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Effects of L-Carnitine during the Storage of Fresh Semen in Miniature Pigs

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ABSTRACT

L-Carnitine is an antioxidant for the transport of fatty acids in mitochondria and breakdown of lipids for metabolic energy. Some studies have suggested that carnitine improves sperm motility in mammals. The objective of this study was to investigate the effect of L-carnitine on the characteristics in fresh semen of miniature pigs. The collected fresh semen was stored in modena B medium with L-carnitine (0, 1.0, 2.0, and 4.0 mg/ml) for 10 days at 18℃. The semen quality of viability, acrosome reaction and mitochondria integrity was analyzed on 0, 3, 7, and 10 day of semen storage. The percentages of live and dying sperm were not different among treatment groups with different concentrations of L-carnitine during the storage period. In acrosome reaction analysis, when the sperm stored for 7 day, the percentages of live sperm with acrosome reaction were significantly (p<0.05) lower in 1 (9.0±0.9%), 2 (7.6±0.2%) or 4 mg/ml (7.9±0.8%) L-carnitine-treated groups than the control group (0 mg/ml L-carnitine) (11.12±0.2%). However, there were no difference in percentages of live sperm with acrosome reaction for 3 and 10 days of storage with each concentrations of L-carnitine. When sperm was stored for 3 and 10 days, the percentages of live sperm with mitochondria integrity were significantly higher in 2 mg/ml of L-carnitine-treated group than control group (p<0.05). In conclusion, the L-carnitine has a positive effect on acrosome reaction and mitochondria integrity in liquid state of fresh semen in miniature pigs.

(Key words: L-Carnitine, Miniature pig, Fresh semen, Sperm viability, Acrosome reaction)

INTRODUCTION

Artificial insemination (AI) requires for maintain population and improve breeding in pig. In South Korea, AI is progress and bigger, and AI centers reported that a use of pig semen (freezing and fresh semen) has increased (Kim *et al*., 2011; Colenbrander *et al*., 1992). Cryopreservation has advantages for preserving long term of semen, easy transport and quality proven of semen (Guimaraes *et al*., 2013). However, freezing semen has many problems and damages, such as viability, acrosome, short live time, cold shock, and membrane damage (Roca *et al*., 2006; Anzar *et al*., 2002; Roca *et al*., 2004). Especially, boar semen is weaker than other domestic animal in the durability (Casas *et al*., 2009). Thus, fresh semen has used to AI for protecting semen quality. Actually, a using of fresh semen at farm is a convenient and popular way, and the semen can store for 5 day, the viability and motility of sperm is decreased during this term (Waberski *et al*., 1994). Therefore, the diluted semen solution is very important for protecting the viability and motility of sperm.

L-carnitine is an antioxidant as stereoisomers of carnitine that is known vitamin B_T, and was originally found as a growth factor for mealworms (Bremer, 1983). The function of L-carnitine is the transport of fatty acids in mitochondria and breakdown of lipids for metabolic energy. Thus, this function is helped metabolism of mitochondria and increased mitochondrial functions (Siliprandi *et al*., 1989).

L-carnitine has been studied in oocyte (Wu *et al*., 2011), sperm (Khademi *et al*., 2012), embryo (You *et al*., 2012), disease (Malaguarnera *et al*., 2011), and metabo-

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lism (Koeth *et al*., 2013). Especially, some study reported that carnitine improves sperm motility (Morgante *et al*., 2010). Also, carnitine helps to the acquisition of sperm motility (Casillas, 1973) and modulates metabolic function (Jeulin and Lewin, 1996) of sperm included to β-oxidation and coenzyme A (Stradaioli *et al*., 2004). Thus, the study of L-carnitine in sperm has been reported in human (Khademi *et al*., 2012), rabbit (Sarıozkan *et al*., 2014), rat (Dehghani *et al*., 2013) and mouse (Moawad *et al*., 2013). However, the diluted solution with L-carnitine for semen storage is not studying in pig. Therefore, the objective of this study was to investigate the effect of L-carnitine on the characteristics in fresh semen storage of miniature pig.

