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Effects of MⅡ stage oocytes zona pellucida birefringence on pregnancy outcome

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ABSTRACT

Objective: To explore the effects of different MⅡ stage oocytes zona pellucida birefringence on pregnancy outcome. **Methods:** A total of 46 couples with infertile which induced by single cause received *in-vitro* fertilization treatment were analyzed retrospectively, and randomly divided into the high zona birefringence (HZB)/HZB group, HZB/low zona birefringence (LZB) group and LZB/LZB group according to different oocytes zona pellucida birefringence. Intracytoplasmic sperm injection outcome was analyzed and compared. **Results:** The proportion of HZB oocytes, implantation rate and the pregnancy rate were decreased in three groups (HZB/HZB group>HZB/LZB group>LZB/LZB group) ($P<0.05$). But there was no significantly different between the number of oocytes and fertilization rate of these groups ($P>0.05$). Logistic regression analysis showed that factors affect MⅡ stage oocytes zona pellucida birefringence were age, basal FSH level and the LH level on the day of HCG injection. Age and FSH levels were negatively correlated with the single oocyte zona pellucida birefringence; While the LH level on the day of hCG injection was positively correlated with the single oocyte zona pellucida birefringence. **Conclusions:** The primary influence factors on MⅡ stage oocytes zona pellucida are age, basal FSH level and the LH level on the day of hCG injection. The birefringence value of zona pellucida can affect the pregnancy outcome.

1. Introduction

Since the first case of healthy test-tube baby was born in 1992, a growing number of test-tube baby has become an ideal way to solve some family's childbearing problem[1]. The success rate of intracytoplasmic sperm injection (ICSI) treatment is still is not ideal, so how to improve it has become the main directions for researchers. Studies have shown that sperm-zona pellucida (ZP) plays an important role in the normal process of fertilization. According to the changes of ZP birefringence, ZP birefringence≥0.5 is high zona birefringence (HZB); ZP birefringence <0.5 is low zona birefringence (LZB)[2]. So if the ICSI is carried out according to different zona pellucida birefringence, it is still a problem whether there is different quality of fertilized embryos and different pregnancy rate. This study was designed to investigate whether ZP can affect pregnancy outcomes.

2. Materials and methods

2.1. Material and groups

The launching of this subject has been approved by the medical ethics committee of the First Affiliated Hospital of Sun Yat-sen University. A total of 46 infertile patients who received ICSI from October 2007 to September 2008 in our hospital were taken as the research object. The

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inclusion criterions were as follows: Male factor is the only reason for infertility; Scheduled for ICSI treatment; 5≤ oocyte number<30. Patients aged <35 and with 2 transferred embryos were included in the study. They were randomly divided into the HZB/HZB group (n=20), HZB/LZB group (n=16) and LZB/LZB group (n=10) according to different oocytes zona pellucida birefringence of embryos transferred before fertilization.

2.2. Agents and method

2.2.1. Main reagents and instruments

Human serum albumin (HSA): Sweden Vitrolife company; Gonal-F injection: recombinant follicle-stimulating hormone (r-FSH), Swiss Serono Company, 75 IU per ramus; Human menopausal gonadotropin (HMG): Lizhu company, 75 IU per ramus. Profasi injection: human chorionic gonadotropin (hCG), Swiss Serono Company, 5 ramus; Triptorelin (GnRH-a) and injection: triptorelin acetate purchased from Beaufour-Ipsen International,France (3.75 mg/ramus). Hyaluronidase: Sigma Company. Polarization microscope (Polscope): OCTAX ICSI Guard TM polarized light system and OCTAX EyeWare TM measuring software (CRI Company, USA); Olympus Ⅸ 71 inverted microscope (Type Ⅸ 70, Japan; with the Hoffman system, Germany); Incubator: French FIV6; CO_2 incubator: American Forma,type 371 3308; Super-clean worktable: Denmark K-SYSTEMS, type *in-vitro* fertilization (IVF)-L26; Sperm counting chambers: Israel Makler Counter chamber, 0.01 mm^2, 10 μm deep; Pasteur pipettes: Sigma Company (S6143), after disinfection.

