A Modified ICSI Technique: Using Zona Pellucida as A Natural Bait

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ABSTRACT
Background: In vivo, the zona pellucida (ZP) of the oocyte can bind to normally functional sperm. The ZP-sperm interaction is one of the final steps of natural selection during their journey in the female reproductive tract.
Aim: In the current study, we evaluated the ability of the ZP of immature oocytes to harvest the fittest sperm.
Method: We compared the embryological outcomes (i.e., rates of fertilization, cleavage, blastocyst formation, and high-quality blastocysts) of intracytoplasmic sperm injection (ICSI) using conventionally selected sperm (control group) and ZP-bound sperm (treated group).
Results: Our results showed that there were no statistically significant differences for the ZP binding technique over the conventional sperm selection with respect to the rates of fertilization and cleavage. However; the rates of blastocyst formation and high-quality blastocysts were significantly higher in the treated group compared to the control group.
Conclusion: These findings imply that the proposed technique (ZP-bound sperm) can serve as a cost-effective and natural sperm selection method that has the potential to improve the embryological and clinical outcomes of intracytoplasmic sperm injection (ICSI).
Keywords: Intracytoplasmic sperm injection (ICSI), zona pellucida (ZP), natural sperm selection, ZP-bound sperm, Acrosomal reaction (AR), Sperm-oocyte interaction.

INTRODUCTION

The quality of the selected sperm used for intracytoplasmic injection (ICSI) plays a detrimental role in embryonic quality and development and, subsequently, in the clinical outcomes of ICSI [1]. In in vitro fertilization (IVF) and during the sperm journey through the female reproductive tract in vivo, sperm interact with the zona pellucida (ZP) of the oocyte, which is the last stage of sperm selection before entering the oocyte [2&3].

The ZP is selective with regard to binding and can bind to normally functioning sperm, especially those with a normal acrosomal region [3]. According to **Liu et al.** [4], only 14 % of the motile spermatozoa in fertile men can bind to the ZP. Only those spermatozoa with relatively normal size and shape of the acrosomal region can bind to and penetrate the ZP and fuse with the plasma membrane of the oocyte (oolemma) and thus are capable of fertilizing the oocyte [5]. Human sperm vary in size, morphology, DNA integrity, motility, membrane composition, etc., and this can be observed even in the same ejaculate [6]. In vivo, sperm pass through various sperm selection checkpoints and barriers to reach the oocyte. Spermatozoa have to overcome obstacles in the vagina, cervix, uterus, fallopian tube (site of fertilization), and finally, the ZP of the oocyte where less than 500 sperm would have made it through [7].

Thus, it is a given fact that thorough sperm selection procedures befall spermatozoa in the female genital tract to filter superior sperm and allow only a small subpopulation of spermatozoa with superior quality to reach the site of fertilization where another sperm selection occurs (i.e. ZP interaction) .

The sperm traits that make in vitro fertilization effective are still debated. ICSI is now the standard practice for most Assisted Reproduction (ART) centers worldwide and accounts for approximately 70% of all in vitro fertilization [4&8]. The routine selection of spermatozoa for ICSI depends on an embryologist subjectively selecting sperm based on their motility and morphology. It is done after an analysis of the seminal fluid, which is a poor predictive tool of male fertility and does not express the fertilization capacity of the sperm [9].

It was assumed that mimicking the natural sperm selection may improve the quality of selected spermatozoa and hence, the clinical outcomes of ICSI. Ideally, a sperm selection method that reduces the number of spermatozoa to a subpopulation with potentially the highest quality can improve fertilization and embryo quality and development and subsequent clinical outcomes of ICSI, including implantation, pregnancy, and live birth rates.

Over the years, several sperm selection techniques have been developed for ICSI. However, these techniques were designed to select sperm based on a single sperm parameter (i.e. motility, density, sedimentation, nuclear integrity, etc.) and ignoring other sperm parameters related to the capability to fertilize the oocyte observed in vivo [10&11]. Sperm selection techniques such as swim-up, microfluidics, and density gradient centrifugation yield a population of highly motile sperm but fail to mimic the rigorous natural sperm selection that considers other sperm parameters. Moreover, most of these methods require centrifugation which may negatively affect the paternal DNA and reduces the quality of sperm by increasing reactive oxygen species [10].

