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Giant panda (*Ailuropoda melanoleuca*) spermatozoon decondensation *in vitro* is not compromised by cryopreservation

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Giant panda spermatozoa decondensation

Natural breeding of giant pandas in captivity is compromised, making artificial insemination and spermatozoa cryopreservation essential for genetic management. This study examined the influence of freeze–thawing on traditional parameters such as motility and spermatozoon functionality, specifically decondensation *in vitro*. Giant panda spermatozoa were assessed before and after rapid cryopreservation (4°C to –130°C over 2 min) in liquid nitrogen vapour. Spermatozoa pre-incubated in medium for 6 h were co-incubated with cat zonae (2 zonae μL^{-1}) for 30 min to effect capacitation and acrosome reaction. Spermatozoa were then mixed with mature cat oocyte cytoplasm (2 cytoplasm μL^{-1}) for 4 h and evaluated for decondensation. Frozen spermatozoa were less motile ($P < 0.05$) than fresh counterparts immediately post-thawing, but not after 6 h incubation. There were more ($P < 0.05$) spermatozoa with completely diffused chromatin post-thaw (10.4 ± I.3%; mean ± s.e.m.) compared with fresh counterparts (5.I ± 1.0%). However, there was no overall difference ($P > 0.05$) in the incidence of decondensation between fresh (4 h, 69.8 ± 5.9%) and thawed (4 h, 71.5 ± 4.9%) spermatozoa after exposure to cat oocyte cytoplasm. It is concluded that the 'rapid' method now used to cryopreserve giant panda spermatozoa has little impact on spermatozoon decondensation.

Introduction

The combination of artificial insemination (AI) and spermatozoa cryopreservation plays an essential role in the *ex situ* genetic management of the giant panda (*Ailuropoda melanoleuca*) (Zheng *et al.* 1997). The need for more research to improve assisted breeding in this species was recognised in 1996 at an international workshop on 'Giant Panda Captive Management Planning' held in China, in part to

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circumvent the common problem of poor reproductive behaviour in males and/or aggressive behaviours towards conspecific females (Zheng *et al.* 1997). The use of assisted breeding is now well recognised in China as a means of ensuring that every genetically valuable individual becomes appropriately represented in the captive population (Wildt 1989; Huang *et al.* 2002; Wildt *et al.* 2002). Additionally, because the giant panda is recognised as an icon for 'species conservation' both within and outside of China, the ability to 'assist' breeding also overcomes biopolitical barriers to moving genes between countries or agencies while avoiding stressful and expensive animal shipments from one location to another (Liu 1981; Hodges *et al.* 1984; Moore *et al.* 1984; Zhang *et al.* 1991; Wildt *et al.* 2001).

Cryopreserved spermatozoa have been used to produce healthy offspring in many domestic and non-domestic animals, including the giant panda (Hu and Wei 1990; Ye *et al.* 1991; Zhang *et al.* 1991; Huang *et al.* 2002). The success of AI is compromised by 15 to 40% in the pig (Johnson *et al.* 1981), cow (Foote and Arriola 1987), horse (Loomis *et al.* 1983) and dog (Silva *et al.* 1996) when using thawed compared with fresh spermatozoa. However, reduced conception does not always occur as a result of using thawed sperm. For example, AI success can be maintained in the sheep (Maxwell *et al.* 1999) and mare (Lindsey *et al.* 2002) using thawed sperm, if total number of spermatozoa inseminated is increased (Cayan *et al.* 2001; Wood *et al.* 2002). Rather than an absolute total spermatozoon number, it seems more likely that AI success with thawed spermatozoa depends on the total number of motile, acrosome-intact spermatozoa in the sample (Thundathil *et al.* 1999), an index that has been rarely studied.

