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Evaluation of Antifreeze Proteins on Miniature Pig Sperm Viability, DNA Damage, and Acrosome Status during Cryopreservation

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ABSTRACT

The cryopreservation of sperm has become the subject of research for successful artificial insemination technologies. Antifreeze proteins (AFPs), one of the factors necessary for effective cryopreservation, are derived from certain Antarctic organisms. These proteins decrease the freezing point of water within these organisms to below the temperature of the surrounding seawater to protect the organism from cold shock. Accordingly, a recent study found that AFPs can increase the motility and viability of spermatozoa during cryopreservation.

To evaluate this relationship, we performed cryopreservation of boar sperm with AFPs produced in the Arctic yeast *Leucosporidium* sp. AFP expression system at four concentrations (0, 0.01, 0.1, and 1 μg/m*l*) and evaluated motility using computer assisted sperm analysis. DNA damage to boar spermatozoa was measured by the comet assay, and sperm membrane integrity and acrosome integrity were evaluated by flow cytometry. The results showed that motility was positively affected by the addition of AFP at each concentration except 1 μg/m*l* ($p<0.001$).

Although cryopreservation with AFP decreased the viability of the boar sperm using, the tail DNA analyses showed that there was no significant difference between the control and the addition of 0.1 or 0.01 μg/m*l* AFP. In addition, the percentage of live sperm with intact acrosomes showed the least significant difference between the control and 0.1 μg/m*l* AFP ($p<0.05$), but increased with 1 μg/m*l* AFP ($p<0.001$). Our results indicate that the addition of AFP during boar sperm cryopreservation can improve viability and acrosome integrity after thawing.

(Key words: Antifreeze protein, Miniature Pig Sperm, Cryopreservation, DNA integrity)

INTRODUCTION

Spermatozoal function needs to be preserved through effective storage for successful artificial insemination (AI) and in vitro fertilisation (Medeiros et al., 2002). The most widely used storage method is cryopreservation; however, this method presents potentially damaging stresses, such as cold, osmotic, and toxic shocks (Watson, 2000), stresses that cause the production of reactive oxygen species, inducing DNA damage (Aitken et al., 1998; Donnelly et al., 1999; Perez-Cerezales et al., 2009; Villani et al., 2010). Because studies have shown that DNA damage occurs during cryopreservation, scientists have made efforts to solve this problem (Boe-Hansen et al., 2005; Cabrita et al., 2005; Fraser and Strzezek, 2007; Perez-Cerezales et al., 2010). The low temperatures at which sperm is preserved induce the formation of sharp ice crystals, which can fatally damage the sperm cell cytoplasm, cytoskeleton, and genome structure (Isachenko et al., 2003). Accordingly, preventing ice crystallisation could help maintain sperm integrity, and regulating the freezing rate and including cryoprotectants could minimize such damage (Johnson et al., 2000).

Antifreeze proteins (AFPs), which are derived from the body fluids of certain Antarctic organisms, have a high affinity for ice crystals (Park et al., 2012). AFPs prevent the growth of the ice crystals, even in subzero temperatures, by binding to the ice through hydrogen bonds (Davies et al., 2002; DeLuca et al., 1998), and organisms expressing AFPs exhibit cold tolerance and can survive at very low temperatures (Bayer-Giraldi et al., 2011; Lee et al., 2012). Thus, the application of such molecules as sperm cryoprotectants could help to maintain spermatozoal survival and function after thawing; indeed, previous studies demonstrated the effects of AFPs in sperm cryopreservation. For instance, the inclusion of low concentrations of AFPs during bull sperm cryopreservation

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improved the post-thaw viability, osmotic resistance, and acrosome integrity relative to the control (Prathalingam et al., 2006). AFP cryoprotectants improve sperm quality in vertebrate spermatozoa and in primordial germ cells: zebra fish primordial germ cell cryopreservation shows that small amounts of AFPs could enhance post thaw viability and DNA integrity (Riesco et al., 2012).

The DNA damage and reductions in cell viability, osmotic resistance, and acrosome integrity induced by freezing stress subsequently cause reductions in fertility (Aitken et al., 1998). Single cell gel electrophoresis (neutral comet assay), a common way to test sperm DNA integrity, detects DNA breakages by separating DNA fragments from undamaged DNA, generating the shape of a tail and head of a comet, respectively (Singh et al., 1988); the length of the comet tail indicates the extent of DNA damage within the cell. A previous study found that freezing resulted in increased DNA in boar sperm in comparison to fresh sperm (Fraser and Strzezek, 2005).

