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Effects of L-Carnitine and Nicotinic Acid on Sperm Characteristics in Miniature Pigs

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ABSTRACT

This study investigated the effects of L-carnitine (LC) and nicotinic acid (NA) on sperm viability during liquid storage at 18℃ in miniature pigs. 10 μM LC and 30 mM NA, combined LC and NA (LN) were treated in fresh semen for 3, 7, and 10 days. In results, sperm survival increased in NA- and LN-treated semen on 7 and 10 days (p<0.05), mitochondrial integrity of live sperm increased in LN-treated semen on 7 days (p<0.05), but not NA-treated semen. In addition, we examined the acrosome reaction of sperm in miniature pigs. LC and NA did not influence on acrosome reaction of boar sperm. In conclusion, LC and NA effectively maintained the viability and quality of sperm during long-term storage in miniature pigs, suggesting that the combined LN may be useful for improving the semen extender for long-term liquid storage in pigs.

(Key words : L-carnitine, Nicotinic acid, Sperm viability, Miniature pigs)

INTRODUCTION

In fertilization, sperm provides genetic material to oocyte, and this genetic material was sufficient to affect the quality of embryos. In previous study, sperm quality was improved on long-term storage of semen (Fang *et al*., 2015) and the modified medium (Dias *et al*., 2014), motility improvement (Aitken *et al*., 2012), and inhibition of DNA damage (Lewis *et al*., 2013).

The storage method of semen is divided cryopreservation and lipid semen storage. First, cryopreservation has advantage for preserving long-term of semen, easy transport and quality proven of semen (Silva *et al*., 2015). However, freezing semen has many problems and damages, such as viability, acrosome, short live time, cold shock, and membrane damage (Anzar *et al*., 2002; Roca *et al*., 2004; Roca *et al*., 2006). Especially, boar semen is weaker than other domestic animal in the durability (Casas *et al*., 2009). In lipid semen storage, semen cannot keep on a long-term. Semen must be used within 5 days, but damages of viability and motility were increased, and DNA damage of sperm is also increased. Thus, the storage of lipid semen storage is very important for protecting the viability and motility of sperm.

Antioxidants have been used as additives to protect sperm damaged by reactive oxygen species (ROS) in diluted solution, cryo-buffer and thawing medium (Chi *et al*., 2008; Michael *et al*., 2007). Antioxidant contains vitamin families and chemical substances. L-carnitine and nicotinic acid includes antioxidant as vitamin B family, and play a role of mitochondria improvement. L-carnitine improves the efficiency of mitochondria, and help the synthesis of coenzyme A (Liu *et al*., 2002). The nicotinic acid is a precursor of the coenzymes NAD and NADP. NAD is participated in beta oxidation, glycolysis and the citric acid cycle on mitochondria mechanism (Stein and Imai, 2012). And, NADP provides regeneration of glutathione and reduction of oxidation from ROS (Rush *et al*., 1985). Also, previous studies reported that L-carnitine (Lee *et al*., 2014a) and nicotinic acid (Lee *et al*., 2014b) improve sperm characteristics in pigs. Therefore, the objective of this study was to investigate the effect of combination of L-carnitine and nicotinic acid on sperm characteristics during

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long-term storage of fresh semen in miniature pigs.

MATERIALS AND METHODS

Boar semen was obtained from miniature pig (PWG ×PWG) at a Kangwon National University farm (Republic of Korea). All animal experiments followed protocols, scientific and ethical regulations proposed by the European Animal Experiment Handling License Textbook (Baumans *et al*., 1997) and board approval (No: KIACUC-09-0139) was attained from the Animal Experiment Ethics Committee in Kangwon National University, Republic of Korea.

Semen Collection

For all experiments, fresh semen was collected using a gloved-hand method from three miniature pigs housed at a Kangwon National University farm (Republic of Korea) and age of the experimental three boars is 24.8±3.8 months. Collected semen was diluted to 1×10^7 spermatozoa/mL using Modena B treated 10 μM L-carnitine (LC), 30 mM nicotinic acid (NC) and LC+NC (LN). Diluted semen stored at 18℃ refrigerator for 10 days, and analyzed at 0 (Fresh), 3, 7 and 10 days after diluted with semen extender. Modena B untreated LC and NC was taken as control.

