Analysis of Membrane Integrity and Mitochondrial Activity in Fresh and Cryopreserved Boar Sperm Using Flow Cytometry

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ABSTRACT

This study was carried out to evaluate the effects of washing medium, breed and washing temperature of fresh and frozen-thawed boar sperm on mitochondrial activity and membrane integrity by flow cytometry. More than 80% of fresh sperm washed with mTLP-PVA medium at 20℃ exhibited an intact membrane and a functional mitochondrion. With frozen-thawed samples, a large number of sperm showed both damaged membrane (36.4~46.9%) and nonfunctional mitochondrion (55.1~71.1%) in the mTLP-PVA and BTS washing media at 20℃. There were no breed effects of fresh and frozen-thawed sperm on mitochondrial activity and membrane integrity. The percentages of damaged membrane of fresh and frozen sperm, respectively, were higher at 4℃ washing temperature than at 20℃ washing temperature in the mTLP-PVA medium. We found that washing medium and washing temperature of fresh and frozen-thawed boar sperm were important for the analyses of mitochondrial activity and membrane integrity by flow cytometry.

(Key words : Membrane integrity, Mitochondrial activity, Boar sperm, Flow cytometry)

INTRODUCTION

The development of flow cytometers has recently increased the potential application of flow cytometric techniques to semen analysis. Flow cytometry has been used to analyze sperm morphology (Pinkel at al., 1979) and to assess sperm cell viability (Matyus *et al.*, 1984; Garner *et al.*, 1986, 1988). The simultaneous analysis of cell viability and mitochondrial activity using flow cytometry has been reported and a correlation between mitochondrial fluorescence intensity and sperm motility established (Evenson *et al.*, 1982; Auger *et al.*, 1989).

Rhodamine 123 (Rh123) has been used to compare mitochondrial potential with sperm motility (Evenson *et al.*, 1982) and to concomitantly evaluated sperm mitochondrial activity by flow cytometry. Recently flow cytometric analysis was performed on fresh and cryopreserved pools of trout sperm after fluorescent staining with Rh123 to quantify mitochondrial function and propidium iodide (PI) to assess plasma membrane integrity (Ogier de Baulny *et al.*, 1997). The application of flow cytometric techniques for general and applied research in reproduction will allow identification of aspects of sperm movement and morphology which are predictive of sperm function and dysfunction. Although not useful for determining the function of fresh and frozen-thawed sperm, mitochondrial activity and membrane integrity analyses may be useful for preliminary screening of sperm in the laboratory.

The purpose of the this study was to evaluate the effects of washing medium, breed and washing temperature of fresh and frozen-thawed boar sperm on mitochondrial activity and membrane integrity by flow cytometry.

MATERIALS AND METHODS

Semen Collection

Semen was collected from August 2004 to July 2005 from adult boars 15~22 months of age. Boars were housed at pig farm of Chungnam National University in Daejeon. Semen was collected one time per week from each of six Duroc, Landrace and Large White boars.

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Determination of Semen Volume, Sperm Concentration, Motility and Acrosome

Semen volumes were determined with a graduated cylinder. Sperm concentrations were estimated by a hemocytometer. The percentage of motile sperm was estimated at 37℃ by light microscope at 250× (Pursel and Park, 1985). The acrosome morphology of 100 sperm per sample was evaluated by phase contrast microscopy at 1000×. Acrosomes were differentially categorized into four morphological classes: normal apical ridge (NAR), damaged apical ridge (DAR), missing apical ridge (MAR) and loose acrosomal cap (LAC) as described by Pursel *et al.* (1972). The sperm-rich fractions of ejaculates with >80% motile sperm and normal apical ridge (NAR) acrosome were used in the experiments.

Frozen Semen Processing

The sperm-rich fraction (30 to 60 mL) of ejaculate was collected into an insulated vacuum bottle. Semen was slowly cooled to room temperature (20 to 23℃) by 2 hours after collection. Semen was transferred into 15 mL tubes, centrifuged at room temperature for 10 min at 800 g and the supernatant solution was poured off. One volume of concentrated sperm was resuspended with 1 volume of lactose, egg yolk and N-acetyl-D-glucosamine (LEN) diluent (the first diluent to provide 1.0×10^9 sperm/ml) at room temperature (Yi *et al.*, 2002).

