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RESEARCH ARTICLE

Is sperm hyaluronic acid binding ability predictive for clinical success of intracytoplasmic sperm injection: PICSI vs. ICSI?

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Abstract

Although intracytoplasmic sperm injection (ICSI) is now a widely-used technique, it is still of interest to improve our knowledge as to which is the best spermatozoon to be selected for ICSI. Infertile men have increased risks of producing aneuploid spermatozoa. Using hyaluronic acid (HA)-binding sperm selection may reduce the genetic risks such as chromosomal aberrations of offspring. In the present study we examined the clinical success of ICSI with HA-selected sperm ('physiologic' ICSI, PICSI) compared to conventional ICSI, as well as the necessity to differentiate patients according to the initial HA-binding assay result (HBA score) and whether the sperm concentration or HBA score can provide additional information. We observed a significantly higher fertilization rate (FR) of the PICSI group with >60% HBA, implantation rate (IR) of the PICSI group with ≤60% HBA, and clinical pregnanacy rate (CPR) in every PICSI group compared to the ICSI groups ($p < 0.01$). We also observed a significantly higher life birth rate (LBR) in the PICSI group with ≤60% HBA compared to ICSI patients with ≤60% HBA ($p < 0.001$). The pregnancy loss rate (PLR) was significanly lower in PICSI patients compared to the ICSI group ($p < 0.0001$). The FR, IR, CPR, and LBR of the PICSI group with <50% HBA were significantly higher and the PLR was lower than in the ICSI group with <50% HBA ($p < 0.01$). A statistically significant correlation was found between the sperm concentration and the HA-binding capacity ($r = 0.62$, $p < 0.001$). We found a closer relationship between HBA score and FR ($r = 0.53$, NS) than between sperm concentration and FR ($r = 0.14$, NS). HBA could be considered for sperm selection prior to ICSI because of its success and apparant ability to reduce genetic complications. However, this must be extended to a larger study.

Abbreviations: CPR: clinical pregnancy rate; FR: fertilization rate; FSH: follicle stimulating hormone; GnRH: gonadotropin releasing hormone; HA: hyaluronic acid; HBA: HA-binding assay, HA-binding ability; hCG: human chorionic gonadotropin; HspA2: heat shock-related 70 kDa protein 2; ICSI: intracytoplasmic sperm injection; IR: implantation rate; IVF: *in vitro* fertilization; LBR: life birth rate; NS: non-significant; PICSI: ICSI with HA-selected sperm ('physiologic' ICSI); PLR: pregnancy loss rate; r: Pearson correlation coefficient; 2PN: two-pronuclear zygote, the appearance of two pronuclei is the first sign of successful fertilization

Keywords

Clinical success, hyaluronic acid (HA)-binding capacity (HBA score), intracytoplasmic sperm injection (ICSI), 'physiologic' ICSI (PICSI), sperm concentration

History

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Introduction

Oligozoospermic men requiring intracytoplasmic sperm injection (ICSI) often carry sperm populations characterised by an increase in chromosomal aberrations and a compromised DNA integrity. A higher incidence of numerical [Palermo et al. 2000; Simpson and Lamb 2001; Van Steirteghem et al. 2002] and structural chromosomal aberrations [Bonduelle et al. 2002] have been associated in studies in the embryos resulting from ICSI.

The selection of sperm for ICSI is commonly done via the microscopic assessment of motility and morphology. Sperm classified as normal morphology are found to host chromosomal aberrations [Celik-Ozenci et al. 2004]. Disomic and diploid sperm have been found in all categories of morphological classification [Zavaczki et al. 2006].

Hyaluronic acid (HA) is thought to be critical within the female reproductive tract when selecting functionally competent sperm during *in vivo* fertilization. The human oocyte is surrounded by the cumulus oophorus, whose major component is HA, a high molecular weight glycosaminoglycan. Developmentally mature sperm were found to bind to HA gels similarly to the binding between sperm and zona pellucida. The binding of sperm to HA *in vitro* is a selection process. In another study it has been demonstrated that there are

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exceptions when motile sperm do not bind to HA [Huszar et al. 2003].

Simultaneously with cytoplasmic extrusion in spermiogenesis, there is also a remodeling of the plasma membrane that facilitates the formation of the zona pellucida- and HA-binding sites [Huszar et al. 1997, 2003]. HA-binding associated with the presence of the HA receptors on the sperm surface is related to sperm development [Huszar and Vigue 1993]. Sperm with HA-binding ability are viable having either intact or slightly capacitated acrosomal status and appear devoid of significant DNA degradation [Huszar et al. 2007; Yagci et al. 2010].

Diminished expression of the heat shock-related 70 kDa protein 2 (HspA2), a testis-specific chaperone protein, part of the meiotic synaptonemal complex, causes meiotic defects leading to aneuploidies [Kovanci et al. 2001]. There is a relationship between diminished sperm development (associated with oligozoo/asthenozoo/teratozoospermia), low levels of HspA2 expression, increased frequency of chromosomal aneuploidies, the presence of apoptotic process, and fragmented DNA [Huszar and Vigue 1993; Huszar et al. 2000, 2003, 2007; Yagci et al., 2010]. *In vitro* solid-state HA-binding facilitates the selection of individual mature sperm with low levels of chromosomal aneuploidies [Jakab et al. 2005]. Based on the percentage of bound sperm, three binding zones were established: excellent (>80%), moderate (60-80%), and low (<60%). The HA sperm selection method for ICSI might reduce the potential genetic complications and adverse public health effects of ICSI [Jakab et al. 2005].

