Background: Centrosomal function and oocyte activation ability of human sperm from a case of globozoospermia was assayed by heterologous ICSI into bovine oocytes. METHODS: Microtubules and chromatin configuration in bovine oocytes were examined by immunofluorescence after heterologous ICSI with human sperm from two fertile donors and from a globozoospermic man. RESULTS: The microtubule array from the sperm centrosome, the ‘sperm aster’ and the male pronucleus were observed in bovine oocytes, following ICSI with round-headed sperm from a globozoospermic man. The rate of sperm aster formation and the rate of male pronuclear formation in the bovine oocytes injected with fertile donor sperm were 57.9 and 92.5% respectively; the respective values for oocytes injected with round-headed sperm without artificial oocyte activation were 15.8 and 31.0%. Ethanol activation after ICSI improved male pronuclear formation (84.9%) but not sperm aster formation rate (32.3%) of the globozoospermic patient. CONCLUSIONS: These data indicated that sperm from this patient with globozoospermia have centrosomal dysfunction and low ability for oocyte activation compared with fertile donor sperm. The centrosomal dysfunction may be one of the reasons for infertility in this patient.

Key words: centrosome/fertilization/globozoospermia/oocyte activation/sperm

Introduction

The human sperm contributes the male genome and the sperm centrosome to the oocyte at fertilization. The centrosome is known as the microtubule-organizing centre, one of the cytoskeletal components. After the sperm enters the cytoplasm of an oocyte, a radial microtubule array, the so called ‘sperm aster’, is organized from the sperm centrosome. The sperm aster formation is essential for pronuclear movement towards the union of male and female genomes (Schatten, 1994; Simerly et al., 1995). Aberrant microtubule organization in ‘fertilization failure oocytes’ in human IVF suggested that centrosomal dysfunction may be a cause of fertilization arrest (Asch et al., 1995). Furthermore, it has been reported that the function of the sperm centrosome varies among bulls during IVF, and this variation affects male fertility (Navara et al., 1996). These reports suggested that the human sperm centrosome dysfunction was related to infertility. However, there has been little information on the relationship between human fertility and human sperm centrosomal function. If sperm centrosomal dysfunction causes fertilization failure, there have been no treatments for such infertile patients. We recently reported that the heterologous ICSI of human sperm into a bovine oocyte can be a tool to assess the human sperm centrosomal function (Nakamura et al., 2001). Globozoospermia is a special feature of teratozoospermia, which is characterized as round head, lack of acrosome and acrosomal enzymes, and disorganized mid-piece (Pedersen and Rebbe, 1974; Singh, 1992). The round-headed sperm cannot penetrate the zona pellucida of an oocyte, resulting in unsuccessful fertilization and leading to infertility (Weissenberg et al., 1983; Von Bernhardi et al., 1990). Some successful pregnancies were reported after ICSI in globozoospermia (Trokoudes et al., 1995; Rybouchkin et al., 1997; Stone et al., 2000), but the fertilization rates were poor (Battaglia et al., 1997; Edirisinghe et al., 1998). Poor fertilization rate has been attributed to the ability of the round-headed sperm to activate an oocyte. One team reported that sperm from globozoospermic men had poor ability for oocyte activation using a heterologous ICSI system in mouse oocytes (Rybouchkin et al., 1996). However, the sperm centrosomal function in cases of teratozoospermia, is unknown until now. In this study, we assessed the sperm centrosomal function and the oocyte activation ability of round-headed sperm from a globozoospermic man by heterologous ICSI using bovine oocytes. Furthermore, we investigated

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whether the sperm centrosomal function was associated with fertilization failure in this case.

**Materials and methods**

All procedures were performed under the approval of the internal review board in Tohoku University School of Medicine.

