

## Effect of Gamma-Oryzanol-Enriched Rice Bran Oil on Quality of Cryopreserved Boar Semen

Kampon KAEOKET<sup>1)\*</sup>, Sarayut DONTOT<sup>1)</sup>, Pinatta NUALNOY<sup>1)</sup>, Jutarat NOIPHINIT<sup>1)</sup> and Panida CHANAPIWAT<sup>1)</sup>

<sup>1)</sup>*Semen Laboratory, Department of Clinical Sciences and Public Health, Faculty of Veterinary Science, Mahidol University, Phuttamonthon, Nakorn-pathom 73170, Thailand*

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**ABSTRACT.** The aim of this study was to determine the effect of gamma-oryzanol-enriched rice bran oil on the quality of cryopreserved boar semen. Ten boars provided semen of proven motility and morphology for this study. The semen was divided into three portions in which lactose-egg yolk (LEY) extender used to resuspend the centrifuged sperm pellet was supplemented with 2 types of rice bran oils, at a gamma-oryzanol concentration of 0 mg/ml of lactose egg yolk (LEY) freezing extender (group A, control), 0.1 mg/ml (0.16 mMol) of freezing extender (group B) and 0.1 mg/ml of freezing extender (group C). Semen suspensions were loaded in medium straws (0.5 ml) and placed in a controlled-rate freezer. After cryopreservation, frozen semen samples were thawed and investigated for progressive motility, viability and acrosomal integrity. There was a significantly higher percentage of progressive motility (34 versus 47.0 and 48.5,  $P<0.001$ ), viability (35.5 versus 48.1 and 50.1,  $P<0.001$ ) and acrosomal integrity (39.8 versus 50.8 and 54.9,  $P<0.001$ ) in the gamma-oryzanol-enriched rice bran oil-supplemented groups (groups B, C) than in the control group (group A), respectively. In conclusion, addition of gamma-oryzanol-enriched rice bran oil to LEY freezing extender is appropriated for improving the quality of frozen-thawed boar semen.

**KEY WORDS:** antioxidant, boar semen, cryopreservation, gamma-oryzanol, rice bran oil.

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Cryopreservation of boar spermatozoa offers an effective way to maintain their valuable genetic material and transport it between countries, thereby minimizing boar transportation. It has been demonstrated that certain processes in cryopreservation including elimination of seminal plasma by centrifugation and reduction in the temperature result in cold shock, owing to a decrease in quantity of antioxidants and an increase in reactive oxygen species (ROS) contents in the semen [6, 8, 43]. The ROS can lead to oxidative stress and lipid peroxidation of the sperm plasma membrane, which have detrimental effects on spermatozoa during cryopreservation, for instance, reduction in progressive motility, acrosomal integrity, sperm-oocyte fusion and fertilizing potential [1–3, 17, 41, 53].

During the past decade, several studies have focused on supplementation of freezing extender for frozen boar semen with a variety of antioxidants (e.g., vitamin E, vitamin C, L-cysteine, glutathione, taurine, pyruvate, SOD, catalase) in an attempt to minimize the detrimental effects of ROS, which occur during the freezing process [7, 10, 16, 23, 26, 38, 39].

Gamma-oryzanol, a phytosteryl ferulate mixture extracted from rice bran oil, has received a great deal of attention, because of its various significant health-promoting functions such as antioxidant activity, inhibition of lipoperoxidation by its scavenging activity [4], reduction in LDL

