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## Impact on Hyperactivated Motility of Cryopreserved Mouse Sperm from Pretreatment with Thimerosal

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### ABSTRACT

Hyperactivated motility of sperm cells is essential to successful fertilization. This study was performed to observe the effect of Thimerosal treatment of cryopreserved mouse sperm on their hyperactivated motility and subsequent development to two-cell embryos after *in vitro* fertilization. Hyperactivated motility was evaluated by curvilinear velocity (VCL, the rate of travel of the sperm head) and Amplitude of Lateral Head Displacement (ALH, degree of side-to-side head movement measured as the mean width of head oscillations) of cells using Computer-assisted Sperm Analysis (CASA). The fresh sperm had higher VCL and ALH than frozen-thawed sperm ( $p < 0.05$ ). Following *In Vitro* Fertilization (IVF), fertilization rate from frozen-thawed sperm was significantly lower than that from fresh sperm (71.6, 94.4%) ( $p < 0.05$ ). Neither VCL nor ALH differed significantly between fresh sperm and frozen-thawed sperm not treated with Thimerosal. The decreased hyperactivated motility of mouse sperm due to cryopreservation may contribute to reduced fertilization rate compared to that achieved with fresh sperm. In addition because Thimerosal treatment of sperm after cryopreservation enhances hyperactivated motility, its use may improve the development of cryopreserved mouse oocytes from cryopreserved sperm.

**Key words:** Cryopreservation, spermatozoa, CASA measurements, hyper activated motility, thimerosal

### INTRODUCTION

Hyperactivated motility is a movement pattern observed in spermatozoa at the site and time of fertilization in mammals (Ho and Suarez, 2001). It is characterized by high-amplitude and asymmetrical flagellar beating of sperm cells which assist them in penetrating the oocyte zona pellucid (Yanagimachi, 1994). In the mouse, hyperactivated sperm can be observed through the wall of the oviduct (Suarez and Pacey, 2006; Abou-Haila and Tulsiani, 2009), pulling off attachments to the mucosal epithelium and rapidly escaping from peripheral storage sites into the central lumen (Suarez and Ho, 2003; Rubinstein *et al.*, 2006; Eisenbach and Giojalas, 2006).

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Ca<sup>2+</sup> signaling in sperm is critical for successful hyperactivated sperm motility. On the one hand, Ca<sup>2+</sup> acts through calmodulin on the axoneme to modulate flagellar curvature (Ho *et al.*, 2002); whereas on the other hand, Ca<sup>2+</sup> influx may regulate ATP homeostasis (Xia *et al.*, 2007). Two mechanisms trigger the rise of intracellular Ca<sup>2+</sup> to initiate and maintain hyperactivation: increased Ca<sup>2+</sup> entry via four plasma membrane CatSper ion channels (Qi *et al.*, 2007) and release of Ca<sup>2+</sup> from an inositol 1,4,5-trisphosphate (IP<sub>3</sub>)-gated internal store at the base of the flagellum (Ho and Suarez, 2001). These CATSPER proteins are from alkaline-sensitive voltage-gated and Ca<sup>2+</sup> channels are located on the principal piece of the flagellum (Ren *et al.*, 2001). Four CATSPER (Casper1, Casper2, Casper3, Casper4) channels have clearly been shown to be required for male fertility in that male mice deficient in any one of the four CATSPERs are completely sterile but exhibit no other apparent abnormalities (Ren *et al.*, 2001; Quill *et al.*, 2001, 2003; Kirichok *et al.*, 2006; Jin *et al.*, 2007). IP<sub>3</sub> receptors localized to a portion of the redundant nuclear envelope on the side of the flagellum have been shown to increase bend amplitude in response to Ca<sup>2+</sup> (Ho and Suarez, 2003). It is evident that release of internal Ca<sup>2+</sup> stores was sufficient to initiate hyperactivation in wild-type sperm, similar to results from bull sperm (Ho and Suarez, 2001).

