Effect of Nicotinic Acid on Sperm Characteristic and Oocyte Development after *In Vitro* Fertilization using Cryopreserved Boar Semen

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ABSTRACT

The objective of this study was to investigate the efficiency of nicotinic acid on sperm cryosurvival and fertilization ability in frozen-thawed boar semen. Boar semen was collected by glove-hand method and was frozen using freezing solution treated to 0, 5, 10 and 20 mM of nicotinic acid. The frozen sperm for sperm characteristic analysis was thawed such as viability, acrosome reaction, and mitochondrial integrity. The frozen-thawed sperm was estimated by SYBR14/PI double staining for viability, FITC-PNA/PI double staining for acrosome reaction and Rhodamine123/PI double staining for mitochondrial integrity using a flow cytometry. The embryo was estimated *in vitro* development and DCFDA staining for reactive oxygen species assessment. As results, frozen-thawed sperm viability was significantly higher in 5 and 10 mM (61.1 ± 1.5%, 64.7 ± 2.0%) of nicotinic acid than other groups (0 mM, 52.1 ± 2.3%; 20 mM, 47.8 ± 5.1%, $P<0.05$). The live sperm with acrosome reaction was significantly higher in 5 and 10 mM of nicotinic acid (26.1 ± 1.8%, 24.9 ± 1.5%) than other groups (0 mM, 35.3 ± 0.8%; 20 mM, 36.5 ± 1.9%, $P<0.05$). The live sperm with mitochondrial integrity was significantly higher in 5 and 10 mM (84.2 ± 3.6%, 88.4 ± 2.3%) of nicotinic acid than other groups (0 mM, 77.3 ± 4.4%; 20 mM, 73.3 ± 3.6%, $P<0.05$). Blastocyst rate of *in vitro* development was significantly higher in 10 mM (17.0 ± 1.3%) of nicotinic acid than other groups (0 mM, 9.4 ± 0.5%; 5mM, 12.6 ± 0.8%; 20 mM, 5.0 ± 1.0%, $P<0.05$). Moreover, total cell number was higher in 5 and 10 mM (53.6 ± 2.9%, 57.9 ± 2.8%) of nicotinic acid than other groups (0 mM, 41.0 ± 1.4%; 20 mM, 23.2 ± 2.8%, $P<0.05$). Hydrogen peroxide in embryos was lower in 5 mM nicotinic acid (0.7 ± 0.1%) than other groups (0 mM, 1.0 ± 0.1%; 10mM, 0.9 ± 0.0%; 20 mM, 1.4 ± 1.0%, $P<0.05$). In conclusion, nicotinic acid-treated semen improves cryosurvival and quality of spermatozoa. Also, the fertilized oocytes with nicotinic acid improve quality of embryo and blastocyst formation.
(Key words : frozen-thawed semen, nicotinic acid, spermatozoa, cryosurvival, embryo)

INTRODUCTION

Cryopreservation technique has various advantages of long-term storage and being powerful tool of gene preservation and restoration of endangered species. Also, frozen semen is widely used for system improvement preservation of superior breed and fertility in domestic animals. However, because of cold shock, oxidative stress and reactive oxygen species (ROS) generation (Baishya *et al.*, 2014) during for freezing and thawing process, frozen-thawed sperm loses its viability, motility (Watson, 2000), mitochondrial integrity and gets more damage of acrosome membrane (Curry, 2000). Also, abnormal morphology increases (Bertoldo *et al.*, 2014).

Several studies has reported that the supplement of antioxidants in the freezing extender was improved the quality of semen for cryopreservation (Sarıozkan *et al.*, 2009). To improve the quality of semen, several methods are used - supplying of extender (Bresciani *et al.*, 2013), controlling of cooling rate (Varisli *et al.*, 2013) and containing the additive (Yeste *et al.*, 2014). Protein (Beirão *et al.*, 2012), sucrose (Chen *et al.*, 1993) and antioxidant (Gadea *et al.*, 2011) were contained into freezing extender for sperm cryopreservation. Addition of antioxidant in freezing extender has reported to improve the viability, motility, plasma membrane integrity and decreased ROS (Gualtieri *et*

al., 2014) of sperm during cryopreservation.