MATERIALS AND METHODS

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, semen of total 3 miniature pigs at the Kangwon National University farm (South Korea) was collected using a gloved-hand method and transported. The Modena B medium used for semen collection and semen was diluted to 1×10^7 spermatozoa/ml. Modena B used for semen conservative solution was separated into 0, 1.0, 2.0, and 4.0 mg/ml of L-carnitine concentration. The diluted semen was stored at 18℃ refrigerator until use. Semen analysis was repeated 3 times.

Sperm Characteristics

Sperm quality was determined by viability, acrosome integrity and mitochondria integrity in spermatozoa. The viability, acrosome and mitochondria integrity were analyzed by flow cytometry. And, flow cytometry methods and assessment of semen were processed using the Lee method (Lee *et al*., 2014).

Sperm Viability

The viability of spermatozoa was analyzed by fluorescent staining using the LIVE/DEAD sperm Viability Kit (Molecular Probes, Eugen, OR, USA). After each semen was diluted with 1×10^8 spermatozoa/ml in Modena B of 1 ml, 1 μl of SYBR-14 (40 nM) labeled live sperm was used at diluted semen for 5 min at 37℃ and 1 μl of propidium iodide (PI, 2 μM) labeled membrane-compromised sperm was added to each diluted semen included SYBR-14 for 10 min at 37℃. Then, stained sperm was centrifuged at 1,500 rpm for 5 min. The pellets were resuspended in 500 μl of PBS, and stained sperm was analyzed by flow cytometry.

Acrosome Integrity

The acrosome integrity of spermatozoa was analyzed by fluorescent staining using the isothiocyanate-conjugated peanut agglutinin (FITC-PNA, Sigma, Saint Louis, Missouri, USA) and PI. After each semen was diluted with 1×10^8 spermatozoa/ml in Modena B of 1 ml, 1 μl of FITC-PNA labeled at damaged acrosome was used at diluted semen for 5 min at 37℃ and 1 μl of PI (2 μM) was added to each diluted semen included FITC-PNA for 10 min at 37℃. Then, stained sperm was centrifuged at 1,500 rpm for 5 min and the pellets were resuspended in 500 μl of PBS. Stained sperm was analyzed by flow cytometry.

Mitochondria Integrity

The mitochondria integrity of spermatozoa was analyzed by fluorescent staining using the Rhodamine 123 (Sigma, steinheim, Germany) and PI. After each semen was diluted with 1×10^8 spermatozoa/ml in Modena B of 1 ml, 1 μl of rhodamine 123 (530 mM) labeled at mitochondria was used at diluted semen for 5 min at 37℃ and 1 μl of PI (2 μM) was added to each diluted semen included rhodamine 123 for 10 min at 37℃. And, the stained sperms were centrifuged at 1,500 rpm for 5 min. Then, after the pellets were re-suspended in 500 μl of PBS, stained sperm was analyzed by flow cytometry.

Flow Cytometry Analysis

Flow cytometry analyses were performed using a FACS canto II flow cytometer (BD FACSCanto™ II). A total of 10,000 gated events were collected per treatment groups. Fluorescence values of SYBR-14, FITC-PNA and Rhodamine 123 were measured by Forward scatter (FSC), side scatter (SSC), green fluorescence (FL1), and fed fluorescence (FL2).

Statistical Analysis

Statistical analysis was performed with analysis of variance (ANOVA) using SAS version 9.1 (SAS Institute Inc., Cary, NC, USA). Data are presented as mean±standard error of the mean (S.E.M) and Duncan's multiple range tests.

RESULTS

Table 1 shows effect of L-carnitine in viable and dying sperm during different storage periods in fresh semen of miniature pigs. The percentages of live and dying sperm were not different among the various concentrations of L-carnitine during storage with periods prolonged.

