Original Article

A Comparative Study on the Efficacy of Four Semen Extenders and Thawing by Seminal Plasma on the Quality of Frozen-thawed Boar Semen

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Abstract

The present study investigated the influence of four different commercial semen extenders used during the holding time and the use of seminal plasma as a thawing medium on the frozen-thawed (FT) boar semen quality. Sperm-rich fractions were collected from 11 boars and were divided into four groups according to the type of semen extender used during the holding time (A, B, C and D). The extended semen was cooled down and centrifuged. The sperm pellets were diluted in lactose-egg yolk extender plus glycerol and Equex STM Paste. The semen were loaded into 0.5 ml straws and frozen. Thawing was achieved by immersing the straws in 50^0C water for 12 sec. The semen was divided into two groups: the control group was diluted in BTS; the treatment group was diluted in seminal plasma. The post-thawed sperm qualities including subjective motility, sperm viability, functional integrity of the sperm plasma membrane and acrosome integrity were evaluated. It was found that semen extended in extender D for 2 hours before cryopreservation yielded a higher individual motility than those extended in extender B (20.9% and 16.9%, p= 0.045) and tended to be higher than those extended in extender A (17.5%, p= 0.090). Thawing of cryopreserved boar semen in BTS yielded a significantly higher subjective motility than thawing in seminal plasma (20.3% and 16.9%, p= 0.021). It could be concluded that both types of extender used during the holding time and the thawing medium significantly influenced FT boar sperm quality.

Keywords: cryopreservation, pig, sperm, thawing medium

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บทคัดย่อ

การศึกษาเปรียบเทียบประสิทธิภาพของสารละลายน้ำเชื้อสุกร 4 ชนิด และการทำละลายด้วยเซมินัลพลาสมาต่อคุณภาพของน้ำเชื้อสุกรแช่แข็ง

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การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาผลของสารละลายน้ำเชื้อพ่อสุกร 4 ชนิดที่ใช้ในการเก็บรักษาน้ำเชื้อหลังรีดเก็บน้ำเชื้อก่อนทำการแช่แข็ง และการใช้เซมินอลพลาสมาสำหรับทำละลายต่อคุณภาพของน้ำเชื้อพ่อสุกรหลังการแช่แข็ง รีดเก็บน้ำเชื้อพ่อสุกรจากส่วนที่มีอสุจิมากจากพ่อสุกร 11 ตัว แล้วนำมาแบ่งเป็น 4 กลุ่ม ตามชนิดของสารละลายน้ำเชื้อ 4 ชนิด (A B C และ D) น้ำเชื้อถูกนำมาเจือจางในสารละลายน้ำเชื้อแต่ละชนิด สารละลายน้ำเชื้อถูกทำให้เย็นลงแล้วนำมาปั่น ส่วนของเซลล์อสุจิที่ตกตะกอนถูกนำมาเจือจางด้วยสารละลายน้ำเชื้อชนิดที่มีไข่แดงและน้ำตาลแลคโต๊สเป็นส่วนประกอบร่วมกับสารกลีเซอรอลและสารอีเควกเอสทีเอ็มเพส น้ำเชื้อที่เจือจางแล้วถูกนำมาบรรจุในหลอดฟางขนาด 0.5 มิลลิลิตร แล้วนำไปแช่แข็ง ทำละลายน้ำเชื้อโดยการจุ่มหลอดบรรจุน้ำเชื้อในน้ำอุ่นอุณหภูมิ 50°ซ. นาน 12 วินาที น้ำเชื้อถูกแบ่งเป็น 2 กลุ่ม กลุ่มควบคุมถูกเจือจางโดยสารละลายบีทีเอส และกลุ่มทดลองถูกเจือจางในเซมินอล พลาสมา สารละลายน้ำเชื้อถูกนำมาประเมินคุณภาพหลังการทำละลายซึ่งประกอบด้วย ร้อยละการเคลื่อนไหวเป็นรายตัว อัตราการมีชีวิตของอสุจิ ความสมบูรณ์ของเยื่อหุ้มเซลล์อสุจิ และความสมบูรณ์ของอะโครโซม ผลการทดลองพบว่าน้ำเชื้อพ่อสุกรที่ถูกทำละลายในสารละลาย D นาน 2 ชั่วโมงก่อนนำไปแช่แข็ง มีอัตราการเคลื่อนไหวเป็นรายตัวสูงกว่าน้ำเชื้อที่ทำละลายในสารละลายน้ำเชื้อชนิด B (20.9% และ 16.9%, p= 0.045) และมีแนวโน้มสูงกว่าสารละลายน้ำเชื้อชนิด A (17.5%, p= 0.090) การทำละลายน้ำเชื้อพ่อสุกรในสารละลายบีทีเอส ได้อัตราการเคลื่อนไหวเป็นรายตัวสูงกว่าการทำละลายน้ำเชื้อพ่อสุกรในเซมินอลพลาสมา (20.3% และ 16.9%, p= 0.021) ผลการศึกษาสรุปได้ว่าทั้งชนิดของสารละลายน้ำเชื้อที่ใช้เก็บรักษาน้ำเชื้อพ่อสุกรก่อนกระบวนการแช่แข็ง และชนิดของสารละลายที่ใช้ในการทำละลายน้ำเชื้อแช่แข็ง มีอิทธิพลต่อคุณภาพของน้ำเชื้อพ่อสุกรหลังการทำละลายอย่างมีนัยสำคัญ

