Acrosomal integrity and capacitation are not influenced by sperm cryopreservation in the giant panda

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Abstract

Sperm cryopreservation and artificial insemination are important management tools for giant panda breeding and the preservation of extant genetic diversity. This study examined the influence of freeze–thawing on sperm function, specifically capacitation. Sperm from nine giant pandas were assessed before and after rapid (–40 and –100 °C/min) cryopreservation by incubation in HEPES-buffered Ham's F10 medium with and without the capacitation accelerators, 3-isobutyl-1-methylxanthine (IBMX) and dibutyryl cyclic AMP (dbcAMP). At 0, 3 and 6 h of exposure, aliquots were assessed for sperm motility traits and capacitation, defined as the proportion of sperm with intact acrosomes following exposure to solubilised zonae pellucidae (ursid or felid) or calcium ionophore subtracted from the proportion of sperm with intact acrosomes before exposure. Although mean $\pm$ S.E.M. sperm motility post-thaw ($56.1 \pm 3.9\%$ at 0 h) was less ($P < 0.05$) than pre-freeze ($71.7 \pm 6.0\%$), there was no difference ($P > 0.05$) in the proportion of acrosome-intact sperm (fresh, $93.0 \pm 1.7\%$ versus cryopreserved–thawed, $81.7 \pm 4.7\%$ at 0 h). Incidence of capacitation was greater ($P < 0.05$) in fresh sperm incubated with capacitation accelerators IBMX and dbcAMP (9 h: $50.9 \pm 1.1\%$) compared with fresh sperm incubated without accelerators (9 h: $41.2 \pm 1.1\%$). Frozen–thawed sperm preincubated without accelerators underwent capacitation ($49.6 \pm 1.1\%$) to a greater extent ($P < 0.05$) compared with these fresh counterparts. Thawed samples with (9 h: $45.9 \pm 1.4\%$) and without accelerators (9 h: $41.2 \pm 1.1\%$) did not differ ($P > 0.05$) during the 9-h incubation. We conclude that giant panda spermatozoa (1) undergo capacitation *in vitro* with or without chemical accelerators and (2) withstand a rapid cryopreservation protocol, including retaining normal acrosomal integrity and functional capacitation ability.

Reproduction (2004) 127 547–556

Introduction

The managed breeding of giant pandas is challenging because males often experience low libido and/or aggressive behaviours toward females (Liu *et al.* 1998, Zhang *et al.* 2004). There is a high level of genetic heterozygosity in the *ex situ* population of >120 giant pandas in China (Lu *et al.* 2001). To avoid potential inbreeding depression in the future, however, it is essential that all the valuable individuals reproduce. Artificial insemination (AI) of fresh semen already plays an important role in breeding giant pandas in captivity, including helping to overcome the problem of sexual incompatibility (Liu 1981, Hodges *et al.* 1984, Moore *et al.* 1984). Most AI successes have occurred using fresh spermatozoa (Liu *et al.* 1979, Moore *et al.* 1984, Masui *et al.* 1989, Huang *et al.* 2001), although a few cubs have been produced by inseminating thawed sperm (Hu & Wei 1990, Ye *et al.* 1991, Zhang *et al.* 1991, Huang *et al.* 2002).

The genetic management of giant pandas in captivity is recognised as one of the highest priorities for *ex situ* conservation action in China (Zheng *et al.* 1997, Yan

et al. 2000). The ability to consistently produce offspring by the efficient use of cryopreserved sperm would allow extending sperm viability from days to years and even generations (Wildt *et al.* 1997). This significantly improves the potential for effective genetic management, and the predicted survival of the species (Harnal *et al.* 2002). The use of AI with thawed sperm also would facilitate maintaining genetic management through the distribution of genes among geographically dispersed panda populations, including those living in remote breeding centres and zoos. Availability of frozen sperm would permit international transport of genetic material without compromising political and legal restrictions, such as policies established by the United States Fish and Wildlife Service (United-States-Fish-and-Wildlife-Service 1998). Finally, effective sperm cryopreservation could theoretically allow securing robust genetic material of wild giant pandas that are occasionally captured for brief periods. Sperm from these individuals could be considered 'insurance' for the gene pool, with aliquots used periodically to infuse heterozygosity into the *ex situ*

population (Wildt *et al.* 1997). This would eliminate the need to ever extract more giant pandas from native habitats to support zoos.