cholesterol and induction of HDL cholesterol [40, 41, 48] and inhibition of platelet aggregation [13]. In humans, many studies have been reported on its potential implications as a UV-A filter in sunscreen cosmetics [14] and as a treatment for type 2 diabetes mellitus [12] and allergic reactions [37]. These data suggest that gamma-oryzanol, especially because of its antioxidant and scavenging activities, can be useful as an antioxidant and lipid peroxidation inhibitor (i.e., membrane stabilizing) during cryopreservation. Rice bran oil is widely used in salad dressing and cooking oil in Asian countries including China, India, Japan and Thailand. At present, it is beginning to gain acceptance in Western countries as well [20, 22, 36, 50]. However, no scientific information is available on its antioxidant and scavenging activities in minimizing the detrimental effects of ROS during the cryopreservation of semen. To our knowledge, supplementation of lactose-egg yolk freezing extender with gamma-oryzanol-enriched rice bran oil for cryopreservation of boar semen has never been reported. Hence, the objective of our study was to investigate the effect of addition of gamma-oryzanol-enriched rice bran oil on the percentage of progressive motility, viability and acrosomal integrity of frozen thawed boar semen.

### MATERIALS AND METHODS

The research proposal of this project was approved by the Faculty of Veterinary Science-Animal Care and Use Committee (FVS-ACUC) of Mahidol University (No. MUVS201031).

*Animals and preparation of boar spermatozoa:* Ten Duroc boars between 1 and 3 years of age, having proven

\*CORRESPONDENCE TO: KAEOKET, K., Semen Laboratory, Department of Clinical Sciences and Public Health, Faculty of Veterinary Science, Mahidol University, Nakorn-pathom 73170, Thailand.  
e-mail: vskkk@mahidol.ac.th

sperm progressive motility and morphology of  $\geq 70\%$  and  $\geq 80\%$ , respectively, and from the same commercial herd were included. The boars were housed in individual pens with an evaporating cooling system. Water was provided *ad libitum* via a water nipple. A feed based on corn, soy bean and fish meal (15–16% protein) was given twice a day (approximately 3 kg/day).

Semen samples from each boar (one ejaculate from each boar) were collected using the gloved-hand technique [23–26]. During collection, the semen was filtered through gauze, and only sperm-rich fractions were collected. Within 30 min after collection, semen volume, pH (by pH paper, Universalindikator (pH 0–14), Merck, Darmstadt, Germany) and progressive motility of spermatozoa (i.e., only spermatozoa that move forward with straight line pattern) determined under a phase contrast microscope (Olympus CX31, New York, NY, U.S.A.) were evaluated. Semen samples of 1 ml were examined after collection into Eppendorf tubes for further analysis of concentration in a Neubauer Haemocytometer (Improved Neubauer's chamber, BOECO, Hamburg, Germany), sperm viability by the living-cell nucleic acid stain SYBR-14/EthD-1 (FertiLight<sup>®</sup>, Sperm Viability Kit; Molecular Probes Europe BV, Leiden, The Netherlands), sperm acrosomal integrity by fluorescein isothiocyanate-labelled peanut (*Arachis hypogaea*) agglutinin (FITC-PNA) staining and sperm morphology by Williams staining and formal-saline solution [26, 27]. The remaining semen was diluted with (1:1 v/v) Extender I (Modena<sup>TM</sup> extender, Swine Genetics International Ltd., Cambridge, IA, U.S.A.) and transported by cell incubator (Micom control system 20Q, Continental Plastic Corp., Delevan, WI, U.S.A.) at 15°C to the semen laboratory. Only ejaculates with motility of  $\geq 70\%$  and  $\geq 80\%$  morphologically normal forms were used for cryopreservation.

**Semen freezing process:** All semen samples were frozen in a controlled-rate freezer (IceCube 14s, Sy-lab, Purkersdorf, Austria).

