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PHYSIOLOGICAL RESPONSE OF ZARAIBI BILLY GOATS SPERMATOZOA TO SOYBEAN LECITHIN ADDITION AS AN ALTERNATIVE TO EGG YOLK VARIETIES IN SEMEN EXTENDERS

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SUMMARY

The present study was designed to determine the influence of cryopreservation action of extenders containing plant (soybean lecithin, SBL) or animal (domestic birds' egg yolk) origins on freezability and fertility of billy goat spermatozoa. Thus, semen ejaculates (thirty-six ejaculates) were collected, evaluated and extended in Tris-citric acid with either SBL or egg yolk (EY) derived from hen (HEY), duck (DEY), goose (GEY), quail (QEY), turkey (TEY) and pigeon (PEY). The diluted semen was packaged in 0.5 ml French straws, equilibrated for 3 hours at 5°C then frozen in liquid nitrogen (at -196°C). The thawing of straws was attained at 37°C for 60 sec. Percentages of progressive sperm motility, viability of sperms and injury of acrosome were evaluated at post - dilution (PD), post - equilibrium (PE) and post - thawing (PT). Fertility rate was carried out with 24 nanny goats divided into two groups (N=12/ group) and inseminated with the best cryosurvival extenders. The results revealed a non-significant difference between plant and bird origin extenders during PD and PE stages. In contrast, the PT was higher (P<0.05) in motility and viability with SBL and DEY extenders than other EY species however, the same trend did not occur between SBL and DEY extenders. The values of acrosomal abnormality was lower (P<0.05) in SBL and DEY extenders than other EY extenders. The nanny goats inseminated by SBL and DEY extenders had a conception rate of 58.33 % and 50.00 %, respectively.

These results indicate that the spermatozoa that were preserved in either SBL or DEY extenders could protect the sperm cells during PD, PE, PT and results in the best fertility rate. Furthermore, the present study demonstrates that SBL may represent a suitable alternative to egg yolk for semen cryopreservation in livestock species.

Keywords: Billy goats' semen, plant and animal cryopreservation origins, fertility rate

INTRODUCTION

Egg yolk (EY) of different domestic birds has been shown to have a beneficial effect on sperm cryopreservation as a protector for the sperm plasma membrane and acrosome against temperature-related injury. The EY Phospholipids, polyunsaturated fatty acids (PUFAs) and cholesterol could prevent cold shock, osmotic stress, ice crystal formation or oxidative damage and loss of sperm fertility (Hu *et al.*, 2010). On the contrary, the EY is representing a potential risk of contamination of artificial insemination (AI) by bacteria and mycoplasma (Rosato & Iaffaldano, 2013). Actually, EY has some problems. Van Reenen and Griffin (1994) reported that the deleterious effects of the aromatic amino acid oxidation (AAAO) is further exacerbated by components of egg yolk which is used as a cryoprotectant for the freezing of mammalian semen. During sperm metabolism, AAAO enzyme becomes active following the death of sperm and increasing hydrogen peroxide (H_2O_2) , a metabolic product of the enzyme, is known to be toxic to the remaining live sperm (Saraswat *et al.*, 2014). Furthermore, EY contains micro elements that result in an increase of extender's viscosity, inhibition of sperm respiration and diminish sperm motility. For the same phenomenon, Salmani *et al.* (2013) stated that the dilution of goat semen using a diluents containing egg yolk can have a detrimental effect on the quality of the sperm cells during extension, freezing and thawing due to the presence of egg yolk - coagulating enzyme (EYCE) and bulbourethral gland secretion glycoprotein (BUSgp60). In this context, reports suggested that EY from avian species have different combinations of PUFAs, phospholipids and cholesterol, which resulted in different cryopreservation effects on the sperm membrane (Kulaksiz, *et al.*, 2010). On the other hand, egg yolk (EY) could attain the greatest spermatozoa protection, but in recent years there was an increasing demands for alternative egg yolk in extenders due to variability composition, risk of microbial contamination and presence of steroid hormones. Hence, the alternative to EY- based extender might be soybean lecithin-based (SBL) extender (Najafi *et al.*, 2014). The extender contains soybean lecithin (SBL) as an egg yolk substitute has become available for the cryoprotection of the animal spermatozoa. This evidence suggests that SBL contains a major phospholipid that plays an important role in building sperm cell membrane and supplies sperm freezability (Emamverdi *et al.* (2013). Finding of Khalifa and Abdel-Hafez (2014) using extenders containing

soybean lecithin at 3.5% cryopreserved the ram sperm and exceeded the conventional hen egg yolk-based in fertility. Therefore, the following experiment was intended to study the effect of either soybean lecithin or egg yolk from different avian domestic species on sperm characteristics of Zaraibi billy goat. In addition, fertility rate was carried out with the best cryoprotective extenders.

