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Effect of Soybean lecithin-based Extender on Sperms Motility in the Chilled and Frozen Semen of Nubian bucks

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Abstract
This study was conducted to investigate the effect of different concentrations of soybean lecithin extenders on motility of sperms in the chilled and frozen semen of Nubian bucks. A total of 59 ejaculates were collected from 6 fertile bucks once a week during 9 weeks interval using artificial vagina. After initial evaluation of ejaculates, the semen samples were divided into three equal groups and diluted at 1:5 (semen: extender) using extenders containing 2% egg yolk with tris –citric acid (TCEY, control group, n= 59), 3% soybean lecithin with tris-citric acid (TCSL 3%, n=59) and 4% soybean lecithin with tris-citric acid (TCSL 4%, n=59).The motility rates of spermatozoa after equilibration period at 5ºC for one and 4 hours; and after freezing at -196 ºC for 30 days were recorded.The results indicated that there were no significant differences in motility rates among the deferent concentrations of TCSL and TCEY at temperature 5 ºC for 1 (very good motile: 80-100%) and 4 (good motile: 60-79%) hours. Overall mean of sperm motility rates was lower after cooling for 4 hours than 1 hour. The motility values of post-thawed semen containing TCSL 3% after cryopreservation at -196 ºC for 30 days were significantly higher (66.36 ± 10.3 %) than those containing TCSL 4% (60.34 ±11.9 %) ($p<0.05$). However, there were no significant differences between motility rates of frozen semen diluted with TCSL and TCEY. It could be concluded that soybean lecithin 3% provided the best motility of frozen-stored spermatozoa than soybean lecithin 4% and egg yolk.

Keywords: Nubian bucks, frozen semen, soybean lecithin, egg yolk.

Introduction
The use of frozen semen in AI protects the animals from the stress caused by transportation for mating and the risk of disease transmission during copulation, in addition to favoring the preservation of high-value genetic material (Silva *et al.*, 2000). Cryopreservation involves semen collection and its dilution with desirable extender (Ray *et al.*, 2015).

One of the most important elements in handling and storage of the semen was the preparation of eligible semen extender, ensuring high survivability and fertility of the spermatozoa for a long period (Purdy, 2006; Mara *et al.,* 2007). In the most cases, extenders used for cooling or freezing of semen included egg yolk, skimmed milk, glycerol or their combination (Sharafi *et al.,* 2009; Kulaksiz *et al.,* 2013).

The main effective component of egg yolk is the lipoprotein fraction, e.g., lecithin, which protects the membrane's phospholipid integrity during cryopreservation (Moussa *et al.,* 2002; Amirat *et al.,* 2004; Forouzanfar *et al.,* 2010). However, egg yolk 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 decreases sperm motility (Sharafi *et al.,* 2009). In goats, dilution of semen using diluents containing egg yolk has harmful effect on quality of sperm cells during dilution, freezing and thawing due to egg yolk coagulating enzyme (EYCE) and glycoprotein secreted by bulburethal gland (Salmani *et al*., 2013).

Recently, many researchers (Phutikanit *et al.,* 2011; Vidal *et al.,* 2013; Salmani *et al.,*2013) have been reported of the replacement of the animal origin components with soybean lecithin. Previous studies suggested that addition of soybean lecithin 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.,* 2010), bull (Akhter *et al*., 2012) and bucks and rams (Khalifa and Abdel-Hafez,2013). Yotov (2015) reported that tris-fructose-citric acid extender containing 1.5 % soybean lecithin and low glycerol (1.5%) provided the best motility and viability of the chilled-stored spermatozoa and preserved their fertilization capacity in Bulgarian bucks.

The aim of the current study was to monitor the effect of two different soybean lecithin concentrations (3% and 4%) on the sperm motility of chilled and cryopreserved semen in Nubian bucks.

Materials and Methods

Study area: This experiment was carried out at the sheep and goats research section in Animals Production Research Center-Animal Resources Research Corporation , Ministry of Animal Resources and Fisheries at Hilt Kuku , Khartoum North (N 15° 37` 11.30", E 32º 33` 51.35").

