# EFFECT OF SOYBEAN LECITHIN ON FREEZABILITY AND FERTILIZING POTENTIALS OF BOVINE SPERMATOZOA

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## ABSTRACT

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Soybean lecithin has been attracted increasing attention and has been used to replace egg yolk in the semen extender. In the present study, effect of soybean lecithin on bovine semen freezability and in vitro fertilizing potentials were evaluated. Semen was cryopreserved in tris-based extender supplemented with different soybean lecithin concentrations (3, 5, 7, 10 and 20%) vs 20% egg yolk (control). Semen post-thawing motility, viability and acrosomal integrity, DNA damage, enzymes leakage, total antioxidant activity (TAC), lipid peroxidation and in vitro fertilizing potentials were assessed. Current results indicated that addition of 7% soybean lecithin to semen extender significantly (P<0.05) improved post-thawing motility, viability and acrosomal integrity (61.25±1.25%, 172.25±5.53 and 10.25±2.39 %, respectively) compared with control (47.50±4.78%, 106.25±16.88 and 22.00±1.47%, respectively). At the same time, a significantly reduced (P<0.05) sperm DNA damage, tail length and tail moment of the cryopreserved semen (1.48±0.27%, 2.08±0.36 µm and 3.14±1.32, respectively) compared with control (3.31±0.17%, 3.93±0.24 µm and 13.09±1.38, respectively). Moreover, extender containing 7% soybean lecithin significantly (P<0.05) increased TAC (0.47±0.04 mµ/ml) and decreased lipid peroxidation (Malondialdehyde) of the cryopreserved spermatozoa (9.18±1.47nmol/ml) with respect to the control (0.19±0.02 mµ/ml, and 21.57±1.45 nmol/ml, respectively). Additionally, 7% soybean lecithin significantly (P<0.05) improved in vitro fertilization rate, cleavage rate, morula and blastocyst development (54.14±6.21, 52.81±3.32, 27.49±2.78 and 18.44±2.11%, respectively) compared with the control (35.10±3.23, 31.75±5.52, 12.2±4.08 and 4.82±2.12 %, respectively). It was concluded that the addition of 7% soybean lecithin to the freezing extender improved freezability and enhanced in vitro fertilizing potentials of bovine spermatozoa through protection of DNA from deterioration and reduction of oxidative stress.

## تأثير ليسيسين الصويا علي قابلية حيامن الأبقار للتجميد وقدرتها الإخصابية معمليا

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اضافة ليسيثين الصويا الى ممدات السائل المنوى كبديل عن صفار البيض لايزال يثير الاهتمام ويتزايد يوما بعد يوم. وحديثا ظهرت أنواع جديدة من ممددات السائل المنوي خالية من الإضافات الحيوانية (صفار البيض أو اللبن) وبالرغم من

ذلك مازالت الممددات التي تحتوى على صفار البيض تستخدم بصورة أساسية لتجميد السائل المنوي البقري. وتهدف الدراسة الحالية إلي دراسة قدرة حيامن الأبقار للتجميد وكذلك قدرتها الإخصابيه معمليا وحقليا عند تجميدها في ممدد مضاف اليه الصويا ليسيسين بتركيزات مختلفة (٣ ، ٥ ،٧، ١٠ و٢٠ %) مقارنة باستخدام ٢٠% صفار البيض. ولقد أوضحت نتائج الدراسة الحالية أن تجميد السائل المنوى البقري في ممدد مضاف اليه صويا ليسيسين بتركيز ٧% نتج عنة زيادة معنوية كبيرة في نسبة الحركة الأمامية بعدالإسالة، معدل الحيوية ونسبة المحافظة علي غشاء القلنسوة (٦١.٢٥% ،١٧٢.٢٥ و١٠.٢٥ % علي التوالي) مقارنة بتلك التي تم تمديدها في ممدد التريس الذي يحتوى على ٢٠% صفار البيض (٤٧.٥٠%، ١٠٦.٢٥ و ٢٢.٠٠% علي التوالى). كما حافظ علي سلامة الحامض النووي للحيامن حيث قلل من تشظي الحامض النووي وطول ذيل المذنب وكثافته (١.٤٨%،٢.٠٨ميكرومول و٣.١٤ علي التوالي) بالمقارنة بالمجموعة الضابطة (٣.٣١%، ٣.٩٣ ميكرومول و13.09علي التوالي). كذلك نتج عنة زيادة معنوية كبيرة فى مستوي مضادات الأكسدة الكلية وانخفاض معنوي كبيرة في معدل أكسده الدهون (٠.٤٧ملليمول/مللي و٩.١٨ نانومول/مللي علي التوالى) مقارنة بالمجموعةالضابطة (٠.١٩ملليمول/مللي و٢١.٥٧ نانومول/مللي علي التوالى). كما أوضحت نتائج الإخصاب المعملى أن تجميد السائل المنوى البقري في ممدد مضاف اليه صويا ليسيسين بتركيز ٧% نتج عنة زيادة معنوية كبيرة في نسبة إخصاب البويضات وكذلك قدرتها علي النمو إلي الطور التوتى وطور البلاستوسيست (٥٤.١٤، ٥٢.٨١ ، ٢٧.٤٩ و١٨.٤٤% علي التوالى) مقارنة بتلك التى تمديدها في ممدد التريس الذي يحتوى على ٢٠% صفار البيض (٣٥.١٠، ٣١.٧٥ ، ١٢.٢٠و ٤.٨٢ % على التوالى). ولهذا يمكن أن نستنتج من نتائج هذه الدراسة أن اضافة الصويا ليسيسين بتركيز ٧% الي ممدد السائل المنوى يمكن أن تكون الاختيار الأمثل لتجميد السائل المنوى البقري في المستقبل.

