EFFECT OF SOYBEAN LECITHIN-BASED SEMEN EXTENDER ON FREEZABILITY AND FERTILITY OF RAHMANI RAM SPERMATOZOA

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ABSTRACT

This experiment was designed to compare the protective action of soybean lecithin and egg yolk on cryopreservation and fertility of ram spermatozoa. The semen ejaculates were collected from three fertile rams, aged over 2.0 years and weighed round 65 kg, using artificial vagina. Two extenders were prepared, 1st served as the control and contain15% egg yolk with Tris-citric acid (TCEY).The second extender used 3.5 % soy lecithin to complete Tris-citric acid (TCSL). After primary evaluation of ejaculates, the semen samples were extended at the rate of 1 semen: 4 dilutes. The packed diluted semen was used to perform three experiments with TCEY and TCSL extenders. In the 1st experiment parameters such as progressive motility, viability and damaged acrosome were assessed after equilibration period at 5°C for 3 hours. Concerning the 2nd experiment, progressive motility, viability and abnormal acrosomes were evaluated after the freezing-thawing process. In the 3rd experiment a fertility test with post-thawing TCEY and TCSL extenders was performed. The results in the 1st experiment indicated that addition of 3.5% soy-lecithin increased (P>0.05) progressive motility, viability and reduced abnormal acrosomes of spermatozoa after equilibration period compared to 15% egg yolk extender. Although, TCEY and TCSL extenders in experiment 2nd showed non-significant differences in post-thawing sperm characteristics, the TCSL had improved post-thawing progressive motility, viability and rate of damaged acrosome of ram spermatozoa. In the 3rd experiment a better pregnancy rate (P<0.05) with TCSL (63.64 %) was observed compared to sperm preserved in TCEY (54.55 %). Based on these results, we conclude that use of a chemically defined, soy-based medium improves long-term motility and capacitation status of frozen–thawed ram' spermatozoa compared with cryopreservation in a traditional protection (egg yolk) extender. Furthermore, the study clarify that soybean lecithin could be able to increase proportions of viable frozen-thawed spermatozoa which reflected positively on fertility rates.

Key Words: Cryopreservation, soybean lecithin, egg yolk, fertility, ram Spermatozoa.

INTRODUCTION

Artificial insemination (AI) is one of the assisted reproductive techniques used to improve the genetic potential of livestock breeds and for exploiting the spermatozoa from superior sires. Preservation of genetically superior male germ cells is very important and useful for improvement of animal productivity as a consequence of improvement of the individuals' genetic make-up (Amirat *et al.,* 2004). Successful preservation of superior male sperm will give the chance for future recalling even in the absence of those males. Currently, egg-yolk (EY) is a common component of most semen preservation extenders used for domestic animals. It has been shown to have a beneficial effect on sperm preservation as a cryoprotectant of plasma membrane and acrosome against temperature (Su *et al.,* 2008). However, EY has represented some problems, as it contains micro elements that might be responsible of increase extender's viscosity, carries microbial, inhibition of sperm respiration and diminishes sperm motility (Sharafi *et al.,* 2009). An alternative to replace the components of animal's origin in semen extenders is the soy lecithin, a natural mixture of phosphatidylcholine and several fatty acids such as stearic, oleic, and palmitic. Such fatty acids are prevailing phospholipids in most of mammalian biological membranes and are known to confer structural stability to cells (Oke *et al.,* 2010). So, the efficiency of soy lecithin as a primary source of lipoproteins in semen extenders was prove (Papa *et al.,* 2010). Previous studies suggested that addition of soy-lecithin (SL) to semen extender improved post-thawing sperm motility, viability, acrosome

integrity and sperm membrane structure in human (Reed *et al.,* 2009), boar (Zhang *et al.,* 2009), stallion (Papa *et al.,* 2011), cat (Vick *et al.,* 2011), bull (Akhter et al., 2012) and billy goats and rams (Khalifa and Abdel-Hafez, 2013).