Nicotinic acid was a type of antioxidant and a precursor of nicotinamide adenine dinucleotide (NAD) source of vitamin B_3. The nicotinic acid is controls the NAD and NAD phosphate (NADP) coenzyme involved in the mitochondria. NAD and its relative NADP help the produce energy by involving in the mechanism of the TCA cycle. Some studies have reported that effect of supplement the nicotinic acid have been used to induced cell death in hepatocytes and induce of hydrogen peroxide in mice (Dou et al., 2013). Also nicotinic acid has been used to improve the viability, mitochondrial integrity in storage of liquid miniature pig sperm (Lee et al., 2014).

However, there is no study about influence of nicotinic acid on boar sperm cryopreservation. Therefore, the objective of this study is to evaluate the characteristic and development of cryopreserved boar sperm with nicotinic acid and assess development of oocytes in embryos in vitro fertilized with the cryopreserved boar sperm.

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 glove-hand method. Collected semen was diluted 1×10^7 spermatozoa using Modena B (30.0 g/l glucose, 2.25 g/l EDTA, 2.50 g/l sodium citrate, 1.00 g/l sodium bicarbonate, 5.00 g/l tris, 2.50 g/l citric acid, 0.05 g/l cysteine and 0.30 g/l gentamicin sulfate) and transported to the laboratory within 2 hours. Semen used in the experiment had normal movement of more than 70% and viability of more than 80% and storaged at 18℃ refrigerator.

2. Preparation of Freezing Solution

1st Lactose Egg-yolk (LEY) freezing solution was made 11% α-lactose (L2643, Sigma, St. Louis, USA) by the addition of 20% egg-yolk and centrifuged at 3,000 rpm for 30 minutes at 4℃. As the 1st freezing solution, different concentration of nicotinic acid 0, 5, 10 and 20 mM were supplied to supernatant and pH was adjusted to pH 6.1, which before addition of nicotinic acid. 2nd freezing solution was produced by 1st freezing solution included 9% glycerol (G6279, Sigma) and 1.5% Orvus Es Paste (OEP, Equex® STM paste, Nova Chemical Sales Inc, Scituate, USA) and storaged at 4℃ refrigerator.

3. Semen Cryopreservation and Thawing

Semen was centrifuged at 1,500 rpm for 5 minutes in 18℃ and remove the supernatant. Semen was diluted 1×10^9 spermatozoa/mL using 1st freezing solution and was cooling at 5℃ within 1.5~2 hours. Semen contained 1st freezing solution was reedited by 2nd freezing solution that one-half volume of 1st freezing solution used to dilute semen. Then, semen diluted by 1st and 2nd freezing solution packed in 0.25 mm straw. 0.25 mm straw was pre-freezing for 10 minutes on a 10 cm from the liquid nitrogen. The frozen semen in 0.25 mm straw was observed the viability and motility where it thawed for 45 seconds at 38℃ in water-bath and cryopreserved.

4. Analysis of Sperm Characteristics

Flow cytometry methods and assessment of semen were processed using the manufacturer's protocol (Lee et al., 2014). The evaluation of frozen-thawed sperm was determined with a LIVE/DEAD sperm viability kit (L7011, Invitrogen, Gaithersburg, USA), Lectin from Arachis hypogaea (FITC-PNA; L7381, Sigma) FITC-PNA and Rhodamine 123 (R8004, Sigma) with Propidium Iodide (PI, 2 μM) by fluorescence staining. The frozen semen thawed for 45 seconds at 38℃ in water-bath and centrifuged at 1,500 rpm for 5 minutes at 18℃. After the supernatant remove, semen was diluted with 1×10^7 spermatozoa/mL using Modena B and was stained with 40 nM of SYBR-14,2 μM of FITC-PNA and 2 μM of Rhodamine 123. After semen diluted for 5 min at 38℃ in dark, 2 μM of PI was added to diluted for 5 minutes at 38℃ in dark. Stained sperms were centrifuged 1,500 rpm for 5 minutes and the supernatant was removed. And pellet was resuspended in 500 μL of PBS (−). Then, stained sperm using dye were analyzed by flow cytometry (BD FACSCanto™II, BD Biosciences, San Diego, USA) which were calculated live, dying, acrosome reaction and mitochondrial integrity of live or all sperm from CELL-Quest, version 6.0 software for influence of nicotinic acid on cryopreservation in boar sperm (Lee et al., 2015).

