The Effect of Epididymal Sperm Cryopreservation on Neonatal Birthweight Following PESA-ICSI

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Research Article

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Abstract

**Purpose:** To compare the neonatal birthweight of singletons derived from ICSI cycles with fresh or frozen-thawed epididymal sperm in patients with obstructive azoospermia

**Methods:** A total of 436 singletons derived from ICSI cycles with fresh (n=220) or frozen-thawed (n=216) epididymal sperm in obstructive azoospermia evaluated from 2012 to 2018 in the retrospective study. Multivariate generalized linear model was used to analyze the association between epididymal sperm cryopreservation and neonatal birthweight.

**Result(s):** The crude birthweight and z-score in neonates derived from frozen-thawed epididymal sperm were significantly lower than those from fresh epididymal sperm (3186.57g vs 3303.61g and -0.18 vs 0.08, respectively), with a mean difference of 117.04 (95%CI: 32.36 to 201.72) g and 0.25 (95%CI: 0.06 to 0.45). Adjusted for confounders including parental age and BMI, maternal ovarian reserve, paternal FSH and T levels, peak E2 during OPU cycles, frozen-thawed embryo transfer, embryo development stage, gestational age, maternal parity and child gender, the multivariate model suggested that singletons conceived from ICSI with fresh epididymal sperm was on average 91.21 g heavier than those conceived from ICSI with frozen-thawed epididymal sperm (95%CI:12.72 to 166.7, P=0.016).

**Conclusion(s):** Cryopreservation of epididymal sperm may negatively affect birthweight

Introduction

Azoospermia, characterized by a complete absence of sperm in the semen, occurs in approximately 5%-10% of infertile men and about 40% of azoospermia cases were diagnosed as obstructive [1, 2]. Beyond surgical reconstruction, the combination of surgical sperm retrieval and ICSI provides an effective therapy option for obstructive azoospermia, yielding comparable pregnancy outcomes than ICSI cycles using ejaculated sperm[3].

During ICSI cycles using epididymal or testicular sperm, cryopreservation of retrieved sperm during diagnostic procedure for ICSI has shown to be valid option to avoid repeating puncture or biopsy. While earlier studies suggested that cycles using frozen-thawed retrieved sperm resulted in comparable pregnancy outcomes than those using fresh retrieved sperm[4, 5], recent evidence suggested a lower pregnancy rate by using frozen epididymal/testicular sperm compared with fresh epididymal/testicular sperm[6, 7], suggesting a potential effect of cryopreservation on injected sperm. Cryopreservation may lead to genetic and epigenetic changes in spermatozoa[8], which affect not only implantation of the transferred embryo but also neonatal outcomes of the offspring. Although the neonatal outcomes of children born after ICSI with epididymal/testicular sperm have been extensively reported[3, 9, 10], less is known about the effect of retrieved sperm cryopreservation on the neonatal outcomes of offspring. The aim of the study was to compare birthweight of neonates conceived from ICSI cycle using fresh and frozen-thawed epididymal sperm retrieved by percutaneous epididymal sperm aspiration (PESA).
Materials And Methods

Patients

We reviewed all PESA-ICSI cycles carried out in the affiliated Chenggong Hospital of Xiamen University in the period between January 2012 and December 2018 for potential inclusion. The inclusion criteria were cycles resulting in singletons. The exclusion criteria included patients receiving PESA-ICSI for reasons other than obstructive azoospermia, such as ejaculation failure.

Patients with obstructive azoospermia were evaluated by andrologists according to anamnesis for obstruction, volume of testis and hormonal level. Included patients met following criteria: 1) The normal testicular volume measured by the Prader orchidometer was $\geq 30$ml bilaterally or $\geq 15$ml unilaterally.2) The serum levels of FSH, LH and T were within a normal range. 3) Sperm were identified from aspiration.

The PESA procedure was routinely scheduled for these patients before the cycle was initiated for both diagnosis and cryopreservation purpose. Cryopreserved epididymal sperm were used if the patient rejected a second PESA on the day of OPU. All patients in this study gave written informed consent. Institutional Review Board approval for this retrospective study was obtained from the Ethical Committee of the Medical College Xiamen University.

