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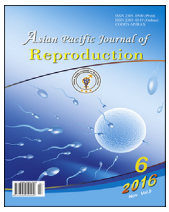

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Substitution of egg yolk with different concentrations of soybean lecithin in tris-based extender during bulls' semen preservability

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ABSTRACT

Objective: To investigate the effect of various concentrations of soybean lecithin as an alternative for egg yolk in bull semen extender on post-chilling and post-thaw sperm quality characteristics.

Methods: Semen ejaculates were collected from three mature bulls' once/week for 5 weeks. After initial evaluation the approved ejaculates were pooled and extended gradually 1:7 with tris-citrate-fructose egg yolk extender (control) and tris-citrate-fructose (TCF) + different concentration of soybean lecithin (0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0%, 3.5% and 4.0%) to ensure 60 million motile spermatozoa/mL, and then proceeded an adopted international cryopreservation protocol. Frozen straws were thawed at 37 °C for 30 s. The parameters studied were sperm motility, viability, abnormality, membrane integrity and normal intact acrosome percentages in chilled and frozen-thawed semen.

Results: The substitution of egg yolk into TCF with 1% soya lecithin significantly ($P < 0.0001$) ameliorated the maintenance of semen characters motility (86.67% ± 0.80%), life (87.60% ± 0.88%), and membrane integrity (82.47% ± 0.94%), meanwhile it had significantly ($P < 0.0001$) reduced the abnormality (13.20% ± 0.60%) of spermatozoa to its least value compared to some other concentrations in use. Moreover, the addition of 1.5% of soya lecithin to TCF had maintained the semen characteristics compared to the control tris-citrate-fructose egg yolk. The significantly higher mean values of post-thawing sperm motility % were observed in 1% soybean lecithin (56.00% ± 1.00%) as compared to the control (41.00% ± 1.00%).

Conclusion: 1–1.5% soya lecithin can effectively alternate egg yolk as cryoprotective additives for cryopreservation extender, without any detrimental effects on post-chilling and post-thaw semen quality in cattle bull.

1. Introduction

Artificial insemination together with perfect progeny testing programs have a fundamental effects on the rate of genetic improvement, enhancing of gene merits and amplifying of chosen reproductive characteristics in farm animals. Cryopreservation is the main essential tool for long-standing storage of semen and control of venereal diseases [1]. However, cryopreservation yields detrimental effects on post-thawing sperm quality and fertilization process [1,2]. Optimization of freezing extender to obtain the best quality of post-thaw semen is crucial. In general, farm animal semen cryopreservation medium includes: A non-penetrating cryoprotectant (a source of lipoprotein to provide protection against cold shock such as egg yolk, milk, or soybean lecithin), a penetrating cryoprotectant (glycerol, ethylene glycol, or dimethyl sulfoxide, *etc.*), ionic or nonionic substances to maintain a suitable osmotic pressure and pH, (buffer such as Tris or Test, *etc.*), energy source substrate (*i.e.*, glucose or fructose), antibiotics (penicillin, streptomycin, *etc.*) and other additives, such as enzymes and antioxidants [3,4]. Egg yolk is the most widely used cryoprotectant in the composition of cryopreservation extenders of mammalian spermatozoa; yet, efforts have been made to find ways to substitute it due to the possibility of transporting pathogenic microorganisms, production of harmful metabolites and toxins, the lack of standardization, and the presence of steroid hormones and substances that inhibit metabolic exchanges or decrease the motility of sperm, all resulting in reduced semen quality [5–9]. In addition, the biosecurity in various countries for semen transport is extremely essential to avoid the hazard of transporting avian influenza through

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egg-based products [5,10]. In this regard, low density lipoproteins (LDL) extracted from egg yolk, gamma-irradiated egg yolk plasma, pasteurized powdered egg yolk or lecithin from non-animal source like soya were tested as a non-permeable cryoprotectant in extender for deep freezing of farm animals spermatozoa [11–13]. The use of non animal origin chemically defined medium is the method of choice in assisted reproductive technology and semen cryopreservation [14,15]. Substitution of egg yolk with soybean lecithin may reduce hygienic risks in extenders. Recently, there are several studies indicating the valuable effects of soybean lecithin for cryopreservation of sperm in bull [3,16], ram [17,18] and goat [19,20]. A soybean lecithin-based extender has been developed and utilized commercially for bull [3], ram [14,21] and buffalo bull [22,23] semen.

