Evaluation of Different Levels of Soybean Lecithin as an Alternative to Egg Yolk for Cryopreservation of Goat and Ram Spermatozoa

Khalifa, E. I. and M. A. M. Abdel-Hafez
Animal Production Research Institute, Sheep and Goat Research Department, Ministry of Agriculture, Dokki, Giza, Egypt

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Abstract: The purpose of the present study was to investigate the effect of different concentrations of soybean lecithin (SL) in goat and ram semen extenders as a substitute for egg yolk. In this study, thirty-six ejaculates were collected from each breed (3 adult goat bucks and 3 adult rams) aged >2.5 years old. Ejaculates were collected during the breeding season by means of an artificial vagina. The semen ejaculates were divided into six equal aliquots and diluted (1semen:4 diluent) to form six different cryoprotectant media (M). The control medium (M0) contained 2.5% and 15% egg yolk for goat bucks and ram semen extenders, respectively. Treatment extender media were M1 contained 1.5% SL, M2 included 2.5% SL, M3 supplemented with 3.5% SL, M4 contained 4.5% SL and M5 contained 5.5% SL. The semen samples were packed in straws (0.50 mL) and equilibrated in refrigeration at 5°C for 3 hours. After the equilibration period straws were warmed and sperm characteristics such as progressive motility, viability and abnormal tails were assayed. For freezing technique, the straws were suspended on a rack 5 cm above the liquid nitrogen vapor for 15 min at −120 °C. Then, straws immersed and stored in liquid nitrogen (−196°C). After thawing at 37°C/60 seconds, post-thawing motility, intact plasma membrane, sperm head damage and abnormal tails were evaluated. Results indicated that soybean lecithin at a level of 3.5 % attained higher ($P < 0.05$) sperm parameters than other concentrations of soybean lecithin and egg yolk in goat and ram semen extenders after the equilibration period. After thawing of goat straws that contained 3.5% soybean lecithin showed better ($P<0.05$) cryopreserving parameters than the egg yolk and the different levels of soybean lecithin. Ram semen extender containing 3.5% soybean lecithin or 15% egg yolk preserved the sperm parameters similar and superiority ($P<0.05$) higher than different soybean lecithin levels. Furthermore, after equilibration and post-thawing process the characteristics of sperm preservation decreased the concentration of soybean lecithin in extender was increased. Based on these results, we concluded that soybean lecithin at a level of 3.5% in semen extenders was more favorable in preserving goat and ram spermatozoa than other soybean lecithin concentrations. Thus, soybean lecithin can be used at a level of 3.5% in extenders of goat bucks and ram semen as a substitute of egg yolk.

Keywords: Goat and ram spermatozoa, cryopreservation, soybean lecithin concentration

INTRODUCTION

Cryoprotectants play a central role in resisting sudden temperature changes, protecting sperm against cold and warm shock. Also, cryoprotectants prevent ice formation during freezing and dissolution during the thawing process. The main causes of sperm injury during cryopreservation include: the oxidative damage, osmotic stress, ice crystal formation and cold shock resulting in damage to spermatozoa viability and a decrease in their fertilizing ability (Anakkul *et al.,* 2010). Egg yolk has since been routinely used with success in chilled and frozen extenders for semen of many domestic animals. The beneficial effect of egg yolk in the cryopreservation of sperm can be attributed to: 1) a resistance factor, which helps to protect the sperm against cold shock and 2) a storage factor in order to maintain viability. The phospholipids, cholesterol and the low density lipoprotein (LDL) content of the egg yolk have been identified as the protective components. These beneficial components are thought to stick to sperm plasma membrane during the cryopreservation process, stop the leakage of membrane phospholipids and enhancing their tolerance to cryopreservation (Bispo *et al.,* 2011). However, the egg yolk may contain cryoprotective antagonists in addition to the hygienic risks associated with its use (Akhter *et al.,* 2011). Recently, research has shown that using proteins of plant origin like soya-lecithin as semen extenders lack these hazards. Furthermore, the soybean lecithin is used as a phospholipids source for the commercial extenders available for preserving semen replacing the traditional membrane protectives of animal origin such as egg yolk (Akhter *et al.,* 2012). Lecithin is extracted during the process of soybean oil extraction by addition of water and centrifugation or steam precipitation. Lecithin contains phosphatidylcholine, phosphatidylinositol and phosphatidylethanolamine. The phosphatidylcholine is a major part of the membranes surrounding the cells. It also keeps the cell structure strong and helps retain its shape (Aires *et al.,* 2003). Lecithin is a natural emulsifier, lubricant and helps to keep the toxins to minimum level (Marie-Josée *et al.,* 2003). Soybean lecithin has similar ingredients to egg yolk used for protection of animal spermatozoa from cold shock in semen cryopreservation (Beccaglia *et al.,* 2009). In ram 1% soybean lecithin with glycerol can be used as a substitute for 20% egg yolk as cryopreservation material (Forouzanfar *et al.,* 2010). On the other hands, Vick et al. (2011) reported that an extender containing soybean lecithin can be used as an alternative for freezing cat semen. Also, soy-based extender resulted in better preservation of long-term motility and capacitation status of frozen-thawed cat sperm than egg yolk-based extenders (Vick et al., 2012). Likewise, Khalifa and Lymberopoulos (2013) reported that soybean lecithin-based semen extenders is superior to egg yolk semen

