Production of normal mice from spermatozoa denatured with high alkali treatment before ICSI

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Abstract

In mammals, ICSI is now a very important tool for both assisted reproductive technology and studying the mechanisms of fertilization. In the latter experiments, it is important to use spermatozoa that have lost their oocyte activation capacity but still retain their developmental potential. In this study, we used high-concentration NaOH to remove oocyte activation potential from spermatozoa, and examined whether normal offspring could be generated from these spermatozoa after ICSI. The spermatozoa were treated with different concentrations of NaOH (1–100 mM) for 1 h and then neutralized with equal amounts of same concentration of HCl. In 10 mM NaOH-treated spermatozoa, the cell membrane was broken and most of them failed to activate oocytes after their injection into the oocytes. However, these spermatozoa did not show strong damage, and after artificial activation with SrCl₂, all of the zygotes were judged as normal by immunostaining to check the methylation status of histone H3 lysine 9, low chromosome damage by karyotype assay and staining with DNA double-strand breaks marker, γ H2AX. Moreover, after transferring those embryos into recipient females, 106 (36.7%) live and healthy offspring were delivered, which is similar to the rate in the fresh control group. By contrast, spermatozoa treated with lower NaOH concentrations retained their oocyte activation capacity and those treated with higher concentrations lost their developmental potential. This suggests that 10 mM NaOH for 1 h is the best treatment to completely destroy the cell membrane and activation capacity of spermatozoa without injuring their developmental potential.

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Introduction

ICSI is now a very important tool for both assisted reproductive technology and the study of fertilization, especially in mammals (Kimura & Yanagimachi 1995a, Yanagimachi 2005). Not only intact spermatozoa but also the abnormal or artificially ‘dead’ spermatozoa can lead to full-term development by ICSI. Nowadays, various methods are employed to remove the plasma membrane of spermatozoa and they have been very valuable in the study of fertilization and in the further applications. Treated spermatozoa can be divided into two groups. The first group includes sonicated sperm, freeze-thawed sperm, and freeze-dried sperm which lack the plasma membrane and acrosome (Goto *et al.* 1990, Kuretake *et al.* 1996, Wakayama & Yanagimachi 1998, Wakayama *et al.* 1998, Tateno *et al.* 2000, Kwon *et al.* 2004, Liu *et al.* 2004). These types of spermatozoa cannot complete fertilization naturally, but they do not lose their sperm-borne oocyte activating factor (SOAF), so their injection into oocytes by ICSI can lead to oocyte activation (Goto *et al.* 1990,

Kuretake *et al.* 1996, Wakayama & Yanagimachi 1998, Wakayama *et al.* 1998, Tateno *et al.* 2000, Kwon *et al.* 2004, Liu *et al.* 2004). However, during the preparation of these samples, their nuclei are damaged, and the developmental potential of these spermatozoa after ICSI is reduced compared with that of fresh spermatozoa (Goto *et al.* 1990, Hoshi *et al.* 1995, Nagy *et al.* 1995, Kuretake *et al.* 1996, Wakayama & Yanagimachi 1998, Ward *et al.* 2003).

On the other hand, the second group, heat-treated, lyssolecithin (LL) and pronase treated spermatozoa or specific-stage male gametes, such as round spermatids, cannot activate oocytes after their injection into oocytes (Ogura *et al.* 1994, Kimura & Yanagimachi 1995b, Cozzi *et al.* 2001, Jiang *et al.* 2005, Yan *et al.* 2008). If these oocytes are activated artificially, such as by Sr²⁺ or electrostimulation treatment (Ogura *et al.* 1994, Bos-Mikich *et al.* 1997, Yan *et al.* 2008), the subsequent embryos generated with these spermatozoa can support embryonic development and produce live offspring (Ogura *et al.* 1994, Kimura & Yanagimachi 1995b, Cozzi *et al.* 2001, Kishigami *et al.* 2004, Jiang

et al. 2005, Yan *et al.* 2008). However, for unknown reasons, the success rate of full-term offspring is significantly reduced in these compared with that of fresh spermatozoa.

The best results were obtained when spermatozoa were treated in alkyltrimethylammonium bromide (ATAB) and dithiothreitol, and then extracted with salt. These treated spermatozoa lost their cell membranes and their oocyte activation potential but did not lose their nuclear integrity, as judged by the full-term development of embryos after ICSI (Ward *et al.* 1999). However, this treatment is not simple, and the oocyte survival rate after ICSI was reduced, which might be attributable to the increased stickiness of the treated spermatozoa, so that ICSI does not proceed smoothly.

From these reports, it can be concluded that if spermatozoa are treated physically or chemically to remove cell components, they can easily lose not only their membrane and activation capacity but also their nuclear integrity. In other words, the treatment of spermatozoa may cause too much damage and the loss of their full-term developmental potential.

Although environmental changes can affect the normal fertilization capacity of spermatozoa, the influence of the pH of the medium is still unknown. Today, in *in vitro* culture, especially of the mouse, the pH of most media (such as TYH, M2, M16, CZB, and KSOM) is maintained as weakly alkaline, near 7.4 (Whittingham 1971, Quinn *et al.* 1982, Chatot *et al.* 1989, Lawitts & Biggers 1991). It is well known that alkali solution is used to extract plasmid DNA from bacteria without injury to the DNA structure, even though the membranes of the bacteria are completely destroyed (Birnboim & Doly 1979). Furthermore, it has been reported that for embryos generated from freeze-dried mouse spermatozoa, the best pH of the medium for the development to the blastocyst stage is pH 8.0–9.0 (Kaneko *et al.* 2003). In this weak alkaline medium, sperm nuclei can be maintained without damage. Also, some papers have reported the fertility of spermatozoa treated with high-pH medium. It has been demonstrated in porcine systems that the treatment of sperm with 100 mM NaOH in Tris-buffered saline results in the destruction of the cell membrane and failure in activating porcine oocytes. (Kim *et al.* 1999). It has been reported that oocyte activation after fertilization is related to phospholipase C ζ (PLC ζ ; Parrington *et al.* 2002). PLC ζ has been identified as an important spermatozoan factor that activates the development of the embryo (Saunders *et al.* 2002, Swann *et al.* 2006). The reason why an alkaline medium abolishes the activation potential of spermatozoa is that the high-pH treatment inhibits PLC ζ , resulting in abrogation of the Ca²⁺ oscillation in oocyte (Kurokawa *et al.* 2005). However, the nuclear integrity of spermatozoa under this condition is still unclear. Moreover, there has been no report of the effects on embryo development when the injected spermatozoa

have been treated with extremely high-pH medium, and no live offspring have been reported using such spermatozoa.