There are reports on giant panda gamete ultrastructure (Chen *et al.* 1984), capacitation (Sun *et al.* 1996), the acrosome reaction (Chen *et al.* 1989a) and oocyte penetration *in vitro* (Moore *et al.* 1984, Chen *et al.* 1989ab), but all using freshly collected spermatozoa. There are no publications on sperm physiology and function in this species occurring during the freezing/thawing process. Functional viability of a spermatozoon generally depends on sperm (1) progressive motility (Drobnis *et al.* 1988), (2) acrosome integrity (Suarez *et al.* 1984, Talbot 1985, Cummins & Yanagimachi 1986) and (3) the ability to undergo capacitation (Yanagimachi 1994), the acrosome reaction (Yanagimachi 1994) and decondensation (Perreault 1990). Capacitation is the final stage of sperm maturation and is essential for the acrosome reaction and subsequent zona pellucida (ZP) penetration and oocyte fertilisation (Yanagimachi 1994).

The present study was one of a series of investigations to assess the influence of seminal processing and cryopreservation/thawing on giant panda sperm function. Here, we focused on the phenomenon of sperm capacitation *in vitro*. Spermatozoa of some species undergo capacitation spontaneously *in vitro*, whereas others require accelerators such as 3-isobutyl-1-methylxanthine (IBMX) (Visconti *et al.* 1999, Dorval *et al.* 2002, Thundathil *et al.* 2002) or dibutyl cyclic AMP (dbcAMP) (Leclerc *et al.* 1996, Visconti *et al.* 1999, Lefevre *et al.* 2000). Our study was designed to determine the dynamics of giant panda sperm capacitation in the presence and absence of IBMX and dbcAMP. Cryopreservation is also known to impede sperm function via damaged acrosomal membranes (Rodriguez-Martinez *et al.* 1997, Burgess *et al.* 2001). Thus, our second objective was to determine if freeze-thawing induced membrane damage which, in turn, disrupted the ability of giant panda sperm to acrosome react.

In mammalian species studied to date, the acrosome reaction is generally readily induced in capacitated sperm by co-culture with conspecific (Uto *et al.* 1988, Yoshimatsu *et al.* 1988, Pukazhenthil *et al.* 1996) or heterospecific (Lee *et al.* 1987, Yoshimatsu *et al.* 1988, Slavik *et al.* 1990) ZP. Due to the lack of giant panda oocytes (and thus ZP), we tested the efficacy of solubilised ZP from felid and ursid oocytes, i.e. other carnivores. These findings were compared with the effectiveness of standard chemical induction (calcium ionophore A23187; CI) commonly used in other taxa including the bovid (Byrd 1981, Parrish *et al.* 1988, 1989), the cervid (Harnal *et al.* 2001) and the felid (Long *et al.* 1996, Pukazhenthil *et al.* 1998). Overall, our approach here was to begin to understand capacitation and the physiological and biophysical consequences of processing and cryopreserving giant panda spermatozoa.

Materials and Methods

Animals and source of ZP for capacitation assessments

One ejaculate was collected from each of nine giant pandas (5–16 years of age; weighing 79.5–130.0 kg each) maintained at the China Research and Conservation Center for the Giant Panda in the Wolong Nature Reserve. Males were housed singly in (1) naturalistic enclosures of approximately 300 m² in size and connected to indoor areas of 8 m², with olfactory and auditory contact with females in adjacent enclosures or (2) concrete and grass enclosures of approximately 35 m² and connected to indoor areas of 8 m², with olfactory, auditory and visual contact with neighbouring females. Males were moved between enclosures of the same or alternate type at irregular intervals, but rarely went more than 1–2 weeks without being in an enclosure immediately adjacent to an adult female. Fresh bamboo (~20 stalks) was provided up to seven times daily and supplemented with high protein concentrate consisting of soybean, bamboo powder, corn, rice and vitamin/mineral additives (M Edwards, personal communication). Water was available *ad libitum*. All semen samples were collected during the spring (February–May) breeding season (Schaller *et al.* 1985).

ZP for functionality assays were collected from oocytes recovered from two species, the domestic cat (*Felis catus*) and the black bear (*Ursus americanus*). The former were collected from ovaries provided by local veterinary clinics in the USA conducting ovariohysterectomies in companion cats (Johnston *et al.* 1989, Wolfe & Wildt 1996). The oocytes underwent *in vitro* maturation (Johnston *et al.* 1989, Wolfe & Wildt 1996), zonae were then separated from cytoplasm by continued pipetting with undersized (slightly smaller than the width of the oocyte) pipettes to rupture the zonae. Zonae were then washed three times in 2 ml Ham's F10 medium plus 5% fetal calf serum and 25 mmol/l HEPES (HF10) before being placed in a cryovial tube (Nunc, Rochester, NY, USA) with HF10 to a concentration of four zonae/μl. These cryovial tubes were then placed in a sonicating bath (Precision Scientific, Winchester, VA, USA) until no solid zona remained (average 45 min). This solution was then divided into 25 μl aliquots and frozen until needed for sperm co-incubation. Ursid oocytes were recovered from freeze-thawed ovarian tissue made available through co-operation with hunters and biologists in the USA and Canada during seasonal culls of local, wild black bears. These ovaries were excised between 2 and 12 h after death, frozen on dry ice and then later transported to the laboratory where the ovaries were thawed and minced (as described previously) (Johnston *et al.* 1994) to collect oocytes. Ursid zonae were prepared as for felid zonae, with the exception that ursid oocytes did not undergo *in vitro* maturation. It is likely that the cytoplasm of these oocytes was no longer functional as a result of ovary handling before oocyte recovery. The number of ursid oocytes available was

