y	AMA	24w	2.84	46,XY	Nil	Normal
#14	23y	Abnormal fetal ultrasound	14w	2.82	46,XX	Megacystitis, hydronephrosis, oligohydramnios	TOP
#15	41y	AMA	16w	2.81	47,XY,+21	Nil	IUFD, TOP
#16	26y	Elective	21w	2.69	46,XX	IUGR, REDV	Delivery at 27 weeks of gestation, SGA
#17	36 y	AMA	17w	2.67	47,XY,+18 [2]/46,XY[29]	Hydrocephalus	IUFD, TOP
#18	30y	Abnormal maternal serum screening	22w	2.60	46,XX	Nil	Normal
#19	39y	AMA	17w	2.59	46,XY	Nil	Maternal preeclampsia, prolonged labor
#20	23y	Abnormal fetal ultrasound	23w	2.47	47,XX,+21	Absent of nasal bone	TOP
#21	35y	AMA	15w	2.46	46,XY	Nil	Normal
#22	37y	AMA	17w	2.42	46,XY	IUGR	Maternal preeclampsia, delivery at 36 weeks of gestation, SGA
#23	28y	Abnormal maternal serum screening	22w	2.42	46,XY	Nil	Delivery at 39 gestational weeks, duodenal obstruction, s/p pediatric surgery at age 1 month
#24	32y	Elective	19w	2.41	46,XY	Nil	PPROM, delivery at 34 weeks of gestation
#25	34y	Abnormal fetal ultrasound	17w	2.38	47,XY+18	ASD,VSD, omphalocele	TOP
#26	34y	AMA	16w	2.37	46,XY	Nil	Normal
#27	35y	AMA	17w	2.37	46,XY	Echogenic foci in left ventricle	Normal
#28	42y	AMA	18w	2.35	47,XY+18	Nil	TOP
#29	37y	AMA	27w	2.34	46,XY	Absent gastric bubble, IUGR	Delivery at 39 weeks of gestation, esophageal atresia, s/p pediatric surgery at age 5 days, SGA
#30	36y	AMA	18w	2.33	46,XY	Nil	Delivery at 39 weeks of gestation, Hirschsprung disease s/p pediatric surgery at age 1 year
#31	34y	AMA	18w	2.31	46,XY	IUGR, AEDV	Maternal preeclampsia, GDM, delivery at 33 weeks of gestation, SGA
#32	24y	Abnormal fetal ultrasound	23w	2.31	46,XY,r(13) [23]/45,XY,-13/46,XY,idic,r(13) [2]	Dextrocardia, IUGR	TOP
#33	30y	Abnormal fetal ultrasound	22w	2.31	46,XY	Para-umbilical cyst	Loss of follow-up
#34	38y	AMA	18w	2.29	46,XY	Nil	Delivery at 38 weeks of gestation, left bifid ureter, right mild pelvic dilatation, follow up to age 3 months
#35	33y	Previous/family history	16w	2.29	46,XY	Nil	Preterm uterine contraction
#36	34y	AMA	18w	2.28	46,XY	Nil	Normal
#37	38y	AMA	27w	2.26	46,XX	Hypoplasia of metatarsal bone of both 4th toes	Hypoplasia of metatarsal bone of both 4th toes
#38	34y	AMA	18w	2.25	46,XY,t(10; 22) (q22.3; q12.3) de novo	Nil	Normal
#39	34y	AMA	17w	2.22	46,XY	Nil	Maternal GDM
#40	33y	Elective	17w	2.21	46,XY	Nil	Normal
#41	36y	AMA	17w	2.20	46,XY	Nil	IUFD, TOP
#42	37y	AMA	17w	2.19	46,XY	Nil	Normal
#43	34y	AMA	18w	2.17	46,XY,inv(9) (p12q13)	Nil	Normal
#44	37y	AMA	20w	2.17	46,XY	Nil	Normal
#45	38y	AMA	17w	2.16	46,XY	Nil	Loss of follow-up
#46	38y	AMA	23w	2.13	46,XY	Nil	Maternal preeclampsia, delivery at 32 weeks of gestation, bilateral subependymal hemorrhage with cyst formation at birth, spontaneously resolved at age 6 months

(*continued on next page*)

Table 1 (*continued*)

Case ID	Age	Indication	GA	AFAFP MoM	Karyotype	Abnormal fetal ultrasound	Perinatal outcome
#47	46y	AMA	17w	2.10	46,XX	Nil	Delivery at 40 weeks of gestation, spinal bifida, tethered cord, involving L3-5 s/p pediatric surgery at age 12 days
#48	39y	AMA	18w	2.06	46,XY	Nil	Normal
#49	33y	Abnormal maternal serum screening	20w	2.05	46,XY	Nil	Normal
#50	39y	AMA	18w	2.05	46,XY	Nil	Normal
#51	35y	AMA	22w	2.05	46,XX	Nil	Normal
#52	35y	AMA	17w	2.03	46,XY	Nil	Normal
#53	39y	AMA	17w	2.03	46,XX	Nil	Maternal gestational hypertension, delivery at 38 weeks of gestation, left subependymal cyst, persistent to age 2 years
#54	29y	Elective	19w	2.02	46,XY	Nil	Normal
#55	31y	Previous/family history	17w	2.01	46,XX	Nil	Normal

GA: gestational age, AFAFP: amniotic fluid alpha-fetoprotein, MoM: multiples of median, y: years, AMA: advanced maternal age, w: weeks, IUFD: intrauterine fetal demise, TOP: termination of pregnancy, ASD: atrial septal defect, VSD: ventricular septal defect, GDM: gestational diabetes mellitus, L: lumbar, s/p: status post.

Table 2
Cases with chromosomal abnormalities.

Case ID	Age	Indication	GA	AFAFP MoM	Karyotype	Abnormal fetal ultrasound	Perinatal outcome
#2	40y	AMA	16w	5.00	47,XY+18	Omphalocele, radius dysplasia	IUFD, TOP
#8	30y	Abnormal maternal serum screening	20w	3.26	47,XY,+21	Nil	TOP
#15	41y	AMA	16w	2.81	47,XY,+21	Nil	IUFD, TOP
#17	36y	AMA	17w	2.67	47,XY,+18 [2]/46,XY[29]	Hydrocephalus	IUFD, TOP
#20	23y	Abnormal fetal ultrasound	23w	2.47	47,XX,+21	Absent of nasal bone	TOP
#25	34y	Abnormal fetal ultrasound	17w	2.38	47,XY+18	ASD,VSD, omphalocele	TOP
#28	42y	AMA	18w	2.35	47,XY+18	Nil	TOP
#32	24y	Abnormal fetal ultrasound	23w	2.31	46,XY,r(13) [23]/45,XY,-13/46,XY,idic,r(13) [2]	Dextrocardia, IUGR	TOP

GA: gestational age, AFAFP: amniotic fluid alpha-fetoprotein, MoM: multiples of median, y: years, AMA: advanced maternal age, w: weeks, IUFD: intrauterine fetal demise, TOP: termination of pregnancy, ASD: atrial septal defect, VSD: ventricular septal defect.

Table 3
Cases with abdominal wall defect.

Case ID	Age	Indication	GA	AFAFP MoM	Karyotype	Abnormal fetal ultrasound	Perinatal outcome
#1	30y	Abnormal fetal ultrasound	17w	12.23	46,XX	Gastroschisis	Loss of follow-up
#2	40y	AMA	16w	5.00	47,XY+18	Omphalocele, radius dysplasia	IUFD, TOP
#4	36y	AMA	18w	3.63	46,XY	Omphalocele	TOP
#12	31y	Abnormal fetal ultrasound	18w	2.86	46,XX	Omphalocele	TOP
#25	34y	Abnormal fetal ultrasound	17w	2.38	47,XY+18	Omphalocele, ASD,VSD	TOP
#33	30y	Abnormal fetal ultrasound	22w	2.31	46,XY	Para-umbilical cyst	Loss of follow-up

GA: gestational age, AFAFP: amniotic fluid alpha-fetoprotein, MoM: multiples of median, y: years, AMA: advanced maternal age, w: weeks, IUFD: intrauterine fetal demise, TOP: termination of pregnancy, ASD: atrial septal defect, VSD: ventricular septal defect.

Table 4
Cases with central nervous system abnormalities.

Case ID	Age	Indication	GA	AFAFP MoM	Karyotype	Abnormal fetal ultrasound	Perinatal outcome
#3	34y	AMA	17w	3.72	46,XY	Oligohydroamnios	Delivery at 38 weeks of gestation, right subependymal cyst, spontaneously resolved at age 6 months
#17	36y	AMA	17w	2.67	47,XY,+18 [2]/46,XY[29]	Hydrocephalus	IUFD, TOP
#46	38y	AMA	23w	2.13	46,XY	Nil	Maternal preeclampsia, delivery at 32 weeks of gestation, bilateral subependymal hemorrhage with cyst formation at birth, spontaneously resolved at age 6 months
#47	46y	AMA	17w	2.10	46,XX	Nil	Delivery at 40 weeks of gestation, spinal bifida, tethered cord, involving L3-5 s/p pediatric surgery at age 12 days
#53	39y	AMA	17w	2.03	46,XX	Nil	Maternal gestational hypertension, delivery at 38 weeks of gestation, left subependymal cyst, persistent to age 2 years

GA: gestational age, AFAFP: amniotic fluid alpha-fetoprotein, MoM: multiples of median, y: years, AMA: advanced maternal age, w: weeks, IUFD: intrauterine fetal demise, TOP: termination of pregnancy, L: lumbar, s/p: status post.

Table 5
Cases with gastrointestinal tract abnormalities.

Case ID	Age	Indication	GA	AFAFP MoM	Karyotype	Abnormal fetal ultrasound	Perinatal outcome
#23	28y	Abnormal maternal serum screening	22w	2.42	46,XY	Nil	Delivery at 39 weeks of gestation, duodenal obstruction, s/p pediatric surgery at age 1month
#29	37y	AMA	27w	2.34	46,XY	Absent gastric bubble, IUGR	Delivery at 39 weeks of gestation, esophageal atresia, s/p pediatric surgery at age 5 days, SGA
#30	36y	AMA	18w	2.33	46,XY	Nil	Delivery at 39 weeks of gestation, Hirschsprung disease s/p pediatric surgery at age 1 year

GA: gestational age, AFAFP: amniotic fluid alpha-fetoprotein, MoM: multiples of median, y: years, AMA: advanced maternal age, w: weeks, s/p: status post, IUGR: intrauterine growth restriction.

Table 6
Cases with cardiovascular abnormalities.

Case ID	Age	Indication	GA	AFAFP MoM	Karyotype	Abnormal fetal ultrasound	Perinatal outcome
#25	34y	Abnormal fetal ultrasound	17w	2.38	47,XY+18	ASD,VSD, omphalocele	TOP
#32	24y	Abnormal fetal ultrasound	23w	2.31	46,XY,r(13) [23]/45,XY,-13/46,XY,idic,r(13) [2]	Dextrocardia, IUGR	TOP

GA: gestational age, AFAFP: amniotic fluid alpha-fetoprotein, MoM: multiples of median, y: years, w: weeks, ASD: atrial septal defect, VSD: ventricular septal defect, TOP: termination of pregnancy, IUGR: intrauterine growth restriction.

Table 7
Cases of genitourinary tract abnormalities.

Case ID	Age	Indication	GA	AFAFP MoM	Karyotype	Abnormal fetal ultrasound	Perinatal outcome
#14	23y	Abnormal fetal ultrasound	14w	2.82	46,XX	Megacystis, hydronephrosis, oligohydramnios	TOP
#34	38y	AMA	18w	2.29	46,XY	Nil	Delivery at 38 weeks of gestation, left bifid ureter, right mild pelvic dilatation, follow up to age 3 months

GA: gestational age, AFAFP: amniotic fluid alpha-fetoprotein, MoM: multiples of median, y: years, w: weeks, TOP: termination of pregnancy.

Regarding the major fetal structural abnormality distribution, elevated AFAFP levels were associated with abdominal wall defect and central nervous system, gastrointestinal tract, cardiovascular, and genitourinary tract abnormalities. These complex structural abnormalities included omphalocele, gastroschisis, para-umbilical cyst, spinal bifida, subependymal cyst with hemorrhage, hydrocephalus, duodenal obstruction, esophageal atresia, Hirschsprung disease, ASD,VSD, dextrocardia, megacystis, hydronephrosis, and bifid ureter. If the fetus had structural defects, including NTD, abdominal wall defect, gastrointestinal tract or genitourinary tract obstruction with lumen defect, the explosion of membrane and vessel surfaces allowed AFP to transudate into the amniotic fluid, leading to increased AFAFP levels. Of interest, one of the four cases with omphalocele in our series, case #4, was conceived by in vitro fertilization. Beckwith–Wiedemann syndrome (BWS), the most common human-assisted reproduction technologies-related imprinting disorders, is observed in 37.5% and 5% of isolated and non-isolated omphaloceles, respectively, after excluding aneuploidy [21,22]. Thus, BWS must to be considered in cases with omphalocele. However, case #4 had not received array-based comparative genomic hybridization; thus, further detailed genetic studies are recommended in future studies.

Elevated AFP level is associated with reduced uteroplacental blood flow in the uterine artery [23]. Adverse pregnancy conditions associated with high level of AFP reportedly include stillbirth, preterm labor, neonatal death, and low birth weight [1]. In our study, the adverse obstetric events observed included preterm delivery, IUFD, SGA, preeclampsia, GDM, gestational hypertension, PPROM, prolonged labor, and preterm uterine contraction.

MSAFP is a common diagnostic tool used in prenatal examination, and its utility can be compared with that of AFAFP. Moreover, adverse fetal outcomes can be associated with elevated MSAFP levels. There is a correlation between the incidence of adverse fetal outcome and MSAFP level. Notably, Hu et al. [24] reported that the incidence of adverse fetal outcome was 42.89% with MSAFP MoM >2.5. In another study, the incidence was 3.4% and 40.3% with MSAFP MoM of >2.5 and > 7.0, respectively [25]. In the present study, the highest incidence of adverse perinatal outcomes was identified in cases with AFAFP MoM >5 (2/2, 100%), followed by 11 of 19 cases (57.8%) with AFAFP MoM >2.5 and 30 of 55 cases (54.5%) with AFAFP MoM >2. Hence, AFAFP has a higher prediction rate for an adverse fetal outcome than MSAFP; moreover, it is a relatively effective indicator for prenatal diagnosis.

Our results supported the notion that AFAFP was a valuable screening tool for predicting adverse perinatal outcomes. AFAFP routinely obtained during amniocentesis may be a valuable additional test providing early clues for advanced prenatal diagnosis without additional risks or costs. This combined screening of AFAFP and ultrasound could help avoid missed NTD, abdominal wall defect, and other fetal defects.

Thus, we recommend that pregnant women with high AFAFP levels (>2.0 MoM) should be alerted of the possibility of fetal chromosomal and/or major structural abnormalities. Further detailed genetic studies and serial sonographic examinations should be performed for advanced prenatal diagnosis.

Conclusions

High AFAFP levels (>2.0 MoM) in midtrimester can be associated with abnormal fetal outcomes, including chromosomal abnormalities, major structural abnormalities, and adverse obstetric events. Women with a prenatal diagnosis of high AFAFP levels (>2.0 MoM)

should be alerted of the possibility of abnormal fetal outcomes, and further detailed genetic studies and serial sonographic examinations are recommended.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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Original Article

Effect of extra-low dose levothyroxine supplementation on pregnancy outcomes in women with subclinical hypothyroidism undergoing in vitro fertilization and embryo transfer

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ABSTRACT

Objective: This study was undertaken to test the therapeutic effect of extra-low dose of levothyroxine (LT4; 25 mcg/day) to preconception and pregnant women with subclinical hypothyroidism (SCH).
Materials and methods: This is a retrospective study, SCH women who succeeded in their first in vitro fertilization (IVF) cycle between January 1, 2018, to December 31, 2020 were included. SCH is defined as normal serum free thyroxine (T4) level and an elevated serum thyroid stimulating hormone (TSH) level >4 mIU/L. Extra-low dose of levothyroxine (LT4; 25 mcg/day) was prescribed to the SCH women from the establish of diagnosis of SCH to the end of pregnancy. The pregnancy outcomes (miscarriage, live birth, preterm birth, and small for gestational age baby) were compared to the euthyroid pregnant women.
Results: Totally, 589 women were screened, and 317 cases received their first time IVF treatment. 167 women were clinically pregnant after IVF treatment, 155 of them were euthyroid and 12 of these women were diagnosed to have SCH. The average age of the participants was 35 years old. There were no significant differences in age, body mass index (BMI), anti-müllerian hormone (AMH), types of embryo transfer, number of embryos to transfer, or embryo stage during transfer between two groups. The live birth rate, miscarriage rate, and preterm birth rate in women with SCH supplemented with extra-low dose of LT4 were non-inferior to euthyroid patients (miscarriage rate: $P = 0.7112$; live birth rate: $P = 0.7028$; preterm delivery: $P = 0.2419$; small for gestational age: $P = 0.2419$).
Conclusion: Our result demonstrated that supplementation with extra-low dose of levothyroxine at 25 mcg/day to SCH women can produce the comparable obstetrical and neonatal outcome as that in euthyroid pregnant women. Accordingly, we suggest extra-low-dose of levothyroxine may be considered as a safe and effective alternative for those SCH pregnant women who were not tolerated to the standard dose of levothyroxine.

Introduction

Subclinical hypothyroidism (SCH) is defined as normal serum levels of free thyroxine (T4) with elevated thyroid stimulating hormone (TSH) without obvious clinical symptoms [1]. The prevalence of SCH in the reproductive-age population is approximately 4–8% [2], while in infertile women, the incidence of SCH can reach 13.9% [3]. Previous studies have demonstrated that women with subclinical thyroid disease before conception or during pregnancy were associated with adverse outcomes such as pregnancy loss, premature delivery, hypertensive disorders, and adverse neurocognitive outcomes (IQ) in their offspring [4–7].

The diagnosis of SCH is based on thyroid function testing. Since different upper limits and the cutoff value of normal TSH reference range have been used in previous publications, the benefits of levothyroxine (LT4) supplementation for SCH have always been controversial. For example, Rao et al., have confirmed the beneficial effects of LT4 supplementation in reducing the risk of pregnancy loss and preterm birth in women with SCH [8,9]. Bein et al. also demonstrated that LT4 supplementation could reduce the risk of

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adverse pregnancy outcomes in women with SCH in their systematic review and meta-analysis [10]. Ding et al. confirmed LT4 treatment could reduce the risk of pregnancy loss, preterm delivery and gestational hypertension in their report [11]. Currently, the American Society for Reproductive Medicine (ASRM) and the European Thyroid Association (ETA) have suggested LT4 treatment in women with SCH (TSH >4.0 mIU/L) to reduce miscarriage rates and improve pregnancy outcome when receiving assisted reproduction technology (ART) [12]. The American Thyroid Association (ATA) also recommends the supplementation of LT4 for infertile women with SCH to improve the fertilization rate of oocytes and clinical pregnancy outcome when receiving ART [4].

However, despite LT4 supplement for preconception and pregnant SCH women has been suggested, the optimal dosage of LT4 supplementation is still undetermined. The dose of LT4 for preconception and pregnant SCH women in previous studies was around 50–100 mcg/day [13–15]. Still, some patients have experienced different degrees of adverse effects, such as increased heart rate, sweating, and anxiety. According to the American Association of Clinical Endocrinologists (AACE) and American Thyroid Association (ATA) guidelines, a low dose of 25–75 mcg/day is usually sufficient for achieving euthyroid levels in SCH patients. Since it carries the potential risk of overtreating the patients with SCH than overt hypothyroidism [16], the ideal dosage of LT4 should be as low as possible while maintaining the therapeutic effect. In this study, we gave our SCH patients an extra-low dose of LT4 (25 mcg/day) and reported the obstetrical and perinatal outcomes as compared to euthyroid pregnant women.

Materials and methods

This is a retrospective, single-center study. Women who underwent their first in vitro fertilization (IVF) retrieval cycle between January 2018 and December 2020 were recruited. Patients over 42 years old with known thyroid dysfunction, newly diagnosed overt hypothyroidism, hyperthyroidism, thyroid autoimmunity, donor eggs, or missing data were excluded. Data were collected from electronic and paper-based medical records.

All patients received a TSH examination before starting their IVF treatment. Patients with normal serum-free T4 but elevated TSH (>4 mIU/L) level were diagnosed to have SCH. SCH patients would receive LT4 supplement at 25 mcg/day for at least one month before ovarian stimulation and continuously during pregnancy. In all the patients, a GnRH antagonist protocol or progestin-primed ovarian stimulation (PPOS) protocol was used for controlled ovarian stimulation. Recombinant human FSH (rhFSH) with or without human menopausal gonadotropin (HMG) or recombinant human LH was administered from the third day of the menstrual cycle. Dual trigger with hCG and GnRH agonist or GnRH agonist alone were given for final oocyte maturation when one or more follicles reached a mean diameter of $\geq$18 mm. Transvaginal ultrasound-guided oocyte retrieval was performed 34–36 h after rhCG or GnRH agonist injection. The harvested oocytes were fertilized either by traditional insemination or intracytoplasmic sperm injection (ICSI). Either fresh or frozen embryo transfer was performed according to the patient's clinical condition. The embryos were transferred at 3 days or 5 days after oocyte retrieval. Luteal phase support was provided by either micronized progesterone 200 mg (Utrogestan) three times daily or 90 mg of vaginal P gel (Crinone gel 8%) with or without 50 mg intramuscular progesterone or 125 mg hydroxyprogesterone caproate. All patients returned approximately 14 days after embryo transfer for a quantitative serum human chorionic gonadotropin (hCG) evaluation to confirm pregnancy. A visible intrauterine sac at 5–6th weeks by transvaginal ultrasound was defined as clinical pregnancy. The miscarriage rate was defined as the ratio of fetal loss before the 20th week of gestation in all pregnant women. The live birth rate was calculated as the percentage of deliveries with at least one infant among all pregnant women. Preterm delivery was defined as babies born alive before 37 weeks of gestation. Small for gestational age (SGA) was defined as weight less than the 10th percentile for gestational age using Hadlock's proportionality formula. The primary outcomes included miscarriage rate, live birth rate, preterm birth, and SGA baby in this study.

The Chi–Square test, or Fisher exact test, and Wilcoxon rank sum test were used to compare the distributions of categorical and continuous variables between subjects with TSH levels of $\leq$4 and >4 mU/L with LT4 treatment, respectively, and expressed as frequency (%) and median. Logistic regression analysis was conducted to estimate the odds ratio (OR) of TSH levels and reproductive outcomes. First, a univariate analysis was conducted to estimate the crude OR of the outcome. Second, age, body mass index (BMI) and anti-müllerian hormone (AMH) were included in the multivariate regression model to adjust for potential confounding factors. A two-tailed P value < 0.05 was considered statistically significant. All statistical analyses were performed using the SAS software (version 9.4; SAS Institute, Cary, NC, USA).

Ethical approval

The study protocol has been reviewed and approved by the Institutional Review Board of Chi Mei Medical Center (11107–007), Tainan, Taiwan.

Results

A total of 589 women were screened, and 317 women were enrolled after excluding patients who did not meet our inclusion criteria. Of the 167 women with clinical pregnancy, the average age was 35 years, among which 92 (55.09%) women were older than 35 years. 92.8% (155/167) of patients had TSH concentrations $\leq$4 mIU/L and 7.2% (12/167) of patients were newly diagnosed with SCH with TSH concentrations >4 mIU/L. There were no statistically significant differences in age, BMI, AMH, types of embryo transfer, number of embryos to transfer, embryo stage during transfer and clinical outcomes between two groups (Tables 1 and 2).

Nine of 12 (75%) women in group I and 124 of 155 (80%) women in group II had live births ($P = 0.7028$). The miscarriage rate was 20% (31/155) in the euthyroid group and 25% (3/12) in the SCH group ($P = 0.7112$). Seventeen (13.7%) euthyroid women and none of the SCH women had preterm delivery ($P = 0.6031$). Thirty-one (25%) euthyroid women and four (44%) women with SCH had small for gestational age baby ($P = 0.2419$). The results were also not statistically significantly different between two groups. Logistic regression was used to estimate the odds ratio (OR) for TSH levels and reproductive outcomes. After adjusting for age, BMI, and AMH level, there was still no statistically significant difference between group I and II (Table 3). The 44% of SGA babies in the SCH group was mainly due to the small sample size. Further analysis of these women revealed some of them had a history of medical problems such as chronic epilepsy in one mother and severe uterine adenomyosis in two.

Discussion

Thyroid hormone (TH) has been shown not only to influence the endometrial and placental physiology but also to be essential for fetal brain development in the embryonic phase. TH receptors (THR) and TSH receptors (TSHR) are widely expressed in the feto-maternal unit during implantation, and both the endometrium

Table 1
Characteristics of 167 pregnant women between TSH level of ≤4 and TSH >4 mIU/L with LT4 treatment.

	Total	TSH ≤4	TSH >4 (LT4 treatment)	p-value
	N[a] = 167	N = 155	N = 12	
Age (y/o)	35.00 (33.00–38.00)	35.00 (33.00–38.00)	34.00 (32.50–36.50)	0.2394
<35	75 (44.91)	67 (42.23)	8 (66.67)	
≥35	92 (55.09)	88 (56.77)	4 (33.33)	
BMI (kg/m^2)	24.44 (21.48–27.41)	24.46 (21.64–27.51)	21.52 (20.04–25.16)	0.1289
BMI subgroup; N (%)				0.1178
<18.5	8 (4.79)	6 (3.87)	2 (16.67)	
18.5 ≤ BMI<24	66 (39.52)	61 (39.35)	5 (41.67)	
≥24	93 (55.69)	88 (56.77)	5 (41.67)	
AMH (ng/ml); N (%)				1.0000
≥2.5 1	89 (53.29)	83 (53.55)	6 (50.00)	
<2.5 0	78 (46.71)	72 (46.45)	6 (50.00)	
Embryo transfer				0.4804
Fresh	40 (23.64)	35 (22.88)	4 (33.33)	
Frozen	127 (76.36)	118 (77.12)	8 (66.67)	
No. of embryos to transfer				0.7256
N = 1	40 (23.95)	37 (23.87)	3 (25.00)	
N = 2	110 (65.87)	101 (65.16)	9 (75.00)	
N = 3	17 (10.18)	17 (10.97)	0	
Embryo stage				0.5458
Day 3	57 (34.13)	52 (33.55)	5 (41.67)	
Day 5	110 (65.87)	103 (66.45)	7 (58.33)	

[a] N: case number.

Table 2
Pregnancy and neonatal outcomes of 167 pregnant women and 133 women who have live birth between TSH level of ≤4 and TSH >4 mIU/L with LT4 treatment.

	Total	TSH ≤4	TSH >4 (LT4 treatment)	p-value
	N = 167	N = 155	N = 12	
Miscarriage; N (%)	34 (20.24)	31 (20.00)	3 (25.00)	0.7112
Live birth; N (%)	133 (79.64)	124 (80.00)	9 (75.00)	0.7028
	Total	TSH ≤4	TSH >4 (LT4 treatment)	
	N = 133	N = 124	N = 9	
Neonatal birth weight (grams); N (%)				0.2419
≥10%	98 (73.68)	93 (75.00)	5 (55.56)	
<10% (SGA[a])	35 (26.32)	31 (25.00)	4 (44.44)	
Gestational age (weeks); N (%)				0.6031
≥37	116 (87.22)	107 (86.29)	9 (100)	
<37(Preterm)	17 (12.78)	17 (13.71)	0 (0)	

[a] SGA: Small for gestational age, defined as below 10th percentile by Hadlock calculator.

Table 3
The odds ratios of selected characteristics between TSH levels of ≤4 and >4 mIU/L with LT4 treatment.

	TSH≤4 vs. TSH>4 (LT4 treatment)			
	Crude OR	p-value	Adjusted ORs[c]	p-value
Miscarriage[a]	0.90 (0.26–3.17)	0.8747	0.81 (0.22–2.94)	0.7502
Live birth[a]	0.75 (0.19–2.94)	0.6793	0.79 (0.19–3.25)	0.7410
Neonatal birth weight (grams)[b] (N = 133)				
≥10%	1.00 (ref.)		1.00 (ref)	
<10% (SGA)	2.40 (0.61–9.51)	0.2121	2.30 (0.57–9.27)	0.2400
Gestational age (weeks)[b] (N = 133)				
≥37	1.00 (ref.)		1.00 (ref)	
<37(Preterm)	0.32 (0.02–6.76)	0.4666	0.35 (0.02–7.13)	0.4973

[a] Only calculated pregnant women (N = 167).
[b] Only calculated women who had live birth (N = 133).
[c] Adjusted ORs were adjusted with age, BMI, and AMH.

and the trophoblast might be influenced by TH either directly or through TH effects on the synthesis and activity of implantation-mediating molecules [17]. Previous reports have indicated that the local action of TH on the endometrium and embryo during the implantation period is crucial for a successful pregnancy [8–11]. Abnormal TH levels in placental trophoblasts followed by diminished trophoblast endocrine function may result in a direct consequence of pregnancy loss. Furthermore, trophoblasts dysfunction also impairs the placental vascular formation and may induce many obstetric complications, such as preterm birth, SGA babies, and preeclampsia [18].

SCH is defined as an elevated TSH level with normal levels of free thyroxine. Previous research has found that it affects 0.25–2.5% of all pregnancies, depending on the definitions used and the populations studied. SCH during pregnancy has been associated with early pregnancy loss, gestational diabetes, hypertension and pre-eclampsia, placental abruption, premature rupture of membranes and neonatal death. LT4 treatment has been used in SCH women who are trying to conceive or already pregnant. LT4 is a synthetic medicine of thyroxine (T4) that mimics its physiologic effects. ATA guidelines suggest TSH levels should be kept at ≤ 2.5 mIU/mL throughout the pregnancy [4]. Guideline from European Thyroid Association suggests TSH should be maintained below 2.5

mIU/mL in the first trimester and 3 mIU/mL in the second and third trimesters [12]. Rahman et al. declared that LT4 at 50–100 mcg/day might decrease miscarriage and increase live birth rate [15]. Kim et al. reported that 50 mcg/day of LT4 could improve embryo quality, decrease miscarriage and increase the live birth rate [14]. Nazarpour et al. also suggested that a dose of 1 mcg/kg/day reduced preterm delivery and newborn admissions to the neonatal unit [9]. Despite the agreement of supplementation of LT4 for SCH pregnant women has been reached, the optimal dosage of T4 for SCH mother has never been concluded [19,20]. For the majority of studies, 50 mcg/day was suggested [2,14], but AACE/ATA guidelines and previous report have declared a potential overtreatment in these women. Indeed, it is prone to overtreat SCH women than women with overt hypothyroidism, since free T4 concentrations were normal before medication in SCH women [16,21].

Despite rare, iatrogenic hyperthyroidism, defined as overtreatment with LT4, has been reported during pregnancy [24]. The first study published in 1998 reported the prevalence of iatrogenic hyperthyroidism [23]; 2.5% was overtly thyrotoxic [22]. Lage et al. reported recently, among hypothyroidism pregnant women treated with levothyroxine, 1.03% were overtreated [24]. Indeed, in the first few weeks of pregnancy, there are physiological changes that might induce gestational transient thyrotoxicosis (GTT). The GTT might originate from the increased thyroid stimulation by endogenous hCG production from the placenta and become deteriorated by an inappropriate exogenous thyroid supplementation [25]. Therefore, the dosage of LT4 for SCH mother should keep effective but remain as low as possible to prevent the potential over-treatment from exogenous T4 supplementation [26]. Korevaar et al. reported abnormal maternal freeT4 concentrations during pregnancy was associated with lower child IQ and lower gray matter and cortex volume [27]. Maraka and Lemieux et al. analyzed 5405 women with SCH and detected overtreatment of thyroid hormones with an increased risk of adverse pregnancy outcomes such as preterm delivery, gestational diabetes, and preeclampsia [28]. Lemieux et al. found the overtreatment with LT4 in pregnant women at any time during pregnancy might be associated with a twice high risk of preterm delivery [29]. Dash et al. suggested while LT4 supplementation has potential beneficial effects but also carries a higher risk of gestational diabetes [30].

In our study, all pregnant women with SCH were prescribed with an extra-low dose of LT4 before conception and during pregnancy, and we found live birth rate is non-inferior to the euthyroid pregnant women. The miscarriage rate, preterm birth rate, and small for gestational age rate were also comparable between the two groups by the analysis of odd ratio under logistic regression. There were some limitations in our study. First, the major pitfall in this retrospective study was the lack of post-treatment TSH level data. Although we administered an extra-low dose of LT4 supplementation and there is no evidence in the published literature that such a dosage can cause iatrogenic hyperthyroidism, the absence of post-treatment TSH level assessment is a notable limitation. Therefore, we recommend measuring TSH levels as a critical criterion for evaluating treatment efficacy, which should be performed six to eight weeks after the initiation of therapy, with subsequent testing every two or three months [4]. It is noteworthy that none of the patients in our study complained of palpitations, tremors, or anxiety while undergoing treatment with 25 mcg/day of LT4. Nevertheless, symptoms alone are not enough for evaluating TSH levels since SCH patients are typically asymptomatic, and mild hyperthyroidism may not result in noticeable symptoms. Therefore, we encourage SCH patients taking LT4 supplements to routinely check their post-treatment TSH levels. In the futures, further extensive studies may be necessary to investigate the changes in TSH levels under extra-low dose of LT4 treatment. Though it is a pity that the post-treatment serum TSH level was not available, the therapeutic effect of extra-low dose of T4 on the SCH pregnant women has been justified by the final obstetrical and neonatal outcomes in this study. To our knowledge, only a limited studies have ever reported the pregnancy outcomes after treatment with an extra-low dose of LT4. We hope to remind clinician that if the patients were not tolerated the standard dose of LT4, then 25 mcg/day of LT4 might be an alternative to avoid overdose effects. The second pitfall in this study is the relatively small size of sample. Despite this, different statistical analysis methods have been conducted to justify our result. Third, the original study design failed to compare the efficacy of LT4 and placebo in SCH patients. Since established guidelines from professional societies strongly recommend LT4 supplements to improve ART and pregnancy outcomes in SCH patients, it might induce some ethical challenges if we refused to give the SCH women purposely.