2.2.2. Experimental method

After 3-7 days abstinence, spermatozoa were fetched by masturbation in the relatively sterile conditions. After routine analysis, semen 1.5-5 mL was placed between 80% lower phase solution and 40% upper phase liquid 1.5 mL after 30 min incubation. It was centrifuged at 200 g × 18 for 20 min, supernatant was removed. And it was added into 2 mL G-FERT medium, well mixed before centrifuge washing (200 g×5 min). Then supernatant were abandoned, the precipitate was break and mixed well then added into the bottom of the 0.4-0.6 mL Falcon tube with G-FERT culture solution which containing 5% HSA. The bottle cap was opened and placed in a 6% CO_2 incubator to hatch and prepare for fertilization.

Oocyte transvaginal was obtained under B ultrasound guidance by superovulation. The collected follicular fluid was poured into a 60 mm petri dish, and the selected eggs were washed in the culture medium containing 5% HAS G-MOPS. OCCC were inhaled into the buffered HTF (G-MOPS) solution Petri dishes (Nunc 35 mm) which contained hyaluronidase (80 IU/mL) and repeated pipetting. The eggs were transferred to a Petri dish with culture solution containing 5% HSA G-MOPS and then washed. OCTAX ICSI Guard TM imaging system was used to observe the zona pellucida. Then them were transferred into prepared Falcon1006 petri dishes according to different groups and received ICSI.

After 16-18 h of ICSI fertilization, morphology and number of the fertilized egg prokaryotic was checked under an inverted microscope. Two pronuclei and two polar bodies within the egg under the inverted microscope marked the normal fertilization. After 72 h of ovum retrieval, the high-quality embryos for artificial insemination was choosed under B-guided .

2.3. Results determination

2.3.1. Pregnancy and follow-up

After 14 d of ET, urine and serum β-hCG level were measured to determine whether there is biochemical pregnancy, if positive, continue to give the corpus luteum support. 28 days after ET, B ultrasound was adopted to detect the fetal heart rate, fetal sac and embryo development, diagnosed clinical pregnancy.

2.3.2. General information

The patient's general information (age, height, weight, etiology, *etc*.) was collected, the dose of GnRH-a down-regulated, basis, hormone level (FSH, LH, E_2, prolactin, testosterone) on the start date and the day of HCG injection, Gn dosage and the time for usage, endometrial thickness, the number of oocytes, MⅡ stage oocytes number, fertilized egg score in pronuclear stage, D3 embryo score, the number of top quality embryos, fertilization rate, cleavage rate, implantation rate, pregnancy rate were measured. Formula of various rates was as following:

Fertilization rate = fertilized egg number/MⅡ oocytes× 100%;

Implantation rate = total number of the plant embryo/total number of transplanted embryonic×100%;

Clinical pregnancy rate = number of pregnancy cycles/ number of transfer cycles ×100%.

2.4. Statistical methods

Data were expressed as mean±SD values, the enumeration data were expressed as frequency and percentage. One sample t-Test was applied in the comparison between two groups. Mean comparison in groups was conducted with single factor variance analysis. Correlation and regression analysis were identified by logistic regression, single factor regression analysis was performed to analyze the impact

of independent variables to the dependent variables, the independent variables which $P<0.05$ were included in multiple regression analysis. All of the data were analyzed by SPSS16.0 statistics software, $P<0.05$ was regarded as statistical significance.

3. Results

3.1. Effect of oocyte zona pellucida birefringence on clinical pregnancy outcome

The average age of cases was (30.00±2.25) years old, and the average period was 1.10±0.31; the retrieved oocyte number was 559, and the average number of oocytes retrieved per cycle was 12.15±3.27.

3.2. Comparison of clinical pregnancy outcome

The result showed signficant differences in he proportion of HZB oocytes, the implantation rate and the pregnancy rate among the groups .From the HZB/HZB group to the LZB/LZB group, all of the above indicators showed a decreasing trend. But the retrieved oocyte number and the fertilization rate had no significant difference among groups, which suggested that the higher oocyte zona pellucida birefringence, the better clinical pregnancy outcome would be. In addition, during the 46 cycles, the number of top quality embryos obtained after fertilization of all HZB oocytes was significantly higher than the LZB oocytes (43.17% *vs* .22.81%, $P<0.05$) (Table 1).

