In the natural human fertilization process, hyaluronic acid (HA) seems to play a pivotal role in physiological sperm selection. In fact, HA is normally present in the extracellular matrix (ECM) of cumulus oophorus surrounding the oocyte. The ECM is a formidable barrier that only mature spermatozoa that have extruded their specific receptors to bind to and digest HA, can overcome to reach and penetrate the zona pellucida and fertilize the oocyte.

Spermatozoa that are able to bind permanently to HA in vitro are mature and have completed the spermiogenetic process of plasma membrane remodeling, cytoplasmic extrusion, and nuclear maturity (1); these mature spermatozoa have a high density of HA receptors. In fact, in normal sperm maturation, elongating spermatids undergo cytoplasmic extrusion and plasma membrane remodeling, leading to the formation of binding sites for HA and oocyte zona pellucida. In contrast, immature spermatozoa with deficient plasma membrane remodeling are not able to bind to HA (2); in spermatozoa with arrested/diminished maturity, there is a deficiency in the zona pellucida and HA binding sites. Immature spermatozoa also show a higher retention of creatine kinase and other cytoplasmic enzymes, increased levels of lipid peroxidation and consequent DNA fragmentation, and an abnormal sperm morphology (3). Furthermore, spermatozoa with arrested maturity have low heat shock protein HspA2 expression, which causes meiotic defects and could be related to chromosomal aneuploidies. This may explain the fact that the frequency of chromosomal aneuploidies is reduced 5.4-fold in HA-bound mature spermatozoa compared with non HA-bound spermatozoa (4).

The selection of ideal spermatozoa before intracytoplasmic sperm injection (ICSI) could help to optimize the outcome of the treatment, because the injection of...
spermatozoa with damaged DNA, for example, may lead to an increased risk of pregnancy loss (5). Effective sperm selection becomes especially critical when a limited number of oocytes are available for injection or when insemination of no more than three gametes is allowed by law, as in Italy (6). The aim of the present study was to better define the role of HA for selection of spermatozoa with normal chromatin content to optimize ICSI outcome, particularly when injecting a limited number of oocytes.

MATERIALS AND METHODS
All these studies were conducted in the Reproductive Medicine Unit, GynePro Medical Centers, Bologna, Italy. The procedure was approved by the Institutional Review Board in this center. All patients were informed of the procedure, and written consent was obtained from each.

Study 1: Determination of Sperm DNA Fragmentation
Sperm DNA fragmentation of 20 patients was studied. Sperm count was carried out 30 minutes after ejaculation. Each semen sample was divided into two aliquots: One aliquot was analyzed for DNA fragmentation (group 1) and the other aliquot was treated via swim-up as described elsewhere (7). After swim-up, the semen sample was subsequently divided into three aliquots: The first aliquot underwent DNA fragmentation analysis (group 2), but one of the remaining two aliquots was put into a polyvinylpyrrolidone (PVP) droplet (PVP Clinical Grade; MediCult, Jyllinge, Denmark) and the other into an HA-containing medium droplet (Sperm Slow; MediCult), both on a Petri dish under oil (Liquid Paraffin; MediCult). Spermatozoa were collected from PVP with an injecting pipette (ICSI Micropipettes; Humagen Fertility Diagnostics, Charlottesville, VA) as for conventional ICSI procedure (8), whereas only the spermatozoa bound to HA (after 15 minutes of incubation at 37°C) were analyzed. Thus, DNA fragmentation rate was observed as follows:

- 30 minutes after ejaculation (group 1: initial semen sperm).
- After sperm treatment via swim-up (group 2: spermatozoa after Swim-Up).
- After recovery in PVP by an injecting pipette (group 3: spermatozoa in PVP).
- After recovery of HA-bound spermatozoa by an injecting pipette (group 4: spermatozoa bound to HA).

Determination of DNA fragmentation was performed with sperm chromatin dispersion (SCD) test using a ready-to-use kit (Halosperm kit; Indas Laboratories, Madrid, Spain). The basis of the SCD test lies in the differential response offered by the nuclei of spermatozoa with fragmented DNA compared with those with their DNA intact (9): When DNA is intact, the controlled denaturation of the DNA, followed by the extraction of the nuclear proteins, gives rise to partially deproteinized nucleoids in which the DNA loops expand, forming large halos of chromatin dispersion. The spermatozoa nucleoids whose DNA is fragmented do not develop a large dispersion halo. After SCD procedure the spermatozoa—fixed on agarose-coated test-slides—were stained and observed at ×40 magnification using an inverted microscope (TE 2000 U; Nikon Instruments Italia, Firenze, Italy) equipped with digital imaging software for measurements and count (NIS Elements BR; Nikon Instruments Italia). Five hundred spermatozoa were analyzed per each test slide in groups 1 and 2, and 200 spermatozoa per test slide in groups 3 and 4 (owing to the lower number of spermatozoa collectable with the injecting pipette). DNA fragmentation rate was calculated as: number of spermatozoa without halo on total number of spermatozoa observed × 100.