Following these techniques, an embryologist has to subjectively select sperm based on their motility and morphology, which does not guarantee DNA integrity and is potentially time-consuming.

One of the developed sperm selection methods to relatively duplicate the natural selection is the hyaluronic acid (HA) binding-based PICSI® dishes. These are based on placing sperm with a dot of HA, a compound found in the extracellular matrix (ECM) of cumulus oophorus layer surrounding the oocyte, mature intact sperm then bind by their acrosome to the AH dot surface. It has been reported that spermatozoa that bind to the HA have higher DNA integrity, normal morphology, and normal HA receptors [12&13]. However, there is conflicting data on the results using PICSI dishes. Several studies showed no statistically significant difference in using HA-bound sperm concerning the clinical outcomes of ICSI [14; 15; &16]. In addition, sperm-ZP binding comprises parameters other than HA that are not featured in PICSI dishes.

Sperm-oocyte interaction is a multi-step process involving physical and molecular interactions [17]. The initial interaction between sperm and ZP is non-specific. It is followed by a more complex and complementary receptor/ligand-based process between the surface proteins expressed on the ZP and the sperm [3]. Research has unveiled several ZP protein candidates postulated to play a role in binding sperm. The main protein of these is the ZP3 glycoprotein, whose O-linked oligosaccharide chains bind to an acrosome-intact sperm and induce an acrosomal reaction [3&17]. An ideal sperm selection technique should be easy to conduct, cost-effective, and enable embryologists to filter a subpopulation of superior sperm.

PATIENTS AND METHODS

The study sample consisted of 200 patients undergoing ICSI cycles at the reproduction unit of the International Islamic Center for Population Studies and Researches, which is affiliated with Al-Azhar University in Cairo, Egypt, between December 2020 and October 2021.
Our inclusion criteria included:
- Female age ≤ 38 years old. Male age ≤ 50 years old.
- Having at least one immature oocyte (i.e. germinal vesicle (GV) or metaphase I (MI) oocyte) for incubation with sperm to preserve mature ones for ICSI.
- At least 10% sperm motility and thus we excluded testicular sperm samples.

The 200 patients were randomly divided based on their male factor (i.e. percentage of abnormal sperm morphology) into normal male factor group (abnormal forms ≤ 96%) and abnormal male factor group (abnormal forms > 96%). Moreover, each group was divided into a control and a treatment group. The patients' own sibling mature oocytes were used for both the control and treatment groups. After retrieval, oocytes were scored and sibling MII oocytes of the patient were randomly divided. Oocytes of the control group were injected with conventionally selected spermatozoa based on sperm morphology and motility after being processed by single wash or swim-up techniques. For both treatment groups, one immature oocyte was incubated with a calculated volume of the processed semen with a concentration of 500,000 sperm/oocyte in a CO_2 incubator for 30 to 60 minutes and then checked for bound sperm under an inverted microscope. Only bound sperm with normal morphology were selected and transferred to a poly vinyl pyrolidone (PVP) drop for immobilization and then injected into the cytoplasm of the MII oocytes of the treatment groups.

Clinical manipulations

1. Controlled ovarian hyperstimulation (COH):

We followed the routine ovarian hyperstimulation [18] and all female patients were injected daily at a fixed time with a subcutaneous follicle-stimulating hormone (Gn, Gonal-F, Merck Serono, USA) from the 3rd to the 5th day of the menstrual cycle.

The follicles were checked for reaching the appropriate diameter (i.e. 18–20 mm) and for their number using an ultrasound device [19]. If two or more follicles reached the appropriate diameter, a trigger (human chorionic gonadotropin (hCG)), (Ovitrelle®; Merck Serono, Switzerland) was administered intramuscularly to encourage final maturation and induce ovulation [20].

2-Sperm preparation:

After male couples had been instructed to abstain from sexual activities for 1 to 7 days, Semen samples were collected by masturbation. Samples were placed at room temperature on warm plates or incubators at 37°c until they were liquefied [21] and the time of liquefaction was recorded.