5. Collection of Oocytes

Porcine ovaries were collected at the butchery and ovary was transported within 2 hours in the laboratory by dipping to saline (0.9%, NaCl) in 34 to 36℃. Ovary washed the Saline (0.85%, NaCl) and 10 ml syringe needle of 18 gauge was collected follicular fluid at 2∼6 mm of follicle. Collected follicular fluid was settle down at room temperature and washed with Phosphate buffer saline-polyvinyl alcohol (PBS-PVA). Cumulus oocytes complexes (COCs) were collected by stereoscopic microscope. Maturation medium of COCs used TCM-199 by the basic culture medium which supplemented with 10% (v/v) porcine follicular fluid (pFF), 10 µl/ml hCG, 1 µl/ml EGF, FSH and LH. After washed three times in PBS- PVA of collected COCs incubated at 39℃, 5% CO_2 for 22 hours. After 22 hours, the maturated COCs were washed three times in TCM-199 which supplemented with 10% (v/v) pFF and 1 µl/ ml EGF and incubated at 39℃, 5% CO_2 for 22 hours.

6. *In Vitro* Fertilization

After *in vitro* maturation, COCs denuded 0.1% hyaluronidase (H3506, Sigma) to remove a cumulus cells. After remove the cumulus cells, oocyte was injected 50 µl of mTBM which supplemented with 2 mg/ml Bovine Serum Albumin (BSA; A6003, Sigma) until fertilized with sperm. Frozen semen trea- ted different concentration of nicotinic acid (0, 5, 10 and 20 mM) thawed for 45 seconds at 38℃ in water-bath and diluted Beltsville thawing solution (BTS) 4 ml. Semen was centrifuged at 1,500 rpm for 5 minutes at 18℃ and remove the supernatant. And semen was diluted to 1×10^5 spermatozoa/ml using mTBM supplemented with 4 mg/ml BSA (A4503, Sigma). Then, diluted semen was infect to mTBM included oocyte and incubated at 39℃, 5% CO_2 for 6 hours.

7. *In Vitro* Culture

Fertilized oocytes used pipet to remove the cumulus cells and sperm by attached to embryos. Embryos were washed three times in Porcine Zygote Medium-3 (PZM-3) which supplemented with 3 mg/ml BSA and incubated at 39℃, 5% CO_2 for 48 hours. After 48 hours, embryos were incubated at 39℃, 5% CO_2 for 144 hours in 4-well dish. Early Blastocyst formation was confirmed after 6∼7 days and total cell counting on 7-day.

8. Assessment of Hydrogen Peroxide Levels in Embryos

Embryonic ROS levels were measured by stained Carboxy- DCFDA (C-369, Invitrogen). 16 cell∼morula cells were collected incubation at 168 hours, and fixed 4% paraformaldehyde at 15 minutes. Fixed 16 cell∼morula cells were stained 20 µm of DCFDA at 30 minutes in dark and washing with PBS-PVA. 16 cell∼morula cells were observed with a fluorescence microscope (ECLIOSE TE 300, Nikon, Tokyo, Japan). The fluorescence level were analysis using Multi Gauge V3.0.

9. Blastocyst Total Cell Counting

Blastocysts were stained with Hoechst 33342 (B2261, Sigma). After blastocysts were immersed to 0.2% PBS-PVA-BSA at 5 minutes, and fixed to 4% paraformaldehyde for 5 minutes. Then, blastocyst was stained with 1 µl/ml Hoechst at 30 minutes in dark. Total cells of each blastocysts were observed with a fluorescence microscope.

10. Statistical Analysis

All the experimental data are presented as mean ± standard error of the mean (S.E.M.). The statistical significance of difference was assessed by General Linear Model (G.L.M.) using SAS version 9.3.

