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Osmotic stress and cryoinjury of koala sperm: an integrative study of the plasma membrane, chromatin stability and mitochondrial function

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Abstract

This study investigated whether cryopreservation-induced injury to koala spermatozoa could be explained using an experimental model that mimics the structural and physiological effects of osmotic flux. DNA labelling after *in situ* nick translation of thawed cryopreserved spermatozoa revealed a positive correlation ($r=0.573$; $P<0.001$; $n=50$) between the area of relaxed chromatin in the nucleus and the degree of nucleotide labelling. While the chromatin of some spermatozoa increased more than eight times its normal size, not all sperm nuclei with relaxed chromatin showed evidence of nucleotide incorporation. Preferential staining associated with sperm DNA fragmentation (SDF) was typically located in the peri-acrosomal and peripheral regions of the sperm head and at the base of the spermatozoa where it appear to be 'hot spots' of DNA damage following cryopreservation. Results of the comparative effects of anisotonic media and cryopreservation on the integrity of koala spermatozoa revealed that injury induced by exposure to osmotic flux, essentially imitated the results found following cryopreservation. Plasma membrane integrity, chromatin relaxation and SDF appeared particularly susceptible to extreme hypotonic environments. Mitochondrial membrane potential (MMP), while susceptible to extreme hypo- and hypertonic environments, showed an ability to rebound from hypertonic stress when returned to isotonic conditions. Koala spermatozoa exposed to 64 mOsm/kg media showed an equivalent, or more severe, degree of structural and physiological injury to that of frozen–thawed spermatozoa, supporting the hypothesis that cryoinjury is principally associated with a hypo-osmotic effect. A direct comparison of SDF of thawed cryopreserved spermatozoa and those exposed to a 64 mOsm/kg excursion showed a significant correlation ($r=0.878$; $P<0.05$; $n=5$); however, no correlation was found when the percentage of sperm with relaxed chromatin was compared. While a cryo-induced osmotic injury model appears to explain post-thaw changes in koala SDF, the mechanisms resulting in relaxed chromatin require further study. A lack of correlation between the percentage of sperm with relaxed chromatin and SDF suggests that the timing of these pathologies are asynchronous. We propose an integrative model of cryo-induced osmotic injury that involves a combination of structural damage (rupture of membrane) and oxidative stress that first leads to the reduction of MMP and the relaxation of chromatin, which is then ultimately followed by an increase in DNA fragmentation.
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Introduction

The cryopreservation process exposes spermatozoa to an extreme range of fluctuating osmotic conditions. These conditions exert physical and biochemical stress on spermatozoa that influences their ability to survive the freeze–thaw process (Watson & Fuller 2001). Extracellular ice crystals formed during sperm cryopreservation result in an increase in solute concentration within unfrozen pockets of liquid and consequently form hypertonic environments so that subsequent equilibration of the solute concentration between intra- and extracellular environments results in osmotic efflux of water across cellular membranes. This in turn leads to a reduction of cellular volume and a distortion of the molecular and cytoplasmic organisation of organelles. During thawing, and removal of the cryoprotective agent, the osmotic environment is restored, and an influx of water across the sperm plasma membrane causes further damage to the already compromised cell (Curry & Watson 1994). Species-specific structural and physiological parameters inherent to the spermatozoa determine their capacity to respond to changes in temperature and osmolality during cryopreservation; particularly important are differences in plasma membrane composition that can affect membrane

permeability to water, ions and solutes and the lipid phase transition temperature (Holt 2000). By contrast, the effect of cryopreservation on DNA structure and stability has received remarkably less attention.

The majority of marsupial spermatozoa have no cysteine residues in their protamines (Retief *et al.* 1995) and consequently lack the stabilising disulphide bonds normally found in the sperm head of eutherian mammals; consequently, marsupial spermatozoa have been shown to be particularly susceptible to damage during air-drying or exposure to high divalent salt concentrations and detergents (Cummins 1980). The koala (*Phascolarctos cinereus*) is one species in which the effects of cryopreservation have been investigated (Johnston *et al.* 1994, 2006, Zee *et al.* 2007, 2008, 2009*a*). For example, koala spermatozoa have been shown to have a post-thaw viability of up to 60–70% if frozen in high levels of glycerol (14% v/v) combined with a relatively slow freezing rate of 6 °C/min. However, extended incubation of thawed koala spermatozoa for a further 2 h rapidly reduces the percentage of live spermatozoa and results in a corresponding increase in the number of cells with swollen or relaxed chromatin (Zee *et al.* 2008, 2009*b*). It is uncertain whether this modified form of chromatin is either a consequence of physical swelling or distortion of the DNA/protamine complex in response to osmotically induced injury or is associated with an increase in the fragmentation of the DNA molecule (Johnston *et al.* 2007, Zee *et al.* 2009*b*).

Mitochondrial membrane potential (MMP) has been shown to be a sensitive indicator of the bioenergetic functions within the mitochondrion (Duchen 2004), and while Zee *et al.* (2007, 2009*a*, 2009*b*) have previously used the JC1 probe (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide) to investigate the effect of cryoinjury on the koala sperm midpiece, the relative detrimental effects of osmotic stress vs structural damage associated with intracellular ice crystal damage remain unresolved. While the response of spermatozoa to the damaging effects of osmotic stress during cryopreservation can be demonstrated artificially by exposure of spermatozoa to a range of osmotically defined media (Curry & Watson 1994, Meyers 2005, Willoughby *et al.* 1996), to our knowledge, this experimental model has never been used before to examine the effect of osmosis on sperm DNA, and certainly not for marsupial sperm chromatin. In fact, the aim of this investigation was to examine whether osmotic flux is one of the primary causes of koala sperm cryoinjury. The first objective was to investigate the phenomenon of koala sperm chromatin relaxation post-cryopreservation associated with a loss of membrane integrity (Johnston *et al.* 2006, Zee *et al.* 2008, 2009*a*) and to determine whether it was linked to a corresponding increase in sperm DNA fragmentation (SDF). Our second objective was to examine the effect of varying anisosmotic media on the MMP of koala spermatozoa and directly compare the observations of spermatozoa from the same ejaculate following cryopreservation. This study was designed to differentiate the integrative effects of cryoinjury on koala spermatozoa that could be attributed to osmotic flux.

