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ASSISTED REPRODUCTION TECHNOLOGIES

Maternal serum levels of angiogenic markers and markers of placentation in pregnancies conceived with fresh and vitrified-warmed blastocyst transfer

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Abstract

Purpose The aim of the study was to compare the levels of angiogenic markers and markers of placentation between pregnancies conceived with fresh (ET) and vitrified-warmed blastocyst transfer (FET).

Methods Women with singleton pregnancies resulting from fresh ET or FET during the period between 2013 and 2017 were included in this prospective observational study. Fresh ET was performed in a stimulated and FET in natural cycle. At 6–7 weeks of gestation, after ultrasound confirmation of a single gestational sac with a viable embryo, serum levels of free β-hCG, pregnancy-associated plasma protein A (PAPP-A), placental growth factor (PIGF) and fms-like tyrosine kinase (sFlt-1) were measured. Data on the patients' characteristics, pregnancy complications and outcomes were collected from a questionnaire and National Perinatal Information System of Slovenia.

Results Among 211 pregnancies, 126 were achieved with fresh ET and 85 with FET. There were no significant differences in perinatal outcome, pregnancy complication and PIGF level between the fresh ET and FET group. Women achieving pregnancy with FET had significant higher levels of free β-hCG (40.20 ± 30.62 IU/L vs. 28.74 ± 23.52, $p = 0.002$), PAPP-A (0.09 ± 0.06 vs. 0.06 ± 0.05 IU/L, $p = 0.004$) and sFlt-1 (596.19 ± 283.06 vs. 436.53 ± 248.23 pg/L, $p < 0.0001$) compared to women having conceived with fresh ET. There were no significant differences in the levels of evaluated biomarkers between patients with different pregnancy outcomes and complications.

Conclusion Levels of angiogenic markers and markers of placentation differ between pregnancies achieved with fresh ET and FET which may reflect altered implantation and early placentation with some forms of assisted reproductive technologies.

Keywords Markers of placentation · Angiogenic markers · Assisted reproductive technologies · Blastocyst transfer

Introduction

In recent years, frozen embryo transfer (FET) has become a very important part of the in vitro fertilization (IVF) programme. With advances in cryopreservation techniques, improved implantation rates were achieved and pregnancy rates after FET are at least comparable with those after the transfer of fresh IVF embryos [1–3]. Some investigators suggest that obstetrics and perinatal outcomes in pregnancies arising from the transfer of cryopreserved embryos are better than those in fresh IVF cycles. Singleton pregnancies resulting from FET were associated with a lower risk of perinatal mortality, placenta previa, placental abruption, small for gestational age baby, preterm birth, low birth weight baby and antepartum haemorrhage, when compared with pregnancies conceived with fresh embryo transfer [4, 5].

Multiple biological mechanisms have been proposed to explain the differences between pregnancy outcomes after FET and fresh embryo transfer (ET). It has been suggested that the supra-physiologic hormonal levels observed during control ovarian stimulation result in a suboptimal endometrial receptivity, whereas more natural uterine environment that occurs in FET cycles is favourable for early placentation and

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embryogenesis [6–10]. This hypothesis is supported by observations that serum levels of some biochemical markers of placental function and angiogenic factors are altered in pregnancies resulting from IVF compared to pregnancies conceived spontaneously. Some studies suggest that first-trimester maternal serum levels of PAPP-A and free beta-human chorionic gonadotropin (β-hCG) are decreased in IVF pregnancies [11, 12]. It was also shown that IVF pregnancies tend to have significantly higher levels of fms-like tyrosine kinase (sFlt-1) and lower levels of placental growth factor (PlGF) throughout gestation [13]. The differences in biomarker levels persisted even after being controlled for adverse obstetric outcomes. These findings are important and should be considered when biomarkers are used for predicting aneuploidy and later pregnancy complications. However, the process of implantation and placentation after pregnancy achieved with natural conception may be different from that after FET, even when it is performed in a natural cycle. Moreover, there is a possibility that freezing and thawing damage the trophoblast and thereby negatively influence this process and biomarker production. Only a few studies have specifically considered the differences in biochemical marker levels between pregnancies conceived after FET compared to fresh ET and presented inconclusive results [11, 14].

