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Assessment of Appropriate *L*-cysteine Concentration for Boar Semen Cryopreservation by Using Flow Cytometry

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Abstract

The present study aims to investigate a suitable *L*-cysteine level to be added the lactose-egg yolk (LEY) extender to improve the quality of boar semen after freezing-thawing using Annexin-V and propidium iodide (PI) assay detected with flow cytometer. Ejaculates from twelve boars were processed in the LEY extender and added with 0, 5, 10 and 15 mM of *L*-cysteine. Semen suspensions were cryopreserved with a controlled-rate freezer and thawed for examination. The surface of sperm plasma membrane was explored by scanning electron microscopy (SEM) while sperm membrane integrity was performed by Annexin-V/PI assay. Of all treatments, the damaged spermatozoa in various patterns were observed whereas the intact spermatozoa were still found by SEM. The viable and non-apoptotic (AN-/PI-) sperms were higher ($p < 0.05$) in the levels of 5 and 10 mM. These results indicated that the damaged spermatozoa were found after freezing/thawing and the *L*-cysteine at concentrations of 5 to 10 mM added to LEY extender was the optimal dose against oxidative stress and acquisition for improving cryopreserved boar sperm quality.

Keywords: freezing, *L*-cysteine, pig, semen extender, spermatozoa

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บทคัดย่อ

การศึกษาหาความเข้มข้นที่เหมาะสมของแอลซีสเตอีนสำหรับกระบวนการแช่แข็งน้ำเชื้อสุกรพ่อพันธุ์ด้วยวิธีโฟลว์ไซโตเมทรี

ไพศาล เทียนไทย¹ พนิดา ขนาภวัฒน์¹ สุภัญญา มณีอินทร์² ดุลยพรรณ กรณธ์แสง³ กัมพล แก้วเกษ²

การศึกษานี้มีวัตถุประสงค์เพื่อตรวจหาความเข้มข้นที่เหมาะสมของแอลซีสเตอีนเติมลงในสารละลายเจือจางน้ำเชื้อ lactose-egg yolk (LEY) เพื่อปรับปรุงคุณภาพของน้ำเชื้อสุกรพ่อพันธุ์ภายหลังการแช่แข็งและทำละลายโดยใช้วิธีย้อม Annexin-V/PI ร่วมกับโฟลว์ไซโตเมเตอร์ น้ำเชื้อสดจากสุกรพ่อพันธุ์จำนวน 12 ตัวผ่านกระบวนการโดยใช้สารละลายเจือจางน้ำเชื้อ LEY และเติมแอลซีสเตอีนที่ความเข้มข้น 0 5 10 และ 15 มิลลิโมลาร์ น้ำเชื้อตัวอย่างนำไปผ่านการแช่แข็งและทำละลายก่อนนำไปศึกษา ลักษณะผิวของเยื่อหุ้มเซลล์สุจิศึกษาด้วยกล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราด (SEM) ขณะที่ความสมบูรณ์ของเยื่อหุ้มเซลล์สุจิศึกษาด้วยวิธีย้อม Annexin-V/PI ผลจากกล้อง SEM พบความเสียหายแบบต่างๆ เกิดขึ้นกับผิวของเยื่อหุ้มเซลล์สุจิในทุกความเข้มข้น ขณะที่ยังพบเซลล์สุจิที่มีเยื่อหุ้มเซลล์สมบูรณ์กระจายอยู่ เมื่อศึกษาด้วยการย้อม Annexin-V/PI พบว่า เซลล์สุจิที่มีเยื่อหุ้มสมบูรณ์ซึ่งมีชีวิตและไม่มีการเสื่อมตาย (AN-/PI-) เพิ่มขึ้น ($p < 0.05$) ที่ความเข้มข้น 5 และ 10 มิลลิโมลาร์ การศึกษานี้บ่งชี้ว่า ความเสียหายของเยื่อหุ้มเซลล์สุจิสามารถพบได้ในน้ำเชื้อที่ผ่านการแช่แข็งและทำละลาย นอกจากนี้ พบว่าแอลซีสเตอีนที่ความเข้มข้น 5 และ 10 มิลลิโมลาร์ใน LEY เป็นความเข้มข้นที่เหมาะสม มีผลต่อต้านภาวะ oxidative stress ซึ่งช่วยให้น้ำเชื้อสุกรพ่อพันธุ์แช่แข็งมีคุณภาพดีขึ้น

