

Effects of adding melatonin on the quality of frozen-thawed boar semen

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Abstract

The aim of this study was to study the effects of adding melatonin at different concentrations on the quality of cryopreserved boar semen. Semen samples ($n = 6$) were collected by hand-glove technique. Semen samples were diluted with Modena[®] and divided into 6 groups. According to the concentrations of melatonin at 0 (control, group A), 0.1 (group B), 0.5 (group C), 1.0 (group D), 1.5 (group E) and 2.0 mM (group F) to the lactose-egg yolk extenders used to freeze boar semen with traditional method. Progressive motility, viability and acrosome integrity were evaluated both before and after cryopreservation. The results showed that there was no significant difference in percentage of progressive motility among groups. However, a higher percentage of progressive motility was found in group D (39.17%). A higher percentage of sperm viability and acrosome integrity were found in group F (28.33%) and group F (34.50%), respectively. In conclusion, the present results suggest that adding melatonin between 0.1 and 1 mM during freezing yield a superior post-thawed semen qualities than freezing without melatonin.

Keywords: antioxidant, boar semen, cryopreservation, melatonin

การศึกษาผลการเติมสารเมลานินในน้ำเชื้อสุกรแช่แข็งต่อคุณภาพน้ำเชื้อ

ณัชชา ทองเรือง นุชนารถ ใช้บางยาง พนิดา ชนาภิวัดน์ กัมพล แก้วเกษ*

ห้องปฏิบัติการน้ำเชื้อ ภาควิชาเวชศาสตร์คลินิกและการสาธารณสุข คณะสัตวแพทยศาสตร์ มหาวิทยาลัยมหิดล

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บทคัดย่อ

การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาผลของการเติมสารเมลานินที่มีความเข้มข้นแตกต่างกันในสารละลายเจือจางน้ำเชื้อต่อคุณภาพน้ำเชื้อสุกรแช่แข็ง โดยใช้ตัวอย่างน้ำเชื้อสุกรจำนวน 6 ตัวอย่าง ที่ทำการรีดเก็บด้วยวิธีการใส่ถุงมือ (hand-glove technique) จากนั้นจึงนำไปเจือจางด้วย Modena™ แล้วแบ่งออกเป็น 6 กลุ่มการทดลอง ตามความเข้มข้นของสารเมลานินในสารละลายเจือจางน้ำเชื้อ lactose-egg yolk (LEY) ซึ่งใช้ในกระบวนการการแช่แข็งน้ำเชื้อสุกร ได้แก่ 0 (กลุ่มควบคุม, กลุ่ม A) 0.1 (กลุ่ม B) 0.5 (กลุ่ม C) 1.0 (กลุ่ม D) 1.5 (กลุ่ม E) และ 2.0 มิลลิโมลาร์ (กลุ่ม F) โดยมีการประเมินคุณภาพน้ำเชื้อทั้งก่อนและหลังกระบวนการแช่แข็ง ผลการศึกษาครั้งนี้แสดงให้เห็นว่าไม่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติของอัตราการเคลื่อนที่ไปข้างหน้าของอสุจิแต่ละกลุ่ม อย่างไรก็ตามพบว่ากลุ่มที่มีอัตราการเคลื่อนที่ไปข้างหน้าของอสุจิสูงที่สุด คือ กลุ่มการทดลอง D (39.17%) กลุ่มที่มีอัตราการรอดชีวิตและความสมบูรณ์ของอะโครโซมสูงที่สุด คือ กลุ่มการทดลอง F (28.33%, 34.50% ตามลำดับ) การศึกษานี้บ่งชี้ว่าการเติมสารเมลานินที่มีความเข้มข้นระหว่าง 0.1 ถึง 1 มิลลิโมลาร์ในสารละลายเจือจางน้ำเชื้อ จะทำให้น้ำเชื้อสุกรแช่แข็งมีคุณภาพดีกว่า เมื่อเปรียบเทียบกับกรณีไม่เติมสารเมลานินในสารละลายเจือจางน้ำเชื้อ

