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Effect of Vitrified Frozen-Thawed Embryo Transplantation on Glycometabolism of the Offspring Mice

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Abstract

Objective: To investigate the change of glycometabolism in the offspring from *in vitro* fertilization through fresh embryo and vitrified embryo transplantation, and evaluate the effect of vitrification technology on the health of offspring.

Materials and method: Offspring mice from fresh embryo transfer (FET) and vitrified embryo transfer (VET) were divided into two groups. Natural mating mice were used as the control. Introperitoneal glucose tolerance (IPGTT) and insulin tolerance test (IPITT) were performed in the adulthood offsprings. Total RNA was extracted from 14.5dpc fetus and neonate livers, and glycometabolism-related genes were detected by quantitative RT-PCR.

Results: The glucose levels of IPGTT at 30 min and 120 min in offspring mice from VET were significantly higher than control, and the glucose level at 120 min was significantly higher than that in FET. In liver tissues from 14.5 dpc embryos, the mRNA expression of Igf1 and Igf2 in FET and VET groups, the mRNA expression of Glut8 in VET was significantly higher than that in the control. In livers of newly born mice, the mRNA expression of Igf1 and Igf2 in FET, Glut1 and Glut2 in FET and VET was significantly lower than that in the control.VET and FET, especially VET, may affect the glycometabolism of offspring mice.

Keywords: *In vitro* fertilization; Fresh embryo; Vitrified embryo; Offspring; Mouse; Glycometabolism

Abbreviations

IVF: *In Vitro* Fertilization; FET: Fresh Embryo Transfer; VET: Vitrified Embryo Transfer; IPGTT: Introperitoneal Glucose Tolerance; IPITT: Insulin Tolerance Test; ICSI: Intracytoplasmic Sperm Injection; PGD: Preimplantation Genetic Diagnosis; IGF2: Insulin-Like Growth Factor 2; GLUT: Glucose Transporter; PMSG: Pregnant Mare Serum Gonadotropin; HCG: Human Chorionic Gonadotropin

Introduction

Since the first child through *in vitro* fertilization (IVF) was born in 1978, assisted reproductive technologies (ART), including IVF, intracytoplasmic sperm injection (ICSI) and preimplantation genetic diagnosis (PGD), have been used for the treatment of infertility [1]. The majority of children born through ART appeared healthy. However, the potential risks of ARTs are still being debated. Increasing new evidences suggest that the culture of pre-implantation mouse embryos may affect the expression of imprinted genes and fetal development and behavior [2]. Aberrant expression of imprinted genes has been observed on day 9.5 of the development after implantation of mouse embryos [3]. It has been reported that *in vitro* fertilization-embryo transfer (IVF-ET) manipulation was associated with low birth weight, Type-II diabetes, obesity, insulin resistance, [4,5]. However, the corresponding mechanisms leading to these issues are poorly known.

The embryo cryopreservation has been widely used in ART since the first child was born by using this technology in 1984 [6]. The remnant embryos were cryopreserved for re-transplantation in women who did not conceive after fresh embryo transplantation, which could avoid the occurrence of ovarian hyper-stimulation syndrome in these women. The vitrification technique may bring higher pregnancy rate and survival rate when compared with traditional cryopreserved techniques [7]. However, the vitrified embryos could lead to abnormal methylation of H19 and insulin-like growth factor 2 (IGF2) [8]. IGFs play important roles in the development of embryos [9]. For example, IGF-1 may promote blastocyst formation and glucose uptake by activating glucose transporter (GLUT) [10] and AMPK [11]. Meanwhile, the decreased expression of IGF-2 could be observed in the placenta of IVF/ICSI [12] and the 10.5 dpc placenta of IVF-ET [13]. Similarly, the expression of IGF-2 in pancreas of the fetuses with Type-II diabetes revealed the decrease by 55% [14], which suggests that parental infertility and ART manipulation may interfere with early development of embryos, and result in abnormal pregnancy and offspring defects [15]. De Rycke et al. have evaluated potential risks of ART children, and found that ART could lead to the change of epigenetic modification-related genes [16]. Moreover, some epidemiological investigations also reveal that ART may induce birth defects and some diseases, such as short gestational age, thyroid dysfunction, cardiovascular diseases, Type-II diabetes, and Beckwith-Wiedemann and Angelman syndromes [17].

