

9-1-2011

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

Kampon Kaeoket

Primsiri Chanapai

Puriwat Junchiyaphoom

Panida Chanapiwat

Follow this and additional works at: <https://digital.car.chula.ac.th/tjvm>



Part of the [Veterinary Medicine Commons](#)

Recommended Citation

Kaeoket, Kampon; Chanapai, Primsiri; Junchiyaphoom, Puriwat; and Chanapiwat, Panida (2011) "The Effect of Using Long Term and Short Term Extenders during Cooling Process on the Quality of Frozen Boar Semen," *The Thai Journal of Veterinary Medicine*: Vol. 41: Iss. 3, Article 4.

Available at: <https://digital.car.chula.ac.th/tjvm/vol41/iss3/4>

This Article is brought to you for free and open access by the Chulalongkorn Journal Online (CUJO) at Chula Digital Collections. It has been accepted for inclusion in The Thai Journal of Veterinary Medicine by an authorized editor of Chula Digital Collections. For more information, please contact ChulaDC@car.chula.ac.th.

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

Kampon Kaeoket* Primsiri Chanapai Puriwat Junchiyaphoom Panida Chanapiwat

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°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

Semen laboratory, Department of Clinical Science and Public Health, Faculty of Veterinary Science, Mahidol University, Phutthamonthon 4 Rd., Salaya, Phuttamonthon, Nakhon-pathom 73170, Thailand

**Corresponding author E-mail: vskkk@mahidol.ac.th*

บทคัดย่อ

การศึกษาผลของการใช้สารละลายเจ็องน้ำเชื้อชนิดระยะสั้นและชนิดระยะยาวเป็นสารละลายเจ็องน้ำเชื้อที่ 1 ต่อคุณภาพของน้ำเชื้อสุกรแช่แข็ง

กัมพล แก้วเกษ* พิมพ์ลิริ ชนะภัย ภูริวัจน์ จันทรชัยภูมิ พนิดา ชนาภวัฒน์

ขั้นตอนการแช่เย็น (cooling process) ในกระบวนการผลิตน้ำเชื้อแช่แข็งนั้นเป็นหนึ่งในปัจจัยที่สำคัญและมีผลกระทบต่อคุณภาพของน้ำเชื้อสุกรแช่แข็ง ดังนั้นการศึกษาในครั้งนี้จึงมีวัตถุประสงค์เพื่อเปรียบเทียบคุณภาพของน้ำเชื้อสุกรแช่แข็งหลังจากการใช้สารละลายเจ็องน้ำเชื้อสำหรับแช่แข็งที่ 1 ที่แตกต่างกัน (BTS Vitasem LD ModenaTM และ Androstar[®] plus) ในขั้นตอนการแช่เย็น โดยในการทดลองครั้งนี้จะใช้น้ำเชื้อสดที่รีดมาจากพ่อสุกรจำนวนแปดตัว โดยน้ำเชื้อสดจากพ่อสุกรแต่ละตัวนั้นจะถูกแบ่งออกเป็น 4 กลุ่มแล้วเติมสารละลายเจ็องน้ำเชื้อสำหรับแช่แข็งที่ 1 ในแต่ละกลุ่มดังนี้ กลุ่มที่ 1 ใช้สารละลายเจ็องน้ำเชื้อชนิดระยะสั้น BTS (กลุ่มควบคุม) กลุ่มที่ 2 ใช้สารละลายเจ็องน้ำเชื้อระยะยาวชนิด Vitasem LD กลุ่มที่ 3 ใช้สารละลายเจ็องน้ำเชื้อระยะยาวชนิด ModenaTM และกลุ่มที่ 4 ใช้สารละลายเจ็องน้ำเชื้อระยะยาวชนิด 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 จากผลการทดลองแสดงให้เห็นว่าการใช้สารละลายเจ็องน้ำเชื้อชนิดระยะยาวเป็นสารละลายเจ็องน้ำเชื้อสำหรับแช่แข็งที่ 1 ในช่วงขั้นตอนการแช่เย็นสามารถรักษาคุณภาพน้ำเชื้อในแง่ของอัตราการเคลื่อนที่ไปข้างหน้าและอัตราการมีชีวิตรอดของตัวอสุจิ ในช่วงขั้นตอนการแช่เย็นได้ดีกว่าการใช้สารละลายเจ็องน้ำเชื้อชนิดระยะสั้น