Results

Sperm chromatin relaxation

Although the koala sperm nucleus can display a high degree of plesiomorphy, the most common shape is one of a 'hooked' or 'sickle' appearance (Fig. 1a). Upon post-thaw incubation at 35 °C for 30 min, ∼14% of the koala spermatozoa demonstrated a massive increase in the size of the sperm head associated with a swelling or an apparent decondensation of the DNA–protamine complex (Fig. 1a, b, c and e). Spermatozoa with an
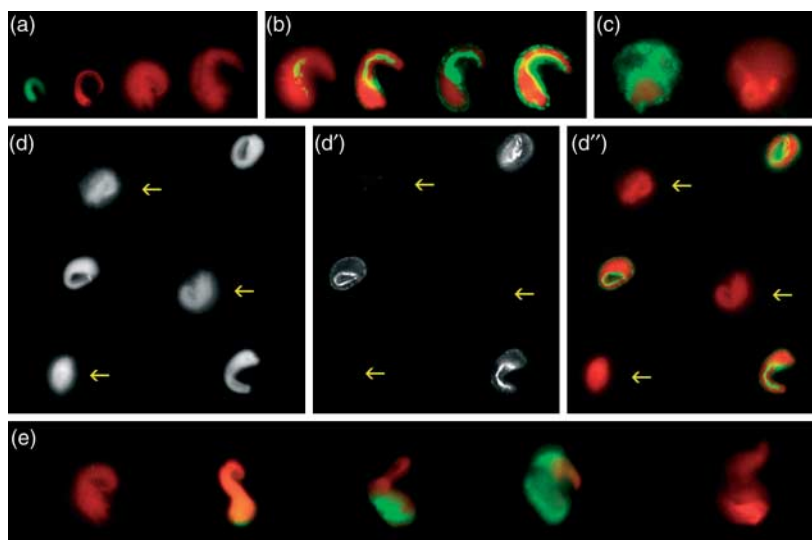

Figure 1 Koala sperm nuclei: (a) range of sperm nuclei showing relaxed chromatin – note small-sized green (SYBR-14) intact nucleus on the left side and the progressively larger red (PI+) membrane damaged nuclei on the right side; (b) sperm nuclei labelled with ISNT but no protein lysis – note nucleotide staining in the peri-acrosomal and nuclear periphery; (c) sperm nuclei with highly relaxed chromatin without protein lysis – nucleus on left side has ISNT staining, whereas the nucleus on the right side shows no evidence of DNA damage; (d) sperm nuclei stained with propidium iodide (PI); (d′) same sperm nuclei stained with ISNT – note partially relaxed nuclei with no evidence of DNA fragmentation (yellow arrow); (d″) same sperm nuclei with ISNT and PI background staining; (e) sperm nuclei with variable degrees of chromatin relaxation and ISNT staining – note nucleotide staining commencing at the base of the head and the large nucleus with relaxed chromatin on right with no evidence of fragmented DNA.

expanded nucleus were defined in this study as possessing a fluorescent signal at least double in area (measured in number of pixels) to that of a spermatozoon with an intact plasma membrane, although in some cases the nucleus expanded up to eight times its original size (Fig. 1a).

Sperm DNA fragmentation

SDF was assessed in this experiment using two different methodologies: the sperm chromatin dispersion (SCD) test and direct labelling of putative DNA breaks by *in situ* DNA extension using DNA polymerase I endonuclease free. After application of the SCD test, sperm nuclei were classified as either non-fragmented (2a: white arrows) or fragmented (2a: yellow and red arrows). Non-fragmented sperm nuclei showed no evidence of chromatin dispersion and in some instances maintained the original shape of the sperm head (Fig. 2a and b); fragmented sperm nuclei displayed a continuum of chromatin fragmentation from a small-to-medium (Fig. 2a and c, yellow arrows)-sized halo of chromatin dispersion to a massive chromatin dispersion of fragmented DNA and a corresponding decrease in size of the nuclear core (Fig. 2a, red arrow, d). It should be noted that chromatin dispersion in this context refers to the movement of the damaged sperm DNA in the SCD microgel following protein lysis. This phenomenon is different to that of chromatin relaxation, which is akin to a form of chromatin unwinding or swelling that is not necessarily associated with fragmented DNA. Spermatozoa identified as presenting with fragmented DNA were strongly labelled following the SCD and *in situ* nick translation (ISNT) procedures (Fig. 2c and d); the high activity of the DNA polymerase in this case was due to the existence of accessible $5'/3'$ ends after protein removal, leading to the production of an intensely labelled halo. The spermatozoa that were not affected by the protein depletion remained unlabelled (Fig. 2b).

Mapping sperm DNA damage

Although the highly compacted nature of the chromatin prevents in some instances the full accessibility of the polymerase for labelling the DNA breaks, ISNT performed directly on koala sperm nuclei without protein depletion was still capable of targeting sperm with DNA breakage. We observed that the ISNT-modified nucleotides were not randomly incorporated in the nucleus but were primarily confined to two specific locations: a peri-acrosomal (Fig. 1b, d, d′, d″) and a basal region (Fig. 1e). While there was a general trend for the ability of the polymerase to increase the level of incorporated labelled nucleotides as the size of the chromatin increased or relaxed (compare Fig. 1b with c and different sized spermatozoa in Fig. 1e), there
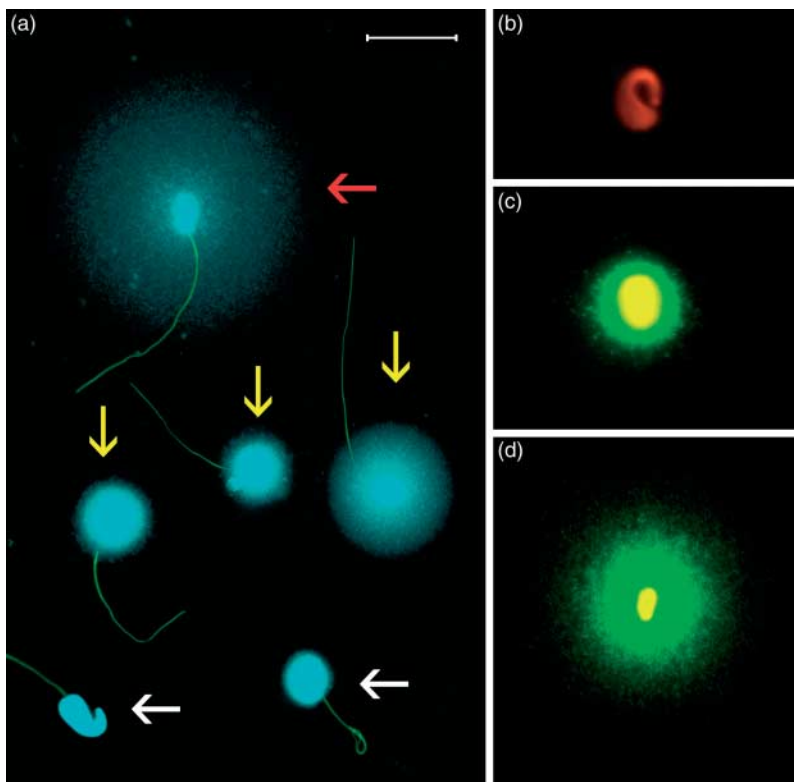