Giant pandas generally produce excellent quality spermatozoa that can be harvested easily by electroejaculation (Platz *et al.* 1983; Moore *et al.* 1984; Howard *et al.* 2001; Spindler *et al.* 2004). Various protocols have been used to cryopreserve spermatozoa from this species (Platz *et al.* 1983; Zhang *et al.* 1991; Chen *et al.* 1992; Fei 1992; Huang *et al.* 2000; Spindler *et al.* 2001). Our laboratories have developed a rapid-rate (–40°C min^{-1} for 1 min, –100°C min^{-1} for 1 min, before plunging into liquid nitrogen) spermatozoa cryopreservation protocol (Spindler *et al.* 2001). This method was developed, in part, to be easily adaptable under remote and primitive conditions, including in the field. Using this protocol, giant panda spermatozoa maintain excellent motility and acrosomal integrity without compromising the ability to undergo capacitation and the acrosome reaction (Spindler *et al.* 2004). The subsequent step in the fertilisation process is decondensation, whereby the male genetic material is made accessible to the oocyte. The phenomenon of giant panda spermatozoa decondensation has not been studied previously, and we were particularly interested in determining if this process was influenced by spermatozoon dilution or the rate of cooling, freezing and thawing.

Our concern emanated from the knowledge that structural changes occur during freeze–thawing that may influence decondensation. At temperatures around –15°C to –60°C, the water in medium

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surrounding the spermatozoon freezes while intracellular water is temporarily supercooled, allowing it to exit the cell in response to an increased osmotic gradient (Mazur 1963). The resulting cell shrinkage and dehydration can help avoid intracellular ice-crystal formation that can cause lethal membrane damage upon thawing (Mazur 1977). However, over protracted time periods, dehydration also can cause intracellular damage, including deranged mitochondrial (Gao *et al.* 1997) and chromatin structure (Martin *et al.* 1988; Rybouchkin *et al.* 1996; Gao *et al.* 1997), both of which have the potential to alter a spermatozoon's ability to undergo decondensation and fertilisation (Perreault 1990).

The disulfide bonds that bind the spermatozoon chromatin in the head region are formed during epididymal maturation by oxidation of free sulfhydryl groups of cysteine residues within spermatozoon protamines, resulting in compaction and nuclear rigidity (Calvin and Bedford 1971; Bedford and Calvin 1974; Evenson *et al.* 1989). Upon oocyte penetration, these disulfide bonds are broken to allow male pronucleus formation (Wiesel and Schultz 1981; Perreault *et al.* 1984, 1988*a*) and the spermatozoon chromatin to become part of the zygotic unit (Usui and Yanagimachi 1976; Crozet and Dumont 1984). Only completely decondensed spermatozoa are capable of fertilisation. Spermatozoon chromatin structure has been shown to be altered by cryopreservation/thawing in the mouse (Watson 2000; Kusakabe *et al.* 2001) and horse (Linfor and Meyers 2002).

Although decondensation depends on spermatozoon disulfide bond and chromatin integrity (Lipitz *et al.* 1992), it is the oocyte's cytoplasm that induces decondensation (Yanagimachi 1994; Sutovsky and Schatten 1997). The study of decondensation often is conducted *in vitro* using chemicals rather than oocytes (Calvin and Bedford 1971; Perreault *et al.* 1988*b*; Samocha-Bone *et al.* 1998; Reichart *et al.* 2000; Martin *et al.* 2003). However, cytoplasmic emulsions from homologous or heterologous oocytes (Yanagimachi 1984; Ohsumi *et al.* 1986) are most likely to be physiologically meaningful as an *in vitro* test of spermatozoon decondensation (Yanagimachi 1994; Li and Gui 2003). In the present study, we established an *in vitro* test of spermatozoon decondensation using mature cytoplasm (from readily available domestic cat oocyte material) to avoid possible artefacts of chemical induction. Our aim was to examine the ability of giant panda spermatozoa to decondense before and after a rapid cryopreservation process that is becoming popular in giant panda breeding centres in China.

