Randomized sibling-oocyte study using recombinant human hyaluronidase versus bovine-derived Sigma hyaluronidase in ICSI patients

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BACKGROUND: The enzyme hyaluronidase from bovine origin is commonly used for oocyte–cumulus cell removal in ICSI. A recombinant human hyaluronidase (rHuPH20) has been introduced as a quality-controlled and safe alternative. METHODS: In order to validate its effectiveness, a non-inferiority trial was started on sibling cumulus–oocyte complexes (135 ICSI patients). Oocyte denudation involved enzyme incubation under Pasteur pipetting, followed by further mechanical stripping. Primary end-points were oocyte intactness after ICSI and fertilization rate. Secondary end-points were oocyte intactness after ICSI and fertilization rate. Secondary end-points were embryo development and positive hCG. RESULTS: Oocyte intactness after ICSI was 89.6% and 92.9% with rHuPH20 and bovine hyaluronidase, respectively [absolute difference 3.3% (7.4 to 10.7)]. The fertilization rate was 73.9% after rHuPH20 and 77.1% after bovine hyaluronidase treatment [absolute difference 3.2% (8.3 to 1.8)]. Embryo development was similar in both treatment groups up till Day 5. Positive hCGs were equally distributed over mixed transfers 21/45 (46.7%) and transfers of only embryos from rHuPH20 treatment 17/35 (48.6%) or transfers of only embryos from bovine hyaluronidase treatment 22/48 (45.8%). CONCLUSIONS: Our results indicate that rHuPH20 is not inferior to bovine hyaluronidase for oocyte denudation, with regard to oocyte survival and fertilization. rHuPH20 treatment of human oocytes is compatible with good embryo development, with positive hCG results and with live birth.

Keywords: cumulus cell removal; hyaluronidase; ICSI prospective study

Introduction

In vitro fertilization (IVF) by means of intracytoplasmic sperm injection (ICSI) involves the removal of the cumulus and corona cells surrounding the oocyte. Since the start of ICSI, the enzyme hyaluronidase from bovine origin has standardly been used for oocyte denudation (Van de Velde et al., 1997; Joris et al., 1998). This enzyme digests the hyaluronic acid interspaced between the cumulus cells (Mahadevan and Trounson, 1985; Dandekar et al., 1992), thus liberating the oocyte for maturity grading and microinjection. Purified preparations of the enzyme are commercially available from bovine and sheep testicular extracts. These animal-derived products have limited purity and accidental transmission of pathogens cannot be fully excluded. In order to reduce unknown harmful effects from the enzyme preparation, low enzyme concentrations and short time incubation of the cumulus–oocyte complexes (COCs) are recommended (Van de Velde et al., 1997). These limitations inevitably imply an increased mechanical decoronization by means of multiple pipetting, which may be harmful to the oocyte.

The use of a plant enzyme preparation (Coronase) has been suggested as an alternative to animal extracted hyaluronidase (Parinaud et al., 1998) for human cumulus cell removal. The time required for total denudation is slightly longer when using Coronase; however, fertilization rates are similar as are percentages of embryos with good morphology. A recently developed recombinant human hyaluronidase (rHuPH20, Cumulase®) may represent a better alternative (Taylor et al., 2006). Owing to its high purity, Cumulase® may be used at higher concentrations and the incubation time becomes less critical. For these reasons, it may reduce, if not eliminate, the risk of damage or trauma done to the oocytes by the mechanical denudation step. Equally important, the risk issue of animal pathogen contamination is alleviated.

Despite promising results on the use of Cumulase® in an ICSI treatment programme (Taylor et al., 2006), a prospective
comparison with the traditional bovine-derived hyaluronidase in order to validate the effectiveness of the new compound in a sufficient number of ICSI cases seems appropriate. Therefore, a non-inferiority trial was started with a prospective target of 1554 sibling oocytes obtained from an estimated 130 ICSI patients (assuming a mean number of COCs of 12 per patient). Oocyte intactness and fertilization rate after ICSI were evaluated as primary outcomes. Secondary outcomes were embryo development and positive hCG.