extenders in preserving chromatin stability and motility ram spermatozoa.

Therefore, the aim of this study was to evaluate effect of soybean lecithin at different concentrations (1.5, 2.5, 3.5, 4.5 and 5.5%) on semen preservation of sperm characteristics and the possibility of using SL as a substitute for egg yolk in goat bucks and ram semen extenders.

MATERIALS AND METHODS

The study was conducted at El-Serw Experimental Farm Animals belonging to Animal Production Research Institute, Ministry of Agriculture, Egypt.

Semen collection and evaluation:

Semen ejaculates were collected from 3 adult Zaraibi goats and 3 adult Rahmani rams (aging >2.5 years) two times weekly up to 6 weeks using an artificial vagina. Experimental animals had clinically normal reproductive tract and donating semen of acceptable quality. Before semen collection, sufficient time was allowed for sexual preparation and 1-2 false mounts were allowed for proper sexual stimulation by an oestrus female (doe or ewe). Immediately, after collection ejaculates were transferred to the laboratory and placed in a water bath at 37°C for assessing quantity and initial semen quality.

Semen dilution with egg yolk or soybean lecithin:

Semen ejaculates achieving progressive sperm motility > 75%, abnormal tail spermatozoa ≤ 15%, intact heads ≥ 85% and sperm cell concentration ≥ 2.8 × 10^9 spermatozoa/ml were selected for cryopreservation process. In each male breed, semen extender was divided into 6 media (M). The control media in goat bucks and ram semen was named M0 contained 2.5% and 15% egg yolk, respectively. While, the 5 trial media /breed were M1, M2, M3, M4 and M5 supplemented with soybean lecithin at levels 1.5, 2.5, 3.5, 4.5 and 5.5 % in semen extenders, respectively. The extension semen rate was performed by one-step method (1semen: 4 extender) at room temperature. Semen dilution for each media was packaged in 0.5ml French straw containing 350- 400x10^6 motility sperm / straw. The full straws were equilibrated in refrigeration for 3 hours at 5°C. After the equilibration period straws were evaluated for sperm progressive motility, viability, abnormal tails using warm straws as described by Evans and Maxwell (1987[a]).

Freezing and thawing techniques:

For freezing process, the straws after equilibration were placed horizontally on metal rack above the surface of liquid nitrogen vapor (-120 °C) at 5 cm in foam box 35×17×20 cm for 15 minutes. Immediately, the frozen straws were immersed in liquid nitrogen at −196°C up to 8 minutes. Then, the frozen straws were transferred into liquid nitrogen container. Thawing technique of frozen straws was obtained in a water bath at 37°C for 60 seconds. After thawing, post-thawing sperm motility and sperm morphology such as intact plasma membrane, damage sperm head and abnormal tails were assayed. The compositions of goat bucks and ram diluents semen were obtained by Evans and Maxwell (1987b) and displayed in Tables 1 and 2, respectively.

Table (1): The composition of diluents of goat bucks semen.