As shown in Table 2, the effect of L-carnitine on acrosome reaction was examined in semen of miniature pigs. When the sperm were stored for 7 day, the percentages of live sperm with acrosome reaction significantly were (p<0.05) higher in 0 mg/ml (11.12±0.2%) than in 1 (9.0±0.9%), 2 (7.6±0.2%) and 4 mg/ml (7.9±0.8 %) treatment groups of L-carnitine. However, there were not different at 3 and 10 days of storage with different concentrations of L-carnitine. There were also striking differences in the rates of all sperm with acrosome reaction among the various concentrations of L-carnitine during the storage of fresh semen in miniature pigs.

In Table 3, when sperm was stored for 3 day, the percentages of live sperm with mitochondria significantly were higher in 2 mg/ml of L-carnitine than in control group (p<0.05). There were higher in groups added with L-carnitine during the storage for 10 day, significantly (p<0.05). On the other hand, when sperm was storage for 3 and 10 days, the percentages of all sperms with mitochondria were higher in 2 mg/ml of L-carnitine than in other groups, significantly (p<0.05).

DISCUSSION

Flow cytometry (FACs) was used to measure in cell (Aghaeepour *et al*., 2012; Walter *et al*., 2011) and sperm (Hossain *et al*., 2011; Escoffier *et al*., 2012) by principle that is reading by laser to suspending cells in a stream of fluid labeled fluorescent (Martinez-Pastor *et al*., 2010). Also, if FACs is using in experiments, the cells can be analyzed counting, cell sorting, biomarker detection, and protein engineering. Especially, in sperm analysis, FACs is essential and popularity methods for determine ability of sperm such as viability (Paynter *et al*., 2014), acrosome reaction (Escoffier *et al*., 2012), mitochondria integrity (Wu *et al*., 2014), and antioxidant ability (Yeste *et al*., 2014) as well as dyeing (Balao da Silva *et al*., 2011) conforming to study objection. In this study, sperm was stained by SYBR-14 for viability, FITC-PNA for acrosome reaction and Rhodamine 123 for mitochondria integrity with PI for check to membrane-compromised.

L-carnitine used in this study is effect to transport of lipid acid for produce energy in mitochondria. The disassembly of lipid acid by L-carnitine made long-chain acyl groups and this long-chain acyl groups entered in mitochondrial matrix. This phenomenon catalyzed metabolism to produce energy in mitochondria. Moreover,

Table 1. Effects of L-carnitine on viability and dying sperm rates (%) during the semen storage in miniature pigs

Sperm state	Concentration of L-carnitine (mg/mL)	Storage periods (days)		
		3	7	10
Live	Fresh	90.8±0.3^{a}	90.8±0.3^{a}	90.8±0.3^{a}
	0	87.7±1.3^{b}	84.5±2.2^{a}	79.1±2.8^{b}
	1	89.2±0.4^{ab}	87.3±1.8^{a}	81.3±3.6^{ab}
	2	90.2±0.7^{ab}	88.0±2.4^{a}	83.7±4.7^{ab}
	4	89.1±0.5^{ab}	87.6±1.8^{a}	80.8±2.7^{ab}
Dying	Fresh	5.1±0.4^{b}	5.1±0.4^{a}	5.1±0.4^{a}
	0	8.9±1.3^{a}	9.3±0.7^{a}	13.4±2.4^{a}
	1	6.9±1.0^{ab}	7.1±1.3^{a}	11.9±4.0^{a}
	2	5.7±0.9^{b}	7.4±3.2^{a}	8.8±3.2^{a}
	4	6.0±0.8^{ab}	8.0±2.6^{a}	11.6±2.2^{a}

^{a,b} Values in the same column with different superscripts are significantly different (p<0.05). Fresh: sperm analyzed in day 0. *All treatment groups were analyzed with 10,000 sperms, n=3.

