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Effects of α-Linolenic Acid and Bovine Serum Albumin on Frozen-thawed Boar Sperm Quality during Cryopreservation

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ABSTRACT

This study was conducted to evaluate effect of α-linolenic acid (ALA) and bovine serum albumin (BSA) on viability, acrosome reaction and mitochondrial intact in frozen-thawed boar sperm. The boar semen was collected by gloved-hand method and cryopreserved using freezing extender containing 3 ng/mL ALA and/or 20 μg/mL BSA. Cryopreserved boar sperms were thawed in 37℃ water-bath for 45 sec to analysis. Viability, acrosome reaction, and mitochondrial intact were analyzed using flow cytometry. In results, viability of frozen-thawed boar sperm was significantly higher in only ALA+BSA supplement group than control group (p<0.05), whereas there was no difference either in ALA or BSA supplement. However, acrosome reacted sperm in both of live and all sperm population were significantly decreased in all treatment groups than control (p<0.05). Interestingly, mitochondrial intact of boar sperm was enhanced in ALA and ALA+BSA groups compared with control (p<0.05). In this study, we showed that supplementation of ALA and BSA in freezing extender enhanced the sperm viability, mitochondrial intact and decrease acrosomal membrane damage. In conclusion, our findings suggest that quality of frozen-thawed sperm in mammalians could improve by using of ALA and BSA.

(Key words : Alpha-linolenic acid, Bovine serum albumin, Cryopreservation, Sperm characteristics, Pigs)

INTRODUCTION

As an important technique for production of domestic animal, artificial insemination (AI) and *in vitro* fertilization (IVF) are used for improvement of animal breed, production of transgenic animal and it require the liquid- or cryo-preserved sperm (Roca *et al*., 2011). Generally, liquid preservation of sperm is used in AI for piglet production. However, these preservation method is difficult to long-term preservation because spermatozoa was exposed to various stress by metabolism (Silva and Gadella, 2006). These damage in spermatozoa during liquid preservation cause to reduce viability and fertility. On the contrary, metabolism of sperm was reduced or stopped during cryopreservation and it enable to long-term preservation (Yoshida, 2000). However, ice crystal formation during freezing and thawing process damaged to spermatozoa by redistribution of phospholipid and protein in membrane, change of plasma membrane structure and function such as permeability (Lessard *et al*., 2000). These cryo-stress induce the generation of reactive oxygen species (ROS) in sperm and functions of frozen-thawed sperm including motility, viability and fertility were reduced (Bailey *et al*., 2003; Grobfeld *et al*., 2008). For resolution of these problem of sperm cryopreservation, a variety of supplement such as antioxidants (Kaeoket *et al*., 2010; Giaretta *et al*., 2015) and cholesterol (Mocé *et al*., 2010) were used during cryopreservation process.

Polyunsaturated fatty acids (PUFAs), which are one of components of phospholipid in plasma membrane of sperm, play an important role in sperm motility, viability and membrane function for oocyte-sperm mem-

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brane fusion and regulation of membrane fluidity (Aksoy *et al.*, 2006). In human and cow, sperm motility was increased by dietary docosahexanoic acid and intake of fish oil that contain amount of omega-3 (n-3) fatty acid enhanced the motility and viability of pigs (Rooke *et al.*, 2001), cows (Khoshvaght *et al.*, 2016) and sheeps (Samadian *et al.*, 2010). As a one of n-3 PUFAs, α-linolenic acid (ALA) in plasma membrane regulate the membrane protein and provide energy to plasma membrane (Shevchenoko and Simons, 2010). These function of ALA maintained the function of plasma membrane and sperm viability. We expect that decreasing of sperm function by cryopreservation process could improve by ALA supplement.

Bovine serum albumin (BSA), which is most abundant in the plasma, has play roles in preventing of damage to sperm. It stabilize a membrane and protect the plasma membrane of sperm by regulating of membrane fluidity (Cheng *et al.*, 2015). Besides, this protein is known to eliminate the free radical through its free radical-trapping property (Roche *et al.*, 2008) and increase the antioxidant activity during freeze-thawing process of bull semen (Schafer and Holzmann, 2000). These function of BSA improve the post-thawed motility and viability of bull (Ashrafi *et al.*, 2013) and ram (Uysal and Bucak, 2007) sperm against heat shock during freeze-thawing process.

