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Original Article

Effect of sperm selection methods on ICSI outcomes in patients with oligoteratzoospermia

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Abstract: Objective: Sperm selection without - or with a low level of - protamine deficiency and DNA fragmentation is a remarkable indicator to increase the success rate of ICSI outcomes. The aim of this study was to compare sperm selection methods in the elimination of sperm with protamine deficiency and DNA fragmentation and their effects on ICSI Outcomes in oligoteratzoospermia patients. Methods: Semen samples were obtained from oligoteratozoospermia patients undergoing ICSI. Sperm selection was conducted using Zona Pellucida (ZP) binding, Hyaluronic Acid (HA) binding, and conventional PVP methods. SCD assay and CMA3 staining were used for the detection of sperm protamine deficiency and DNA fragmentation. Good quality of the embryo, blastocyst formation, chemical, and clinical pregnancy rates among studied groups was evaluated and compared. Results: Our results indicated the percentage of sperm DNA fragmentation and protamine deficiency were lower significantly in the HA- and ZP-bound sperm. Although no significant differences were observed in the fertilization rate among studied methods, good quality of cleavage embryo rates were increased using ZP and HA methods versus the conventional PVP method. However, there were no significant differences in cleavage and embryo quality between the HA compared to the ZP method. Blastocyst formation, chemical and clinical pregnancy rates increased in the HA method. Conclusions: Overall, the HA method for sperm selection due to high sensitivity in selecting sperm with a low level of DNA fragmentation and protamine deficiency is a very useful method to increase the success rate of ICSI outcomes in oligoteratozoospermia patients.

Keywords: Oligoteratozoospermia, sperm-zona pellucida (ZP), sperm hyaluronic acid (HA), ICSI

Introduction

Oligoteratozoospermia is defined as a low sperm count and normal morphology, which are the leading cause of about 90% of male infertility [1]. Sperm DNA fragmentation has a crucial role in male infertility for achieving reproduction success [2]. Increasing evidence demonstrates a negative affecting of DNA fragmentation and protamine deficiency on fertilization, embryo development, implantation, and pregnancy rates [3, 4]. Among the various tests available to diagnose sperm DNA damage, the sperm chromatin dispersion (SCD) test is recognized as a reliable and fast test with high accuracy [5]. Chromomycin A3 staining (CMA3) is also a reliable tool for assessing protamine deficiency [6]. The increase in the percentage of CMA3 positive sperm is related to loss of DNA condensation and reduced protamine contents [7]. Nowadays, to childbearing in male infertility treatment, the most commonly used technique is ICSI [8].

Thus, sperm selection of an effective method without - or low high level of DNA fragmentation for ICSI in oligoteratozoospermia is greatly needed [9, 10]. In the last decades, Due to conventional method of sperm selection does not diagnose DNA fragmentation; thus, various methods have been developed to select the best sperm before using them for ICSI [7, 11, 12]. The literature on some researches confirmed the sperm-ZP and HA-binding as useful

methods to select sperm with high quality and improve the success rate of Intra Cytoplasmic Sperm Injection (ICSI) outcome [9, 13].

However, other researches did not demonstrate significant differences between these methods and the conventional sperm selection method for improving reproductive success [14-17]. However, the diversity in these findings could be that due to only a few studies have been included patients with male factors, and most of these were aimed at healthy men. Additionally, the use of a HA method as a useful method to select sperm with low-level sperm DNA fragmentation is still in conflict. With a focus on conflict results, the aim of this study was to compare sensitivity sperm selection methods in the elimination of sperm with protamine deficiency and DNA fragmentation and their effects on ICSI outcomes in patients with Oligoteratzoospermia.

Patients and methods

Ethical approval

The study was initially approved by the ethical and scientific committee of Kurdistan University of medical sciences (code: IR.MUK.REC.1396/76).

Study design and subjects

Semen samples obtained from patients referred to the infertility treatment center of the besat hospital, Sanandaj, Iran due to male factor infertility with oligoteratozoospermia. The inclusion criteria for Oligoteratozoospermia according to strict criteria last WHO 2010, were as follows: sperm count $<39\times10^6$/ml, Sperm concentration $<15\times10^6$/ml, normal morphology <4% [18]. All the patients attended infertility treatment from August 2016 to Jun 2020. The exclusion criteria were those with normal semen analysis, asthenozoospermia, seminal infection, and patients with systemic diseases or a history of cryptorchidism. Additionally, cycles that were used for sperm recovery including, testicular sperm aspiration (TESA), percutaneous sperm aspiration (PESA), or testicular sperm extraction (TESE) procedures were excluded from the study. All patients signed consent forms that permit the use of their immature oocytes and unfertilized OOCYTES or sperm samples for research. The study was approved by the ethics committees of Kurdistan University of medical sciences. The 50 oligoteratozoospermia patients undergoing ICSI, and based on sperm selection methods were divided into three groups: 1) ICSI using ZP-bound sperm (n=16) and 2) ICSI using HA-bound sperm (n=16) 3) ICSI using conventional PVP method (n=18).