In conclusion, we found supplementation with an extra-low dose of levothyroxine at 25 mcg/day for SCH women might produce the same clinical outcome as that in euthyroid patients. Extra-low dose levothyroxine may be administered as a safe and effective alternative for patients who were not tolerated the standard dose of levothyroxine.

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Author contribution

Dr. YC Tsai designed this study, provided data and revised this article. Dr. YT Chen collected data and wrote this article; Mr. CH Ho conducted statistics analysis; the other authors provided their opinions and joined the discussion.

Declaration of competing interest

The authors declare no conflicts of interest relevant to this article.

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Original Article

The no-observed-adverse-effect level of phthalates promotes proliferation and cell cycle progression in normal human breast cells

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ABSTRACT

Objective: The data on the association between phthalates and breast cancer risk remains inconsistent. This study aimed to explore the possible mechanism of low-dose exposures of phthalates, including Butyl benzyl phthalate (BBP), di(n-butyl) phthalate (DBP), and di(20ethylhexyl) phthalate (DEHP), on breast tumorigenesis.

Methods and methods: MCF-10A normal breast cells were treated with phthalates (10 and 100 nM) and 17β-estradiol (E_2, 10 nM), which were co-cultured with fibroblasts from normal mammary tissue. Cell viability, cycle, and apoptosis were detected by MTT assay, flow cytometry, and TUNEL assay respectively. The expression levels of related proteins were determined by Western blot.

Results: Like E_2, both 10 nM and 100 nM phthalates exerted significantly higher cell viability, lower apoptosis, and increased cell numbers in the S and G2/M phases with up-regulation of cyclin D/CDK4, cyclin E/CDK2, cyclin A/CDK2, cyclin A/CDK1, and cyclin B/CDK1, compared with the control group. Significant increase in PDK1, P13K, p-AKT, p-mTOR, and BCL-2 expression and a decrease in Bax protein, cytochrome C, caspase 8, and caspase 3 levels were noted in cells treated with 10 nM and 100 nM phthalates and E2, compared with the control group and MCF-10A cells co-cultured with fibroblasts. The effects of the three phthalates were noted to be dose-dependent.

Conclusions: The results indicate that phthalates at a level below its no-observed-adverse-effect concentration, as defined by the current standards, still induce cell cycle progression and proliferation as well as inhibit apoptosis of normal breast cells. Thus, the possibility of breast tumorigenesis through chronic phthalate exposure should be considered.

Introduction

In 2020, female breast cancer surpassed lung cancer to become the leading diagnosed cancer worldwide [1]. Despites increasing life expectancy and personal risk factors, the role of environment endocrine disruptors, such as phthalates, in breast carcinogenesis should be considered. Phthalates, including Butyl benzyl phthalate (BBP), di(n-butyl) phthalate (DBP), and di(2-ethylhexyl) phthalate (DEHP), are used in many consumer products, such as pharmaceuticals, medical devices, and food packaging [2], which results in human exposure. Although epidemiological studies are limited, associations between phthalate urinary concentrations and breast cancer risk have been reported in Mexican and Alaska-Native women [3,4]. In addition, breast cancer incidence associated with cumulative exposure to phthalates has been demonstrated in previous literature: a Canadian case-control study from the automotive and food-canning industries [5], as well as in a Danish nationwide cohort study [6]. Thus, the effects of phthalates on breast cancer risk should not be ignored.

The association between phthalates exposure and the risk of breast cancer is still under debate as results from epidemiologic studies using urinary phthalate metabolites as a proxy for systemic exposure to parent compounds have been inconsistent [3,4,7,8]. Although phthalate clearance from the body is rapid and is done mainly via urinary excretion with only slight cumulative potential, measurement of urinary metabolites only captures recent exposure. A recent study found that phthalates with long alkyl chains, such as DEHP, undergo further metabolism prior to excretion [9]. Although both primary and secondary phthalates metabolites have

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biological activity, the influence of cumulative and no-observed-adverse-effect concentrations of phthalates on breast cells require further evaluation.

The European Food Safety Authority (EFSA) has established a tolerable daily intake (TDI) of 50 μg/kg body weight per day for DBP, BBP, DEHP, and diisononyl phthalate (DINP) collectively by utilizing the "no-observed-adverse-effect level" and dividing it by an uncertainty factor of 100 (EFSA 2005a) [10]. Our previous studies revealed that BBP, DBP, and DEHP at concentrations of 100 nM (a level comparable to TDI) and 10 nM (a level lower than TDI) were capable of inducing a proliferative effect, not only on breast cancer cells [11,12] but also on normal breast cells (MCF-10A) co-cultured with fibroblasts from estrogen receptor (ER) positive breast cancers [13]. These results revealed that tumorigenesis could potentially be induced by phthalates at levels lower than the no-observed-adverse-effect concentrations. As suggested from the California Breast Cancer Research Program [14] and the Breast Cancer and Chemicals Policy (BCCP) project [15], cell cycle changes include increased cell replication or decreased apoptosis. Both endpoints are widely recognized as markers of increased cellularity, which could signify limitless cell replication, a hallmark of cancer. Thus, to further identify the potential impact on breast cancer risk, we investigated and compared the effects of BBP, DBP, and DEHP at both 10 and 100 nM concentrations on cell cycle changes, as well as on the signaling pathway for cell apoptosis and the proliferation of normal breast cells.

Materials and methods

Ethics

This study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethical Medicine Committee of Chang Gung Memorial Hospital (IRB 201901918B0 and date of approval: 03/01/2020-07/31/2021).

Reagents and concentrations of phthalates

Phthalates (10 and 100 nM) including butyl benzyl phthalate (BBP), di(n-butyl) phthalate (DBP), and di(20ethylhexyl) phthalate (DEHP) were purchased from SUPEL Co. (Bellefonte, PA, USA). 17β-estradiol (E2, 10 nM) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The compounds were reconstituted according to the manufacturer's instructions as stated on the package insert and were stored in aliquots at −20 °C. The European Food Safety Authority (EFSA) has established a tolerable daily intake (TDI) of 50 μg/kg body weight per day for DBP, BBP, and DEHP collectively [10]. Despites this reference dose, the concentrations of phthalates used in this study were determined using a concentration-response curve, as demonstrated in our previous study using MCF-7 breast cells. In that study, MCF-7 breast cancer cells were exposed at concentrations of 10^{-10} to 10^{-4} M for 24, 48, 72, and 96 h respectively [11]. DEHP> 10^{-5} M and both BBP and DBP at 10^{-4} M significantly decreased cell survival in the MTT assay. Cell proliferation significantly increased at concentrations of 10^{-8} to 10^{-5} M of BBP and DBP and at 10^{-8} to 10^{-6} M of DEHP. Therefore, 10 nM and 100 nM phthalates were chosen to represent decreased and comparable concentrations to TDI respectively.

Cell culture

MCF-10A cells

MCF-10A cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The MCF-10A normal mammary epithelial cell line was cultured in Dulbecco modified Eagle medium/F12 (1:1) media containing 10% fetal bovine serum, 20 ng/mL epidermal growth factor, 0.5 mg/mL hydrocortisone, 100 ng/mL cholera toxin, 10 μg/mL insulin, 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin in humidified air (5% CO2) at 37 °C. Cell cultures were periodically checked for mycoplasma using PCR (Biological Industries, Connecticut, USA) and DNA fluorochrome staining (DAPI, Roche, Mannheim, Germany) (with the most recent test in 09/2020). The cell lines were also authenticated using short tandem repeat (STR) profiling by scientific services at Genomics BioSci. & Tech. Co., LTD, Taiwan.

The cell lines were tested and found to be free of mycoplasma and were cultured for no more than 10 passages, per the manufacturer's recommendations. The cell types were checked for proper morphology prior to every experiment and consistently monitored for changes in cell replication that might suggest mycoplasma contamination.

Isolation of primary fibroblast

After obtaining informed consent, consecutive breast tissue samples were obtained from fresh surgical tissue at the time of mastectomy procedures at Keelung Chang Gung Memorial Hospital. Histological assessment and tumor typing were performed at the Department of Pathology at Keelung Chang Gung Memorial hospital. Three ER (+) stromal cells were collected from six women. Data related to the patients' characteristics, sample size, and type of surgery were recorded. According to the procedure as described by Proia and Kuperwasser [16] with minor modifications, breast tissue was carefully dissected under a stereo-microscope to exclude as much adipose tissue as possible and then cut with fine scissors to 3–5 mm^3. 1–2 g of tissue was subsequently transferred to a 15-ml conical polypropylene tube filled with 10 ml of working collagenase solution and incubated on a rotator at 37 °C until the large tissue fragments were dissociated. The tubes were removed from the incubator and were left to stand for 2–5 min to allow the organoids to settle. The supernatant was decanted to a fresh tube and then washed with PBS and centrifuged (300g on a tabletop centrifuge at 4 °C) for 5 min. The washing and centrifuging were repeated three or more times with 10 ml of PBS. The fibroblastic nature of the isolated cells was confirmed through microscopic determination of the morphology portrait and immunofluorescence characterization using antibodies against the fibroblast surface protein (FSP) (Abcam, Cambridge, MA, USA). The primary fibroblast cells were maintained in DMEM/F12, supplemented with 10% fetal bovine serum, 1.5 mg/L sodium bicarbonate, and 100 IU/ml penicillin with 100 g/ml streptomycin and cultured at 37 °C in 5% humidified CO2 environment.

Co-culture system

For construction of the cell–cell interactive environment, MCF-10A cells were plated on the bottom of the six-well transwell cell culture system (Corning, NY, USA) at a density of 4×10^5 cells/well. Contrastingly, primary fibroblasts at a density of 4×10^5 cells/well on the membrane of the transwell cell culture inserts (Pore size 0.4 μm; Corning, NY, USA) were placed into the six-well pates containing MCF-10A cells to initiate the experiments. Co-culture was continuously cultured for 120 h. The co-cultures were incubated with serum-free DMEM/F12 1.5 mg/L sodium bicarbonate, 100 IU/ml penicillin, and 100 μg/ml streptomycin, and were also treated separately with phthalates (BBP, DBP, and DEHP at both 10 nM and 100 nM concentrations) and estradiol (E2 at 10 nM) alone.

Methods of evaluation

Cell viability assay

Cell viability was determined using a 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (Sigma Chemical Co, St. Louis, MO, USA) [17] after completing the aforementioned

co-culture experiments. For the assay, 100 μL of the prepared MTT (0.5 mg/ml in no phenol red and serum-free DMEM/F12) (Sigma Chemical Co, St. Louis, MO, USA) was added to each well (5000 cells/well) and cells were incubated for 4 h at 37 °C and 5% CO2. Then, 100 μl DMSO (Sigma Chemical Co, St. Louis, MO, USA) was added into each well to dissolve the formazan crystals. The plates were incubated overnight at 37 °C and 5% CO2. The optical density in each well was determined with a microplate reader (Molecular Device Spectramax M3, Sunnyvale, CA, USA) using an absorption spectrum of 570 nm. The corresponding cell-free culture media was also examined during the MTT incubation for proper background subtraction. The quantity of the formazan product was directly proportional to the number of viable cells in the culture medium.

Cell apoptosis assay

Cell death (apoptosis) was measured by terminal deoxynucleotidyl transferase mediated dUTP nick end labelling (TUNEL). MCF-10A cells grown on a 6-mm plate were washed twice with PBS and fixed for 30 min in 4% buffered paraformaldehyde. The cells were then incubated with 0.1% Triton X-100 in 0.1% sodium citrate solution for 8 min, washed in phosphate-buffered saline, and incubated with terminal deoxynucleotidyl transferase for 90 min and then fluorescein isothiocyanate-dUTP for 30 min at 37 °C using an apoptosis detection kit (Roche Applied Science, IN, USA). Samples were analyzed in a drop of PBS under a fluorescent UV light microscope at this state, using an excitation wavelength in the range of 450~500 nm and detection in the range of 515~565 nm (green).

Cell cycle analysis

Cells were harvested and washed in PBS three times and then fixed in cold 70% ethanol for 30 min at 4 °C. Cells were washed with PBS again thrice. Propidium iodide (PI, Sigma–Aldrich P4170, St. Louis, MO, USA) was added into cells with a final concentration of 10 μg/ml. DNA contents of the cells were analyzed using a Gallios flow cytometer (Beckman Coulter, Indianapolis, IN, USA) and their cell cycle distribution patterns were determined using Kaluza 2.1 software (Beckman Coulter, Indianapolis, IN, USA). 10 000 cells in each sample were analyzed and the percentages of cells at each of the various cell cycle stages were determined.

Western blot analysis

The cell pellets were lysed for 30 min in lysis buffer (50 mM Tris, pH 7.5, 0.5 M NaCl, 1.0 mM EDTA, pH 7.5, 10% glycerol, 1 mM basal medium Eagle, 1% Igepal-630, and proteinase inhibitor cocktail tablet) (Roche Applied Science, IN, USA) and then centrifuged at 12 000 g for 10 min. The supernatants were removed and placed in new Eppendorf tubes for western blot analysis. Proteins from the MCF-10A cells were separated in 12% gradient SDS–PAGE and transferred onto nitrocellulose 10 membranes. Nonspecific protein binding was blocked using a blocking buffer at RT for 1 h (5% milk, 20 mM Tris–HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween 20). The membranes were blotted with cyclin A (Merck Millipore, Darmstadt, DE.), cyclin B (Merck Millipore, Darmstadt, DE.), cyclin D (Merck Millipore, Darmstadt, DE.), cyclin E (Upstate, Lake Placid, NY), CDK 1 (Merck Millipore, Darmstadt, DE.), CDK 2 (Merck Millipore, Darmstadt, DE.), CDK4 (Merck Millipore, Darmstadt, DE.), phosphatidyl-inositol 3-kinase (PI3K) (Merck Millipore, Darmstadt, DE.), phospho-AKT Ser473 (Upstate, Lake Placid, NY), Phosphoinositide-dependent Kinase-1 (PDK-1) (Merck Millipore, Darmstadt, DE.), mammalian target of rapamycin (mTOR) (Merck Millipore, Darmstadt, DE.), B-cell lymphoma-2 (BCL-2) (BD Pharmingen, San Diego, CA), Bcl-2-associated X protein (BAX) (Merck Millipore, Darmstadt, DE.), Caspase-3 (Chemicon, Temecula, CA, USA), Caspase-8 (Merck Millipore, Darmstadt, DE.), Cytochrome C (BD Pharmingen, San Diego, CA), Vinculin (Abcam, Cambridge, MA, USA), and β-actin (Thermo, Rockford, IL, USA) antibodies and then incubated in 4 °C blocking buffer overnight. Densitometric analysis of immunoblots was performed using the Bio Rad molecular imager versadoc MP 4000 system (Bio Rad, Hercules, CA, USA). Experiments were performed in triplicate.

Statistical analysis

Cell proliferation data was expressed using percentages and then compared with vehicle-treated control cells, which were arbitrarily assigned 100%. All data was measured versus controls (MCF-10A breast cells alone), as well as versus controls with fibroblasts (MCF-10A co-cultured with fibroblasts from ER (+) patients). Statistical significance of difference was calculated using Student's *t*-test for paired data with a level of significance selected at $p < 0.05$. All data was analyzed using Excel statistical software.

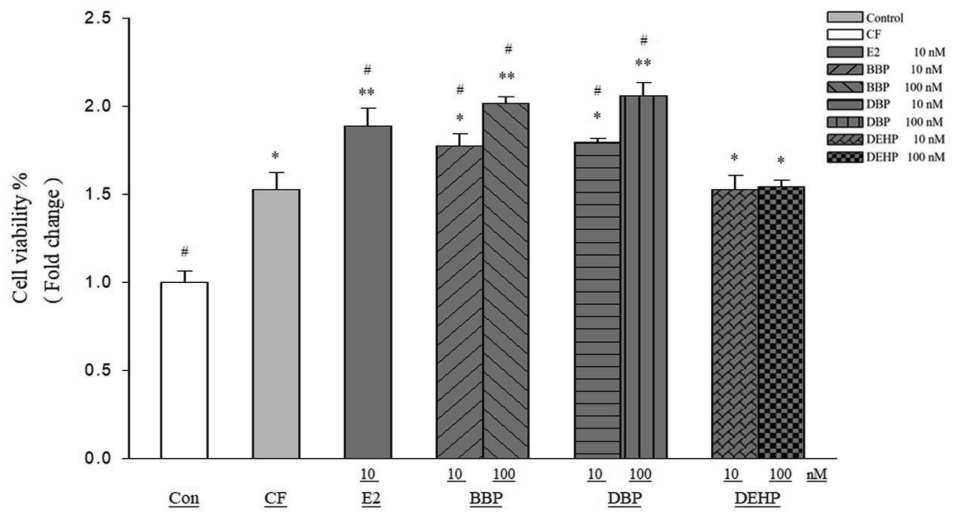

Fig. 1. Effects of phthalates and estradiol on cell viability in MCF-10A cells. Phthalates (BBP, DBP, and DEHP at 10 nM and 100 nM), 17β-estradiol (E_2 at 10 nM), and MCF-10A cells co-cultured with fibroblasts from ER (+) primary breast cancers significantly increased cell viability of MCF-10A cells. Con: control (MCF-10A alone), CF: control fibroblast (MCF-10A co-cultured with fibroblast), *: $P < 0.05$ vs. control, **: $P < 0.001$ vs. control, #: $P < 0.05$ vs. CF.

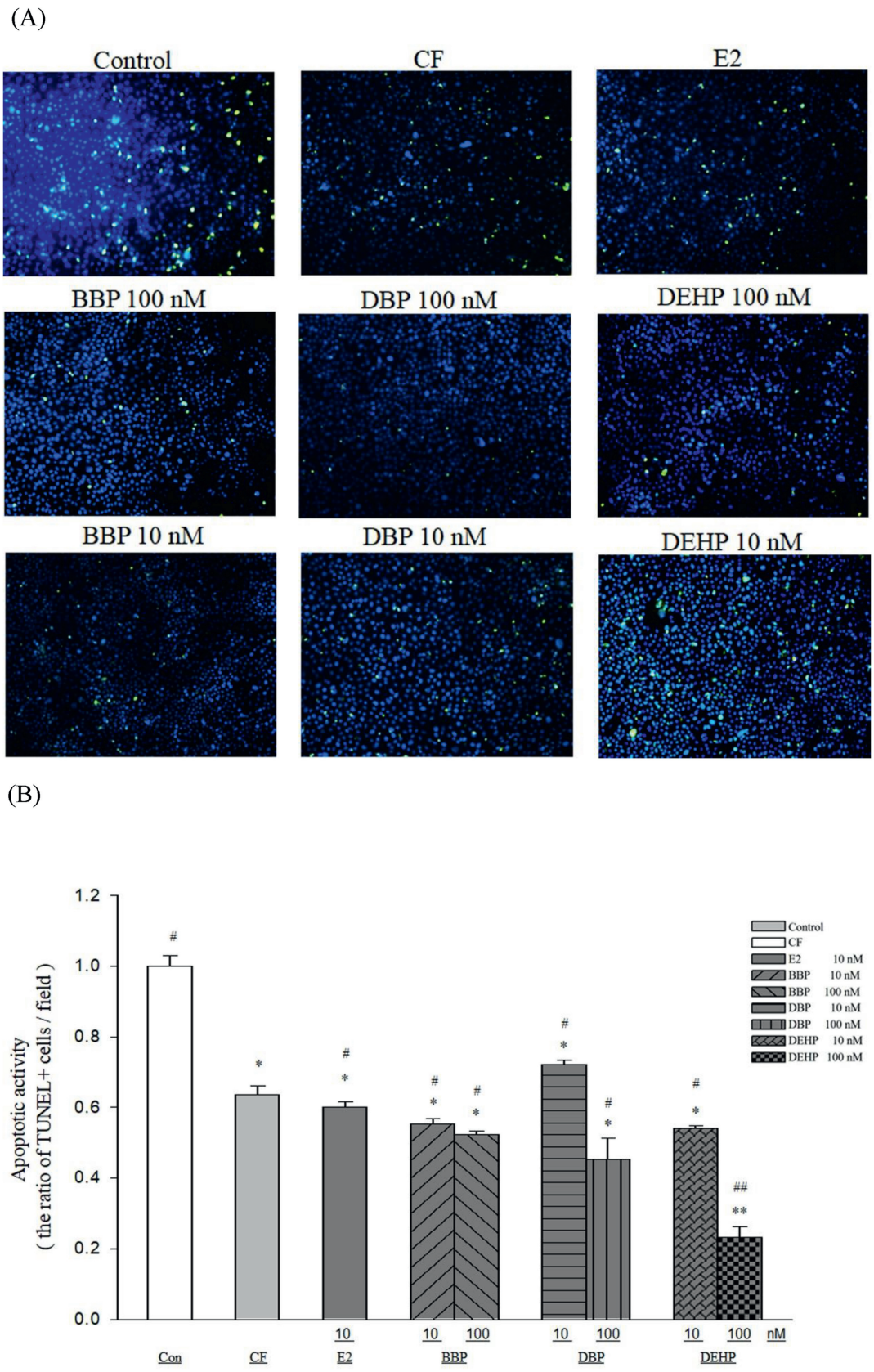

Fig. 2. Effects of phthalates and estradiol on cell apoptosis in MCF-10A cells. (A) Compared with cell treated with phthalates, TUNEL-positive cells (green) were widely distributed in the control group (magnification, ×200). (B) The rate of apoptosis was significantly lower in cells treated with BBP, DBP, and DEHP at both 10 nM and 100 nM and 17β-estradiol (E_2) at 10 nM, as well as MCF-10A co-cultured with fibroblasts, compared with the control group. Con: control (MCF-10A alone), CF: control fibroblast (MCF-10A co-cultured with fibroblast), *: $P < 0.05$ vs. control, **: $P < 0.001$ vs. control, #: $P < 0.05$ vs. CF, ##: $P < 0.001$ vs. CF.

Results

Viability of MCF-10A normal breast cells following phthalates exposure

Viability of breast cells was significantly higher after exposure to both 10 nM and 100 nM of BBP, DBP, and DEHP, as well as 10 nM E_2, compared with the control group (Fig. 1). BBP and DBP at 100 nM both had superior effects on cell viability compared to phthalates at 10 nM. However, in contrast to MCF-10A cells co-cultured with fibroblasts from ER (+) primary breast cancers, BBP and DBP at both 10 nM and 100 nM and 10 nM of E2 also significantly increased cell viability ($P < 0.05$).

Effects of phthalates on apoptosis of MCF-10A normal breast cells

We also examined whether the increased viability could be attributed to an anti-apoptotic effect. As such, the rate of apoptosis was significantly lower in cells treated with BBP, DBP, and DEHP at both 10 nM and 100 nM and E_2 at 10 nM, as well as in MCF-10A co-cultured with fibroblasts, compared with the control group (Fig. 2A and B). Anti-apoptotic effects were higher in phthalates at 100 nM than in those at 10 nM.

Effects of phthalates on cell cycle progression

Regulation of the cell cycle is imperative to the growth and development of cancer.

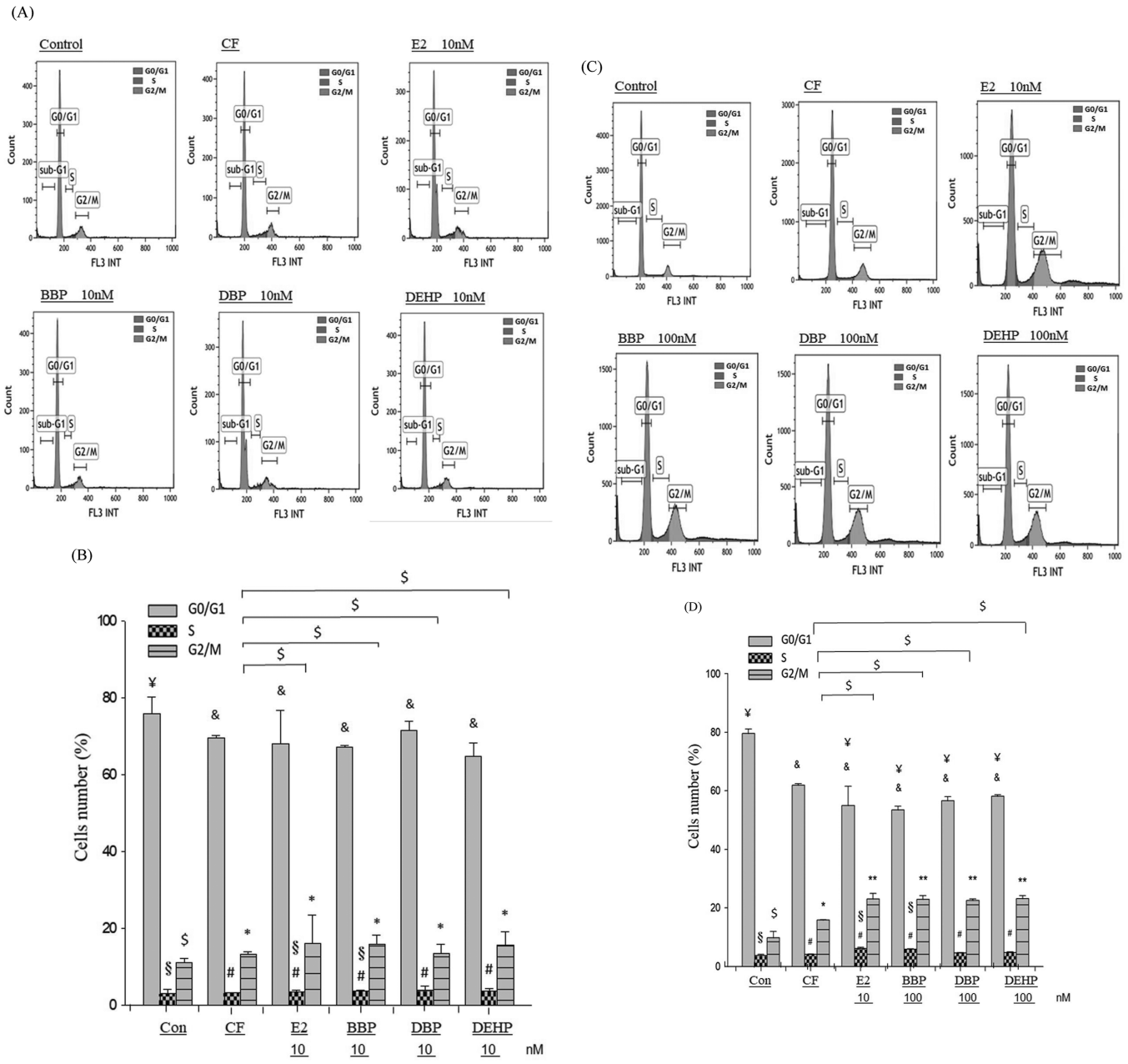

Fig. 3. Effects of phthalates and estradiol on cell cycle distribution. Phase distribution of MCF-10A cells treated with BBP, DBP, and DEHP at 10 nM (A, B) and at 100 nM (C, D) and with E_2 at 10 nM, as well as MCF-10A cells co-cultured with fibroblasts by flow cytometry analysis. Con: control (MCF-10A alone), CF: control fibroblast (MCF-10A co-cultured with fibroblast), &: $p < 0.05$ Vs. control at G0/G1 phase, #: $p < 0.05$ VS. control at S phase, *: $P < 0.05$ VS. control at G2/M phase, **: $P < 0.001$ VS. control at G2/M phase, ¥: $p < 0.05$ Vs. CF at G0/G1 phase, §: $p < 0.05$ VS. CF at S phase, $: $P < 0.05$ VS. CF at G2/M phase.

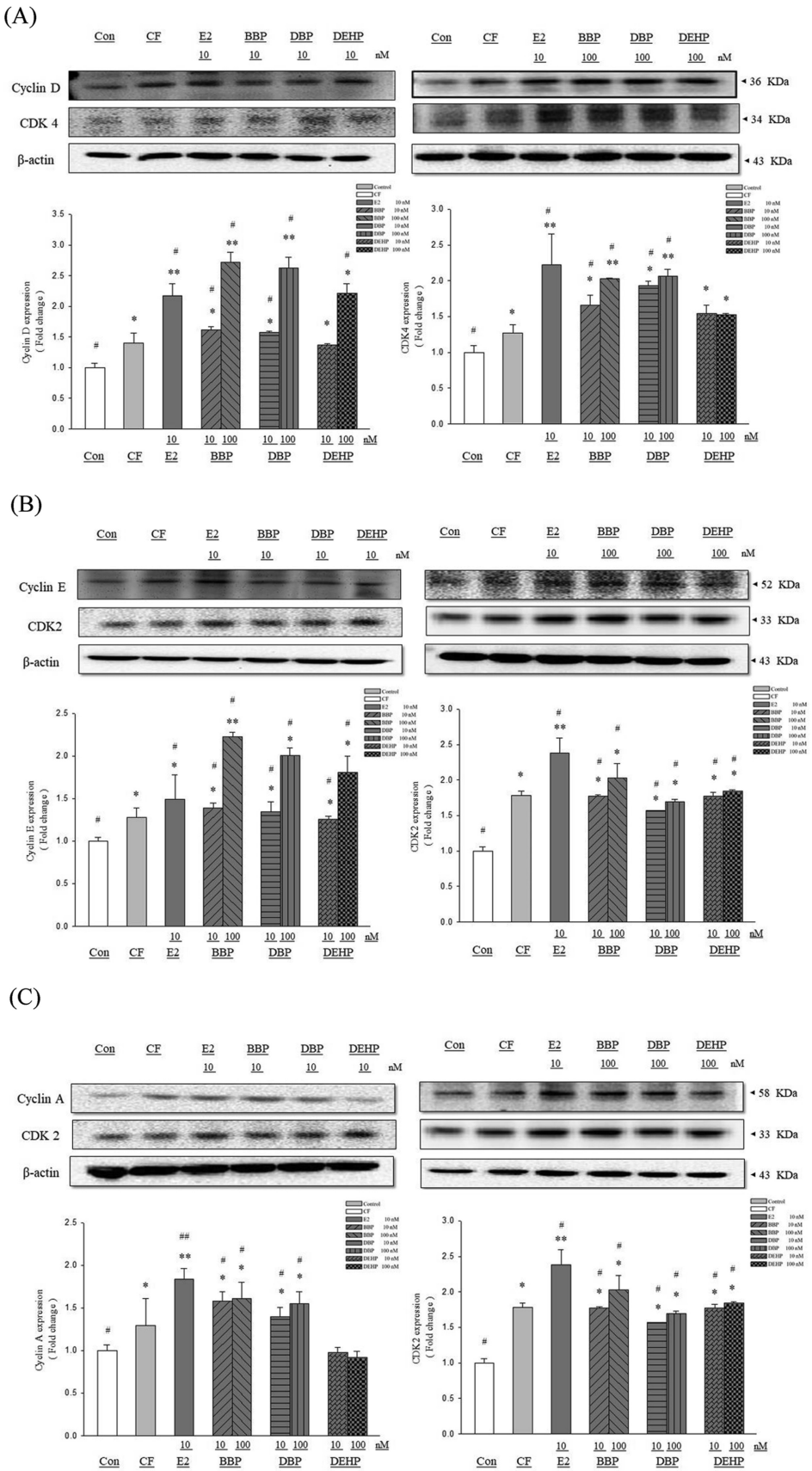

Fig. 4. Effects of phthalates and estradiol on protein expression of cyclin and cyclin-dependent kinases (CDKs). After MCF-10A cells were treated with BBP, DBP, and DEHP (at both 10 nM and 100 nM) and 17β-estradiol (E_2 at 10 nM), Western blot was utilized to analyze the relative levels of key proteins involved in the cell cycle progression, including (A) cyclin D/CDK4, (B) cyclin E/CDK2, (C) cyclin A/CDK2, (D) cyclin A/CDK1, and (E) cyclin B/CDK1. β-actin was used to normalize the amount of protein in each lane. Representative blots are illustrated above each diagram. Con: control (MCF-10A alone), CF: control fibroblast (MCF-10A co-cultured with fibroblast), *: $P < 0.05$ vs. control, **: $P < 0.001$ vs. control, #: $P < 0.05$ vs. CF, ##: $P < 0.001$ vs. CF.

