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Osmotic characteristics and fertility of murine spermatozoa collected in different solutions

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Abstract

Osmotic stress is an important factor that can result in cell damage during cryopreservation. Before ejaculation or collection for cryopreservation, murine spermatozoa are stored in epididymal fluid, a physiologically hyperosmotic environment (~415 mmol/kg). The objectives of this study were to determine the osmotic tolerance limits of sperm motion parameters of ICR and C57BL/6 mouse spermatozoa collected in isosmotic (290 mmol/kg) and hyperosmotic (415 mmol/kg) media, and the effect of the osmolality of sperm collection media on sperm fertility after cryopreservation. Our results indicate that murine spermatozoa collected in media with different osmolalities (290 and 415 mmol/kg Dulbecco's phosphate buffered saline (DPBS)) appeared to have different osmotic tolerances for the maintenance of sperm motility and other motion parameters in both mouse strains. The hypo- and hyperosmotic treatments decreased motility and affected other motion parameters of spermatozoa collected in 290 mmol/kg DPBS. The extent of the change of motion parameters after treatments corresponded with the levels of osmotic stress. However, for spermatozoa collected in 415 mmol/kg DPBS, exposure to 290 mmol/kg DPBS tended to increase sperm motility and the quality of their motion parameters. The osmolality of sperm collection medium can affect murine sperm fertility. Spermatozoa collected in 415 mmol/kg medium showed higher fertility compared with spermatozoa collected in 290 mmol/kg as assessed by *IVF*. Results characterizing murine sperm osmotic tolerance collected in media with different osmolalities from different strains and the effect of collection media osmolality on sperm fertility after cryopreservation will be useful in designing cryopreservation protocols.

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Introduction

Cryopreservation of murine spermatozoa has been of great interest in the biomedical community due to an exponential increase in genetically modified mouse models of human disease (Critser & Mobraaten 2000). Banking murine spermatozoa of all existing strains by efficient sperm cryopreservation protocols would significantly relieve the burden and the cost of conventional maintenance of these strains/lines as live animals (Sharp & Mobraaten 1997, Knight & Abbott 2002). Currently, the protocol developed by Nakagata (2000*a*, 2000*b*) is widely used as a method for the banking of murine spermatozoa of various strains at laboratories around the world. However, there has been no significant improvement since the development of that protocol (Critser & Mobraaten 2000) until recently when Stacy *et al*. (2006) improved the cryosurvival of mouse spermatozoa by optimizing the cooling rate of Nakagata's mouse sperm protocol and Bath (2003) achieved 38–88% fertilization rate by removing non-motile spermatozoa and cell debris from thawed sperm suspension of C57BL/6J mouse.

The cryobiological behavior of murine spermatozoa appears to be quite different from other types of cells and even spermatozoa from other species (Critser & Mobraaten 2000). Previous work has demonstrated that a permeating cryoprotectant such as glycerol may not be needed and may even be detrimental to murine spermatozoa during cryopreservation (Katkov *et al*. 1998); the most successful protocol for murine sperm cryopreservation requires media consisting of only non-permeating cryoprotectants (for example, raffinose and skimmed milk; Nakagata 2000*a*, 2000*b*). Because of the uniqueness of murine spermatozoa in response to cryopreservation, it is necessary to gain an in-depth understanding of their fundamental cryobiological properties in order to improve current murine sperm cryopreservation protocols and to develop an optimized

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protocol that could be consistently applied to the cryopreservation of murine spermatozoa from inbred strains as well as genetically modified lines.

During equilibrium cryopreservation, cells are dehydrated to a certain extent to prevent the formation of intracellular ice, a lethal condition that can result in the loss of viability of the cells. Usually chemical cryoprotective agents (CPAs) are used to achieve this purpose. Subsequently, the CPA(s) must be removed from the cells and their media after warming. During the processes of CPA addition and removal, cells experience osmotically driven volume changes. It has been demonstrated that murine spermatozoa are very sensitive to osmotic stress (Willoughby *et al*. 1996, Agca *et al*. 2002, Walters *et al*. 2005). Because of this, murine spermatozoa have a very limited ability to swell or shrink in response to osmotically driven volume excursions. In order to maintain $\geq$90% motility of murine spermatozoa from the B6C3F1 strain, volume excursions of the spermatozoa must be kept between 90 and 103% of isotonic volume (Willoughby *et al*. 1996).

For *in vitro* manipulation of murine spermatozoa, such as *IVF*, intracytoplasmic sperm injection as well as sperm biology studies, the media used for sperm collection have typically had an osmolality of ~290 mmol/kg (Nagy 2003). However, recent studies examining the osmolality of cauda epididymal fluid found that murine spermatozoa are actually stored in a hyperosmotic milieu in cauda epididymides ranging from 375 to 500 mmol/kg (Yeung *et al*. 1999, Cooper & Barfield 2006, Cooper *et al*. 2008). Even after ejaculation, spermatozoa are still at a slightly hyperosmotic condition in the female reproductive tract (uterine fluid is 330 mmol/kg; Yeung *et al*. 2000). From this we can see that murine spermatozoa are maintained in hyperosmotic conditions during nearly their entire life.