In the present study we examined (1) the clinical success of ICSI with HA-selected sperm ('physiologic' ICSI, PICSI) compared to the conventional ICSI, (2) the necessity to differentiate patients according to the initial HA-binding assay result, and (3) whether the sperm concentration or the HA-binding ability can give more information about fertilization outcome. For this purpose, (i) we analyzed the clinical outcome (fertilization rate (FR), implantation rate (IR), clinical pregnancy rate (CPR), life birth rate (LBR), and pregnancy loss rate (PLR)) of 250 infertile couples (idiopathic infertile couples or infertility caused by male factor infertility) conceived by PICSI or ICSI, (ii) we carried out initial HA-binding score of all male partners, formed different groups according to the results, and then analyzed the clinical outcome, and finally (iii) we studied the correlation between the sperm concentration, HA-binding capacity, and fertilization rate.

Results

Clinical outcome of PICSI vs. ICSI

An average of 10.6 Metaphase II oocytes and 7.9 2PN zygotes were produced. The average fertilization rate was 62.7%. The male patients demonstrated average sperm concentration of 33.3×10^6/mL with 52.6% HBA score. In all cases the morphology of the embryos was normal (<30% fragmentation; [WHO 2010]). The results of Study 1 are summarized in Figure 1.

In the ICSI group the average sperm concentration proved to be 39.2×10^6/mL, the HBA score 62.5%, the FR 56.5%, the IR 17.12%, the CPR 29.22%, the LBR 0.42%, and the PLR 5.14%, respectively. In the ICSI group, where the HBA score was >60%, the average sperm concentration was 47.4×10^6/mL, HBA score was 75.7%, FR was 60.14%, IR was 21.47%, CPR was 31.85%, LBR was 0.58%, and PLR was 8.37%. In the ICSI group with HBA score ≤60% the parameters found were as follows: average sperm concentration: 20.1×10^6/mL, the HBA score: 31.7%, the FR: 52.85%, the IR: 12.76%, the CPR: 26.6%, the LBR: 0.27%, and the PLR: 1.9%.

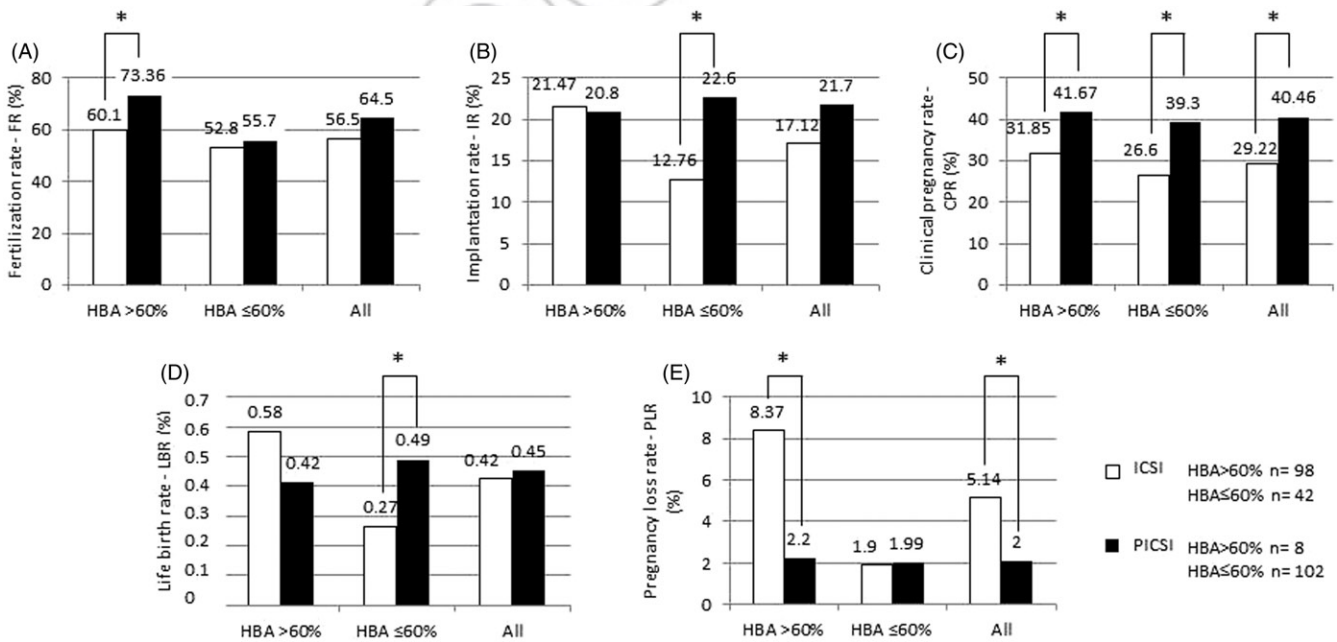

Figure 1. Clinical outcome of PICSI vs. ICSI. (A) Fertilization rate (FR) of patients with HBA >60%, patients with HBA ≤60%, and all patients; (B) Implantation rate (IR) of patients with HBA >60%, patients with HBA ≤60%, and all patients; (C) Clinical pregnancy rate (CPR) of patients with HBA >60%, patients with HBA ≤60%, and all patients; (D) Life birth rate (LBR) of patients with HBA >60%, patients with HBA ≤60%, and all patients; (E) Pregnancy loss rate (PLR) of patients with HBA >60%, patients with HBA ≤60%, and all patients. Statistical significance (*) is indicated at $p < 0.05$. HBA: hyaluronic acid (HA) binding ability;ICSI: intracytoplasmic sperm injection; PICSI: ICSI with HA-selected sperm ('physiologic' ICSI).

In the PICSI group the average sperm concentration was 25.6×10^6/mL associating with HBA score of 34.8%; the FR was 64.5%; the IR 21.7%, the CPR 40.46%, the LBR 0.45%, and the PLR 2%. In the PICSI group, where the HBA score was >60%, the average sperm concentration was measured as 35.5×10^6/mL, the HBA score 66.7%, the FR 73.36%, the IR 20.8%, the CPR 41.67%, the LBR 0.42%, and the PLR 2.2%. In the PICSI group with HBA score ≤60% the same parameters are as follows: average sperm concentration: 24.8×10^6/mL, the HBA score: 32.3%, the FR: 55.7%, the IR: 22.6%, the CPR: 39.3%, the LBR: 0.49%, and the PLR: 1.99%.