Table 1

Comparison of the general situation, hormone levels, situation of medication and clinical pregnancy outcome of the patients.

Parameters	HZB/HZB group	HZB/LZB group	LZB/LZB group	*P* value
Retrieved oocyte number (*n*)	11.58±4.94	12.33±2.82	11.67±3.87	0.332
HZB oocytes proportion (%)	45.08	30.20	17.35	0.002
Fertilization rate (%)	82.92	79.87	80.61	0.757
Implantation rate (%)	40.00	25.00	10.00	0.046
Pregnancy rate (%)	65.00	37.50	110.00	0.013

Table 2

Factors affecting the MⅡ stage oocytes zona pellucida birefringence (single factor analysis).

Parameters		B	SE	Wald	Sig.	Exp(B)	95% *CI* for EXP(B)	
							Lower	Upper
	Age (years old)	−0.071	0.025	8.073	0.004	0.931	0.887	0.978
	BMI (kg/m^2)	0.013	0.041	0.095	0.758	1.013	0.935	1.097
	cycle (*n*)	0.282	0.213	1.746	0.186	1.326	0.873	2.014
	GnRH-a dose (mg)	0.650	0.783	0.690	0.406	1.916	0.413	8.882
Basic hormone levels	FSH (IU/L)	−0.138	0.060	5.329	0.021	0.871	0.775	0.979
	LH (IU/L)	−0.070	0.046	2.271	0.132	0.932	0.851	1.021
	FSH/LH	0.092	0.065	2.005	0.157	1.097	0.965	1.246
	E_2 (pg/mL)	−0.004	0.005	0.765	0.382	0.996	0.987	1.005
	Testosterone	−0.105	0.389	0.072	0.788	0.901	0.420	1.931
	PRL	0.027	0.010	7.646	0.056	1.027	1.008	1.047
Hormone levels on the start-up day	FSH (IU/L)	−0.051	0.128	0.163	0.686	0.950	0.740	1.220
	LH (IU/L)	−0.092	0.150	0.376	0.540	0.912	0.680	1.224
	E_2 (pg/mL)	−0.011	0.006	3.112	0.078	0.989	0.976	1.001
	Gn application time (d)	−0.239	0.064	14.040	0.000	0.787	0.695	0.892
	Gn dose (ramus)	−.022	0.009	6.172	0.013	0.979	0.962	0.995
Hormone levels on the day of hCG injection	LH (IU/L)	0.437	0.187	5.448	0.020	1.548	1.073	2.234
	E_2 (pg/mL)	0.000	0.000	13.766	0.000	1.000	1.000	1.000
	Progestin	−0.637	0.200	10.117	0.001	0.529	0.357	0.783
	Retrieved oocyte number (*n*)	0.021	0.019	1.243	0.265	1.022	0.984	1.061
	MⅡ stage oocytes number (*n*)	−0.019	0.023	0.713	0.398	0.981	0.939	1.025

3.3. Factors affecting MⅡ stage oocytes zona pellucida birefringen

The results were shown that age, basal FSH level, hormone application time and dose, the LH level and P level on the day of hCG injection all affected the single oocyte zona pellucida birefringence (Table 2). The independent variable with $P<0.05$ in single factor analysis were included in the multivariate regression analysis. The result shown that age, basal FSH level, the LH level on the day of hCG were still the factors which affect edthe single oocytes zona pellucida birefringence (Table 3).

Table 3
Factors affecting the MⅡ stage oocytes zona pellucida birefringence (multivariate analysis).

Parameters	B	SE	Wald	Sig.	Exp(B)	95% CI for EXP(B)	
						Lower	Upper
Age (years old)	−0.066	0.027	5.753	0.016	0.936	0.887	0.988
Basic FSH (IU/L)	−0.235	0.066	12.863	0.000	0.790	0.695	0.899
HCG day LH (IU/L)	0.619	0.207	8.946	0.003	1.857	1.238	2.785