Study 2: Sperm Nucleus Normalcy Assessment
This study was carried out on 15 patients. Spermatozoa were first treated with a two-layer density gradient system (Sil Select, FertiPro, Beernem, Belgium) (10). Then one aliquot of prepared sperm suspension was put into a PVP (PVP Clinical Grade, MediCult) droplet and another aliquot into an HA-containing medium (Sperm Slow, MediCult) droplet, both on a glass-bottom Petri dish (WillCo-dish; WillCo Wells, Amsterdam, The Netherlands) under oil (Liquid Paraffin; MediCult). The nucleus normalcy rate of spermatozoa in PVP and of spermatozoa bound to HA (after 15 minutes of incubation at 37°C) was analyzed.

The nucleus normalcy was assessed in real time according to motile sperm organelle morphology examination (MSOME) criteria defined by Bartoov et al. (11). The examination was performed using an inverted light microscope (TE 2000 U; Nikon Instruments Italia) equipped with high-power Nomarski optics enhanced by digital imaging to achieve a magnification up to ×6,300. According to MSOME criteria, normally shaped sperm nucleus is smooth, symmetric, and with oval configuration. Average lengths and widths (±SD) must be 4.75 ± 0.28 μm and 3.28 ± 0.20 μm, respectively. Nuclear chromatin content is considered to be abnormal if sperm head contains one or more vacuoles (diameter of 0.78 ± 0.18 μm) that occupy more than 4% of the normal nuclear area. To be considered morphologically normal, a sperm nucleus has to have both normal shape and normal chromatin content (12). In this study, for rapid evaluation of nuclear normalcy, a fixed, transparent, celluloid form of a sperm nucleus fitting MSOME criteria for length and width was superimposed on the examined cell: the nuclear shape was considered to be abnormal if it differed in length or width by two standard deviations from the normal mean axis values; vacuoles were examined using a similar celluloid form (12). Furthermore, spermatozoa were then measured for nuclear length, width, and vacuoles with digital imaging software (NIS Elements BR, Nikon Instruments Italia). One hundred spermatozoa per patient were observed and measured in each group. The nucleus normalcy rate was calculated as number of spermatozoa with normal nucleus on total number of spermatozoa analyzed × 100.
Controlled ovarian stimulation was achieved using gonadotropin-releasing hormone analogues in combination with a graded gonadotropin administration (13). Oocyte retrieval was performed 35 hours after ovulation induction with either 5,000 or 10,000 IU of hCG. After retrieval, oocytes were cultured at 37°C in an atmosphere of 5% CO₂ before the complete removal of cumulus mass and corona cells by enzymatic digestion of recombinant hyaluronidase (SynVitro Cumulase; MediCult) and by gentle mechanical aspiration with plastic pipettes (Stripper Tips; MidAtlantic Diagnostic, Mount Laurel, NJ). The denuded oocytes were then evaluated to assess their nuclear maturation stage. The oocytes that had released the first polar body (metaphase II [MII]) underwent decumulation rate (5.3%; P < .001) after swim-up (11.0%, group 2) compared with initial semen sperm (Table 1).

Conventional PVP-ICSI procedure was executed as described elsewhere (8). For HA-ICSI, spermatozoa were selected for their ability to bind to HA: A 2-μL droplet with suspension of spermatozoa was connected with a pipette tip to a 5-μL droplet of HA-containing medium (Sperm Slow; MediCult) and allowed to incubate for 15 minutes at 37°C under oil (Liquid Paraffin; MediCult). Spermatozoa bound to HA in the junction zone of the two droplets were selected and easily detached by injecting pipette (ICSI Micropipette; Humagen Fertility Diagnostics) and subsequently injected into oocytes.