In this study, we examined spermatozoal fertility and nuclear integrity after treating with highly alkaline medium. The sperm were treated with different concentrations of NaOH solution (1–100 mM) for 1 h. The morphological changes and vitality of the NaOH-treated spermatozoa were analyzed by live/dead assay, transmission electron microscopy (TEM), acridine orange (AO) staining, and the TUNEL assay. The oocyte activation potential of NaOH-treated spermatozoa was investigated by ICSI. The normality of male pronuclei in zygotes was examined by immunostaining of histone H2AX phosphorylation at serine 139 (γ H2AX), which is a DNA double strand breaks (DSBs) marker, and karyotype analysis after artificial activation. Finally, the nuclear integrity of NaOH-treated spermatozoa was demonstrated by the production of healthy offspring after embryo transfer into recipient females. These results showed that a suitably high pH can destroy both the plasma membrane and oocyte activation factors but does not injure the spermatozoal nucleus in terms of full-term embryonic development. From these results, we suggest that NaOH treatment is a simple method of spermatozoal pretreatment, and that these spermatozoa can be widely used in future research, investigating issues such as oocyte activation, sperm preservation, and transgenesis.

Results

Morphology and vitality of NaOH-treated spermatozoa

Although it has been reported that highly alkaline medium can inhibit the oocyte activation of porcine spermatozoa (Kim *et al.* 1999, Kurokawa *et al.* 2005), it is still unclear whether high pH values affect the morphology of spermatozoa. First, epididymal spermatozoa were treated with different concentrations of NaOH (1–100 mM) for 1 h. All the spermatozoa treated with NaOH lost their motility. Using the live/dead assay, we showed that 42.8% of the fresh spermatozoa and 100% of the NaOH-treated spermatozoa were biologically 'dead' (Fig. 1e–h, $n=3$). Next, to analyze the damage to the spermatozoa in detail, TEM was used to observe sagittal sections of the sperm heads after NaOH treatment. Only 13% of the fresh spermatozoa showed a dissolved membrane, and the acrosome was invisible in 6% of them (Fig. 1i). However, these rates were 43 and 65% respectively, in 1 mM NaOH-treated spermatozoa; 100 and 80% respectively, in 10 mM NaOH-treated spermatozoa (Fig. 1j and k); and 100 and 100% respectively, in 100 mM NaOH-treated spermatozoa (Fig. 1l). Importantly, in spermatozoa treated with 10 mM

Figure 1 Vitality and morphological alteration of spermatozoa after NaOH treatment. (a–d) The morphology of normal and NaOH-treated spermatozoa. (a) Fresh spermatozoa; (b) 1 mM NaOH-treated spermatozoa; (c) 10 mM NaOH-treated spermatozoa; (d) 100 mM NaOH-treated spermatozoa. Note that in (c) and (d), in spermatozoa treated with stronger NaOH, the head is separated from the tail. (a–d, scale bar = 20 μ m). (e–h) The pH of the medium and the vitality of the NaOH-treated spermatozoa assessed with the live/dead assay. Green fluorescence shows living spermatozoa, and red fluorescence shows dead spermatozoa. (e) Living spermatozoa constituted 57.2% of the fresh control group. However, all NaOH-treated spermatozoa were dead: (f) 1 mM NaOH-treated, (g) 10 mM NaOH-treated and (h) 100 mM NaOH-treated (e–h, scale bar = 200 μ m). (i–l) TEM images of spermatozoa heads after NaOH treatment. (i) The normal spermatozoa structure, with the plasma membrane and acrosome; (j–l) after NaOH treatment, the plasma membrane and acrosome were invisible: (i) 1 mM NaOH-treated spermatozoa, (k) 10 mM NaOH-treated spermatozoa and (l) 100 mM NaOH-treated spermatozoa (scale bar = 1 μ m). ac, acrosome; p, plasma membrane; and n, nucleus.

NaOH or stronger, the tails of most spermatozoa had detached from the heads (Fig. 1c and d), which made it easier to compare them with the tail-bearing intact fresh spermatozoa in ICSI.

Activation capacity of NaOH-treated spermatozoa after ICSI

To determine whether the spermatozoa treated with alkaline medium maintained their activation capacity, NaOH-treated spermatozoa were injected into MII oocytes using ICSI. As shown in Table 1, 1 mM NaOH-treated spermatozoa retained their oocyte activation potential, and most of the oocytes were activated after ICSI. However, the development rate to morula/blastocyst (M/B) stage was reduced (44%) compared with that of the control group (76.5%). Immunostaining showed that the zygotes fertilized by 1 mM NaOH-treated spermatozoa had normal H3K9 methylation status at both 6 and 24 h after ICSI (Fig. 2Aa–b'' and Ba–b''). However, 10 mM or stronger NaOH-treated spermatozoa lost their oocyte activation potential because most of the injected oocytes did not form either

male or female pronuclei (Fig. 2Ac–e''). The spindles derived from the sperm nuclei were clearly visible in these injected oocytes, even 24 h after ICSI (Fig. 2Bc–e''). This result is comparable with that of the heat-shock experiment described previously (Cozzi *et al.* 2001), in which the heat-treated spermatozoa had a morphology similar to that of the NaOH-treated spermatozoa.

In vitro development after artificial activation of NaOH-treated spermatozoa injected oocytes

It is well known that calcium-free medium containing strontium can activate oocytes, and that the resultant embryo fertilized with a defective sperm, such as a heat-treated spermatozoon or round spermatid (Ogura *et al.* 1994, Kimura & Yanagimachi 1995b, Cozzi *et al.* 2001), can develop to full-term. Therefore, we artificially activated oocytes to determine whether oocytes injected with NaOH-treated spermatozoa could develop normally. In all the NaOH-treated spermatozoa groups, most embryos formed male and female pronuclei following activation with Sr^{2+} for 1 h (Table 2). Immunostaining showed normal pronucleus formation

Table 1 Embryonic development after ICSI with NaOH-treated spermatozoa.