much lower than that of felid oocytes, limiting the number of time-points that sperm could be exposed to this treatment. Thus, the 6-h time-point was chosen to compare the effects of ursid with felid zona on giant panda sperm because it was assumed that this would be the time of maximum capacitation and sperm viability and, therefore, offer the greatest sensitivity. All procedures used in this study were submitted to and approved by the Smithsonian's National Zoological Park's Institutional Animal Care and Use Committee.

Semen collection/analysis

Semen was collected by electroejaculation after each male was induced into a surgical plane of anaesthesia by an intramuscular injection of 10 mg/kg ketamine HCl (Keta-set; Fort Dodge Laboratories Inc., Fort Dodge, IA, USA). Deep anaesthesia was maintained with 0–5% isoflurane gas, as needed. The electroejaculation technique has been described (Platz *et al.* 1983, Howard 1993, 1999, Spindler *et al.* 2001) and briefly relied on using a 2.6 cm diameter rectal probe with three longitudinal, stainless-steel electrodes and a 60 Hz sine wave stimulator (P.T. Electronics, Boring, OR, USA). A standardised set of low voltage stimulations (2–8 V) over three series of 20–30 stimuli each was adequate to elicit ejaculation. The entire semen collection interval generally required <20 min.

Penile erection occurred during stimulation, and semen was collected into a sterile glass container. Seminal volume was immediately recorded by pipetting, and pH was measured using <5 µl semen on Color pHast indicator strips (EM Science, Gibbstown, NJ, USA). A seminal pH value in the normally basic range (7.5–9.0) indicated that urine contamination had not occurred. An additional 10 µl sample from each electroejaculation series was used to determine sperm concentration using a haemocytometer (Howard *et al.* 1990). A 5 µl seminal aliquot was examined at 200× (Olympus BX40 microscope) for a subjective estimate of sperm motility traits, including percent sperm motility and progressive motility (i.e. forward progression on a 0–5 scale, with 5 indicative of rapid forward sperm movement and 0 indicating no forward progression (Howard 1993). Within each ejaculation series, samples differing from each other by <10% in sperm motility and progressive status were combined. Spermatozoa were maintained at 37°C throughout assessment and processing using a water bath or dry-bath incubator (Fisher, Hanover Park, IL, USA).

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA). A 5 µl aliquot of the combined electroejaculate was removed and added to 100 µl fixative (0.3% glutaraldehyde in phosphate-buffered serum) for morphologic examination of spermatozoa (200/sample) by phase-contrast microscopy (× 630) (Howard 1993). Spermatozoa were categorised as normal or as having one of the following anomalies, including an abnormal head (i.e. macrocephaly,

Figure 1 Acrosome intact sperm appear blue (a) whereas damaged acrosomes are evident by mottled or patchy staining (b) or a detached membrane (c). Sperm with a missing acrosome have a white or pink cap, with no blue staining evident (d).

microcephaly and bicephaly), abnormal acrosome, abnormal midpiece, coiled flagellum, bent midpiece with cytoplasmic droplet, bent midpiece without cytoplasmic droplet, bent flagellum with cytoplasmic droplet, bent flagellum without cytoplasmic droplet, proximal cytoplasmic droplet and distal cytoplasmic droplet (Howard 1993). The proportion of spermatozoa with abaxial attachment of the midpiece was noted but not considered an abnormality (Moore *et al.* 1984). Acrosomal integrity was evaluated using the rose bengal/fast green stain (Pope *et al.* 1991). Spermatozoa were stained for 90 s, smeared on a glass slide, allowed to air-dry, and a minimum of 100 spermatozoa were examined by light microscopy (× 630). Cells then were categorised as having: (1) an intact acrosome whereby the acrosomal membrane stained blue and remained in contact with the sperm head (Fig. 1a); (2) a damaged acrosome whereby the membrane was damaged (Fig. 1b), or was separate from the sperm head (Fig. 1c); or (3) a missing acrosome (Fig. 1d).

