Original Article

Comparison of Effects of Different Antioxidants Supplemented to Long-term Extender on Boar Semen Quality Following Storage at 17°C

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Abstract

The use of antioxidants as semen additives in the extender has been proposed to improve sperm function during cool storage. The objective of this study was to compare the effects of different antioxidant supplementation in extender on boar semen quality during storage for 10 days at 17°C. Semen samples (n=18) were divided into 10 aliquots and diluted with Reading extender supplemented with three different concentrations of water-soluble glutathione, L-carnitine, and vitamin E analogue Trolox (0.1, 1 and 5mM). All samples were stored in a refrigerator at 17°C for 10 days. Sperm motility was assessed at Days 1, 4, 7 and 10 of storage under a phase-contrast microscopy. Percentage of live sperm with normal apical ridge and high mitochondrial energy status was assessed at Days 1, 5 and 10 of storage by fluorescent multiple staining using propidium iodide (PI), fluorescein isothiocyanate-labeled peanut (Arachis hypogaea) agglutinin (FITC-PNA) and 5,5′,6,6′-tetrachloro-1,1′,3,3′ tetraethylbenzimidazolyl-carbocyanine iodide (JC-1), respectively, under fluorescent microscopy. The sperm motility was greater in 1 mM of glutathione supplementation than the others (p<0.01). However, the percentage of live sperm with normal apical ridge and high mitochondrial energy status did not differ among the groups (p>0.05). The supplementation of semen extender with 1 and 5 mM of vitamin E had detrimental effect on the quality of stored semen (p<0.01). In conclusion, the addition of 1 mM dose of glutathione can be recommended as an alternative component of boar semen extender for long-term cool storage.

Keywords: glutathione, L-carnitine, liquid semen, vitamin E

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Introduction

Currently, the number of female pigs inseminated by means of artificial insemination (AI) using cooled semen preserved at 15-20°C has increased (Riesenbeck, 2011). Even though cryopreservation of boar semen has been set up, acceptable fertility after AI is limited because of low cryosurvival, and also the method being costly (Rodriguez-Martinez and Wallgren, 2010). The use of semen is possibly carried out either on the day of semen ejaculation or after semen processing (Johnson et al., 2000). In case that the preservation time is less than 3 days, the use of short-term extender is appropriate for AI on farm, while long-term extender, a higher complex extender, is generally used in case that the semen production center is quite far from female pig farms especially for smallholder farms in rural areas. This latter type of extender has been expected to extend the lifetime of boar semen. However, the fertility of extended semen gradually deteriorates with the increase in storage time, probably related to oxidative stress during storage (Gadea, 2003; Johnson et al., 2000; Kumaresem et al., 2009).

Sperm plasma membrane has abundant polyunsaturated fatty acid (PUFA), which renders cell very susceptible to the harmful effect of reactive oxygen species (ROS), especially in boar sperm which contain a high concentration of PUFA (Awda et al., 2009; Waterhouse et al., 2004). The excess of ROS causes a pathological response leading to damage to sperm membranes. The consequences of membrane damage are decrease in sperm motility and viability, and alternations of membrane permeability and fertility ability (Cummings et al., 1994). In male reproductive tract, seminal plasma normally plays responsibility for producing antioxidants, for instance vitamin E, vitamin C, and glutathione. However, once ejaculated semen is collected, the antioxidants thoroughly diminish (Brezezinska-Slevbodzinska et al., 1995). Therefore, a variety of antioxidants have been proposed as semen additives in the extender to improve liquid semen preservation in boars (Chankitisakul, 2014; Funahashi and Sano, 2005; Roca et al., 2004; Zhang et al., 2012). Glutathione, which prevents damage to important cellular components caused by ROS, could improve the viability and functional status of boar sperm during long-term liquid preservation and cryopreservation (Funahashi and Sano, 2005; Kaeoket et al., 2008). The addition of vitamin E, which acts as a peroxyl radical scavenger, could increase sperm resistance to ROS, resulting in improvement in the quality of boar sperm in both short-term liquid preservation and cryopreservation in boars (Breininger et al., 2005; Kaeoket et al., 2008; Mendez et al., 2013). However, there is no report available on the use of vitamin E supplemented during long-term liquid preservation. L-carnitine has antioxidant and free radical scavenging abilities, and as a result can reduce intracellular concentrations of ROS (Ilhami, 2006; Kerner and Hoppel, 2000). Supplementation of oocytes/embryos culture medium with L-carnitine was used to improve cryotolerance after freezing (Chankitisakul et al., 2013; Takahashi et al., 2013). In addition, there are recent evidences of using L-carnitine successfully in rabbit and cat as a supplement in liquid semen storage and semen cryopreservation, respectively (Manee-In et al., 2014; Sarıözkan et al., 2014). However, as far as our knowledge, there are no data available about the influence of L-carnitine supplement used as an antioxidant during liquid preservation to improve sperm quality in boar semen.

