Rescuing Genetic Material of Unexpectedly Die Animal

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ABSTRAK

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Kauda epididimis tikus dikoleksi dan dimasukkan kedalam tabung sentrifus ukuran 1,5 ml berisi 1 ml air (milli-Q) atau NaCl fisiologis (0,9% NaCl) sebelum dibekukan pada suhu -196°C dan disimpan selama 7 hari tanpa krioprotektan. Setelah kauda epididimis diencerkan kembali (*thawing*) dan spermatozoa pada kauda epididimis dikoleksi, tidak ditemukan adanya spermatozoa yang motil. Spermatozoa yang dikoleksi dari kauda epididimis yang disimpan dalam salin (NaCl fisiologis) setelah thawing dan intinya diinjeksikan (dimasukkan) kedalam sel telur, seluruh sel telur mengalami aktivasi (100%) dan menurun (P<0,05) secara bertahap kauda epididimis yang dibekukan dalam media air (milli-Q) pada suhu -196°C (86%) dan kontrol (69%). Pada sel telur yang teraktivasi, sebagian besar kepala sperma mengalami transformasi membentuk pronukleus jantan (66-78%). Embrio 1 sel yang dikultur selama 120 jam berkembang menjadi tahap blastosis sebanyak 7%. Hasil penelitian ini menunjukkan bahwa materi genetik spesies (paling tidak pada tikus) yang mati mendadak dapat diselamatkan dengan metode sederhana.

Kata Kunci: ICSI, Kepala Spermatozoa, Piezo-Injection, Beku, Tikus

ABSTRACT

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Rat cauda epididymides were kept in 1.5-ml centrifuge tubes containing 1 ml milli-Q water or physiological saline (0.9% NaCl) and stored and freezed at -196°C without cryoprotectant for up to 7 days. After thawing of the cauda epididymis, all spermatozoa were non-motile immediately after collection. All oocytes injected with sperm heads (nuclei) of spermatozoa collected from frozen-thawed cauda epididymis in saline were activated (100%) and gradually decreased (P<0.05) in cauda epididymis frozen in milli-Q water at -196°C (86%), and in control (69%). In activated oocytes, a large proportion of sperm heads had transformed into male pronuclear formation (66-78%). When 1-cell embryos were cultured for 120 h, 7% developed to blastocyst stages. These results indicate that genetic materials of species (at least in the rat) that had unexpectedly die can be saved by a simple method.

Key words: ICSI, Sperm Heads, Piezo-Injection, Frozen, Rat

INTRODUCTION

Because many mammalian species are threatened or in danger of becoming extinct within the next few decades with loss of biological diversity, ultimately resulting in mayor changes of all living organisms (LEIBO and SONGSASEN, 2002). Developing assisted reproduction techniques including *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and embryo culture could play an important role in managing captive and natural populations as well as in sustaining both genetic and global biodiversity (WILDT *et al.*, 1992). As enumerated by SOULE (1991), six major classes of human interference that contribute to degradation of biological diversity are 1) loss of habitat; 2) fragmentation of habitat; 3) overexploitation; 4) spread of exotic species and diseases; 5) air, soil, and water pollution; and 6) climate change. To maintain biological diversity, the concept of genome resource banks, something called frozen zoos, has been gaining strength and acceptance, not the least because of thoughtful and reasoned advocacy by several committed proponents of this concept (BAINBRIDGE and JABBOUR, 1998; COMIZZOLI *et al.*, 2000; WILDT *et al.*, 1993). A central theme of genome resource banking has been that the reproductive sciences (WILDT and WEMMER, 1999), assisted reproductive techniques (ART; BAINBRIDGE and JABBOUR, 1998), or reproductive biotechnologies (COMIZZOLI *et al.*, 2000) have the potential to prevent or at least to lessen the degradation of biodiversity.

The ability of spermatozoa regardless of motility and then fertilize *in vitro* may provide one approach for rescueing genetic material from animals that dies or undergoes a vasectomy of medical reasons. Intracytoplasmic sperm injection (ICSI) is the most direct micromanipulation method, for which sperm motility is unnecessary. If the recovery of sperm motility after storage is unnecessary, it may be possible to store spermatozoa using a simplified method such as storage of cauda epididymis in the freezer without a cryoprotection.