RESULTS

The effect of nicotinic acid on viability of frozen-thawed sperm shows in Fig. 1. The percentages of live sperm were higher in 5 (61.1 ± 1.5%) and 10 mM (64.7 ± 2.0%) than 0 (52.2 ± 2.3%) and 20 mM (47.8 ± 5.1%). And, 10mM of nicotinic acid was significantly higher than 0 and 20 mM in live sperm percentage ($P<0.05$). The percentages of dying sperm were significantly lower in 10 mM (20.5 ± 2.5%) of nicotinic acid than 0 (35.2 ± 2.9%), 5 (29.4 ± 1.7%), and 20 mM (36.6 ± 2.9%, $P<0.05$).

The effect of nicotinic acid on acrosome reaction with live sperm and all sperm of frozen-thawed sperm is shows in Fig. 2. The percentages of live sperm with acrosome reaction were lower in 5 (26.1 ± 1.8%) and 10 mM (24.9 ± 1.5%) of nicotinic acid than 0 (35.3 ± 0.8%) and 20 mM (36.5 ± 1.9%). And, 10 mM of nicotinic acid was significantly lower than 0 and 20 mM in live sperm with acrosome reaction ($P<0.05$). The percentages of all sperm were lower in 5 (32.2 ± 1.9%) and 10 mM (35.6 ± 1.1%) than 0 (42.3 ± 2.1%) and 20 mM (45.0 ± 3.2%) of nicotinic acid. And, 5 mM of nicotinic acid was significantly lower than 0 and 20 mM in all sperm with
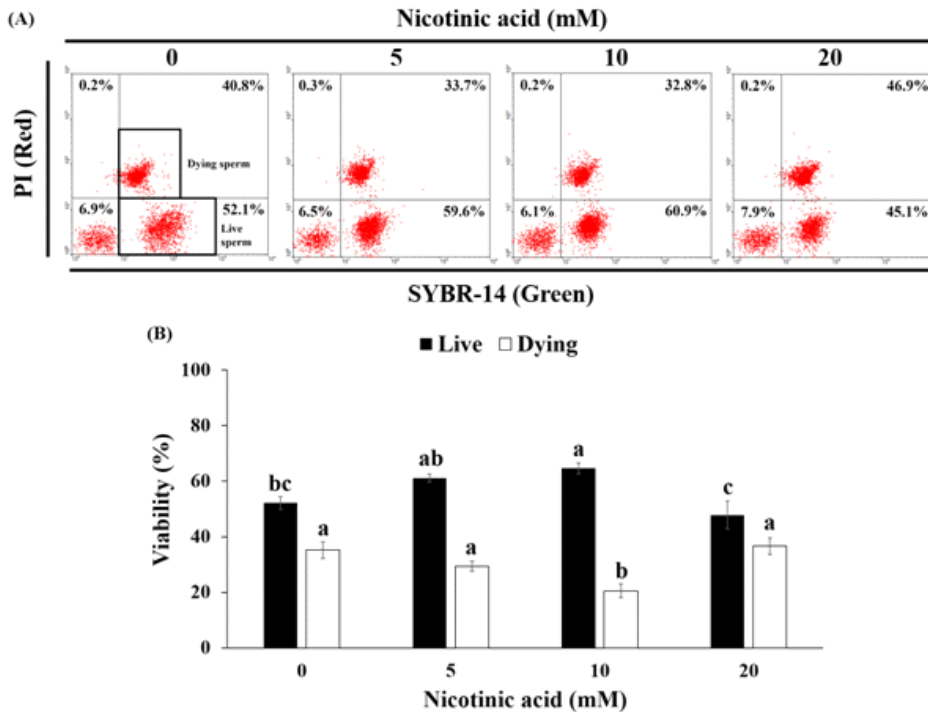
Fig. 1. Flow cytometric dot-plot of boar sperm population cryopreserved in freezing extender supplemented with nicotinic acid, effect of nicotinic acid on live (black bar) and dying (white bar) in frozen-thawed boar sperm (A), (B). [a~c] Values with differents uper scripts in the same column with live sperm and dying sperm are significantly different ($P<0.05$, n=4).
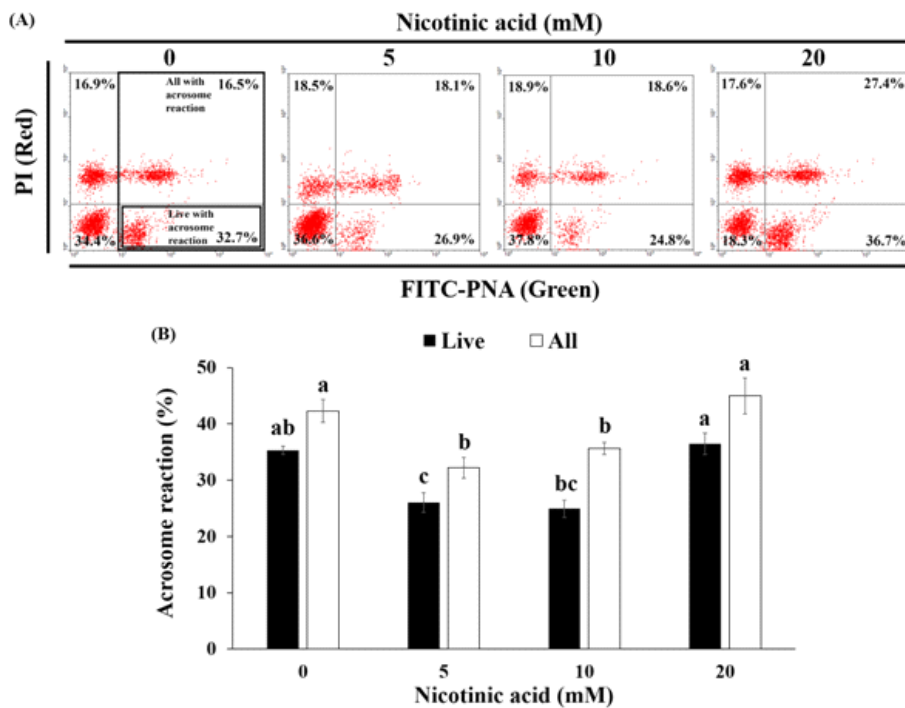