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Introduction

Cryopreservation of boar semen can damage the sperm and lead to various types of cryoinjury, e.g. membrane disorder, acrosome defect and cholesterol efflux (Vadnais and Althouse, 2011). Previous report found that the individual motility, plasma membrane integrity and acrosome integrity of the boar sperm significantly reduced after cryopreservation (Buranaamnuay et al., 2009; Chanapiwat et al., 2009[a]). It is known that the boar sperm are more sensitive to temperature shock than that of other species. On average, the use of FT boar semen for artificial insemination in swine industry resulted in a 20% lower conception rate than the use of extended fresh semen (Chanapiwat et al., 2009[b]; Buranaamnuay et al., 2010). Therefore, additional researches are required to investigate the ways to minimize cryoinjury and enhance the qualities FT boar semen.

It is well established that cryopreservation process significantly affects post-thawed semen qualities in all species, especially the boar. The cryoinjury is mainly affected by the formation of ice crystal within the sperm cell during cryopreservation, while the temperature shock is caused by a rapid reduction of the temperature during either cooling and freezing processes. Therefore, a cryoprotective agent is necessary. Currently, the most common cryoprotectant of the boar sperm is 3.0% glycerol (Kim et al., 2011). During the present time, the FT boar semen technology is rapidly developed with special emphasis on genetic preservation and the distribution of a good genetic resource worldwide. Our recent study has demonstrated that the modification of the semen extender can significantly improve the post-thawed boar semen qualities (Chanapiwat et al., 2009[b]). Generally, the sperm-rich fraction of the ejaculated boar semen is extended in a commercial extender for at least 2 hours at 15°C before cryopreservation, so called "holding time"(Eriksson et al., 2001). It has been demonstrated that the length of the holding time significantly influences post-thawed sperm qualities (Guthrie and Welch, 2005). However, the effect of the type of the semen extender used

during the holding time on the FT boar semen qualities has not been investigated.

A supplementation of 10% v/v seminal plasma or 50% v/v seminal plasma to the thawing media has been shown to improve the FT sperm motility, viability and pregnancy rate (Garcia et al., 2009; Okazaki et al., 2009). However, Abad et al. (2007) found that 10% v/v seminal plasma supplementation for conventional AI with FT boar semen failed to improve both litter size and farrowing rate in multiparous sows. Therefore, studies on the FT semen qualities in relation to the seminal plasma supplementation still need further investigation.

The objectives of the present study were to investigate the influence of four different commercial extenders used during the holding time on the FT boar semen quality and to determine the FT semen quality after using seminal plasma as a thawing medium.

Materials and Methods

Animal: The experiment was conducted at artificial insemination center of a commercial swine herd in Chonburi province, Thailand, during May and August 2010. In total, 11 fertility-proven boars routinely used in the farm were included in the experiment. The boars were selected based on previous information on their semen quality and freezing ability. The ejaculates included in the trial were required to have ≥70% subjective motility, ≥75% normal morphology, ≥75% sperm viability with a volume of ≥100 ml per ejaculate. The boars were housed in an evaporative cooling system and each was allocated in individual pens with space allowance of 2.0 m^2/pen. Feed was provided twice a day with a corn-soybean-fish based commercial feed, containing approximately 15.0% crude protein. Water was provided *ad libitum* via water nipples.