Figure 2 Sperm DNA fragmentation of koala spermatozoa: (a) non-fragmented (white arrows) and fragmented koala sperm nuclei (small to medium – yellow arrows and large – red arrow); (b, c and d) ISNT following protein lysis – (b) non-fragmented; (c) small to medium halo fragmented and (d) large halo fragmented with highly dispersed chromatin.

were also spermatozoa that showed a high degree of chromatin relaxation but remained unlabelled (Fig. 1c and arrow in d–d″). Conversely, there were other sperm nuclei showing no evidence of massive chromatin relaxation that were labelled by ISNT (Fig. 1d–d″).

To assess a possible relationship between the degree of chromatin relaxation and the extent of DNA damage, a Pearson's *r* correlation analysis was conducted. Figure 3 depicts the relationship between the area of the sperm chromatin (red fluorescence, measured in pixels as visualised in Fig. 1b, relaxed chromatin) and the incidence of DNA ISNT label (green fluorescence as visualised in Fig. 1b, SDF) for 50 thawed incubated spermatozoa. There was positive correlation ($r = 0.573$; $P < 0.001$) between the degree of chromatin relaxation and the extent of DNA labelling following ISNT. It is interesting to note that the primary regional sperm mapping of DNA labelling commenced on the chromatin around the acrosomal region but expanded from this region to the whole periphery of the spermatozoon (Fig. 1b and d). The selective incorporation of nucleotides to the basal region of the spermatozoon was also observed in some cells (Fig. 1e). In these latter nuclei, the expansion of the DNA labelling occurred from the base of the sperm head to the distal extremity (Fig. 1e), and there was also a tendency towards increased DNA labelling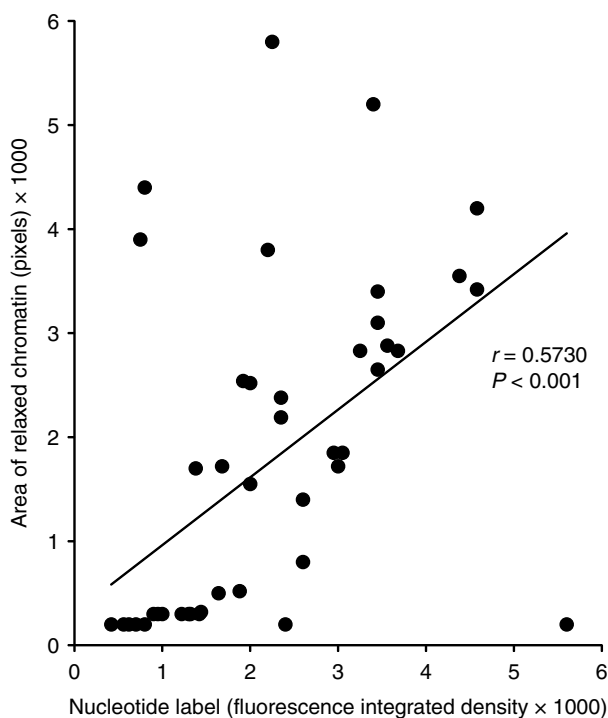 as the chromatin became more relaxed; note, however, that in other sperm nuclei, the highly relaxed nucleus was devoid of DNA labelling (Fig. 1e; far right).

Mitochondrial membrane potential

In this study, it is possible to recognise three levels of MMP (Fig. 4). High MMP was characterised by the majority of the mitochondria in the midpiece of the spermatozoon showing evidence of red/orange fluorescence (Fig. 4a and d); mitochondria of low MMP were characterised by a green fluorescence (Fig. 4b and e), while in other rare occasions, an absence of MMP (Fig. 4c and f) was also noted. Thus, MMP could be co-localised with the membrane viability, producing a total of six different classes of spermatozoa as shown in Fig. 4. Interestingly, a small fraction of spermatozoa that showed evidence of damaged plasma membranes (propidium iodide (PI)-positive sperm head) was still capable of possessing high or low MMP (Fig. 4d and e). Subsequent experiments in this study have only reported the effect of anisotonic excursions and cryopreservation on the percentage of spermatozoa with high MMP.