Medium	No. of oocytes survived (no. of replicates)	No. of oocytes activated (%) ^a	No. of two-cell embryos (%) ^a	No. of morula/blastocysts (%) ^a
HEPES–CZB	68 (5)	67 (98.5)	64 (94.1)	52 (76.5)
1 mM NaOH	95 (5)	83 (87.4)	79 (83.2)	42 (44.2)
10 mM NaOH	91 (5)	3 (3.3)	3 (3.3)	2 (2.2)
20 mM NaOH	81 (5)	0	0	0
50 mM NaOH	70 (5)	1 (1.4)	1 (1.4)	1 (1.4)

^aCompared with surviving oocytes.

Figure 2 Pronuclei formation and embryonic development check after injection of NaOH-treated spermatozoa by immunostaining. (A) Six hours after injection. (a–e'') Zygotes were stained for H3K9me3, β -tubulin and with DAPI at the pronucleus stage. (a–e) Pronuclei were stained with DAPI. (a'–e') Female pronuclei stained brightly for H3K9me3. (a''–e'') Staining for β -tubulin. Spindles are clearly visible in (c''–e''). (a) Oocytes were microinjected with fresh spermatozoa ($n=62$); (b) with 1 mM NaOH-treated spermatozoa ($n=30$); (c) with 10 mM NaOH-treated spermatozoa ($n=74$); (d) with 20 mM NaOH-treated spermatozoa ($n=20$); and (e) with 50 mM NaOH-treated spermatozoa ($n=20$). a–e'', scale bar=20 μ m. (B) Twenty-four hours after injection. (a–e'') *In vitro* development of zygotes at the two-cell stage. (a–e) Nuclei were stained with DAPI. Enlargement of (e) shows the remaining spermatozoal head 24 h after ICSI. (a'–e') Stained for H3K9me3. Only female-derived chromatin stained for H3K9me3. (a''–e'') β -Tubulin-directed staining of the cytoskeleton. In normal embryonic development, β -tubulin is located at the cell membrane (a'', b''), whereas in arrested zygotes, β -tubulin is still observed surrounding the nucleus (c''–e''). (a) ICSI using fresh spermatozoa ($n=13$); (b) 1 mM NaOH-treated spermatozoa ($n=17$); (c) 10 mM NaOH-treated spermatozoa ($n=35$); (d) 20 mM NaOH-treated spermatozoa ($n=13$) and (e) 50 mM NaOH-treated spermatozoa ($n=15$). a–e'', scale bar=20 μ m.

and the normal methylation status of H3K9 in all groups (Fig. 3a–e''). However, the *in vitro* development rate was quite different in each group. Although a slight reduction occurred, there was no significant difference between fresh, 1 mM NaOH-treated, and 10 mM NaOH-treated spermatozoa at the two-cell stage or the M/B stage (88.8–99.2 and 67.4–83.5% respectively). However, the

oocytes injected with spermatozoa treated with higher NaOH concentrations (20 or 50 mM) showed a low developmental rate at both the two-cell and M/B stages (63.4–7.7 and 30.5–0% respectively).

Because artificial activation can lead to the parthenogenetic development of oocytes without a male genome, it is necessary to examine whether those

Table 2 *In vitro* development after artificial activation.

Medium	No. of oocytes survived (no. of replicates)	No. of oocytes activated (%) ^a	No. of two-cell embryos (%) ^a	No. of morula/ blastocysts (%) ^a
HEPES–CZB	122 (5)	121 (99.2)	120 (99.2)	101 (83.5)
1 mM NaOH	67 (4)	67 (100)	61 (91.0)	48 (71.6)
10 mM NaOH	89 (4)	89 (100)	79 (88.8)	60 (67.4)
20 mM NaOH	85 (4)	82 (96.5)	52 (63.4)	25 (30.5)
50 mM NaOH	81 (5)	78 (96.3)	6 (7.7)	0

^aCompared with surviving oocytes.

Figure 3 Pronucleus formation in zygotes obtained by ICSI with NaOH-treated spermatozoa followed by artificial activation. Pronucleus formation 6 h after ICSI following injection of artificially activated NaOH-treated spermatozoa (a–e''). (a–e) DAPI staining of pronuclei; (a'–e') H3K9me3 staining of female pronucleus; (a''–e'') β -tubulin-directed staining of the cytoskeleton. There was no significant difference between any group injected with NaOH-treated spermatozoa (b: 1 mM ($n=26$); c: 10 mM ($n=30$); d: 20 mM ($n=21$); e: 50 mM ($n=20$)) and the group injected with fresh spermatozoa (a ($n=30$)). a–e'', scale bar=20 μ m.

blastocysts were derived from embryos fertilized with the NaOH-treated spermatozoa. After ICSI with NaOH treated green fluorescent protein (GFP) transgenic (Tg) mice-spermatozoa, 100% (26/26) of blastocysts micro-injected with the NaOH-treated GFP sperm exhibited GFP expression under u.v. light (Fig. 4a–c'). As expected, no GFP-positive blastocysts were found in the parthenogenetic group.

Influence of NaOH treatment of spermatozoa DNA

Previous research has reported that spermatozoal chromosomal damage occurs in some extreme environments (Tateno *et al.* 2000, Ward *et al.* 2003, Tateno & Kamiguchi 2004). Furthermore, in our study, fewer embryos that had been injected with NaOH-treated spermatozoa reached the M/B stage than did those injected with fresh spermatozoa. Therefore, it was necessary to analyze whether alkali treatment injures the DNA of spermatozoa. First, AO staining was used to examine the changes in spermatozoal DNA. Like fresh spermatozoa, spermatozoa treated with a lower concentration of NaOH had normal DNA, visible as distinct

green fluorescence (Fig. 5a–c''), whereas spermatozoa treated with higher concentrations of NaOH displayed abnormal DNA structures, in which AO combined with single-stranded DNA (ssDNA) to produce red fluorescence (Fig. 5d–e''; $n=3$). DNA fragmentation was also analyzed with TUNEL. Unlike the DNaseI-treated spermatozoa (positive control), none of the NaOH-treated spermatozoa were stained by 3,3'-diaminobenzidine (DAB; Fig. 5f–j'), meaning that there was no DNA fragmentation.

