Original Article

The Effect of Using Long Term and Short Term Extenders during Cooling Process on the Quality of Frozen Boar Semen

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Abstract

Cooling process of cryopreservation is one of the important factors that affect the qualities of frozen-thawed boar semen. The objective of this study was to compare the qualities of frozen-thawed boar semen after using different extenders (i.e. BTS, Vitasem LD, Modena™ and Androstar® plus) during cooling process. Eight sperm-rich fractions from 8 boars were employed. The ejaculated semen sample from each boar was divided into four groups and extended in different freezing extender I as follows: group I (control, short term, BTS), group II (long term, Vitasem LD), group III (long term, Modena™) and group IV (long term, Androstar® plus) and kept at 15^{o}C for 2 hours (so-called cooling process) before cryopreservation. Thereafter, the semen samples were further evaluated for semen qualities at 2 hours post-cooling and also after post-thawing. For post-cooling, the highest percentage of motility and viability were found in treatment groups (II, III and IV) compared with control group (p<0.05). For post-thawing, the highest percentage of motility was found in groups I and II. A tendency of higher percentage of viability was found in treatment group IV than control group. In conclusion, in the term of progressive motility and viability, the results indicate that using long term extenders as freezing extender I during the cooling process yields a superior semen quality at post-cooling than using short term extender.

Keywords: boar semen, cooling time, cryopreservation, long term extenders, short term extender

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บทคัดย่อ

การศึกษาผลของการใช้สารละลายเจือจางน้ำเชื้อชนิดระยะสั้นและชนิดระยะยาวเป็นสารละลายเจือจางน้ำเชื้อที่ 1 ต่อคุณภาพของน้ำเชื้อสุกรแช่แข็ง

กัมพล แก้วเกษ พิมพ์สิริ ชนะภัย ภูริวัจน์ จันทร์ชัยภูมิ พนิดา ชนาภิวัตน์*

ขั้นตอนการแช่เย็น (cooling process) ในกระบวนการผลิตน้ำเชื้อแช่แข็งนั้นเป็นหนึ่งในปัจจัยที่สำคัญและมีผลกระทบต่อคุณภาพของน้ำเชื้อสุกรแช่แข็ง ดังนั้นการศึกษาในครั้งนี้จึงมีวัตถุประสงค์เพื่อเปรียบเทียบคุณภาพของน้ำเชื้อสุกรแช่แข็งหลังจากการใช้สารละลายเจือจางน้ำเชื้อสำหรับแช่แข็งที่ 1 ที่แตกต่างกัน (BTS Vitasem LD Modena™ และ Androstar® plus) ในขั้นตอนการแช่เย็น โดยในการทดลองครั้งนี้จะใช้น้ำเชื้อสดที่รีดมาจากพ่อสุกรจำนวนแปดตัว โดยน้ำเชื้อสดจากพ่อสุกรแต่ละตัวนั้นจะถูกแบ่งออกเป็น 4 กลุ่มแล้วเติมสารละลายเจือจางน้ำเชื้อสำหรับแช่แข็งที่ 1 ในแต่ละกลุ่มดังนี้ กลุ่มที่ 1 ใช้สารละลายเจือจางน้ำเชื้อชนิดระยะสั้น BTS (กลุ่มควบคุม) กลุ่มที่ 2 ใช้สารละลายเจือจางน้ำเชื้อระยะยาวชนิด Vitasem LD กลุ่มที่ 3 ใช้สารละลายเจือจางน้ำเชื้อระยะยาวชนิด Modena™ และกลุ่มที่ 4 ใช้สารละลายเจอจางน้ำเชื้อระยะยาวชนิด Androstar® plus จากนั้นเก็บตัวอย่างไว้ที่อุณหภูมิ 15 องศาเซลเซียสนาน 2 ชั่วโมงก่อนเริ่มกระบวนการแช่แข็ง โดยทำการตรวจคุณภาพน้ำเชื้อทั้งในช่วงหลังขั้นตอนการแช่เย็นและภายหลังจากการแช่แข็ง โดยผลการตรวจคุณภาพน้ำเชื้อภายหลังขั้นตอนการแช่เย็น พบว่าในกลุ่มทดลอง (2 3 และ 4) มีค่าอัตราการเคลื่อนที่ไปข้างหน้าและอัตราการมีชีวิตรอดของตัวอสุจิมากกว่ากลุ่มทดลอง (p<0.05) สำหรับคุณภาพน้ำเชื้อภายหลังจากผ่านกระบวนการแช่แข็งพบว่าอัตราการเคลื่อนที่ไปข้างหน้ามากที่สุดในกลุ่มที่ 1 และ 2 และพบว่ามีแนวโน้มของอัตราการมีชีวิตรอดของตัวอสุจิในกลุ่มที่ 4 สูงกว่ากลุ่มควบคุม จากผลการทดลองแสดงให้เห็นว่าการใช้สารละลายเจือจางน้ำเชื้อชนิดระยะยาวเป็นสารละลายเจือจางน้ำเชื้อสำหรับแช่แข็งที่ 1 ในช่วงขั้นตอนการแช่เย็นสามารถรักษาคุณภาพน้ำเชื้อในแง่ของอัตราการเคลื่อนที่ไปข้างหน้าและอัตราการมีชีวิตรอดของตัวอสุจิ ในช่วงขั้นตอนการแช่เย็นได้ดีกว่าการใช้สารละลายเจือจางน้ำเชื้อชนิดระยะสั้น

