J Assist Reprod Genet (2008) 25:197–203
DOI 10.1007/s10815-008-9223-4

ANDROLOGY

Evaluation of sperm selection procedure based on hyaluronic acid binding ability on ICSI outcome

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Received: 15 November 2007 / Accepted: 23 April 2008 / Published online: 16 May 2008

Abstract
Purpose To compare the efficiency of routine sperm selection method with HA-selection procedure for fertilization rate, embryo development, implantation and pregnancy rates as well as evaluating the relationship between HA-binding ability with sperm protamine deficiency and DNA fragmentation.
Methods Semen samples were obtained from the 50 couples undergoing ICSI. The percentage of fertilization rate, cleavage and quality of embryos compared between two procedures (routine sperm selection and HA-binding selection). The semen samples were assessed for DNA fragmentation and protamine deficiency by sperm chromatin dispersion (SCD) test and Chromomycin A3 (CMA3) staining, respectively.
Results A significant inverse correlation was observed between percentage of HA binding with protamine deficiency, DNA fragmentation and abnormal sperm morphology ($P<0.05$). Furthermore, in current study, oocytes inseminated by HA sperm selection procedure had significantly higher fertilization rate ($P<0.05$). While the pregnancy and implantation rates were insignificantly increased.
Conclusion The results suggest that normal sperm have higher chance to bind HA and therefore, HA sperm selection procedure may select sperm with normal protamine content and low DNA fragmentation, but to confirm the effect of HA sperm selection on the ICSI outcome requires further studies.

Keywords Sperm selection · HA-binding · Protamine deficiency · DNA fragmentation · ICSI

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Introduction

For successful in vivo or in vitro fertilization to take place, sperm has to pass natural fertilization barriers. However, ICSI circumvents natural barriers. The success rate of fertilization in this technique is greatly affected by the quality of sperm used for insemination [1, 2].

During ICSI, sperm is selected based on sperm morphology and motility. However, this selection procedure is not discriminatory with respect to the identification of spermatozoa with normal haploid chromosome, intact chromatin or DNA [3–5]. In addition, in sever male infertility cases, sperm used for ICSI may have a detrimental effect and lead to production of potentially defective embryos, low pregnancy rate and high abortion rate [6]. Therefore, an effective procedure for selection of normal sperm based on membrane maturity for ICSI is greatly needed [7, 8].

Since the introduction of assisted reproductive techniques in treatment of infertility, different sperm functional tests have been developed or used for understanding the mechanism underlying male infertility, in hope of designing

sperm selection procedures. Sperm functional tests have been mainly based on assessing nuclear, membrane and cytoplasmic maturity [9–11].

In a consecutive series of studies on sperm surface marker, Huszer et al have shown that similar to zona binding site(s), HA receptor formation is associated by the remodeling of the plasma membrane during spermiogenesis [12]. Therefore, mature spermatozoa may selectively bind to solid state HA, as with sperm binding to zona pellucida [13–15]. Capacitated or acrosome reacted spermatozoa do not exhibit HA-binding [10].

Characteristics of HA-bound mature spermatozoa indicated that HA-selected spermatozoa are viable (non-viable spermatozoa do not bind), devoid of cytoplasmic retention, persistent histones, DNA fragmentation, and apoptotic markers such as caspase 3 [7, 8, 10]. Furthermore, expression of HSPA2 (a testis-expressed chaperone which is part of the synaptonemal complex that directs and supports the meiotic process) has been shown to be lower in immature sperm with increased cytoplasmic retention [16–18]. Therefore, selection of HA-bound mature sperm has been shown to have lower frequency of chromosomal aneuploidies [19].

Based on these features of HA-bound spermatozoa, it is expected that the method will facilitate the selection of single mature spermatozoa for ICSI. Therefore, the aim of this study was to compare efficiency of the routine sperm selection method with HA-selection procedure in terms of fertilization rate, embryo development, implantation, and pregnancy rates. In addition during this study, the relationship between HA-binding ability with sperm protamine deficiency and DNA fragmentation were assessed.

Materials and methods

This study was initially approved in the ethical and scientific committee of Isfahan Fertility and Infertility Center and Royan Institute.

Patient selection

During this study 50 couples undergoing ICSI for the first time due to male infertility were informed regarding routine sperm selection procedure and HA-sperm selection method. Fifty couples voluntarily accepted to have half of their oocytes to be inseminated by HA procedure. Couples were informed that HA is a natural substance present in the body fluids, including in human follicular fluid. Furthermore, they were informed that HA used in this study was prepared for human usage. However, considering the fact that there was no literature study regarding the effect of HA on pregnancy and implantation rate, thus the ethical committee allowed the couples to have the choice to receive embryos transfer from HA-sperm selected procedure, or the routine ICSI procedure or from both procedures (HA/ICSI). Written information consent forms were signed by couples.

