Fertilization rates are improved after IVF if the corona radiata is left intact in vitrified-warmed human oocytes

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BACKGROUND: Before human MII oocytes are vitrified they are usually denuded from their cumulus cells. In this study we wanted to investigate the effects of an intact corona radiata on the vitrification and fertilization of human oocytes.

METHODS: The study comprised two different parts. In Part 1, 36 MII stage oocytes, from 6 patients, were randomly assigned into a control group, a group of vitrified-warmed oocytes without a corona radiata and a group of vitrified-warmed oocytes with an intact corona radiata. In each group of 12, 6 oocytes were used for evaluation of the zona pellucida solubility (hardening) and another 6 oocytes were used for the analysis of their ultrastructure. In addition, six polyspermically fertilized oocytes were used as positive controls for zona pellucida hardening. In Part 2, 16 patients in total produced 107 fresh and 98 vitrified-warmed oocytes, with or without an intact corona radiata. All oocytes were fertilized via conventional IVF and embryos were transferred according to our standard ET routines. The oocyte survival and fertilization rates, embryo quality and pregnancy and implantation rates were evaluated.

RESULTS: There were no differences in oocyte survival, zona pellucida solubility (hardening) or the number of cortical granules between the vitrified-warmed and fresh oocytes. There were also no differences in the zona pellucida solubility and the number of cortical granules between vitrified-warmed oocytes with or without an intact corona radiata. However, the oocytes with an intact corona radiata had a higher fertilization rate after conventional IVF insemination. No differences were seen in the survival and cleavage rates, the percentage of high-quality embryos or the clinical outcome.

CONCLUSIONS: Zona hardening and ultrastructural damage do not seem to occur in vitrified human oocytes. An intact corona radiata in vitrified-warmed oocytes retains their fertilization capacity in conventional IVF, but does not improve the embryo quality. Poor fertilizing capacities of vitrified-warmed oocytes without an intact corona radiata seem to have been due to the complete removal of the cumulus cells.

Key words: vitrification / human oocytes / cumulus cells / zona pellucida hardening / fertilization

Introduction

Human oocyte cryopreservation remains an elusive task for centers of reproductive medicine and infertility. Many research groups continue to make improvements in human oocyte cryopreservation, although the vitrification of oocytes has been shown to provide higher pregnancy rates in recent years (Fabbri, 2006; Boldt, 2011).

An intact corona radiata seems to protect MII oocytes against structural damage during slow freezing as shown for human oocytes (Imoedemhe and Signe, 1992) and during vitrification as shown for horse MII oocytes (Tharasanit et al., 2009). Vitrification of denuded MII horse oocytes was shown to lead to a high incidence of spindle abnormalities and very poor embryo development (Tharasanit et al., 2006). Normally, human MII oocytes are completely denuded from their protective cumulus cells before vitrification and little is known about the effects of cumulus cells on human MII oocytes during vitrification. Could the cumulus corona radiata layer protect human oocytes during the vitrification process?

† These authors contributed equally to this work.
In the current study, cumulus–oocyte complexes (COCs) were obtained from donors undergoing conventional IVF treatment. For human oocytes with or without an intact corona radiata, the survival rate, zona pellucida solubility (hardening) and ultrastructure were examined post-vitrification and the fertilization rate, embryo quality and implantation and pregnancy rates were analyzed for both fresh and vitrified-thawed oocytes.

### Materials and Methods

After complete explanation of the study, written informed consent was obtained from each participant. The study was conducted in compliance with the Declaration of Helsinki and Ethics Committees on Human Research of Anhui Provincial Hospital, an affiliation to the Anhui Medical University.

#### Study design

Women were stimulated with the long gonadotrophin-releasing hormone agonist (GnRH-a, Diphereline; Ipsen Pharma, Biotech, Signes, France) protocol combined with recombinant FSH (Gonal-f, Merk Serono SA, Geneva, Switzerland). Oocytes were retrieved by transvaginal ultrasound 36 h after administration of 10,000 IU human chorionic gonadotrophin (hCG, LiZhu Pharma, ZhouHai, China). Only MII oocytes with an extruded first polar body were included in this study. The study comprised three different parts.