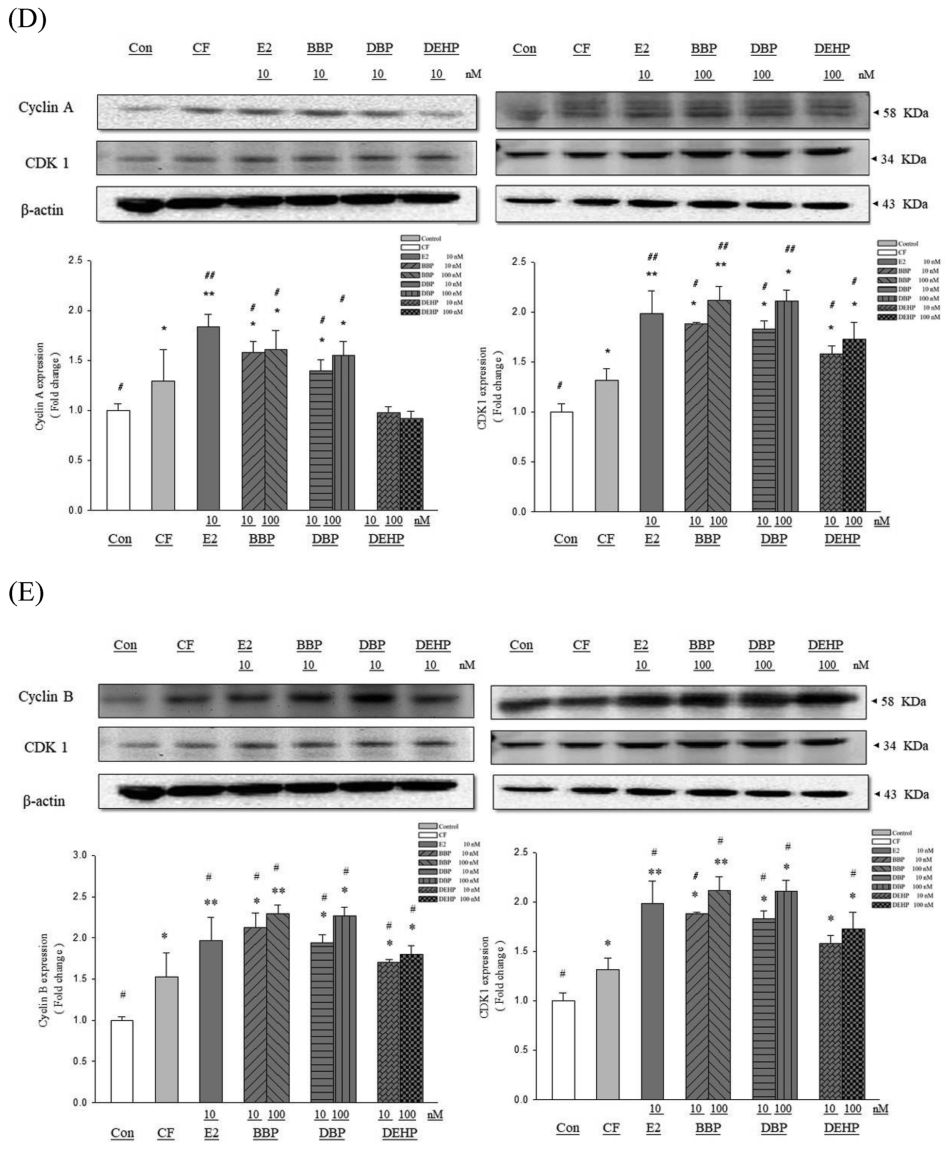

Fig. 4. (*continued*).

The cell cycle distribution after cells were exposed to fibroblasts, E_2, and phthalates is shown in Fig. 3A, B, C, and D. Compared with the control group (MCF-10A alone), breast cells co-cultured with fibroblasts or treated with E_2 at 10 nM and BBP, DBP, and DEHP at both 10 and 100 nM had significantly increased cell numbers in the S and G2/M phases with a corresponding decrease in the G0/G1 phase ($P < 0.05$). The effects on the G2/M phase were more prominent in cells treated with phthalates at 100 nM than those treated with phthalates at 10 nM, compared with the control group or with MCF-10A co-cultured with fibroblasts. Additionally, we examined the mechanism by which phthalates promoted cell cycle progression, DNA synthesis and cell proliferation by measuring the expression of cell cyclins and CDKs. As expected, BBP, DBP, and DEHP at both 10 and 100 nM concentrations promoted cell cycle progression and DNA synthesis by significantly up-regulating cyclin D/CDK4 (Fig. 4A), cyclin E/CDK2 (Fig. 4B), and cyclin A/CDK2 (Fig. 4C) as E2 (at 10 nM) did. The expression of cyclin A/CDK1 (Fig. 4D) and cyclin B/CDK1 (Fig. 4E), which are necessary for G2/M passage, were also positively regulated by these three phthalates at both 10 and 100 nM concentrations, as well as by E2 (at 10 nM). However, the effects of these three phthalates on the cell cycle progression of MCF-10A breast cells were dose-dependent.

Phthalates induce cell cycle progression and cell proliferation by regulating the P13K/AKT/mTOR pathway

We also aimed to further verify whether the P13K/AKT/mTOR signaling pathway was involved in the phthalates' induction of cell proliferation and cell cycle progression of breast cells. As shown in Fig. 5A, B, C, D, and E, the expressions of PDK1, P13K, p-AKT, p-mTOR, and BCL-2 were all significantly elevated in cells treated with BBP, DBP, and DEHP at both 10 nM and 100 nM, as well as with E2 at 10 nM, compared with the control group or with MCF-10A co-cultured with fibroblasts. The up-regulation induced by the three phthalates occurred in a dose-dependent manner. As compared with the control group and with MCF-10A co-cultured with fibroblasts, there was a significant reduction in Bax protein, cytochrome C, caspase 8, and caspase 3 levels (Fig. 5F, G, H, I) after exposure to BBP, DBP, and DEHP at both 10 nM and 100 nM, as well as with E2.

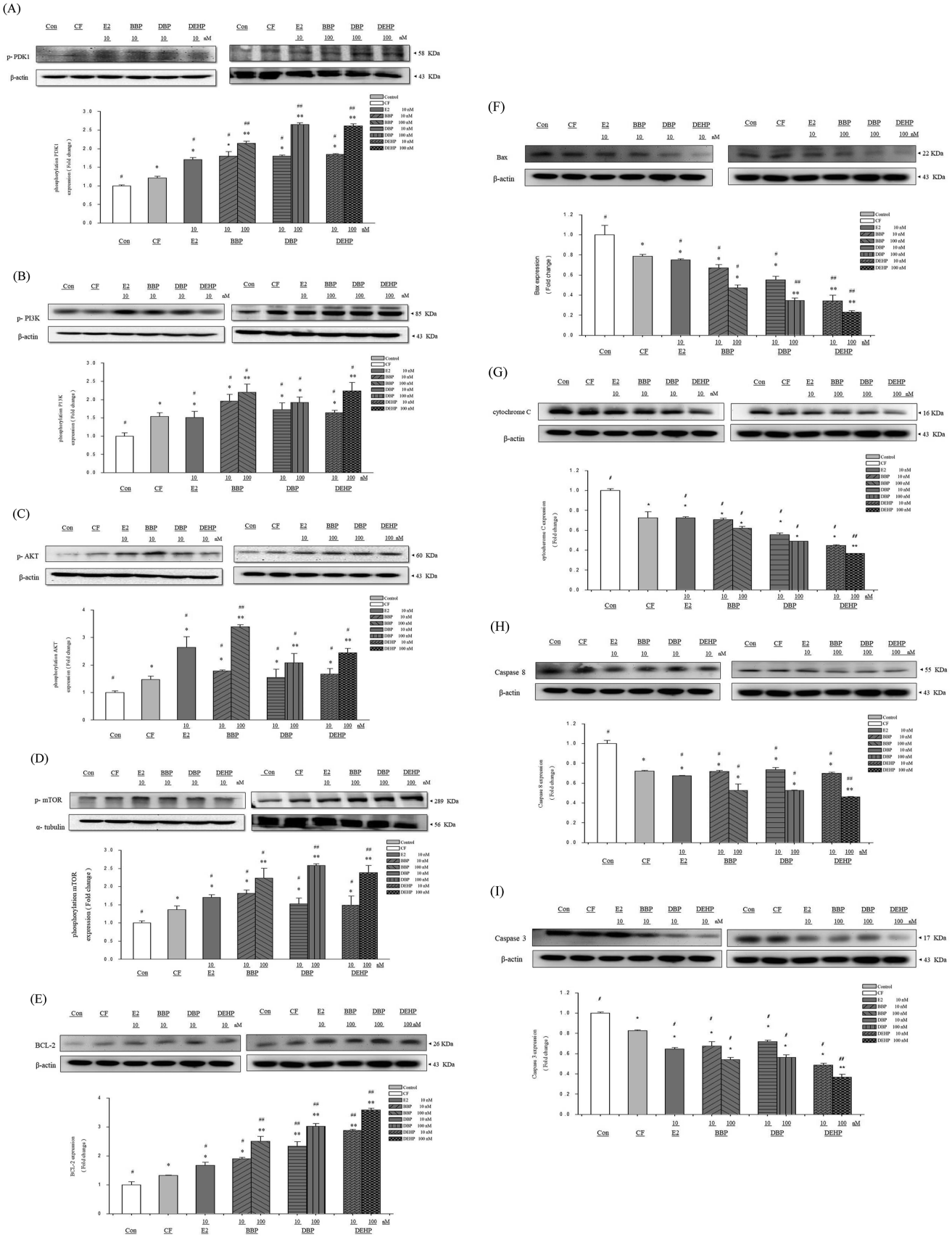

Fig. 5. Phthalates and estradiol induce MCF-10A cell proliferation and inhibit apoptosis by regulating the P13K/AKT/mTOR signaling and extrinsic apoptosis pathways. The protein expression of signaling pathways in MCF-10A cells treated with BBP, DBP, and DEHP at both 10 nM and 100 nM and 17β-estradiol (E_2) at 10 nM, as well as MCF-10A cells co-cultured with fibroblasts, were evaluated using Western blot. Data from the Western blot image (upper panel) and densitometric analysis (bottom panel) of PDK1 (A), PI3K (B), p-AKT (C), p-mTOR (D), Bcl-2 (E), Bax (F), cytochrome C (G), caspase 8 (H), and caspase 3 (I) are displayed. The amount of each protein was normalized in comparison with β-actin. Con: control (MCF-10A alone), CF: control fibroblast (MCF-10A co-cultured with fibroblast), *: $P < 0.05$ vs. control, **: $P < 0.001$ vs. control, #: $P < 0.05$ vs. CF, ##: $P < 0.001$ vs. CF.

The down-regulation induced by the three phthalates was also more prominent at 100 nM than at 10 nM.

Discussion

This study revealed that phthalates, including BBP, DBP, and DEHP, should be considered endocrine disruptors for breast cancer risk, even at concentrations recognized as no-observed-adverse-effect level by current standards. The effects of these three phthalates on cell cycle progression were dose-dependent. BBP, DBP, and DEHP at both 10 and 100 nM concentrations induced breast cell proliferation through activation of the P13K/AKT/mTOR pathway and decreased MCF-10 A cell apoptosis via both the extrinsic and intrinsic pathways. Thus, it should be considered that BBP, DBP, and DEHP have the potential to promote the onset of breast cancer.

It is generally recognized that phthalates promote cancer progression. The European Union (EU) has also imposed restrictions on phthalate plasticizers in products since July 7, 2020. Although phthalates are easily metabolized and excreted in the urine, their pervasiveness and potential endocrine-disrupting effects present a concern for chronic "low-dose" exposure [18]. In the present study, increased MCF-10A cell viability and suppressed apoptosis were observed after treatment with BBP, DBP, and DEHP at the dose of 10 nM and effects were even more prominent at 100 nM. Thus, the effects of phthalates on breast tumorigenesis, even at low concentrations, needs to be considered.

To investigate the possible mechanism for increasing cell viability, the cell cycle distribution of MCF-10A cells was determined. This study found that BBP, DBP, and DEHP at concentrations of 10 and 100 nM induced a decreased percentage of cells at the G0/G1 phase and increased the percentage of cells at the S and G2/M phases. The increased percentage of cells at the S or G2/M phases may be due to arrest and/or acceleration of the transition from the G1 to S phase or the S to G2 phase [19]. Progression in the cell cycle is controlled by the sequential, transient activation of a protein complex composed of cyclins and CDK, which allows an ordered succession of the cell-cycle phases G1, S, G2, and M [20,21]. In the present study, significantly up-regulated cyclin D/CDK4, cyclin E/CDK2, cyclin A/CDK2, cyclin A/CDK1, and cyclin B/CDK1 complexes after treatment with the three phthalates at both low doses promoted progression in G1, the transition to DNA replication in S, progression in S and transition to G2, and finally the G2/M transition, allowing entry into mitosis. Cyclin/CDK complex expression was increased in a dose-dependent manner. Therefore, this alteration of cell cycle distribution indicates that the three phthalates at low concentrations increased MCF-10A cell proliferation by promoting cell cycle progression at the S and G2/M phases.

PI3K/AKT/m-TOR cascade is a signal transduction pathway that regulates cell metabolism, cell growth, proliferation, apoptosis, and angiogenesis [22]. Increasing evidence suggests that abnormal activation of the P13K/AKT/mTOR pathway is a frequent event in numerous malignant tumors, including prostate cancer [23], gastrointestinal cancer [24], breast cancer [25], non-small cell lung cancer [26], acute myeloid leukemia [27], and liver cancer [28]. Our study shows that PDK1, P13K, p-AKT, and p-mTOR protein levels were up-regulated following treatment with BBP, DBP, and DEHP at both 10 nM and 100 nM. In addition, the three phthalates also induced upregulation of Bcl-2 expression and reduction of Bax, cytochrome C, caspase 8, and caspase 3 expression for apoptosis inhibition. Despite the inhibition of the extrinsic apoptosis pathway via suppressed caspase 8 expression, the three phthalates induced anti-apoptotic effects through the P13K/AKT/mTOR pathway, driving the mitochondrial apoptosis pathway. Collectively, our results indicate that BBP, DBP, and DEHP at doses of 10 and 100 nM induce cell viability, inhibit apoptosis, and promote cell cycle progression by modulating the P13K/AKT/mTOR pathway to promote cell proliferation of normal breast cells.

As aforementioned, despite further restrictions imposed on the amount of phthalates allowed in consumer products, the pervasiveness and potential endocrine-disrupting effects of phthalates present a concern for "chronic" exposure in terms of current no-observed-adverse-effect-level doses. This study is also not without limitations. In vitro short-term exposure cannot represent chronic exposure. Further research into whether the in vitro effects of phthalates, especially at such low doses, are equal to in vivo effects is required, even though long-term bioaccumulations should also be considered. However, most of the research on the effects of phthalates on breast carcinogenesis have used breast cancer cells, unlike our study which focused on normal breast cells. Since phthalates are rapidly metabolized and excreted, phthalates at lower doses, like in the present study, may actually mirror real world exposure. However, additional animal studies and/or long-term epidemiological research are needed.

Conclusion

This study revealed that BBP, DBP, and DEHP at no-observed-adverse-effect concentrations still exhibited proliferation effects in normal breast cells in a dose-dependent manner. The underlying potential mechanism may be related to activation of the PI3K/AKT/m-TOR signaling pathway, subsequent inhibition of mitochondrial apoptosis, and progression of the cell cycle, in addition to the inhibition of the extrinsic apoptosis pathway. This study provides a theoretical basis to support concern regarding the possibility of breast tumorigenesis through chronic phthalate exposure at current established safety standards.

Disclosure

The authors declare that they have no conflicts of interest relevant to this article.

Declaration of competing interest

None.

Acknowledgments

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Original Article

A practical method for prenatal diagnosis of anal atresia by second trimester ultrasound screening - A retrospective study

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ABSTRACT

Objectives: The study aimed to demonstrate the performance of anal atresia ultrasound screening in the second trimester and to describe associated experiences in a primary care fetal medicine clinic.
Materials and methods: We retrospectively analyzed the medical records of fetuses who underwent a second-trimester screening at the Taiji clinic between November 2019 and May 2022. Fisher's exact test was conducted to investigate potential risk factors.
Results: There were 28 459 fetuses screened in our clinic during the study period; eventually, 6 cases were diagnosed with anal atresia after birth. The incidence of anal atresia in our sample was 2.11 in 10 000. Based on our findings, potential risk factors significantly associated with anal atresia included: multiple pregnancies (p-value = 0.0185) and in-vitro fertilization (p-value = 0.038). Half of the anal atresia cases were associated with abnormalities affecting other organ systems, most frequently the genitourinary system (66.7%) and cardiovascular system (66.7%), especially persistent left superior vena cava (2 cases).
Conclusion: Anal atresia is a malformation that requires extensive care; the clinical management after the prenatal discovery of its signs should include testing for chromosomal abnormalities and close monitoring of the amniotic fluid volume. Therefore, prenatal ultrasound screening for anal atresia in the second trimester is critical, particularly in the cases of multiple and IVF pregnancies, and multiple abnormalities. The fetuses with ultrasound signs of anal atresia should be followed at a later gestational period and referred to a specialized institution for postnatal management planning and parental counseling.

Introduction

Anal atresia is a rare congenital anomaly presenting with an abnormal opening of the anus. An estimated incidence ranges between 1 in 4000 and 1 in 5000 live births [1]. A fistulous tract is established on adjacent structures instead of the normal anal opening. In males, recto-urethral fistulas are more common. On the other hand, perineal fistulas or recto-vestibular fistulas are more common in females [1]. About half of the patients with anal atresia also have associated anomalies of other organ systems [2]. Anomalies associated with anal atresia most commonly involve genitourinary and musculoskeletal systems. Frequently, anal atresia occurs as a part of the VACTERL association, including vertebral defects, cardiac defects, tracheoesophageal fistula, renal anomalies, and limb abnormalities [3]. Isolated anal atresia is rare and might be challenging to detect prenatally. The abnormal urinary system or rectum can be indirect signs of anal malformations on prenatal ultrasound [4–6].

This group of malformations often requires postnatal surgery to ensure a basic quality of life [1]. Despite that, prenatal identification of anal atresia is still a challenge. Additionally, anal atresia may co-occur with chromosomal abnormalities or tracheoesophageal fistulas, resulting in polyhydramnios [7]. Therefore, prenatal screening for anal atresia might be crucial for parental counseling and postnatal management planning. Detection of ultrasound signs of anal atresia can necessitate karyotyping and initiate measures for managing preterm birth in case of polyhydramnios [8]. The “target sign” is the most commonly used direct ultrasound indicator for prenatal evaluation of the anus [8–11]. The typical anal structure appears as hyperechoic anal mucosa surrounded by a thick hypoechoic anal sphincter ring in the tangential view of the fetal

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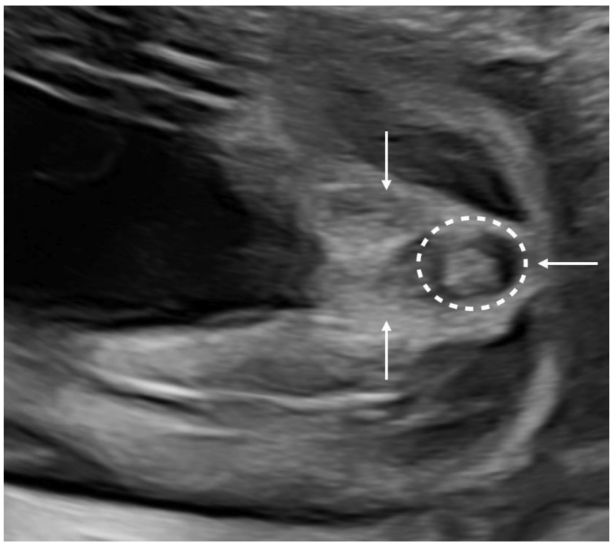

Fig. 1. 2D grayscale ultrasound image of "target sign" in the tangential view of fetal perineum.
In the tangential view of the fetal perineum, in the absence of the bilateral ischial tuberosities, appears as the low-high concentric circular rings representing the anal sphincter and the anal mucosa (dashed line circle) called the "target sign". The perianal tissue forms the solid hyperechoic region around the center of the "target sign" (arrows).

perineum at the level below bilateral ischial tuberosities (Fig. 1). The objectives of the study were to share our experiences with prenatal detection of anal atresia during the second-trimester ultrasound screening and to report the incidence and associated risk factors for anal atresia in a primary care fetal medicine clinic in Taiwan.

Methods

Sample

This study is a retrospective data analysis of the medical records. The sample included pregnant women who underwent a mid-trimester screening at a private primary care center specialized in fetal ultrasound medicine between November 2019 and May 2022. The advanced mid-pregnancy fetal anatomical screening took place at 20–24 weeks of gestation. The excluded cases were those above 24 gestational weeks at the first screening and those with unavailable outcome data. Birth outcomes were obtained by reviewing hospital records and via phone interviews.

Ultrasound examinations

All examinations were performed using Aloka Prosound Alpha 6 (Hitachi Aloka Medical, Japan) and GE Voluson E10 (GE Healthcare, Austria) systems. Two evaluated elements of the anal structure included the anal sphincter and anal mucosa that appears as low-high echoic concentric circles on ultrasound - typical "target sign" (direct finding) (Fig. 2). Also, the appearance and symmetry of hyperechoic perianal tissue were assessed. In addition, the bowel malformation or calcified meconium were considered indirect findings suggestive of anal atresia (Fig. 3). In cases when perianal muscular complex (PAMC) had an equivocal appearance or when combined with other indirect signs, the patients had additional follow-ups in 2–4 weeks. In cases of multiple anomalies, fetal MRI (magnetic resonance imaging) was advisable (see Fig. 4).

Statistical methods

Frequency was described by count (n) and percentage (%). Due to the low incidence of anal atresia and small counts, we performed Fisher's exact test to evaluate association between potential risk factors and anal atresia (IVF, multiple pregnancy). The calculations were performed using Microsoft Excel (2023). A-priori level of statistical significance was assumed at a p-value <0.05.

Results

A total of 28 459 fetuses: 26 422 singletons, 1005 twins, and 9 triplets underwent mid-trimester screening during the study period (Fig. 4). In 21 cases, visualization of the anal structure was inconclusive during the first visit due to the poor fetal position or maternal factors such as uterine contractions, current physical discomfort (morning sickness or difficulty maintaining the fixed

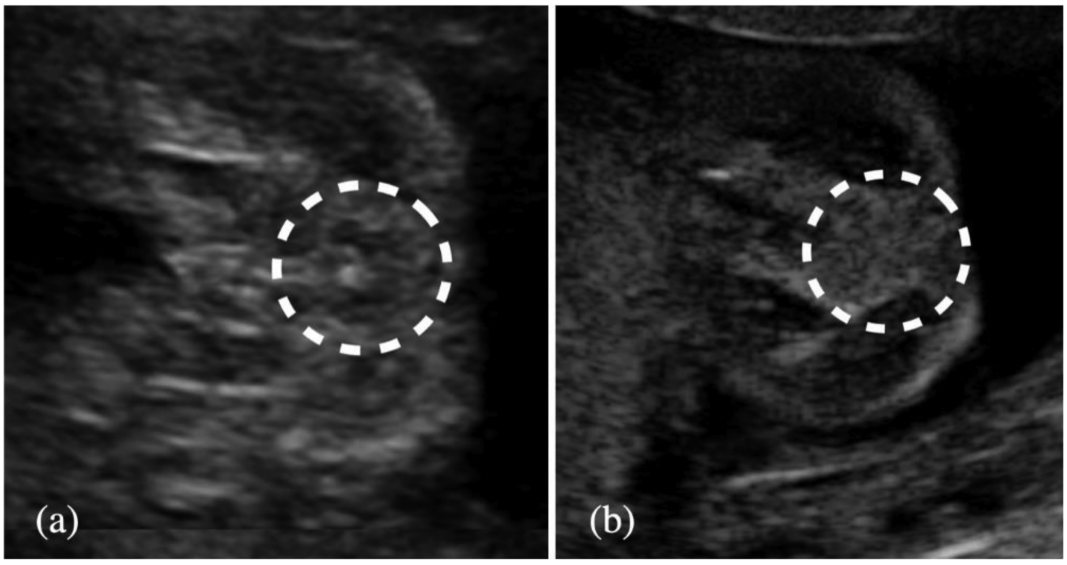

Fig. 2. Appearance of direct findings of anorectal malformations in 2D ultrasound.
a. Absent anal mucosa: No obvious anal mucosa or small anal mucosa with shallow hypoechoic anal sphincter (dashed line circle).
b. Absent anal sphincter and anal mucosa: No hypoechoic sphincter and hyperechoic mucosa inside (dashed line circle).

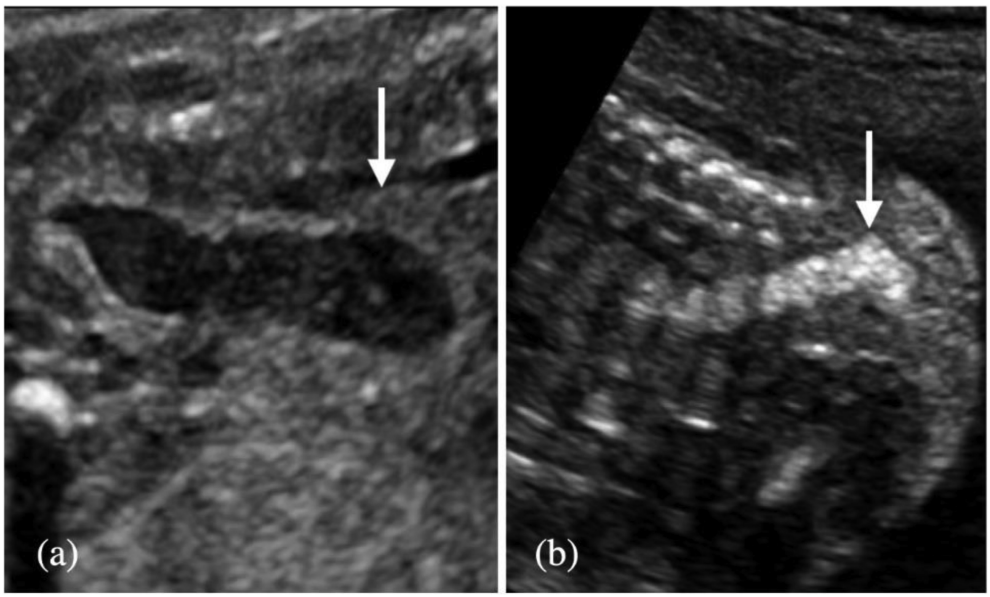

Fig. 3. Appearance of indirect findings of anorectal malformations on 2D ultrasound.
a. Dilated distal bowel segments: U or V-shaped bowel in presacral space and no extension to perineum (arrow) (dilated colon space can be considered normal in 3rd trimester).
b. Calcified intraluminal meconium: Mixture of alkaline urine and meconium (arrow).

supine position) during the scanning, or time constraints; eventually, in all of these cases, the scanning was completed within two weeks, without abnormal findings. There were 6 postnatally confirmed cases of anal atresia, with 4 detected by the described ultrasound screening in our cohort. Table 1 illustrates observations in 15 fetuses with equivocal or abnormal anal structure impressions during the first visit. Eleven of 15 cases had a follow-up at later gestation after the primary findings. After the follow-up scanning, 3/11 still presented an absent "target sign" (Fig. 2). It is worth mentioning that there were 2 cases discovered postnatally that had negative findings on prenatal ultrasound (Table 1, Cases 16 and 17, the cases are described elsewhere in more detail [12]). Overall, prenatal diagnosis was suspected in around 67% (4/6) of confirmed anal atresia patients. It appears that additional follow-up scanning reduced the number of uncertain cases from 9 to 1. Among the diagnosed cases of anal atresia, three were isolated anomalies. One of these cases was associated with a mildly dilated renal pelvis, with the dilation falling within the normal range. Of the 6 anal atresia cases, 2 were multiple pregnancies and 3 were conceived by IVF (in-vitro fertilization). Based on the Fisher's exact test, both factors were significantly associated with anal atresia with respective p-values <0.05 at 0.0185 and 0.038.

Three fetuses with anal atresia, including case 17 in our sample, had associated anomalies (Table 2). One had a VACTERL association

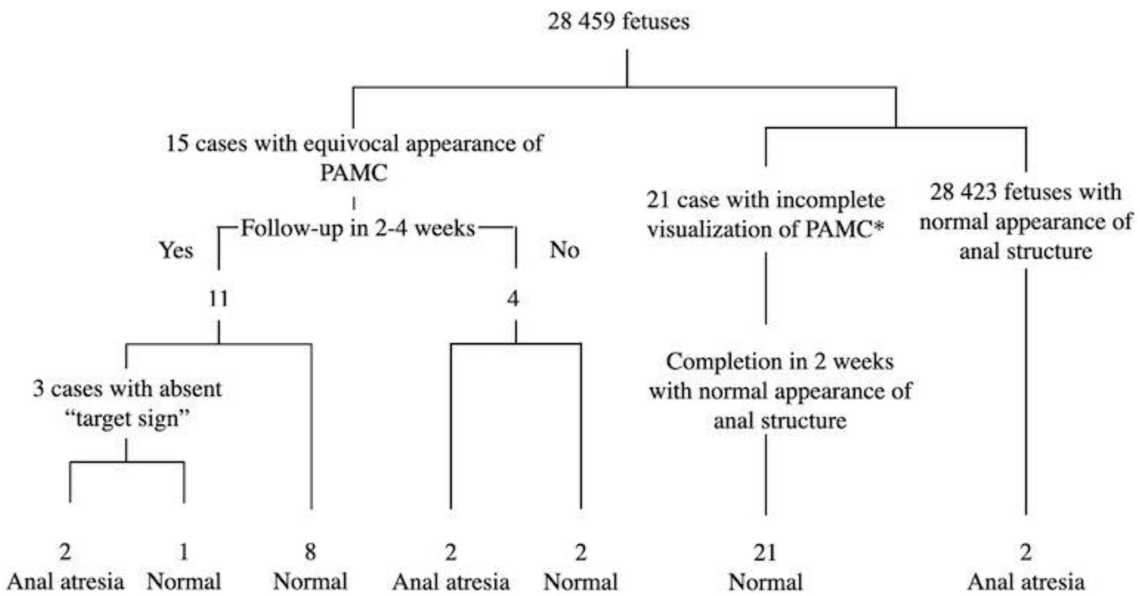

Fig. 4. Process flow chart in suspicted anal atresia cases.
*Factors which possibly affected the completion of the scan: poor fetal position, maternal uterine contractions, anhydramnios. If due to the abovementioned factors, the screening was partially incomplete, the woman was invited for a follow-up visit in two weeks. PAMC: perianal muscular complex.

Table 1
Fetal ultrasound characteristics and postnatal findings in suspected or confirmed anal atresia cases.

Case/Sex	GA[a]	Method of conception	Ultrasound findings at first visit	Ultrasound findings at the latest follow-up	Review diagnosis[d]	Anal atresia (prenatal)	Anal atresia (postnatal)	Anal atresia type
1/M	21/24	spontaneous	shallow anal sphincter with normal anal mucosa	visible target sign	visible target sign	Negative	Negative	
2/M	22/30	IVF	1.right clubfoot 2.sacral agenesis and conus medullaris ends between L3-L4 3.left hand pre-axial polydactyly 4.small anal mucosa and shallow anal sphincter	1.right clubfoot 2.sacral agenesis and conus medullaris ends at the level of L3 3.left hand pre-axial polydactyly 4.visible target sign	visible target sign	Negative	Negative	
3/F	22/26	spontaneous	small anal mucosa and shallow anal sphincter	visible target sign	visible target sign	Negative	Negative	
4/M	22/26	spontaneous	irregular anal sphincter wall and dilated rectum (14.3 × 8.3 mm)	visible target sign	visible target sign	Negative	Negative	
5/M	23/27	spontaneous	equivocal position of the anal pit, relatively anterior to the normal position	visible target sign	visible target sign	Negative	Negative	
6/F	22/27	spontaneous	equivocal position of the normal anal sphincter and mucosa, much higher than the normal position	visible target sign	visible target sign	Negative	Negative	
7/F	23/32	spontaneous	1. Umbilical–portal–systemic venous shunt (umbilical vein drained into RA, absent DV and portal venous system) Cardiomegaly (C/T ratio: 0.64) 2. right hand pre-axial polydactyly 3. shallow anal sphincter but normal anal mucosa	1. umbilical vein drained into right atrium, absent ductus venosus, cardiomegaly 2. hypoplastic portal venous system (main portal vein seen) 3. right hand pre-axial polydactyly 4. visible target sign	visible target sign	Negative	Negative	
8/F	20/23	spontaneous	small anal mucosa and shallow anal sphincter	visible target sign	visible target sign	Negative	Negative	
9/M	23/26	spontaneous	normal rectal peristalsis but small anal mucosa, low type anal atresia could not be excluded	relatively small anal mucosa, low type anal atresia could not be excluded	small anal mucosa	Positive	Negative	
10/M	23/25	IVF	1. right ectopic kidney. D/D: left solitary kidney) 2. no hyperechoic anal sphincter and mucosa	1. right ectopic kidney. D/D: left solitary kidney) 2. no hyperechoic anal sphincter and mucosa (target sign not seen)	absent target sign	Positive	Positive	high
11/F[b]	22/27	IVF	No hyperechoic anal sphincter and mucosa	small anal sphincter and mucosa in fetal perineum	small anal sphincter and mucosa	Positive	Positive	low
12/M	22/-	spontaneous	shallow anal sphincter	–	visible target sign	Negative	Negative	
13/M	22/-	IVF	small anal mucosa and shallow anal sphincter	–	shallow anal sphincter	Negative	Negative	
14/M[b]	22/-	IVF	No hyperechoic anal sphincter and mucosa	–	no obvious anal mucosa	Positive	Positive	high
15/F	22/-	spontaneous	1. Persistent right umbilical vein (PRUV) with DV agenesis, umbilical vein directly drained into the right atrium, without hydrops 2. Persistent left superior vena cava (PLSVC), drained into enlarged coronary sinus 3. Cardiac position: mesoposition cardiac angle: 8° 4. r/o T10-T12 hemivertebrae with scoliosis 5. Single umbilical artery (only left side)	–	small anal sphincter and mucosa	Positive	Positive	low

(*continued on next page*)

Table 1 (*continued*)

Case/Sex	GA[a]	Method of conception	Ultrasound findings at first visit	Ultrasound findings at the latest follow-up	Review diagnosis[d]	Anal atresia (prenatal)	Anal atresia (postnatal)	Anal atresia type
			6. small anal mucosa and shallow anal sphincter					
			VACTERL association should be considered					
16/M[c]	23/-	spontaneous	mildly dilated renal pelvis	–	small target sign and densely packed bilateral perianal tissue	Negative	Positive	high
17/M[c]	22/-	spontaneous	1.Persistent left superior vena cava (PLSVC), drained into enlarged coronary sinus 2.Mild cardiac effusion r/o hypertrophic cardiomyopathy of bilateral ventricles. CT ratio: 0.54 3.r/o micropenis or relatively smaller penis penile length: 5.1 mm (reference range at 22 weeks: 6.72–10.82 mm) 4.IUGR	–	no hyperechoic mucosa seen inside the small hypoechoic anal sphincter	Negative	Positive	unknown

[a] gestational age at the first visit/gestational age at latest follow-up (weeks).
[b] twin pregnancy with one of the fetuses having prenatal findings suggestive of anal atresia.
[c] cases with no findings suggestive of anal atresia on second-trimester ultrasound with consequent postnatal diagnosis were described elsewhere [12].
[d] review of prenatal images after postnatal diagnosis of anal atresia.

with vertebral defects and cardiac defects. The most frequently associated anomalies involved cardiovascular (66.7%) and genitourinary systems (66.7%). The most recurrent cardiac abnormality was persistent left superior vena cava (2 cases). The genitourinary anomalies included micropenis and ectopic kidney. Concerning the group of the musculoskeletal system (33.33%), the case was associated with hemivertebrae. In one fetus, anal atresia co-occurred with severe intrauterine growth restriction.