Successful cryopreservation of semen is important not only for the preservation of genetic resources but also for artificial insemination (Salamon and Maxwell, 2000; Dumpala *et al.*, 2006; Sundararaman and Edwin, 2008; Shakeri *et al.*, 2008). Although much progress has been made since this technique was introduced in 1949 (Polge *et al.*, 1949), cryopreservation of mammalian sperm remains more difficult than cryopreservation of diploid cells. Previous studies have shown that cryopreservation induces ultrastructural damage to plasma (Gillan *et al.*, 1995) and to acrosomal membranes (Purdy, 2006; Saad *et al.*, 2011) and that it lowers mitochondrial membrane potential (Pena *et al.*, 2009; Nauk, 1991). However, systematic investigations of hyperactivated sperm motility following cryopreservation have not been reported.

This study has been designed to observe: (1) hyperactivation motility and fertilization rate from frozen-thawed sperm and (2) the effect of Thimerosal, Ca<sup>2+</sup> or procaine pretreatment on hyperactivation motility.

## **MATERIALS AND METHODS**

This research project was conducted from February 2009 to March 2010 in Beijing. All chemicals and media were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated.

**Animals:** Kunming (KM) mice (Academy of Military Medical Sciences, Beijing, China) were used in the present study. Mice were housed under temperature- (20-22°C) and light-controlled conditions (lights-on, 06:00-20:00) with free access to food and water. Semen samples were collected from 50 mature males weighing between 35 and 40 g and ranging between 10 and 15 week of age.

**Solutions for freezing sperm:** Cryopreservation medium contained 18% (w/v) raffinose and 3% (w/v) skim milk (Nakagata, 2000).

**Sperm freezing and thawing:** Fresh sperm: Caudal epididymides were excised and rinsed with HS medium (Wennemuth *et al.*, 2000):

- **Freezing sperm:** Sperm were loaded into 0.25 mL straws (I.V.M., L'Aigle, France) with cryopreservation solution. After equilibrating for 30 min, straws were placed 2 cm above liquid nitrogen (LN<sub>2</sub>) for 10 min, then plunged into LN<sub>2</sub> and stored for at least 1 week

- **Thawing:** Frozen straws were removed from liquid nitrogen and put into a water bath maintained at 37°C for no longer than 15 min. The spermatozoa suspensions were then added to 75 µL of HTF medium with a final concentration of approximately  $5 \times 10^5$  cells mL<sup>-1</sup> and incubated for 1-2 h at 37°C

**Partial zona pellucida incised by a piezo manipulator (ZIP) of oocytes:** Procedures for ZIP were performed as described by Kawase *et al.* (2002). After ZIP, the oocytes were cultured in HTF medium for at least 1 h before IVF.

**In vitro fertilization (IVF):** Oocytes were placed into 75 µL drops of HTF medium under mineral oil, in which a small drop of spermatozoa suspension was added for insemination. Five hours after IVF, eggs were removed from the fertilization drop, washed in HTF medium and cultured in 50 µL drops of HTF medium.

About 24 h after fertilization, oocytes were examined and those that had cleaved into two equal blastomeres were considered to be fertilized.

**Analysis of sperm motility:** Sperm motility was also evaluated using Computer-assisted Sperm Analysis (CASA) (Sundararaman *et al.*, 2007). Sperm movement was imaged using a 4×Olympus negative phase objective (Hi-Tech Instruments, Philadelphia, PA) and recorded using a Panasonic AG-7300 Super VHS video recorder. The video images were digitized (30 frames at 60 Hz) and analyzed using HTM-IVOS (Version 12.3, Hamilton Thorne Research, Beverly, MA). Motion parameters measured were curvilinear velocity (VCL, the rate of travel of the sperm head), amplitude of lateral head displacement (ALH, degree of side-to-side head movement measured as the mean width of head oscillations) and beat/cross frequency (BCF, number of times the head crossed the averaged path per second). Because movement of the sperm head is dependent on flagellar activity, it can be used as an indicator of flagellar bending patterns. Increased VCL and ALH are indicative of hyperactivation (Mortimer and Mortimer, 1990). For each treatment sample, 400-450 motile sperm were analyzed and for each experiment, replicate tests were performed.

