Brief co-incubation of gametes benefits the outcomes of newborns

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Abstract

Purpose The objective of this study was to determine whether ammonium accumulates in IVF media during fertility process and whether the brief co-incubation of gametes (bIVF) benefited the outcomes of newborns.

Methods Ammonium levels in IVF media during gamete co-incubation were measured and the effects of bIVF on neonatal outcomes were evaluated retrospectively in this study.

Results A total of 609 live newborns cycles were included in this study. The results showed that ammonium levels in the conventional IVF (cIVF) media was significantly increased than that in bIVF and control media (27.32 ± 5.60 vs 20.71 ± 3.89 , $P = 0.03$; 27.32 ± 5.60 vs 19.46 ± 1.31 , $P = 0.01$, respectively). In the cIVF group, the mean gestational age was significantly lower (37.36 ± 2.29 vs. 37.74 ± 1.94 weeks, $P = 0.031$) and the incidence of preterm birth (< 37 weeks) was higher than that in the bIVF group (25.80 vs. 17.63%, $P = 0.015$). Singleton cycles and twin cycles were then analyzed respectively. The gestational age and birth weight of the singleton cycles were similar between the two groups. However, of the twin cycles, the gestational age was significantly decreased and the rate of preterm birth was increased significantly in the cIVF group (35.76 ± 2.31 vs. 36.48 ± 1.73 , $P = 0.013$; 53.33 vs. 31.52%, $P = 0.002$, respectively).

Conclusions There is an ammonium accumulation in IVF media during co-incubation of gametes. And bIVF reduces the risk of preterm birth (< 37 weeks), especially with regard to preterm birth of the twin cycles, and seems to be a safe alternative method for improving the neonatal outcomes compared with cIVF.

Keywords Ammonium · Neonatal outcomes · Preterm birth · Brief incubation

Introduction

It is important that culture media provide a proper environment to support embryo development in vitro. However, recent studies in humans indicated that the culture media used during IVF treatment could influence intrauterine growth and birth weight of newborns [1, 2]. A number of studies have

reported that, compared with natural conception, IVF was associated with an increased risk of low birth weight and preterm birth [1, 3–5]. Current studies have shown that the types of culture media [6] or the in vitro culture period [7, 8] of embryos could influence neonatal outcomes such as birth weight and gestational age.

Kleijkers et al. [9, 10] reported that the birth weight of singletons born after IVF showed an inverse association with age of the embryo culture media, which may be caused by the ammonium accumulation during storage and incubation. Our previous study showed that ammonium levels increased significantly greater in the non-pregnant group than the pregnant group during the period of embryo culture [11]. Lane and Gardner [12] reported that the presence of ammonium in culture media had significant detrimental effects on the regulation of embryo physiology and genetics, such as increasing apoptosis, perturbing metabolism, even altering the expression of the imprinted gene *H19*. Therefore, we can speculate that neonatal outcomes would be altered by the ammonium accumulated in culture media.

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Brief co-incubation of gametes (bIVF), reducing the insemination time from 18 to 1–2 h, has been used to improve the IVF outcomes by reducing the detrimental effects of reactive oxygen species (ROS) on embryo viability compared with conventional IVF (cIVF) [13–17]. However, whether ammonium would be accumulated during IVF and the effects of bIVF on the outcome of newborns are not well documented yet. So, the purpose of this study was to examine the varieties of ammonium in IVF media during IVF and to retrospectively evaluate bIVF on gestational age, birth weight, and birth defects.

Material and methods

Patients

Patients who underwent a gonadotropin-releasing hormone (GnRH) agonist or GnRH antagonist protocol at the Reproductive Medicine Center of Sun Yat-sen Memorial Hospital between 2011 and 2013 were enrolled in this study. Fresh embryos were transferred on day 3. Patients for whom pre-implantation genetic diagnosis (PGD) had been performed and patients with donor oocytes or donor sperm were excluded. When we analyzed the singleton cycles, patients who were diagnosed with more than one fetal sac but delivered singletons (vanishing twin [18]) were excluded. This study was approved by the Ethics Committee of Sun Yat-Sen Memorial Hospital. All patients gave written informed consent.

Sperm preparation and insemination procedures

Semen samples were collected by masturbation after 3 days of sexual abstinence. Samples were assessed for sperm concentration, motility, and vitality before and after selection, according to WHO guidelines (WHO 1999, 2010). After liquefaction, sperm were selected by gradient centrifugation (90 and 45%, SpermGrad; Vitrolife). The oocytes were inseminated with $1\text{--}5 \times 10^5/\text{mL}$ sperm 3–5 h after oocyte retrieval.