The widely used murine sperm freezing media, either consisting of raffinose and PBS (Koshimoto & Mazur 2002) or raffinose, skimmed milk, and water (Nakagata *et al*. 1997), have an osmolality of 418–480 mmol/kg – close to the osmolality of epididymal fluid (Yeung *et al*. 1999, 2006). However, previous studies defining the osmotic tolerance limits (OTLs) of mouse strains have used a sperm collection media with osmolality of 290 or 300 mmol/kg (Willoughby *et al*. 1996, Songsasen & Leibo 1997, Agca *et al*. 2002, Walters *et al*. 2005). Because the osmolality of the cauda epididymides ranges from 375 to 500 mmol/kg (Yeung *et al*. 2006, Cooper *et al*. 2008), spermatozoa have been subjected to a hyposmotic challenge during collection, and under hyposmotic conditions, sperm regulatory volume decreases, and osmolyte loss occurs, which are related to sperm fertility (Yeung *et al*. 2006, Cooper *et al*. 2008). Because of the extreme sensitivity of murine spermatozoa to osmotically driven volume excursions, we were interested in looking at the OTLs of murine spermatozoa when collected at 415 mmol/kg and challenged with anisosmotic solutions using 415 mmol/kg as the nominal isosmotic point and the osmolalities of sperm collection medium on sperm fertility after cryopreservation. Furthermore, a previous study has found that volume regulation of spermatozoa after exposure to hypotonic conditions is related to the influx of calcium and the subsequent achievement of fertility (Rossato *et al*. 1996). In addition to motility, other sperm motion parameters are also important for the assessment of murine sperm quality and fertility (Toth *et al*. 1991). Therefore, the effects of anisosmotic treatments on other sperm motion parameters have been associated with sperm fertility were also investigated in this study.

The objective of the present study was to investigate the OTLs of murine spermatozoa from ICR and C57BL/6 strains collected in an isosmotic solution (290 mmol/kg) and a hyperosmotic solution with an osmolality similar to epididymal fluid (415 mmol/kg) and their fertilization capabilities after cryopreservation.

Results

Experiment 1. The comparison of motion parameters of spermatozoa collected and assessed in 290 and 415 mmol/kg DPBS

The motion parameters (motility, average path velocity (VAP), straight line velocity (VSL), curvilinear velocity (VCL), lateral head displacement (ALH), and linearity (LIN)) of spermatozoa collected in 290 and 415 mmol/kg Dulbecco's phosphate buffered saline (DPBS) are shown in Fig. 1A–F. A significant difference was found for motility between the spermatozoa collected in 290 and 415 mmol/kg DPBS within each strain in both strains ($P<0.05$). In ICR mice, the motility of spermatozoa collected in 290 mmol/kg DPBS was significantly lower than that of spermatozoa collected in 415 mmol/kg DPBS (64.6 vs 70.5% respectively; $P<0.05$). In C57BL/6 mice, however, the motility of spermatozoa in 290 mmol/kg DPBS was significantly higher than that of spermatozoa collected in 415 mmol/kg DPBS (73.3 vs 45.6% respectively; $P<0.05$). In both strains, the values of VAP, VSL, VCL, and LIN of motile spermatozoa collected in 290 mmol/kg DPBS were found to be significantly higher than those of spermatozoa collected in 415 mmol/kg DPBS ($P<0.05$). The value of ALH of motile spermatozoa collected in 290 mmol/kg DPBS was found to be significantly higher than that of spermatozoa collected in 415 mmol/kg DPBS only in the C57BL/6 strain ($P<0.05$).

Experiment 2. The effect of anisosmotic treatments on sperm motion parameters (motility, VAP, VSL, VCL, ALH, and LIN)

Significant main effects ($P<0.05$) of osmolalities of sperm collection media (290 and 415 mmol/kg respectively) and genetic background were found on the

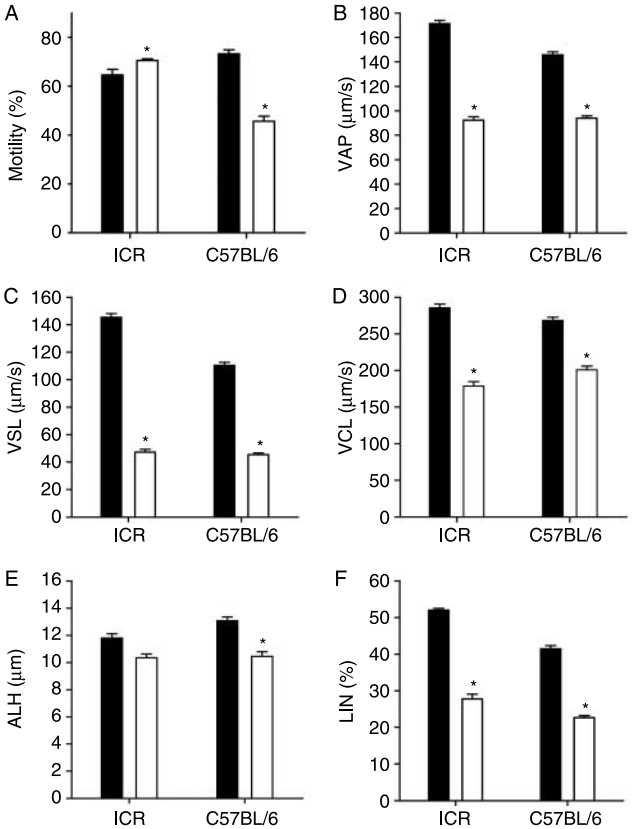

Figure 1 (A) Motility, (B) average path velocity (VAP), (C) straight line velocity (VSL), (D) curvilinear velocity (VCL), (E) lateral head displacement (ALH), and (F) linearity (LIN) of ICR and C57BL/6 mice spermatozoa collected in 290 (solid bar) and 415 (open bar) mmol/kg DPBS. *Significantly different between 290 and 415 mmol/kg DPBS within same strain.