The soybean lecithin is a valuable plant-based phospholipids source that included in commercial extenders used for freezing mammalian sperm without clear levels and adjustments due to trade protection. Therefore, this study was designed to investigate the effect of various levels of soybean lecithin as an alternative for egg yolk in bull semen extender on post-chilling and post-thaw sperm quality including motility, viability, abnormalities, and plasma membrane integrity and intact acrosome.

2. Materials and methods

2.1. Semen collection and initial evaluation

Five mature genetically improved cattle-bulls with superior quality semen characteristics maintained at The Semen Freezing Center, General Organization for Vet. Services, Ministry of Agriculture, Abbasia, Egypt, were used for this study as semen source. Semen ejaculates were collected from bulls using an artificial vagina at weekly intervals for 5 weeks. The semen samples were initially evaluated for volume (in graduated tube), concentration using Thoma rulling of the Neubaur hemocytometer and sperm motility. The neat semen samples with more than 70% motility and 80% morphologically normal spermatozoa were admitted to freezing procedure. The ejaculates were pooled in order to have sufficient semen for a replicate and to eliminate the bull effect. The semen was given a holding time for 10 min at 37 °C in a water bath before dilution and reevaluated for sperm motility, viability, total abnormalities, and acrosome and membrane integrities before processing.

2.2. Semen processing

The control cryopreservation extender was Tris-citric acid-egg yolk-fructose (TCFY) diluent [24]. Semen samples were extended gradually 1:7 with TCFY extender (control) and TCFY + different concentration of soya lecithin (0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0%, 3.5% and 4.0%) to ensure 60 million motile spermatozoa/mL, then cooled slowly (approximately for 2 h) up to 5 °C and equilibrated for 4 h. Semen was packed into 0.25 mL polyvinyl French straws (IMV, France). After equilibration periods, the straws were placed horizontally on a rack and frozen in a vapor 4 cm above liquid nitrogen for 10 min and were then dipped stored in liquid nitrogen at −196 °C.

2.3. Assessment of semen quality parameters

Frozen straws were thawed individually at 37 °C for 30 s in a water bath for microscopic evaluation [25]. The parameters studied were sperm motility, sperm viability, sperm abnormality, sperm membrane integrity, percent of normal intact acrosome in chilled and frozen-thawed semen.

2.3.1. Sperm motility (%)

Subjective motility was observed using phase contrast microscope (Olympus Optical Co. Ltd., Japan). Visual motility was assessed microscopically with closed circuit television [26].

2.3.2. Live and abnormal spermatozoa (%)

The viability and abnormalities % of sperm were evaluated using eosin-Nigrosin stained smear as described by Sidhu *et al.* [27].

2.3.3. Sperm membrane integrity (%)

Sperm membrane integrity was assessed using the hypo-osmotic swelling test [28]. Two hundred spermatozoa were assessed and the percentage of spermatozoa with curled tails (swollen/intact plasma membrane) was calculated.

2.3.4. Intact normal acrosome (%)

Acrosome integrity was evaluated using giemsa stain as described by Watson [29]. The intact acrosome was recorded for 200 spermatozoa that were randomly examined under an immersion objective (×1 000) using phase contract microscope.

2.4. Statistical analysis

Output data were analyzed by one-way analysis of variance (ANOVA), followed by Duncan test to determine significant differences in all the parameters among all groups, with SPSS Version 14.0 for Windows [30]. Differences with values of $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. The chilling

Data output in Table 1 referred that the substitution of egg yolk into TCF with soya lecithin at a concentration of 1% significantly ($P < 0.000\,1$) ameliorated the maintenance of semen characters (motility, life, and membrane integrity %), meanwhile it had significantly ($P < 0.000\,1$) reduced the abnormality % of spermatozoa to its least value compared to some other concentrations in use. In the same consent, the addition of 1.5% of soya lecithin to TCF had maintained non significantly ($P < 0.000\,1$) the semen characteristics compared to the control TCFY. The addition of soya lecithin less than 1% or more than 1.5% had lowered significantly ($P < 0.000\,1$) the semen characteristics compared to the control TCFY.

