Outcomes of intracytoplasmic sperm injection using the zona pellucida-bound sperm or manually selected sperm

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Abstract

Purpose Zona pellucida (ZP)-bound sperm used for intracytoplasmic sperm injection (ICSI) enhances embryo quality, implantation, and clinical pregnancy rates. This study aimed to assess the pregnancy outcomes and clinical significance of ICSI with ZP-bound sperm.

Method A total of 84 infertile couples who underwent cycles of ICSI following failed in vitro fertilization between June 2012 and February 2014 were enrolled and randomized (1:1): in the treatment group, ICSI was performed using ZP-bound sperm; in the control group, ICSI was performed in a standard manner. Rates of fertilization, cleavage, high-quality embryos, and clinical pregnancy were compared between the two groups.

Results There were no significant differences in age, infertile period, gonadotrophin dose, number of metaphase II oocytes, and number of embryo transfers between the two groups ($P > 0.05$). The clinical pregnancy rate was higher in the treatment group than in the control group, but without statistical significance (60.5 vs. 47.6 %, $P > 0.05$). No significant differences in the rates of fertilization and cleavage were observed (83.0 vs. 81.6 %, and 96.3 vs. 96.5 %, both $P > 0.05$), but

higher rates of high-quality embryos and useable embryos were observed with ZP-bound sperm compared with controls (66.1 vs. 50.8 % and 76.0 vs. 66.3 %, both $P < 0.05$).

Conclusions ICSI using ZP-bound sperm might increase the embryo quality and number of useable embryos, possibly improving the clinical pregnancy outcome of ICSI.

Keywords In vitro fertilization · Intracytoplasmic sperm injection · Sperm-zona pellucida binding test · Embryo transfer · Clinical pregnancy rate · Fertilization rate · Cleavage rate · High-quality embryo rate

Introduction

Intracytoplasmic sperm injection (ICSI) is an important treatment for severe oligoasthenospermia [1]. It is performed as part of an in vitro fertilization cycle and has proven to be an effective method for infertility issues in men with suboptimal semen parameters or with no or low fertilization rates after conventional in vitro fertilization [2]. In addition, this technique spares all the initial and natural steps of fertilization and sperm selection including sperm-zona pellucida (ZP) binding, fusion, and penetration of the vitelline membrane. Novel indications of ICSI appeared in recent years and now include severe oligoasthenospermia, requirement for sperms from the testis or epididymis, in vitro fertility failure, appearance of antisperm antibodies, requirement for pre-implantation genetic diagnosis, and patients with poor ovarian response. Data from national and regional registers suggested an increased use of ICSI from 39.6 % of the assisted reproductive technology (ART) cycles in 1997 to 58.9 % in 2004 [3]. Nevertheless, due to insufficient safety evidence from in vivo studies and because most of the male infertilities are caused by genetic factors, there is still a possibility that ICSI could be associated

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with an increased risk of congenital malformations and genetic abnormalities in the babies.

Ideally, sperm-ZP binding is crucial for the initiation of the normal fertilization process, and it can only be accomplished by a sperm with a normal morphology and intact acrosome [4]. During ICSI, a sperm is subjectively selected by the lab technician (based on morphology and activity) and is injected into the oocyte cytoplasm, sparing the natural selection between a sperm and the ZP [5]. Thus, the selection of a functioning sperm without defect is crucial for improving the safety of this technique. Previous studies by Liu et al. [6, 7] showed that the use of ZP-bound sperms resulted in high-quality embryos. A study by Casciani et al. [8] showed that the use of sperms able to bind to the ZP resulted in good clinical outcomes after ICSI.

In this study, we aimed to provide an objective and natural evaluation using sperm-ZP binding for sperm selection, to explore the pregnancy outcomes with ICSI using ZP-bound sperm, and to offer a novel approach for sperm optimization for ICSI.

Material and methods

Subjects

This study selected and recruited 84 infertile couples (excluding those with testicular or epididymal sperm retrieval) undergoing ICSI at the Reproductive Center of Yinchuan Maternal and Child Care Service Center between June 2012 and February 2014 due to failures of *in vitro* fertilization. Patients were randomized 1:1 to two groups by their oocyte retrieval dates: those with retrieval on odd-numbered days were allocated to the treatment group and those with retrieval on even-numbered days were allocated to the control group. Oocytes from the treatment group (42 cycles) were evaluated after a 2-h incubation, and one to three immature oocytes or those with a low score were selected to bind the sperms. Two hours later, sperm-ZP binding was performed to select high-quality sperms for ICSI. Only those with successful binding to the ZP were selected. In the control group (42 cycles), ICSI was performed directly 4 h after oocyte retrieval.