Therefore, the present study was planned to compare the efficiency of 3.5% soy lecithin with 15% egg yolk based extender on quality of ram spermatozoa after equilibration period, post-thaw sperm viability and fertility.

MATERIALS AND METHODS

Experimental Animals

The present study was conducted at El-Serw Experimental Farm belonging to Animal Production Research Institute (APRI), Ministry of Agriculture, Egypt. Three sexually mature Rahmani rams aged over 2.0 years and weighed over 65.0 kg were used in the current study. All rams were healthy and clinically free of internal and external parasites. The animals were kept under natural photoperiod and balanced nutritional status. The rations offered to rams adjusted to meet maintenance requirements during breeding season according to NRC (2007). The rams had received free access to salt lick and fresh water during the experimental period.

Semen collection and handling

Semen samples were collected twice a week using artificial vagina after stimulating with an estrus ewe during breeding season. Then, ejaculates immediately transported to the laboratory and placed in a water bath at 37°C. Ejaculates were evaluated for volume (ml) using gradual test tube, progressive motility (%), sperm concentration (n×10^9 sperm/ml) by hemocytometer and evaluated for viability (%) using eosin- nigrosin staining. Then, abnormal acrosome (%) using formal citrate method (2.9 g tri-sodium citrate dihydrate and 1 ml of 37% solution of formaldehyde, dissolved in 100 ml of distilled water where 100 µL semen sample was fixed in 500 µL of 1% formal citrate). Only fresh semen samples with adequate individual motility more than 80%, viability less than 15%, abnormal acrosome less than 15% and sperm concentration more than 2.8×10^9sperm/ml were used for the cryopreservation procedure.

Semen extenders

Semen was extended at a rate of 1(semen) to 4 (extender) where the extender components were dissolved in 100 ml distilled water for the control group semen media. In control media, Tris- citric acid was supplemented with 15% egg yolk (TCEY). While, in the trial semen media 3.5 gm soybean lecithin was added to the Tris- citric acid (TCSL). The ingredients of TCEY and TCSL semen extenders were adjusted as reported by Evans and Maxwell (1987) and presented in Table 1.

Experimental design

Semen samples were diluted with TCEY and TCSL extenders using one step extension method. The dilute semen was loaded in 0.5 ml French straws to reach a sperm concentration of 350-400x10^6 sperm/straw then, the open end of the filled straws was sealed with polyvinyl chloride powder. Three experiments were operated after packed straws kept at 5°C for 3 hours as equilibration period.

The first experiment

It was designed to investigate the effect of semen extender (TCEY and TCSL) on parameters of preserved sperm quality after equilibration period. Semen straws were placed in the water bath at 37°C for one minute. Then post equilibration period, progressive motility, viability and acrosomal injuries were assessed.

Table 1: The composition of TCEY and TCSL ram semen extenders.

Ingredients	Semen extenders	
	TCEY	TCSL
Tris (g)	3.634	3.634
Glucose (g)	0.500	0.500
Citric acid (g)	1.990	1.990
Soybean lecithin (g)	-	3.5
Egg yolk,%	15	-
Glycerol, %	5.000	5.000
Penicillin (IU/ml)	100	100
Streptomycin (mg/ml)	100	100
Distilled water up to	100 ml	100 ml

The second experiment

It was conducted to study the influence of either TCEY or TCSL semen extenders on sperm physical characteristics after frozen-thawed step. After equilibration straws were held on metal rack in foam box (35×17×20 cm) 5 cm above the surface of the liquid nitrogen vapor for 15 minutes. Immediately, straws immersed in liquid nitrogen at −196°C for 8 minutes then, the frozen straws plunged into liquid nitrogen container for storage. After storage, the straws were thawed in water bath at 37°C for 60 seconds. The frozen-thawed semen samples were assessed for progressive motility, viability and acrosomal injuries.