The effect of anisotonic media on sperm integrity

The effects of initial exposure on koala sperm plasma membrane integrity (PMI) MMP, chromatin relaxation and %SDF to a range of anisotonic media (64, 170, 350, 680 and 1330 mOsm/kg) (E) are shown in Fig. 5a, b, c and d. Figure 5 also illustrates the subsequent change in sperm integrity following processing of the diluted sperm suspension and its return to isotonic conditions (350 mOsm/kg) (R). Figure 5a shows that the sperm plasma membrane was significantly damaged when spermatozoa were initially exposed to extreme hypotonic (64 and 170 mOsm/kg) and hypertonic (1332 mOsm/kg) media. Interestingly, there was no further statistically significant detrimental change in membrane integrity once the spermatozoa were returned to the 350 mOsm/kg Tris–citrate–glucose (TCG) buffer. Figure 5b indicates that high MMP declined in hypotonic (64 and 170 mOsm/kg) and extreme hypertonic (1330 mOsm/kg) media, but the loss of MMP in the 64 mOsm/kg medium was greater than that observed in the 1332 mOsm/kg medium. On return of spermatozoa to the isotonic media, spermatozoa initially diluted in the 64 mOsm/kg medium showed a trend towards a significant increase in MMP ($P = 0.08$), while the MMP of spermatozoa initially diluted in 1330 mOsm/kg, and then returned to isotonic conditions, was not significantly different from that of the initial sample. Figure 5c indicates how initial exposure of koala spermatozoa to the 64 mOsm/kg medium resulted in a significant increase in spermatozoa with relaxed chromatin; on the return of these spermatozoa to isotonic conditions, there was a further increase in spermatozoa with
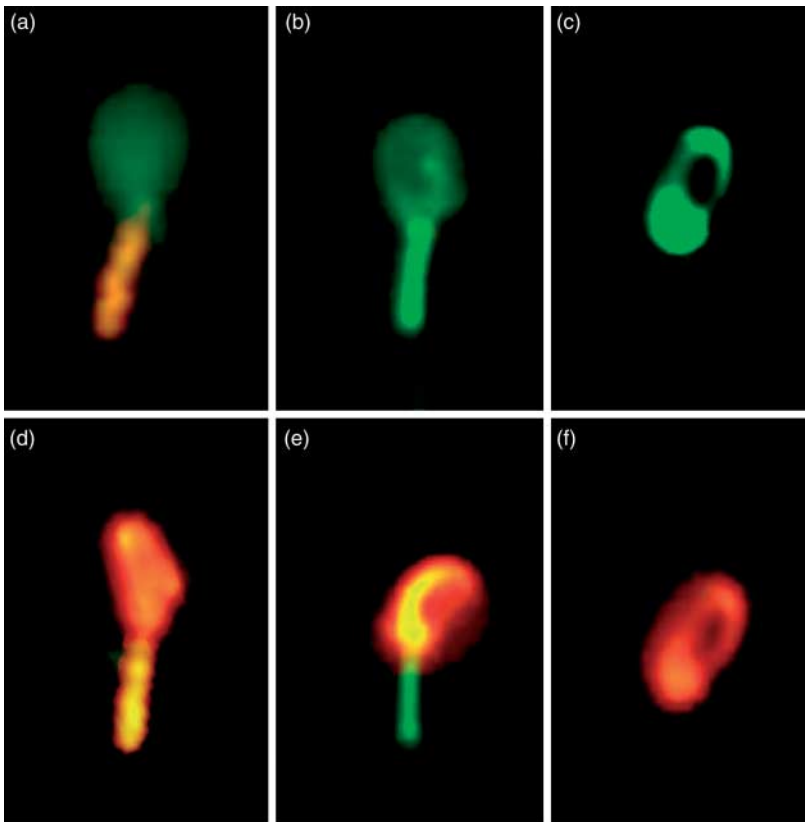

Figure 3 Correlation between chromatin area measured in pixels (red fluorescence) and the integrated density of the DNA ISNT label (green fluorescence) for 50 thawed incubated spermatozoa from two koalas.

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Figure 4 (a) Intact plasma membrane with high mitochondrial membrane potential (MMP); (b) intact plasma membrane with low MMP; (c) intact plasma membrane with no MMP; (d) damaged plasma membrane with high MMP; (e) damaged plasma membrane with low MMP and (f) damaged plasma membrane with no MMP.

relaxed chromatin. Spermatozoa initially diluted in the 170 mOsm/kg media showed no evidence of increased relaxed chromatin, but when these spermatozoa returned to the isotonic conditions, there was a significant increase in this parameter when compared with those found in the initial diluted sample. Figure 5d also shows how initial exposure of koala spermatozoa to the 64, 170 and 1330 mOsm/kg media resulted in significant increases in the percentage of spermatozoa with fragmented DNA when compared with the initial sample. Exposure of spermatozoa to the 64 mOsm/kg medium resulted in the highest level of SDF, and the subsequent return of these spermatozoa to isotonic conditions resulted in a further significant increase in %SDF.

Comparative effects of anisotonic media and cryopreservation on sperm integrity

When compared with the sperm integrity of the initial sample, the cryopreservation procedure significantly reduced PMI and MMP but increased the percentage of spermatozoa with relaxed chromatin and damaged DNA (Fig. 5a, b, c and d). The proportion of spermatozoa with intact plasma membranes 30 min after cryopreservation was not significantly different from those exposed to the 64 or 170 mOsm/kg media or exposed and then returned to the isotonic media after exposure to the

1330 mOsm/kg medium. The PMI of thawed spermatozoa was lower than that of spermatozoa exposed to the 350 and 680 mOsm/kg media and of spermatozoa following their initial exposure to the 1330 mOsm/kg media. The MMP of thawed spermatozoa was significantly higher than the spermatozoa initially exposed to the 64 mOsm/kg media and lower than that of spermatozoa initially exposed to the 350 mOsm/kg media; there were no other statistical differences noted between the thawed spermatozoa and sperm diluted in any other of the anisotonic diluents. The thawed spermatozoa, along with the spermatozoa exposed to the 64 and 170 mOsm/kg diluents, showed higher levels of relaxed chromatin compared with those in other anisotonic media. The highest proportion of spermatozoa with SDF was found in the semen samples exposed to the 64 mOsm/kg media; the proportion of sperm with SDF in this diluent was higher than that found in the thawed semen sample.