The main objectives of this study were, therefore, to optimize the concentrations of different antioxidants (glutathione, water-soluble vitamin E analogue Trolox, and L-carnitine) supplemented in extender and compare the effects of those on boar semen quality in terms of sperm motility and percentage of live sperm with normal apical ridge and high mitochondrial energy status during long-term storage for 10 days at 17°C.

Materials and Methods

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

Animals: Semen samples were collected at a commercial AI center (Khon Kaen province, Thailand) which is approved by Department of Livestock Development of Thailand. Eighteen boars (Duroc, 1-2 years old) were used in this study. The experiment protocol was approved by Animal Ethics Committee of Khon Kaen University (Record No. AEKKU54/2557). The boars were raised in individual pens in an evaporative housing system. Fresh clean water was provided *ad libitum* with automated watering and feed levels were adjusted to meet semen production requirements. The boars have been used for routine semen collection for artificial insemination.

Semen extender: Reading extender (without cysteine) composed of 11.5 g glucose, 11.6 g sodium citrate, 2.35 g EDTA, 1.75 g sodium bicarbonate, 0.75 g potassium chloride, 5.5 g Tris, 4.1 g citric acid, 1 g trehalose, 133 mg lincomycin, 330 mg spectinomycin in 1,000 ml double distilled water was used in the experiment.

Semen collection and preparation: Semen collection was performed using a gloved-hand technique by the same technician. The first portion of ejaculate was discarded and the remaining ejaculate was filtered through sheath cloth. Immediately after collection, microscopic semen evaluation was carried out under a microscope with computer software (CASA; Dynamic swine sperm; Optika). Only samples with sperm progressive motility and normal morphology at least 85% were used in the experiment.

After evaluation, the fresh boar semen was divided into 50 ml centrifuge tube and diluted to 1:1 with the Reading extender supplemented with different concentrations of antioxidants at 35°C. The semen samples were then kept at 25-28°C and immediately transported to the laboratory within an hour after collection for further processing. At the laboratory, the boar semen was further diluted with antioxidants supplemented in Reading extender similar to the former to a final concentration of 3×10^7

sperm/ml. Then, the samples of each experimental group were aliquot in 5 ml into five 15 ml centrifuge tubes which were then placed in a refrigerator at 17°C and stored for 10 days. The temperature was monitored constantly by a digital thermometer throughout the experiment.

Experimental design: Effective concentrations of glutathione, water-soluble vitamin E analogue Trolox, and L-carnitine supplements in long-term liquid extender for preservation at 17°C was determined and compared. Different concentrations of those antioxidants at 0.1, 1 or 5 mM were added to the Reading extender. The extender supplemented without antioxidant was served as control. Thus, the semen from each boar was divided and assigned to 10 different treatment groups. An aliquot of each sample was evaluated for sperm characteristics after storage at Days 1, 4, 7 and 10 for sperm motility and at Days 1, 5, and 10 for sperm viability, acrosome integrity and mitochondrial function as described below. The experiment was repeated four times (n=18).

Sperm quality assessment: A 5 ml aliquot of each diluted boar semen sample was taken on each experimental time. The semen samples were warmed at 37°C for 30 min before assessment.