Fig. 2. Flow cytometric dot-plot of boar sperm population cryopreserved in freezing extender supplemented with nicotinic acid, effect of nicotinic acid on live (black bar) and all (white bar) in frozen-thawed boar sperm (A), (B). [a~c] Values with differents uper scripts in the same column with live sperm and all sperm are significantly different ($P<0.05$, n=4).

acrosome reaction ($P<0.05$).

The effect of nicotinic acid on mitochondrial integrity with live sperm and all sperm of frozen-thawed sperm is shows in Fig. 3. The percentages of live sperm with mitochondrial integrity were higher in 5 (84.1 ± 3.6%) and 10 mM (88.4 ± 2.3 %) than 0 (77.3 ± 4.4%) and 20 mM (73.3 ± 3.6%) of nicotinic acid in live sperm with mitochondrial integrity. And, 10 mM of nicotinic acid was significantly higher than 0 and 20 mM in live sperm percentage ($P<0.05$). The percentages of all sperm with mitochondrial integrity were higher in 5 (58.1 ± 6.8%) and 10 mM (66.8 ± 3.4%) of nicotinic acid than 0 (45.3 ± 5.4%) and 20 mM (41.7 ± 5.6%) in all sperm with mitochon-

drial integrity. And, 10 mM of nicotinic acid was significantly higher than 20 mM in live sperm percentage ($P<0.05$).

