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ASSISTED REPRODUCTION

Efficiency of hyaluronic acid (HA) sperm selection

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Abstract
Purpose Hyaluronic Acid (HA) has a role as "physiologic selector" for spermatozoa prior to intracytoplasmic sperm injection (ICSI). The objective of this study is to analyze the results achievable by the introduction of a routine HA-ICSI programme.
Methods We retrospectively observed 293 couples treated with HA-ICSI versus 86 couples treated with conventional PVP-ICSI (historical control group). ICSI was performed on a limited number of oocytes per patient (1–3) according to Italian IVF law at the time of the study. Main outcome measures observed were: fertilization, embryo quality, implantation and pregnancy.
Results This study showed that Injection of HA-bound spermatozoa (HA-ICSI) significantly improves embryo quality and implantation.
Conclusions If wider multi-center randomized studies will confirm these beneficial effects on ICSI outcome, HA could be considered as a routine choice for "physiologic" sperm selection prior to ICSI.

Keywords Hyaluronic Acid selection · HA-ICSI · ICSI · Physiologic ICSI · Sperm selection

Capsule Injection of Hyaluronic Acid (HA)-bound spermatozoa (HA-ICSI) significantly improves embryo quality and implantation.

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Introduction

In nature, human oocytes are surrounded by Hyaluronic Acid (HA), which is then involved in the mechanism of sperm selection. In fact, only mature spermatozoa which have extruded their specific receptors to bind to and digest HA can reach the oocyte and fertilize it. HA's role as "physiologic selector" is now well recognized also in vitro: it has been demonstrated that spermatozoa able to bind HA in vitro are those that have completed plasma membrane remodelling, cytoplasmic extrusion and nuclear maturation [1–3]. Furthermore, these HA-bound spermatozoa show low chromosomal aneuploidies and DNA fragmentation, and good nuclear morphology [4, 5]. Thus, HA-sperm selection prior to intracytoplasmic sperm injection (ICSI) helps to optimize the outcome of the treatment, especially when insemination of only a limited number of oocytes is allowed by law [6].

In a previous prospective-randomized study, we demonstrated that the injection of HA-bound spermatozoa (HA-ICSI or Physiologic ICSI) improved embryo quality and development, by favouring selection of spermatozoa with normal nucleus and intact DNA [5]. The aim of the present study is to verify the role of HA for sperm selection on a larger number of HA-ICSI treatments.

Materials and methods

Patient population

This study was conducted in our Reproductive Medicine Unit—GynePro Medical Centers- Bologna, Italy; the procedure was approved by the Institutional Review Board of our center. All patients were informed of the procedure and

written consent was obtained from each. In our previous prospective randomized study we compared HA-ICSI to conventional PVP-ICSI [5]; for the HA-CSI procedure spermatozoa were selected for their ability to bind to HA using a HA-containing product (Sperm Slow™, MediCult, Jyllinge, Denmark), whereas for the conventional PVP-ICSI procedure sperm motility was reduced by PVP (PVP Clinical Grade, MediCult). This preliminary study demonstrated that HA sperm selection improved embryo quality when injecting a limited number of oocytes. Encouraged by these results, we decided to perform HA sperm selection routinely and from January 2005 all our treatments were switched from conventional PVP-ICSI to Physiologic HA-ICSI. The present study analyzes retrospectively the outcome of 331 consecutive HA-ICSI performed from January 2005 to January 2009 by a single embryologist, in order to avoid variability between different operators. As a control group, we considered 97 conventional PVP-ICSI performed by the same operator (in the same laboratory conditions) during the preliminary randomized study. To minimize the influence of female age on ICSI outcome we included in this analysis only the ICSI treatments performed on women with age≤39 at oocyte retrieval. As AH selection is effective on motile spermatozoa, we introduced inclusion criteria for semen: presence of ejaculate motile spermatozoa with total sperm number ≥ 1×10^6 and sperm motility ≥ 5%.

Controlled ovarian stimulation and oocyte selection

Controlled ovarian stimulation (COS) was achieved using gonadotropin-releasing hormone analogs in combination with a graded gonadotropin administration [7]. After decumulation and quality evaluation, the three best available MII oocytes were inseminated by ICSI, according to the Italian law regulating Assisted Reproductive Technology, at the time of this study [6].

