pISSN 1225-4991, eISSN 2288-0178
J. Emb. Trans. (2014) Vol. 29, No. 4, pp. 385～391
http://dx.doi.org/10.12750/JET.2014.29.4.385

Effect of Nicotinic Acid on Fresh Semen Characteristics in Miniature Pigs

Yeon-Ju Lee[1], Sang-Hee Lee[1], Eunsong Lee[2], Seung Tae Lee[1], Hee-Tae Cheong[2], Boo-Keun Yang[1], Seunghyung Lee[3] and Choon-Keun Park[1,†]

[1]*College of Animal Life Science, Kangwon National University, Chuncheon 200-701, Republic of Korea*
[2]*College of Veterinary Medicine, Kangwon National University, Chuncheon 200-701, Republic of Korea*
[3]*Institute of Animal Resources, Kangwon National University, Chuncheon 200-701, Republic of Korea*

ABSTRACT

Objective of this study was to investigate the effect of nicotinic acid (NA) on the characteristics in fresh semen of miniature pig. We evaluated viability, acrosome reaction and mitochondrial integrity of sperm on 0, 3, 7 and 10 days during storage period with nicotinic acid. As results, the survival rate of sperm in 15 mM NA (day 3, 87.8 ± 1.2%; day 5, 84.0 ± 2.7%; day 7, 82.2 ± 0.9%) and 30 mM NA (day 3, 87.7 ± 0.3%; day 5, 84.4 ± 2.5%; day 7, 82.3 ± 0.7%) groups were higher than control and 5 mM NA groups in 3, 7 and 10 days of semen storage. The NA-treated sperm on 10 day was used day for observing acrosome integrity. The survival sperm with acrosome reaction was higher in 30 mM NA group (day 3, 2.7 ± 0.2%; day 5, 3.3 ± 0.6%; day 7, 11.4 ± 0.3%) than in the control, significantly ($P<0.05$). Moreover, the live sperm with mitochondrial integrity was higher in whole treatment groups of NA than control group, significantly ($P<0.05$). Specially, most mitochondrial integrity on 10 day of semen storage was significantly higher in 30 mM NA group (90.2 ± 1.6%) than other treatment groups (control, 81.8 ± 3.1%; 5 mM NA, 83.4 ± 3.0%; 15 mM NA, 89.1 ± 0.7%, $P<0.05$). In conclusion, supplement of NA in liquid semen of miniature pig can improve and maintain semen quality, such as viability, acrosome reaction, and mitochondria integrity.

(Key words: flow cytometry, nicotinic acid, fresh semen, mitochondria, miniature pig)

INTRODUCTION

Artificial insemination (AI) is a common fertilization method that is infection the diluted sperm to female domestic animals used by farmers. Sperm used to AI is usually fresh semen stored liquid state at 15～18℃. However, because the fresh sperm can not keep a long period of time, the collected semen has the disadvantage that should be used within 0～5 days. And even if sperm used within 5 days, the abilities such as viability and motility of fresh sperm are reduced.

Sperm emitted from the body of the male is move and fertility to adenosine triphosphate (ATP) produced by to mitochondria. To produce energy mitochondria plays an important role in motility and fertility in sperm (O'connell *et al.*, 2002). Thus, several experiments have been made to improve the mitochondrial function. The experiments were made chemical substances like to dimethyl sulfate (Me_2SO_4) and glycine (He and Woods Iii, 2004), antioxidants like to carnitine (Lenzi *et al.*, 2003) and vitamin E (Breininger *et al.*, 2005), hormone like to melatonin (Li *et al.*, 2012) and inhibitor (Huynh *et al.*, 2000). And methods of study is addition in diluted medium (Gravance *et al.*, 2003) or diet (Marin-Guzman *et al.*, 2000).

