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ASSISTED REPRODUCTION TECHNOLOGIES

The role of hyaluronic acid binding assay in choosing the fertilization method for patients undergoing IVF for unexplained infertility

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Abstract
Purpose Patients with unexplained infertility may have fertilization problems. Split fertilization (ICSI and conventional IVF on sibling oocytes) is often used to avoid poor fertilization. Our aim was to assess the ability of hyaluronic acid binding (HA-binding) assay to predict spontaneous fertilization during IVF.
Methods Prospective, blinded, controlled trial. Patients undergoing their first IVF cycle for unexplained infertility were eligible. Split fertilization was used. IVF and ICSI fertilization rates and embryo development based on 3 HA-binding cut-offs (< 60%; 60–80%; >80%) were compared.
Results ICSI fertilization was higher than IVF, but none of the HA-binding cut-off levels predicted those cases where IVF was less effective, therefore ICSI only would have lead to improved outcome. Embryo development and morphology were similar in all cut-off groups.
Conclusions HA-binding did not predict spontaneous fertilization in patients with unexplained infertility undergoing IVF treatment. When it was used for "screening" it did not help to select the method of fertilization.

Capsule HA binding did not help in selecting IVF or ICSI fertilization in those cases when the first IVF cycle was initiated following failed IUIs and unexplained infertility.

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Introduction

Infertility affects about 10–15% of the reproductive age population. In about 20% of infertility cases standard evaluation techniques will not identify any pathology and so-called unexplained infertility is diagnosed. Controlled ovarian hyperstimulation (COH) in combination with intrauterine insemination (IUI) is usually the first line of treatment for mild male factor infertility, unexplained infertility and early stage endometriosis [1–3]. The per cycle pregnancy rate is influenced by various factors and is typically in the range of 5–20%. Cumulative pregnancy rate after 3-4 treatments is in the range of 25–40% [1, 3–7].

Approximately half of the couples will not achieve a pregnancy after insemination and they proceed to in vitro fertilization (IVF) as the next step. In the case of unexplained infertility, several pathomechanisms could play a role. Endometriosis, immunologic factors, and undiagnosed genetic causes could all be responsible [8]. Low fertilization rate or complete fertilization failure, despite the normal or close to normal semen parameters, are other potential causes. Standard semen analysis techniques assessing number, motility and morphology are inadequate to evaluate the sperm's functional (fertilizing) capacity therefore new tests are needed [9, 10].

Huszar et al., have evaluated various biochemical markers of sperm maturity and functional capacity. They have shown that sperm creatine kinase (CK) and testis-expressed heat shock protein (HspA2) concentrations correlated with sperm maturity and had a predictive value for pregnancy [11, 12]. During spermiogenesis binding sites

for hyaluronic acid (HA) develop in the plasma membrane. The sperm's ability to bind to HA can also be used to assess sperm maturity. HA bound sperm was found viable, had normal morphology, had low rates of DNA fragmentation and aneuploidy [11–13]. In addition, based on the percent of bound sperm, three binding zones were established (excellent: > 90%; moderate: 60–90%; low: < 60%) and a recommendation was made to proceed with ICSI in the low binding group, while IUI could be attempted in those where the binding was over 60% [14].

To avoid low fertilization or complete fertilization failure split fertilization of sibling oocytes (IVF half and ICSI the other half) is often used during IVF. We have been using ICSI fertilization on half of the eggs and conventional IVF fertilization on the other half in cases of unexplained infertility. Other groups have recommended this approach as well [15–18].

The aim of this study was to evaluate whether HA binding results could be used to decide the method of fertilization during IVF cycles in patients with unexplained infertility. The secondary aim was to see whether higher HA binding indicating better quality sperm was associated with improved in vitro embryo development.

Materials and methods

The study was a prospective, blinded, controlled trial. IRB approval was obtained for the study (SZTE 12/2008). All participating patients signed an informed consent (at study entry). The study was initiated in 2008 and was completed early 2009. Couples starting their first IVF treatment following at least 3 failed IUI cycles were recruited to participate. Women under the age of 40 with regular (21–35 days) menstrual cycles, with normal baseline follicle stimulating hormone (FSH) level (≤ 12 IU/l) and at least 4 mature eggs at the retrieval were eligible.