***Key words**: Soybean lecithin, semen cryopreservation, DNA integrity, IVF, antioxidant.*

## INTRODUCTION

Semen cryopreservation has profound effects on spermatozoa, many of which result in sublethal damage to the cells, and subsequent reduction of fertilizing ability. The sperm plasma membrane serves as the main physical barrier to the outside environment and is a primary site of freeze-thaw damage. Such damage includes membrane destabilization due to lateral lipid rearrangement (De Leeuw *et al.,* 1990), loss of lipids from the membrane (Buhr *et al.,* 1994 and Golal *et al.,* 1998), and peroxidation of membrane lipids as a result of formation of reactive oxygen species (ROS) (Aitken 1995; Flesch and Gadella, 2000; Badr *et al.,* 2010). These events can affect sperm motility, response to osmotic stress, and signaling pathways; therefore, the fertilizing ability is compromised (Holt, 2000). Defining causes of damage to sperm during cryopreservation is further complicated because the processing of semen for cryopreservation is not standardized and there is a wide variety of freezing diluents in use. Semen extender contain some forms of lipids, the most common being egg yolk lipids (Watson, 1995). Egg yolk, a common protectant in cryopreservation media, has been used for providing protection against cold shock in the cryopreservation of mammalian semen for over half a century (Gousset *et al.,* 2004). However, there have been frequent arguments against the use of animal-originated ingredients, egg yolk, milk or even low density lipoprotein (LDL) extracted from egg yolk, one of which is the wide variability of composition that make it difficult to analyze the beneficial effects of a particular compound on sperm cryopreservation. Furthermore, they could introduce possible sanitary risks (viruses, bacteria and fungi), with the subsequent production of endotoxins capable of damaging the fertilizing capacity of spermatozoa (Bousseau *et al.,* 1998; Jiang *et al.,* 2007).

As one of phospholipids, lecithin (or phosphatidylchline) is distributed widely in plants and it plays an important role in the regulation of the physiological function of animal cells bio-membrane (Thun *et al.,* 2002). Soy bean lecithin has similar ingredients to egg yolk used for protection of animal spermatozoa from cold shock in semen cryopreservation (Aires *et al.,* 2003). It has been suggested that soybean lecithin may play a better protective role for spermatozoa than egg yolk during the

cryopreservation process and therefore reduce the risk of introducing bacterial and mycoplasma into freezing extenders (Fukui *et al.,* 2008). The main goal for the present study was to determine the effect of soybean lecithin as cryoprotective on bull spermatozoa, through investigating the quality parameters of the cryopreserved spermatozoa, DNA integrity, antioxidant activities and in vitro fertilizing potentials of frozen-thawed bull semen.