Chromosomal abnormalities in embryos injected with NaOH-treated spermatozoa

To examine whether chromosome damage can occur in NaOH-treated spermatozoa, karyotypes were analyzed 20 h after ICSI, when the zygotes were treated with vinblastine to inhibit the first mitotic cleavage. As shown in Table 3, over 80% of fresh-spermatozoa-injected zygotes had normal chromosomes. However, in those injected with NaOH-treated spermatozoa, the rate of abnormal chromosomes increased to 52% for 1 mM NaOH- and 33% for 10 mM NaOH-treated spermatozoa (Table 3 and Fig. 6). Moreover, most karyotypes showed abnormalities after 20 and 50 mM NaOH treatments (Table 3). Freeze-thawed and heat-treated spermatozoa were also tested as controls. Heat-treated spermatozoa showed more severe damage (63%) than NaOH-treated

Figure 4 Comparison of blastocysts produced by ICSI with NaOH-treated spermatozoa and parthenogenesis. (a–c') *In vitro* development assessed with GFP transgenic (Tg) mouse spermatozoa. Embryos were observed at the blastocyst stage. 100% of NaOH-treated Tg spermatozoa displayed GFP fluorescence (a and a'), as in the fresh-spermatozoa-injected embryos (b and b'), whereas no fluorescence was observed in the parthenogenetic oocytes (c and c'). a–c', scale bar=100 μ m.

Figure 5 Chromosomal DNA damage in NaOH-treated spermatozoa. (a–e'') Spermatozoa chromatin injury, apparent as single-stranded DNA (ssDNA) after NaOH treatment, visualized with AO stain. Normal DNA shows distinct green fluorescence, whereas the sperm head displaying red fluorescence contains ssDNA. (a–a'') Fresh spermatozoa; (b–b'') 1 mM NaOH-treated spermatozoa; (c–c'') 10 mM NaOH-treated spermatozoa; (d–d'') 50 mM NaOH-treated spermatozoa and (e–e'') 100 mM NaOH-treated spermatozoa. a–e'', scale bar = 10 µm. (f–j') TUNEL assay for NaOH-treated spermatozoa. Fragmented spermatozoal DNA was stained with DAB. (f and f') Fresh spermatozoa; (g and g') DNaseI-treated spermatozoa (positive control); (h and h') 1 mM NaOH-treated spermatozoa; (i and i') 10 mM NaOH-treated spermatozoa, and (j and j') 100 mM NaOH-treated spermatozoa. f–j', scale bar = 10 µm.

spermatozoa, and there was no significant difference between frozen and NaOH-treated spermatozoa. Although this result suggests that NaOH treatment causes more chromosomal damage than that is observed

in fresh spermatozoa, it has been reported that the DNA repair mechanism can partly rescue chromosomal damage during early embryonic development (Derijck *et al.* 2008).

Table 3 Ratios of normal karyotypes in zygotes injected with differently treated spermatozoa.

Type of spermatozoa	No. of zygotes examined (no. of replicates)	No. of normal chromosomes (%)	No. of abnormal chromosomes of different types		
			Fragment	Clump	Others ^a
Fresh	27 (4)	22 (81)	2	1	2
Heat-treated	35 (4)	13 (37)	8	10	4
Freeze-thawed	47 (4)	23 (49)	16	5	3
1 mM NaOH	56 (5)	27 (48)	25	2	2
10 mM NaOH	61 (5)	41 (67)	13	6	1
20 mM NaOH	45 (4)	12 (27)	14	13	5
50 mM NaOH	32 (2)	0	0	0	32

^aOthers included ring, abnormally configured, longer, and straighter chromosomes.

Figure 6 Karyotype analysis of zygotes obtained after ICSI with NaOH-treated spermatozoa. (a) Normal and (b) abnormal karyotypes of zygotes after ICSI with 10 mM NaOH-treated spermatozoa. (a) Forty normal chromosomes. (b) Twenty normal chromosomes and abnormal chromosomes with visible fragmentation (arrow). Scale bar = 10 μ m.

At the two-cell stage, DSBs were examined using immunostaining by the DSB marker γ H2AX. Embryos containing DSBs showed γ H2AX expression, and in each group, three patterns were observed: the normal type, the 1-cell arrest type and DSBs positive type, which positively expressed γ H2AX foci. In the fresh spermatozoa group, few DSBs positive type zygotes (7.1%) were observed (Table 4 and Fig. 7a–a''), whereas a significantly higher rate of the DSBs positive type was observed in the embryos obtained with frozen (58.3%) or heat-treated spermatozoa (45.8%; Table 4 and

Fig. 7b–c''). Although the number of DSBs expressing embryos generated from 1 mM NaOH- (54.5%) or 10 mM NaOH-treated spermatozoa (35.9%) was more than the number of DSBs embryos generated from fresh spermatozoa, about half the embryos were still the normal type, notably 64.1% in the 10 mM NaOH-treated group (Table 4). However, embryos generated by spermatozoa treated with 20 mM NaOH (65.8%) and most embryos injected with 50 mM NaOH-treated spermatozoa showed severe DSBs (Table 4 and Fig. 7f–g''). All the DSBs were identified as paternally derived because no γ H2AX expression was observed from the maternal chromosome contained for H3K9me3. Considering these results together, we suggest that although the DNA damage in the NaOH-treated spermatozoa was significant compared with that in the fresh spermatozoa, the appropriate concentration of NaOH treatment still maintained a low level of abnormalities compared with those generated any other methods used to dissolve the plasma membrane.

Full-term development of embryos injected with NaOH-treated spermatozoa

Two-cell-stage embryos that had been injected with NaOH-treated spermatozoa were transferred into recipient mice. Table 5 shows that 32% (20/64) of embryos injected with 1 mM NaOH-treated spermatozoa and 36.7% (106/289) of those injected with 10 mM NaOH-treated spermatozoa supported full-term development, whereas only 6% (5/98) of those injected with 20 mM NaOH-treated spermatozoa did so. All the offspring survived and were healthy. To examine whether the offspring remained fertile, we randomly selected a few offspring and mated them with normal ICR partners. As shown in Table 6, all offspring tested grew to adulthood and demonstrated their reproductive ability within three months after birth. These data indicate that although 10 mM alkali medium can completely reduce the fertility of spermatozoa, oocytes injected with these spermatozoa still retain their developmental potential after artificial activation.