Recently, SYBR-14 and propidium iodide (PI) have been used as viability probes for mammalian sperm (Foster et al., 2011; Klimowicz-Bodys et al., 2012; Niu et al., 2011). Garner et al. discovered that this simple staining protocol was useful to determine the viability of spermatozoa by flow cytometry (Garner and Johnson, 1995). The unlabeled (PI- SYBR-) spermatazoa were considered debris because the expression indicated that these cells did not contain DNA, whereas the spermatozoa stained with SYBR14 but not PI (PI- SYBR+) were considered viable because the expression indicated that the plasma membrane was intact. The PI+ SYBR- spermatozoa were considered dead, and the PI+ SYBR+ spermatozoa were considered to be dying (Partyka et al., 2010).

The acrosomal status has been evaluated using florescent probes that recognise the targets inside the acrosome, thus labelling sperm with damaged or reacted acrosomes (Martinez-Pastor et al., 2010). Fluorescein isothiocyanate-conjugated pea (*Pisum sativum*) agglutinin (FITC-PSA), which binds to the glucosidic residues of the acrosomal membrane, has been used in studies of various species (Bencharif et al., 2012; Nagy et al., 2003; Nur et al., 2010; Orgal et al., 2012).

In contrast, there have been no previous studies about the effect of AFPs on the post-thaw DNA and membrane integrity of boar sperm. Thus, to improve the cryopreservation protocols for sperm, we examined the effect of AFPs on sperm viability and DNA damage after a freeze thaw cycle.

MATERIALS and METHODS

Ejaculate collection and processing

Ejaculates were collected from miniature pigs in Kangwon National University (aged 8 years). After ejaculation, the semen was diluted with modified Modena B extender (mMB, 6 g glucose, 0.45 g EDTA, 1.38 g sodium citrate, 0.2 g sodium bicarbonate, 1 g Tris base, 0.5 g citric acid, 0.01 g cysteine, 0.8 g BSA, and 0.06 g kanamycin sulfate; pH 7). The diluted semen was packed and delivered to the laboratory in Incheon at 17°C for 3 hrs.

Preparation of AFPs

AFP (24.9 kDa, Korea Polar Research Institute) was produced using a *Leucosporidium* sp. expression system. Recombinant LeIBP (*Leucosporidium* ice-binding protein) was produced using a *Pichia* expression system, and purified as described in a previous study (Park et al., 2012). Briefly, the codon-optimised mature LeIBP gene was synthesised and cloned into the pPICZαA expression vector (Invitrogen, USA). The plasmid was transformed into *P. pastoris* strain X33(Invitrogen Co., CA, USA), and the recombinant cells were incubated in 24 m*l* of BMGY (Buffered Glycerol-complex Medium, 1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5}% biotin and 0.5% methanol) medium at 28°C for 48 hrs and 5 m*l* methanol was added daily to induce the expression of recombinant LeIBP. The eluted LeIBP was further purified using a Superdex 200 size-exclusion column (Thermo Fisher Co., USA). The purified LeIBP fractions, according to SDS-PAGE and Western blot analyses, were pooled. UV spectrophotometry was used for determining the concentration of LeIBP at an extinction coefficient of 26,930 M^{-1} cm^{-1} at 280 nm

Boar sperm cryopreservation

The semen diluted with mMB extender was centrifuged at 400 × *g* for 10 min at 17°C; for the analyses, the samples were washed 3 times with mMB extender to isolate the spermatozoa. The supernatant was discarded and the pellet was

diluted in lactose-egg yolk solution (LEY extender). The purified sperm pellet was suspended in cooling extender (LEY solution: 80% v/v lactose solution [310 mM] and 20% v/v egg yolk with 100 μg/m*l* kanamycin sulfate) at a density of approximately 1.5×10^9 sperm/m*l*. The sperm suspensions was subjected to a gradual cooling stage of 1°C every 5 min in ice water. Freezing extender (LEYGO solution: 89.5% v/v LEY extender; 9% v/v glycerol; 1.5% v/v Orvus ES Paste [OEP]; 100 mM trehalose; and 0, 0.01, 0.1, or 1 μg/m*l* AFP [KOPRI, Incheon, Korea]) was added to the suspensions when a temperature of 5°C had been reached. The sperm suspensions were loaded into 0.25 m*l* straws and stored in liquid nitrogen.