คำสำคัญ: น้ำเชื้อสุกร ระยะพัก กระบวนการแช่แข็ง สารละลายเจือจางน้ำเชื้อชนิดระยะสั้น สารละลายเจือจางน้ำเชื้อชนิดระยะยาว

ห้องปฏิบัติการน้ำเชื้อ ภาควิชาเวชศาสตร์คลินิกและการสาธารณสุข คณะสัตวแพทยศาสตร์ มหาวิทยาลัยมหิดล ถนนพุทธมณฑล 4 ต. ศาลายา อ. พุทธมณฑล จ. นครปฐม 73170

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Introduction

Cryopreservation process plays an important role in frozen-thawed boar semen qualities in that the viability of spermatozoa in frozen-thawed semen is reduced to more than 50% after freezing, which results in low conception rate and small litter size after artificial insemination (Almlid and Hofmo, 1996; Eriksson et al., 2002). The low fertility of frozen-thawed semen is mainly due to cold shock during cooling and freezing procedures as well as the fluctuation of the temperature during thawing, which cause the damage of highly sensitive plasma membrane of boar spermatozoa (Holt, 2000; Watson, 2000). However, the viability and fertility of the frozen-thawed boar semen is significantly improved when the extended fresh semen is held at 15°C (cooling process) for over 3 hours before cryopreservation (Almlid and Johnson, 1988; Eriksson et al., 2001).

In pig industry, the boar semen used for artificial insemination is extended with semen extender and kept in cold storage at 18-20°C for few days before artificial insemination. It has recently been reported that using long term extenders (i.e. Androstar®Plus, Modena™, Vitasem LD) to preserve fresh semen for 7 days yields a superior fresh boar semen qualities compared with using short term extender (Kaeoket et al., 2010[d]). In addition, the difference in extended fresh semen qualities was also found depending on each type of long term extender used. This indicates that some constituents in each long term extender may assist sperm in overcoming cold shock during cold storage. Generally, the difference between the short term and long term extenders are the ingredients contained in the extenders. Long term extenders contain complex buffering agent (i.e. HEPES, Tris, TES and MOPs) and antioxidants (i.e., bovine serum albumin (BSA), beta-carotene, cysteine, taurine, vitamin E and ascorbic acid) (Alvarez and Storey, 1995; Gadea, 2003;

Funahashi and Sano, 2005), which can maintain semen qualities during cold storage for a longer period than short term extender. For these reasons, it can be hypothesized that the constituents in long term extenders which are employed during cooling process may improve the post-cooling fresh semen qualities and also post-thawing semen qualities. The objective of this study was to compare the post cooling and post thawing semen qualities after using different semen extenders (short term extender versus long term extender) as freezing extender I during cooling process.

Materials and Methods

The research proposal of this project was approved by the Institution of Animal Care and Use Committee (FVS-ACUC)-protocol no. MUVS-2010-13.

Animals: Eight boars, consisting of Landrace (n=3) and Duroc (n=5), aged between 1-4 years old, having fertility and being routinely used for semen collection for AI were included in the present study. The boars were housed in individual pens in an evaporative cooling system. Water was provided ad libitum via a water nipple. A corn-soyabean-fishmeal based feed (15-16% protein) was given twice a day (approximately 3 kg/day).