Table 4 Chromosome abnormalities in two-cell-stage embryos injected with differently treated spermatozoa.

Type of spermatozoa	No. of embryos examined	No. of embryos arrested at 1-cell stage (%)	No. of zygotes developed to 2-cell stage		
			Embryos	γ H2AX posi (%)	Normal embryos (%)
Fresh	42	0	42	3 (7.1)	39 (92.9)
Heat-treated	24	1 (4.2)	23	10 (41.6)	13 (54.2)
Freeze-thawed	24	5 (20.8)	19	9 (37.5)	10 (41.7)
1 mM NaOH	33	6 (18.2)	27	12 (36.3)	15 (45.5)
10 mM NaOH	39	3 (7.7)	36	11 (28.2)	25 (64.1)
20 mM NaOH	38	16 (42.1)	22	9 (23.7)	13 (34.2)
50 mM NaOH	41	38 (92.7)	3	3 (7.3)	0

Figure 7 DSBs in embryos produced by ICSI with NaOH-treated spermatozoa, stained for γ H2AX. The two-cell-stage zygotes, which had been injected with different concentrations of NaOH-treated, freeze-thawed or heat-treated spermatozoa, were stained for the DSB marker γ H2AX (a–g), DAPI (a'–g') or H3K9me3 (a''–g''). Oocytes injected with fresh (a), freeze-thawed (b), heat-treated (c) or NaOH-treated spermatozoa (1 mM (d), 10 mM (e), 20 mM (f), 50 mM (g)). Arrow shows an ACS zygote, which is stained for γ H2AX. The numbers of zygotes examined are shown in Table 4. a–g'', scale bar = 20 μ m.

Discussion

In this study, we examined the nuclear integrity and the developmental potential of spermatozoa treated with highly alkaline medium. The concentration of NaOH was very important in the treatment of spermatozoa; 10 mM NaOH dissolved the membrane and destroyed the SOAF of the spermatozoa, and separated the tail from the head, so that ICSI could be performed easily. Moreover, 36.7% healthy pups were delivered after embryo transfer into recipient mice, and all of them maintained a normal reproductive capacity.

'Dead' spermatozoa after treatment with NaOH still retain developmental potential

Our data show that after treatment with NaOH, all spermatozoa lost their motility, and all NaOH-treated spermatozoa were 'dead' according to the live/dead

assay (Fig. 1e–h). However, the plasma membrane and acrosome are not essential to support normal embryonic development. So far, two types of treated spermatozoa have been reported that can still support normal embryonic development after ICSI. Type I has no membrane but retains its activation capacity, as do frozen spermatozoa and freeze-dried spermatozoa (Wakayama & Yanagimachi 1998, Wakayama *et al.* 1998, Ogonuki *et al.* 2006). Type II has no membrane or SOAF, like heat-treated spermatozoa, LL and pronase treated spermatozoa, round spermatids, and ATAB-treated spermatozoa (Ogura *et al.* 1994, Kimura & Yanagimachi 1995b, Ward *et al.* 1999, Cozzi *et al.* 2001, Yan *et al.* 2008). In the present study, we suggested that treatment of spermatozoa with NaOH results in the increased full-term development compared with other methods. The phenotype of zygotes can be divided into the above two types after treating with different

Table 5 Full-term development from NaOH-treated spermatozoa after artificial activation.

Medium	No. of oocytes survived (no. of replicates)	No. of oocytes activated (%) ^a	No. of two-cell embryos (%) ^b	No. of embryos transferred (recipients)	No. of offspring (%) ^c	No. of implanted embryos (%) ^c
HEPES-CZB	124 (8)	124 (100)	116 (93.5)	109 (8)	52 (48)*	N/A
1 mM NaOH	75 (3)	75 (100)	64 (85.3)	64 (6)	20 (32)*	28 (43.8)
10 mM NaOH	352 (11)	344 (97.7)	295 (85.8)	289 (22)	106 (36.7)*	169 (58.5)
20 mM NaOH	175 (7)	173 (98.9)	98 (56.0)	98 (7)	5 (6) [†]	11 (11.2)
50 mM NaOH	113 (4)	84 (74.3)	16 (14.2)	16 (4)	0 [†]	0

Significant difference between * and [†] ($P < 0.01$).

^aCompared with surviving oocytes. ^bCompared with activated oocytes. ^cCompared with the number of transferred oocytes.

concentrations of NaOH. Spermatozoa treated by weak alkaline (1 mM NaOH pH: 8.8) retains the fertility after ICSI (Table 1 and Fig. 2b–b''), which has already been demonstrated in freeze-dry experiments (Kaneko *et al.* 2003). On the other hand, high alkali (10 mM NaOH pH: 11.3) treated spermatozoa were similar to the type II because high pH treatment completely destroyed the plasma membrane of spermatozoa (Fig. 1i–l), and these spermatozoa cannot cause oocyte activation naturally (Table 1 and Fig. 2c–e'). These results are similar to a previous report by Kim *et al.* who showed that 100 mM NaOH-treated porcine spermatozoa lose fertility. It has also been reported that PLC ζ , which is regarded as an important spermatozoan factor that activates the development of the oocyte (Swann *et al.* 2006), is inhibited by high alkaline treatment (Kurokawa *et al.* 2005); however, there was no evidence that the loss of PLC ζ reduced the developmental rate of the spermatozoa, when we used strontium to artificially activate oocytes, as normal male and female pronuclei were formed, as assessed by immunostaining (Table 2 and Fig. 3a–e). In type II treated spermatozoa, although embryonic development can be rescued using artificial activation, the full-term development rate was significantly low. To our surprise, 36.7% of transferred embryos using NaOH-treated spermatozoa developed to full-term, which is three times higher than that by heated spermatozoa (12.5%; Cozzi *et al.* 2001) and, two times higher than that by LL and pronase treated spermatozoa (17%; Yan *et al.* 2008). After mating with ICR partners, all of the offspring from NaOH-treated spermatozoa retained reproductive ability (Table 6).

Table 6 The fertility of the offspring from NaOH-treated spermatozoa.