The third experiment

It was carried out to determine success rate of artificial insemination for post-thaw cryopreserved semen in TCEY or TCSL extenders. Fertility trial was administered with twenty-two adult and healthy ewes with no difference in reproductive performance or in submitted estrous cycle. The experimental ewes divided into two groups (N=11 in each). The 1st group considered as control and 2nd group employed as trial. The insemination procedure was implemented with both semen samples cryopreserved in TCEY or TCSL. The insemination was performed at the os-cervix using speculum, after ewe displayed natural estrous cycle. When ewe came in heat, it received two frozen- thawed within 12 hours as interval period. The pregnancy diagnosis attained at 35 days after semen inseminated without returned to heat. The pregnancy rate was calculated as number of conceived ewes/ number of inseminated ewes. Single rate calculated as the number of ewes lambing single per total number of ewes lambed. Twins rate calculated as number of ewes lambing twin per total number of ewes lambed. Sexing lambs % (male: female) as the number of born lambs in each sex per total number of born lambs was estimated. Litter size was calculated as number of total lambs born per number of ewes lambed.

Statistical analysis

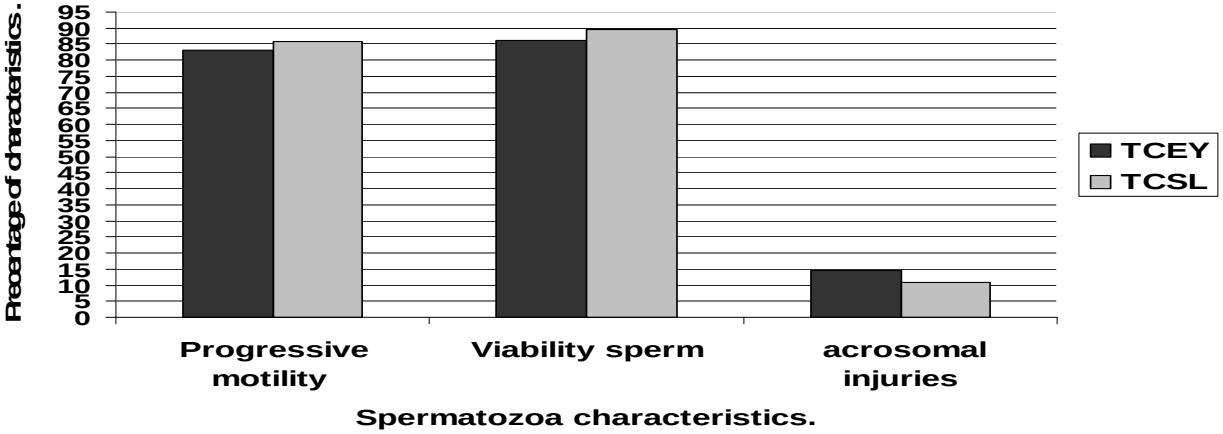
The data were subjected to statistical analysis with independent t-test of SPSS for Windows, Version 20.0 (2011). Probability values of less than 0.05 ($P < 0.05$) were considered significant. Results are expressed as means ± SE. The differences between TCEG and TCSL extenders in sex ratio were statistically analyzed by x^2.

RESULTS AND DISCUSSION

First experiment

Influence of semen extenders (TCEY or TCSL) on post equilibration sperm physical characteristics are shown in Figure 1. Generally, the current extenders (TCEY or TCSL) had no significant ($P>0.05$) difference on progressive motility, viability and acrosomal damage after equilibration period. Whereas, the results indicate that TCSL extender had higher progressive motility, viability and lower acrosomal damage after equilibration period than TCEY extender. The percentages of progressive motility, viability and damage acrosomes were 85.70, 89.57 and 10.76% compared to 83.21, 86.07 and 14.43% in TCSL and TCEY extenders, respectively. This finding is in accordance with Futino *et al.* (2010) who revealed a reduction of sperm characteristics during refrigeration which was attributed to changes in the pH of extension, osmolarity and growth of bacteria in diluent. Also, Ortega *et al.* (2003) found that equilibration is an important step prior to semen cryopreservation for safeguard biological and functional sperm cells. The lowest sperm characteristics observed with using TCEY may be attributed to the risk of contamination of microorganisms such as bacteria and fungi that are present in egg yolk-based extender. The contamination releases endotoxins that reduce the liveliness of sperm (Manjunath, 2012). However, increasing vitality of sperm obtained with TCSL is generally caused by phosphatidylcholine from soy-lecithin that restore phospholipids of membranes, preserving the integrity of the membrane and maintaining sperm motility at low temperature. This observation is also consisted with Baliarti *et al*. (2012) who concluded that extender contained soy- lecithin at a rate of 3% had the best cold survival motility and viability of ram spermatozoa stored at 5°C. Furthermore, soy-lecithin plays an