Materials and methods

Animals

The study population included eight giant pandas of reproductive age (5 to 16 years, weighing 79.5 to 130.0 kg each) maintained at the China Conservation and Research Center for the Giant Panda in the Wolong Nature Reserve. Males were housed singly in: (*1*) naturalistic outdoor enclosures of ~300 m^2 and connecting indoor areas of 8 m^2; or (*2*) concrete and grass outdoor enclosures of ~35 m^2 and connecting indoor areas of 8 m^2. Both situations afforded

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test males 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 week without being in an enclosure immediately adjacent to an adult female. Fresh bamboo (~20 stalks) was provided up to seven times daily, along with a high-fibre supplement consisting of soybean, bamboo powder, corn, rice and vitamin and mineral supplements (M. Edwards, personal communication). Water was provided *ad libitum*. All semen samples were collected during the spring (February through May) breeding season (Schaller *et al.* 1985). All procedures were approved by the Smithsonian's National Zoological Park's Institutional Animal Care and Use Committee, 2001.

Semen collection and analysis

All chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA) unless otherwise stated. Semen was collected as described previously (Platz *et al.* 1983; Howard *et al.* 1993; Spindler *et al.* 2004). Briefly, a surgical plane of anaesthesia was initiated by an intramuscular injection of 10 mg kg^{-1} ketamine HCl (Ketaset, Fort Dodge Laboratories, Fort Dodge, IA, USA) and maintained with 0–5% isofluorane gas, as needed. The electroejaculation technique relied on 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 no more than 20 min.

Semen was collected into a sterile glass container. Seminal volume was immediately measured by pipetting, and pH was determined using Colour 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 did not occur. A 5-μL seminal aliquot was examined using phase-contrast microscopy (200×; Olympus BX40 microscope, Olympus, city, country) for a subjective estimate of spermatozoon motility traits, including an estimate of spermatozoon motility and progressive motility (i.e. forward progression on a 0-to-5 scale with a '5' status being indicative of rapid forward movement of spermatozoa and a '0' status indicating no forward progression) (Howard 1993). A 5-μL sample was removed and added to 100 μL of fixative (0.3% glutaraldehyde in phosphate-buffered saline) for morphologic examination of spermatozoa (200 per sample) by phase-contrast microscopy (630×) (Howard 1993). Spermatozoa were categorised as normal or as having one of the following anomalies: abnormal head (including macrocephaly, microcephaly, 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 that displayed an abaxial attachment of the midpiece was noted, but not considered abnormal (Moore *et al.* 1984). Spermatozoon acrosomal integrity was evaluated using the rose bengal/fast green stain (Pope *et al.* 1991). In brief, spermatozoa were stained for 90 s, smeared on a glass slide, allowed to air-dry and examined (minimum 100 sperm) under brightfield microscopy (630×). Spermatozoa were categorised as having an intact acrosome (acrosomal membrane stained blue) or non-intact acrosome (damaged; missing or loose acrosomal membrane, appearing pink or white) (Spindler *et al.* 2004). Within each ejaculate, the

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results from the three seminal series were assessed, and ejaculate aliquots differing from each other by no more than 10% in both spermatozoon motility and progressive status estimates were combined. An additional 10-μL sample from each electroejaculate was used to determine spermatozoon concentration using a haemocytometer (Howard *et al.* 1990). Spermatozoa were maintained at 37°C throughout assessment and processing using a water-bath or dry-bath incubator (Fisher, Hanover Park, IL, USA).