In the mouse, successful cryopreservation of spermatozoa using raffinose (18%) plus skim milk (3%) (OKUYAMA *et al.*, 1990), and raffinose (18%) plus glycerol (1.75%) (TADA *et al.*, 1990) were reported. In the original study of ICSI onto mammalian oocytes, UEHARA and YANAGIMACHI (1976) showed that human spermatozoa frozen in isotonic alone decondensed and formed pronuclei when injected into hamster oocytes indicating that the sperm nucleus is an extremely stable organelle. Furthermore, the genetic integrity of mouse spermatozoa and their isolated heads was shown to be maintained after freezing and thawing in media containing glycerol by the birth of live young after injection into oocytes (KURETAKE *et al.*, 1996). Normal offspring has also been reported after freezing spermatozoa in the absence of cryoprotectant and injection into oocytes after thawing (WAKAYAMA *et al.*, 1998). In the rat, NAKATSUKASA *et al.* (2001) reported live offspring after intrauterine insemination with epididymal spermatozoa cryopreserved at -196°C with various concentration of glycerol (0, 3 and 6%) either in presence or absence of Equex Stem as cryoprotective agents. More recently, the birth of live rat offspring has been reported after freezing spermatozoa at -20°C without cryoprotection and injection of their sperm heads into oocytes after thawing (HIRABAYASHI *et al.*, 2002).

In the present study, we report that not only showed a simple method for rescueing of genetic material from animals that unexpectedly die, but also showed that rat spermatozoa derived from frozen-thawed cauda epididymis without a cryoprotection were fertile when the sperm heads were injected microsurgically into oocytes.

MATERIALS AND METHODS

Media

The basic medium used for manipulation of oocytes and spermatozoa was modified Krebs-Ringer bicarbonate solution containing Hepes (mKRB-Hepes). This medium, consisting of 94.6 mM NaCl, 4.78 mM KCl, 1.71 mM $CaCl_2$, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4$, 5.0 mM $NaHCO_3$, 5.56 mM glucose, 21.58 mM sodium lactate, 0.5 mM sodium pyruvate, 4 mg ml^{-1} BSA (A-7638; Sigma Chemical Co., St Louis, MO), 75 $\mu g ml^{-1}$ potassium penicillin G, 50 μg ml^{-1} streptomycin sulphate and 20 mM Hepes (Sigma), was essentially the same as that used by TOYODA and CHANG (1974) for *in vitro* fertilization of rat oocytes, except that the concentration of $NaHCO_3$ was reduced from 25.07 mM to 5.0 mM and 20 mM Hepes was added. The medium used for the culture of embryos was mR1ECM composed of 76.7 mM NaCl, 3.2 mM KCl, 2.0 mM $CaCl_2$, 0.5 mM $MgCl_2$, 25.0 mM $NaHCO_3$, 10.0 mM sodium lactate, 0.5 mM sodium pyruvate, 7.5 mM glucose, 1.0 mg ml^{-1} polyvinylalcohol, 2% (v/v) Minimal Essential Medium (MEM) essential amino acid solution (No. 11130-051; Gibco Laboratories, Grand Island, NY), 0.1 mM glutamine (Sigma), and 1% (v/v) MEM nonessential amino acid solution (No. 11140-050; Gibco) (MIYOSHI *et al.*, 1995a, b).

Preparation of oocytes

Adult (8-12 weeks old) female Wistar rats were induced to superovulate by i.p. injections of 25-30 IU eCG between 19:50 and 20:00 h on the day of estrus and 25-30 IU hCG 48 h later. The females were killed 15-16 h after hCG injection and the excised oviducts were placed in a small drop (100-200 µl) of mKRB-Hepes supplemented with 0.1% (w/v) hyaluronidase from bovine testes (Sigma) in a polystyrene culture dish (35 x 10 mm; Falcon No. 1008, Becton Dickinson Labware, Lincoln Park, NJ) at 37°C. Oocytes with cumulus cells were released by dissecting the ampullar portion of the oviducts and kept in the medium for about 5 min. The oocytes freed from cumulus cells were washed three times in Hepes-free mKRB containing an increased concentration (25.07 mM) of $NaHCO_3$, placed into 100 µl of the same medium, and kept in a CO_2 incubator (5% CO_2 in air at 37°C) for up to 1 h until spermatozoa were microinjected.