Capacitation assessment

After combining series into a single ejaculate per male, a 0.5 ml aliquot was removed immediately, subdivided into two and diluted with (1) HF10 or (2) HF10 containing an additional supplement of 200 µmol/l IBMX and 1 mmol/l dbcAMP (CHF10). These diluted aliquots were centrifuged (200 g 8 min) and the supernatants discarded before each pellet was resuspended in either HF10 or CHF10 (keeping medium consistent before and after centrifugation). Spermatozoa were resuspended in 0.5 ml of either HF10 or CHF10 at a final concentration of 50×10^6 cells/ml and incubated at 37°C for 6 h.

At 0, 3 and 6 h after resuspension, 5 µl samples were removed and assessed for motility and forward progressive

status. Additional 10 μ l aliquots were removed and diluted with an equal volume of one of four treatments: (1) HF10 only (control for ZP); (2) HF10 containing solubilised cat (all time-points) or bear (6-h time-point only) ZP to a final concentration of 2 zonae/ μ l; (3) HF10 containing 5% dimethylsulfoxide (as a control for CI); or (4) HF10 containing 1 μ mol/l CI. Each of the four aliquot/treatment suspensions was incubated under mineral oil for 30 min at 37°C. At the end of the 30-min incubation, acrosomal integrity was assessed (as above). Percentage of capacitated spermatozoa was defined as the proportion of acrosome intact sperm in treatments no. 1 and no. 3 (controls) minus the percentage of sperm with intact acrosomes in treatments no. 2 and no. 4.

Sperm cryopreservation and thawing

The remainder of the ejaculate not used for the capacitation study was diluted immediately (within 15 min of original collection) with commercially available TEST egg yolk buffer (Irvine Scientific, Santa Ana, CA, USA) modified to contain 5% glycerol. Semen was diluted to give a final concentration of 400×10^6 motile sperm/ml in 15 ml conical, plastic tubes (Falcon, Bedford, MA, USA) and then placed in a water jacket (400 ml 37°C water) before being placed in a refrigerator to cool slowly to 4°C over 4 h, as verified by a thermocouple (Brandt, Prairieville, LA, USA). Cooled semen was pipetted into 0.25 ml sterile, plastic straws (Veterinary Concepts, Spring Valley, WI, USA) and sealed using a Nyclave impulse heat sealer (The Lorvic Corporation, St Louis, MO, USA). Straws were placed 7.5 cm over liquid nitrogen (LN) for 1 min and then 2.5 cm above LN for 1 min to achieve a rapid cryopreservation rate of $-40^\circ\text{C}/\text{min}$ and $-100^\circ\text{C}/\text{min}$ respectively (as determined by previous control thermocouple testing). Frozen samples were plunged into the liquid phase of LN and stored for at least 24 h before thawing. Spermatozoa were thawed by exposing the straw to air for 10 s, followed by plunging the straw into a 37°C waterbath for 30 s. Both ends of the straw were cut off and the thawed sample allowed to flow into a 5 ml sterile plastic tube (Falcon) where it was diluted immediately in 2 ml HF10 at 37°C. The thawed sample was split into two equal aliquots, diluted in HF10 and each centrifuged simultaneously in a 1.5 ml Eppendorf microcentrifuge tube (200 g 8 min). The supernatant was discarded, and one sperm pellet was resuspended in HF10, whereas the other was suspended in CHF10 (both at a final concentration of 50×10^6 cells/ml in 0.5 ml) at 37°C. Thawed spermatozoa then were assessed for capacitation exactly as described above for fresh counterparts.

Statistical analysis

Differences among treatments were evaluated by ANOVA and Dunnett's multiple comparison testing (Miller 1981). Percentage data were arcsine transformed before analysis.

Data are expressed as means $\pm$ S.E.M. Correlation coefficients among sperm traits were determined using the Pearson-product moment correlation curve (Box *et al.* 1978).

Results

Seminal volume (2.8 ± 0.4 ml) and sperm concentration ($1.1 \pm 0.3 \times 10^9$ sperm/ml), initial motility ($76.1 \pm 4.4\%$), forward progression rating (3.4 ± 0.2) and acrosomal integrity ($93.6 \pm 1.7\%$ intact) for giant panda ejaculates collected here were consistent with previous reports (Platz *et al.* 1983, Chen *et al.* 1994). Most spermatozoa were morphologically normal ($84.2 \pm 3.0\%$), although many ($74.9 \pm 8.2\%$) displayed the interesting abaxial attachment trait for the head/midpiece region as noted previously (Moore *et al.* 1984). There is no evidence that this is an abnormal morphotype for the giant panda spermatozoon; thus, these sperm were categorised as normal if other abnormalities were absent. Furthermore, there were no differences ($P > 0.05$) among males in any of the ejaculate traits measured (data not shown). In short, all subject males produced prodigious amounts of motile, high quality spermatozoa.