Sperm preparation

Semen samples from the 50 couples were collected by masturbation after 3–4 days of abstinence on the day of oocyte recovery. All the assessments were performed after liquefaction of the semen. Sperm motility and concentration were assessed according to WHO criteria [20]. Sperm morphology was assessed according to Kruger's strict criteria [21].

Part of semen samples were processed using discontinuous Pure sperm gradients (80:40; Nidacon, Gothenburg, Sweden). Sperm were finally washed in G-Rinse (Vitrolife, Gothenburg, Sweden). For ICSI or HA-ICSI procedure, sperm insemination was carried out within 30–45 min after completion of semen processing. The remaining semen was used for assessment of protamine deficiency and DNA fragmentation.

Preparation of HA coated dishes and ICSI procedure

All of the media were purchased from Vitrolife, Gothenburg, Sweden, G3 series plus, unless otherwise stated. A single stimulation protocol was used for all the patients. Briefly, Ovarian stimulation and ovulation induction were induced using, Burserelin (Aventis, Germany) from day 21 of previous cycle. Human menopausal gonadotropin (hMG, Menogon, Ferring, Germany) in combination with recombinant FSH (Gonal-F, Serono, Swiss) was administered daily from second day of the cycle. Ovulation was induced by 10,000 IU hCG (Organon, Holland). Oocytes were retrieved by trans-vaginal needle-guided ultrasound at 34–36 h post hCG. After oocyte collection, the oocytes were treated in hyaluronidase (Hyase) in G-MOPS medium. Oocytes were then washed in fresh G-MOPS and randomly divided into two groups: 1) inseminated using routine ICSI procedure (Control) and 2) sperm selected by HA procedure (HA). Oocytes from each group were transferred to G-oocyte dishes under oil in the prepared Falcon 1006 dish as described below, for ICSI procedure.

For preparation of HA dishes, hyaluronic acid (Juvederm30, LEA Derm, Paris, France), was diluted 1:40. Fifty-microliter drops were placed in Falcon 1006 dish and allowed to dry in a sterile condition. The dishes were stored and used on the day of ICSI. Concomitantly with preparation of ICSI dishes with G-oocyte and ICSI-100 (a viscous sperm handling solution) for routine ICSI, the HA spot was also covered by 50 μl of G-oocyte. HA drops were washed twice with G-oocyte to remove any unbounded HA. As stated, denuded oocytes were randomly divided into

HA or control group in G-oocyte drops under oil in a HA-coated dish prepared for ICSI. Processed semen sample was introduced both into ICSI-100 and HA coated drops. For HA-ICSI, in addition to best sperm morphology and motility, sperm were also selected according to ability to bind solid HA. The selected sperm were then washed twice both in G-oocyte and ICSI-100 before insemination. Eppendorf micro-manipulator mounted on a Nikon inverted microscope was used to perform ICSI. The injected oocytes from each group were cultured in G1 plus, separately. Around 16–18 h post ICSI, fertilization was assessed by presence of pronuclei. The percentage of fertilization was calculated by the ratio of fertilized oocytes to the total number of survived injected metaphase II (MII) oocytes multiplied by 100 in both groups. Immature, deformed and post-mature oocytes, or any oocyte with certain types of abnormality, were excluded.

Assessment of sperm HA-binding

Briefly, separate dishes with HA drops were also prepared. To determine the percentage of motile spermatozoa that bound to HA, numbers of bounded and unbounded motile sperm and immotile sperm were determined in different microscopic fields, and then the percentage of total bonded motile spermatozoa was determined. HA bounded spermatozoa exhibited vigorous beating with increased tail cross beat frequency.

Chromomycin A3 staining

Processed semen samples were fixed in Carnoy's solution [methanol: glacial acetic acid 3:1 (Merck, Germany)] at 4° C for 5 min. Smears were prepared and each slide was treated for 20 min with 100 µl of CMA3 solution [Sigma, USA; 0.25 mg/ml in McIlvaine buffer (7 ml citric acid 0.1 M+32.9 ml $Na_2HPO_4 \cdot 7H_2O$, 0.2 M, pH 7.0, containing 10 mM MgCl2)]. The slides were then rinsed in phosphate buffer saline and mounted with buffered glycerol (1:1). Microscopic analysis of the slides was performed on an Olympus fluorescent microscope (BX51, Tokyo, Japan) with the appropriate filters (460–470 nm). On each slide, 100 sperm were evaluated. Evaluation of CMA3 positivity was carried out using Olysia software. Pixel intensity of each sperm was recorded. Sperm with pixel intensity of higher than 100 was considered as CMA3 positive or protamine deficient, while those with pixel intensity of lower than 100 were considered as CMA3 negative or with normal amount of protamine [11].