The aim of our study was to evaluate if the levels of free β-hCG, PAPP-A, PIGF and sFlt-1 differ between fresh and frozen embryo transfers during the early period of placentation.

Materials and methods

In this prospective observational study, 211 women with singleton pregnancies resulting from fresh or vitrified-warmed blastocyst transfer at the Department of Reproductive Medicine and Gynaecological Endocrinology, University Medical Centre Maribor during the period between 2013 and 2017 were analysed. Only women with one gestational sac with a viable embryo at 6–7 weeks of pregnancy were included. Each woman was included in the study only once. Patients without available data on the pregnancy outcome and complete measurements of biochemical markers as well as women with induced abortion were excluded.

The indication for IVF/ICSI was female infertility in 30.80%, male infertility in 33.17%, female and male infertility in 19.43% and unexplained infertility in 17.06% of couples. All patients underwent ovarian stimulation using protocols with a combination of GnRH agonist/GnRH antagonist and recombinant FSH (Gonal-F, Merc-Serono, Darmstadt, Germany)/HMG (Menopur, Ferring Pharmaceuticals Inc., Saint-Prex, Switzerland) that were previously described in detail [15]. The GnRH antagonist protocol of ovarian stimulation was carried out in 88 cycles and the GnRH agonist protocol in 37 cycles. The mean number of retrieved oocytes was 10.06 ± 52.56.

In the study population, IVF was performed in 36.49% and intracytoplasmic sperm injection ICSI in 63.51% of cycles. After oocyte fertilization, embryos were cultured in the Blast Assist extended culture media (Origio, Denmark). Day 5 blastocyst transfer was performed if more than three optimal embryos were available on day 3 due to our standard policies. Blastocysts were graded according to our established grading system 5 days after oocyte fertilization [16]. The blastocyst was considered optimal if it was fully expanded and the blastocoel completely filled the embryo. It contained a cohesive trophectoderm and a compact inner cell mass (ICM). According to the doctor-patient agreement, one or two blastocysts were transferred. Surplus blastocysts, not selected for transfer, were cryopreserved using the standard embryo vitrification procedure [16]. After fresh ET, patients received luteal supplementation using Crinone 8% gel 90 mg/day (Merck Serono, Darmstadt, Germany) until about 12 weeks of gestation. Transfer of vitrified-warmed blastocyst stage embryos was performed in natural menstrual cycles monitored by ultrasound and urinary LH surge tests. According to our routine clinical practice, progesterone supplementation with 400 mg of micronized vaginal progesterone per day (Utrogestan, Ferring Pharmaceuticals Inc., Saint-Prex, Switzerland) was started after FET and finished at the time of a positive pregnancy test. Serum β-hCG level measurement was scheduled 12–16 days after blastocyst ET. All patients underwent transvaginal ultrasound examination at 6–7 weeks of gestation to determine the pregnancy status. The embryo viability was confirmed if heartbeat was present. The crown–rump length (CRL) measurements were obtained as described in the WHO Manual of diagnostic ultrasound, volume 2 [17]. Gestational age was calculated from CRL.

Maternal serum levels of free β-hCG, PAPP-A, PIGF and sFlt-1 were measured in previously frozen serum specimens collected on the day of ultrasound examination. Samples were stored at − 70 °C in a repository. Levels of all biomarkers were determined by the electrochemiluminescence method (ECLIA) (Roche Diagnostics, Mannheim, Germany) in the same laboratory.

To obtain clinical data, patients completed a questionnaire on the day of the ultrasound examination. Data on patients' characteristics, pregnancy complications and outcomes were collected from our database and National Perinatal Information System of Slovenia.

All patients were divided in 4 groups according to pregnancy outcome and complications: spontaneous abortion, preterm delivery, complications of placental origin (preeclampsia/eclampsia, intrauterine growth restriction, gestational hypertension) and term birth without complications group.