**Materials and Methods**

**Study design and outcome measures**

A randomized sibling-oocyte trial was started in order to evaluate the effectiveness of cumulus–oocyte separation by Cumulase® when compared with traditionally used bovine-derived hyaluronidase. In view of the small differences to be expected, classical testing would necessitate a too high number of oocytes to be included. Therefore, this study was set up as a non-inferiority trial.

Randomization was performed at the moment of oocyte retrieval. For each patient with 8–16 COCs, the COCs were allocated to two separate oocyte collection dishes. By using a pair of dishes for sibling oocytes from each patient in a paired study design, differences in patient characteristics, such as female age, rank of trial, duration and origin of infertility, were eliminated. The dishes were allocated by the person who performed the cumulus removal to Cumulase® or to standard separation according to a computer-generated random list, issued in balanced blocks of six numbers. Disclosure (which dish, which protocol) only occurred after introduction of the unique patient cycle number on the list.

The primary efficacy end-points were non-inferiority in intactness of oocytes after ICSI and fertilization rate (primary outcome measures). Intact oocytes are those not showing signs of degeneration when assessed 16–18 h after micro-injection. Secondary efficacy measures included embryo development and positive hCG. Embryo development up till Day 3 was evaluated for all patients included, whereas Day 5 blastocyst development was only available for some patients who were scheduled for Day 5 transfer. In addition, the following baseline characteristics were documented: women’s age, enzyme incubation time, level of mechanical stripping and availability for ICSI. The level of mechanical stripping was quantified by counting the number of times individual oocytes had to be pipetted in and out of a 135 μm inner diameter pipette. Availability for ICSI was expressed as percentage intact metaphase-II oocytes per number of COCs retrieved.

**Target population**

The study started in May 2006 and ended in February 2007. Consecutive women not older than 38 years of age, presenting with 8–16 COCs at ovum pick-up, and undergoing ICSI treatment with ejaculated spermatozoa were considered for inclusion. Surgically extracted sperm preparations and embryo biopsy cycles were excluded from the study. Similarly, patients older than 37 years of age or presenting with fewer than 8 or with more than 16 COCs were excluded. The study and the informed consent were approved by the Medical Review Board of the University Hospital Brussels.

**Ovarian stimulation**

Two ovarian stimulation protocols were used for patients in the present study. One was the long GnRH agonist protocol using Buserelin (Suprefact, Hoechst, Frankfurt, Germany) combined with HMGs (Menopur, Ferring Pharmaceuticals A/S) (Van de Velde et al., 1998). The other protocol involved a combination of GnRH antagonist (Orgalantrin, NV Organon, Oss, The Netherlands) and recombinant gonadotrophins (recombinant FSH, Puregon, NV Organon) (Kolibianakis et al., 2004). Final oocyte maturation was achieved by 5000 or 10 000 IU of hCG (Pregnyl, NV Organon) when at least three follicles of minimum 17 mm diameter were present on ultrasound. Oocyte retrieval was carried out by vaginal ultrasound-guided puncture of the ovarian follicles, 36 h after hCG was administered. Intravaginally administered progesterone (Utrogestan, Piette, Brussels, Belgium) was used for luteal phase supplementation.

**Oocyte collection and denudation**

At the time of oocyte retrieval, sibling COCs were allocated to two separate oocyte collection dishes in an alternating way. A computer-generated randomization list (balanced blocks of six) was used for allocation of the first dish to either Cumulase® or bovine hyaluronidase denudation.