Table 2. Effects of L-carnitine on acrosome reaction rates (%) during the semen storage in miniature pigs

Sperm state	Concentration of L-carnitine (mg/mL)	Storage periods (days)		
		3	7	10
Live with acrosome reaction	Fresh	3.4±1.6[a]	3.4±1.6[c]	3.4±1.6[b]
	0	5.4±0.5[a]	11.12±0.2[a]	16.3±0.6[a]
	1	5.2±0.3[a]	9.0±0.9[ab]	15.8±0.6[a]
	2	3.8±0.8[a]	7.6±0.2[b]	14.5±0.7[a]
	4	4.6±0.5[a]	7.9±0.8[b]	18.4±3.0[a]
All with acrosome reaction	Fresh	6.6±1.7[b]	6.6±1.7[b]	6.6±1.7[b]
	0	11.8±1.2[a]	18.9±0.4[a]	28.4±1.7[a]
	1	11.2±1.1[ab]	17.9±1.8[a]	30.1±2.6[a]
	2	7.9±0.8[ab]	17.4±1.2[a]	26.1±1.2[a]
	4	9.7±1.9[ab]	16.2±3.4[a]	31.7±5.1[a]

[a,b] Values in the same column with different superscripts are significantly different (p<0.05). Fresh: sperm analyzed in day 0. *All treatment groups were analyzed with 10,000 sperms, n=3.

Table 3. Effects of L-carnitine on mitochondria integrity rates (%) during the semen storage in miniature pigs

Sperm state	Concentration of L-carnitine (mg/mL)	Storage periods (days)		
		3	7	10
Live with mitochondria	Fresh	95.3±0.1[a]	95.3±0.1[a]	95.3±0.1[a]
	0	81.5±0.8[c]	83.1±2.8[b]	76.1±0.2[c]
	1	86.0±1.4[bc]	87.0±4.3[ab]	79.2±0.6[b]
	2	88.6±2.6[b]	90.1±4.7[ab]	80.8±0.2[b]
	4	86.1±1.5[bc]	91.0±2.9[ab]	79.8±0.6[b]
All with mitochondria	Fresh	92.9±0.3[a]	92.9±0.3[a]	92.9±0.3[a]
	0	70.7±2.2[c]	73.5±1.6[b]	67.1±2.2[c]
	1	76.5±2.3[bc]	84.8±5.4[ab]	71.3±2.4b[c]
	2	79.2±3.1[b]	82.5±6.3[ab]	73.3±1.2[b]
	4	72.9±2.4[bc]	80.2±5.3[ab]	70.8±0.6b[c]

[a~c] Values in the same column with different superscripts are significantly different (p<0.05). Fresh: sperm analyzed in day 0. *All treatment groups were analyzed with 10,000 sperms, n=3.

L-carnitine catalyzed motility in sperm (Lenzi *et al*., 2004).

In this study, we determined the effects of L- carnitine during the semen storage of miniature pigs. The concentrations of 0, 1.0, 2.0, and 4.0 mg/ml of L-carnitine were examined for improving sperm ability and analyzing fresh semen stored liquid state on 3, 7 and 10 days. The viability and dying sperm were tended to a positive effect in 2.0 mg/ml of L-carnitine treatment. We suggest that L-carnitine influences on sperm membrane and viability in miniature pig. The effects of an tioxidants increased on sperm viability and membrane integrity during liquid storage (Donoghue and Donoghue, 1997), and influenced in motility, acrosome reaction (Ball *et al*., 2001; Baumber *et al*., 2000; Cerolini *et al*., 2000). Also, the above results suggest that a decrease of acrosome reaction in treatment of L-carnitine-treated semen solution can decrease the acrosome reaction of sperm.

The analyzed ability of mitochondrial integrity on 10 days of semen storage was increased by L-carnitine, suggesting L-carnitine-treated medium protects mitochondria (Arrigoni-Martelli and Caso, 2000) and helps mitochondria mechanism (Lysiak *et al*., 1988).