Statistical analysis was performed with Statistica 8.0 data analysis software (Stat Soft Inc., Tulsa, OK, USA). Patients'

characteristics, pregnancy outcome and serum levels of biochemical markers were compared between the fresh ET and FET group. The normal distribution of numeric variables was determined by the Shapiro-Wilk test. Student's *t* test or the Mann–Whitney *U* test was used to assess these variables, depending on the data distribution. Chi-squared test was used to compare differences in frequencies. Significant findings between the two groups were further evaluated using logistic regression in order to adjust for covariates. The relation between the PAPP-A levels and birth weights in both groups was tested using the Spearman correlation coefficient. Differences in levels of biochemical markers between 4 groups with different pregnancy outcome were calculated by Kruskal–Wallis ANOVA test. Statistical significance was set at $p < 0.05$.

The study was approved by our institutional review board.

Results

Among 211 singleton pregnancies, 126 were achieved with fresh and 85 with vitrified-warmed blastocyst transfer. There were 18 (8.53%) spontaneous abortions and 193 (91.47%) live births. No stillbirth, neonatal death or chromosomal aneuploidies were observed. Women conceived with fresh ET were statistically significantly older (34.01 ± 3.98 vs. 32.65 ± 3.87 years, $p < 0.015$) and more common primigravid (60.32% vs. 36.47%, $p = 0.003$) and had less previous pregnancies (0.58 ± 0.99 vs. 0.92 ± 0.91, $p = 0.01$) compared to women having conceived with FET. There were no statistically significant differences between these two groups in infertility causes, the proportion of ICSI cycles, body mass index, the proportion of tobacco smokers, the proportion of women with spontaneous abortion, preterm delivery in previous pregnancies and the proportion of ET of optimal blastocysts (Table 1). Timing from embryo transfer to 6–7-week blood work was similar between both groups. There were also no statistically significant differences in gestational age at blood sampling and serum cryopreservation for later measurements of biochemical markers (Table 1).

There were no statistically significant differences in perinatal outcome, pregnancy complications and PIGF level between the fresh ET and FET group. However, women who achieved pregnancy with FET had statistically significant higher levels of free β-hCG, PAPP-A and sFlt-1 compared to women who conceived with fresh ET (Table 2). The differences in levels of free β-hCG, PAPP-A and sFlt-1 remained statistically significant after the adjustment for age, primigravid and number of previous pregnancies using logistic regression models. There was no relationship between PAPP-A levels and birth weights in the fresh ET (Spearman $R = 0.09$, $p = 0324$) and FET group (Spearman $R = 0.10$, $p = 0382$).

All 211 patients were divided into 4 groups according to pregnancy outcomes and complications. Eighteen (8.53%) pregnancies ended in spontaneous abortion. Term birth without complications were observed in 159 (72.51%) women, preterm birth in 23 (10.90%) and complications of placental origin, such as preeclampsia/eclampsia, intrauterine growth restriction and gestational hypertension, in 17 (8.06%) patients. There were no statistically significant differences in free β-hCG, PAPP-A, PIGF and sFlt-1 levels between these 4 groups (Table 3).

There were also no significant differences in the levels of free β-hCG (32.54 ± 31.52 vs. 33.66 ± 25.51 IU/L, $p = 0.797$), PAPP-A (0.07 ± 0.05 vs. 0.08 ± 0.06 IU/L, $p = 0.282$), PIGF (14.03 ± 3.76 vs. 13.65 ± 3.85 pg/L, $p = 0.537$) and sFlt-1 (452.25 ± 247.72 vs. 518.00 ± 278.06 pg/L, $p = 0.132$) between the group of patients with and without pregnancy complications.