Preparation of boar spermatozoa: Semen samples from each boar (one ejaculate from each boar) were collected by using gloved-hand method (Kaeoket et al., 2002[a], 2005, 2010[a]). The semen was filtered through gauze and only sperm rich fraction was collected. Within 30 min after semen collection, semen volume and progressive motility of spermatozoa were determined by a phase contrast microscope (Olympus CX31, New York, NY, USA). The semen sample of 1 ml was examined after collection into Eppendorf tubes for further analysis of concentration by using Neubauer hemocytometer (improved Neubauer's chamber, BOECO, Humburg, Germany), sperm viability by using the SYBR-14 staining (Fertilight;Sperm Viability Kit, Molecular Probes Europe BV, Leiden, the Netherlands), sperm acrosome integrity by using FITC-PNA staining and sperm morphology by using Williams staining and formal-saline solution (Kaeoket, et, al., 2008, 2010*b*). Only ejaculates with motility of ≥ 70% and normal morphology of ≤ 80% were used for cryopreservation.

The fresh semen sample was divided into 4 groups (I, II, III and IV) for dilution (1:1 v/v)with freezing extender I, i.e. Beltsville Thawing Solution (BTS, Minitübe, Abfüll-und Labortechnik GmbH & CO. KG, Tiefenach, Germany), Vitasem LD (Magapor S.L., Zaragoza, Spain), Modena™ (Swine Genetics International Ltd., Iowa, USA) and Androstar® plus (Minitübe, Abfüll-und Labortechnik GmbH & CO. KG, Tiefenach, Germany) respectively, according to the experimental design and transported by cell incubator (Micom control system 20Q, Continental Plastic CORP, Delevan, WI, USA) at 15°C to semen laboratory, Faculty of Veterinary Science, Mahidol University.

Semen freezing process: All semen samples were frozen in a controlled-rate freezer (Icecube 14s, Sylab, Purkersdorf, Austria). After collection and evaluation, the fresh semen was divided into 4 groups for dilution (1: 1 v/v) with BTS (gr. I, control), Vitasem LD (gr. II), Modena™ (gr. III) and Androstar® plus (gr.IV) respectively. All diluted semen was transferred to 50 ml centrifuge tubes, cooled at 15°C for 120 min and then centrifuged at 800xg at 15°C for 10 min (Hettich Rotanta 460R, Tuttlingen, Germany). The supernatant was discarded and the sperm pellet was re-suspended (1-2:1) with lactose-egg yolk (LEY) extender (80 ml of 11% lactose solution and 20 ml egg yolk, extender II) to a concentration of 1.5×10^9 sperm/ml (Kasetrtut and Kaeoket, 2010).

The diluted semen was incubated at 5oC for 90 min. The four groups of semen were each mixed with a half volume of extender III (89.5% lactose-egg yolk (LEY) extender with 9% (v/v) glycerol and 1.5% (v/v) Equex-STM paste (Novo chemical sale Inc., Scituate, MA, USA).

The final semen concentration was approximately 1.0×10^9 spermatozoa/ml. The sperm suspension was loaded into 0.5 ml medium-straws (Bio-Vet, Z.I. Le Berdoulet, France) and sealed by plasticine. All straws were placed horizontally on rack and put into a chamber of the controlled-rate freezer set to +5°C. The cooling/freezing rate was perform according to Kaeoket et al. (2008, 2010[c]). Then, the straws were immediately plunged into liquid nitrogen (-196°C) for storage and further analysis.

Semen thawing process: Thawing of straw was carried out in warm water at 50°C for 12 sec (Selles et al., 2003). Frozen semen samples were thawed in different post-thawing solutions depending on their freezing extender I. The diluted thawed semen samples were incubated in a 37°C water bath for 15 min before post-thawed semen quality assessment (Kasetrtut and Kaeoket et al., 2010).

Sperm qualities assessment

Sperm motility: Progressive sperm motility was subjectively evaluated at 37°C in phase contrast microscope at x100 and x400 magnification (Berger et al., 1985). Visual estimation was done by the same person throughout the study, who was unaware of the treatments. Progressive motility was expressed as the percentage of motility sperm cells.

Sperm viability: The percentages of sperm viability were evaluated with SYBR-14/EthD-1 (Fertilight, Sperm Viability Kit, Molecular Probes). Ten µl of diluted spermatozoa were mixed with 2.7 µl of the working solution of SYBR-14 and 10 µl of EthD-1. After incubation at 37°C for 20 min, a total of 200 spermatozoa were assessed (x400) in fluorescence microscope (Carl Zeiss Inc., Axioskop 40, Oberkochen, Germany). The nuclei of spermatozoa with intact plasma membranes stained green with SYBR-14, while those damaged membranes stained red with EthD-1. Spermatozoa were classified into three types as earlier describe by Kaeoket et al., (2010[a,b,c]). The results were scored as the percentage of viability spermatozoa and non-viability (damaged and dead spermatozoa).