Sperm Viability

The viability of sperm was analyzed by fluorescent staining using the LIVE/DEAD sperm Viability Kit (Molecular Probes, Eugen, USA). Live sperm was stained as green fluorescent by SYBR-14, and membrane-compromised sperm was stained as red fluorescent by PI. Each semen was diluted to 1×10^8 spermatozoa/mL in 1 mL Modena B and 1 μL SYBR-14 (40 nM, v/v), and incubated at 37℃ for 5 min. Then, the sample was incubated with 1 μL PI (2 μM, v/v) for 10 min. Stained samples were centrifuged at 400 g for 5min, and the pellets were re-suspended with 500 μL PBS. The stained sperm was analyzed by flow cytometry (FACs).

Acrosome Integrity of Sperm

The acrosome integrity of sperm was analyzed by fluorescent staining using the FITC-PNA (SIGMA) and PI. Activated acrosome was stained as green fluorescent by FITC-PNA and membrane-compromised sperm was stained as red fluorescent by PI. Each semen was diluted to 1×10^8 spermatozoa/mL in 1 mL Modena B, and added 1 μL FITC-PNA (2 ng/mL, w/v). After incubation for 5 min at 37℃, 1 μL PI (2 μM, v/v) was added and incubated for 10 min. The samples were centrifuged at 400 g for 5 min, and the pellets were re-suspended with 500 μL PBS. The sperm was analyzed by FACs.

Mitochondria Integrity

The mitochondria integrity of sperm was analyzed by fluorescent staining using the Rhodamine 123 (Steinheim, SIGMA) and PI. The activated mitochondria stained as green fluorescent by Rhodamine 123 and membrane-compromised sperm was stained as red fluorescent by PI. Each semen was diluted to 1×10^8 spermatozoa/mL in 1 mL Modena B, 1 μL Rhodamine 123 (530 mM, v/v) was added into the samples, and incubated for 5 min at 37℃. After incubation, the samples incubated with 1 μL PI (2 μM, v/v) for 10 min at 37℃. Stained samples were centrifuged at 400 g for 5 min, and the pellets were re-suspended with 500 μL PBS. The stained sperm was analyzed by flow cytometry (FACs).

Statistical Analysis

Statistical analysis was performed with analysis of variance (ANOVA) using SAS version 9.3 (SAS Institute). All data are presented as mean±standard error of the mean (S.E.M) and Duncan's multiple range tests.

RESULTS

Effects of L-carnitine and nicotinic acid on sperm viability, acrosome reaction and mitochondrial integrity during *in vitro* storage of fresh semen in miniature pigs

Sperm viability was not significantly difference among treatment groups until 3 days of storage. But, the viability of sperm was significantly ($p<0.05$) higher in the NA (day 7, 87.6±2.9%; day 10, 84.5±2.3%) and LN (day 7, 86.2±2.3%; day 10, 89.0±0.3%) at days 7 and 10, however, there were no significantly in viability between NA and LN groups.

In addition, percentage of live with acrosome reaction (Fig. 2A) and all with acrosome reaction (Fig. 2B) sperm were not significantly difference among the treatment groups until 10 days of storage.

Rate of live sperm with mitochondrial integrity was significantly ($p<0.05$) higher in LN than Cont at 7 day of storage, but there were no significantly difference in mitochondrial integrity among the treatment groups at 3 and 10 days (Fig. 3A). However, all with mitochondrial integrity was no significantly difference among the treatments until 10 days of storage (Fig. 3B).