Co-culture regimes

In the bIVF group, the gametes were incubated for 2 h, and the oocytes were then removed, washed gently in fresh culture media, and placed into another culture dish filled with IVF media. The oocytes in the cIVF group were co-incubated with sperm for 16–18 h. All oocytes were denuded and assessed for fertilization about 18 h later in both groups.

Embryo transfer

Embryos with the best morphology were transferred using a soft cook transfer catheter on day 3 after oocyte retrieval. Two embryos were transferred for the patients <35 years. Three

Fig. 1 Ammonium concentration in different groups. *There was a significant difference compared with other groups ($P < 0.05$)

embryos were transferred if the age of the patient was ≥ 35 years or if the patient had experienced one or more failed IVF cycles. The embryo transfer was canceled if the patients did not have embryos available for transfer.

Outcome measures

The data on neonatal outcomes were obtained after birth, by telephone interview. The questionnaire included data on gestational age, sex, birth weight, and congenital malformations. Diagnosis of congenital malformations were made by the hospitals where the neonate was born.

Ammonium level measurement

For the measurement of the ammonium levels, patients were included if the oocytes retrieved more than 10. And the oocytes were divided into bIVF and cIVF groups equally. The

Fig. 2 Ammonium concentrations in different oocytes groups. *There was a significant difference compared with other groups ($P < 0.05$)

Fig. 3 Ammonium levels in different sperm concentration groups

IVF media of bIVF and cIVF were collected 2 and 18 h later after insemination respectively. The ammonium levels in the media of the two groups was then measured immediately. The control media was the IVF media that did not contain any gametes and placed in the same incubator with the bIVF and cIVF culture media. Ammonium level in the culture media was measured by Ammonium Test Kit II (Arkray, Japan) at room temperature. twenty microliters specimen (culture media) was used for ammonium measurement every time. Every specimen measured twice and the average was used for the final analysis. When a specimen is applied to the sample-receiving layer of the test area, the alkaline buffer agent (borate buffer) impregnated in the layer dissolves making the specimen alkaline. The ammonium ions in the specimen are gasified by the alkaline state into ammonium gas. The ammonium gas passes through the pores of the spacer to indicator

(bromocresol green) triggering color development. As the degree of color development is proportional to the concentration of ammonium gas produced, the specimen ammonium levels is quantitatively determined by measuring the color with the AMMONIUM CHECKER II (Arkray, Japan). The measurement range is 7–286 μM.

AMMONIUM CHECKER II can continuously measure the ammonia concentration. The interval between the samples was about 5 s. There is no fixed order in the detection of samples, which could avoid the influence of time interval on the results of ammonium test.

Statistical analyses

The data were analyzed using SPSS statistical software (version 17.0). Continuous variables were presented as the mean ± SD, and differences in the means between groups were determined using a two-sample *t* test. Differences in ammonium concentrations among groups were analyzed using ANOVA test. For categorical variables, a Pearson χ^2 test was used to analyze the differences between groups. The adjusted odd ratios (ORs) for preterm births (< 37 weeks), early preterm births (< 25 weeks), low birth weight (< 2.5 kg), and very low birth weight (< 1.5 kg) were calculated by logistic regression analysis. Statistical significance was determined at a *P* value of < 0.05.

Results

A total of eight patient samples were tested for ammonium levels. The ammonium levels in bIVF and control groups was

Table 1 Comparison of patient characteristics in the two groups

	cIVF (<i>N</i> = 314)	bIVF (<i>N</i> = 295)	<i>P</i>
Maternal age (years)	30.86 ± 3.91	31.35 ± 3.95	0.123
Paternal age (years)	33.32 ± 4.92	33.67 ± 4.77	0.370
Body mass index (BMI)	21.10 ± 2.62	21.07 ± 2.39	0.869
Infertility duration (years)	4.36 ± 3.03	4.71 ± 3.07	0.162
NO. of treatment cycles	1.17 ± 0.46	1.27 ± 0.64	0.027
NO. of oocytes retrieved	11.38 ± 4.82	11.61 ± 5.08	0.564
2PN rate (%)	68.01 (2330/3426)	66.98 (2394/3574)	0.360
Rate of top-quality embryos (%)	24.01 (522/2174)	25.99 (616/2370)	0.124
Embryo transfer number	2.19 ± 0.45	2.24 ± 0.48	0.160
Type of infertility			
Primary	111 (35.35%)	114 (38.64%)	0.400
Secondary	203 (64.65%)	181 (61.36%)	
Infertility factor			
Female	234 (74.52%)	223 (75.59%)	0.760
Male	13 (4.14%)	6 (2.03%)	0.135
Mix	53 (16.88%)	54 (18.31%)	0.644
Unknown	14 (4.46%)	12 (4.07%)	0.812