Percutaneous epididymal sperm aspiration and cryopreservation

PESA was performed under local anesthesia with 2 ml 1% lidocaine. A 25-G needle was attached to a 1-ml syringe and inserted vertically through the skin to the caput epididymidis for epididymal fluid collection. Negative pressure was applied until a fluid presented the syringe. Then the needle was gently removed and the aspirate was flushed with warm gamete buffer (KSIGB, COOK MEDICAL, Bloomington, IN). Presence of motile sperm was identified by an immediate microscopic examination.

For cryopreservation, 1 part of sperm freezing medium (ORIGO, Malov, Denmark) was added to 1 part of the sperm sample, mixing after adding each drop of medium. The mixture was transferred to a cryovial and left in 4-5°C for 30 min, followed by a suspension in nitrogen vapor for 30 min. Then the cryovial was merged in liquid nitrogen for storage.

ART procedures

Female patients received agonist or antagonist protocol with recombinant FSH (Gonal-F; Merck-Serono) or hMG (urofollitropin for injection; Livzon Pharma) for ovarian stimulation. HCG triggering was given when at least 3 follicles reach 17mm in diameters. Oocyte retrieval under transvaginal ultrasound guidance was scheduled 34-36 hours after hCG administration.

For both fresh and frozen-thawed PESA-ICSI, the epididymal sperm was washed (400G, 5min) and re-suspended in gamete buffer. The retrieved cumulus-corona-oocyte complexes were denuded by mechanical pipetting with 80 IU/ml hyaluronidase solution (Irvine, Santa Ana, CA) 2 hours after OPU.
After an examination of nuclear maturity, all MII oocytes were injected with standard ICSI in pre-warmed gamete buffer droplets with oil overlay (OVOIL, Vitrolife, Göteborg, Sweden). Prepared sperm was placed in a separated droplet under the same oil overlay, where the sperm for injection was selected and transferred to 7% polyvinyl prolidone (KSIPV, COOK MEDICAL, Bloomington, IN) for immobilization.

Injected oocytes were cultured in a single droplet of culture media (KSICM, COOK MEDICAL, Bloomington, IN) with oil overlay in traditional incubators (C200, Labotect, Göttingen, Germany) at 37°C, 6%CO₂, 5%O₂. Fertilization was checked 18 hours post insemination. Embryo morphology was evaluated on day 3 and embryos were transferred to blastocyst medium (KSIBM, COOK MEDICAL, Bloomington, IN) if prolonged culture was required.

In FET cycles, a vitrification protocol employing 15% dimethyl sulfoxide, 15% ethylene glycol, and 0.6M sucrose as cryoprotectants was used. Blastocoelic volume was reduced before cryopreservation using a laser system (SATURN, RI, Falmouth, UK).

The number of embryos transferred and the stage of ET was determined based on the patient’s preferences, with written consent. Luteal support continued until 10 weeks of pregnancy in all transfer cycles.

Sample size calculation

We performed a post-hoc power calculation with currently available sample size. In a previous study, the singleton birthweight in Chinese population was normally distributed with standard deviation about 400. With 220 subjects in each group, we will be able to detect a true difference in the birthweight of -107.087 or 107.087 with probability (power) 0.8. The Type I error probability associated with this test of the null hypothesis that the population means of the experimental and control groups are equal is 0.05.

Statistical analysis

Calculation of z-score was based on a birth weight reference for Chinese population generating from National Population-based Birth Defects Surveillance System\[11]\). Z-score was defined as the weight of the individual child minus the media weight of the reference population of children born at the same gestational age and of the same gender divided by the standard deviation from the same reference population. SGA newborns were those with a birth weight <10th percentile for that gestational age and gender. LBW newborns were those with a birth weight <2500g.

Generalized linear model was used to analyze the association between frozen PESA and birthweight outcomes. We included the following covariates to the model because they might affect the birthweight of offspring: parental BMI, male smoking status, parity, PCOS diagnosis, type of transfer cycle (frozen or fresh), stage of embryo transfer (cleavage or blastocyst), endometrial thickness, vanishing twin and peak E₂ during ovarian stimulation. Female smoking was not included because none of the patients were reported as smoker. We also adjusted for parental age, female AFC, male testosterone and FSH levels,
duration of infertility and year of delivery, because they may stand for potential bias that may affect the estimation. For absolute birthweight, offspring gender and gestational age was also included to the model.