Antioxidant associated with mitochondrial is nicotinic acid called vitamin B_3 and niacin. Nicotinic acid is an organic compound with the formula $C_6H_5NO_2$ and precursors of the coenzymes nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) *in vivo*. NAD and NADP are coenzymes for many dehydrogenases containing many hydrogen transfer processes. This hydrogen transfer processes are responded using NADH dehydrogenase called complex I and act in the mitochondria. In mitochondria, this process is essential process because of enzyme of the mitochondrial electron transport chain and enzyme made to electrochemical potential difference used to produce ATP (Krauss, 2001). In the same function, nicotinic acid has been used in monocyte of human (Digby *et al.*, 2012), egg of sea urchin (Walseth *et al.*, 2012), guinea pig trachea (Aley *et al.*, 2013) and muscle of pig (Khan *et al.*, 2013). But, nicotinic acid was not used in the

* This research was supported by Bio-industry Technology Development Program (IPET 312060-05-1-CG000), Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea.
† Correspondence : parkck@kangwon.ac.kr

semen in pig. Therefore, the objective of this study was to investigate the effect of nicotinic acid on the characteristics in fresh semen of boar.

MATERIALS AND METHODS

All experiment procedures that included animals followed the 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.

1. Semen Collection

Fresh semen samples from 3 miniature pigs housed at a Kangwon National University farm (Republic of Korea) were collected using a gloved-hand method. Collected semen was diluted 1 × 10^7 spermatozoa/ml using Modena B. In this process, Modena B used semen conservative solution was divided as 0, 5, 15 and 30 mM of nicotinic acid concentration. Diluted semen was storage at 18℃ refrigerator to analysis and every semen analysis was repeated 3 times.

2. Analysis of Sperm Characteristics

Flow cytometry methods and assessment of semen were processed using the manufacturer's protocol (Lee *et al.*, 2014). The viability of sperm was analyzed by fluorescent staining using the LIVE/DEAD sperm Viability Kit (Molecular Probes, Eugen, OR, USA). Live sperm were stained as green fluorescent by SYBR-14 and membrane-compromised sperm was stained as red fluorescent by propidium iodide (PI). Each semen were diluted 1 × 10^8 spermatozoa/ml in Modena B of 1 ml and 1 µl of SYBR-14 (40 nM) was used in diluted semen for 5 min at 37℃. Then, 1 µl of PI (2 µM) was added to diluted semen included SYBR-14 for 10 min at 37℃. Stained sperms were centrifuged at 1,500 rpm for 5 min and the pellets were re-suspended in 500 µl of PBS. And, stained sperm was analyzed by flow cytometry.

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

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

3. Statistical Analysis

The analysis of variance (ANOVA) using SAS version 9.1 (SAS Institute Inc., Cary, NC, USA) was used statistical analysis. Data are presented as mean ± standard error of the mean (S.E.M). Differences in the mean values of sperm condition after treatment were analyzed using Duncan's multiple range tests.

RESULTS

The effect of nicotinic acid on viability and dying in pig's sperm is show in Fig. 1 and 2. In Fig. 1, the percentages of live sperm stained by SYBR-14 were higher in 15 mM (87.8 ± 1.2%, 84.0 ± 2.7%, 82.2 ± 0.9%) and 30 mM (87.7 ± 0.3%, 84.4 ± 2.5%, 82.3 ± 0.7%) of nicotinic acid than in control group (83.3 ± 1.2%, 78.8 ± 1.9%, 72.5 ± 0.9%) and 5 mM (82.7 ± 1.1%, 82.2 ± 1.7%, 74.1 ± 2.4%) in 3, 7 and 10 days of semen storage. The 3 and 10 days of semen storage were significantly (P<0.05) higher in treatment of nicotinic acid, but there were not significantly different in 7 days of semen storage. In Fig. 2, the percentages of dying sperm stained by SYBR-14 and PI were not significantly different among the control (8.8 ± 1.2%, 10.4 ± 0.8%, 19.9 ± 3.2%), 5 mM (12.0 ± 2.0%, 10.9 ± 2.2%, 20.3 ± 3.7%), 15 mM (6.6 ± 1.9%, 8.6 ± 1.6%, 12.0