The FR of the PICSI group with >60% HBA was significantly higher than that in the ICSI group with >60% HBA ($p < 0.01$). The IR of the PICSI group with ≤60% HBA proved to be significantly higher than that in the ICSI group with ≤60% ($p < 0.001$). The CPR was significantly higher in every PICSI group compared to the ICSI groups ($p < 0.01$). We have observed a significantly higher LBR in the PICSI group with ≤60% HBA compared to ICSI patients with the same HBA ratio (≤60%; $p < 0.001$). PLR was significanly lower in PICSI patients and in the PICSI group with above 60% HBA compared to the ICSI group and the ICSI patients with >60% HBA, respectively ($p < 0.0001$).

Characterization according to the HBA score

Patients were further differentiated into two groups: HA-excellent (>70%) and HA-low bound sperm (<50%) groups based upon their HA binding capacity (%). The ICSI group with excellent HBA consisted of 69 couples and the ICSI group with <50% HBA contained 32 patients. The PICSI group with excellent HBA consisted of six couples and the PICSI group with <50% HBA contained 87 patients. The results of Study 2 are summarized in Figure 2.

In the ICSI group, where the HBA score was >70%, the average sperm concentration was 54.1×10^6/mL, the HBA score 84.1%, the FR 70.14%, the IR 21.5%, the CPR 35.8%, the LBR 0.58%, and the PLR 8.3%, respectively. In the ICSI group with <50% HBA the average sperm concentration proved to be 16.13×10^6/mL, the HBA score 24%, the FR 47.24%, the IR 12.5%, the CPR 30.8%, the LBR 0.26%, and the PLR 9.15%. In the PICSI group, where the HBA score was >70%, the average sperm concentration was 54.1×10^6/mL, the HBA score 83.1%, the FR 73.4%, the IR 20.8%, the CPR 41.7%, the LBR 0.4%, and the PLR 2.2%. In the PICSI group with <50% HBA the average sperm concentration was 24.1×10^6/mL, the HBA score 28.5%, the FR 55.42%, the IR 24.02%, the CPR 41.2%, the LBR 0.5%, and the PLR 4.65%.

The FR, IR, CPR, and LBR of the PICSI group with <50% HBA were significantly higher and the PLR was significantly lower than those in the ICSI group with <50% HBA ($p < 0.01$). The PLR of the PICSI group with >70% HBA proved to be significantly lower than that in the ICSI group with >70% HBA ($p < 0.0001$).

Correlation analysis between sperm concentration, HA-binding capacity, and fertilization rate

The Pearson correlation (r) between the sperm concentration and HA-binding capacity was determined by comparing all samples in the ICSI and PICSI groups. The Pearson correlation between the sperm concentration and FR and between the HBA score and FR independent of treatment (ICSI or PICSI), were respectively calculated. The results of the correlation analysis are summarized in Figure 3.

A statistically significant positive correlation was found between the sperm concentration and the HA-binding