Discussion

In our study, we found that women having achieved pregnancy with FET had higher levels of free β-hCG, PAPP-A and sFlt-1 compared to women conceiving with fresh ET. There were no differences in concentrations of PIGF between both groups. Only a few published studies compared the levels of biomarkers of placentation between FET and fresh ET cycles. Some of them evaluated β-hCG in at a very early pregnancy stage and presented inconsistent results [18, 19]. This is not surprising, since several parameters that have impact on embryo growth can also alter β-hCG levels, for instance embryo culture, duration of cultivation and cryopreservation methods [20–22]. Therefore, it is important to compare studies with similar methodological approach. Our previous study has not shown any statistically significant difference in β-hCG levels 13 days after transferring fresh or vitrified-warmed blastocyst. However, recent data suggest that the rate of β-hCG increase is higher following FET [23], so there is a possibility that the difference becomes apparent later in the pregnancy. In the present study, blood sampling was performed approximately 15 days later than in the previous study, which may explain disparity in results. The second possible explanation is that in this study free β-hCG was measured, which represents about 0.3–4% of the total hCG and better reflects placental function [24, 25]. Our results are in accordance with the data published by Hui et al. [12], who have demonstrated that midtrimester β-hCG levels are considerably higher in pregnancies resulting from FET compared with fresh ET, leading to a higher false positive rate in aneuploidy screening tests.

Lower PAPP-A in IVF pregnancies compared to spontaneously conceived pregnancies has been reported by most studies on this topic [11, 26–30]. It has been suggested that hormonal treatment is the most important cause of altered biochemical

Table 1 Characteristics of women conceived with fresh and vitrified-warmed blastocyst transfer

	Fresh ET ($N = 126$)	FET ($N = 85$)	p value
Age (years, $X \pm$ SD)	34.01 ± 3.98	32.65 ± 3.87	0.015
Age ≥ 35 years (%, N)	32.54 (41)	23.53 (20)	NS
Body mass index (kg/m^2, $X \pm$ SD)	25.01 ± 5.30	24.08 ± 5.27	NS
Body mass index ≥ 25 kg/m^2 (%, N)	32.54 (41)	30.58 (26)	NS
Female infertility (%, N)	32.53 (41)	27.23 (24)	NS
Male infertility (%, N)	34.12 (43)	31.76 (27)	NS
Female and male infertility (%, N)	17.46 (22)	22.35 (19)	NS
Unexplained infertility (%, N)	15.87 (20)	18.82 (16)	NS
Intracytoplasmic sperm injection (%, N)	65.87 (83)	60.00 (51)	NS
Primigravid (%, N)	60.32 (76)	36.47 (31)	0.003
No. of previous pregnancies ($X \pm$ SD)	0.58 ± 0.99	0.92 ± 0.91	0.01
Spontaneous abortion in previous pregnancies (%, N)	54.00 (27/50)	50.00 (27/54)	NS
Preterm delivery in previous pregnancies (%, N)	12.00 (6/50)	18.52 (10/54)	NS
Smokers (%, N)	5.55 (7)	4.71 (4)	NS
Blood sampling after ET/FET (days, $X \pm$ SD)	28.27 ± 2.06	28.68 ± 2.02	NS
Gestation at blood sampling (days, $X \pm$ SD)	45.87 ± 1.98	46.11 ± 2.06	NS
ET of optimal blastocysts (%, N)	40.48 (51)	37.65 (32)	NS

X ± SD mean ± standard deviation, *ET* blastocyst transfer, *FET* vitrified-warmed blastocyst transfer, *NS* not significant

markers in IVF pregnancies. The supra-physiologic oestrogen level and premature progesterone secretion due to ovarian stimulation may result in a non-synchronous uterine environment, a suboptimal placenta-endometrial interface and impaired placentation [6, 8, 11, 31]. This hypothesis was supported by findings that first-trimester maternal serum PAPP-A levels are affected by the oestradiol level at the time of oocyte retrieval and by the number of oocytes in IVF cycles [27, 32]. Moreover, it was demonstrated that the levels of biochemical markers were not significantly different between women pregnant after assisted conception without ovarian stimulation (transfer of frozen-thawed embryo or in spontaneous cycle) and women after spontaneous conception [27]. On the contrary, it was shown in some studies that PAPP-A levels in FET pregnancies are lower than in spontaneous conceived pregnancies [11, 31]. Only a few published studies compare PAPP-A levels between pregnancies achieved by fresh and frozen-thawed embryo transfer and presented conflicting results. Amor et al. [11] reported lower PAPP-A levels in pregnancies conceived with FET compared to spontaneous conceived pregnancies, but these levels were higher than in the fresh ET group. They suggest that lower PAPP-A levels may