After collection and evaluation, the semen was diluted (1:1 v/v) with Extender I (Modena<sup>TM</sup> extender, Swine Genetics International Ltd, Cambridge, IA, U.S.A.). Diluted semen was transferred to 50 ml centrifuge tubes, cooled at 15°C for 120 min and later centrifuged at  $800 \times g$  15°C for 10 min (Hettich Rotanta 460R, Andreas Hettich GmbH & Co., KG, Tuttlingen, Germany). The supernatant was discarded, and the sperm pellet was re-suspended (about 1–2:1) with extender II (80 ml of 11% lactose and 20 ml egg yolk, LEY) to a concentration of  $1.5 \times 10^9$  spermatozoa/ml [9, 23, 24, 29]. To prepare LEY freezing Extender II supplemented with gamma-oryzanol, two types of gamma-oryzanol-enriched rice bran oil, i.e., from a dietary supplement product (Biogrow Natural<sup>TM</sup>, Biogrow (TH) Co., Ltd., Bangkok, Thailand) and from a salad dressing and cooking oil (King<sup>TM</sup> rice bran oil, Thai Edible Oil Co., Ltd., Bangkok, Thailand) were used and prepared as follows to provide a concentration of gamma-oryzanol of 0.1 mg/ml of Extender II. This concentration was considered according to the original gamma-oryzanol concentration of these products. Fifty milliliters of Extender II was mixed with rice bran oil from Biogrow Natural<sup>TM</sup> (0.5

ml) or King<sup>TM</sup> rice bran oil (1.0 ml) containing 4 mg of gamma-oryzanol to produce semen supplemented with 0.1 mg/ml (0.16 mM) of gamma-oryzanol in Extender II. Thus, there were three groups of semen samples. Group A was not supplemented with gamma-oryzanol (control); Group B was supplemented with gamma-oryzanol at 0.1 mg/ml (Biogrow Natural<sup>TM</sup>) of Extender II; Group C was supplemented with gamma-oryzanol at 0.1 mg/ml (King<sup>TM</sup> rice bran oil) of Extender II.

The diluted semen was incubated at 5°C for 90 min [9, 10, 23]. The three groups of semen were each mixed with a half volume of Extender III (89.5% LEY extender with 9% v/v glycerol and 1.5% v/v Equex-STM<sup>®</sup>; Nova Chemical Sales Inc., Scituate, MA, U.S.A.). The final sperm concentration was  $1.0 \times 10^9$  spermatozoa/ml. The sperm suspensions were loaded into 0.5 ml polyvinyl chloride medium-straws (Bio-Vet, Fleurance, France) and sealed with plasticine. All straws were placed horizontally on a rack and put into a chamber of the controlled-rate freezer set to +5°C. [23, 27, 29, 46, 49]. The cooling/freezing rate was as follows: 3°C/min from +5 to -5°C, 1 min of holding time and then 50°C/min from -5 to -140°C. Then, the straws were immediately plunged into liquid nitrogen (-196°C) for storage and further analysis. Thawing of straws was carried out in a thermos flask at 50°C for 12 sec [23, 26]. After thawing, the samples were diluted (1:4) with Extender I in a test tube and incubated in a water bath at 37°C for 15 min before evaluation.

**Evaluation of spermatozoa: Progressive sperm motility.** Progressive sperm motility was subjectively evaluated at 37°C under a phase-contrast microscope at  $100 \times$  and  $400 \times$  magnification [5]. Visual estimation was performed by the same person, who was unaware of the treatments. Progressive motility was expressed as the percentage of motile sperm cells.

**Assessment of sperm viability.** Viability was evaluated with SYBR-14/Ethidiumhomodimer-1 (EthD-1; FertiLight<sup>®</sup>; Sperm Viability Kit; Molecular Probes Europe BV, Leiden, The Netherlands). Ten microliters of diluted spermatozoa were mixed with 2.7  $\mu$ l of the working solution of SYBR-14 and 10  $\mu$ l of EthD-1. After incubation at 37°C for 20 min, a total of 200 spermatozoa were assessed ( $\times 400$ ) under a fluorescence microscope (Axioskop 40, Carl Zeiss Inc., Oberkochen, Germany). The nuclei of spermatozoa with intact plasma membranes were stained green with SYBR-14, while those with damaged membranes were stained red with EthD-1 [23–27]. Spermatozoa were classified into three types: viable, having intact plasma membranes (stained green); damaged, having damaged plasma membranes but an intact acrosome (stained red and green) and dead, having both plasma membranes and acrosomal damage stained red. The results were recorded as the percentage of nonviable (damaged and dead spermatozoa) and viable spermatozoa.

