Removal of Acrosomal Membrane from Sperm Head Improves Development of Rat Zygotes Derived from Intracytoplasmic Sperm Injection

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Abstract. When intracytoplasmic sperm injection (ICSI) is applied in the rat, sperm chromatin is introduced into the oocyte together with the acrosome, which does not enter the cytoplasm of the oocyte during normal fertilization, resulting in the rat giving birth to pups. Since successful ICSI was reported in rats, but with low efficiency, it has been observed that the acrosome of the sperm head seems to have detrimental effects on the embryonic development of ICSI oocytes. To improve ICSI in rats, the effects of removal of the acrosomal membrane from rat sperm on the development of ICSI oocytes were examined. While most control (non-treated) sperm had an intact acrosomal membrane, the Triton X-100 (TX)- and lysolecithin (LL)-treated groups showed high percentages of sperm with a removed acrosomal membrane. The timing of pronuclear formation in ICSI-oocytes using TX- or LL-treated sperm was significantly accelerated compared with that of the control sperm (P<0.05). However, neither TX nor LL treatment affected amounts of PLCζ in rat sperm. The rates of offspring derived from TX- (20.3 ± 4.4%) and LL-treated sperm (19.0 ± 2.8%) were also significantly higher than that of the control group (7.6 ± 2.3%; P<0.05). Our data clearly indicate that removal of acrosomal membranes from sperm by reagents is effective for generation of offspring via ICSI in rats.

Key words: Acrosomal membranes, Intracytoplasmic sperm injection, Rat

In rats, intracytoplasmic sperm injection (ICSI) is the only way to routinely obtain offspring routinely derived from oocytes fertilized ‘in vitro’ using fresh and cryopreserved sperm [1], although we have previously succeeded in generation of rat offspring using cryopreserved sperm via artificial insemination (‘in vivo’ fertilization) [2]. Because a reliable and reproducible protocol of in vitro fertilization (IVF) using cryopreserved sperm has not been widely applied for rats, although we have established an IVF protocol very recently [3]. Successful ICSI was first reported in mice [4] and has subsequently been applied to many mammalian species [5–10]. Moreover, ICSI has been used for efficient production of not only wild-type animals but also transgenic animals [11]. In the case of rats, the protocol of ICSI has been improved by using an injection pipette with a very small outer diameter (2–4 μm), resulting in successful generation of offspring derived from the ICSI oocytes [12–14]. However, the efficiency is still low even though improvements such as using a small injection pipette have been applied to rat ICSI. These results imply that further improvement is required.

Recently, Morozumi and Yanagimachi [15] reported that removal of both the plasma and acrosomal membranes from mouse spermatozoa before ICSI accelerates the onset of oocyte activation. They also suggested that removal of acrosomal membranes prior to ICSI is recommended for animals with large sperm acrosomes in view of the potential harmful effects of acrosomal enzymes on embryonic development. Moreover, it has been shown that injection of hamster sperm having a large acrosome has detrimental effects on oocytes [16]. These results suggest that the removal of acrosomal membrane is effective for ICSI even in rats because rat sperm also has a large head, probably including an abundantly large acrosome. However, such information is very limited in rat ICSI.

The objective of the present study was to improve the fertility of rat ICSI oocytes injected with sperm after removal of the acrosomal membranes by using two different plasma membrane-disrupting agents (Triton X-100 and lysolecithin). We also evaluated in vitro and in vivo development of ICSI oocytes using these pretreated sperm. Moreover, we investigated whether the plasma membrane-disrupting agents affected the amounts of rat PLCζ which is thought to be a strong candidate sperm-derived oocyte activating factor [17].

Materials and Methods

All chemicals and reagents were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA) unless otherwise stated. All procedures for handling and treatment of the animals were conducted according to the guidelines established by the Animal Research Committee of Azabu University.

Animals

The rats used in this study were Crlj:Wistar rats and were purchased from Charles River Laboratories Japan (Kanagawa, Japan). The rats were housed in an environmentally controlled room with a 12-h dark/12-h light cycle at a temperature of 23 ± 2 C and humidity of 55 ± 5% with free access to a laboratory diet and filtered water. Mature male rats (12–24 weeks old) and immature female rats (4–5 weeks old) were used in the present experiment.
weeks old) were used as sperm and oocyte donors, respectively. Mature female rats (10–14 weeks old) were also used as recipients of embryo transfer. To induce pseudopregnancies, the females were naturally mated with vasectomized male rats (15–30 weeks old).