Sperm chromatin dispersion test

Semen samples were washed with Ham's F10 and then diluted to 5 to 10 million per milliliter. Sperm chromatin dispersion test was carried out according to Fernández et al. [22] procedure. Slides were stained with Wright for light field microscopy. A minimum of 500 sperm per sample were scored under the ×100 objective of the microscope.

Five SCD patterns were established: (1) Sperm cells with large halos (SCBH): those whose halo width is similar or higher than the minor diameter of the core; (2) Sperm cells with medium size halos (SCMH): their halo size is between those with high and with very small halo; (3) Sperm cells with very small size halo (SCSH): the halo width is similar or smaller than one-third of the minor diameter of the core; (4) Sperm cells without a halo (SCWH); and (5) Sperm cells without a halo-degraded (DC): similar to SCWH but weakly or irregularly stained. Sperm cells with very small halos, without halos and without halo degradation, contain fragmented DNA, thus the percent of DNA fragmentation was determined for each semen sample [22].

Statistical analysis

The relation between parameters were analyzed using Spearmen Rank Correlation and Pearson Correlation. The mean difference between variables was assessed by Student's *t* test using Statistical Package for the Social Studies Software (SPSS11.5) and pregnancy and implantation rate were compared using Chi square method with logic function using Statistic Analysis Software (SAS).

Results

Tables 1 and 2 show the descriptive analysis and clinical characteristic of 50 couples involved in this study, respectively. The mean ages of male and female in this study were 36.5±7 and 30±6 respectively, with a mean duration of infertility 8.4±5 years.

Table 3 reveals the mean number of oocytes, percentage of fertilization, embryo cleavage and quality score on day 2 and 3 post ICSI as well as the number of embryos transferred per transfer in Hyaluronic acid (HA) and control groups. As

Table 1 Descriptive information of semen parameters, chromatin status and duration of infertility

	Minimum	Maximum	Mean±SD
Concentration ($\times 10^6$/ml)	1.5	90	40.56±21.91
Motility (%)	5	60	35.41±13.73
Abnormal morphology (%)	60	100	86.34±8.75
CMA3 positivity (%)	20	88	52.10±13.96
DNA fragmentation (%)	5	83	38.52±15.84
Duration of infertility (years)	2	20	8.47±5.24

Table 2 Clinical characteristics of 50 couples undergoing ICSI

Parameters	Routine ICSI NO (%)	HA Group NO (%)
Polycystic ovarian syndrome	1 (6.6)	1 (6.6)
Endometriosis	1 (6.6)	–
Tubal factor	3 (20)	3 (20)
Male factor	6 (40)	6 (40)
Unexplained	2 (13.3)	3 (20)
Repeated cycles	2 (13.3)	2 (13.3)

the results show, the mean numbers of oocytes allocated to each group were similar. However, the percentage of fertilization was significantly higher in the HA group compared to the control group. Although, the percentage of cleavage and good quality embryo were not significantly different between the two groups on day 2 and 3 post ICSI, the cleavage rate on day 2 was close to be significant (P= 0.066). The number of embryos derived from HA selection or routine ICSI and transferred per individual were not significantly different. Furthermore, the mean number of embryos per transfer in the HA/ICSI group was 1.97±1.13 and was insignificantly different from the HA or control group.

During ICSI procedure and embryo development no specific side effects was observed with HA-ICSI procedure.

Table 4 shows the correlation between different parameters with the percentage of sperm HA-bounded sperm, DNA fragmentation and fertilization rate. The results show a significant positive correlation between sperm concentration and motility with percentage HA-bounded sperm while a negative correlation with percentage abnormal morphology. Furthermore, the percentage HA-bounded sperm showed a negative significant correlation with protamine deficiency assessed by CMA3 staining and DNA fragmentation assessed by sperm chromatin dispersion test. The results also show a significant positive correlation between DNA fragmentation with CMA3 positivity and percentage of abnormal morphology. In addition the percentage of DNA fragmentation shows a significant negative correlation with sperm motility. It is of interest to note that percentage of DNA fragmentation showed no correlation with percentage of fertilization. However, percentage fertilization post ICSI showed only a significant negative correlation with protamine deficiency.