The motility of fresh (control) sperm samples declined over time (Fig. 2) to about 20% after a 9-h incubation in HF10. The gradual decline in sperm motility was not accelerated or otherwise altered ($P > 0.05$) by the presence of IBMX and dbcAMP in the medium. For example, whereas overall sperm motility in HF10 at 9 h was $27.0 \pm 5.6\%$, this same trait was similar ($P > 0.05$) in CHF10 containing the combined IBMX and dbcAMP ($13.3 \pm 5.4\%$). There also was a loss ($P < 0.05$) in sperm motility immediately after thawing ($56.1 \pm 3.9\%$ motile) compared with pre-freeze motility ($71.7 \pm 6.0\%$; Fig. 2). Nonetheless, subsequent sperm motility values for fresh and frozen-thawed aliquots were comparable within time-points ($P > 0.05$) (Fig. 2). Overall motility in thawed samples did not ($P > 0.05$) decline more rapidly than

Figure 2 Motility of giant panda spermatozoa incubated *in vitro* in HF10. Different superscripts within and across time-periods differ ($P < 0.05$).

fresh counterparts (the gradient of decline in motility was $-4.91\%/h$ for frozen-thawed versus $-4.97\%/h$ for fresh aliquots). A similar observation was made for progressive motility ratings in that no differences ($P > 0.05$) were measured between the same time-periods for fresh (0 h, 3.4 ± 0.2 ; 3 h, 3.4 ± 0.2 ; 6 h, 3.1 ± 0.2 ; 9 h, 1.3 ± 0.6) versus thawed (0 h, 3.1 ± 0.3 ; 3 h, 3.3 ± 0.2 ; 6 h, 2.8 ± 0.2 ; 9 h, 1.1 ± 0.4) aliquots.

There was a comparable ($P > 0.05$) percentage of fresh ($93.0 \pm 1.7\%$) and thawed ($81.7 \pm 4.7\%$) spermatozoa with intact acrosomes at 0 h. The proportion of fresh spermatozoa with intact acrosomes decreased ($P < 0.05$), albeit modestly ($\sim 10\%$) within 3 h of incubation and regardless of medium (HF10 or CHF10) (Fig. 3). There was a similar trend in acrosomal integrity for thawed sperm with an overall decline ($P > 0.05$) of $\sim 6\%$ from 0 to 3 h of culture. Within time-period and medium, there were no differences ($P > 0.05$) in the proportions of fresh versus thawed sperm with intact acrosomes (Fig. 3). However, acrosomal integrity degraded ($P < 0.05$) more severely in cells that were both cryopreserved and incubated in CHF10 for 6 and 9 h compared with fresh sperm incubated without capacitation accelerators (Fig. 3).

The number of spermatozoa with intact acrosomes decreased markedly ($P < 0.05$) in response to the addition of heterologous cat or bear ZP to the culture medium compared with spermatozoa incubated in control medium (indicating acrosome reaction and therefore successful capacitation) (Fig. 4). For example, because 87% of spermatozoa from male 4 incubated in control HF10 had intact acrosomes in the control treatment and only 30% of cat ZP-exposed spermatozoa had intact acrosomes at 6 h, then 57% of spermatozoa at this time-period in HF10 were considered capacitated.

Compared with 0 h, percentage of capacitated spermatozoa was greater ($P < 0.05$) in all treatments after 3 h and increased ($P < 0.05$) further by 6 h (Fig. 5). No further increase ($P > 0.05$) was observed at 9 h. Fresh sperm incubated in CHF10 had a greater ($P < 0.05$) incidence of capacitation than HF10-incubated sperm at 3, 6 and 9 h (Fig. 5). Fewer frozen-thawed CHF10-incubated sperm completed capacitation by 6 h compared with fresh counterparts. On the contrary, more frozen-thawed sperm had undergone capacitation after a 9-h incubation in HF10 compared with fresh counterparts (Fig. 5). The extent and rate of capacitation of thawed sperm was not otherwise different ($P > 0.05$) compared with fresh counterparts. Percentage of capacitated spermatozoa immediately post-thaw (0 h) was $< 5\%$ in all treatments, but increased ($P < 0.05$) to $27.7 \pm 1.2\%$ and $32.1 \pm 1.5\%$ after a 3-h incubation in HF10 and CHF10 respectively (Fig. 5). After a 6-h incubation, an even greater number ($P < 0.05$) of thawed sperm incubated in HF10 ($43.3 \pm 1.1\%$) and CHF10 ($40.4 \pm 1.4\%$) were capacitated. An additional 3-h incubation resulted in more capacitated sperm only in the frozen-thawed HF10-incubated sperm. The presence of IBMX and dbcAMP in the medium had no impact ($P > 0.05$) on the overall incidence or rate at achieving sperm capacitation in thawed sperm aliquots (Fig. 5).