Collection and denudation of the oocytes was done in Heps-buffered HTF medium (Lonza, Verviers, Belgium) supplemented with 0.5% (w/v) synthetic serum supplement (SSS, Irvine Scientific, Wicklow, UK). Cumulase® denudation (Halozyme Therapeutics Inc., San Diego, CA, USA) involved incubation in 80 USP Units/ml, including Pasteur pipetting of all allocated COCs together, with the time of incubation to be determined during the study. (A USP unit is a unit defined and adopted by the United States Pharmacopeia. It is used to measure the potency of a substance, that is, its expected biological effects. In most cases, the USP unit is equal to the international unit, IU). The 80 USP Units/ml dose for Cumulase® was chosen because in the USA, all hyaluronidase products approved for ICSI are approved at the 80 IU dose, and for Cumulase®, which was approved under the 501k route of FDA approval, the predicate device was the Medi-Cult hyaluronidase product, which was 80 IU. When most cumulus cells were removed, oocytes were rinsed twice in HTF medium prior to further mechanical decoronization in the absence of the enzyme. The level of additional mechanical stripping was quantified by counting the number of times that individual oocytes had to be pipetted in and out of a narrow hand-made pipette (inner diameter 135 μm) using a Swemed pipette holder (Vitrolife, Kungsbacka, Sweden).

Denudation in bovine hyaluronidase (Type VIII, Sigma Chemical Company, St Louis, MO, USA) was done similarly except for using a 40 IU/ml concentration of the enzyme, due to long-term laboratory experience with this concentration. Incubation time, including Pasteur pipetting, and level of additional mechanical stripping were evaluated similarly.

Microscopic observation of the denuded oocytes included assessment of the zona pellucida and the oocyte, and the presence or absence of a germinal vesicle or a first polar body. Available for ICSI were intact metaphase-II oocytes (excluding immature oocytes, bad morphology oocytes, cracked or empty zonae).

**ICSI procedure**

Sperm handling for ICSI has been described before (Van de Velde et al., 1997). After denudation, metaphase-II oocytes were immediately injected with a single spermatozoan into the ooplasm as described earlier (De Vos and Van Steirteghem, 2005; Van Landuyt et al., 2005). The injected oocytes were incubated at 37° C (6% CO₂, 5% O₂ and 89% N₂) in 25 μl droplets of sequential media (BlastAssist® System, Medicult, De Pinte, Belgium) placed under oil suitable for embryo culture (Irvine Scientific). BlastAssist Medium 1 (cleavage-stage medium) was changed for BlastAssist Medium 2 (blastocyst culture) on Day 3 of culture.
Fertilization and embryo development

At 16–18 h post-injection, fertilization was assessed and further development of the embryos was then evaluated on a daily basis. For embryo evaluation on Day 3, the developmental stage was registered and the embryos were further classified as type A, B or C according to the percentage of fragmentation (Van Landuyt et al., 2005). Signs of compaction in the embryos was scored as compacting (C1, cell borders are still visible) or compact (C2, a dense morula is formed). On Day 3, good quality embryos were defined as presenting at least 8 cells and no more than 10% of fragmentation. Day 5 blastocyst evaluation was done as described by Gardner et al. (2000). Blastocysts 1 and 2 were considered as early blastocysts, good quality blastocysts included all other expanded stages (3–6, at least type BA or BB) and excellent quality blastocysts included stages 3–6, types AA or AB. The first index refers to the quality of the inner cell mass and the second index refers to the quality of the trophectoderm.

Clinical outcome

A biochemical pregnancy was indicated by a rise in serum hCG (>20 IU), measured 14 days after oocyte aspiration, and repeated 3 days later. A clinical pregnancy was defined by the presence of a gestational sac at ultrasound performed at 7 weeks of gestation (Zegers-Hochschild et al., 2006).

Sample specifications and analysis plan

This study was set up as a non-inferiority trial. The non-inferiority margin was set at 10% because this threshold was considered to indicate clinically important differences based on clinical judgement from standard day-to-day laboratory practice in our Centre for Reproductive Medicine. With an average of 12 oocytes per patient, with six assigned to each separation protocol, 10% would amount to less than one damaged oocyte per patient. In practice, more than 90% of oocytes are intact after the standard separation procedure, and for the Cumulase® procedure the lower confidence limit should be no <10% below this, that is not <81% of oocytes should be intact. Approximately 70% of injected oocytes have 2PN. Therefore, the lower confidence limit should not be <63% 2PN.