HA-ICSI

Spermatozoa were treated with a two-layer density gradient system or via Swim-Up [8, 9]. For HA-ICSI: on a Petri dish, a 1 μL to 2 μL droplet with suspension of treated spermatozoa (concentration 1×10^6) was connected with a pipette tip to a 5 μL droplet of fresh culture medium (FertiCult Flushing Medium, FertiPro NV, Beernem, Belgium). Simultaneously, a 5 μL droplet of HA-containing medium (Sperm Slow™, MediCult) was connected with a pipette tip to the droplet of fresh culture medium. The spermatozoa on this Petri dish were incubated for 15 min at 37°C under oil (Liquid Paraffin, MediCult). Spermatozoa bound to HA in the junction zone of the droplets were selected and easily detached by injecting pipette (ICSI Micropipets, Humagen Fertility Diagnostics) and subsequently injected into oocytes. Conventional PVP-ICSI procedure was executed as described elsewhere [10]. Fertilization and embryo development were examined by inverted microscope. Embryos were graded 1–5 (1 best—5 worst), with grade 1 assigned to the best quality embryos containing equally sized symmetrical blastomers with no fragmentation, according to the criteria previously described by Veeck [11]. Embryo transfer was carried out after 2 days (day 2) or 3 days (day 3) from ICSI. All available embryos were transferred in accordance with Italian law. Clinical pregnancy was defined as the presence of a gestational sac with or without Fetal Heart Beat (FHB) at ultrasound examination, 2 weeks after positive hCG testing.

Statistical analysis

Continuous variables are presented as mean and standard deviation (SD). Categorical variables are presented as percentage. Normality of distribution of continuous variables was assessed with a Kolmogorov-Smirnov test (with Lillefor correction). Between-group differences of normally distributed continuous variables were assessed with parametric statistic (Student's *t*-test), whereas non parametric statistics (Mann-Whitney Rank Sum Test) were employed when the normality test was not passed. Between-group differences in non-continuous variables were assessed using the χ^2-method with Yates correction if needed or with Fisher's exact test. Difference was considered significant when a *P*-value was <0.05.

Results

331 HA-ICSI treatments were performed on 293 patients, compared with 97 conventional ICSI treatments on 86 patients. No significant differences were observed in mean male age, motile sperm number, female age at oocyte recovery and number of oocytes injected. Mean male age ± SD was 38.3±5.5 (*median* 38) for group of Physiologic HA-ICSI and of 38.2±5.4 (*median* 38) for group of conventional PVP-ICSI. Mean total motile sperm number was 11.4 (± 4.6)×10^6 in group HA-ICSI and 10.6 (± 4.5)×10^6 group PVP-ICSI. Mean female age ± SE was 34.8±3.4 (*median* 36) for group HA-ICSI and of 35.0±3.6 (*median* 35) for group PVP-ICSI. Mean number of injected oocytes was 2.8±0.8 and 2.6±0.6, respectively. The best quality embryo rate (grade 1) in group HA-ICSI was significantly higher (35.2%—P=0.011) than in group PVP-ICSI (22.3%). Implantation rate was significantly improved when performing Physiologic HA-ICSI (17.1% versus 10.3%—P=0.047). Although the difference is not statistically significant, a clear trend towards a better pregnancy rate per transfer (32.8% versus 21.6%) was found in the Physiologic HA-ICSI group (Table 1).

Table 1 HA-ICSI versus conventional ICSI

	HA-ICSI	PVP-ICSI	P
Fertilized oocytes (%)	874/936 (93.4)	223/256 (87.1)	*0.533*
Grade 1 embryos (%)	274/778 (35.2)	48/215 (22.3)	**0.011***
Clinical pregnancy rate per transfer (%)	107/326 (32.8)	21/96 (21.6)	*0.158*
Clinical pregnancy rate per cycle (%)	107/331 (32.3)	21/97 (21.6)	*0.163*
Implantations (%)	133/778 (17.1)	22/213 (10.3)	**0.047***
Abortions (%)	19/107 (17.7)	3/21 (14.3)	*0.990*
No. Ectopic pregnancies	3	0	
No. Gestational sacs without FHB	3	0	
Live births (babies born)	53 (62)	18 (19)	
Ongoing pregnancies	29	0	