***Sperm progressive motility*:** Sperm motility was assessed by visual estimation by the same person throughout the study. For progressive motility, 15 µl of stored semen was dropped on a clean slide at 37°C, covered with a cover slip and examined under a phase-contrast microscopy at x 400 magnification. Motility was expressed as percentage of progressively motile sperm.

Sperm viability, acrosome integrity and mitochondrial function: Viability, normal apical ridge of acrosome and high potential mitochondrial membrane of sperm were evaluated by fluorescent multiple staining (FMS). The FMS protocol followed that by Andrade et al. (2007) with minor modification. Briefly, 150 µl sperm suspension was mixed with 2 µl propidium iodide (PI; 0.5 mg/mL; Live/dead® sperm viability kit L7011 Invitrogen USA), 5 µl fluorescein isothiocyanate-labeled peanut (Arachis hypogaea) agglutinin (FITC-PNA) and 2 µl 5,5′,6,6′-tetrachloro-1,1′,3,3′ tetraethylbenzimidazolyl-carbocyanine iodide (JC-1). Then, the samples were incubated in a dark moist chamber at room temperature for 10 min. For fluorimetric assessment, small drop of suspension was placed on a clean slide, covered with a cover slip and evaluated immediately by a fluorescence microscope in a triple filter, showing a set of 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 x 400 magnification. Colorless head with red mitochondria sperm were classified as living and undamaged sperm with high membrane potential.

Statistical analysis: Progressive motility and sperm viability, acrosome integrity, and mitochondrial function were analyzed by repeated measurement in completely randomized design and statistical analysis of data was performed by the General Linear Model (GLM) procedure of SAS (1996). Single treatments consisting of 10 treatments with 4 replications for progressive motility and 3 replications for viability, acrosome integrity, and mitochondrial function were tested. The full statistical model was as follows:

$$y_{ijk} = \mu + \alpha_i + \delta_{(k)i} + \tau_j + \alpha\tau_{ij} + \Phi_{ijk}$$

y_{ijk} = observation of progressive motility and viability, acrosome integrity, and mitochondrial function on treatment i and time (days of storage) j at replication k; k = 1,…,r

μ = overall mean

α_i = effect of treatment i; i = 1 to 10

$\delta_{(k)i}$ = effect of animal k; k = 1,…,r

τ_j = effect of time (days of storage) j; j = 1 to 4

$\alpha\tau_{ij}$ = interaction effect between treatment i and time j

Φ_{ijk} = effect of correlated error ijk ~N(0, σ_e^2)

Results

The average (±SD) progressive motility of fresh boar semen after ejaculation was 91.85 ± 1.95%. The levels of significance for the influence of days of storage and treatments on progressive motility, and sperm viability, acrosome integrity and mitochondrial function are demonstrated in Table 1. The interaction effect between days of storage and treatment was significant in the progressive motility (p = 0.0001). Meanwhile, only days of storage effect was significant in the sperm viability, acrosome integrity and mitochondrial function.

The effect of antioxidants on progressive motility is shown in Table 2. Minor decreases in progressive motility within the first 24 h of storage were observed except in 5 mM dose of vitamin E (p<0.01). The supplementation of semen extender with 5 mM vitamin E had detrimental toxicity effect on sperm as the sperm motility was less than 60% at Day 1 and the sperm were totally dead since Day 4. Therefore, data of such level were not included in any Tables.

Since Day 4 of storage, the extended semen supplemented with 1 mM dose of vitamin E exhibited the lowest motility (p<0.01). On Days 7 and 10, the highest motility was found in the extender supplemented with 1 mM dose of Glutathione compared with the control (p<0.01).

For live sperm with normal apical ridge and high mitochondrial energy status, the antioxidants did not seem beneficial to sperm viability, acrosome integrity and mitochondrial function (p>0.05, Table 3).

Discussion

In the present study, the attempt was made to prolong the cool storage time by the addition of various antioxidants at different concentrations into the extender. The results of this experiment suggested that almost all antioxidants at optimal concentrations tended to improve boar semen quality during storage for 10 days at 17°C. The 1 mM dose of glutathione was the most effective for improving the long-term storage of boar semen in terms of sperm motility. Meanwhile, higher doses of vitamin E seemed toxic to boar sperm. This study demonstrated that the percentage of sperm plasma membrane integrity, acrosome integrity and mitochondrial potential was maintained similarly among the groups within the same day of examination.