Acrosome integrity in live spermatozoa: The integrity of the sperm acrosome was evaluated using FITC-PNA staining (Carvajal et al., 2004, Chanapiwat et al., 2009, 2010). Ten µl of diluted semen with 140 µl phosphate buffered saline (PBS) were mixed with 10 µl of EthD-1 and incubated at 37^{o}C for 15 min. Then, 5 µl of suspension were smeared on glass slides and fixed with 95% ethanol for 30 sec and air-dried. In the next step, 50 µl Fit-C-PNA (diluted FitC-PNA with PBS 1:10 v/v) were spread over the slide and incubated in a moist chamber at 4^{o}C for 30 min. After being incubated, the slide was rinsed with cold PBS and air-dried. A total of 100 spermatozoa were assessed in fluorescence microscope at x1000 magnification, and classified as those with intact acrosomes and those with non-intact acrosome (reacted and loose acrosome).

Statistic analysis: Data were analyzed by using general linear model (GLM) (SPSS 18.0; SPSS Inc, Chicago, IL, USA) and expressed as the mean±SD. The normality of dependent variables was determined by Kolmogorov-Smirnov test. The specific treatment was modeled according to the Factorial Experiments in randomized complete block design (RCBD) and analyzed with general linear model. When the GLM revealed a significant effect, the mean values were compared by Duncan test with $p < 0.05$.

Results

Fresh semen analysis parameters: The percentages of normal sperm morphology, progressive motility, viability and acrosome integrity of fresh semen samples were 88.7±5.5, 74.0±4.4, 72.1±10.3 and 43.0±18.4, respectively.

Post-cooling semen qualities

Progressive motility: The percentage of progressive motility in groups II, III and IV were significantly higher (p<0.05) than group I (Fig 1).

Viability of spermatozoa (alive and non-alive spermatozoa): The percentage of live spermatozoa in groups II, III and IV were significantly higher (p<0.05) than group I (Fig 2).

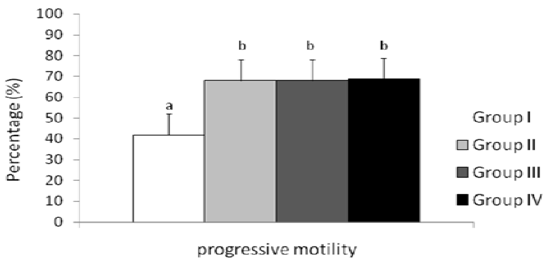

Figure 1 Post cooling progressive motility of semen samples in different extenders: Group I (BTS), Group II (Vitasem LD), Group III (Modena™), Group IV (Androstar® plus) presented as bar (mean±SD). Bar marked by different letters are significantly different (p<0.05).

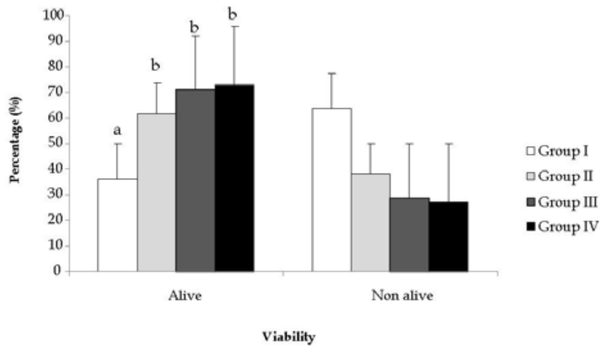

Figure 2 Post-cooling viability of semen samples in different extenders: Group I (BTS), Group II (Vitasem LD), Groups III (ModenaTM) and group IV (Androstar® plus) presented as bar (mean ± SD). Bar marked by different letters are significantly different (p<0.05).

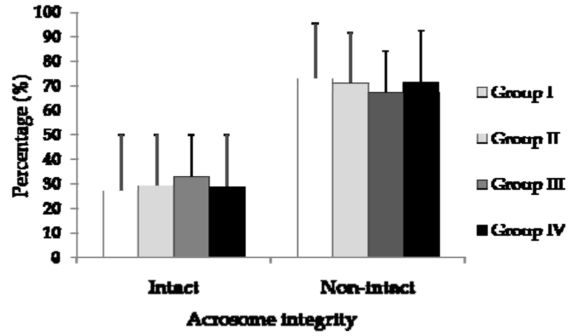

Figure 3 Post-cooling acrosome integrity of semen samples in different extenders: Group I (BTS), Group II (Vitasem LD), Group II (Modena™) and Group IV (Androstar® plus) presented as bar (mean±SD).

