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Apoptotic-like changes in epididymal spermatozoa of soft-shelled turtles, *Pelodiscus sinensis*, during long-term storage at 4 ºC

Hong Chen, Yufei Huang, Xuebing Bai, Ping Yang, Imran Tarique, Waseem Ali Vistro, Noor Samad Gandahi, Sarfaraz Ali Fazlani, Qiusheng Chen*

MOE Joint International Research Laboratory of Animal Health and Food Safety, College of Veterinary Medicine, Nanjing Agricultural University, Nanjing, Jiangsu Province, 210095, China

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ABSTRACT

Apoptosis is a physiological phenomenon that has been recognized as a cause of sperm death during cryopreservation in endothermic mammals. There is, however, no data on its role in sperm death during cooled storage in ectothermic animals. In this study, spermatozoa from the epididymis of soft-shelled turtle were investigated to identify the mechanism of spermatozoa apoptotic-like changes during storage at 4 °C. In this study, there was survival of spermatozoa for more than 40 Days when stored at 4 °C. During cooled storage, sperm kinematics was evaluated using CASA system. Values for all sperm motility variables decreased during the period of storage; while for velocity curvilinear (VCL) there was a further decrease after 20 Days of storage. Results from flow cytometry analysis indicated that there was a significant increase in the percentage of apoptotic spermatozoa, but there was no change in the percentage of necrosis. Furthermore, the concentration of cellular ROS increased after 20 Days of storage at 4 °C. The results using JC-1 staining indicated there was a decrease in MMP of spermatozoa as the duration of storage at 4 °C increased. Nuclear fragmentation of spermatozoa was observed using TEM on Day 30 of storage. There were large amounts of pro-apoptotic cytochrome c (Cytc) and cleaved caspase-9/3 proteins detected using western blot analysis after 30 days of spermatozoa storage at 4 °C. These findings indicate ROS generation induces mitochondria damage after 20 days of storage at 4 °C, which can induce spermatozoa apoptotic-like changes during storage of soft-shelled turtle spermatozoa.

1. Introduction

Cryopreservation of sperm is an effective procedure for sperm storage when artificial insemination and *in vitro* fertilization procedures are utilized for animal production (Dong et al., 2008). Mammal spermatozoa, however, remain viable after freezing and thawing for only a few days or even a few hours, and rapidly lose fertilization capacity (Martin et al., 2004; Bolaños et al., 2012). The soft-shelled turtle, *Pelodiscus sinensis*, is one of the reptiles that hibernates from December to April in China. Spermatogenesis in this turtle has a seasonal pattern (Zhang et al., 2008). After spermiation in October, immature spermatozoa are transferred into the epididymis and stored there during the hibernation period until the subsequent mating season (June to August; Bian et al., 2013). Sperm storage *in vivo* is common in reptilian species resulting in a relatively greater longevity of spermatozoa until the period of reproduction is initiated in females (Reviewed by Holt et al., 2010). The soft-shelled turtle may have unique physiological

* Corresponding author.
E-mail address: chenqsh305@njau.edu.cn (Q. Chen).

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characteristics for elucidating the mechanism whereby long-term viable sperm storage can occur in males.

During cryopreservation, the cooling process results in imposing several stresses on the spermatozoa, termed “cold shock”, which ultimately leads to cell damage or death (Chantler et al., 2000; Naresh, 2016). The rapid change in temperature during freezing results in membrane bound phospholipid reorientation, which affects membrane functionality and permeability, and results in physical damage to spermatozoa structures (Sieme et al., 2015). Other detrimental outcomes from the freezing process is: an increase in reactive oxygen species (ROS), lipid peroxidation (Moran et al., 2008), changes in pH (Purdy et al., 2010) and ATP depletion (Ortega-Ferrusola et al., 2009a). Because of the marked loss of sperm viability during cryopreservation, several approaches have been used to increase the duration of storage without compromising fertility (Gallardo Bolanos et al., 2012; Karimfar et al., 2015). It has been proposed that the study of molecular damage may be a useful approach to determine methods for improving semen cryopreservation and to extend the lifespan of the spermatozoa *in vitro* (Holt, 2011). One of the intracellular pathways that has been studied to the greatest extent during sperm cryopreservation is apoptosis (Said et al., 2010). Apoptosis is activated by stressful conditions or extrinsic stimuli inducing cell death, which is characterized by the activation of caspases, externalization of phosphatidylserine, alteration of mitochondrial membrane potential and DNA fragmentation.

Cell death resulting from apoptosis that has been induced by cryopreservation has been described in the ejaculated human spermatozoa (Said et al., 2010). The same mechanism has also been investigated in bull and boar sperm cryo-injury (Martin et al., 2004; Zeng et al., 2014). The soft-shelled turtle may have some important physiologic characteristics that would make it a unique animal for exploration of molecular mechanisms of the long-term sperm storage. There are no data regarding the mechanisms leading to soft-shelled turtle sperm death during cryopreservation. The objective of the present study, therefore, is to investigate the sperm apoptotic-like changes of the soft-shelled turtle at different times of the cryopreservation period.

2. Materials and methods

2.1. Animals

Adult male soft-shelled turtles, *Pelodiscus sinensis*, (average body weight and plastron length of 1.62 ± 0.15 kg and 18.34 ± 1.25 cm, respectively), aged 4–5 years, were purchased from Yangcheng Lake in Suzhou (31 °N, 120 °E), southeastern China, during January (n = 9). All procedures with the *P. sinensis* were conducted according to the Animal Research Institute Committee guidelines of Nanjing Agricultural University, China. The *P. sinensis* were anaesthetized by using an intraperitoneal injection of pentobarbital sodium (20 mg/kg) and killed by exsanguination. All efforts were made to minimize animal suffering. The protocol was approved by the Science and Technology Agency of Jiangsu Province (SYXK (SU) 2010-0005).