Relationship between relaxed chromatin and percentage of SDF

Figure 6A and B report the Pearson's *r* correlation analyses of %SDF and % relaxed chromatin between two aliquots of spermatozoa from the same koala ($n=5$) that were, respectively, cryopreserved and exposed to a 64 mOsm/kg hypotonic excursion. Figure 6C and D show the respective Pearson's *r* correlation analyses
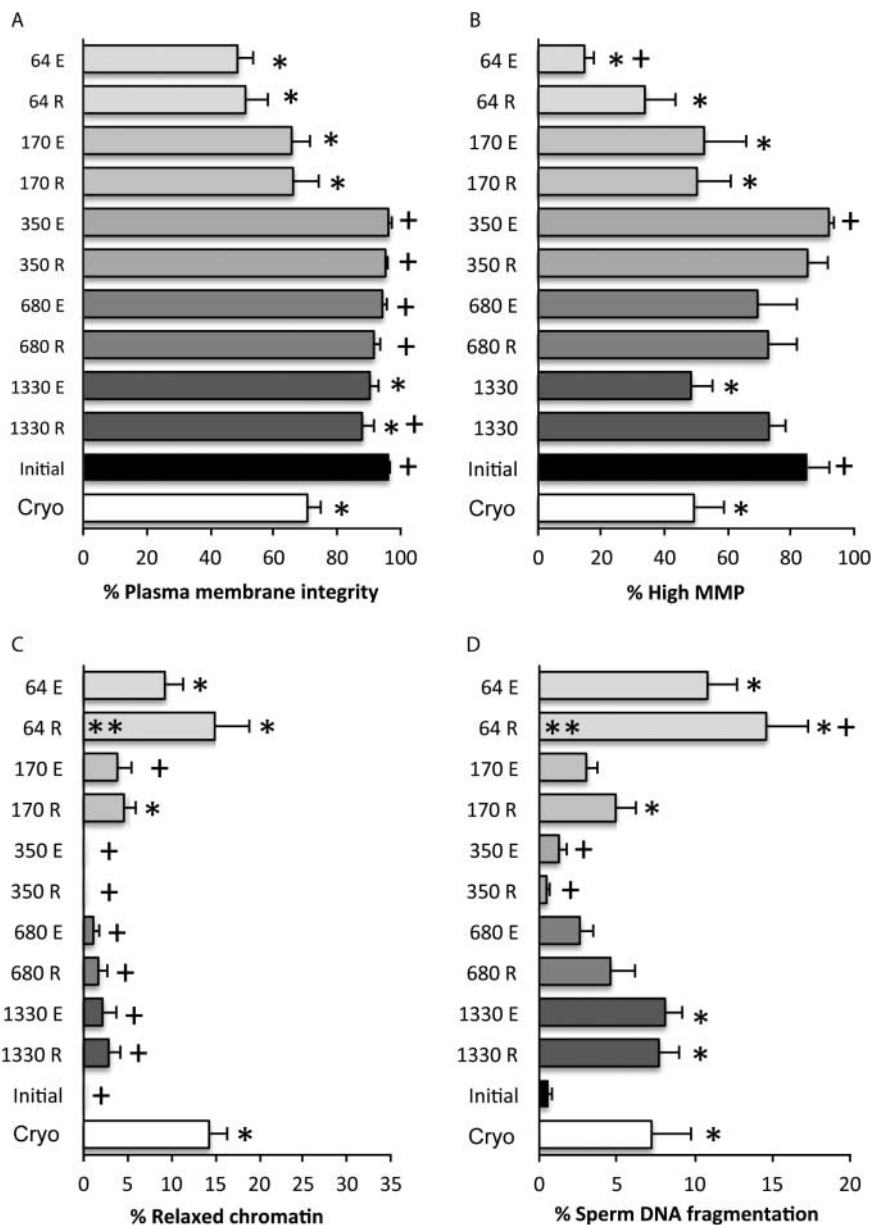

Figure 5 The effect of anisotonic media and cryopreservation (Cryo: post-thaw not washed) on the percentage of spermatozoa with (A) intact plasma membranes (PMI), (B) high mitochondrial membrane potential (HMMP), (C) relaxed chromatin and (D) sperm DNA fragmentation (SDF). Key: E - initial osmotic excursion; R – return osmotic excursion back to an isotonic environment; * statistical difference ($P<0.05$) when compared with the initial sample; ** statistical difference between E and R samples of the same osmolality; + statistical difference ($P<0.05$) when compared with the post-thaw semen sample Cryo.

between %SDF and % relaxed chromatin of sperm following cryopreservation and after a 64 mOsm/kg hypotonic excursion. Figure 6A and B show that exposure of koala sperm DNA to an extreme hypotonic excursion may be a potential model for explaining SDF-induced cryopreservation damage ($r=0.878$; $P=0.049$), but it does not appear to be an explanation for relaxed chromatin ($r=-0.350$; $P=0.564$). Figure 6C and D fail to support a strong relationship between relaxed chromatin and SDF.

Discussion

One of the primary objectives of this study was to examine whether pathology associated with osmotic flux of the non-frozen koala sperm could help better explain

cryoinjury. The impact of osmotic changes on ejaculates, which are *ex vivo* handled, has been examined in a range of species, with particular attention to the effects of anisotonic media on the sperm plasma membrane and motility (Willoughby *et al.* 1996, Meyers 2005, Johnston *et al.* 2006), but this is the first time that this experimental approach has been used to examine the effect on SDF. An important feature of our experimental design was that spermatozoa extended from the same ejaculate were concurrently examined following exposure to anisotonic media and cryopreservation so that these effects could be directly compared. Results from this study have shown that the koala spermatozoa exposed to extreme anisotonic environments show a loss of membrane integrity and MMP and an overall increase in chromatin relaxation and SDF. The extent of these effects was
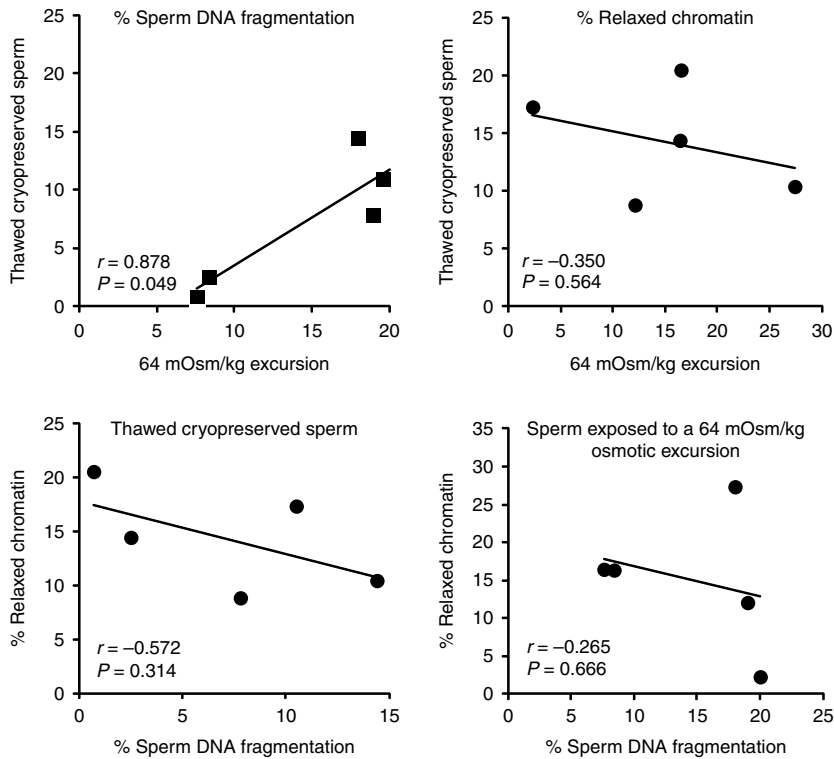
Figure 6 Pearson's *r* correlations comparing the percentage of sperm with SDF and relaxed chromatin following cryopreservation and exposure to a 64 mOsm/kg osmotic excursion. (A) correlation of the % of sperm with fragmented DNA following cryopreservation and induced osmotic damage; (B) correlation of the % of sperm with relaxed chromatin following cryopreservation and induced osmotic damage; (C) correlation of the percentage of sperm with relaxed chromatin and %SDF following cryopreservation and (D) correlation of the percentage of sperm with relaxed chromatin and %SDF following exposure to a 64 mOsm/kg excursion.