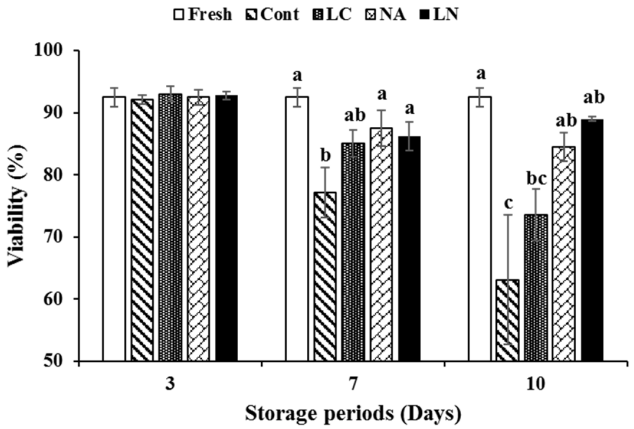

Fig. 1. Effect of L-carnitine and/or nicotinic acid on viability rate during *in vitro* storage in the semen of miniature pigs. [a~c] Values in the same columnf with different superscripts are significantly different($p<0.05$). Fresh: semen of storage 0 day, Cont: control, LC: 10 μM L-carnitine, NA: 30 mM Nicotinic acid, LN: LC+NA (LN), n=3.

DISCUSSION

Ejaculated boar sperm is diluted with semen extender to use artificial insemination (AI) for many sows. Semen extender accomplishes basic functions such as providing nutrients for sperm metabolism, stabilizing membranes, preventing of capacitation, neutralizing metabolic waste products, maintaining osmotic equilibrium, and retarding bacterial growth for successful AI during liquid preservation (Johnson *et al*., 2000). In this study, we investigated effect of L-carnitine and/or nicotinic acid on sperm characteristics during liquid storage in miniature pig.

The viability is one of important factor for sperm fertility, however excessive ROS by sperm metabolism during liquid storage term can damage sperm membranes and organelles, resulting in a low conception rate (Johnson *et al*., 2000). In this study, viability of LN -treated sperm was increased compared to control group, and the results suggest that combination of LC and NA improve extension of preservation time of boar semen. The optimal level of lipid peroxidation and antioxidant enzymes maintain sperm morphology, function, and fertility (Partyka *et al*., 2012). In addition, sperm viability can maintain a higher by antioxidants such as Vitamin E (Pena *et al*., 2003), glutathione (Funahashi and Sano, 2005), cysteine (Thuwanut *et al*., 2008), taurine (Michael *et al*., 2009) and selenium (Contri *et al*., 2011). Thus, antioxidant influence of combination of LC and NA may be improved boar sperm characteristics during long-term liquid preservation.

Generally, acrosome reaction is important physiological phenomenon for capacitation which is induced by ROS in mammal (Lamirande *et al*., 1998). In this study, LC, NA and LN did not influence acrosome reaction. Thus, combination of LC and NA may be not influenced acrosome reaction of boar sperm during long-term storage.

Sperm motility source is energy from mitochondria (Cardullo and Baltz, 1991), and damage of mitochondria in sperm is one of infertility (Piomboni *et al*., 2012). The oxidative compound of semen extender is generated by sperm metabolic activity during long-term liquid preservation that can damage mitochondrial integrity in pigs (Lee *et al*., 2015). Thus, stable sperm mi-