Implantation and pregnancy rates are presented in the Fig. 1. Implantation and clinical pregnancy rate are 20%, 16%, 22% and 46%, 40%, 55% in patients receiving embryos from HA selected sperm (HA), routine ICSI (control) and from both procedures (HA/ICSI), respectively. The differences between these groups were not significant (P>0.05).

During analysis of data, we also divide patients into three groups according to the sperm density 0–10, 10.1–30 and >30 million per milliliter. We analyzed the fertilization, pregnancy and implantation rates in these groups between HA, routine ICSI and HA/ICSI group. The only significant differences were observed in the group with higher than 30 million sperm per milliliter for the fertilization and implantation rate at significance level of 5% and 10% between the HA and routine ICSI (Control) respectively. The statistical analyses for other groups were not significantly different, possible due to low sample size (data not shown).

Follow up of patients revealed two miscarriages, one in the HA and the other in the HA/ICSI group.

Discussion

The development of the novel sperm selection method with HA binding is based on the recognition that during spermatogenesis and plasma membrane remolding, the formation of the zona pellucida-binding and HA-binding sites are commonly regulated. Furthermore, it has been shown that the HA-selected mature sperm have a frequency of chromosomal aberrations comparable to that of sperm selected by the zona pellucida in conventional fertilization [23]. Therefore, the aim of this study was to evaluate the efficiency of this procedure on ICSI outcome. However, in order to justify the HA-binding procedure used in this study, the relation between HA-binding ability of the 50 semen samples with different parameters were evaluated.

Table 3 Comparison of the mean number of oocytes, percentage of fertilization, cleavage and good quality embryo on day 2 and 3 post ICSI and mean number of embryos transferred per patient in Hyaluronic acid (HA) and control group

Parameters	HA mean±SD	Control mean±SD	P value
Number of oocyte	4.98±2.57	5.34±2.42	0.465
Percentage of fertilization	79.4±26.0	67.7±23.5	0.020
Percentage of cleavage day 2	99.4±04.5	95.2±15.8	0.066
Percentage of cleavage day 3	88.8±22.2	89.8±20.8	0.817
Percentage of good quality embryo day 2	97.8±09.2	99.4±04.6	0.279
Percentage of good quality embryo day 3	96.4±14.1	97.6±08.7	0.597
Number of embryos per transfer	1.44±0.89	1.7±1.31	0.539

Table 4 Shows the correlation between different parameters with the percentage of sperm hyaluronic acid (HA) binding, DNA fragmentation and fertilization rates

Parameters	%HA-binding r (P-Value)	%DNA Fragmentation r (P-Value)	% Fertilization r (P-Value)
% Abnormal morphology	−0.431 (0.001)	+0.36 (0.008)	−0.10 (0.790)
% Motility	+0.47 (0.000)	−0.28 (0.045)	−0.04 (0.787)
Density (million per milliliter)	+0.43 (0.001)	−0.12 (0.389)	−0.05 (0.745)
%CMA3 Positivity[a]	−0.30 (0.028)	+0.28 (0.045)	−0.30 (0.038)
% DNA fragmentation	−0.29 (0.035)	–	−0.16 (0.292)
% Fertilization	−0.20 (0.157)	−0.16 (0.292)	–
% HA-binding	–	−0.29 (0.035)	−0.20 (0.157)

[a] Percent CMA3 presents protamine deficiency.

The results reveal a significant correlation between semen parameters and HA-binding ability (Table 4), suggesting that sperm from semen samples with higher sperm density, motility and lower sperm abnormality, have a higher ability to bind solid state HA. Ye et al. [24] also showed that the ability of sperm in a semen sample to bind HA was highly correlated with the total, progressive motility and normal sperm morphology, suggesting that HA binding is likely to reflect the semen quality revealed by the routine semen analysis. However, Ye et al. [24] observed no significant correlation between HA binding ability and sperm concentration [24]. This difference could be due to different population or the fact that in this study HA binding was carried out immediately after semen samples were processed.

During this study, the relation between protamine deficiency, as a maker of sperm chromatin maturity [11] with the HA binding ability was also assessed. The results showed a negative correlation between these two parameters. Thus, revealing that semen samples with chromatin immaturity have lower HA-binding ability. Similar results were also observed by Huszar et al assessing presence of excessive histones by aniline blue staining as a marker of sperm chromatin maturity [10]. Previous study also revealed a strong positive correlation between presence of excessive histone and protamine deficiency [11] suggesting that sperm with excessive amount of histone or absence of adequate amount of protamine have a lower binding ability to solid state HA.