Semen collection: Each boar was allowed to mount a dummy and the sperm-rich fraction of the ejaculates was collected with the glove-handed method. The boars rested for at least 5 days before each semen collection.

Semen cryopreservation: The sperm-rich fraction of the ejaculated semen was divided into four groups according to the type of commercial semen extender used during the holding time of semen freezing process (extender I) [i.e., extender A, Beltsville Thawing Solution (BTS) (Minitüb GmbH Tiefenbach, Germany), extender B, Optim® (Magapor, Zaragoza, Spain), extender C, MIII® (Minitüb, Tiefenbach, Germany) and extender D, Vitasem® (Magapor, Zaragoza, Spain)]. Each group of the semen sample was cryopreserved using the straw freezing procedure as described by Buranaamnuay et al. (2009). Briefly, the semen was extended (1:1, v/v) in extender I (four groups). The extended semen was cooled down to 15°C and held for 2 hours. Thereafter, the semen was centrifuged at 15^0C, 800x g for 10 min. The supernatant (i.e., 50% v/v seminal plasma and extender I) was collected and kept at -20^0C in order to use as the thawing medium in the seminal plasma treatment group (see below). The sperm pellets were diluted in lactose-egg yolk (LEY) extender (80 ml of 11% lactose solution and 20 ml egg yolk) (extender II) to a concentration of $1.5x10^9$ cells per ml. After cooling to 4^0C for 1.5 hours period, the diluted spermatozoa were re-suspended with extender III (LEY extender, 10% glycerol and 1.5% Equex STM Paste; Nova Chemical Sales Inc., MA, USA) to a final concentration of $1x10^9$ sperm per ml. The processed semen were packed into 0.5 PVC-French straws (Bio-Vet, Z.I. Le Berdoulet, France) and frozen by placing in liquid nitrogen (LN_2) vapor approximately 4 cm above the level of liquid nitrogen for 15 min (-120^0C). The straws were plunged into liquid nitrogen (-196^0C) until thawing.

Thawing: Thawing was achieved by immersing the straws in 50^0C water for 12 sec. Immediately after thawing, the semen was divided into two groups: the control group was diluted in BTS® extender 1:4 v/v and the seminal plasma treatment group was diluted 1:4 v/v in 50% seminal plasma. The extended thawed semen was incubated in a 37^0C water bath for 15 min and the post-thawed sperm qualities were evaluated.

Semen evaluation

Sperm concentration: The sperm concentration of each ejaculate of fresh semen was measured by a photometer (Spermacue®, Minitübe GmbH, Tiefenbach, Germany). The sperm concentration of the FT semen was evaluated by direct cell count using Bürker haemocytometer (Boeco, Germany).

Subjective motility: The subjective sperm motility was assessed at 37^0C under light microscope using 400x magnification. The sperm motility was evaluated by the same person throughtout the experiment.

Sperm viability: The sperm viability was determined by eosin-nigrosin staining and was evaluated under a light-field microscope at 1000x magnification. A total of 200 sperm was evaluated for each smear (Buranaamnuay et al., 2009).

Acrosome integrity: Acrosome integrity was assessed using fluorescein isothiocyanate–labeled peanut (Arachis hypogaea) agglutinin (FITC-PNA) staining. Ten µl of the diluted semen were mixed with 10 µl of EthD-1 and were incubated at 37^0C for 15 min. Five µl of the mixture were smeared on a glass slide and fixed with 95% ethanol for 30 sec. Fifty µl of FITC-PNA (diluted FITC-PNA with PBS 1:10 v/v) spreaded over the slide and incubated in a moist chamber at 4^0C for 30 min. After incubation, the specimen was rinsed with cold PBS and air dried. In total, 200 sperm were assessed under fluorescent microscope at 1000x magnification and classified as intact acrosome, reacted acrosome and loose acrosome (Chanapiwat et al., 2009[a]).