dependent on the intensity of the osmotic stress, but exposure to hypotonic environments was particularly harmful to sperm integrity, except that of MMP which showed some degree of recovery when returned to an isotonic environment. The response of sperm integrity to hypotonic conditions essentially mimicked that described for spermatozoa following cryopreservation. These results are consistent with other studies which show that the osmotic stress associated with thawing is likely to be most detrimental to the spermatozoa during the cryopreservation procedure (Holt *et al*. 1992). The post-thaw membrane integrity reported in this study was higher than that reported in other studies on koala sperm examined immediately after cryopreservation (Johnston *et al*. 2006, Zee *et al*. 2008, 2009*a*) and reflects the fact that spermatozoa in the current study were examined 30 min after thawing; however, it should be noted that these studies have also reported a rapid decline in PMI of these same spermatozoa after 120 min of incubation at 35 °C. These observations draw attention to the importance of induced damage associated with *ex vivo* handling (so-called 'iatrogenic damage') and the variable survival time of spermatozoa when incubated within *in vitro* environments that attempt to mimic the physiology of the female reproductive tract. Additionally, it also reinforces the idea of using the spermatozoa as soon as possible for assisted reproductive technology, once the thawed samples have been processed.

The effect of osmosis on MMP is particularly interesting and suggests that koala sperm mitochondria

are sensitive to hypotonic environments; clearly, changes in water volume of the koala spermatozoon during osmosis interfere with the physiological mechanisms that generate and maintain membrane potential. Spermatozoa exposed to hypertonic environments also showed a reduction in MMP, but this effect could be reversed if the spermatozoa were re-equilibrated back into an isotonic environment. This observation suggests that the physiology that drives mitochondrial function is capable of tolerating hypertonic environments better than they can cope with hypotonic changes. Interestingly, the percentage of koala spermatozoa with high MMP following cryopreservation (this study; Zee *et al*. 2007, 2009*a*) was similar to that of spermatozoa exposed to hypotonic conditions and, therefore, suggests that the thawing component of cryopreservation may be most detrimental to koala sperm mitochondria.

Koala spermatozoa showed a significant increase in chromatin relaxation when exposed to hypotonic conditions and following cryopreservation. On the face of it, these observations appear similar and suggest that chromatin relaxation is associated with an increase in the structural damage connected with changes in water flux across the plasma membrane. However, subsequent analysis of the data (Fig. 6) revealed no significant correlation between the incidence of spermatozoa with relaxed chromatin following cryopreservation and exposure to a 64 mOsm/kg osmotic excursion. It appears, therefore, that the respective causes of an

increased incidence in relaxed chromatin following koala sperm cryopreservation and osmotic injury are not directly related to SDF, or more likely occur asynchronously. We offer at least two possible explanations to account for this phenomenon: 1) the nuclear membrane is somehow physically attached to the chromatin – then it is possible that water flux into the nucleus and/or general ice crystal damage to the sperm cell could result in a subsequent physical 'tearing' of the tertiary structure of the DNA–protamine complex, leading to an increase in the incidence of relaxed chromatin, and 2) the massive presence of alkali labile sites in koala sperm DNA, together with the absence of disulphide bonding to cross link protamines, predisposes the chromatin to relax, probably associated with the rapid production of single-stranded DNA motifs emerging from unpaired single-stranded stretches of either apurinic or apyrimidinic sites. The synergistic coexistence of both processes cannot be discarded. With respect to the first explanation, the existence of a physical connection between chromatin and the nuclear membrane is not well established, but there is evidence in human spermatozoa of a telomere-binding protein complex, identified as hSTBP (a variant of the H2B histone) which has a possible role in membrane attachment when associated with the telomeres (Gineitis et al. 2000). A possible relationship between sperm chromatin and the nuclear membrane could also involve certain lipophilic structures such as those described in sea urchin sperm nuclei, which together may function as pronuclear envelope organising centres (Collas & Poccia 1995). Interestingly, these lipophilic structures are located at the acrosomal and centriolar poles of sea urchin sperm and initiate ATP-dependent vesicle binding in fertilised egg extracts. In some sense, these same sperm domains have also been identified in koalas as 'hot spots' to initiate sperm DNA damage. Breed et al. (2001) have noted the presence of a thin segment of the koala acrosome that runs from the main body of the acrosome towards the attachment of the tail and also laterally around the edges of the nucleus; again these areas are consistent with the DNA fragmentation 'hot spots' identified in the current study. Whatever the mechanism of chromatin relaxation, the koala sperm must have its own peculiar chromatin organisation, because relaxation of the chromatin in this species is not as pronounced as in the closely related wombat. Johnston et al. (2006) have shown that sperm chromatin relaxation in the wombat, following post-thaw incubation, is significantly lower than that found in the koala if the spermatozoa are frozen under the same conditions. Similarly, Breed et al. (2001) have also noted that the koala sperm nucleus is more prone to dispersion with Triton X-100 detergent than wombat spermatozoa, and this may be related to the presence of a large nuclear vacuole in the base of the koala sperm nucleus, which is not apparent in wombat. With respect to the second possibility, we have previously shown and proposed the notion that alkali-labile sites (ALS) present on the DNA (Zee et al. 2009b) facilitate the DNA bending necessary to configure the tertiary and ultimate structure of the DNA–protamine complex (Johnston et al. 2007). These ALS could be present in the mature spermatozoa in the form of unpaired single-stranded DNA stretches or simply as apurinic or apyrimidinic sites. This suggestion was made on the basis of the prominent presence of alkaline comets in normal spermatozoa (Zee et al. 2009b). It is possible that further structural damage and/ or oxidative attack to these potentially weakened areas may lead to chromatin relaxation. In addition, we cannot rule out the formation of newly formed single-stranded breaks leading to chromatin relaxation arising directly from osmotic membrane rupture (plasma membrane and mitochondria) and subsequent oxidative damage. The mechanism that leads to increased DNA breaks following exposure to hypotonic media requires further investigation but is likely to be similar to that resulting from cryoinjury. This was supported by the observation of a significant correlation of SDF between thawed cryopreserved spermatozoa and those exposed to a 64 mOsm/kg excursion ($r=0.878$; $P<0.05$).

