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Cryopreservation and quality assessment of boar semen collected from bulk samples

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Abstract: The purpose of this study was to examine sperm quality after cryopreservation of ejaculates collected as a bulk sample, which is routinely part of semen collection, and to compare this quality with the sperm-rich fraction in boars. Ejaculates were collected as sperm-rich fractions (SRF) and bulk samples (BE) using a gloved-hand technique. Fresh semen quality in terms of semen volume, sperm concentration, total sperm motility and pH were conventionally evaluated. Then, semen was cryopreserved using the liquid nitrogen vapour method. The post-thaw sperm quality was evaluated by assessing sperm motility, live sperm with normal apical ridge and high mitochondrial energy status, lipid peroxidation was evaluated using CASA and fluorescent multiple staining and MDA levels were determined using a spectrophotometer, respectively. In terms of fresh semen quality, sperm motility in fresh semen did not differ significantly between the two groups. The treatment with the greater mean volume (BE; $P < 0.05$) had a lower mean sperm concentration ($P < 0.05$); meanwhile, the mean ejaculate pH collected as BE was more basic compared with SRF ($P < 0.05$). However, there were no significant post-thaw quality changes between sperm-rich fractions and bulk samples of semen. In conclusion, ejaculates can be collected as bulk samples without the need to classify fractions for boar semen cryopreservation.

Keywords: acrosome integrity; freezing boar semen; lipid peroxidation; mitochondria function; sperm viability; sperm-rich fraction

Boar semen cryopreservation is an acceptable technology for the long-term storage of good boar genetic resources, especially for endangered species (Gomez-Fernandez et al. 2012). Moreover, the use of cryopreserved semen is also carried out for international exchange and genetic improvement (Rodriguez-Martinez and Estrada 2013). However, most artificial insemination (AI) centres currently use liquid semen instead of frozen semen (Yeste 2015), as the fertilising ability in terms of the farrowing rate and the total number of births after AI is quite limited (Roca et al. 2015; Yeste 2015); sperm cryodamage is the main problem, which results in low sperm viability after cryopreservation (Rodriguez-Martinez and Wallgren 2011). Many researchers have conducted experiments aimed at achieving satisfactory reproductive performance, which could be practically applied, as in the artificial insemination of cattle.

Even though some research has proven that boar semen from the first 10 ml of sperm-rich fractions could provide the best quality of post-thaw semen when compared with the rest of the ejaculate (Saravia et al. 2005; Saravia et al. 2010), the

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sperm-rich fraction, which is recovered using the traditional gloved-hand technique, is the fraction commonly collected for sperm freezing in research (Kaeoket et al. 2008; Blanch et al. 2014; Chanapiwat et al. 2014). However, currently, in modern industrial pig farming, automated semen collection systems such as Collectis® and Boarmatic® have successfully replacing the gloved-hand technique for ejaculate collection, which has advanced the process of collection by improving labour conditions, reducing human injuries, increasing hygienic procedures and most importantly, improving the productivity and quality of semen. It is important to note that the automated boar collection machine does not allow the collection of separate ejaculate fractions; in other words, the bulk ejaculate is collected, and the amount of seminal plasma in the collected semen samples is concurrently increased as well. Besides, under current semen collection conditions on local pig farms, many operators cannot classify semen fractions in their routine assessments; therefore, not only is the sperm-rich semen fraction collected, but the other semen fractions are also extended with the collected semen. Whether the bulk ejaculates could be collected without the need to classify the fraction for further freezing procedures remains unknown.