Experimental animals: A total of six fertile Nubian bucks were used in this study. Their ages ranged between 1 to 3 years and body weights were between 30 to 45 kg. All bucks were healthy and clinically free of internal and external parasites.

Semen collection and evaluation: A total of 59 ejaculates were collected once a week for 9 weeks interval using an artificial vagina. Collected semen was placed in a water path (37°C) and immediately evaluate for colour, volume (ml) using gradual test tube, wave motion (0-5 scale), percentage of motile spermatozoa and viability (%) using eosin-nigrosin staining (Noakes *et al.,* 2009). Fresh semen with a thick consistency, rapid wave motion (3-5 on a 0-5 scale), more than 70% individual motility and viability less than 15% were used for chilling and cryopreservation.

Semen extenders and dilution: Three extenders includes; egg yolk with tris –citric acid (TCEY), 3% soybean lecithin with tris-citric acid (TCSL 3%) and 4% soybean lecithin with tris-citric acid (TCSL 4%) were prepared for semen dilution according to khalifa and Abdel-Hafez (2014) and components of all extenders presented in table 1. All extenders were stored in a refrigerator until use for dilution.

After initial evaluation, the approved ejaculates were divided into three equally groups (n= 59) and extended gradually to ratio of 1:5 (semen:extender) with TCEY (control group), TCSL 3% and TCSL 4%.

Storage of semen: Semen samples stored at 5 °C and sperm motility was assessed after one and 4 hours of cooling using high power heated microscopic stage (37 ºC). Motility rates were calculated according to Noakes *et al*. (2009). Furthermore, equilibrated semen was packed into French straws (IMV, L, A. Agle, France) and sealed with poly vinyl alcohol powder. Then, the straws were placed horizontally on a rack and frozen in vapor 4 cm above liquid nitrogen (-120 ºC) for 15 minutes and then dipped stored in liquid nitrogen at -196 ºC for one month.

Thawing of frozen semen: Frozen straws were thawed in water bath at 37 ºC for 30 seconds and individual motility of post-thaw sperm was evaluated.

Table1: The composition TCEY and TCSL extenders used for semen dilution of Nubian bucks:

	Semen extenders	
Ingredients	**TCEY**	**TCSL**
Tris (g)	3.7	3.7
Glucose (g)	0.500	0.500
Citric acid (g)	1.9	1.9
Soybean lecithin (g)	-	3 ,4
Egg yolk (%)	2.0	-
Glycerol (%)	5.0	5.0
Penicillin (IU/ml)	100	100
Streptomycin (mg/ml)	100	100
Distilled water (ml)	100	100

Results

Means of sperm motility (%) of semen diluted with TCEY, TCSL 3% and TCSL 4% extenders during the storage at temperature 5 ºC for 1 hour shown in table 2. The motility rates of spermatozoa in semen diluted with egg yolk and different concentrations of soybean lecithin extenders after cooling for 1 hour were very good motile (80-100%). The type and concentration of extenders did not influence significant on the sperm motility. At temperature 5 ºC for 4 hours, motility values of sperm at all extenders were good motile (60-79%) and there were slightly high in semen diluted with TCSL 3% (79.49 ± 9.8 %) compared with TCSL 4% (77.03±10.7 %) and (TCEY 77.80 ± 11.1 %). There were no significant differences between motility rates in all diluents (table 3). There was negative relationship between overall mean of sperm motility and interval of cooling (5 ºC). Overall mean of sperm motility was reduced after 4 hours (78.11 ± 10.6) of cooling compare with 1 hour (85.11 ± 9.8).

Table 2: Mean of sperm motility of Nubian bucks semen diluted with TCEY, TCSL 3% and TCSL 4% extenders after the storage at 5 ºC for 1 hour.

Diluents	No of samples (n)	Mean ± SD %	Sig
TCSL 3%	59	85.85 ± 10.3	Ns
TCSL 4%	59	85.25 ± 9.2	Ns
TCEY	59	84.24 ± 10.1	Ns
Overall mean	177	85.11 ± 9.8	Ns

Ns=non significant.