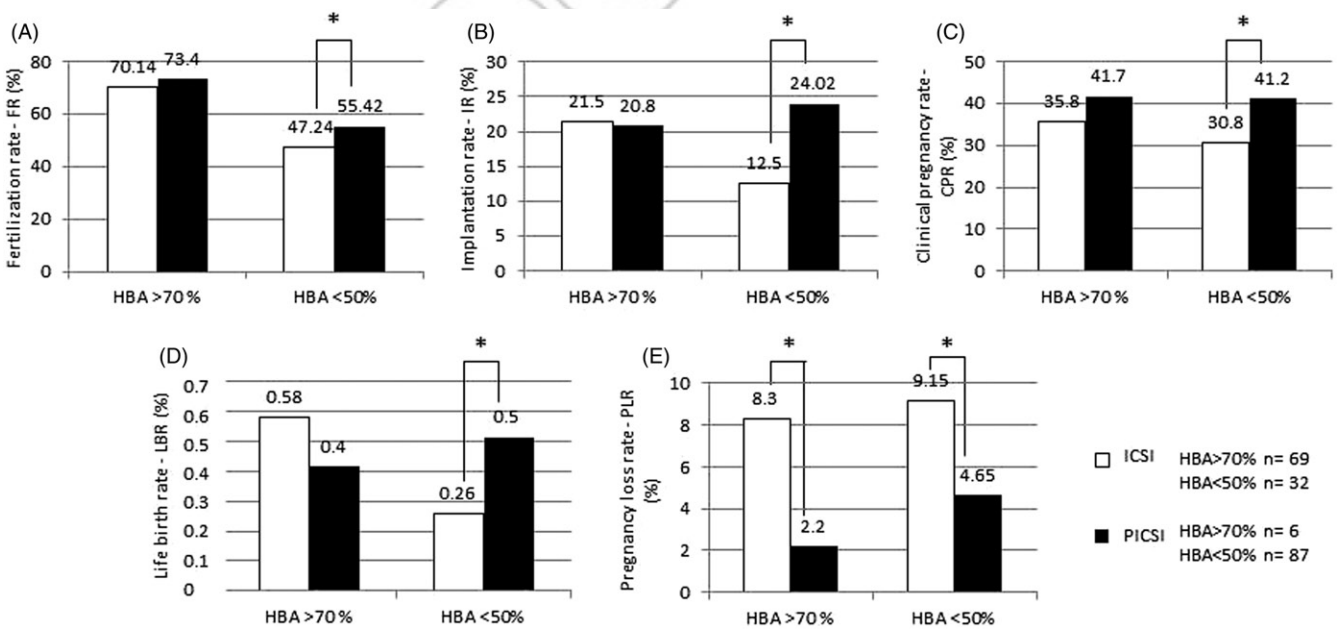

Figure 2. Clinical outcome of PICSI vs. ICSI of patients with HA-excellent (HBA score >70%) and HA-low bound sperm (HBA score <50%). (A) Fertilization rate (FR) of patients with HBA >70% and patients with HBA <50%; (B) Implantation rate (IR) of patients with HBA >70% and patients with HBA <50%; (C) Clinical pregnancy rate (CPR) of patients with HBA >70% and patients with HBA <50%; (D) Life birth rate (LBR) of patients with HBA >70% and patients with HBA <50%; (E) Pregnancy loss rate (PLR) of patients with HBA >70% and patients with HBA <50%. Statistical significance (*) is indicated at $p < 0.05$. HBA: hyaluronic acid (HA) binding ability; ICSI: intracytoplasmic sperm injection; PICSI: ICSI with HA-selected sperm ('physiologic' ICSI).

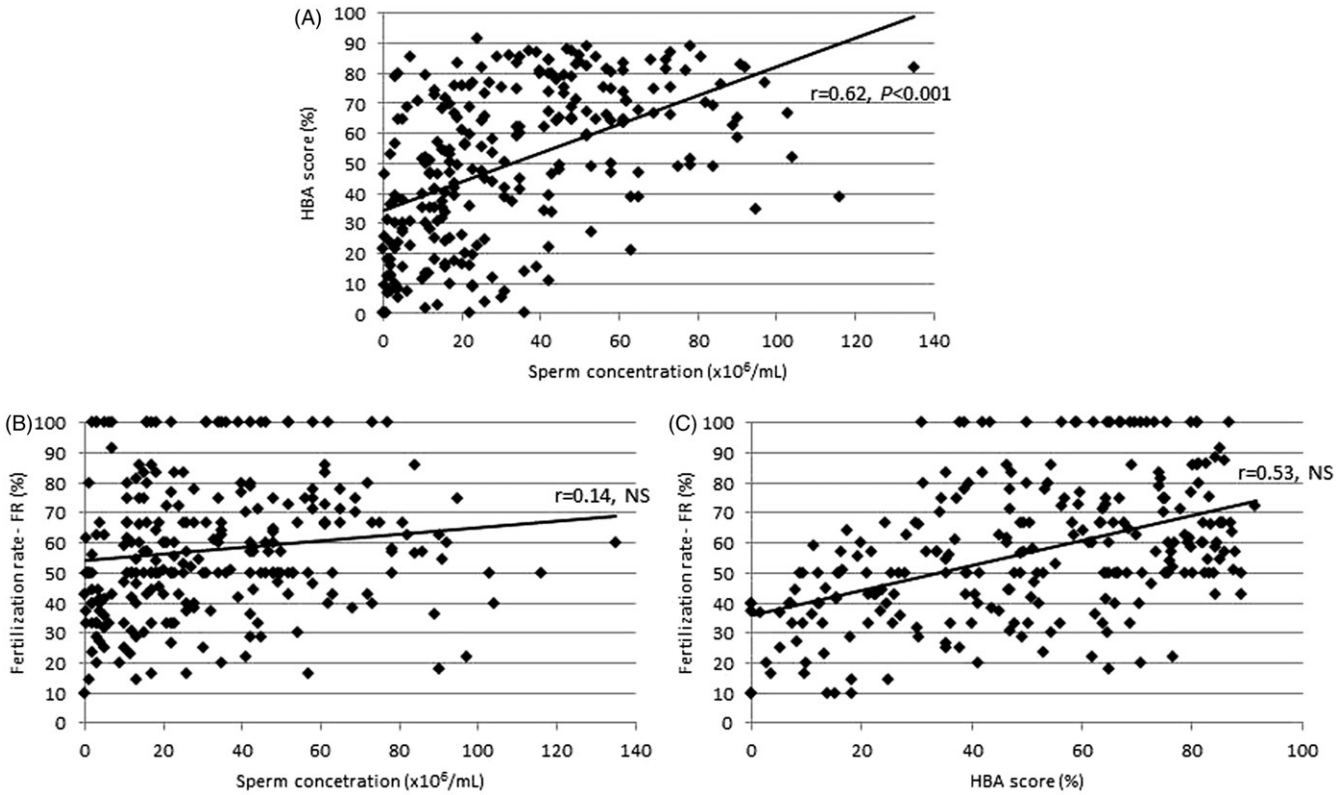

Figure 3. Correlation analysis between the sperm concentration and HBA score (A), between the sperm concentration and FR (B) and between the HBA score and FR (C). A statistically significant correlation was found between the sperm concentration and the HA-binding capacity. Higher positive correlation was found between HBA score and FR than between sperm concentration and FR. HBA: hyaluronic acid (HA) binding ability; FR: fertilization rate; r: Pearson correlation coefficient; NS: non-significant.

capacity ($r = 0.62$, $p < 0.001$). We found a higher positive correlation between HBA score and FR ($r = 0.53$, NS) than between sperm concentration and FR ($r = 0.14$, NS), but the difference was not statistically significant. In the ICSI and in the PICSI groups a higher positive correlation between HBA score and FR ($r = 0.51$ and $r = 0.49$, NS) than between sperm concentration and FR ($r = 0.22$ and $r = 0.19$, NS) was observed. This association was not statistically significant.