Table 2 Comparison of pregnancy outcome, pregnancy complications and levels of biochemical markers between women conceiving with fresh and vitrified-warmed blastocyst transfer

	Fresh ET ($N = 126$)	FET ($N = 85$)	p value
Spontaneous abortion (%, N)	9.5 (12)	7.05 (6)	NS
Caesarean section (%, N)	26.89 (34)	25.88 (22)	NS
Birth weight (g, $X \pm$ SD)	3251.25 ± 562.52	3236.62 ± 694.47	NS
Gestational age at birth (weeks, $X \pm$ SD)	38.61 ± 2.23	38.22 ± 2.95	NS
Preterm birth (%, N)	8.7 (11)	14.11 (12)	NS
Small for gestational age (%, N)	3.17 (4)	4.71 (4)	NS
Gestational hypertension (%, N)	0.79 (1)	4.71 (4)	NS
Preeclampsia/Eclampsia (%, N)	2.38 (3)	1.17 (1)	NS
Gestational diabetes (%, N)	12.30 (16)	14.12 (12)	NS
Free β-hCG (IU/L, $X \pm$ SD)	28.74 ± 23.52	40.20 ± 30.62	0.002
PAPP-A (IU/L, $X \pm$ SD)	0.06 ± 0.09	0.09 ± 0.06	0.004
PIGF (pg/L, $X \pm$ SD)	14.00 ± 4.00	13.37 ± 3.50	NS
sFlt-1 (pg/L, $X \pm$ SD)	436.53 ± 248.23	596.19 ± 283.06	< 0.0001

X ± SD mean ± standard deviation, *ET* blastocyst transfer, *FET* vitrified-warmed blastocyst transfer, *NS* not significant

Table 3 Levels of angiogenic and placental markers in group or women with different pregnancy outcomes

	Spontaneous abortion	Preterm birth	Pregnancy complication	Term birth, no complication	*p* value
Number	18	23	17	153	
Free β-hCG (IU/L, $X \pm SD$)	25.52 ± 20.74	23.73 ± 29.02	19.69 ± 13.23	27.06 ± 25.51	0.433
PAPP-A (IU/L, $X \pm SD$)	0.03 ± 0.03	0.05 ± 0.06	0.055 ± 0.05	0.06 ± 0.05	0.067
PIGF (pg/L, $X \pm SD$)	12.43 ± 3.76	14.82 ± 2.76	15.04 ± 4.22	13.55 ± 3.85	0.163
sFlt-1 (pg/L, $X \pm SD$)	326.50 ± 177.80	330.00 ± 285.30	431.50 ± 244.67	467.00 ± 278.94	0.567

$X \pm SD$ median ± standard deviation

result from exogenous hormonal therapy used in artificial FET cycles [11]. In natural FET cycles, a significantly higher live birth rate and a significantly lower clinical spontaneous abortion rate than in artificial FET were found reported by some authors. They suggest that a suboptimal oestrogen environment or a suboptimal ratio between progesterone and oestradiol may have a negative impact on implantation and placentation processes [33, 34]. Therefore, inconsistent results of studies may be the consequence of a different protocol for endometrial preparation and freezing technique. In our study, we have performed FET in a natural cycle and the vitrification technique was used for cryopreservation. This technique causes less damage to the embryo than the slow freezing method.

It has also been suggested that lower PAPP-A levels in ART pregnancies might be the result of metabolic impairments related to infertility in the mother and method of fertilization. Significantly lower PAPP-A was observed in pregnancies conceived in ICSI cycles [26, 31]. However, there were no differences in our study in the causes of infertility and the proportion of ICSI procedures between the fresh ET and FET group. The results could be biased because in the FET group only women with normal ovulatory menstrual cycle were included.