The other issue of interest emerging from our results is that DNA damage associated with cryoinjury occurred in a non-randomised manner. Thawing of koala spermatozoa results in the production of highly localised DNA damage that then extends to other chromosomal domains. ISNT revealed that the chromatin is first affected in the peri-acrosomal and basal regions. DNA labelling then subsequently expanded to other regions of the nucleus. It is also possible that peripheral DNA damage observed in this study, after loss of membrane stability and chromatin relaxation, is due to the presence of non-orthodox DNA conformations such as putative unpaired single-stranded DNA motifs or even abasic sites affecting one of the DNA strands. The presence of single-stranded DNA breaks in the periphery of the spermatozoa may act as putative DNA nicks that facilitate the production of further single-stranded DNA motifs and ultimately manifest as double-stranded breaks in highly affected spermatozoa, such as those presenting in this study as massive haloes of chromatin dispersion of the microgel following the SCD test. While this concept has not been thoroughly investigated, there are some observations where the sperm nuclei may exhibit discrete and preferable peripheral distribution of foci containing single-stranded DNA motifs and this fact has been linked to infertility in men (Zhang et al. 2007). A highly ordered form of DNA damage has also been reported in short-beaked echidna (Johnston et al. 2009). In this case, the combined use of ISNT and alkaline comets revealed the presence of a directional DNA nicking co-localised with the presence of highly sensitive ALS (putative single-stranded DNA motif generators after alkaline DNA denaturation) along the

length of the sperm nucleus. While there was evidence of DNA damage starting from the base of the koala spermatozoa in this study, there was also apparent DNA damage adjacent to the acrosome; no such observations were made in echidna spermatozoa. The release of chromatin damaging agents such as proteases might be expected from the acrosome, particularly when associated with damaged plasma membranes. Although the marsupial acrosome is generally thought to be resistant to cryoinjury (Sistina *et al.* 1993, Breed *et al.* 2001, Johnston & Holt 2001), this might still be a plausible explanation for co-localisation of initial DNA damage and the peri-acrosomal region in this study. Although the marsupial acrosome is known to be highly stable, even to the repeated effects of a freeze–thaw procedure (Sistina *et al.* 1993), rupture or more subtle damage of the underlying nuclear or acrosomal membranes might still occur in the peri-acrosomal region during cryo-preservation. These events could plausibly induce digestion of proteins associated with these chromatin domains and may then lead to secondary structural changes in the adjacent DNA. The other region where DNA may be directly attacked due to the presence of localised oxidative stress is in close proximity to the mitochondria within the proximal midpiece at the base of the sperm head. The combined loss of plasma membrane function and damaged mitochondria following cryopreservation could be contributing to an elevated REDOX environment that is detrimental to sperm DNA.

On summarising, we propose an integrative model of cryo-induced osmotic injury for koala sperm that initially involves damage to membranes, which in combination with oxidative stress, leads to reduction of MMP and relaxation of chromatin in the short term followed by non-randomised DNA fragmentation.

Materials and Methods

Animals and semen collection

A total of seven sexually mature, clinically healthy captive male koalas were used to conduct this study. All animals and experiments were approved and conducted under permit SAS/297/08/UQFRG of the University of Queensland Animal Ethics Committee. Anaesthesia and the electroejaculation procedure in the koala have previously been described (Johnston *et al.* 1994, McGowan *et al.* 1995).

Sperm preparation

TCG buffer was used as the base diluent in this study and was composed of 3.0 g Tris base (Sigma–Aldrich), 1.7 g citric acid (Ajax Finechem, Taren Point, NSW, Australia), 1.25 g glucose (Ajax Finechem) made up to 100 ml with Milli-Q Ultrapure water (Millipore Australia Pty Ltd., North Ryde, NSW, Australia) and adjusted to a pH of 7.4 and an osmolality of 350 mOsm/kg (Johnston *et al.* 2006). The 'control', a 350 mOsm/kg TCG buffer, was based on the osmolality of undiluted koala semen. Hypertonic solutions used in this study were prepared by adjustment of osmolality by addition of sucrose (BDH Chemicals Australia Pty Ltd., Melbourne, Vic, Australia) to the TCG buffer (680 and 1330 mOsm/kg), whereas hypotonic solutions were prepared by dilution of TCG buffer with Milli-Q Ultrapure water (64 and 170 mOsm/kg). TCG buffer with 28% (v/v) glycerol was prepared as the cryopreservation medium and had a final working glycerol concentration of 14% (v/v) (Johnston *et al.* 2006).

Koala semen cryopreservation has been described previously (Johnston *et al.* 2006, Zee *et al.* 2008). Briefly, each ejaculate was initially diluted (1:1) with warm (35 °C – normal koala body temperature) TCG buffer in a pre-warmed 1.5 ml microcentrifuge tube (Eppendorf AG, Hamburg, Germany) and allowed to cool to room temperature (25 °C) over a period of 10 min. The extended semen sample was placed into a 10 ml flask containing ∼2 ml of water at 25 °C and subsequently placed into a refrigerator to equilibrate to 4 °C over ∼2 h. This chilled, equilibrated sample was assessed as the initial (T0) sample. The semen sample was then analysed for PMI, MMP, chromatin relaxation and SDF. Chilled (4 °C) cryopreservation media was added, drop-wise, to the extended chilled-equilibrated semen. Semen samples were drawn into pre-chilled 0.25 ml straws (IMV, L'Aigle, France), sealed and frozen in a programmable freezer at 6 °C/min (Freeze Control CL-863, Cryologics Pty Ltd., Mulgrave, Vic, Australia) from 4 °C to −80 °C. Straws were then removed from the freezer, plunged into liquid nitrogen (LN; −196 °C) and stored. Straws were thawed in a 35 °C water bath for 1 min and semen samples then dispensed into pre-warmed 1.5 ml microcentrifuge tubes.