Based on these findings, we have conducted the investigation to explore the effect of vitrification technique on body weight and body fat of the off-spring. In the present study, we further investigated the glycometabolism of the offspring mice from vitrified embryo and fresh embryo transplantation, which will provide the evidence for the selection of transplantation methods.

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Materials and Methods

Animals

All Kunming mice were acclimatized for 2 weeks under a standard environment (room temperature: 20-24°C, relative humidity: 50-60%) with a 12 h light-dark cycle before experiment. They may adlibitum access to water and food containing 20% protein and 9% fat. All animals and procedures performed in this study were approved by animal ethics committee of Nanjing Jinling Hospital.

Experimental grouping

The experiment included control, fresh embryo transfer (FET) and vitrified frozen-thawed embryo transfer (VET) groups. In the control group, 6-week old oestrous female mice were naturally mated with 8-week old male mice overnight. Then, vaginal plug of female mice was checked in the following morning and this was considered as day 0.5 of pregnancy. The offspring mice were born after approximately 3 weeks.

In FET and VET groups, 6-week old dioestrous female mice were subjected to the injection of 10 IU pregnant mare serum gonadotropin (PMSG, the Second Hormone Factory, Ningbo, China), and after 48 h, these mice were injected with 10 IU human chorionic gonadotropin (HCG, the Second Hormone Factory, Ningbo, China) for superovulation.

In vitro fertilization and embryo culture: Sperms from cauda epididymis of male mice were collected and incubated in 100 μL of HTF medium (Sage Quinn's, USA) covered with paraffin oil (Sigma, USA) for 1 h (37°C,5% CO_2, saturated humidity). Cumulus-oocyte complexes were recovered from oviducts of female mice after hCG injection for 14 h. Then, sperms were co-cultured with cumulus-oocyte complexes for 4-6 h for IVF. Fertilized eggs were transferred to 30 μL of cleavage medium (CM, Sage Quinn's, USA) covered with paraffin oil and cultured for 36-48 h to obtain embryos at 4-cell stage. The 4-cell embryos were transferred to 30 μL of blastula medium (BM, Sage Quinn's, USA) covered with paraffin oil and cultured for 24-36 h to obtain blastocysts. The blastocysts were randomly divided into two groups, FET for direct blastocyst transfer and VET for vitrified freezing. The blastocysts for vitrified freezing were incubated in equilibration solution (ES, Kitazato, Japan) for 10 min, then in vitrification solution (VS, Kitazato, Japan) for 30 s. Ten blastocysts were loaded onto each cryotop (Kitazato, Japan) and immediately stored in liquid nitrogen with the preservation time of 1 month. Before thawing, all media including thawing solution (TS, Kitazato, Japan), diluent solution (DS, Kitazato, Japan), washing solution 1 (WS1, Kitazato, Japan) and washing solution 2 (WS2, Kitazato, Japan) were prepared. The cryotop with blastocysts was taken out from liquid nitrogen and directly immersed in TS for 60 s, and then DS for 60 s, WS1 for 3 min, WS2 for 3 min, and finally transferred into 30 μL of BM covered with mineral oil for 1.5-2.5 h (37°C, 5% CO_2, saturated humidity).

Blastocyst transfer: Ten-week old male mice were vasectomized, and then healed for 2 weeks before experiment. Oestrous female mice were mated with vasectomized male mice. The female mice with copulatory plugs in the following morning were used as the recipients for 0.5 day of pseudopregnancy. Then, fresh blastocysts and vitrified frozen-thawed blastocyst were transferred into these recipients at 2.5 days of pseudopregnancy. The offspring mice were obtained after approximately 3 weeks.

Collection of livers from fetus and newly born mice

On day 14.5 of pregnancy, a small number of female mice were sacrificed, and fetus livers were collected and immerged into liquid nitrogen, and then stored at -80°C for further analysis. The livers from newly born mice were collected as above-mentioned operation. Determination of glycometabolism-related genes by quantitative RT-PCR (qRT-PCR)

Total RNA was extracted with RNAprep Pure Tissue Kit (Tiangen, China) from livers, and cDNA was synthesized using PrimeScript™ RT-PCR Kit (Takara, Japan). qRT-PCR was performed using the SYBR-Green Kit (Vazyme, China). The β-actin was served as an internal reference gene for data normalization. The primers used in this study are listed in Table 1. Data were analyzed using the $2^{-\Delta\Delta CT}$ method.