Different seminal plasma fractions are main factors that should be considered as they are necessary elements for sperm function and metabolism in the freezing procedure. Boar semen is mainly divided into three parts: a pre-sperm fraction, a sperm-rich fraction (SRF) and a post-sperm fraction. Each semen fraction has different components of seminal plasma, secreted by both the caudal epididymis and accessory glands before and during ejaculation; this is obviously related to semen quality (Garcia et al. 2009; Lopez Rodriguez et al. 2013). In the female reproductive tract, the seminal plasma components play important roles not only in terms of immunological and physiological responses in the female genital tract after insemination (Bischof et al. 1994; O'Leary et al. 2004), but are also beneficial for sperm capacitation and acrosome reactions (Calvete et al. 1994; Maxwell and Johnson 1999). However, compared with artificial insemination, sperm are combined with seminal plasma longer in the case of *in vitro* semen storage. For sperm freezing, semen (as sperm and seminal plasma) is extended with an extender and kept at 15 °C for a couple of hours before centrifugation; then, the supernatant is discarded and there is a further cooling process. It is documented that the holding time prior to cooling could improve the sperm's resistance to cold shock (Pursel et al. 1973; Maxwell et al. 1999; Vadnais et al. 2005; Juarez et al. 2011; Yeste et al. 2014); meanwhile, there is other evidence of detrimental effects of storage on the sperm plasma membrane prior to cooling (Kawano et al. 2004; Okazaki et al. 2009). In addition, different seminal plasma fractions may have noticeable effects on the function of the stored sperm, and seminal plasma from SRF appears to promote sperm survival. Seminal plasma proteins (BSP proteins) appear to be beneficial for sperm storage by stimulating cholesterol and phospholipid efflux from the sperm membrane in a time- and concentration-dependent manner to provide protection against cryostorage in the liquid or frozen states (Manjunath et al. 2007). On the other hand, seminal plasma from post-SRF seems to be harmful (Garcia et al. 2009; Alkmin et al. 2014).

We therefore hypothesised that in ejaculates collected as bulk samples, which is routinely part of semen collection instead of SRF, we might see signs of detrimental effects of seminal plasma on sperm quality after semen cryopreservation. This approach has not been previously comparatively evaluated; the present research was therefore designed to examine sperm quality after cryopreservation of ejaculates collected as a bulk sample, which is routinely part of semen collection, and to compare its quality with the sperm-rich fraction in boars.

MATERIAL AND METHODS

All chemicals used in this study were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), unless otherwise stated.

Experimental design. Semen samples were collected at a commercial AI centre (Khon Kaen province, Thailand), which is approved by the Department of Livestock Development of Thailand. Twenty commercial breed boars (BA81 breed, a commercial species modified by Betagro Agro Industry Co. Ltd.) were studied. The boars' ages ranged between two and three years, and they were raised in individual pens in an evaporative housing system. Fresh clean water was provided *ad libitum* with automated watering; feed levels were adjusted

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to meet the semen production requirements. Boars had been used for routine semen collection for artificial insemination. The experimental protocol was approved by the Animal Ethics Committee of Khon Kaen University (Record No. 0514.1.75/62).

Ejaculates collected as sperm-rich fractions (SRF, $n = 8$) or bulk samples (BE, $n = 12$) were collected using a gloved-hand technique. Then, semen volume, sperm concentration, total sperm motility and pH were conventionally evaluated and recorded. Only ejaculates with ≥ 70% motility were selected for cryopreservation. Semen samples were extended in Modena extender (1 : 1, (v/v)) (Modena™ extender, Swine Genetics International Ltd, Cambridge, IA, USA) and cooled down to 15 °C for 120 min before the freezing procedure was performed.

Sperm cryopreservation. The freezing procedure used in the present study was that described by Chanapiwat et al. (2014), with a few modifications by Ratchamak et al. (2017). After cooling at 15 °C for 120 min, extended semen was centrifuged at 800 × *g* for 10 min at 15 °C; the pellets were diluted in freezing extender I (1–2 : 1 v/v, 80 ml 11% lactose solution lactose, 20 ml egg yolk), and the approximate concentration was 1.5×10^9 sperm/ml. After cooling for 90 min to 5 °C, they were re-suspended in freezing extender II (2 : 1 v/v, 89.5% freezing extender I, 9% glycerol, 1.5% Equex), yielding a final concentration of approximately 1×10^9 sperm/ml. The sperm were then loaded into 0.5 ml plastic straws and sealed with polyvinylpyrrolidone powder (PVP). Semen straws were cryopreserved using the liquid nitrogen (LN_2) vapor method and were laid horizontally on a rack 11 cm and 5 cm above the surface of LN_2 (–35 °C and –120 °C) for 5 min and 10 min, respectively, before plunging into LN_2 for storage at –196 °C until analysis. Straws were thawed at 5 °C for 5 min, as described by Ratchamak et al. (2017). The thawed semen samples were diluted in Modena™ (1 : 4 v/v) and incubated in a water bath at 37 °C for approximately 30 min prior to being evaluated.

Sperm quality assessments. Fresh semen quality was evaluated by assessing semen volume, sperm concentration, total sperm motility and pH.

Semen volume. Semen volume was assessed using a 250 ml cylinder. For each sample, the volume was measured and recorded.