**In-vitro maturation of bovine oocytes**

Bovine ovaries were obtained at a local slaughterhouse and oocytes were recovered by aspiration from 2–8 mm follicles. Oocytes were matured for 22–24 h in HEPES-buffered TCM-199 supplemented with 10% (v/v) fetal calf serum (FCS), 0.12 IU/ml pFSH (Antrin; Denka pharmaceutical, Kanagawa, Japan) and 50 ng/ml recombinant human epidermal growth factor (Toyobo, Tokyo, Japan) at 38.5°C, 5% CO₂ in air. Cumulus cells were removed by a brief incubation in 2 mg/ml hyaluronidase (Sigma, St Louis, MO, USA) in M2 culture medium (Irvine Scientific Co., Santa Ana, CA, USA), and thawed in a water bath at 37°C. Sperm were then washed with modified human tubal fluid (hTF) medium (Irvine Scientific) supplemented with 10% (v/v) fetal bovine serum, 0.12 IU/ml pFSH, 0.12 IU/ml hCG, and 1 mmol/l EDTA, and 1 mmol/l 2-mercaptoethanol, pH 6.8 containing 5% (v/v) methanol and 1% (v/v) Triton X-100 detergent and fixed in cold methanol for 10 min according to a published method (Simerly and Schatten, 1993). Fixed oocytes were then permeabilized overnight in 0.1 mol/l phosphate-buffered saline containing 0.1% (v/v) Triton X-100. Microtubules were labelled with a mixture of monoclonal antibody against β-tubulin (clone 2-28-33; diluted 1:100; Sigma) and acetylated α-tubulin (clone 6-11-B1; diluted 1:100; Sigma). The primary antibodies were detected by fluorescein-conjugated goat anti-mouse immunoglobulin G (IgG, diluted 1:40; Zymed, San Francisco, CA, USA). DNA was detected after labelling with 10 mg/ml Hoechst 33342. Sperm samples from fertile donors and a globozoospermic man were fixed in modified HTF medium with 2% (v/v) formaldehyde at 37°C for 40 min. Fixed sperm samples were labelled with a mixture of monoclonal antibody against α-tubulin (clone 2-28-33, diluted 1:100; Sigma) and acetylated α-tubulin (clone 6-11-B1, diluted 1:100; Sigma). The primary antibodies were detected by fluorescein-conjugated goat anti-mouse immunoglobulin G (IgG, diluted 1:40; Zymed). Sperm nucleous was detected after labelling with 10 mg/ml Hoechst 33342. Coverslips were mounted in anti-fade medium ( Vectashield; Vector Labs, Burlingame, CA, USA) and were examined with a Nikon Optiphot-2 epifluorescence-equipped microscope. The images were acquired using a Hamamatsu C-4742 Digital Camera (Hamamatsu Photonics K.K., Hamamatsu, Japan), which was operated with Fluoro-Pro 3.0 software (Media Cybernetics, MD, USA). In addition, the images were recorded digitally, archived on magnetic optical disks and processed using Adobe Photoshop software (Adobe Systems Inc., Mountain View, CA, USA). Data on sperm aster formation rate at 6 h post ICSI, and on male pronuclear formation rate at 12 h post ICSI, were compared for oocytes with and without ethanol activation by χ²-test. P < 0.05 was considered to indicate statistical significance.

**Results**

Round-headed sperm from a globozoospermic man in comparison with a fertile donor are shown in Figure 1. Sperm from a globozoospermic man contained a round head, and the sperm tail was shorter than sperm from fertile men. Microtubule organization and chromatin configuration in bovine oocytes after ICSI with sperm from globozoospermia are shown in Figure 2. The unfertilized bovine oocyte had an anastral barrel-shaped meiotic spindle (Figure 2A, microtubules; green, DNA; zona-free oocytes were extracted for 15 min by buffer M [25% (v/v) glycerol, 50 mmol/l KCl, 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 and fixed in cold methanol for 10 min according to a published method (Simerly and Schatten, 1993). Fixed oocytes were then permeabilized overnight in 0.1 mol/l phosphate-buffered saline containing 0.1% (v/v) Triton X-100. Microtubules were labelled with a mixture of monoclonal antibody against β-tubulin (clone 2-28-33; diluted 1:100; Sigma) and acetylated α-tubulin (clone 6-11-B1, diluted 1:100; Sigma). The primary antibodies were detected by fluorescein-conjugated goat anti-mouse immunoglobulin G (IgG, diluted 1:40; Zymed). Sperm nucleous was detected after labelling with 10 mg/ml Hoechst 33342. Coverslips were mounted in anti-fade medium ( Vectashield; Vector Labs, Burlingame, CA, USA) and were examined with a Nikon Optiphot-2 epifluorescence-equipped microscope. The images were acquired using a Hamamatsu C-4742 Digital Camera (Hamamatsu Photonics K.K., Hamamatsu, Japan), which was operated with Fluoro-Pro 3.0 software (Media Cybernetics, MD, USA). In addition, the images were recorded digitally, archived on magnetic optical disks and processed using Adobe Photoshop software (Adobe Systems Inc., Mountain View, CA, USA). Data on sperm aster formation rate at 6 h post ICSI, and on male pronuclear formation rate at 12 h post ICSI, were compared for oocytes with and without ethanol activation by χ²-test. P < 0.05 was considered to indicate statistical significance.
Figure 2. Microtubule (green) and chromatin (blue) configurations in bovine oocytes after ICSI with human round-headed sperm by piezo-driven pipette. (A) The unfertilized bovine oocyte has microtubules only in the second meiotic spindle (arrowhead). (B) At 6 h post ICSI, male and female pronucleus can be observed. Furthermore, radial array of microtubules (the sperm aster; arrow) has been organized from the sperm centrosome. Microtubules are not organized around the female pronucleus (arrowhead). (C) At 12 h post ICSI, male and female pronuclei are adjacent and they are surrounded by a microtubule array. Mpn = male pronucleus; Fpn = female pronucleus. Bar = 25 µm.