คำสำคัญ: น้ำเชื้อสุกร ระยะพัก กระบวนการแช่แข็ง สารละลายเจ็องน้ำเชื้อชนิดระยะสั้น สารละลายเจ็องน้ำเชื้อชนิดระยะยาว

ห้องปฏิบัติการน้ำเชื้อ ภาควิชาเวชศาสตร์คลินิกและการสาธารณสุข คณะสัตวแพทยศาสตร์ มหาวิทยาลัยมหิดล ถนนพุทธมณฑล 4 ต.

ศาลายา อ. พุทธมณฑล จ. นครปฐม 73170

*ผู้รับผิดชอบบทความ E-mail: vskkk@mahidol.ac.th

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, ModenaTM, 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 $\geq 70\%$ and normal morphology of $\leq 80\%$ 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 5°C 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 performed 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 μ l of diluted spermatozoa were mixed with 2.7 μ l of the working solution of SYBR-14 and 10 μ l 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 μ l of diluted semen with 140 μ l phosphate buffered saline (PBS) were mixed with 10 μ l of EthD-1 and incubated at 37°C for 15 min. Then, 5 μ l of suspension were smeared on glass slides and fixed with 95% ethanol for 30 sec and air-dried. In the next step, 50 μ l Fit-C-PNA (diluted Fit-C-PNA with PBS 1:10 v/v) were spread over the slide and incubated in a moist chamber at 4°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 \times 1000 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 \pm 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 \pm 5.5, 74.0 \pm 4.4, 72.1 \pm 10.3 and 43.0 \pm 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).

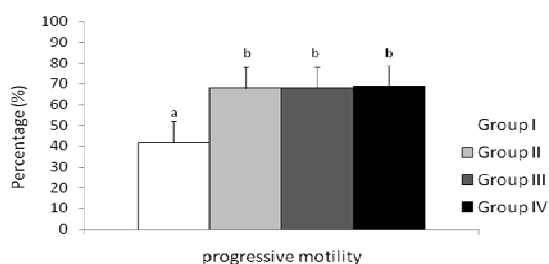


Figure 1 Post cooling progressive motility of semen samples in different extenders: Group I (BTS), Group II (Vitasem LD), Group III (Modena™), Group IV (Androstar® plus) presented as bar (mean \pm SD). Bar marked by different letters are significantly different ($p < 0.05$).

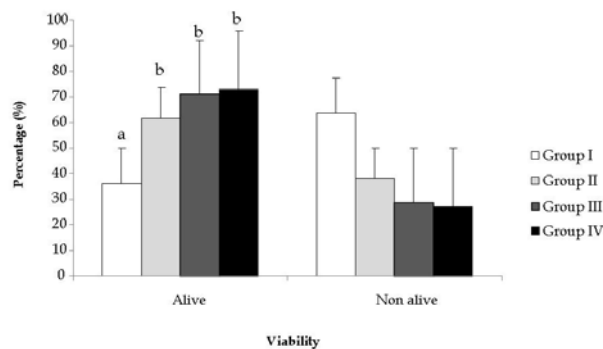


Figure 2 Post-cooling viability of semen samples in different extenders: Group I (BTS), Group II (Vitasem LD), Groups III (Modena™) and group IV (Androstar® plus) presented as bar (mean \pm SD). Bar marked by different letters are significantly different ($p < 0.05$).

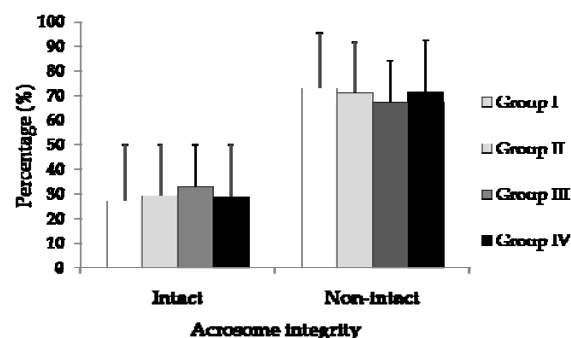


Figure 3 Post-cooling acrosome integrity of semen samples in different extenders: Group I (BTS), Group II (Vitasem LD), Group II (Modena™) and Group IV (Androstar® plus) presented as bar (mean \pm SD).