**Media**

The base medium used for sperm collection and incubation was modified rat 1-cell embryo culture medium (mR1ECM) containing 110 mM NaCl, 3.2 mM KCl, 0.5 mM MgCl₂, 2.0 mM CaCl₂, 25.0 mM NaHCO₃, 7.5 mM D-glucose, 0.5 mM sodium pyruvate, 10.0 mM sodium lactate, 0.1 mM glutamine, 2% (v/v) minimal essential medium (MEM) essential amino acid solution (50×; Gibco BRL, Grand Island, NY, USA), 1% (v/v) MEM nonessential amino acid solution (100×; Gibco BRL) and 4 mg/ml of fatty acid free BSA [18]. The culture medium for in vitro development of the ICSI oocytes was mR1ECM-reduced NaCl (76.7 mM) supplemented with 1 mg/ml PVA instead of BSA [19]. For sperm cryopreservation, the freezing medium contained 23% (v/v) egg yolk, 8% (w/v) lactose monohydrate, antibiotics (1000 IU/ml penicillin G potassium, 100 IU/ml streptomycin sulphate) and 0.7% (v/v) Equex Stem (Nova Chemical Sales, Scituate, MA, USA) [2].

**Preparation of frozen/thawed sperm for intracytoplasmic sperm injection (ICSI)**

The sperm collection and freezing/thawing procedures were performed according the methods described in our previous report [2] with some modifications. In brief, epididymal sperm were counted by hemacytometer and then diluted to 5.0 × 10⁷ sperm/ml with modified Niwa and Sasaki Freezing medium (mNSF). Sperm samples were loaded into 0.25-ml plastic straws (Fujihira Industry, Tokyo, Japan) and placed into a programmable freezer (Fujihira Industry) at 23 C. The straws were cooled to 5.0 C for 40 min (0.5 C/min) and held for 5 min. After that, the straws were exposed to liquid nitrogen vapor for 10–15 min and then plunged into liquid nitrogen and stored for at least 1 week. The spermatozoa in straws were thawed in a 37 C water bath for 15 sec, and diluted in 1 ml of R1ECM containing 22 mM Hepes and 5 mM NaHCO₃ (Hepes-R1ECM). The spermatozoa were then separated into heads and tails by sonication treatment, as described previously [17].

**Morphological evaluation of acrosomal membranes in sperm**

Acrosomal membrane staining was performed according to the procedure described previously [20] with some modifications. In brief, the sperm samples treated with TX or LL were smeared onto microscope slides, air-dried, and fixed with absolute methanol. Fixed samples were treated with 100 μg/ml FITC-labeled peanut agglutinin (FITC-PNA) in PBS at 37 C for 30 min. After washing with PBS, the samples were stained with propidium iodide for 5 min and then evaluated using a fluorescence microscope (IX-71, Olympus, Tokyo, Japan). The sperm were classified into two groups: sperm with intense fluorescence of the acrosomal cap, indicating an intact outer acrosomal membrane (Intact, Fig. 1A(a)), and sperm with no fluorescence, indicating partial or complete loss of the outer acrosomal membrane (Removed, Fig. 1A(b)).

**Sperm immunofluorescence**

After treatment with TX or LL, the sperm samples were fixed in 3.7% paraformaldehyde for 30 min at 4 C followed by permeabilization with 0.1% (v/v) Triton X-100-DPBS for 10 min at room temperature. The sperm suspension was then spotted as 50-μl drops onto glass slides and allowed to attach to the slides for 20 min at 37 C. The sperm samples were incubated in 5% normal goat serum (NGS; Cedarlane Laboratories Limited, Hornby, ON, Canada) in DPBS for 3 h at 4 C and then incubated overnight at 4 C with anti-mouse PLCζ (mPLCζ) (1:100) in 5% NGS. Anti mPLCζ antibody against a 19-mer sequence (GYYRVPFLFSKSGANLEPSS) on the C-terminus of mPLCζ (accession no. NP_473403 [17]) was raised [21]. The 19-mer sequence of mPLCζ corresponded to that on the C-terminus of rat PLCζ. After several washes with 0.1% (v/v) Tween 20-DPBS (DPBS-T), a secondary Alexa Fluor 488-labeled goat anti-rabbit antibody (1:200; Molecular Probes, Eugene, OR, USA) was added for 1 h at room temperature. After several washings in DPBS-T, the samples were counterstained with propidium iodide as described above and mounted using Vectashield mounting media (Vector Laboratories, Burlingame, CA, USA). Fluorescence images were obtained using a fluorescence microscope. As a negative control, sperm were immunostained by the abovementioned protocol but without primary antibody treatment.