Sperm evaluation

Post-thaw sperm motility

The sperm motility was evaluated using a computer-assisted sperm analysis (CASA) system (Hamilton Thorne, Inc., Beverly, MA, USA). The cryopreserved sperm samples were incubated in a 50°C water bath for 20 sec, and the thawed sperm was transferred to 15 m*l* tubes and diluted with 10 m*l* mMB extender pre-warmed to 37°C. After dilution, the sperm cells were centrifuged at 300 × *g* for 5 min at room temperature. The sperm concentrations were adjusted to 5×10^7 sperm/m*l* with mMB. For analysis, 3 μl of the sperm sample was placed in a counting chamber (20μm deep) and observed using a microscope; at least 10 predetermined fields were analysed. The CASA evaluation yielded the path velocity (VAP), velocity of straight-line motion (VSL), track speed (VCL), and lateral amplitude of the sperm.

Post-thaw sperm DNA integrity

The cryopreserved sperm samples were thawed and washed three times, similar to the procedure for the CASA. The diluted sperm cells were incubated with a 2% 1-mercaptoethanol solution for 2 hr at 4°C, centrifuged (400 × *g*, 5 min), and resuspended in 500 μl Ca^{2+}- and Mg^{2+}-free phosphate buffered saline. The sperm samples (10 μl) were mixed with 50 μl 0.7% low-melting agarose (Bio Rad, Berkeley, CA, USA), and 50 μl of the sperm agarose mixture was coated onto a prepared comet slide (Trevigen, Maryland, USA). The slides were incubated at 4°C with CometAssay lysis solution (Trevigen) in a Coplin jar. After a 2 hr incubation, an RNase and proteinase treatment (2.5 M NaCl, 5 mM Tris, and 0.05% SLS, pH 7.5 with 20 μg/m*l* RNase A and 1 mg/ml proteinase K) was performed for 4 h to remove the RNA and proteins. After draining off the treatment solution, the slides were placed in a Coplin jar with 33 ml alkaline solution (0.6 g NaOH and 250 μ*l* 200 mM EDTA; pH 13) in the dark for 30 min at room temperature. The slides were placed in an electrophoresis tray filled with electrophoresis solution (10.8 g Tris base, 0.93 g EDTA, and 5.5 g boric acid; 1000 m*l*). Electrophoresis was performed for 1 hr at 12 V and 300 mA, following which, slides were placed in distilled water for 20 min and 70% ethanol for 5 min and then stained with SYBR Green fluorescent dye and incubated in the dark for 5 minutes at 4°C. Each sperm comet shape was imaged using a fluorescent microscope, and each image was analysed by a comet analysing program (COMETSCORE, TriTek, Sumerduck, Virginia, USA).

Plasma membrane integrity of boar sperm

To stain the sperm with SYBR14 and PI (LIVE/DEAD Sperm Viability Kit, Invitrogen, Eugene, OR, USA), the samples were first diluted with BTS extender to a concentration 1×10^6 spermatozoa/m*l*. Aliquots of 500 μ*l* were mixed with SYBR14 and PI at final concentrations of 100 nM and 12 μM, respectively, prior to the flow cytometric analysis, which was performed using an FC 500 series cytometer (Beckman Coulter, Inc., Miami, FL, USA). The flow cytometric data were analysed using the CXP program (Beckman Coulter, Inc.).

Acrosome integrity of boar sperm

The sperm acrosome integrity was assessed using a fluorescein isothiocyanate (FITC-PNA; Lectin from *Arachis hypogaea*, Sigma-Aldrich, St. Louis, MO, USA)/ PI fluorescent staining technique (Nagy et al., 2003). Aliquots of 500 μl of the post-thaw sperm samples (1×10^6/m*l* spermatozoa) were mixed with 10 μl of FITC-PNA (50 μg/m*l*) and PI (12 μM) working solution and the mixture was incubated at 37°C for 5 min before flow cytometric analysis.

Statistical analysis

The data were processed using the R statistical program (R

Development Core Team). The data were expressed as the means ± SD and analysed using ANOVA and Tukey statistical tests to determine the limit of detection for the viability and DNA damage after cryopreservation. Differences were considered significant at $P<0.05$.

RESULTS AND DISCUSSION

Motility and viability

The effects of AFP on the motility and viability were measured with a CASA system (Table 1). The sperm motility was positively affected by the addition of the lower concentrations of AFP (excepting 1 μg/m*l*). The percentage of both motility and progressive motility were higher for the sperm cryopreserved in the presence of 0.01μg/m*l* AFP than for the control sperm and sperm cryopreserved with 0.1 μg/m*l* AFP but was not significantly different between the control and 0.1 μg/m*l* AFP; in contrast, these properties were lower for the sperm cryopreserved with 1 μg/m*l* AFP. In particular, the percentage of progressive motility was not significantly different between the fresh sperm and the sperm with 0.01 μg/m*l* AFP. Most of the motion parameters analysed (VAP, VSL, and VCL) were decreased by the addition of AFP compared to the control, whereas the lateral amplitude was increased. The analyses of all of the motion parameters, except for the VCL ($p<0.01$), showed that there were significant differences among the frozen semen samples ($p<0.001$).