Freezing of cauda epididymis tract

Cauda epididymis tract were removed from a mature wistar rat (4-8 months old). The blood and adipose tissue were removed from the surface and washed in a petri dish (35 x 10 mm; Falcon No. 1008) containing 5 ml milli-Q water or physiological saline (0.9% NaCl in milli-Q water). Each Cauda epididymis was then placed in a 1.5-ml tapered centrifuge tubes containing in 1 ml milli-Q water or saline. Each tube was tightly capped and placed directly into liquid nitrogen (-196°C). All samples were stored for periods up to 7 days.

Thawing Cauda Epididymis and preparation of spermatozoa

Tubes were removed from the freezer or liquid nitrogen and placed in water at 37°C for about 1 min. The small part of thawed cauda epididymis excised with a fine scissors and a small drop of sperm mass was placed in 1 ml mKRB-Hepes in a 1.5-ml tapered centrifuge tubes and vortexing for about 30 sec. The tubes were placed in ice water and the spermatozoa were sonicated for 3 sec (ten 0.3 sec bursts at 0.7 sec interval) using a 20% power output of a Branson Sonifier Model 250 (Branson Ultrasonies, Danbury, CT). Separation into heads and tails was successful in more than 80% of the spermatozoa. A portion (100 µl) of the sonicated suspension was transferred into another centrifuge tube containing 1 ml mKRB-Hepes and the diluted sperm suspension was centrifuged at 1800 *g* for 3 min. The pelleted material was resuspended in 1 ml mKRB-Hepes containing 5 µg ml^{-1} cytochalasin B.

Microinjection of Sperm Heads

A small drop (5 µl) of the suspension containing 20-40 sperm heads and some tails was placed in a cover of a petri dish (50 x 4 mm; Falcon No. 1006) and covered with paraffin oil (Nacalai Tesque Inc., Kyoto, Japan). The oocytes which had been kept in a CO_2 incubator were initially transferred into 100-200 µl mKRB-Hepes containing 5 µg ml^{-1} cytochalasin B and then 8-10 oocytes in about 1 µl medium were introduced into a drop containing sperm heads. The addition of cytocalasin B in the manipulation medium intends to reduce spontaneous activation in rat oocytes. Microinjection of sperm heads into oocytes was performed at 37°C using a piezo- driven pipette that was prepared from Borosilicate glass capillary tubes (Sutter Instrument Co., Novato, CA). The external and internal diameters of the tip of the pipette were 10-11 µm and 7-8 µm, respectively. A sperm head was aspirated into the injection pipette so that the apex of sperm head was positioned facing the opening of the pipette. The tip of the pipette was brought in contact with the zona pellucida of the oocyte which was held by a holding pipette with an external diameter of 100-110 µm and opening of 15-20 µm. The zona was drilled by applying a few piezo pulses. The tip was introduced into the perivitelline space and forced slightly onto the oolemma, and then a few piezo pulses were applied in order to puncture the oolemma. The sperm head, in a minimal amount of medium, was expelled into the ooplasm.

Experimental Design

Experiment 1 examined whether sperm heads of spermatozoa derived from frozen cauda epididymis at -196°C in milli-Q water or saline after thawing and injection into oocytes can fertilize normally. The oocytes successfully injected with sperm heads were washed four times with Hepes-free mKRB containing 25.07 mM $NaHCO_3$. The washed oocytes were transferred into 100 µl of the same medium covered with paraffin oil in a culture dish (35 x 10 mm; Falcon No. 1008) and cultured in a CO_2 incubator (5% CO_2 in air at 37°C). At 9-12 h after the start of culture, the number of morphologically normal oocytes was then mounted, fixed with 2.5% (v/v) glutaraldehyde in phosphate buffer (pH 7.4) followed by 10% (v/v) neutral formalin and stained with 0.25% (w/v) lacmoid in 45% (v/v) acetic acid (TOYODA and CHANG, 1974). The stained oocytes were examined for the activation and morphological changes of the penetrated sperm heads under a phase-contrast microscope. For the control, fresh sperm heads were also injected into the oocytes in the same condition.