Discussion

We compared conventional ICSI ($n = 140$) to ICSI in which the spermatozoa were selected for their capacity to bind to HA (PICSI, $n = 110$). We observed a significantly higher FR in the PICSI group with >60% initial HBA; IR of the PICSI group with ≤60% HBA, and CPR in every PICSI group compared to the ICSI groups ($p < 0.01$). We also observed a significantly higher LBR in the PICSI group with ≤60% HBA compared to ICSI of patients with ≤60% HBA ($p < 0.001$). PLR was significantly lower in PICSI patients compared to the same parameter in the ICSI group ($p < 0.0001$). When the outcome was assessed as a function of the HBA score, the FR, IR, CPR, and LBR of the PICSI group with <50% HBA were significantly higher and the PLR was significantly lower than in the ICSI group with <50% HBA ($p < 0.01$). A statistically significant positive correlation was found between the sperm concentration and the HA-binding capacity ($r = 0.62$, $p < 0.001$). We found a closer relationship between HBA score and FR ($r = 0.53$, NS) than between sperm concentration and FR ($r = 0.14$, NS).

Previous studies regarding the development and function of biochemical and molecular markers of human sperm are supported by the above clinical results. A relationship between HA selected sperm and increased levels of developmental maturity [Cayli et al. 2004; Huszar et al. 1994, 2003], as well as nuclear [Kovanci et al. 2001; Jakab et al. 2005], and cytoplasmic integrity [Huszar et al. 1997; Sakkas et al. 1999] have been demonstrated.

A similar increase in IR, CPR, and lower PLR values was found by Worrilow and colleagues [Worrilow et al. 2006; Worrilow et al. 2007; Worrilow et al. 2012]. Others compared conventional sperm selection and the use of sperm selected from a liquid source of HA and an increased IR was found [Parmegiani et al. 2010]. The same positive trend was observed comparing polyvinylpyrrolidone-ICSI ($n = 110$) and PICSI ($n = 92$) treatments [Ménézo and Nicollet 2004]. In a study of 50 couples, a higher FR was observed when HA-selected spermatozoa were injected into oocytes [Nasr-Esfahani et al. 2008]. These studies, in accordance with ours, did not demonstrate any negative effect on embryogenesis using HA sperm selection for ICSI, but they all was 'in-house' developed HA slides.

In two further reports, no association was found between HA binding and FR, fragmentation, and embryo quality though they used washed sperm [Choe et al. 2012; Tarozzi et al. 2009]. In another report the clinical outcome of sperm functional assays including HBA was studied [Nijs et al. 2009]. A correlation of HA-binding was found with morphology, but it did not predict FR and CPR. Another recent study did not find any differences in FR, IR, and CPR

between ICSI and PICSI patients. The only benefit of injecting HA selected sperm was a lower PLR which consequently translated to a higher LBR, both of which were not statistically significant [Majumdar and Majumdar 2013].

No visual integrity of the DNA in selected sperm can be assessed which can basically determine the overall success of ICSI. When natural and assisted reproduction fails defects in sperm chromatin have been blamed [Bungum et al. 2007; Carrell et al. 2007]. Sperm DNA damage was found to be positively correlated with PLR when 11 studies involving 1,549 *in vitro* fertilization (IVF) and ICSI cycles was systematically reviewed [Zini et al. 2008]. It is well known that the proportion of immature sperm closely correlates with chromosomal disomies [Kovanci et al. 2001]. The relationship between the frequencies of chromosomal aneuploidies and diminished sperm maturity is thought to reflect that cytoplasmic retention and diminished maturity in sperm are associated with a low expression of the HspA2 [Eddy 1999; Huszar et al. 2000]. The relationship between sperm zona pellucida binding competence and maturity has been identified earlier. In the semen samples there were sperm with various degrees of cytoplasmic retention, but all sperm bound to the zona pellucida were mature as characterized with the absence of any cytoplasmic retention. Diminished HspA2 chaperone activity found in developmentally immature sperm is thought to be connected with a diminished presence of DNA repair enzymes, causing DNA chain breaks and fragmentation [Dix et al. 1996; Eddy 1999; Huszar et al. 2000]. There is a correlation between the decreased levels of expression of the HspA2 chaperone and sperm cellular development as well as IVF success [Ergur et al. 2002; Huszar et al. 1992, 2000]. Van Steirteghem et al. [2002] found increased rates of *de novo* numerical and cytogenetically detectable structural chromosomal aberrations following ICSI. The low concentration of HspA2 in the undeveloped spermatozoa likely suggests numerical chromosomal aberrations in sperm of oligozoospermic or severely oligozoospermic men [Huszar et al. 2007].

An enhancement of DNA and chromosomal integrity was demonstrated in HA-bound sperm by Yagci et al. [2010] when they analyzed HA-bound sperm with acridine orange fluorescence and they did not find DNA fragmentation. Selecting individual mature sperm with low levels of chromosomal disomy, diploidy, and sex chromosome disomy is facilitated by HA-binding and might reduce the potential genetic complications in male candidates for ICSI [Jakab et al. 2005]. It has been observed that almost all HA-bound spermatozoa are devoid of persistent histones, which correlated with DNA strand breakage [Sati et al. 2004].

After ICSI, no sperm function tests were well correlated with FR. These results are in line with the data of several studies [Bakos et al. 2008; Henkel et al. 2003; Nasr-Esfahani et al. 2008] but contradictory to the data presented above, where we found PICSI proved to be significantly more effective than ICSI in respect of clinical success for patients with a low initial HBA score (≤50%). Based on our results HA selection becomes an important factor in cases with low binding scores, where the expected number of normal sperm is much lower. It has been observed in a single study where a correlation was found between sperm HA-binding capacity and FR after IVF [Pregl Breznik et al. 2013]. Our results indicate that sperm selection by HA binding is promising and significantly improves the success of the result in patients with a low HBA score. We conclude that HBA screening prior to ICSI may be useful to increase clinical success. It has been demonstrated that injection of spermatozoa recovered from HA-containing products had no negative effects on post-injection zygote development [Balaban et al. 2003; Barak et al. 2001]. A statistically significant reduction in PLR was observed in patients with a low HBA score. The use of HA sperm selection may be considered in patients with an initial HBA score of ≤50%. To determine the use of HA-bound sperm in ICSI, the use of HBA score would be beneficial since it could offer a balance to unnecessary treatment.