Figure 3. Premature condensation of chromosomes of human sperm in bovine oocyte. Microtubule (green) organization and chromatin (blue) configuration are associated with defects in chromatin decondensation (arrow: sperm nucleus). The oocyte nucleus remains arrested in metaphase II (arrowhead). Bar = 10 µm.

Table I. Human sperm aster formation in bovine oocytes at 6 h post ICSI

<table>
<thead>
<tr>
<th>Sperm source</th>
<th>Oocyte activation</th>
<th>No. of oocytes (%)</th>
<th>Sperm aster formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient</td>
<td>–</td>
<td>19</td>
<td>3 (15.8)a</td>
</tr>
<tr>
<td>Patient</td>
<td>+</td>
<td>34</td>
<td>11 (32.3)b</td>
</tr>
<tr>
<td>Fertile donor</td>
<td>–</td>
<td>38</td>
<td>22 (57.9)c</td>
</tr>
</tbody>
</table>

*a,bP < 0.05; a,cP < 0.05.

Table II. Human male pronuclear configuration in bovine oocytes at 12 h post ICSI

<table>
<thead>
<tr>
<th>Sperm source</th>
<th>Oocyte activation</th>
<th>No. of oocytes (%)</th>
<th>Intact</th>
<th>PCC</th>
<th>Male pronucleus</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient</td>
<td>–</td>
<td>6 (14.3)</td>
<td>23 (54.7)b</td>
<td>13 (31.0)a</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>Patient</td>
<td>+</td>
<td>5 (9.4)</td>
<td>3 (5.7)b</td>
<td>45 (84.9)b</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>Fertile donor</td>
<td>–</td>
<td>1 (2.5)</td>
<td>2 (5.0)b</td>
<td>37 (92.5)b</td>
<td>40</td>
<td></td>
</tr>
</tbody>
</table>

*a,bP < 0.05.

At 6 h post ICSI, the sperm aster, a radial microtubule array extending from the sperm centrosome, was organized (Figure 2B). At 12 h post ICSI, male and female pronuclei were observed in bovine oocytes. Microtubules elongated throughout the cytoplasm of the bovine oocyte, and both the male and female pronuclei, were surrounded by a microtubule array (Figure 2C). The sperm aster configuration rates at 6 h post ICSI are shown in Table I, and chromatin configuration at 12 h post ICSI are presented in Table II. The sperm aster formation rate at 6 h post ICSI in oocytes injected with fertile donor sperm without ethanol activation was 57.9% (22/38 oocytes); the male pronuclear formation rate at 12 h post ICSI was 92.5% (37/40 oocytes). The sperm aster formation rate in oocytes injected with round-headed sperm without ethanol activation was only 15.8% (3/19 oocytes) and the male pronuclear formation rate was 31.0% (13/42 oocytes). Prema-
tecture condensation of chromosomes (PCC) (Figure 3) was observed in 54.7% (23/42 oocytes) of these oocytes. The sperm aster formation rate and the male pronuclear formation rate of oocytes injected with round-headed sperm without artificial activation were significant lower than these rates of oocytes injected with fertile donor sperm. The sperm aster formation rate of oocytes injected with round-headed sperm with ethanol activation was 32.3% (11/34 oocytes) and the male pronuclear formation rate was 84.9% (45/53 oocytes). Ethanol activation significantly improved the rate of male pronuclear formation in oocytes injected with round-headed sperm ($P < 0.001$), but the sperm aster formation rate was not significantly improved by ethanol activation.