All calculations were performed with SPSS (version 19; IBM).

Results

We reviewed 1078 PESA-ICSI cycles, using either frozen-thawed (n=525) or fresh (n=553) epididymal sperm. Five hundred and eighty-one live births were achieved from these cycles (292 in fresh PESA cycles and 289 in frozen thawed PESA cycles), resulting in 132 twins and 449 singletons. Among the cycles leading to singletons, ten cycles were excluded due to ejaculation failure and 3 cycles were excluded due to incomplete follow-up record. Finally, 216 PESA-ICSI cycles and 220 PESA-ICSI cycles which resulted in singleton were included for evaluation.

The characteristics of the cycles were shown in Table 1. The mean female age was 28.6 years and the mean male age was 31.2 years in the cohort. The mean age for both men and women was significantly lower in cycles using frozen-thawed epididymal sperm. In addition, men receiving epididymal sperm cryopreservation had a higher serum FSH and testosterone level. Higher parity was observed in women from PESA-ICSI cycles using fresh epididymal sperm. There was no significant difference observed in parental BMI, maternal ovarian reserve markers, peak E₂ during ovarian stimulation and endometrial thickness.

When comparing the neonatal outcomes, the gestational age and sex ratio of the offspring were not significantly different among groups. However, both absolute birthweight and z-score normalized according to gestational age and sex were significant lower in singletons conceived from PESA-ICSI cycles using frozen-thawed sperm. The odds ratio for SGA comparing frozen-PESA versus fresh PESA was 1.56 (95%CI: 0.82 to 2.99), and the odds ratio for LBW was 1.42(95%CI: 0.56 to 3.61).

Adjusted for aforementioned covariates and factors, the association between frozen PESA-ICSI and reduced birthweight remained significant (Table 2). The coefficient suggested that the birthweight of singleton was 91.21 (95%CI: 15.72 to 166.7) g lower following PESA-ICSI using frozen sperm in comparison with that following PESA-ICSI using fresh sperm. Similarly, the z-score was decreased by 0.27 (95%CI: 0.07 to 0.46) in singletons following PESA-ICSI using frozen sperm in comparison with that following PESA-ICSI using fresh sperm.

Discussion

In the present study, we observed a decrease of 117.04 (95%CI: 32.36 to 201.72) g in birthweight of singletons conceived from PESA-ICSI cycles using frozen-thawed sperm, in comparison with those from cycles using fresh epididymal sperm. Adjusted for confounders, the mean difference was 91.21 (95%CI: 15.72 to 166.7)g in birthweight and 0.27 (95%CI: 0.07 to 0.46) in z-score. The magnitude of the
differences approximated the size of effect of several well-known factors that might affect the birthweight, such as parity and maternal overweight[12, 13]. For instance, a difference of 0.2 in z-score was also observed in a study comparing birthweight in children of primiparas and nulliparas[12] and a 119 g difference in mean birthweight was reported in children of normal weight and overweight women[13].

Birthweight is not only an important predictor of newborn survival, but also observationally associated with health risks and developmental issues in later life. An approximately 100 g decrease in birthweight is associated with subtle but significant changes in risk markers for diabetes and cardiovascular diseases [14, 15]. For women, a 100 g decrease in birthweight may be associated with a 3% risk increase for hypertensive disorders of pregnancy in later life [16]. Therefore, the magnitude of changes in birthweight in the present study might suggest a subtle effect on long-term health.

There have been several studies investigating the neonatal outcome in children born after ICSI using epididymal sperm and the cumulative data suggested a neutral effect of using epididymal sperm on birthweight outcomes[3, 9, 10, 17-19]. However, only a few studies mentioned whether cryopreserved epididymal sperm were included. Belva et al. included pregnancies derived from both fresh and frozen epididymal sperm in their study, and about 40% of children was born after the use of frozen sperm in cycles using non-ejaculated sperm[17]. However, whether cryopreservation of epididymal sperm affects neonatal outcomes was not reported in their study. Similarly, Oldereid et al. included pregnancies obtained from ICSI using frozen epididymal sperm, leaving the potential effects of cryopreservation unmeasured[19]. Our data may add to the understanding of neonatal outcomes after ICSI with non-ejaculated sperm under context of sperm cryopreservation.