Fertilization and embryo development were examined by inverted microscope. Embryos were graded 1–5, with grade 1 assigned to the best-quality embryos containing equally sized symmetric blastomers with no fragmentation, according to the criteria previously described by Veeck (18). The embryo development rate (EDR) as described by Cummins et al. (19) was calculated to define the growth rate of transferred embryos. The formula for calculating the EDR was as follows: EDR = (TE/TO) × 100 (TE = time expected; TO = time observed). The ideal EDR is 100; this value is obtained when a hypothetic “normally” growing embryo is at the two-cell stage at 33.6 hours, at the four-cell stage at 45.5 hours, and at eight-cell stage at 56.4 hours. Embryo transfer was carried out 2 (day 2) or 3 (day 3) days after ICSI. Clinical pregnancy was defined as the presence of a gestational sac with or without fetal heart beat at ultrasound examination, two weeks after positive hCG testing.

Statistical Analysis
Continuous variables are presented as mean and standard error (SE). Categoric variables are presented as percentage. Normality of distribution of continuous variables was assessed with a Kolmogorov-Smirnov test (with Lilliefors correction). Between-group differences of normally distributed continuous variables were assessed with parametric statistic (Student t test), whereas nonparametric statistics (Mann-Whitney rank sum test) were used when the normality test was not passed. Between-group differences in noncontinuous variables were assessed using the χ² method with Yates correction if needed or with Fisher exact test. Difference was considered to be significant when the P value was < .05.

RESULTS

Study 1: Determination of Sperm DNA Fragmentation
Mean patient age was 38.7 ± 1.0 years (median 39 years). Mean total motile sperm number was 39.4 (±7.3) × 10⁶; 12 patients were normozoospermic (≥20 × 10⁶/mL spermatozoa) and 8 oligozoospermic (<20 × 10⁶/mL spermatozoa) according to the World Health Organization (WHO) criteria (20).

Sperm DNA fragmentation rate was significantly lower (P ≤ .001) after swim-up (11.0%, group 2) compared with initial semen sperm (16.5%, group 1). Spermatozoa collected in PVP (group 3) showed the same DNA fragmentation rate as spermatozoa of group 2 (spermatozoa after swim-up). The HA-bound spermatozoa (group 4) had the lowest fragmentation rate (5.3%; P ≤ .001 vs. groups 1, 2, and 3), resulting in a threefold reduction compared with initial semen sperm (Table 1).

Study 2: Sperm Nucleus Normalcy Assessment
Mean patient age was 39.5 ± 1.2 years (median 40 years). Mean total motile sperm number was 42.6 (±9.1) × 10⁶; nine patients were normozoospermic (≥20 × 10⁶/mL spermatozoa) and 12 patients were oligozoospermic (<20 × 10⁶/mL spermatozoa) according to the World Health Organization (WHO) criteria (20).
spermatozoa) and six oligozoospermic (<20 × 10^6/mL spermatozoa) according to the WHO criteria (20).

Nucleus normalcy rate according to MSOME criteria was significantly higher (P = 0.013) in HA-bound spermatozoa than in spermatozoa in PVP (14.5% vs. 11.0%; Table 2).

**DISCUSSION**

The selection of ideal spermatozoa before injection may optimize the outcome of ICSI treatments. It has been demonstrated that sperm dimension and shape, when observed with conventional magnification for ICSI (×40), are not reliable attributes for prediction of chromatin integrity or the absence or presence of numerical chromosomal aberrations (21). The injection of aneuploid spermatozoa may be the cause of an increased incidence of sex chromosome aberrations in ICSI offspring (22, 23). Furthermore, oocyte fertilization with spermatozoa with damaged DNA may lead to an increased risk of pregnancy loss (5). Sperm selection becomes critical when a limited number of oocytes are available for injection, as in Italy where insemination of no more than three gametes is allowed by law (6).

Given that HA has a natural sperm-selective function during human fertilization, a method for in vitro selection of mature spermatozoa based on sperm-HA binding can be effective (1, 4). The HA-bound spermatozoa are easily

**TABLE 1**

<table>
<thead>
<tr>
<th>Study 1: Determination of sperm DNA fragmentation.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1: Initial semen sperm</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Spermatozoa with fragmented DNA</td>
</tr>
<tr>
<td>Total no. of spermatozoa analyzed</td>
</tr>
<tr>
<td>DNA fragmentation rate</td>
</tr>
</tbody>
</table>

*P ≤ .001 versus groups 2, 3, and 4.
**P ≤ .001 versus group 4.
***P ≤ .001 versus group 4.

Note: HA = hyaluronic acid; PVP = polyvinylpyrrolidone.