Semen was cooled in a refrigerator to 5℃ over a 2 hours period and 1 volume a LEN+4% glycerol diluent (the second diluent) was added to 1 volume of cooled semen. Straws (Minitub Gmbh, Landshut, Germany) ware immediately filled with 5 mL of semen and steel or glass balls were used to seal the ends of the straws. The air bubble was adjusted to the center of the straws and the straws were horizontally placed on an aluminum rack and set into a liquid nitrogen tank containing liquid nitrogen (LN). The straws were situated 5 cm above the LN, and kept at that level for 20 min before the straws were transferred into LN storage. Straws were thawed in 52℃ water bath for 45 S, which brings the temperature of the sperm to 15℃.

Rh123 and PI Staining

Ten or twenty μL of fresh semen and frozen-thawed semen to obtain a final concentration of 5×10^6 sperm/mL were used. Sperm samples were washed with 400 μL mTLP-PVA (Yoshida *et al.*, 1992) and BTS (Pursel and Johnson, 1975) media at 20℃ and 4℃ (1,500 rpm, 3 min), respectively, then incubated with 5 μg/mL of Rh 123 (Sigma, St. Louis, MD, USA). After 20 min, the samples were washed two times with 400 μL mTLP-PVA and BTS at 20℃ and 4℃ (1,500 rpm, 3 min), respectively, and then the samples were incubated with 5 μg/mL of PI (Sigma, Sigma, St. Louis, MD, USA) for 10 min before analysis.

Flow Cytometric Analysis

Sperm sample analysis was performed at a rate of 1,000 events per second in a sheath fluid using a FACStar Plus Analyzer (Becton-Dickinson, CA, USA) equipped with an argon laser operated at 488 nm with a power of 100 mW. Rh 123 fluorescence was measured through a 530 nm band pass filter. PI fluorescence was measured through a 630 nm longpass filter, and the two fluorescence emissions were separated by a 560 nm dichroic mirror. For each sample, 10,000 events were analyzed for the two fluorescence and 5,000 events for the PI population. Percentages of cells and mean values of fluorescence were calculated by windows defined on the bi-parametric analysis (Rh 123 vs. PI fluorescence) and on the mono-parametric analysis for the PI population.

Statistical Analysis

Analyses of variance (ANOVA) were carried out using the SAS package (SAS, 1996) in a completely randomized design, student's *t*-test was used to compare mean values of individual treatment, when the *F*-value was significant ($p<0.05$).

RESULTS

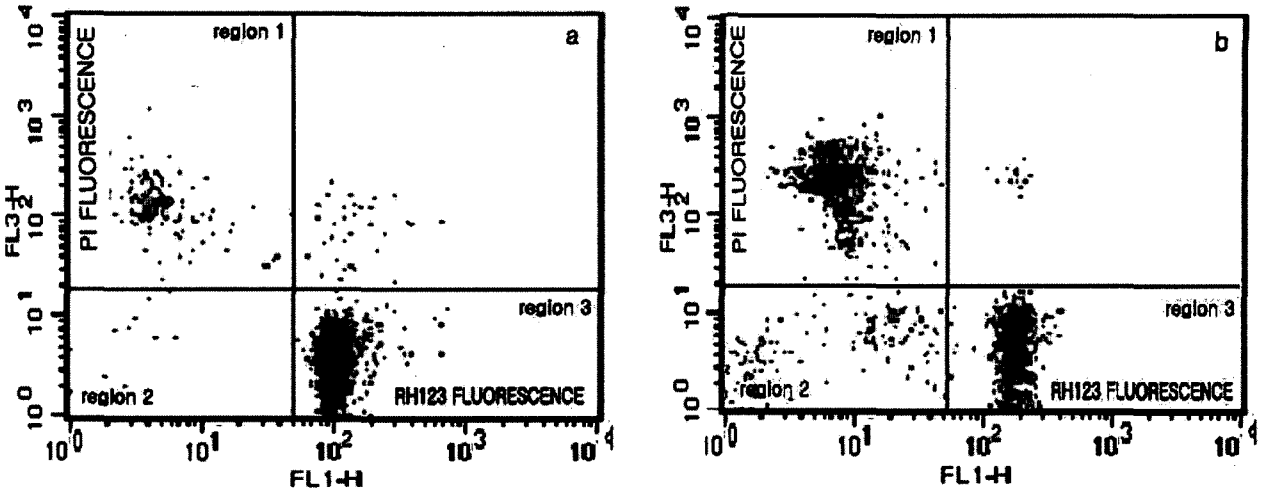
As shown in Fig. 1, the percentage of moribund or dead sperm, as identified by PI staining, was localized and quantified as region1 while the percentage of sperm with a functional membrane and mitochondrion, as identified by Rh 123 staining and no PI staining, was localized and measured as region 3. Sperm with an intact membrane but without mitochondrial activity were measured as region 2. Very few cells were found in region 4 and they were not taken into account.