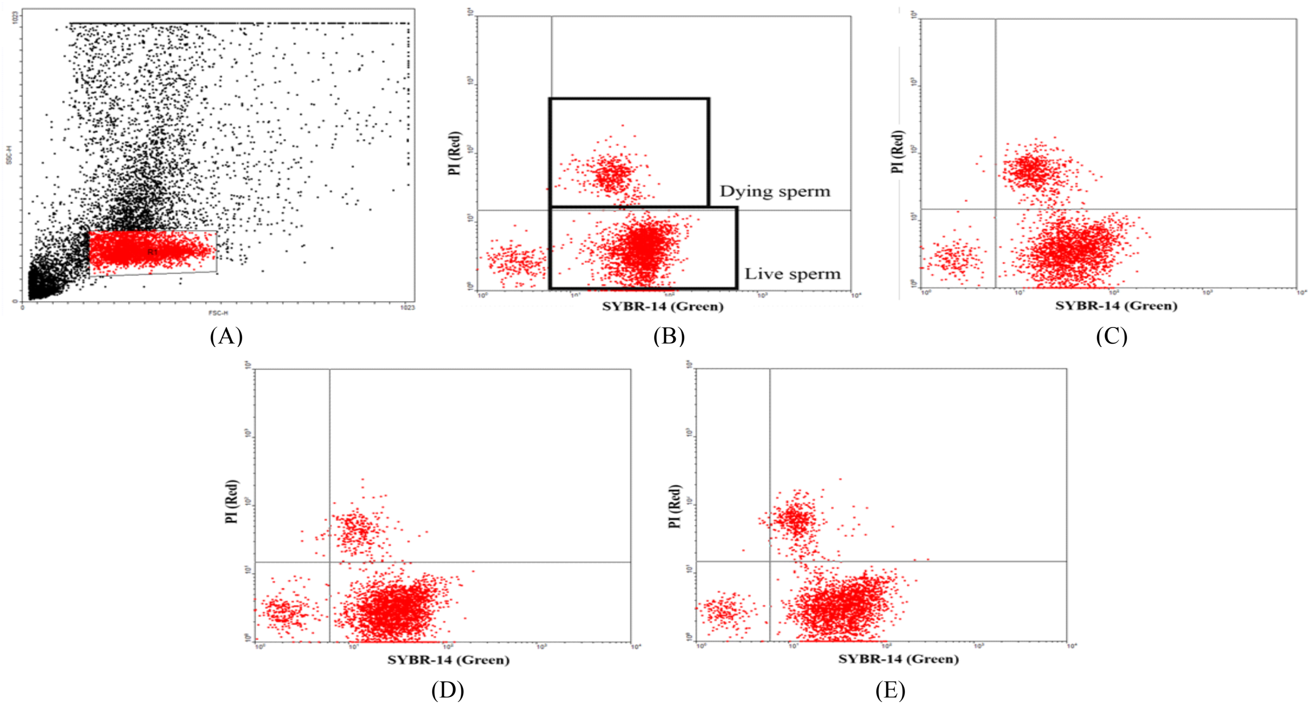

Fig. 1. Flow cytometric anaysis on sperm viability using SYBR-14 and propidium idodie (PI) double staining during liquid storage in semen extender with nicotinic acid (A) in miniature pigs. (B) control group, (C) 5 mM treatment group, (D) 15 mM treatment group, (E) 30 mM treatment group.