The effect of *in vitro* development on embryo fertilized using frozen-thawed semen treated 0, 5, 10 and 20 mM of nicotinic acid shows in Table 1. Cleavage rates were significantly lower in 20 mM (71.0 ± 5.2%) than 0 (83.8 ± 2.1%), 5 (85.4 ± 2.4 %) and 10 mM (88.9 ± 1.2) of nicotinic acid ($P<0.05$). Blastocyst rates were significantly higher in 10 mM (17.0 ± 1.3) than 0 (9.4 ± 0.5), 5 (12.6 ± 0.8%) and 20 mM (5.0 ± 1.0) ($P<0.05$). And, Degeneration rates of embryos were significantly lower in 20mM (28.7 ± 3.5%) than 0 (16.2 ± 2.1%), 5 (14.6 ± 2.4%), and 10 mM (11.1 ± 1.2%) of nicotinic acid ($P<0.05$).
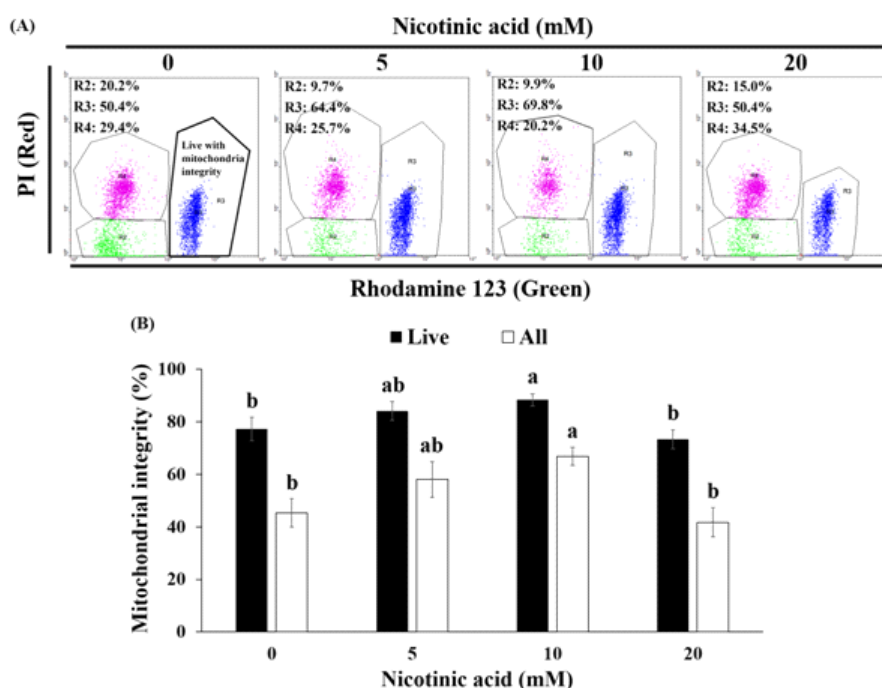

Fig. 3. Flow cytometric dot-plot of boar sperm population cryopreserved in freezing extender supplemented with nicotinic acid, effect of nicotinic acid on live (black bar) and all (white bar) in frozen-thawed boar sperm (A), (B). [a,b] Values with differents uper scripts in the same column with live sperm and all sperm are significantly different ($P<0.05$, n=4).

Table 1. *In vitro* development of oocytes of 168 hour after fertilized with frozen-thawed semen supplemented with nicotinic acid in pigs

Nicotinic acid (mM)	No. of oocytes examined	Cleavage (%)	No. of embryo development to (%)		Degeneration (%)
			2 cell~morula	Blastocyst	
0	222	187 (83.8±2.1)[a]	166 (74.4±2.1)	21 (9.4±0.5)[c]	35 (16.2±2.1)[b]
5	215	183 (85.4±2.4)[a]	156 (72.8±2.1)	27 (12.6±0.8)[b]	32 (14.6±2.4)[b]
10	223	198 (88.9±1.2)[a]	161 (71.9±1.8)	37 (17.0±1.3)[a]	25 (11.1±1.2)[b]
20	219	159 (71.0±5.2)[b]	149 (72.8±9.3)	10 (5.0±1.0)[d]	60 (28.7±3.5)[a]

[a~d] Values in the same column with different superscripts are significantly different ($P<0.05$, n=4).

The effect of total cell number on blastocyst formation of embryos fertilized using frozen-thawed semen treated 0, 5, 10 and 20 mM of nicotinic acid is show in Fig. 4. The percentages of total cell number rate were higher in 5 ($53.6 \pm 2.9\%$) and 10 mM ($57.9 \pm 2.8\%$) than 0 ($41.7 \pm 1.4\%$) and 20 mM ($23.2 \pm 2.8\%$) of nicotinic acid. Then, 5 and 10 mM of nicotinic acid was significantly lower than 0 and 20 mM in total cell number rate of blastocyst ($P<0.05$). Moreover, we determined whether hydrogen peroxide regulates embryo production in frozen-thawed sperm. As Fig. 5, the percentage of ROS generation was significantly lower in 5 mM ($0.7 \pm 0.1\%$) of nicotinic acid than 0 ($1 \pm 0.1\%$), 10 ($0.9 \pm 0.0\%$), and 20 mM ($1.4 \pm 0.1\%$, $P<0.05$).

