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Outcomes of Hyaluronic Acid and HOS Test on Sperm Selection for Intracytoplasmic Sperm Injection: A Comparative Study

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ABSTRACT

Background: The outcome of intracytoplasmic sperm injection (ICSI) and embryo development are significantly influenced by sperm quality.

Objective: The aim of the present study is to find the different outcomes of ICSI by using hyaluronic acid (HA) and hypo-osmotic swelling (HOS) test in sperm selection so as to obtain the best results of ICSI outcomes and the best embryos with high quality to increase pregnancy rate in cases of infertility.

Patients and Methods: This study was carried out on 100 patients in ICSI cycles with confirmed diagnosis with unexplained infertility or repeated failure of ICSI cycles, and patients with HOS test positive semen profile. Statistics have been made for the study results.

Results: There was significant difference between the HOS and HA groups regarding the fertilization rates, cleavage stage, and blastulation quality (63.7% VS 85.9%; 54.3% VS 80.7% and 50.8% VS 66.6% respectively). However, no statistically significant difference was found between the HOS and HA groups regarding oocytes injected (6.46 % VS 6.56%; *P* =0.870). They were almost equal in number in ICSI, to avoid any factors which may affect the results.

Conclusion: HA could be thought of as the best routine on a regular basis for sperm selection before ICSI. Finally, we have achieved our main goal from the study, which is getting the best protocol for sperm selection in ICSI, which gives the best embryos quality for embryo transfer and increase pregnancy rate in cases of infertility.

Keywords: Hyaluronic acid, HOS test, Sperm selection, ICSI.

INTRODUCTION

A single sperm cell is injected directly into the cytoplasm of an egg during an in vitro fertilisation (IVF) process known as intracytoplasmic sperm injection (ICSI). The gametes are prepared using this method in order to obtain embryos that may be transplanted to a mother uterus. ICSI and conventional IVF differ from one another in a number of ways. The procedures that must be carried out both before and after insemination are the same. ICSI requires just one sperm cell per oocyte for insemination [(1)].

The effectiveness of ICSI has been compared to that of in vitro fertilisation (IVF), a less complex procedure in which the oocytes are picked up and overnight left with the prepared sperm sample, where fertilisation takes place after a kind of spontaneous sperm selection [(2)]. Today, ICSI has supplanted IVF as the method of choice for treating infertility in wealthy nations [(3)].

For ICSI, there are many sperm selection methods. Sperm in vitro selection and capacitation must be completed prior to ICSI. In addition to the most popular in vitro sperm capacitation methods (swim-up, density gradients, filtering, and simple wash) [(4)], the traditional techniques, such as swim up (SU) and density gradient centrifugation (DGC), which rely on separation processes reliant on morphology and motility, have produced disappointing results [(5)]. For sperm cell preparation techniques to be used in assisted reproductive technology (ART) ART routine procedures, they must be easy, inexpensive, and quick, enabling a highly effective selection that separates motile and morphologically normal spermatozoa from other cell types, leukocytes, bacteria, and toxic substances, preventing the production of ROS [(6)].

Additionally, growing research indicates that sperm DNA integrity issues lead to worse ART results [(7)]. Some new methods are far superior than the old ones and highly helpful [(8)].

According to **Casper *et al.* [(9)],** who recommended using the traditional hypo-osmotic swelling (HOS) test in these circumstances, fertilisation and cleavage rates were 43 and 39%, respectively, when the spermatozoa were selected using the HOS test as opposed to 26 and 23% when the spermatozoa were selected randomly. **Smikle and Turek [(10)]** discovered in an in vitro investigation that the traditional HOS test could precisely determine the viability of functioning spermatozoa, and occasional pregnancies have been documented [(11)]. **Liu *et al.* [(12)]**, who chose viable spermatozoa using a NaCl hypo-osmotic solution, obtained similar outcomes.

Based on the functional sperms' propensity to enlarge when exposed to hypoosmotic fluid, the HOS test measures this propensity. The tips of the tails of spermatozoa with defective cell membrane function do not enlarge or invaginate [(13)].

To assess the structural and functional integrity of spermatozoa, a low-cost hypo-osmotic swelling test is helpful. It is predicated on the idea that, in hypoosmotic situations, fluids move across cell membranes to reach equilibrium on both sides of the membrane [(14)]. This behavior is frequently referred to as "tail curling". The ballooning of the plasma membrane is what causes the curl [(15)].