Sperm DNA integrity

The addition of AFP significantly improved the sperm DNA integrity, as based on the tail DNA, tail length, and olive moment parameters (Table 2). The tail DNA analysis showed that there was no significant difference between the control semen and semen cryopreserved 0.1 or 0.01 μg/m*l* AFP, but the percentage of tail DNA was higher for the semen cryopreserved with 1 μg/m*l* AFP than for the semen cryopreserved with 0.01 μg/m*l* AFP (13.4% ± 9.6 vs. 6.5 ± 7.5; $p<0.05$). There was no significant difference in the tail length between the groups ($p=0.324$). The olive moment was higher for the sperm cryopreserved with 1 μg/m*l* AFP than for the sperm cryopreserved 0.01 μg/m*l* AFP, but there were no significant differences between the control and 0.1 or 0.01 μg/m*l* AFP. Conversely, there was a significant difference in

Table 1. Mean values of the sperm quality analysis of boar sperm cryopreservation added AFP

	Motility(%)	Progressive(%)	VAP(μm/s)	VSL(μm/s) (μm/s)	VCL(μm/s)	ALH (μm)
Fresh	80.4±2.5^{c}	71.2±2.2^{c}	54.48±1.88^{c}	33.84±1.15^{bc}	100.62±4.67^{a}	6.20±0.23^{b}
Control	68.2±2.4^{a}	61.7±2.3^{a}	64.97±3.25^{b}	42.97±2.69^{a}	100.78±2.38^{a}	5.62±0.30^{a}
AFP(0.01μg/ml)	74.6±2.6^{d}	68.0±3.2^{c}	58.16±1.51^{ac}	33.78±0.75^{bc}	99.92±3.51^{a}	6.40±0.21^{b}
AFP(0.1μg/ml)	67.8±3.9^{a}	61.4±4.4^{a}	60.86±5.51^{ab}	37.44±6.23^{ab}	99.58±4.92^{a}	6.08±0.35^{ab}
AFP(1μg/ml)	47.0±1.6^{b}	40.6±1.7^{b}	52.42±1.06^{c}	28.06±0.67^{c}	91.54±3.20^{b}	6.42±0.24^{b}
p-Value	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.01$	$p < 0.001$

The parameters are Path Velocity (VAP), Velocity of Straight-line motion (VSL), Track Speed (VCL), Lateral Amplitude (ALH). Results are expressed as means ± SD of 5 or 6 different experiments with a total number of anlaysed sperm of 6764 (Fresh semen), 7389 (Control), 5996 (0.01mg/ml AFP added semen), 6558 (0.1mg/ml AFP added semen), and 6621 (1mg/ml AFP added semen). Different letters (a, b, c, d) show significant differences ($p < 0.01$ or $p < 0.001$).

Table 2. Effects of AFP on the DNA integrity of boar sperm

Group	Tail DNA (%)	Tail Length (μm)	Olive Moment
Control	7.1 ± 8.1^{ab}	2.2 ± 2.4	0.75 ± 0.87^{ab}
AFP(0.01μg/ml)	6.5 ± 7.5^{a}	1.9 ± 1.5	0.59 ± 0.63^{a}
AFP(0.1μg/ml)	11.6 ± 7.5^{ab}	2.5 ± 1.4	1.04 ± 0.72^{ab}
AFP(1μg/ml)	13.4 ± 9.6^{b}	3.0 ± 1.8	1.29 ± 0.92^{b}
p-Value	p <0.05	p=0.324	p <0.05

Each data represents the means ± SD, with a minimum of 20 cells scored per experiment for each samples. Different letters (a, b) show significant differences ($p < 0.05$).

the olive moment between 1 and 0.01 μg/ml AFP (p<0.05).

Plasma membrane integrity

The SYBR/PI analysis using flow cytometry revealed the membrane integrity of the thawed boar sperm (Figure 1 and Table 3). The percentage of live spermatozoa was higher for the control sperm than for the sperm cryopreserved with AFP (p<0.001). However, there were no significant differences in most of the other parameters between the control semen and the semen cryopreserved with AFP, except that the addition of 1 μg/ml AFP (p<0.001) resulted in dead. Nevertheless, the analyses of all of the parameters indicated a difference between the control and experimental groups (p<0.001).