Assessment of semen quality

PMI of the spermatozoa was determined using a dual fluorescent staining procedure; 100 nM SYBR-14 and 12 µM PI from the Live/DeadSperm viability kit (L-7011; Invitrogen Australia Pty Limited) was used according to the manufacturer's instructions (Zee *et al.* 2007). MMP was determined using a lipophilic cationic fluorescent probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide (JC-1; Invitrogen Australia Pty Ltd.) that has an excitation range of 485 to 535 nm (J-aggregates only). Each sample was stained with final concentration of 2 µM JC-1, counterstained using the PMI technique above and incubated for 30 min. The JC-1 probe indicated a potential accumulation in the mitochondria where the monomeric molecules (emission at 530 nm) accumulate to form J-aggregates with increasing mitochondrial potential (emission at 590 nm). Spermatozoa were categorised into three levels of mitochondrial potential: none, low (LMMP) and high (HMMP) with no fluorescence, green fluorescence and orange fluorescence respectively. Fluorescence was assessed using an epifluorescence microscope (Nikon Eclipse E400; FITC 465–495 excitation filter; DM 505 dichroic mirror; BA 515–555 barrier filter).

SDF was determined using a Halomax kit (Halotech SL, Madrid, Spain) previously validated and described for koala spermatozoa first by Johnston *et al.* (2007) and then refined by Zee *et al.* (2009*b*). ISNT of DNA breaks was determined on cryopreserved semen samples ($n=2$). Samples were incubated

for 2 h at 35 °C post-thaw. ISNT was conducted on samples with and without protein lysis. Spermatozoa were mixed into low-density agarose, and microgels were prepared onto glass slides, then dehydrated in a series of increasing ethanol solutions (70, 90 and 100%), dried for 5 min, and the glass coverslip was removed before ISNT. Reaction buffer containing 5 units of DNA polymerase I endonuclease free (Kornberg polymerase; Roche Diagnostics GmbH) and 10 µM digoxigenin-11-dUTP (Roche Diagnostics GmbH) was dispensed onto the microgel, covered with a plastic coverslip and incubated in a humidified chamber for 5, 10, 20 and 30 min at 37 °C. Slides were washed in Tris–borate–EDTA buffer (pH 8), dehydrated in sequential ethanol solutions (70, 90 and 100%) and air-dried. Digoxigenin-11-dUTP was detected using anti-digoxigenin-fluorescein, Fab Fragments (Roche Diagnostics GmbH) incubated for 30 min. The slides were counterstained with PI (2 µg/ml) in Vectashield (H 1100, Vector, Burlingame, CA, USA). In order to control for background binding, designated areas of the microgels were incubated with the reaction buffer alone, omitting the DNA polymerase I, and a small section of the microgels between those areas was removed to avoid diffusion of the enzyme. Following ISNT, spermatozoa within the microgels were imaged at ×600 magnification, using Leica DMRB microscope (Leica Microsystems, Germany) to determine degrees of chromatin relaxation and the proportion of the nucleus positively stained with the DNA label. The ISNT-unlabelled spermatozoa typically stained red if the nucleotides were not incorporated but exhibited green fluorescence if traces of DNA damage were present. The area of the nucleus represented by the red fluorescence was then correlated with the integrated density of the green DNA label. Fluorescent images were captured as.tiff 12-bit files using a black and white cooled Leica DCF 300 camera with single band pass filters (FITC-3540B-536/617; TxRed-4040C-000; Semrock, Rochester, NY, USA). Image analysis was performed using Leica Q-Win software (Leica-Microsystems, Barcelona, Spain). Colour coding was selected to improve visual discrimination between both fluorescent channels. Images were transformed into colour by converting.tiff 12-bit images into.tiff 8-bit images and colour assignment, red/blue for DNA and the green for proteins, to the 8-bit grey level images using Adobe Photoshop 7.0 (Adobe Systems Incorporated).

The effect of anisotonic media on sperm integrity

This study examined the potential role of osmotic flux associated with the loss of koala sperm integrity following cryopreservation. Initial assessments were carried out on ejaculated semen samples ($n=5$) that were diluted 1:1 with TCG and stored chilled (4 °C) before use. These samples were then equilibrated at 25 °C for 5 min and incubated for a further 10 min at 35 °C; subsamples were stained for assessment of PMI, MMP and chromatin integrity. A 10 µl aliquot of the initial semen sample was then extended into 190 µl of the anisotonic test media and incubated at 35 °C for 10 min (sufficient time to induce osmotic stress (Johnston *et al.* 2006)). This exposure represented the first or initial osmotic excursion (E); subsamples were stained for assessment of PMI, MMP and chromatin

integrity. The remaining semen sample was washed by centrifugation at 200 **g** for 30 s, the supernatant was removed and the pellet was resuspended in TCG medium (350 mOsm/kg) and incubated at 35 °C for 10 min. This exposure represented the second osmotic excursion and a return to an isotonic environment (R); subsamples were stained for assessment of PMI, MMP and chromatin integrity.

Effect of post-thaw dilution on sperm integrity

Concurrent to the aforementioned exposure of spermatozoa to varying osmotic stress, an aliquot of the initial sample was cryopreserved and thawed in order to examine post-thaw PMI, MMP and chromatin integrity in order to compare the results with non-frozen spermatozoa exposed to the anisotonic media. Two frozen semen straws were thawed and pooled and incubated with no further processing so that spermatozoa were left in 14% glycerol v/v. All cryopreserved koala sperm samples were assessed for PMI, MMP and chromatin integrity after 10 min of incubation at 35 °C within 30 min of thawing.

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences 17 (SPSS, Inc., Chicago, IL, USA). All percentage data were arcsine transformed before analysis. Non-parametric statistics for a non-Gaussian population including pairwise comparisons using exact one-sided Wilcoxon tests were performed. Correlation analysis was conducted using a Person's *r* test. Image analysis was performed to compare the fluorescence intensities obtained after ISNT and the area of the spermatozoa (measured in pixels) as an index of chromatin relaxation. The analysis was performed on 50 different sperm nuclei with protein depletion exhibiting different levels of chromatin relaxation and DNA labelling. For this purpose, integrated density (a correlation between the area of interest and fluorescence intensity after background subtraction) was calculated for analysis of fluorescence intensity. Areas of the nuclei were represented as the number of pixels after using automatic image analysis threshold algorithms based on background analysis against the regions of interest. Leica Q-Win image analysis software (Leica-Microsystems Germany) was used.

Declaration of interest

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

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