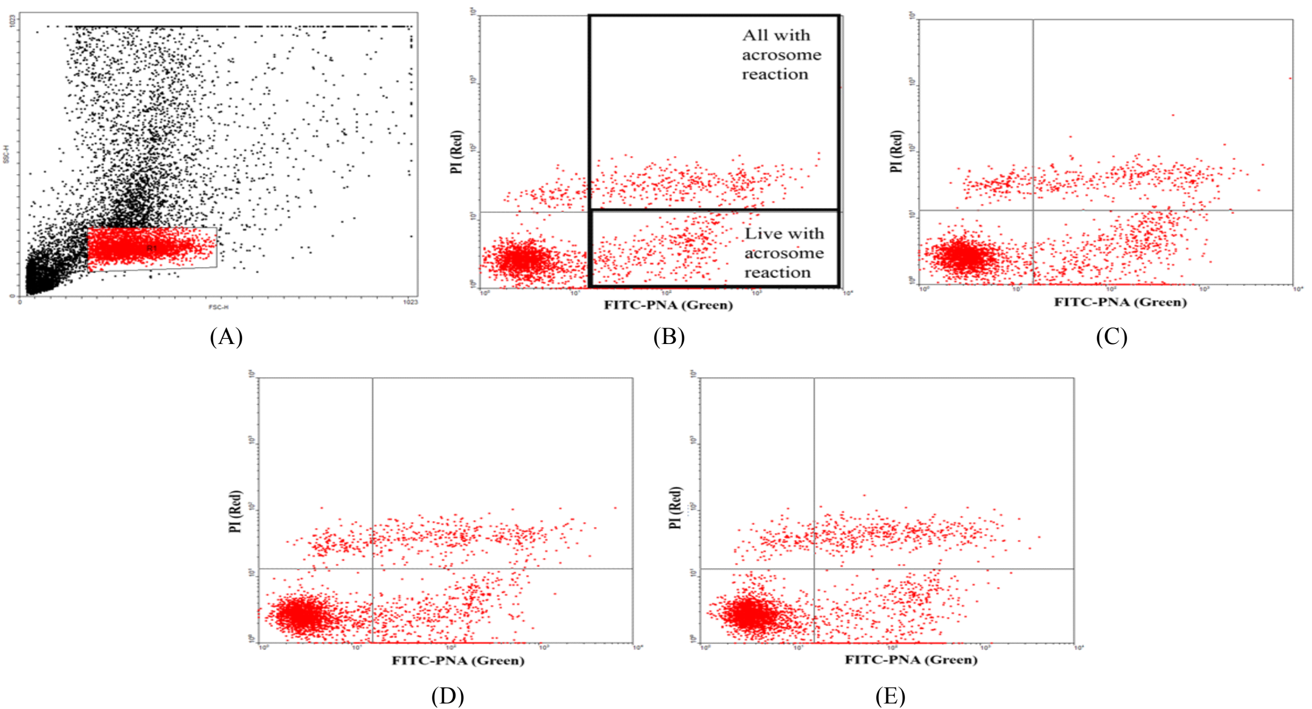

Fig. 2. Flow cytometric anaysis on acrosome reaction using FITC-PNA and PI double staining during liquid storage in semen extender with nicotinic acid (A) in miniatrue pigs. (B) control group, (C) 5 mM treatment group, (D) 15 mM treatment group, (E) 30 mM treatment group.

± 3.0%), and 30 mM (8.8 ± 1.2%, 7.9 ± 1.4%, 13.1 ± 0.3%) groups during 10 days of semen storage. When acrosome integrity was analyzed in treatment groups of nicotinic acid during 10 days of semen storage (Table 1), the live sperm with acrosome reaction were significantly higher in 30 mM (2.7 ± 0.2%, 3.3 ± 0.6%, 11.4 ± 0.3%) than in the control group (4.50 ± 0.7%, 6.9 ± 0.7%, 16.3 ± 0.6%, $P<0.05$). And, the all with acrosome reaction was significantly higher in treatment groups of nicotinic acid than in control group in 3 and 10 days of semen storage ($P<0.05$). On the other hand, when mitochondria integrity was analyzed after treatment of nicotinic acid in semen storage during 10 days (Table 2), the live sperm with mitochondrial integrity was significantly higher in groups treated with nicotinic acid than in the control group ($P<0.05$). Most of all, mitochondrial integrity at 10 day of semen storage was significantly higher in the 30 mM (90.2 ± 1.6%) group than control (81.8 ± 3.1%), 5 mM (83.4 ± 3.0%), 15 mM (89.1 ± 0.7%) of nicotinic acid groups ($P<0.05$). An all sperm with mitochondrial integrity was not significantly different among the all treatment groups. But, there were significantly higher in the treatment groups of nicotinic acid than in control at 7 days of semen storage ($P<0.05$).