The highest mean value of motility % was observed in 1% and 1.5% SL. These values were significantly ($P < 0.000\,1$) higher than 0.5%, 2% and 2.5%, 3%, 3.5% and 4% SL post-chilling. Similarly, the highest mean values of life sperm % were observed in 1% and 1.5% SL as compared to the control. These values were significantly ($P < 0.000\,1$) higher than 0.5%, 2% and 2.5%, 3%, 3.5% and 4% SL post-chilling. The total sperm abnormalities mean values were apparently lower in 0.5%, 1% and 1.5% SL as compared to the control. These values were lower than 2%, 2.5%, 3%, 3.5 and 4% post-chilling. The membrane integrity % mean values were significantly higher in 1% and 1.5% SL as compared to the control. These values are lower than in 0.5%, 2%, 2.5%, 3%, 3.5% and 4% SL post-chilling (Table 1).

Table 1

Effect of different concentrations of soybean lecithin in extender on post-chilling bull semen characteristics ($P < 0.000\,1$).

Parameter	TCFY control	Soya lecithin concentration % in TCF							F-cal
		0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	
Motility %	$81.67^{b} \pm 0.63$	$77.67^{c} \pm 0.67$	$86.67^{a} \pm 0.80$	$82.00^{b} \pm 0.95$	$73.67^{d} \pm 0.91$	$70.67^{e} \pm 0.96$	$67.33^{f} \pm 0.55$	$67.00^{f} \pm 1.07$	$65.67^{f} \pm 0.96$	80.81
Life %	$83.20^{b} \pm 0.53$	$78.67^{c} \pm 0.96$	$87.60^{a} \pm 0.88$	$83.40^{b} \pm 1.01$	$76.20^{c} \pm 0.91$	$72.80^{d} \pm 0.78$	$68.87^{e} \pm 1.15$	$67.80^{e} \pm 0.91$	$67.00^{e} \pm 1.10$	66.06
Abnormality %	$13.40^{c} \pm 0.40$	$14.13^{c} \pm 0.69$	$13.20^{c} \pm 0.60$	$13.60^{c} \pm 0.70$	$15.87^{b} \pm 0.43$	$14.13^{c} \pm 0.46$	$15.87^{b} \pm 0.52$	$15.80^{b} \pm 0.57$	$17.60^{a} \pm 0.53$	7.33
HOST %	$74.93^{c} \pm 0.62$	$73.60^{cd} \pm 0.69$	$82.47^{a} \pm 0.94$	$80.20^{b} \pm 0.76$	$71.67^{de} \pm 0.78$	$70.47^{ef} \pm 0.68$	$71.87^{de} \pm 0.77$	$68.13^{f} \pm 0.77$	$68.33^{f} \pm 1.06$	38.98

Means with the different superscripts are significantly different using the Duncan multiple range test ($P < 0.05$).

Table 2

Effect of different concentration of soybean lecithin in extender on post-thawing bull semen characteristics ($P < 0.000\,1$).