To demonstrate non-inferiority of Cumulase® based on a maximum difference of 10% between the two procedures with a power of 90% and a type I error of 10% (non-inferiority design with tests performed one-side) would require 583 oocytes per group if pairing is taken into account. In order to err on the side of conservatism and plan for up to 25% for non-evaluable oocytes, a sample size of 777 oocytes per group was selected. The total of 1554 oocytes corresponds to 130 patients with 8–16 (mean of 12) COCs. We decided to include 135 patients in the study in order to reach the calculated number of COCs.

Baseline characteristics (continuous data) are presented as means with standard deviation (SD). The primary and secondary efficacy end-points (oocyte and embryo outcomes) were aggregated to the level of each patient for each arm and then a paired t-test of the 135 arm-pairs of aggregated data was performed, with patient characteristics such as female age, rank of trial, duration and origin of infertility, being accounted for in the sibling oocyte (paired) study design. The primary and secondary efficacy end-points (oocyte and embryo outcomes) within each treatment arm are presented as means with corresponding two-sided normal approximation 95% confidence intervals (95% CIs). The results are summarized comparatively as differences between the two arms with a 95% CI for each between-arm difference for each comparison made (Altman, 1991). In doing so, we allow the reader to quantify how similar the treatment arms actually are. Clinical data on positive hCG and clinical pregnancy rates are presented as percentages with the corresponding 95% CI.

Results

Baseline outcome measures

One hundred and thirty-five patients met the inclusion criteria and signed informed consent to participate in the study. The mean female age was 31.7 years (SD 3.6; range 21–37) and the mean rank of trial was 2.1 (SD 1.3; range 1–7). In total, 1559 COCs were collected at ovum pick-up [i.e. 11.5 per patient (SD 2.5)]. There were 778 COCs allocated to Cumulase® denudation, whereas the other 781 were denuded with bovine hyaluronidase (Table I). The enzyme incubation time (including Pasteur pipetting) for cumulus removal was not different for the two enzymes: 61.9 s (SD 21.0) for Cumulase® and 62.8 s (SD 29.2) for bovine hyaluronidase (Table I). The level of additional mechanical decoronization was also similar: cumulase®-treated oocytes were pipetted in and out of the 135 μm inner diameter pipette 17.2 times (SD 7.6), whereas this parameter for bovine hyaluronidase-treated oocytes was 16.8 times (SD 7.6). Similar percentages of metaphase-II oocytes, expressed per number of COCs retrieved, were available for ICSI in the two groups.

Primary outcome measures: oocyte intactness and fertilization rate after ICSI

Table II shows that the percentage of intact oocytes after ICSI was not different between the two treatment groups 89.6% (86.0–93.2) per injected oocyte for Cumulase® and 92.9% (90.5–95.3) for bovine hyaluronidase, representing an absolute between-arms difference of –3.3% (–7.4 to 10.7). Similarly, the fertilization rate per injected oocyte was not different: 73.9% (69.5–78.3) after Cumulase® treatment of the oocytes and 77.1% (73.3–80.9) after bovine hyaluronidase treatment, representing an absolute between-arms difference of –3.2% (–8.3 to 1.8). The lower limit of the confidence intervals for both primary outcome measures excluded the predefined non-inferiority margins of 81% and 63% for intact oocytes and fertilization rate after ICSI, respectively. Likewise, non-inferiority of Cumulase® to bovine hyaluronidase was demonstrated for both primary outcome measures based on the values of the lower 95% CI limits for the absolute differences between arms (being less than –10%).

Embryo development

Paired results for Day 3 development were available for all 130 patients included. Day 3 development was not available

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<th>Table I. Baseline outcome measures.</th>
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<td>Enzyme incubation time&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Mechanical denudation&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Availability for ICSI&lt;sup&gt;d&lt;/sup&gt;</td>
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</tbody>
</table>

COCs, cumulus–oocyte complexes.

All values represent mean ± standard deviation (SD).