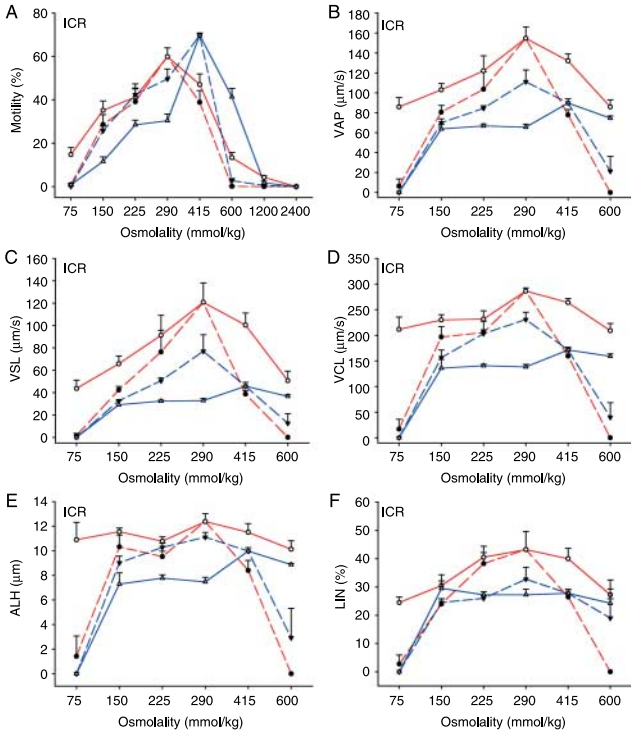

Figure 2 The motion parameters (A–F) of sperm from ICR mice collected in 290 (red lines) and 415 mmol/kg (blue lines) DPBS that were abruptly exposed to different osmotic conditions (dash lines) and abruptly returned to initial conditions (solid lines).

maintenance of sperm parameters after the anisosmotic treatments and upon return to the initial osmotic conditions.

The effects of the anisosmotic treatments on sperm motion parameters of the ICR and C57BL/6 mice are shown in Figs 2 and 3 respectively. As shown in Fig. 2A, the motility of ICR murine spermatozoa collected either in 290 mmol/kg or 415 DPBS was decreased significantly after all of the anisosmotic treatments ($P<0.05$). After a return to anisosmotic condition (290 mmol/kg), the motility of spermatozoa collected in 290 mmol/kg was not significantly increased ($P>0.05$). However, for spermatozoa collected in 415 mmol/kg DPBS, the motility of spermatozoa treated with 150, 225, and 290 mmol/kg solutions was significantly decreased after being returned to initial osmolality (415 mmol/kg; $P<0.05$). Only the motility of spermatozoa treated with 600 mmol/kg was significantly increased after being returned to 415 mmol/kg ($P<0.05$). As shown in Fig. 3A, the motility of C57BL/6 mouse spermatozoa collected in 290 mmol/kg DPBS was significantly decreased after being exposed to anisosmotic solutions ($P<0.05$). However, for the spermatozoa collected in 415 mmol/kg DPBS, the motility of spermatozoa exposed to 225 and 290 mmol/kg solutions was not decreased but was significantly increased when compared with spermatozoa kept in 415 mmol/kg DPBS ($P<0.05$). After being returned to 415 mmol/kg, the motility of spermatozoa treated with 150, 225, and 290 mmol/kg was significantly decreased to a level lower than those kept in 415 mmol/kg DPBS ($P<0.05$).

The effects of the anisosmotic treatments on other parameters (VAP, VSL, VCL, ALH, and LIN) of motile spermatozoa collected in 290 and 415 mmol/kg DPBS are summarized in Figs 2B–F and 3B–F. Since the motility of spermatozoa collected in 290 or 415 mmol/kg DPBS and treated with 1200 and 2400 mmol/kg solutions were less than 5% in both strains (Figs 2A and 3A), the spermatozoa treated with 1200 and 2400 mmol/kg were excluded from the following studies of the ability of motile spermatozoa maintaining VAP, VSL, VCL, ALH, and LIN during anisosmotic treatments.

In general, for spermatozoa collected in 290 mmol/kg DPBS, the responses of VAP, VSL, VCL, ALH, and LIN of motile spermatozoa to anisosmotic treatments were very similar between ICR and C57BL/6 mice. The exposure to anisosmotic solutions decreased the values of these motion parameters of motile spermatozoa and a return to initial osmolality recovered those motion

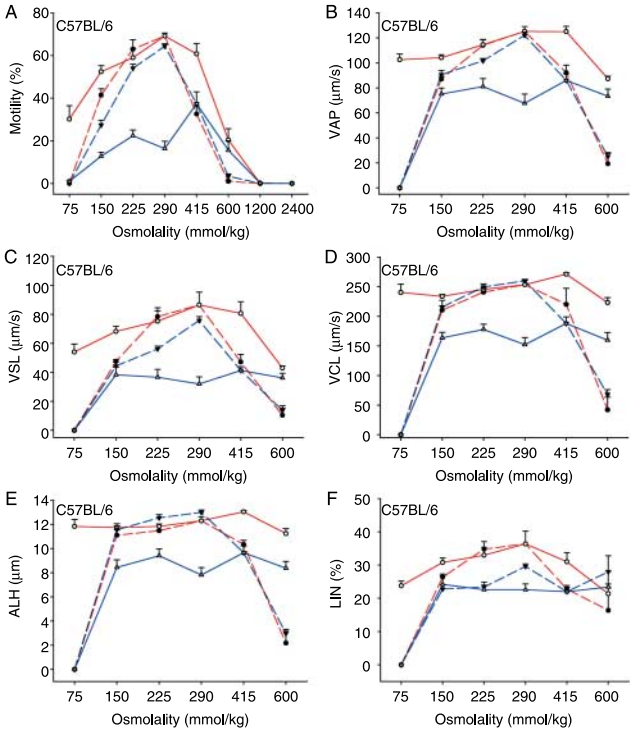

Figure 3 The motion parameters (A–F) of spermatozoa from C57BL/6 mice collected in 290 (red lines) and 415 mmol/kg (blue lines) DPBS that were abruptly exposed to different osmotic conditions (dash lines) and abruptly returned to initial conditions (solid lines).