2.2. Reagents

The following antibodies and reagents were used: Rabbit anti-β-actin (Bioworld, AP0060, Nanjing, China), rabbit anti-Caspase-9 (Abcam, ab25758, Cambridge, MA, USA), rabbit anti- cleaved caspase-3 (Merck Millipore, AB3623, Darmstadt, Germany), mouse anti-Cytc antibody (Abcam, ab13575, Cambridge, MA, USA). FITC Annexin V Apoptosis Detection Kit (BD Pharmingen, 556547, New Jersey, USA), cationic dye tetraethylbenzimidazolylcarbocyanine iodide (JC-1, Abcam, ab113850, Cambridge, MA, USA), MDA kit (Nanjing Jiancheng Bioengineering Institute, A003-4, Nanjing, China,), SOD kit (Nanjing Jiancheng Bioengineering Institute, A001-3, Nanjing China,), ROS kit (Nanjing Jiancheng Bioengineering Institute, E004, Nanjing, China,), BCA protein assay kit (Thermo Fisher Scientific, 23252, Massachusetts, USA), and PVDF membranes (Merck Millipore, IPVH08130, Darmstadt, Germany).

2.3. Sperm collection and processing

Samples of epididymides from *P. sinensis* were placed in phosphate buffer and allowed to exsanguinate for 5–10 min. After transfer to fresh PBS, connective tissues surrounding the epididymis were removed, the inner tubule areas were accessed and the spermatozoa were allowed to drain into the PBS for 10–15 min. Buffer containing spermatozoa was transferred to centrifuge tubes and concentrated by centrifugation at 700 *g* for 10 min at 4 °C. The spermatozoa were suspended in sterilized PBS and then storage at 4 °C. Spermatozoa motility was recorded at 23 °C using light microscopy. The samples of spermatozoa were collected and evaluated on Days 10, 20, 30, and 40 of cooled storage.

2.4. Soft-shelled turtle sperm kinematics during the period of storage at 4 °C

The sperm kinematics was assessed using a Computer-assisted sperm analysis (CASA) system (ISAS® Proiser Valencia Spain). The analysis was based on the examination of consecutive, digitalized images obtained from a single field using × 10 microscope objective. With respect to the setting parameters for the analysis, spermatozoa with a VAP (Velocity average path) > 15 μm/s were considered motile. Sperm kinematics measured by CASA included the following: Velocity curvilinear (VCL, μm/s), Velocity straight line (VSL, μm/s), Linearity (LIN, VSL/VAP), Wobble (WOB, VAP/VCL), BCF (Beat cross frequency).

2.5. Flow cytometric analysis of apoptosis by Annexin V-propidium iodide double staining

Apoptotic spermatozoa were stained and analyzed using the FITC Annexin V Apoptosis Detection Kit, according to the

manufacturer's instructions. Briefly, the 400 μL spermatozoa (4×10^6 spermatozoa/mL) were collected during periods of storage, rinsed twice with PBS, and subsequently re-suspended in 1 × Binding Buffer. The suspension was stained with FITC Annexin V and propidium iodide (PI) at 23 °C in the dark. After 15 min of incubation, 400 μL of the Binding Buffer was added into tube, and the samples were then analyzed using Flow cytometry.

2.6. Transmission electron microscopy (TEM)

Spermatozoa samples were fixed in 2.5% (v/v) glutaraldehyde in phosphate-buffered saline for 24 h. The samples were rinsed in PBS, then post-fixed in 1% (w/v) osmium tetroxide for 1 h at room temperature and washed with PBS. Subsequently, the samples were dehydrated in ascending concentrations of ethyl alcohol, infiltrated with a propylene oxide-araldite mixture (50% propylene oxide: araldite), and then embedding in araldite. Ultrathin sections (50 nm) were mounted on Formvar-coated grids, stained with uranyl acetate, and then with lead citrate (20 min each step). The sections were examined and electron micrographs obtained using a transmission electron microscope (TEM; Hitachi H-7650, Japan).

2.7. Isolation of cytoplasmic droplets (CDs)

The spermatozoa-containing supernatants were collected at different stages of cooled storage and subjected to cytoplasmic droplets (CDs) purification using a discontinuous sucrose density gradient centrifugation method (Yuan et al., 2013). Briefly, ~2 mL of spermatozoa suspension was layered over a discontinuous sucrose gradient composed of 2 mL of 0.25 M sucrose on top of 3 mL of 1 M sucrose. The upper 1 mL of the 0.25 M sucrose layer was removed after centrifugation for 20 min at 1500 × *g*, at 4 °C. The remainder of the 0.25 M sucrose layer and materials located at the interface with the 1 M sucrose layer were carefully transferred to a new 1.5 mL centrifuge tube followed by centrifugation at 1200 × *g* for 10 min, at 4 °C. The CD-enriched supernatant was transferred to a new 1.5 mL centrifuge tube followed by centrifugation at 12,500 × *g* for 20 min at 4 °C to yield the CD pellet.

2.8. Western blotting

The spermatozoa were homogenized in ice-cold RIPA-like buffer. The protein concentration was quantified using a BCA protein assay. Equal amounts of proteins (28 μg/well) were subjected to 10% SDS-PAGE and subsequently transferred onto PVDF membranes. After blocking with 5% nonfat milk in Tris-buffered saline with Tween 20 (TBST) for 1 h at room temperature, the stripped membranes of interest were incubated with primary antibodies at 4 °C overnight. The β-actin served as an internal standard protein. Protein bands were assessed using the ECL detection system (Vazyme Biotech, China) and immunoreactive bands were quantified with Quantity One software (Bio-Rad Laboratories). To detect the released Cytc in cytosol, there was an initial isolation of CD from spermatozon. Western blot analysis was performed to detect the released Cytc in CD; and coomassie blue-stained gels of protein extracts from isolated CD were used to standardize the data. The validity of the primary antibodies used in present study was confirmed using negative and positive control analysis. The proteins from mouse testis was the positive control. The negative controls were determined by replacing primary antibodies with 0.01 M PBS.