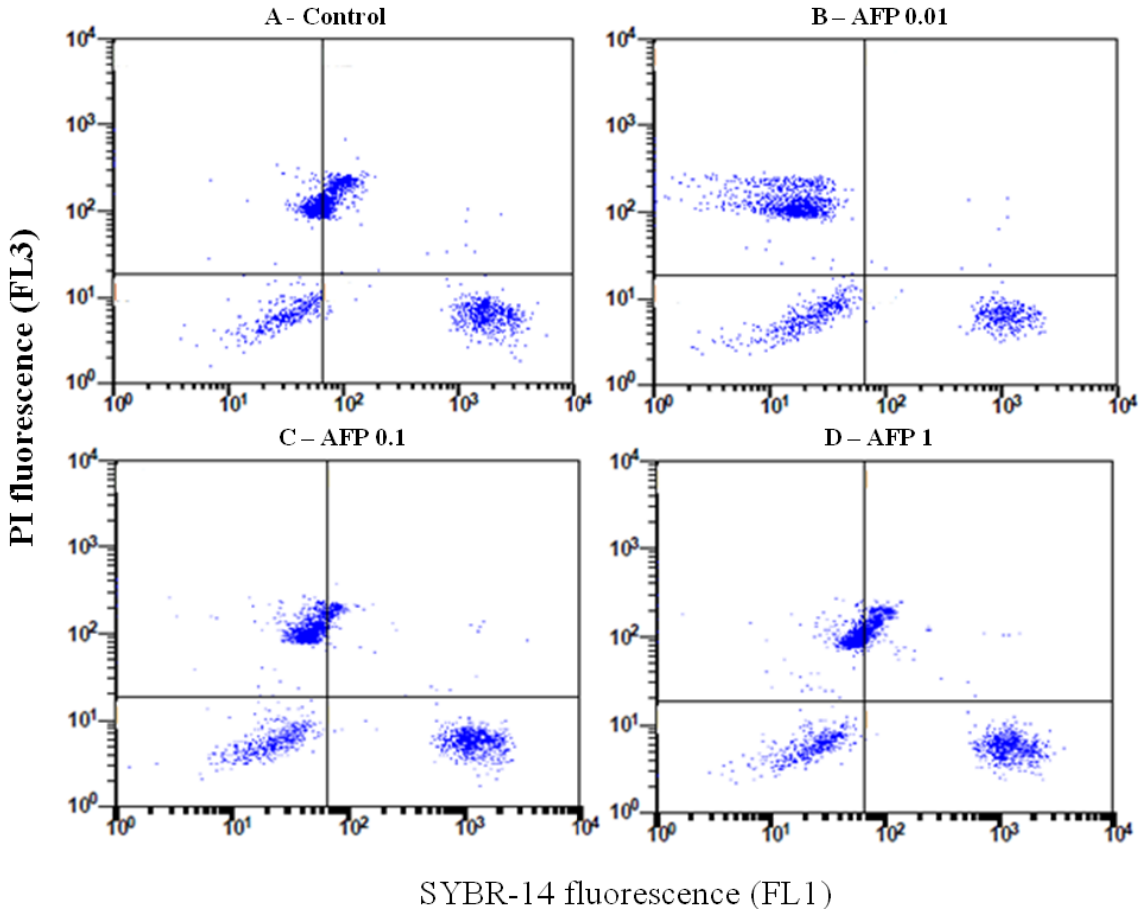

Figure 1. Flow cytometric analysis of boar sperm labeled with PI and SYBR. Panel (A) Control; (B-D) (0.01, 0.1, and 1mg/ml), AFP supplemented frozen-thawed sperm groups. PI+ SYBR- quadrant contains dead spermatozoa; PI+ SYBR+ quadrant contains dying spermatozoa; PI- SYBR- quadrant contains debris; PI- SYBR+ quadrant contains live spermatozoa.

Table 3. Effects on plasma membrane integrity of post-thawed boar sperm in LEYGO extender added AFP

Spermatozoa	Dead (PI+ SYBR-)	Dying (PI+ SYBR+)	Live (PI- SYBR+)
Control	51.9 ± 5.5^{a}	9.3 ± 5.0^{ab}	38.8 ± 0.8^{c}
AFP(0.01μg/ml)	45.2 ± 7.6^{a}	18.4 ± 7.3^{a}	36.4 ± 0.9^{d}
AFP(0.1μg/ml)	47.8 ± 19.0^{a}	18.8 ± 19.3^{a}	33.4 ± 0.7^{a}
AFP(1μg/ml)	72.0 ± 0.9^{b}	0.46 ± 0.1^{b}	27.5 ± 0.9^{b}
p-Value	p < 0.001	p < 0.001	p < 0.001

Different letters (a, b, c, d) show significant differences (p < 0.001).