MATERIALS AND METHODS

This experiment was carried out at El-Serw Experimental Farm, Damietta governorate belonging to Animal Production Research Institute (APRI), Ministry of Agriculture, Egypt. The duration of the trial was between June and November (2013).

Semen collection

Thirty - six semen ejaculates were collected from three adult billy goats (as two ejaculates / week / up to six weeks) using an artificial vagina method. Each ejaculate was observed visually, semen samples that were having a motility > 75%, viability > 85% and integrity acrosome > 85% were utilized in the extension protocol.

Preparation of diluents and freeze - thawing process

The soybean lecithin (SBL) was supplemented at 2.5 % using Tris - citric glucose (TCG) buffer. However, 2.5% ml egg yolk (EY) of avian specie birds such as hen, duck, goose, quail, turkey and pigeon was supplemented in TCG buffer to perform HEY, DEY, GEY, QEY, TEY and PEY diluents applying the same rate, respectively. The extension rate designed to be used is one - step method at room temperature. The extender was regulated by Evans and Maxwell (1987) and either plant or birds origin extenders as demonstrated in Table 1.

Table 1. The composition of extenders with either plant or bird origins for Zaraibi billy goats semen

Ingredients	*SBL	Egg yolk of avian species					
		HEY	DEY	GEY	QEY	TEY	PEY
Tris (g)	3.786	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	0. 625
Citric acid (g)	2.172	2.172	2.172	2.172	2.172	2.172	2.172
Soybean lecithin,%	2.500	-	-	-	-	-	-
Hen egg yolk, %	-	2.500	-	-	-	-	-
Duck egg yolk , %	-	-	2.500	-	-	-	-
Goose egg yolk, %	-	-	-	2.500	-	-	-
Quail egg yolk, %	-	-	-	-	2.500	-	-
Turkey egg yolk, %	-	-	-	-	-	2.500	-
Pigeon egg yolk, %	-	-	-	-	-	-	2.500
Glycerol (v/v)	5.000	5.000	5.000	5.000	5.000	5.000	5.000
Penicillin (IU/ml)	100.000	100.000	100.000	100.000	100.000	100.000	100.000
Streptomycin (mg /ml)	100.000	100.000	100.000	100.000	100.000	100.000	100.000
Distilled water to	100 ml	100 ml	100 ml	100 ml	100 ml	100 ml	100 ml

SBL: soybean lecithin, HEY: hen egg yolk, DEY: duck egg yolk, GEY: goose egg yolk, QEY: quail egg yolk, TEY: turkey egg yolk and PEY: pigeon egg yolk.
*SBL: Sigma Chemical Co. (St. Louis, MO, USA).

After dilution, the sperm was drawn into 0.50 ml French straws and sealed with polyvinyl chloride powder; each straw was adjusted to contain 350 - 400x10^6 motile sperm. Straws were equilibrated at 5°C for 3 hours and after equilibration period; the refrigerated straws were suspended on metal rack in foam box (35×17×20 cm) above the surface of the liquid nitrogen vapor for 15 minutes by 5 cm. Then, frozen straws were plunged into the liquid nitrogen at -196°C for 8 min. Subsequently, frozen straws were collected in foam box and stored in liquid nitrogen container until thawing steps. After storage for a period of 24 hours, the semen straws were thawed in a water bath at 37°C for 60 sec.

Semen Evaluation

In different stages of cryopreservation technique such as post - dilution (PD), post - equilibration (PE) and post - thawing (PT), the percentages of sperm motility, viability of spermatozoa and acrosomal abnormality were implemented according to Khalifa and Abdel - Hafez (2013).