4. Discussion

Oocytes zona pellucida birefringence is an important indicator to assess oocyte quality, it also reflect the complex structure and integrity of the zona. However, this has not yet been fully confirmed, and there were even some controversy. This study found that embryo developed from egg with high zona pellucida birefringence may obtain better clinical pregnancy outcome, the implantation rate and pregnancy rate were significantly higher than the egg with low birefringence. That suggested oocyte zona pellucida birefringence had good prediction of clinical pregnancy prognosis, which is consistent with the related studies[3]. In addition, this study found that there was no statistical difference between the fertilization rate of the egg with high zona pellucida birefringence and the egg with low birefringence, which maybe related to the ICSI treatment, but has nothing to do with the natural fertilization. And the number of top quality embryos obtained after fertilization of all HZB oocytes was significantly higher than the LZB oocytes, which suggested that eggs with higher birefringence has better egg quality and embryo development[4,5].

A number of studies have confirmed the success rate of *in vitro* fertilization-embryo transfer is directly related to the quality of transferred embryos. While the IVF center at home and abroad both evaluate the embryo quality based on the embryo morphology score, but its limitations can't be ignored[6,7]. So find a reliable and stable detection indicators can not only assess egg quality and predict the developmental potential of embryos accurately, but also help to improve the clinical pregnancy outcome. OCTAX ICSI Guard TM system is a device which can automatically detect and analyze the molecular structure within the egg by the polarized light principle, it can record and analyze the zona pellucida data quickly and efficiently, and doesn't need more than one angle measurement and additional calculation[8,9]. Moreover, this study determined that using polarized light to measure photoresist strength of zona pellucida and then to predict the oocyte quality and the vitality after fertilization is more accurate. Combined with some foreign studies, this study suggest that zona pellucida birefringence may be a reliable and stable indicator that can predict the potential of the egg and the development of embryos, and reduce the number of transferred embryos, and thus can avoid the occurrence of multiple pregnancies. During the study process, we found that this method does not damage the eggs nor affect its activity[10].

The study found that the patient's age, basal FSH levels, the LH level on the day of hCG injection can be independent factor that influencing the single oocyte zona pellucida birefringence. There was a significant negative correlation between the patient's age, basal FSH levels and the single oocyte zona pellucida birefringence; while the LH level on the day of hCG injection had a positive correlation with it. The main reason we consider is that with the increasing of the age, the pregnancy rate decreased, the number of follicles in the ovary and the number of oocytes were significantly reduced, so was the quality of the dominant follicle. The age with the most reproductive potential is 18-25, it began to decline since 30, and after 35 the ovarian follicles storage decreased significantly[11]. We also think that there was a negative correlation between the patient's age and oocytes zona pellucida birefringence. The elder the patient is, the lower the zona pellucida birefringence will be. The oxygen free radicals in the follicle increased with the age, which made the egg apoptosis increased, thereby further reduce the pregnancy rate. Some studies suggest that basal FSH levels is often considered to be a trend of reducing the follicles and indicators of reducing ovarian reserve function, which led to the decreased quality of oocytes and not ideal clinical pregnancy outcome. However, this conclusion is still controversial[12].

Some studies think that there are estrogen receptors in human oocyte. By paracrine and autocrine-estrogen involved

in the development and maturation process of the follicle and oocyte, therefore excessively low LH levels may lead to the follicle developmental disorders and immature eggs. The oocyte zona pellucida birefringence increases with the increase of LH levels, which indicating better quality of the egg. Therefore in order to obtain better clinical pregnancy outcome, it is very important to reduce the the GnRHa dose or increase the LH dose or maintain an appropriate level of LH of HMG, prevent them from being too high or too low[13].

In summary, this study found oocytes zona pellucida birefringence and clinical pregnancy outcome was positively correlated by analyzing the zona pellucida birefringence of patients with ICSI and the clinical pregnancy outcomes and the related factors. Using high birefringence zona eggs can obtain better pregnancy outcomes, which suggested oocyte zona pellucida birefringence is a reliable indicator to predict the developmental potential of the egg and embryonic. This study also found that the patient's age, basal FSH levels, the LH level on the day of hCG injection can affect the oocyte zona pellucida birefringence to different degree, thereby affecting the quality of the egg. Therefore take positive measures to control the controllable factors to improve egg quality will improve the patient's clinical pregnancy outcomes, and then improve the implantation rate and pregnancy rate.

Conflict of interest statement

We declare that we have no conflict of interest.

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