Table 1. Effect of nicotinic acid on sperm acrosome reaction rate (%) in miniature pig

	Treatment (mM)	Storage periods (days)			
		0	3	7	10
Live with acrosome reaction	0	3.3 ± 1.5	4.5 ± 0.7^{a}	6.9 ± 0.7^{a}	16.3 ± 0.6^{a}
	5		2.5 ± 0.3^{b}	4.5 ± 0.9^{ab}	10.8 ± 1.2^{b}
	15		3.5 ± 0.6^{ab}	4.0 ± 0.8^{b}	12.0 ± 0.3^{b}
	30		2.7 ± 0.2^{b}	3.3 ± 0.6^{b}	11.4 ± 0.3^{b}
All with acrosome reaction	0	7.5 ± 1.2	11.7 ± 1.3^{a}	18.2 ± 6.3^{a}	28.4 ± 1.7^{a}
	5		6.7 ± 1.2^{b}	13.6 ± 6.2^{a}	21.0 ± 2.3^{b}
	15		7.0 ± 0.9^{b}	9.4 ± 1.3^{a}	16.0 ± 1.5^{b}
	30		6.4 ± 0.9^{b}	8.3 ± 1.5^{a}	19.8 ± 1.0^{b}

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

Table 2. Effect of nicotinic acid on sperm live mitochondrial integrity rate (%) in miniature pig

	Treatment (mM)	Storage periods (days)			
		0	3	7	10
Live with acrosome reaction	0	95.3 ± 0.1	95.7 ± 0.3^{a}	83.1 ± 2.8^{b}	81.8 ± 3.1^{b}
	5		95.5 ± 0.1^{a}	94.6 ± 1.8^{a}	83.4 ± 3.0^{ab}
	15		96.2 ± 0.3^{a}	94.4 ± 0.3^{a}	89.1 ± 0.7^{ab}
	30		95.6 ± 0.9^{a}	95.7 ± 0.9^{a}	90.2 ± 1.6^{a}
All with acrosome reaction	0	93.2 ± 0.3	88.1 ± 2.0^{a}	73.5 ± 1.6^{b}	71.3 ± 6.0^{a}
	5		88.4 ± 2.1^{a}	87.3 ± 3.1^{a}	73.0 ± 6.4^{a}
	15		90.8 ± 0.9^{a}	86.8 ± 1.3^{a}	83.4 ± 3.0^{a}
	30		90.5 ± 1.0^{a}	87.8 ± 1.5^{a}	86.4 ± 1.8^{a}