คำสำคัญ: การแช่แข็ง แอลซีสเตอีน สุกร สารละลายเจือจางน้ำเชื้อ เซลล์สุจิ

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Introduction

The principal limitation of using cryopreserved semen is the possibility of lower fertility in comparison with fresh semen due to cold shock and the condition of the suspension media (Johnson et al., 2000). In boars, sperm plasma membrane contains high level of polyunsaturated fatty acids (PUFA) and there is low anti-oxidant competence in the seminal plasma (Roca et al., 2005). For these reasons, boar spermatozoa are absolutely more vulnerable to oxidative damage by reactive oxygen species (ROS) than other livestock animals (Parks and Lynch, 1992; Flesch and Gadella, 2000). Excessive production of ROS during cryopreservation procedures is able to diminish sperm motility and viability (Armstrong et al., 1999) and plasma membrane function (Chatterjee and Gagnon, 2001), and increase DNA damage (Lopes et al., 1998; Baumber et al., 2003).

With the properties of boar semen, corroboration to defend boar sperms by oxidative stress through the addition of anti-oxidants has a more beneficial outcome than in animals that contain high levels of the innate resistance to ROS (Bathgate, 2011). Among

the anti-oxidants, L-cysteine has been used during boar semen cryopreservation to adjust the quality of sperm parameters (Chanapiwat et al., 2009; Kaeoket et al., 2010; Malo et al., 2010). The effective concentration of L-cysteine varied from 1 to 10 mmol/l and these studies increased by use of conventional sperm evaluation techniques. At present, the capability of Annexin-V/PI binding assay combined with flow cytometer is a better and faster assessment to detect the early deleterious changes of sperm plasma membrane in boars (Pena et al., 2005). The present study, therefore, aimed to investigate the optimal concentration of L-cysteine in LEY extender using Annexin-V/PI flow cytometry to develop the qualities of post-thaw boar spermatozoa.

Materials and Methods

Semen processing and experimental design: Semen were collected by gloved-hand method from 12 boars comprising Duroc (n= 4), Landrace (n= 4) and Large White (n= 4) of proven fertility, 1-3 years of age and routinely used for semen collection. The sperm membrane morphology of the fresh spermatozoa was assessed by scanning electron microscopy (SEM).

Only semen samples with $\geq 70\%$ motile and $\geq 80\%$ morphologically normal sperms were used for cryopreservation. All semen samples were cryopreserved in a controlled-rate freezer (Icccube 14s, Sylab, Purkersdorf, Austria) according to the previous study by Kaeoket et al. (2008). In brief, fresh boar semen was diluted (1:1 v/v) with Extender I, i.e. Modena extender (Swine Genetics Int Ltd.). Diluted semen samples were cooled at 15°C for 120 min and centrifuged at $800\times g$ at 15°C for 10 min. The supernatant was removed and the sperm pellet was re-suspended with Extender II, consisting of 80 ml of 11% lactose and 20 ml egg yolk (LEY), to a concentration of 1.5×10^9 spermatozoa/ml. Four groups of varied concentrations of L-cysteine (Fluka Chemie GmbH, Sigma-Aldrich, Buchs, Switzerland) were 0, 5, 10 and 15 mM supplemented in LEY extender. The diluted semen was incubated at 5°C for 90 min and mixed with a half volume of Extender III, composing of 89.5% LEY extender with 9% (v/v) glycerol and 1.5% (v/v) Equex-STM (Nova Chemical Sale Inc., Scituate, MA, USA). The semen suspensions were adjusted to a final concentration of 1.0×10^9 sperms/ml, contained in 0.5 ml polyvinyl chloride medium-straws (Bio-Vet, Fleurance, France). All straws were put into the controlled-rate freezer and promptly plunged into liquid nitrogen (-196°C) for storage and investigation. Thawing was done in a thermos flask at 50°C for 12 sec.

