A trial to restore defective human sperm centrosomal function

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BACKGROUND: In human fertilization, sperm centrosome function is essential for male and female pronuclear movement and fusion. In this study, we investigated the possibility of restoring human sperm centrosomal function in sperm exhibiting abnormalities in microtubule organization. METHODS: Semen was obtained from both a fertile donor and a patient with dysplasia of the fibrous sheath (DFS). Following heterologous ICSI using human sperm, we examined microtubules and chromatin configuration in bovine oocytes. Sperm were treated with dithiothreitol (DTT) prior to ICSI, while the oocytes were treated with the cytoskeletal stabilizer paclitaxel after ICSI. RESULTS: The combination of DTT and paclitaxel treatment induced microtubule organization in dead sperm from the fertile donor following heterologous ICSI. This treatment, however, was not effective for DFS sperm. In addition, expression of centrin, a protein functioning within the sperm centrosome, was reduced in DFS sperm from that of the normal levels observed in fertile donor sperm. CONCLUSION: These results indicate that sperm centrosomal function could be induced by the treatment of sperm with DTT before ICSI and of oocytes with paclitaxel after ICSI. DFS sperm are likely to exhibit such severe dysfunction of sperm centrosome that cannot be compensated for by this treatment; therefore, this method may be a practical way to discern the degree of sperm centrosomal dysfunction.

Key words: cytoskeleton/drug/fertilization/infertility/sperm centrosome

Introduction

ICSI is an innovative treatment for male infertility (Palermo et al., 1992), though a significant number of clinical cases of fertilization failure remain, even after ICSI (Rawe et al., 2003). Although only limited information is available on the molecular and cellular mechanisms of fertilization following the entry of sperm into the oocyte cytoplasm, sperm centrosomal function is critical in human fertilization. After sperm entry into the oocyte cytoplasm, the sperm centrosome acts as the microtubule organizing centre (MTOC) to organize radiating microtubules called the ‘sperm aster’. This organization of microtubules from the sperm centrosome is essential for the movement and fusion of male and female pronuclei (Schatten, 1994; Simerly et al., 1995). Therefore, proper function of the human sperm centrosome is thought to be essential for human fertility, although direct assessment of human sperm centrosomal function is difficult. Recently, a number of novel methods have been reported to examine sperm centrosomal function using the heterologous ICSI system, in which human sperm was microinjected into either a rabbit (Terada et al., 2000, 2004) or bovine (Nakamura et al., 2001, 2002) oocyte. The microtubule organization in these systems derives from the paternal centrosome during fertilization; these microtubule organizations are similar to those functioning in human fertilization. In rabbit oocytes, human sperm aster formation rate correlated with cleavage rates, but did not reflect the rate of pronuclear formation in clinical IVF (Terada et al., 2004). These studies touch on the intimate relationship between infertility and human sperm centrosomal dysfunction.

Dysplasia of the fibrous sheath (DFS), a rare form of teratozoospermia, results in infertility. DFS sperm, which are immotile due to deformities from midpiece to tail (Chemes et al., 1987, 1998) also exhibit sperm centrosomal dysfunction; both of these abnormalities may be causes of infertility (Rawe et al., 2002). Failure of either fertilization or embryo...
development continued to occur in several patients, even after performing ICSI (Chemes and Rawe, 2003). We believe that artificial methods are available to restore the sperm centrosomal dysfunction of DFS sperm.

In this study, we treated human sperm that demonstrated deficiencies in centrosomal function with two drugs. Human sperm have disulphide bonds within the head and pericentriolar regions (Seligman et al., 1994; Simerly et al., 1999; Tateno and Kamiguchi, 1999). Dithiotreitol (DTT), which induces the reduction of disulphide bonds, was determined to be effective for unravelling the sperm centrosome. Paclitaxel (Taxol®), which acts as a cytoskeleton stabilizer, is frequently used for studying cytoskeletal dynamics (Hewitson et al., 1997; Mailhes et al., 1999). Taxol enhances microtubule polymerization within the oocyte cytoplasm and may restore the recondite function of impaired human sperm centrosomes. Centrin, a ubiquitous, calcium-sensitive, bipolarly contributed centrosomal component, severs axonemal microtubules from their associated basal bodies, potentially functioning in centrosome duplication. Centrin abnormalities may lead to fertilization failure or incomplete embryonic development (Salisbury, 1995; Schiebel and Bornens, 1995; Levy et al., 1996).