Extender components	Soybean lecithin media					
	M0	M1	M2	M3	M4	M5
Tris (g)	3.786	3.786	3.786	3.786	3.786	3.786
Glucose (g)	0.625	0.625	0.625	0.625	0.625	0.625
Citric acid (g)	2.172	2.172	2.172	2.172	2.172	2.172
***Soybean lecithin,%**	-	1.500	2.500	3.500	4.500	5.500
Egg yolk,%	2.500	-	-	-	-	-
Glycerol, %	5.000	5.000	5.000	5.000	5.000	5.000
Penicillin (IU)	100.00	100.00	100.00	100.00	100.00	100.00
Streptomycin (mg)	100	100	100	100	100	100
Distilled water to	100 ml	100 ml	100 ml	100 ml	100 ml	100 ml

* Sigma Chemical Co. (St. Louis, MO, USA).

Table (2): The composition of diluents of ram semen.

Extender components	Soybean lecithin media					
	M0	M1	M2	M3	M4	M5
Tris (g)	3.634	3.634	3.634	3.634	3.634	3.634
Glucose (g)	0.500	0.500	0.500	0.500	0.500	0.500
Citric acid (g)	1.990	1.990	1.990	1.990	1.990	1.990
***Soybean lecithin, %**	-	1.500	2.500	3.500	4.500	5.500
Egg yolk, %	15.000	-	-	-	-	-
Glycerol, %	5.000	5.000	5.000	5.000	5.000	5.000
Penicillin (IU)	100.00	100.00	100.00	100.00	100.00	100.00
Streptomycin (mg)	100	100	100	100	100	100
Distilled water to	100 ml	100 ml	100 ml	100 ml	100 ml	100 ml

* Sigma Chemical Co. (St. Louis, MO, USA).

The compositions of goat bucks and ram diluents semen were obtained by Evans and Maxwell (1987[b]) and displayed in Tables 1 and 2, respectively.

Semen evaluation at post-thawing:

The post-thawing sperm motility was assessed using 5 µl semen placed directly on a warm slide and covered. For each sample, at least five microscopic fields were examined by three trained observers at a magnification of 400×. The mean of the three successive evaluations was recorded as the final post-thawing motility score.

The hypo-osmotic swelling test (HOST) was used to evaluate the functional intact sperm plasma membrane, based on swollen tails. The hypo-osmotic solution consisted of 7.35 g sodium citrate and 13.51 g fructose dissolved in one liter of distilled water. Concisely, 500 µl of hypo-osmotic solution was mixed with 50 µl of frozen-thawed semen and was incubated at 37°C for 1 hour. Then a drop of well mixed semen was placed on a glass slide and covered with a cover slip. At least 200 spermatozoa were counted in different fields under 400x phase contrast microscope.

For examining the sperm morphologically, frozen-thawed semen sample (100 µl) was fixed in 500 µl of 1% formal citrate (2.9g tri-sodium citrate dihydrate and 1 ml of 37% solution of formaldehyde, and dissolved in 100 ml of distilled water). Morphological abnormalities of at least 200 spermatozoa were assessed using phase contrast microscope under oil immersion and the percentages of spermatozoa with damage head sperm and abnormal tails were determined.

Statistical analysis

Data were subjected to statistical analysis using general linear model (GLM) procedure of SAS (2009). Significance was declared using Duncan's multiple rang-tests (Duncan, 1955).

RESULTS AND DISCUSSION

The quality of goat and ram semen equilibrated with different levels of soybean lecithin (SL) or egg yolk extenders:

The quality of goat and ram semen diluted in egg yolk or different concentration of soybean lecithin after equilibration is summarized in Table 3. Extenders supplemented with different levels of soybean lecithin preserved sperm morphology during cold storage higher than egg yolk in goat and ram diluents. The decrease preservation of sperm parameters with egg yolk after equilibration period may be related to poor protection when used with low levels of egg yolk (Aboagla and Terada, 2004) and components of egg yolk that inhibit respiration of spermatozoa with high concentration (Amirat *et al.*, 2004). However, goat and ram extenders contained 3.5% of soybean lecithin showed higher success ($P<0.05$) to maintain sperm characteristics than those of other lecithin medium levels and egg yolk. This finding was in accordance with de-Paz *et al.* (2010) who revealed that semen extender with 3.5% soybean lecithin showed a high content of phospholipids that refluxed the best sperm ram motility and viability through cold storage. Furthermore, Phutikanit *et al.* (2011) concluded that the addition of soybean lecithin in the extender could preserve goat sperm characteristics during equilibration process. In the current study, soybean lecithin at a level of 4.5 or 5.5% failed to preserve sperm quality. This result confirms with Del-Valle *et al.* (2012) who indicated that lecithin may affect the inner mitochondrial membrane in spermatozoa and causes sublethal damages that seriously affect sperm functionality.