parameters in some treatments (Figs 2B–F and 3B–F). The extent of the decrease of motion parameters after treatments corresponded with the levels of osmotic stress, which is in accord with the response of sperm motility to anisosmotic treatments. The responses of VAP, VSL, VCL, ALH, and LIN of motile spermatozoa collected in 415 mmol/kg DPBS to anisosmotic treatments were also very similar between the two mouse strains. The exposure of spermatozoa to low osmotic solutions especially to 290 mmol/kg DPBS increased the VAP, VSL, VCL, ALH, and LIN values of motile spermatozoa when compared with the spermatozoa kept in 415 mmol/kg DPBS in both ICR and C57BL/6 strains. However, the increase in VAP, VSL, VCL, ALH, and LIN was only significant in C57BL/6 ($P<0.05$). After being returned to initial osmotic conditions (415 mmol/kg), the VAP, VSL, VCL, ALH, and LIN values of motile spermatozoa treated with 290 mmol/kg solutions in both strains were significantly reduced to a similar level or lower than the spermatozoa kept in 415 mmol/kg DPBS (Figs 2B–F and 3B–F; $P<0.05$). The exposure of spermatozoa to hyperosmotic solution (600 mmol/kg) decreased the VAP, VSL, VCL, and ALH values of motile spermatozoa when compared with the spermatozoa kept in 415 mmol/kg DPBS in both ICR and C57BL/6 strains ($P<0.05$). After being returned to initial osmotic conditions (415 mmol/kg), the VAP, VSL, VCL, and ALH values of motile spermatozoa treated with 600 mmol/kg solution in both strains were significantly increased ($P<0.05$). However, the differences between LIN values of spermatozoa in hyperosmotic solution (600 mmol/kg) and after being returned to initial osmotic conditions (415 mmol/kg) were not significantly different in both strains ($P>0.05$).

Experiment 3. The effects of collection media osmolality on sperm cryopreservation and IVF

As shown in Tables 1 and 2, the motility, VAP, VSL, VCL, ALH, and LIN of spermatozoa collected either in 290 or 415 mmol/kg raffinose medium were significantly decreased after cryopreservation compared with fresh spermatozoa from both strains ($P<0.05$). However, no significant difference in LIN values was observed between fresh spermatozoa and frozen–thawed spermatozoa from C57BL/6 that were collected either in 290 or 415 mmol/kg raffinose medium before cryopreservation ($P>0.05$). Similar results were also obtained for spermatozoa from ICR ($P>0.05$). In C57BL/6 strain, the VAP of frozen–thawed spermatozoa that were collected in 415 mmol/kg medium before cryopreservation, was significantly higher than that of spermatozoa collected in 290 mmol/kg medium ($P<0.05$). However, this difference was not observed in ICR mice ($P>0.05$).

Cryopreserved spermatozoa from ICR mice were further used for *IVF* to assess the effect of the osmolalities of sperm collection media on sperm fertilizing ability. As shown in Table 3, the percentages of cleavage and blastocysts by frozen–thawed spermatozoa that were collected in 290 mmol/kg medium before cryopreservation were significantly lower than those by spermatozoa collected in 415 mmol/kg medium and fresh spermatozoa ($P<0.05$).

Table 1 Motion parameters of fresh spermatozoa (non-frozen) and frozen–thawed spermatozoa from ICR mice that collected in 290 and 415 mmol/kg group media for cryopreservation.

Collection medium	**Motility** (%)	**VAP** (μm/s)	**VSL** (μm/s)	**VCL** (μm/s)	**ALH** (μm)	**LIN** (%)
Non-frozen group	78 ± 4^{a}	168 ± 12^{a}	120 ± 13^{a}	326 ± 18^{a}	16 ± 1^{a}	36 ± 3^{a}
290 mmol/kg group	30 ± 2^{b}	117 ± 8^{b}	89 ± 8^{b}	224 ± 10^{b}	12 ± 1^{b}	38 ± 2^{a}
415 mmol/kg group	28 ± 4^{b}	118 ± 7^{b}	88 ± 8^{b}	233 ± 6^{b}	13 ± 1^{b}	36 ± 2^{a}

Different superscripts in a column indicate significant difference, $P<0.05$. *Sperm motion parameters were measured in 290 mmol/kg DPBS containing 2 mg/ml BSA after thawing.

Table 2 Motion parameters of fresh spermatozoa (non-frozen) and frozen–thawed spermatozoa from C57BL/6 mice that collected in 290 and 415 mmol/kg group media for cryopreservation.

Collection medium	Motility (%)	VAP (μm/s)	VSL (μm/s)	VCL (μm/s)	ALH (μm)	LIN (%)
Non-frozen group	73 ± 2^{a}	119 ± 4^{a}	84 ± 1^{a}	232 ± 16^{a}	14 ± 1^{a}	37 ± 3^{a}
290 mmol/kg group	28 ± 4^{b}	76 ± 4^{b}	48 ± 3^{b}	154 ± 6^{b}	9 ± 1^{b}	32 ± 1^{a}
415 mmol/kg group	29 ± 4^{b}	90 ± 2^{c}	52 ± 2^{b}	179 ± 2^{b}	11 ± 1^{b}	31 ± 1^{a}

Different superscripts in a column indicate significant difference, $P<0.05$. *Sperm motion parameters were measured in 290 mmol/kg DPBS containing 2 mg/ml BSA after thawing.