Mammalian sperm contain high concentration of PUFAs in plasma membrane and is sensitive to attack by ROS (Alvarez and Storey, 1995). Generation of ROS in spermatozoa induce lipid peroxidation (LPO) and produced lipid peroxides effuse PUFAs in plasma membrane via activation of phospholipase A2 (Wathes *et al.*, 2007). We expect that supplement of ALA and BSA to freezing extender could prevent cryo-damage such as ROS generation and effusion of PUFAs in plasma membrane of sperm. Therefore, this study was conducted to evaluate effect of ALA and BSA on viability, acrosome reaction and mitochondrial intact in frozen-thawed boar sperm.

MATERIALS AND METHODS

Semen Collection

All procedures that involved the use of animals were approved by the Kangwon National University Institutional Animal Care and Use Committee (KIACUC-09-0139). The ejaculate was collected by gloved-hand methods and diluted with modified Modena B (30.0 g/L glucose, 2.25 g/L EDTA, 2.50 g/L sodium citrate, 1.00 g/L sodium bicarbonate, 5.00g/L tris, 2.50 g/L citric acid, 0.05 g/L cysteine, and 0.30 g/L gentamicin sulfate).

Samples were transported to laboratory within 1 h and stored at 18℃ for 24 h. Ejaculated semen in this study was used with more than 80% viability.

Semen Freezing and Thawing Process

1st freezing extender, which mixed 11% α-lactose solution (Sigma, St. Louis, MO, USA) with 20% egg-yolk, was centrifuged for 30 min (3000 rpm, 4℃) and collected supernatant. 3 ng/mL ALA and/or 20 μg/mL BSA were added to 1st freezing extender (control: no addition; ALA: only ALA addition; BSA: only BSA addition; ALA+BSA: both of ALA and BSA addition) and boar sperm (1.5×10^9 spermatozoa/mL) were diluted with freezing extender. Subsequently, it were cooled down to 4℃ for 2 h and 2nd freezing extender that was 1st freezing extender containing 9% Glycerol (Sigma) and 1.5% (OEP; Nova Chem, USA) mixed with diluted sperm to 1×10^9 spermatozoas/mL concentration. Prepared sperm samples were loaded into 0.5 mL straw and pre-freezing to −120℃ for 10 min. finally, sperm samples were preserved in liquid nitrogen and frozen sperms were thawed in 37.5℃ water bath for 45 sec to analyze.

Analysis of Viability, Acrosome Reaction, and Mitochondrial Intact

Analysis of sperm characteristics were carried out using methods previously described (Lee *et al.*, 2016). Briefly, SYBR-14 (40 nM; Live/Dead sperm viability kit, Molecular probes, USA), Lectin from *Arachis hypogagea* (2 μM; FITC-PNA; Sigma), and Rhodamine123 (2 μM; Sigma) were added to frozen-thawed boar sperm (1×10^6 spermatozoas/mL) for viability, acrosome reaction, and mitochondrial intact, respectively. The sperm samples were incubated for 5 min at 38℃ in dark room and propidium iodide (2 μM; PI; Sigma) was stained for 5min in same condition. After stain, total 10,000 stained sperm were measured using flow cytometry (FACSCaliber, BD, USA) and measured data from flow cytometry were analyzed by CELLQuest version 6.0(Becton Dickinson).

Statistical Analysis

Date were analyzed using Statistical Analysis System software (SAS®, version 9.3). All obtained data were analyzed using general linear model (GLM) and treatment groups were compared for differences though use of Duncan's modified multiple range test ($p<0.05$).