Experiment 2 examined the *in vitro* developmental ability of oocytes injected with sperm heads of spermatozoa derived from frozen cauda epididymis at -196°C in milli-Q water or saline after thawing. Embryos having two pronuclei 10 h after injection were washed three times with mR1ECM and cultured for up to 120 h in the same medium (100 µl) in 5% CO_2 in air at 37°C. For the control, 1-cell embryos having two pronuclei derived from sperm head injection of fresh spermatozoa were also cultured in the same conditions.

Statistical Analysis

The percentage data in each replicate were arcsin-transformed and subjected to one-way ANOVA. The means were compared by Fisher's protected least significant difference test using Stat View program (Abacus Concepts, Inc., Berkeley, CA). A value of $P < 0.05$ was chosen as an indication of statistical significance.

RESULTS AND DISCUSSIONS

All spermatozoa obtained from frozen cauda epididymis at -196°C in milli-Q water or saline kept up to 7 days after thawing were non-motile immediately after collection. As shown in Table 1, all oocytes

Table 1. Status of rat oocytes following intracytoplasmic injection of sperm heads of fresh spermatozoa and spermatozoa derived from frozen-thawed cauda epididymis at -196°C up to 7 days

Treatment	Storage medium	No. of oocytes examined (n)	Activated oocytes having female pronucleus			
			Total n (%) [a]	Condensed sperm heads n (%) [b]	Decondensed sperm heads n (%) [b]	Male pronucleus n (%) [b]
Fresh	-	95	66 (69) [c]	7 (10)	3 (5) [c]	56 (85) [cd]
Frozen	Milli Q water	92	79 (86) [d]	11 (14)	16 (20) [d]	52 (66) [c]
	Saline*	90	90 (100) [e]	0	20 (22) [d]	70 (78) [d]

[a] Percentage of number of oocytes examined.
[b] Percentage of activated oocytes.
[c,d,e] Values with different superscripts in each column are significantly different ($P<0.05$).
* 0.9% NaCl in milli-Q water.

injected with sperm heads of spermatozoa collected from frozen cauda epididymis at -196°C had activated 100% (90/90) and gradually decreased ($P<0.05$) in cauda epididymis frozen in milli-Q water 86% (79/92) and control 69% (66/95). In activated oocytes, sperm heads had transformed into male pronuclear formation 78% (70/90) in saline, no significantly different compared to either cauda epididymis kept in milli-Q water 66% (52/79) or control 85% (56/66), but male pronucleus formation in milli-Q water was significantly lower ($P<0.05$) compared than control.

As shown in Table 2, when 1-cell embryos were cultured in mR1ECM, the cleavage rates and blastocyst rates of embyos were not different between embryos obtained from sperm heads injection of spermatozoa derived from frozen cauda epididymis at -196°C in saline, 59% (49/83) and 7% (6/83), respectively, and in milli-Q water, 51% (44/87) and 7% (6/87), respectively. However, the cleavage rates and blastocyst rates of both of them, were significantly lower ($P<0.05$) than control 73% (52/71) and 23% (16/71), respectively.