คำสำคัญ: สารต้านอนุมูลอิสระ น้ำเชื้อสุกร น้ำเชื้อสุกรแช่แข็ง สารเมลานิน

Introduction

Cryopreservation processes including the rapid change in temperature, difference of osmolarity and the attack of free radicals, impaired the spermatozoa which affect sperm membrane damage and consequently a reduction in motility, viability and fertilizing capacity (Chatterjee et al., 2000). Boar spermatozoa are very susceptible to reactive oxygen species (ROS), residue substances of lipid peroxidation (LPO), because of the high quantities of polyunsaturated fatty acids in their plasma membrane. LPO of sperm plasma membrane is considered to be the key mechanism of oxidative stress (Kadirvel et al., 2009). Oxidative stress is caused by imbalance between the formation of ROS and scavenging activities of antioxidant system responsible for their neutralization and removal (Sikka et al., 1995). In order to minimize the ROS, the effect of supplementation of antioxidant such as alpha-tocopherol (Penä et al., 2003, Breininger et al., 2005, Kaeoket et al., 2008), glutathione peroxidase (Gadea et al., 2004; Roca et al., 2005), L-cysteine (Kaeoket et al., 2010) and curcumin (Chanapiwat and Kaeoket, 2015) have been performed and demonstrated that these antioxidant have beneficial effect on post-thawed semen qualities.

Melatonin (N-acetyl-5-methoxy tryptamine) is a derivative of tryptophan, which has high efficacy as a hydroxyl radical (.OH) scavenger (Reiter et al., 2000). It has been reported in human that melatonin also stimulates the activities of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase (Rodriguez et al., 1999; Okatani et al., 2000). In animal, melatonin has been used to improve the quality of frozen semen in ram (Kaya et al., 2001; Succu et al., 2011) and buffalo (Li et al., 2012). In boar, it has been reported that melatonin showed its ability to minimized the oxidative damage of spermatozoa induced by ROS and hydroxyl

radical (.OH) (Jang et al., 2010). To our knowledge, there is no report on effects of melatonin on the qualities of frozen-thawed boar semen. Therefore, the aim of this study was to investigate the effects of adding melatonin at different concentrations on the qualities of frozen thawed boar semen.

Materials and methods

This research project was approved by the Faculty of Veterinary Science ñ Animal Care and Use Committee, Mahidol University, Phuttamonthon, Thailand (FVS-ACUC-Protocol No. MUVS-2015-05-09).

Animals

Six healthy Duroc boars age 1-3 years old were selected. They were kept in individual pens in an evaporative cooling system. Feed with commercial feed twice a day (approximately 2.5 kg per day) and water was provided ad libitum via a nipple.

Chemical

1. Antioxidant : Melatonin; (M5250; Sigma-Aldrich Chemical, Buchs, Switzerland)
2. Solvent : Dimethyl sulfoxide (DMSO) 0.5%

Semen extenders preparation

In this study, there are three semen extenders were used for boar semen cryopreservation as follows:

1. Freezing extender I (Modena™ extender, Swine Genetics International, Ltd, Cambridge, IA, USA)
2. Freezing extender II composed of 20% of egg yolk and 11% lactose solution supplemented with different concentration of melatonin (Group A-F: 0, 0.1, 0.5, 1.0, 1.5 and 2.0 mM) in 0.5% dimethyl sulfoxide (DMSO)

3. Freezing extender III composed of 85% freezing extender II, with 9% (v/v) glycerol and 1.5% (v/v) Equex-STM® (Nova Chemical Sales Inc., Scituate, MA, USA)

Semen collection and preparation

Boar semen samples were collected by using glove-hand technique (Chanapiwat et al., 2009; Kaeoket et al., 2010). The semen was filtered through gauze and only sperm rich was collected and evaluated with parameters including semen volume, pH, sperm motility, concentration, sperm viability and morphology. Only sperm-rich fraction ejaculates with a motility of 70% and 80% morphologically normal were used for cryopreservation. After that, semen was diluted with Modena™ extender to 1:1 (v/v) and transported by cell incubator (Micom control system 20 Q, Continental plastic CORP, Delavan, WI, USA) at 15°C to the semen laboratory, Faculty of Veterinary Science, Mahidol University.