RESULTS

The viability of frozen-thawed boar sperm was pre-

sented in Fig. 1. Sperm viability in ALA+BSA treatment group was significantly increased than control group (p<0.05). Both of ALA group and BSA group increased viability than control (57.83% and 57.6% vs 54.57%, respectively; p>0.05). Acrosome reacted boar sperm in both of all and live sperm population were significantly reduced by treatment of ALA and/or BSA than non-treatment group (Fig. 2; p<0.05). Mitochondrial intact level in all sperm population was no difference among the all group, however, ALA and ALA+BSA treatment in live sperm population enhanced mitochondrial intact compare with control group (Fig. 3; p<0.05).

DISCUSSION

This study was conducted to confirm effects of ALA and BSA in freezing extender on characteristics of frozen-thawed boar sperm. During freeze-thawing process, viability, motility and lipid composition of sperm were negatively influenced by physical and chemical damage such as ice crystal formation, change of temperature and osmotic pressure (Watson, 2000; Martinez-Soto *et al*., 2013). Because mammalian sperms contain amount of PUFAs in plasma membrane, it were sensitive to LPO by ROS and PUFAs in plasma membrane were effused by LPO (Alvarez and Storey, 1995; Wathes *et al*., 2007). These fatty acids were reduced and generation of ROS was increased during freeze-thawing process (Parks and Lynch, 1992; Kim *et al*., 2011). Kaka *et al*. (2015b) reported that concentration of ALA in bull spermatozoa was increased by supplement of ALA in freezing extender. Thus, these absorption of ALA and antioxidant property of BSA may enhance quality of frozen-thawed sperm via reducing effusion of PUFAs and ROS generation.

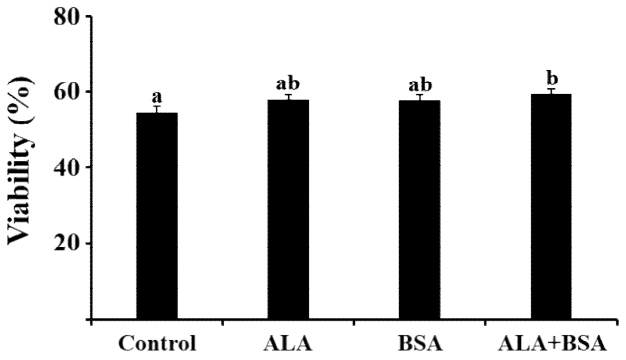

Fig. 1. Effect of α-linolenic acid (3 ng/mL; ALA) and/or bovine serum albumin (20 μg/mL; BSA) on viability of frozen-thawed boar sperm. [a,b] Values with different superscripts indicate significant difference within a live column (p<0.05).

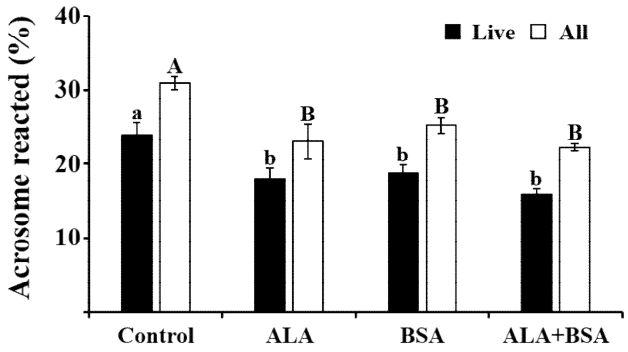

Fig. 2. Effect of α-linolenic acid (3 ng/mL; ALA) and/or bovine serum albumin (20 μg/mL; BSA) on acrosome reaction of frozen-thawed boar sperm. [a,b] Values with different superscripts indicate significant difference within a live column (p<0.05), [A,B] Values with different superscripts indicate significant difference within all column (p<0.05).