In this study, we attempted to restore the sperm centrosome function of aster formation, which does not occur naturally in patients with DFS. We also examined the differences in centrin expression between the sperm from a fertile donor and a patient with DFS.

Materials and methods
All procedures were performed with the approval of the internal review board of the Tohoku University School of Medicine.

Sperm samples
DFS sperm were collected from a 36 year old man, complaining of primary infertility, the patient whose sperm morphology and clinical progress were reported previously (Rawe et al., 2002). Normal fertile sperm were collected from a 37 year old healthy man with children.

Heterologous ICSI with human sperm into bovine oocyte
Heterologous ICSI was performed by injecting human sperm into bovine oocytes, as previously described (Nakamura et al., 2001). Briefly, bovine oocytes were recovered from small follicles and allowed to mature for 22–24 h. Cyopreserved sperm from a patient with DFS sperm were thawed in a water bath at 37°C. Human sperm from a fertile donor were processed in a similar manner for use as normal controls. Human sperm centrosomal function was examined in motile and dead sperm from the fertile donor and DFS sperm. Sperm viability assessment in the fertile donor was made using eosin-Y staining (World Health Organization, 1999). Sperm were immobilized by a brief piezo-pulse. Then, a Piezo-micromani- pulator (MB-U; PRIM TECH, Japan) was used to inject the sperm into bovine metaphase II (MII) oocytes with the cumulus cells removed. The injected oocytes were then cultured for 6 h in tissue culture medium TCM-199 supplemented with 10% fetal calf serum in 5% CO2 at 38.5°C. All of the oocytes injected with human sperm were treated with 5% ethanol in TCM-199 at 4 h post-ICSI for 5 min to artificially induce activation (Horiiuchi et al., 2002).

**DTT treatment of human sperm and taxol treatment of bovine oocytes following ICSI**
Both fertile donor sperm and DFS sperm were treated for 1 h with 5 mmol/l DTT (Sigma Chemical Co., USA) in modified HTF medium (Irvine Scientific Co., USA). After washing in modified HTF supplemented with 10% serum substitute supplement (SSS) (Irvine Scientific), we injected either the dead sperm from a fertile donor or the DFS sperm into bovine oocytes. Six hours after the injection, a subset of the oocytes was treated with 2 μmol/l Taxol in TCM-199 for 1 h. All oocytes were fixed at 7 h post-ICSI. Examinations were performed at each time of fixation; the experiment was repeated three times.

**Immunocytochemical detection of microtubules and DNA**
Zonae pellucidae were removed using M2 culture medium (Sigma) supplemented with 0.75% protease (Actinase E; Kaken Chemical Co., Japan). After a 30 min recovery period, zona-free oocytes were extracted using buffer M [25% (v/v) glycerol, 50 mmol/l KCI, 0.5 mmol/l MgCl2, 0.1 mmol/l EDTA, 1 mmol/l EGTA, 50 mmol/l imidazole hydrochloride, and 1 mmol/l 2-mercaptoethanol, pH 6.8] containing 5% (v/v) methanol and 1% (v/v) Triton X-100 detergent for 15 min. After fixation in cold methanol for 10 min (Simerly and Schatten, 1993), microtubules were labelled with a mixture of monoclonal antibody specific for β-tubulin (clone 2-283; Sigma) and acetylated α-tubulin (clone 6-11-B1; Sigma). Primary antibodies were detected using a fluorescein-conjugated goat anti-mouse IgG (Zymed, USA). DNA was detected by labelling with 10 mg/ml Hoechst 33342. After mounting in anti-fade medium ( Vectashield; Vector Labs, USA), oocytes were examined using an epifluorescence microscope (DMRXA/IC; Leica, Germany). Images, acquired using Leica Q550FW (Leica, UK), were digitally recorded using Adobe Photoshop software (Adobe Systems Inc., USA). These data were used to compare the rate of sperm aster formation for each group of injected oocytes using the χ²-test. *P < 0.01 were considered to be statistically significant.*