Fertility test

The fertility performance was managed using twenty - four Zaraibi nanny goats satisfactorily homogeneous in live body weight, age and body conditions. The nanny goats were divided into two groups (N=12/ group) and inseminated by frozen - thawed sperm cryopreserved in either SBL or DEY extenders. The insemination semen straws of either SBL or DEY were collected and extended from the same billy goat to avoid the semen characteristics variance. The insemination protocol started after 12 hours of nanny goat displayed oestrous cycle using two frozen - thawed straws within 12 hours as an interval period between received 1st and 2nd frozen - thawed straws as described by (Khalifa and Abdel -

Hafez, 2014). The pregnancy rate was attained after doe passed an oestrous cycle without returning to heat. The fertility performance was calculated as the pregnancy rate (number of does conceived/ number of does inseminated), percentage of does kidded (number of does kidded / number of does conceived), twins rate (number of does kidded twins / total number of does kidded), triplet rate (number of does kidded triplet / total number of does kidded). Sexing kids % (male: female) = number of born kids in each sex/Total number of born kids and litter size = number of total born kids / number of does kidded.

Statistical analysis

The statistical analysis was performed using one-way analysis of variance of IBM SPSS (2013) version 22.0. The data were represented as mean ± SE and a P value of < 0.05 considered as statistically significant using Duncan multiple range test of the same program of SPSS.

The following model was used for analysis of variance:

$$Y_{mdqtk} = \mu + E_m + S_d + S_q + S_t + e_{mdqtk}$$

Y_{mdqtk}= observation, μ= overall mean, E_m= extender media (m=7), S_d= sperm parameters post - dilution (d=36), S_q= sperm parameters post - equilibration (q=36), S_t= sperm parameters post - thawing (t= 36), e_{mdqtk}= experimental errors.

RESULTS AND DISCUSSION

The sperm motility, viability and acrosomal abnormality at the steps of cryopreservation are shown in (Figures 1, 2 and 3, respectively). The results indicate that there were differences ($P>0.05$) between SBL, HEY, DEY, GEY, QEY, TEY and PEY extenders on the measured physiological sperm parameters during PD and PE. Actually, using extenders contain either DEY or SBL resulted in higher ($P<0.05$) physiological sperm characteristics (motility, viability and acrosomal abnormality) than HEY, GEY, QEY, TEY and PEY extenders. Intriguingly, the recovery rate of the total frozen-thawed motility, viable sperm and integrity acrosome were higher ($P<0.05$) using SBL than avian species egg yolks extender. Theoretically, the degree of sperm viability deterioration depends on several factors, e.g., the nature and concentration of cryoprotectants and component of the diluents used for freezing and thawing (Emamverdi *et al.*, 2013). The current study corroborative SBL extender could be attained post-thaw progressive sperm motility (53.93%), post-thaw viability sperm (69.58%) and the lowest post-thaw acrosomal abnormality (37.08%). Similar results were found by de-Paz *et al.* (2010) who stated that soybean lecithin is an efficient ingredient for the protection of the animal spermatozoa from cold shock and freezing process. Furthermore, these authors stated that semen extender with soybean lecithin at 3.5% showed a higher concentration of phospholipids which optimized to preserve the individual sperm motility, viability and intact acrosomes. In addition, Yotov (2015) concluded that extenders containing soybean lecithin at 1.5% (w/v) provided the best motility and viability of the chilled-stored goats' spermatozoa. In recent decades, soybean lecithin was demonstrated to be safer than egg yolk in terms of biosecurity and it is used for sperm cryopreservation in ram (Najafi *et al.*, 2014), goats (Chelucci *et al.*, 2015). It is known that extender contained soybean lecithin could change mitochondrial inner membrane function by increasing outer membrane permeability which releases soluble intermembrane proteins to activate metabolic pathways. Similar trend was observed by Del valle *et al.* (2012) who established that lecithin protect sperm cells; activate mitochondrial functionality and lengthy sperm motility. The lecithin was reported to have neither cytotoxic effect nor negative effect on sperm motility, whereas lysophosphatidylcholine and other fatty acids in egg yolk have inhibitory effects on sperm motility and induce acrosomal damage (Alvarez - Rodríguez *et al.*, 2013). Likewise, Marisa Bezjian *et al.* (2013) indicated that sperm in soy - based Bioxcell® displayed more viability and intact acrosomes than sperm in Tris - egg yolk. The present results clearly demonstrate that DEY extender has the best cryoprotective action after frozen - thawed in motility, viability and the lowest acrosomal abnormality; it achieved 52.78, 68.42 and 37.67 % compared to the other avian egg yolk, respectively. In the present study, the percentages of thawing sperm characteristics in DEY extender provided excellent cryoprotective action for sperm compared to other egg yolk birds. These findings are supported by Kulaksiz *et al.* (2010) who recommended that the basic components of duck egg yolk had more ratios of fatty acids, phospholipids classes, monounsaturated fatty acid and phosphotidylionsitol than egg yolk obtained from avian specie birds. Finding of, Gholami *et al.* (2012) demonstrated that duck egg yolk might be superior to chicken egg yolk for cryopreservation of ram spermatozoa in Tris-citric yolk extender. On the other hand, Waheed *et al.* (2012) stated an improvement of sperm parameters when the buffalo semen frozen in extenders containing duck egg yolk. Thus, the improvement or decline in post - thawing quality of mammalian spermatozoa with egg yolk of different avian species in the freezing extender may be attributed to the differences in the biochemical composition of yolk (El-Sharawy *et al.*, 2012). The preserving action of birds' egg yolk depends on cholesterol and lecithin that prevented the ice crystal formation, protected sperm plasma membranes during cold shock and freeze-thawing process. This finding was coincided with Hu *et al.* (2010) who assured that cholesterol modulates fluidity of sperm membranes by interacting with the fatty acyl chains of the phospholipids, maintains phospholipids in a random and lamellar arrangement as temperature decreases. Duck egg yolk cholesterol could reduce the