DISCUSSION

In this study, we evaluated the efficiency on cryopreservation of sperm with nicotinic acid and *in vitro* development of embryos fertilized by frozen-thawed semen with nicotinic acid in pigs. In boar sperm, cryopreservation was adverse effect
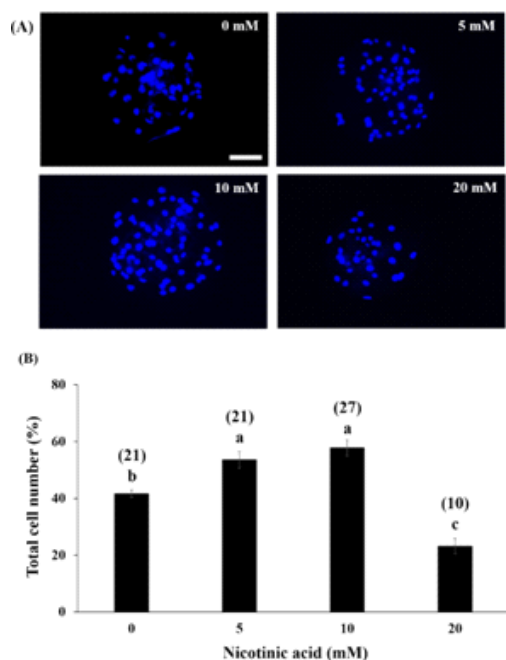
Fig. 4. Image of nucleus (A) and total cell number (B) of blastocysts at 168 h after *in vitro* fertilization using frozen-thawed semen supplemented with nicotinic acid in pigs. [a~c] Differents uper script letters indicate a significant difference ($P<0.05$). Number of brackets were presented total blastocyst. Scale bar, 50 μm; Blue, Hoechst 33342.
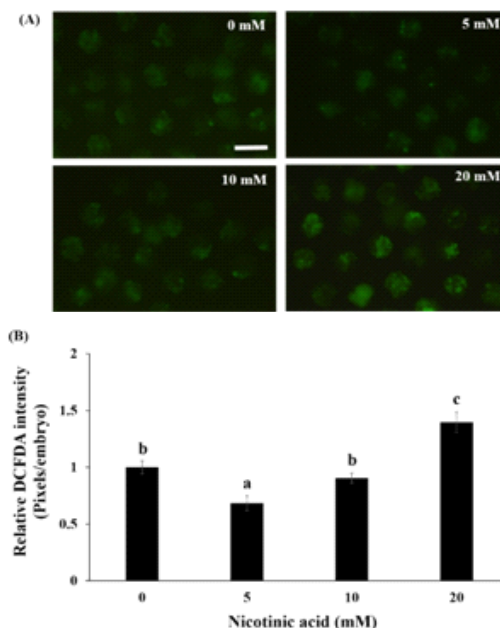
Fig. 5. Fluorsecence photomicrograph (A) and relative intensity level (B) of hydrogen peroxide in embryos after 168 h at *in vitro* fertilization using frozen-thawed semen supplemented with nicotinic acid in pigs. [a~c] Differents uper script letters indicate a significant difference ($P<0.05$). Scale bar: 100 μm, Green: DCFDA stained embryo, n=3.

such as reduce of motility, increasing of membrane damage and increasing of DNA damage (Eriksson and Rodriguez-Martinez, 2000), because of cold shock by oxidative stress and ROS generation during freezing and thawing process (Gadea *et al.*, 2004). To prevent the adverse effect widely used supplement of antioxidant method in frozen semen (Chanapiwat *et al.*, 2014). Moreover, high quality of frozen-thawed semen can improve antioxidant ability and development of embryos in human (Donnelly *et al.*, 1998), cattle (Petrunkina *et al.*, 2007), pigs (El Shourbagy *et al.*, 2006).