Clinical manipulations

1. Controlled ovarian hyper-stimulation. Controlled ovarian hyper-stimulations in the two groups were performed by experienced clinicians with a title higher than associated chief. Abdominal subcutaneous injections of follicle-stimulating hormone (Gn, Gonal-F, Merck Serono, USA) were given daily at fixed time from the third to the fifth days of menstruation until two to three dominant follicles with a diameter of 18–20 mm were observed by B-mode ultrasound. The timing of intramuscular injections of human chorionic gonadotropin (hCG, Livzon,

China) was determined according to the blood hormone levels; 34–36 h after hCG injection, oocytes (mature and immature ones in the treatment group and only mature ones in the control group) were retrieved under the guidance of vaginal B-mode ultrasound (GE Healthcare, Waukesha, WI, USA). Mature and immature oocytes were treated in the exact same way.

2. Treatment of the oocytes and evaluation of nuclear maturity. After retrieval, oocytes were incubated at 37 °C with 5 % CO₂. Two hours later, oocytes were treated with a fertilization medium (ART-1020, SAGE, USA) containing 80 IU/L of hyaluronidase (SAGE, USA) to separate the cells. Oocyte-corona-cumulus complexes (OCCCs) were pipetted repeatedly to remove the granulosa cells on the oocytes. The oocytes were then transferred to dishes containing ART-1020 (SAGE, USA) for morphological observation and evaluation. Nuclear maturity of the oocytes was evaluated with the following four phases: germinal vesicle phase (GV), metaphase I (MI), metaphase II (MII), and MII in degeneration.
3. Evaluation standards were as follows [9]: (1) oocytes in the GV phase are without cumulus, but are with dense corona radiata (sometimes absent), while the oocytes are closely enveloped by granulosa cells and reproductive bubbles can be found inside the oocyte cytoplasm after removing the granulosa cells; (2) in oocytes in the MI phase, granulosa cells at the corona radiata are enlarged, the first polar bodies have not been discharged, and the reproductive bubbles inside the oocyte cytoplasm are small; (3) in oocytes in the MII phase, the corona radiata cells are expanded around the oocytes while the granulosa cells are expanded into translucent cells with indefinite forms, and the first polar bodies can be found; and (4) in hypermature oocytes, oocytes are pale and sphere-shaped, with detached corona radiata cells around them and are surrounded by granulosa cells, forming small and black droplets.
4. Preparation of sperms [6, 7, 10]. After retrieval, the sperms were placed at a constant temperature of 37 °C for 30–60 min. After liquefaction, the sperms were treated as follows: (a) centrifugation in 80 and 40 % upper phase gradients (PureCeption, SAGE, USA) at 1800 rpm for 20 min; (b) washing of the sperm pellets with the sperm washing solution (SAGE, USA) after removing the supernatant; (c) centrifugation at 1500 rpm for 15 min; and (d) after removal of the supernatant, culture of the pelleted sperms in the fertilization medium (ART-1020, SAGE, USA) for 30 min. Sperms in the control group were treated in the same way.
5. Sperm-ZP binding. Sperms were co-incubated with immature or degenerating oocytes (5000 sperms/oocyte) in a center-well dish (#3037, BD Biosciences, Franklin Lake, NJ, USA) at 37 °C with 5 % CO₂ for 2 h. Sequentially, the

oocytes were transferred onto dishes with ART-1023 culture medium and covered with mineral oil. After that, sperms remotely binding to the ZP were collected with a microneedle (Sunlight Medical, Jacksonville, FL, USA) after about 10 s and transferred in a 7 % polyvinylpyrrolidone (PVP) solution (SAGE, USA) for further experiments. Immature oocytes used for ZP-sperm binding were discarded.