**Ca<sup>2+</sup> imaging:** Sperm were loaded with 10 µM Fluo-3 AM and 0.05% pluronic F-127 for 30 min at room temperature in the dark, followed by 2 washes in HS medium, each with a 5 min spin at 400 g. Washed sperm were resuspended in HS medium, plated onto cover slips coated with Cell Tak and allowed to attach for approximately 10 min.

Fluorescence intensities were quantified using EZ-C1 Free Viewer software (Nikon), as described previously (Aoki *et al.*, 1997) with some modifications.

**The treatment of sperm:** Fresh and cryopreservation sperm were exposed to 50 µM Thimerosal, 2.4 mM Ca<sup>2+</sup>, 5 mM Procaine or 10 mM 1,2-bis (2-aminophenoxy) ethane-N,N, N9, N9-tetraacetic acid (BAPTA), after which hyperactivated motility was measured using Computer-assisted Sperm Analysis (CASA) as previously described.

**Statistic analyses:** Data were analyzed using Minitab statistical software (Minitab Inc., State College, PA). Treatment effects were detected using analysis of variance, followed by Tukey's test for individual post-hoc comparisons. Differences at  $p < 0.05$  were considered statistically significant.

**RESULTS**

**Effect of ZIP treatment on the fertilization rates of mouse oocytes after IVF with cryopreserved spermatozoa:** According to Table 1, the cleavage rate (71.6%) of oocytes fertilized by post-thawed spermatozoa was significantly lower than that of fresh oocytes fertilized by fresh sperm (94.4%) and of ZIP (Partial zona pellucida incised by a piezo manipulator)-zona oocytes fertilized with either fresh (94.5%) or frozen sperm (89.3%) (p<0.05).

**Computer-assisted sperm analysis (CASA) measurements of post-thawed spermatozoa treated with thimerosal:** As shown in Table 2, the VCL and ALH in post-thawed sperm were lower than in fresh sperm (301.5 vs.216.0, 16.2 vs.10.4, p<0.05). There was significantly higher in VCL or ALH between post-thawed sperm exposed to thimerosal and that of those without Thimerosal treatment (277.6 vs. 216.0, 15.9 vs 10.4, p<0.05). It is similar to the situation with fresh sperm (357.2 vs 301.5. 21.3 vs. 16.2, p<0.05). Stimulation by thimerosal of post-thawed sperm was not blocked by lowering extracellular Ca<sup>2+</sup> with 10 mM 1,2-bis (2-aminophenoxy) ethane-N,N, N9, N9-tetraacetic acid (BAPTA) to approximately 30 nM.

**Computer-assisted sperm analysis (CASA) measurements of post-thawed spermatozoa treated with Ca<sup>2+</sup>:** Addition of 2.4 mM Ca<sup>2+</sup> increased the VCL and ALH in post-thawed sperm and the VCL were higher (373.5, p<0.05) than that in non-treated fresh sperm (314.6). But there is a significant difference in ALH between the post-thawed sperm exposed to Ca<sup>2+</sup> and the fresh sperm (13.8 vs.15.3, p<0.05) (Table 3).

**Casa measurements of post-thawed spermatozoa treated with procaine:** Exposure of post-thawed sperm to Procaine visibly increased the fresh sperm hyperactivated motility as

Table 1: Effect of ZIP treatment on the fertilization rates of mouse oocytes after IVF with cryopreserved spermatozoa

Group	No. of oocytes	2-cell (%)
Fresh sperm+fresh oocyte	90	85(94.4) <sup>a</sup>
Frozen sperm+fresh oocyte	95	68(71.6) <sup>b</sup>
Fresh sperm+fresh oocyte (ZIP)	91	86(94.5) <sup>a</sup>
Frozen sperm+fresh oocyte (ZIP)	93	83(89.3) <sup>a</sup>