2.9. Detection of reactive oxygen species (ROS) in spermatozoa

Intracellular ROS production was determined by DCFH-DA, and the fluorescent intensity was quantified using a Fluorescent Microplate Reader. Briefly, the spermatozoa (5×10^6 spermatozoa/mL) were collected and incubated with 25 μM of DCFH-DA for 30 min in dark at 37 °C. The spermatozoa samples were then re-suspended in appropriate amounts of PBS. The fluorescent intensity was measured by comparing two fluorescence emission at 480 nm/530 nm using a Full wavelength Fluorescent Microplate Reader (CLARIOstar, BMG Labtech, Germany) and each experiment was technically repeated three times.

2.10. Evaluation of mitochondrial membrane potential (MMP)

The cationic dye tetraethylbenzimidazolylcarbocyanine iodide (JC-1) is widely used to detect MMP in the stage of apoptosis (Smiley et al., 1991). The spermatozoa suspension (5×10^6 spermatozoa/mL) was incubated with the JC-1 for 15 min in a darkened area at 37 °C. The fluorescence of the JC-1 monomer and aggregate was measured by a Full wavelength Fluorescent Microplate Reader (CLARIOstar, BMG Labtech, Germany) with excitation/emission settings at 485/535 nm and 560/595 nm, respectively. The ratio of JC-1 aggregate to monomer (the 590:530 ration) was calculated. Each experiment was technically repeated three times.

2.11. Oxidative damage and antioxidant capacity assay

The activity of SOD (Superoxide Dismutase) in spermatozoa was measured using the xanthine oxidase method provided by the standard assay kit (Nanjing Jiancheng Bioengineering Institute, China). With the commercial kit, there was use of the xanthine-oxidase system to produce superoxide ions, which reacted with 2,3,5-phenlyltetrazolium chloride to form a colored compound and the absorbance at 450 nm was determined by Microplate Reader. The values were expressed as units per mg protein (U/mgprot). One unit of SOD was defined as the amount of SOD inhibiting the rate of reaction by 50% at 25 °C. Lipid peroxidation was determined by measuring cellular MDA (Malondialdehyde) concentrations using the thiobarbituric acid (TBA) method as commercially

recommended (Nanjing Jiancheng Bioengineering Institute, China). The method was based on the spectrophotometric measurement of the colored product during the reacton to TBA with MDA. MDA concentrations were calculated using an absorbance at 530 nm by Microplate Reader. The values were expressed as nmol/mgprot.

2.12. TdT-mediated dUTP nick-end labeling (TUNEL) assay

The TUNEL analysis was performed using an *in situ* cell Apoptosis Detection Kit (S7100, Millipore, Billerica, MA, USA) according to the manufacturer's instructions. In brief, the spermatozoa (5×10^6 spermatozoa/mL) were collected and spread onto Superfrost Plus slides (CITOGLAS). The spermatozoa smear that was applied to slides was fixed using 4% paraformaldehyde for 30 min at room temperature. After washing with PBS, the slides treated with proteinase K at 20 μg/mL for 20 min at room temperature. Sections were then incubated with reaction buffer containing dUTP and TdT enzyme at 37 °C for 60 min. After washing with stop buffer, sections were incubated with Streptavidin-HRP solution for 30 min at room temperature. Subsequently, the sections were stained with DAB solution to distinguish the DNA-fragmented spermatozoa from normal spermatozoa.

2.13. Statistical analysis

All numerical results were expressed as the means ± standard error of the mean (SEM). The quantified values obtained in this study were from biological duplicates that were conducted in triplicate. All values were first examined using the Kolmogorov-Smirnov test to determine the distribution, and homogeneity of variance was assessed using Levene's test. For data that were normally distributed, there was analysis using a one-way ANOVA with the Tukey Multiple Comparison Test using Graph Pad Prism software. If data were not normally distributed, the non-parametric Mann-Whitney *U* test was directly used to compare pairs of values. The differences were considered significant at $P < 0.05$.

3. Results

3.1. Soft-shelled turtle sperm motility and kinematics during storage at 4 °C

Spermatozoa with vigorous progressive motility had oscillation movements of the tail flagellum (Movie S1). The total percentage of motile spermatozoa decreased as the time of storage at 4 °C increased (Movies). The percentage of progressive motile spermatozoa was less on the Day 20 of storage at 4 °C as compared to earlier in the storage period (Fig. 1). All values for variables of sperm motility and kinematics decreased as duration of storage increased (Table 1). The percentage of total motile and progressively motile sperm