Functional integrity of sperm plasma membrane: The functional integrity of sperm plasma membrane was evaluated by short hypo-osmotic swelling test (sHOST) as described by Buranaamnuay et al. (2009). Briefly, the semen sample (100 ml) was incubated at 38^0C for 30 min with 1,000 ml of hypo-osmotic solution (75 mOsm/kg, 0.368% Na-citrate and 0.675% fructose in distilled water). After incubation, 200 µl of

semen and hypo-osmotic solution was fixed in 1000 µl of hypo-osmotic solution plus 5% formaldehyde (Merck, Germany). Sperm coiling was assessed by placing 20 µl of sample on a warm slide with cover slip before evaluation. A total of 200 sperm was evaluated under light-field microscope at 400x magnification. The positive result (coiled tail) was defined as sperm with plasma membrane function.

Statistical analyses: The statistical analyses were carried out using SAS version 9.0 (SAS Inst. Inc., Cary, NC. USA.). Descriptive statistics were calculated by group of extenders and by type of thawing medium. The data included semen concentration, semen volume, total number of sperm per ejaculate, subjective motility, sperm viability, acrosome integrity and functional integrity of the sperm plasma membrane. All of the outcome variables were tested for normality by using residual plot under general linear model procedure of SAS. Shapiro-Wilk statistic, normality plot, kurtosis and skewness were carefully determined for each dependent variable. Since all outcome variables were normally distributed, the continuous outcome variables were analyzed by general linear mixed model procedure of SAS (PROC MIXED). The statistical models included type of extender, thawing medium and interaction between types of extender and thawing medium as fixed effect and boar was assigned as a random effect. Least-squares means were obtained from each class of the factor and were compared by least significant different test. $P<0.05$ were considered statistically significant.

Results

Fresh and frozen-thawed boar semen quality: On average, the total number of sperm per ejaculate was 84,300 x 10^6 sperm (range 42,330–122,877 x 10^6 sperm/ejaculate). The volume, concentration and subjective motility of the ejaculated fresh semen were 254±80 ml (range 163–369 ml), 334±33 sperm/ml (range 255–371 sperm/ml) and 77.3±3.4% (range 70.0-80.0%), respectively. The sperm viability and the functional integrity of the sperm plasma membrane of the fresh semen were 77.5±2.9% (range 73.0-81.5%) and 52.3±6.1% (range 41.5-60.0%), respectively. Across the treatment groups, the post-thawed sperm concentration, subjective motility, sperm viability, acrosome integrity and functional integrity of the sperm plasma membrane were 840 x 10^6 sperm/ml (range 412-1,282 x 10^6 sperm/ml), 18.6% (range 0-45%), 26.2% (range 6-50%), 20.1% (range 14-28%) and 28.4% (range 14-39%), respectively.

Effect of semen extender: semen extended in extender D for 2 hours before cryopreservation yielded a higher individual motility than those extended in extender B (20.9% and 16.9%, p= 0.045) and tended to be higher than those extended in extender A (17.5%, p= 0.090) (Table 1). Sperm viability and functional integrity of the sperm plasma membrane did not differ significantly among extenders (Table 1). Acrosome integrity of the semen extended in extender C was higher than those extended in extender A and D (Table 1).

Effect of thawing medium: Across the treatments, thawing of cryopreserved boar semen in BTS yielded a significantly higher subjective motility than thawing in seminal plasma (20.3% and 16.9%, p= 0.021). Nevertheless, the sperm viability (26.0% and 26.4%, p= 0.876), functional integrity of the sperm plasma membrane (27.1% and 29.7, p= 0.342) and acrosome integrity (20.5% and 19.6%, p= 0.411) did not differ significantly between BTS and seminal plasma. Subjective motility of the cryopreserved boar semen thawed in BTS compared to thawing in seminal plasma are presented in Fig 1. As can be seen from the figure, the post-thawed subjective motility was maximized when the semen was diluted and kept in extender D during the holding time and was thawed by using BTS. Furthermore, the difference between BTS and seminal plasma was more pronounced in semen that were extended in either extender C or D than those extended in extender A or B.

