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Improvement in the Development of Oocytes from C57BL/6 Mice after Sperm Injection

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The C57BL/6 mouse strain is used widely for producing transgenic and knockout strains. Sperm motility is extremely low after a freeze–thaw process. Although intracytoplasmic sperm injection (ICSI) can be used to produce embryos from sperm with low or even no motility, its success rate is poor in the C57BL/6 strain. In particular, the survival of C57BL/6 oocytes after ICSI is extremely low compared with that of hybrid strains. We found that the survival percentages of C57BL/6J oocytes (63% and 64%) were lower than those of B6D2F1 oocytes (80% and 80%) when B6D2F1 and C57BL/6J sperm were injected, respectively. For C57BL/6J mice, 87%, 72%, 64%, 56%, and 59% of oocytes survived after ICSI in media containing 61.62, 71.62, 81.62, 91.62, and 101.62 mM NaCl, respectively. In addition, 64%, 81%, and 79% of oocytes survived after ICSI in media with 4.83, 14.83, and 24.83 mM KCl, respectively. Our results suggest that the survival of C57BL/6J oocytes after ICSI is improved by using Na^+-deficient and K^+ -rich media.

Abbreviation: ICSI, intracytoplasmic sperm injection.

The C57BL/6 mouse is used widely for producing transgenic and knockout strains. Although the sperm of these strains have been maintained by cryopreservation as genetic resources,[9] sperm motility is extremely low after a freeze–thaw process.[15,19] Partial dissection of the zona pellucida by using a steel needle is one technique to facilitate fertilizing oocytes with sperm showing poor motility.[14] However, this technique requires a considerable degree of technical skill. Microdissection of the zona pellucida by using a laser beam is an easy and simple technique to produce a large number of embryos from poorly motile sperm.[4,10] These are useful tools unless the sperm are immotile.

Intracytoplasmic sperm injection (ICSI) is a technique to produce embryos regardless of sperm motility.[3,20] Moreover, using the ICSI technique yielded offspring from oocytes injected with sperm that had been freeze-dried without cryoprotectants.[5-8,21] Reports of successful ICSI have been published for many species of mammals.[16,22] Since the technique was first reported,[12] various improvements have been applied to mouse ICSI to increase its success rates.[13,18]

Although the C57BL/6 strain is important for biomedical research, the success rate of ICSI in this strain remains low.[17] In particular, the survival of C57BL/6 oocytes after ICSI is extremely low, because the oocytes have very poor tolerance to the damage caused by injection, compared with that of oocytes from hybrid strains. Hybrid strains have been used in most previous studies.[12,13,18,20] However, improving the success rate of ICSI in the C57BL/6 strain is important for biomedical research using transgenic or knockout mice derived from C57BL/6 so that they can be maintained as genetic resources. Here we investigated the culture media used for ICSI with the aim of improving the survival of C57BL/6 oocytes after sperm injection.

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Materials and Methods

Animals. C57BL/6J and B6D2F1/Crlj mice (male, older than 11 wk; female, 8 to 16 wk of age) were purchased from Charles River Laboratories Japan (Kanagawa, Japan). All animals were maintained in an air-conditioned (temperature, 22 ± 2 °C; humidity, 60% ± 10%) and light-controlled (lights on 0700 to 1900) room. Microbial monitoring was carried out monthly for mouse hepatitis virus, Sendai virus, *Citrobacter rodentium*, *Clostridium piliforme*, *Corynebacterium kutscheri*, *Helicobacter bilis*, *Helicobacter hepaticus*, *Mycoplasma* spp., *Pasteurella pneumotropica*, *Salmonella* spp., *Aspiculuris tetraptera*, *Syphacia* spp., *Giardia muris*, *Spironucleus muris*, trichomonads, and ectoparasites. All mice had negative test results throughout this microbial monitoring. The Animal Care and Use Committee of Kumamoto University School of Medicine approved all procedures performed in this study.