We observed higher levels of antiangiogenic factor sFlt-1 in the FET group compared with the fresh ET group, but the levels of angiogenic marker PlGF were comparable in both groups. This is contrary to our expectations, since PlGF has been identified as having an important role in promoting neovascularization while sFlt-1 acts as a growth inhibitory factor. It was shown in a previous study that pregnancies conceived with IVF had an increased antiangiogenic profile (elevated sFlt-1 and decreased PlGF) at 18, 26 and 35 weeks of gestation when compared with spontaneously conceived pregnancies [13]. In FET in a natural cycle, a more physiological uterine environment and better regulation of angiogenesis during the placentation process is predicted than in fresh ET. However, Woo et al. [14] found no difference in sFlt-1:PlGF ratios at 5 weeks of gestation between fresh ET and frozen FET. They suggest that the altered uterine hormonal milieu in fresh embryo transfers does not influence the angiogenic factors and that these factors do not drive early placentation [14]. Higher levels of sFlt-1 in the FET group in our study are in concordance with this opinion.

Placental and angiogenic biomarkers reflect placental dysfunction which is associated with adverse pregnancy complications and perinatal outcome [35]. It was observed in some studies that obstetrics and perinatal outcomes in pregnancies arising from the transfer of cryopreserved embryos are better than those in fresh IVF cycles [4, 5, 7]. Therefore, different levels of biomarkers might be a consequence of different perinatal outcome. However, in our study, the incidence of pregnancy complications and perinatal outcome was comparable. Our results are in concordance with findings of some other researchers who demonstrated that levels of biomarkers are altered in pregnancies resulted from fresh ET irrespectively to maternal comorbidities and perinatal outcome [11, 13].

Multiple biomarkers are used in early pregnancy for the prediction of foetal aneuploidy, pregnancy outcome and later pregnancy complications, such as preeclampsia, preterm birth and foetal growth restriction. However, no later pregnancy complication can be predicted with sufficient specificity and sensitivity by any single biomarker [33]. In the present study, we did not find differences in levels of evaluated biomarkers between patients with different pregnancy outcome and maternal morbidity. The possible explanation might be that the groups of patients were too small and heterogeneous. Blood sampling in our studies was performed at 6–7 weeks of pregnancy, which is much earlier than in most other studies that evaluated the prognostic value of biochemical markers during the late first trimester.

Our study is limited by the small sample size, particularly in the group of patients conceived with FET. Due to low number of patients in some groups with different pregnancy outcome, comparation of biomarker levels between these groups additionally divided to pregnancies followed fresh ET and FET was not reasonable. There is also a possibility of biased selection since in the FET group only patients with regular ovulatory cycles were included. From our point of view, it is important that FET was done in a natural cycle without exogenous hormone supplementation because we believe that hormonal imbalance may affect the implantation and placentation process.