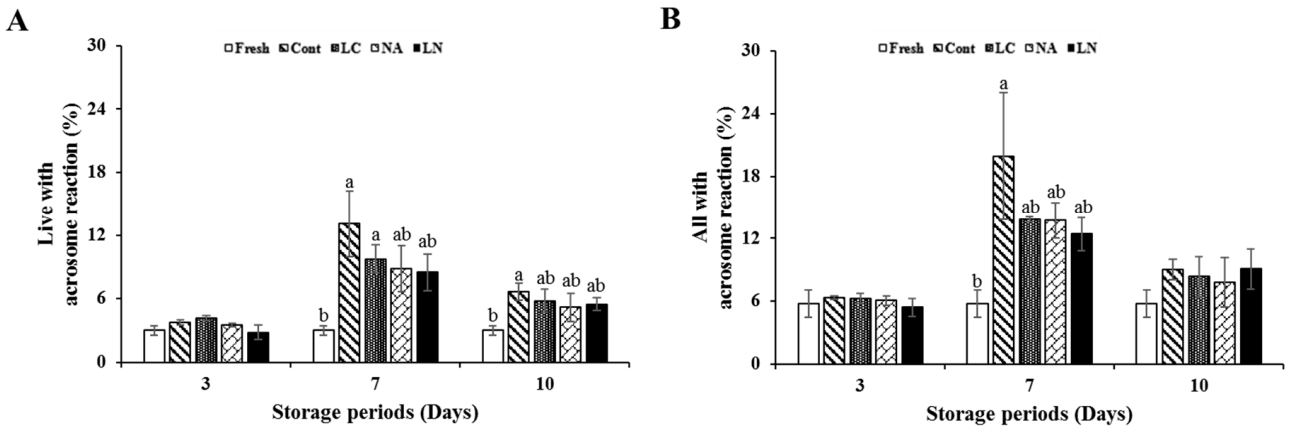

Fig. 2. Effect of L-carnitine and/or nicotinic acid on acrosome reaction rate during *in vitro* storage in the semen of miniature pigs. Rate of live sperm with acrosome reaction (A) and all sperm with acrosome reaction (B) during storage under 18℃, [a,b] Values in the same column with different superscripts are significantly different ($p<0.05$). Fresh: semen of storage 0 day, Cont: C, LC: 10 μM L-carnitine, NA: 30 mM Nicotinic acid, LN: LC+NA (LN), n=3.

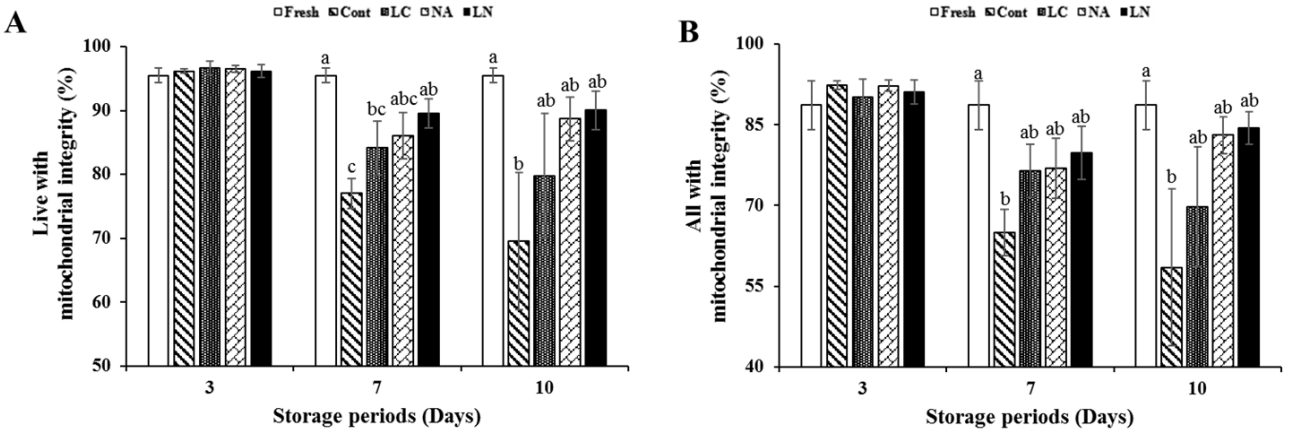

Fig. 3. Effect of L-carnitine and/or nicotinic acid on mitochondria integrity rate during *in vitro* storage in the semen of miniature pigs. Rate of live sperm with mitochondrial integrity (A) and all sperm with mitochondrial integrity (B) during storage under 18℃, $^{a\sim c}$ Values in the same column with different superscripts are significantly different(p<0.05). Fresh: semen of storage 0 day, Cont: C, LC: 10 μM L-carnitine, NA: 30 mM Nicotinic acid, LN: LC+NA (LN), n=3.

tochondrial integrity during long-term preservation is important for successful AI in pigs. Antioxidant can inhibit the oxidative compound and eliminate free radical in mammal cell, and improve motility and characteristics (Bansal and Bilaspuri, 2010; Spinaci *et al*., 2005; Yue *et al*., 2010). In this study combined LC and NA-treated semen extender may have antioxidant ability which influence mitochondrial integrity until 7 days for liquid storage in pigs.

The combined LC and NA have a positive effect to sperm viability and mitochondrial integrity during long-term liquid storage in pigs. In conclusion, LN-added semen extender is useful for long-term liquid preservation of boar semen.

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