The hypothesis that cryopreservation of epididymal sperm affects the offspring birthweight may be supported by evidence from pregnancies using donor sperm, where sperm cryopreservation is inevitable. A 2018 meta-analysis including 5358 donor sperm pregnancies and 637843 spontaneous pregnancies suggested that sperm donation may increase the risks of low birthweight in offspring in comparison with spontaneous conceptions, without a concomitant effect on preterm delivery[20]. A more recent meta-analysis comparing pregnancies using donor sperm with those using paternal sperm also demonstrated a mild increase in the risks of SGA in the sperm donation offspring[21]. Controversially, however, several studies in specific populations demonstrated associations of an opposite direction. Based the data of the Society of Assisted Reproductive Technology Clinic Outcome Reporting System, a 42 g increase in birthweight was reported in sperm donation offspring comparing with that in partner sperm offspring[22]. Similarly, Chen et al. reported an increase in birthweight in children born after frozen donor sperm intrauterine insemination comparing with those after paternal sperm intrauterine insemination[23]. The heterogeneous results may warrant further investigation on unmeasured or unknown factors that may affect the birthweight in cooperated with sperm cryopreservation.

The postulated effect of sperm cryopreservation on the next generation may be explained by the transgenerational epigenetic information carried by spermatozoon. Previous studies have shown
destructive effects of cryopreservation on sperm, including increasing DNA integrity, causing structural damage, and disrupting mitochondrial function [24]. In addition, subtle changes in sperm epigenetic may play a role in the transmission of the phenotype. Cryopreservation may result in a significant alternation in RNA profiles of sperm [25], and thus may disrupt the RNA based transmission of paternal information. Sperm borne small RNA was known an epigenetic mediator between paternal environmental stressor and offspring phenotypes [26]. Paternal information that reflects the environmental exposure and health status can be encoded in specific subsets of sperm RNA and induce specific phenotype in offspring. Injection of sperm tRNA from high-fat-diet mice into zygotes is sufficient to generate metabolic disorders in offspring [27]. When content of sperm borne RNAs was reduced by RNases, the body weight of offspring was decreased[28]. Taken together, the growing evidence in animals may justify the concerns regarding the transgenerational effects of disrupted sperm borne RNA profile in men.

The study was limited by the retrospective nature which allows no statement of a casual relationship. The fact that selection of using fresh or frozen epididymal sperm was determined by the patients’ preference may introduce bias into the study. Unmeasured or unknown factors may also skew the conclusion. In addition, the relatively small number of participants is another reason to take caution. The sample size has not enough power to detect a significant difference in SGA or LBW, as the events were relatively rare in the population.

In conclusion, the data have demonstrated an association between epididymal sperm cryopreservation and decreased offspring birthweight, suggesting an additional technique factor that might potentially affect children’s development. While earlier studies have provided reassurance for the using of frozen PESA/TESA in terms of pregnancy rates, our study highlighted the needs for focusing on its effects on neonatal outcomes.

Declarations

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Conflicts of interest/Competing interests

The authors declare that they have no conflict of interest.

Availability of data and material

Not applicable

Code availability
Authors' contributions

Jiali Cai: Protocol/project development, Data collection or management, Manuscript writing/editing.
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Haixiao Chen: Data collection or management, Manuscript writing/editing.
Jianzhi Ren: Data analysis, Manuscript writing/editing.

Ethics approval

Institutional Review Board approval for this retrospective study was obtained from the Ethical Committee of the Medical College Xiamen University.

Consent to participate

Informed consent

Signed informed consents were obtained from all the patients prior to the study.