Both macroscopic and microscopic assessments were performed using the 2021 WHO manual as a reference. After that, samples were treated by one of the following techniques depending on the male factor:

1. Swim-up: this method was applied for the normal male factor group. samples were centrifuged at 1200 rpm` for 4 minutes (Heraeus 400, Osterode, Germany) then the supernatant was discarded and an overlying layer of 0.25 ml of Ham F10 medium (Sigma, UK) was placed over the semen sample in a round bottom tube and incubated at 37° C in a 45° position, for 30-60 minutes. After that, the supernatant layer containing the spermatozoa was harvested and transferred to a different tube [22]. The transferred volume was recorded.

2. Simple wash: this method was applied in cases with abnormal male factor.   1 ml of

sperm gradient medium was added to the fresh sample in a 14 ml falcon tube. The mix was then centrifuged for 10 minutes at 1800 rpm. The supernatant was discharged and the remaining pellet was supplemented with 2 ml of sperm washing medium (SAGE, USA). After that, the mix was centrifuged for 10 minutes at 1800 rpm [23]. The supernatant was discarded and the pellet was transferred into a different tube and its volume was recorded.

For the treatment groups, a specific volume of the processed semen was co-incubated with an immature oocyte in an injection dish containing 10 µl micro drops of the total global media (LifeGlobal, Europe) covered with 3 ml of sterile equilibrated mineral oil at 37 °C with 6 % CO^2 for 10 to 30 minutes. The volume taken contained 500000 sperm per oocyte according to the following equation:

$$x = \frac{pellet\ volume\ x\ 0.5\ x\ 100}{motility\ x\ count\ (after\ processing)}$$

3-Oocyte retrieval:

Oocyte retrieval was performed approximately 36 hours following the administration of the ovulation trigger [24]. Under ultrasound guidance, a single lumen gauge needle (Reproline, Germany) had been used to aspirate the follicles for fast oocyte pick-up with a negative pressure of 115-120 mm Hg [25]. At the same time, the follicular fluid had been collected in round bottom sterile 14 ml falcon tubes. Under a stereo microscope, the oocyte-cumulus complexes (COCs) were identified, washed, and transferred into fertilizing global total media (LifeGlobal, Europe) and incubated at 6% CO2 at 37°C until denudation [25].

4- Oocyte denudation and scoring:

The COCs were denudated by placing them into a 100 µl drop of buffered media containing hyaluronidase enzyme 80 IU/ml (LifeGlobal, Europe) for 30 to 45 seconds. Then the oocytes were gently aspirated in and out by a sterile stripper pipette resulting in the removal of the coronal cells [26]. Following that, a global total w/HEPES Buffer (LifeGlobal, Europe) was used to wash the denudated oocytes. An inverted microscope equipped with automatic manipulators, Narishige, hot stage, and Hoffman optics (Olympus 1x71) was used for assessing the oocytes' maturity [27]. The oocyte maturation assessment was as follows: mature oocytes in the metaphase II (MII) characterized by the extrusion of the polar body, and immature oocytes were either in the germinal vesicle phase (GV) characterized by a centrally located germinal vesicle or in the Metaphase I (MI) characterized by the absence of both the polar body and the germinal vesicle [26]. Mature oocytes were then incubated in a culture medium in a Labotect incubator with 6% Co_2 at 37 °C until the time of the intracytoplasmic sperm injection. In contrast, sibling immature oocytes were incubated in a culture medium (50 µ) with a **500,000** concentration of spermatozoa/oocyte and placed in the Labotect incubator for 10-30 minutes till the time of sperm selection.

5- Intracytoplasmic sperm injection:

Mature oocytes were placed in injection dishes containing 10 µl micro drops of the global total w/HEPES Buffer (LifeGlobal, Europe) covered with 3 ml of pure equilibrated mineral oil for ICSI [28].