Discussion

Anal atresia remains challenging for prenatal diagnosis, and the current article presents our experiences. Despite challenges, prenatal diagnosis is feasible and might be improved by raising awareness and gaining clinical experience. We were able to visualize anal structure in most of the fetuses during the second-trimester anatomical ultrasound scan. Based on our findings, the incidence of anal atresia was 2.11 per 10 000 births in our sample, comparable to the previously reported postnatal incidence of 2.15 per 10 000 in Taiwan [13,14]. Additionally, we ascertained a significant association between multiple pregnancies (p-value = 0.0185), IVF conception (p-value = 0.038) and anal atresia.

There was a high completion rate (~100%) of anal structures imaging in our cohort, in contrast to some previous studies reporting rates around 58–81% [8,15]. One possible explanation might be that as a specialized fetal ultrasound clinic, we could allocate an extended duration for the exam to account for the poor fetal position, oligohydramnios, or other factors influencing the scan. We also organized additional follow-ups in the cases when we were unable to visualize anal structure (21 cases) and provided additional training and education for our personnel. Previous study by Bischoff et al. also supports additional follow-up visits to improve the rate of visualization and predictive value of the screening [8]. Our experiences show that the prenatal visualization of the direct and indirect signs of anal atresia was generally feasible in the second trimester, and any abnormal findings before 24 weeks require follow-ups. Additional prenatal follow-up scanning can improve the accuracy of the screening.

Detecting anal atresia is still challenging, even following the step-by-step screening protocol by visualizing the anal structure and the indirect findings [9,15,16]. We encountered 2 cases of anal atresia with a reportedly normal appearance on prenatal ultrasound [12]. One case of high-type anal atresia was observed postnatally. A retrospective review of the images revealed that the perianal tissue appeared asymmetric and too densely packed to differentiate the anal structure inside, and it resembled a low-high concentric circle structure similar to a target sign. Another postnatally diagnosed anal atresia was associated with severe intrauterine growth restriction (IUGR). Anal atresia was associated with multiple anomalies, including cardiovascular and genitourinary systems. Based on the prenatal image of the anal structure in this case, the anal mucosa appeared smaller and had lower echogenicity in comparison to the normal mucosa, and the peripheral anal sphincter was shallow. Screening for anal atresia of appropriate for gestational age (AGA) fetuses around 20 weeks is challenging, as a result, making it even more difficult in severe IUGR cases with the fetal size too small to allow delineation of these structures on the scan [11,17].

There were two twin pairs with one of the fetuses having suspected anal atresia on prenatal ultrasound in our study; both were eventually confirmed after birth (Fisher's exact test p-value = 0.0185). The association between malformations and twin pregnancies is the object of multiple studies [18–20]. A higher incidence was noted among twins in the rate of atresias, notably esophageal and anal atresia [18,20]. One of the hypotheses

Table 2
The distribution of the associated prenatal findings in postnatally confirmed anal atresia cases.

System	Ultrasound findings	Number of cases (n)	Percentage (%)
Cardiovascular		2	66.67
	mesocardia	1	33.33
	cardiomegaly	1	33.33
	single umbilical artery	1	33.33
	umbilical–portal –systemic venous shunt	1	33.33
	persistent left superior vena cava	2	66.67
Musculoskeletal		1	33.33
	hemivertebrae	1	33.33
Genitourinary		2	66.67
	micropenis	1	33.33
	ectopic kidney	1	33.33
Other		1	33.33
	Intrauterine growth restriction	1	33.33

proposed that primitive streak anomalies resulting in monozygotic twins could also affect axial mesoderm development and elevate the risk for this group of malformations [18,20]. Additionally, we noted that half of the anal atresia patients in our sample were conceived through IVF (Fisher's exact test p-value = 0.038). IVF might be a risk factor for anal atresia, however, assisted reproductive techniques likely were not directly associated with anal atresia. The association between ARTs and increased risk of anal atresia may be due to the potential causes of infertility [21]. Specific maternal factors, including obesity and diabetes, were identified as risk factors for anal atresia in previous publications and considered common causes of infertility [22].

As mentioned above, anal malformation may co-occur with the associated multisystemic anomalies. Further clinical management and multidisciplinary consultation should be defined based on the presence of multiple anomalies. A previous study regarding associated abnormalities in children with anal atresia reported that the most frequently affected systems were genitourinary and cardiovascular [22,23]. The most common anomaly in their study was vesicoureteric reflux, but in our sample, there was no vesicoureteric reflux during the second-trimester screening. One possible explanation for this difference was the timing of the diagnosis, as the abnormal signs of vesicoureteric reflux usually develop during the later gestation and are rarely diagnosed prenatally. If the prenatal findings suggest the VACTERL association, close attention to the anal structure and additional follow-ups are required. In general population, visualization of anal structures in every case might not be feasible, therefore focusing on the fetuses with multiple anomalies, especially VACTERL associations or indirect abnormal findings (bowel dilatation and calcified meconium) [9,15,16] could be a straightforward way to improve cost-effectiveness and predictive value. Our results suggest that IVF and multiple pregnancies also require closer monitoring for anal atresia.

Recent developments in MRI allow its use as a second-line screening for anal atresia. Rohrer et al. reported advantages of using MRI over the ultrasound, such as dynamic data acquisition and evaluation of the fluid-filled pelvic organs (vagina, uterus, rectum, bladder), and additionally, the perineum can be seen in different planes simultaneously on MRI. Although currently, we have limited experience in this area, it might be a valuable tool for prenatal diagnosis of anal atresia.

Prenatal diagnosis of anal atresia remains challenging but unified protocol and quality control measures might improve the detection rates. We illustrate that the prenatal diagnosis during the second-trimester ultrasound screening is feasible. Additional follow-ups appeared helpful to avoid false positive results in cases of absent or equivocal "target sign". The group with elevated risk for anal atresia: multiple pregnancies, IVF, or associated multiple abnormalities require monitoring and, if needed, a referral to a specialized institution. The rapid development of ultrasound and MRI techniques and increasing awareness regarding anal atresia provides a basis for early detection, which may lead to improved postnatal outcomes.

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Declaration of competing interest

Authors declare no conflicts of interest.

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Case Report

Low-level mosaic trisomy 21 at amniocentesis in a pregnancy associated with cytogenetic discrepancy in various tissues, perinatal progressive decrease of the trisomy 21 cell line and a favorable fetal outcome

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ABSTRACT

Objective: We present low-level mosaic trisomy 21 at amniocentesis in a pregnancy associated with cytogenetic discrepancy in various tissues, perinatal progressive decrease of the trisomy 21 cell line and a favorable fetal outcome.

Case report: A 36-year-old, gravida 2, para 1, woman underwent amniocentesis at 18 weeks of gestation because of advanced maternal age, and the result was 47,XY,+21 [8]/46,XY [26]. Prenatal ultrasound findings were unremarkable. She was referred for genetic counseling, and repeat amniocentesis performed at 23 weeks of gestation revealed the result of 47,XY,+21 [3]/46,XY [21]. The parental karyotypes were normal. At repeat amniocentesis, quantitative fluorescent polymerase chain reaction (QF-PCR) analysis using the DNA extracted from uncultured amniocytes and parental bloods excluded uniparental disomy (UPD) 21, array comparative genomic hybridization (aCGH) analysis on uncultured amniocytes revealed the result of arr 21q11.2q22.3 × 2.4, consistent with 40% mosaicism for trisomy 21, and fluorescence *in situ* hybridization (FISH) analysis on uncultured amniocytes revealed 67% (67/100 cells) mosaicism for trisomy 21. The woman was advised to continue the pregnancy, and a 1370-g male baby was delivered prematurely at 29 weeks of gestation without phenotypic abnormalities. The karyotypes of umbilical cord and placenta were 47,XY,+21 [13]/46,XY [27] and 47,XY,+21 [40], respectively. QF-PCR determined maternal origin of the extra chromosome 21 of trisomy 21 in the placenta. When follow-up at age 8½ months, the neonate was normal in appearance and development. The peripheral blood had a karyotype of 47,XY,+21 [1]/46,XY [39], and FISH analysis on buccal mucosal cells showed 9.7% (11/113 cells) mosaicism for trisomy 21, compared with 2% (2/100 cells) in the normal control.

Conclusion: Low-level mosaic trisomy 21 at amniocentesis can be associated with cytogenetic discrepancy in various tissues, perinatal progressive decrease of the trisomy 21 cell line and a favorable fetal outcome.

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Introduction

We previously reported prenatal diagnosis of low-level mosaic trisomy 21 by amniocentesis in seven consecutive cases with favorable fetal outcomes of which two cases was associated with uniparental disomy (UPD) 21 [1–8]. Here, we present an additional case. Our case adds to the list of mosaic trisomy 21 at amniocentesis with favorable fetal outcomes. The information provided in this case along with our previous reports is very useful for genetic counselors, obstetricians and the parents who have very advanced maternal age, who have undergone difficult assisted reproductive technology and who wish to keep the babies under such a circumstance.

Case report

A 36-year-old, gravida 2, para 1, woman underwent amniocentesis at 18 weeks of gestation because of advanced maternal age,

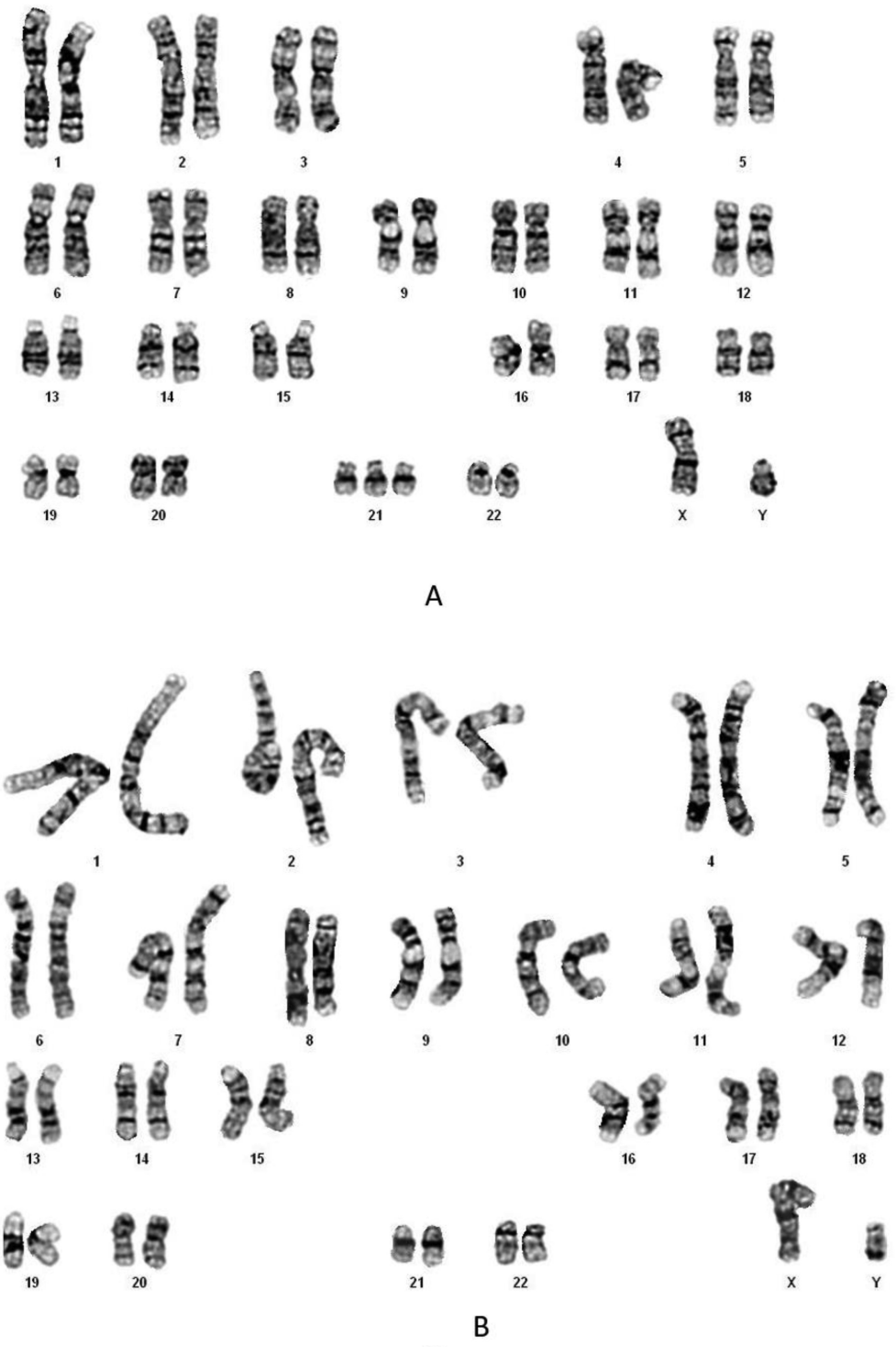

Fig. 1. (A) A karyotype of 47,XY,+21 and (B) a karyotype of 46,XY.

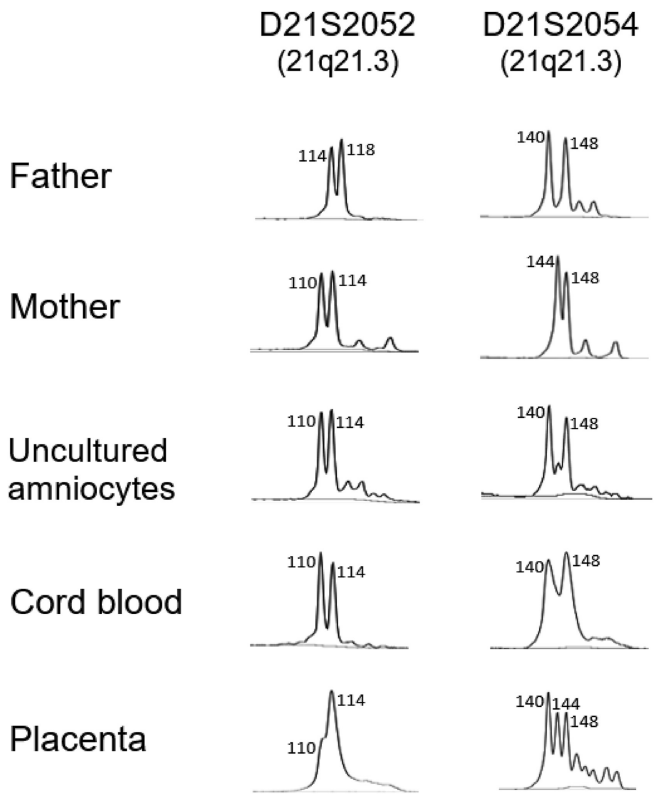

Fig. 2. Quantitative fluorescent polymerase chain reaction (QF-PCR) analysis on the DNA extracted from the parental bloods and uncultured amniocytes shows biparental inheritance of the informative markers in the uncultured amniocytes and cord blood, and a maternal origin of the extra chromosome 21 of trisomy 21 in the placenta.

and the result was 47,XY,+21 [8]/46,XY [26]. Prenatal ultrasound findings were unremarkable. She was referred for genetic counseling, and repeat amniocentesis performed at 23 weeks of gestation revealed the result of 47,XY,+21 [3]/46,XY [21] (Fig. 1). The parental karyotypes were normal. At repeat amniocentesis, quantitative fluorescent polymerase chain reaction (QF-PCR) analysis using the DNA extracted from uncultured amniocytes and parental bloods excluded UPD 21 (Fig. 2), array comparative genomic hybridization (aCGH) analysis on uncultured amniocytes revealed the result of arr 21q11.2q22.3 × 2.4 (Fig. 3), consistent with 40% mosaicism for trisomy 21, and interphase fluorescence *in situ* hybridization (FISH) analysis on uncultured amniocytes revealed 67% (67/100 cells) mosaicism for trisomy 21 (Fig. 4). The woman was advised to continue the pregnancy, and a 1370-g male baby was delivered prematurely at 29 weeks of gestation without phenotypic abnormalities. The karyotypes of umbilical cord and placenta were 47,XY,+21 [13]/46,XY [27] and 47,XY,+21 [40], respectively. QF-PCR determined maternal origin of the extra chromosome 21 of trisomy 21 in the placenta (Fig. 2). When follow-up at age 8½ months, the neonate was normal in appearance and development. The peripheral blood had a karyotype of 47,XY,+21 [1]/46,XY [39], and FISH analysis on buccal mucosal cells showed 9.7% (11/113 cells) mosaicism for trisomy 21, compared with 2% (2/100 cells) in the normal control.

Discussion

The present case provides evidence for perinatal progressive decrease of the trisomy 21 cell line and cytogenetic discrepancy between cultured amniocytes and uncultured amniocytes in case of mosaic trisomy 21 at amniocentesis. In the present case, at the first amniocentesis at 18 weeks of gestation, the cultured amniocytes had the karyotype of 47,XY,+21 [8]/46,XY [26], consistent with 23.5% mosaicism for trisomy 21. At the repeat amniocentesis at 23 weeks of gestation, the cultured amniocytes had the karyotype of 47,XY,+21 [3]/46,XY [21], consistent with 12.5% mosaicism for trisomy 21, aCGH on the DNA extracted from uncultured amniocytes revealed 40% mosaicism for trisomy 21, and interphase FISH on uncultured amniocytes revealed 67% (67/100 cells) mosaicism for trisomy 21. After birth, the umbilical cord and placenta had the karyotypes of 47,XY,+21 [13]/46,XY [27] (32.5% mosaic trisomy 21) and 47,XY,+21 (full trisomy 21), respectively. At age 8½ months, the peripheral blood had the karyotype of 47,XY,+21 [1]/46,XY [39] (2.5% mosaic trisomy 21), and buccal mucosal cells had 9.7% (11/113 cells) mosaic trisomy 21.

Our observation is in accordance with our previous reports that evaluating mosaic trisomy 21 at amniocentesis simply based on the result obtained in the early second trimester is not reliable, and repeat amniocentesis should be performed in late second trimester or in early third trimester because the mosaic level of trisomy 21 in the cultured amniocytes may significantly decrease in the late gestation. For instance, in this present case, 23.5% mosaicism for trisomy 21 at 18 weeks of gestation and 12.5% mosaicism for trisomy 21 at 23 weeks of gestation.

Our observation also shows that conventional cytogenetic analysis on cultured amniocytes is more reliable than molecular analysis on the uncultured amniocytes if there is cytogenetic discrepancy between uncultured amniocytes and cultured amniocytes in case of mosaic trisomy 21 at amniocentesis. For instance, in this present case, aCGH analysis on the DNA extracted from uncultured amniocytes revealed 40% mosaicism for trisomy 21, and interphase FISH on uncultured amniocytes revealed 67% mosaicism for trisomy 21, whereas the cultured amniocytes revealed 12.5% mosaicism for trisomy 21. This indicates that during repeat amniocentesis for furthermore investigation of mosaic trisomy 21 at amniocentesis, molecular study of uncultured amniocytes may result in misleading interpretation because there may be many dead trisomy cells in the amniotic fluid. Therefore, we suggest that at repeat amniocentesis for confirmation of true level of mosaic trisomy 21 at amniocentesis, conventional cytogenetic analysis should be included, and molecular cytogenetic analysis cannot replace conventional cytogenetic analysis.

In the present case, the placenta had full trisomy 21, and this result is in accordance with our previous observations that in case of trisomy rescue, the placenta is always trisomic or partially trisomic because placenta, unlike the fetus, always fails to achieve a successful trisomy rescue. Therefore, non-invasive prenatal testing (NIPT) plays a very important role in the early identification of mosaic trisomy and the possible associated UPD.

In summary, low-level mosaic trisomy 21 at amniocentesis can be associated with cytogenetic discrepancy in various tissues, perinatal progressive decrease of the trisomy 21 cell line and a favorable fetal outcome.

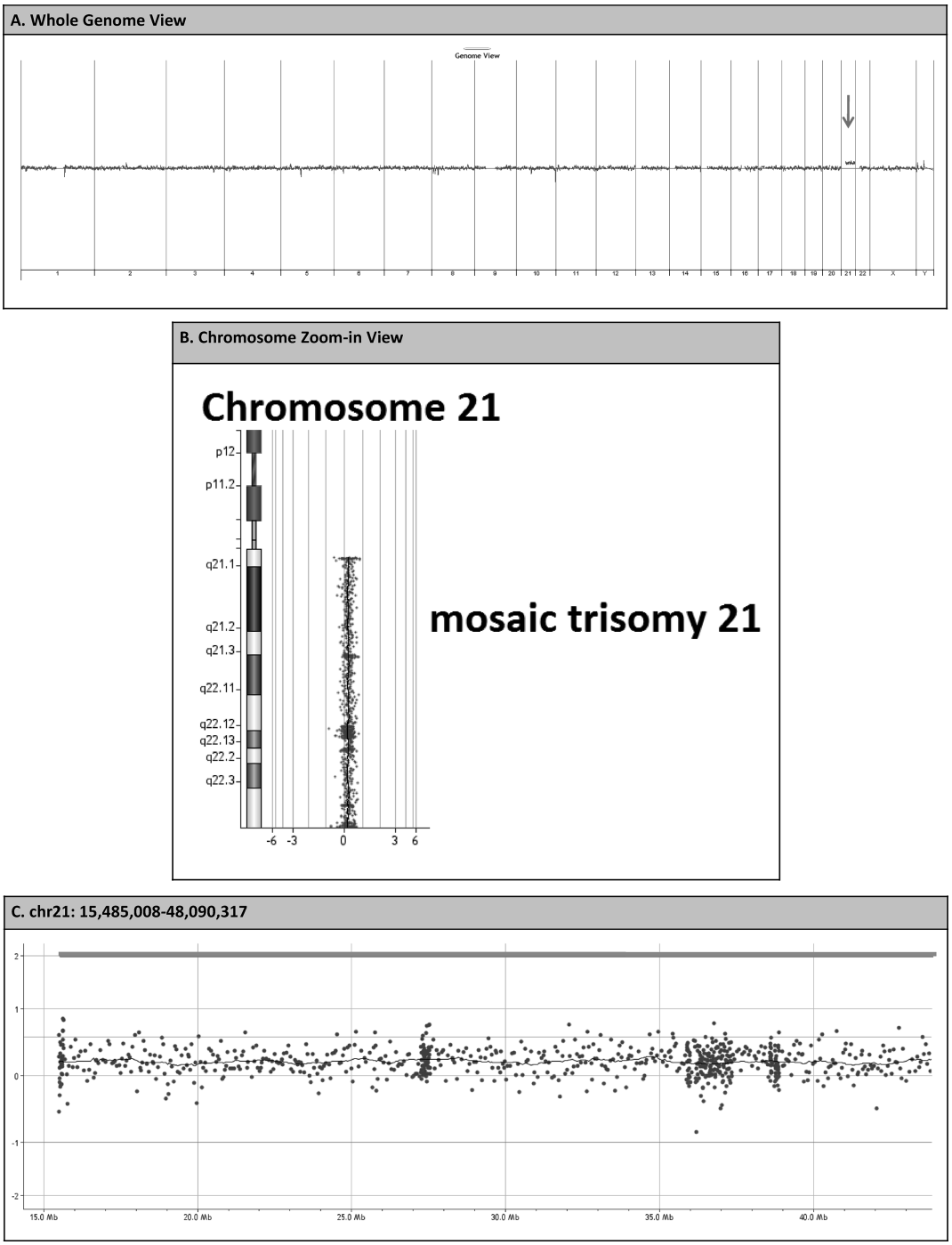

Fig. 3. (A) (B) and (C) Array comparative genomic hybridization (aCGH) analysis by SurePrint G3 Unrestricted CGH ISCA v2, 8′60 K (Agilent Technologies, Santa Clara, CA, USA) on the DNA extracted from uncultured amniocytes shows the result of arr [GRCh37 (hg19)] 21q11.2q22.3 (15,485,008–48,090,317) × 2.4 ($\log_2$ ratio = 0.25–0.3) consistent with 40% mosaicism for trisomy 21.

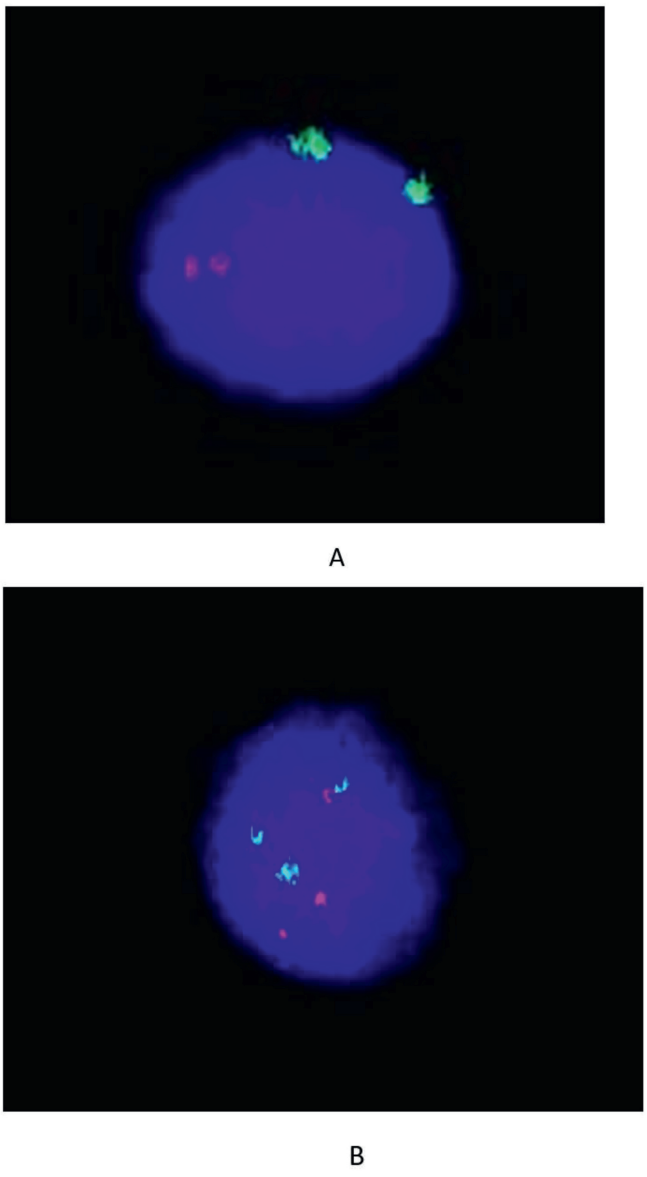

Fig. 4. Interphase fluorescence *in situ* hybridization (FISH) analysis on buccal mucosal cells using bacterial artificial chromosome (BAC) probes of RP11-139O21 [21p11.1; fluorescein isothiocyanate (FITC), spectrum green] and RP11-161H21 (21q11.2; Texas Red, spectrum red) shows (A) a disomy 21 cell with two red signals and two green signals and (B) a trisomy 21 cell with three red signals and three green signals.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

Acknowledgements

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Case Report

High-level mosaic trisomy 21 at amniocentesis in a pregnancy associated with positive NIPT for trisomy 21, prenatal progressive decrease of the trisomy 21 cell line, acute fatty liver of pregnancy and intrauterine fetal death in late gestation

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IUFD
Mosaic trisomy 21

ABSTRACT

Objective: We present high-level mosaic trisomy 21 at amniocentesis in a pregnancy associated with positive non-invasive prenatal testing (NIPT) for trisomy 21, prenatal progressive decrease of the trisomy 21 cell line, acute fatty liver of pregnancy and intrauterine fetal death (IUFD) in late gestation.

Case report: A 32-year-old, primigravid woman underwent amniocentesis at 17 weeks of gestation because of positive NIPT for trisomy 21 at 12 weeks of gestation. This pregnancy was conceived by *in vitro* fertilization. She did not have obesity, diabetes mellitus, hepatic biliary disorders and preeclampsia. Amniocentesis revealed a karyotype of 47,XY,+21[10]/46,XY[11], and array comparative genomic hybridization (aCGH) analysis on uncultured amniocytes revealed the result of arr (21) × 2–3. She was referred for genetic counseling, and repeat amniocentesis performed at 21 weeks of gestation revealed the karyotype of 47,XY,+21[10]/46,XY[28]. The parental karyotypes and fetal ultrasound findings were normal. Simultaneous molecular analysis on uncultured amniocytes showed no uniparental disomy 21, but a maternal origin of trisomy 21 by quantitative fluorescent polymerase chain reaction (QF-PCR) and the result of arr 21q11.2q22.3 × 2.5 by aCGH analysis. At 27 weeks of gestation, she underwent a third amniocentesis, of which conventional cytogenetic analysis revealed the result of 47,XY,+21[5]/46,XY[17] in cultured amniocytes, and aCGH analysis revealed arr 21q11.2q22.3 × 2.48, and interphase fluorescence *in situ* hybridization (FISH) analysis revealed 39% (39/100 cells) mosaicism fro trisomy 21 in uncultured amniocytes. At 36 weeks of gestation, the woman suffered from a sudden onset of acute fatty liver and IUFD. A 3522-g male baby was delivered without Down syndrome phenotype. The umbilical cord had a karyotype of 47,XY,+21[10]/46,XY[30]. aCGH analysis on the skin and placenta showed arr 21q11.2q22.3 × 2.73 and arr 21q11.2q22.3 × 2.75, respectively. QF-PCR analysis of umbilical cord, placenta and skin showed a maternal origin of trisomy 21.

Conclusion: High-level mosaic trisomy 21 at amniocentesis can be associated with prenatal progressive decrease of the trisomy 21 cell line in cultured amniocytes and perinatal fetal mortality and maternal morbidity.