Table 3: Mean of sperm motility of Nubian bucks semen diluted with TCEY, TCSL 3% and TCSL 4% extenders after the storage at 5 ºC for 4 hours.

Diluents	No of samples (n)	Mean ± SD %	Sig
TCSL 3%	59	79.49 ± 9.8	Ns
TCSL 4%	59	77.03±10.7	Ns
TCEY	59	77.80 ± 11.1	Ns
Overall mean	177	78.11 ± 10.6	Ns

Ns=non significant.

Mean of sperm motility (%) in post-thaw semen diluted with TCEY, TCSL 3% and TCSL 4% extenders after cryopreservation at -196 ºC for 30 days presented in table 4. The higher percentage of sperm motility was recorded in semen diluted with TCSL 3% (66.36 ± 10.3) followed by that semen diluted by TCEY (64.24 ± 12.3). While the lower percentages sperm motility was reported in semen diluted with TCSL 4% (60.34 ±11.9). There was significant deference between motility in semen extended with TCSL 3% and TCSL 4% ($p<0.05$).

Table 4: Mean of sperm motility of Nubian bucks semen diluted with TCEY, TCSL 3% and TCSL 4% extenders after freezing at -196 ºC for 30 days.

Diluents	No of samples (n)	Mean ± SD %	Sig
TCSL 3%	59	66.36 ± 10.3[a]	*
TCSL 4%	59	60.34 ±11.9 [b]	*
TCEY	59	64.24 ± 12.3 [ab]	*
Overall mean	177	63.64 ± 11.8	*

* = The mean deference is significant at ($p<0.05$).

a,b: The mean values having different letters within the same column showed significant differences ($P<0.05$).

Discussion

This study compared the effect of different concentrations of soybean lecithin and egg yolk containing tris-based extender on post thawing sperm motility.

Most semen extenders contain egg yolk and skim milk as a source of lipoprotein that protect sperm cells from cold shock and other damage (Moussa *et al*, 2002; Amirat, *et al*, 2004). However, the possible disadvantages of using egg yolk, including its potential to be a cause of allergic reactions, the risk of bacterial contamination and its variable effect on semen have been reported (Bousseau *et al*., 1998; Aries et al., 2003; Amirat, *et al*, 2004; Fukui *et al*., 2008). Extenders containing soybean lecithin could be an alternative to the conventional extenders that include egg yolk (Forouzanfar *et al.*, 2010).

In this study, percentages of sperm motility in the semen stored at 5ºC for 1 hour, diluted with TCEY, TCSL 3% and TCSL 4% extenders, were very good (80-100 %) and there were no significant differences in motility rates between all diluents. This finding is agreement with Yotov (2015) who recorded that type of extender did not influence significant on the sperm motility (at 0-4 ºC) between 0 and 3 hours in semen of Bulgarian bucks.

In the present results, all extenders (TCEY, TCSL 3% and 4%) had no significant difference ($P>0.05$) on individual motility (ranged between 77-79%) after cooling at 5ºC for 4 hours. Whereas, the results showed that TCSL 3% (79.49 ± 9.8%) had higher sperm motility than TCEY (77.80 ± 11.1 %) and TCSL 4% (77.03 ± 10.7) extenders. This observation is consisted with Salmin *et al*. (2012) who registered 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. Also, Khalifa and Abdel-Hafez (2014) who recorded that lowest sperm characteristics in

semen diluted with 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 involves endotoxins that decrease the vaibility of sperm (Manjunath, 2012).

Yotov (2015) who showed that time of storage at 0-4 ºC had not significant effect on motility values between 0 and 3 hours. This observation is similar to that finding in current experiment. Overall mean of sperm motility stored at 5ºC was slightly reduced after 4 hours (78.11 ± 10.6) of cooling compare with 1 hour (85.11 ± 9.8).