sensitivity of sperm membranes to cooling damage by eliminating or at least minimizing the lateral phase separation of the lipids. Subsequently, this observation is harmonious with Szafner *et al.* (2012) who suggested that cholesterol concentration (mg/g yolk) in yolks among different avian species such as chicken, ducks, turkey, quail and goose were 15.4, 18.8, 16.7, 7.78 and 17.2, respectively. Furthermore, Wu *et al.* (2012) indicated that average lecithin concentration in hen and duck egg yolk was 2.94 and 3.81%, respectively. On the other hand, it is well documented that duck egg yolk consists 0.52% of Docosahexaenoic acid (DHA) which higher than chicken (0.28%) and quail (0.39%) which appeared to have an important role to active ingredient for the cryoprotection morphological spermatozoa (Nasiri *et al.* 2012). The DHA has critical role in maintaining fluidity of sperm plasma membrane during cryopreservation which in turn prevents lipid peroxidation that causes sperm plasma membrane damage. Similarly, Kaeoket and Chanapiwat (2013) observed that DHA in duck egg yolk has a similar manner of lactose - egg - yolk (LEY) in based freezing extender that improves the quality of frozen semen thus; DHA of duck egg yolk could achieve improvement in frozen-thawed semen qualities instead of chicken egg yolk.

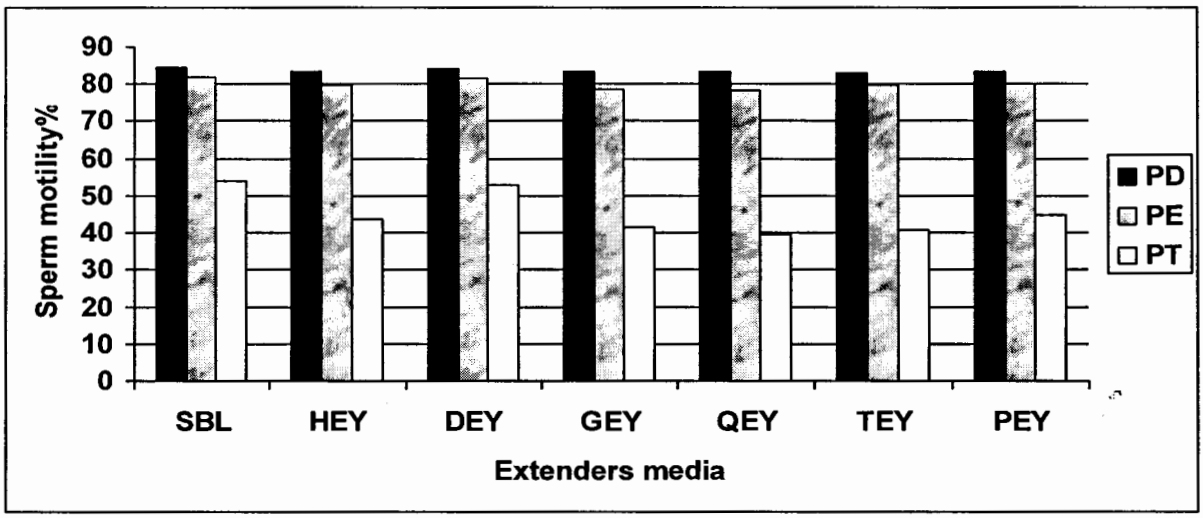

Fig. 1. Sperm motility in billy goats semen during cryopreservation stages using soybean lecithin and different avian species of egg yolk.

