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The importance of intact sperm DNA in development of oocytes injected with sperm heads in rats and pigs

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ABSTRACT

The intracytoplasmic sperm injection (ICSI) is an established technique in many mammals for generating live offspring even if their spermatozoa lack motility. In this paper, we described the developmental ability of ICSI oocytes to improve the ability of ICSI oocytes in rats and pigs. As rat spermatozoa have long tails, sonicated sperm heads have been used for ICSI to improve injection efficiency. However, it was reported that bovine spermatozoa were damaged by sonication. We evaluated that the effect of separation of rat spermatozoa by sonication on *in vitro* development of oocytes injected with sperm heads. The sonication treatment for separation of sperm heads did not affect *in vitro* development to the 2-cell stage of ICSI oocytes in rats. In addition, the effects of sonication and holding medium for spermatozoa on development of ICSI oocytes were examined. Although K^+-rich medium promoted pronuclear formation, full-term developmental ability was not influenced by the ratio of Na^+ to K^+ in medium for sperm separation in rats. In pigs, development *in vivo* and *in vitro* of oocytes injected with freeze-dried sperm heads was investigated. The developmental rate *in vitro* to the blastocysts of oocytes injected with freeze-dried sperm heads was 18.6%. The average cell number of the blastocysts was 37. As results of transfer of the oocytes injected with freeze-dried sperm heads, two aborted fetuses were obtained on day 39 after the transfer. Therefore, it was suggested that oocytes injected with freeze-dried sperm heads have the competence to grow to day 39 after oocyte transfer. Also, effect of chelating agents on DNA fragmentation after rehydration of freeze-dried boar spermatozoa was studied. The results indicate that fragmentation of DNA in freeze-dried sperm decreases the developmental ability of injected oocytes. In conclusion, the presence of structurally intact DNA in sperm for ICSI is quite important for normal embryogenesis.

Key words: *freeze-dried sperm heads; intracytoplasmic sperm injection (ICSI); pig ; rat; sperm sonication;*

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INTRODUCTION

The intracytoplasmic sperm injection (ICSI) is an established technique in many mammals for generating live offspring even if their spermatozoa lack motility. However, there is currently a debate about the risk of sperm with abnormalities achieving fertilization (completion of male pronucleus formation), because physiological selection processes such as binding to zona pellucidae, acrosomal reaction, and fusion to the ooplasm, are bypassed [1,2]. DNA fragmentation in human spermatozoa is one of the causes of failure of embryonic development and successful pregnancy [3]. This suggests that the presence of structurally intact DNA in the sperm is quite important for normal embryogenesis after fertilization. In this paper, we described the developmental ability of ICSI oocytes to improve the ability of ICSI oocytes in rats and pigs.

Rats

The effect of separation of spermatozoa by sonication on in vitro development of oocytes injected with sperm heads

As rat spermatozoa have long tails [4], sonicated sperm heads have been usually used for ICSI to improve injection efficiency [5,6]. On the other hand, bovine spermatozoa were reported to be damaged by sonication, since oocytes injected with sperm heads developed more poorly than those injected with whole sperm [7]. So we investigated the effect of separation of sperm heads by sonication or Piezo-pulse on development to the 2-cell stage *in vitro* of oocytes injected with sperm heads in rats [8]. The percentages of formation of two pronuclei (64.4% *vs* 68.7%) and development to the 2-cell stage (31.6% *vs* 39.6%) did not differ significantly between the sonication and Piezo-pulse groups. From the results, it was suggested that the sonication treatment for separation of sperm heads did not affect *in vitro* development to the 2-cell stage in rats.

The effect of sonication and holding medium of rat spermatozoa on development of oocytes injected with sperm heads

It was suggested that the ratio of Na^+ to K^+ in medium used for sonication and holding affects the incidence of structural chromosome aberrations of mouse and human spermatozoa [9]. We examined whether the ratio of Na^+ to K^+ in sonication and holding medium for rat spermatozoa influences *in vivo* and *in vitro* development of oocytes injected with sperm heads or not [8]. The spermatozoa were suspended and sonicated for 10 sec in NIM ($Na^+ < K^+$) [9], NaCl (K^+-free) [10] and KRB ($Na^+ > K^+$) [11] media. The sonicated sperm

heads were injected into rat oocytes within 2 h after sonication, and the injected oocytes were cultured in KRB medium. The percentage of pronuclear formation in NIM group (83.1%) was significantly ($P < 0.05$) higher than those of NaCl (68.2%) and KRB (65.5%) groups. The rate of development to the 2-cell stage in NaCl group (19.3%) was significantly ($P < 0.05$) lower than those of NIM (36.1%) and KRB (41.4%) groups. However, there was no significantly difference among groups in the offspring rates (7.2% in NIM group, 6.6% in NaCl group, 6.3% in KRB group). These results suggested that K^+-rich medium such as NIM medium promoted pronuclear formation, and that full-term developmental ability was not influenced by the ratio of Na^+ to K^+ in medium for sperm separation in rats.