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Introduction

We previously reported prenatal diagnosis of low-level mosaic trisomy 21 by amniocentesis in seven consecutive cases with favorable fetal outcomes of which two cases was associated with uniparental disomy (UPD) 21 [1–8]. Here, we present an unusual case of mosaic trisomy 21 at amniocentesis with adverse perinatal outcome. The information provided in this case along with our previous reports is very useful for genetic counselors, obstetricians and the parents who have very advanced maternal age, who have

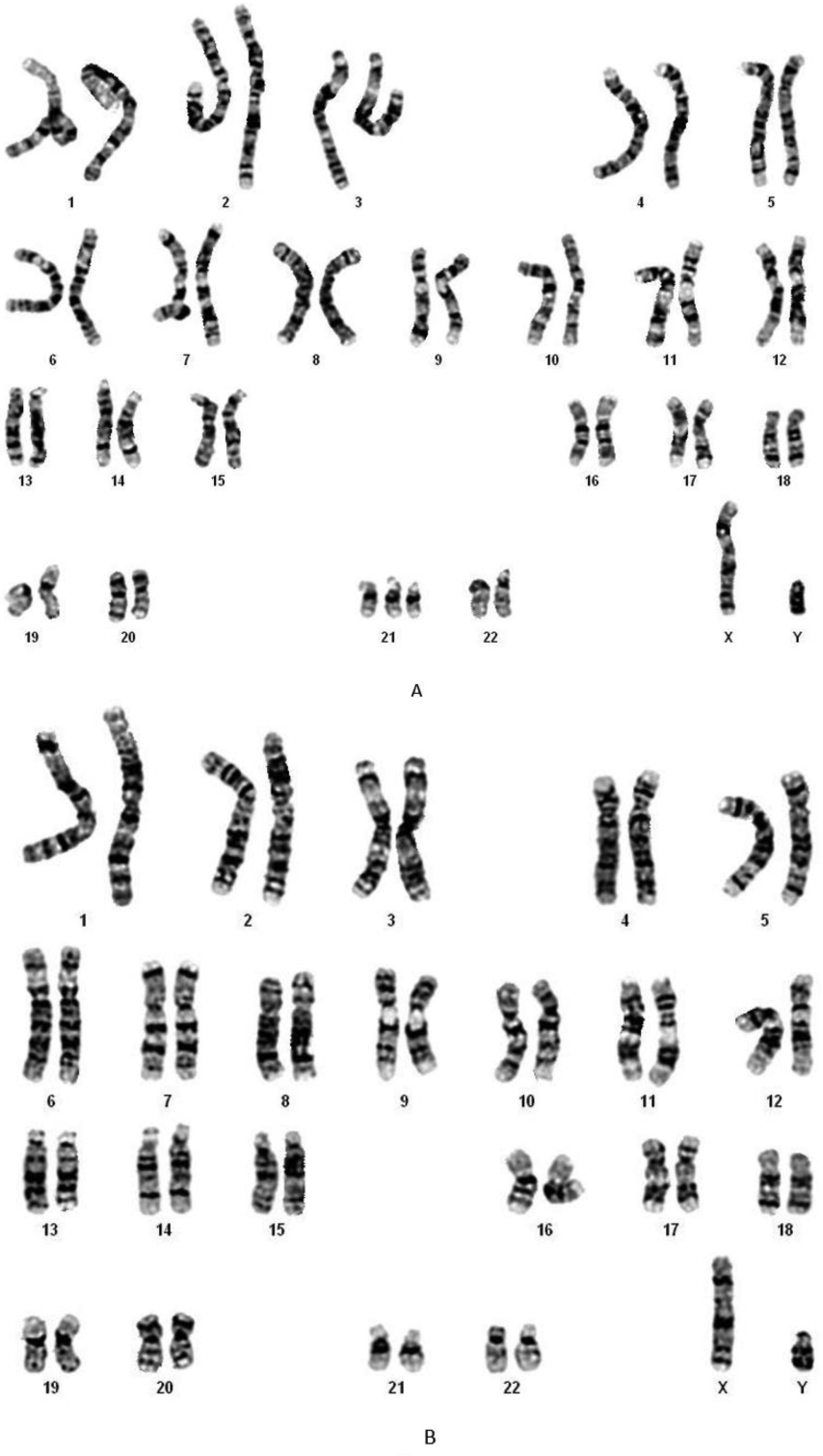


Fig. 1. (A) A karyotype of 47,XY,+21 and (B) a karyotype of 46,XY.

undergone difficult assisted reproductive technology and who wish to keep the babies under such a circumstance.

Case report

A 32-year-old, primigravid woman underwent amniocentesis at 17 weeks of gestation because of positive non-invasive prenatal testing (NIPT) for trisomy 21 at 12 weeks of gestation. This pregnancy was conceived by *in vitro* fertilization (IVF). She did not have obesity, diabetes mellitus, hepatic biliary disorders and pre-eclampsia. Amniocentesis revealed a karyotype of 47,XY,+21[10]/46,XY[11], and array comparative genomic hybridization (aCGH) analysis on uncultured amniocytes revealed the result of arr (21) × 2–3. She was referred for genetic counseling, and repeat amniocentesis performed at 21 weeks of gestation revealed the karyotype of 47,XY,+21[10]/46,XY[28] (Fig. 1). The parental karyotypes and fetal ultrasound findings were normal. Simultaneous molecular analysis on uncultured amniocytes showed no UPD 21 but a maternal origin of trisomy 21 by quantitative fluorescent polymerase chain reaction (QF-PCR) (Fig. 2), and the result of arr 21q11.2q22.3 × 2.5 by aCGH analysis (Fig. 3). At 27 weeks of gestation, she underwent a third amniocentesis, of which conventional cytogenetic analysis revealed the result of 47,XY,+21[5]/46,XY[17] in cultured amniocytes, and aCGH analysis revealed arr 21q11.2q22.3 × 2.48, and interphase fluorescence *in situ* hybridization (FISH) analysis revealed 39% (39/100 cells) mosaicism for trisomy 21 in uncultured amniocytes (Fig. 4). At 36 weeks of gestation, the woman suffered from a sudden onset of acute fatty liver and IUFD. A 3522-g male baby was delivered without Down syndrome phenotype. The umbilical cord had a karyotype of 47,XY,+21[10]/46,XY[30]. aCGH analysis on the skin and placenta showed arr 21q11.2q22.3 × 2.73 (Fig. 5) and arr 21q11.2q22.3 × 2.75 (Fig. 6), respectively. QF-PCR analysis of umbilical cord, placenta and skin showed a maternal origin of trisomy 21 (Fig. 2).

Discussion

The present case was associated with high-level mosaicism for trisomy 21 at amniocentesis and prenatal progressive decrease of the mosaic trisomy 21 levels in cultured amniocytes. In the present case, at the first amniocentesis at 17 weeks of gestation, the cultured amniocytes had the karyotype of 47,XY,+21[10]/46,XY[11], consistent with 47.6% (10/21 colonies) mosaicism for trisomy 21, at the second amniocentesis at 21 weeks of gestation, the cultured amniocytes had the karyotype of 47,XY,+21[10]/46,XY[28], consistent with 26.5% (10/38 colonies) mosaicism for trisomy 21, and at the third amniocentesis at 27 weeks of gestation, the cultured amniocytes had the karyotype of 47,XY,+21[5]/46,XY[17], consistent with 22.7% (5/22 colonies) mosaicism for trisomy 21.

The present case also manifested cytogenetic discrepancy between cultured amniocytes and uncultured amniocytes. For instance, at the second amniocentesis at 21 weeks of gestation, the cultured amniocytes had 26.5% (10/38 colonies) mosaicism for trisomy 21, whereas the uncultured amniocytes showed 50% mosaicism for trisomy 21 by aCGH analysis, and at the third amniocentesis at 27 weeks of gestation, the cultured amniocytes

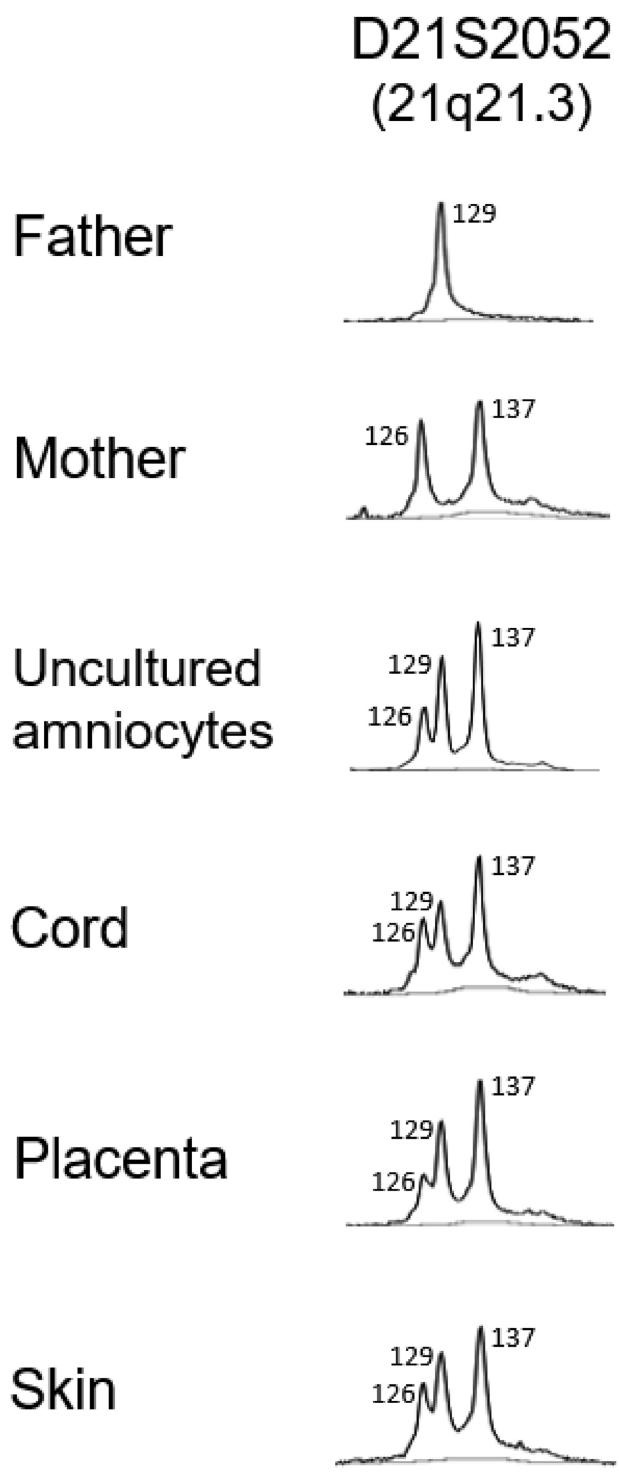


Fig. 2. Quantitative fluorescent polymerase chain reaction (QF-PCR) analysis on the DNA extracted from the parental bloods, uncultured amniocytes, umbilical cord, placenta and skin excludes uniparental disomy 21 and shows a maternal origin of mosaic trisomy 21, *i.e.*, a small peak of 126 bp derived from the maternal allele.

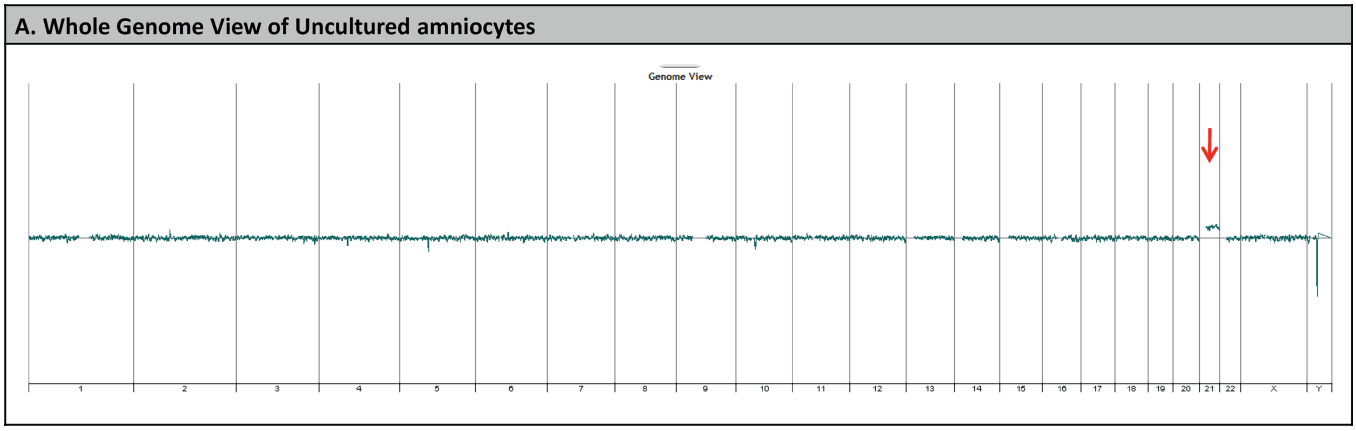
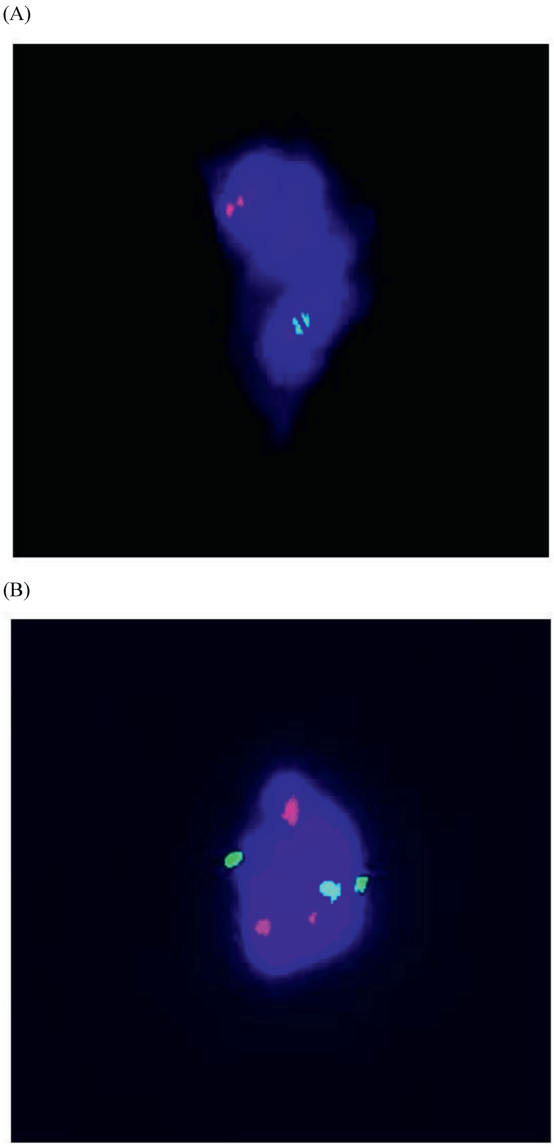


Fig. 3. Array comparative genomic hybridization (aCGH) analysis by SurePrint G3 Unrestricted CGH ISCA v2, 8 × 60 K (Agilent Technologies, Santa Clara, CA, USA) on the DNA extracted from uncultured amniocytes at 27 weeks of gestation shows the result of arr [GRCh37 (hg19)] 21q11.2q22.3 (15,502,446–48,090,317) × 2.48 consistent with 48% ($\log_2$ ratio = 0.309) mosaicism for trisomy 21.



had 22.7% (5/22 colonies) mosaicism for trisomy 21, whereas the uncultured amniocytes showed 48% mosaicism for trisomy 21 by aCGH analysis and 39% (39/100 cells) mosaicism for trisomy 21 by interphase FISH analysis. This observation is in accordance with our previous reports that molecular analysis on uncultured amniocytes may reveal higher levels of mosaicism for trisomy 21, compared with conventional cytogenetic analysis on cultured amniocytes.

The present case was conceived by IVF in a mother of 32 years old. In the present case, NIPT at 12 weeks of gestation showed a positive result suspicious of trisomy 21. The present case also had a maternal origin of the mosaic trisomy 21 and a trisomic placenta. The present case shows that women who undergo IVF will have the benefit of early diagnosis of mosaic trisomies by NIPT.

The peculiar aspect of the present case is the association of acute fatty liver of pregnancy and IUFD at 36 weeks of gestation. The risk factors of acute fatty liver of pregnancy include multifetal gestation, nulliparity, a male fetus, fatty acid oxidation disorders, maternal obesity, diabetes mellitus, hepatic and biliary disorders, and pre-eclampsia [9]. Whether the mosaic trisomy 21 placenta is associated with the development of acute fatty liver of pregnancy as presented in this case is unclear and will require more cases to investigate.

In summary, we present high-level mosaic trisomy 21 at amniocentesis in a pregnancy associated with positive NIPT for trisomy 21, prenatal progressive decrease of the trisomy 21 cell line, acute fatty liver of pregnancy and IUFD in late gestation. High-level mosaic trisomy 21 at amniocentesis can be associated with prenatal progressive decrease of the trisomy 21 cell line in cultured amniocytes and perinatal fetal mortality and maternal morbidity.

Fig. 4. Interphase fluorescence *in situ* hybridization (FISH) analysis on uncultured amniocytes using bacterial artificial chromosome (BAC) probes of RP11-138O15 [21q11.2; fluorescein isothiocyanate (FITC), spectrum green] and RP11-345F15 (21q22.3; Texas Red, spectrum red) shows (A) a disomy 21 cell with two red signals and two green signals and (B) a trisomy 21 cell with three red signals and three green signals.

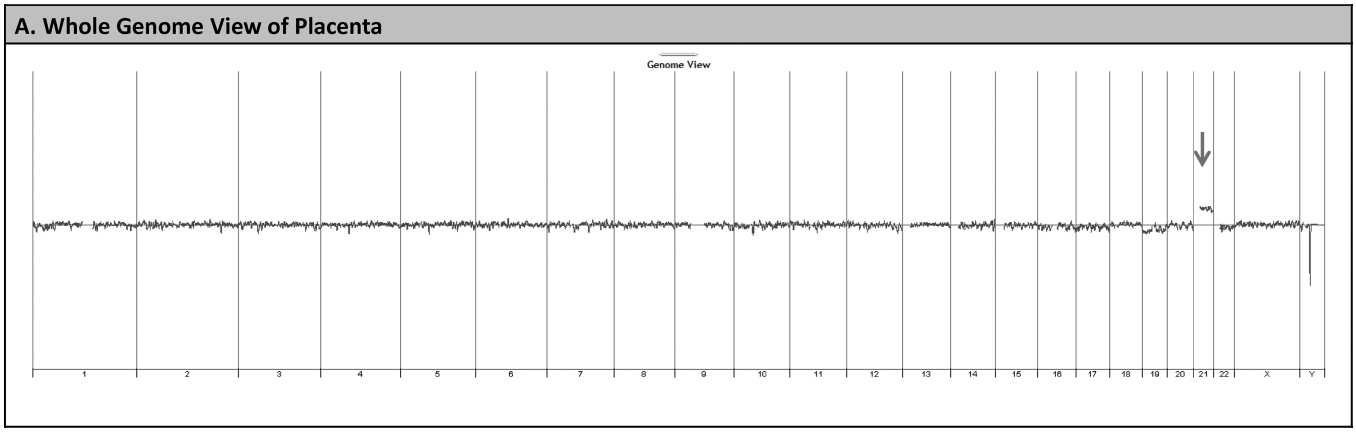


Fig. 5. aCGH analysis by SurePrint G3 Unrestricted CGH ISCA v2, 8 × 60 K (Agilent Technologies, Santa Clara, CA, USA) on the DNA extracted from placenta shows the result of arr [GRCh37 (hg19)] 21q11.2q22.3 (15,485,008–48,090,317) × 2.75 consistent with 75% ($\log_2$ ratio = 0.46) mosaicism for trisomy 21.

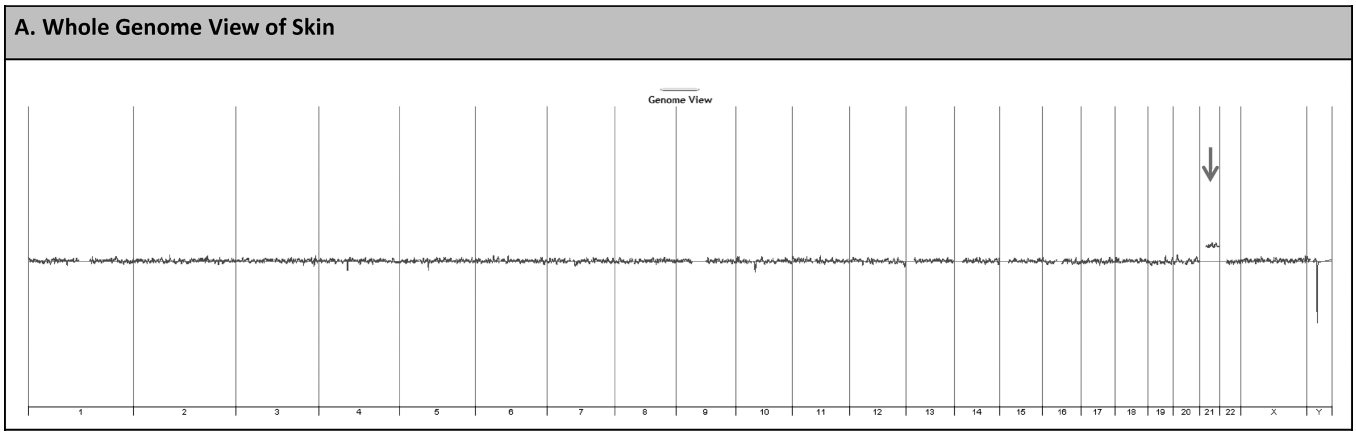


Fig. 6. aCGH analysis by SurePrint G3 Unrestricted CGH ISCA v2, 8 × 60 K (Agilent Technologies, Santa Clara, CA, USA) on the DNA extracted from skin shows the result of arr [GRCh37 (hg19)] 21q11.2q22.3 (15,499,847–48,090,317) × 2.73 consistent with 73% ($\log_2$ ratio = 0.447) mosaicism for trisomy 21.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

Acknowledgements

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Case Report

45,X/46,XX at the first amniocentesis, and 45,X/47,XXX/46,XX at the repeat amniocentesis and at birth in a pregnancy associated with a favorable fetal outcome, perinatal progressive decrease of the 45,X cell line and cytogenetic discrepancy between cultured amniocytes and uncultured amniocytes

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45,X/46,XX
45,X/47,XXX/46,XX
Amniocentesis

ABSTRACT

Objective: We present 45,X/46,XX at the first amniocentesis, and 45,X/47,XXX/46,XX at the repeat amniocentesis and at birth in a pregnancy associated with a favorable fetal outcome, perinatal progressive decrease of the 45,X cell line and cytogenetic discrepancy between cultured amniocytes and uncultured amniocytes.

Case report: A 43-year-old, gravida 3, para 1, woman underwent amniocentesis at 18 weeks of gestation because of advanced maternal age. Amniocentesis revealed a karyotype of 45,X[4]/46,XX[20]. Simultaneous array comparative genomic hybridization (aCGH) analysis on the DNA extracted from uncultured amniocytes revealed arr (X) × 3 [0.24], consistent with 24% mosaicism for triple X. Repeat amniocentesis at 20 weeks of gestation revealed the result of 45,X[17]/47,XXX[8]/46,XX[121]. She was referred for genetic counseling, and the third amniocentesis performed at 30 weeks of gestation revealed the result of 45,X[3]/47,XXX[2]/46,XX[16]. The mother had a karyotype of 46,XX. aCGH analysis on the DNA extracted from uncultured amniocytes showed arr Xp22.33q28 × 2.2 ($\log_2$ ratio = 0.15), consistent with 20% mosaicism for triple X. Interphase fluorescence *in situ* hybridization (FISH) analysis on 100 uncultured amniocytes showed that 11 cells (11%) were monosomy X, seven cells (7%) were triple X, and the others were disomy X. At 39 weeks of gestation, a 3,620-g phenotypically normal female baby was delivered without any phenotypic abnormality. The karyotypes of cord blood, umbilical cord and placenta were 47,XXX[7]/45,X[1]/46,XX[32], 47,XXX[13]/46,XX[27] and 47,XXX[2]/46,XX[38], respectively. When follow-up at age one month, the neonate was phenotypically normal, and FISH analysis on 106 buccal mucosal cells showed that eight cells (7.5%) were monosomy X, seven cells (6.6%) were triple X, and the others were disomy X.

Conclusion: Mosaic 45,X/46,XX at amniocentesis may be in fact mosaic 45,X/47,XXX/46,XX and can be associated with a favorable fetal outcome and perinatal progressive decrease of the 45,X cell line.

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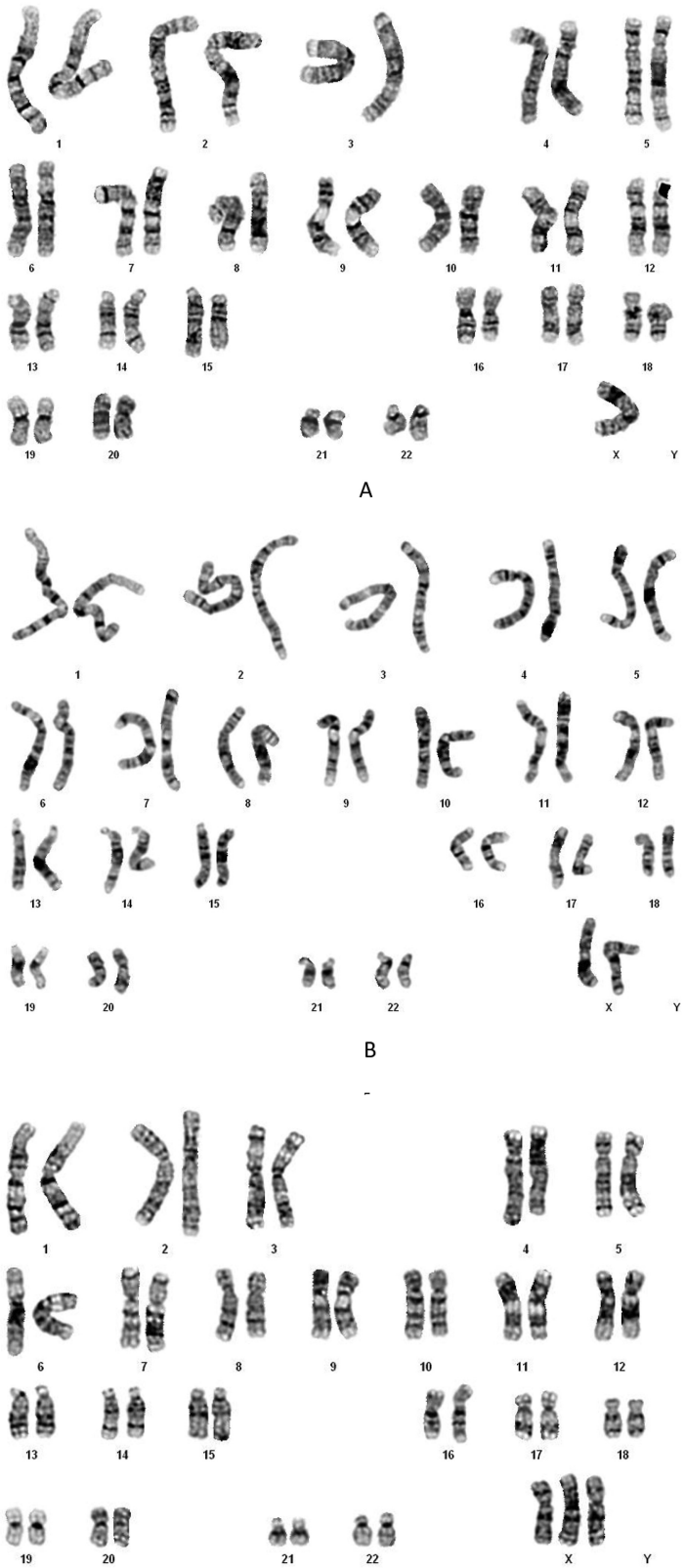
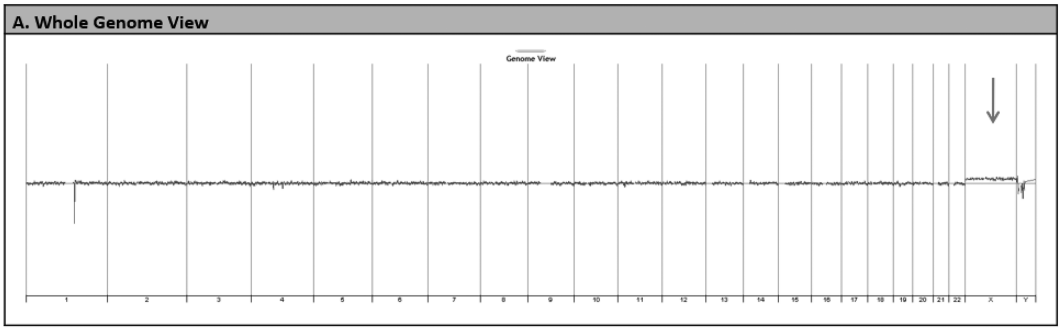
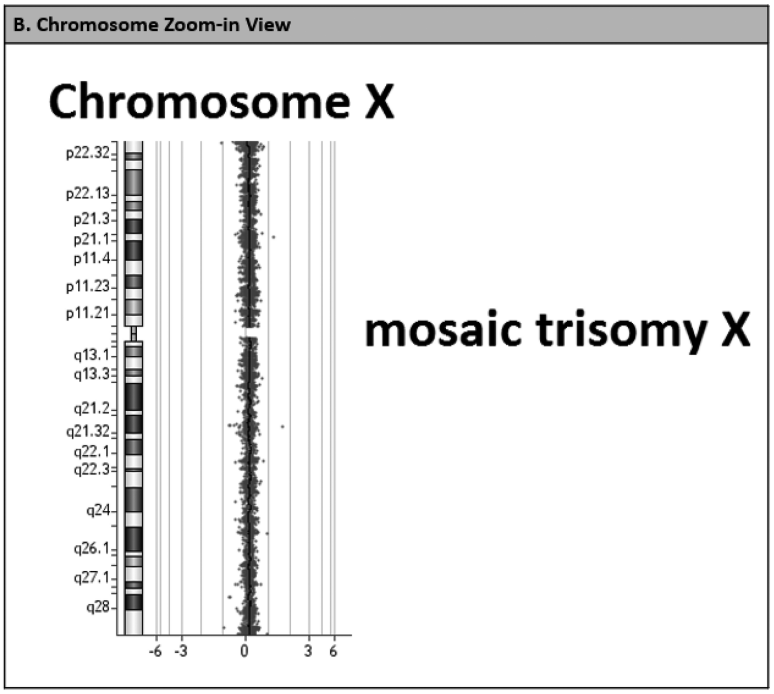


Fig. 1. (A) A karyotype of 45,X, (B) a karyotype of 46,XX and (C) a karyotype of 47,XXX.

(A)



(B)



(C)

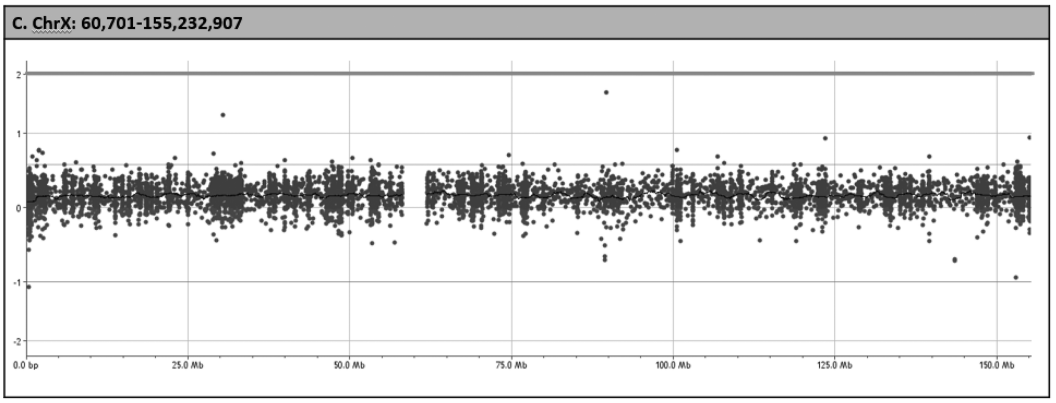
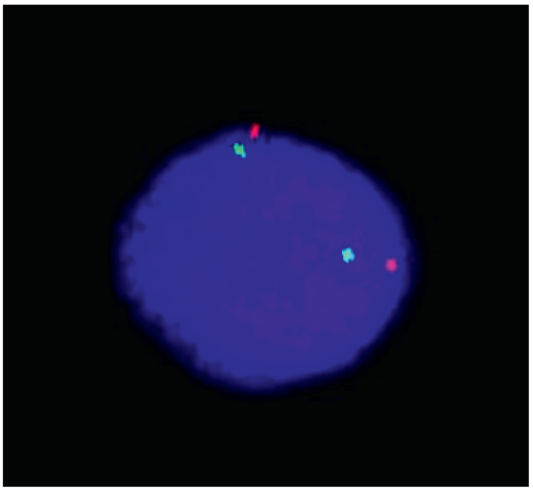


Fig. 2. Array comparative genomic hybridization (aCGH) analysis using SurePrint G3 Unrestricted CGH ISCA v2, 8 × 60K (Agilent Technologies, Santa Clara, CA, USA) on the DNA extracted from uncultured amniocytes shows the result of arr [GRCh37 (hg19)] Xp22.33q28 (60,701–155,232,907) × 2.2, consistent with 20% ($\log_2$ ratio = 0.15) mosaicism for triple X.