Intraperitoneal glucose tolerance test (IPGTT) and insulin tolerance test (ITT)

The adult offspring mice were subjected to IPGTT or ITT at the age of 12 weeks old. After fasting for 16 h, the mice were injected with glucose at the dose of 2 g/kg or insulin with 1 IU/kg according to body weight. Then, the glucose levels in blood samples from mouse tails were measured at 0, 15, 30, 60 and 120 min post-injection by a glucometer (Johnson, USA) for IPGTT, and at 0, 30, 60, 90, and 120 min for ITT, respectively.

Statistical analysis

All data were expressed as mean ± standard deviation (SD) and analyzed using SPSS 16.0 software (SPSS, USA). The comparison among three groups was conducted by One-way ANOVA and the comparison between two groups was conducted by Independent-Samples *t* test. A P-value of 0.05 was considered to be statistically significant.

Results

In the control group, 7 pregnant mice were obtained, and delivered 65 off-springs. In FET group, 7 pregnant mice were obtained, and delivered 59 off-springs, while in VET group, 8 pregnant mice were obtained and delivered 65 off-springs, respectively. There was no significant difference in litter size among FET group (8.42 ± 2.30), VET group (8.13 ± 1.36) and control group (9.29 ± 1.80) ($P > 0.05$).

For IPGTT, the glucose levels at 30 min and 120 min in VET group were significantly higher than those in the control group, and the glucose level at 120 min was also higher than that in FET group (Figure 1A). However, there was no significant difference in glucose levels at 0, 15, 30, 60 and 120 min between FET group and control group. In addition, the area under the curve (AUC) from IPGTT in VET group was significantly higher than that in FET and control groups (Figure 1B).

For ITT, there was no significant difference in glucose levels at 30, 60, 90 and 120 min among FET, VET and control groups (Figure 2A).

Gene	Primer sequence 5- 3	Product size (bp)
Glut1	F: TCTTAAGTGCGTCAGGGCGT	133
	R: GTCACCTTCTTGCTGCTGGGAT	
Glut2	F: ATCAGGACTGTATTGTGGGCT	110
	R: CAAGGGCCAGTTGGTGAAGA	
Glut8	F: TGCCTCTTCATTGCTGGCTT	111
	R: GAGGACACAGATGCCGGTAG	
Igf1	F: GCCTATTTTTCTATGGCAGCCTC	128
	R: CTGAAGTACAAAGTCTTCGCTC	
Igf2	F: CTCACCTTCAGCAGCGTCCA	111
	R: ATTGGTACCACAAGGCCGAAGG	
β-actin	F: GGCTGTATTCCCCTCCATCG	245
	R: TCCTATGGGAGAACGGCAGA	

Table 1: Primers for qRT-PCR.

Meanwhile, there was also no significant difference in AUC from ITT among three groups (Figure 2B).

In livers of 14.5 dpc embryos, the mRNA expression of Igf1 and Igf2 in FET and VET groups were significantly lower than that in the control group ($P < 0.01$, Figure 3), while there was no significant difference between FET and VET groups. Similarly, there was no significant difference in mRNA expression of Glut1 and Glut2 among FET, VET and control groups ($P > 0.05$, Figure 3). However, the mRNA expression of Glut8 in VET group was significantly higher than that in the control group ($P < 0.05$, Figure 3), but there was no significant difference between FET and control groups ($P > 0.05$).

In livers of newly born mice, the mRNA expression of Igf1 in FET group was significantly lower than that in the control group ($P < 0.05$, Figure 4), while there was no significant difference between VET and control groups and between VET and FET groups. The mRNA expression of Igf2 in FET and VET groups was significantly lower than that in the control group, while there was no significant difference between FET and VET groups (Figure 4). Similarly, the mRNA expression of Glut1 and Glut2 in FET and VET groups was significantly lower than that in the control group, while there was no significant difference between FET and VET groups (Figure 4). However, there was no significant difference in mRNA expression of Glut8 among FET, VET and control groups.