In Part 1, a total of 36 MII stage oocytes were obtained from 6 different patients, from whom at least >20 COCs were retrieved. The women were younger than 30 years and the infertility was due to male or tubal factors. Only fully expanded COCs were chosen. With the help of a micromanipulation system, the presence of the polar body in the oocytes with or without an intact radiata was determined under microscope. The oocytes were randomly assigned into three different groups with 12 oocytes per group. The control group comprised fresh oocytes with an intact corona radiata. The second group comprised vitrified-warmed denuded oocytes without a corona radiata. The third group comprised vitrified-warmed oocytes with an intact corona radiata. In each group, 6 oocytes were used for evaluation of the zona pellucida solubility (hardening) and another 6 oocytes were used for analysis of their ultrastructure. In addition, 6 polyspermically fertilized oocytes were used as positive control for zona pellucida hardening.

In Part 2 of the study, there were four groups: one group of vitrified-warmed oocytes without a corona radiata, a second group of vitrified-warmed oocytes with a corona radiata, a third group of fresh oocytes without a corona radiata and a fourth group of fresh oocytes with a corona radiata. The vitrified-warmed oocytes (n = 98) were obtained from eight different patients where the husband could not produce a semen sample on the day of oocyte collection. Half of these oocytes (n = 49) were completely denuded and the other half retained an intact corona radiata before vitrification. After warming, the oocytes were inseminated via conventional IVF. The fresh oocytes were donated from an additional eight patients. In total, 107 fresh and 98 vitrified-warmed oocytes from 16 different patients were randomly assigned for insemination with or without an intact corona radiata. All oocytes were fertilized via conventional IVF and embryos were transferred according to our standard ET routines. Early rescue ICSI was done 6–8 h after conventional insemination to prevent fertilization failure. The oocyte survival and fertilization rates, embryo quality and pregnancy and implantation rates were evaluated.

#### Human oocytes with and without intact corona radiata

For human oocytes with an intact corona radiata (Fig. 1), the outer layers of cumulus cells of the COCs were partially denuded using two disposable needles and two 1-ml plastic disposable syringes. These oocytes and complete denuded oocytes without a corona radiata were washed three times in the fertilization medium (William A. Cook Australia Pty. Ltd, Queensland, Australia) and kept in the medium (37°C, 6% CO₂) for no more than 20 min before vitrification was undertaken.

#### Oocytes vitrification and warming

Vitrification was carried out using a two-step CryoTop method (Kitazato Biopharma Co., Ltd. Japan). Briefly, the oocytes were treated for 5 min in equilibration solution (7.5% ethylene glycol and 7.5% dimethylsulfoxide), then transferred in a minimal volume of equilibration solution into a drop (100 µl) of the vitrification solution (15% ethylene glycol plus 15% dimethylsulfoxide plus 0.5 M sucrose). The oocytes were briefly rinsed three times (<30 s every time) in three drops of vitrification solution, then loaded onto the CryoTop. After loading, most of the solution was removed so that only a thin layer covered the oocytes. Once the oocytes were transferred into the vitrification solution, they were plunged into liquid nitrogen within 90 s.

When the oocytes were warmed, the CryoTop with vitrified oocytes, was taken out of the liquid nitrogen and warmed by submerging it into 1.5 ml of thawing solution 1 (TS1, 1 M sucrose) at 37°C for 1 min.

**Figure 1** Human oocytes with intact corona radiata before (a) and after (b) vitrification.
The oocytes were immediately transferred into diluent solution (DS1, 0.5 M sucrose) and washing solution 1 (0 M sucrose) for 2 min in each solution at room temperature, and then transferred into washing solution 2 (0 M sucrose) for 5 min. Finally, the oocytes were kept in the fertilization medium, under mineral oil, and cultured at 37 °C and 6% CO₂ in the incubator for 2 h before treatment.

Assessment of zona pellucida solubility (hardening)

Oocytes with the corona radiata were treated with 40 IU/ml of hyaluronidase (SAGE In Vitro Fertilization, Inc. Trumbull, USA) in HEPES-buffered Gamete medium (William A. Cook Australia Pty. Ltd) for complete denudation of the oocytes. Each of the denuded oocytes were placed individually in 25-μl micro-drops of 0.1% (w/v) Pronase E solution (Sigma-Aldrich, St. Louis, MO, USA) in PBS, covered with mineral oil and incubated at 37 °C (Bijttebier et al., 2008). Zona pellucidae were continuously observed for dissolution under an inverted microscope (Leica 3000B) equipped with a warm plate set at 37 °C. The zona pellucida dissolution time of each oocyte was assessed at 200× magnification. The end-point for zona dissolution was complete digestion of the zona.