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Another new technique is hyaluronic acid that considers biomarker of functional sperm as the hyaluronan is one of the cumulus oophorous complex components which surrounds the oocyte and is regarded as an important biomarker for sperm maturity and quality [16]. Only fully mature sperm that have completed the last crucial stages of spermatogenesis have fully functioning receptors for hyaluronan. Increased levels of cellular viability, maturity, and acrosomal unreaction are seen in human sperm that binds HA [2,17]. In response to hyaluronan, mature spermatozoa immobilise their sperm and wriggle their tails erratically. The Hyaluronan binding biomarker can be used to find the best spermatozoa in the ejaculate [18]. Additionally, sperm that can cling to HA is said to have a lower likelihood of being anueploid [19] or having DNA fragments [7,8]. A reduced rate of IVF pregnancy, abnormal preimplantation development, early pregnancy loss, and a lesser percentage of infants conceived with ART have all been associated with poor DNA integrity [20, 21].

The aim of this study is to obtain the best results of ICSI outcomes and the best embryos with high quality to increase pregnancy rate in cases of infertility. So, this study compared the effect of hyaluronic acid and HOS test on sperm selection for ICSI following by observation cleavage and blastulation stage of embryos to get the best quality embryos, which are selected to transfer to infertility woman.

PATIENTS AND METHODS

The study was carried out on 100 patients in ICSI cycles.

Inclusion criteria:

The study included male patients aged between 20 to 60 years and female patients aged between 20 to 40 years with good hormonal profile and considered by laboratory and imaging studies as fertile females at the beginning of the study period, confirmed diagnosis with unexplained infertility or repeated failure of ICSI cycles and patients with HOS test positive semen profile.
Firstly, we prepare the semen sample according to the two protocols in our study as following:

HOS test procedures:

0.05 mL of semen and 0.5 mL of HOS (0.735 g sodium citrate, 1 g fructose, and 100 mL distilled water) were combined for this test, which was incubated for 60–120 minutes.

A 40X microscope was used to look at 100 spermatozoa at a temperature of 37°C. The number of spermatozoa with coiled tails and HOS positivity was counted; samples with 40% or more coiled spermatozoa were thought to be harmful for reproduction. When 60% of the spermatozoa had coils on them, it was regarded as typical. Thus, the HOS positive spermatozoa were selected and applied for intracytoplasmic sperm injection (ICSI) refers to the process used in laboratories when each egg was injected with one sperm picked up with a tiny glass needle. Utilizing specialised equipment, this was carried out in a lab setting by skilled embryologists.

Hyaluronic acid procedure:

Ready-to-use media were removed from storage at 2–8 ℃ and left to equilibrate with room temperature for 10 minutes. 2x10 µl drops of ready-to-use media were pipetted, one at the center of the dish and another at the rim of the dish. 5 µl of already prepared and washed sperm was added close to the central drop and it was made sure to create a junction between the sperm drop and the media central drop. After that, incubation for 10–15 minutes was carried out at 37 °C. After incubation, oocyte holding media drops was added around the center drop with corresponding 5–10 µl per holding media drop. 10–20 mm of the sperm preparation medium were aspirated into the holding pipette and 2 – 5 mm of the sperm preparation medium into the injection pipette. It was released after 1 min in order to coat the pipettes before aspirating mature sperm select media. 2–6 mm were aspirated of the mature sperm select media of the outer rim drop into the injection pipette. A spermatozoon was selected from the junction between the prepared sperm droplet and the mature sperm select central drop was selected and directly injected.

Mature spermatozoa should exhibit tail movement but no forward motility/progression (to be selected and applied for intracytoplasmic sperm injection (ICSI)). Immature spermatozoa should be moving freely (are not to be selected). Secondly, the oocytes were prepared where retrieval and denudation to get oocytes ready for ICSI.

ICSI procedures:

Equipment must be in place to maintain oocytes close to 37°C. ICSI in 38 to 40 hours after the HCG trigger. The injection dish was made with buffer media drops for oocytes and PVP line for sperm. Line of sperm processed with HOS test was made and drop of sperm processed with HA. Sperms were selected from HOS line and HA drop by injection pipette to PVP drop for immobilization them. Several micromanipulators and a microscope were used to complete the procedure (micromanipulator, microinjectors and micropipettes). The mature oocyte was stabilised by a holding pipette using the gentle suction of a microinjector. A thin, hollow glass micropipette (injection pipette) was used to extract a single sperm from the other side after first immobilising it by severing its tail. The oolemma was torn, allowing sperm to enter the inside of the egg (cytoplasm). After that, the sperm was discharged into the oocyte. The mature oocyte has an extruded polar body at around 12 o'clock. To avoid disrupting the spindle of the egg, the polar body was positioned at 12 or 6 o'clock.