Zona pellucida and cytoplasmic emulsion preparation

Zona pellucida (ZP) and cytoplasmic emulsion (CYTEM) treatments were prepared in the USA using cat oocytes recovered from ovariohysterectomy material collected fresh from local veterinary clinics and processed as described previously (Spindler and Wildt 2002). Briefly, oocytes were recovered by mincing the ovaries with a scalpel blade to liberate oocytes into Ham's F10 medium plus 5% fetal calf serum and 25 mM HEPES, hereafter designated as HF10. Grade I oocytes with uniform, dark cytoplasm and an intact cumulus cell investment (Wood and Wildt 1997) were selected for maturation and rinsed three times in HF10. Oocytes were incubated (10 oocytes in 50 μL) in maturation medium (MEM) containing 1.0 mM glutamine, 1.0 mM pyruvate, 100 IU mL^{-1} penicillin, 100 mg mL^{-1} streptomycin, 4 mg mL^{-1} bovine serum albumin, 1 μg mL^{-1} follicle-stimulating hormone (1.64 IU mL^{-1}; NIDDK-ovine follicle-stimulating hormone-17, lot 3082; National Hormone and Pituitary Program, Rockville, MD, USA), 1 μg mL^{-1} luteinising hormone (1.06 IU mL^{-1}; NIDDK-ovine luteinising hormone-25, lot 3502; National Hormone and Pituitary Program), and 1 μg mL oestradiol (Sigma Chemical Co.). Oocytes were cultured in this medium under mineral oil for 32 h at 38°C in a humidified environment of 5% CO_2 in air (Wolfe and Wildt 1996). Oocytes were denuded of cumulus cells, rinsed and placed in small volumes of HF10 medium, and punctured with a glass Pasteur pipette that had been heated and 'pulled' so that the internal diameter was equivalent to the outer diameter of the oocyte. Oocytes were pipetted up and down through the pipette until broken and the zonae separated from the cytoplasm. Zonae used to induce the acrosome reaction were then rinsed through several drops of medium and finally taken up in medium at a concentration of 4 ZP $μL^{-1}$. A separate group of oocytes were used to provide cytoplasm for the decondensation assessment. The zonae and cytoplasm of these oocytes were similarly separated, but the zonae were removed, leaving the cytoplasm–medium emulsion (~4 cytoplasm $μL^{-1}$ of medium). These ZP and CYTEM solutions were frozen at –80°C and transported to China for future incubation with sperm.

Pre-incubation and acrosome reaction

To emulate peri-fertilisation events, capacitation was induced in spermatozoa before testing for decondensation. One semen sample (500 μL) was removed immediately after combining all seminal series and diluted with HF10. Samples were centrifuged (200*g*, 8 min) and the supernatants were discarded before each aliquot was resuspended in HF10 and incubated at 37°C for 6 h (final concentration, 50×10^6 mL^{-1}; volume, 500 μL). This medium and preparation protocol was identical to those known to induce capacitation in giant panda spermatozoa before and after cryopreservation (Spindler *et al.* 2004). An aliquot (100 μL) was removed and incubated in 0.5-mL Eppendorf microcentrifuge tubes with an equal volume of solubilised cat ZP solution (final concentration 2 ZP $μL^{-1}$)

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at 37°C for 30 min, a treatment known to induce the acrosome reaction in the giant panda (Spindler *et al.* 2004). The percentage of spermatozoa undergoing acrosome reaction was determined by examining the state of the acrosomal cap before and after addition of the zona pellucida emulsion. 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 spermatozoon head; (*2*) a damaged acrosome whereby the membrane was damaged, or was separate from the spermatozoon head; or (*3*) a missing acrosome. The percentage of acrosome-reacted spermatozoa was defined as the proportion of spermatozoa with missing acrosomes after the addition of ZP compared with the proportion of spermatozoa without acrosomes in control medium (HF10).

Assessment of decondensation

Following ZP exposure, each treatment (HF10 and CHF10) was divided into two equal-volume aliquots, centrifuged (100*g*, 8 min) and the supernatant discarded. Pellets of these samples were resuspended in 100 μL of HF10 medium (control) or cat oocyte cytoplasm emulsion (CYTEM) under mineral oil at 37°C for 4 h. During the 4-h incubation in CYTEM, giant panda spermatozoa samples (5 μL) were removed at 0, 2 and 4 h and assessed for decondensation using brightfield microscopy (630×). Spermatozoa were categorised as: (*1*) normal spermatozoa (fully opaque) with condensed cytoplasm; (*2*) partially decondensed (spermatozoon head appearing more transparent than undecondensed counterparts, but not noticeably expanded); (*3*) completely decondensed (spermatozoon head appearing transparent and expanded in size compared with normal condensed spermozoon head); or (*4*) diffuse spermatozoon head (only post-acrosomal region remaining) (Fig. 1). Spermatozoa that had undergone partial and complete decondensation were combined for analyses of total spermatozoon decondensation. The spermatozoa with completely diffused chromatin were not included in this category because the significance of this spermatozoon type has yet to be established.