**TABLE 2**

<table>
<thead>
<tr>
<th>Study 2: Sperm nucleus normalcy (MSOME criteria).</th>
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<tbody>
<tr>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Spermatozoa in PVP</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Spermatozoa with normal nucleus</td>
</tr>
<tr>
<td>Total no. of spermatozoa measured</td>
</tr>
<tr>
<td>Nucleus normalcy rate</td>
</tr>
</tbody>
</table>

*P = .013, PVP versus HA.

Note: MSOME = motile sperm organelle morphology examination; other abbreviations as in Table 1.

recovered by an injecting pipette, so this method may at least represent a physiologic alternative for slowing sperm motility before ICSI, avoiding any potential damaging effect of synthetic plastic PVP (24–26). Polyvinylpyrrolidone is routinely used to reduce sperm motility during ICSI procedure in the majority of assisted reproduction centers. It has been demonstrated that injection of spermatozoa recovered from HA-containing products had no negative effects on post-injection zygote development (27, 28). Furthermore, a selection method based on sperm-HA binding seems to be useful in reducing the potential genetic complications and adverse public health effects of ICSI (4). 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) or a viscous medium containing HA (Sperm Slow; MediCult). Both systems have received the CE mark, indicating their conformity with health and safety requirements in the European Economic Area; the PICSI system has also been cleared for marketing by the U.S. Food and Drug Administration. These systems, in which the spermatozoa are selected for their capacity to bind to HA (HA-ICSI), allow the execution of a more “physiologic” ICSI than conventional PVP-ICSI (8).

The purpose of the present study was to better define the role of HA for selection of spermatozoa with a normal chromatin content, and to assess the effect of their injection when performing a “physiologic” HA-ICSI on a limited number of oocytes.

The role of HA in selection of spermatozoa with intact DNA was assessed in study 1 (Table 1), where HA-bound spermatozoa showed a significant twofold reduction in the fragmentation rate compared with spermatozoa recovered from PVP (and a threefold reduction compared with the basal sample). Only 5.3% of HA-bound spermatozoa showed DNA fragmentation. This observation confirms the findings of Huszar et al. that observed that almost all HA-bound spermatozoa are devoid of DNA fragmentation (2) and persistent histones (1), which are correlated to DNA chain breakage (29). It is interesting to observe that, in the present study, spermatozoa collected in PVP showed the same DNA fragmentation rate as spermatozoa after swim-up, demonstrating that PVP has no selective function. It should be remembered, however, that basal sperm DNA fragmentation rate was significantly reduced after swim-up, confirming that this simple semen treatment improves the percentage of spermatozoa with normal chromatin structure, as previously demonstrated by Spanò et al. (30). For the assessment of sperm DNA fragmentation, we decided to perform the SCD test. We believe that for the small number of spermatozoa collectable with injecting pipette this was the most easily executable test. Furthermore, the SCD test is a simple and cost-effective procedure, showing similar predictive values to other chromatin assays for DNA fragmentation (31). In a recent study, a negative correlation between sperm-HA binding with DNA fragmentation (performed via SCD test) was observed, validating further the hypothesis that sperm with DNA fragmentation have a lower potential to bind to HA (32).

A new method of unstained, real-time, high-magnification MSOME has been developed by Bartoov et al. (12). This examination was performed using an inverted light microscope equipped with high-power Nomarski optics enhanced by digital imaging to achieve a magnification up to ×6,300. Application of this method to patients undergoing ICSI demonstrated that fine morphologic integrity of the human sperm nuclei is an important parameter associated with pregnancy rate (12, 33). In fact, ICSI outcome was significantly improved by the exclusive microinjection into the oocyte of spermatozoa with a strictly defined morphologically normal nucleus in couples with previous ICSI failures (33–36); this