Pigs

Development in vivo and in vitro of oocytes injected with freeze-dried sperm heads

Storage of semen in frozen form requires expenditure on liquid nitrogen and space for storing containers. Freeze-drying method has been paid attention, because freeze-dried materials are easy to store and transport without special equipment. Viable offspring have been produced by ICSI of freeze-dried spermatozoa in mice [12-15], rabbits [16], and rats [17]. However, in pigs [18] and cattle [19], it has been reported only that oocytes resulting from ICSI with freeze-dried spermatozoa have developed to the blastocyst stage. Thus, we examined the *in vivo* and *in vitro* developmental ability of oocytes injected with freeze-dried sperm heads in pigs.

Ejaculated spermatozoa were sonicated for 1 min to isolate the sperm heads from the tails and suspended in Pig-FM medium [20]. Sonicated sperm were placed as a 100-μl suspension into a glass ampoule, pre-cooled at –40 °C for 6 h and attached to a freeze-drying system for 6 h. The ampoule was then closed by heat from a gas burner and stored at 4 °C. Freeze-dried sperm samples were rehydrated by adding 100 μl of distilled water. Sperm heads were injected into oocytes and the injected oocytes were stimulated as described previously [21-23]. The developmental rate *in vitro* to the blastocyst of oocytes after injection with the freeze-dried sperm heads was 18.6%. The average number of cells in the blastocysts was 37. As results of transfer of the oocytes generated after freeze-dried sperm head injection to the 12 recipients, two aborted fetuses were obtained from one of the recipients on day 39 after the transfer. Therefore, it was suggested that oocytes injected with freeze-dried sperm heads have the competence to grow to day 39 after oocyte transfer.

Effect of chelating agents on DNA fragmentation after rehydration of freeze-dried boar spermatozoa

It has been suggested that endonucleases are among the causes of DNA fragmentation in spermatozoa [13]. Sperm endonucleases are activated with divalent cation such as Ca^{2+} and Mg^{2+} [24]. We therefore examined the effect of the addition of chelating agents such as ethylene glycol-bis [beta-aminoethyl ether]-*N,N,N',N'*-tetraacetic acid (EGTA) or ethylenediamine-*N,N,N',N'*-tetraacetic acid, disodium salt (EDTA) to freeze-drying buffer on preventing fragmentation of boar sperm nuclear DNA [23].

The sonicated spermatozoa were suspended in four different types of freeze-drying buffer: 1) 50 mM EGTA was added to the basic solution [50 mM NaCl and 10 mM Tris-HCl (50-mM EGTA)] [13]; 2) 50 mM EDTA was added to the basic solution (50 mM EDTA); 3) 10 mM EDTA and 0.117 M sorbitol were added to the basic solution (10 mM EDTA); and 4) 0.15 M sorbitol was added to the basic solution (non-chelated medium). As a control, the spermatozoa were suspended in a Pig-FM. The osmolarity and pH of the buffers 1–4 were 265 mOsm/kg and pH 8.0, respectively. Those of the control buffer were to 305 mOsm/kg and pH 7.4, respectively. Each group spermatozoa were then freeze-dried by the above mentioned method. Firstly, we examined the proportion with DNA fragmentation in each group was assessed by the TUNEL method as soon as the sperm had been rehydrated, centrifuged and resuspended in Pig-FM. The percentage of sperm with DNA fragmentation in the Pig-FM group was significantly higher ($P < 0.05$) than those of fresh spermatozoa which were not freeze-dried, 50 mM EGTA, 50 mM EDTA, and 10 mM EDTA groups. Secondly, we investigated the fluctuations in the percentages of DNA-fragmented spermatozoa during the incubation (0-180 min) after rehydration. The percentage of spermatozoa with DNA fragmentation in the Pig-FM group increased gradually with incubation time. On the other hand, the rates were not significantly different among other groups. The rate of DNA fragmentation in the Pig-FM group at 180 min (12.2%) was significantly higher ($P < 0.05$) than those in the other groups (0.7–4.1%). These results show that the addition of chelating agents in a freeze-drying medium is effective to prevent the increase of sperm DNA fragmentation during freeze-drying process and incubation after rehydration.

Effect of the addition chelating agents in freeze-drying medium on development in vitro of freeze-dried sperm head-injected oocytes

We examined the influences of time

elapsed after rehydration and chelating agents on the *in vitro* developmental ability of oocytes injected with freeze-dried sperm heads from each group [23]. At 120 180 min of rehydration, the percentage in the Pig-FM group (6.0%) was significantly lower ($P < 0.05$) than those in the 50 mM EGTA group and the 10 mM EDTA group (23.1% and 22.6 %, respectively). The average numbers of cells in the 50 mM EGTA group had the highest cell number of the blastocysts, regardless of the incubation time after rehydration. These results suggest that fragmentation of DNA in freeze-dried sperm decreases the developmental ability of injected oocytes. Furthermore, it was suggested that EGTA would be suitable chelating for addition to freeze-drying buffer.

Conclusion

We believe that the presence of structurally intact DNA in the sperm is quite important for normal embryogenesis. Therefore, we should pay attention to preservation of sperm DNA when we operate spermatozoa for ICSI.

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