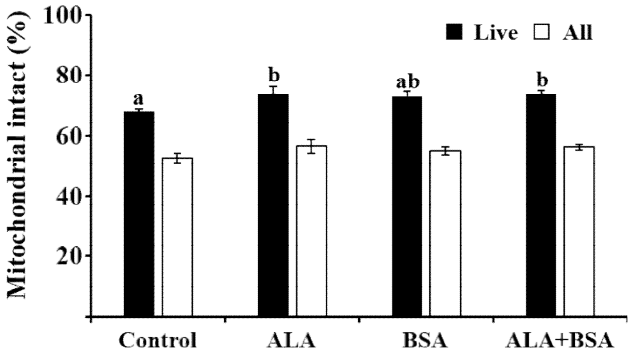

Fig. 3. Effect of α-linolenic acid (3 ng/mL; ALA) and/or bovine serum albumin (20 μg/mL; BSA) on mitochondrial intact of frozen-thawed boar sperm. [a,b] Values with different superscripts indicate significant difference within a live column (p<0.05).

The function of plasma membrane, which play important role in viability, motility and cell death in sperm, was inhibited by various stress during cryopreservation (Watson, 2000). For reducing of membrane damage, many researchers added a sugar (Bucak *et al*., 2007), antioxidants (Memon *et al*., 2011) and fatty acids (Kaka *et al*., 2015a) during cryopreservation process of sperm. Our previous study (2016) shown that supplement of 3 ng/mL ALA in freezing extender prevented the plasma membrane damage by ethanol in frozen-thawed boar sperm. Also, this present result show that both of ALA and BSA treatment in freezing extender enhance the sperm viability compared with non-treatment group (Fig. 1; p<0.05), however, treatment of ALA or BSA were not effect. ALA supplement during cryopreservation process of sperm induced LPO that caused membrane damage (Kaka *et al*., 2015a) and BSA

was known to effuse cholesterol of the plasma membrane in sperm, which induce the generation of ROS (Radomil *et al.*, 2011). Despite positive effect of ALA and BSA, treatment of only ALA or BSA may not enhanced to viability of frozen-thawed sperm by LPO and effusion of cholesterol. During liquid storage of boar sperm, supplement of 3~5 g/L BSA decreased LPO level at day 3 and day 7 (Zhang *et al.*, 2015). Thus, BSA treatment with ALA may increase sperm viability through preventing of ALA-induced LPO.

Acrosome of spermatozoa contain an enzyme including acrosin and hyaluronidase and acrosome reaction is essential phenomenon for fertilization. During cryopreservation process, acrosome reaction was induced by cryo-damage (Watson, 2000; O'Flaherty *et al.*, 2005) and acrosome reacted sperms have low fertility. Treatment of H_2O_2 and sodium nitroprusside increase acrosome reaction in boar spermatozoa (Kim *et al.*, 2009). In this study, acrosome reacted sperm in both of live and all sperm population were significantly decreased in ALA and/or BSA supplement group than control group (Fig. 2; $p<0.05$) and this result is similar to sperm viability. Kaka *et al* (2015b) reported that acrosome integrity in frozen-thawed bull sperm was enhanced by 5 ng/mL ALA supplement. Therefore, ALA could protect the acrosome membrane via preventing damage of plasma membrane and BSA decrease acrosome membrane damage by antioxidant ability.

Mitochondrial metabolism plays an important role for production of energy (Ramio-Lluch *et al.*, 2011) and mitochondrial function in spermatozoa is important for motility, viability and fertilization of sperm. In present study, mitochondrial intact was higher in ALA and ALA+BSA treatment groups than control (Fig. 3; $p<0.05$). During energy production, ROS such as superoxide ($O_2\cdot$), hydrogen peroxide (H_2O_2), or hydroxyl radical ($OH\cdot$) and it damaged to mitochondrial DNA (Kumar *et al.*, 2009). ALA and BSA treatment may reduce the mitochondrial damages by ROS through reduction of damage in plasma membrane. In this study, we showed that supplementation of ALA and BSA in freezing extender enhanced the sperm viability, mitochondrial intact and decrease acrosomal membrane damage. In conclusion, our findings suggest that quality of frozen-thawed sperm in mammalians could improve by using of ALA and BSA.