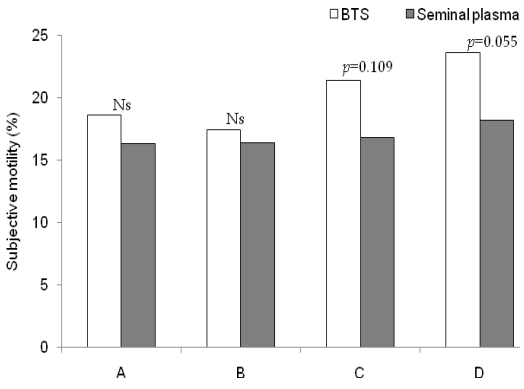

Figure 1 Subjective motility of frozen-thawed boar semen extended in four semen extenders and thawed with Beltsville Thawing Solution (BTS) compared with seminal plasma; NS: non-significant

Table 1 Mean±SEM of sperm concentration (x10^6 sperm/ml, subjective motility (%), sperm viability (%), functional integrity of sperm plasma membrane (sHost, %) and acrosome integrity (%) of frozen-thawed boar semen (n=11) by semen men extender used during holding time

Sperm parameters	Extenders			
	A	B	C	D
Concentration (x 10^6 sperm/ml)	827.5±42.3[a]	828.0±49.2[a]	845.5±36.4[a]	858.6±55.5[a]
Motility (%)	17.5±2.6[ab]	16.9±2.3[a]	19.1±2.7[ab]	20.9±2.7[b]
Viability (%)	25.8±2.6[a]	25.1±2.3[a]	27.0±2.6[a]	26.9±2.2[a]
Acrosome integrity (%)	18.5±2.6[a]	21.0±1.5[ab]	22.8±2.0[b]	18.0±1.1[a]
sHost (%)	25.4±3.8[a]	28.1±1.5[a]	29.5±4.3[a]	30.5±1.5[a]

[a,b] Different superscript within row differed significantly (p< 0.05)

Discussion

The present study demonstrated that the type of semen extender used for pre-dilution of the boar semen before cryopreservation significantly influenced post-thawed sperm motility. This is in agreement with Chanapiwat et al. (2008), who found that the boar semen pre-diluted with Modena™ extender had a higher sperm motility and sperm viability than with BTS. The reason might be due to the fact that both extender D and Modena™ extender (Chanapiwat et al., 2008) contained antioxidant, which may help reduce the occurrence of reactive oxygen species (ROS) during the holding period. It is well established that ROS could damage the sperm plasma membrane and hence reduced sperm motility (Chanapiwat et al., 2009). These studies implied that when the semen had to be transported from a certain distance before cryopreservation, the type of extender used during the holding period should be concerned. Based on the results of the present study, extender D is recommended.

Previous studies demonstrated that the supplementation of seminal plasma in the cryopreserved boar sperm reduced the occurrence of cryoinjury of the boar sperm and improved some post-thawed sperm parameters, e.g. acrosome integrity and membrane disorder (Vadnais et al. 2005; Vadnais and Althouse 2011). However, the present study found that the use of seminal plasma as a thawing medium failed to improve the FT boar sperm quality. Vadnais et al. (2005) found that the supplementation of 20% v/v could decrease the percentage of capacitated sperm from 59.5% to 26.8%. However, no effect of seminal plasma was observed when egg yolk remained in the freezing semen or when the temperature was maintained at 17^0C. Therefore, the effect of seminal plasma on sperm capacitation depend on both temperature and composition of the semen extender. In the present study, egg yolk was not washed before dilution and some of the egg yolk might remain during seminal plasma supplementation. In addition, Caballero et al. (2008) found that >90% of protein in the boar seminal plasma was in the spermadhesin family, including both heparin-binding and non-heparin-binding proteins. Of the non-heparin-binding proteins, PSP-I and PSP-II accounted for >50% of seminal plasma proteins. These seminal plasma proteins enhanced the boar sperm cryosurvival and stabilized the sperm acrosomal membrane. Furthermore, the beneficial effects of PSP-I and PSP-II on sperm viability, motility and mitochondrial membrane have been demonstrated (Novak et al., 2010). Additional study should be carried out to evaluate the effect of specific protein content of the seminal plasma or different fractions of the seminal plasma for a better understanding of their contributions to fertility potential of the boar as well as the roles of these proteins in the female reproductive tract. It could be concluded that both types of extender used during the holding time and the thawing medium significantly influenced FT boar sperm quality.

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