Sperm-ZP binding

For the determination and selection of ZP-bound sperm, at least motile sperm (2×10^6) per 1 ml were co-incubated at 37°C in 5% CO_2 for 2 h to allow the sperm to bind to ZP from unfertilized OR IMMATURE OOCYTES. Following this period, attached sperm to the surface of the ZP oocytes were removed, and ICSI using them was performed [19].

Sperm-HA binding

Assessment of the sperm HA-binding method was carried out according to the Renata Fabiane Perrelli et al protocol. Briefly, a PICSI (Midatlantic Diagnoses-Origio, Denmark) dish was used for sperm selection. 1 µl of prepared sperm (2×10^6) per 1 ml was added into the hydrated HA of the PICSI dish, and was placed at room temperature for 2 hours and kept at 36.8°C for at least 5 minutes. The HA-bound sperm were removed, and ICSI was performed [20].

Ovarian stimulation

Ovarian stimulation was started according to the GnRH agonist long protocol using depot triptorelin (Decapeptyl® Sr, 3.75 Mg; Ferring, Malmo, Sweden) injection. After pituitary down-regulation, gonadotropin (Gonal-F®; Serono, Geneva, Switzerland) and menotropin (Menopur®; Ferring) were added to reach follicles with a mean diameter of 17 or 18 mm in diameter. Then, oocytes retrieval was conducted 36 hours after administration of a recombinant human chorionic gonadotropin (ovidrel®, 250 µg; Serono) [21].

ICSI was performed using sperm recovery of all methods and then injected oocytes were cultured at 6% CO_2, 5% O_2, and 89% N_2 in Global® mediums (Life Global, USA) from day 1 to day 5 and in Global® medium. The presence of two pronuclei (2PN), cleavage, and blastocysts of

Table 1. Comparison of the 2PN zygote, cleavage (A and B grades), and Blastocysts formation rates of 491 oocytes with the use of ZP- and HA-bound sperm, and conventional methods

variable	Control n=165	ZP n=163	HA n=163	95% CI Conventional vs. ZP	95% CI Conventional vs. HA	95% CI ZP vs. HA
2PN zygote	137±1.4	141±0.9	143±1.2	0.6741 to 0.4341 P=0.8	-0.7341 to 0.3741 P=0.7	-0.6141 to 0.4941 P=0.1
Cleavage (A and B grades)	95±1.3	123±0.8	130±0.9	-1.065 to -0.09453 P=0.01*	-1.225 to -0.2545 P=0.001**	-0.6455 to 0.3255 P=0.7
Blastocysts	23±0.7	39±0.8	45±0.5	-0.6195 to -0.02051 P=0.03*	-0.7395 to -0.1405 P=0.002**	-0.4195 to 0.1795 P=0.6

All data are presented as mean ± SD; Asterisks are indicated significant Differences (*P<0.05, **P<0.01, one-way ANOVA with Tukey test).

embryos were assessed at 19 hours post-ICSI and at day 3, 5; respectively. The quality of the cleavage embryos was evaluated based on existing observations into four grades. Briefly, Grade A: top-quality embryo with 6-8 even cells in size and fragmentation <10%, Grade B: good quality embryos with 6-8 uneven cells in size and with fragmentation <26%. Grade C: impaired embryo quality with 6-8 uneven cells in size and with fragmentation <35%. Grade D: cells and were significantly uneven in size and shape and multinucleated embryos with 1-6 uneven cells in size with fragmentation >35% [22]. Embryos with C and D grades were not able to form blastocysts on day 3 or day 5, they were considered low-quality embryos. Embryos with grade A and B on day 3 were transferred into the uterine cavity. Chemical and clinical pregnancy rates were evaluated by the measurement of serum βeta-HCG concentration on day 12 and observation of a gestational sac at 5 weeks after embryo transfer, respectively.