<sup>a</sup>seconds.

<sup>b</sup>i.e. in/out pipetting.

<sup>c</sup>Mean percentage per cycle of metaphase-II oocytes expressed per number of COCs retrieved.
for one patient because of Day 2 transfer, and four patients obtained no fertilization in either arm of the comparison. For one patient, intra-Fallopian transfer of two zygotes (out of six available) was performed (providing Day 3 development only on four remaining zygotes). As shown in Table III, the mean % per cycle of good quality embryos per oocyte fertilized was 19.5% (14.8–24.1) for Cumulase® treatment and 22.4% (17.4–27.4) when using bovine hyaluronidase, representing an absolute between-arms difference of −2.9% (−8.2 to 2.4). Day 5 embryo development was available only for the 52 patients scheduled for Day 5 blastocyst transfer. Full blastocysts (at least stage 3, Gardner classification) of excellent or good quality were expressed per number of oocytes fertilized. The mean % per cycle was not different (Table III): 15.8% (10.1–21.5) for Cumulase®, representing an absolute between-arms difference of −3.3% (−7.4 to 10.7).

### Clinical outcome

Of 135 cycles started, 128 resulted in embryo transfer. The mean number of embryos replaced was 1.6 (SD 0.7). Mainly Day 3 transfers were performed (n = 80) and 46 transfers were on Day 5. Two patients received transfer earlier (one zygote transfer on Day 1 and one embryo transfer on Day 2). Seven cycles remained without embryo transfer due to insufficient embryo quality on Day 3 (n = 1) or on Day 5 (n = 6).

The overall rate of positive hCG was 60/128 (46.9%, 95% CI 35.8–60.3) per embryo transfer or 60/135 (44.4%; 95% CI 33.9–57.2) per started cycle. Embryo transfers of mixed nature (n = 45) or otherwise only embryos resulting from Cumulase®-treated oocytes (n = 35) or only embryos resulting from hyaluronidase-treated oocytes (n = 48) were transferred. Positive hCGs were reported in all three groups: 21/45 (46.7%; 95% CI 28.9–71.3), 17/35 (48.6%; 95% CI 28.3–77.8) and 22/48 (45.8%; 95% CI 28.7–69.4), respectively. These results show that Cumulase treatment of the oocytes is compatible with positive hCG.

Out of 60 positive hCGs, 52 were clinical pregnancies as evidenced by a gestational sac on ultrasound. Therefore, the clinical pregnancy rate was 52/127 (40.9%; 95% CI 30.6–71.3) per transfer with known outcome on clinical pregnancy (one transfer with positive hCG was lost for follow-up after hCG measurement). Per started cycle this figure was 52/134 (38.8%; 95% CI 29.0–50.9; one cycle with unknown outcome on clinical pregnancy). There were 47 patients who delivered (respectively, 15, 15 and 17 in the three different types of transfer, as mentioned above, mixed, Cumulase and hyaluronidase), whereas three pregnancies remained without result on delivery outcome. There was one ectopic pregnancy (in the mixed transfer group) and one clinical abortion (in the hyaluronidase transfer group).

Seven positive hCGs remained without clinical evidence of pregnancy by ultrasound visualization of a gestational sac (biochemical pregnancies), respectively, 3, 2 and 2 in the three different transfer groups (mixed, Cumulase and hyaluronidase). One positive hCG in the mixed transfer group was lost to further follow-up.

### Discussion

The objective of this study was to validate the effectiveness of the recombinant human enzyme Cumulase® for cumulus–oocyte denudation in human ICSI. A randomized non-inferiority trial on sibling oocytes was set up for Cumulase® versus bovine-derived hyaluronidase. Oocyte survival, fertilization and embryo development were similar in both treatment groups.

Cumulase® was used at 80 USP Units/ml, as recommended by the company, in our standard oocyte denudation protocol. The incubation time needed to loosen the cumulus cells did not differ from the time needed when bovine-derived hyaluronidase was used at 40 IU/ml, indicating similar effectiveness at the level of COC disruption. Oocytes were then immediately further denuded mechanically. The mechanical force on Cumulase®-treated oocytes was similar compared to bovine hyaluronidase.