### TABLE 3

<table>
<thead>
<tr>
<th>Study 3: PVP-ICSI versus HA-ICSI.</th>
<th>PVP-ICSI</th>
<th>HA-ICSI</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. treatments</td>
<td>107</td>
<td>125</td>
</tr>
<tr>
<td>Mean female age ± SE at oocyte retrieval</td>
<td>37.1 ± 0.4</td>
<td>37.5 ± 0.4</td>
</tr>
<tr>
<td>Fertilized oocytes (%)</td>
<td>236/275 (85.8)</td>
<td>304/332 (91.6)</td>
</tr>
<tr>
<td>Grade 1 embryos (%)</td>
<td>55/228 (24.1)(^a)</td>
<td>101/282 (35.8)(^b)</td>
</tr>
<tr>
<td>Mean embryo development rating ± SE</td>
<td>84.0 ± 1.1(^c)</td>
<td>95.0 ± 0.8(^d)</td>
</tr>
<tr>
<td>No. of embryo transfers</td>
<td>105</td>
<td>125</td>
</tr>
<tr>
<td>Clinical pregnancy rate per transfer (%)</td>
<td>22/105 (20.9)</td>
<td>31/125 (24.8)</td>
</tr>
<tr>
<td>Implantations (%)</td>
<td>23/226 (10.2)</td>
<td>35/282 (12.4)</td>
</tr>
<tr>
<td>Abortions (%)</td>
<td>4/22 (18.2)</td>
<td>6/31 (19.3)</td>
</tr>
<tr>
<td>No. of live births</td>
<td>19</td>
<td>29</td>
</tr>
</tbody>
</table>

**Note:** ICSI = intracytoplasmic sperm injection; other abbreviations as in Table 1.

\(^a\)\(^b\)P = .046, PVP-ICSI versus HA-ICSI.

\(^c\)\(^d\)P ≤ .001, PVP-ICSI versus HA-ICSI.

modified ICSI procedure was named intracytoplasmic morphologically selected sperm injection (IMSI) (33). In the present study 2, the sperm nucleus normalcy rate (according to MSOME criteria) of spermatozoa in PVP and of spermatozoa bound to HA was analyzed. We found that nucleus normalcy rate was significantly higher in HA-bound spermatozoa than in spermatozoa in PVP (Table 2). Based on this finding, it could be hypothesized that HA may help to select a subpopulation of spermatozoa with normal nucleus, thus speeding up the time-consuming IMSI procedure: in fact, selecting a normal spermatozoon according to MSOME criteria may require 60–120 minutes (34).

In study 3 we analyzed 232 ICSI treatments performed on a limited number of oocytes (1 to 3) per patient, as limited by IVF law in Italy, with the obligation to reimplant all available embryos. The overall results in terms of fertilization, cleavage, pregnancy, implantation, and abortion rates were similar to other ICSI studies carried out on women with a mean age of 37 years under the same law (6). In the present study we compared conventional PVP-ICSI to ICSI in which the spermatozoa were selected for their capacity to bind to HA (HA-ICSI), and we observed a trend toward better fertilization, pregnancy, and implantation in the HA-ICSI group (Table 3). The same positive trend—when injecting HA-bound spermatozoa—was observed by Ménézo et al. comparing 110 PVP-ICSI and 92 HA-ICSI treatments (37). Furthermore, a statistically significant improvement in fertilization rate and embryo quality and a reduction in the number of miscarriages were observed by Worrilow et al. performing PICS versus conventional ICSI in a study of 240 patients (38). A possible explanation for the lack of improvements in abortion rate with the HA-selected sperm is due to the Italian legal obligation to reimplant all available embryos. Otherwise, in another small study on HA-ICSI (18 treatments) no differences in fertilization, pregnancy, and implantation rates were observed compared with PVP-ICSI (39). All of these studies were meeting reports. Recently, Nasr-Esfahani et al. (32) have published a study (performed on 50 couples) observing a higher fertilization rate when injecting oocytes with HA-selected spermatozoa. To our knowledge, the present study is the widest prospective-randomized study comparing conventional PVP-ICSI and HA-ICSI presented for publication. This study revealed that injection of HA-bound spermatozoa (HA-ICSI) determines a statistically significant improvement in embryo quality and development (Table 3) when performing ICSI on a limited number of oocytes (between 1 and 3). This finding confirms the positive effect of HA sperm selection on ICSI outcome observed by other authors (32, 37, 38).

The present studies have demonstrated that spermatozoa bound to HA show a significant reduction in DNA fragmentation and nuclear anomalies compared with spermatozoa recovered from PVP, and their injection determines improvement of embryo quality after ICSI. We can conclude that, by favoring selection of spermatozoa with intact DNA and normal nucleus, HA may optimize ICSI outcome. If further studies confirm these beneficial effects on ICSI outcome, HA could be considered as the recommended choice for sperm “physiologic” selection before ICSI because of its potential capacity to reduce genetic complications and for its total lack of toxicity. Furthermore, HA may also be used to speed up the selection of spermatozoa with normal nucleus during the IMSI procedure.

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