Acrosome integrity in live spermatozoa (intact and non-intact spermatozoa): There is a tendency of higher percentage of acrosome integrity in group III than the other groups (Fig 3).

Post-thawing semen qualities

The percentage of progressive motility was significantly higher in groups I and II compared to groups III and IV (p<0.05, Fig 4). In addition, there is a tendency of higher percentage of live spermatozoa in group III than the other groups, and also a tendency of acrosome integrity in group IV than the other groups (Fig 4).

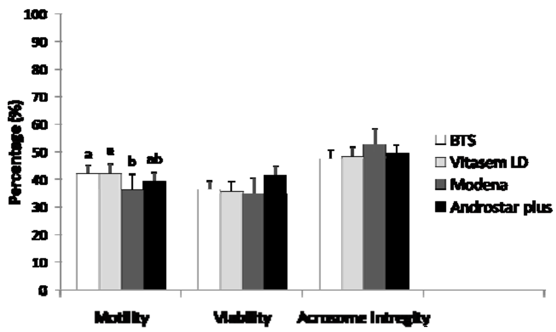

Figure 4 Post-thawing semen qualities in four different freezing extenders I: Group I (BTS), Group II (Vitasem LD), Group III (Modena™) and Group IV (Androstar® plus) presented as bar (mean±SD). Bar marked by different letters are significantly different (p<0.05).

Discussion

The present results showed that using long term extenders as freezing extender I could maintain boar spermatozoa qualities after cooling and post-thawing processes better than short term extender (BTS). This is in agreement with Guthrie and Welch (2005) who reported that the storage of semen prior to cryopreservation in different extenders had a significant effect on spermatozoa qualities after-cooling and post-thawing. Nevertheless, the different results between short term and long term extenders seen in the present study might be explained by the different constituents of short term and long term extenders, in which long term extenders are composed of a more complex buffering agents which are an effective pH regulators, and also antioxidants (i.e. bovine serum albumin, BSA) in which BSA protects sperm against cold shock and inhibits lipid peroxidation (Alvarez and Storey, 1995; Gadea, 2003; Funahashi and Sano, 2005). In addition, it is well documented that during cold storage, glycolytic metabolism of spermatozoa leads to a reduced intracellular pH, subsequently cell metabolism is suppressed, which in turn, affect both the metabolism and motility of spermatozoa (Medeiros et al., 2002). However, these detrimental effects can be overcome by buffering agents which contain in each semen extender (Gadea, 2003).

Besides, long term extenders used in the present study is also composed of EDTA and cysteine. During cooling or cryopreservation, an increase in intracellular calcium level can induce sperm capacitation and acrosome reaction, subsequently reduces the motility and freezing ability of spermatozoa. However, this reaction can be solved by EDTA which is a chelating agent that blocks the action of calcium (McLaughlin and Ford, 1994). Cysteine (L-cysteine and N-acetyl-cysteine), an antioxidant, has ability to maintain plasma membrane by its membrane stabilizer property and inhibits sperm capacitation (Johnson et al., 2000). The positive effect of L-cysteine as an antioxidant for cold storage of boar spermatozoa has been earlier demonstrated by Funahashi and Sano (2005). Recently, Kaeoket et al. (2010[d]) demonstrated that changes in motility, viability and acrosome integrity during cold storage of fresh boar semen as long as 7 days were depended upon the extender utilized, particularly its constituents, in which long term extenders maintained a superior sperm quality compared with short term extender.

Furthermore, it has been reported that reactive oxygen species (ROS) produced by oxidative stress during cold storage and cryopreservation have a detrimental effect on sperm plasma membranes, particularly lipid peroxidation (LPO) (Sikka et al., 1995; Uysal and Bucak, 2007). This reaction had negative effects on spermatozoa such as inhibiting respiration and causing intracellular enzymes leakage (White, 1993), which resulted in irreversible loss of motility and sperm permeability. In recent times, it has been shown that supplementing the freezing extender with a variety of antioxidants (i.e. L-cysteine, vitamin E, glutathione) improves the viability, acrosome integrity and motility of cryopreserved boar spermatozoa (Kaeoket et al., 2008; Chanapiwat et al., 2009, 2010; Kaeoket et al. 2010[a,b,c,d]) and also frozen-thawed bull semen (Bilodeau et al., 2001). The important role of antioxidants which constituent in particular long term extenders can also be seen, in the present results, in that a higher post-thaw boar semen quality was observed when long term extender was employed as freezing extender I.

Conclusion

In conclusion, in the term of progressive motility and viability, the results indicate that using long term extender as freezing extender I during the cooling process yields a superior semen quality at post-cooling than using short term extender.

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