Semen concentration. The sperm concentration was assessed using Sperma Cue® (Minitube of America, Inc., Verona, WI, USA). A 25 µl sample was loaded into the semen sample well of a new cuvette. The cuvette was placed in the slider and the slider was pushed into the machine. The number displayed was in millions of sperm/ml.

Total sperm motility. Total sperm motility was assessed using a phase contrast microscope (Olympus, CH30, Tokyo, Japan). For each assessment, a 3–5 ml sample of fresh semen was dropped onto a glass slide and covered by a cover slip. A minimum of 300 sperm per sample were evaluated.

pH. The pH was assessed using a pH meter (OHAUS, Starter2100, Medford, New York, USA). Before use, it was calibrated with standard buffer solution. A two-point calibration, which adjusts the pH range to be measured with two buffers, such as pH 4 and 7 or pH 7 and 10, was used in order to obtain the correct value. Then, the electrode was cleaned with deionised or distilled water and the liner was dried. The electrode was immediately dipped into the semen, and the value was read and recorded.

Post-thaw semen quality was evaluated by assessing sperm motility, viability, acrosome integrity, mitochondrial function and lipid peroxidation.

Sperm motility. Total sperm motility and progressive motility were assessed using a Computer Assisted Semen Analyzer, CASA (Hamilton Thorne Biosciences, version 12 TOX VIOS, Beverly, USA). For each assessment, a 3–5 ml sample of frozen-thawed semen was dropped into a pre-warmed (37 °C) counting chamber. Evaluations of at least five fields with a minimum of 300 sperm per sample were performed.

Sperm viability, acrosome integrity and mitochondrial function. The viability, normal apical ridge of the acrosome and the mitochondrial membrane potential of sperm were evaluated by fluorescent multiple staining using propidium iodide (PI; 0.5 mg/ml; Live/dead® sperm viability kit L7011 Invitrogen, USA), fluorescein isothiocyanate-labelled peanut (*Arachishypogaea*) agglutinin (FITC-PNA) and 5,5',6,6'-tetrachloro1,1'3,3'tetramethylbenzi midazolyl-carbocyanine iodide (JC-1) staining, respectively, according to Lange-Consiglio et al. (2013) with minor modifications. Briefly, a 300 µl sample of freeze-thawed semen was mixed with 2.5 µl PI and incubated at 37 °C for 5 min. Then, the mitochondria were labelled using 2 µl JC-1 and incubated at 37 °C for 10 min. Finally, the acrosomes were labelled using 2 µl FITC-PNA and incubated

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Table 1. Effect of differences in the type of ejaculate collection (SRF vs BE) on fresh semen quality after collection

Treatment	Volume (ml)	Concentration (× 10^6 sperm/ml)	Total motility (%)	pH
SRF	120.00 ± 07.63[b]	757.00 ± 14.65[b]	85.00 ± 0.58	7.35 ± 0.15[b]
BE	247.00 ± 11.72[a]	417.00 ± 3.94[a]	80.00 ± 0.91	7.40 ± 0.04[a]
P-value	0.0320	0.0358	0.3058	0.0481

Within each column, means ± standard error (SE) with different superscripts differed significantly ($P < 0.05$)
BE = bulk sample; SRF = sperm-rich fraction

at 37 °C for 15 min. All procedures were conducted in the dark. Then, 3–5 µl mixtures were dropped onto a glass slide and covered by a cover slip. Three hundred sperm were assessed immediately using a fluorescence microscope in a triple filter, with the following parameters: UV-2E/C (excitation 340–380 nm and emission 435–485 nm), B-25/C (excitation 465–495 nm and emission 515–555 nm) and G-2E/C (excitation 540–525 nm and emission 605–655 nm), at × 400 magnification. Sperm with colourless heads and red mitochondria were classified as living and undamaged sperm with high membrane potentials (Vongpralub et al. 2016).

Lipid peroxidation. The concentration of malondialdehyde (MDA) is an index of the lipid peroxidation in the semen samples, which can be measured using the thiobarbituric acid (TBA) reaction, according to Chuaychu-Noo et al. (2017). Semen samples were added to 0.25 ml ferrous sulphate (0.2 mM) and 0.25 ml ascorbic acid (1 mM), which were then incubated at 37 °C for 60 min in a water bath. Then, 1 ml trichloroacetic acid (15% (w/v)) and 1 ml thiobarbituric acid (0.375% (w/v)) were added before boiling for 10 min. The samples were cooled down to 4 °C to stop the reaction. Finally, the samples were centrifuged at 800 × *g* for 10 min at 4 °C. The 2 ml of supernatant were used for analysis using a UV-visible spectrophotometer (Analytikjena Model Specord 250 plus) at 532 nm.