## MATERIALS and METHODS

**Semen collection and processing:**
Semen samples were collected from six fertile bovine bulls. Only semen samples at least 70% initial motility and $800x10^6$ sperm cells/ml were used. After collection, semen samples were pooled, split into 6 portions and diluted at 30°C with Tris-based extender supplemented with different concentrations of soybean lecithin (3, 5, 7, 10 and 20%) vs 20% egg yolk (control). The extended semen was cooled to 5°C throughout 60 minute in a cold cabinet. The cooled semen was loaded into 0.25 ml French straws (IMV, L'Aigle, France), then suspended into liquid nitrogen vapor inside foam box before immersed into liquid nitrogen. Frozen semen straws were thawed in a water bath at 37°C for 30 second. Post-thawing sperm motility, viability and acrosomal integrity were assessed according to Mohammed *et al.* (1998).

**Assessment of sperm DNA integrity:**
DNA integrity and the incidence of DNA strand breaks or fragmentation was detected using alkaline comet assay according to Boe-Hansen (2005). Briefly, DNA status of individual cells was determined by the neutral single cell gel electrophoresis (comet) assay. For this assay, frozen-thawed spermatozoa were diluted in phosphate buffer saline (PPS), embedded in agarose, followed by cell lysis, DNA decondensation, electrophoresis and DNA staining with 50 µl of 20 µg/ml ethidium bromide (Sigma). The cells were then visualized by fluorescent microscopy. Intact nuclei in the comet assay appeared to have compact and brightly fluorescent heads; in contrast, strand breaks in damaged cells allow DNA migration during electrophoresis, and a tail of DNA could be seen behind the head, giving the appearance of a comet (Hughes *et al.,* 1996). After subjecting spermatozoa to the comet assay, sperm nuclei were analyzed by computer software program.

**Biochemical analysis:**
Extra-cellular aspartate-aminotransferase (AST); alanine-aminotransferase (ALT) and alkaline phosphatase (ALP) enzymes leakage during cryopreservation was assessed spectrophotometrically according Tietz (1976) to evaluate the membrane stability of spermatozoa. Additionally, total antioxidant capacity and membrane lipid peroxidation was estimated by the end point generation of malondialdehyde (MDA) determined by the thiobarbituric acid (TBA) test, of the cryopreserved spermatozoa were measured as described by Cortassa *et al.* (2004).

**Evaluation of in vitro fertilizing potential of the treated semen:**
The fertilizing potentials of the treated semen were assessed using *in vitro* fertilization technology, as demonstrated by Totey *et al.* (1992). Three straws from each treatment were thawed in a water bath at 37°C for 30 sec. The most motile spermatozoa were separated by swim up technique in the fertilization medium, modified Tyrode's Albumin-Lactate-Pyruvate (TALP) containing 6 mg/ml bovine serum albumin (BSA), for 1 hour as recorded by Parrish *et al.* (1988). The uppermost layer of the medium containing the most motile spermatozoa was collected and washed twice by centrifugation at 2000 rpm for 10 minutes. The sperm pellet was resuspended in the fertilization TALP medium containing 10 µg/ml heparin. After appropriate dilution, 2 µl (final concentration $2 x10^6$ sperm cell/ml) of sperm suspension was added to the fertilization drops, containing in *vitro* matured oocytes. Gametes were co-incubated in the fertilization drops under sterile mineral oil for 18 hour at 39°C in an atmosphere of 5% $CO_2$ in air with

maximum humidity. The inseminated oocytes were freed from extra cumulus cells and attached spermatozoa by gentle pipetting and then cultured in TCM-199 medium for seven days in the same previous conditions. The proportional of cleaved oocytes was recorded 48 hour after insemination and those developed to the morula and blastocyst stages were recorded at 5-7 day post-insemination.

**Statistical analysis:**
All data were analyzed by using Costat Computer Program (1986) Cottort Software, and were compared by the least significant difference least (LSD) at 5% levels of probability. The results were expressed as means ±SE.

## RESULTS

The results presented in Table 1 revealed that, addition of soybean lecithin to the freezing extender improved the freezability of bull spermatozoa compared with the control semen in a dose-dependent trend. Addition of 7% soybean lecithin to semen extender, appeared to be the best concentration that increased ($P<0.05$) significantly the post-thawing sperm motility; viability index and maintained acrosomal integrity (61.25±1.25%, 172.25±5.53 and 10.25±2.39 %, respectively) compared to the control semen (47.50±4.78%, 106.25±16.88 and 22.00±1.47%, respectively).