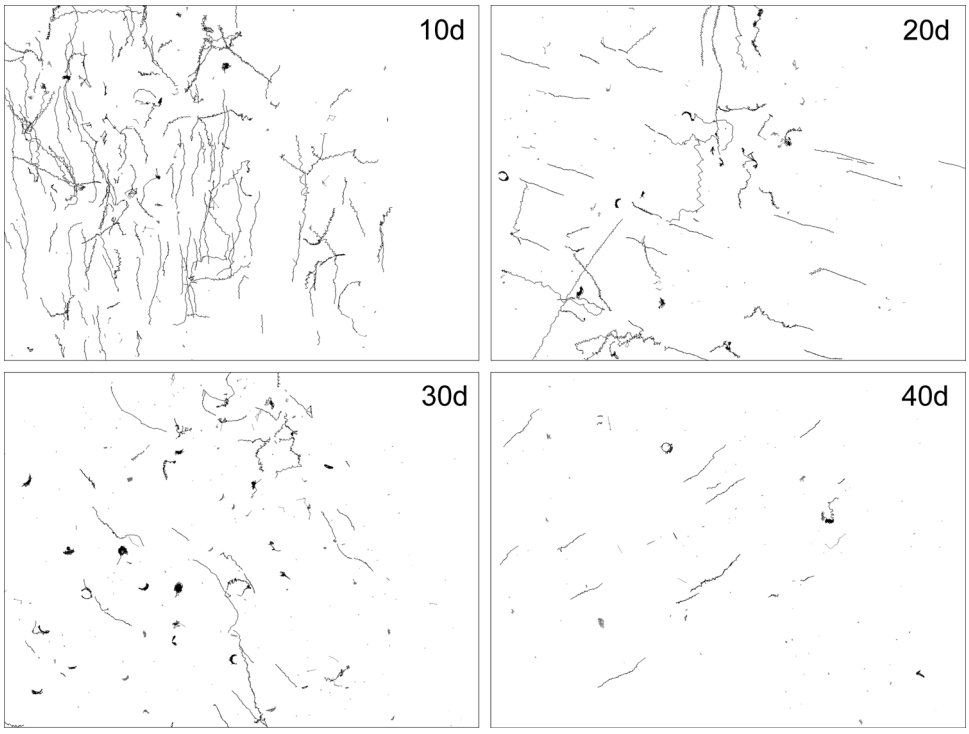

Fig. 1. Motion path of spermatozoa analyzed by CASA system during long-term *in vitro* storage at 4 °C; Motion path of spermatozoa was recorded on Days 10, 20, 30, and 40 of storage based on video analyses.

Table 1
Sperm motility and kinematics were analyzed using CASA system during long-term storage at 4 °C.

	Days				
	10	20	30	40	45
TM %	81.3 ± 4.1[a]	64.3 ± 4.2[b]	40.1 ± 0.8[c]	32.1 ± 3.2[d]	7.9 ± 3.5[e]
VCL μm/s	106.5 ± 11.2[a]	93.9 ± 5.6[a,b]	79.5 ± 4.6[c]	65.4 ± 3.8[d]	37.8 ± 2.8[e]
VSL μm/s	32.7 ± 5.8[a]	28.6 ± 4.5[a]	26.4 ± 3.2[a]	25.8 ± 2.8[a]	12.7 ± 3.4[b]
VAP μm/s	45.8 ± 1.5[a]	38.5 ± 3.1[b]	32.6 ± 2.4[c]	30.6 ± 1.6[c]	21.3 ± 1.1[d]
LIN	0.71	0.74	0.81	0.84	0.6
WOB	0.43	0.41	0.41	0.47	0.56
BCF Hz	15.6 ± 1.5[a]	13.7 ± 2.1[a,b]	11.6 ± 1.4[b]	10.8 ± 1.1[b]	5.6 ± 1.3[c]

TM%: total motile spermatozoa, VCL: velocity curvilinear, VSL: velocity straight line, VAP: velocity average path, Lin: linearity, WOB: wobble, BCF: beat cross frequency; Values are presented as mean ± SEM; Within a row values with different superscripts (a–e) differ ($P < 0.01$).

decreased after Day 20 of storage ($F = 212.1$, $P < 0.01$). The VCL was less on Day 30 as compared with earlier in the storage period ($F = 53.55$, $P < 0.01$), however, the VCL on Day 10 was not different from that on Day 20. The BCF was not less until the end of the storage period ($F = 18.54$, $P < 0.01$).

3.2. Detection of phosphatidylserine (PS) translocation/Annexin assay

As depicted in Fig. 2, flow cytometry analysis indicated that the percentage of viable spermatozoa (Q4) decreased as duration of storage increased. There was no change in the percentage of necrotic spermatozoa (Q1) as duration of storage increased ($F = 5.313$, $P > 0.01$). The percentage of Annexin V single-positive spermatozoa (Q3) increased after 30 days of storage ($F = 75.83$, $P < 0.01$). The percentage of Annexin V/PI double-positive spermatozoa (Q2) were, however, about 35% after storage for 40 days, and there were almost no viable spermatozoa observed on Day 45 (Table 2).