The bear ZP emulsion was less effective ($P > 0.05$) at inducing the acrosome reaction (by 6 h) in fresh (HF10, $26.0 \pm 2.8\%$) and thawed sperm (HF10, $21.7 \pm 5.2\%$) compared with either cat ZP (fresh sperm, HF10, 6 h: $43.6 \pm 1.1\%$; thawed, HF10, 6 h: $43.3 \pm 1.1\%$) or CI (fresh sperm, HF10, 6 h: $42.1 \pm 1.8\%$; thawed, HF10, 6 h: $41.4 \pm 2.1\%$). Sperm categorised as not intact could be either damaged or missing (see Fig. 1). A significant

Figure 3 Acrosomal integrity of fresh giant panda spermatozoa incubated in HF10 and CHF10 and frozen-thawed spermatozoa incubated in HF10 and CHF10. Different superscripts within and between time-periods differ ($P < 0.05$).

Figure 4 Acrosome-intact fresh giant panda spermatozoa after incubation in HF10 control (solid bars) versus solubilised cat or bear ZP (open bars) at 6 h. The difference between these treatments is the percentage of capacitated sperm.

proportion of sperm categorised as having damaged acrosomes in control sperm increased from $5.3 \pm 2.1\%$ (HF10) at 0 h to $14.3 \pm 1.9\%$ at 9 h. There was a higher proportion of damaged sperm in HF10 at 0 h following cryopreservation (HF10: $13.1 \pm 2.1\%$), but at 3 h this difference was no longer evident (fresh: $9.4 \pm 1.7\%$ versus thawed: $16.4 \pm 2.6\%$). Fresh and frozen sperm sustained this damage regardless of whether or not incubation medium contained capacitation accelerators. However, there was a trend ($P > 0.05$) toward greater damage to frozen-thawed sperm after incubation with capacitation accelerators for 9 h ($25.5 \pm 0.8\%$) compared with thawed sperm incubated without accelerators ($20.2 \pm 1.4\%$). The number of sperm assessed to be capacitated after incubation with cat ZP was less ($P > 0.05$) than when CI was used to induce the acrosome reaction at 9 h (Fig. 6). There was no difference in the ability of CI and ZP to induce the acrosome reaction at any other time-point (Fig. 6).

Figure 5 Percent capacitated fresh and thawed giant panda spermatozoa after incubation in HF10 and CHF10 as determined by incubation with cat ZP. Within each treatment, different superscripts differ over time ($P < 0.05$). *Within the time-period, the value is different ($P < 0.05$) from the HF10 counterpart.

Damage to fresh and thawed sperm over time was not different ($P > 0.05$) between sperm exposed to cat ZP versus CI (Table 1).

Discussion

Giant panda spermatozoa were capable of capacitating *in vitro* over a 6-h interval of incubation with or without the use of the accelerators IBMX and dbcAMP. Although there was a modest loss of cellular motility post-thaw, the methods described here allowed freeze–thawing of giant panda sperm without compromising acrosomal integrity. Both ursid and felid zonae emulsions elicited the acrosome reaction in giant panda spermatozoa, supporting data indicating that the triggers to this phenomenon are not species specific (Yanagimachi 1994). Most importantly, the methods utilised here for freeze–thawing these cells did not compromise the ability of giant panda sperm to undergo capacitation.

Given the importance of sperm motility (Drobnis *et al.* 1988) and acrosome integrity (Suarez *et al.* 1984, Talbot 1985, Cummins & Yanagimachi 1986) to successful fertilisation, the discovery of a high proportion of motile, acrosome-intact sperm after cryopreservation was encouraging, but perhaps still not sufficient for normal fertilisation. For example, the incidence of damaged acrosomes found in thawed, CHF10-incubated and even control sperm over time may have indicated that these cells were becoming damaged due to contact with other sperm or the plastic incubation tube. It is possible that damage or premature ‘capacitation-like’ changes were occurring to membranes spontaneously, during freeze–thawing, or exposure to medium containing the accelerators (Gillan *et al.* 1997, Gillan & Maxwell 1999, Green & Watson 2001, Samper 2001). Alternatively, these sperm labelled damaged may have actually been partially

Figure 6 Estimated capacitation of fresh and cryopreserved spermatozoa as determined by incubation with solubilised cat ZP versus CI. Within each treatment, different superscripts differ over time ($P < 0.05$). *Within the time-period, the value is different ($P < 0.05$) from the ZP counterpart.

capacitated sperm as has been found in felids (Long *et al.* 1996). This appears to have been supported by the finding of negligible damage early in incubation, followed by an increase over time, paralleling the observed incidence of capacitation. In either case, an acrosome that reacts or that is damaged before interacting with the oocyte will likely result in a non-functional spermatozoon (Kopf & Gerton 1991). However, the percentage of intact acrosomes does not always correlate positively with embryo development after insemination *in vitro* (O'Brien & Roth 2000). Further, fertility may be unaffected, as shown in a recent study of sheep where pregnancy success was similar using inseminates containing a high versus a low percentage of acrosome-intact sperm, if adjustments were made to ensure that an equal number of intact sperm were inseminated (Gil *et al.* 2002).