In our study, we established that levels of some angiogenic markers and markers of placentation differ between pregnancies resulting from fresh and vitrified-warmed blastocyst transfer. These data suggest that early placentation may be altered with some forms of assisted reproductive technologies and that not all biochemical markers reflect this process. There are also several possible explanations for these finding; however, future studies are required to elucidate all factors that may affect implantation and early placentation in ART pregnancies.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Herrero L, Martinez M, Garcia-Velasco JA. Current status of human oocyte and embryo cryopreservation. Curr Opin Obstet Gynecol. 2011;23:245–50.
2. Shapiro BS, Daneshmand ST, Garner FC, Aguirre M, Hudson C, Thomas S. Similar ongoing pregnancy rates after blastocyst transfer in fresh donor cycles and autologous cycles using cryopreserved bipronuclear oocytes suggest similar viability of transferred blastocysts. Fertil Steril. 2010;93:319–21.
3. Roque M, Lattes K, Serra S, Sola I, Geber S, Carreras R, et al. Fresh embryo transfer versus frozen embryo transfer in in vitro fertilization cycles: a systematic review and meta-analysis. Fertil Steril. 2013;99:156–62.
4. Sha T, Yin X, Cheng W, Massey IY. Pregnancy-related complications and perinatal outcomes resulting from transfer of cryopreserved versus fresh embryos in vitro fertilization: a meta-analysis. Fertil Steril. 2018 Feb;109(2):330–42.
5. Maheshwari A, Pandey S, Shetty A, Hamilton M, Bhattacharya S. Obstetric and perinatal outcomes in singleton pregnancies resulting from the transfer of frozen thawed versus fresh embryos generated through in vitro fertilization treatment: a systematic review and meta-analysis. Fertil Steril. 2012;98:368–77.
6. Shapiro BS, Daneshmand ST, Garner FC, Aguirre M, Hudson C, Thomas S. Evidence of impaired endometrial receptivity after ovarian stimulation for in vitro fertilization: a prospective randomized trial comparing fresh and frozen-thawed embryo transfer in normal responders. Fertil Steril. 2011;96:344–8.
7. Kansal Kalra S, Ratcliffe SJ, Milman L, Gracia CR, Coutifaris C, Barnhart KT. Perinatal morbidity after in vitro fertilization is lower with frozen embryo transfer. Fertil Steril. 2011;95:548–53.
8. Imudia AN, Awonuga AO, Doyle JO, Kaimal AJ, Wright DL, Toth TL, et al. Peak serum estradiol level during controlled ovarian hyperstimulation is associated with increased risk of small for gestational age and preeclampsia in singleton pregnancies after in vitro fertilization. Fertil Steril. 2012;97:1374–9.
9. Barnhart KT. Introduction: are we ready to eliminate the transfer of fresh embryos in in vitro fertilization? Fertil Steril. 2014;102:1–2.
10. Weinerman R, Mainigi M. Why we should transfer frozen instead of fresh embryos: the translational rationale. Fertil Steril. 2014;102:10–8.
11. Amor DJ, Xu JX, Halliday JL, Francis I, Healy DL, Breheny S, et al. Pregnancies conceived using assisted reproductive technologies (ART) have low levels of pregnancy-associated plasma protein-A (PAPP-A) leading to a high rate of false-positive results in first trimester screening for Down syndrome. Hum Reprod. 2009;24:1330–8.
12. Hui PW, Lam YH, Tang MH, NG EH, Yeung WS, Ho PC. Maternal serum pregnancy-associated plasma protein-A and free beta-human chorionic gonadotrophin in pregnancies conceived with fresh and frozen-thawed embryos from in vitro fertilization and intracytoplasmic sperm injection. Prenat Diagn. 2005;25(5):390–3.
13. Lee MS, Cantonwine D, Little SE, et al. Angiogenic markers in pregnancies conceived through in vitro fertilization. Am J Obstet Gynecol. 2015;213(2):212.e211–8.
14. Woo I, Chan Y, Sriprasert I, Louie K, Ingles S, Stanczyk F, et al. The role of angiogenic markers in adverse perinatal outcomes: fresh versus frozen embryo transfers. J Assist Reprod Genet. 2017;34(12):1639–43.
15. Vlaisavljević V, Kovačič B, Gavrić-Lovrec V, Reljič M. Simplification of the clinical phase of IVF and ICSI treatment in programmed cycles. Int J Gynaecol Obstet. 2000;69:35–42.
16. Kovačič B, Vlaisavljević V. Importance of blastocyst morphology in selection for transfer. In: Wu B, editor. Advances in embryo transfer. Rijeka: Intech; 2012. p. 161–77.
17. WHO. WHO manual of diagnostic ultrasound, vol. 2. 2nd ed. Geneva: Switzerland World Health Organization: World Health Organization & World Federation for Ultrasound in Medicine and Biology; 2013.
18. Keane KN, Mustafa KB, Hinchliffe P, Conceicao J, Yovich JL. Higher β-HCG concentrations and higher birthweights ensue from single vitrified embryo transfers. Reprod BioMed Online. 2016;33(2):149–60.
19. Reljič M, Knez J, Vlaisavljević V. Human chorionic gonadotropin levels are equally predictive for pregnancy outcome after fresh and vitrified-warmed blastocyst transfer. J Assist Reprod Genet. 2013;30(11):1459–63.
20. Kolibianakis EM, Venetis CA, Tarlatzis BC. Cryopreservation of human embryos by vitrification or slow freezing: which one is better? Curr Opin Obstet Gynecol. 2009;21:270–4.
21. Dumoulin JC, Land JA, Van Montfoort AP, Nelissen EC, Coonen E, Derhaag JG, et al. Effect of in vitro culture of human embryos on birthweight of newborns. Hum Reprod. 2010;25:605–12.
22. Kathiresan ASQ, Cruz-Almeida Y, Barrionuevo MJ, et al. Prognostic value of beta-human chorionic gonadotropin is dependent on day of embryo transfer during in vitro fertilization. Fertil Steril. 2011;96:1362–6.
23. Grin L, Indurski A, Leytes S, Rabinovich M, Friedler S. Trends in primeval β-hCG level increment after fresh and frozen-thawed IVF embryo transfer cycles. Gynecol Endocrinol. 2018 Oct;9:1–6.
24. Evans MI, Krantz DA, Hallahan TW, Galen RS. Meta-analysis of first trimester Down syndrome screening studies: free beta-human chorionic gonadotropin significantly outperforms intact human chorionic gonadotropin in a multimarker protocol. Am J Obstet Gynecol. 2007;196(3):198–205.
25. Fernando MR, Donato D, Felice P. Predictive value of hormone measurements in maternal and fetal complications of pregnancy. Endocrine. 2006;23(2):230–57.
26. Bellver J, Casanova C, Garrido N, Lara C, Remohi J, Pellicer A, et al. Additive effect of factors related to assisted conception on the reduction of maternal serum pregnancy-associated plasma protein A concentrations and the increased false-positive rates in first trimester Down syndrome screening. Fertil Steril. 2013;100(5):1314–20.
27. Tul N, Novak-Antolic Z. Serum PAPP-A levels at 10-14 weeks of gestation are altered in women after assisted conception. Prenat Diagn. 2006;26(13):1206–11.