Media. All chemicals were purchased from Sigma Aldrich (St Louis, MO) unless otherwise specified. The medium used for manipulation, including collection and handling of oocytes and injection of sperm, was H-mCZB medium, a modified CZB medium[1,2] containing 20 mM HEPES-Na, 5 mM $NaHCO_3$, and 0.1 mg/mL polyvinyl alcohol (cold-water–soluble; M_r 30,000 to 70,000) instead of bovine serum albumin.[12] CZB medium supplemented with 5.56 mM D-glucose (mCZB medium) was used for oocyte culture after sperm injection. H-mCZB medium was used under air, and mCZB medium was used under 5% CO_2 in air.

Collection of sperm and oocytes. Both cauda epididymides of each male mouse were removed by using a pair of small scissors. Blood and adipose tissue were removed, and a dense mass of sperm was squeezed out by using sharply pointed forceps. This sperm mass was placed in 1 mL H-mCZB medium in a 1.5-mL microcentrifuge tube. The sperm suspension was left at 37 °C for 10 min to allow the spermatozoa to disperse into the medium.

Female mice were induced to superovulate by intraperitoneal injection of 7.5 IU pregnant mare serum gonadotropin (Teikokuzoki, Tokyo, Japan), followed by injection of 7.5

Table 1. Development of oocytes after sperm injection according to pairing of mouse strains

Strain (male:female)	No. of oocytes injected	No. (%) of oocytes survived/ no. injected	No. (%) of oocytes fertilized/ no. survived	No. (%) of 2-cell embryos/ no. fertilized	No. (%) of morulae/ no. 2-cell embryos	No. (%) of blastocysts/ no. 2-cell embryos
B6D2F1:B6D2F1	224	179/224 (80)[a]	177/179 (99)	167/177 (94)	155/167 (93)[c]	117/167 (70)[e]
C57BL/6J:B6D2F1	219	174/219 (80)[a]	169/174 (97)	163/169 (97)	158/163 (97)[c]	119/163 (73)[e]
B6D2F1:C57BL/6J	232	147/232 (63)[b]	147/147 (100)	138/147 (94)	99/138 (72)[d]	55/138 (40)[f]
C57BL/6J:C57BL/6J	220	140/220 (64)[b]	139/140 (99)	128/139 (92)	100/128 (78)[d]	47/128 (37)[f]

Within each column, percentages with different letters were significantly ($P < 0.05$) different.

Table 2. Development of C57BL/6J oocytes according to NaCl concentration of the media

NaCl (mM)	No. of oocytes injected	No. (%) of oocytes survived/ no. injected	No. (%) of oocytes fertilized/ no. survived	No. (%) of 2-cell embryos/ no. fertilized	No. (%) of morulae/ no. 2-cell embryos	No. (%) of blastocysts/ no. 2-cell embryos
61.62	142	123/142 (87)[a]	118/123 (96)	114/118 (97)[e]	102/114 (89)[g]	25/114 (22)[k]
71.62	140	101/140 (72)[b]	100/101 (99)	93/100 (93)	82/93 (88)[h]	40/93 (43)[l]
81.62[m]	220	140/220 (64)[c]	139/140 (99)	128/139 (92)	100/128 (78)[i]	47/128 (37)[l]
91.62	128	71/128 (56)[d]	68/71 (96)	59/68 (87)[f]	49/59 (83)	21/59 (36)
101.62	128	75/128 (59)[d]	72/75 (96)	69/72 (96)	50/69 (73)[j]	27/69 (39)[l]

Within each column, percentages with different letters were significantly ($P < 0.05$).
[m]Data from Table 1.

IU human chorionic gonadotropin (Teikokuzoki) 48 h later. Cumulus–oocyte complexes were collected from oviducts 13 to 15 h after injection of human chorionic gonadotropin. Oocytes were freed from cumulus cells by treatment with 0.1% hyaluronidase in H-mCZB medium. Oocytes then were rinsed in fresh H-mCZB medium and kept at room temperature before ICSI.

ICSI. ICSI was carried out by slightly modifying the procedure described previously.[12,13] A small volume (1 to 2 µL) of sperm suspension was mixed thoroughly with a droplet of H-mCZB medium containing 12% (w/v) polyvinylpyrrolidone (M_r 360,000; ICN Pharmaceuticals, Costa Mesa, CA). Sperm with normal morphology were selected and placed into another droplet of H-mCZB medium containing 12% (w/v) polyvinylpyrrolidone. A single spermatozoon was drawn from the tail into the injection pipette. The sperm head was separated from the tail by applying a few piezo pulses when the junction between the head and tail was at the opening of the pipette. Sperm heads were injected immediately into each oocyte in a droplet of H-mCZB medium.