In the control group, the mRNA expression levels of Igf1, Igf2, Glut2, and Glut8 in livers of 14.5 dpc embryos were significantly lower than those in newly born mice ($P < 0.01$, Figure 5A), while there was no significant difference in mRNA expression level of Glut1 between 14.5 dpc embryos and newly born mice. In FET group, the mRNA expression levels of Igf1 and Igf2 in livers of 14.5 dpc embryos were significantly lower than those in newly born mice ($P < 0.01$, Figure. 5B), and the mRNA expression level of Glut1 was significantly higher than that in newly born mice ($P < 0.01$), but there was no significant difference in mRNA expression levels of Glut2 and Glut8 between them (Figure 5B). In VET group, the mRNA expression levels of Igf1, Igf2 and Glut2 in livers of 14.5 dpc embryos were significantly lower than those in newly born mice ($P < 0.01$, Figureure 5C), and the mRNA expression level of Glut1 was significantly higher than that in newly born mice ($P < 0.01$), but there was no significant difference in mRNA expression level of Glut8 between them (Figure 5C).

Between FET and VET groups, for IPGTT, the glucose level at 120 min and the area under the curve (AUC) from IPGTT in VET group was higher than that in FET group (Figures 1A and 1B). For ITT, there

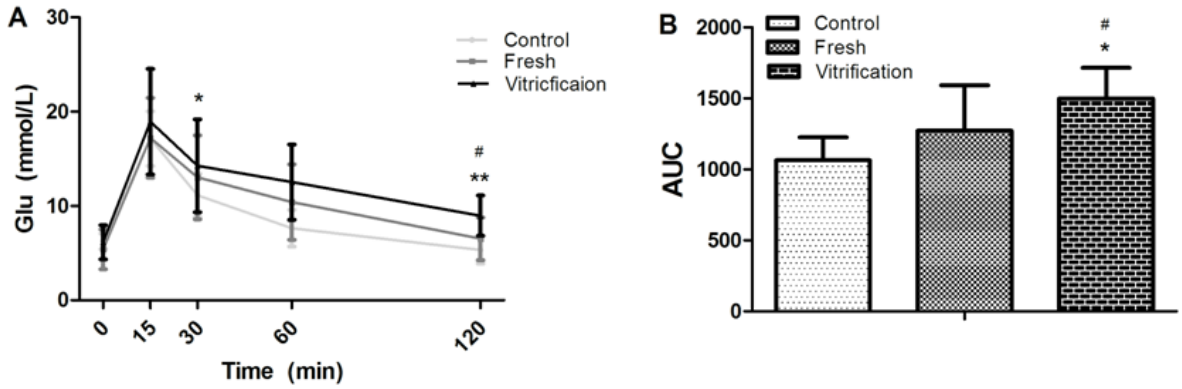


Figure 1: The results of IPGTT in 12-week old offspring mice from FET (n = 9), VET (n = 12) and control (n = 12) groups. A: IPGTT; B: Area under the curve (AUC) from IPGTT. *P < 0.05, **P < 0.01 versus control group; #P < 0.05 versus fresh group.