Offspring ID no.	Gender of the offspring	No. of pups born	Sex ratio (M/F)
1	Male	13	5/8
2	Male	9	6/3
3	Male	16	7/9
4	Male	8	3/5
5	Female	15	8/7
6	Female	14	6/8
7	Female	11	4/7
8	Female	12	7/5

We suggest that the offspring of NaOH-treated spermatozoa are not different from those stemming from fresh spermatozoa.

Chromosome damage in NaOH-treated spermatozoa

In the present study, compared with the fresh spermatozoa, the offspring success rate was somewhat low (36.7 vs 48%) which may be due to some extent chromosomal damage by high pH. In general, a highly alkaline environment can convert double-stranded chromosomal DNA into ssDNA (Mala *et al.* 1999). Moreover, it has been reported that a surprisingly large number of ssDNA breaks can be detected after high-alkaline treatment (Singh *et al.* 1989); thus, the assessment of the chromosomal DNA integrity of spermatozoa treated with various concentrations of NaOH becomes crucial. AO staining is an easy method with which to assess the proportion of ssDNA in abnormal spermatozoa (Tejada *et al.* 1984, Hammadeh *et al.* 2001). Based on AO staining, 1 and 10 mM NaOH-treated spermatozoa showed normal double-stranded DNA structures, like those of fresh spermatozoa, whereas in 50 and 100 mM NaOH-treated spermatozoa, ssDNA (red fluorescence) was apparent (Fig. 2a–e''). DSBs in the spermatozoal chromosome are another form of DNA damage that influences normal embryonic development (Derijck *et al.* 2006, 2008). In this study, both karyotype analysis and immunostaining for the DSB marker, γ H2AX, showed that 1 and 10 mM NaOH-treated spermatozoa had more chromosomal fragments and DSBs than those observed in fresh spermatozoa. However, more highly alkaline medium led to significantly more severe damage, which must have certainly resulted in injury to the spermatozoal DNA and explains why no embryos reached the M/B stage and no offspring were produced with 50 mM NaOH-treated spermatozoa (Table 2). This indicates that the alkali treatments destroyed a part of the spermatozoal developmental capacity and this was concentration dependent.

Although in the TEM images of NaOH-treated spermatozoa the phenotype is similar to those of ATAB-treated, heat-treated and freeze-dried spermatozoa (Tejada *et al.* 1984, Wakayama & Yanagimachi 1998, Ward *et al.* 1999, Cozzi *et al.* 2001), the percentage of

abnormal chromosomes in the NaOH-treated spermatozoa was still low compared with 63% in heat-treated spermatozoa and 51% in freeze-thawed spermatozoa. Similar results were obtained with staining for γ H2AX (Table 4 and Fig. 7). Considering these data together, we believe that the alkaline treatment of spermatozoa caused less damage than other methods, allowing safer nuclear denudation of the spermatozoa.

On the other hand, it was suggested that sperm-borne RNAs may play an important role in development and may affect the phenotype of offspring (Ostermeier *et al.* 2004, Rassoulzadegan *et al.* 2006). Recently, Yan *et al.* reported that the removal of perinuclear materials from spermatozoa heads using LL and pronase treatment led to a large decrease in RNA and miRNA when compared with fresh spermatozoa. Although some of those spermatozoa retained full-term developmental potential after ICSI and artificial activation, the birth rate of offspring was significantly reduced (only 17%) when compared with offspring of fresh control spermatozoa. By contrast, in our study, NaOH-treated spermatozoa did not lose their developmental potential, which may suggest that NaOH treatment has a lesser effect on RNA species. We plan to address this issue in future work.

Advantages of NaOH-treated spermatozoa and their future applications

To date, several methods have been exploited in research into the fertilization mechanism. Freeze-thawing spermatozoa without cryoprotectant can remove their membranes but reduces the developmental rate to offspring. Another shortcoming is that these spermatozoa still retain their activation capacity. Heat-treated spermatozoa completely lose their SOAF, but the subsequent low offspring rate has restricted correlation analysis. The treatment of spermatozoa with ATAB seems the best method because SOAF are lost, but the normal production rate of offspring is still retained after transfer. However, this treatment is not simple and might increase the stickiness of the treated spermatozoa so that ICSI cannot be performed smoothly. The reason why our apparently harsh treatment can result in comparatively high birth rates is that other methods, which include enzyme treatment or freeze-thaw, may result not only in membrane removal but also in DNA or RNA damage. Although it is known that DNA is susceptible to alkali treatment, our results show that chromosomal damage could be suppressed to a low level via the treatment of spermatozoa using optimal alkaline conditions; therefore, this method may lead to higher birth rates when compared with others. In the present study, NaOH-treated spermatozoa, especially those treated with 10 mM NaOH, demonstrated several advantages. 1) Spermatozoa treatment is very simple, insofar as the spermatozoal suspension need only to be mixed with NaOH solution and then neutralized with

the same concentration of HCl added to the medium, ensuring low cost and no need for special equipment. 2) Using this method, the spermatozoal head can be easily separated from the tail, so no Piezo pulse is required to cut the tail, which can contribute to paternal chromosome damage (Szczygiel *et al.* 2003, Tatenio & Kamiguchi 2007). In this study, we also showed that 1 mM NaOH-treated spermatozoa, in which Piezo pulses are required to cut the sperm tail, had greater damage and a lower offspring rate than spermatozoa treated with 10 mM NaOH. 3) There was only slight damage to the spermatozoal nucleus and a high rate of offspring production, all of which were healthy, including their germ cells, as demonstrated by natural mating.

The wide use of these spermatozoa in sperm preservation, and transgenesis is expected in future research. We anticipate that this method will allow the development of a novel preservation method for spermatozoa. Several methods have been developed for preserving live spermatozoa using cryoprotectants (Wakayama & Yanagimachi 1998, Wakayama *et al.* 1998, Ward *et al.* 2003, Ogonuki *et al.* 2006). The difficulty in preserving live sperm is that some cryoprotectants, such as sucrose, cannot pass through the sperm membrane. However, we and others have demonstrated that the membranes of spermatozoa are not necessary for the production of offspring. If these spermatozoa are used, any cryoprotectant can be used for their longer preservation because the sperm nuclei are exposed directly to the cryoprotectant. In our study, we easily obtained naked spermatozoa with negligible nuclear damage. We intend to test whether, using this simple method, spermatozoa can be readily treated with protectant for long-term preservation.