Data regarding the effect of soybean lecithin addition to the freezing extender on the total antioxidant capacity (TAC) and lipid peroxidation of the cryopreserved semen which indicated by malondialdehyde ((MDA) are presented in table 2. In vitro provision of semen extender with 7% soybean lecithin significantly ($P<0.05$) increased the total antioxidant and diminished lipid peroxidation of the frozen-thawed semen (0.47±0.04 mμ/ml, and 9.18±1.47 nmol/ml, respectively) compared with the control extender (0.19±0.02 mμ/ml, and 21.57±1.45 nmol/ml, respectively). Moreover, data presented in table 2 clarified that, addition of 7% soybean lecithin to the semen extender maintained sperm cell membrane integrity and this appeared through reduction of extracellular enzymes (AST, ALT and ALP) leakage (70.00±4.56, 11.5±1.04 and 14.65±2.79 U/L, respectively) compared with the control extender (106.00±5.95, 23.00±2.48 and 23.27±3.75 U/L, respectively)

With respect to the effect of soybean lecithin addition to the freezing extender on the DNA integrity of the frozen-thawed bovine spermatozoa are demonstrated in Table 3. The present data indicated that, in vitro provision of semen extender with 7% soybean lecithin significantly ($P<0.05$) decreased the DNA fragmentation, tail length and tail moment of the frozen-thawed semen (1.48±0.27%, 2.08±0.36 μm and 3.14±1.32, respectively) as compared with the control extender (3.31±0.17%, 3.93±0.24 μm and 13.09±1.38, respectively).

Data concerning the effect of replenishing of semen extender with soybean lecithin on the in vitro fertilizing potentials and embryo development are presented in tables 4 and 5. The current results revealed that, addition of 7% soybean lecithin to the freezing extender had a positive effect ($P < 0.05$) on the in vitro fertilization rate and embryo developmental rate compared with the control semen. When 7% soybean lecithin was added to the freezing extender, a higher proportion of in vitro fertilized oocytes, cleavage rate, morula and blastocyst development (54.14±6.21, 52.81±3.32, 27.49±2.78 and 18.44±2.11%, respectively) compared with the control semen (35.10±3.23, 31.75±5.52, 12.2±4.08 and 4.82±2.12 %, respectively).

**Table 1:** Effect of different soybean lecithin concentrations on bovine spermatozoa freezability.

| Treatment | Pre-freeze motility (%) | Post-thaw motility (%) | Viability index | Acrosomal integrity (%) |
|---|---|---|---|---|
| Control | 78.75±3.12 $^{a}$ | 47.50±4.78 $^{b}$ | 106.25±16.88 $^{b}$ | 22.00±1.47 $^{a}$ |
| 3% soy lecithin | 78.75±1.25 $^{a}$ | 53.75±2.39 $^{ab}$ | 131.88±10.38 $^{b}$ | 15.5±1.33 $^{ab}$ |
| 5% soy lecithin | 77.5±3.23 $^{a}$ | 56.25±2.40 $^{ab}$ | 140.5±12.04 $^{ab}$ | 15.5±2.55 $^{ab}$ |
| 7% soy lecithin | 82.25±1.44 $^{a}$ | 61.25±1.25 $^{a}$ | 172.25±5.53 $^{a}$ | 10.25±2.39 $^{b}$ |
| 10% soy lecithin | 81.25±2.39 $^{a}$ | 51.25±5.54 $^{ab}$ | 116.87±9.92 $^{b}$ | 15.25±1.92 $^{ab}$ |
| 20% soy lecithin | 80.00±2.04 $^{a}$ | 48.75±3.75 $^{b}$ | 113.00±7.72 $^{b}$ | 20.25±2.93 $^{a}$ |

Means in the same column with different superscripts are significantly differ at P<0.0