**Immunocytochemical detection of centrin expression in human sperm**
Fertile donor and DFS sperm samples, mounted on coverslips, were fixed using 2% formaldehyde in modified HTF at 37°C. Centrin was detected with a polyclonal antibody (anti-centrin-1; Sigma). Primary antibody was detected with an affinity-purified goat anti-rabbit immunoglobulin (whole molecule) conjugated to tetra-methylrhodamine isothiocyanate (TRITC; Sigma). Microtubules and DNA were detected as described for the fixed bovine oocytes. Sperm samples on coverslips were mounted in anti-fade medium and were examined on an epifluorescence microscope. A total of 1000 sperm was analysed in each sample.

**Results**
After thawing, the proportions of living sperm remaining in samples were 56% from a fertile donor and 36% from the DFS patient, as detected by eosin-Y staining. We examined the organization of microtubules and the chromatin configuration in bovine oocytes after ICSI with human sperm at 7 h post ICSI (Figure 1). We also investigated the formation of sperm aster, a radial microtubule array extending from the sperm centrosome, after the injection of human sperm into bovine oocytes (Table I). Sperm aster formation was observed at a high rate (38/46: 82.6%) in oocytes injected
with motile sperm from a fertile donor (Figure 1A), but was not observed in most of the oocytes injected with DFS sperm (4/42: 9.5%) (Figure 1B). Sperm aster formation was not observed in oocytes injected with dead sperm (0/37: 0.0%). Thus, dead sperm are unable to activate bovine oocytes; the female pronucleus was not observed upon injection of dead sperm (data not shown). A subsequent activation of these oocytes with ethanol, however, could induce the formation of

Figure 1. Microtubule (green) and chromatin (blue) configuration in bovine oocytes following ICSI with human sperm samples (A–G). All bovine oocytes were artificially activated with 7% ethanol in TCM-199 4 h after ICSI and fixed 7 h after ICSI. MPN = male pronucleus; FPN = female pronucleus. (A) Bovine oocytes injected with motile sperm from a fertile donor without dithiothreitol (DTT) treatment and/or Taxol treatment exhibited a radial array of microtubules, ‘the sperm aster’, organized from the sperm centrosome. Microtubule organization was not observed around the female pronucleus. The formation of both male and female pronuclei was observed in the oocyte. (B) The majority of the bovine oocytes injected with DFS (dysplasia of the fibrous sheath) sperm did not exhibit any sperm aster formation or the organization of microtubules within the oocyte cytoplasm in the absence of DTT and/or Taxol treatment. The female pronucleus and decondensed sperm nucleus, however, were readily observed. (C) Oocytes injected with dead sperm from fertile donors (dead sperm) did not form a sperm aster, lacking any microtubule organization in the absence of DTT and/or Taxol treatment. The injected sperm remained intact; only female pronucleus was observed in the bovine oocyte. (D) Bovine oocytes injected with DTT-treated dead sperm exhibited male pronuclear formation; however, no microtubule organization from the sperm centrosome was observed in the oocyte. (E) After ICSI with dead sperm, Taxol treatment could induce microtubule organization within the oocyte cytoplasm; however, microtubule organization from the sperm centrosome was not observed; the sperm head remained to be condensed. (F) Following injection with DTT-treated dead sperm, bovine oocytes treated with Taxol displayed sperm aster formation with the creation of male and female pronuclei. Microtubule formation was also observed around the female pronucleus. (G) Bovine oocytes injected with DTT-treated DFS sperm that were given Taxol after ICSI. No sperm aster formation was observed; only a minor amount of microtubule organization originated from the oocyte cytoplasm; both male and female pronuclei formation was observed. A small number of bovine oocytes exhibited both sperm aster formation and microtubule organization around female pronucleus, in a manner similar to that seen in F. Bar = 25 μm. (H) Centrin expression was observed at the sperm centrosome in sperm from fertile donors. (I) DFS sperm only exhibited centrin expression in ~2% of the sperm samples. Centrin: red, arrow. Bar = 10 μm.
Table I. Human sperm aster formation in bovine eggs