### Table II. Primary outcome measures.

<table>
<thead>
<tr>
<th></th>
<th>Cumulase® (n = 621 injected M-IIs)</th>
<th>Bovine hyaluronidase (n = 611 injected M-IIs)</th>
<th>Absolute difference in percentages between arms</th>
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<tbody>
<tr>
<td>ICSI intact oocytes</td>
<td>89.6% (86.0–93.2)</td>
<td>92.9% (90.5–95.3)</td>
<td>−3.3% (−7.4 to 10.7)</td>
</tr>
<tr>
<td>Fertilized oocytes (2-PN)</td>
<td>73.9% (69.5–78.3)</td>
<td>77.1% (73.3–80.9)</td>
<td>−3.2% (−8.3 to 1.8)</td>
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</table>

aM-II, mature metaphase-II oocyte.
bMean percentages per cycle, expressed per injected M-II oocyte.
cValues between brackets indicate 95% confidence interval.

### Table III. Embryo development Day 3 and Day 5.

<table>
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<tr>
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<th>Cumulase®</th>
<th>Bovine hyaluronidase</th>
<th>Absolute difference in percentages between arms</th>
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<tbody>
<tr>
<td>Day 3 development</td>
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<tr>
<td>≥8-cell, ≤10% fragments</td>
<td>19.5 (14.8–24.1)</td>
<td>22.4 (17.4–27.4)</td>
<td>−2.9% (−8.2 to 2.4)</td>
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<tr>
<td>Day 5 development</td>
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<tr>
<td>Excellent or good Q blastocyst</td>
<td>15.8 (10.1–21.5)</td>
<td>13.1 (7.0–19.3)</td>
<td>2.7% (−4.8 to 10.1)</td>
</tr>
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</table>

aMean percentages per cycle, expressed per oocyte fertilized.
bValues between brackets indicate 95% confidence interval.
with hyaluronidase-treated oocytes. In other words, no gain at the level of mechanical stress towards the oocytes was observed with the recombinant enzyme. It is presently unknown whether this could be obtained by incubating the COCs longer in the presence of the enzyme or by an incubation without enzyme prior to mechanical denudation in contrast to immediate mechanical denudation (as done by Taylor et al., 2006). However, in the latter protocol, no difference in time needed for complete denudation of the oocytes was observed between Cumulase® and the bovine-derived preparation.

The present study did not confirm the improved fertilization rate as described by Taylor et al. (2006). It would be hard to explain why Cumulase® denuded oocytes would fertilize better, especially because no differences can be found in the denudation procedure (similar incubation time and similar mechanical denudation stress). One difference that could be raised is the purity of the Cumulase® product when compared with bovine-derived preparations. Typical animal extracts do contain impurities that may interfere with the fertilization process of the oocyte. In this study, however, a high fertilization rate was obtained when oocytes were exposed to the bovine-derived preparation (77%). Our results indicate that Cumulase® exposed oocyte fertilization is not inferior (74%). Likewise, further embryo development was irrespective of the enzyme used for denudation.

The present data illustrate that the recombinant enzyme Cumulase® is not inferior to bovine-derived hyaluronidase for oocyte denudation, and can be used within an ICSI programme with similar fertilization and embryo developmental outcome. Cumulase® treatment of human oocytes before ICSI is also compatible with positive hCG results and with live birth. Cumulase® has the strict advantage of being a non-animal-derived recombinant product for use in human IVF. In contrast to slaughterhouse-derived hyaluronidase enzymes, it is animal DNA free and eliminates the risk of animal pathogen contamination, in particular the risk of transmitting transmissible spongiform encephalopathies. This is important in view of the European Commission quality and safety measures to eliminate threats to public health (European Cell and Tissue Directives 2004/23/EC, 2006/17/EC and 2006/86/EC; Commission Directive 2003/32/EC).

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