Parameter	TCFY control	Soya lecithin concentration % in TCF							F-cal
		0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	
Motility %	$41.00^{b} \pm 1.00$	$23.00^{e} \pm 0.65$	$56.00^{a} \pm 1.00$	$41.00^{b} \pm 0.53$	$31.00^{c} \pm 0.53$	$27.00^{d} \pm 1.36$	$21.00^{e} \pm 1.77$	$12.00^{f} \pm 0.65$	$8.00^{g} \pm 0.65$	235.60
Life %	$80.60^{a} \pm 0.95$	$67.60^{bc} \pm 2.09$	$63.60^{cd} \pm 0.52$	$70.00^{b} \pm 2.93$	$78.60^{a} \pm 0.55$	$66.40^{bc} \pm 1.73$	$59.00^{de} \pm 1.12$	$57.00^{e} \pm 2.62$	$65.00^{c} \pm 1.69$	20.07
Abnormality %	$18.40^{d} \pm 1.02$	$19.20^{d} \pm 0.90$	$19.00^{d} \pm 0.29$	$19.00^{d} \pm 0.53$	$24.80^{b} \pm 0.68$	$22.00^{c} \pm 1.17$	$27.00^{ab} \pm 0.74$	$27.00^{ab} \pm 1.07$	$28.00^{a} \pm 1.36$	18.93
HOST %	$71.20^{d} \pm 0.62$	$79.00^{b} \pm 0.61$	$82.20^{a} \pm 0.46$	$79.40^{b} \pm 0.50$	$82.60^{a} \pm 0.43$	$77.00^{c} \pm 0.34$	$76.60^{c} \pm 0.36$	$76.80^{c} \pm 0.35$	$61.20^{e} \pm 0.35$	205.29
Acrosome integrity %	$81.80^{a} \pm 0.57$	$67.60^{c} \pm 1.39$	$72.20^{b} \pm 1.76$	$67.60^{c} \pm 1.43$	$62.60^{d} \pm 1.17$	$67.60^{c} \pm 1.61$	$60.60^{d} \pm 2.14$	$56.20^{e} \pm 1.30$	$53.00^{e} \pm 0.79$	36.90

Means with the different superscripts are significantly different using the Duncan multiple range test ($P < 0.05$).

3.2. The post-thawing

Concerning the data output in Table 2, the substitution of egg yolk with 1% soya lecithin showed significantly higher ($P < 0.000\,1$) semen characteristics concerning the motility and membrane integrity % compared to control TCFY. The significantly ($P < 0.000\,1$) higher mean values of post-thawing sperm motility % were observed in 1% SL as compared to the control. These values were significantly ($P < 0.000\,1$) higher than 0.5%, 2% and 2.5%, 3%, 3.5% and 4% SL (Table 2). Meanwhile, the addition of 1.5% soybean lecithin had a similar effect as the egg yolk addition to TCF (Table 2).

The post-thawing life sperm % mean values were significantly ($P < 0.000\,1$) lower in 1.5% and 2% SL (Table 2) as compared to the control. These values were significantly ($P < 0.000\,1$) higher than 0.5%, 1%, 2.5%, 3%, 3.5% and 4% SL (Table 2).

The post-thawing sperm abnormalities % mean values were significantly ($P < 0.000\,1$) higher in 0.5%, 1% and 1.5% SL (Table 2) compared to the control (18.40% ± 1.02%). These values were significantly ($P < 0.000\,1$) lower than 2%, 2.5%, 3%, 3.5% and 4% SL (Table 2).

Concerning the acrosome integrity %, mean values were significantly ($P < 0.000\,1$) higher in 1% (Table 2) than all SL concentrations.

4. Discussion

Egg yolk is the most widely used advantageous cryoprotectants during freezing and thawing process in the majority of animal species. Although favorable efficiency of egg yolk in extender, the usage of egg yolk facing many protests mainly attributed to hygiene concerns and risk of bacterial contaminations [3,5,14], consequently, its interference with semen quality [31]. The use of non animal origin cryoprotectant instead of egg yolk in semen cryopreservation is of growing interest in the last years [14,15,18,32–34].

The results of the current study revealed that among the wide range (0.5–4%) range of tested concentrations of soya-lecithin, the most beneficial levels of soybean lecithin for cooling and cryopreservation of cattle bull semen were 1% and 1.5%. These concentrations showed best semen quality studied parameters after both post-chilling and post-thawing.

In the present experimental work we observed that the range of soy lecithin level from 1% to 1.5% in the extender yielded the best semen characteristics post preservation.

The reported optimal concentrations of soybean lecithin in extender used for cryopreservation in the literature were ranged from 0.8% in canine [35] to 1% in ram [17], human [36] and cat [37] and 1.5% in goat [2,34].

These species different may be interrelated to the variations in of sperm plasma membrane composition between species which may determine the required level of soybean lecithin in the extender [38]. Another cause which affected the optimum level of soybean lecithin in the extender is species differences in seminal plasma composition specially the concentration of bovine seminal plasma proteins. The bovine seminal plasma proteins envelop the sperm membrane and stimulate cholesterol and phospholipid efflux and damage the sperm membrane [39].