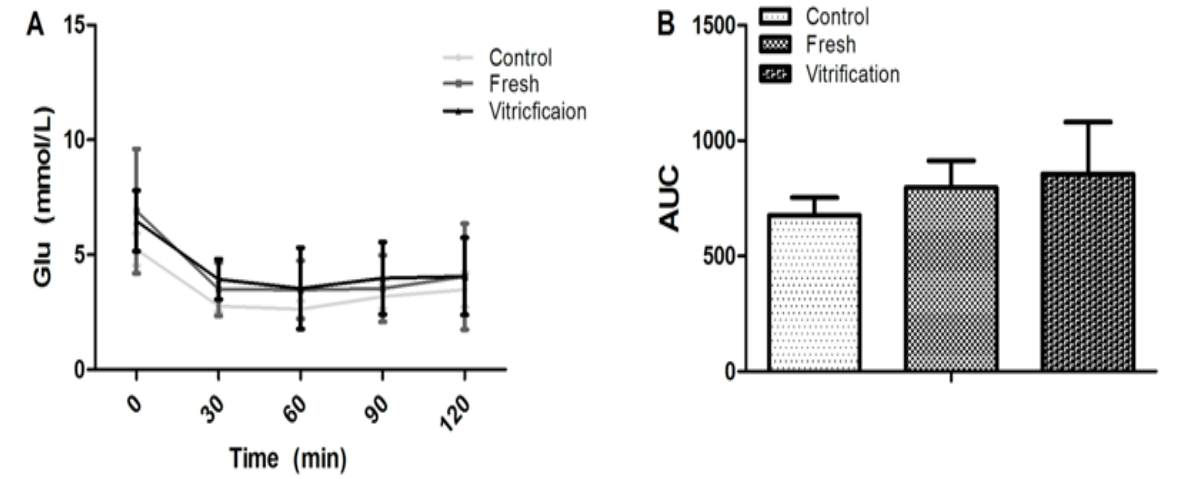


Figure 2: The results of ITT in 12-week old offspring mice from FET (n = 9), VET (n = 12) and control (n = 12) groups. A: ITT; B: Area under the curve (AUC) from ITT.

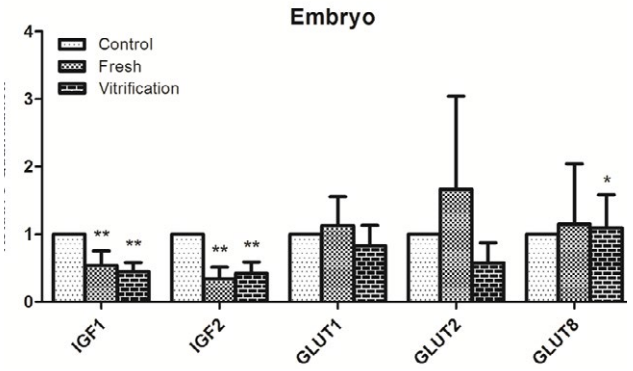


Figure 3: Expression of Igf1, Igf2, Glut1, Glut2 and Glut8 genes in livers of 14.5 dpc embryos from FET (n = 11), VET (n = 12) and control (n = 12) groups. *P < 0.05, **P < 0.01 versus control group.

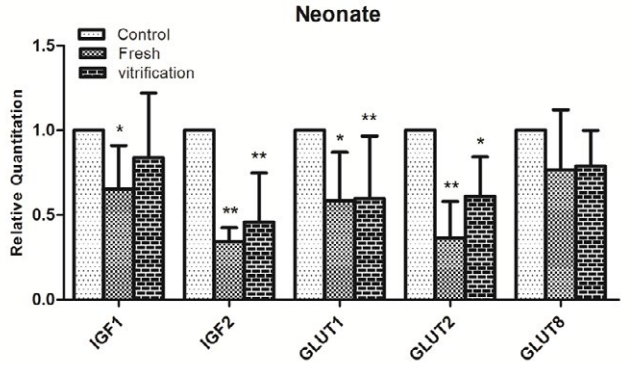


Figure 4: Expression of Igf1, Igf2, Glut1, Glut2 and Glut8 genes in livers of newly born mice from FET (n = 12), VET (n = 12) and control (n = 12) groups. *P < 0.05, **P < 0.01 versus control group.

was no significant difference in glucose levels and AUC from ITT among the two groups (Figures 2A and 2B). No matter in livers of 14.5 dpc embryos or newly born mice, there was no significant difference between FET and VET groups in mRNA expression of Igf1, Igf2, Glut1, Glut2 and Glut8. (Figures 3 and 4).