Sperm membrane morphology: The aliquots from fresh semen and all frozen/thawed semen groups were fixed in a solution of 2.5% glutaraldehyde in phosphate-buffered saline (pH 7.4) at 4°C for 24 hours. The fixed samples were centrifuged at $400\times g$ for 5 min and rinsed in distilled water. Suspensions of semen were placed on a glass slide, post-fixed for 1 hour in 1% osmium tetroxide (Merk, Darmstadt, Germany) in PBS (pH 7.4) and washed again. The semen samples were dehydrated in graded ethanol (30-100%) and then subjected to critical point drying using liquid CO_2 substitution. Glass slides with dehydrated sperm suspensions were cut and mounted on stubs, coated with gold-palladium in a sputter coater, and evaluated using a JEOL 5800 LV (JEOL, Tokyo, Japan) SEM.

Sperm membrane stability: To detect the initial deleterious sperm membrane changes, a fluorescein isothiocyanate (FITC) annexin-V apoptosis detection kit II (BD Pharmingen, San Diego, CA, USA) was used and the staining protocol was performed as suggested by the manufacturer with slight modifications. After thawing, the boar semen samples were gently washed twice in PBS and resuspended with Annexin-V binding buffer (0.1 M HEPES/NaOH (pH 7.4), 1.4 M NaCl, 25 mM CaCl_2) at room temperature to 1.0×10^7 sperms/ml. Aliquots of 100 μl extended semen (1.0×10^6 sperms/ml) were transferred to a 5 ml culture tube and incubated in the dark with 5 μl FITC Annexin-V and 5 μl PI for 15 min at room temperature. A quantity of 400 μl of binding buffer was added to each tube and flow cytometric analysis was performed within 10 min. The semen samples were evaluated on an LSR flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with

standard optics using the instruments Argon-ion (488 nm) and Helium-Cadmium (325 nm) laser. For each cell, FSC, SSC, FITC fluorescence (FL1) and PI (FL3) were evaluated using CellQuest version 3.3 (Becton Dickinson, San Jose, CA, USA). For the gated cells, the percentages of viable spermatozoa with stable plasma membrane (AN-/PI-), unstable spermatozoa but intact plasma membrane (AN+/PI-) while membrane-damaged spermatozoa (AN-/PI+) and double positive (AN+/PI+) were also evaluated (Fig 1).

Statistical analyses: All data were analyzed by the general linear model (GLM) procedure of the Statistical Analysis Software (SAS Institute Inc, Cary, NC, USA) and expressed as mean \pm SD. The mean values were compared by Student's *t*-test with $p < 0.05$.

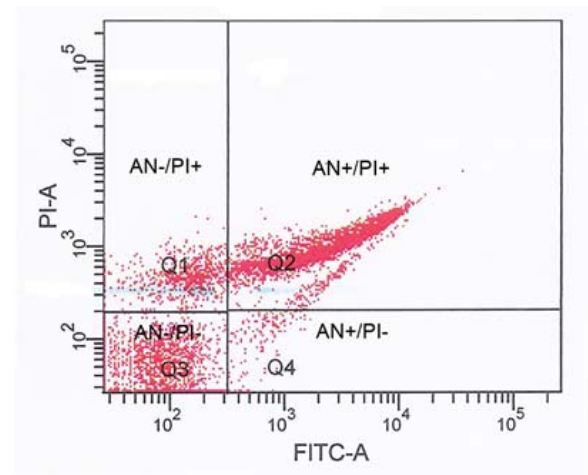


Figure 1 Flow cytometry analysis of a sperm subpopulation by Annexin-V/PI fluorescent staining. Each quadrant for evaluation is indicated as AN-/PI-: viable spermatozoa with no signs of PS translocation; AN+/PI-: viable spermatozoa showing PS translocation. AN+/PI+: dead spermatozoa showing PS translocation. AN-/PI+: dead spermatozoa with no signs of PS translocation.