Those couples who had poor semen parameters on the day of the retrieval (sperm count < 10 million/ml, and/or rapid progressive motility < 25%) were excluded. We chose 10 million/ml and 25% forward motility (A type motility) as the inclusion cut-off for sperm parameters, since we would attempt IUI treatment in cases with such values, and therefore we considered it acceptable for any type of fertility treatment. In all of these cases the plan was to divide the eggs evenly into two groups and to use both conventional IVF and ICSI for fertilization.

Standard stimulation protocols (gonadotropin releasing hormone [GnRH] agonist long [n=24]; GnRH short [n=23]; gonadotropin releasing hormone antagonist [n=13]) were used. The stimulation protocol and dose of gonadotropins were not standardized for the study; the decision was made by the treating physician. For the long protocol, GnRH agonist (buserelin 0.5 mg, Suprefact, Aventis) was started in the midluteal phase. At suppression the dose was reduced to half and stimulation using recombinant FSH (Gonal F, Merck Serono), human menopausal gonadotropin (Merional, IBSA) or the combination were used. For the short protocol, the GnRH agonist was started on cycle day 2 and gonadotropin stimulation was initiated on day 3. In the case of the antagonist protocol, stimulation was started on day 2 of the cycle and the GnRH antagonist (cetrorelix 0.25 mg, Cetrotide, Merck Serono) was started once the largest follicles had reached the 13-14 mm in size. When at least two follicles reached 17 mm in diameter human chorionic gonadotropin (hCG) was used to trigger ovulation (250 μg rHCG, Ovitrelle, Merck Serono). Transvaginal oocyte retrieval was performed 35-36 h later.

On the day of the retrieval the male partner was asked to provide a fresh semen sample. Once the sample was liquefied a standard Makler chamber analysis was performed to assess sperm number and motility. In addition, a drop of the sample was placed on the HA coated slide (HBA, Sperm Hyaluronan Binding Assay, MidAtlantic, USA) and on average 100–200 sperm were analyzed to assess HA binding (unbound sperm moves freely while bound sperm is anchored down). HA binding is expressed as the percent of the bound motile sperm in relation to all sperm analyzed. Gradient centrifugation (300 g for 20 min) was used to separate the cellular components (Spermgrad solution, Vitrolife, Scandinavia). Following centrifugation the supernatant was removed and the sediment was washed twice (GSperm, Vitrolife, Scandinavia; 300 g for 10 min). The supernatant was removed again and the sediment was diluted. The mature oocytes (assessment was based on cumulus expansion and structure) were evenly split between conventional IVF and ICSI. For conventional IVF, 100–150,000/ml motile sperm were placed in the culture dish next to the egg. ICSI was performed with micropipettes (Humagen, USA). The embryologist performing the fertilization was blinded to the HA binding result. Those oocytes that were injected were denuded prior to the procedure and the MII stage development was confirmed. In the case of IVF, fertilization took place in four-well dishes (Nunc, Denmark) in a volume of 500 μl of G-Fert solution (Vitrolife, Scandinavia). We check for fertilization 16–18 h post insemination. Fertilization was confirmed when two pronuclei were present. At this stage the embryos were transferred to G1.3 culture medium (Vitrolife, Scandinavia) and in groups of 3–5 were cultured in microdroplets of 30–50 μl under paraffin oil (Ovoil-100, Vitrolife, Scandinavia) until the 6–8 cell stage, when embryos subjected to blastocyst culture were placed to G2.3 (Vitrolife, Scandinavia) medium. On day 3 or 5 after fertilization, two or three embryos were transferred. The couple decided, taking into consideration the physician's

advice, how many embryos were transferred. The embryos for the transfer were selected based on their morphology. The method by which the egg had been fertilized did not influence the decision. In the minority of the cases, we transferred embryos where the eggs had been fertilized with the same method (IVF or ICSI) but in the majority of the cases we transferred embryos with mixed fertilization (one ICSI the other IVF). The luteal phase was supported with vaginal micronized progesterone (3×200 mg Utrogestan, Lab Besius Int., France). Two weeks after the transfer, either serum hCG was measured or a urinary pregnancy test was performed.

Data was obtained for baseline characteristics (age, baseline FSH/LH and estradiol), stimulation parameters (protocol, dose of gonadotropin, number of follicles, number of oocytes, sperm count/motility, HA binding), embryology parameters (fertilization rate in sibling oocytes with IVF and ICSI, number of embryos with IVF and ICSI, number of top quality day 3 embryos with conventional IVF vs ICSI [≥ 6 cells and < 20% fragmentation on day 3] the proportion of top quality embryos among all embryos with IVF and ICSI, availability of surplus embryos for cryopreservation) and for cycle outcome. Fertilization rates were calculated separately for IVF and ICSI for each patient based on the number of eggs that were fertilized with the given method. Three cut-off levels for HA binding were tested: ≥ 60% or < 60%; ≥ 70% or < 70%; and ≥ 80% or < 80%. In addition, the outcomes in cycles with low binding (< 60%) and in cycles with high binding (> 80%) were compared.