Spermatozoa cryopreservation and thawing

The remainder of the ejaculate that was not used to determine decondensation of fresh spermatozoa 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. The semen was diluted slowly over a 3-min period. Semen was diluted in a 15-mL conical, plastic tube (Falcon, Bedford, MA, USA) to give a final concentration of 400×10^6 motile spermatozoa mL^{-1}. Each tube then was placed in a water jacket (400 mL of 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) in an identical tube in the same waterbath. Cooled semen was pipetted into 0.25-mL sterile, plastic straws (Veterinary Concepts, Spring Valley, WI, USA), each of which was sealed using a Nyclave impulse heat sealer (Lorvic Corporation, St Louis, MO, USA). Each straw was placed 7.5 cm above liquid nitrogen (LN) for 1 min and then 2.5 cm above LN for 1 min to achieve a rapid cryopreservation rate of –40°C min^{-1} and –100°C min^{-1} respectively (as determined by previous control thermocouple testing). Frozen samples were plunged into the

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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 was allowed to flow into a 5-mL sterile plastic tube (Falcon) where it was diluted slowly over a 3-min period with 2 mL HF10 at 37°C. The thawed sample was centrifuged in a 1.5-mL Eppendorf microcentrifuge tube (200*g*, 8 min), the supernatant discarded and the spermatozoa pellet resuspended in HF10 (final concentration of 50×10^6 cells mL^{-1} in 500 μL) at 37°C. Thawed spermatozoa then were pre-incubated in HF10 for 6 h and ZP for 30 min, then CYTEM for 4 h and assessed for decondensation exactly as described above for fresh counterparts.

Statistical analysis

Differences among treatments were evaluated using analysis of variance (ANOVA) and Dunnett's multiple comparison testing (Miller 1981). Percentage data were arcsine transformed before analysis. Data are expressed as means ± s.e.m. Correlation coefficients among spermatozoa traits were determined using the Pearson-product moment correlation curve (Box *et al.* 1978).

Results

The eight giant panda ejaculates were similar ($P > 0.05$) in assessed measures, each having high concentrations of motile spermatozoa with relatively few pleiomorphic forms. Semen volume (2.6 ± 0.5 mL), spermatozoon concentration ($1.2 \pm 0.3 \times 10^9$ spermatozoa mL^{-1}), initial motility (75.6 ± 4.6), forward progression (3.4 ± 0.2) and acrosomal integrity (93.0 ± 1.9% intact) also were consistent with previous reports (Platz *et al.* 1983; Chen *et al.* 1994). Most spermatozoa were morphologically normal (83.3 ± 3.3%), although many (71.7 ± 8.8%) displayed an abaxial attachment to the neck/midpiece region that has been categorised by a previous investigator as normal for this species (Moore *et al.* 1984).

From the initial (freshly collected) motility value (75.6 ± 4.6), spermatozoon motility decreased ($P < 0.05$) after slow cooling to 4°C (63.7 ± 4.1% motile sperm) 3.5 h later. There was no further loss ($P > 0.05$) of motility upon thawing (57.1 ± 3.1%). This similarity ($P > 0.05$) in motility values was maintained throughout the 6-h pre-incubation (fresh, 47.2 ± 4.8%; thawed, 42.8 ± 5.7%). After 30-min incubation with ZP solution, spermatozoon motility was <10% in all treatments.

The incidence of spontaneous decondensation (partial and complete) in thawed spermatozoa incubated in HF10 medium did not differ ($P > 0.05$) from fresh spermatozoa under identical conditions at any time (total decondensation, fresh = 16.9 ± 4.1%; frozen = 14.9 ± 3.7%) (Fig. 2). Spermatozoa with completely diffused chromatin were also observed in samples incubated in medium alone (Fig. 2). The percentage of partially decondensed spermatozoa pre-incubated in HF10 increased ($P < 0.05$) to ~25% after only a 2-h incubation with CYTEM, but did not increase thereafter (Fig. 3). In contrast, the proportion of completely decondensed spermatozoa in the HF10 pre-incubation group increased ($P < 0.05$) after 2 h and again after

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4-h incubation in CYTEM (Fig. 3). The number of diffuse-headed spermatozoa was greater ($P < 0.05$) after a 4-h incubation in CYTEM compared with 0 h (Fig. 3).