More than 80% of fresh sperm washed with mTLP-PVA medium at 20℃ exhibited an intact membrane and a functional mitochondrion. With frozen-thawed samples, cells were localized in regions 1, 2 or 3, and a large number of sperm showed both damaged membrane and non-functional mitochondrion.

The effects of washing medium of fresh and frozen-thawed on mitochondrial activity and membrane integrity were shown in Table 1. The percentages of mitochondrial activity and intact membrane of fresh and frozen-thawed sperm, respectively, were higher in the mTLP-PVA medium than in the BTS medium.

As shown in Table 2, there were no breed effects of fresh and frozen-thawed sperm on mitochondrial activity and membrane integrity.

The effects of washing temperature of fresh and frozen-thawed sperm on mitochondrial activity and membrane integrity were shown in Table 3. The percentages of damaged membrane of fresh and frozen sperm, respec-



(a) (b)

Fig. 1. Flow cytometric dot plots of boar sperm analyzed for rhodamine 123 and propidium iodide fluorescence. Fresh and frozen-thawed sperm washed with mTLP-PVA medium at 20℃ are presented in the (a) and (b).

Table 1. Effects of washing medium of fresh and frozen-thawed sperm on mitochondrial activity and membrane integrity

Washing medium[1]	Fresh sperm[2]		Frozen-thawed sperm[2]	
	Rh 123 (%)[3]	PI (%)[3]	Rh 123 (%)[3]	PI (%)[3]
mTLP-PVA	81.6±1.8[b]	14.8±1.8[a]	44.9±2.6[b]	36.4±1.8[a]
BTS	73.1±2.2[a]	21.9±2.1[b]	28.9±1.8[a]	46.9±2.1[b]

[1] Washing of fresh and frozen-thawed sperm was done at 20℃.

[2] Means ± S.E. for six ejaculates from each of six Duroc, Landrace and Yorkshire boars.

[3] Rhodamine 123 (Rh 123) and propidium iodide (PI) are percentages of mitochondrial activity and damaged membrane, respectively.

[a, b] Means ± S.E. in the same column with different letters differ significantly ($p<0.05$).

Table 2. Effects of breed of fresh and frozen-thawed sperm on mitochondrial activity and membrane integrity[1]

Breed	Fresh sperm[2]		Frozen-thawed sperm[2]	
	Rh 123 (%)[3]	PI (%)[3]	Rh 123 (%)[3]	PI (%)[3]
Duroc	85.8±3.4	11.0±2.7	45.1±2.5	38.7±2.7
Landrace	78.2±3.3	17.9±3.9	48.3±3.6	32.3±2.4
Yorkshire	80.8±2.5	15.5±2.1	41.4±6.7	38.2±3.7

[1] Washing of fresh and frozen-thawed sperm was done at 20℃.

[2] Means ± S.E. for six ejaculates from each of six Duroc, Landrace and Yorkshire boars.