Statistical analysis. Data were first tested for the normal distribution of residuals from the statistical models using the UNIVARIATE procedure with the NORMAL option. A group T-test was used to compare results between treatment groups. The overall differences between treatment means were considered significant when $P < 0.05$. The full statistical model was as follows:

$$y_{ij} = \mu + \tau_i + \varepsilon_{ij}$$

where:

y_{ij} – observation of volume, concentration, motility, pH, total motility, progressive motility, viability, acrosome integrity and mitochondrial function and MDA of treatment i (i = 1 to 2) at replication j (j = 1 to 12);
μ – overall mean;
τ_i – effect of treatment i (i = 1 to 2);
ε_{ij} – error term of experiment.

RESULTS

The average (± SE) values for semen volume, sperm concentration, total sperm motility and the pH of fresh boar semen after ejaculation, which were collected as sperm-rich fractions (SRF) and bulk samples (BE), are shown in Table 1. Except for the total sperm motility, all variables were significantly different between groups. The mean ejaculate volume as a bulk sample was greater than the

Table 2. Effect of differences in the type of ejaculate collection (SRF vs BE) on total motility, progressive motility, viability, acrosome integrity, mitochondrial function and MDA of freeze-thawed sperm

Treatment	Total motility (%)	Progressive motility (%)	Viability, acrosome integrity, mitochondrial function (%)	MDA (µm/ml/250 × 10^6 spz)
SRF	34.33 ± 1.51	27.75 ± 1.43	31.57 ± 4.56	3.67 ± 0.40
BE	34.65 ± 2.39	23.50 ± 1.51	32.84 ± 4.83	3.14 ± 0.54
P-value	0.3078	0.7149	0.7106	0.6326

BE = bulk sample; MDA = malondialdehyde; spz = spermatozoa; SRF = sperm-rich fraction

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volume as a sperm-rich fraction ($P < 0.05$); meanwhile, the mean sperm concentration as a bulk sample was lower than as a sperm-rich fraction ($P < 0.05$). There was a minor increase in the mean ejaculate pH collected in bulk samples ($P < 0.05$).

With respect to post-thaw semen quality after cryopreservation, neither sperm motility, nor live sperm with normal apical ridge and high mitochondrial energy status nor MDA differed between the types of ejaculate collection ($P > 0.05$; Table 2).

DISCUSSION

In the present study, we investigated the effect of differences in the type of ejaculate collection, in terms of sperm-rich fractions compared to bulk samples, for semen cryopreservation of freeze-thawed sperm quality in boars. In terms of fresh semen quality, there were no differences in sperm motility between the two groups. The semen groups with a greater mean volume (ejaculates collected as bulk samples) had a lower mean sperm concentration. Meanwhile, the mean ejaculate pH collected as bulk samples was more basic when compared with sperm-rich fractions. However, there were no significant differences in any sperm quality parameters after cryopreservation.

Good sperm quality of fresh semen is required for further semen cryopreservation. The semen volume was, however, undoubtedly inversely related to sperm concentration, and concurrently was significantly different between ejaculates collected as sperm-rich fractions and bulk samples. Importantly, the increase in the dilution of seminal plasma in the bulk sample was not so pronounced as to be harmful to the sperm motility of ejaculate semen, known as the "dilution effect" (Mann 1964). Generally, more than 70% total motile sperm was the main recommended criterion by which to evaluate sperm quality for further semen cryopreservation, as it is an indicator of both unimpaired metabolism and the intactness of the membranes (Johnson et al. 2000); in the present study, both the sperm-rich fraction and bulk ejaculate met this criterion. Similar to the report by Pena et al. (2003), all fresh sperm fractions had above-standard criteria for sperm motility and viability, and post-thaw semen quality, evaluated using simple operator-based observation and PI staining, was not different. Therefore, we inferred that the noticeable difference in semen volume and sperm concentration, as occurred in our study, did not affect further semen cryopreservation whether or not the sperm motility met the mentioned criterion.