(A)



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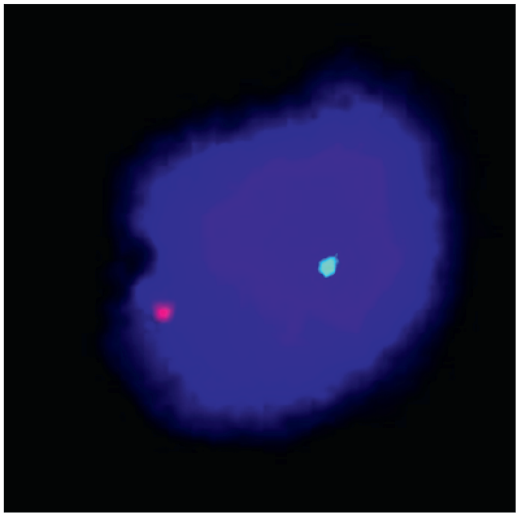

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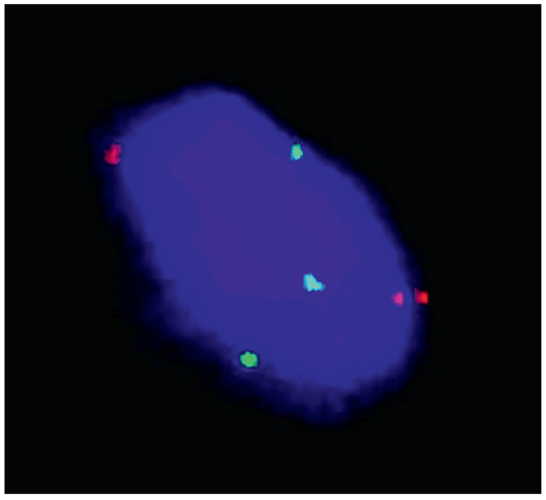

Introduction

We previously reported 45,X/46,XX at amniocentesis with s favorable outcome [1–5]. Here, we present an additional case with cytogenetic discrepancy between cultured amniocytes and uncultured amniocytes and in different amniocenteses and a favorable fetal outcome with a karyotype of 45,X/47,XXX/46,XX at birth. The information provided in this presentation is useful for obstetricians, genetic counselors and the parents who wish to keep the babies under such a circumstance.

Case report

A 43-year-old, gravida 3, para 1, woman underwent amniocentesis at 18 weeks of gestation because of advanced maternal age. Amniocentesis revealed a karyotype of 45,X[4]/46,XX[20]. Simultaneous array comparative genomic hybridization (aCGH) analysis on the DNA extracted from uncultured amniocytes revealed arr (X) $\times$ 3 [0.24], consistent with 24% mosaicism for triple X. Repeat amniocentesis at 20 weeks of gestation revealed the result of 45,X [17]/47,XXX[8]/46,XX[121]. She was referred for genetic counseling, and the third amniocentesis performed at 30 weeks of gestation revealed the result of 45,X[3]/47,XXX[2]/46,XX[16] (Fig. 1). The mother had a karyotype of 46,XX. aCGH analysis on the DNA extracted from uncultured amniocytes showed arr Xp22.33q28 $\times$ 2.2 ($\log_2$ ratio = 0.15), consistent with 20% mosaicism for triple X (Fig. 2). Interphase fluorescence *in situ* hybridization (FISH) analysis on 100 uncultured amniocytes showed that 11 cells (11%) were monosomy X, seven cells (7%) were triple X, and the others were disomy X (Fig. 3). At 39 weeks of gestation, a 3,620-g phenotypically normal female baby was delivered without any phenotypic abnormality. The karyotypes of cord blood, umbilical cord and placenta were 47,XXX[7]/45,X[1]/ 46,XX[32], 47,XXX[13]/ 46,XX[27] and 47,XXX[2]/46,XX[38], respectively. When follow-up at age one month, the neonate was phenotypically normal, and FISH analysis on the buccal mucosal cells showed that eight cells (7.5%) were monosomy X, seven cells (6.6%) were triple X, and the others were disomy X.

Discussion

The peculiar aspect of the present case is the cytogenetic discrepancy between cultured amniocytes and uncultured amniocytes 4 in mosaic 45,X/46,XX at amniocentesis. During the first amniocentesis, the karyotype was mosaic 45,X/46,XX, whereas aCGH analysis on uncultured amniocytes revealed mosaic triple X. In the present case, at the first amniocentesis at 18 weeks of gestation, conventional cytogenetic analysis revealed the karyotype of 45,X[4]/46,XX[20], consistent with 16.7% mosaicism for 45,X. However, in aCGH analysis on the DNA extracted from uncultured amniocytes, the result was arr (X) $\times$ 3 [0.24], consistent with 24% mosaicism for triple X. At the second amniocentesis at 20 weeks of gestation, the karyotype was 45,X[17]/47,XXX[8]/46,XX[121], consistent with 11.6% mosaicism for 45,X and 5.5% mosaicism for 47,XXX. At the third amniocentesis at 30 weeks of gestation, the

Fig. 3. Interphase fluorescence *in situ* hybridization (FISH) analysis on uncultured amniocytes using bacterial artificial chromosome (BAC) probes of RP11-383I22 [Xp22.31; fluorescein isothiocyanate (FITC), spectrum green] and RP11-943J20 (Xq11.1-q11.2; Texas Red, spectrum red) shows (A) a disomy X cell with two red signals and two green signals, (B) a monosomy X cell with one red signal and one green signal, and (C) a triple X cell with three red signals and three green signals.

karyotype was 45,X[3]/47,XXX[2]/46,XX[16], consistent with 14.2% mosaicism for 45,X and 9.5% mosaicism for 47,XXX, and aCGH analysis on the DNA extracted from uncultured amniocytes revealed 20% mosaicism for triple X. Interphase FISH analysis on uncultured amniocytes revealed 11% (11/100 cells) mosaicism for 45,X and 7% (7/100 cells) mosaicism for triple X. At birth, the placenta had the karyotype of 47,XXX[2]/46,XX[38], and the umbilical cord had the karyotype of 47,XXX[13]/46,XX[27]. Both the placenta and the umbilical cord did not have mosaic 45,X but had mosaic triple X. However, the cord blood had the karyotype of 47,XXX[7]/45,X[1]/ 46,XX[32], consistent with 17.5% mosaicism for triple X and 2.5% mosaicism for 45,X.

The present case provides evidence for perinatal progressive decrease of the 45,X cell line in mosaic 45,X at amniocentesis. For instance, at the first amniocentesis, the cultured amniocytes had 16.7% mosaicism for 45,X, at the second amniocentesis, the cultured amniocytes had 11.6% mosaicism for 45,X, and at birth, the cord blood had only 2.5% mosaicism for 45,X, and the placenta and the umbilical cord did not have the 45,X cell line in conventional cytogenetic analysis. When follow-up at age one month, the buccal mucosal cells had 7.5% (8/106 cells) mosaicism for monosomy X,

The present case also provides evidence that 45,X/46,XX at amniocentesis can be in fact 45,X/47,XXX/46,XX due to postzygotic mitotic non-disjunction and can be associated with a favorable fetal outcome. This information is very useful for genetic counseling under such a circumstance.

In summary, we present 45,X/46,XX at the first amniocentesis, and 45,X/47,XXX/46,XX at the repeat amniocentesis and at birth in a pregnancy associated with a favorable fetal outcome, perinatal progressive decrease of the 45,X cell line and cytogenetic discrepancy between cultured amniocytes and uncultured amniocytes.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

Acknowledgements

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Case Report

Perinatal detection of disomy X cell line by fluorescence *in situ* hybridization in a pregnancy with 45,X/47,XXX at amniocentesis, cytogenetic discrepancy in various tissues and a favorable outcome

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ABSTRACT

Objective: We present perinatal detection of disomy X cell line by fluorescence *in situ* hybridization (FISH) in a pregnancy with 45,X/47,XXX at amniocentesis, cytogenetic discrepancy in various tissues and a favorable outcome.

Case report: A 34-year-old, gravida 3, para 1, woman underwent amniocentesis at 17 weeks of gestation because of advanced maternal age. Amniocentesis revealed a karyotype of 45,X[22]/47,XXX[10]. Simultaneous array comparative genomic hybridization (aCGH) analysis on the DNA extracted from uncultured amniocytes revealed the result of arr (X) × 1–2, (1–22) × 2, consistent with 32% mosaicism for monosomy X. She was referred for genetic counseling at 19 weeks of gestation. Prenatal ultrasound findings and parental karyotypes were normal. Repeat amniocentesis at 29 weeks of gestation revealed a karyotype of 45,X[36]/47,XXX[4] (Fig. 1) in cultured amniocytes. Simultaneous molecular analysis on uncultured amniocytes revealed the result of arr (1–22) × 2, Y × 0 by aCGH with no genomic imbalance, and 15% (15/100 cells) mosaicism for disomy X, 61% (61/100 cells) mosaicism for monosomy X and 24% (24/100 cells) mosaicism for triple X by interphase fluorescence *in situ* hybridization (FISH) analysis. The pregnancy was encouraged to continue and at 37 weeks of gestation, a 2834-g phenotypically normal female baby was delivered. The karyotypes of cord blood, umbilical cord and placenta were 45,X[33]/47,XXX[7], 45,X[30]/47,XXX[10] and 47,XXX[38]/45,X[2], respectively. When follow-up at age three months, the neonate was normal in development. FISH analysis on 99 buccal mucosal cells showed 49% (48/99 cells) mosaicism for monosomy X, 8% (8/99 cells) mosaicism for triple X and 43% (42/99 cells) mosaicism for disomy X (Fig. 2). Peripheral blood had a karyotype of 45,X[38]/47,XXX[2]. When follow-up at age nine months, the neonate was normal in development. FISH analysis on 102 buccal mucosal cells showed 11% (11/102 cells) mosaicism for monosomy X, 12% (12/102 cells) mosaicism for triple X and 77% (79/102 cells) mosaicism for disomy X. Peripheral blood had a karyotype of 45,X[30]/47,XXX[10].

Conclusion: 45,X/47,XXX at amniocentesis may detect disomy X cell line by FISH analysis and can be associated with postnatal progressive decrease of the aneuploid cell lines, increase of the disomy X cell line and a favorable outcome.

Introduction

We previously reported 45,X/46,XX at amniocentesis with a favorable outcome [1–5]. Here, we present an additional case with cytogenetic discrepancy between cultured amniocytes and

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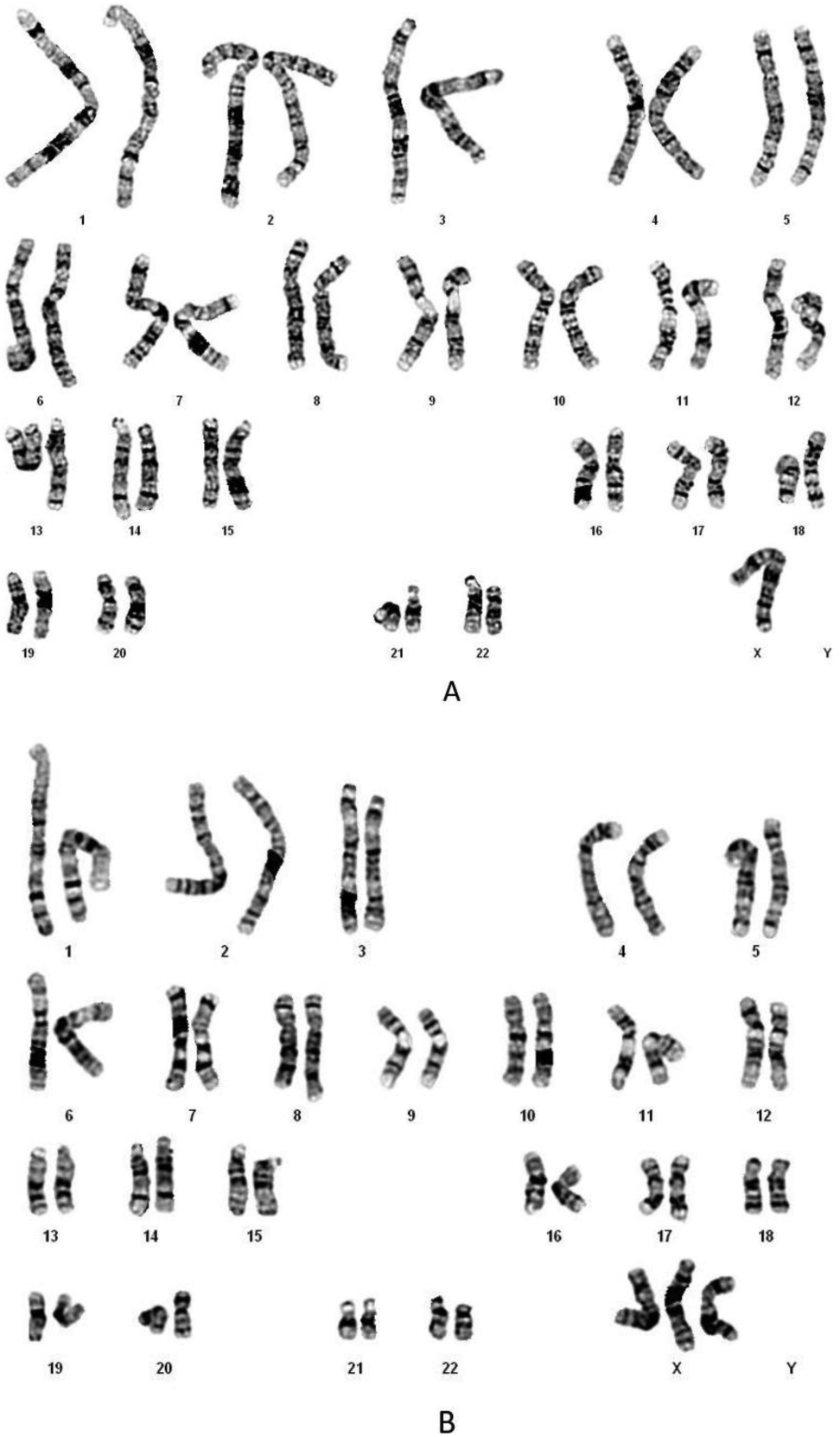

Fig. 1. (A) A karyotype of 45,X and (B) a karyotype of 47,XXX.

uncultured amniocytes, in different tissues and in different amniocenteses and a favorable fetal outcome with a karyotype of 45,X/47,XXX at amniocentesis. The information provided in this presentation is useful for obstetricians, genetic counselors and the parents who wish to keep the babies under such a circumstance.

Case Report

A 34-year-old, gravida 3, para 1, woman underwent amniocentesis at 17 weeks of gestation because of advanced maternal age. Amniocentesis revealed a karyotype of 45,X[22]/47,XXX[10]. Simultaneous array comparative genomic hybridization (aCGH) analysis on the DNA extracted from uncultured amniocytes revealed the result of arr (X) $\times$ 1–2, (1–22) $\times$ 2, consistent with 32% mosaicism for monosomy X. She was referred for genetic counseling at 19 weeks of gestation. Prenatal ultrasound findings and parental karyotypes were normal. Repeat amniocentesis at 29 weeks of gestation revealed a karyotype of 45,X[36]/47,XXX[4] (Fig. 1) in cultured amniocytes. Simultaneous molecular analysis on uncultured amniocytes revealed the result of arr (1–22) $\times$ 2, Y $\times$ 0 by aCGH with no genomic imbalance, and 15% (15/100 cells) mosaicism for disomy X, 61% (61/100 cells) mosaicism for monosomy X and 24% (24/100 cells) mosaicism for triple X by interphase fluorescence *in situ* hybridization (FISH) analysis. The pregnancy was encouraged to continue and at 37 weeks of gestation, a 2834-g phenotypically normal female baby was delivered. The karyotypes of cord blood, umbilical cord and placenta were 45,X[33]/47,XXX[7], 45,X[30]/47,XXX[10] and 47,XXX[38]/45,X[2], respectively. When follow-up at age three months, the neonate was normal in development. FISH analysis on 99 buccal mucosal cells showed 49% (48/99 cells) mosaicism for monosomy X, 8% (8/99 cells) mosaicism for triple X and 43% (42/99 cells) mosaicism for disomy X (Fig. 2). Peripheral blood had a karyotype of 45,X[38]/47,XXX[2]. When follow-up at age nine months, the neonate was normal in development. FISH analysis on 102 buccal mucosal cells showed 11% (11/102 cells) mosaicism for monosomy X, 12% (12/102 cells) mosaicism for triple X and 77% (79/102 cells) mosaicism for disomy X. Peripheral blood had a karyotype of 45,X[30]/47,XXX[10].

Discussion

Genetic counseling of 45,X/47,XXX at amniocentesis remains difficulty because there is no normal euploid cell line, and the parents may make the decision to terminate the pregnancy because over-emphasis of the associated Turner syndrome and triple X syndrome by genetic counselors during genetic counseling. The peculiar aspect of the present case is the perinatal detection of disomy X cells in case of 45,X/47,XXX by FISH, and our case provides evidence that 45,X/47,XXX at amniocentesis may be in fact 45,X/47,XXX/46,XX, which will make genetic counseling different. In the present case, at the first amniocentesis at 17 weeks of gestation, the cultured amniocytes had a karyotype of 45,X[22]/47,XXX[10], consistent with 68.75% mosaicism for 45,X and 31.25% mosaicism for triple X. Simultaneous aCGH analysis on the DNA extracted from uncultured amniocytes revealed 32% mosaicism for 45,X. However, at the repeat amniocentesis at 29 weeks of gestation, FISH analysis on uncultured amniocytes revealed 61% mosaicism for 45,X, 24% mosaicism for triple X and 15% mosaicism for disomy X. Nonetheless, the karyotype of cultured amniocytes was 45,X[36]/47,XXX[4], consistent with 90% mosaicism for 45,X and 10% mosaicism for 47,XXX. At birth, the

Fig. 2. Interphase fluorescence *in situ* hybridization analysis on buccal mucosal cells using bacterial artificial chromosome (BAC) probes of RP11-383I22 [Xp22.31; fluorescein isothiocyanate (FITC), spectrum green] and RP11-943J20 (Xq11.1-q11.2; Texas Red, spectrum red) shows (A) a disomy X cell with two red signals and two green signals, (B) a monosomy X cell with one red signal and one green signal, and (C) a triple X cell with three red signals and three green signals.

cord blood had a karyotype of 45,X[33]/47,XXX[7], consistent with 82.5% mosaicism for 45,X and 17.5% mosaicism for. 47,XXX. At age three months, the peripheral blood had a karyotype of 45,X[38]/47,XXX[2], consistent with 95% mosaicism for 45,X and 5% mosaicism for 47,XXX. However, interphase FISH analysis on buccal mucosal cells revealed 49% mosaicism for 45,X, 8% mosaicism for triple X and 43% mosaicism for disomy X. At age nine months, the peripheral blood had a karyotype of 45,X[30]/47,XXX[10], consistent with 75% mosaicism for 45,X and 25% mosaicism for 47,XXX. However, interphase FISH analysis on buccal mucosal cells revealed 11% mosaicism for 45,X, 12% mosaicism for triple X and 77% mosaicism for disomy X.

The present case provides evidence that 45,X/47,XXX at amniocentesis may be in fact 45,X/47,XXX/46,XX with presence of euploid disomy X cell line undetected by conventional cytogenetic analysis. In the present case, the euploid disomy X cell line perinatally increased in the mosaic level from 15% at 29 weeks of gestation to 43% at age three months and to 77% at age nine months by FISH, and the monosomy X cell line perinatally decreased in the mosaic level from 61% at 29 weeks of gestation to 49% at age three months and to 11% at age nine months by FISH.

In summary, we present perinatal detection of disomy X cell line by FISH in a pregnancy with 45,X/47,XXX at amniocentesis, cytogenetic discrepancy in various tissues and a favorable outcome. 45,X/47,XXX at amniocentesis may detect disomy X cell line by FISH analysis and can be associated with postnatal progressive decrease of the aneuploid cell lines, increase of the disomy X cell line and a favorable outcome.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

Acknowledgements

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Case Report

Low-level mosaic trisomy 21 at amniocentesis in a pregnancy associated with cytogenetic discrepancy between cultured amniocytes and uncultured amniocytes, perinatal progressive decrease of the aneuploid cell line and a favorable fetal outcome

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ABSTRACT

Objective: We present low-level mosaic trisomy 21 at amniocentesis in a pregnancy with a favorable fetal outcome.

Case report: A 34-year-old, primigravid woman underwent amniocentesis at 17 weeks of gestation because of advanced maternal age. Amniocentesis revealed a karyotype of 47,XY,+21 [7]/46,XY [33]. At 23 weeks of gestation, repeat amniocentesis revealed a karyotype of 47,XY,+21 [4]/46,XY [22], and cord blood sampling revealed the karyotype of 47,XY,+21 [5]/46,XY [35]. The parental karyotypes were normal. Quantitative fluorescent polymerase chain reaction (QF-PCR) analysis on uncultured amniocytes and parental bloods excluded UPD 21, array comparative genomic hybridization (aCGH) analysis on uncultured amniocytes revealed the result of arr 21q11.2q22.3 × 2.3, consistent with 30% mosaicism for trisomy 21. Interphase fluorescence *in situ* hybridization (FISH) analysis on uncultured amniocytes revealed 43.8% (35/80 cells) mosaicism for trisomy 21. The woman was advised to continue the pregnancy, and a phenotypically normal 3,340-g male baby was delivered at 39 weeks of gestation. The cord blood had a karyotypes of 46,XY (40/40 cells). QF-PCR on placenta showed mosaic trisomy 21. When follow-up at age three months, the neonate was normal in phenotype and development. FISH analysis on buccal mucosal cells showed 9% (10/101 cells) mosaicism for trisomy 21, compared with 0% (0/100 cells) in the normal control.

Conclusion: Low-level mosaic trisomy 21 at amniocentesis can be associated with cytogenetic discrepancy between cultured amniocytes and uncultured amniocytes, perinatal progressive decrease of the aneuploid cell line and a favorable fetal outcome.

Introduction

We previously reported prenatal diagnosis of low-level mosaic trisomy 21 by amniocentesis in seven consecutive cases with favorable fetal outcomes of which two cases was associated with uniparental disomy (UPD) 21 [1–8]. Here, we present an additional case. Our case adds to the list of mosaic trisomy 21 at amniocentesis with favorable fetal outcomes. The information provided in this case along with our previous reports is very useful for genetic counselors, obstetricians and the parents who have very advanced maternal age, who have undergone difficult assisted reproductive

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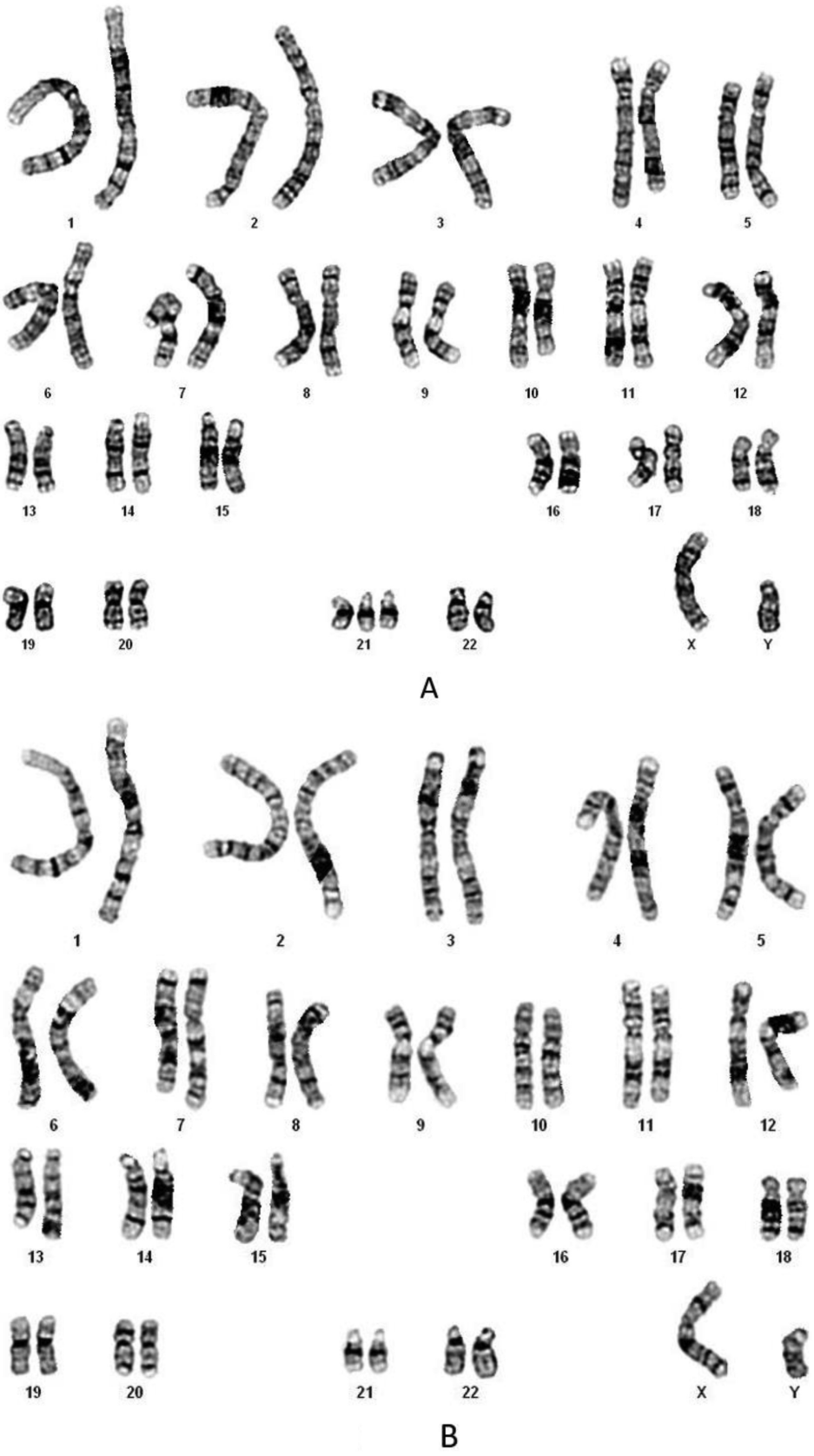

Fig. 1. (A) A karyotype of 47,XY,+21 and (B) a karyotype of 46,XY.

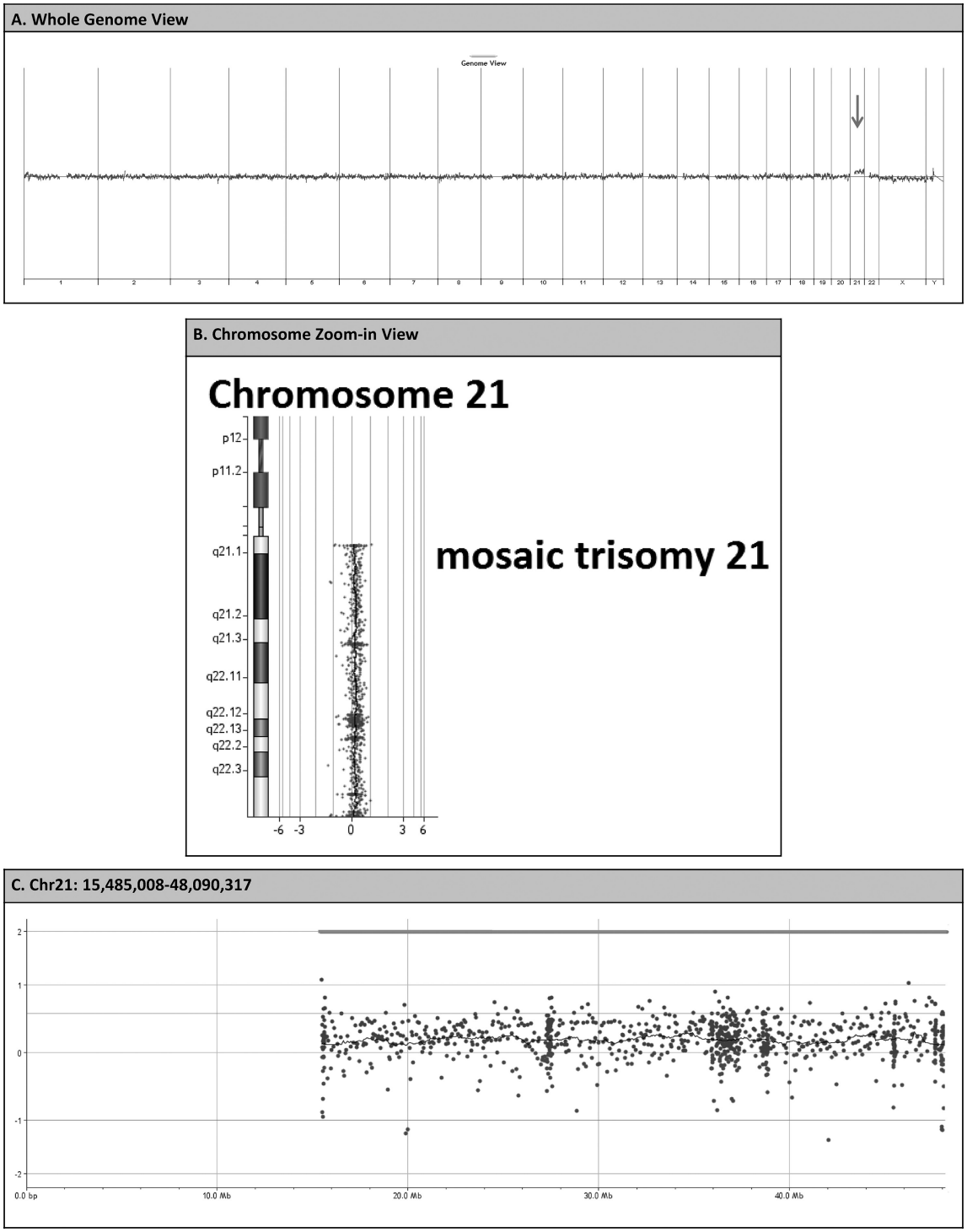

Fig. 2. Array comparative genomic hybridization (aCGH) analysis by SurePrint G3 Unrestricted CGH ISCA v2, 8 × 60K (Agilent Technologies, Santa Clara, CA, USA) on the DNA extracted from uncultured amniocytes shows the result of arr [GRCh37 (hg19)] 21q11.2q22.3 (15,485,008–48,090,317) × 2.3 consistent with 30% ($\log_2$ ratio = 0.2) mosaicism for trisomy 21.

technology and who wish to keep the babies under such a circumstance.

Case Report

A 34-year-old, primigravid woman underwent amniocentesis at 17 weeks of gestation because of advanced maternal age. Amniocentesis revealed a karyotype of 47,XY,+21 [7]/46,XY [33]. At 23 weeks of gestation, repeat amniocentesis revealed a karyotype of 47,XY,+21 [4]/46,XY [22] (Fig. 1), and cord blood sampling revealed the karyotype of 47,XY,+21 [5]/46,XY [35]. The parental karyotypes were normal. Quantitative fluorescent polymerase chain reaction (QF-PCR) analysis on uncultured amniocytes and parental bloods excluded UPD 21, array comparative genomic hybridization (aCGH) analysis on uncultured amniocytes revealed the result of arr 21q11.2q22.3 × 2.3 (Fig. 2), consistent with 30% mosaicism for trisomy 21. Interphase fluorescence *in situ* hybridization (FISH) analysis on uncultured amniocytes revealed 43.8% (35/80 cells) mosaicism for trisomy 21. The woman was advised to continue the pregnancy, and a phenotypically normal 3,340-g male baby was delivered at 39 weeks of gestation. The cord blood had a karyotypes of 46,XY (40/40 cells). QF-PCR on placenta showed mosaic trisomy 21. When follow-up at age three months, the neonate was normal in phenotype and development. FISH analysis on buccal mucosal cells showed 9% (10/101 cells) mosaicism for trisomy 21 (Fig. 3), compared with 0% (0/100 cells) in the normal control.

Discussion

In the present case, at the first amniocentesis at 17 weeks of gestation, the cultured amniocytes had the karyotype of 47,XY,+21 [7]/46,XY [33], consistent with 17.5% mosaicism for trisomy 21. At the repeat amniocentesis at 23 weeks of gestation, the cultured amniocytes had the karyotype of 47,XY,+21 [4]/46,XY [22], consistent with 15.3% mosaicism for trisomy 21, and the cord blood sampling revealed the karyotype of 47,XY,+21 [5]/46,XY [25], consistent with 12.5% mosaicism for trisomy 21. aCGH on the DNA extracted from uncultured amniocytes revealed 30% mosaicism for trisomy 21, and interphase FISH on uncultured amniocytes revealed 43.8% (35/80 cells) mosaicism for trisomy 21. At birth, the karyotype of the cord blood was 46,XY. At age three months, the buccal mucosal cells had 9% (10/101 cells) mosaic trisomy 21.

The present case provides evidence for perinatal progressive decrease of the trisomy 21 cell line and cytogenetic discrepancy between cultured amniocytes and uncultured amniocytes in case of mosaic trisomy 21 at amniocentesis. For instance, in the cord blood analysis, there was 12.5% mosaicism for trisomy 21 at 23 weeks of gestation in comparison with 0% mosaicism for trisomy 21 at birth at 39 weeks of gestation. In the present case, the mosaic trisomy 21 levels in uncultured amniocytes detected by aCGH (30%) and interphase FISH (43.8%) were significantly higher than that acquired by conventional cytogenetic analysis (15.3%). Therefore, we suggest that during repeat amniocentesis for further investigation of mosaic trisomy 21 at amniocentesis, molecular study of uncultured amniocytes alone is not adequate and should include conventional cytogenetic analysis. It is likely that many dead triosmic cells in the amniotic fluid may result in overestimation of the real mosaic level and lead to a misleading interpretation resulting in termination of the pregnancy.

In summary, we present low-level mosaic trisomy 21 at amniocentesis in a pregnancy with a favorable fetal outcome. Low-level mosaic trisomy 21 at amniocentesis can be associated with cytogenetic discrepancy between cultured amniocytes and uncultured amniocytes, perinatal progressive decrease of the aneuploid cell line and a favorable fetal outcome.