Table 1 Summary of analysis of variance for sperm motility and sperm viability, acrosome integrity, and mitochondrial function of semen supplemented with various antioxidants and stored for 10 days in Reading medium at 17°C

Source of variation	Level of significance Progressive motility (*p*-value)	Level of significance viability, acrosome integrity, and mitochondrial function (*p*-value)
Days of storage	0.0001	0.0001
Treatment	0.0001	0.9703
Days of storage x Treatment	0.0001	0.8122

Table 2 Effect of glutathione, vitamin E analog (Trolox), and L-carnitine supplementation at various concentrations in Reading medium on progressive motility percentage of boar sperm at Days 1, 4, 7 and 10 after storage at 17°C (n=18)

Antioxidants	Concentrations (mM)	Days of storage			
		1	4	7	10
Control	-	84.06±5.34^{a}	79.89±4.76^{a}	74.33±7.77^{b}	67.44±10.26^{b}
Glutathione	0.1	84.94±5.66^{a}	82.06±4.18^{a}	79.61±5.77^{ab}	73.89±4.89^{ab}
	1	86.28±5.38^{a}	83.78±4.82^{a}	81.72±3.91^{a}	77.72±4.90^{a}
	5	86.61±5.99^{a}	82.67±5.98^{a}	78.94±7.07^{ab}	71.78±7.42^{ab}
Vitamin E	0.1	83.72±6.88^{a}	81.22±5.68^{a}	76.17±8.46^{ab}	70.56±9.22^{ab}
	1	83.56±6.72^{a}	73.61±21.70^{b}	63.22±24.88^{c}	51.33±27.16^{c}
	5	58.33±30.53^{b}	NA	NA	NA
L-Carnitine	0.1	85.00±4.87^{a}	81.78±5.60^{a}	77.50±8.18^{ab}	70.94±9.28^{ab}
	1	85.94±5.29^{a}	83.06±5.88^{a}	77.33±9.02^{ab}	73.39±9.91^{ab}
	5	86.94±5.82^{a}	83.00±6.00^{a}	76.11±11.29^{ab}	70.61±11.98^{ab}
p-value		----------<0.01----------			

^{a,b,c} Different superscripts within column indicate a highly significant difference (p<0.01). ±Standard deviation (SD); NA: unavailable as sperm were totally dead

Good sperm quality is required for the improvement in AI efficiency as it plays an important role in influencing the fertilization of oocytes. Progressive motility is the most commonly used parameter to evaluate sperm quality as it is an indicator of both unimpaired metabolism and intactness of membranes (Johnson et al., 2000). Except for the 1 and 5 mM doses of vitamin E that seemed toxic to sperm, the progressive motility in all groups decreased significantly as the storage time increased. However, differences in sperm motility among the groups within the same day were not significantly different until Day 7 of storage, at which it was found significantly higher in the group supplemented with 1 mM dose of glutathione compared to the control. This dose has previously been documented to improve boar sperm cryosurvival when supplemented in freezing media (Kaeoket et al., 2008). Recently, some studies have reported that the addition of 2 and 5 mM doses of glutathione in freezing media increased freeze ability and enhanced farrowing rates and litter size (Estrada et al., 2014; Yeste et al., 2014). For liquid storage of boar semen, Funahashi and Sone (2005) reported that supplementation of 5 mM glutathione had beneficial effect on long-term preservation of boar semen. However, from this study, in which 0.1, 1 and 5 mM were compared, it could be inferred that the progressive motility was similar for all three concentrations of glutathione but tended to be higher in the 1 mM.

Taken together with glutathione, in this study, the addition of different L-carnitine concentrations to semen extender did not improve sperm motility, even though L-carnitine is an important antioxidant and previous studies reported its positive effect on cryotolerance after oocytes and sperm freezing in other species (Chankitisakul et al., 2013; Manee-In et al., 2014; Sarıözkan et al., 2014; Takahashi et al., 2013). In addition, it was reported that the supplementation of L-carnitine in extended semen of chicken improved semen quality during storage at 4°C (Tabatabaei and Aghaei, 2012).