In the control groups, each MII oocyte was injected with a conventionally selected sperm based on normal morphology and motility after being processed by simple wash or swim-up. However, in the treatment groups, ZP-bound sperm were selected from the surface of the immature oocytes through the use of a microneedle (Sunlight Medical, Jacksonville, FL, USA) and transferred in a 10 % polyvinylpyrrolidone (PVP) solution (SAGE, USA), immobilized, and then used to inject sibling MII oocytes [28]. The procedure was carried out under an inverted microscope equipped with a holding pipette with slight negative pressure for handling the oocyte and an injection needle for injecting the sperm [29]. The immature oocytes used for sperm selection were discarded. In all groups, the injection needle containing a single sperm was steadily and slowly moved through the cytoplasm of the MII oocyte and dropped the sperm 1 to 3µl from the center of the oocyte [28].

6- Outcome measures:

All the embryological parameters (i.e. fertilization rates, cleavage, blastocyst formation, and blastocyst quality) were recorded, assessed, and analyzed for the four groups. Signs for fertilization were observed 16-18 hours post intracytoplasmic injection. The fertilization rate had been calculated by dividing the total number of fertilized oocytes by the total number of injected MII oocytes [29]. Furthermore, 48 and 72 h after ICSI, the cleavage rate was assessed by dividing the number of cleaved embryos by the total number of injected MII oocytes. The blastocyst formation rate had assessed on day five post-ICSI by dividing the number of formed blastocysts by the total number of injected MII oocytes [30]. Embryos with high-quality blastocyst formation were classified according to Gardner's blastocyst grading system [31]. This system assigns grades to the quality of a blastocyst according to three components: 1- the expansion status of the blastocyst, 2- the inner cell mass (ICM), 3- and the trophectoderm (TE) quality, see Table 1. We classified Grade A blastocysts as those with AA, AB, or BA grade regardless of their expansion degree. Grade B blastocysts were those with BB grade regardless of the expansion degree.

Table 1: Blastocyst scoring according to the Gardner blastocyst grading system [31]

Inner cell mass (ICM)	
A	Tightly packed large number of cells
B	Smaller number of cells loosely grouped
C	Rare cells
Trophectoderm (TE)	
A	Various cells cohesively group in one layer
B	Loose epithelium cells
C	Unorganized layer of few cells
Blastocysts expansion degree	
3	Thick ZP, large blastocoel
4	Thin ZP with a blastocoel expanded larger than the embryo
5	Embryo hatching out of the ZP
6	Hatched embryo

7-Statistical analysis

The rates of fertilization, cleavage, blastocyst formation, and high-quality blastocysts were reported as percentages for each group. Characteristics of male and female patients (age, sperm count, sperm motility, sperm morphology, number of retrieved oocytes, and number of mature and immature oocytes) were expressed as mean ± standard deviation (SD). The Student t-test was employed to compare continuous variables (fertilization rates, cleavage, blastocyst formation, and high-quality blastocysts). Statistical analysis was performed with SPSS 13.0. P-value ≤ 0.05 was considered statistically significant.

8-Ethical approval:

The ethical research committee affiliated with Al-Azhar University in Cairo, Egypt, has approved the current study. This was done following the ethical standards of the 1964 Helsinki Declaration and its later comparable ethical standards or amendments, as well as the ethical standards of the national and/or the institutional research committee. All couples filled out the informed consent forms for this study.

RESULTS

In each group, we used sibling oocytes; therefore, the female age, the male age, infertile period, gonadotrophin dose, pre-existing conditions, and seminal fluid parameters were all the same between the control and the treatment groups.

1-Results of the normal male factor group (abnormal forms ≤ 96%):

Tables 2&3: male and female patients' characteristics for the normal male factor group (normal forms ≤ 96%) are presented as mean ± standard deviation:

Table 2:

Male Patients' characteristics		WHO (2021) reference range
Sperm count (million/ml)	33.8±19.88	15 (12-16)
Sperm motility (%)	56.53±18.45	40 (38-42)
Progressive motility (PR, %)	31±5.28	32 (31-34)
Sperm normal forms (%)	2.17±1.64	4 (3.9-4)

Table 3:

Female Patients' characteristics	
Female ages (years old)	29±5.02
Number of retrieved oocytes	9.58±3
Number of MII oocytes	6.35±2.22
Number of immature oocytes	3.23±2.2

As illustrated in tables 2& 3, the means of the female age were (29±5.02 years old), and sperm count (33.8±19.88 million/ml), sperm motility (56.53±18.45%), sperm progressive motility (31±5.28%), sperm normal forms (2.17±1.64%), number of retrieved oocytes (9.58±3 oocytes), number of retrieved MII (6.35±2.22 oocytes), and number of immature oocytes (3.23±2.2 oocytes). The ranges of the 6th edition of the WHO (2021) Manuel for normal semen parameters were used as a reference [32].