The percentage of spermatozoa with a diffused head was greater ($P < 0.05$) in post-thaw spermatozoa (10.4 ± 1.3%) compared with fresh spermatozoa (5.1 ± 1.0%) after a 4-h incubation in CYTEM. Frozen–thawed spermatozoa had more ($P < 0.05$) partially and completely decondensed and headless spermatozoa after a 2- and 4-h incubation in CYTEM compared with 0 h (Fig. 3). The incidence of partial spermatozoon decondensation did not differ ($P > 0.05$) between 2 and 4 h in either medium. The total percentage of spermatozoa that underwent partial and complete decondensation (71.5 ± 4.9%) after 4 h incubation in CYTEM did not differ ($P > 0.05$) from fresh counterparts.

Discussion

Giant panda spermatozoa decondensed in response to culture in the presence of felid (heterologous) oocyte cytoplasm after ZP exposure. This ability of giant panda spermatozoa to undergo decondensation was not impaired by the rapid-rate freezing protocol that already has been found to be innocuous to post-thaw spermatozoon motility and viability (as measured by acrosomal integrity and ability to undergo capacitation) (Spindler *et al.* 2004). These studies add to growing evidence that cryopreserved giant panda spermatozoa are not compromised in fertilisability if suitable post-thaw techniques are used.

The response of giant panda spermatozoa to cat cytoplasm supports the assertion that there is limited species specificity in induction of decondensation (Yanagimachi 1984, 1988; Ohsumi *et al.* 1986). Further, human spermatozoa will undergo apparently normal decondensation and pronuclear formation after penetrating zona-free hamster eggs (Yanagimachi 1984) and even after being injected into the eggs of an evolutionarily distant genus, *Xenopus* (Ohsumi *et al.* 1986). In fact, cytoplasm from cells other than oocytes (such as fibroblasts) can induce membrane and nuclear swelling and even decondensation in a small proportion (~2%) of human spermatozoa (Van Meel and Pearson 1979). Conversely, gibel carp oocyte emulsions induced decondensation in conspecific spermatozoa, but were insufficient to induce decondensation in the closely related red common carp (Li and Gui 2003). Thus, species specificity of spermatozoon decondensation appears to be strict in some taxa and not in others. There also is variability in rate of decondensation among species (Yanagimachi 1994).

There appears to be quite a bit of variation in rates of spermotozoon decondensation among species. For example, decondensation of human spermatozoa is generally complete in 1 to 2 h (Dozortsev *et al.* 1995; Hammadeh *et al.* 2001), whereas decondensation in the golden hamster is complete within 40 min (Yanagimachi and Noda 1970). Mouse spermatozoa undergo decondensation within 1 h (Urner and Sakkas 1999), although this rate will extend up to 4 h depending on post-ovulatory age of the oocytes

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used to induce the response (Fraser 1979). After intracytoplasmic spermatozoon injection, decondensation in sheep spermatozoa occurs within 1 to 2 h (Gomez *et al.* 1998) and between 3 and 6 h in horse spermatozoa injected into heterospecific oocytes (Li *et al.* 2003). Given this variation, it is possible in the present study that cat oocyte cytoplasm induced decondensation (2–4 h) in a differing temporal pattern than would have been elicited using giant panda or ursid oocytes. Cytoplasm used for this assay was heterospecific and also diluted in medium (cytoplasm from 2 oocytes μL^{-1}), so each spermatozoon was exposed to less concentrated cytoplasm than if the spermatozoon had penetrated an oocyte. Thus, it was difficult to estimate if the rate of giant panda spermatozoa decondensation observed here was typical of what would occur during the normal course of oocyte fertilisation.

The progression of giant panda spermatozoa from normal, through expanded or partially decondensed to fully decondensed appeared consistent with descriptions of this same spermatozoon phenomenon in other species, including the human (Dozortsev *et al.* 1995), sheep (Dozortsev *et al.* 1995) and mouse (Fraser 1979). However, complete detachment of the chromatin from the spermatozoa calyx has rarely been described. Spermatozoa with completely diffused chromatin previously may have gone largely unnoticed because most decondensation assessments have been by flow cytometry (Molina *et al.* 1995; Samocha-Bone *et al.* 1998; Rens *et al.* 2001), agarose gel electrophoresis (Ohsako *et al.* 1997) or cellular assessments after intra-cytoplasmic spermatozoon injection (Tateno *et al.* 2000; Kusakabe *et al.* 2001), rather than by examining individual spermatozoa by microscopy.