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

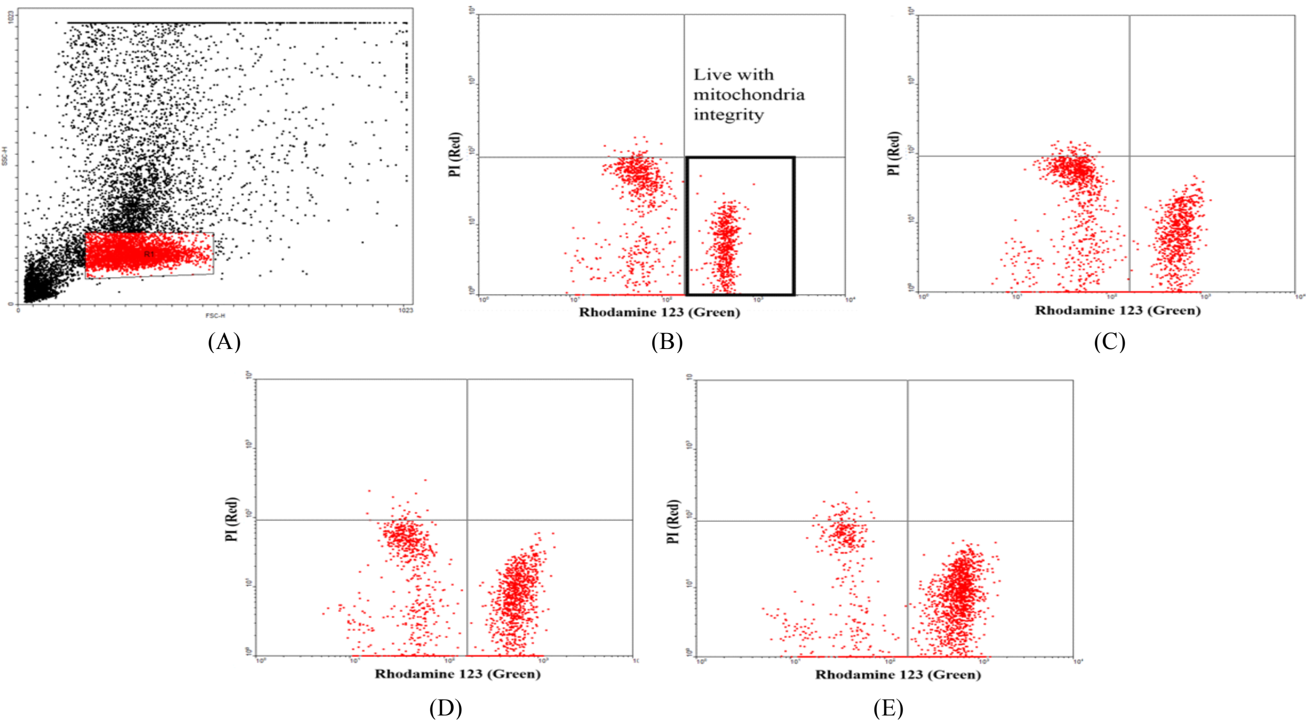

Fig. 3. Flow cytometric anaysis on mitochondria integrity using Rhodamine 123 and PI double staining during liquid storage in semen extender with nicotinic acid (A) in miniature pigs. (B) control group, (C) 5 mM treatment group, (D) 15 mM treatment group, (E) 30 mM treatment group.

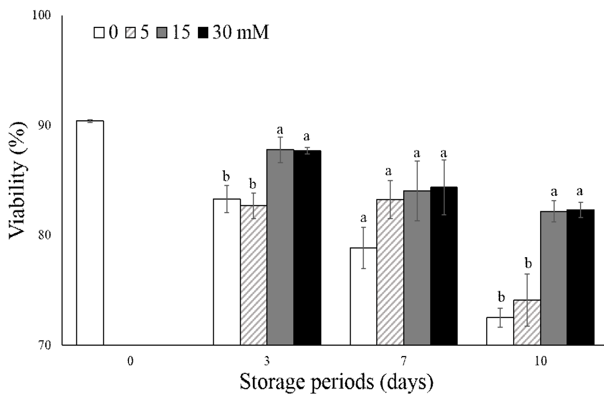

Fig. 4. Effect of nicotinic acid on sperm viability during various semen storage periods in miniature pig. [a,b] Values in the same column with different superscripts are significantly different (p<0.05), n=3.