ZIP: Partial zona pellucida incised by a piezo manipulator, IVF: *In vitro* fertilization, Values with different superscripts within the same column differ significantly (p<0.05)

Table 2: CASA measurements of post-thawed spermatozoa treated with thimerosal

Group	Motile (%)	VCL (µm sec <sup>-1</sup> )	ALH (µm)	BCF (sec <sup>-1</sup> )
Fresh sperm	85.7 <sup>a</sup>	301.5 <sup>b</sup>	16.2 <sup>b</sup>	33.2
Fresh sperm+BAPTA	84.9 <sup>a</sup>	250.7 <sup>c</sup>	12.4 <sup>f</sup>	31.7
Fresh sperm+thimerosal	81.8 <sup>a</sup>	357.2 <sup>a</sup>	21.3 <sup>a</sup>	32.6
Fresh sperm+thimerosal+BAPTA	85.4 <sup>a</sup>	360.9 <sup>a</sup>	20.5 <sup>a</sup>	30.4
post-thawed sperm	43.5 <sup>b</sup>	216.0 <sup>d</sup>	10.4 <sup>f</sup>	31.2
post-thawed sperm+BAPTA	42.1 <sup>b</sup>	165.3 <sup>e</sup>	8.7 <sup>g</sup>	28.4
post-thawed sperm+thimerosal	44.3 <sup>b</sup>	277.6 <sup>c</sup>	15.9 <sup>b</sup>	35.7
post-thawed sperm+thimerosal+BAPTA	43.7 <sup>b</sup>	269.7 <sup>c</sup>	16.3 <sup>b</sup>	34.9

Values with different superscripts within the same column differ significantly (p<0.05). VCL: The rate of travel of the sperm head, ALH: Amplitude of lateral head displacement, degree of side-to-side head movement measured as the mean width of head oscillations, BCF: Beat/cross frequency, No. of times the head crossed the averaged path per second, CASA: Computer-assisted sperm analysis

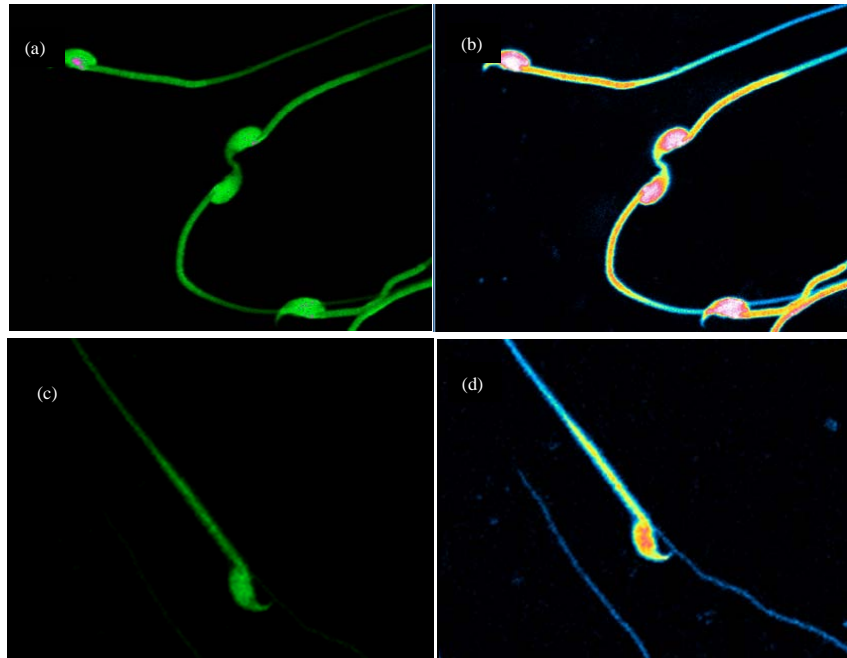


Fig. 1(a-d):  $Ca^{2+}$  imaging in the cryopreserved sperm.  $Ca^{2+}$  (green) were found to be distributed at the head and the flagellar in the mouse fresh sperm (Fig. 1a, b) and their color turned cooler (Fig. 1c, d) when the sperm were cryopreserved