Over our 9-h incubation, up to one-half of the sperm in each sample were intact and capacitated (Fig. 4). It is not known if this time-line is consistent with the course of events *in vivo*; however, the rate and extent of capacitation

were similar to that reported for bovine (Byrd 1981, Parrish *et al.* 1988), porcine (Hunter & Hall 1974), feline (Andrews *et al.* 1992) and human (Lee *et al.* 1987) spermatozoa. Previous studies have performed ultrastructural and chemical studies of giant panda sperm capacitation (Chen *et al.* 1989b Sun *et al.* 1996), but a detailed report of the expected time-course for capacitation has not been performed before. However, our results concur with those of Sun *et al.* (1996) who report that at least some giant panda sperm undergo capacitation by 5.5 h and 6.5 h of incubation.

It is somewhat surprising that CI and zonae emulsions were almost equally capable of eliciting the acrosome reaction, as a previous report has found that CI is able to induce capacitation in up to 40% more sperm than solubilised zona (Long *et al.* 1996). However, this may be accounted for by the fact that the giant panda sperm studied here were exposed to twice the concentration of emulsified zonae and one-quarter the CI concentration of that used by Long *et al.* (1996). It should be noted that this lower concentration of CI has been used successfully by other investigators (Garde *et al.* 1997); further, the incidence of acrosome reaction in this study increased over time, indicating that this concentration is sufficient to induce the acrosome reaction in capacitated sperm.

In comparing the efficacy of inducing the acrosome reaction with heterologous, solubilised ZP, it was somewhat surprising to discover that ZP of the cat were more effective than those of the panda's closer relative, the black bear. However, this result may simply be more a reflection of oocyte quality than a species effect. For example, whereas the cat oocytes were harvested within only 2–5 h of ovarian excision, bear ovaries were frozen after excision, sometimes 12 h postmortem. A delay between death and harvest plus the tissue cryopreservation no doubt resulted in a higher incidence of oocyte degeneration in black bear than

Table 1 Percentage of fresh and thawed giant panda spermatozoa categorised as having missing and damaged acrosomes after exposure to cat ZP or CI. Values are means $\pm$ S.E.M.

	Fresh aliquots		Thawed aliquots	
	Missing	Damaged	Missing	Damaged
0 h, ZP	3.0 $\pm$ 0.4 ^a	7.0 $\pm$ 2.2 ^a	6.0 $\pm$ 2.0 ^a	17.7 $\pm$ 2.8 ^a
0 h, CI	2.5 $\pm$ 0.9 ^a	9.4 $\pm$ 2.1 ^a	5.6 $\pm$ 3.2 ^a	20.4 $\pm$ 3.1 ^a
3 h, ZP	18.1 $\pm$ 4.2 ^b	24.8 $\pm$ 3.2 ^b	26.2 $\pm$ 4.7 ^b	25.2 $\pm$ 3.3 ^{a,b}
3 h, CI	20.7 $\pm$ 3.6 ^b	28.1 $\pm$ 3.1 ^b	23.8 $\pm$ 4.2 ^b	33.6 $\pm$ 5.9 ^b
6 h, ZP	40.3 $\pm$ 7.4 ^c	22.1 $\pm$ 2.3 ^b	43.4 $\pm$ 3.7 ^c	27.8 $\pm$ 4.7 ^{a,b}
6 h, CI	43.7 $\pm$ 8.4 ^c	25.2 $\pm$ 3.6 ^b	43.4 $\pm$ 7.1 ^c	30.2 $\pm$ 5.2 ^{a,b}
9 h, ZP	52.0 $\pm$ 4.6 ^c	18.9 $\pm$ 2.2 ^b	62.4 $\pm$ 4.9 ^c	22.2 $\pm$ 5.3 ^{a,b}
9 h, CI	63.0 $\pm$ 2.8 ^d	23.4 $\pm$ 3.1 ^b	61.6 $\pm$ 5.7 ^c	25.4 $\pm$ 5.9 ^{a,b}

Different superscripts within the same column differ significantly ($P < 0.05$).

cat oocytes, perhaps compromising ZP composition. Additionally, cat oocytes underwent *in vitro* maturation prior to zonae harvesting, which may alter the acrosome reaction-inducing properties of the zonae. Regardless, it was important to recognise the efficacy of ZP from an evolutionarily distant family (i.e. the Felidae) inducing the acrosome reaction in a unique species in the family Ursidae. This finding substantiated earlier assertions for a lack of species specificity in the ability of the ZP to induce the acrosome reaction (Lee *et al.* 1987, Yoshimatsu *et al.* 1988, Slavik *et al.* 1990).