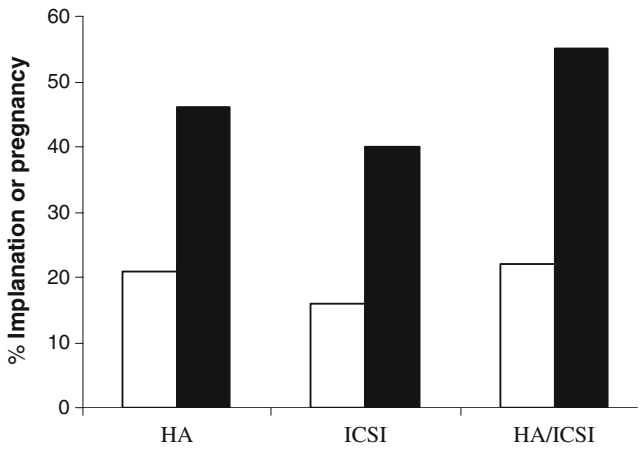

Fig. 1 Shows percentage of implantation (*white columns*) and pregnancy (*black columns*) in patients receiving embryos derived from HA selection, routine ICSI or from both procedure (HA/ICSI)

Risk of successful fertilization and subsequent development using DNA-damaged spermatozoa has always remained a concern, especially during ICSI procedure when natural barriers to fertilization are bypassed [25]. The results of present study also show a negative significant correlation between HA binding with DNA fragmentation, suggesting that sperm with DNA fragmentation have a lower potential to bind to HA and thus such sperm are likely to be excluded form insemination population during HA-ICSI procedure. The results of this study are in agreement with Huszar et al report showing that HA-bounded sperm are devoid of DNA fragmentation and apoptotic markers [26]. In this study a significant positive correlation was observed between DNA fragmentations with sperm abnormal morphology and sperm protamine deficiency, but a significant negative correlation was obtained with sperm motility (Table 4). Taken together the results of this part of study verify our HA-sperm selection procedure.

Thus, it can be concluded that the above parameters may have a confounding effect on each other, but among these factors protamine deficiency has a significant negative correlation with fertilization rate and HA binding ability. Thus, the HA sperm procedure may select mature sperm in terms of chromatin and DNA integrity. Therefore, this preliminary study tries to evaluate the effect of HA sperm selection on unselected population of infertile patients.

The results of the present study show that sperm HA selection improved fertilization rate but did not have a significant effect on cleavage and embryo quality on day 2 and 3 post ICSI. Among the latter parameters, cleavage rate on day 2 was close to be significantly different from the control group. The fact that embryo quality were not significant different between HA and control group, is in

accordance to pervious studies which suggest that paternal DNA anomalies may become manifested post genomic activation and during or following implantation [27].

Considering the fact the number of embryo per transfer and causes of infertility were similar in the two groups, however, the results revealed that those patients which received embryos derived from HA-ICSI procedure have higher pregnancy and implantation rates compared to patients who received embryos from the control group. However, this difference was not insignificant. Furthermore, the pregnancy and implantation rates were also higher in those patients receiving embryos derived from both HA and routine ICSI procedure. The insignificant difference is likely to be due to the low number of patients which warrants further study to be carried out as a clinical trial.

In this study we also divided patients according to their sperm density. The percentage fertilization, pregnancy and implantation was compared between HA, routine ICSI and HA/ICSI groups. Only significant difference was detected in the group with higher than 30 million sperm per milliliter for the fertilization and implantation rate at significance level of 5% and 10% between the HA and routine ICSI (Control) respectively. However, to make a conclusion that this procedure might be suitable for particular patients, further trial with higher number of patients is required.

Survey of literature reveals two meeting reports on using HA-ICSI procedure. In one report, unlike our results, no significant difference in fertilization rate was found, although a significant increase was observed in pregnancy rate [28]. Comparison of these results to our data, reveal a similar pregnancy rate in patients who received embryos from HA (57.1% vs 46%) or both HA/ICSI (57.1% vs 40%) procedure. However, they had a substantial lower pregnancy in their control group (25% vs 40%), which has lead to significant difference in pregnancy rate between HA and control groups in their study [28]. In another study by Janssens et al., a higher fertilization rate was reported, but no further results were available [29].

Although a substantial amount of work has been carried out by Huszar and their colleagues in this filed, especially for producing HA coated FDA approved dishes called “PICSI” [26].To our knowledge this is the first study, presented for publication, to compare the HA selection procedure with routine ICSI, concluding that HA sperm selection may improve the ICSI outcome. This conclusion, of course, awaits further confirmation.

Acknowledgement The authors would like to express their gratitude to the Royan Institute for its financial support and the staff of Isfahan Fertility and Infertility Center for their kind collaboration.

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