6. Sperm immobilization test and microinjection. Six droplets of culture medium (each containing 5 μ L of ART-1023, SAGE, USA) and one droplet of PVP solution were added onto the microinjection dish (Falcon #1006; BD Biosciences, Franklin Lake, NJ, USA). During ICSI, one oocyte was placed into each ART-1023 droplet with 1 μ L of sperm. Microinjection was performed using the Hoffman Microscope System, pre-heated at 37 °C. In the treatment group, only the sperms that had been washed off by the ZP were used for ICSI. In the control group, ICSI was conducted using sperms with good mobility and no obvious morphological deformities.
7. Embryo quality assessment criteria [11–13]. Embryos were cultured in cohorts, fertilization features were observed 17–20 h after fertilization, and the morphology of the embryos was observed 48 and 72 h after oocyte retrieval. Based on the morphological parameters, early embryos were classified into four grades. Grade I (four points): cells were even in size and regular in shape, with intact ZP; the cytoplasm was homogenous and clear, without granule phenomenon; debris was 0–5 %. Grade II (three points): cells were slightly uneven in size and shape; granule phenomenon might be seen in the cytoplasm; debris was 6–20 %. Grade III (two points): cells were obviously uneven in size and sometimes in shape; the granule phenomenon could be seen in the cytoplasm; debris was 21–50 %. Grade IV (one point): cells were significantly uneven in size and in shape; a severe granule phenomenon could be seen in the cytoplasm; debris was over 50 %. Two high-quality (at least grade II) embryos with six to eight cells were selected and transferred into the uterine cavity 72 h after oocyte retrieval.
8. Pregnancy check. Blood and hCG tests (Cobas e601; Roche Diagnostics, Basel, Switzerland) were performed 14 days after transfer. B-mode ultrasound (GE Healthcare, Waukesha, WI, USA) was conducted 40 days after transfer. If a gestational sac was observed, a clinical pregnancy was confirmed.

Statistical analysis

Statistical analysis was performed with SPSS 13.0. Data are expressed as mean $\pm$ standard deviation. The Student *t* test was used for the comparison of continuous variables (numbers of

oocyte injected, fertilization rate, percentage of top-quality embryos, and number of transferred embryos). The categorical variables included the rates of pregnancy per cycle and implantation per pregnancy. Analyses of the categorical variables between the matched groups were performed using the chi-square test.

To examine the performance of ZP-bound sperm to predict a pregnancy after an ICSI cycle, receiver-operating characteristic curve (ROC) analysis was carried out using SAS 9.3. An optimized threshold was determined. The discriminative performance of the model was assessed by the area under the ROC curve. Sensitivity was defined as the fraction of cycles that resulted in a pregnancy that was predicted correctly, and specificity was defined as the fraction of cycle not resulting in a pregnancy that was predicted correctly. Tests for normality and homogeneity of variance were applied to all the data in advance. For data that met the aforementioned tests, one-way analysis of variance (ANOVA) was used for intergroup comparisons. A *P* value < 0.05 indicated statistical significance.

Results

In this study, 84 infertile couples were included. Mean age of the wives and husbands was 30.42 ± 0.54 (range 23–41) years and 34.5 ± 2.9 (range 24–43) years, respectively. No significant differences (all *P* > 0.05) were found between the two groups for the age of the wives (31.8 ± 0.7 vs. 32.3 ± 0.9 years), infertile period (5.67 ± 0.52 vs. 5.04 ± 0.46 years), Gn dose (31.35 ± 2.56 vs. 30.16 ± 2.00 injections), number of MII oocytes (9.70 ± 0.85 vs. 9.38 ± 0.61), and number of embryo transfers (2.16 ± 0.13 vs. 2.20 ± 0.08) (Table 1). Four cycles in the treatment group were excluded because of failed sperm-ZP binding and adoption of the traditional ICSI fertilization,

Table 1 Characteristics of the patients

	Treatment group	Control group
<i>N</i>	38	42
Age (years)	31.8 ± 0.7	32.3 ± 0.9
Body mass index (kg/m ²)	22.7 ± 3.8	22.2 ± 2.3
Infertile period (years)	5.7 ± 0.52	5.0 ± 0.5
Gonadotrophin dose (injections)	31.4 ± 2.6	30.2 ± 2.0
Number of MII oocytes/woman	9.7 ± 0.9	9.4 ± 0.6
Total number of mature oocytes	229	245
Embryos after ICSI	183	193
High-quality embryos	121	98
Number of embryos ^a	2.2 ± 0.1	2.2 ± 0.1

^a Number of embryos that were transferred during a single embryo transfer session

All *P* > 0.05

while the remaining 38 cycles showed successful sperm-ZP binding. After the ICSI, no significant differences were found in the rates of fertilization and cleavage between the two groups (both $P > 0.05$), while significantly higher rates of high-quality embryos and useable embryos were observed in the treatment group compared with the control group ($P < 0.05$). The clinical pregnancy rate in the treatment group (60.5 %, 23/38) was higher than in the control group (47.6 %, 20/42), but the difference was not statistically significant ($P > 0.05$). The implantation rate was higher in the treatment group compared with the control group (39.3 vs. 23.1 %, $P = 0.045$) (Table 2).