Giant panda sperm were able to capacitate and acrosome react in HEPES-buffered medium. Others have reported that HEPES is detrimental to capacitation (Visconti *et al.* 1999), but only if it contains no bicarbonate (Shi & Roldan 1995). Our capacitation-supporting, HEPES-based medium contained bicarbonate that maintained the spermatozoa at the requisite pH. This relatively simple finding is quite important when working in situations (as we were in this remote centre) where there was an absence of a CO₂-regulated incubator. We expect that this finding will also be applicable when we eventually collect samples under even more rigorous conditions in the field.

Cryopreservation of giant panda spermatozoa did not alter the subsequent pace or overall incidence of capacitation compared with fresh counterparts until the final time-point, and only in the HF10 treatment. This similarity among treatments most likely was an artifact of the increase in spontaneous acrosome reaction or damage in the control groups. Because we specifically defined a capacitated spermatozoon as one that was intact and capable of undergoing the acrosome reaction (by subtracting the percentage of sperm with intact acrosomes in treatment groups from control groups), the potential number of cells falling into this category decreased over time as more sperm were damaged or underwent acrosome reaction spontaneously. Nonetheless, the similarity of fresh and thawed sperm was surprising. In general, it is considered that the components of the egg yolk extenders used to protect the sperm during cryopreservation accelerate the capacitation process as determined by their ability to penetrate zona-free oocytes (Ijaz & Hunter 1989, Ijaz *et al.* 1989, Bielfeld *et al.* 1990, Cormier *et al.* 1997), but detection of capacitation in sperm exposed to an egg yolk buffer using CI may be suppressed compared with more physiological assays (Bielfeld *et al.* 1990). Bielfeld *et al.* (1990) found that CI induced acrosome reaction in sperm that were incubated in a salt-based medium but not those exposed to egg yolk, whether the egg yolk was removed before testing or not (Bielfeld *et al.* 1990). So it is possible that a difference between the capacity of fresh and thawed sperm to capacitate existed but was masked by our assessment technique. However, if this was the case, we would have expected the ZP treatment to demonstrate any increased capacitation in thawed sperm.

Cryopreservation and thawing of sperm also appears to accelerate capacitation by inducing calcium flux and

changes in lipid bilayer structure and tyrosine phosphorylation (Green & Watson 2001). These spermatozoa often are capable of fertilisation and progressing into hyperactivity associated with capacitation (Eriksson *et al.* 2001); however, this suite of changes are of a destabilising nature and may contribute to reduced sperm longevity post-thawing (Samper 2001). Whether giant panda spermatozoa are less susceptible than other species to these perturbations (therefore explaining their ability to stay motile *in vitro* for long periods (Moore *et al.* 1984)) remains in question and requires further study. A logical priority would be to determine fresh versus frozen-thawed sperm longevity *in vitro* and *in vivo*. Thus, fresh and frozen giant panda sperm appear to spontaneously capacitate to an equivalent extent. It is possible that simply removing seminal plasma was sufficient to elicit membrane-destabilising effects similar to capacitation in the giant panda as it is in boar sperm (Maxwell & Johnson 1999).

In summary, giant panda spermatozoa are readily induced to capacitate *in vitro*, and the acrosome reaction can be elicited using CI or heterologous (felid or ursid) ZP. Most importantly, there is no detrimental effect on this requisite step to fertilisation using spermatozoa subjected to a rapid rate cryopreservation and thawing protocol. These results provide new fundamental information on a high profile, endangered species while suggesting that the fertility of cryopreserved-thawed giant panda spermatozoa will be similar to that following the use of fresh spermatozoa. This is critically important, as AI with fresh sperm has resulted in the birth of more than six cubs in the past 2 years alone (Xie Zhong, personal communication). The data presented here will likely increase the confidence of giant panda managers in testing frozen-thawed sperm to achieve genetic management goals (Wildt *et al.* 2002).

Acknowledgements

We thank Lena May Bush for technical assistance, Dr Budhan Pukazhenthil for valuable advice, Jonathan Aaltonen and Abby Mathewson for technical assistance. Financial support was provided by Friends of the National Zoo, Morris Animal Foundation and the Smithsonian Institution's Women's Committee. The China Wildlife Conservation Association is a co-operative partner in the Smithsonian's National Zoological Park's research and conservation programme for giant pandas.

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Received 7 October 2003

First decision 7 January 2004

Accepted 2 February 2004