Another application of NaOH-treated spermatozoa is in the generation of transgenic mice. As we know, the first ICSI-mediated transgenic mouse was delivered using freeze-thawed, freeze-dried and Triton X-100-treated spermatozoa (Perry *et al.* 1999). However, the low rate of GFP-positive pups indicated a lethal shortcoming. To improve the success rate, several original plasmids and constructs have been designed to combine the transgene with the spermatozoa more easily (Kaneko *et al.* 2005, Moreira *et al.* 2005, Suganuma *et al.* 2005, Shinohara *et al.* 2007). However, there have been no advances in treating the spermatozoa. Here, naked spermatozoal chromosomes seemed to bind instantly to the transgene. We have already tried to use NaOH-treated spermatozoa mixed with GFP gene to create the transgenic mice, and succeeded in getting high percentage of GFP-positive offspring (C Li, unpublished observations). This indicates great scope for future research.

On the other hand, although the birth rate was higher and the percentage of chromosome damage was lower when compared with any other methods (Table 3), some unavoidable limitations remain. Specifically, a high

percentage of abnormal karyotypes were observed in the NaOH-treated spermatozoa when compared with fresh spermatozoa. If this method is used for other purposes, such as human clinical applications, this high risk may be apparent; thus, at the present stage, the NaOH treatment method should be limited to the study of basic biology to remove the plasma membrane and activate factors in laboratory animals.

In conclusion, we have reported here for the first time the effects of high pH on mouse spermatozoa. Although the plasma membrane and acrosome are destroyed and the SOAF lost, the spermatozoal chromosomes still retain their developmental potential. This may be an important pretreatment method for reproductive biology.

Materials and Methods

Animals

B6D2F1 (C57BL/6J×DBA/2) and ICR mice were obtained at 8–10 weeks of age from SLC (Hamamatsu, Japan). Transgenic mice carrying GFP (ICR background) were kindly provided by Dr Masaru Okabe (Okabe *et al.* 1997). All animals were maintained in accordance with the Animal Experiment Handbook at RIKEN, Center for Developmental Biology, Kobe, Japan.

Media

HEPES-buffered CZB (Kimura & Yanagimachi 1995a) medium was used for gamete handling and ICSI in air. CZB (Chatot *et al.* 1989) was used for embryo culture in an atmosphere of 5% CO₂ in air. Ca²⁺-free CZB containing 10 mM SrCl₂ (Wako, Osaka, Japan) was used to activate the NaOH-treated spermatozoa artificially (Kishigami & Wakayama 2007). For embryo incubation, CZB and SrCl₂-containing Ca²⁺-free CZB were overlaid with mineral oil (Wako).

Collection of spermatozoa and NaOH treatment

Epididymides were removed from male B6D2F1 mice. After both epididymal ducts were cut with sharp scissors, a few drops of the dense sperm mass were placed into an Eppendorf tube with 100 µL HEPES–CZB and incubated for 30 min at 37 °C. The spermatozoa suspension (10 µL) was mixed with 100 µL 1, 10, 20, 50 or 100 mM NaOH solution (Wako) in Eppendorf tubes and was left at room temperature for 1 h. HEPES–CZB significantly neutralized the 1 mM NaOH solution and reduced the pH value from 10.4 to 8.8, whereas only a minor change in the pH value was observed (11.3 in 10 mM NaOH-supplemented medium versus 11.5 in 10 mM NaOH solution). When the medium was supplemented with 50 mM or 100 mM NaOH, there was no difference in the pH of the NaOH-supplemented medium and the corresponding NaOH solution. The suspension was then neutralized by the same concentration of HCl (Wako). For the freeze-thaw treatment, 10 µL spermatozoal suspension in HEPES–CZB was directly placed at –30 °C. For heat treatment, 100 µL motile spermatozoal

suspension in a 1.5 ml tube was heated in a water bath for 30 min at 58 °C. The treated spermatozoa were used for analysis and ICSI. In a few control experiments, the spermatozoa collected from GFP–ICR mice were used to confirm the experimental procedure.

Oocyte preparation

Female B6D2F1 mice were caused to superovulate by the injection of 5 IU equine chorionic gonadotropin, followed 48 h later by 5 IU of human chorionic gonadotropin (hCG). Fourteen to sixteen hours after injecting hCG, cumulus–oocyte complexes (COCs) were collected from the oviducts and moved into HEPES–CZB in a Falcon dish. To disperse the cumulus, the COCs were transferred into a HEPES–CZB drop containing 0.1% bovine testicular hyaluronidase (Sigma Chemical Co.) for 3 min. The cumulus-free oocytes were washed twice and then moved to a new CZB droplet for culture.

ICSI and embryos transfer

To inject fresh spermatozoa, ~1 µL of the incubated spermatozoal suspension was mixed with a drop of HEPES–CZB containing 10% (w/v) polyvinylpyrrolidone (PVP; Irvine Scientific, Santa Ana, CA, USA). The sperm head was separated from the tail by the application of several Piezo pulses, and the head was then injected into the oocyte according to the method described by Kimura & Yanagimachi (1995a). To inject the NaOH-treated spermatozoa, 2 µL spermatozoal suspension was moved into another HEPES–CZB–PVP drop. Several spermatozoa heads were picked up and moved into a new HEPES–CZB–PVP drop for washing and were then injected into oocytes with the same method. The sperm suspension was replaced every 30 min during the ICSI experiment. The oocytes that survived after ICSI were incubated in CZB medium at 37 °C under an atmosphere of 5% CO₂. For the oocytes injected with NaOH-treated and heated spermatozoa, a Ca²⁺-free CZB medium containing 10 mM SrCl₂ was used to artificially activate these oocytes. Twenty microliters of 100 mM SrCl₂ stock solution dissolved in distilled water (stored at RT) was added to the 180 µL Ca²⁺-free CZB medium. Oocytes were incubated for at least 15 min after collection in the CZB media and were then placed in the activation medium for 1 h. After washing twice, oocytes were transferred to the CZB medium to resume incubation. Parthenogenetic activation was induced using the same activation medium in the presence of 5 µg/ml cytochalasin B.