**Oocyte collection and ICSI**

The females were superovulated as previously reported [22]. Fourteen h after the hCG injection, cumulus-oocyte complexes (COCs) were collected from the oviductal ampullae of the donor females using mR1ECM supplemented with 0.1% hyaluronidase. After the cumulus cells were removed, intracytoplasmic injection of sperm heads was carried out according to the method described in our previous study [13] with some modifications. In brief, about 10 oocytes were transferred into 20-μl drops of Hepes-R1ECM. A small volume (0.5 μl) of the sperm heads suspension was transferred to 2-μl drops of Hepes-R1ECM supplemented with 12% polyvinylpyrrolidone (MW 400,000), which was prepared close to the oil-covered drops for the oocytes. Microinjection of sperm heads into oocytes was performed using a Piezo-driven pipette (Prime Tech, Ibaraki, Japan) that was prepared from borosilicate glass capillary tubes (Sutter Instrument, Novato, CA, USA). The external diameter of the pipette tip was 2–4 μm. After injection, the oocytes were cultured in mR1ECM up to 120 h. The rate of for-
Improvement of Development in Rat ICSI-Oocytes

The sperm were divided into two categories: sperm with intense fluorescence of the acrosomal cap, indicating an intact outer acrosomal membrane (Intact, Fig. 1A(a)), and sperm with no fluorescence, indicating partial or complete loss of the outer acrosomal membrane (Removed, Fig. 1A(b)). The percentages of acrosomal membrane-removed sperm by different treatments are shown in Fig. 1B. In the control sperm, the proportion of acrosomal membrane-removed sperm was only 24.0 ± 4.5%. On the other hand, the proportions of acrosomal membrane-removed sperm after treatment with TX and LL were 76.0 ± 9.9% (TX) and 89.0 ± 3.8% (LL), respectively. These rates were significantly higher than that of the control group (P<0.05).

Fisher’s protected least significant difference test using the StatView software (Abacus Concepts, Berkeley, CA, USA). A value of P<0.05 was chosen as an indication of statistical significance. Data are shown as means ± standard error of means (SEM).

Results

The sperm were divided into two categories: sperm with intense fluorescence of the acrosomal cap, indicating an intact outer acrosomal membrane (Intact, Fig. 1A(a)), and sperm with no fluorescence, indicating partial or complete loss of the outer acrosomal membrane (Removed, Fig. 1A(b)). The percentages of acrosomal membrane-removed sperm by different treatments are shown in Fig. 1B. In the control sperm, the proportion of acrosomal membrane-removed sperm was only 24.0 ± 4.5%. On the other hand, the proportions of acrosomal membrane-removed sperm after treatment with TX and LL were 76.0 ± 9.9% (TX) and 89.0 ± 3.8% (LL), respectively. These rates were significantly higher than that of the control group (P<0.05).

The results of PLCζ immunostaining in rat spermatozoa are shown in Fig. 2. PLCζ was not detected in the samples treated without primary antibody (Fig. 2a). In the control sperm (Fig. 2b), PLCζ was localized in the acrosomal region. Furthermore, the TX- (Fig. 2c) and LL-treated sperm (Fig. 2d), PLCζ was localized in the acrosomal region of the sperm heads.

The time-dependent changes of the 2PN formation rate after intracytoplasmic injection of sperm heads treated with TX or LL are shown in Fig. 3A. At 4 h after ICSI, the percentages of 2PN formation in the TX (76.0 ± 10.3%, 38/50) and LL (70.0 ± 3.8%, 38/50) groups were determined hourly up to 6 h. In total, 56 (control), 50 (TX) and 60 oocytes (LL) were used for evaluation of 2 PN formation. The rate of blastocyst formation was determined at 120 h. In total, 77 (control), 79 (TX) and 59 oocytes (LL) were used for evaluation of blastocyst formation.

Embryo transfer

To evaluate the developmental competence in vivo of embryos derived from ICSI, 1-cell embryos were transferred into the oviducts of recipients after induction of pseudopregnancy as described previously [13]. Female recipient rats were mated with vasectomized males on day 0 to induce pseudopregnancy. On day 1, nine to ten 2PN oocytes were transferred into each oviduct of the recipients. On day 22, the transferred females underwent Caesarean section to confirm pregnancy and normality of the offspring.