Acrosome intrgrity

The boar sperm acrosome integrity was examined by FITC-PNA/PI staining (Figure 2 and Table 4). The percentage of live sperm with intact acrosomes (FITC-PNA-/PI-) showed a slightly significant difference between the control and 0.1 μg/ml AFP (22.9% ± 0.5 vs. 21.9% ± 0.5; $p<0.05$) and increased for the sperm with 1 μg/ml AFP ($p<0.001$). Also the percentage of live sperm with reacted acrosome (FITC-PNA+/PI-) showed the least significant difference between the control and 0.1 μg/ml AFP (11.4% ± 0.4 vs. 10.6% ± 0.3; $p<0.05$) and decreased with 1 μg/ml AFP and 0.01 μg/ml AFP ($p<0.001$).

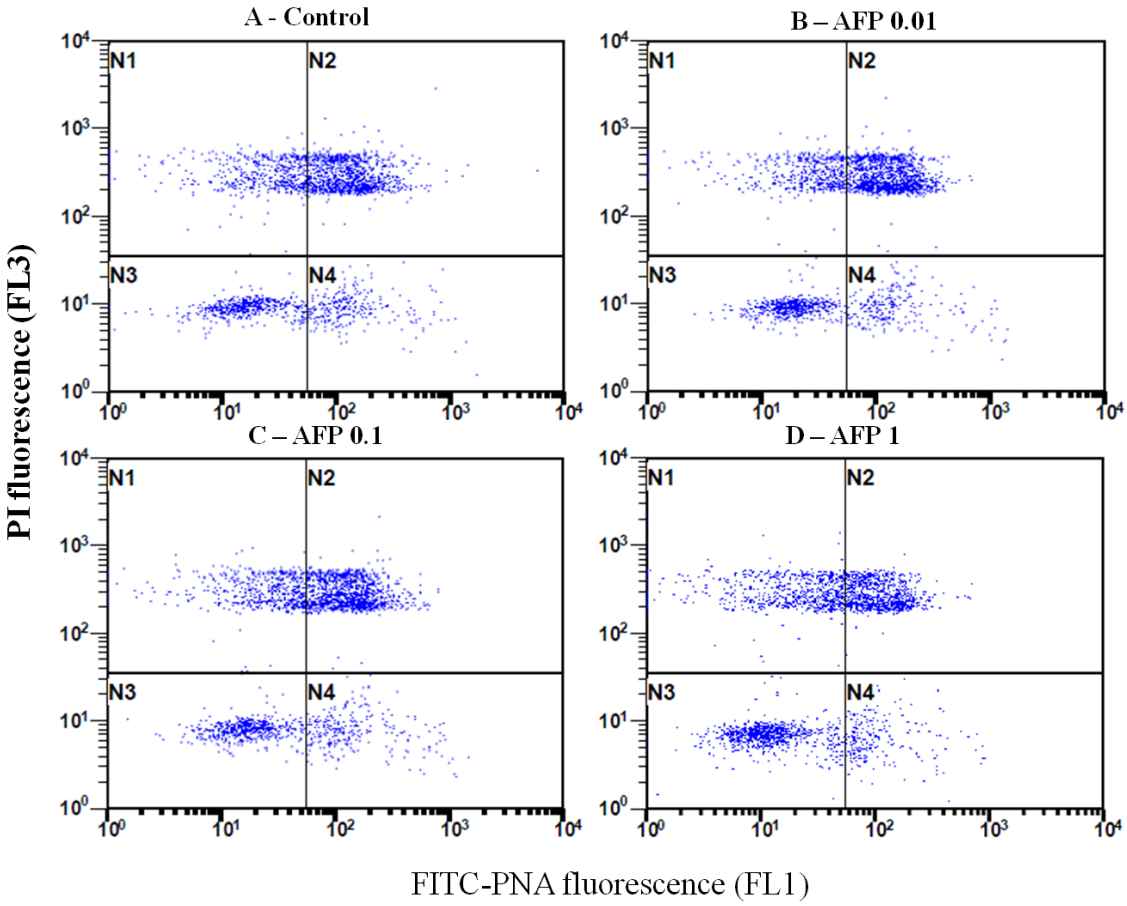

Figure 2. Flow cytometric analysis of boar sperm labeled with PI and FITC-PNA. Panel (A) Control; (B-D) (0.01, 0.1, and 1mg/ml), AFP supplemented frozen-thawed sperm groups. PI+ PNA- quadrant contains dead acrosome-intact; PI+ PNA+ quadrant contains dead acrosome-damaged; PI- PNA- quadrant contains viable acrosome-intact; PI- PNA+ quadrant contains viable acrosome-reacted.