Discussion

Using the crucial step of sperm-ZP binding that is usually observed during normal fertilization [14], mobile and functioning sperms with normal morphology were used for ICSI. The differences in the rates of fertilization, cleavage, high-quality embryos, useable embryos, and clinical pregnancy after ICSI were examined. Results indicated that ICSI with ZP-bound sperm could significantly increase the rates of high-quality embryos and useable embryos and slightly improve the clinical pregnancy rate. Liu et al. [10] reported that better pregnancy rates could be obtained using ICSI with sperms crossing the ZP. Adopting a similar method, the present study also showed that ICSI with sperms binding to the ZP or ZP-bound sperms could significantly improve embryo quality and increase the useable embryo rate, compared with traditional ICSI with subjectively selected sperms [15]. This method allows for the rapid retrieval of the sperms after binding to the ZP [16–19].

Recent advances in human-assisted reproductive technologies allowed for the wide application of ICSI, making more and more couples with male infertility being able to achieve successful pregnancy [20]. However, the safety of the method, mainly concerning the infants resulting from ICSI, remains an important issue. Traditionally, sperms are subjectively selected by lab technicians, therefore avoiding the natural

elimination of unfit sperms by the oocyte. Although the performance of the microscopes has been greatly improved, most centers still use $\times 400$ microscopes, making it impossible to have a clear view of eventual microstructural defects such as debris and DNA denaturation [21]. In addition, there is a risk of injecting a defective sperm into the oocyte, in particular those with normal morphologies but abnormal or defective DNA, which may result in adverse outcomes for the viability of the embryo or the infant [22]. Moreover, objective evaluation criteria of clinical ICSI treatment are lacking due to non-uniform criteria among different technicians and different centers for the selection of sperms with normal morphology and strong mobility. During natural fertilization, only structurally sound sperms can complete the whole process [23] because sperms must have an intact acrosome to bind the ZP and trigger acrosomal exocytosis, and this process exposes specific regions at the sperm surface to interact with the oocyte [24, 25]. Therefore, as suggested by Braga et al. [25], sperm-ZP binding might be the best way to identify the most competent sperms for ICSI. Nevertheless, it does not prevent the selection of sperms harboring genetic defects.

Based on this natural selection theory, during controlled ovarian hyperstimulation and sequential ICSI treatment, the present study used one or two immature oocytes that lost their potentiality for fertilization to perform sperm-ZP binding (2 h of binding) to screen out high-quality sperms with normal morphology for ICSI. Hence, natural selection of sperm-ZP binding for ICSI was used to provide objective criteria for sperm selection for ICSI. Results indicated that ICSI with ZP-bound sperm could increase the numbers of high-quality and useable embryos and slightly improve the clinical pregnancy rate and clinical pregnancy outcome, which are superior to traditional ICSI.

This study is not without limitations. The sample size was relatively small and from a single center. The method itself might benefit from some improvements. Among others, some techniques are available for the selection of sperms with high DNA integrity [26], and these methods could be used in combination with sperm-ZP binding to select the best sperms for ICSI. Additional studies are still needed to optimize the method and to assess the long-term safety of the method.

In conclusion, ICSI using ZP-bound sperm might increase the embryo quality and number of useable embryos, and thus improve the clinical pregnancy outcome.

Table 2 Comparison of the rates of fertilization, cleavage, high-quality embryos, useful embryos, and clinical pregnancy between the two groups

	Treatment group	Control group
Fertilization rate (%)	83.0 (190/229)	81.6 (200/245)
Cleavage rate (%)	96.3 (183/190)	96.5 (193/200)
High-quality embryo rate (%)	66.1 (121/183) [#]	50.8 (98/193)
Useable embryo rate (%)	76.0 (139/183) [#]	66.3 (128/193)
Clinical pregnancy rate (%)	60.5 (23/38)	47.6 (20/42)
Implantation rate	39.3 (24/61) [#]	23.1 (18/78)

[#] $P < 0.05$

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Compliance with ethical standards

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

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the Ethical Committees of Maternal and Children Health Care Hospital of Yinchuan and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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