CMA3 staining

Briefly, for each sample, fixation was carried out in carney's solution. 200 µl of CAM3 solution was added to the prepared smear. Then, the slides were rinsed in buffer and mounted with buffered glycerol. Microscopic analysis of the slides was assessed on an Olympus fluorescent microscope with the appropriate filter (460-470 nm). On each slide, at least 200 sperm were evaluated [23].

SCD test

The SCD test was conducted using the Halosperm kit (Halotech DNA, SL, Madrid, Spain) according to the manufacturer's instructions; and was assessed according to the criteria of Fernández and colleagues [24]. Sperm with DNA fragmentation showed big or medium-sized halos, whereas those without DNA fragmentation appeared with a small or no halo. The halo was evaluated under bright field microscopy with Diff-Quik staining.

Statistical analysis

Considering the different methods of sperm selection in the elimination of sperm with protamine deficiency and DNA fragmentation, measured values of CMA3+ and DNA fragmentation as percentages were expressed. Therefore, analysis of variance (ANOVA) followed by Tukey's test was used to compare the means of these data by expressed as mean ± SD. Moreover, Statistical significance for the effect of sperm selection methods on ICSI outcome was also analyzed by one-way ANOVA followed by Tukey's multiple comparison test, and P<0.05 was considered significant. GraphPad Prism 8.2.1 software (GraphPad Software, Inc., La Jolla, CA) was used for statistical analyses and graphic drawings.

Results

A total of 491 metaphase II (MII) oocytes from 50 ICSI cycles were collected and injected sperm. Men and women's age (20-35 years-old) were observed normal distribution and no significant differences in average age. The number of transferred embryos was similar among the three methods. As shown in **Table 1**, there was no significant difference in the fertilization rate (2PN) among study methods (conventional PVP vs. ZP P=0.8, conventional PVP vs. HA P=0.7, and ZP vs. HA P=0.1). Good quality cleavage embryos (A, B grades) rates increased in the ZP and HA methods when compared to the conventional PVP method (P=0.01 and P=0.001, respectively); but blastocyst formation for ZP and HA methods were also higher than conventional PVP method (P=0.03 and P=0.002, respectively). As shown in **Table 2**, chemical and clinical pregnancy rates were significantly higher in the HA method compared with the conventional PVP method

Table 2. Comparison of chemical and clinical pregnancy of 50 ICSI cycles among used three methods

Variable	Conventional (n=18)	ZP (n=16)	HA (n=16)	Coventional vs. ZP	Conventional vs. HA	ZP vs. HA
Chemical pregnancy per transfer (%)	9 (18%)	13 (26%)	13 (26%)	0.3	0.008*,**	0.09
Clinical pregnancy per transfer (%)	7 (14%)	11 (22%)	17 (34%)	0.6	0.02*	0.2

All data are presented as mean ± SD; Asterisks are indicated significant Differences (*P<0.05, **P<0.01, one-way ANOVA with Tukey test).

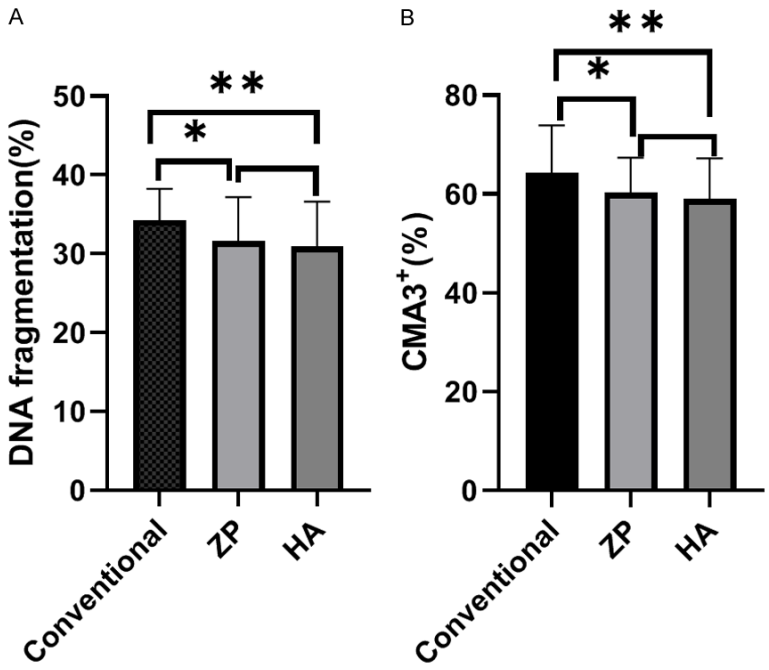

Figure 1. (A) Sperm DNA fragmentation, and (B) chromomycin A3 positive (CMA3+) among sperm selection methods include, ZP = Zona Pellucid; HA = Hyaluronic Acid, Conventional methods. All data are presented as mean ± SD; Asterisks are indicated significant Differences (*P<0.05, **P<0.01, one-way ANOVA with Tukey test).