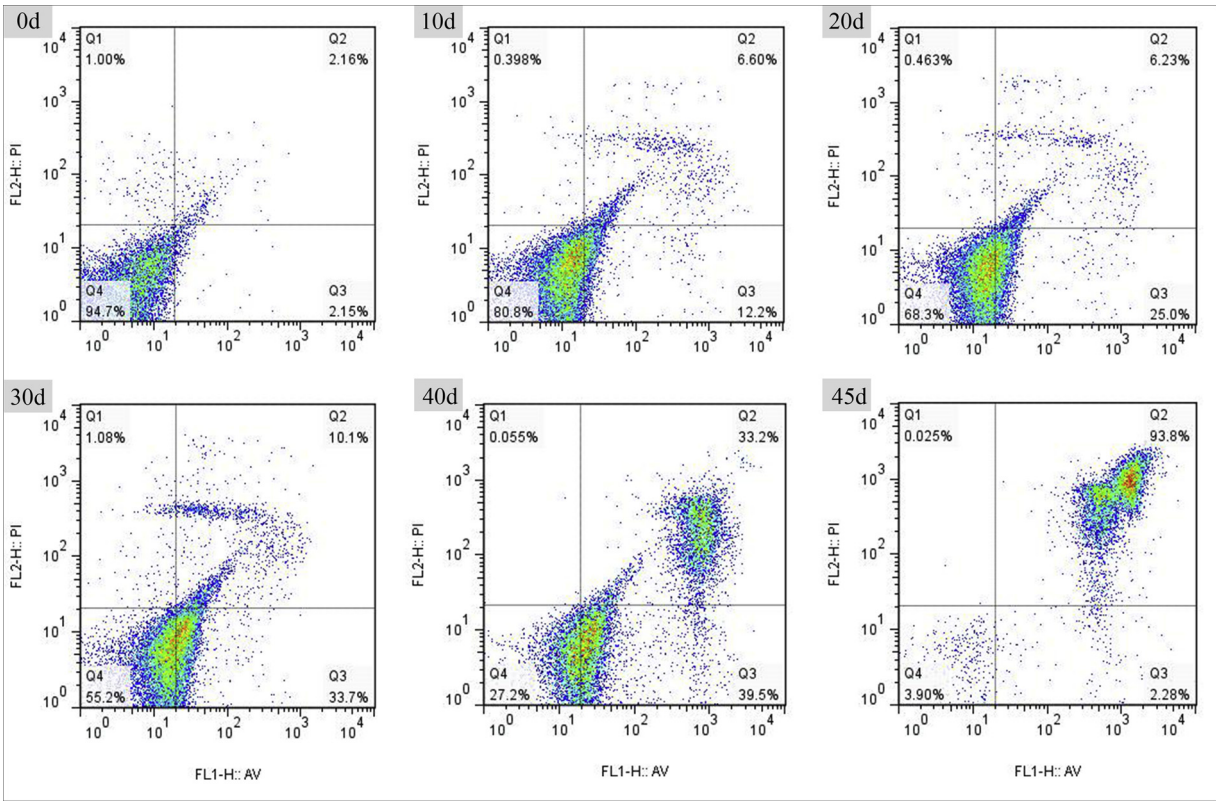

Fig. 2. Spermatozoa of *P. sinensis* were analyzed for phosphatidylserine translocation/Annexin assay using flow cytometry at various times during storage of spermatozoa at 4 °C; Quadrant regions indicate the percentage of spermatozoa in each sub-population, and events in the upper-left quadrant (Q1) represent necrotic spermatozoa; Spermatozoa in the upper-right (Q2) and lower-right (Q3) quadrants represent apoptotic spermatozoa, events in the lower-left quadrant (Q4) are viable spermatozoa.

Table 2
Annexin-V assay of spermatozoa during the periods of storage at 4 °C.

	Days				
	10	20	30	40	45
Viable (A-/PI-) %	81.2 ± 3.2[a]	67.8 ± 5.5[b]	54.4 ± 5.3[b]	30.0 ± 3.5[c]	2.3 ± 1.8[d]
A+/PI- %	11.8 ± 2.3[a]	23.5 ± 2.9[b]	32.8 ± 3.6[c]	38.8 ± 1.8[c]	7.5 ± 2.3[a]
A+/PI+ %	6.4 ± 1.3[a]	8.0 ± 3.2[ab]	11.5 ± 4.6[b]	30.6 ± 3.2[c]	89.4 ± 4.5[d]
A-/PI+ %	0.6 ± 0.2[a]	0.7 ± 0.1[a]	1.3 ± 0.3[b]	0.6 ± 0.3[a]	0.8 ± 0.1[a]

Viable %: the percentage of viable sperm(Q4), A+/PI− %: Annexin V single−positive spermatozoa(Q3), A+/PI+ %: Annexin V/PI double−positive spermatozoa(Q2), A−/PI+: the percentage of necrotic spermatozoa (Q1); All values are reported as the means ± SEM, and within a row values with different superscript (a–d) differ ($P < 0.01$).

3.3. Reactive oxygen species (ROS) and mitochondrial membrane potential (MMP)

Intracellular ROS generation was measured using DCFH-DA, and ROS production was markedly greater on Days 20 and 30 ($F = 54.35$, $P < 0.01$) as compared to values on Day 10 of storage at 4 ºC (Fig. 3A). As the storage time increased, SOD activity was at a peak on Day 20 (35.05 ± 4.5 U/mgprot) and subsequently decreased as duration of storage increased (Fig. 3B). The content of MDA, however, was markedly increased after 10 days of storage at 4 ºC ($F = 63.32$, $P < 0.01$; Fig. 3C). In general, the antioxidant capacity of spermatozoa was less and the damage to spermatozoa was greater as duration of storage increased. The mitochondrial membrane potential (MMP) in spermatozoa was evaluated during storage using JC-1 staining. The MMP of spermatozoa decreased as duration storage increased, which indicates that there was a loss MMP as storage duration increased (Fig. 3D).

3.4. Detection of morphological changes and active caspases in spermatozoa

Apoptosis involves a series of biochemical events that induce cellular morphological alterations eventually leading to cell death. The TEM was performed to detect the morphological changes of spermatozoa during storage at 4 °C. The onion-like shaped mitochondria were detected with eight to 15 concentric laminated membranes around a dense substrate core (Fig. 4A). After 20 days of