The other significant finding in this study was that the pH of bulk ejaculate (pH 7.40) was significantly increased compared with that of the sperm-rich fractions (pH 7.35; Table 1). The elevation of the pH of the bulk sample was explained by the addition of bicarbonate-rich fluid from the seminal vesicles (Rodriguez-Martinez et al. 1990). Boar-ejaculated semen has been reported to form three different fractions (the pre-sperm fraction, the sperm-rich fraction (SRF) and the post-sperm fraction), with pH values of 7.61, 7.32 and 7.96, respectively; meanwhile, the pH of ejaculated semen as a bulk sample was 7.61 (Rodriguez-Martinez et al. 1990), which is consistent with our results. After ejaculation, however, the mean pH of storage semen should be decreased in order to reduce sperm metabolism and motility (Gadea 2003). The presence of buffers in the extender is necessary for pH variation control; subsequently, semen was therefore diluted with extender after semen collection and was kept at 15–20 °C for a couple of hours before the freezing procedure.

Besides controlling the pH, the incubation of boar sperm samples, which has the effect of surrounding the seminal plasma with semen extender before freezing, had the beneficial effect of improving the resistance of sperm to cold shock (Pursel et al. 1973; Maxwell et al. 1999; Vadnais et al. 2005; Juarez et al. 2011; Yeste et al. 2014). In any case, the duration of holding boar semen samples before freezing influenced the sperm cryotolerance in different portions; it seemed that longer holding times before freezing resulted in lower sperm freezability in bulk ejaculates compared with sperm-rich fractions (including the first 10 ml of the sperm-rich fraction) (Alkmin et al. 2014). In theory, the exposure of sperm to a variety of sources of stress, including dilution and cooling incubation (Cummins et al. 1994; Waberski et al. 2011), is hypothesised to result in damage to the sperm plasma membrane through lipid peroxidation from increased levels of reactive oxygen species (ROS) (Walczak-Jedrzejowska et al. 2013). Therefore, sperm quality from all portions gradually deteriorates, with declines in motility and viability regardless of whether they were kept at cooling temperatures for longer periods of time. Furthermore, increasing the bicarbonate concentration in any

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sperm fraction (as mentioned above) results in increased ROS production as well; therefore, sperm held in seminal plasma from sperm-rich fractions were described to be of a better quality than those from the other fractions (Saravia et al. 2009; Saravia et al. 2010; Barranco et al. 2015). However, in the present study, sperm were cooled at 15 °C for only 2 h, so sperm might not have been exposed to the seminal plasma for long enough to be detrimentally affected. Thus, post-thaw semen quality, in terms of sperm motility, live sperm with normal apical ridges and high mitochondrial energy status, and MDA in bulk ejaculate compared with sperm-rich fractions, were not affected by incubation time. In addition, seminal plasma was eventually removed by centrifugation in order to concentrate the amount of sperm before re-diluting with a freezing extender; therefore, there was no adverse effect of seminal plasma on sperm freezing. Kawano et al. (2004) reported that the cryosurvival of sperm was improved when seminal plasma was removed from the cryopreservation process after collection.

The post-thaw semen quality in this study was comparable with that of previous studies, and qualities such as progressive motility and viability had standard values similar to the control groups in previous reports (Chanapiwat et al. 2012; Chanapiwat and Kaeoket 2015). This revealed that the environment in our laboratory was reliable. In order to improve sperm function and survival after thawing, different additives may be recommended as supplements to freezing and thawing extenders. For example, antioxidants have been supplemented to extenders, as high levels of ROS are generated during the cryopreservation process (Kaeoket et al. 2008; Amidi et al. 2016). Additionally, plasma membrane fluidity during cooling and freezing was increased by cholesterol-loaded cyclodextrin supplementation to freezing extenders (Blanch et al. 2012).

In conclusion, the present study examined the effect of differences in the type of ejaculate collection, in terms of sperm-rich fractions compared to bulk samples, for cryopreservation of boar sperm and its effects on quality. The findings demonstrate that no sperm quality parameters were significantly different after cryopreservation; therefore, ejaculates can be collected as bulk samples without a need to classify the fraction for boar semen cryopreservation; this would be useful for the current conditions of semen collection on farms, especially for local farming in which many semen collectors cannot not classify semen fractions and almost all semen are collected as bulk samples. Besides, for modern industrial pig farming, in which the automated boar collection machine is implemented in AI centres instead of manual collection, only bulk samples are collected. Further study is required to improve the post-freezing quality and usability of semen.

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