Discussion

The protocol for murine sperm cryopreservation developed by Nakagata (2000*a*, 2000*b*) has been widely used in different laboratories all over the world. The fertilizing ability of cryopreserved murine spermatozoa, however, is less than that of fresh spermatozoa in general and is especially low in many inbred strains (Sztein *et al*. 2000, Nishizono *et al*. 2004). Understanding the fundamental cryobiology of murine spermatozoa will assist in the development of improved freezing protocols for murine spermatozoa.

Studies have found that spermatozoa of many mammalian species are stored in a hyperosmotic environment in the epididymal fluid, and during the transit along the male genital tract (seminiferous tubules, epididymides, and vas deferens) spermatozoa are continuously exposed to high osmolality (Yeung *et al*. 1999, 2006, Cooper & Yeung 2003). The osmolality of human testicular tubular fluid is 312–380 mmol/kg and the osmolality of human fresh seminal plasma is reported to be 380 mmol/kg (Polak & Daunter 1984), and the osmolality of epididymal fluid of C57BL/6 mouse is 415 mmol/kg (Yeung *et al*. 1999, 2000, Cooper & Barfield 2006, Cooper *et al*. 2008), for example. Therefore, the examination of the effects of osmolalities of collection media on sperm fertility and the osmotic characteristics of murine spermatozoa collected in media with the osmolality of seminal fluid with a comparison of spermatozoa collected in isosmotic media will be useful to determine optimal media properties and cryopreservation protocols for murine spermatozoa.

Experiment 1. The comparison of motion parameters of spermatozoa collected in 290 and 415 mmol/kg DPBS

Our present study showed that the osmolality of the solution used for sperm collection and the genetic background of mice can affect the motion parameters of the spermatozoa. For the ICR strain, the motility of spermatozoa collected in isosmotic solution (290 mmol/kg) was slightly but significantly lower than that of spermatozoa collected in 415 mmol/kg solution, but for the C57BL/6 strain, the motility of spermatozoa collected in isosmotic DPBS (290 mmol/kg) was significantly higher than that of spermatozoa collected in 415 mmol/kg DPBS. These results indicate that a hyperosmotic environment (415 mmol/kg) may depress sperm motility of C57BL/6 mice but not of ICR mice. In this study, most of the other sperm motion parameters including VAP, VSL, VCL, and LIN of motile spermatozoa collected in 290 mmol/kg DPBS were found to be significantly higher compared with those of spermatozoa collected in 415 mmol/kg solutions in both strains. These results suggest that the exposure of murine spermatozoa to isosmotic conditions (290 mmol/kg) may activate murine spermatozoa by increasing the velocity of motile spermatozoa. By contrast, the exposure of murine spermatozoa to osmotic conditions similar to epididymal fluid seems to keep spermatozoa quiescent by depressing the activation of spermatozoa. Studies have shown that human spermatozoa collected in a medium with the same osmolality as seminal plasma (380 mmol/kg) results in an influx of calcium from the extracellular solution upon exposure to isotonic medium (300 mmol/kg), and this osmotically sensitive calcium influx may have a crucial regulatory role in the cellular events of sperm activation and fertilization (Rossato *et al*. 1996). Therefore, the physiological role of maintaining spermatozoa at a hypertonic surrounding in males is believed to prevent capacitation before spermatozoa are ejaculated into the female genital tract (Fisch *et al*. 1990, Rossato *et al*. 1996).

Experiment 2. The effect of anisosmotic treatments on sperm motion parameters (motility, VAP, VSL, VCL, ALH, and LIN)

Murine spermatozoa are very sensitive to osmotic volume excursions (Willoughby *et al*. 1996, Songsasen & Leibo 1997, Walters *et al*. 2005). For *in vitro* work, spermatozoa are usually collected in solutions with an osmolality of

Table 3 Fertility of fresh spermatozoa (non-frozen group) and frozen–thawed spermatozoa that were collected in 290 and 415 mmol/kg group media before cryopreservation and the development of embryos.

		Developmental stage reached (%)	
Spermatozoa for IVF	Total no. of oocytes	2-Cell	Blastocyst
Non-frozen group	72	48 (66.7%)[a]	30 (62.5%)[a]
290 mmol/kg group	63	26 (41.3%)[b]	13 (50.0%)[b]
415 mmol/kg group	70	49 (70.0%)[a]	35 (71.4%)[a]

Different superscripts in a column indicate significant difference, $P<0.05$.