**Table 2:** Effect of different soybean lecithin concentrations on biochemical activity of bovine spermatozoa.

| Treatment | AST (U/L) | ALT (U/L) | ALP (U/L) | TAC (mμ/ml) | MDA (nmol/ml) |
|---|---|---|---|---|---|
| Control | 106.00±5.95 $^{a}$ | 23.00±2.48$^{a}$ | 23.27±3.75 $^{a}$ | 0.19±0.02 $^{c}$ | 21.57±1.45 $^{a}$ |
| 3%soy lecithin | 90.75±7.48 $^{ab}$ | 17.75±1.25 $^{ab}$ | 15.48±2.08 $^{b}$ | 0.31±0.08 $^{b}$ | 16.1±1.64 $^{ab}$ |
| 5%soy lecithin | 74.75±8.78 $^{bc}$ | 15.25±2.75 $^{bc}$ | 16.43±1.10 $^{b}$ | 0.42±0.04 $^{a}$ | 14.39± 2.6 $^{bc}$ |
| 7%soy lecithin | 70.00±4.56$^{c}$ | 11.5±1.04 $^{c}$ | 14.65±2.79 $^{b}$ | 0.47±0.04 $^{a}$ | 9.18±1.47 $^{c}$ |
| 10%soy lecithin | 100.25±0.55$^{a}$ | 22.25±1.49 $^{a}$ | 18.55±1.34 $^{ab}$ | 0.29±0.02 $^{b}$ | 16.79±2.59 $^{ab}$ |
| 20%soy lecithin | 91.25±2.17 $^{ab}$ | 21.00±1.47$^{a}$ | 19.9±1.38 $^{ab}$ | 0.27±0.03 $^{b}$ | 17.94±1.69 $^{ab}$ |

Means in the same column with different superscripts are significantly differ at P<0.05
AST: Aspartate-aminotransferase ALT: Alanine-aminotransferase ALP: Alkaline phosphatase
TAC: Total antioxidant capacity MDA: Malondialdehyde

**Table 3:** Effect of different soybean lecithin concentrations on DNA integrity of bovine spermatozoa.

| Treatment | DNA integrity (%) | Tail length (μm) | Tail moment |
|---|---|---|---|
| Control | 3.31±0.17 $^{a}$ | 3.93±0.24 $^{a}$ | 13.09±1.38 $^{a}$ |
| 3% soy lecithin | 2.06±0.11 $^{bc}$ | 2.28±0.45 $^{ab}$ | 4.80±1.22 $^{c}$ |
| 5% soy lecithin | 2.17±0.25$^{bc}$ | 2.38±0.20 $^{ab}$ | 5.31± 1.47 $^{bc}$ |
| 7% soy lecithin | 1.48±0.27$^{c}$ | 2.08±0.36 $^{b}$ | 3.14±1.32$^{c}$ |
| 10% soy lecithin | 2.34±0.16 $^{b}$ | 2.63±0.15 $^{ab}$ | 7.11±2.41 $^{bc}$ |
| 20% soy lecithin | 3.06±0.32 $^{a}$ | 3.22±0.20 $^{ab}$ | 10.05±1.72 $^{ab}$ |

Means in the same column with different superscripts are significantly differ at P<0.05

**Table 4:** Effect of different soybean lecithin concentrations on the in vitro fertilizing potentials of bovine spermatozoa.

| Treatment | No. of oocytes | No. of Penetrated oocytes | Penetration rate (%) | No. of fertilized oocytes | Fertilization rate (%) |
|---|---|---|---|---|---|
| Control | 87 | 54 | 63.07±8.34 [a] | 31 | 35.10±3.23 [b] |
| 3% soy lecithin | 75 | 49 | 65.03±1.72 [a] | 30 | 41.47±5.42 [ab] |
| 5% soy lecithin | 63 | 40 | 63.62±5.14 [a] | 28 | 43.84±5.63 [ab] |
| 7% soy lecithin | 84 | 62 | 73.55±3.66 [a] | 56 | 54.14±6.21 [a] |
| 10% soy lecithin | 71 | 46 | 63.41±4.13[a] | 33 | 46.03±3.44 [ab] |
| 20% soy lecithin | 63 | 43 | 68.19±6.27 [a] | 22 | 34.71±2.28 [b] |

Means in the same column with different superscripts are significantly differ at P<0.05