Our data are in agreement with Salmani *et al.* [20] in goat, Forouzanfar *et al.* [17] and Masoudi *et al.* [34] in ram, Vick *et al.* [37] in cat, and Reed *et al.* [36] in human whom reported that 1% to 1.5% SL in extender has beneficial effects and that higher concentrations of soybean lecithin above 1.5% have a harmful and toxic effect on sperm during cryopreservation and resulted in reduction in sperm motility and viability.

Aires *et al.* [3] reported a significantly higher post-thaw bovine sperm motility in soybean lecithin based extender than Tris-egg yolk diluents. Masoudi *et al.* [34] recorded higher life sperm % in 1% SL contained extender as compared to egg yolk and did not detect any significant variations in sperm motility, membrane and acrosome integrity. However, De Leeuw *et al.* [40] and Celeghini *et al.* [41] reported that egg

yolk-containing diluents is more efficient in preserving survivability of bull sperm during freezing than diluents containing soybean lecithin. va Wagtendonk-de Leeuw *et al.* [42] noticed that higher concentrations of lecithin in the freezing medium produced particular debris and increased viscosity and decreased osmotic pressure of extenders which could have adverse effect on semen fertility [42]. Moussa *et al.* [11] noticed a significant decline in extender osmotic pressure when LDL concentration increase due to the precipitation of fructose and salts included in the extender and this decreased osmotic pressure had a damaging effect to sperm cell. On the contrary, de Paz *et al.* [18] noticed a significant improvement in frozen-thawed motility when 2–3.5% soy bean lecithin was included in ram freezing extender.

In the current study, the sperm motility was significantly improved by addition of the 1% and 1.5% SL to TCF medium as compared with TCF with egg yolk. This improvement of sperm motility may be attributed to the low viscosity of these concentrations of soya lecithin as compared with egg yolk [3,42] which may facilitate the movement of spermatozoa. Salmani *et al.* [20] indicated that soya-lecithin was more efficient in protecting goat sperm against destructive lipid peroxidation during cryopreservation compared to egg yolk which can be attributed to egg yolk composition that is containing more unsaturated fatty acids susceptible to lipid peroxidation. They also noticed a significant reduction in MDA level in the goat semen extender containing different concentrations of soybean lecithin than extender containing egg yolk.

The post-thawing life sperm % mean values were significantly ($P < 0.000\,1$) higher in 1.5% and 2% SL compared to 0.5%, 1%, 2.5%, 3%, 3.5% and 4% SL. These results are in agreement with Salmani *et al.* [20] and Masoudi *et al.* [34] whom notice that SL extender yield a significantly ($P < 0.000\,1$) higher live sperm % than egg yolk extender in ram. It appears that SL had extra protective action on plasma membrane of sperm against destruction of intercellular sperm structures [43]. Moreover, Masoudi *et al.* [34] and Emamverdi *et al.* [44] reported that SL can prevent apoptotic cascades in goat and ram spermatozoa during cryopreservation.

It has been previously evidenced that the LDL fraction off egg yolk is the main cryoprotective component of egg yolk during the cryopreservation of spermatozoa [11,39,45,46]. The mechanisms for cryoprotective effects of soybean lecithin may be due to an interaction between seminal plasma proteins and LDL in extenders as suggested by Bergeron and Manjunath [45] and also, may be related to the protecting film of lecithin at the surface of spermatozoa membranes against of ice crystal [11]. Furthermore, it has been suggested that exogenous phospholipids could replaces some phospholipids of sperm membrane, refurbishing the damaged plasma membrane [47], and improving tolerance against freezing process [3,11,48].

In conclusion, 1–1.5% soya lecithin can effectively alternate egg yolk as a cryoprotective additive for cryopreservation extender, without any detrimental effects on post-chilling and post-thaw semen quality in cattle bull. Further studies are necessary for evaluation *in vivo* fertility of frozen-thawed cattle bull semen in extender including these soybean lecithin concentrations.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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