SBL: soybean lecithin, HEY: hen egg yolk, DEY: duck egg yolk, GEY: goose egg yolk, QEY: quail egg yolk, TEY: turkey egg yolk and PEY: pigeon egg yolk.

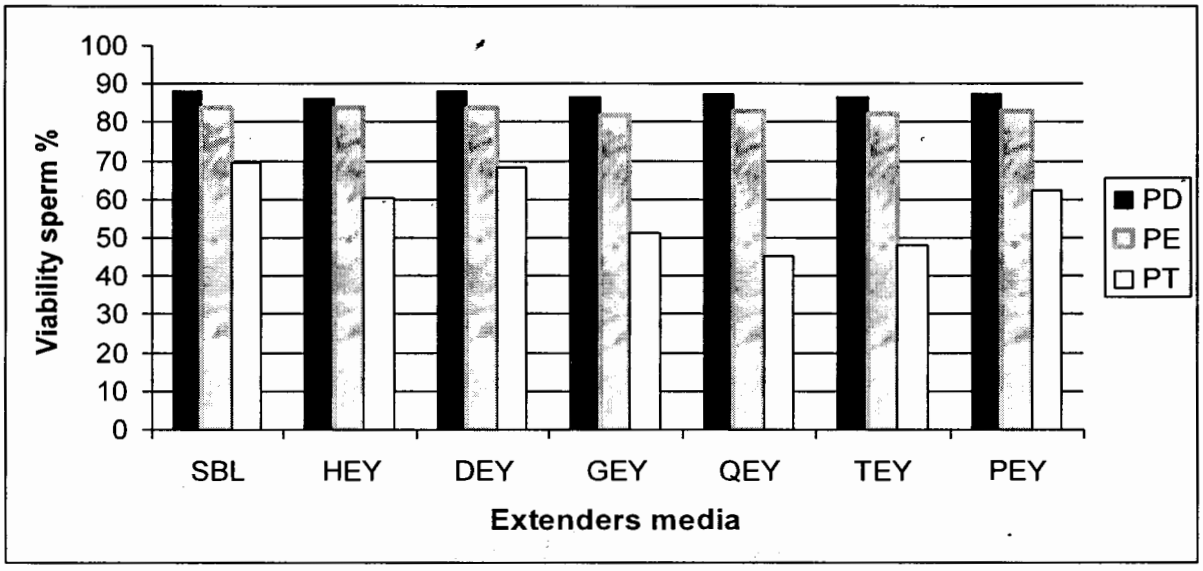

Fig. 2.Viability sperm in billy goats semen during cryopreservation stages using soybean lecithin and different avian species of egg yolk.

SBL: soybean lecithin, HEY: hen egg yolk, DEY: duck egg yolk, GEY: goose egg yolk, QEY: quail egg yolk, TEY: turkey egg yolk and PEY: pigeon egg yolk.