**Table 5:** Effect of different soybean lecithin concentrations on the bovine embryo development in vitro.

| Treatment | No. of oocytes | Cleavage rate No. ( %) | Morula stage No. (%) | Blastocyst stage No. (%) |
|---|---|---|---|---|
| Control | 83 | 26 (31.75±5.52)[b] | 10 (12.2±4.08)[b] | 4 (4.82±2.12)[c] |
| 3% soy lecithin | 94 | 39 (41.67±1.28)[ab] | 16 (16.59±3.16)[ab] | 10 (9.42±1.09)[bc] |
| 5% soy lecithin | 70 | 32 (45.32±5.39)[ab] | 13 (18.85±2.99)[ab] | 9 (12.83±1.79)[ab] |
| 7% soy lecithin | 89 | 47 (52.81±3.32)[a] | 24 (27.49±2.78) [a] | 16 (18.44±2.11)[a] |
| 10% soy lecithin | 88 | 36 (40.97±1.52)[b] | 15 (16.35±3.92) [ab] | 7 (7.28±3.03)[bc] |
| 20% soy lecithin | 83 | 29 (34.87±8.93)[b] | 7 (8.3±2.55) [b] | 4 (3.53±1.73)[c] |

Means in the same column with different superscripts are significantly differ at P<0.05

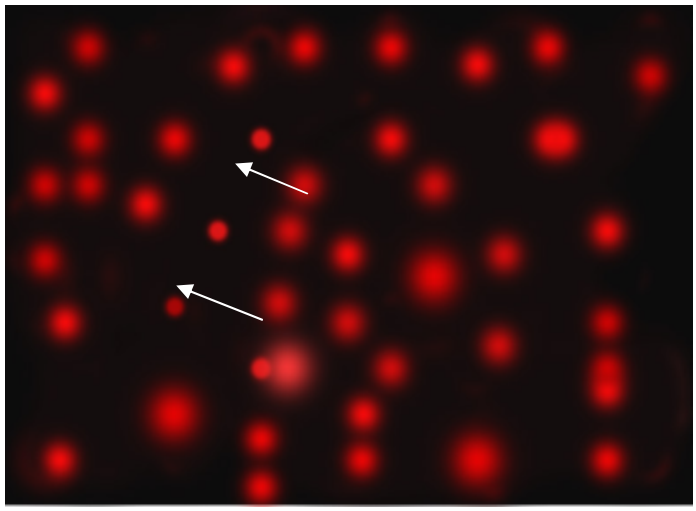

**Fig 1:** Bovine spermatozoa cryopreserved in tris-7% soybean lecithin. The single cell gel electrophoresis (comet) assay showed reduction in the DNA fragmentation as represented by a limited amount of DNA present in the comet tail.

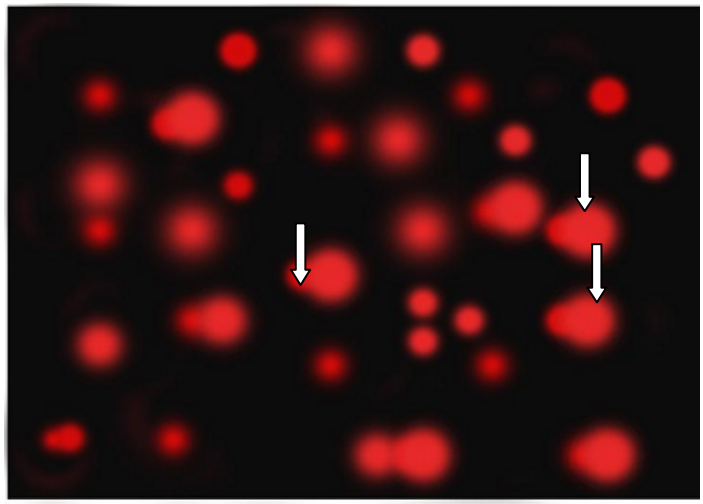

**Fig 2:** Bovine spermatozoa cryopreserved in tris-egg yolk extender. The single cell gel electrophoresis (comet) assay showed increased in the DNA fragmentation as represented by an increasing amount of DNA present in the comet tail.