<table>
<thead>
<tr>
<th>Sperm source</th>
<th>Treatment</th>
<th>Total</th>
<th>Sperm aster formation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>fertile donors sperm*</td>
<td>no treatments</td>
<td>46</td>
<td>38 (82.6)</td>
</tr>
<tr>
<td></td>
<td>DTT</td>
<td>43</td>
<td>5 (11.6)</td>
</tr>
<tr>
<td></td>
<td>Taxol</td>
<td>25</td>
<td>2 (8.0)</td>
</tr>
<tr>
<td></td>
<td>DTT + Taxol</td>
<td>57</td>
<td>40 (70.1)</td>
</tr>
<tr>
<td>dead sperm**</td>
<td>no treatments</td>
<td>37</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td></td>
<td>DTT</td>
<td>42</td>
<td>4 (9.5)</td>
</tr>
<tr>
<td></td>
<td>Taxol</td>
<td>39</td>
<td>3 (7.7)</td>
</tr>
<tr>
<td>DFS sperm***</td>
<td>no treatments</td>
<td>57</td>
<td>40 (70.1)</td>
</tr>
<tr>
<td></td>
<td>DTT</td>
<td>57</td>
<td>40 (70.1)</td>
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*motile sperm from fertile donors.
**dead sperm from fertile donors.
***sperm from dysplasia of the fibrous sheath patient

1996). In this study, male pronuclear formation and sperm centrosomal function in bovine oocytes were absent from dead human sperm. While sperm from fertile donors activated bovine MII oocytes at a high rate of ~80% without artificial activation (Nakamura et al., 2001), dead sperm could not activate bovine MII oocytes. Therefore, artificial activation of injected oocytes was achieved by ethanol treatment. However, artificial oocyte activation did not improve sperm centrosomal function. Normally, the reducing environment of the mammalian oocyte facilitates the breaking of disulphide bonds, allowing pronuclear decondensation and possibly centrosomal decondensation (Mellon and Rebhun, 1976; Oliver et al., 1976; Sanders and Salisbury, 1994). The DTT priming of dead sperm promoted the decondensation of the sperm nucleus, but was ineffective to restore the deficient sperm centrosomal function. Thus, we attempted to reinstate the centrosomal function of dead sperm using a cytoskeletal accelerator. Taxol treatment alone induced microtubule organization in the bovine oocyte cytoplasm; however, the organization of microtubules by the dead sperm centrosome was not observed. We then attempted the priming of sperm by DTT in conjunction with Taxol treatment of injected bovine oocytes. This treatment was effective to restore the centrosomal function of dead human sperm. We could observe the sperm aster, an astral microtubule array, at a significantly higher rate than that seen with the other treatments. We speculated that DTT induced the decondensation of the sperm nucleus and centrosome, accelerating the separation of the tail microtubule from the basal body after ICSI. Upon oocyte activation, the naked sperm centrosome is easily affected by Taxol for use as an accelerator of microtubule organization. Dead sperm may have some alterations in their centrosomes that make the release of their disulphide bonds difficult, thereby affecting centrosomal function. It appears likely, however, that these defects are not severe alterations of the sperm centrosome, because these structures could introduce sperm aster formation following treatment with DTT and Taxol. Interestingly, bovine oocytes treated with Taxol after ICSI exhibited astral microtubule organization within the oocyte cytoplasm. It is possible that bovine oocytes have a few MTOC in their cytoplasm that may function in parthenogenetic development (Navara et al., 1994). Such structures, however, are not observed in normal fertilization. Taxol may induce the microtubule organization from maternal MTOC, as observed in this study.