Ultrastructure observations with transmission electron microscopy

Transmission electron microscopy analysis provides valuable and objective criteria to assess cell injury from cryopreservation (Coticchio et al., 2010). The oocytes were fixed and processed for TEM (transmission electron microscopy) analysis as previously described by Nottola et al. (2007). Ultra-thin section (60–80 nm) were cut with a diamond knife, the sections were mounted on a copper grid and contrasted with saturated uranyl acetate followed by lead citrate. They were examined and photographed using a JEOL-1230 Transmission Electron Microscope. Cortical granule density was evaluated through collection of transmission electron microscopy microphotographs at ×5000 magnification. Images were further enlarged on the PC screen in order to easily recognize and count the number of cortical granules. Values were expressed as the number of cortical granule per 10 μm of the oocyte linear surface profile.

Fertilization, embryo quality and clinical outcome

The oocytes were cultured together in a large drop of fertilization medium (William A. COOK Australia Pty. Ltd, Queensland, Australia) under mineral oil in 35-mm Petri dishes (Falcon 1008; Becton & Dickinson, Lincoln Park, NJ). Motile sperm prepared using the swim-up technique were added to oocytes at a concentration of 100,000 sperm/ml. After co-incubation with spermatozoa for 6 h, the oocytes were transferred into microdrops of fertilization medium without the sperm and were completely denuded by pipetting.

The oocyte was considered fertilized if the second polar body was extruded or if pro-nuclei were seen 16 h after insemination. Those with two pronuclei (2PN) and a second polar body were identified as normally fertilized. The oocytes with a single pronucleus or three pronuclei were considered as abnormally fertilized.

The normally fertilized oocytes were transferred into drops of cleavage medium (William A. Cook Australia Pty. Ltd). Two or three good quality embryos were selected for clinical transfer at Day 3. Day 3 embryos that had 7–10 non-multinucleated blastomeres and <20% fragmentation were classified as high-quality embryos (Vergouw et al., 2008). Pregnancy was diagnosed by a positive blood test for hCG at 14 days after ET. A clinical pregnancy was confirmed by observation on transvaginal scanning of a gestational sac with fetal heart beat ~4 weeks after the positive pregnancy test. The implantation rate was defined as the number of gestational sacs visible on ultrasound divided by the total number of replaced embryos, and expressed as a percentage. All the pregnancies were followed up to the time of delivery.

Statistical analysis

The analyses were performed blind. Statistical data were shown as the mean ± SE. P-value and statistical significance were evaluated by one-way ANOVA. Survival, fertilization and cleavage rates, the percentage of high-quality embryos and implantation and pregnancy rates were evaluated with χ² test. Significance was defined as P < 0.05.

Results

The morphology of human oocytes with corona radiata before and after vitrification

As Fig. 1 shows, the macroscopic structures of human oocytes with a corona radiata were not significantly altered after vitrification and warming.

Zona pellucida solubility (hardening)

As expected, the mean time required for zona pellucida digestion in the positive control group (polyspermic fertilized oocytes) was increased significantly (P < 0.001) compared with the control group of fresh oocytes. The results presented in Table I and Fig. 2 show that the vitrification-warming process does not affect the solubility of the zona pellucida, whether or not the oocytes have an intact corona radiata (P > 0.05).

Oocyte ultrastructure

Both the control and vitrified-warmed oocytes had regular oolemma with numerous microvillus formations (Fig. 3a–c). The area was rich in cortical granules aligned peripherally in a continuous sub-oolemma array. The mean numbers ± SE of cortical granule per 10 μm of the oocyte linear surface profile.

Table I Dissolution time of the zona pellucida of human oocytes.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dissolution time (s)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>868.00 ± 11.76</td>
</tr>
<tr>
<td>Polyspermically fertilized oocytes</td>
<td>1083.17 ± 31.55***</td>
</tr>
<tr>
<td>Vitrified-warmed oocytes with an intact corona radiata</td>
<td>870.67 ± 12.15</td>
</tr>
<tr>
<td>Vitrified-warmed oocytes without a corona radiate</td>
<td>868.50 ± 15.35</td>
</tr>
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The presented data are the mean ± SE (n = 6). ***P < 0.001 versus control group.
The survival, fertilization and developmental competence of the vitrified-warmed oocytes

Vitrified-warmed oocytes, with or without the corona radiata, had the same survival rates (98%, Table II). These oocytes were subsequently inseminated via conventional IVF, and 77% (n = 37) of the vitrified-warmed oocytes with an intact corona radiata were normally fertilized, whereas only 18.8% (n = 9) of the completely denuded vitrified-warmed oocytes were normally fertilized (P < 0.001). Also the fresh oocytes without a corona radiata showed a low fertilization rate (12/53, 22.6%), while the fresh oocytes with an intact corona radiata had a normal fertilization rate (40/54, 74.1%; P < 0.001).