ICR mice were used as the recipients of the embryos. Two-cell-stage zygotes were transferred to a day 0.5 pseudo-pregnant mouse, which had been mated with a vasectomized male the night before transfer. Six to ten embryos were transferred into each oviduct. At day 18.5, the offspring were delivered by caesarean section and allowed to mature.

Live/dead assay

The LIVE/DEAD Sperm Viability Kit (Molecular Probes Inc., Eugene, OR, USA) was used to analyze the viability of the spermatozoa. The procedures were performed according to the

manufacturer's protocol. Briefly, fresh spermatozoa and NaOH-treated spermatozoa were washed three times with HEPES–CZB. A 50-fold dilution of the SYBR 14 stock solution (component A) was prepared in HEPES–CZB just before use. Diluted SYBR 14 dye (5 µl) was added to a 1 ml sample of spermatozoa, which was then incubated for 10 min at 37 °C. Propidium iodide (5 µl, component B) was then added to the 1 ml sample of spermatozoa, to a final propidium iodide concentration of 12 µM, and incubated for a further 10 min. An aliquot (10 µl) of the spermatozoa sample was moved onto a glass slide (Matsunami, Osaka, Japan). The sample was observed under a BX51 fluorescence microscope (Olympus) equipped with a 488 nm filter. Images were captured with an Olympus DP70 digital camera.

Transmission electron microscopy

Fresh sperm suspension (200 µl) and neutralized NaOH-treated sperm suspension, as described above, were examined to assess the damage to the cell membrane. TEM analysis was performed by the Electron Microscope Laboratory, RIKEN, Center for Developmental Biology, Kobe, Japan.

AO staining

The AO staining method was based on previous reports (Tejada *et al.* 1984, Hammadeh *et al.* 2001). Each sample was prepared by spreading a 5 µl sample of spermatozoa onto slide glass and air drying it, before it was fixed in Carnoy's solution (methanol:glacial acetic acid = 3:1) overnight at room temperature. AO (Sigma Chemical Co.) was diluted to 1 g/l and stored at 4 °C in the darkness. The buffer solution contained 40 ml 0.1 M citric acid (Wako) and 2.5 ml 0.3 M Na₂HPO₄ (Wako). Diluted AO solution (10 ml) was mixed with buffer, at a final pH of 2.5. The slide glass was placed into the staining solution for 5 min in the darkness, gently rinsed with deionized water, and mounted with VectaShield Mounting Medium for fluorescence (Vector, Burlingame, CA, USA). The samples were observed by phase-contrast microscopy with 488 and 530 nm excitation filters, using an Olympus BX51 fluorescence microscope, and the images were captured with an Olympus DP70 digital camera.

TUNEL staining

The *In situ* Apoptosis Detection Kit (Takara Bio, Shiga, Japan) was used to analyze the DNA fragmentation of the spermatozoal chromosomes. The procedures were performed according to the manufacturer's protocol. The NaOH-treated spermatozoa were washed three times with PBS (Sigma Chemical Co.), spotted onto slide glass, air dried and fixed with 4% paraformaldehyde–PBS (Wako) for 30 min at ambient temperature. The samples were then treated with permeabilization buffer for 5 min on ice. As a positive control, spermatozoa were treated with 100 µg/ml DNaseI in buffer (50 mM Tris/HCl (pH 7.4; Nacalai, Kyoto, Japan), 5 mM MgSO₄ (Wako), 0.25 M sucrose (Wako)) for 1 h at 37 °C. The spermatozoa were covered with the reaction solution (5 µl terminal deoxynucleotidyl transferase, 45 µl labelling safe buffer), reacted for 90 min

at 37 °C, and the reaction was terminated by washing with 1% BSA–PBS. The samples were treated with HRP-conjugated anti-FITC antibody at 37 °C, washed, and stained with DAB–H₂O₂ (50 mg/l DAB in Tris–HCl (pH 7.6), 0.1 ml of 0.3% hydrogen peroxide) for 10 min. The slides were washed and observed by phase-contrast and bright-field microscopy using an Olympus BX51 microscope. Data were captured with an Olympus DP70 digital camera.

Karyotype analysis

After ICSI, the fertilized oocytes were cultured for 6 h at 37 °C and then transferred into CZB containing 0.006 µg/ml vinblastine (Sigma Chemical Co.) to arrest the oocytes at the M phase of the cell cycle. Culture was continued for 14 h, and then the zygotes were treated with acid tyrode solution to remove the zona pellucidae. The oocytes were then fixed with hypotonic solution (mixed with equal volumes of 1% sodium citrate (Wako) and 30% foetal bovine serum (Sigma Chemical Co.)) for 10 min at room temperature. The oocytes were then spread onto a slide glass, according to the gradual fixation/air-drying method described previously (Mikamo & Kamiguchi 1983). The samples were stained with 2% Giemsa (Merck) in PBS. The stained chromosomes were observed by bright-field microscopy using a BX41 microscope (Olympus). Normal zygotes were considered to contain 40 metaphase chromosomes.

Immunofluorescence microscopy

Pronucleus-stage and two-cell-stage embryos were fixed in 4% paraformaldehyde–PBS for 30 min, washed twice with 1% BSA–PBS, and transferred into 1% BSA–PBS containing 0.1% Triton X-100 (Nacalai) and incubated overnight at 4 °C. The oocytes were then rinsed twice with 1% BSA–PBS and incubated with the primary antibody at room temperature for 2 h. Here, the primary antibodies used were rabbit polyclonal antibody directed against histone H3 (trimethyl K9; ChIP grade; ab8898, Abcam), purified mouse anti-tubulin (BD Biosciences, San Jose, CA, USA), and anti-phospho-histone (Ser139; Upstate, Temecula, CA, USA). After the embryos had been washed twice in 1% BSA–PBS for 15 min each, they were incubated for 1 h with fluorochrome-conjugated secondary antibodies: Alexa-Fluor-488-labeled chicken anti-rabbit IgG (Molecular Probes Inc.) or Alexa-Fluor-568-labeled goat anti-mouse IgG (Molecular Probes Inc.). The embryos were washed, and their DNA was stained with 4, 6-diamidino-2-phenylindole (2 µg/ml; Molecular Probes Inc.). Finally, the embryos were washed twice and mounted on slides with VectaShield Mounting Medium. All the embryos were observed with a confocal scanning laser microscope (FV-1000, Olympus).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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