Table 4: Comparison of the embryological outcomes between the control and the treatment groups in patients with normal male factor (abnormal forms ≤96%):

Study outcomes			
	Control n=438	Treatment n=184	p-values
Fertilization (%)	415 /438 94.75%	177/184 96.20%	0.4414
Cleavage (%)	398/438 90.87%	172/184 93.48%	0.2834
Blastocyst formation (%)	271/438 61.87%	131/184 71.20%	0.0265*
Grade A blastocyst (%)	21/271 7.74%	19/131 14.50%	0.0340*
Grade B blastocyst (%)	6/271 2.21%	7/131 5.34%	0.0966
N= total number of injected MII oocytes			
* Refers to a statistically significant *p-value* (< 0.05)			

Figure 1: Illustration of the embryological outcomes of the control and treatment groups in patients with normal male factor (abnormal forms ≤ 96%):
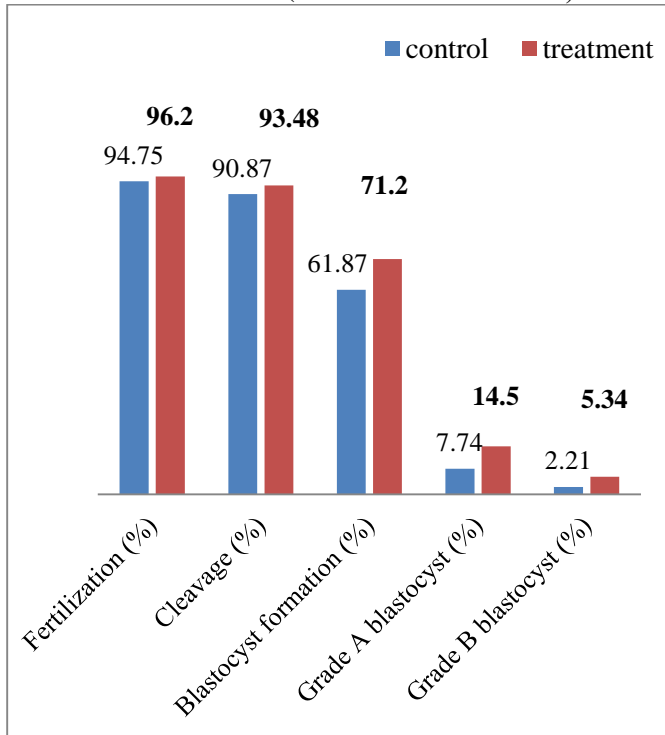

Table 4 and Figure 1 show that the rates of fertilization and cleavage were higher in the treatment group compared to the control groups but without statistical significance (96.20 vs. 94.75% and 93.48 vs. 90.87%), p-values (0.4414 and 0.2834), respectively. The blastocyst formation rate was statistically significantly higher in the treatment group than in the control group (71.20 vs. 61.87%, P-value= 0.0265).

Moreover, the treatment group showed higher rates of high-quality embryos Grade A and B. However, only the rate of Grade A blastocysts was statistically significant (14.50 vs. 7.74% and 5.34 vs. 2.21, p- value= 0.0340 and 0.0966).