Once the steps of ICSI were complete and fertilization was successful, the embryo start in divisions to form cleavage then blastocyst, which was recorded.

Ethical approval:

This experiment was ethically approved by the Ain Shams University Faculty of Medicine. After being fully informed, all participants provided written consent. The study was conducted out in line with the Helsinki Declaration.

Statistical analysis

Statistical analysis was carried out using SPSS version 22. (SPSS Inc., Chicago, IL, USA). Categorical data are reported as frequencies and percentages, whereas continuous variables are given as mean±standard deviation. The Shapiro-Wilk test was used to determine whether something is normal. The Student's t test was used to examine how different continuous data differed from each other. P value of 0.05 or below was regarded as statistically significant.

RESULTS

The comparison outcome of HA and HOS test of sperm selection in ICSI cycles

Table (1) shows the application of the inclusion criteria on the patients as wife ages range (20-40) and husband ages range (21-45). The semen parameters (count and motility) ranged (10-85 M) and (25-80%), numbers of oocytes picked up range (2-30) and the mature oocyte range (2-26).

Table (1): Range and mean of wife age, husband age, semen parameters (count, motility), number of oocytes picked up and mature oocyte in the study

		Range	Mean ± S. D
Wife age (year)		20 – 40	29.3 ± 0.9
Husband age (year)		21 – 45	32.7 ± 1.0
Semen parameters	Count (M)	10 -85	33.8±2.56
	Motility	25-80	53.8±14.27
No. of oocytes picked up		2-30	15.48±6.9
MII (mature oocyte)		2-26	13.0±6.1

The injection of oocytes by semen prepared by HA and HOS:

There was no statistically significant difference between the HOS and HA groups regarding oocytes injected. They were almost equal in number, to avoid any factors, which may affect the results (Table 2).

Table (2): Ratio between HOS and HA groups regarding the oocytes injected with simple ratio of standard deviation

		HOS	HA
Oocytes injected	**Range**	1 – 13	1 – 13
	Mean± SD	6.46 ± 0.42	6.56 ± 0.44
	P value	0.870	

The observation stage between the two groups

1- Fertilization rate:

The percent of fertilization of HA group was significantly lower than that of HOS group (Table 3).

Table (3): Comparison between the HOS and HA groups regarding percentage of fertilization

		HOS	HA
Percentage of Fertilization (%)	**Range**	0 – 100	33.3 – 100
	Mean± SD	63.7 ± 23.8	85.9 ± 17.3
	P value	0.0001*	

2- Cleavage stage quality: We recorded the quality of embryos in cleavage stage between two groups HA versus HOS. Grading the embryos with A, B and C according to their quality**.** As showed in table (4).

Table (4) shows that there was statistically significant difference between the HOS and HA groups regarding cleavage stage.

Table (4): Comparison between the HOS and HA groups regarding cleavage stage

Cleavage stage	HOS Mean± SD	HA Mean± SD	Chi-square	*P* value
Grade A (%) (Good embryo)	54.3±4.2	80.73±2.6	14.87	0.001*
(%) Grade B	30.0±4.4	10.0±2.39		
Grade C (%)	9.1±2.67	9.2±2.2		

3- Blastocyst stage quality: We recorded the quality of embryos in blastulation stage between two groups HA versus HOS. Grading the embryos with (4AA. 3AA. Early) (good blastocyst), morula (moderate stage) and compacted arrested cells (bad quality) according to **David Gardner's** scoring as showed in table (5).

There was significant difference between the group of HA and group of HOS regarding grading the embryo in the blastocyst stage (Table 5).