Capsule: Cryopreservation of epididymal sperm decreases birthweight of children conceived through PESA-ICSI

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References

[1] Practice Committee of the American Society for Reproductive Medicine in collaboration with the Society for Male R, Urology. Electronic address aao. The management of obstructive azoospermia: a committee opinion. Fertil Steril. 2019;111:873-80. doi: 10.1016/j.fertnstert.2019.02.013.
[2] Practice Committee of the American Society for Reproductive Medicine. Electronic address aao. Management of nonobstructive azoospermia: a committee opinion. Fertil Steril. 2018;110:1239-45. doi: 10.1016/j.fertnstert.2018.09.012.

[3] Kawwass JF, Chang J, Boulet SL, Nangia A, Mehta A, Kissin DM. Surgically acquired sperm use for assisted reproductive technology: trends and perinatal outcomes, USA, 2004-2015. J Assist Reprod Genet. 2018;35:1229-37. doi: 10.1007/s10815-018-1178-5.

[4] Tournaye H, Merdad T, Silber S, Joris H, Verheyen G, Devroey P, et al. No differences in outcome after intracytoplasmic sperm injection with fresh or with frozen-thawed epididymal spermatozoa. Hum Reprod. 1999;14:90-5. doi: 10.1093/humrep/14.1.90.

[5] Nagy Z, Liu J, Cecile J, Silber S, Devroey P, Van Steirteghem A. Using ejaculated, fresh, and frozen-thawed epididymal and testicular spermatozoa gives rise to comparable results after intracytoplasmic sperm injection. Fertil Steril. 1995;63:808-15. doi: 10.1016/s0015-0282(16)57486-x.

[6] Liu H, Xie Y, Gao L, Sun X, Liang X, Deng C, et al. Impact on using cryopreservation of testicular or epididymal sperm upon intracytoplasmic sperm injection outcome in men with obstructive azoospermia: a systematic review and meta-analysis. J Assist Reprod Genetics. 2020;37:2643-51. doi: 10.1007/s10815-020-01940-1.

[7] Javed A, Ramaiah MK, Talkad MS. ICSI using fresh and frozen PESA-TESA spermatozoa to examine assisted reproductive outcome retrospectively. Obstet Gynecol Sci. 2019;62:429-37. doi: 10.5468/ogs.2019.62.6.429.

[8] Hezavehei M, Sharafi M, Kouchesfahani HM, Henkel R, Agarwal A, Esmaeili V, et al. Sperm cryopreservation: A review on current molecular cryobiology and advanced approaches. Reprod Biomed Online. 2018;37:327-39. doi: 10.1016/j.rbmo.2018.05.012.

[9] Fedder J, Loft A, Parner ET, Rasmussen S, Pinborg A. Neonatal outcome and congenital malformations in children born after ICSI with testicular or epididymal sperm: a controlled national cohort study. Hum Reprod. 2013;28:230-40. doi: 10.1093/humrep/des377.

[10] Jin L, Li Z, Gu L, Huang B. Neonatal outcome of children born after ICSI with epididymal or testicular sperm: A 10-year study in China. Sci Rep. 2020;10:5145. doi: 10.1038/s41598-020-62102-y.

[11] Dai L, Deng C, Li Y, Zhu J, Mu Y, Deng Y, et al. Birth weight reference percentiles for Chinese. PloS one. 2014;9:e104779. doi: 10.1371/journal.pone.0104779.

[12] Hinkle SN, Albert PS, Mendola P, Sjaarda LA, Yeung E, Boghossian NS, et al. The association between parity and birthweight in a longitudinal consecutive pregnancy cohort. Paediatr Perinat Epidemiol. 2014;28:106-15. doi: 10.1111/ppe.12099.
[13] Stamnes Koepp UM, Frost Andersen L, Dahl-Joergensen K, Stigum H, Nass O, Nystad W. Maternal pre-pregnant body mass index, maternal weight change and offspring birthweight. Acta Obstet Gynecol Scand. 2012;91:243-9. doi: 10.1111/j.1600-0412.2011.01321.x.

[14] Nightingale CM, Rudnicka AR, Owen CG, Newton SL, Bales JL, Donin AS, et al. Birthweight and risk markers for type 2 diabetes and cardiovascular disease in childhood: the Child Heart and Health Study in England (CHASE). Diabetologia. 2015;58:474-84. doi: 10.1007/s00125-014-3474-7.