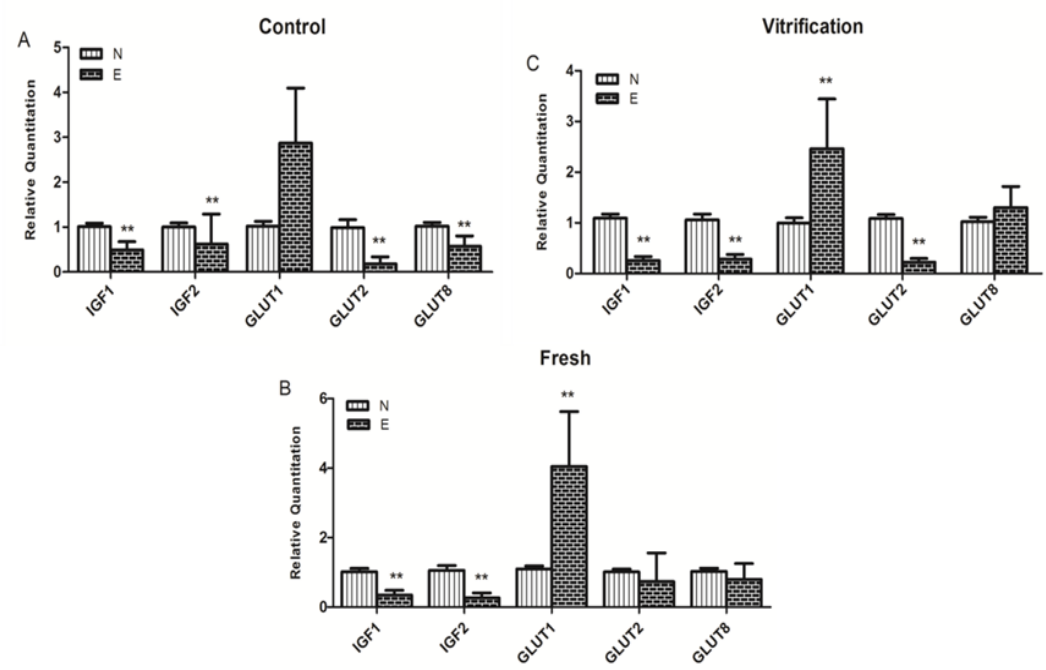
Discussion

With the improvement of pregnancy rate of ART, the number of transferred embryos is reduced. The cryopreservation of embryos is important for IVF laboratories. The surplus embryos are available for cryopreservation. Vitrification technique has become the first-selected freezing technology due to its higher frozen-recovery rate and survival rate of embryos. Mamo et al. have explored the effect of vitrified freezing on 8-cell stage mouse embryos by high-density oligonucleotide microarray, and found that the abnormal expression genes were correlated with metabolism, regulatory role and stress response [18]. Abnormal imprinting genes may lead to a variety of syndromes. Similarly, the change of epigenetics induced by ART may also influence the development of the fetus, and even the structure of chromatin [19].

According to previous report, there is no significant difference in the distribution of body fat from 4 to 14 years old children between IVF and natural mating [20]. Donjacur et al. have detected the distribution of body lean and fat mass in IVF offspring by dual energy X-ray absorptiometry (DEXA) scanning, and found that the fat mass exhibits an obvious increase as the extension of age [5]. Similarly, Tamashiro et al. have reported that the mice derived from *in vitro* cultured embryos have many obesity-associated characteristics [21]. Moreover, the change in distribution of body fat has been found in the ICSI children [22]. It is widely accepted that the increased body fat can lead to the onset of metabolic diseases in adults, such as insulin resistance, obesity, diabetes, and cardiovascular diseases. In order to investigate the effect of vitrified freezing on glycometabolism of the offspring, we have conducted IPGTT and ITT in adult offspring. The IPGTT results showed that the glucose level at 120 min in VET group was significantly higher than that in the control group but no difference with FET group, indicating that the offspring mice from VET need more time to clear serum glucose. The evidence in humans shows that ART may lead to vascular dysfunction in offspring children [23]. Moreover, the adult mice from IVF-FET may present impaired glucose metabolism and insulin resistance [5]. Although these changes have not occurred in our investigation, the increased glucose level has been observed. It is possible that the 12-week old offspring mice will develop to impaired glucose metabolism and insulin resistance, or that the limited sample size may affect our observation. Therefore, although our investigation indicated that vitrified freezing may influence the glycometabolism of offspring mice, more samples and long-term investigations to explore the effects of IVF and vitrified freezing on their offsprings are required.