REFERENCES

1. Aksoy Y, Aksoy H, Altinkayanak K, Aydin HR, Ozkan A (2006): Sperm fatty acid composition in subfertile men. Prostaglandins Leukot Essent Fatty Acids 75:75-79.
2. Alvarez JG, Storey BT (1995): Differential incorporation of fatty acids into and peroxidative loss of fatty acids from phospholipids of human spermatozoa. Mol Reprod Dev 42:334-336.
3. Ashrafi I, Kohram H, Nasrabadi HT (2013): Antioxidant effects of bovine serum albumin on kinetics, microscopic and oxidative characters of cryopreserved bull spermatozoa. Span J Agric Res 11:695-701.
4. Bailey JI, Morrie A, Cormier N (2003): Semen cryopreservation: success and persistent problems in farm species. Can J Anim Sci 83(3):393-401.
5. Bucak MN, Atessahin A, Varisli O, Yuce A, Tekin N, Akcay A (2007): The influence of trehalose, taurine, cystramine and hyaluronan on ram semen: microscopic and oxidative stress parameters afer freeze-thawing process. Theriogenology 67(5):1060-1067.
6. Cheng S, Sheng Z, Zhu JQ, Wu XF (2015): Effects of trehalose, bovine serum albumin, and sucrose on the integrity of the plasma membrane of *Pseudosciaena crocea* semen after cryopreservation. Isr J Aquacult Bamid 67.
7. Giaretta E, Estrada E, Bucci D, Spinaci M, Rodríguez-Gil JE, Yeste M (2015): Combining reduced glutathione and ascorbic acid has supplementary beneficial effects on boar sperm cryotolerance. Theriogenology 83:399-407.
8. Grobfeld R, Sieg B, Struckmann C, Frenzel A, Maxwell WMC, Rath D (2008): New aspects of boar semen freezing strategies. Theriogenology 70:1225-1233.
9. Kaeoket K, Chanapiwat P, Tummaruk P, Techakumphu M (2010): Supplemental effect of varying L-cysteine concentrations on the quality of cryopreserved boar semen. Asian J Androl 12:760-765.
10. Kaka A, Wahid H, Rosnina Y, Yimer N, Khumran AM, Behan AA, Ebrahimi M (2015a): Alpha-linolenic acid supplementation in tris extender can improve frozen-thawed bull semen quality. Reprod Dom Anim 50:29-33.
11. Kaka A, Wahid H, Rosnina Y, Yimer N, Khumran AM, Sarsaifi K, Behan AA, Kaka U, Ebrahimi M (2015b): α-Linolenic acid supplementation in Bio-Xcell extender can improve the quality of post-cooling and frezen-thawed bovine sperm. Anim Reprod Sci 153:1-7.
12. Khoshvaght A, Towhidi A, Zare-shahneh A, Noruozi M, Zhandi M, Davachi ND, Karimi R (2016): Dietary n-3 PUFAs improve fresh and post-thaw semen quality in Holstein bulls via alteration of sperm fatty acid composition. Theriogenology 85:807-812.
13. Kim S, Lee YJ, Kim YJ (2011): Changes in sperm membrane and ROS following cryopreservation of liquid boar semen stored at 15℃. Anim Reprod Sci