Materials and Methods

Patients

A total of 250 couples referred to the Assisted Reproduction Center, Kaali Institute, Medical and Health Science Center, University of Debrecen for ICSI were studied. The study was done between January 2012 and March 2013. In this period, 140 ICSI and 110 PICSI were carried out on the basis of the sperm HA-binding ability of the male partner (HBA score): when initial HBA score was >60% ICSI was carried out ($n = 98$), in cases with HBA score ≤60% PICSI was performed ($n = 102$). The ICSI group included 42 couples where the sperm number and the HBA score was low and unsuitable for PICSI. We carried out eight control PICSI where the HBA score was >60%.

Women under the age of 40 (mean: 33.18, range: 22–40) with regular (21–35 days) menstrual cycles, with normal baseline follicle stimulating hormone (FSH) level (≤12 IU/L) were eligible. Within the overall studied population the average male age was 35.8 years (range: 23–45). Patients excluded from the study were as follows: those from whom testicular sperm were taken, who got donor or cryopreserved gametes, received preimplantation genetic diagnosis, underwent sperm sorting procedures, patients whose maternal age was >40 years, and those who demonstrated a sperm count <10,000 motile sperm/mL.

Prior to the study, all patients were given detailed information about the aim and method of investigation and their consents were obtained. All protocols had to be approved by the author's respective Institutional Review Board (IRB) for human subjects (IRB reference number: 2976/2012-EHR).

Stimulation protocols

Standard stimulation protocols, gonadotropin releasing hormone (GnRH) agonist long ($n = 75$), short ($n = 120$), and GnRH antagonist ($n = 55$), were used. The stimulation protocol and dose of gonadotropins were not standardized for the study; the decision was made by the physician.

For the long protocol, GnRH agonist was started in the midluteal phase. During suppression the dosage was reduced to half and stimulation with either recombinant FSH or human menopausal gonadotropin, or the combination of the two 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 was started when the largest follicles had reached 13–14 mm in size. When at least two follicles reached 17 mm in diameter, recombinant human chorionic gonadotropin (hCG) was used to trigger ovulation. Transvaginal oocyte retrieval was performed 35–36 h later.

Semen analysis and hyaloronic acid binding assay

Semen specimens were collected after a requested abstinence of two to three days on the day of the oocyte retrieval. The sperm sample was maintained at room temperature (18–28 °C) for 30 to 60 min to allow it to liquefy. Semen analysis was performed manually according to WHO guidelines and morphology was examined using strict criteria [WHO 2010]. The HBA-test (hyaloronic acid binding assay) (MidAtlantic Diagnostics, Martlon, NJ, USA) was carried out at room temperature: the sample was mixed and a pipette of 7–10 µl was placed near the center of the chamber. The CELL-VU gridded cover slip was located over the chamber to avoid air bubble formation. The chamber was incubated at room temperature for at least 10 min, but not more than 20 min: this period proved to be necessary for sperm to bind to HA (according to the HBA-test protocol). The number of bound, motile sperm and the totality of motile sperm was scored. At least 200 spermatozoa in the same square or the entire 100 squares were counted. The ratio of hyaluronic binding motile sperm was calculated as follows:

$$\%\text{Bound} = 100 \times \text{Bound Motile}/\text{Total Motile}.$$

Fertilization

Gradient centrifugation (600 g for 10 min) was used to separate the cellular components of semen (PureCeption™ Sperm Washing Solution, SAGE, Pasadena, CA, USA). Following centrifugation the supernatant was removed and the sediment was washed twice (Quinn's Advantage® Sperm Washing Medium, SAGE, Pasadena, CA, USA; 600 g for 10 min). The supernatant was removed again and the sediment was diluted.

In order to select the morphologically 'best' spermatozoon, sperm were placed into standard ICSI dishes which were later injected into oocytes. We placed the final sperm suspension of PICSI patients upon microdots of hyaluronic acid in the PICSI® Sperm Selection Device (Biocoat, Inc., Horsham, PA, USA) and then overlaid it with oil (SAGE, Pasadena, CA, USA). After an incubation period of 5 to 10 min, HBA sperm were selected as per the manufacturer's instructions. We selected spermatozoa bound to HA in the junction zone of the two droplets and it was easy to detach then by an injecting pipette (ICSI Micropipette; ORIGIO, Charlottesville, VA, USA) and subsequently injected into oocytes.

Embryo culture

In the presence of two pronuclei fertilization was confirmed. The embryos were transferred to Quinn's Advantage® Protein Plus Cleavage Medium at this stage (SAGE) and in microdroplets of 20–25 µL under Washed Oil for Tissue Culture, groups of 3–5 were cultured until the 6–8 cell stage (SAGE). After this, embryos were put to Quinn's Advantage® Protein Plus Blastocyst Medium (SAGE).

One, two, or three embryos were transferred following 3 or 5 d of fertilization. It was the couple's decision of how many embryos to be transferred after consulting with their physician. The morphology of the embryos was the basis for the transfer.

Statistical analysis

Statistical analyses were performed with commercial software SigmaStat and SPSS. Sample normality was assessed using Shapiro-Wilk test, sample homogeneity using Barlett test. Differences in the sperm concentration, HA-binding ability, FR, IR, CPR, LBR, and PLR between the ICSI and PICSI groups were analyzed using Mann-Whitney/Wilcoxon Two-Sample Test, Kruskal-Wallis test (when normality does not exist), and Two-sample t-probe (when normality exists). A value of $p < 0.05$ was considered a significant difference. Correlation analyses between the sperm concentration, HA-binding capacity, and FR using all samples in the two groups were examined with Pearson correlation test.