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).

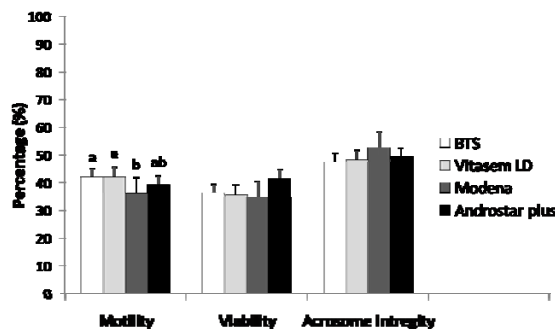


Figure 4 Post-thawing semen qualities in four different freezing extenders I: Group I (BTS), Group II (Vitasem LD), Group III (Modena™) and Group IV (Androstar® plus) presented as bar (mean \pm SD). Bar marked by different letters are significantly different ($p < 0.05$).

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. (2010⁴) 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.

Acknowledgements

The authors are grateful to Dr. Surasak Jittakhot, Faculty of Veterinary Science, Mahidol University for his advice in statistical analysis. Research grant was provided by Faculty of Veterinary Science, Mahidol University.

References

- Almlid, T. and Hofmo, P.O. 1996. A brief review of frozen semen application under Norwegian AI service conditions. *Reprod Dom Anim.* 31(1): 169-173.
- Almlid, T. and Johnson, L.A. 1998. Effects of glycerol concentration, equilibration time and temperature of glycerol addition on post-thaw viability of boar spermatozoa frozen in straws. *J Anim Sci.* 66(11): 2899-2905.
- Alvarez, J.G. and Storey, B.T. 1995. Differential incorporation of fatty acids into and peroxidative loss of fatty acids from phospholipids of human spermatozoa. *Mol Reprod Dev.* 42(3): 334-346.
- Bamba, K. and Cran, D.G. 1992. Effects of treatment with butylated hydroxytoluene on the susceptibility of boar spermatozoa to cold stress and dilution. *J Reprod Fertil.* 95(1): 69-77.
- Berger, T., Marrs, R.P. and Moyer, D.L. 1985. Comparison of techniques for selection of motile spermatozoa. *Fertil Steril.* 43(2): 268-273.
- Bilodeau, J.F., Blanchette, S., Gagnon, C. and Sirard, M.A. 2001. Thiols prevent H₂O₂-mediated loss of sperm motility in cryopreserved bull semen. *Theriogenology.* 56(2): 275-286.
- Breining, E., Beorlegui, N.B., O'Flaherty, C.M. and Beconi, M.T. 2005. Alpha-tocopherol improves biochemical and dynamic parameters in cryopreserved boar semen. *Theriogenology.* 63(8): 2126-2135.
- Brzezińska-Ślebodzińska, E., Ślebodziński, A.B., Pietras, B. and Wiczorek, G. 1995. Antioxidant effect of vitamin E and glutathione on lipid peroxidation in boar semen plasma. *Biol Trace Elem Res.* 47(1-3): 69-74.
- Buranaamnuay, K., Tummaruk, P., Singlor, J., Rodriguez-Martinez, H. and Techakumphu, M. 2009. Effects of straw volume and Equex-STM® on boar sperm quality after cryopreservation. *Reprod Dom Anim.* 44(1): 69-73.
- Carvajal, G., Cuello, C., Ruiz, M., Vazquez, J.M., Martinez, E.A. and Roca, A. 2004. Effects of centrifugation before freezing on boar sperm cryosurvival. *J Androl.* 25(3): 389-396.
- Cerolini, S., Maldjian, A., Surai, P. and Noble, R. 2000. Viability, susceptibility to peroxidation and fatty acid composition of boar semen during liquid storage. *Anim Reprod Sci.* 58(1-2): 99-111.
- Chanapiwat, P., Kaeoket, K. and Tummaruk, P. 2009. Effects of DHA-enriched hen egg yolk and L-cysteine supplementation on quality of cryopreserved boar