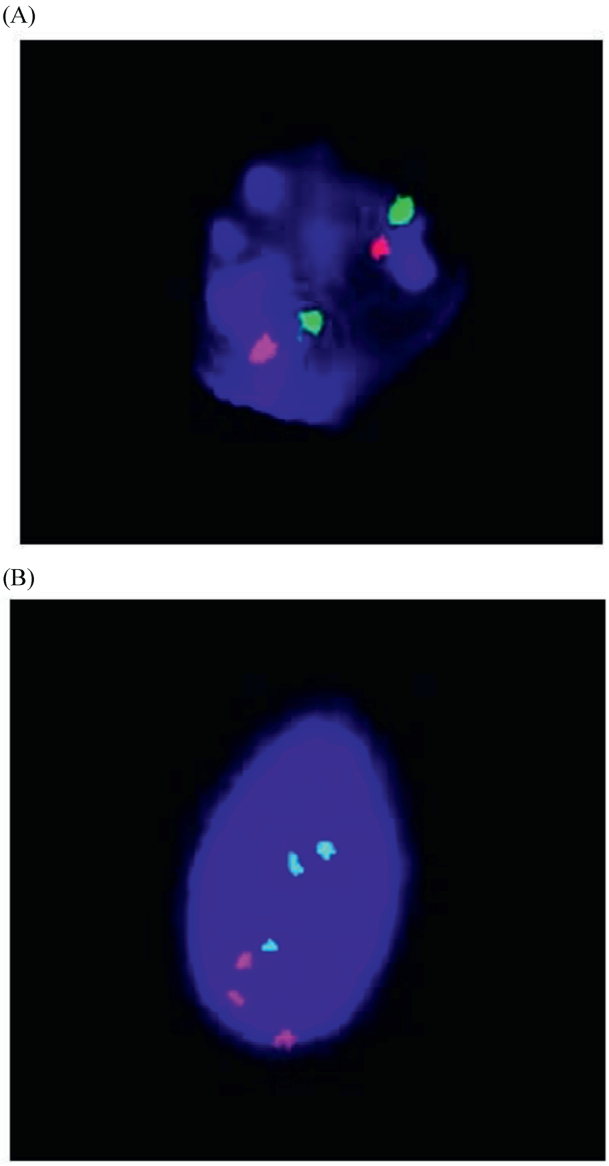

Fig. 3. Interphase fluorescence *in situ* hybridization (FISH) analysis on buccal mucosal cells using bacterial artificial chromosome (BAC) probes of RP11-138O15 [21p11.2; fluorescein isothiocyanate (FITC), spectrum green] and RP11-1115G12 (21q22.3; Texas Red, spectrum red) shows (A) a disomy 21 cell with two red signals and two green signals and (B) a trisomy 21 cell with three red signals and three green signals.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

Acknowledgements

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Case Report

Coincidental spontaneous perforation of the small intestine following operative hysteroscopy: A case report

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ABSTRACT

Objective: Operative hysteroscopy is a common gynecologic procedure, but it carries the risk of complications. Spontaneous small intestine perforation is rare and fatal, especially in young adults. We present a spontaneous small intestine perforation after operative hysteroscopy with mimicking sign of uterine perforation after operation hysteroscopy.

Case report: A 30-year-old nulligravida woman underwent Truclear® hysteroscopic polypectomy in the morning in LMD. She suffered from upper abdominal pain in the afternoon. Subsequently, progressive abdominal distention and imminent shock occurred the next morning. Initially, it was supposed to be a case of uterine rupture with internal bleeding. She was transferred to the emergency department of our hospital. Complete biochemistry data and abdominal CT were performed. The CT revealed pneumoperitoneum and ascites. Emergent laparoscopy was arranged. The abdominal cavity was full of intestinal fluid and the myomatous uterus was intact. The surgeon performed a laparotomy, two sites of spontaneous perforation of the small intestine were detected. The patient underwent laparotomic segmental resection and anastomosis and was discharged 14 days after surgery without incident.

Conclusions: The risk of uterine perforation during hysteroscopy is up to 1.6%. The use of non-thermal intrauterine morcellator device (Truclear®) has been shown to significantly reduce the risk of perforation and thermal injury. As this case highlights, we suspected the possibility of uterine perforation immediately after hysteroscopic surgery. However, it happened to be rare spontaneous perforation of small bowel. The patient recovered well after timely transfer and management. Hysteroscopy is a very common procedure in gynecologic clinics, but even relatively safe intrauterine morcellator devices carry risk of complications. As a healthcare provider, we should beware of any comorbidity, for sometimes it would be catastrophic.

Introduction

Hysteroscopic surgery is a commonly performed procedure due to its wide range of indications. In addition to office hysteroscopy, operative hysteroscopy presents a minimally invasive alternative for patients, obviating the need for profound anesthesia or hospitalization, thereby regarded as a cost-effective surgical intervention. However, despite its benefits, hysteroscopic surgery is also accompanied by potential risks, including complications related to anesthesia, infections, and the procedure itself [1]. Truclear®, an intrauterine morcellator device, received FDA approval in 2005 and has gained popularity in Taiwan in recent years [2]. It is associated with a significant reduction in surgical time and associated risks, without causing thermal damage, and is recommended for women with a desire to preserve fertility [3].

Nontraumatic intestinal perforation is a rare event associated with high mortality rates and often challenging to diagnose preoperatively, especially in healthy young adults [4]. Although the clinical symptoms of acute abdomen or peritonitis and the presence of pneumoperitoneum on CT imaging may aid in diagnosis, surgical intervention is often necessary to accurately identify the location of the perforation and repair it.

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Here we present the case where it was accidently found that a spontaneous perforation of the small intestine occurred after hysteroscopic polypectomy.

Case presentation

A 30-year-old nulligravida woman was found to have an endometrial polyp during infertility surveillance. She underwent hysteroscopic polypectomy with Truclear®, an intrauterine morcellator device, in an infertility clinic. The procedure was performed under intravenous general anesthesia and transient bradycardia was observed during surgery.

On the same afternoon of surgery, the patient reported epigastric pain that was relieved with medication. However, the following morning the patient complained of severe abdominal pain and was unable to lie supine or tolerate oral intake. On physical examination, the physician noted significant rebound pain and suspected peritonitis, prompting a transfer to our emergency department. In addition to tachycardia (heart rate of 125), the patient's blood pressure and body temperature were within normal ranges.

Laboratory data showed an elevated level of C-reactive protein (171 mg/L), but there was no anemia or leukocytosis. Non-contrast CT showed the presence of pneumoperitoneum and ascites (Fig. 1).

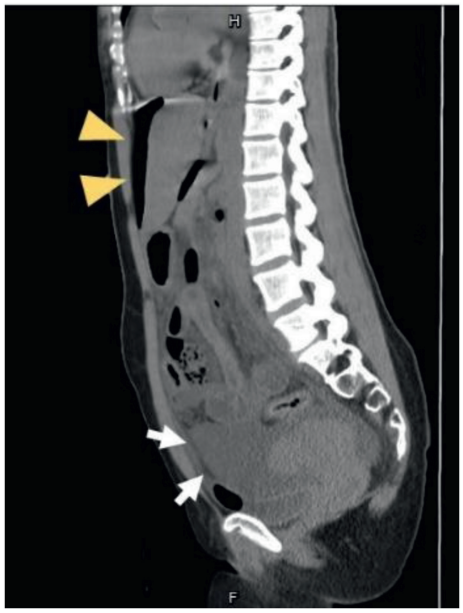

Fig. 1. Non-contrast CT scan of abdomen demonstrated pneumoperitoneum and massive ascites.

Due to a high suspicion of hollow organ perforation, the patient underwent laparoscopic examination.

During surgery, in addition to the presence of massive yellowish-colored turbid ascites, the omentum and bowel exhibited signs of inflammation and adhesions. Although multiple leiomyomas were identified on the uterus, no apparent lesions were detected (Fig. 2). Due to severe adhesion, the operation was converted to laparotomy. Following adhesiolysis, two perforations in the small intestine were identified, located 90 and 40 cm from the ileocecal valve, respectively (Fig. 3). The patient underwent segmental resection and anastomosis.

The patient reported experiencing severe abdominal pain after consuming a beverage two days before her hysteroscopic surgery. She also noticed a decrease in appetite since then, but denied any fever or other obvious discomfort. The pathology report indicated perforation and peritonitis of the jejunum, with ulcerated mucosa, necrosis, and inflammatory infiltrate and granulation tissue observed at the site (Fig. 4). The patient was discharged 14 days after surgery without complications.

Discussion

The annual prevalence of spontaneous perforation of the small intestine is estimated to be 1 in 350,000, with a mortality rate ranging from 8.5 to 47% [4,5]. Bowel perforation indicates the complete disruption of the bowel wall, leading to the release of bowel contents. It is primarily caused by inflammatory changes or disorders that weaken the tissue [6]. Inflammatory bowel disease, autoimmune disorders, malignancy, infections, vascular conditions, and iatrogenic factors are commonly associated with small bowel ulceration and related stenosis. Nonsteroidal anti-inflammatory drugs (NSAIDs) enteropathy is widely recognized as an important cause of intestinal ulcers [7,8]. While only 15% of nonspecific small bowel ulcers are found in the jejunum, 78% of ulcer related perforations occur in this region [5]. Gastrointestinal symptoms do not always correlate with the presence of a small intestinal ulcer [9]. In most cases, the exact diagnosis remains unknown preoperatively.

One of the complications of hysteroscopy is uterine perforation. Iatrogenic uterine perforation is rare, but can be fatal. The risk of uterine perforation during a gynecological procedure ranges from 0.1% to 4% and estimated to be up to 1.6% during operative hysteroscopy, with a higher risk during synechiolysis [10,11]. It can lead to sepsis, hemorrhage, adverse outcomes outcome in reproduction and obstetrics, or even death. Thermal injuries to the ureter or intestine can be difficult to diagnose, particularly if they occur indirectly in the bowel that adhered to the uterus during resectoscopic surgery [12].

The intrauterine morcellator device was originally developed from orthopedic endoscopy. It utilizes a reciprocating blade mechanism to morcellate the protruding lesions. The instrument

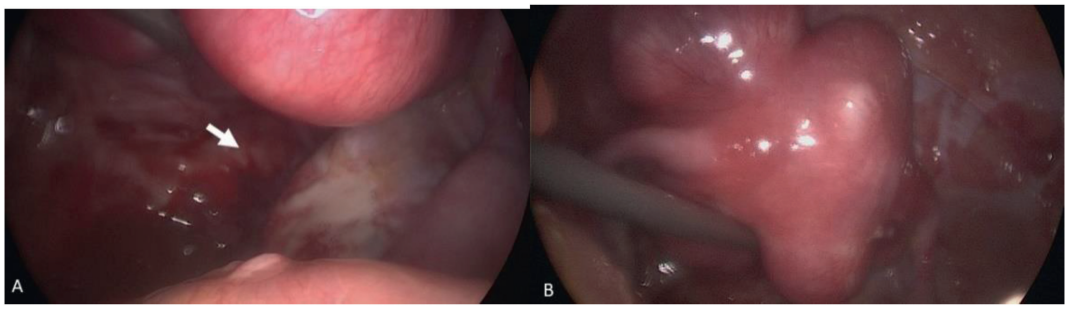

Fig. 2. Laparoscopic examination (2a)Absence of bleeding in cul-de-sac (2b)Multiple leiomyomas without defect on uterus.

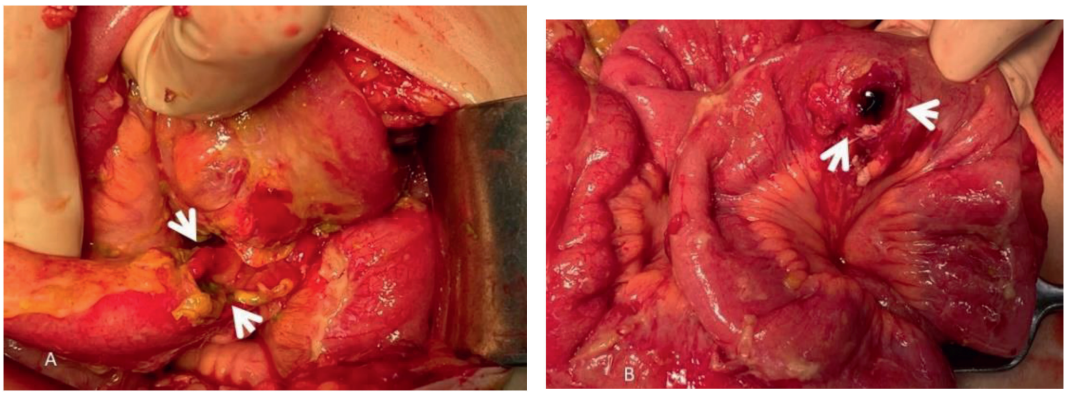

Fig. 3. Two sites of small intestine perforations.

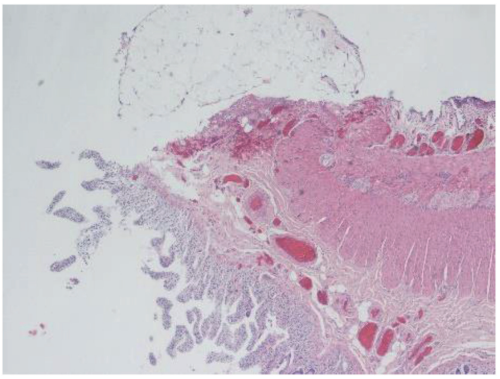

Fig. 4. Microscopic section of small intestine show ulcerated intestinal mucosa with necrosis and granulation tissue.

operates independently of the electrocautery system, with the reciprocating blade safely concealed within a blunt instrument. This design mitigates the risk of excessive tissue excision or damage to normal tissue. Additionally, the device incorporates simultaneous aspiration of the excised tissue during the morcellation process, resulting in enhanced visualization and a clearer operative field during surgery [13]. Consequently, apart from effectively reducing surgical time and minimizing the risk of fluid overload, the nonthermal nature of the shaver reduces the likelihood of perforation and virtually eliminates the risk of thermal injury [14]. The reported complication rates for Truclear® use in polypectomy were 0.02% among inpatients and 1.6% in office settings [15].

The patient underwent a hysteroscopic polypectomy using a nonthermal intrauterine morcellator device (Truclear®) without experiencing uterine perforation, as confirmed by both laparoscopic and laparotomic inspections. The patient experienced transient bradycardia, progressive abdominal pain, and abdominal distention, but there were no of signs of hemorrhage and associated anemia. Preoperatively, there were no discernible indicators predicting bowel perforation. Notably, the patient reported epigastric pain following the consumption of a beverage two days prior to the surgery. In the present case, the pathological report revealed the presence of ulcers, due to the absence of iatrogenic effect, the risk factors for her might be inflammatory bowel disease, autoimmune disorders, infections, vascular conditions. And we should consider the common cause of nonsteroidal anti-inflammatory drugs (NSAIDs) enteropathy. Hysteroscopy is a prevalent procedure in the field of gynecology. Even the relatively safe intrauterine morcellator device carries a potential risk of complications. This case is presented to highlight the importance of recognizing unusual but severe clinical conditions in addition to the expected complications of hysteroscopic surgery.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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Case Report

Safe delivery planning of patients with moyamoya disease in pregnancy: Case series of a single center

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ABSTRACT

Objective: Moyamoya disease (MMD) is a rare cerebral vascular disease and there is limited clinical experience for pregnant women. Cerebrovascular condition might deteriorated during pregnancy. Management and mode of delivery is challenging for obstetrics specialist.
Case report: Three cases of parturients with moyamoya disease delivered in National Taiwan University Hospital are presented. All were previously diagnosed and one had stroke incidence before current pregnancy course. Two delivered with Cesarean section and one with vaginal delivery, and all delivered at term without maternal or neonatal complication.
Conclusion: Although delivery method of parturients with MMD has been debating, vaginal delivery may be suitable for certain cases under adequate monitoring and case selection.

Introduction

MMD is a rare occlusive cerebrovascular disease, while its etiology remain unknown [1]. The character of the disease is narrowing and occlusion of internal carotid artery, with distinct collateral bypass vessels extending from skull base to middle cerebral arteries (MCAs), and particular involvement of the arteries of the circle of Willis [2]. The symptoms vary widely from mild transient ischemic attacks to severe intracranial hemorrhage, seizures, coma and death [3].

MMD mostly affects children and young adults with a female predominance. In pregnancy, the risk of ischemia and hemorrhage might be increased during pregnancy due to vascular dynamic changes. Vascular dilatation with probably weakness of structure is caused by estrogen and progesterone. Arterial occlusion happened during the second and third trimester of pregnancy or the first week postpartum [4]. For women who diagnosed MMD during pregnancy, the operation choice is similar comparing with normal population. However, their efficacy in preventing morbidity and mortality has not been proven, and there is no evidence that bypass surgery before cesarean section is beneficial to clinical outcomes if blood pressure and ventilation were properly managed [5].

The most concerned issue is the mode of delivery. Cesarean section is recommended to avoid hypertension caused by labor in the second stage of vaginal delivery and ischemia caused by hyperventilation, while vaginal delivery with assisted tools and epidural anesthesia may be used to reduce stress [2]. According to a nationwide survey in Japan, the cesarean section rate was 76.3% among women with moyamoya disease, and the rate of vaginal delivery with and without epidural anesthesia was 18.6% and 5.1%, respectively [6]. Cesarean section was used to be considered the safer way, but Takahashi et al. found out similar outcomes for both delivery methods in two recent nationwide surveys in Japan [6].

We reviewed three pregnant cases with MMD delivered in our hospital, and discussed their obstetric management and delivery methods in details respectively.

Case presentation

Case 1

A 30-year-old primigravida Asian was initially presented with dizziness and right hand weakness at the age of 21 years and her magnetic resonance angiography (MRA) was diagnostic for MMD. The patient felt dizziness and general weakness 2 years laterthus she received extracranial-intracranial (EC-IC) bypass combining left encephalo-pericranio-synangiosi (EPS) with encephalo-duro-

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Table 1
Operation condition of patients with Moyamoya disease, prognosis and delivery method in different reports.

Reference	Total pregnancies (case N)	Diagnosis before pregnancy, case N (%)	Operation before pregnancy, N (%)	Bypass	Indirect bypass	Direct bypass	Neurological event during pregnancy	Newly diagnosed peripartum	C/S[a], N (%)	VD[b], N (%)	Complications
Takahashi [6]	64 (64)	59 (92.2)	34 (53.1)	34 (53.1)	N/A	N/A	8 (12.5)	5 (7.8)	50 (78.1)	14 (21.9)	
Jung [14]	28 (22)	20 (90.9)	6 (27.3)	4 (18.2)	4 (18.2)	0 (0)	5 (17.9)	2 (9.1)	25 (89.3)	2 (7.1)	
Tanaka [3]	27 (19)	14 (79)	14 (79)	19 (100)	N/A	N/A	10 (37)	5 (26.3)	7 (25.9)	20 (74.1)	
Sato [15]	14 (12)	12 (100)	9 (75)	9 (75)	N/A	N/A	2 (14.3)	0 (0)	4 (28.6)	10 (71.4)	
Fujimura [16]	6 (6)	4 (66.7)	N/A	N/A	N/A	2 (33.3)	2 (33.3)	2 (33.3)	6 (100)	0 (0)	2 newly diagnosed cases developed stroke, one during pregnancy, one postpartum
Our series	3 (3)	3 (100)	3 (100)	3 (100)	3 (100)	0 (0)	0 (0)	0 (0)	2 (67)	1 (33)	No

[a] C/S: Cesarean section.
[b] VD: vaginal delivery.

arterio-synangiosis (EDAS) at the age of 27. The patient recovered well without any residual neurological deficits.

After she got pregnant, a multidisciplinary team consisting of a consultant obstetrician, a neurologist and an anesthesiologist were involved and the patient was followed up at the clinic closely. Prenatal exam were uneventful. Due to relative patent blood vessels after surgery, vaginal delivery was not contraindicated. The team took this decision after detailed discussions with the patient. She underwent low-vacuum assisted vaginal delivery under epidural anesthesia at 38 + 6 weeks of gestational age. The postpartum course was smooth except one episode of transient dizziness was noted and brain MRI done after one week postpartum reported no active lesion. The mother was discharged under stable conditions with neurologist and obstetrics follow up.

Case 2

A 26-year-old Asian primigravida was diagnosed with MMD at the age of 17 years.

Intermittent headache and left hand weakness were noted for over one year in her seventeenth. Cerebral angiography showed occluded right distal internal carotid artery, proximal middle and anterior cerebral arteries with prominent collaterals. The image of brain MRI was compatible with MMD. Right side temporal encephalo-duro-arterio-synangiosis (EDAS), frontal duropexy and left side parieto-temporal EDAS were performed at the age of 20.

During her prenatal exam, no medical complication of the mother was found in the serial follow-up, only one episode of syncope was noted at 20 weeks of gestational age. Though her brain perfusion was decent at present, a cesarean section was recommended in order to avoid a potential risk of intracranial hemorrhage due to the force of bearing-down during parturition. The patient received a scheduled Cesarean section with combined spinal epidural anesthesia at 38 weeks, and the postpartum course was uneventful without complication.

Case 3

A 43-year-old Asian primigravida was diagnosed with MMD at the age of forties presenting with dizziness and right hemiparesis.

MRI and angiography showed multiple infarction and left distal internal carotid artery narrowing and occlusion. Indirect revascularization with encephalomyosynangiosis (EMS) was performed without complications.

This time she got pregnant from donated-oocyte at a private infertility center. Cervical cerclage was performed at 12 weeks of gestational age due to short cervical length. Gestational diabetes was diagnosed and Pshe was admitted several times for tocolysis due to preterm uterine contraction.

In order to avoid a potential risk of intracranial hemorrhage, she received Cesarean section with combined spinal epidural anesthesia at 37 weeks without further complications.

Discussion

For patients with moyamoya disease who wants to be pregnant, preconception evaluation of cerebral circulation status is important. During antepartum period, strict blood pressure control to prevent intracranial hemorrhage is important. Infarction Intracranial hemorrhage could be prevented by avoiding dehydration, especially at or beyond 24 weeks, while cerebral infarction tends to occur postpartum.

During pregnancy, the blood pressure of MMD patients should be strictly controlled, even puerperium. All of our cases were diagnosed before their pregnancy. For patients who developed neurological symptoms during pregnancy, MRA is still the primary method of diagnosis of MMD [4]. There was no definite treatment option between surgery or conservative management for pregnant women. Recently, an European cohort and a Korean cohort showed that surgical treatment is beneficial to avoid cerebral ischemia during pregnancy and delivery [7,8]. The bypass surgery is also proven to reduce intracerebral hemorrhage recurrence with low complication rates [8,9]. Thus bypass surgery should be considered for patients before pregnancy.

There are several revascularization methods for MMD, including direct and indirect bypass. All of the three cases received indirect bypass prenatally, and the operation methods were slightly different. However, there were no consensus between different operation methods and obstetric outcomes. Reviewing previous literature, different operation methods and their pregnancy outcomes were listed in Table 1.

In patients diagnosed with MMD before pregnancy, the risk of hemorrhage was not increased during pregnancy. Cerebrovascular accident risk has not been increased during the pregnancy but increased in the first 6 weeks postpartum and might be accounted for 25% of perinatal cerebral hemorrhage [10].

The delivery method has always been controversial. There is no strong evidence of routine cesarean delivery in this population. Cesarean delivery has been thought to be the safer way due to avoid straining during labor, but activation of coagulation system should also be taken into consideration. The hypercoagulable state is at its highest during delivery and immediately postpartum, which is also more activated after operation, leading to more incidence of postpartum cerebral infarction. The amount of blood loss during delivery has been reported to be greater with cesarean section than

with vaginal delivery. Thus patients who undergo operation are more likely to experience event [1]. A recent systemic review showed in patients diagnosed prenatally, 64% received cesarean section with mostly good maternal outcomes (95%). Of those diagnosed during pregnancy due to cerebrovascular accident during late pregnancy, mostly (80%) received cesarean section with a 13.6% of maternal mortality [5]. Thus the timing of diagnosis also effects the prognosis of delivery. Owing to above reasons, more caregivers attempted to offer vaginal delivery as a choice for this population.

Considerable number of women with undiagnosed MMD have probably undergone delivery without severe neurological complications, the risk of stroke during delivery does not appear to be high when the disease is stable, regardless of the mode of delivery [3,11]. Therefore, vaginal delivery with epidural anesthesia appears to be favorable and fully feasible for pregnant women with MMD who is stable [1]. Several studies have also proven vaginal delivery with epidural anesthesia has been suggested feasible in adequate cases. A recent retrospective study in Japan also suggested vaginal delivery may be viable in patients with good cerebral circulation [3]. Currently, vaginal delivery is more common in the USA, while 60–70% of patient still underwent cesarean section in Japan [12,13]. Although aiming to avoid unnecessary operation is the current trend, health care practitioners should provide objective statistics and make a thorough discussion with the patients about the benefits and risks of delivery planning.

Declaration of competing interest

There is no conflict of interest.

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Case Report

Amniotic fluid embolism: A case report of good outcome with timely intensive multidisciplinary team involvement

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ABSTRACT

Objective: Amniotic fluid embolism is one of the most serious pregnancy complications. It can cause sudden maternal collapse with high mortality and morbidity. We present a case report regarding the important of prompt decision making and multidisciplinary team work for management of amniotic fluid embolism to yield favorable maternal and neonatal outcome.

Case report: This is a 35-year-old, gravida 2, para 1, woman underwent labor induction at gestational age of 37 + 6 weeks due to elective induction. She had sudden facial cyanosis and shortness of breath right after artificial rupture of membrane. Prompt decision of urgent cesarean section, aggressive and timely massive blood transfusion and multidisciplinary team work had spared patient from extracorporeal membrane oxygenation placement and prolonged hospitalization. A male infant was born with Apgar score 3' -> 5' with estimate body weight of 2958 gm; he was hospitalized for 10 days and no other complications was found at follow up pediatric outpatient clinic.

Conclusion: One of the most dreadful, but rare pregnancy complications is amniotic fluid embolism (AFE). It can cause serious maternal and neonatal morbidity and mortality. Rapid recognition and multidisciplinary team management are essential to maternal and neonatal prognosis.

Introduction

Amniotic fluid embolism (AFE) is a rare but serious complication of pregnancy. High mortality and morbidity are noted [1]. It can cause sudden maternal collapse with classic presentation of hypoxia, hypotension and coagulopathy, or even other variants. Rapid disease progression can cause maternal death within 2 h [2]. Thus, prompt resuscitation should start as early as possible before diagnosis with AFE for better prognosis. In 2021, Society for Maternal-Fetal Medicine (SMFM) updates a sample checklist for management of most conditions of amniotic fluid embolism [3]. Modification of this checklist according to facility-specific circumstances can shortened response time and improve prognosis. We will present a case report of a patient involved suspected amniotic fluid embolism with favorable maternal and neonatal outcome.

Case report

This 35-year-old female was pregnant with 37 + 6 weeks, G2P1, without other medical history. She received regular antepartum care (ANC) at obstetric outpatient clinic department of Changhua Christian Hospital. Her last menstrual period was 2020–07-30; estimated date of delivery was 2021–05-06. During ANC, no obstetric complications were noted. Follow-up fetal ultrasound revealed no anomaly. Estimated fetal weight by sonography was 3 kg with vertex presentation on 2021-04-14. Due to elective induction, she was admitted for labor induction.

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At delivery room, cardiotocography revealed normal fetal heart rate (FHR) with good variability. Uterine contraction was once per 10 min. Per vaginal examination showed closed cervical os with poor effacement. Induction with Dinoprostone 3 mg vaginal tablet was given 10 min after performing epidural anesthesia. After 7 h of induction, her cervical os dilated to 4 cm. Cardiotocography showed FHR were 135–155 beats per minute (bpm) with good variability, and uterine contraction was 4 times per 10 min. Artificial rupture of membrane (ARM) was performed. Thirty-five minutes after ARM, the patient's husband alarmed the nurse that the patient felt dizziness, chest tightness, shortness of breath; meanwhile, FHR was decelerating. The nurse visited the patient immediately, whom GCS was E4M6V5, but her face and lip were cyanosed. Oxygen mask with 10 L per 1 min was given. Her blood pressure decreased to 81/42 mmHg, heart rates was 63 bpm, and SPO2 revealed 96%. Cardiotocography showed FHR decelerated from 130 to 150 bpm to 60 bpm without recovery (Fig. 1). Urgent cesarean section (CS) was arranged by attending physician within 1 min after visiting the patient. Endotracheal tube was inserted in operation room. Decision to incision time was within 10 min. Blood loss was 1000 cc during operation. Pathology of placenta reported 3% of retroplacental hemorrhage. A male fetus was born with Apgar score 3'->5′ and inserted with endotracheal tube due to poor oxygen saturation. Then, he was sent to neonatal ICU for further care. His birth weight was 2958 gm. During operation, uterine atony presented. Uterotonic agents (oxytocin, carbetocin, methylergonovine, misoprostol) were given to improve uterine contraction. Lab data during operation revealed acute anemia (7.9 g/dL), thrombocytopenia (9600 μL), prolonged prothrombin time (16.5sec), prolonged activated-partial thromboplastic time (48.6sec), Prothrombin time I.N.R. (1.43) and hypofibrinogenemia (90.5 mg/dL). Disseminated intravascular coagulation (DIC) was impressed; massive transfusion protocol (MTP) was started immediately. Meanwhile, extracorporeal membrane oxygenation (ECMO) team was on standby. Luckily that she didn't need ECMO after evaluation. After 6 h with blood products resuscitation (cryoprecipitates 40 units, fresh frozen plasma 16 units, packed red blood cell 10 units, and apheresis platelet 1 unit), fibrinogen level was normalized and other lab data were gradually improved. Endotracheal tube was removed 13 h later. She was transferred from ICU to ordinary ward on the 2nd day after surgery. Followed up Cardiac ultrasound reported normal left ventricle (ejection fraction 68.4%) and slight hypokinesia of right ventricular wall without obvious pulmonary hypertension. She was discharged 6 days after surgery with smooth hospital course. The whole clinical course was summarized in Fig. 2.

Discussion

AFE or a more appropriated name as “anaphylactoid syndrome of pregnancy” is a rare and unpredictable, but devastating disease of pregnant women. The incident rate of AFE in United States was 1 in 12,953 deliveries [4] and 1.7 in 100,000 deliveries in the United Kingdom regardless of fatal or nonfatal cases, respectively. UK obstetric surveillance system stated the fatality rate was 19% during 2005–2014 [5]. The other 9 systemic reviews revealed that the fatality rate was 24.8% [6]. Risk factors with strong evidences proposed for AFE are induction of labor by any means, assisted delivery, and CS [7]. Other factors might increase the risk of AFE such as maternal age >35 years old, male fetus, multiple pregnancy, polyhydramnios, eclampsia, uterine rupture, cervical trauma, placenta previa or abruption, and ethnic minority. The clinical diagnostic criteria of AFE proposed by Clark et al.'s [1] comprised of six clinical symptoms (hypotension, cardiac arrest, acute hypoxia, cyanosis, dyspnea, coagulopathy) and timing (onset during labor, CS, dilation and evacuation, within 30 min postpartum). Our patient meets the criteria of hypotension, hypoxia and coagulopathy.

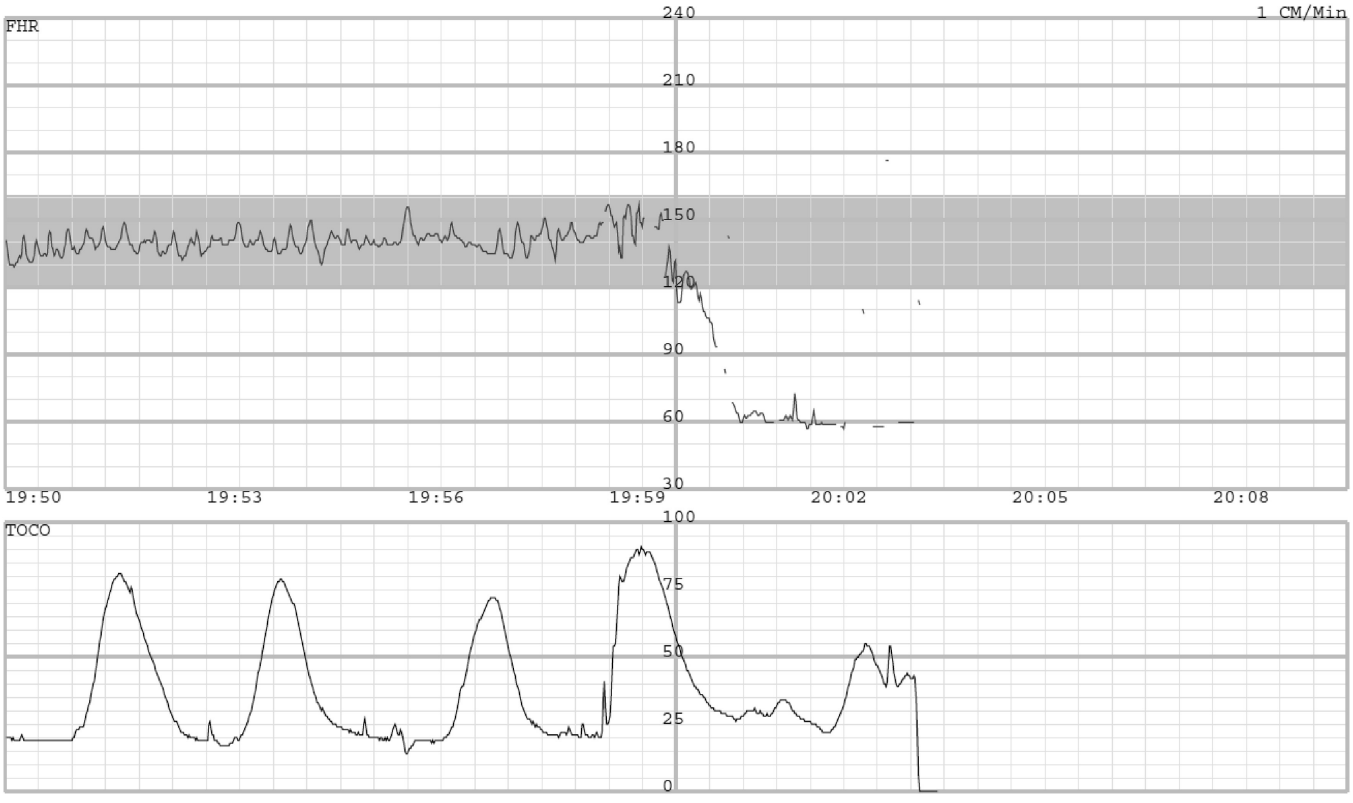

Fig. 1. Fetal heart rates deceleration without recovery.