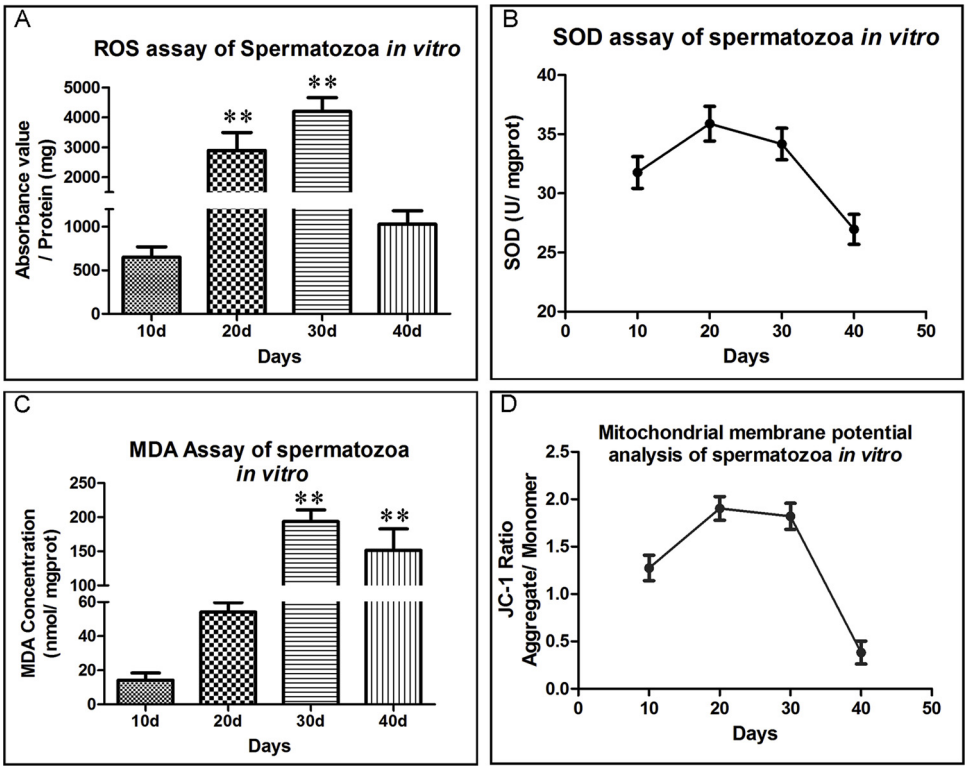

Fig. 3. Extent of spermatozoa injury greater as duration of spermatozoa storage at 4 °C increased; ROS generation (A), antioxidant capacity (B), oxidant damage (C) and MMP (D) of spermatozoa were tested during storage at 4 °C; All values are presented as mean ± SEM, data obtained in the experiment were technical duplications in triplicate; '**'indicates difference compared to the 10 day of spermatozoa storage at $P < 0.01$.

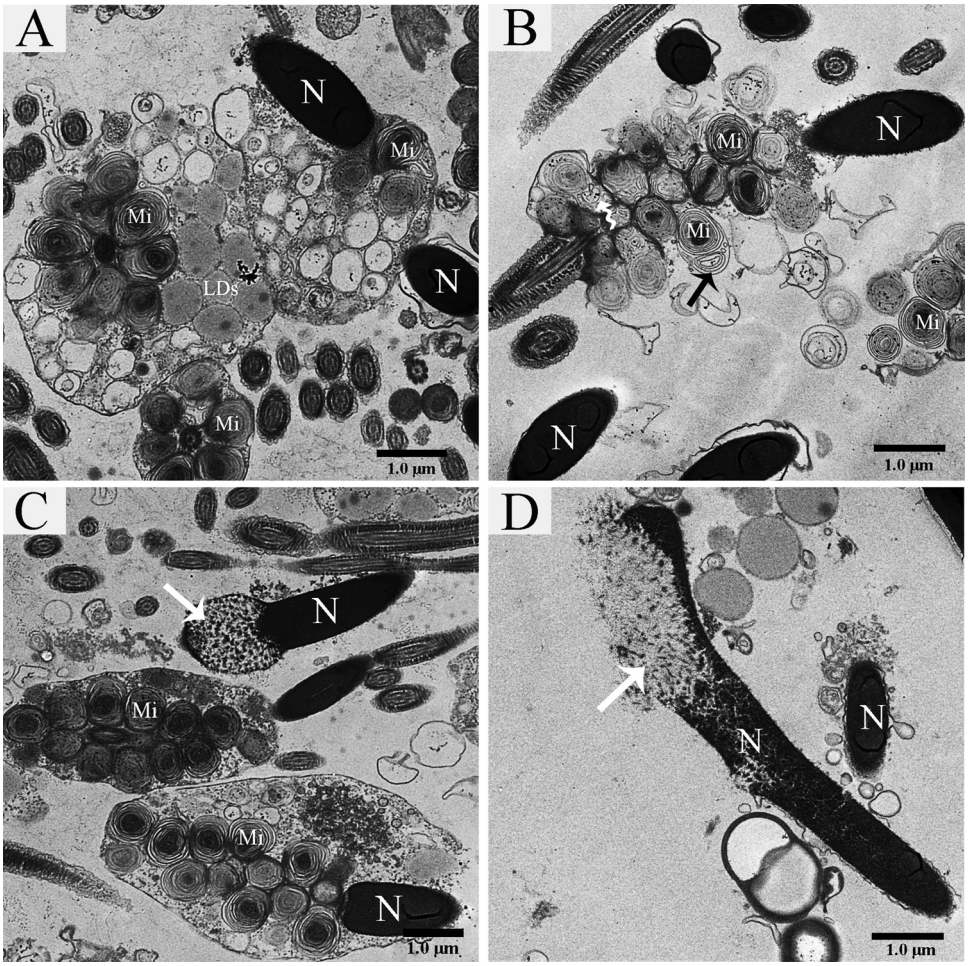

Fig. 4. Morphological changes of mitochondria and nucleus in spermatozoa; Spermatozoa were observed using TEM on Days of 10 (A), 20 (B), 30 (C) and 40 (D) of sperm storage at 4 °C; N, nucleus; Mi, mitochondria; LDs, lipid droplets; White curved arrow (B): the dispersion of mitochondrial membrane; Black arrow: the mitochondrial swelling; White arrow (C and D): the fragmented nucleus; Scale Bars = 1.0 μm.