**Assessment of acrosomal integrity.** The integrity of the sperm acrosome was evaluated with fluorescein isothiocyanate-labelled peanut (*Arachis hypogaea*) agglutinin (FITC-PNA) staining [15, 30, 34, 52]. Ten microliters of diluted semen with 140  $\mu$ l phosphate buffered saline (PBS) was mixed 10  $\mu$ l of ethidiumhomodimer-1 and incubated at 37°C

for 15 min. Then, 5  $\mu$ l of the suspension was smeared on glass slides fixed with 95% ethanol for 30 sec and air-dried. In the next step, 50  $\mu$ l FITC-PNA (FITC-PNA diluted with PBS 1:10 v/v) was spread over the slide and incubated in a moist chamber at 4°C for 30 min. After incubation, the slide was rinsed with cold PBS and air-dried. A total of 200 spermatozoa were assessed under a fluorescence microscope at 1.000 X magnification, and classified as those with intact acrosomes and non-intact acrosomes (reacted and loose acrosome) [34].

**Statistical analysis:** Prior to analysis of variance (ANOVA), all data for each parameter were checked for normal distribution with the Shapiro-Wilk test. All data were subjected to a 2-way ANOVA according to a Randomized Complete Block Design, with treatments as a fixed factor, parameters observed as dependent variables and boars as a random block factor. When the effect of treatment was significant, multiple comparisons with Duncan's new multiple range test were used to compare means with different effects of those parameters observed. All statistical analyses were performed as a General Linear Model using the Univariate procedure of SPSS version 18.0 (SPSS 18.0; SPSS Inc, Chicago, IL, U.S.A.). Data of parameters observed for sperm motility, viability and acrosome integrity were reported as means of percentages. Overall differences between treatment means were considered significant when  $P < 0.05$ .

## RESULTS

**Fresh semen analysis:** The mean percentages of progressive motility, viability and acrosomal integrity were 82.9, 76.4 and 66.7, respectively. The pH, volume, morphology, and concentration of fresh semen samples ranged between 7.0–7.5, 50–190 ml, 85–90%, and  $180\text{--}639 \times 10^6$  spermatozoa/ml, respectively.

**Post-thaw semen analysis: Progressive motility.** There was a significantly higher percentage of progressive motility in the treatment groups than in the control group ( $P < 0.001$ ). A higher percentage of progressive motility was found in groups B and C (Table 1) than in group A.

**Viability of spermatozoa.** The percentage of viable spermatozoa was significantly higher in the treatment groups than in the control group ( $P < 0.001$ ). A higher percentage of viable spermatozoa was found in groups B and C than in group A (Table 1).

**Acrosomal integrity in live spermatozoa.** The percentage of cells with intact acrosomes was significantly higher in the treatment groups than in the control group ( $P < 0.001$ ). A higher percentage of intact acrosome was observed in groups B and C than in group A (Table 1).

## DISCUSSION

In the present study, it was clearly shown that the addition of rice bran oil to LEY freezing extender, which contained gamma-oryzanol at a concentration of 0.1 mg/ml (0.16 mM), produced better results in terms of progressive motility, viability and acrosomal integrity compared with the control

group. To the best of our knowledge, this is the first study showing the positive effects of gamma-oryzanol during the cryopreservation of boar semen, which may be explained by its antioxidant and scavenging activities in minimizing the detrimental effects of ROS as shown in another study [4]. It might also be possible that boar spermatozoa absorb and utilize gamma-oryzanol from the freezing extender to neutralize ROS, which in turn protects their plasma membrane and inner organelles during freezing [9, 23, 27, 44].