Fig. 1:Effect of TCEY and TCSL semen extenders on ram spermatozoa physical characteristics after equilibration period.

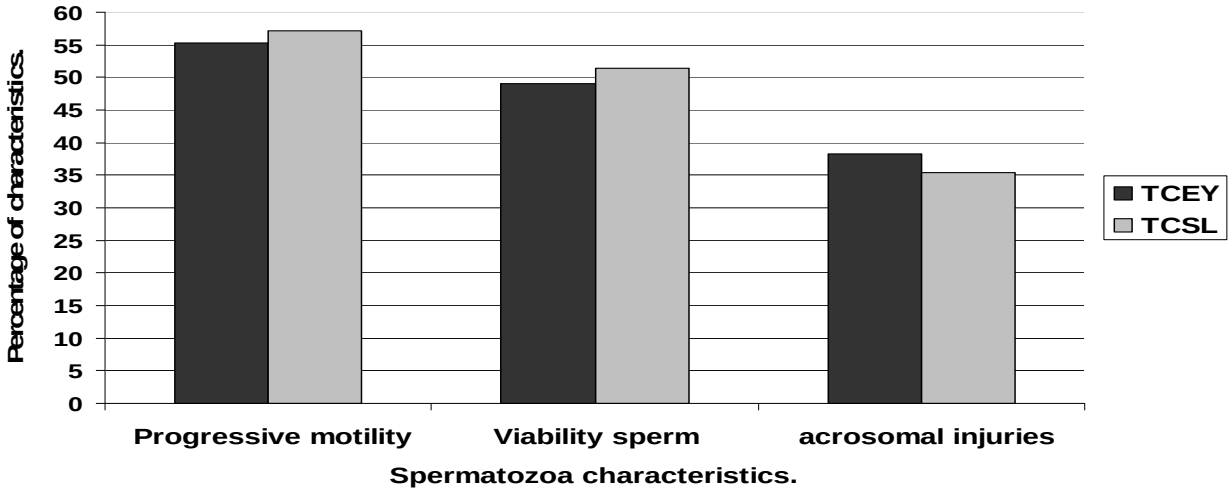


important physiological function in reducing the cooling point and minimizing the replacement of plasmalogens to reduce the possible mechanical injuries of the sperm membrane (Singh *et al.*, 2012).