Another unexpected finding in this study was the lack of positive effect of various antioxidants on sperm viability, acrosome integrity and mitochondrial membrane potential during cold storage for 10 days. According to theory, it is commonly hypothesized that sperm cells exposed to a number of potential sources of stress including dilution and cooling incubation (Cummings et al., 1994; Waberski et al., 2011) results in the damage to sperm plasma membrane through lipid peroxidation from increased level of ROS (Walczak-Jedrzejowska et al., 2013). Therefore, sperm quality gradually deteriorated with decline in motility and viability. Besides, another organelle involved with ROS generation in the spermatozoa is the mitochondria, which is more sensitive at either cooling or freezing temperatures (Kopper et al., 2008). Therefore, the use of antioxidants as semen additives in the extender has been proposed to improve the sperm function (Pena et al., 2003; Roca et al., 2004; Funahashi and Sano, 2005). Surprisingly, the addition of various antioxidants did not improve semen quality in our study. A possible explanation for the lack of positive effect of various antioxidants on sperm quality in terms of motility, viability, acrosome integrity and mitochondrial membrane potential may be due to one of two notices including the storage temperature and potential agents in the Reading medium.

Table 3 Effect of glutathione, vitamin E analog (Trolox), and L-carnitine supplementation at various concentrations in Reading medium on viability, acrosome integrity, and mitochondrial function percentage of boar sperm at Days 1, 5, and 10 after storage at 17°C (n=18)

Antioxidants	Concentrations (mM)	Days of storage		
		1	5	10
Control	-	81.89±3.09	79.68±3.94	73.10±6.74
Glutathione	0.1	82.28±4.12	81.33±4.60	73.84±7.97
	1	83.40±3.13	81.55±4.62	76.73±6.87
	5	81.22±3.62	78.75±4.18	75.67±5.42
Vitamin E	0.1	82.96±4.03	80.54±4.20	74.26±8.16
	1	81.43±4.20	79.34±4.42	74.62±9.00
	5	74.65±9.11	NA	NA
L-Carnitine	0.1	81.95±1.63	80.56±2.20	75.76±5.64
	1	82.79±2.11	80.92±3.52	76.53±5.17
	5	82.42±3.76	78.63±5.23	71.58±5.61
p-value		----------->0.05----------		

±Standard deviation (SD), NA: unavailable as sperm were totally dead

Boar sperm are well-known for being more sensitive to cooling, with less tolerance to decreased temperature than bull (De Leeuw et al., 1990). However, the practical storage temperature of extended boar semen between 15 and 20°C is acceptable. The critical cold shock temperature for extended semen is possibly lower than 12°C as expressed by decreased sperm motility (Althouse et al., 1998). It is, therefore, noted that the storage temperature at 17°C in this study would not negatively affect the sperm quality.

For the latter notice, it seems plausible that Reading medium is suitable for long-term storage without effect on sperm quality (Gadea, 2003). Good extenders commonly act as an energy source for sperm metabolism and provide pH buffering from sperm cell waste, ions for membrane and cell balance (Knox, 2011). Reading medium contains EDTA, a potential chelating agent, and has a significant function to block the action of calcium as a mediator of sperm capacitation and the acrosome reaction during cold storage (Kaeoket et al., 2010). Besides, the composition of trehalose in Reading medium possibly has positive effect on sperm quality. Trehalose is known as a cryoprotectant which protects the sperm membrane from oxidative and cold shock damage (Aisen et al., 2005; Hu et al., 2009). Reading medium itself is, therefore, suitable for long-term liquid preservation without any addition of antioxidant.

It should be noted that this experiment was carried out with conventional procedures similar to the practices on the farm. Therefore, seminal plasma was still retained in the extended semen. Seminal plasma contains natural antioxidants, for instance vitamin E, vitamin C, and glutathione (Brezezinska-Slevbodzinska et al., 1995); these antioxidants could be an advantage for cool preservation.