In experiment 1, ICSI combined different strains. Either B6D2F1 or C57BL/6J sperm heads were injected into either strain of oocytes. In experiment 2, B6D2F1 and C57BL/6J oocytes were collected and injected with sperm heads in H-mCZB medium containing different concentrations of NaCl: 61.62, 71.62, 81.62 (control), 91.62, or 101.62 mM. In experiment 3, C57BL/6J oocytes were collected and injected with sperm heads in H-mCZB medium with different concentrations of KCl: 4.83 (control), 14.83, or 24.83 mM. Each experiment was repeated 5 times.

Embryo culture. After ICSI, oocytes were cultured in mCZB medium. Oocytes containing 2 distinct pronuclei at 5 h after sperm injection were recorded as being fertilized. Fertilized oocytes were cultured to blastocysts at 37 °C under 5% CO_2 in air.

Analysis of data. All data were analyzed by using χ^2 tests with Yates correction for continuity.

Results

The survival of C57BL/6J oocytes after ICSI was significantly ($P < 0.05$) lower than that for B6D2F1 oocytes (Table 1). For C57BL/6J oocytes, 63% and 64% survived after ICSI using B6D2F1 and C57BL/6J sperm, respectively. However, 80% of B6D2F1 oocytes survived after sperm injection regardless of the sperm source. Overall, more than 97% of all oocytes that survived after ICSI were fertilized normally, and more than 92% of fertilized oocytes developed to the 2-cell stage. The percentages of development to the morula and blastocyst stages of embryos derived from C57BL/6J oocytes were significantly ($P < 0.05$) lower than those derived from B6D2F1 oocytes. The percentages of development to blastocyst of the embryos derived from C57BL/6J oocytes were 40% and 37% with B6D2F1 and C57BL/6J sperm, respectively. In comparison, the percentages of development to blastocyst of embryos derived from B6D2F1 oocytes were 70% and 73% with B6D2F1 and C57BL/6J sperm, respectively.

Survival percentages of oocytes after ICSI with C57BL/6J sperm in media containing 81.62 mM NaCl or less were significantly ($P < 0.05$) higher than those obtained by using media containing 91.62 or 101.62 mM NaCl (Table 2). A high proportion (more than 73%) of embryos developed to the morula stage in all replicates, and 43% of embryos developed to blastocysts when injected by using 71.62 mM NaCl. A similar study using B6D2F1 sperm was conducted (Table 3). The survival of oocytes after ICSI of B6D2F1 sperm injection was high (more than 80%) except when the media contained 61.62 mM NaCl (63%). A high proportion (more than 67%) of embryos developed to blastocysts overall.

Table 4 shows that survival percentages of oocytes undergoing ICSI in media containing 14.83 or 24.83 mM (81% or 79%) were significantly ($P < 0.05$) higher than when 4.83 mM KCl was used (64%). In addition, percentages of embryo development from the 2-cell to blastocyst stage were significantly ($P < 0.05$) higher in media containing 14.83 or 24.83 mM KCl (53% and 52%, respectively) than 4.83 mM KCl (37%).

Table 3. Development of B6D2F1 oocytes according to NaCl concentration of the media

NaCl (mM)	No. of oocytes injected	No. (%) of oocytes survived/ no. injected	No. (%) of oocytes fertilized/ no. survived	No. (%) of 2-cell embryos/ no. fertilized	No. (%) of morulae/ no. 2-cell embryos	No. (%) of blastocysts/ no. 2-cell embryos
61.62	155	98/155 (63)[a]	95/98 (97)	84/95 (88)[e]	79/84 (94)	56/84 (67)
71.62	171	150/171 (88)[b]	150/150 (100)	148/150 (99)[f]	142/148 (96)	109/148 (74)
81.62[i]	224	179/224 (80)[c]	177/179 (99)	167/177 (94)[h]	155/167 (93)	117/167 (70)
91.62	157	136/157 (87)[b]	134/136 (99)	133/134 (99)[g]	127/133 (96)	100/133 (75)
101.62	159	142/159 (89)[d]	142/142 (100)	140/142 (99)[f]	137/140 (98)	102/140 (73)

Within columns, percentages with different letters were significantly ($P < 0.05$) different.
[i]Data from Table 1.