124:118-124.
14. Kim TS, Sa SJ, Shin MY, Jang DM, Kwon SH, Kwon EH, Cho KH, Park CK, Lee DS (2009): Stimulation of plasminogen activator activity by free radicals in boar spermatozoa. Anim Reprod Sci 114(1):228-237.
15. Kumar R, Venkatesh S, Kumar M, Tanwar M, Shasmsi MB, Kumar R, Gupta NP, Sharma RK, Talwar P, Dada R (2009): Oxidative stress and sperm mitochondrial DNA mutation in idiopathic oligoasthenozoospermic men. Indian J Biochem Biophys 46(2): 172-177.
16. Lee WH, Hwangbo Y, Lee SH, Tang JW, Kim HY, Lee YR, Park JE, Cheong HT, Yang BK, Park CK (2016): Effects of α-linolenic acid in frozen-thawed boar spermatozoa. Reprod Dev Biol 40(3):27-31.
17. Lessard C, Parent S, Leclerc P, Bailey JI, Sullivan R (2000): Cryopreservation alters the levels of the bull sperm surface protein P25b. J Androl 21:700-707.
18. Martinez-Soto JC, Landeras J, Gadea J (2013): Spermatozoa and seminal plasma fatty acids as predictors of cryopreservation success. Andrology 1:365-375.
19. Memon AA, Wahid H, Rosnina Y, Goh YM, Ebrahimi M, Nadia FM, Andrey G (2011): Effect of butylated hydroxytoluene on cryopreservation of boer goat semen in Tris egg yolk extender. Anim Reprod Sci 129:44-49.
20. Mocé E, Blanch E, Tomás C, Graham JK (2010): Use of cholesterol in sperm cryopreservation: present moment and perspectives to future. Reprod Dom Anim 45(Suppl. 2):57-66.
21. O'Flaherty C, Breininger E, Beorlegui N, Beconi MT (2005): Acrosome reaction in bovine spermatozoa: Role of reactive oxygen species and lactate dehydrogenase C4. Biochimica et Biophysica Acta (BBA)-General subjects 1726(1):96-101.
22. Parks JE, Lynch DV (1992): Lipid composition and thermotropic phase behavior of boar, bull, stallion, and rooster sperm membranes. Cryobiology 29(2):255-266.
23. Radomil L, Pettitt MJ, Merkies KM, Hickey KD, Buhr MM (2011): Stress and dietary factors modify boar sperm for processing. Reprod Domest Anim 46(Suppl. 2):39-44.
24. Ramio-Lluch L, Fernandez-Novell JM, Pena A, Colas C, Cebrian_perez JA, Muino-Blanco T, Ramirez A, Concha II, Rigau T, Rodriguez-Gil JE (2011): '*In vitro*' capacitation and acrosome reaction are concomitant with specific changes in mitochondrial activity in boar sperm: evidence for a nucleated mitochondrial activation and for the existence of a capacitation-sensitive subpopulational structure. Reprod Domest Anim 46:664-673.
25. Roca J, Parrilla I, Rodriguez-Martinez H, Gil MA, Cuello C, Vazquez JM, Martinez EA (2011): Approaches towards efficient use of boar semen in the pig industry. Reprod Domest Anim 46:79-83.
26. Roche M, Rondeau P, Singh NR, Tarnus E, Bourdon E (2008): The antioxidant properties of serum albumin. F.E.B.S.. Letters 582:1783-1787.
27. Rooke JA, Shao CC, Speake BK (2001): Effects of feeding tuna oil on the lipid composition of pig spermatozoa and *in vitro* characteristics of semen. Reproduction 121:315-322.
28. Samadian F, Towhidi A, Rezayazdi K, Bahreini M (2010): Effects of dietary n-3 fatty acids on characteristics and lipid composition of ovine sperm. Animal 4:2017-2022.
29. Schafer S, Holzmann A (2000): The ise of transmigration and spermac stain to evaluate epididymal cat spermatozoa. Anim Reprod Sci 59:201-211.
30. Shevchenko A, Simons K (2010): Lipidomics: coming to grip with lipid diversity. Nat Rev Mol Cell Biol 11(8):593-598.
31. Silva PFN, Gadella BM (2006): Detection of damage in mammalian sperm cells. Theriogenology 65(5): 958-978.
32. Uysal O, Bucak MN (2007): Effects of oxidized glutathione, bovine serum albumin, cysteine and lycopene in quality of frozen-thawed ram semen. Acta Vet Brno 76:383-390.
33. Wathes DC, Abayasekara DRE, Aitken RJ (2007): Polyunsaturated fatty acids in male and female reproduction. Biol Reprod 77:190-201.
34. Watson PF (2000): The causes of reduced fertility with cryopreserved semen. Anim Reprod Sci 60-61: 481-492.
35. Yoshida M (2000): Conservation of sperms: current status and new trends. Anim Reprod Sci 60-61:349-355.
36. Zhang XG, Yan GJ, Hong JY, Su ZZ, Yang GS, Li QW, Hu JH (2015): Effects of bovine serum albumin on boar sperm quality during liquid storage at 17℃. Reprod Domest Anim 50:263-269.

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