[15] Wang F, Hua Y, Whelton PK, Zhang T, Fernandez CA, Zhang H, et al. Relationship Between Birth Weight and the Double Product in Childhood, Adolescence, and Adulthood (from the Bogalusa Heart Study). Am J Cardiol. 2017;120:1016-9. doi: 10.1016/j.amjcard.2017.06.037.

[16] Ushida T, Kotani T, Kinoshita F, Imai K, Nakano-Kobayashi T, Nakamura N, et al. Maternal low birth weight and hypertensive disorders of pregnancy. Pregnancy Hypertens. 2020;23:5-10. doi: 10.1016/j.preghy.2020.10.010.

[17] Belva F, De Schrijver F, Tournaye H, Liebaers I, Devroey P, Haentjens P, et al. Neonatal outcome of 724 children born after ICSI using non-ejaculated sperm. Hum Reprod. 2011;26:1752-8. doi: 10.1093/humrep/der121.

[18] Woldringh GH, Horvers M, Janssen AJ, Reuser JJ, de Groot SA, Steiner K, et al. Follow-up of children born after ICSI with epididymal spermatozoa. Hum Reprod. 2011;26:1759-67. doi: 10.1093/humrep/der136.

[19] Oldereid NB, Hanevik HI, Bakkevig I, Romundstad LB, Magnus O, Hazekamp J, et al. Pregnancy outcome according to male diagnosis after ICSI with non-ejaculated sperm compared with ejaculated sperm controls. Reprod Biomed Online. 2014;29:417-23. doi: 10.1016/j.rbmo.2014.06.009.

[20] Adams DH, Clark RA, Davies MJ, de Lacey S. Update on: a meta-analysis of sperm donation offspring health outcomes - 2018 update. J Dev Orig Health Dis. 2018;9:561-2. doi: 10.1017/S2040174418000272.

[21] Allen CP, Marconi N, McLernon DJ, Bhattacharya S, Maheshwari A. Outcomes of pregnancies using donor sperm compared with those using partner sperm: systematic review and meta-analysis. Hum Reprod Update. 2021;27:190-211. doi: 10.1093/humupd/dmaa030.

[22] Yu B, Fritz R, Xie X, Negassa A, Jindal S, Vega M, et al. The impact of using donor sperm in assisted reproductive technology cycles on perinatal outcomes. Fertil Steril. 2018;110:1285-9. doi: 10.1016/j.fertnstert.2018.08.012.

[23] Chen L, Zhu L, Cai C, Yan G, Sun H. Clinical and neonatal outcomes of intrauterine insemination with frozen donor sperm. Syst Biol Reprod Med. 2018;64:240-5. doi: 10.1080/19396368.2018.1453563.

[24] Ezzati M, Shanaehbandi D, Hamdi K, Rahbar S, Pashaiasl M. Influence of cryopreservation on structure and function of mammalian spermatozoa: an overview. Cell Tissue Bank. 2020;21:1-15.
Tables