Table 2 Neonatal outcomes of live newborns from cIVF and bIVF

	cIVF (N = 314)	bIVF (N = 295)	P
Gestational age (weeks)	37.36 ± 2.29	37.74 ± 1.94	0.031
Mean birth weight (kg)	2.85 ± 0.60	2.88 ± 0.56	0.412
No. of singletons	209 (66.56%)	203 (68.81%)	0.553
No. of twins	105 (33.44%)	92 (31.19%)	
Male/female ratio	200/219 (1.11)	204/183 (0.91)	0.158
No. of preterm deliveries (< 37 weeks)	81 (25.80%)	52 (17.63%)	0.015
No. of very preterm deliveries (< 32 weeks)	9 (2.87%)	3 (1.02%)	0.101
Low birth weight (< 2.5 kg) ^a	88 (28.03%)	77 (26.10%)	0.593
Very low birth weight (< 1.5 kg) ^a	6 (1.91%)	4 (1.36%)	0.590
No. of birth defects	5 (1.59%)	0 (0.0%)	0.062

^a The average birth weight of twins was used to compare the birth weight of all cycles in different groups

similar (20.71 ± 3.89 vs 19.46 ± 1.31 , $P = 0.54$). However, in cIVF group, ammonium levels was significantly increased than that in bIVF and control groups (27.32 ± 5.60 vs 20.71 ± 3.89 , $P = 0.03$; 27.32 ± 5.60 vs 19.46 ± 1.31 , $P = 0.01$) (Fig. 1). We also examined the relationship between ammonium concentrations and the number of oocytes and sperm concentration. The results showed that ammonium concentrations in the group with more than 5 oocytes (> 5) was significantly higher than the group with the number of oocytes less than 5 (≤ 5) and the control group (29.89 ± 7.81 vs 18.83 ± 2.11 , $P = 0.001$; 29.89 ± 7.81 vs 19.43 ± 3.13 , $P = 0.003$, respectively) (Fig. 2). However, the ammonium concentrations in different sperm concentration groups were similar (Fig. 3).

A total of 609 live birth cycles, which included 412 singleton cycles and 197 twin cycles, were analyzed. As shown in Table 1, the characteristics of patients in the two groups were similar except the number of cycles treated. Table 2 shows the neonatal outcomes in the two groups. The mean gestational age was significantly lower (37.36 ± 2.29 vs. 37.74 ± 1.94 weeks, $P = 0.031$), and the incidence of preterm birth (< 37 weeks) was significantly higher (25.80 vs. 17.63%, $P = 0.015$) in the cIVF group than that in the bIVF group. The incidence of early preterm birth, low birth weight, and

very low birth weight were higher in the cIVF group than in the bIVF group, but the difference was not significant (2.87 vs. 1.02%, $P = 0.101$; 28.03 vs. 26.10%, $P = 0.593$; and 1.91 vs. 1.36%; $P = 0.590$, respectively).

On a separate note, five newborns in the cIVF group were born with congenital malformations, including congenital heart disease, scoliosis and harelip, intestinal deformity, harelip, chromosome abnormality (47, xxx). The age of the five birth defect-newborn mothers were 27, 41, 26, 36, and 32 years old, respectively. Of the newborns with congenital malformation in this study, two were singletons and one of them was a female baby with congenital heart disease who had a normal sister, born 8 years before. The other three cases were twins and all of them included a normal brother or sister. None of the parents of these newborns with birth defects had a chromosomal examination before entering the IVF program. There were other three congenital malformations in the cIVF group (not enrolled in this study because they did not meet the inclusion criteria; not shown in the tables), including cleft lip and palate, agenesis of corpus callosum, and right toe defect. No congenital malformation was seen in the bIVF group. However, the rate of congenital malformation was not significantly different between the two groups (1.59 vs. 0.0%, $P = 0.062$) (Table 2).

Table 3 The effect of different co-incubation time of gametes on neonatal outcomes of live born singletons and twins

	Singleton cycles		P	Twin cycles		P
	cIVF (N = 168)	bIVF (N = 163)		cIVF (N = 105)	bIVF (N = 92)	
Gestational age (weeks)	38.17 ± 1.87	38.23 ± 1.75	0.762	35.76 ± 2.31	36.48 ± 1.73	0.013
< 37 weeks	18 (10.71%)	20 (12.27%)	0.657	56 (53.33%)	29 (31.52%)	0.002
Mean birth weight (kg) ^a	3.146 ± 0.48	3.143 ± 0.46	0.958	2.29 ± 0.42	2.38 ± 0.41	0.125
< 32 weeks	2 (1.19%)	1 (0.61%)	1.00	6 (5.71%)	2 (2.17%)	0.209
< 2.5 kg	13 (7.74%)	14 (8.59%)	0.777	70 (66.67%)	59 (64.13%)	0.709
< 1.5 kg	1 (0.60%)	1 (0.61%)	1.00	4 (3.81%)	3 (3.26%)	1.00
Z-score	0.149 ± 0.99	0.126 ± 1.07	0.845	—	—	—