NAD/NADP regulates mitochondrial biogenesis in rodents (Stein and Imai, 2012). NAD/NADP ratio was essential for mitochondrial function and mitochondria which was produced by ATP activity (Stein and Imai, 2012). Also mitochondrial membrane potential of sperm was related with early apoptosis, reduce of motility and decrease of fertilize function (Amaral *et al.*, 2013). The survival of the sperm was associated with ROS and mitochondria in the sperm preventing the generation of oxidative stress (Fang *et al.*, 2014). These results suggest that nicotinic acid can stimulate mitochondrial activity and protect ROS-mediated damage of sperm as antioxidants. (Ben Abdallah

et al., 2012). Sperm capacitation, acrosome reaction, and outer acrosomal membrane damage are increased by intracellular Ca^{2+} (Breitbart et al., 2002). Nicotinic acid adenine dinucleotide phosphate (NAADP) was regulated with intracellular Ca^{2+} in human jurkat T cells (Berg et al., 2000) and NADH keep the sperm plasma membrane, mitochondria, and scavenger ROS (Delmas et al., 2005).

Effect of nicotinic acid on sperm motility maintained a large amount of ATP activity (Halangk et al., 1990). Actually, half of the ATP was used to maintain the motility in cattle and sheep (Halangk et al., 1990). The activation of ATP signaling pathway was released mitochondrial by metabolites such as NAD, NADH and nicotinamide adenine dinucleotide phosphate (NADPH, Verdin et al., 2010). NAD, NADP and NADPH could belong to the fundamental common mediators of various biological process (Ying, 2008). NAD and NADP produce impact factors such as mitochondrial activates, oxidative stress, prevent of cell death and aging process. Also NADPH was important in cellular antioxidants systems and prevented of ROS (Fleury et al., 2002). Therefore, the supplemented nicotinic acid helps intact mitochondria by metabolism produce mitochondrial integrity by these enzymes.

During the semen freezing, nicotinic acid was activated by the coenzyme. Adjusting the Ca^{2+} and capacitation was inhibited by coenzyme. Therefore, to increase the survival rate of sperm, acrosome reaction was in hibited, and the coenzyme was increasing mitochondrial integrity. Thus, we will study about Ca^{2+}, capacitation and mitochondria coenzyme by sperm with nicotinic acid.

The calcium signal was controlled in NAADP that release Ca^{2+} from organelles (Lee, 2001). Excessive nicotinic acid caused intracellular calcium overload don't control NAADP (Peng and Jou, 2010). Also, excessive concentration of intracellular Ca^{2+} accelerates acrosome reaction in sperm. Eventually, improper acrosome reaction in pre-fertilization was being about apoptosis and unable to fertilize the oocyte (Breitbart, 2002). Therefore, excessive nicotinic acid affect sperm characteristic, we determined that 20 mM nicotinic acid was negative control group.

In this study, frozen-thawed sperm using 5 and 10 mM nicotinic acid supplemented in freezing extender was increased to blastocyst formation. This result suggests that addition of nicotinic acid in freezing extender was improved fertility of frozen-thawed sperm. High quality sperm was fertilized with

oocyte which was development by healthy fetal (Gualtieri et al., 2014) and the higher the cleavage rate was to help development of blastocyst and hatching blastocyst stage (Cruz et al., 2011). Therefore, frozen-thawed semen supplemented with nicotinic acid was beneficial for growth of the embryos such as, the blastocyst rate and total cell number of blastocyst.

Unstable oxygen molecules produce ROS which are hydrogen peroxide, superoxide ion and hydroxyl radical that affect the mammal cells (Chaube et al., 2014). The growth of embryos was related with ROS, and supplement of antioxidants can scavenge of ROS (Zini and Al-Hathal et al., 2011). Generally, mitochondria of sperm produce energy though ATP pathway, which was involved in sperm-oocyte interaction, development of embryo and formation of blastocysts (Sawosz et al., 2012). This study was observed that mitochondria integrity in embryo of 5 and 10 mM nicotinic acid groups were higher than other groups. We also observed that sperm mitochondria regulate cleavage rate, blastocyst rate, and blastocysts of embryos.

Finally, supplement of 5 and 10 mM nicotinic acid in freezing extender has positive effect on survival, acrosomal intact and mitochondrial integrity in frozen-thawed boar semen. Especially 10 mM group have a greater effect of frozen-thawed semen quality than other groups. Moreover, in vitro development of oocytes after fertilized with 10 mM of nicotinic acid has enhanced oocyte cleavage, blastocyst rate and total cell number of blastocyst. We suggest that this result will help study of freezing sperm and development in vitro.

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