All normally fertilized oocytes cleaved (100% cleavage rate) and two embryos were transferred to each patient.

In the group with vitrified-warmed oocytes with an intact corona radiata, four patients had a positive test of which pregnancies three continued. The implantation rate per embryo transfer was 31.5% (5/16) and three babies were born.

Figure 2 Representative dissolution process of oocytes. The figure shows the solubility of zona pellucida in the control group (a–c), in vitrified-warmed oocytes without an intact corona radiata group (d–f), in vitrified-warmed oocytes with a corona radiata (g–i) and in polyspermic fertilized oocytes (j–m).

In the group with fresh oocytes with an intact corona radiata, four patients conceived (50%) and one case spontaneous abortion. The implantation rate per embryo transfer was 37.5% (6/16) and three babies were born.

There were no differences seen between fresh oocytes with an intact corona radiata and vitrified-warmed oocytes with an intact corona radiata, with respect to fertilization, cleavage, implantation and baby take home rates.

The absence of a second polar body 6 h after insemination indicates that the oocyte is not fertilized. Early rescue ICSI was done to prevent poor fertilization, 6–8 h after insemination when the fertilization rate was <30%. Most of the early rescue ICSI was done in fresh and vitrified-warmed oocytes without a corona radiata. No differences were seen in fertilization and cleavage rates or development of high-quality embryos between fresh and vitrified-warmed oocytes with or without a corona radiata, after rescue ICSI (data not shown).

The vitrified-warmed oocytes were from women whose average age was 29.3 ± 3.24 years. The fresh oocytes were from women
whose average age was 28.6 ± 3.12 years. There was no significant difference in ages between the groups ($P > 0.05$).

**Discussion**

The objective of this study was to evaluate the effects of the corona radiata on human oocytes during vitrification. The major findings were that no zona hardening occurred in oocytes with or without an intact corona radiata after vitrification and there were differences between these groups in the ultrastructure of the oocytes. However, the fertilizing capability of vitrified-warmed oocytes with an intact corona radiata was better than that of vitrified-warmed oocytes without the corona radiata upon insemination in conventional IVF. There were however no differences between vitrified-warmed oocytes with or without the corona radiata in the survival and cleavage rates, and the development of high-quality embryos and the clinical outcomes. The low fertilization rates of vitrified-warmed oocytes were only observed when the corona radiata had been removed.

It has been suggested that freezing procedures may induce rupture and release of the content of the cortical granules, resulting in a hardening of the zona which impairs or prevents the sperm from fertilizing the oocytes during conventional insemination (Vincent et al., 1990;
Reducing the fertilization rate in IVF (Tanghe et al., 2003). This finding is consistent with reports showing that the removal of all cumulus, with impure hyaluronidase, shortly before insemination is necessary for obtaining normal fertilization chances in conventional IVF, while those with an intact corona radiata showed normal fertilizing capabilities. The result indicated that an intact corona radiata is the best choice for fertilization in this case.

Our study showed the oocytes without the corona radiata, both in fresh and vitrified warmed, had a lower fertilizing capability with an intact corona radiata, normal fertilization rates were obtained. The difference in results between different studies is most likely to depend upon the freezing method, slow freezing versus vitrification. It is well-known that vitrification of oocytes significantly improves the outcome in comparison to that of slow freezing of human oocyte (Boldt, 2011). It has also been suggested that slow freezing induces zona hardening and re-arrangements and function of the cortical granules, indicating that ICSI would be the best choice for fertilization in this case.

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The present study indicates that human oocytes with an intact corona radiata do not develop hardening of the zona pellucida after vitrification with the CryoTop method and that poor penetration of the zona pellucida after vitrification has probably been due to the removal of the cumulus cells.

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**Authors’ roles**

X.-H.T. and L.-M.W. collected and processed the cumulus complexes, carried out experimental work, processed the data and wrote the manuscript. R.-T.J. collected the cumulus complexes. L.-H.L. and H.-B.L. selected suitable patients, retrieved oocytes and transferred embryos. Y.-S.L. designed the study and edited the manuscript.

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**Conflict of interest**

None declared.
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