^a The average birth weight of twins was used to compare birth weight

Table 4 Adjusted risk of adverse outcomes in 806 newborns after cIVF and bIVF

Outcomes	<i>P</i>	OR (95% CI) cIVF versus bIVF ^b
Preterm births (< 37 weeks)	0.001	2.783 (1.496–5.176)
early preterm births (< 32 weeks)	0.065	6.173 (0.896–42.521)
Low birth weight (< 2.5 kg) ^a	0.736	1.118 (0.586–2.130)
Very low birth weight (< 1.5 kg) ^a	0.348	2.288 (0.406–12.901)

^a The average birth weight of twins was used to compare birth weight

^b Adjusted for parents' age, cycles of treatment, BMI, parity, type of infertility, infertility diagnosis category, duration of infertility, number of oocytes retrieved, number of embryos transferred, and implantation number

We further analyzed the singleton and twin cycles (Table 3). In the singleton cycles, the gestational age, birth weight, and the gender and gestational age-adjusted birth weight (Z-scores) were similar in the two groups. On the contrary, the gestational age was decreased significantly and the preterm birth rate was increased significantly in the twin cycle subgroup. We then adjusted for confounding factors that may have affected the clinical results; the risk of preterm birth still differed significantly between the two co-culture regimes in the twin cycles (Table 4).

Discussion

This study showed that ammonium accumulated in IVF media during gamete co-culture and bIVF improved neonatal outcomes, especially with regard to preterm birth of twin cycles.

Kleijkers et al. [9, 10] reported that birth weight of singletons born after IVF showed an inverse association with ammonium levels accumulated in ready-to-use embryo culture media incubation. Similarly, our study also showed that ammonium levels increased during the IVF procedure. Zander et al [19] revealed that embryos, in particular between the zygote to two-cell, was extremely sensitive to ammonium. Some studies also showed that ammonium in the culture environment could alter the expression of imprinted genes related to metabolism and neural development [20–22]. Lane and Gardner [12] revealed that ammonium in culture media had significant detrimental effects on the regulation of embryo physiology and genetics, such as altering the expression of the imprinted gene *H19*, which associated with intrauterine growth of fetals. This means that ammonium accumulated during IVF procedure has an adverse effect on embryo development and/or neonatal outcomes.

A systematic review and meta-analysis indicated that when compared with natural pregnancies, IVF singletons have poorer obstetric and prenatal outcomes [23]. These include increased rates of preterm birth, low birth weight, and congenital malformations. Moreover, recent studies have indicated that higher rates of preterm birth and low birth weight were observed in singletons resulting from blastocyst transfer vs. non-blastocyst transfer [1, 7]. Kallen et al. [24] showed that the risk for early preterm delivery (< 32 weeks) of gestation

was significantly increased among dizygotic twin pairs born after IVF compared spontaneously conceived twins. For the first time, we show that compared with cIVF, bIVF significantly improved the gestational age, especially for the twin cycles. This means that bIVF is safer than cIVF in ART.

Congenital malformations were observed in five children conceived after cIVF (1.59%). The rate of congenital malformation was higher than that seen in another study (0.6%) [8] but was lower than the results of Anthony et al. (3.2%) [25]. However, none of the parents of the newborns with congenital malformations underwent chromosomal examinations, so genetic factors cannot be completely excluded for these congenital malformations, especially for the singletons with birth defects.

It has been well established in a number of studies that many factors determine neonatal outcomes, such as maternal age [26], infant gender [27, 28], culture methods, and parental characteristics, including age, BMI [29], infertility style, and parity. After we adjusted for these confounding factors, the risk of preterm birth still showed a significant difference between the two groups. Lifestyle is also associated with neonatal outcomes [28], but these data were not available in our database, which may be one of our study limitations.

In conclusion, there is ammonium accumulation during IVF and bIVF seemed to be a safe alternative method for improving the neonatal outcomes. This result needs further study for verification, and the impact of co-incubation time of gametes on epigenetic changes in the embryos needs further study.

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Author's contribution Ruiqi Li analyzed the data and drafted the manuscript. Songbang O, Nengyong Ouyang, Lingyan Zheng, and Meiqi Mai collected the data. Qingxue Zhang revised the manuscript. Dongzi Yang and Wenjun Wang conceived and designed the study. All the authors interpreted the data.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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