In this study, progressive motility in post-thaw semen extended with TCSL 3% and stored at -196 ºC for 30 days was higher (66.36 ± 10.3%) compared with that diluted with TCSL 4% (60.34 ±11.9 %) and TCEY (64.24 ± 12.3 %). There was significant deference ($p<0.05$) between sperm motility in semen extended with TCSL 3% and TCSL 4%. These results are in accordance with Khalifa and Abdel-Hafez (2014) who reported that frozen sperm in semen diluted by TCSL was higher post–thawing motility and viability rate (57.14% and 51.42%) than sperm cryopreserved in TCEY media (55.35% and 49.07%) in Rahmani rams. Also, Emamverdi *et al.* (2013) indicated that soybean lecithin extender improved motility, plasma membrane acrosome integrity; apoptosis status and mitochondrial activity after thawing ram spermatozoa. Moreover, Singh *et al.* (2013) recorded that newly developed lecithin-tris extender could maintain comparable semen quality and improve the freezability as compared to egg yolk-tris extender.

Conversely, these findings are dissimilar with Ustuner *et al*. (2014) who noted that post-thaw sperm motility was significantly greater in semen containing egg yolk as compared to different concentrations (1%, 3% and 6%) of soybean lecithin extender. These authors indicated that there were no significant differences between groups of soybean lecithin in terms of post-thaw motility ($P>0.05$).Also, De Leeuw et al. (1993) has found that bull sperm survive freezing more effectively in egg yolk-containing diluents than in soybean lecithin.

In conclusion, dilution with extender containing 3% soybean lecithin provided the best individual motility of frozen sperm in semen of the buck.

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تاثير مخفف ليسيثين فول الصويا علي حركة النطف في السائل المنوي المبرد والمجمد للتيوس النوبية

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المستخلص

اجريت هذه الدراسة للتحقق من تاثير تراكيز مختلفة من مخفف ليسيثين فول الصويا علي حركة النطف في السائل المنوي المبرد والمجمد للتيوس النوبية .جمعت عدد 59 قذفة من 6 تيوس نوبية معلومة الخصوبة مرة واحدة كل اسبوع لمدة 9 اسابيع باستخدام المهبل الاصطناعي. بعد التقييم الاولي للسائل المنوي تم تقسيمه الي ثلاثة مجموعات متساوية (كل مجموعة تتكون من 59 عينة) و تخفيفه بنسبة 5:1 (السائل منوي: المخفف) باستخدام ثلاثة مخففات تتكون من 2% صفار البيض مضاف له حامض الستريك , 3% ليسيثين فول الصويا ومضاف له حامض الستريك و 4% ليسيثين فول الصويا ومضاف له حامض الستريك. رصدت حركة الحيوانات المنوية بعد فترة موازنة في درجة حرارة 5 مئوية لمدة ساعة و 4 ساعات من التبريد, وبعد 30 يوما من التجميد في درجة حرارة 196- مئوية. اثبتت النتائج بأن ليس هنالك اختلافات معنوية مابين معدلات حركة الحيونات المنوية في التراكيز المختلفة لمخفف ليسيثين فول الصويا وصفار البيض للسائل المنوي المبرد في درجة 5 مئوية لمدة ساعة (100%-80) و 4 ساعات (% 79-60). المتوسط الكلي لمعدلات حركة النطف اقل بعد التبريد لمدة 4 ساعات مقارنة بالتبريد لمدة ساعة. حركة الحيوانات المنوية في السائل المنوي المخفف باستخدام 3% ليسيثين فول الصويا وبعد التجميد في درجة حرارة 196- مئوية لمدة 30 يوم كانت اعلي معنويا (10.3% ± 66.36) من الحركة في مخفف 4% ليسيثين فول الصويا (% 11.9± 60.34). كما ان ليس هنالك اختلافات معنوية مابين معدلات الحركة في السائل المجمد والذي تم تخفيفه بصفار البيض وليسيثين فول الصويا. نخلص من هذه الدراسة بأن استخدام 3% ليسيثين فول الصويا يعطي حركة افضل للحيوانات المنوية المجمدة مقارنة ب 4% ليسيثين فول الصويا وصفار البيض.