One technology that, even today, provides a temporary expedient to alleviate the problem is cryopresevation. It has been shown that bovine spermatozoa remain alive and functional even after cryopreservation in LN_2 up to 37 years (LEIBO *et al.*, 1994). In the mouse, successful cryopreservation of spermatozoa using raffinose (18%) plus skim milk (3%) (OKUYAMA *et al.*, 1990), and raffinose (18%) plus glycerol (1.75%) (TADA *et al.*, 1990) were reported. NAKATSUKASA *et al.* (2001) was reported live rat offspring after intrauterine insemination with epididymal spermatozoa cryopreserved at -196°C with various concentration of glycerol (0, 3 and 6%) either in presence or absence of Equex Stem as cryoprotective agents. More recently, the birth of live rat offspring has reported after freezing spermatozoa at -20°C without cryoprotection and injection of their sperm heads into oocytes after thawing (HIRABAYASHI *et al.*, 2002). Overall, the previously reported cryopreservation and injection procedures were very complicated. In the present study, we demonstrated a simplified method for cryopreservation of spermatozoa and injection procedure. Cryopreservation of spermatozoa was conducted by inserting cauda epididymis tract in a centrifuge tube containing 1 ml water or saline and kept it in -196°C. In this procedure, the cryoprotectant and medium were not necessary. Almost all studies in ICSI, spot of spermatozoa and oocytes were separated for avoiding the oocytes directly contact with PVP that presence in the spot medium spermatozoa. In this experiment, PVP in manipulation medium was not used, so that spermatozoa and oocytes were easily combined in a spot, hence, ICSI could be carried out easily and quickly. Usually, an oocyte could be injected in less than 20 sec.

Utilizing PVP in manipulation medium is to reduce the motility of sperm and increase the viscosity of medium, which makes it easier to precisely control the injection of sperm. However, injecting PVP, a potentially harmful agent, into an oocyte during injection of spermatozoa is unavoidable. Improvement of the rate of pronuclear formation following ICSI using medium with reduced concentration of PVP has been reported in the cattle (WEI and FUKUI, 2000). Since separated sperm heads are not motile, it was easy to pick up a sperm head with an injection pipette in the present study. Furthermore, since the apex of the sperm head was out of the tip of the opening of the pipette at injection, sperm head can easily be expelled into the ooplasm.

Rat oocytes have a high rate of spontaneous activation (KEEFER and SCHUETZ, 1982). The causes of spontaneous activation of rat eggs are complicated. Previous studies had reported that although all rat oocytes injected with whole spermatozoa survived and were able to undergo activation, only a small proportion

Table 2. *In vitro* development of rat oocytes following intracytoplasmic injection with sperm heads of fresh spermatozoa and spermatozoa derived from frozen-thawed cauda epididymis at -196°C up to 7 days.

Treatment	Storage medium	Embryos cultured (n)	Number (%) of embryos developed to			
			2-cell (24) [a]	≥ 4-cell (72) [a]	≥ Morula (96) [a]	Blastocyst (120) [a]
Fresh	-	71	52 (73) [b]	36 (51) [b]	22 (31) [b]	16 (23) [b]
Frozen	Milli Q water	87	44 (51) [c]	25 (29) [c]	7 (8) [c]	6 (7) [c]
	Saline*	83	49 (59) [c]	28 (34) [c]	8 (10) [c]	6 (7) [c]

[a] Numbers in parentheses indicate the time of examination (h after the start of culture)
[b,c] Values with different superscripts within each column are significantly different ($P<0.05$)
* 0.9% NaCl in milli-Q water

of the activated oocytes (10%) appeared to be fertilized normally, having two pronuclei and a second polar body. In many oocytes the extrusion of the second polar body was suppressed and multiple pronuclei were formed (DOZORTSEV *et al.*, 1998). The formation of multiple pronuclei is probably due to fertilization after spontaneous activation. The comparatively high proportion of normal fertilization (66-78%) we have obtained in this study may reflect our addition of cytocalasin B in the microinjection medium to reduce spontaneous activation in rat oocytes. Furthermore, treatment with cytocalasins makes the plasma membrane less rigid and more elastic so that microfilaments are not disrupted during micromanipulation (MCGRATH and SOLTER, 1983).

Although no live births were reported in this study, the fact that eggs were developed to blastocyst stages, indicating that it may eventually be possible to obtain live young if these embryos were transferred to the recipients (HIRABAYASHI *et al.*, 2002; SAID *et al.*, 2003).

CONCLUSION

In conclusion, the present study, shows that not only rat spermatozoa derived from frozen-thawed cauda epididymis without a cryoprotection are fertile when the sperm heads are injected microsurgically into oocytes, but also a simple method for possible rescue of genetic material from animals immediately after unexpectedly die.

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