Table (5): Comparison between the HOS and HA groups regarding blastocyst stage

Blastocyst	HOS Mean± SD	HA Mean± SD	Chi-square	*P* value
4AA +3AA+ Early (%)	50.8± 18.4	66.6± 24.0	11.8	0.002*
Morula (%)	54.0± 28.4	29.1± 5.9		
Compact (%)	47.9± 25.4	29.8±1 5.2		

According to these statistics there are significant difference between HA and HOS groups, we obtained that HA has better results than HOS test in terms of embryos quality and HA outperformed HOS according to fertilization, cleavage stage and blastocyst stage. Therefore, if broader, multi-infertility center randomized studies confirm these favorable impacts on embryo quality and ICSI outcomes, HA could be thought of as the best routine on a regular basis for sperm selection before ICSI.

Finally, we have achieved our main goal from the study, which is getting the best protocol for sperm selection in ICSI, which gives the best embryos quality for embryo transfer and increases pregnancy rate in cases of infertility.

DISCUSSION

All studies carried on the effect of HOS test and HA on sperm selection for ICSI outcomes both separately, however, in this study a comparison was made between all of them to obtain which of them had the best results and outcomes. From many studies of HOS test on sperm selection for ICSI the following was found: The inexpensive hypo osmotic swelling test aids in assessing the structural and functional integrity of spermatozoa. It is predicated on the idea that fluid moves across the cell membrane under hypoosmotic circumstances to achieve balance on both sides of the membrane, and as a result of this movement, loose sides expand **(14).** This behavior is frequently referred to as "tail curling". The ballooning of the plasma membrane is what causes the curl **(15).**

The HOS test is more than just a test of the spermatozoa's metabolic health; it also serves as a test of their capacity to develop, among other things. It is reasonable to anticipate that a nonfunctional membrane, as shown by abnormal (negative) HOS test findings, would significantly reduce fertility **(22, 23).**

As an exterior cell structure that serves as a physiological barrier and whose integrity is necessary for its regular functions, the integrity of the sperm membrane has received increased attention in research. Additionally, the integrity of the sperm membrane plays a crucial part in fertilisation **(24)**. As a result, compared to healthy controls, infertile men have a larger percentage of sperm with damaged membranes **(25)**. Through the previous results of HOS-ICSI, high quality embryos were obtained, which increase pregnancy rate for infertile woman.

Many previous studies of HA on sperm selection for ICSI, which agreed with our study in its effect on sperm selection for ICSI and enhance the ICSI outcomes and increase the embryo quality. Hyaluronan is one of the cumulus oophorous complex components which surrounds the oocyte and is regarded as an important biomarker for sperm maturity and quality **(26)**. Only fully mature sperm that have completed the last crucial stages of spermatogenesis have fully functioning receptors for hyaluronan. Mature spermatozoa response to hyaluronan results in sperm immobilization and tail vigorous movement. The highest quality of spermatozoa possible within the ejaculate can be identified using hyaluronan binding biomarker **(27).** So, hyaluronic acid (HA) thereby encourages the selection of spermatozoa without DNA breakage and with a normal nucleus, improving the quality of the embryo **(28)**.

Hyaluronic acid (herein termed hyaluronan) is a biologically active molecule that is also a major component of the extracellular matrix surrounding the oocyte-cumulus complex **(29).** Several small clinical studies **(29,30)** including three randomised trials **(26,31,32)** reported that ICSI with hyaluronan-selected sperm improved embryo quality and livebirth rates and decreased miscarriage rates compared with ICSI with sperm selected using standard methods.

In a previous study, HA ICSI has been mostly accepted in much research. Since it is beneficial to increase fertilization rate; helping the embryo to reach cleavage stage and blastocyst stage more efficiently **(29).** Furthermore, the use of HA ICSI can increase embryo implantation rate and pregnancy rate as well as reduce abortion rate, compared to ICSI with standard methods of sperm selection. By favouring the selection of spermatozoa with a normal nucleus and undamaged DNA, it was shown that the injection of HA-bound spermatozoa enhanced embryo quality and development **(5)**. Additionally, it has been noted that sperm that can attach to HA are less likely to be aneuploidy**(6)** or contain DNA fragments **(7,8)**. Poor DNA integrity is associated with early pregnancy loss, abnormal preimplantation development, a lower rate of in vitro fertilisation pregnancy, and a lower proportion of ART-conceived kids **(20)**. Injection of sperm that has been shown to have strong DNA integrity and is coupled to HA **(21)**.

CONCLUSION

Hyaluronic acid may serve as the best technique for sperm selection in ICSI, resulting in the greatest quality embryos for embryo transfer and a higher pregnancy rate in infertile patients.

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