storage, some of the mitochondria were swollen and the shape changed from onion-like to elliptic. Meanwhile, the concentric membranes of mitochondria were dispersed (Fig. 4B). Nuclear fragmentation is a marker for late-stage apoptotic-like changes in spermatozoa. Nuclear fragmentation of spermatozoa was observed on Day 30 of storage at 4 °C, and the fragmented nucleus was dispersed inside the nuclear membrane. These fragmented nuclear components were dispersed due to the disruption of nuclear membrane on Day 40 (Fig. 4C and D). The apoptotic spermatozoa after different durations of storage at 4 °C was investigated using the TUNEL assay. Consistent with TEM results, the result of TUNEL analysis indicated that the apoptotic spermatozoa were greater on Days 30 and 40 than earlier in the storage period (Fig. 5C and D). Furthermore, western blot analysis was performed to detect the relative abundance of Cytc and Cleaved caspase-9/-3 proteins at different times during the storage period. As depicted in Fig. 6, the Cytc content in the CDs isolated from spermatozoa was greater after storage for 30 Days as compared to earlier in the storage period (Fig. 6D). The relative abundances of Cleaved caspase-9/-3 proteins in spermatozoa were also greater at the same time of storage at 4 °C (Fig. 6A).

4. Discussion

In the present study, the epididymal spermatozoa of *P. sinensis* were investigated for sperm viability and motility characteristics to determine apoptotic-like changes during cooled storage. Results from *in vitro* studies indicate that the spermatozoa (collected from hibernation) of *P. sinensis* were viable for more than 40 Days during storage at 4 °C. There were similar results in turtles in a previous study, however, the mechanism of loss of sperm viability during refrigerated storage was not assessed (Gist et al., 2002). Sperm motility is used as the primary indicator of sperm quality and loss of motility is correlated with the loss of sperm viability (Ortega-Ferrusola et al., 2009b; Salehi et al., 2018; Xin et al., 2018). Results from studies using the CASA system indicate that the values for all variables of sperm kinematics markedly decreased as duration of storage increased (Table 1). Results from flow cytometric analysis indicate mortality percentage of spermatozoa was about 30% on Day 40 of storage in the present study, and there were almost no

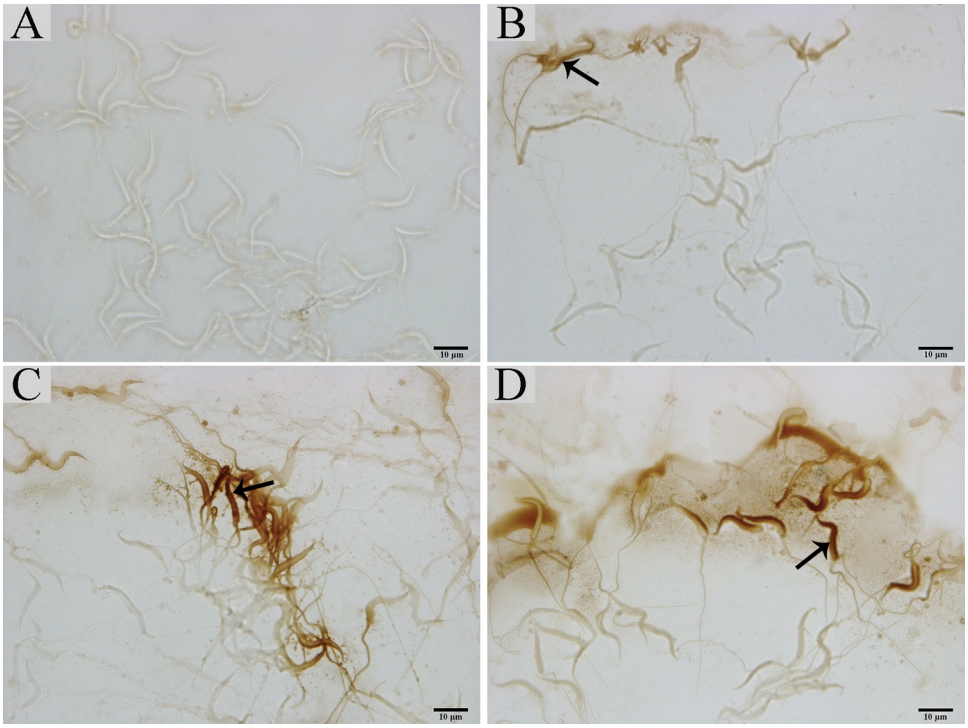

Fig. 5. Spermatozoa photographs with TUNEL staining after different durations storage at 4 °C; Spermatozoa were collected on Day 10 (A), 20 (B), 30 (C) and 40 (D) of storage; Apoptotic spermatozoa stained yellow brown and the results indicate the larger proportion of TUNEL-positive spermatozoa on Days 30 and 40 of storage; Arrow: apoptotic spermatozoa; Scale Bars = 10 μm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

viable spermatozoa on Day 45. Overall, results of the present study indicate percentage of sperm death was positively correlated with the decrease of total motile and progressively motile spermatozoa.