2-Results of the abnormal male factor group (abnormal forms > 96%):

Tables 5&6: Patients' characteristics for the abnormal male factor group (abnormal forms > 96%) presented as mean ± standard deviation:

Table 5:

Male Patients' characteristics		WHO (2021) reference range
Sperm count (million/ml)	21.7±20	15 (12-16)
Sperm motility (%)	37.47±24.89	40 (38-42)
Progressive motility (PR, %)	10.5±5.24	32 (31-34)
Sperm normal forms (%)	0.76±1.06	4 (3.9-4)

Table 6:

Female Patients' characteristics	
Female ages (years old)	29±5.39
Number of retrieved oocytes	8.94±2.92
Number of MII oocytes	6.67±2.77
Number of immature oocytes	2.28±2.12

Tables 5&6 show patients' characteristics presented as means ± standard deviation as follows: the female age (29±5.39 years old) years, sperm count (20±21.7 million/ml), sperm motility (37.47±24.89%), percentage of sperm abnormal forms (99.24±1.06%), number of retrieved oocytes (8.94±2.92 oocytes), number of retrieved MII oocytes (6.67±2.77 oocytes), and number of immature oocytes (2.28±2.12 oocytes). The ranges of the 6th edition of the WHO Manuel for normal semen parameters were used as a reference [32].

Table 7: Comparison of the embryological outcomes between the control and the treatment groups in patients with abnormal male factors (abnormal forms >96%):

Study outcomes			
	Control n=487	Treatment n=167	p-values
Fertilization (%)	452/487 92.81%	159/167 95.21%	0.2806
Cleavage (%)	441/487 90.55%	155/167 92.81%	0.3758
Blastocyst formation (%)	298/487 61.19%	118/167 70.66%	0.0283*
Grade A blastocyst (%)	54/298 18.12%	25/118 21.18%	0.4737
Grade B blastocyst (%)	7/298 2.35%	8/118 6.87%	0.0265*
N= number of MII oocytes that were injected in each group *Refers to a statistically significant *p-value* (< 0.05)			

Figure 2: Illustration of the embryological outcomes of the control and treatment groups in patients with abnormal male factors (abnormal forms > 96%):
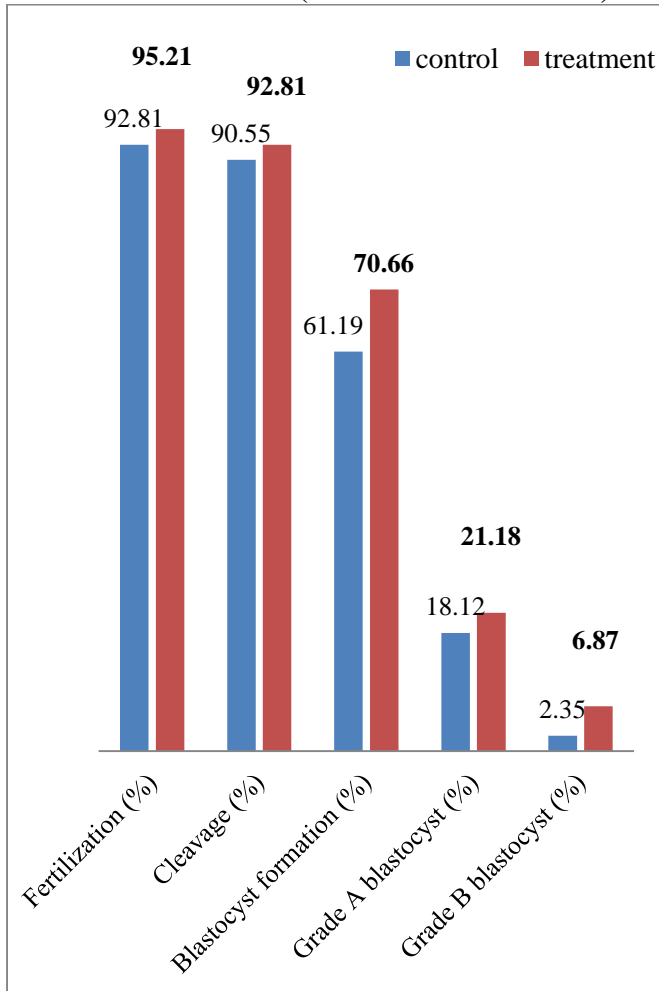

Table 7 and Figure 2 illustrate that the rates of fertilization and cleavage were higher in the treatment group than in the control group but without statistical significance (95.21vs. 92.81%, and 92.81 vs. 90.55% P-values=0.2806 and 0.3758, respectively). However, we found a statistically significant difference in favor of the treatment group in the rates of blastocyst formation and the number of Grade B blastocysts (75.35 vs. 61.19%, and 6.87 vs. 2.35%, P-values=0.0283and 0.0265). Moreover, although the rates of Grade A blastocysts were higher in the treatment group, they were not statistically significant (21.18 vs. 18.12%, P-value=0.4737).