Table 3: CASA measurements of post-thawed spermatozoa treated with  $Ca^{2+}$

Group	Motile (%)	VCL ( $\mu\text{m sec}^{-1}$ )	ALH ( $\mu\text{m}$ )	BCF ( $\text{sec}^{-1}$ )
Fresh sperm	83.9 <sup>a</sup>	314.6 <sup>b</sup>	15.3 <sup>b</sup>	30.6 <sup>a</sup>
Fresh sperm+ $Ca^{2+}$	81.4 <sup>a</sup>	373.5 <sup>a</sup>	18.5 <sup>a</sup>	31.3 <sup>a</sup>
post-thawed sperm	41.8 <sup>b</sup>	221.7 <sup>c</sup>	11.0 <sup>c</sup>	33.5 <sup>a</sup>
post-thawed sperm+ $Ca^{2+}$	32.5 <sup>b</sup>	280.6 <sup>b</sup>	13.8 <sup>c</sup>	21.4 <sup>b</sup>

Values with different superscripts within the same column differ significantly ( $p < 0.05$ )

Table 4: CASA measurements of post-thawed spermatozoa treated with Procaine

Group	Motile (%)	VCL ( $\mu\text{m sec}^{-1}$ )	ALH ( $\mu\text{m}$ )	BCF ( $\text{sec}^{-1}$ )
Fresh sperm	83.1 <sup>a</sup>	308.7 <sup>b</sup>	15.9 <sup>b</sup>	28.7
Fresh sperm+Procaine	89.9 <sup>a</sup>	358.3 <sup>a</sup>	19.3 <sup>a</sup>	34.6
Post-thawed sperm	40.3 <sup>b</sup>	230.1 <sup>c</sup>	11.3 <sup>c</sup>	30.9
Post-thawed sperm+Procaine	42.4 <sup>b</sup>	264.8 <sup>c</sup>	12.4 <sup>c</sup>	29.7

Values with different superscripts within the same column differ significantly ( $p < 0.05$ )

measured by VCL (308.7 vs. 358.3,  $p < 0.05$ ) but the VCL and ALH were significantly lower ( $p < 0.05$ ) in post-thawed sperm than in fresh sperm irregardless of whether they were treated with procaine (230.1 vs. 308.7, 264.8 vs. 308.7; 11.3 vs. 15.9, 12.4 vs. 15.9) (Table 4).

**Effect of cryopreservation on  $Ca^{2+}$  concentration in mouse sperm:** In the present study, the changes of  $Ca^{2+}$  imaging of fluo3-loaded sperm were also examined. As shown in Fig. 1,  $Ca^{2+}$  were found to be distributed at the head and the flagellar (Fig. 1a, d). However, in some

cryopreserved sperm, we observed the concentration of the  $\text{Ca}^{2+}$  were decreased (Fig. 1b, d) because warmer colors indicate higher fluorescent intensities and increased intracellular  $\text{Ca}^{2+}$  (Fig. 1b, d).

## DISCUSSION

In this study, we compared fertilization rate and hyperactivated motility of fresh vs. frozen and thawed mouse sperm. The sperm had been cryopreserved with a medium containing raffinose and skim milk, followed after thawing by incubation with fresh oocytes. Results showed that cleavage rate following use of frozen-thawed sperm (71.6%) was significantly lower than that from fresh sperm (94.4%) (Table 1). It seems that the frozen-thawed sperm did not have enough power to penetrate the oocyte zona pellucida which is suggested by our following result. When the oocytes with partial zona pellucida removal were *in vitro* fertilized with frozen-thawed sperm, cleavage rate was improved to 89.3% (Table 1). Such decreased penetration of sperm due to cryopreservation was also observed by Salamon and Maxwell (1995) and may have resulted from reduced hyperactivated motility, a normal level which is necessary for sperm cells to penetrate the cumulus matrix and zona pellucida (Suarez and Pacey, 2006).