290 mmol/kg. It has been demonstrated that the change of osmolality from ~415 mmol/kg to 290 mmol/kg results in active cell volume regulation and defects in the regulatory mechanism will result in male infertility (Yeung *et al*. 1999, Cooper *et al*. 2008). This change may have some profound effects on spermatozoa's ability to tolerate subsequent osmotic challenge, such as the addition and removal of CPAs during cryopreservation. In addition, much of the loss of sperm fertilizing ability during cryopreservation is associated with osmotic injury (Meyers 2005). Sperm motion parameters such as motility, VAP, VSL, VCL, ALH, and LIN have been found to be highly related to *IVF* rate and are useful in fertility prediction (Toth *et al*. 1991, Verstegen *et al*. 2002), In this study, spermatozoa from ICR and C57BL/6 mice were collected in (1) 290 mmol/kg DPBS, which is considered as the nominal isosmolality in many studies (Willoughby *et al*. 1996, Songsasen & Leibo 1997, Agca *et al*. 2002, Walters *et al*. 2005) and (2) 415 mmol/kg DPBS, which has been shown to be the physiological osmolality of epididymal fluid of C57BL/6 mouse (Yeung *et al*. 1999, 2000). When spermatozoa were collected in 290 mmol/kg DPBS, we found that both hyposmotic and hyperosmotic treatments resulted in sperm motility loss in both strains, and a return to 290 mmol/kg did not result in significant recovery in sperm motility. Similar to the response of sperm motility, the anisosmotic treatments also resulted in a decrease of VAP, VSL, VCL, ALH, and LIN of motile spermatozoa collected in 290 mmol/kg DPBS in both mouse strains. However, unlike motility, certain motion parameters could be recovered after return to 290 mmol/kg. When murine spermatozoa were collected in DPBS with the osmolality (415 mmol/kg) of epididymal fluid, however, the responses of spermatozoa to the anisosmotic treatments were clearly different compared with the spermatozoa collected in isosmotic DPBS (290 mmol/kg). In this study, the responses of motion parameters (VAP, VSL, VCL, ALH, and LIN) of spermatozoa collected in 415 mmol/kg DPBS were similar between the two mouse strains: the exposure of spermatozoa to hyposmotic (225 mmol/kg) and isosmotic solution (290 mmol/kg) resulted in an increase of the motion parameter values of motile spermatozoa in general. After a return to 415 mmol/kg, the motion parameter values significantly dropped to the same level or below of those spermatozoa kept in 415 mmol/kg solutions. These results are consistent with the theory that the exposure of murine spermatozoa to an osmotic condition similar to the epididymal fluid keeps spermatozoa quiescent by depressing the motility and velocity of spermatozoa, but that the exposure of murine spermatozoa to an isosmotic condition (290 mmol/kg) or hyposmotic condition (225 mmol/kg) could activate murine spermatozoa. The responses of spermatozoa to hyperosmotic exposure were different between ICR and C57BL/6. In the ICR mouse, we found that the motility of spermatozoa collected in 415 mmol/kg DPBS maintained $41.4 \pm 3.9\%$ of their original motility after exposure to 600 mmol/kg solution and upon return to 415 mmol/kg conditions, while the motility of spermatozoa collected in 290 mmol/kg DPBS decreased to $13.4 \pm 2.4\%$ after exposure to 600 mmol/kg solution and being returned to isotonic condition. These results indicate that the collection of spermatozoa in 415 mmol/kg DPBS could improve the maintenance of sperm motility upon exposure to hypertonic treatments (600 mmol/kg) in the ICR mouse. However, similar results were not observed in spermatozoa from C57BL/6 mice. The effect of the osmolality of spermatozoa collecting medium seems to be related to the genetic background of mouse. The failure to extend the resistance to hyperosmotic stress in C57BL/6 spermatozoa by collecting them in 415 mmol/kg solutions may be a reason why sperm cryopreservation of inbred mice, especially the C57BL/6 strain, is not as successful as it is in hybrid or outbreed strains.

In this study, despite of the increase of other motion parameters such as VAP, VSL, VCL, and LIN, the exposure of ICR mouse spermatozoa collected in 415 mmol/kg DPBS to 290 mmol/kg DPBS did not increase sperm motility, a phenomenon observed in C57BL/6 mouse. This may be in part due to the fact that the osmolality of epididymal fluid used here was from measurements on C57BL/6 strain (Yeung *et al*. 1999, 2000). Because we see differences in other membrane parameters and sensitivities (Willoughby *et al*. 1996), it is reasonable to expect that the osmolality of epididymal fluid could be different for different mouse genetic backgrounds. Measurements of these osmolalities would be illustrative. Recent studies have shown that the physical osmolalities of mouse cauda epididymides ranges from 375 to 500 mmol/kg depending on strains or lines (Cooper & Barfield 2006, Cooper *et al*. 2008). We suspect that the different response of sperm motility between ICR and C57BL/6 mouse may be caused by a higher physiological osmolality of epididymal fluid in the ICR strain. Therefore, when ICR mouse spermatozoa are exposed to 415 mmol/kg medium they are still hyposmotically challenged and the depression to the velocity of spermatozoa is less marked compared with spermatozoa from C57BL/6 strain, and a high percentage of spermatozoa are still evaluated as motile using the Hamilton Thorne.

It has been shown that the freezing/thawing processes can result in an acceleration of sperm capacitation (Critser *et al*. 1987, Watson 1995) and spermatozoa in this state display hyperactivated motion parameters – a reduction in progressive motility and LIN, with a concomitant increase in beat cross frequency (BCF) and ALH (Ohmuro & Ishijima 2006, Muiño *et al*. 2008). However, it seems that osmotic stress may not be a contributing factor to this phenomenon because there was not a significant decrease in ALH and significant increase in BCF (data not shown) in both strains.