In conclusion, the L-carnitine has a positive effect on

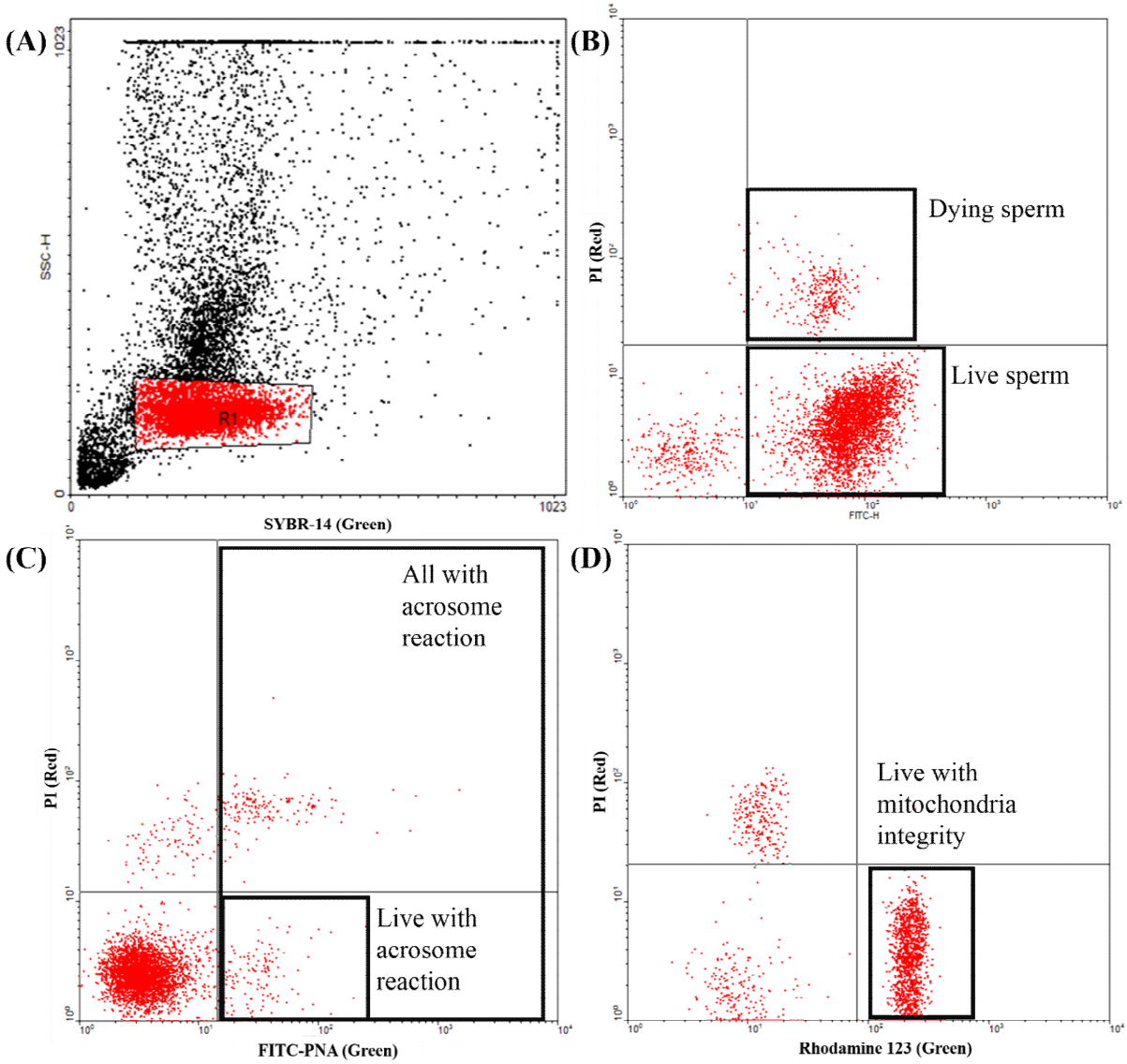

Fig. 1. Experimental of flow cytometric anaysis on sperm during liquid storage in semen extender with L-caritine (A), Viability was analysis using SYBR14 and propidium idodie (PI) double staining (B), Acrosome reaction (C) detected using FITC-PNA and PI double staining. Mitodhoncrial integrity (D) was detected by Rhodamine 123 and PI double staining.

acrosome reaction and mitochondria integrity at liquid state in fresh semen of miniature pig. 2.0 mg/ml of L-carnitine was improved the sperm ability during storage periods of fresh semen in miniature pigs.

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