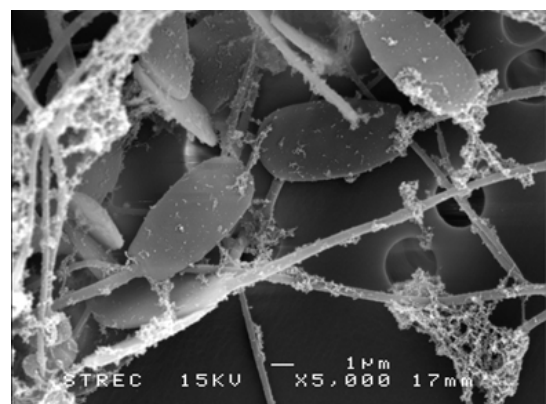


Figure 2 SEM micrograph of ejaculated boar spermatozoa demonstrates a normal plasma membrane surface without any damage. Scale bars represent 1 μm .

Results

Morphological observation by SEM of most fresh boar spermatozoa depicted the normal and smooth surface of the plasma membrane (Fig 2) whereas boar spermatozoa with various damaged degrees of plasma membrane were seen in arbitrary frozen-thawed spermatozoa from three different breeds of all treatments (Fig 3a-d). Among these injured cells, the intact spermatozoa were frequently seen in all *L*-cysteine concentration applied.

For Annexin-V/PI staining assay with flow cytometer, the percentages of viable spermatozoa without apoptosis or necrosis (AN-/PI-) were revealed to be significantly higher ($p < 0.05$) in LEY extender supplemented with 5 and 10 mM *L*-cysteine than other concentrations (Table 1). Additionally, the average percentages of dead spermatozoa (AN+/PI+) were apparently shown significantly higher ($p < 0.05$) in the groups of 0 mM and 15 mM concentrations.

Discussion

In the present study, the *L*-cysteine at doses of 5 mM and 10 mM added in the LEY extender (extender II) increased sperm viability that demonstrated complete plasma membrane stability of spermatozoa compared with the other groups corresponding to previous study (Kaeoket et al., 2010). This is the first report to determine the optimal concentrations of *L*-cysteine for boar semen cryopreservation by Annexin-V/PI assay with flow cytometer.

Semen cryopreservation processes cause oxidative stress by producing ROS that damage various cellular structures of spermatozoa (O'Flaherty et al., 1997; Bilodeau et al., 2001) and high amounts of ROS were able to decrease sperm motility and viability (Armstrong et al., 1999) as well as to damage DNA (Baumber et al., 2003). However, in the latter, a recent study reported that DNA detriment of frozen-thawed boar spermatozoa was relatively low (Chanapiwat et al., 2010). Therefore, the major injury to boar spermatozoa during cryopreservation could be focused on the sperm plasma membrane that is affected by lipid peroxidation in accordance to our study by SEM which demonstrated the laceration of the frozen-thawed sperm plasma membrane in varying patterns. It is reported that lipid peroxidation releases PUFA from the plasma membrane, altering membrane fluidity, permeability and cellular capacity to regulate intracellular ions and, in this manner, disrupting membrane structure and function resulting in reducing motility (Baumber et al., 2003; Awda et al., 2009). Additionally, lipid peroxidation of PUFA also causes cell dysfunction associated with the loss of membrane functions, integrity and, of course, cell viability (Bansal and Bilaspuri, 2010).

In this study, *L*-cysteine at levels of 5 and 10 mM in LEY extender notably improved sperm viability corresponding to earlier studies (Chanapiwat et al., 2009; Kaeoket et al., 2010) which were performed using the conventional staining procedures to assess sperm quality. On the contrary, the viable