In this study, the significantly negative influence of high vitamin E dosages (1 and 5 mM) on the sperm quality was noted. In earlier studies in boar, the addition of less than 0.2 mM of vitamin E to the freezing or cooling extender could improve sperm quality (Breininger et al., 2005; Kaeoket et al., 2008; Mendez et al., 2013). High concentration of vitamin E was reported as detrimental to the integrity of acrosome and sperm membrane on rooster after freezing process (Amini et al., 2015). Therefore, our study suggests that high concentrations of vitamin E are not recommended for supplementation in long-term liquid preservation of boar semen.

In conclusion, the sperm motility during long-term storage was affected by the type and dose of

antioxidants. However, the addition of 1 mM dose of Glutathione is recommended as an alternative component of boar semen extender to improve the motility of long-term storage. Moreover, Reading medium itself could maintain viability, acrosome integrity and mitochondrial membrane potential.

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บทคัดย่อ

การศึกษาเปรียบเทียบผลของการเสริมสารต้านอนุมูลอิสระชนิดต่างกันในน้ำยาเจือจางชนิดเก็บรักษาในระยะยาวต่อคุณภาพของน้ำเชื้อสุกรที่เก็บรักษาที่อุณหภูมิ 17 องศาเซลเซียส

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การใช้สารต้านอนุมูลอิสระช่วยให้คุณภาพตัวอสุจิดีขึ้นภายหลังการเก็บรักษาน้ำเชื้อแบบแช่เย็น การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อเปรียบเทียบผลของการเสริมสารต้านอนุมูลอิสระต่างชนิดกันในสารละลายเจือจางน้ำเชื้อต่อคุณภาพของน้ำเชื้อสุกรในระหว่างการเก็บรักษาแบบยาวนานที่อุณหภูมิ 17°C แบ่งน้ำเชื้อสดเจือจาง (18 ตัวอย่าง) ออกเป็น 10 กลุ่มทดลอง และเสริมสารต้านอนุมูลอิสระ 3 ชนิดที่แตกต่างกัน ได้แก่ กลูตาไธโอน แอล-คาร์นิทีน และ วิตามิน-อี ที่ความเข้มข้น 0.1, 1 และ 5mM นำน้ำเชื้อเก็บรักษาในตู้เย็นที่อุณหภูมิ 17°C เป็นเวลา 10 วัน ประเมินการเคลื่อนที่ของตัวอสุจิที่วันที่ 1, 4, 7 และ 10 ของการเก็บรักษาภายใต้กลองจุลทรรศน์ และประเมินอัตราการมีชีวิตรอดของตัวอสุจิที่มีอะโครโซมสมบูรณ์และไมโตคอนเดรียสภาพปกติโดยวิธีการย้อมสีฟลูออเรสเซนต์ 3 สี ได้แก่ propidium iodide (PI), fluorescein isothiocyanate-labeled peanut (Arachis hypogaea) agglutinin (FITC-PNA) และ 5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) ตามลำดับ การศึกษาพบว่า การเสริมกลูตาไธโอนขนาด 1 mM ส่งผลดีต่อการเคลื่อนที่ของตัวอสุจิมากที่สุด ($p<0.01$) อย่างไรก็ตามไม่พบความแตกต่างของอัตราการมีชีวิตรอดของตัวอสุจิที่มีอะโครโซมสมบูรณ์และไมโตคอนเดรียสภาพปกติ ($p>0.05$) ส่วนการเสริมวิตามิน-อีขนาด 1 และ 5 mM ส่งผลเสียต่อคุณภาพน้ำเชื้อ ($p<0.01$) จากการศึกษานี้สรุปได้ว่าการเสริมกลูตาไธโอนขนาด 1 mM ในน้ำยาเจือจางมีผลดีและเป็นทางเลือกในการเก็บรักษาน้ำเชื้อสุกรแช่เย็นเป็นระยะเวลานาน

คำสำคัญ: กลูตาไธโอน แอล-คาร์นิทีน การเก็บรักษาน้ำเชื้อสด วิตามิน-อี

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