Also, Emamverdi *et al.* (2013) indicated that 1.5 % soybean lecithin in extender caused better live spermatozoa and actives mitochondria than other semen ram extenders.

On other hand, Akhter *et al.* (2011) concluded that motility, viability and plasma membrane integrity of buffalo bull spermatozoa remained similar in soya-lecithin and egg yolk semen extender at first and third days of storage at 5°C. Also, these authors reported that the values for the aforementioned parameters in soya-lecithin were higher compared with egg-yolk extender at fifth day of storage at 5°C.

Table (3): The quality of goat buck and ram semen equilibrated with different levels of soybean lecithin or egg yolk extenders.

Male breeds	Percentage of sperm characteristics after equilibration period %			
	Extender media	Progressive motility	Sperm Viability	Abnormal tails
Goat bucks	M 0	84.33±7.33[b]	86.27±7.12[b]	14.06±3.25[b]
	M 1	85.33±6.55[b]	87.07±7.11[b]	12.87±3.11[b]
	M 2	85.67±8.21[b]	87.73±6.95[b]	12.13±2.12[b]
	M 3	89.00±6.48[a]	91.13±6.67[a]	8.80±1.33[a]
	M 4	84.67±6.59[b]	87.47±6.89[b]	12.53±2.12[b]
	M5	84.00±6.74[b]	87.53±5.98[b]	12.93±3.15[b]
Rams	M 0	85.00±7.77[b]	86.73±6.54[b]	13.87±2.33[b]
	M 1	86.00±6.65[b]	87.87±5.89[b]	12.47±2.98[b]
	M 2	86.33±8.11[b]	87.93±7.52[b]	12.33±2.11[b]
	M 3	89.67±8.21[a]	91.47±6.98[a]	8.53±1.01[a]
	M 4	85.67±7.65[b]	87.80±6.58[b]	12.73±2.11[b]
	M 5	85.33±7.24[b]	87.87±7.56[b]	12.80±2.61[b]

Data within the same columns with different superscripts are statistically different at ($P<0.05$).
Mo male goat= 2.5 % egg yolk, Mo male ram=15% egg yolk, M1= 1.5% SL, M2= 2.5% SL, M3=3.5% SL, M4= 4.5%SL and M5=5.5 % SL for goat and ram.

The quality of goat and ram semen frozen with different levels of soybean lecithin (SL) or egg yolk extenders:

The values of post- thawing samples in either goat or ram semen extended in egg yolk or soybean lecithin supplemented with different concentrations are shown in Table 4. After thawing, it was observed that the sperm motility values in goat semen were lower ($P<0.05$) in M0 and other SL concentrations than those of M3. The rapid loss of motility after freezing/thawing process is related to the reduction of metabolic activity, the loss of intracellular components, and injury to mitochondrial sheath and axoneme (Melo *et al.*, 2007). However in rams, the post-thawing motility did not differ significantly between the samples frozen with M0 that contained 15% egg yolk or soy lecithin supplemented with 3.5% compared to other soya-lecithin mediums. Similarly, de-Paz et al. (2010) found that the semen extender obtained with a concentration of soybean up to 3.5% showed a higher content of phospholipids which was optimized to preserve the sperm motility and viability. Increasing the amount of egg yolk in ram diluent, improved post-thawing sperm parameters because of the beneficial effects of egg yolk components. This observation is in consistent with Manjunath *et al.* (2012) who suggested that the interaction between BSP (family of proteins in seminal plasma called Binder of sperm) and low density lipoproteins (LDL) present in egg yolk could be the basis sperm protection by forming a protective film on the sperm surface and replacing sperm membrane phospholipids that are lost or damaged during the cryopreservation process. In the current study, sperm morphology (intact plasma membrane, damage of sperm head and abnormal tails) showed that frozen ram semen samples with the 15% egg yolk extender or soy lecithin at 3.5 % were significantly ($P<0.05$) higher than other soybean lecithin samples. However, in goat semen samples frozen semen extender plus soy lecithin at 3.5% presented higher ($P < 0.05$) percentages of sperm morphological traits in comparison with those straws frozen with 2.5% egg yolk and other soybean lecithin concentration. Zhang et al. (2009) demonstrated that phospholipids in soya-lecithin being the major component of sperm membrane which play important physiological functions in reducing the freezing point, avoiding the formation of large ice crystals and reduce the possible mechanical damage to the sperm membrane. Furthermore, phospholipids of soya-lecithin do not enter the membrane to alter phospholipids concentration but may form a protective film around the cell to prevent the formation of intracellular ice crystals during freezing/thawing process (Reed *et al.*, 2009). In addition, it is believed that the lecithin component protects the plasma membrane by restoring the phospholipids lost during heat shock. These results are supported by the results of (Futino *et al.*, 2010) who reported that lecithin may have played a protective role during cryopreservation due to its low viscosity, lower presence of debris, improves the kinematics of sperm cells and rearrangements structure of the plasma membrane. Moreover, Papa *et al.* (2011) explained that lecithin maintains progressive motility and plasma membrane integrity in stallion sperm samples that were similar to samples frozen in egg yolk.

On the other hand, Akhter *et al.* (2012) suggest that 10% soybean lecithin in extender improves the freezability of buffalo bull spermatozoa and can be used as an alternate to egg yolk in cryopreservation. In fish semen, Yildiz *et al.* (2013) concluded that extender containing soybean lecithin had similar cryoprotective actions to conventional egg yolk-based extender against freezing damages and fertilization.

Table (4): The quality of goat buck and ram post-thawing semen with different levels of soybean lecithin or egg yolk extenders.

Male breeds	Extender media	Percentage of sperm characteristics post-thawing %			
		Post-thawing motility	Intact plasma membrane	Damage sperm head	abnormal tails
Goat bucks	M 0	41.67±9.35^{b}	38.73±8.62^{b}	35.87±2.22^{b}	35.60±2.29^{b}
	M 1	42.67±8.59^{b}	40.20±7.77^{b}	33.73±2.71^{b}	33.67±3.16^{b}
	M 2	43.33±8.27^{b}	41.80±9.74^{b}	33.47±3.05^{b}	32.47±3.42^{b}
	M 3	54.33±7.88^{a}	47.73±8.55^{a}	29.87±1.17^{a}	26.07±3.53^{a}
	M 4	42.67±9.79^{b}	40.87±7.86^{b}	33.67±3.19^{b}	33.13±4.72^{b}
	M5	42.33±9.71^{b}	39.73±9.55^{b}	34.13±3.10^{b}	34.27±4.55^{b}
Rams	M 0	55.33±9.71^{a}	47.87±9.01^{a}	28.67±2.55^{a}	24.93±2.39^{a}
	M 1	44.33±9.15^{b}	41.73±8.95^{b}	33.73±3.19^{b}	29.87±3.26^{b}
	M 2	45.67±8.22^{b}	42.53±8.54^{b}	32.93±2.57^{b}	29.93±3.14^{b}
	M 3	56.00±9.25^{a}	48.20±9.25^{a}	27.07±2.18^{a}	23.67±2.61^{a}
	M 4	43.67±8.69^{b}	40.47±7.98^{b}	34.67±3.28^{b}	31.53±3.01^{b}
	M 5	42.33±8.27^{b}	39.27±8.87^{b}	35.13±3.51^{b}	32.87±3.22^{b}

Data within the same columns with different superscripts are statistically different at ($P<0.05$).
Mo male goat= 2.5 % egg yolk, Mo male ram=15% egg yolk, M1= 1.5% SL, M2= 2.5% SL, M3=3.5% SL, M4= 4.5%SL and M5=5.5 % SL for goat and ram.