Discussion

In 1994, Schatten (1994) reasoned that calcium oscillations during oocyte activation cause the release of the tail microtubule from the basal body by centrin-induced cleavage. Subsequently, the sperm centrosome is phosphorylated, while the disulphide bonds are simultaneously reduced. The sperm centrosome can bind additional γ-tubulin derived from the oocyte cytoplasm, forming a centriole. The resulting halo of γ-tubulin nucleates microtubules, which assemble into the sperm aster (Schatten, 1994). Simerly et al. (1999) reported the phosphorylation of human sperm centrosome following DTT priming in a cell-free extract system.

In cases of human necrozoosperma, oocytes injected with dead sperm exhibit fertilization failure (Tournaye et al., 1996). In this study, male pronuclear formation and sperm centrosomal function in bovine oocytes were absent from dead human sperm. While sperm from fertile donors activated bovine MII oocytes at a high rate of ~80% without artificial activation (Nakamura et al., 2001), dead sperm could not activate bovine MII oocytes. Therefore, artificial activation of injected oocytes was achieved by ethanol treatment. However, artificial oocyte activation did not improve sperm centrosomal function. Normally, the reducing environment of the mammalian oocyte facilitates the breaking of disulphide bonds, allowing pronuclear decondensation and possibly centrosomal decondensation (Mellon and Rebhun, 1976; Oliver et al., 1976; Sanders and Salisbury, 1994). The DTT priming of dead sperm promoted the decondensation of the sperm nucleus, but was ineffective to restore the deficient sperm centrosomal function. Thus, we attempted to reinstate the centrosomal function of dead sperm using a cytoskeletal accelerator. Taxol treatment alone induced microtubule organization in the bovine oocyte cytoplasm; however, the organization of microtubules by the dead sperm centrosome was not observed. We then attempted the priming of sperm by DTT in conjunction with Taxol treatment of injected bovine oocytes. This treatment was effective to restore the centrosomal function of dead human sperm. We could observe the sperm aster, an astral microtubule array, at a significantly higher rate than that seen with the other treatments. We speculated that DTT induced the decondensation of the sperm nucleus and centrosome, accelerating the separation of the tail microtubule from the basal body after ICSI. Upon oocyte activation, the naked sperm centrosome is easily affected by Taxol for use as an accelerator of microtubule organization. Dead sperm may have some alterations in their centrosomes that make the release of their disulphide bonds difficult, thereby affecting centrosomal function. It appears likely, however, that these defects are not severe alterations of the sperm centrosome, because these structures could introduce sperm aster formation following treatment with DTT and Taxol. Interestingly, bovine oocytes treated with Taxol after ICSI exhibited astral microtubule organization within the oocyte cytoplasm. It is possible that bovine oocytes have a few MTOC in their cytoplasm that may function in parthenogenetic development (Navara et al., 1994). Such structures, however, are not observed in normal fertilization. Taxol may induce the microtubule organization from maternal MTOC, as observed in this study.
In conclusion, we examined human sperm centrosomal function using the heterologous ICSI system in an attempt to restore deficient sperm centrosomal function by treatment with DTT and Taxol. Although untreated dead sperm exhibited centrosomal dysfunction, they responded well to DTT treatment before ICSI in combination with the Taxol treatment of oocytes after ICSI. Sperm from a patient with DFS, a teratozoospermia resulting in infertility, displayed centrosomal dysfunction and abnormal centrin expression. DTT and Taxol treatments were not effective at restoring DFS sperm centrosomal function, indicating that the deficiency of DFS sperm in centrosomal function may be too severe to be saved by this treatment method. The heterologous ICSI system of sperm priming by DTT followed by oocyte Taxol treatment after sperm injection, however, may be a new method to assess sperm centrosomal function. Needless to say, this assessment system cannot be a tool for clinical rescue of sperm centrosomal dysfunction, because we are presently unable to assess the safety of this cytoskeletal manipulation.

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References


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