Statistical analyses

Each experiment had at least three replicates. All percentage data were subjected to arcsine transformation before statistical analysis. Data were compared by one-way analysis of variance and Fisher’s protected least significant difference test using the StatView software (Abacus Concepts, Berkeley, CA, USA). A value of P<0.05 was chosen as an indication of statistical significance. Data are shown as means ± standard error of means (SEM).
In mammals, the sperm head contains sperm factor, which is indispensable for oocyte activation [24], and it is now well understood that PLCζ is a strong candidate the sperm factor [17, 25, 26]. It has previously been that PLCζ is localized in the perinuclear theca in the post-acrosomal region [27, 28]. Our present study confirmed, for the first time, that rat PLCζ is also localized in the acrosomal region in sperm (Fig. 2). Moreover, TX and LL did not affect the intensity of the immunofluorescence of PLCζ in rat spermatozoa, although TX and LL treatments significantly induced removal of acrosomal membranes from spermatozoa compared to the control. These acrosomal membranes-removing treatments hastened the timing of 2PN formation, suggesting the possibility that the sperm plasma membrane quickly disintegrates with the oocytes and that sperm factor then leaks out of the spermatozoon. As a result, 2PN formation, which was strongly correlated with termination of oocyte activation, was accelerated. Why is the timing of oocyte activation important? In rats, ovulated oocytes collected from the oviduct are easily activated in vitro in a process called ‘spontaneous activation’ [29, 30]. Spontaneously activated oocytes showed low developmental ability in vitro after artificial activation [22]. The incidence of spontaneous activation is dependent on the strains and commercial sources [31, 32]. The rats used in this study (Crlj:Wistar) were the same as those used in a previous study [32]. It has been demonstrated that Wistar oocytes are likely to spontaneously activate in vitro because they have relatively lower cytostatic factor activity than that oocytes of Slc:Sprague-Dawley rats [32]. In the present study, we demonstrated that removal of the acrosomal membrane had a positive effect on the timing of 2PN formation, which may be due to early termination of oocyte activation.

Another possibility is that sperm chromatin condensation was physically prevented by introduction of an intact acrosome and sperm plasma membrane to the ooplasm. In domestic species, it has been reported that failed or abnormal decondensation of sperm chromatin is frequency observed in oocytes injected with intact sperm [33, 34]. Katayama et al. [35] showed that oocyte activation and decondensation of sperm chromatin are delayed after injection of intact spermatozoa, which is due to the existence of the sperm plasma membrane. Although the kinetics of chromosomes in acrosome-capped sperm were not evaluated in the present study, it is likely that injection of non-treated sperm after sonication had detrimental effects on decondensation of sperm chromosomes in oocytes. It was considered that these positive effects of acrosome

Table 1. Effect of removal of acrosomal membrane on in vivo development of ICSI oocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of transferred embryos</th>
<th>No. of recipients</th>
<th>No. of offspring (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>123</td>
<td>7</td>
<td>11 (7.6 ± 2.3)</td>
</tr>
<tr>
<td>TX</td>
<td>106</td>
<td>7</td>
<td>20 (20.3 ± 4.4)</td>
</tr>
<tr>
<td>LL</td>
<td>119</td>
<td>7</td>
<td>22 (19.0 ± 2.8)</td>
</tr>
</tbody>
</table>

Contorl: Sonicated sperm. TX: Sonicated sperm was treated with 0.04% (v/v) Triton X-100 for 1 min. LL: Sonicated sperm was treated with 0.04% (v/v) lysolecithin for 1 min. a,b Different superscripts within columns denote significant differences (P<0.05).

In the present study, we improved the efficiency of ICSI in rats using sperm pretreated with reagents for removal of the acrosomal membranes. Our present results were in complete agreement with previous papers showing that the contents of the acrosome are potentially harmful to oocytes in mice [15, 23] and hamsters [16]. One of the beneficial effects of pretreatment is to accelerate the timing of PN formation by ICSI.
removal contributed to improvement of the efficiency of ICSI in rats.

In mouse ICSI, the best results are obtained by using pretreated sperm with TX because LL is a product of hydrolysis of membrane phospholipids by phospholipase A and is not ‘an obstacle’ to spermatozoa [23]. However, in this study, there was no significant difference in the percentage of offspring between the TX and LL treatment groups. The difference in results between mice and rats may be resolved in accordance with the following explanation. Morozumi et al. [23] demonstrated that sperm treated with TX as a group showed a lower percentage of offspring than LL-treated sperm individually. In the present study, we briefly treated sperm with TX or LL and completed injection of the sperm head within 15 min after the treatment to minimize the detrimental effects of TX and LL. Further experiments will be required to optimize the treatments in rat ICSI.

Taken together, we showed here the positive effect of removal of acrosomal membranes from sperm on the improvement of rat ICSI. Very recently, we succeeded in establishment of an in vitro fertilization system using rat cryopreserved spermatozoa [3]. Since the in vitro fertilization protocol has not been widely used yet, our present data will contribute to efficient production of rat offspring derived from cryopreserved sperm via ICSI.

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