[3] Rhodamine 123 (Rh 123) and propidium iodide (PI) are percentages of mitochondrial activity and damaged membrane, respectively.

tively, were higher at 4℃ washing temperature than at 20℃ washing temperature. However, the percentages of mitochondrial activity were not exhibited at 4℃ washing temperature by flow cytometry.

DISCUSSION

Deep frozen boar sperm had poorer motility, acrosomal morphology and viability than fresh sperm (Clarke and Johnson, 1987; Almlid and Johnson, 1988; Hofmo and Almlid, 1991), and the accompanying poor farrowing rates (40~50%) and low litter size have made frozen boar semen impractical for the commercial swine producer (Johnson, 1985; Crabo and Dial, 1992).

Table 3. Effects of washing temperature of fresh and frozen-thawed sperm on mitochondrial activity and membrane integrity[1]

Washing temperature	Fresh sperm[2]		Frozen-thawed sperm[2]	
	Rh 123 (%)[3]	PI (%)[3]	Rh 123 (%)[3]	PI (%)[3]
20℃	81.6±1.8	14.8±1.8[a]	44.9±2.6	36.4±1.8[a]
4℃	-	68.9±3.4[b]	-	77.8±3.7[b]

[1] Washing of fresh and frozen-thawed sperm was done by mTLP-PVA medium.

[2] Means ± S.E. for six ejaculates from each of six Duroc, Landrace and Yorkshire boars.

[3] Rhodamine 123 (Rh 123) and propidium iodide (PI) are percentages of mitochondrial activity and damaged membrane, respectively.

[a, b] Means ± S.E. in the same column with different letters differ significantly ($p<0.05$).

Several procedures using frozen-thawed boar semen have been reported for *in vitro* fertilization of *in vitro* or *in vivo* matured porcine oocytes. However, the results have not been consistent sufficiently because of day to day variations between ejaculates, even under identical laboratory conditions. Yi *et al.* (2004) reported that the rate of blastocysts from the cleaved oocytes were higher in the mTLP-PVA treatment than in the unwashed, BTS and mTBM treatments. Kramer *et al.* (1993) reported that flow cytometric analyses were performed on 24 h extended and cryopreserved samples after fluorescent staining with Rh 123 to quantify mitochondrial function and PI to assess plasma membrane integrity. The percentages of sperm with functional mitochondria and intact membranes along with the proportion of dead cells were identified and quantified by flow cytometry.

This study showed that the percentages of mitochondrial activity and intact membrane of fresh and frozen-thawed sperm, respectively, were higher in the mTLP-PVA medium of porcine embryo culture than in the BTS medium of frozen-thawed boar sperm extender. From this result, we found out that washing medium before flow cytometric analysis at 20℃ was important for the mitochondrial activity and intact membrane of fresh and frozen-thawed sperm, respectively.

Also, we analyzed the breed effects of fresh and frozen-thawed sperm on mitochondrial activity and membrane integrity by mTLP-PVA medium at 20℃. However, we did not find out the breed effects of fresh and frozen-thawed sperm on mitochondrial activity and membrane integrity by flow cytometry.

Cellular injury resulting from rapid cooling to temperatures in the range of 0~10℃ or 15℃ is surprisingly widespread (Morris and Watson, 1984). Freshly ejaculated boar sperm in the whole ejaculate do not survive even slow cooling below 15℃, but they acquire resistance to cooling stress upon incubation. The organelles most frequently damaged by cold shock are the plasma membrane, the acrosome and the mitochondria. Membrane damage is progressive involving usually breakage or loss of the plasma membrane followed by damage to the acrosome, loss of its contents and finally shedding of the outer acrosomal membrane (Watson and Plummer, 1985). In this study, we found out the percentages of damaged membrane of fresh and frozen-thawed sperm after staining with PI, respectively, were higher at 4℃ washing temperature than at 20℃ washing temperature. However, the percentages of mitochondrial activity after staining with Rh 123 were not analyzed at 4℃ washing temperature. Further studies should be focused on the analysis of mitochondria at 4℃.

In conclusion, we found out that mitochondrial activity and membrane integrity of fresh and frozen-thawed boar sperm using flow cytometry could be analyzed by mTLP-PVA medium at 20℃.

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