DISCUSSION

Successful pregnancy has been made possible through the advancement in assisted reproduction technologies (ART) which has led to the development and breakthrough of ICSI [33&21]. Although ICSI enabled embryologists to overcome the mechanical barriers, especially for cases of male-related infertility, it has bypassed natural checkpoints of sperm selection [33&21]. In conventional ICSI, an embryologist selects spermatozoa subjectively, discarding the role of oocyte-based sperm selection. So, it does not guarantee the quality of the selected sperm and, subsequently, the quality of the resulting embryos [33].

Individual selection of sperm is very inconsistent and subjective. We used sperm-ZP binding as an objective, uncostly sperm selection technique that is based on the natural sperm selection observed in vivo. The outcome measures after ICSI consisted of the embryological results, including the rates of fertilization, cleavage, blastocyst formation rate, and high-quality blastocysts. Due to the use of sibling oocytes, we could not measure the clinical outcomes, including implantation and clinical pregnancy rates. The use of ZP-bound sperm for couples with normal male factor (abnormal forms $\leq$ 96%) yielded statistically significantly higher rates of blastocyst formation in the treatment group (71.20% vs. 61.87% p-value=0.0265) as well as grade A blastocyst rate (14.50 vs. 7.74, p-value=0.0340). It was reported that there is an association between embryological outcomes (i.e. fertilization, cleavage, blastocyst formation, and high-quality embryo rates) and sperm quality [34] which could explain our observations.

In addition, the rates of blastocyst formation and Grade B blastocysts were significantly higher in the treatment group as opposed to the control group in the abnormal male factor group (70.66 and 6.87% vs. 61.19% and 2.35%, p-value=0.0283 and 0.0265, respectively). The rate of Grade A blastocysts was also higher in the treatment group than in the control. However, it was not statistically significant.

According to Liu & Baker [4], using spermatozoa that have crossed the ZP for ICSI increased pregnancy rates. Similarly, one study reported higher implantation and pregnancy rates after using ZP-bound sperm [35]. Moreover, in a cohort study conducted by Jin et al. [33] on 84 infertile couples, the authors observed a significant increase in the rates of useable embryos and embryonic development and quality through the use of sperm bound to the ZP in ICSI.

It is almost impossible for the embryologist to examine or evaluate the genetic quality or the microstructural defects of the selected sperm even with the aid of high magnification ($\times$400) microscopes [33]. Moreover, it is possible for spermatozoa to show normal morphology but have genetic defects that may be related to fertilizing the oocyte; accordingly, the embryologist may inject the oocyte with a defective or abnormal sperm which may adversely affect fertilization or subsequent embryonic quality and development.

Binding to the zona pellucida induces an acrosomal reaction leading to the exocytosis of enzymes which enable the sperm to penetrate the oocyte and exposes certain sperm regions that enable the sperm surface to interact with the ZP3 of the oocyte [33]. In this study, we employed the ZP of sibling immature oocytes as a trap to capture competent sperm to inject the MII oocytes for ICSI. However, this method does not guarantee 100% selection of sperm with genetic integrity. We incubated one or more immature oocytes of the wife with a specific volume of the husband's semen for 10 to 30 minutes. With a microneedle, we

removed ZP-bound sperm with normal morphology and injected it into the cytoplasm of the sibling mature oocytes.

The study had some limitations. For example, using sibling oocytes prevented measuring clinical outcomes such as implantation and pregnancy rates. In addition, some may assume that the oocyte repair capacity may have interfered with the results, but this is not the case as we used sibling oocytes. Another limitation is that some patients had no immature oocytes and thus were excluded. Moreover, some rare cases may have no sperm bound to the ZP and conventionally selected sperm may be required. Furthermore, this method is not applicable in cases of sperm obtained through epididymal aspiration or surgically obtained from the testis.

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