The results in the present study are in agreement with those of Bilodeau *et al.* [6], Kaeoket *et al.* [23, 27] and Jeong *et al.* [21] in that a supplement of antioxidant (e.g., L-cysteine, Taurine, vitamin E, vitamin C) could improve progressive motility in frozen-thawed bull semen and the quality of frozen boar and canine semen in terms of sperm survival [35, 42]. The improvement in the qualities of frozen-thawed boar semen found in the present study might be explained by the studies of Ismail *et al.* [19] and Wang *et al.* [45] in that gamma-oryzanol prevents peroxide formation and possesses antioxidant activity, thereby stabilizing lipid components in the sperm plasma membrane [22]. Xu *et al.* [50] demonstrated that gamma-oryzanol exhibits a higher level of antioxidant activity compared with vitamin E, even though they utilize the same antioxidant mechanism. Recently, it has also been suggested that gamma-oryzanol, a group of phytosterol ferulates, is responsible for the antioxidant activity in living cells via ROS scavenging and inhibition of ROS production [18]. In addition, gamma-oryzanol has the ability to prevent lipoperoxidation that produces free radicals (e.g., lipid peroxide, hydroperoxyl radical, hydroperoxide, alkoxyl radical) [31] and thus protect spermatozoa from ROS [2, 11, 32, 47]. Furthermore, gamma-oryzanol may also account for a protective effect in the extracellular milieu, predominantly due to its antioxidant properties [22]. However, it is worth noting that other antioxidants including tocopherols, tocotrienols and phytosterols are also present in rice bran oil [36, 50, 51]; we cannot therefore exclude the possibility that they may contribute independently or synergistically with gamma-oryzanol to the improvement of antioxidant capacity [28].

In the present study, it was shown that the qualities of frozen-thawed boar semen were improved, even though we used a trial concentration of gamma-oryzanol of 0.1 mg/ml of LEY freezing extender. Nevertheless, high doses of gamma-oryzanol additives may not be advantageous, if spermatozoa have a limited capacity for gamma-oryzanol uptake [33, 49], as shown by a study on the supplementation of L-cysteine or DHA (fish oil) in LEY freezing extender for cryopreservation of boar semen [23, 26]. Therefore, a further investigation is needed in order to obtain an optimal concentration of gamma-oryzanol for cryopreservation of boar semen.

In conclusion, gamma-oryzanol-enriched rice bran oil can be added to lactose-egg yolk freezing extender to improve the quality of frozen-thawed boar semen.

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Table 1. The mean ( $\pm$ SEM) percentages of progressive motility, viability and acrosomal integrity of frozen-thawed boar semen in the different groups (group A=control, group B=Biogrow Natural™, group C=King™)

Groups	Progressive motility (%)	Viability (%)		Acrosomal integrity (%)	
		Viable	Nonviable	Intact	Nonintact
A (n=10)	34.0 $\pm$ 3.0 <sup>a</sup>	35.5 $\pm$ 2.7 <sup>a</sup>	64.5 $\pm$ 2.7	39.8 $\pm$ 1.8 <sup>a</sup>	60.2 $\pm$ 2.2
B (n=10)	47.0 $\pm$ 2.9 <sup>b</sup>	48.1 $\pm$ 3.1 <sup>b</sup>	51.9 $\pm$ 3.1	50.8 $\pm$ 2.3 <sup>b</sup>	49.2 $\pm$ 2.3
C (n=10)	48.5 $\pm$ 3.6 <sup>b</sup>	50.1 $\pm$ 3.2 <sup>b</sup>	49.9 $\pm$ 3.2	54.9 $\pm$ 3.1 <sup>b</sup>	45.1 $\pm$ 2.9
Significant differences	P<0.001	P<0.001	NA	P<0.001	NA

Values in each row marked with different superscript letters differ significantly ( $P<0.001$ ).

NA=Not analyzed.

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