Experiment 3. An investigation of the effects of collection media osmolality on sperm fertility after cryopreservation

Since the OTLs of murine spermatozoa collected in 290 and 415 mmol/kg medium are different, we examined the effect of osmolalities of collection media on sperm fertility after cryopreservation. When we collected ICR mouse spermatozoa into 290 and 415 mmol/kg DPBS medium and further diluted with cryopreservation medium, however, the post-thaw sperm motility was 15 and 3% respectively. These results made us consider that the extremely low cryosurvival might be due to the toxic effect of sodium chloride during sperm freezing and thawing. Therefore, we used raffinose instead of sodium chloride to prepare the 290 and 415 mmol/kg medium to collect spermatozoa and performed the consequent sperm cryopreservation. In our study, after collection in 100 μl 290 or 415 mmol/kg medium, spermatozoa were further diluted at a ratio of 1–9 with regular murine sperm cryopreservation medium for cryopreservation. Note that the difference in raffinose concentration between the two groups was negligible. Post-thaw motility after collection in raffinose containing media at 290 and 415 mmol/kg was 30 and 28% respectively for ICR mice and 28 and 29% respectively for the C57BL/6 mice. We did not examine the differences in kinematics of spermatozoa after freezing–thawing between the two strains because cryopreservation was done without penetrating CPA and no hypotonic volume change would be expected. Instead, we performed IVF using frozen/thawed spermatozoa from ICR. In addition to the increased ability to resist hyperosmotic challenge based on the OTL experiment, spermatozoa collected in 415 mmol/kg medium also had higher fertility and developmental competence than those collected in 290 mmol/kg medium, as demonstrated by the cleavage and blastocyst rates. The increased fertility and developmental competence of spermatozoa collected in 415 mmol/kg may be partially due to the reduced chance of cell swelling during cryopreservation. For spermatozoa collected in 290 mmol/kg medium, the direct exposure from epididymides (hyperosmotic) to isosmotic condition subjected them to cell swelling, and volume regulation driven by this osmotic change might initiate sperm capacitation and activation (Rossato *et al*. 1996). However, after further dilution with hyperosmotic sperm cryopreservation medium and subsequent extracellular ice formation during freezing spermatozoa were subjected to cell shrinkage. After thawing spermatozoa experienced swelling during the removal of CPAs and the process of *IVF*. By contrast, spermatozoa collected in 415 mmol/kg medium experienced cell swelling only after cryopreservation and thawing. Since sperm swelling leads to the loss of osmolytes and the ability of spermatozoa to regulate volume, both of which are related to sperm capacitation and fertility (Rossato *et al*. 1996, Cooper & Barfield 2006), and dysfunction of the volume regulatory ability of spermatozoa to hyposmotic condition causes infertility (Yeung *et al*. 2006, Cooper & Barfield 2006, Cooper *et al*. 2008), the extra experience of osmotic challenges and the associated cell swelling of the group of spermatozoa collected in 290 mmol/kg medium might lead to reduced responses to the hyposmotic challenges when spermatozoa from this group are subjected to *IVF* and result in lower fertility. However, more studies are needed in this direction.

In conclusion, the osmolalities of sperm collecting solutions affected the OTLs of spermatozoa. Spermatozoa collected in a hyperosmotic solution with an osmolality similar to epididymal fluid (415 mmol/kg) resulted in an increase in sperm osmotic tolerance to higher osmolality compared with those collected in 290 mmol/kg and also resulted in high fertility of spermatozoa after cryopreservation. These results are useful when designing protocols that prevent or reduce osmotic damage and loss of fertility during handling and cryopreservation.

Materials and Methods

Animals

Mature male mice between 10 and 12 weeks old from ICR and C57BL/6 genetic backgrounds (Harlan, Indianapolis, IN, USA) were used as sperm donors, and female ICR mice between 4 and 5 weeks old were used as oocyte donors for IVF in this study. The mice were housed in a room with a 12h light:12h darkness cycle and provided with sterile food and water *ad libitum*. The temperature was controlled at 22 °C. All animals were handled in accordance with the policies of the University of Missouri Animal Care and Use Committee, and the Guide for the Care and Use of Laboratory Animals of Association for Assessment and Accreditation of Laboratory Animal Care International.

Media

All chemicals were from Sigma Chemical unless otherwise stated. In experiment 1, DPBS, (Gibco #14287-080; Invitrogen) was used to prepare solutions with different osmolalities. Hyposmotic solutions (75, 150, and 225 mmol/kg) were prepared by diluting the isosmotic DPBS medium (290 mmol/kg) with water, and hyperosmotic solutions (415, 600, 1200, and 2400 mmol/kg) were prepared by adding appropriate amounts of sodium chloride to isotonic DPBS medium. Osmolalities of the solutions were measured using a vapor pressure osmometer (VAPRO 5520, Wescor, Logan, UT, USA) with an accuracy of ± 5 mmol/kg. Prior to use, 2 mg/ml BSA was supplemented into all of the solutions.

In experiment 2, murine sperm cryopreservation medium containing 18% raffinose and 3% skimmed milk was prepared as described by Nakagata (2000*a*, 2000*b*). Media at osmolalities of 290 and 415 mmol/kg prepared by dissolving

appropriate amounts of raffinose in water containing 3% skimmed milk were used as sperm collection media before cryopreservation in experiment 2.

Sperm motion parameters analysis

Computer-assisted sperm analysis (CASA; Hamilton Thorne IVOS v 12.2c, Beverly, MA, USA) was used to analyze murine sperm motion parameters: sperm motility (%), VAP (μm/s), VSL (μm/s), VCL (μm/s), ALH (μm), and LIN (%). Spermatozoa were analyzed according to the following parameters described by Hamilton Thorne: frames acquired 30, frame rate 60 Hz, minimum contrast 50, minimum cell size 5 pixels, default cell size 8 pixels, default cell intensity 90, VAP cut off 5 μm/s, intensity gate 0.50–1.30, size gate 0.30–1.95, elongation gate 0–87, magnification 0.79, video frequency 60, temperature set 37 °C, and chamber depth 80 μm.