It was the number of eggs fertilized with the given method that determined the fertilization rate (FR) for each patient. Implantation rate (IR) was calculated from the number of intrauterine sacs/the number of embryos transferred in each patient. There is an agreement that clinical pregnancy means that fetal cardiac activity is present within an intrauterine gestational sac. Vaginal ultrasound was used to assess pregnancy loss rate (PLR) and it means the proportion of patients demonstrating an intrauterine sac at 5–7 w of gestation and those where no fetal cardiac activity was present at 8–10 w of gestation.

Declaration of interest

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Author contributions

Collected the data, conceived and carried out the statistical analyses, evaluated the results, and wrote the first draft of the manuscript: AM; Collected the data, conceived and designed the analyses: EVT; Involved in clinical examination and follow up of the patients: BB, ZT;Conceived and designed the analyses: ZM, AJ, AU; Made substantial contribution to the design and interpretation of data, critically revised the manuscript, and approved the final version to be published: EO.

References

Bakos, H.W., Thompson, J.G., Feil, D. and Lane, M. (2008) Sperm DNA damage is associated with assisted reproductive technology pregnancy. Int J Androl **31**:518–26.

Balaban, B., Lundin, K., Morrell, J.M., Tjellström, H., Urman, B. and Holmes, P.V. (2003) An alternative to PVP for slowing sperm prior to ICSI. Hum Reprod **18**:1887–9.

Barak, Y., Menezo, Y., Veiga, A. and Elder, K. (2001) A physiological replacement for polyvinylpyrrolidone (PVP) in assisted reproductive technology. Hum Fertil (Camb) **4**:99–103.

Bonduelle, M., Van Assche, E., Joris, H., Keymolen, K., Devroey, P., Van Steirteghem, A., et al. (2002) Prenatal testing in ICSI pregnancies: incidence of chromosomal anomalies in 1586 karyotypes and relation to sperm parameters. Hum Reprod **17**:2600–14.

Bungum, M., Humaidan, P., Axmon, A., Spano, M., Bungum, L., Erenpreiss, J., et al. (2007) Sperm DNA integrity assessment in prediction of assisted reproduction technology outcome. Hum Reprod **22**:174–9.

Carrell, D.T., Emery, B.R. and Hammoud, S. (2007) Altered protamine expression and diminished spermatogenesis: what is the link? Hum Reprod Update **13**:313–27.

Cayli, S., Sakkas, D., Vigue, L., Demir, R. and Huszar, G. (2004) Cellular maturity and apoptosis in human sperm: creatine kinase, caspase-3 and Bcl-XL levels in mature and diminished maturity sperm. Mol Hum Reprod **10**:365–72.

Celik-Ozenci, C., Jakab, A., Kovacs, T., Catalanotti, J., Demir, R., Bray-Ward, P., et al. (2004) Sperm selection for ICSI: shape properties do not predict the absence or presence of numerical chromosomal aberrations. Hum Reprod **19**:2052–9.

Choe, S.A., Tae, J.C., Shin, M.Y., Kim, H.J., Kim, C.H., Lee, J.Y., et al. (2012) Application of sperm selection using hyaluronic acid binding in intracytoplasmic sperm injection cycles: a sibling oocyte study. J Korean Med Sci **27**:1569–73.

Dix, D.J., Allen, J.W., Collins, B.W., Mori, C., Nakamura, N., Poorman-Allen, P., et al. (1996) Targeted gene disruption of Hsp70-2 results in failed meiosis, germ cell apoptosis, and male infertility. Proc Natl Acad Sci USA **93**:3264–8.

Eddy, E.M. (1999) Role of heat shock protein HSP70-2 in spermatogenesis. Rev Reprod **4**:23–30.

Ergur, A., Dokras, A., Giraldo, J., Habana, A., Kovanci, E. and Huszar, G. (2002) Sperm maturity and treatment choice of in vitro fertilization (IVF) or intracytoplasmic sperm injection: diminished sperm HspA2 chaperone levels predict IVF failure. Fertil Steril **77**:910–18.

Henkel, R., Kierspel, E., Hajimohammad, M., Stalf, T., Hoogendijk, C., Mehnert, C., et al. (2003) DNA fragmentation of spermatozoa and assisted reproduction technology. Reprod Biomed Online **7**:477–84.

Huszar, G., Vigue, L. and Morphed, M. (1992) Sperm creatine phosphokinase M-isoform ratios and fertilizing potential of men: a blinded study of 84 couples treated with in vitro fertilization. Fertil Steril **57**:882–8.

Huszar, G. and Vigue, L. (1993) Incomplete development of human spermatozoa is associated with increased creatine phosphokinase concentration and abnormal head morphology. Mol Reprod Dev **34**: 292–8.

Huszar, G., Vigue, L. and Oehninger, S. (1994) Creatine kinase immunocytochemistry of human sperm-hemizona complexes: selective binding of sperm with mature creatine kinase-staining pattern. Fertil Steril **61**:136–42.

Huszar, G., Sbracia, M., Vigue, L., Miller, D.J. and Shur, B.D. (1997) Sperm plasma membrane remodeling during spermiogenetic maturation in men: relationship among plasma membrane beta 1,4-galactosyltransferase, cytoplasmic creatine phosphokinase, and creatine phosphokinase isoform ratios. Biol Reprod **56**:1020–4.

Huszar, G., Stone, K., Dix, D. and Vigue, L. (2000) Putative creatine kinase M-isoform in human sperm is identifiedas the 70-kilodalton heat shock protein HspA2. Biol Reprod **63**:925–32.

Huszar, G., Ozenci, C.C., Cayli, S., Zavaczki, Z., Hansch, E. and Vigue, L. (2003) Hyaluronic acid binding by human sperm indicates cellular maturity, viability, and unreacted acrosomal status. Fertil Steril **79**: 1616–24.