Second experiment

The influence of semen extenders on post-thawing sperm physical characteristics are presented in Figure 2. The TCSL extender had boost the cryoprotective effect in terms of post-thawing sperm characteristics compared to TCEY extender. Generally, there were no significant effect between TCEY and TCSL extenders on post-thawing progressive motility, viability and acrosomal abnormality. However, sperm frozen-thawed in TCSL recorded a higher post–thawing motility and viability rate (57.14% and 51.42%) than sperm cryopreserved in TCEY media (55.35% and 49.07%), respectively. Furthermore, the percentage of acrosomal abnormality after thawing was lower in TCSL (35.42%) than TCEY (38.21%) extenders. The improvement of sperm physical characteristics with TCEY extender might be related to concentration of cholesterol (13-16%) presented in egg yolk. Cholesterol is thought to be the main component responsible for the protective role of egg yolk during freezing spermatozoa. These results are in accordance with Moraes *et al.* (2010) who found that sperm with cholesterol before freezing process could reduce the sensitivity of sperm membranes to damage by eliminating or at least minimizing the lateral phase separation of the lipids. Furthermore, Mocé *et al.* (2010) concluded that treating ram sperm with cholesterol increases sperm cryosurvival rates, sperm longevity after thawing, osmotic tolerance, zona-binding capabilities of sperm and provide suitable microenvironments (chemical and ⁄or physical) for membrane-associated proteins. Otherwise, the lowest cryosurvival of sperm contained egg yolk may be related to microbial sanitary risk (Hu *et al.*, 2010) which might fail to maintain sperm motility and affect artificial insemination success (Forouzanfar *et al.*, 2010). On the other hand, the mammalian sperm contain a high concentration of polyunsaturated fatty acids in the plasma membrane which make sperm susceptible to lipid peroxidation (LPO) when egg yolk supplemented to extender. The LPO lead to the deterioration of sperm functions through oxidative stress and the production of components such as malondialdehyde (MDA) that is one of the most prevalent by-products of lipid peroxidation. These results are also agree with Camara *et al.* (2011) who reported that reactive oxygen species (ROS) in semen decreases energy metabolism, progressive motility, viability rate and DNA integrity in sperm. Nevertheless, lecithin of plant origin (soy bean) has successfully substitute egg yolk in field trials for livestock semen. These results are supported by Fukui *et al.* (2008) who reported that lecithin may have played a

Fig. 2: Effect of TCEY and TCSL semen extenders on sperm physical characteristics after freezing- thawing process.



protective role during freezing due to low viscosity, improvement of the kinematics of sperm membrane and rearrangements phospholipid of the sperm cells membrane. Furthermore, the influential antioxidant compounds in soy lecithin as glutathione may protect the sperm viability via scavenging the LPO and prevention of MDA formation during cooling or freezing process (Salmani *et al.,* 2013). Also, Emamverdi *et al.* (2013) showed that soybean lecithin extender recovered motility, plasma membrane acrosome integrity, apoptosis status and mitochondrial activity after thawing ram spermatozoa. On the other hand, in bovine frozen semen, Crespilho *et al. (*2012) reported higher sperm motility and acrosomal integrity for semen cryopreserved using lecithin based commercial extenders. Moreover, Singh *et al.* (2013) concluded that newly developed lecithin-tris extender could maintain comparable semen quality and improve the freezability as compared to egg yolk-tris extender.

Third experiment

Influence of semen extenders (TCEY or TCSL) on fertility of ewes artificially inseminated with frozen- thawed ram semen extended in TCEY or TCSL are presented in Table 2. The pregnancy rate of frozen-thawed semen that was cryopreserved in TCSL (63.64 %) was greater ($P < 0.05$) than those frozen in TCEY (54.55 %). Diluents containing soybean lecithin might maintain the sperm motility, viability and plasma membrane integrity greater than diluent containing egg yolk that repercussion on lambing rate. These results are different from those obtained by Ricker *et al.* (2006) who reported that fertility rate of stallion semen frozen in diluent containing egg yolk was higher (55%) than soybean phosphatidylcholine (36%). Also, Fukui *et al.* (2008) established that lambing rates were 64.5 and 56.7% for sperm cryopreserved in egg yolk and commercial soybean lecithin extenders, respectively. Also, these authors indicated that extender derived from non-animal compounds could be used for frozen-thawed ram semen without reducing fertility. Conversely, Gil *et al.* (2003) concluded that commercial soybean lecithin extenders Bioxcell® (IMV Technologies, L'Aigle, France) appears to be an alternative to the conventional extenders as milk and egg yolk and provides similar fertility results (33.2, 32.6 and 27.2%), respectively. Indeed, soybean lecithin based extender has been used for cryopreservation of semen and produced acceptable fertility rates after artificial insemination compared with egg yolk based extender. These findings are also confirmed by Akhter *et al*. (2012) who demonstrated that fertility rate recorded in buffalo cows inseminated with semen containing 10% soya-lecithin (56.7%) compared with 20% egg yolk

Table 2: The fertility rate of frozen-thawed ram semen extended with TCEY and TCSL.