discussion

Sperm centrosomal function plays an important role in the process of human fertilization after sperm entry, and sperm centrosomal dysfunction is a possible cause of infertility. Assessing the sperm centrosomal function may identify one cause of infertility. However, it has been difficult to assay the human sperm centrosomal function. A heterologous ICSI system using oocytes of a species with paternal centrosomal inheritance provides a tool to assess sperm centrosomal function. Mouse and hamster are common experimental animals. However, the centrosome is maternally derived in mouse fertilization (Schatten et al., 1985). Furthermore, a sperm aster was absent when human sperm were introduced into a hamster oocyte (Hewitston et al., 1997). These reports suggested that the rodent oocyte could not be a tool for assessment of human sperm centrosomal function. In bovine fertilization, the centrosome is derived from the sperm and the centrosomal inheritance pattern in fertilization is similar to humans (Navara et al., 1996). We reported the observation of sperm aster formation and male pronuclear formation in bovine oocytes using ICSI with human sperm obtained from fertile donors (Nakamura et al., 2001). Therefore, this heterologous ICSI system using bovine oocytes makes it possible to assay human sperm centrosomal function and oocyte activation ability of human sperm. In this investigation, we examined the ability of oocyte activation of sperm and the sperm centrosomal function of a globozoospermic man, a feature of teratozoospermia that commonly causes fertilization failure. In bovine oocytes injected with human sperm from a patient with globozoospermia, we could observe both the male and the female pronuclear formation. However, the rate of male pronuclear formation in the oocytes without ethanol activation was significantly lower (31.0 versus 92.5%, $\chi^2$-test: $P < 0.001$) and the PCC rate was significantly higher (54.7 versus 5.0%, $P < 0.001$), compared with those of fertile donor sperm. It is difficult to activate bovine oocytes by ICSI (Goto et al., 1990; Motoishi et al., 1996; Rho et al., 1998). Activation can be improved by using a piezo-driven pipette and ethanol after ICSI (Horiuchi and Numabe, 1999). We examined the effect of activation in the same way in this heterologous ICSI, using human sperm and bovine oocytes. Bovine oocytes were highly activated by injection of human fertile donor sperm alone using a piezo-driven pipette without ethanol activation. However, sperm from a man with globozoospermia have poor ability for oocyte activation compared with fertile donor sperm. One team reported that sperm from a patient with globozoospermia had low or absent oocyte-activating capacity after injection into mouse oocytes, and ethanol oocyte activation improved the oocyte activation rate (Ryubouchkin et al., 1996). Therefore, we examined the ethanol activation of bovine oocytes injected with sperm from a man with globozoospermia, and obtained oocyte activation similar to oocytes injected with fertile donor sperm. Artificial oocyte activation by ethanol improved the male pronuclear formation rate remarkably; however, the sperm aster formation rates in the ethanol activation group (32.3%) and the untreated oocytes injected with sperm from a globozoospermic patient (15.8%) were significantly lower than those of fertile donor sperm (57.9%). The sperm aster formation rate of round-headed sperm was not improved by ethanol activation. These results suggest that sperm from this patient, a globozoospermic man, had a dysfunctional centrosome.

In conclusion, we examined the sperm centrosomal function and the oocyte activation ability of human sperm from a case of globozoospermia using a heterologous ICSI system in bovine oocytes. This is a valuable method for assaying human centrosomal function and oocyte activation ability of human sperm at the same time. In this patient with globozoospermia, the function of the sperm centrosome was lower as compared with fertile donors and the centrosomal dysfunction might be one reason for infertility in this case.

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