Semen freezing and thawing process

After incubation at 15°C for 120 min, diluted semen was divided to 6 parts and transferred into 50 ml centrifuge tubes and centrifuged at 2000 rpm, 15°C for 10 min (Hettich Universal 32R, Tuttlingen, Germany). After centrifugation, supernatant was discarded and freezing extender I to a concentration of 1.5×10^9 sperm/ml (Chanapiwat et al., 2009). After cooling at 5°C for 90 min, diluted semen was mixed with freezing extender II to concentration of 1.0×10^9 sperm/mL. The processed semen were loaded into 0.5 ml polyvinyl chloride medium straws (Bio-Vet, Z.I. Le Berdoulet, France) and placed on a rack in an expandable polystyrene box (20.5 cm x 31 cm x 18.5 cm) which contained liquid

nitrogen. The straws were placed in contact with nitrogen vapor at 4 cm above the liquid nitrogen level for 20 min and then plunged into liquid nitrogen tank (at 196°C) for storage. Frozen semen samples were thawed at 50°C for 12 sec. The thawed semen sample was diluted with Modena™ extender (1:6 v/v) and kept in an incubator at 37°C until semen evaluation (Chanapiwat et al., 2009).

Evaluation of sperm

Progressive motility

Progressive sperm motility was evaluated at 37°C under a phase contrast microscope at 100 x magnification. Progressive motility was expressed as the percentage of motile spermatozoa.

Concentration

Sperm concentration was assessed by a spermacue™ at farm and assessed by Neubauer hemocytometer (Improved Neubauer's chamber, BOECO, Humburg, Germany) after diluting semen with distilled water to 1:100 (v/v) and evaluation by phase contrast microscopy at 400 x magnification. Sperm concentration was expressed as sperm $\times 10^6$ sperm/mL.

Viability (viable and non-viable spermatozoa)

The sperm viability was assessed, 10 μ L of diluted semen was mixed with 2.7 μ L of SYBR-14 and 10 μ L of Ethidiumhomodimer-1 (EthD-1). After incubation at 37°C for 15 min, at least 200 sperms were assessed under fluorescence microscope at 400 x magnification. The nuclei of live sperm was stained as a green color with SYBR-14, and sperm with damaged plasma membrane or dead sperm stained as a red color with EthD-1. The sperm viability was expressed as the percentage of live sperm with intact plasma membrane.

Acrosome integrity

The evaluation of sperm acrosome integrity was conducted by using fluorescein isothiocyanate labeled peanut (*Arachis hypogaea*) agglutinin (FITC-PNA) staining, 10 μ L of diluted semen was mixed with 10 μ L of EthD-1 and incubated at 37°C for 15 min before smearing 5 μ L of the mixture on a glass slide and air drying. It was fixed with 95% ethanol for 30 s and air dried. Fifty microliters of FITC-PNA solution (diluted FITC-PNA with phosphate buffered saline (PBS) 1:10 v/v) was spread over the slides and kept in a moist chamber at 4°C for 30 min. Then, slides were rinsed with cold PBS and air dried. At least 200 sperm were assessed by fluorescence microscope at 1000x magnification (Axioskop 40; Carl Zeiss, Inc., Oberkochen, Germany) and classified as intact acrosomes and non-intact acrosomes. The results were presented as the percentage of live sperm with intact acrosome.

Sperm morphology

Head abnormality evaluated by using William's stain and tail abnormality evaluated by formal saline. At least 200 sperm were assessed by phase contrast microscopy at 400x magnification. The results were presented as the percentage of abnormal sperm morphology with normal sperm.

Statistical analyses

The statistical analysis was performed using Statistical Analysis Systems software package (Version 9.0, SAS Institute Inc., 1996, Cary, N.C., USA). Normality of the data was evaluated using UNIVARIATE procedure option NORMAL PLOT. The sperm parameters not normally distributed (sperm motility, sperm viability and acrosome integrity) were transformed using arcsine

transformation. All sperm parameters were analyzed using General Linear Mixed Model (MIXED) procedure of the SAS. The model included group of extenders (control, treatment groups) as a fixed effect and included boar as random effects. Statistically significant difference was defined as $P < 0.05$.