Items	Semen extenders	
	TCEY	TCSL
Number of ewes inseminated	11	11
Number of ewes conceived	6	7
Pregnant rate, %	54.55[b]	63.64[a]
Number of ewes lambing	6	7
Total number of lambs	7	9
Number of ewes lambing single	5	5
Single rate, %	83.33[a]	71.43[b]
Number of ewes lambing twins	1	2
Twins rate, %	16.67[b]	28.57[a]
Number of female lambs	2	6
Female rate, %	28.57[b]	66.67[a]
Number of male lambs	5	3
Male rate, %	71.43[a]	33.33[b]
Litter size	1.17	1.29

Values with different superscripts in the column, differ significantly (P<0.05).

(41.5%). The lesser fertility rates observed in sperm frozen in TCEY compared to those frozen in TCSL may be mediated by convolution of the soy-lecithin around surface of sperm membrane and to changes in the composition of sperm membrane by interaction lipids of soy-lecithin during the freezing and thawing process. These results are similar to those obtained by Khalifa *et al.* (2013) who affirmed that soy-lecithin was used in protecting sperm from cryodamage and it can contribute to satisfactory fertility rate (71.0%) with preserved ram spermatozoa in Bioxcell®.

CONCLUSION

The results of this study revealed that soy-lecithin extender could be used to preserve sperm motility, viability and plasma membrane integrity during freezing-thawing procedures. Subsequently, these alterations became more evident along with thawing time and cannot be adversely affecting sperm fertilizing capacity.

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الملخص العربى

تأثير مخفف ليسيثين فول الصويا على قدرة التجميد وخصوبة السائل المنوي للأغنام الرحمانى

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هذه الدراسة صممت لمقارنة الأثر الواقى للصويا ليسيثين بصفار البيض لحفظ وخصوبة السائل المنوى المجمد للأغنام. حيث تم تجميع السائل المنوى من ثلاثة كباش عمرها أكبر من سنتين ووزنها أكبر من 65 كجم بإستخدام طريقة المهبل الصناعى. ومن ثم تم تجهيز مخففين الأول استخدم للمقارنة ويحتوى على 15% صفار البيض مع مخفف الترس (Tris) والثانى للإختبار وإستخدم 3.5% صويا ليسيثين مع مخفف الترس. بعد الفحص الأولي للسائل المنوى تم التخفيف بمعدل 1 سائل منوى إلى 4 مخفف.وتم إجراء ثلاثة تجارب مع مخفف السائل المنوى المعبأ:- التجربة الأولى لقياس الحركة- الحيوية- والأكروسوم المحطم بعد فترة الإتزان على 5 °م لمدة 3ساعات. التجربة الثانية لقياس الحركة- الحيوية - والأكروسوم الغير طبيعى بعد التجميد والإسالة. التجربة الثالثة لقياس معدلات الخصوبة مع مخفف صفار البيض وصويا ليسيثين.

أظهرت النتائج فى التجربة الأولى أن أضافة 3.5% صويا ليسيثين اعطى زيادة غيرمعنوية للحركة والحيوية وقلة فى الأكروسوم المحطم مقارنة بالمخفف 15% صفار البيض. وقد أتضح من النتائج فى التجربة الثانية أن تأثير التجميد والإسالة على الصفات الطبيعية للسائل المنوى لم تختلف نتيجة إضافة الصويا ليسيثين أو صفار البيض. فى التجربة الثالثة قد تحسن معدل الخصوبة مع مخفف صويا ليسيثين 46و 63% عن مخفف صفار البيض 55و54%. ومن نتائج هذه الدراسة يتضح أن مخفف صويا ليسيثين يحسن من الحركة والحيوية ومعدلات الخصوبة للسائل المنوى المجمد مقارنة بصفار البيض كما أن مخفف الصويا ليسيثين قادر على زيادة نسب الحيوية للحيوانات المنوية المجمدة مما ينعكس بالموجب على معدلات الخصوبة.