CONCLUSION

Our results indicated that supplementation of soybean lecithin at 3.5% in the semen extender could better preserve spermatozoa characteristics after equilibration period and thawing. In addition, our results suggested that lecithin as the lipid/lipoprotein source can be used as a substitute for egg yolk in either goat or ram semen extenders. However, more studies are necessary to determine the effects of using this semen extender on fertility characteristics of the cryopreserved goat and ram spermatozoa.

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تقييم مستويات مختلفة من ليسيثين فول الصويا كبديل لصفار البيض لحفظ الحيوانات المنوية للماعز والكباش

عزالدين إبراهيم خليفة، محمد عبد الحافظ

معهد بحوث الإنتاج الحيوانى ،مركز البحوث الزراعية، الدقى، الجيزة، مصر

الغرض من هذه الدراسة هودراسة تأثير تركيزات مختلفة من ليسيثين فول صويا كبديل لصفار البيض لحفظ الحيوانات المنوية للماعز والكباش بعد فترة الإتزان والإسالة .استخدم في هذه الدراسة، ستة وثلاثين قذفة جمعت القذفات من ٣ ذكورماعز، ٣ ذكوركباش أعمارها أكبر من سنتين ونصف في موسم التلقيح عن طريق المهبل الاصطناعي .تم تقسيم قذفات المني إلى ستة بيئات متساوية بمعدل تخفيف ١ ملى سائل منوى إلى ٤ ملى مخفف .وكانت البيئة M0 بيئة التحكم تحتوي على ٢,٥٪ و ١٥٪ صفار البيض لكلا من مخففات السائل المنوى للماعز والكباش على التوالي .والبيئات لكل من الماعز والكباش M1 تحتوى على ١,٥٪ ليسيثين الصويا ، M2 تحتوى على ٢,٥٪ ليسيثين الصويا، M3 تحتوى على ٣,٥٪ ليسيثين الصويا، M4 تحتوى على ٤,٥٪ ليسيثين الصويا، M6 تحتوى على ٥,٥٪ ليسيثين الصويا .وتم تعبئة السائل المنوى المخفف في القش (٠,٥ مل) وتم إجراء فترة الإتزان فى الثلاجة على ٥ م° لمدة ٣ ساعات .وبعد فترة الأتزان تم تقييم خصائص الحيوانات المنوية مثل الحركة التقدمية، الحيوية والذيل الغيرطبيعى .ولإجراء التجميد علقت القش على حامل معدنى يبعد ٥ سم من سطح بخار النيتروجين السائل) لمدة ١٥ دقيقة عند ١٢٠- درجة منوية (وبعد التجميد تم غمرالقش وتخزينها في النيتروجين السائل -١٩٦ م°. وتم عمل الإسالة للقشة على درجة 37 م° لمدة ٦٠ دقيقة .وبعد ذوبانها قيمت حركة الحيوان المنوى، غشاء البلازما السليم ، رأس الحيوانات المنوية المحطم والذيل الشاذ. والنتائج المتحصل عليها أثبتت أن مستوى ليسيثين فول الصويا بنسبة ٣,٥٪ أعلى معنويا ($P < 0.05$) لصفات الحيوانات المنوية من تركيزات ليسيثين فول الصويا الأخرى وصفار البيض في مخففات الماعز والكباش بعد فترة الإتزان. وحققت قش الماعزالمحتوى ٣,٥٪ ليسيثين فول الصويا أفضل ($P < 0.05$) من صفار البيض والمستويات المختلفة من ليسيثين فول الصويا الأخرى. بالعكس بعد ذوبان السائل المنوي يحتوي ٣,٥٪ ليسيثين فول الصويا وصفار البيض ١٥٪ تفوق ($P < 0.05$) على المستويات المختلفة من ليسيثين فول الصويا .وإستنادا إلى هذه النتائج، تبين أن مستوى ليسييثين فول الصويا عند 3.5٪ في مخففات السائل المنوى المني أكثر حفاظ على الحيوانات المنوية للماعز والكباش الحيوانات المنوية أفضل من تركيزات ليسيثين فول الصويا الأخرى. وبالتالي فإن استخدام مخففات السائل المنوي مع ليسيثين فول الصويا تعتبر الأمثل كبديل عن صفار البيض لتجميد الحيوانات المنوية للماعزوالكباش.