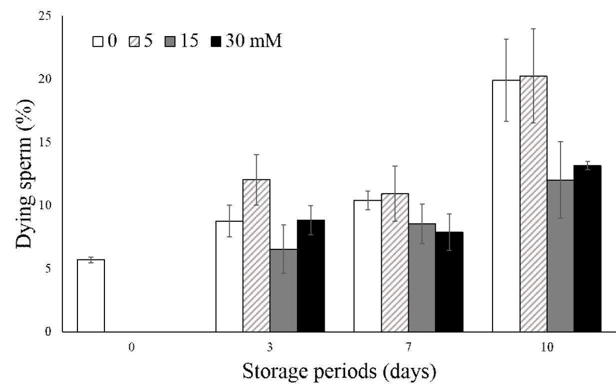

Fig. 5. Effect of nicotinic acid in dying sperm during various semen storage periods in miniature pig. n=3.

DISCUSSION

In this study, we tried to determine effect of nicotinic acid in boar fresh semen of miniature pig. A fresh semen is used with a short period to keep the sperm collected from the male, but lower in damage of sperm than in other storage method such as cryopreservation (Bailey *et al.*, 2000). A semen stored liquid state is most affected by medium and basic conditions such as pH and temperature. So, many studies tried to improve for quality of semen. Antioxidants already demonstrated ability (Contri *et al.*, 2011) play a role to inhibit the oxidation in cells included germ cell, cell organelle and other many cells. This oxidation reaction produce free radicals that have a bad influence like to cell damage, diseases of animal concluded human

(Karthikeyan *et al.*, 2011), cell cycle and aging (Mukherjee *et al.*, 2004). For this reason, use of antioxidants that can minimize the damage caused by oxidation is effective to improve to ability of semen stored liquid state.

In the present study, semen stored during 10 days was treated nicotinic acid of 0, 5, 15 and 30 mM and analyzed at 0, 3, 7 and 10 days after semen storage. First, viability in fresh semen was significantly different in treatment of nicotinic acid in 3 and 10 days (P<0.05). The viability in 7 day of semen storage and dying sperm during the 10 days of semen storage is not significantly different among treatment groups. These results suggest that nicotinic acid was protecting sperm as antioxidants, which were decreased lipid peroxidation caused membrane damage (Contri *et al.*, 2011) and loss of the membrane function, integrity and fertilizing ability in sperm (Partyka *et al.*, 2012).

In fresh semen analysis, live sperm with acrosome reaction was significantly lower in groups of 15 and 30 mM of nicotinic acid than in the control group in 3, 7 and 10 days of semen storage (P<0.05). And, the all with acrosome reaction was significantly lower in treatment groups of nicotinic acid than in control in only 3 and 10 days of semen storage (P<0.05). In other words, acrosome reaction was showed lower reaction rates with nicotinic acid treatment. This phenomenon is comparability that hamster sperm response to H_2O_2 induced acrosome reactions, while this reaction is inhibited by antioxidants (Bize *et al.*, 1991). Also, in human the acrosome reaction was reported association with reactive oxygen species (ROS) (De Lamirande *et al.*, 1997). This ROS catalyzed acrosome reaction and regulated acrosome reaction (De Lamirande *et al.*, 1997).

The mitochondrial integrity was significantly higher in the treatment groups with nicotinic acid than in the control group (P<0.05). In the case of sperm, mitochondria produced energy source plays a very important role because of motility of sperm. In this study, the nicotinic acid treated semen is precursors 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 reduced glutathione and oxidation-reduction concluded in protecting from ROS (Rush *et al.*, 1985) and was used lipid and nucleic acid synthesis for NADPH (Igamberdiev and Gardeström, 2003). A metabolism to addition of nicotinic acid supposed protection of mitochondrial integrity during 10 days in boar semen.

In conclusion, the nicotinic acid has a positive effect in fresh semen stored in liquid state in pig. Especially, the 30 mM supplementation of nicotinic acid can improve quality of semen such as viability, acrosome reaction and mitochondria integrity.

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Received November 30, 2014, Revised December 3, 2014, Accepted December 17, 2014