Table 4. Effects on acrosome integrity of frozen-thawed boar sperm in LEYGO extender added AFP

Spermatozoa	Acrosomal membrane			
	PI+/PNA- (%)	PI+/ PNA+ (%)	PI-/ PNA- (%)	PI-/ PNA+ (%)
Control	23.1 ± 3.9^{b}	42.49 ± 3.5^{c}	22.9 ± 0.5^{c}	11.4 ± 0.4^{c}
AFP(0.01μg/ml)	17.9 ± 0.8^{a}	51.9 ± 1.1^{d}	21.0 ± 0.4^{d}	9.2 ± 0.6^{d}
AFP(0.1μg/ml)	20.8 ± 1.1^{ab}	46.6 ± 0.9^{a}	21.9 ± 0.5^{a}	10.6 ± 0.3^{a}
AFP(1μg/ml)	27.7 ± 0.6^{c}	33.9 ± 0.8^{b}	30.8 ± 0.5^{b}	7.5 ± 0.2^{b}
p-Value	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$

Different letters (a, b, c, d) show significant differences ($p < 0.001$).

DISCUSSION

We have demonstrated that AFP, used during the freezing of miniature pig sperm, has effects on DNA damage and membrane integrity. Although the membrane integrity decreased in the AFP treatment groups compared with the control, the motility significantly improved with the addition of 0.01 μg/m*l* AFP. However, the DNA damage was not significantly reduced according to the comet assay. These results support previous findings concerning the positive effects of AFPs (Jo et al., 2011; Kawahara et al., 2009; Lee et al., 2012).

Our experiments were performed to evaluate which concentration of AFP would provide the most effective protection against cold shock during the cryopreservation of boar sperm. After AFPs were discovered, antifreeze glycoproteins (AFGPs) and several other AFPs were found (Logsdon and Doolittle, 1997). Several studies have used AFP and the expression of AFP genes to inhibit the growth of ice crystals to protect cells due to characterization of AFP (Bayer-Giraldi et al., 2011; Gwak et al., 2010). The main function of AFPs is to inhibit both recrystallisation and ice crystal formation, reducing cellular damage (Chaytor and Ben, 2010; Rubinsky et al., 2010). Because AFPs inhibit the formation of ice crystals, the freezing and melting points of water become different. This phenomenon is called the thermal hysteresis effect and is a quantitative indicator representing the effect of AFPs; this effect can be measured using a nanoliter osmometer (Kristiansen et al., 2011). AFPs, which are components of certain Antarctic organisms, depress the freezing point of water below the temperature of the surrounding seawater to protect the organism from freezing, and AFP has been applied to the cryopreservation of red blood cells, mesenchymal stem cells, germ cells, and even tissues, such as cardiac valves, bones, and corneas

Sperm cryopreservation has been used in many important ways in the swine industry: enhancing herd productivity, assisting biosecurity measures, encouraging international exchange, facilitating gender selection technology, and permitting rational gene banking (Bailey et al., 2008). However, it is difficult to prevent cold shock, which, through morphological and biochemical effects, may results in damage to the cytoskeleton and cellular organelles, loss of embryo viability, and cell death (Makarevich et al., 2010). Thus, cryoprotectants have been examined as agents that could be used to avoid cold shock damage during cryopreservation (Almlid and Johnson, 1988; Dong et al., 2009; Jeong et al., 2009; Pena et al., 2003).