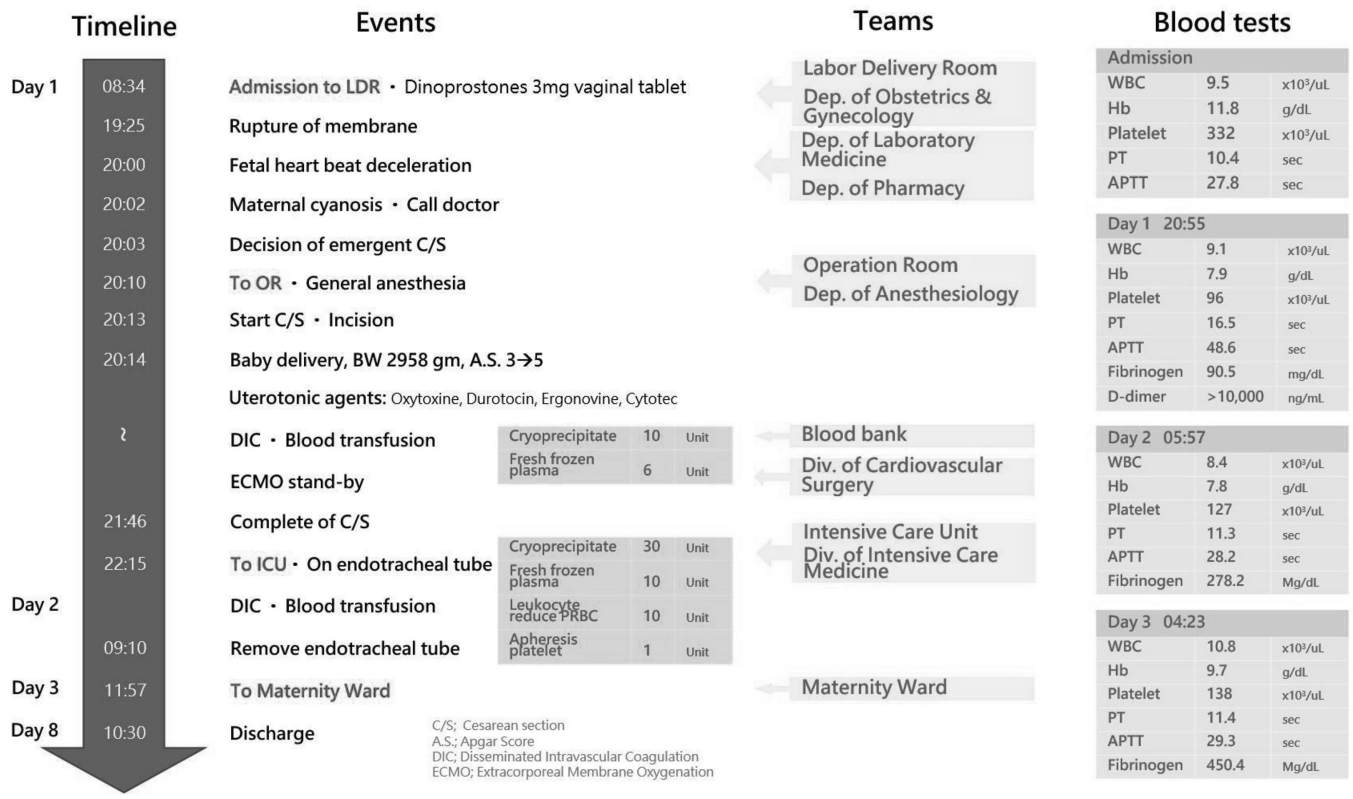

Fig. 2. The timeline, events, and management of the case.

However, the clinical manifestations of AFE are variable [8]. Even the serum markers such as monoclonal antibody TKH-2 or zinc coproporphyrin are proven to be the sensitive methods for diagnosis of AFE [1], these markers won't be immediately available for diagnosis of AFE. In the other hand, tryptase has been suggested to be a more convenient serum marker for diagnosis of AFE. Mast cell degranulation is incited during anaphylactoid reaction; tryptase is also released during mast cell degranulation. Serum tryptase won't be detectable during the first 30 min so the best time to obtain serum tryptase will be between 30 min and 2 h after the onset of anaphylaxis. The peak of serum tryptase is one to 2 h; its half-life is 2 h with fairly stable concentration in serum. Thus, Benson et al. suggest tryptase is a more convenient serum marker for anaphylactoid reaction as compared to histamine, whose half-life is 2 min. Hence, tryptase is proposed to be one of the serum marker of AFE; however, further study is still needed to prove it [9]. Therefore, the diagnosis of AFE currently mainly by exclusion based on clinical presentation; early recognition and prompt managements are important for yield better prognosis. Based on clinical presentations, the other possible differential diagnosis of sudden cardiorespiratory collapse in the laboring or recently delivered woman are thrombotic embolus, septic shock, acute myocardial infarction, peripartum cardiomyopathy, anaphylaxis, placental abruption, transfusion reaction, local anesthetic toxicity [3,10].

According to SMFM check list of initial management of AFE, the most important treatments are focused on adequate oxygenation, circulatory support, correction of coagulopathy and management of pulmonary hypotension. Adequate oxygenation includes prompt endotracheal tube insertion and proper used of diuretics. CPR might perform promptly for circulatory support and consider necessity of resuscitative hysterotomy. In addition, MTP should start as early as possible to correct coagulopathy (packed RBC: fresh frozen plasma: platelet = 1:1:1). The last but not the least, pulmonary vasodilators (nitric oxide, inhaled prostacyclin etc) and improved ventricular contractility with inotropes should be considered [3,10]. Prompt management started once cardiopulmonary compromise presented. In our case, hypoxia was found initially with clinical presentation of facial cyanosis. Prompt endotracheal tube inserted for airway protection and oxygen support are important before the pathophysiological changes of AFE such as bronchospasm and pulmonary vasoconstriction. These hemodynamic changes lead to pulmonary hypertension, right ventricular enlargement, intraventricular septum bow into left ventricle and cause systolic dysfunction. Right ventricle failure happened first; then, left ventricle failure ensued. In one study, 87% of patients presented with cardiovascular collapse or cardiac arrest [5]. Therefore, anesthesiologist is needed in helping with maternal cardiopulmonary and hemodynamic stabilization. Furthermore, ECMO increased survival rate reported in some studies when severe cardiovascular compromise presented. However, the use of anticoagulation during ECMO may worsen the bleeding tendency if the patients had proceeded to disseminated intravascular coagulation (DIC). The delicate balance between pros and cons of ECMO in AFE needed to be carefully examined. Due to the controversy, ECMO is not routinely recommended in the management of AFE [10]. Thus, ECMO wasn't performed in our patient after thorough evaluation by cardiac surgeons. To evaluation of cardiac function after AFE, transesophageal echocardiography is preferred than transthoracic echocardiography (TTE). In studies, post-AFE cardiac morphologies might show right ventricular dilatation, hypokinesis and overload, tricuspid regurgitation, and right atrial enlargement [11]. TTE was arranged in our patient on postoperative day 2. The cardiac echography reported normal wall function with 68.4% ejection fraction and slightly hypokinesia of right ventricle wall motion without obvious pulmonary hypertension. Luckily, our patient was treated in time that there was only mild cardiovascular

compromise. Her cardiac function is still required for long-term follow-up.

80% of patients presented DIC during AFE [11]. Since the risk of coagulopathy was high, aggressive blood products replacement should start simultaneously during operation before lab test results [12]. In our case, MTP was initiated within 10 min after incision. The second round of MTP were adjusted according to lab tests until coagulopathy resolved. Administration of recombinant VIIa (rVIIa) has successfully treated patients with postpartum hemorrhage, preeclampsia, or HELLP variant of preeclampsia. However, the most serious complications associated with rVIIa is thrombosis in major organs. Patients with AFE have elevated level of circulating tissue factor concentration [2]. While tissue factors exposed to rVIIa, excessive diffused thrombosis and multiorgan failure might happen. Therefore, rVIIa should be as last resource for AFE patients whom hemorrhage cannot be stopped by massive component replacement [13] Our patient's coagulopathy had been corrected within 8 h so Recombinant VIIa was not administered.

One of the leading causes of death directly from pregnancy is AFE, which accounts for 5–15% of cases worldwide [2]. Mortality rates exceeding 60% if classical triad symptoms of AFE present [1]. In patients who survive from AFE, 7% has permanent neurological damage, and 17% has other major morbidities without neurological damage [5]. Perinatal mortality rate is between 7 and 38% [1,14]. In women with AFE at or before delivery, the perinatal mortality rate of infant was higher compared with after delivery [5]. Surviving children manifest persistent neurological deficits in a rate of 24–50% [1,14]. In our case, AFE occurred before delivery so FHR decelerated once hypoxia and hypotension presented in patient. The infant was on endotracheal tube since birth and kept for four days; he was discharged in 10 days. Follow-up brain and heart echography of infant revealed normal appearance and functions during hospitalization. Long-term follow up on his neurological development is needed.

AFE is catastrophic emergency with sudden onset and rapid progression. Timely management and multidisciplinary team (MDT) involvement are the key elements to rescue the patient in facing this critical disease. SMFM proposed a checklist for initial management of AFE, which involved a multidisciplinary and rapid-response team [3]. In our case, prompt decision of emergent CS was made when doctor was called to the bedside. The time of decision to incision was within 10 min. The baby was delivered in 1 min since start of incision, and 14 min since FHR decelerated. Blood products transfused promptly during the operation, including complete transfusion of cryoprecipitate 10 units and fresh frozen plasma 6 units in 2 h. A total of cryoprecipitates 40 units, fresh frozen plasma 16 units, packed red blood cell 10 units, and apheresis platelet 1 unit were transfused within 10 h. The involved teams included seven departments (Obstetrics & Gynecology, Anesthesiology, Intensive Care, Cardiovascular Surgery, Pharmacy, Medicine Laboratory, and Blood bank), and four units (Labor Delivery Room, Operation Room, Intensive Care Unit, and Maternity Ward). AFE is a devastating condition with high maternal mortality could occur in first 2 h following the acute event in 56% patient as record by US registry [1]. Therefore, early recognition with prompt resuscitation and MDT involvement are the leading factors to successful maternal and fetal outcome.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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Research Letter

Identification of a familial balanced chromosomal rearrangement in a couple presenting with recurrent pregnancy loss

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Dear Editor

A 35-year-old, gravida 3, para 0, woman was referred for genetic analysis because she had suffered from three fetal losses. During genetic counseling, the woman was noted that she had a younger sister who had also suffered from repeated pregnancy loss, and cytogenetic analysis revealed a balanced reciprocal translocation of t(1; 8) (q44; p22). Cytogenetic analysis of the woman showed that her karyotype was 46,XX,t(1; 8) (q44; p22) (Fig. 1).

In all couples suffering from two or more pregnancy losses, about 5% carry a balanced chromosomal rearrangement in one of the partners which represents a significant increase in the incidence in the general population [1,2]. In a review of 1743 couples with recurrent pregnancy loss, Fryns and Van Buggenhout [1] found that 5.34% had chromosomal rearrangements with autosomal balanced translocations accounting for 2/3 of the cases which is a 30-fold increase compared to the general population. In a review of 440 couples with recurrent pregnancy loss, Sudhir et al. [2] found that 3.41% had chromosomal aberrations, and 60% were balanced reciprocal translocations. In a review of 2324 couples with repeat pregnancy loss, Ozawa et al. [3] found that 4.91% (114 cases) had chromosome abnormalities including reciprocal translocations (74/114 = 64.9%), Robertsonian translocations (23/114 = 20.2%) and inversions (10/114 = 8.8%), and there is an increase odds of 3.6 fold (odds ratio: 3.6, 95% confidence interval: 1.8–7.1) to have subsequent pregnancy loss compared to normal couples who have had repeat pregnancy loss. However, Sugiura-Ogasawara et al. [4] found that no infant with an unbalanced translocation in 29 cases of successful pregnancy following recurrent miscarriage in the groups of 129 couples of parental carries with a structural chromosomal rearrangement. Barber et al. [5] found only four unbalanced rearrangements after referral for prenatal diagnosis because of a balanced parental translocation ascertained for recurrent miscarriages in 406 out of 20,432 parents that had experienced miscarriage. Although the risk for a viable unbalanced chromosomal rearrangement is very low in the previous study, Knibbs et al. [6] suggested that karyotyping couples experiencing recurrent miscarriage really is worth the cost.

In summary, detection of a balanced chromosomal rearrangement in a couple presenting with recurrent pregnancy loss should alert the possibility of familial inheritance and prompt cytogenetic analysis of the family members.

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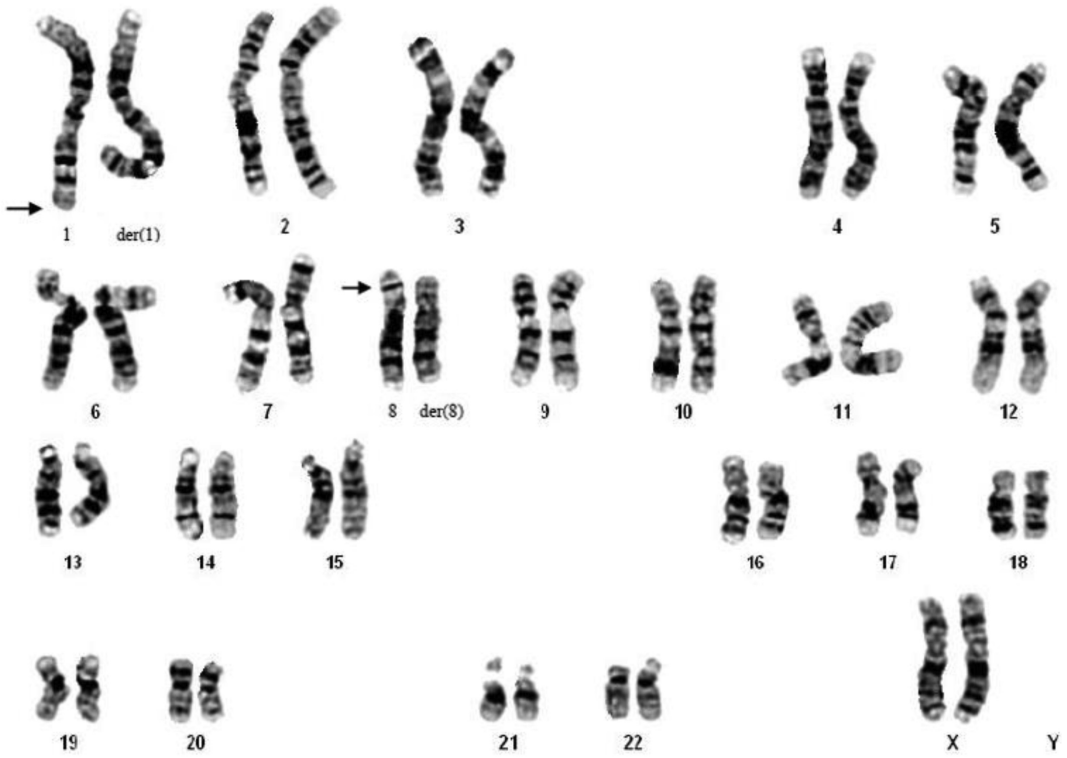

Fig. 1. A karyotype of 46,XX,t(1; 8) (q44; p22). The arrows indicate the breakpoints.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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Research Letter

Low-level mosaic trisomy 6 at amniocentesis can be a culture artefact and associated with a favorable outcome and cytogenetic discrepancy between cultured amniocytes and uncultured amniocytes

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Dear Editor

We previously reported low-level mosaic double trisomy involving trisomy 6 and trisomy 20 (48,XY,+6,+20) at amniocentesis without uniparental disomy (UPD) 6 and UPD 20 in a pregnancy associated with a favorable outcome [1]. Here, we present an additional case of low-level mosaic trisomy 6 associated with a favorable fetal and cytogenetic discrepancy between cultured amniocytes and uncultured amniocytes.

A 31-year-old, gravida 2, para 0, woman underwent amniocentesis at 16 weeks of gestation because of an increased nuchal thickness (NT) of 3.9 mm. Amniocentesis revealed a karyotype of 47,XY,+6[2]/46,XY[18]. Among 20 colonies of cultured amniocytes, two colonies had the karyotype of 47,XY,+6, whereas the rest had the karyotype of 46,XY. Array comparative genomic hybridization (aCGH) analysis on the DNA extracted from uncultured amniocytes revealed the result of arr (1–22) × 2, (X,Y) × 1 with no genomic imbalance. Prenatal ultrasound findings were normal. She was referred for genetic counseling at 18 weeks of gestation, and repeat amniocentesis performed at 22 weeks of gestation revealed a karyotype of 46,XY in 20/20 colonies. The parental karyotypes were normal. At repeat amniocentesis, aCGH analysis on the DNA extracted from uncultured amniocytes revealed the result of arr (1–22) × 2, (X,Y) × 1 and detected no genomic imbalance, and quantitative fluorescent polymerase chain reaction (QF-PCR) analysis on the DNA extracted from the uncultured amniocytes and parental bloods excluded UPD 6. The woman was encouraged to continue the pregnancy, and a healthy 2980-g male baby was delivered with no phenotypic abnormalities. aCGH analysis on the cord blood revealed the result of arr (1–22) × 2, (X,Y) × 1 and detected no genomic imbalance. The neonate was normal in development during postnatal follow-ups at 5½ years. The peripheral blood had a karyotype of 46,XY in 40/40 cells, and fluorescence *in situ* hybridization (FISH) analysis on 50 buccal mucosal cells revealed disomy 6 in all cells.

The present case along with our previous report [1] shows cytogenetic discrepancy between cultured amniocytes and uncultured amniocytes in mosaic trisomy 6 at amniocentesis, *i.e.*, low-level mosaic trisomy 6 in cultured amniocytes, whereas no genomic imbalance in uncultured amniocytes. This finding indicates that low-level mosaic trisomy 6 can be a culture artefact which is also common in mosaic trisomy 20 at amniocentesis.

However, prenatal diagnosis of mosaic trisomy 6 at amniocentesis should raise a suspicion of pathogenic UPD 6 and include a differential diagnosis of UPD 6 by polymorphic DNA marker analysis such as QF-PCR using the DNA extracted from the uncultured amniocytes and parental bloods. Paternal UPD 6 is associated with transient neonatal diabetes mellitus (TNDM) (OMIM 601410) which is characterized by transient neonatal diabetes mellitus and intrauterine growth restriction.

In summary, low-level mosaic trisomy 6 at amniocentesis can be a culture artefact and associated with a favorable fetal outcome and cytogenetic discrepancy between cultured amniocytes and uncultured amniocytes.

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Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

Acknowledgements

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Research Letter

False positive non-invasive prenatal testing (NIPT) for trisomy 21 in vanishing twin syndrome pregnancy: A comparison of the NIPT results performed in different gestations

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Dear Editor,

A 43-year-old woman received non-invasive prenatal testing (NIPT) for aneuploidy at 12 weeks of gestation following *in vitro* fertilization (IVF) and transfer of two embryos affected by early single fetal demise. The NIPT result showed a Z-score of 9.23 (normal: −3.0~3.0) for trisomy 21 (fetal DNA content = 10.8%). She underwent amniocentesis at 17 weeks of gestation, and the karyotype was 46,XX. The parental karyotypes were normal. Polymorphic DNA marker analysis using the DNA extracted from parental bloods and uncultured amniocytes excluded uniparental disomy 21. She received the same NIPT for aneuploidy at 31 weeks of gestation, and the NIPT result showed a Z-score of 1.27 (normal: −3.0~3.0) for trisomy 21 (fetal DNA content = 21.28%). A 3114-g healthy female baby was delivered at 38 weeks of gestation. Cytogenetic analysis of the cord blood, umbilical cord and placenta revealed the karyotype of 46,XX in all sample examined.

The present case represents the occurrence of false positive NIPT for trisomy 21 in vanishing twin syndrome pregnancy conceived by assisted reproductive technology. The present case provides evidence for that in case of vanishing twin syndrome pregnancy with a false positive NIPT for trisomy 21, a repeat NIPT can achieve a negative result in the later gestation. In the present case, the first NIPT performed in the first trimester at 12 week of gestation was positive, but the subsequent NIPT performed in the third trimester at 31 weeks of gestation became negative. Balaguer et al. [1] suggested that the vanishing twin syndrome pregnancy could be included in the cell-free DNA NIPT testing as long as it is applied after the 14th week of pregnancy. In a study of 579 cases of vanishing twin syndrome pregnancy, Zou et al. [2] detected 12 positive NIPT at 11–13 weeks of gestation, and only one true positive was confirmed, giving a PPV (positive predictive rate) of 8%, and among the 536 cases with NIPT negative results, no false-negative result was reported. Zou et al. [2] suggested that NIPT has the potential to be used in prenatal screening for vanishing twin syndrome pregnancies, and for the pregnant women who obtain positive and testing failure results, resampling after 15 weeks of gestation is recommended. In a systematic review of NIPT and vanishing twins, van Eekhout et al. [3] concluded that NIPT can successfully detect common autosomal aneuploidies in pregnancies affected by a vanishing twin but with a higher false positive rate.

Our case demonstrates that false positive NIPT for trisomy 21 can occur in the vanishing twin pregnancy conceived by assisted reproductive technology, and resampling after 15 weeks of gestation can achieve a true negative result.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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Research Letter

Mosaic 46,XX,der(9)t(9;13)(p24;q12)/46,XX at amniocentesis can be a culture artefact and associated with a favorable fetal outcome

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Dear Editor,

We previously reported mosaicism for an aberrant chromosome with a normal cell line at amniocentesis in association with favorable outcomes such as mosaicism for a small supernumerary marker chromosome (sSMC) derived from chromosome 16q (16q11.2-q22.1) [1], mosaicism for an sSMC derived from chromosome 9q (9q13-q21.33) [2], mosaic 46,XX,dup(9)(q22.3q34.1)/46,XX [3], mosaic tetrasomy 18p [4–7], mosaic 46,X,der(X)dup(X)(q22.1q22.2)dup(X) (q25q22.3)/46,XX [8], mosaic 46,XY,der (15)t (6;15) (q25.1;p12)/46,XY [9], mosaic 46,XY, dup (14) (q12q22.3)/46,XY [10], mosaicism for a 15q11.2 microduplication with a normal euploid cell line [11] and mosaicism for a 12p12.1p12.2 microdeletion [12]. Here, we present an additional case with mosaic 46,XX,der(9)t (9;13) (p24;q12)/46,XX in a pregnancy associated with culture artefact and a favorable fetal outcome. The information provided is useful for genetic counselors, obstetricians and the parents who have very advanced maternal age, who have undergone difficult assisted reproductive technology and who wish to keep the babies under such a circumstance.

A 37-year-old, primigravid woman underwent amniocentesis at 17 weeks of gestation because of advanced maternal age. Amniocentesis of one co-twin revealed the result of 46,XX,der(9)t (9;13) (p24;q12)[3]/46,XX [16]. Among 19 colonies of cultured amniocytes, three colonies had the karyotype of 46,XX,der(9)t (9;13) (p24;q12), whereas the rest 16 colonies had a normal karyotype of 46,XX. Prenatal ultrasound findings were unremarkable. She was referred for genetic counseling, and repeat amniocentesis performed at 22 weeks of gestation revealed the result of 46,XX (20/20 colonies) in one co-twin and 46,XY in the other co-twin. Both twins had normal array comparative genomic hybridization (aCGH) results with no genomic imbalance in the uncultured amniocytes. The pregnancy continued, and a phenotypically normal 2,248-g female baby and a normal 1,984-g male baby were delivered at 35 weeks of gestation. The cord blood of the female baby had a karyotype of 46,XX (20/20 cells).

The present case provides evidence that low-level mosaicism for an unbalanced reciprocal translocation at amniocentesis can be a culture artefact. In the present case, at the first amniocentesis at 17 weeks of gestation, the cultured amniocytes had 15.8% (3/19 colonies) mosaicism for der(9)t (9;13) (p24;q12) of which three colonies of cultured amniocytes had the unbalanced reciprocal translocation involving partial monosomy 9p (9p24→pter) and partial trisomy 13q (13q12→qter). However, at repeat amniocentesis at 22 weeks of gestation, both the cultured and uncultured amniocytes were normal in genetic analysis.

Prenatal diagnosis of mosaic unbalanced reciprocal translocation at amniocentesis is very rare. We previously reported mosaic 46,XY,der (15)t (6;15) (q25.1;p12)/46,XY at amniocentesis in a pregnancy associated with a favorable fetal outcome and postnatal decrease of the aneuploid cell line with the unbalanced translocation [9]. The present case adds to the list of clinical cases with mosaic unbalanced reciprocal translocation with a normal cell line at amniocentesis and shows that such a mosaic unbalanced reciprocal translocation can be a culture artefact due to *in vitro* culture process, and repeat amniocentesis is necessary for the confirmation under such a circumstance.

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Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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Research Letter

Incidental detection of partial Xq deletion (Xq21→qter), or 46,X,del(X)(q21) in a 17-year-old girl with irregular menstrual cycle

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Dear Editor

A 17-year-old girl was referred for genetic analysis because of irregular menstrual cycle. Her body weight was 63 Kg, and body height was 158 cm. She had normal intelligence. Her menarche came at age 12 years. Blood analysis revealed anti-Müllerian hormone (AMH) = 0.04 ng/mL, follicle stimulating hormone (FSH) = 25.36 mIU/mL, estradiol (E2) < 10 pg/mL, prolactin = 6.79 ng/mL, luteinizing hormone (LH) = 1.00 mIU/mL and normal *FMR* gene CGG repeats (29:29). Abdominal ultrasound revealed a normal uterus. Cytogenetic analysis revealed a karyotype of 46,X,del(X) (q21) (Fig. 1).

The present case manifested irregular menstrual cycle and an Xq deletion encompassing Xq21→qter. Four Xq premature ovarian failure (POF) susceptibility regions have been identified, *i.e.*, *POF2A* (Xq21.33; X-linked dominant, *DIAPH2*), *POF2B* (Xq21.1; X-linked recessive, *FLJ22792*), *POF1B* (Xq21.1; X-linked recessive) and *POF1* (Xq27.3; X-linked, *FMR1*).

POF2A (OMIM 300511) is caused by mutation in the *DIAPH2* gene (OMIM 300108) at Xq21.33. Philippe et al. [1,2] reported a 17-year-old girl with POF and a balanced translocation of t(X;12) (q21;p13) with a breakpoint at Xq21. The girl had secondary amenorrhea but no other associated features at the age 17 years. Her mother had the same balanced translocation of t(X;12) (q21;p13) and manifested premature menopause at age 32 years. Both the patients had high gonadotropin levels. Sala et al. [3] and Bione et al. [4] demonstrated a *POF* gene at Xq21 and confirmed that breakpoint of t(X;12) (q21;p13) in the patients with POF reported by Philippe et al. [1993, 1995] was at the *DIAPH2* gene.

The present case demonstrates that conventional cytogenetic analysis may incidentally detect X chromosome abnormalities in women with irregular menstrual cycle or POF.

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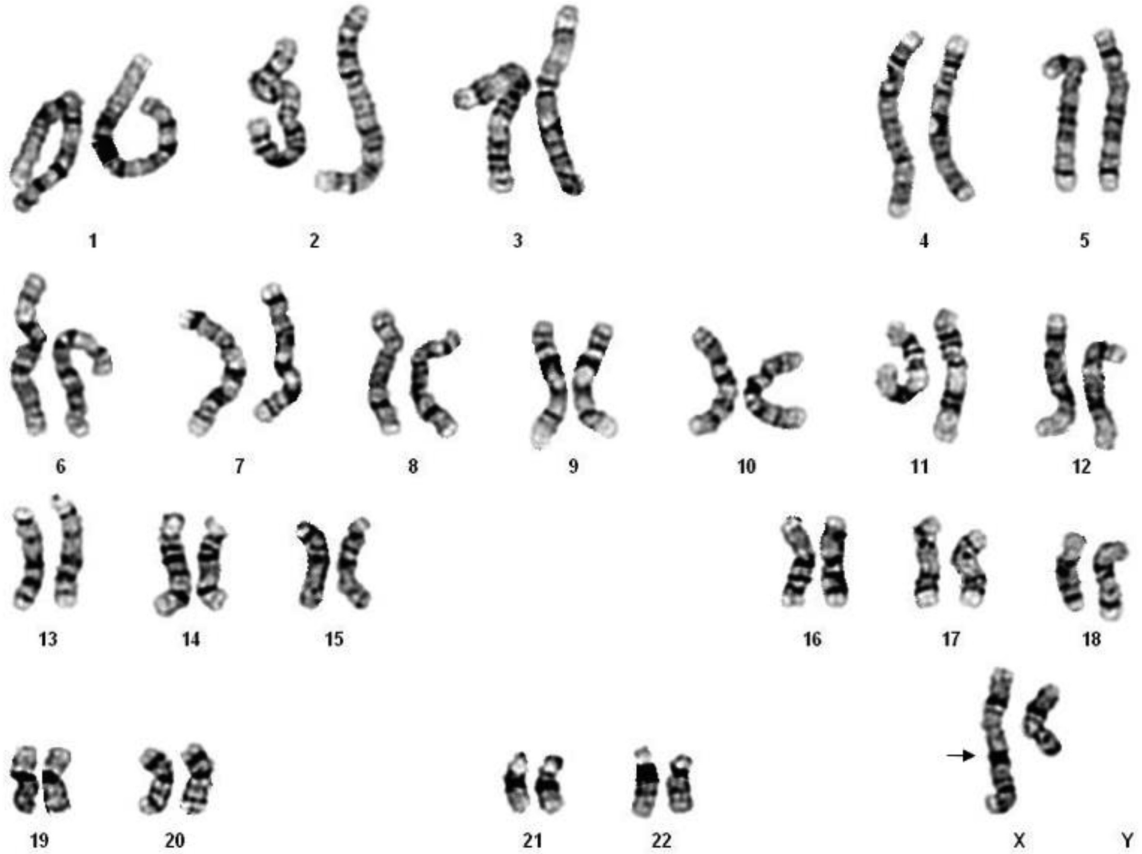

Fig. 1. A karyotype of 46,X,del(X) (q21). The arrow indicates the breakpoint.

Declaration of competing interest

The author has no conflicts of interest relevant to this article.

Acknowledgements

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Retraction notice to "Maternal blood concentration of tadalafil in pregnancy: Comparison of pregnant and non-pregnant women" [Taiwan J Obstet Gynecol 61 (2022) 230–233]

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This article has been retracted: please see Elsevier Policy on Article Withdrawal (https://www.elsevier.com/about/policies/article-withdrawal).

This article has been retracted at the request of the Editors.

As the result of the submission of a correspondence article discussing concerns with the current study [1], the journal was made aware of allegations that some of the data presented in this article appeared to be identical to data presented in a previous publication by some of the same authors as in the present work [2].

Upon further investigation and assessment of the article, the journal found multiple instances of data in the current article that indeed appear to be duplicated. Specifically, Table 1 (Clinical parameters of the participants), Table 2 (Estimated tadalafil pharmacokinetic parameters in pregnant and non-pregnant women), and Figure 1 (Average concentration–time profiles of tadalafil and unbound tadalafil in pregnant and non-pregnant women) appear to have been reproduced from the previous publication without appropriate citation [2].

Additionally, reexamination of the article by the Editors has led them to conclude firstly that the side effects evaluated by the authors were overly subjective, and secondly that the authors failed to show the outcomes of fetuses in their study, but nonetheless claimed safety in the use of tadalafil in pregnant women, with both points raising questions about the reliability of the study's conclusions.

The authors were contacted for comment but did not provide an explanation for the concerns listed above.

The degree of redundant publication detailed above represents a misuse of the scientific publishing system. This, in addition to concerns regarding the reliability of the article's conclusions, has led to the decision to retract the article. The scientific community takes a very strong view on this matter and apologies are offered to readers of the journal that this was not detected during the submission process.

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