It is likely that this spermatozoon type is merely a natural progression from the chromatin swelling and disruption of the subacrosomal region whereby the s-bonds within the spermatozoon were broken with the chromatin becoming more loosely bound, and eventually dispersing into the cytoplasm (Longo *et al.* 1987). However, there also are reports of damage (including protamine extrusion from the nucleus during decondensation (Bezanehtak and Swan 1999) and chromatin structural abnormalities after cryopreservation (Cordova *et al.* 2002) and processing (Molina *et al.* 1995). Thus, the probability that the number of spermatozoa with this level of detachment is increased as a result of damage cannot be discounted.

It was apparent that the number of diffused chromatin spermatozoa increased with time, particularly in thawed cohorts (Fig. 3). Normal spermatozoa that already had undergone complete decondensation may have been damaged by the assessment process itself, either while being aspirated into the transfer pipette and/or being covered with a coverslip, both of which could have physically separated the fragile decondensing chromatin from the base of the spermatozoon head. It was possible that our observations were made well beyond the time normally taken for decondensation to fully occur. However, this was not likely because the percentage of spermatozoa that underwent both partial and complete decondensation

continued to increase through this time point. Therefore, it is unlikely that the induction or examination processes alone caused the increased incidence of spermatozoa with diffuse chromatin.

Chromatin integrity can be maintained in thawed spermatozoa following suitable cryopreservation techniques (Ohsako *et al.* 1997; Steele *et al.* 2000). We have previously demonstrated the resilience of giant panda spermatozoa to membrane damage after rapid rate cryopreservation followed by thawing (Spindler *et al.* 2004). The present data indicated that giant panda spermatozoon chromatin also may be resistant to injury. Immature (testicular) spermatozoa appear to sustain more chromatin damage during freeze–thawing than epididymal and ejaculated spermatozoa (Wood *et al.* 2002). This is most likely because spermatozoon chromatin is structurally stabilised during the epididymal maturation process when single s-bonds between protamines become disulphide bonds (Calvin and Bedford 1971; Bedford and Hoskins 1990). Thus, ejaculates with a high proportion of immature spermatozoa (as indicated by presence of cytoplasmic droplets) may be less stable and, therefore, more likely to sustain damage during cryopreservation than ejaculates from a species such as the giant panda, which consistently produces excellent quality, mature sperm.

In summary, we have determined that induction of giant panda spermatozoon decondensation was possible under *in vitro* conditions using the physiological assessment tool of heterologous cytoplasm. Most importantly, a rapid-rate spermatozoa cryopreservation protocol that is recognised as 'field friendly' appeared to impose little or no detrimental effect on the critical fertilisation process of decondensation in the giant panda. All current evidence has supported the conclusion that this gamete preservation technique maintains the functionality of frozen–thawed giant panda sperm.

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Fig. 1. (*a*) 'Normal' giant panda spermatozoa before decondensation were opaque. (*b*) 'Partially decondensed' giant panda spermatozoa were comparatively more transparent. (*c*) 'Fully decondensed' spermatozoa were transparent and enlarged. (*d*) Spermatozoa with only the calyx remaining were categorised as diffuse headed sperm. Scale bars = 10 μm.

Fig. 2. Percentage of fresh (□) and frozen–thawed (■) giant panda spermatozoa ($n = 8$ ejaculates) undergoing spontaneous decondensation in control medium.

Fig. 3. Percentage of (*a*) fresh and (*b*) frozen–thawed spermatozoa undergoing decondensation after incubation in cytoplasmic emulsion ($n = 8$ ejaculates). ^{a,b,c}Within categories, different superscripts differ ($P < 0.05$). *Different from fresh spermatozoa at same timepoint ($P < 0.05$).