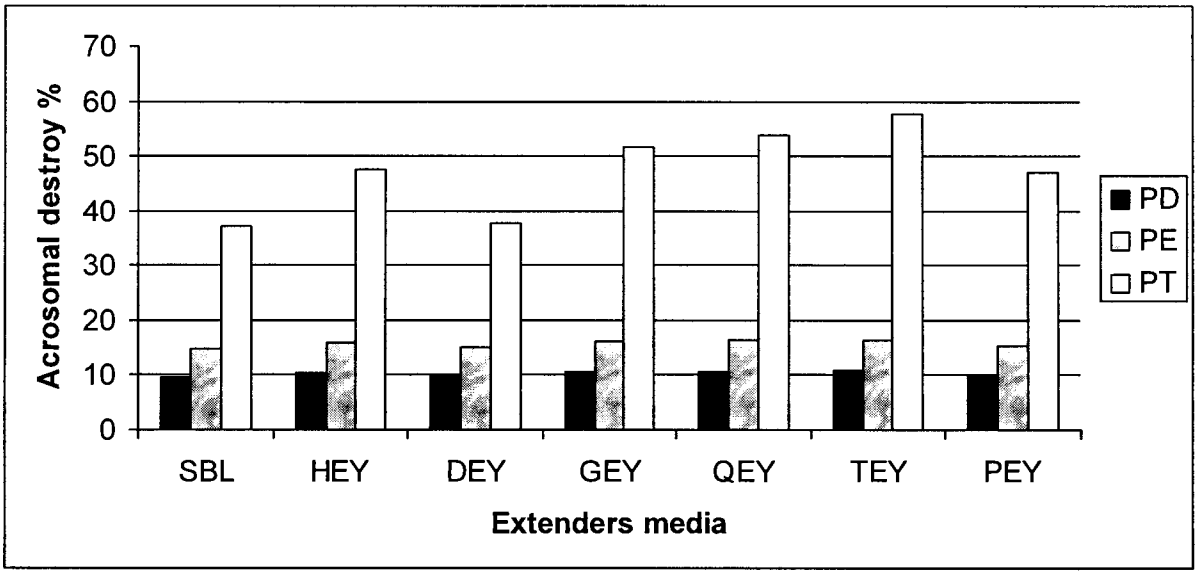

Fig. 3. Acrosomal destroy in billy goats semen during cryopreservation stages using soybean lecithin and different avian species of egg yolk

SBL: soybean lecithin, HEY: hen egg yolk, DEY: duck egg yolk, GEY: goose egg yolk, QEY: quail egg yolk, TEY: turkey egg yolk and PEY: pigeon egg yolk.

Fertility rate

Results in Table (2) show the influence of either SBL or DEY as semen extenders on the fertility rate parameters. The pregnancy rate was higher for spermatozoa extended by SBL (58.33 %) dilute than that in DEY (50.00 %) dilute. This discrepancy of pregnancy rate with DEY extender may be due to an increase of aromatic amino acid oxidase (AAAO) enzyme that is released from dead spermatozoa which increased reactive oxygen species (ROS) and reduced PUFAs in extender. Similar conclusions have come from Agarwal *et al.* (2005) who found that PUFAs in egg yolk decreased dramatically when the dead spermatozoa released AAAO enzyme and ROS attacked survival spermatozoa which reflected on decreasing progressive motility, increasing mid - piece abnormalities and inhibited sperm oocyte fusion. On the other hand, bacterial contamination which is present in egg yolk can release endotoxins that reduce the fertilization capacity of sperm and ATP synthesis. Similar results have been obtained by Sukho *et al.* (2013) who defined that growth of microbes egg yolk inhibition glycolysis cycle which forces the sperm mitochondria to work at a higher level to generate energy lead to diminish pyruvic acid in seminal plasma appeared to be a marked short - term of viable sperm too long . In the present study, the best fertility rate was obtained with SBL spermatozoa compared to those spermatozoa in the DEY extender. This is in accordance with a previous study by Emamverdi *et al.* (2013) who reported that SBL has a low density lipoprotein, conserve phospholipids membrane during cryopreservation which is reflected on sperm motility and fertility. Independently of extender used SBL increased viscosity of the extender, which has been complicated motility and cell membrane integrity (Najafi *et al.*, 2014). The highest pregnancy rate occurred with SBL was related to possibly displacement cardiolipin (kind of diphosphatidylglycerol lipid activated mitochondria enzymes) to mitochondrial membrane that reflected a high motility value after thawing (Pen˜a *et al.*, 2009). Likewise, the mitochondria may play a tender role in the energy maintenance for sperm motility one of the major parameters related to fertility. These observations were explained with Shahani *et al.* (2012) who suggested that lecithin provides better protection of mitochondria than animal originated, by playing a protective role for mitochondria during cryopreservation due to its low viscosity and improves the kinematics of mitochondria. According to this information, Del valle *et al.* (2012) reported that effectiveness of soybean lecithin to protect spermatozoa against cryoinjury is because it increased proportions of viable spermatozoa post - thawed that reflected on sperm motility and fertility. Otherwise, Chelucci *et al.* (2015) demonstrated that lecithin can be considered as a suitable alternative to egg yolk in semen cryopreservation, because it ensures higher fertilization rates and a better protection from membrane damage by cold shock. Also, the last authors showed that lecithin and egg yolk extenders obtained 66.2 and 38.7 % in vitro fertilization test, respectively. On the other hand, Khalifa and Abdel - Hafez, (2014) concluded that SBL diluent could ameliorate the quality of sperm cryopreservation and heighten fertility rate of ram spermatozoa (63.64 %) compared to extender contained hen egg yolk (54.55 %).