- semen. *Asian J Androl.* 11(5): 600-608
- Eriksson, B.M., Vazquez, J.M. and Martinez, E.A. 2001. Effects of holding time during cooling and of type of package on plasma membrane integrity, motility and *in vitro* oocyte penetration ability of frozen-thawed boar spermatozoa. *Theriogenology.* 55(8): 1593-1605.
- Eriksson, B.M., Petersson, H. and Rodriguez-Martinez, H. 2002. Field fertility with exported boar semen frozen in the new flatpack container. *Theriogenology.* 58(6): 1065-1079.
- Foote, R., Brockett C. and Kaproth, M. 2002. Motility and fertility of bull sperm in whole milk extender containing antioxidants. *Anim Reprod Sci.* 71(1-2): 13-23.
- Funahashi, H. and Sano, T. 2005. Select antioxidants improve the function of extended boar semen stored at 10 degrees C. *Theriogenology.* 63(6): 1605-1616.
- Gadea, J. 2003. Semen extenders used in artificial insemination of swine. *Spanish Agric Res.* 1(2): 17-27.
- Gadea, J., Selles, E., Marco, M.A., Coy, P., Matas, C., Romar, R. and Ruiz, S. 2004. Decrease in glutathione content in boar sperm after cryopreservation, effect of the addition of reduced glutathione to the freezing and thawing extender. *Theriogenology.* 62(3): 690-701.
- Gilmore, J.A., Junying, D., Jun, T., Peter, A.T. and Crister, J.K. 1996. Osmotic properties of boar spermatozoa and their relevance to cryopreservation. *J Reprod Fertil.* 107(1): 87-95.
- Guthrie, H.D. and Welch, G.R. 2005. Impact of storage prior to cryopreservation on plasma membrane function and fertility of boar sperm. *Theriogenology.* 63(2): 396-410.
- Hammerstedt, R.H., Graham, J.K. and Nolan, J.P. 1990. Cryopreservation of mammalian sperm: What we ask them to survive. *J Androl.* 11(1): 73-88.
- Holt, W.V. 2000a. Fundamental aspects of sperm cryobiology: The importance of species and individual differences. *Theriogenology.* 53(1): 47-58.
- Holt, W.V. 2000b. Basic aspects of frozen storage of semen. *Anim Reprod Sci.* 62(1-3): 3-22.
- Johnson, L.A., Weitze, K.F., Fiser, P. and Maxwell, W.M.C. 2000 Storage of boar semen. *Anim Reprod Sci.* 62(1-3): 142-172.
- Kaeoket, K. and Tummaruk, P. 2002^a. Seminal plasma: Its function and importance for pig artificial insemination. *Thai J Vet Med.* 32(4): 15-25.
- Kaeoket, K., Persson, E. and Dalin, A.M. 2002^b. The influence of pre- and post-ovulatory insemination on sperm distribution in the oviduct, accessory sperm to the zona pellucida, fertilisation rate and embryo development in sows. *Anim Reprod Sci.* 71(3): 239-248.
- Kaeoket, K., Tantasuparak, W. and Kunavongkrit, A. 2005. The effect of post-ovulatory insemination on the subsequent oestrous cycle length, embryonic loss and vaginal discharge in sows. *Reprod Domest Anim.* 40(5): 492-494.
- Kaeoket, K., Tantiparinyakul, K., Kladaew, W., Chanapiwat, P. and Techakumphu, M. 2008 Effect of different antioxidants on quality of cryopreserved boar semen in different breeds. *Thai J Agri Sci.* 41(1-2): 1-9.
- Kaeoket K., Chanapiwat P., Tummaruk P. and Techakumphu M. 2010^a. Supplemental effect of varying L-cysteine concentration on qualities of cryopreserved boar semen. *Asian J Androl.* 12(5): 760-765.
- Kaeoket, K., Chanapiwat, P., Wongtawan, T. and Kunavongkrit, A. 2010^b. Successful intrauterine insemination (IUI) with frozen boar semen: effect of dose, volume and fixed-time AI on fertility. *Thai J Agric Sci.* 43(1): 31-37.
- Kaeoket, K., Sang-urai, P., Thamniyom, A., Chanapiwat, P. and Techakumphu, M. 2010^c. Effect of docosahexaenoic acid (DHA) on quality of cryopreserved boar semen in different breeds. *Reprod Dom Anim.* 45(3): 458-463.