Student's *t*-test and chi square test were used where appropriate. A $p<0.05$ was considered significant.

Results

Over the study period a total of 69 couples were enrolled. In 9 cases the sperm parameters were insufficient for conventional IVF on the day of the retrieval (count was below 10 million/ml and/or the rapid progressive motility was below 25%) and in these cases only ICSI was used and therefore were excluded from the analysis.

Data based on the remaining 60 cycles were analyzed. Baseline characteristics are shown in Table 1. While overall ICSI fertilization was higher than conventional IVF fertilization (68.0% vs 55.1%, $p<0.01$), none of the three tested HA binding cut-off levels were able to identify those cases where conventional IVF was significantly less effective, therefore ICSI-only fertilization would have lead to more improved outcome. (Table 2.)

Looking at conventional IVF fertilization only, we compared HA binding results in the below mean and above mean IVF fertilization groups (mean IVF fertilization was 55%). In those cases where the IVF fertilization rate was ≤ 55% the mean HA binding was 60.9%, while in those cases where the IVF fertilization rate was ≥ 56% the HA binding was 61.6% (p = NS).

Table 1 Baseline and stimulation characteristics

	Mean±SD
Female age (yrs)	32.9±4.4
Baseline FSH (IU/l)	7.0±1.6
Baseline E2 (pmol/l)	141.4±55.2
Amount of gonadotropin (IU)	1542.9±581.3
Oocytes	10.8±4.1
Sperm count (million/ml)	49.5±23.3
Sperm motility (%)	58.6±17.2
HA binding (%)	61.2±18.1
ICSI fertilization rate (%)	68.0±26.6
IVF fertilization rates (%)	55.1±27.7
Number of top quality embryos with ICSI	2.6±1.7
Number of top quality embryos with IVF	1.9±1.6

Values are expressed as mean±standard deviation

Embryo development and morphology were not associated with better binding results either. On average 68% of the 2PN embryos developed into top quality day 3 embryos. HA binding was similar in the below 68% development and above 68% development (2PN to top quality day 3 development) groups.

When we analyzed HA binding based on the proportion of 2PN embryos that progressed to the blastocysts stage no correlation was found between HA binding and 2PN to blastocyst stage development rate. (Table 3.)

The overall pregnancy rate was 43.3% (26/60) in the study population. There were no significant differences in the clinical pregnancy rates when the different subgroups were compared based on the three HA binding cut-off levels.

Discussion

Based on our results, the HA binding assay does not help the identification of those cases where ICSI should be used to avoid suboptimal fertilization among patients with unexplained infertility undergoing their first IVF cycle.

To avoid low fertilization or complete fertilization failure many centers offer split fertilization for those couples who undergo IVF following failed inseminations. This approach is often recommended despite the data that shows that fertilization rate and treatment outcome should be comparable with IVF and ICSI when the semen parameters are in the normal range and ICSI improves fertilization rates only when the semen parameters are borderline or when the morphology is poor [19–22].

Table 2 Fertilization rate of sibling oocytes with ICSI vs IVF based on 3 different HA binding cut-off levels

	HA binding					
	< 60% (*N*=21)	≥60% (*N*=39)	<70% (*N*=37)	≥70% (*N*=23)	<80% (*N*=49)	≥80% (*N*=11)
IVF fertilization (%)	53.9±6.7	55.8±4.1	55.5±4.7	54.6±5.4	55.7±3.9	52.2±7.7
ICSI fertilization (%)	70.8±5.9	66.4±4.2	71.1±4.2	63.1±5.9	65.4±3.8	80.8±6.3

Values are expressed as mean±standard deviation

Several studies have reported lower fertilization rates with IVF versus ICSI and a higher risk for complete fertilization failure with IVF among couples with unexplained infertility undergoing IVF following failed IUIs [15–18, 23]. Ruiz et al., on the other hand found no significant difference between IVF and ICSI fertilization rates among women with unexplained infertility in their prospective randomized trial [24].