Huszar, G., Jakab, A., Sakkas, D., Ozenci, C.C., Cayli, S., Delpiano, E., et al. (2007) Fertility testing and ICSI sperm selection by hyaluronic acid binding: clinical and genetic aspects. Reprod Biomed Online **14**: 650–63.

Jakab, A., Sakkas, D., Delpiano, E., Cayli, S., Kovanci, E., Ward, D., et al. (2005) Intracytoplasmic sperm injection: a novel selection method for sperm with normal frequency of chromosomal aneuploidies. Fertil Steril **84**:1665–73.

Kovanci, E., Kovacs, T., Moretti, E., Vigue, L., Bray-Ward, P., Ward, D.C., et al. (2001) FISH assessment of aneuploidy frequencies in mature and immature human spermatozoa classified by the absence or presence of cytoplasmic retention. Hum Reprod **16**:1209–17.

Majumdar, G. and Majumdar, A. (2013) A prospective randomized study to evaluate the effect of hyaluronic acid sperm selection on the intracytoplasmic sperm injection outcome of patients with unexplained infertility having normal semen parameters. J Assist Reprod Genet **30**:1471–5.

Ménézo, Y. and Nicollet, B. (2004) Replacement of PVP by hyaluronate (SpermSlow) in ICSI - impact on outcome. Eighteenth World Congress on Fertility and Sterility, International Federation of Fertility Societies.

Nasr-Esfahani, M.H., Razavi, S., Vahdati, A.A., Fathi, F. and Tavalaee, M. (2008) Evaluation of sperm selection procedure based on hyaluronic acid binding ability on ICSI outcome. J Assist Reprod Genet **25**:197–203.

Nijs, M., Creemers, E., Cox, A., Franssen, K., Janssen, M., Vanheusden, E., et al. (2009) Chromomycin A3 staining, sperm chromatin structure assay and hyaluronic acid binding assay as predictors for assisted reproductive outcome. Reprod Biomed Online **19**:671–84.

Palermo, G.D., Neri, Q.V., Hariprashad, J.J., Davis, O.K., Veeck, L.L. and Rosenwaks, Z. (2000) ICSI and its outcome. Semin Reprod Med **18**:161–9.

Parmegiani, L., Cognigni, G.E., Bernardi, S., Troilo, E., Ciampaglia, W. and Filicori, M. (2010) ''Physiologic ICSI'': hyaluronic acid (HA) favors selection of spermatozoa without DNA fragmentation and with normal nucleus, resulting in improvement of embryo quality. Fertil Steril **93**:598–604.

Pregl Breznik, B., Kovačič, B. and Vlaisavljević, V. (2013) Are sperm DNA fragmentation, hyperactivation, and hyaluronan-binding ability predictive for fertilization and embryo development in in vitro fertilization and intracytoplasmic sperm injection? Fertil Steril **99**: 1233–41.

Sakkas, D., Mariethoz, E. and St. John, J.C. (1999) Abnormal sperm parameters in humans are indicative of an abortive apoptotic mechanism linked to the Fas-mediated pathway. Exp Cell Res **251**: 350–5.

Sati, L.G., Ovari, L., Demir, R., Ward, D.C., Bray-Ward, P. and Huszar, G. (2004) Persistent histones in immature sperma are associated with DNA fragmentation and affect paternal contribution of sperm: a study of aniline blue staining, fluorescence in situ hybridization (FISH) and DNA nick translation. Fertil Steril **82**:S52.

Simpson, J.L. and Lamb, D.J. (2001) Genetic effects of intracytoplasmic sperm injection. Semin Reprod Med **19**:239–49.

Tarozzi, N., Nadalini, M., Bizzaro, D., Serrao, L., Fava, L., Scaravelli, G., et al. (2009) Sperm-hyaluronan-binding assay: clinical value in conventional IVF under Italian law. Reprod Biomed Online **19**:35–43.

Van Steirteghem, A., Bonduelle, M., Devroey, P. and Liebaers, I. (2002) Follow-up of children born after ICSI. Hum Reprod Update **8**:111–16.

WHO (2010) WHO laboratory manual for the examination and processing of human semen. World Health Organization, 5th edition. p. 271.

Worrilow, K.C., Huynh, H.T., Bower, J., Peters, A.J. and Johnston, J.B. (2006) The clinical impact associated with the use of PICSI-derived embryos. Fertil Steril **86**:S62.

Worrilow, K.C., Huynh, H.T., Bowers, J.B., Anderson, A., Schillings, W. and Crain, J. (2007) PICSI versus ICSI: Statistically significant improvement in clinical outcomes in 240 in vitro fertilization (IVF) patients. Fertil Steril **88**:S37.

Worrilow, K.C., Eid, S., Woodhouse, D., Perloe, M., Smith, S., Witmyer, J., et al. (2012) Use of hyaluronan in the selection of sperm for intracytoplasmic sperm injection (ICSI): significant improvement in clinical outcomes–multicenter, double-blinded and randomized controlled trial. Hum Reprod **28**:306–14.

Yagci, A., Murk, W., Stronk, J. and Huszar, G. (2010) Spermatozoa bound to solid state hyaluronic acid show chromatin structure with high DNA chain integrity: an acridine orange fluorescence study. J Androl **31**:566–72.

Zavaczki, Z., Celik-Ozenci, C., Ovari, L., Jakab, A., Sati, G.L., Ward, D.C., et al. (2006) Dimensional assessment of X-bearing and Y-bearing haploid and disomic human sperm with the use of fluorescence in situ hybridization and objective morphometry. Fertil Steril **85**:121–7.

Zini, A., Borman, J.M., Belzile, E. and Ciampi, A. (2008) Sperm DNA damage is associated with an increased risk of pregnancy loss after IVF and ICSI: Systematic review and meta-analysis. Hum Reprod **23**: 2663–8.