Experiment 1. Determination of the OTLs of ICR and C57BL/6 spermatozoa collected in 290 and 415 mmol/kg DPBS

Spermatozoa from ICR mice ($n=8$) and C57BL/6 mice ($n=6$) were used for the experiment, and all of the experiments were performed at room temperature. Mice were killed and the cauda epididymidum were excised using eye scissors. Blood and adipose tissue were removed. One cauda epididymis of each mouse was placed into 500 μl DPBS (290 mmol/kg), and the contralateral cauda epididymis was placed into 500 μl DPBS (415 mmol/kg). Cauda epididymidum were well minced, and murine spermatozoa were allowed to swim out for 10 min at 37 °C in each solution. Sperm suspensions ($\sim 2\times 10^7$ cells/ml) in 20 μl aliquots collected in 290 mmol/kg DPBS were added to each of seven 1.5 ml Eppendorf centrifuge tubes containing DPBS solutions with different osmolalities (75, 150, 225, 290, 415, 600, and 1200 mmol/kg) respectively. Spermatozoa were equilibrated in these DPBS solutions with different osmolalities for 5 min, and the sperm motion parameters of each treatment were evaluated immediately using the Hamilton Thorne IVOS CASA system. The anisosmotically treated spermatozoa, which were collected in 290 mmol/kg DPBS solution, were then returned to osmolality (290 mmol/kg) by adding appropriate amounts of DPBS solutions at other corresponding osmolalities without centrifugation (Willoughby *et al.* 1996, Walters *et al.* 2005). The treatments of sperm suspension collected in 415 mmol/kg DPBS solution were essentially the same as those collected in 290 mmol/kg DPBS except that the anisosmotically treated spermatozoa were returned to 415 mmol/kg rather than 290 mmol/kg. The CASA evaluation for spermatozoa collected in 415 mmol/kg DPBS also followed the procedure of spermatozoa collected in 290 mmol/kg DPBS.

Experiment 2. Sperm cryopreservation and IVF

Sperm cryopreservation

Spermatozoa from ICR mice ($n=6$) and C57BL/6 mice ($n=4$) were used for the experiment. Sperm collection was performed as described in Experiment 1 except that cauda epididymidum of each mouse was collected in 100 μl medium prepared by dissolving different amounts of raffinose in water containing 3% skimmed milk with osmolalities of 290 and 415 mmol/kg respectively rather than DPBS. Spermatozoa were allowed to swim out of the cauda epididymides at 37 °C for 10 min and were further diluted with 1 ml sperm cryopreservation medium prepared by dissolving 18% raffinose and 3% skimmed milk in water (Nakagata 2000*b*). Diluted sperm suspensions (100 μl each) were loaded in 0.25 ml cryostraws (Conception Technologies, San Diego, CA, USA) and frozen in liquid nitrogen vapor according to the method described by Nakagata (2000*b*). Straws were thawed by directly transferring from the liquid nitrogen into a 37 °C water bath. Frozen–thawed sperm suspensions were diluted with 1 ml DPBS medium supplemented with 2 mg/ml BSA and centrifuged at 300 ***g*** for 5 min. The soft pellets were resuspended in DPBS medium with 2 mg/ml BSA and an aliquot was taken for sperm motion parameter examination.

IVF

Since the fertilization rate of C57BL/6 strain is typically low when cryopreserved spermatozoa are used to inseminate oocytes using a standard murine IVF protocol (Sztein *et al.* 2000), IVF was only used for assessing the fertility of cryopreserved spermatozoa from ICR mice collected in media with osmolalities of 290 and 415 mmol/kg (four replicates).

Three 4 to 5 week-old ICR female mice were superovulated by i.p. injection of 5 IU pregnant mare serum gonadotropin followed by 5 IU human chorionic gonadotropin (hCG) for each replicate experiment. Thirteen hours after the hCG injection, the females were killed and the oviducts were removed. The oocyte–cumulus complexes (COCs) were isolated in flushing holding medium (Lawitts & Biggers 1993) containing 0.4% BSA. Cumulus cells were removed by treating the COCs with 1 mg/ml hyaluronidase.

Fresh or washed frozen/thawed spermatozoa were added to 100 μl human tubal fluid (Quinn *et al.* 1985) medium with 4 mg/ml BSA at the concentration of 1×10^6 motile spermatozoa/ml and were cultured at 37 °C under 5% CO_2 in air for 1 h respectively. Oocytes were randomly divided into three groups and inseminated with fresh or frozen/thawed spermatozoa that were collected in 290 or 415 mmol/kg raffinose medium before cryopreservation respectively. After 6 h of co-culture with spermatozoa, the oocytes of each group were washed to eliminate excess spermatozoa and then cultured in 30 μl drops of potassium simplex optimization medium (KSOM, Lawitts & Biggers 1993) with 1 mg/ml BSA. The next morning, the number of two-cell embryos was scored, and the embryos were transferred to fresh drops of KSOM medium with 1 mg/ml BSA for culture until the blastocyst stage.

Statistical analysis

All data are expressed as mean ± S.E.M. A paired two-tail *t*-test was used to analyze the difference between sperm motion parameters of spermatozoa collected in 290 and 415 mmol/kg

DPBS in Experiment 1. ANOVA using the General Linear Model univariate procedure of the SPSS software (SPSS INC., Chicago, IL, USA) and a Tukey multiple comparison test was used to determine the effects of osmolalities (290 and 415 mmol/kg) of sperm collecting media and genetic backgrounds on sperm motion parameters in experiment 1. An ANOVA followed by Tukey's test was used to analyze the differences of sperm motion parameters, the fertilization rate and a Dunnet's *post hoc* test was used to compare embryo development among fresh sperm controls and cryopreserved spermatozoa collected in 290 and 415 mmol/kg media in experiment 2. Differences were considered significant using an α value of 0.05.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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