In order to choose the right fertilization method after failed intrauterine inseminations better in vitro sperm functional assays need to be developed. The HA binding assay is able to select mature sperm with normal morphology. In addition sperm with lower aneuploidy rate is selected by the method [13]. These abilities of the test are useful when the individual sperm is selected for ICSI. Our aim was to assess the assay's ability to identify those couples where, despite the normal semen parameters, conventional IVF would be less effective and therefore ICSI should be used. We included cases with unexplained infertility following failed IUIs. We used split fertilization of sibling oocytes and the embryologist performing the actual fertilization was blinded to the HA assay result performed on the day of the retrieval. Overall, higher fertilization rates were obtained with ICSI. The semen parameters do not explain this difference as they were in the normal range. One possible explanation is that in this specific group of patients (unexplained infertility, failed IUIs) there is indeed a problem with fertilization. The other is that for conventional IVF the maturity of the oocytes based on the morphology of cumulus oophorus complex (COC) cannot be reliably assessed on the day of the fertilization and there is a possibility that some of the oocytes were immature in this group. HA binding results, however, were unable to identify those cases where conventional IVF was less effective and therefore the use of ICSI fertilization only could have significantly impacted outcome. Prior to starting the study we hypothesized that in cycles with lower binding, fertilization rate with conventional IVF would be lower too. IVF fertilization rate with low binding (< 60%) was 53.9% and with high binding (> 80%) was 52.2% however respectively.

HA binding is associated with improved sperm maturity and morphology and lower aneuploidy rate. We hypothesized that cases with higher binding, indicating overall better quality sperm, would be associated with improved embryo in vitro development. However, HA binding scores were similar in the below and above mean 2PN to cleavage stage development and in the below and above mean 2PN to blastocyst stage development groups.

In our study, we did not use the test for identifying the individual sperm for fertilization but we used it for an overall assessment of the semen sample as our aim was to assess it as a screening tool. Had we had positive findings the test could have been introduced as an initial screening test to complete the quantitative numeric and morphologic analysis of the semen sample with a functional parameter. Such information could affect the clinical management and could help in selecting the right fertility treatment or fertilization method if IVF is needed. It is possible that using HA-bound sperm for fertilization during ICSI (in this case the test is used for selection) could have lead to improved fertilization and potentially better embryo quality but this cannot be answered based on our data due to our study design.

Our results are in accordance with that of Ye et al., who reported a correlation between hyaluronan-binding assay and sperm motility and morphology but was unable to use the test to identify those with poor fertilization [25]. Similarly to us, in a recent report Tarozzi et al., found no association between HA binding and fertilization rate, cleavage rate and embryo quality [26]. Nijs et al., studied

Table 3 2 PN to blastocyst development rates based on different HA binding categories (embryos from IVF and ICSI fertilization combined)

	HA binding					
	< 60%	≥60%	<70%	≥70%	<80%	≥80%
% blastocyst development from 2PN embryos	55.5±8.2	48.7±4.8	54.8±5.2	45.1±7.0	51.1±4.6	50.8±9.9

Values are expressed as mean±standard deviation

the clinical role of sperm functional assays including hyaluronan-binding assay during IVF, ICSI or combined IVF-ICSI (unexplained infertility) cycles. HA-binding did correlate with morphology (weak association) but had no predictive value for fertilization or pregnancy rate. They found no diagnostic or clinical role for HA binding assay in the case of unexplained infertility [27]. In our study morphology was not assessed on the day of fertilization. In all cases the morphology was in the normal range (i.e.: > 30% according to WHO 1999 criteria) when the initial assessment during the infertility work-up of these patients was made. In addition our aim was not to look for correlations of quantitative of qualitative parameters of the semen sample but to assess whether the sperm's functional capacity can be evaluated using the assay. This would have been the information that could have altered the clinical management.

In summary, our study failed to find a benefit with HA binding assay in the care of patients with unexplained infertility who are undergoing IVF treatment. We were hoping to be able to identify those cases where unnecessary ICSI could be avoided or low fertilization could be prevented by using ICSI only. When the test is used for "screening" it does not help in selecting the method of fertilization. Since HA binding is associated with better sperm characteristics and lower rate of aneuploidy it still could be used to choose the individual sperm for fertilization during ICSI. Further functional tests need to be developed that could be used during the work-up of infertile couples with otherwise unexplained infertility. A test that is able to assess the overall fertilizing capacity could be used to choose treatment and fertilization methods for these couples. The ideal management of fertilization in IVF cycles initiated for unexplained infertility is still unknown and the use of the split fertilization of sibling oocytes remains one good option for now.

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