Findings in the present study indicate that the percentage of non-viable spermatozoa increased after 20 Days of storage at 4 °C. The determinations as to the molecular mechanisms leading to sperm death, however, vary depending on the biotechnology procedures used in processing the spermatozoa. Apoptosis is a primary cause of sperm death during *in vitro* storage in refrigerated conditions (Rao et al., 2016). Apoptosis results from a series of biochemical events that induce cellular morphological alterations eventually leading to cell death. In the present study, MMP of spermatozoa decreased as storage duration increased. The TEM results indicate that the mitochondrion was swollen and the concentric membranes were dispersed after 20 Days of storage. Mitochondria have a central role in cell death regulation. When mitochondria dysfunction, a channel traversing across the outer and inner mitochondrial membranes termed 'PT (permeability transition) pore' is induced to open (Green and Reed, 1998; Clavier et al., 2016). Solute and water in the cytosol then enter the mitochondrial matrix through this channel, causing the mitochondria to swell and its outer membrane to rupture. As a result, Cytc and other proteins in the inter-membrane space are released (Green and Reed, 1998). Recently, there have been reports in cultured Hela cells that Cytc release during apoptosis is not a result of mitochondrial swelling (Gao et al., 2001). The results of the present study, however, indicate mitochondrial swelling occurred and the shape changed from onion-like (Haseeb et al., 2018) to elliptic. At the same time, the relative protein abundance of Cytc in CDs isolated from spermatozoa was greater after Day 30 of cooled storage. The nuclear fragmentation of spermatozoa was observed using TEM on Days 30 and 40 (Fig. 4). The results from TUNEL staining indicate there were more apoptotic spermatozoa on Days 30 and 40 which is consistent with TEM results. Furthermore, there was a greater abundance of Cleaved caspase-9/3 proteins detected after 30 Days of storage. Taken together, results of the present and previous studies indicate soft-shelled turtle spermatozoa undergo apoptotic-like changes during refrigerated storage *in vitro*.

5. Conclusions

In conclusion, results of the present study provide the first cytological and molecular evidence about the spermatozoa apoptotic-like changes during long-term storage at 4 °C in ectothermic animals. Results indicate ROS generation by soft-shelled turtle spermatozoa induce mitochondrial damage, which in turn induce the release of the Cytc protein from mitochondria. Furthermore, Cytc release would activate a series of caspase cascades and then induce the reactions of the apoptotic pathway.

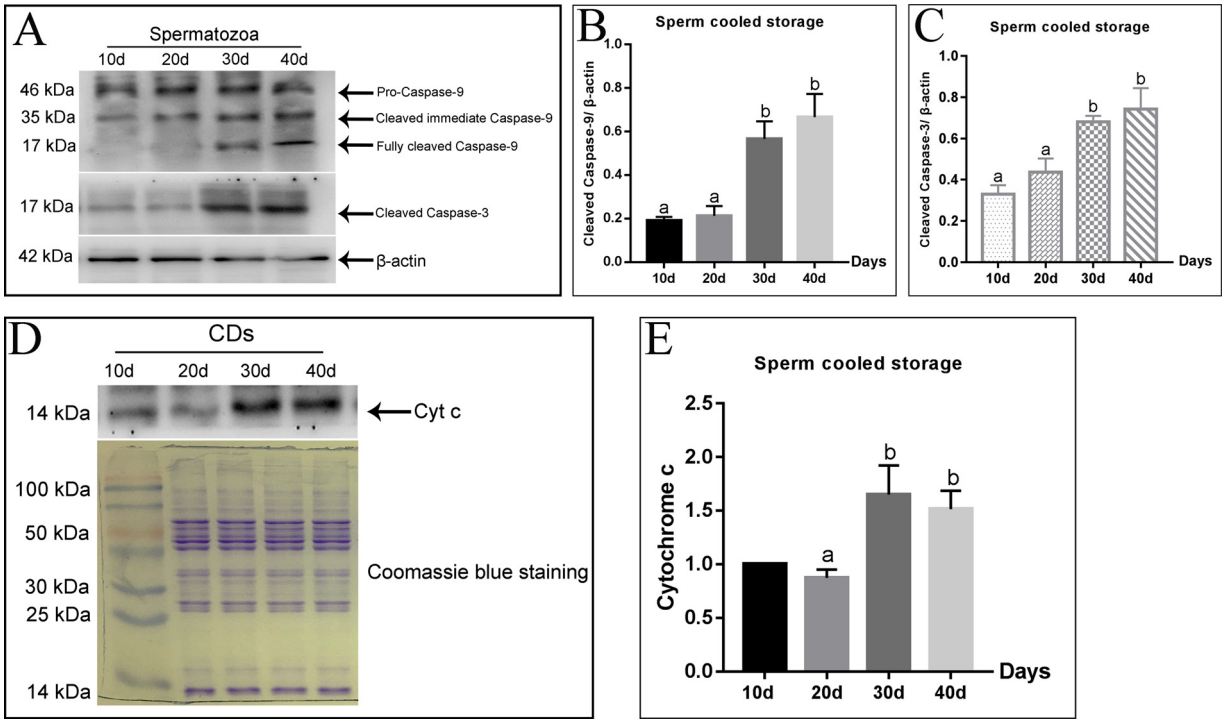

Fig. 6. Relative abundance of apoptosis-related proteins in spermatozoa after different durations storage at 4 °C; (A) Relative abundance of apoptosis-related proteins, Cleaved caspase-9, Cleaved caspase-3, were detected using Western blot analysis. β-actin was used as loading control; (B, C) Protein quantification of cleaved caspase-9/3 was evaluated by assessing band density compared with the control, and the results were expressed as mean ± SEM; Values with different letters (a–b) differ ($P < 0.05$, $n = 3$); (D) Relative abundance of Cytc in spermatoza; Coomassie Blue-stained gels, proteins extracted from isolated CD, was used to standardize the data; (E) Quantification of the Cytc protein abundance occurred by assessing band density, and the data are presented as mean ± SEM with three technical repetitions' Values with different letters (a–b) differ compared with values on Daye 10 storage at 4 °C; Error bars represent the s. e. m (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Conflicts of interest

There are no potential conflicts of interest that need to be disclosed.

Authors' contributions

H. C. designed the experiments and drafted the manuscript. Y. H., X. B., P. Y., I. T., W. A. V., S. A. F and N S., G. participated in the study design and performed data analysis. Prof. Q. C. conceived the study and participated in its design and coordination and helped draft the manuscript. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.anireprosci.2019.04.014.

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