28. Gjerris AC, Loft A, Pinborg A, Christiansen M, Tabor A. First trimester screening markers are altered in pregnancies conceived after IVF/ICSI. Ultrasound Obstet Gynecol. 2009;33(1):8–17.
29. Engels MA, Kooij M, Schats R, Twisk JW, Blankenstein MA, van Vugt JM. First-trimester serum marker distribution in singleton pregnancies conceived with assisted reproduction. Prenat Diagn. 2010;30(4):372–7.
30. Sahraravand M, Laitinen P, Järvelä I, Ryynänen M. First-trimester maternal serum ADAM12-s and PAPP-A levels are altered in pregnancies conceived after assisted reproduction techniques (ART). Prenat Diagn. 2016;36(2):163–9.
31. Hunt LP, McInerney-Leo AM, Sinnott S, Sutton B, Cincotta R, Duncombe G, et al. Low first-trimester PAPP-A in IVF (fresh and frozen-thawed) pregnancies, likely due to a biological cause. J Assist Reprod Genet. 2017;34(10):1367–75.
32. Giorgetti C, Vanden Meerschaut F, De Roo C, Saunier O, Quarello E, Hairion D, et al. Multivariate analysis identifies the estradiol level at ovulation triggering as an independent predictor of the first trimester pregnancy-associated plasma protein-A level in IVF/ICSI pregnancies. Hum Reprod. 2013;28(10):2636–42.
33. Tomás C, Alsbjerg B, Martikainen H, Humaidan P. Pregnancy loss after frozen-embryotransfer—a comparison of three protocols. Fertil Steril. 2012;98(5):1165–9.
34. Morozov V, Ruman J, Kenigsberg D, Moodie G, Brenner S. Natural cycle cryo-thaw transfer may improve pregnancy outcome. J Assist Reprod Genet. 2007;24:119–23.
35. Kane SC, Costa FS, Brennecke S. First trimester biomarkers in the prediction of later pregnancy complications. Biomed Res Int. 2014;2014:807196.