Liver is the most important organ to regulate glucose homeostasis. A plenty of evidences suggest the fetal origin of diabetes [24]. In order to further investigate the effect of vitrified freezing on glycometabolism in embryo stage and the possible cause leading to abnormal glycometabolism in adulthood, we compared the expression of Igf1 and Igf2 genes in livers of 14.5 dpc embryo and newly born mice from VET and FET technology. Igf1 was closely related to individual growth and development, glucose uptake and glycogen synthesis. When an adult was famished for 7-10 days, the expression of Igf1 mRNA in liver revealed a significant decrease. Moreover, lower serum Igf1 level is also detected in ART children [25], and the increased expression of Igf1 after birth could improve brain development restriction induced by malnutrition [26]. It is reported that the expression of Igf2 gene in 9.5 dpc fetus and placenta from IVF-ET is decreased [3], and the abnormal expression of Igf2 may lead to abnormal embryonic development and congenital malformation [27]. Our study showed that IVF-ET might lead to the decreased expression of Igf1 and Igf2 genes in livers of off-springs. Therefore, the nutritional status plays an important role in mRNA expression of Igf1 in livers [28]. In the model of intrauterine growth retardation, the growth and development of fetus could be delayed, the expression of Igf2 gene could be decreased, and the metabolism syndrome can be formed in the adult stage [29]. All these findings indicate that IVF-ET has great influence on the growth of offspring embryo and may lead to fetus intrauterine growth retardation. Igf2 plays a critical role in glucolipid metabolism [30]. In our investigation, we found that IVF-ET could induce the decreased expression of Igf2 gene in offspring, and that the expression of Igf1 gene was improved with the birth of mice.

Glucose may maintain the metabolism and growth of embryos after 4-cell stage. The fetal glycometabolism is dependent on plasma glucose and insulin levels, and fetal glucose utilization is directly dependent on insulin produced by pancreas. Abnormal glycometabolism may be associated with abnormal glucose transporters. Therefore, we also determined the expression of glucose transporters such as Glut1, Glut2 and Glut8 genes in livers of 14.5 dpc embryos and newly born mice from VET, FET and control groups. The embryo glycometabolism



E: 14.5 dpc embryo; N: Newly born mice. A: Control group; B: FET group; C: VET group. **P < 0.01 versus newly born mice.

Figure 5: The expression of Igf1, Igf2, Glut1, Glut2, Glut8 genes in livers of 14.5 dpc offspring and newly born mice from FET, VET and control groups.

is dependent on glucose uptake, which is mediated by glucose transporters. Glucose homeostasis is also maintained by glucose transporters. The mutation of Glut1 gene may result in the lack of energy supply for brain tissue and cerebral dysgenesis. Chronic fetal hypoglycemia may produce a subsequent decline of Glut1 in liver [31]. Compared with adult rats, the expression of Glut2 mRNA and protein in livers of fetal and suckling rats reveals an obvious decrease. The expression of Glut2 mRNA is also dependent on glucose level [32]. In our investigation, we found that the expression of Glut1 and Glut2 genes in livers of 14.5 dpc embryos from VET and FET groups was similar to that in the control group, but the expression in newly born mice was significantly lower than that in the control group, suggesting that IVF-ET may influence the expression of Glut1 and Glut2 genes in neonates, and may further influence the glycometabolism of offspring mice. Hyperglycaemia in blastocyst stage is related to the decreased expression of Glut1 mRNA and protein [33]. Glut2, accounting for more than 97% of glucose transporters in liver tissue, is responsible for transporting glucose. Its mRNA and protein expression is decreased in diabetic rats [34]. Moreover, the expression of Glut2 mRNA in fetus and neonate with intrauterine growth restriction is significantly lower than that in controls [35]. The expression of Glut8 mRNA presents an increase in diabetes rats [36], The Glut8 expression in the fetuses of the VET increased significantly compared with the control group in our study. This result may be suggested that vitrification offspring have the risk of diabets. And FET is slightly better than VET in glycometabolism.

Conclusion

The vitrification technology may influence the glycometabolism of off-springs from FET and VET, and VET could increase risk of diabetes rather than FET.. First, in adult offspring, serum glucose level in VET mice is increased significantly. Next, the expression of Igf1, Igf2, Glut1, Glut2 and Glut8 in livers of embryos and newly born mice from VET and FET presents significant changes when compared with the control group. Therefore, VET and FET, especially VET, may influence the glycometabolism of off-springs. This animal study indicated that VET may have less negative impact in glycometabolism of off-springs than FET, which can have certain guiding significance in choice of embryo transplantation in human ART. However, there are still some shortcomings in our investigation, such as the small sample size, short observation time, and limited markers. The long-term effect of vitrified freezing on the development and growth of the offspring needs to be explored in the future.

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