- Kaeoket, K., Srisowanna, T., Wichaidit, U., Chanapiwat, P. and Manee-in, S. 2010^d. Comparative study on six different long term commercial extenders for fresh boar semen. *Thai J Vet Med.* 40(3): 257-263.
- Kasetrut C. and Kaeoket, K. 2010. Effect of using supernatant for post-thawing solution and semen extender prior to insemination on sow reproductive performance. *Thai J Vet Med.* 40(2): 171-178.
- Lin, D. S., Neuringer, M. and Connor, W.E. 2003. Selective changes of docosahexaenoic acid-containing phospholipid molecular species in monkey testis during puberty. *J Lipid Res.* 45(3): 529-235.
- McLaughlin, E.A. and Ford, W.C.L. 1994. Effects of cryopreservation on the intracellular calcium concentration of human spermatozoa and its response to progesterone. *Mol Reprod Dev.* 37(2): 241-246.
- Medeiros, C.M.O., Forell, F., Oliveira, A.T.D. and Rodrigues, J.L. 2002. Current status of sperm cryopreservation: Why isn't it better? *Theriogenology.* 57(1): 327-344.
- Ollero, M., Powers, R.D. and Alvarez, J.G. 2000. Variation of docosahexaenoic acid content in subsets of human spermatozoa at different stages of maturation implications for sperm lipoperoxidative damage. *Mol Reprod Dev.* 55(3): 326-334.
- Penã, F. J., Johannisson, A., Wallgren, M. and Rodriguez Martinez, H. 2003. Antioxidant supplementation *in vitro* improves boar sperm motility and mitochondrial membrane potential after cryopreservation of different fractions of the ejaculate. *Anim Reprod Sci.* 78(1-2): 85-98.
- Pursel, V.G., Schulman, L.L. and Johnson, L.A. 1987. Effect of Orvus ES Paste on acrosome morphology, motility and fertilizing capacity of frozen-thawed boar sperm. *J Anim Sci.* 47(1): 198-202.
- Pursel, V.G. 1979. Effect of cold shock on boar sperm treated with butylated hydroxytoluene. *Biol Reprod.* 21(2): 319-324.
- Roca, J., Gil, M.A., Hernandez, M., Parrilla, I., Vazquez, J.M. and Martinez, E.A. 2004. Survival and fertility of boar spermatozoa after freeze-thawing in extender supplemented with butylated hydroxytoluene. *J Androl.* 25(3): 397-405.
- Roca, J., Rodriguez, J.M., Gil, M.A., Carvajal, G., Garcia, E.M., Cuello, C., Vazquez, J.M. and Martinez, E.A. 2005 Survival and *in vitro* fertility of boar spermatozoa frozen in the presence of superoxide dismutase and/or catalase. *J Androl.* 26(1): 15-24
- Satorre, M.M., Breininger, E., Beconi, M.T. and Beorlegui, N.B. 2007. Alpha-tocopherol modifies tyrosine phosphorylation and capacitation-like state of cryopreserved porcine sperm. *Theriogenology.* 68(7): 958-965.
- Selles, E., Gadea, J., Romar, R., Matas, C. and Ruiz, S. 2003. Analysis of *in vitro* fertilizing capacity to evaluate the freezing procedures of boar semen and to predict the subsequent fertility. *Reprod Dom Anim.* 38(1): 66-72.
- Sikka, S.C., Rajasekaran, M. and Hellstrom, W.J. 1995. Role of oxidative stress and antioxidants in male infertility. *J Androl.* 16(6): 464-481.
- Uysal, O. and Bucak, M.N. 2007. Effects of oxidized glutathione, bovine serum albumin, cysteine and lycopene on the quality of frozen-thawed ram semen. *Acta Vet Brno.* 76(3): 383-390.
- Watson, P.F. 2000. The causes of reduced fertility with cryopreserved semen. *Anim Reprod Sci.* 60-61(1-2): 481-492.
- White, I.G. 1993. Lipids and calcium uptake of sperm in relation to cold shock and preservation: A review. *Reprod Fertil Dev.* 5(6): 639-658.