Table 2. The fertility rate of Zaraibi billy goats frozen using either SBL or DEY extenders

Item	Semen extenders	
	SBL	DEY
Number of does inseminated	12.00	12.00
Number of does conceived	7.00	6.00
Pregnant rate, %	58.33	50.00
Number of does kidded	7.00	6.00
Does Kidded, %	100	100
Total number of kids at birth	20.00	15.00
Number of does kidded twins	1.00	3.00
Twins rate, %	14.29	50.00
Number of does kidded triplet	6.00	3.00
Triplet rate, %	85.71	50.00
Number of female kids	10.00	6.00
Female rate, %	50.00	40.00
Number of male kids	10.00	9.00
Male rate, %	50.00	60.00
Litter size	2.88	2.50

CONCLUSION

Based on the results of this study, semen extenders containing either a source of plant origin as soybean lecithin or bird origin as duck egg yolk remarkably cryoprotected Zaraibi billy goat spermatozoa examined post-diluent, post - equilibration and post - thawing compared to other different egg yolk of other birds. Additionally, SBL extender as cryosurvival materials is considered a new strategy to protect spermatozoa and also improve sperm fertility compared to DEY extender.

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الإستجابة الفسيولوجية للحيوانات المنوية لذكور الماعز لليسيثين فول الصويا كبديل لأصناف صفارالبيض فى مخففات السائل المنوى

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أجريت هذه الدراسة لتقييم تأثير الفعل الواقى للواقيات ذات الأصول النباتية أو الحيوانية (مثل صفار البيض للطيور المنزلية) على قدرتها التجميدية والإخصابية للحيوانات المنوية لذكور الماعز. ومن أجل هذا الغرض: تم تجميع 36 قذفة من السائل المنوى من ذكور زرايبى بإستخدام المهبل الصناعى وخففت القذفات بمعدل 1 مل سائل منوى: 4مل مخفف فى مخفف الترس الذى يحتوى على واقيات من أصل نباتى أو داجنى. والواقيات من أصل نباتى كانت صويا ليسيثين (SBL). بينما واقيات من أصل داجنى كانت صفار البيض وتم الحصول عليه من الدواجن (HEY)، البط (DEY) ، الأوز(GEY)، السمان (QEY)، الرومى (TEY)، الحمام (PEY) وعبئ السائل المنوى بعد التخفيف فى القشة الفرنساوى 5و0 مل وعرضت لفترة الإتزان لمدة 3ساعات على درجة 5 منوية وجمدت فى النتروجين السائل على درجة -196 منوية والإسالة على درجة 37 درجة منوية لمدة 60ثانية. وتم تقييم الحركة التقدمية، والحيوية ، والأكروسوم المصاب للحيوانات المنوية بعد فترة التخفيف (PE) ،الأتزان (PE) ، والإسالة (PT). كما اجرى التلقيح الصناعى بإستخدام 24 عنزة قسمت الى 12 عنزة بكل مجموعة لقحت المجموعة الأولى بمخفف SBL والمجموعة الثانية بمخفف DEY. والنتائج اظهرت عدم وجود فروق معنوية بين انواع المخففات بعد PE، PE. بينما ظهرت الفروق المعنوية بعد الإسالة لصالح مخفف SBL،DEY مقارنة بباقى المخففات الأخرى. ومن ناحية أخرى لم تظهر أى فروق معنوية بين مخفف SBL،DEY بعد الإسالة. وكان معدل الحمل لكلا من مخفف SBLومخفف DEY 33و58، 00و50 % على التوالى. وتوصى التجربة أن استخدام مخفف صويا ليسيثين ومخفف صفار بيض البط لها القدرة على حماية وحفظ الحيوانات المنوية دون فقد لإعاشة الحيوانات المنوية وأيضا مخفف صفار بيض البط افضل من صفارالبيض للسلالات الطيور الأخرى لحفظ وجودة السائل المنوى لذكور الماعز بعد التخفيف ، بعد التوزان، بعد الإسالة.