Table 1. Patients characteristics and neonatal outcomes
|                                | Frozen-thawed (n=216) | Fresh (n=220) | Difference (95%CI) | P     |
|--------------------------------|-----------------------|---------------|--------------------|-------|
| Female age, year               | 28.19 (3.72)          | 29.03 (3.94)  | -0.84 (-1.56 to -0.12) | 0.022 |
| PCOS                           | 13 (6)                | 10 (4.5)      | 1.34 (0.58 to 3.14)  | 0.491 |
| Parity                         |                       |               |                    | 0.034 |
| 0                              | 200 (92.6)            | 190 (86.4)    | 1.97 (1.04 to 3.74)  |       |
| ≥1                             | 16 (7.4)              | 30 (13.6)     | 0.51 (0.27 to 0.96)  |       |
| Duration of infertility, year  | 4.08 (3.15)           | 4.04 (2.92)   | 0.04 (-0.53 to 0.61) | 0.893 |
| Female BMI, kg/m²               | 20.93 (2.36)          | 20.68 (2.31)  | 0.25 (-0.19 to 0.69) | 0.262 |
| Female basal FSH, IU/L          | 7.06 (2.1)            | 7.05 (2.54)   | 0.01 (-0.43 to 0.45) | 0.96  |
| AFC                            | 11.46 (4.92)          | 11.32 (4.61)  | 0.15 (-0.75 to 1.04) | 0.751 |
| Male age, year                 | 30.71 (4.78)          | 31.65 (5.07)  | -0.95 (-1.87 to -0.02) | 0.046 |
| Male smoker                    | 114 (52.8)            | 119 (54.1)    | 0.95 (0.65 to 1.38)  | 0.783 |
| Male BMI, kg/m²                | 23.75 (3.17)          | 23.68 (3.35)  | 0.08 (-0.54 to 0.69) | 0.802 |
| Male FSH, IU/L                 | 5 (2.67)              | 5.51 (3.33)   | -0.51 (-1.08 to 0.06) | 0.079 |
| Male testosterone, ng/ml       | 4.33 (1.66)           | 5.39 (5.64)   | -1.06 (-1.85 to -0.28) | 0.008 |
| Peak estradiol level, pg/ml    | 3969.69 (2780.1)      | 3738.16 (2341.98) | 231.52 (-252.01 to 715.06) | 0.347 |
| Endometrial thickness, mm      | 10.4 (2.38)           | 10.38 (2.4)   | 0.03 (-0.42 to 0.48)  | 0.9   |
| Stage of ET                    |                       |               |                    | 0.582 |
| Cleavage                       | 124 (57.4)            | 132 (60)      | 0.9 (0.61 to 1.32)   |       |
| Blastocyst                     | 92 (42.6)             | 88 (40)       | 1.11 (0.76 to 1.63)  |       |
| FET                            | 101 (46.8)            | 97 (44.1)     | 1.11 (0.76 to 1.62)  | 0.576 |
| Vanishing twin                 | 23 (10.6)             | 19 (8.6)      | 1.26 (0.67 to 2.39)  | 0.477 |
| Year of delivery               |                       |               |                    | 0.558 |
| 2013                           | 23 (10.5)             | 19 (8.8)      | 0.83 (0.44 to 1.56)  |       |
| 2014                           | 24 (10.9)             | 36 (16.7)     | 1.63 (0.94 to 2.84)  |       |
| Year | Offspring gender | Male | Female | Mean difference (95% CI) for continuous variables and odds ratio (95% CI) for categorized variables |
|------|-----------------|------|--------|----------------------------------------------------------------------------------|
| 2015 | Offspring gender | 113(52.3) | 119(54.1) | 0.93(0.64 to 1.36) |
| 2016 | male            | 103(47.7) | 101(45.9) | 1.07(0.74 to 1.56) |
| 2017 | female          | 39.09(1.44) | 39.27(1.45) | -0.18(-0.45 to 0.09) |
| 2018 | Gestational age, week | 39.09(1.44) | 39.27(1.45) | -0.18(-0.45 to 0.09) |
| 2019 | Birthweight, g | 3186.57(440.72) | 3303.61(458.55) | -117.04(-201.72 to -32.36) |
| 2020 | Z-score         | -0.18(1.06) | 0.08(1.01) | -0.25(-0.45 to -0.06) |
| 2021 | SGA             | 25(11.6) | 17(7.7) | 1.56(0.82 to 2.99) |
| 2022 | LGA             | 15(6.9) | 23(10.5) | 0.64(0.32 to 1.26) |
| 2023 | LBW             | 11(5.1) | 8(3.6) | 1.42(0.56 to 3.61) |

Data were presented as median (interquartile range) or n (proportion).

a Mean difference (95%CI) for continuous variables and odds ratio (95%CI) for categorized variables.