Difference was considered significant when a *P*-value was <0.05

**P*-value<0.05

Discussion

Hyaluronic Acid (HA) has a natural sperm-selective function. It has been demonstrated that a method for in-vitro selection of mature spermatozoa based on sperm-HA binding results in spermatozoa for ICSI with low incidence of aneuploidies and DNA damage [1–5]. Since the chromosomal status and the chromatin integrity are not predictable by the observation of sperm dimension and shape [12] when performing conventional ICSI, the injection of aneuploid spermatozoa may generate chromosome aberrations in ICSI offspring [13, 14]; likewise, oocyte fertilization with spermatozoa with damaged DNA may lead to an increased risk of pregnancy loss [15]. Nowadays, viscous media containing synthetic plastic polyvinylpyrrolidone (PVP) are routinely used to reduce sperm motility during ICSI procedure in the majority of AR centers. Nevertheless, some authors maintain that PVP may be toxic for the gametes and the developing embryo [16–18]. HA-bound spermatozoa are easily recovered by an injecting pipette; furthermore, HA-containing products have no negative effects on post-injection zygote development and can be metabolised by the oocyte [19–21].Thus, the HA-sperm selecting method may represent at least a physiological alternative for slowing sperm motility prior to ICSI. Currently, two ready-to-use systems specially designed for sperm-HA binding selection are available: a plastic culture dish with microdots of HA hydrogel attached to the bottom interior of the dish (PICSI® Sperm Selection Device, MidAtlantic Diagnostic—FDA approved and CE-marked) or a viscous medium containing HA (Sperm Slow™, MediCult—CE marked).

In a previous study, we demonstrated that the injection of HA-bound spermatozoa (HA-ICSI or Physiologic ICSI)—when using the viscous medium Sperm Slow™ (MediCult)—improved embryo quality and development, by favouring selection of spermatozoa with normal nucleus and intact DNA [5]. Furthermore, we demonstrated that HA may speed up the time-consuming Intracytoplasmic Morphologically Selected Sperm Injection (IMSI) [22, 23]. In the present study we assessed the clinical outcome of a larger number of HA-ICSI treatments. In accordance with IVF law in Italy at the time of the study, the ICSI treatments were performed on a limited number of oocytes per patient (between 1 and 3) with the obligation to re-implant all available embryos [6]. Confirming the observation of our previous study, we found a trend towards better fertilization and pregnancy in the study group (Physiologic HA-ICSI; Table 1). The same positive trend—when injecting HA-bound spermatozoa—was observed by Menezo et al. comparing 92 HA-ICSI versus 110 PVP-ICSI treatments [24]. A statistically significant improvement in fertilization rate, embryo quality and a reduction in the number of miscarriages were observed by Worrilow et al. performing PICSI ® (MidAtlantic Diagnostic) versus conventional ICSI, in a study of 240 patients [25]. Against this, in two studies with a small number of patients involved [44 patients Van Den Berg et al. [21] and 18 patients Sanchez et al. [26]] no differences in fertilization [21, 26], pregnancy and implantation rates [26] were observed when comparing HA-ICSI to PVP-ICSI. Recently, Nasr-Esfahani et al. have published a study (performed on 50 couples) observing a higher fertilization rate when injecting oocytes with HA-selected spermatozoa [27].

Our present study is just a retrospective comparison of HA-ICSI versus an historical control group of PVP-ICSI, however this is the widest study to date comparing HA-ICSI to conventional PVP-ICSI. This study revealed that injection of HA-bound spermatozoa (HA-ICSI) determines a statistically significant improvement in embryo quality and implantation (Table 1) when performing ICSI on a limited number of oocytes (between 1 and 3). We did not observe a statistical significant difference in fertilization rate, however it is difficult to analyze the contribution of the spermatozoa in fertilization when ICSI was performed on a limited number of oocytes. In the same way, it may be that we did not observe a reduction of miscarriage rate with the HA-selected sperm due to the legal obligation to transfer all available embryos. However, the finding that the injection of HA-bound spermatozoa significantly

improves embryo quality and implantation confirms the benefit on clinical outcome of HA-ICSI when using the viscous medium Sperm Slow™ (MediCult) as previously demonstrated by our group, and in general the positive effect of HA sperm selection on ICSI outcome observed by other authors [28, 29, 31]. Even though in our study the patients were treated with the same COS regimen [7] and ICSI was performed by the same embryologist—using the same instruments and media for gamete handling and culture—nevertheless, due to the retrospective nature of this study, it could be hypothesized that the observed difference may be related to other factors (sonographers, catheters, etc.). For this reason, wider multi-center randomized studies (without any limitation on number of injected oocytes) are required to confirm these beneficial effects of HA-sperm selection on ICSI outcome. In this article for the first time we have described accurately the procedure for droplet preparation ("Materials and methods" section) which we have developed to optimize the selection of HA-bound spermatozoa using the viscous medium Sperm Slow™ (MediCult), with the intention of helping embryologists working in this field. However, since different sperm-HA binding selection systems are available, efficient and approved for IVF use [5, 28, 29], every IVF center can choose the one best suited to its needs.

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