Results

Fresh semen analysis

On average, the percentage of progressive motility, sperm viability, acrosome integrity, head and tail morphology were 85.83 (range 75-95), 71.75 (range 41.5-88), 82.25 (range 53-95), 94.33 (range 88.5-98.5), and 80.67 (range 59-91), respectively. The concentration and volume of fresh semen were varied between 354-585 x 10⁶ sperm/ml and 100-190 ml, respectively.

Post-thawed semen analysis

The results of post-thawed semen qualities are showed in Table 1. There was no significant difference in percentage of progressive motility among groups. However, a higher percentage of progressive motility was found in group D (39.17%). No significant difference for percentage of sperm viability among groups was found. However, a higher percentage of sperm viability was found in group F (28.33%). Although, no significant difference in percentage of acrosome integrity among groups was found, a higher percentage of acrosome integrity was found in group F (34.50%).

Table 1 Percentages of progressive motility, sperm viability and acrosome integrity in frozen-thawed boar semen (means \pm SD).

Groups	Progressive motility (%)	Sperm viability (%)	Acrosome integrity (%)
A (Control)	28.33 \pm 5.16 ^{ab}	22.00 \pm 10.52 ^a	29.33 \pm 16.06 ^a
B (0.1 mM)	35.00 \pm 8.49 ^a	24.25 \pm 6.55 ^a	32.67 \pm 8.1 ^a
C (0.5 mM)	30.83 \pm 8.01 ^{ab}	19.50 \pm 9.72 ^a	29.05 \pm 9.81 ^a
D (1.0 mM)	39.17 \pm 15.30 ^a	20.67 \pm 10.40 ^a	26.28 \pm 14.68 ^a
E (1.5 mM)	18.33 \pm 8.16 ^b	16.75 \pm 11.04 ^a	24.75 \pm 10.49 ^a
F (2.0 mM)	29.17 \pm 10.21 ^{ab}	28.33 \pm 18.59 ^a	34.50 \pm 11.71 ^a

Values in the same column with different letters means significant difference (P< 0.05).

Discussion

Nevertheless, in the present study, there was no statistically significant difference in percentage of progressive motility between control and treatment groups, however, adding melatonin between 0.1 and 1.0 mM in treatment groups (30.83-39.17%) showed a higher percentage of progressive motility than in control group (28.33%). This indicated that freezing boar semen without melatonin may yield an inferior post-thawed semen quality. In contrast, Albring et al. (2016) reported that adding melatonin between 1.25-5 mM did not have a protective effect on boar sperm motility and acrosomal membrane after freezing and thawing process. A lower concentration of melatonin used in our study may explained this difference. Melatonin has previously been used in chilled boar semen and showed that chilled boar semen need only small amount

of melatonin (i.e. 0.1 μ M) to improve the sperm quality parameters including sperm motility, viability, membrane integrity, mitochondrial activity and survival rate during hydrogen peroxide-induced oxidative stress (Jang et al., 2010). In addition, melatonin supplementation at 1 μ M resulted in an improvement of proportion of live sperm with intact acrosome during long term storage at 17°C (Martín et al., 2011). Considering the concentration of melatonin used for fresh semen storage and during freezing semen, the latter need rather high concentration of melatonin to diminish the effect of cryodamage. The result in present study also in agreement with other studies in ram (Succu et al., 2011), bull (Iraj et al, 2013) (Karimfar et al., 2015) and mouse (Chen X et al., 2016) in that melatonin has a beneficial effect on post-thawed semen qualities. The reason might be that melatonin with its antioxidant activity, able to neutralize free radical

and ROS, stimulates antioxidant enzyme including superoxide dismutase, glutathione peroxidase and glutathione dismutase (Reiter et al., 2000). In addition, adding too high concentration of melatonin in the freezing extender can cause detrimental effect on the sperm which is also found in other studies (Kaeoket et al., 2008; 2010; Chanapiwat et al., 2009). To be honest, a high variation of results in each parameter found in the present study may explained by individual boar variation in term of good and poor freezability as has been earlier described by Chanapiwat et al. (2009) and Kaeoket et al. (2011).

In conclusion, the present results suggest that adding melatonin between 0.1 and 1 mM during freezing yield a superior post-thawed semen qualities than freezing without melatonin.

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