**Table 2.** Multivariate analyses for birthweight and z-score
| Variables                                      | Birthweight          | Z-score            |
|-----------------------------------------------|----------------------|--------------------|
|                                               | B 95%CI)             | P                  | B 95%CI)             | P                  |
| **Sperm cryopreservation**                     |                      |                    |                    |
| no                                            | Ref                  | Ref               |                     |                    |
| yes                                           | -91.21(-166.7 to -15.72) | 0.018             | -0.27(-0.46 to -0.07) | 0.007             |
| **Transfer cycles**                           |                      |                    |                    |
| Fresh                                         | Ref                  | Ref               |                     |                    |
| FET                                           | 105.45(-6.25 to 217.16) | 0.064             | 0.34(0.06 to 0.63)  | 0.019             |
| **Vanishing twin**                            |                      |                    |                    |
| no                                            | Ref                  | -0.17(-0.5 to 0.15)|                     |                    |
| yes                                           | -74.84(-201.35 to 51.67) | 0.246             |                     |                    |
| **Stage of transferred embryo(s)**             |                      |                    |                    |
| Cleavage                                      | Ref                  | Ref               |                     |                    |
| Blastocyst                                    | 11.77(-94.58 to 118.12) | 0.828             | 0.01(-0.26 to 0.29) | 0.926             |
| **PCOS**                                      |                      |                    |                    |
| no                                            | Ref                  | Ref               |                     |                    |
| yes                                           | -135.46(-313.41 to 42.48) | 0.136             | -0.43(-0.88 to 0.03) | 0.068             |
| **Duration of infertility, year**             | -12.47(-26.85 to 1.91) | 0.089             | -0.03(-0.06 to 0.01) | 0.174             |
| **Female BMI, kg/m²**                         | 24.4(7.07 to 41.74)  | 0.006             | 0.07(0.03 to 0.12)  | 0.002             |
| **Female age, year**                          | -4.75(-19.8 to 10.29) | 0.536             | -0.02(-0.06 to 0.02) | 0.28              |
| **Male age, year**                            | -1.09(-12.01 to 9.83) | 0.845             | 0(-0.03 to 0.03)   | 0.864             |
| **Female basal FSH, IU/L**                    | -12.71(-29.85 to 4.43) | 0.146             | -0.04(-0.08 to 0)  | 0.076             |
| **Male BMI,**                                 | 1.3(-10.55 to 13.15) | 0.83              | 0(-0.03 to 0.03)   | 0.94              |
| **Female basal AFC**                          | 1.44(-7.4 to 10.27)  | 0.75              | 0.01(-0.02 to 0.03) | 0.641             |
| **Male FSH, IU/L**                            | 4.4(-8.08 to 16.88)  | 0.49              | 0.02(-0.02 to 0.05) | 0.37              |
| **Male T, ng/ml**                             | 4.06(-5.07 to 13.18) | 0.384             | 0.01(-0.01 to 0.03) | 0.436             |
| **Endometria thickness, mm**                  | -1.1(-19.21 to 17.01) | 0.905             | -0.01(-0.05 to 0.04) | 0.817             |
| **Peak E2, log transferred**                  | -61.92(-126.73 to 2.88) | 0.061             | -0.17(-0.33 to 0)  | 0.049             |
| Parity       | Ref | Ref |
|-------------|-----|-----|
| 0           |     |     |
| ≥1          | 33.21(-98.4 to 164.82) | 0.621 -0.02(-0.35 to 0.32) | 0.921 |

| Smoker      | Ref | Ref |
|-------------|-----|-----|
| no          |     |     |
| yes         | 133.59(59.47 to 207.7) | <0.001 0.36(0.17 to 0.55) | <0.001 |

| Year of delivery | Ref |
|------------------|-----|
| 2013             |     |
| 2014             | -95.69(-247.86 to 56.48) | 0.218 0.17(-0.56 to 0.22) | 0.39 |
| 2015             | -107.7(-259.27 to 43.88) | 0.164 -0.25(-0.64 to 0.14) | 0.201 |
| 2016             | -69.07(-221.1 to 82.96) | 0.373 -0.15(-0.54 to 0.24) | 0.454 |
| 2017             | -15.1(-163.88 to 133.67) | 0.842 0(-0.39 to 0.38) | 0.985 |
| 2018             | -219.98(-371.27 to -68.69) | 0.004 -0.55(-0.94 to -0.16) | 0.006 |
| 2019             | -202.63(-379.48 to -25.78) | 0.025 -0.55(-1 to -0.09) | 0.018 |

| Offspring gender | - |
|------------------|---|
| Female           | Ref |
| Male             | 23.69(-51.44 to 98.83) | 0.537 |

| Gestational age, week | <0.001 |
|-----------------------|--------|