Table 4. Development of C57BL/6J oocytes according to KCl concentration of the media

KCl (mM)	No. of oocytes injected	No. (%) of oocytes survived/ no. injected	No. (%) of oocytes fertilized/ no. survived	No. (%) of 2-cell embryos/ no. fertilized	No. (%) of morulae/ no. 2-cell embryos	No. (%) of blastocysts/ no. 2-cell embryos
4.83[i]	220	140/220 (64)[a]	139/140 (99)	128/139 (92)[c]	100/128 (78)[e]	47/128 (37)[g]
14.83	182	148/182 (81)[b]	148/148 (100)	147/148 (99)[d]	127/147 (86)	78/147 (53)[h]
24.83	165	130/165 (79)[b]	130/130 (100)	128/130 (99)[d]	118/128 (92)[f]	67/128 (52)

Within columns, percentages with different letters were significantly ($P < 0.05$) different.
[i]Data from Table 1.

Discussion

The survival of oocytes after ICSI and their subsequent development into embryos were low when C57BL/6J oocytes were used, regardless of the sperm strain used (Table 1). However, the survival of C57BL/6J oocytes after ICSI improved when media contained low concentrations of NaCl (81.62 mM and lower; Table 2). The survival percentage of B6D2F1 oocytes after ICSI was high except when media containing 61.62 mM NaCl was used (Table 3). Development of C57BL/6J embryos to blastocyst stage was particularly low in the medium containing 61.62 mM NaCl. Although the lowest concentration of NaCl used in this study showed a positive influence on the survival of oocytes after sperm injection, it had a negative effect on the subsequent development of embryos in vitro. Most previous studies using the medium with 81.62 mM NaCl in hybrid strains showed good results in terms of the survival of oocytes after ICSI and subsequent embryo development.[12,13,18,20] Our present study suggests that the survival of both C57BL/6J and B6D2F1 oocytes after ICSI and the numbers of subsequent blastocysts could be increased by using medium containing 71.62 mM NaCl.

Mouse strain,[11] temperature during sperm injection[12] and oocyte conditions[17] have been reported as factors that affect the survival of oocytes after ICSI. Live offspring were obtained from oocytes injected with sperm at 17 to 18 °C;[12] the authors surmised that damage to the oolemma was minimized at this low temperature. However, the B6D2F1 strain used in that report and our present study showed high survival of oocytes even when injected with sperm at normal (ambient room) temperature. The survival of C57BL/6J oocytes after sperm injection reportedly was increased significantly for frozen–thawed oocytes compared with fresh oocytes;[17] the authors suggested that the wound-healing ability of the oocyte plasma membrane after ICSI was increased by the freeze–thaw process. However, oocyte freezing is time-consuming and requires a considerable degree of technical skill.

We found here that using media with decreased concentrations of NaCl (Table 2) or increased KCl (Table 4) is a simple technique to improve the survival of C57BL/6J oocytes after ICSI. In a previous study, [13] high concentrations of Na^+ were thought to be detrimental to the nuclear DNA of mouse sperm, and the influx of extracellular Na^+ into oocytes through the hole made by the injection pipette in the oolemma was postulated to be damaging to oocytes, resulting in decreased survival after sperm injection. A K^+-rich medium appears to enhance the wound-healing ability of the oolemma and development to blastocyst in C57BL/6J oocytes after ICSI. Therefore, the media's ionic composition seems to affect the survivability and developmental ability of oocytes after ICSI.

We found here that the survival rates of C57BL/6J oocytes after ICSI were improved by using Na^+-deficient or K^+-rich medium. Sperm injection is an important technique for the maintenance of genetic resources when only immotile sperm are available. Moreover, ICSI is indispensable for the production of offspring from freeze-dried sperm, which can be preserved for a long time under refrigeration.[5-8,21] The modified ICSI methods described in the present study may improve success rates for C57BL/6-derived mouse lines and likely will improve available tools for conserving genetic resources.

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