Increases in  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) often are associated with sperm hyperactivated motility (Yanagimachi, 1994). In addition,  $\text{Ca}^{2+}$  acts through calmodulin on the axoneme to modulate flagellar curvature (Ho *et al.*, 2002). Hyperactivated motility is characterized by high amplitude, asymmetrical flagellar bends and a circular or erratic swimming trajectory when intracellular  $\text{Ca}^{2+}$  is approximately 200B1000 nM in sperm (Ho *et al.*, 2002; Suarez and Dai, 1995). Cytosolic  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) can be raised by either  $\text{Ca}^{2+}$  influx through plasma membrane ion channels (Yanagimachi, 1982) or by  $\text{Ca}^{2+}$  release from intracellular stores (Ho and Suarez, 2001). When Thimerosal was added to the medium of fresh sperm, flagellar bend amplitude and VCL were increased (Table 2). From this result, we infer that Thimerosal, a sulfhydryl reagent, can induce  $\text{Ca}^{2+}$  release from IP3R (Bindoli and Fleischer, 1983). The VCL and ALH of frozen-thawed sperm treated with Thimerosal, similar to the situation with fresh sperm, were higher than that of those without Thimerosal treatment (Table 2), suggesting that Thimerosal can stimulate release of  $\text{Ca}^{2+}$  from internal stores and increase hyperactivated motility of mouse frozen-thawed sperm.

Incubation of fresh or frozen-thawed sperm for 90 min under 2.4 mM  $\text{Ca}^{2+}$  condition led to hyperactivation (Table 3). The VCL and ALH in fresh sperm exposed to  $\text{Ca}^{2+}$  were significantly higher than those in fresh sperm not exposed to  $\text{Ca}^{2+}$  and in frozen-thawed sperm that did have  $\text{Ca}^{2+}$  exposure. There was no difference in ALH between the frozen-thawed sperm and the frozen-thawed sperm exposed to  $\text{Ca}^{2+}$ . At the same time, Procaine treatment did not improve the hyperactivated motility of frozen-thawed sperm (Table 3), possibly because plasma membranes had been damaged during sperm cryopreservation (Gillan *et al.*, 1995). Frozen-thawed sperm showed a swollen flagellar base and had coiled flagella (Nishizono *et al.*, 2004) which likely damaged the Catspers channels, leading to impaired hyperactivated motility. Similarly, Marquez *et al.* (2007) reported that Procaine (5 mM) did not hyperactivate CATSPER1 or CATSPER2<sup>-/-</sup> sperm.  $\text{Ca}^{2+}$  entry through the principal piece-localized CATSPER channels has been implicated in the activation of hyperactivated motility (Xia *et al.*, 2007). Mice deficient in any of the four CATSPERs are completely sterile but exhibit no other apparent abnormality (Quill *et al.*, 2001). In this study there was no difference in  $\text{Ca}^{2+}$  concentration of flagella between untreated cryopreserved sperm and cryopreserved sperm treated with  $\text{Ca}^{2+}$  or with Procaine (data not shown).

Fluorescent imaging of fluo3-loaded sperm showed that frozen-thawed sperm had lower fluorescent intensities ( $[\text{Ca}^{2+}]_i$ ) than those of fresh sperm (Fig. 1). The  $[\text{Ca}^{2+}]_i$  decrease may be

associated with un-hyperactivated sperm motility which is suggested by the finding that hyperactivated motility of sperm after cryopreservation was significantly lower than that of fresh sperm (Table 2).

## CONCLUSION

To sum up, in the present study, the decreased hyperactivated motility of mouse sperm due to cryopreservation may impair fertilization and Thimerosal treatment of mouse sperm after cryopreservation may increase hyperactivated motility.

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