## DISCUSSION

In the present study, freezing extender supplemented with 7% soybean lecithin could provide better cryoprotective action for bovine spermatozoa during cryopreservation compared with control group (20% egg yolk). These results are in accordance with the previous reports in the cryopreservation of bovine (Moussa *et al.,* 2002 and Amirat *et al.,* 2005), buffalo (Badr 2008) and sheep (Fukui *et al.,* 2008) semen. Contrary, other researchers reported that the cryoprotective effect of soybean lecithin extenders on bovine sperm freezability was similar or slightly inferior to that of 20% egg yolk extender (Aires *et al.,* 2003). The precise mechanism by which soybean lecithin protects spermatozoa during cryopreservation remains unclear. It was generally accepted that cold shock and cryodamage might impair the physiological function of spermatozoa membrane due to the change of the lipid composition of its bilayer and the fluidity of the plasma membrane during freeze-thawing process as a result of formation ROS (Johnson *et al.,* 2000). Excessive generation of ROS negatively affect the fluidity of sperm plasma membrane and integrity of DNA in the sperm nucleus (Cocuzza *et al.,* 2007). The beneficial effect of soybean lecithin extender on the sperm function may be attributed to the ability of the lecithin to maintain the integrity of cell membranes, facilitating the movement of fluids inside and outside the cell; and without lecithin cell membranes would harden and would no longer stay semi-permeable (Zeisel, 2000). It is believed that phospholipids from egg yolk or soybean lecithin might integrate with sperm membrane to form a protective film against the formation of lethal intracellular ice crystal and protect the sperm membrane from mechanical damage during freeze-thawing process (Quinn *et al.,* 1985). These beneficial effect of soybean lecithin dose dependent on plasma membrane stability leads to reduction of extracellular enzymes leakages (AST, ALT and ALP) which appeared clearly in our findings mainly at concentration of 7%. Additionally, soybean lecithin could play a protective role for sperm during cryopreservation due to its low viscosity and less debris which enhance the sperm motion characteristics and fertilizing ability, compared with egg yolk which provided higher viscosity and the presence of particulate debris in extenders (Van Wagtendonk-de Leeuw *et al.,* 2000).

In a way, soybean lecithin is also thought as a better emulsifier that might promote cryoprotectants to distribute uniformly and

reduce its local concentration, which led to relieve the toxicity of cryoprotectants during the freeze-thawing process (Trotta *et al.,* 2002). Moreover, soybean lecithin might reduce the cholesterol/phospholipids ratio of sperm cell membranes by permeating into the sperm membrane, so capacitation like changes during the freezing process were restrained to improve the fertilizing ability of frozen-thawed spermatozoa (Galantino-Homer *et al.,* 2006). The present results evidently revealed that soybean lecithin decreased significantly membrane lipid peroxidation, throughout reduction of malondialdehyde (MDA) production and increased total antioxidant capacity (TAC) in the frozen-thawed spermatozoa that maintain a suitable level of ROS which play a significant role in many physiological processes of the sperm such as capacitation, hyper-activation and sperm-oocyte fusion (Sies, 1993; Lewis *et al.,* 1995). However, ROS must be continuously inactivated to keep only a small amount necessary to maintain normal cell function, as a result the excessive generation of ROS in semen can cause damage to sperm that increased by extruding of cytoplasm during the maturation process which is the major source of antioxidants. Therefore, the improved freezability and in vitro fertilizing potentials of the semen that extended in soybean lecithin extender may be attributed to the ability of the lecithin to protect the spermatozoa from the destructive effects of oxidative stress during cryopreservation (Chatterjee and Gagnon, 2001). Additionally, soybean lecithin may act as a stabilizer and protectant of proteins and cell membranes, whose fluidity decreases during temperature downshift. This may emphasize the current results which indicated that soybean lecithin provision to the freezing extender diminished the enzymes leakage.

Furthermore, the beneficial effect of soybean lecithin extender on semen cyropreservation may be attributed to the high concentration of linoleic acid in its constituent, which is the precursor of prostaglandin E. Addition of prostaglandin E to the semen extender increassed the life span of the spermatozoa and the cleavage rate of the inseminated oocytes (Kolev and Dimov 1998). Accordingly, in our results, post-thaw motility and viability index appeared significantly higher ($P<0.05$) in soybean additive diluent pariculary at 7% than 20% egg yolk addition.

In conclusion, the present study provides novel evidence that addition of 7% soybean lecithin to the freezing extender improved freezability and enhanced in vitro fertilizing potentials of bovine spermatozoa through protection of DNA from deterioration, reduction of the oxidative stress. We suggest that consistent with quality standards that should be required for cryoprotectant extender, soy lecithin-based extender in recommended dose (7%) is a viable alternative to conventional egg-yolk-based freezing diluents for cryopreserving of bovine bull spermatozoa.

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