(P=0.008, P=0.02); whereas no significant difference was between ZP and conventional PVP (P=0.3 and P=0.6, respectively) as well as between ZP and HA methods (P=0.09 and P=0.2, respectively). There were significant differences in the percentage of sperm DNA fragmentation among all studied methods (conventional PVP vs. ZP P=0.01, Conventional PVP vs. HA P=0.001 and ZP vs. HA P=0.8), and significant differences also were found in CMA3+ of ZP and HA methods (conventional PVP vs. ZP P=0.04, Conventional PVP vs. HA P=0.006 and ZP vs. HA P=0.7) (**Figure 1A**, **1B**).

Discussion

Sperm selection for ICSI technique has a direct impact on the success of its outcomes. Sperm DNA fragmentation has been indicated significantly higher in male infertility patients [25, 26]. Therefore, the use of an effective method to select sperm with reduced DNA fragmentation can be lead to improve the success of ICSI outcomes [4]. Sperm protamine is essential to keep up highly compacted sperm chromatin and DNA packaging for providing structural stability [27]. Some reports have confirmed a reduction of sperm protamine leading to the susceptibility of sperm to DNA damage [27, 28]. The current results showed no statistically significant differences in the fertilization rates among studied methods. This result could be explained by the fact that oocytes can be able to repair sperm with a 10% fragmented chromatin [29]. Moreover, Ye and colleagues in the evaluation of 75 IVF patients using HA method found a significant correlation between the use of the HA method and fertilization rate [30]. Contrary to the current results, other authors have reported that the fertilization rate increases when the use of the HA method for sperm selection [10, 27]. Nonetheless, the current study indicated that increase good quality cleavage embryos (grade A, B) and blastocyst formation rates were accompanied by a significant decrease of the sperm DNA fragmentation and CMA3 positive in ZP- and HA-bound sperm. These results demonstrate that low levels of sperm DNA fragmentation and protamine deficiency are closely associated with the increase in ICSI success outcomes. The present results strongly showed an increase in chemical and clinical pregnancy

rates when the HA method was compared to other methods. Inconsistent with current findings, associations between the use of PICSI dish, and HA-bound sperm without DNA fragmentation in a study by parmegiani and colleagues were documented [14]; while the study by Petersen and colleagues didn't unable to confirm this association [31]. They suggested the HA method has limited efficacy in the selecting best sperm, and there was no correlation between the HA-bound sperm, and low levels of DNA fragmentation. Another report also indicated that additional exposure to nonphysiologic conditions during the HA method leads to damaged sperm and an even lower fertilization and cleavage rate than those in the conventional method [17]. In contrast to these results, other evidence indicated that HA-bound sperm have little or no effect on clinical pregnancy [32]. This result was similar to the result of the study by Tarozzi and colleagues [33], they demonstrated no relationship between using the HA method and fertilization, cleavage, good-quality embryos, implantation, clinical pregnancy. Moreover, Huszar's studies on plasma membrane biomarkers of sperm maturity, such as heat shock protein A2 (HspA2) synthesis, and nuclear histoneprotamine replacement demonstrated that HA-bound sperm show features of maturity and DNA integrity [34]. Thus, HA-bound sperm are mature sperm, it seems that these sperm have value clinical to improve successes of ICSI outcomes. In agreement with the present study, Castillo-baso and colleagues reported that the development of embryos using the HA method increase in patients with male factor infertility [15]. It is suggested that intact DNA in the HA method resulted in good quality embryos. The main limitation in this study was the small sample size used for sperm selection methods. Therefore, a large sample size is recommended to increase the validity and reliability of results.

Conclusion

The current study showed that the use of the HA method has a high sensitivity degree for sperm selection with low levels of DNA fragmentation and protamine deficiency; it significantly improves embryo development and increases the pregnancy rate. Hence, this method with respect to other methods (ZP, conventional PVP) is a very useful method in the selecting of undamaged sperm for ICSI, and that should be used for giving a better chance to successful treatment of oligoteraozoospermia patients.

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Disclosure of conflict of interest

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