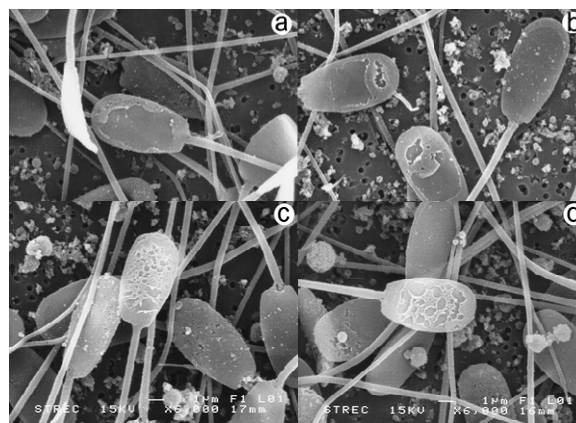


Figure 3 SEM micrographs of thawed spermatozoa from random breeds of boar after cryopreservation with concentrations of (a) 0 mM, (b) 5 mM, (c) 10 mM and (d) 15 mM of *L*-cysteine supplemented in the LEY extender showing diversity degrees of injury to the sperm plasma membrane. Scale bars represent 1 μ m.

Table 1 Percentages (mean \pm SD) of plasma membrane stability of frozen-thawed boar spermatozoa in different concentrations of *L*-cysteine supplemented in LEY extender using Annexin-V/PI detected with flow cytometry.

Annexin-V/PI staining (%)	Treatments			
	0 mM (n= 12)	5 mM (n= 12)	10 mM (n= 12)	15 mM (n= 12)
Alive (AN-/PI-)	19.7 \pm 2.1 ^a	31.3 \pm 7.4 ^b	36.4 \pm 9.6 ^b	16.6 \pm 6.7 ^a
Alive (AN-/PI+)	10.3 \pm 2.0 ^a	11.7 \pm 2.9 ^a	9.5 \pm 2.6 ^a	10.5 \pm 3.1 ^a
Dead (AN+/PI-)	2.9 \pm 4.7 ^a	2.6 \pm 5.3 ^a	2.4 \pm 8.7 ^a	3.9 \pm 5.4 ^a
Dead (AN+/PI+)	67.1 \pm 13.7 ^a	53.4 \pm 5.7 ^b	50.7 \pm 15.1 ^b	69.0 \pm 11.3 ^a

Values followed by different letters within the same row against each sperm parameter are significantly different ($p < 0.05$)

spermatozoa in this study indicated the spermatozoa with stable plasma membrane because the equipment, Annexin-V/PI via flow cytometer, can detect the earliest plasma membrane changes in spermatozoa (Pena et al., 2005). It is known that the stability and integrity (from previous study) of sperm plasma membrane are important for cell viability and the capability of sperm to interact with the environment during sperm transport throughout female reproductive tract including penetrating the cumulus cells and zona pellucida. Therefore, we ensured that *L*-cysteine added to the extender would have a beneficial effect on frozen-thawed boar semen protocol by protecting the sperm plasma membrane and improving sperm survival (Szczeniak-Fabianzyk, 2003; Uysal and Bucak, 2007).

Concerning the properties of *L*-cysteine, we found that cysteine is a precursor of glutathione and it has a low-molecular weight which easily penetrates the cell membrane (Uysal and Bucak, 2007). Cysteine also increases intracellular glutathione sulfhydryl or reduced glutathione (GSH) biosynthesis and protects

the membrane lipids because it acts as a free radical scavenger (Aruoma et al., 1989; Hendin et al., 1999). However, there have been several studies suggesting that cysteine can be effective to cell structures depending on the relative concentrations added (Whitaker and Knight, 2010) and the higher doses of antioxidants supplemented to semen extender can raise the cryopreserved sperm injury (Whitaker et al., 2008) corresponding to our investigation. Gadea et al. (2004) suggested that the levels of GSH were decreased during freezing-thawing boar semen and there were no effective results to improve the sperm parameters when GSH was added to the semen extender. Therefore, the indirect mechanism to increase intracellular GSH can be performed by supplementation of *L*-cysteine in the freezing extender and the doses of *L*-cysteine between 5 to 10 mM might compensate for the GSH content which is lost during cryopreservation. In conclusion, the *L*-cysteine at concentrations of 5 and 10 mM supplemented in the LEY freezing extender increased sperm viability (intact plasma membrane stability without apoptosis/necrosis) and could be confirmed to be the optimal concentration for boar semen cryopreservation protocols.

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