In a recent study, special concern was given to efficient cryopreservation protocols for human embryonic stem cells (hESCs) (Malpique et al., 2012) and, indicated that the surface-based vitrification of hESCs is the most efficient technique for the cryopreservation of intact hESC colonies. According to another report, freezing may be useful to preserve tissue for transplantation because the survival of the resident innate immune cells in adrenal gland tissue decreases after cryopreservation (Alabedalkarim et al., 2012), with the allogenic grafts of adrenal gland tissue prompting weaker rejection responses due to the decreased leukocytes in the tissue after cryopreservation. Additionally, ice-free cryopreservation instead of conventional frozen cryopreservation was considered for the preservation of allogeneic heart valves (Huber et al., 2012), and many scientists are studying the cryopreservation of germ cells and other cells, tissues, and organs. Cryopreservation, as confirmed by the evaluation of damage caused to the bull sperm cytoskeleton after thawing, alters perinuclear theca proteins, such actin and dystrobrevin, and also the nucleoskeleton (Felipe-Perez et al., 2012); such damage could cause fertilisation failure. Forero-Gonzalez et al. (Forero-Gonzalez et al., 2012) compared the effects of two freezing techniques (conventional and automated) and three cryoprotectants (glycerol, ethylene glycol, and dimethyl formamide) on post thaw sperm motility and the integrity of acrosomal, plasma, and mitochondrial membranes. Indeed, cryopreservation garners attention in various fields because of its wide applicability.

In the present study, we evaluated quality characteristics, such as motility, DNA integrity, membrane integrity, and acrosome integrity, to confirm the effect of AFP on the effects of cold shock during cryopreservation. Our results indicate the addition of AFP at a specific concentration (0.01 μg/m*l*) results in the highest motility and progressive motility after thawing, findings that support the idea that AFP can help to maintain cell viability during the freeze-thaw process (Jo et al., 2011(Prathalingam et al., 2006; Younis et al., 1998). Additionally, it is important to note that extenders, along with AFP, have been effective for the maintenance of germ cell viability (Martinez-Paramo et al., 2009; Palasz and Mapletoft, 1996). In addition to boar sperm (Inglis et al., 2006), it has been reported that somatic cells also benefit from the

decreased cold shock damage conferred by AFP (Beirao et al., 2012; Makarevich et al., 2010). The high motility of sperm cryopreserved with AFP was observed using a CASA after thawing, reflecting the cryoprotectant properties of AFP. However, excessive AFP concentrations decreased the motility and others parameters, such as the progressive motility, VAP, VSL, and VCL. Therefore, high concentrations of AFP may be detrimental to boar sperm motility and may be explained by decreased DNA integrity and membrane integrity. According to the comet assay results, the DNA integrity is maintained using AFP at 0.01 μg/m*l* but not at 1 μg/ml. The present study analysed several DNA integrity parameters, including the tail DNA (%), tail length (μm), and olive moment, to reveal damage to the sperm DNA. The DNA integrity of the sperm in extender with 0.01 μg/m*l* AFP was better than that of the sperm in extender with 1 μg/ml AFP. However, the DNA integrity of the sperm cryopreserved with 0.1 or 1 μg/m*l* AFP was not significantly different compared to the control sperm, a result that is in agreement with previous reports (Riesco et al., 2012). The plasma membrane integrity was also analysed with PI and SYBR14 staining to provide information about the effect of AFP. Partyka et al.; Partyka et al., 2010) made a similar observation using the same fluorescent dye for the assessment of fresh fowl semen. We observed that more boar sperm in the control group were alived when compared to the sperm cryopreserved with AFP. Thus, AFP at high concentration has a cytotoxic effect on boar spermatozoa after thawing. Our results related to acrosome integrity are in agreement with the study by Younis et al. (Younis et al., 1998) which indicated an increased proportion of chimpanzee sperm intact acrosomes post-thaw in the presence of AFPIII, suggesting that the AFPs helped to maintain the chimpanzee sperm acrosomal membranes by inhibiting capacitation and the subsequent acrosomal reaction. However, Rubinsky et al. (Rubinsky et al., 1990) suggested that AFPs might stabilize the transmembrane electrolyte gradients by blocking calcium ion channels, thereby partly inhibiting the capacitation reaction. Prathalingam et al. (Prathalingam et al., 2006) proposed that the reason for their discrepant results was because of the media and the different cooling curves. The results for bovine sperm showed no significant difference between the control sperm and sperm in extender with AFPs. Despite these experiments, the effects of AFPs on acrosome integrity during cryopreservation are unclear; however, our results showed that the acrosome integrity of sperm in extender with 1 μg/m*l* AFP was much better than the other treatment.

The addition of AFP in the cryopreservation of boar sperm acts as an effective cryoprotectant and might maintain the acrosome integrity of cryopreserved sperm. Although the addition of AFP causes a disruption of the plasma membrane, the concentration of 0.01 μg/m*l* AFP is optimal because it retains a high motility. However, our results of the acrosome integrity revealed the benefit of AFP (1 μg/m*l*). Thus, further studies will be necessary to confirm the effect of AFP on the fertility of cryopreserved boar sperm and to determine the reasons for the different acrosome integrity results between the present and previous studies.

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