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EFFECT OF SOME EXTENDERS ON CHILLED RABBIT SEMEN STORED AT 5°C FOR 48 HOURS

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ABSTRACT: With the global demand for rabbit meat steadily increasing, commercial rabbit breeding has become more dependent on artificial insemination (AI) rather than traditional natural mating. The study aimed to compare coconut milk (CME) as a novel extender for rabbit semen with Tris Yolk-Glucose (TYG) and Lactose Yolk-Citrate (LYC), to evaluate the *in vitro* presrevability of rabbit spermatozoa stored at 5°C for 48 hr. Twelve New Zealand White (NZW) rabbit bucks were used to collect semen. Semen was pooled, partioned to three portions and then diluted with one of three different extenders; Tris Yolk-Glucose, Lactose Yolk-Citrate or Coconut Milk. The dilution rate was 1:4 (volume: volume) and stored at 5°C for 48 hr., the experiment lasted for 8 weeks. Sperm motility, dead sperms, abnormal sperms and acrosome loss percentage were evaluated in chilled semen at 0, 24 and 48hr. of storage. The results showed that sperm motility was the highest at both 24 and 48hr. storage with TYG compared with LYC and CME without significant statistical differences between them. There were significant differences between extenders at 24hr. of storage with respect to the percentage of dead spermatozoa; the lowest percentages were recorded in semen stored with TYG. The CME extender gave significantly the highest values of abnormal sperms (29.16%) at 48hr. than TYG and LYC (23.16 and 26.33%, respectively). At 48hr. of storage, higher acrosome loss values were found with CME (27%) compared with TYG and LYC extenders (21.16 and 21.50%), respectively. The lowest values for the acrosome loss (21.16%), during the 48 hr., storage were obtained with TYG.

Key words: Extenders, chilled rabbit semen.

INTRODUCTION

The farming systems for commercial rabbit meat production are based almost on AI programmes, which are currently performed with fresh diluted semen within 6-12 hr., from semen collection, and are mostly limited to does in farms where bucks are kept (Daniel and Renard, 2010). It is technically easier to chill than to freeze semen, the major problem of frozen semen is the low sperms survival after freezing, in addition, freezing technique is more expensive and is time consumer than the chilling technique (Di lorio *et al.,* 2014). Maintaining the fertilizing capacity of stored rabbit semen for longer time than 48 to 72 hr., remains an important target to reach for rabbit industry. Therefore, attempts are needed to improve semen extenders and storage conditions to prolong the time during which stored semen can maintain its functional status.

The composition of extenders play a key role in long term storage of rabbit semen, because they provide the nutrients needed for the metabolic maintenance of sperm cells, control pH and osmotic pressure of the medium. Different extenders have been used over time to protect the sperm during processing and storage in chilled and frozen semen. At present, Tris Yolk-Glucose (TYG) based extender is generally the most applied for liquid rabbit semen storage. As because of animal protein content, TYG may be considered of high contamination risk (Kasimanickam *et al.,* 2011). While, coconut milk (CME) has been tested as

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an extender in some animal species semen such as goats, West African dwarf buck (Sule *et al.,* 2007), boar (Bottini-Luzardo *et al.,* 2013) and cock (Ogbu *et al.,* 2014). Studies on its inclusion in rabbit semen are limited

Coconut milk (the liquid extract from fresh coconut meat or kernel) has been shown to contain several nutrients compounds and minerals, vitamins, enzymes (Ogbu *et al.,* 2014), antiviral, antibacterial, antifungal, antiparasitic, and antioxidant properties, in addition to other medicinal and health benefits (Manisha and Shyamapada, 2011).The use of coconut water-based extender has been proposed also, as an alternative semen extender that is non-toxic, buffering, low cost, practical, and effective (Cardoso *et al.,* 2005). To the best of our knowledge CME has never been used as an extender for storage of rabbit semen. Therefore, this work was designed to study the potential role of CME, in comparison with TYG and LYC extenders to assess the *in vitro* preservability of rabbit sperms at 5°C.

MATERIALS AND METHODS

The present study was carried out in the Rabbit Farm, Department of Animal Production, Faculty of Agriculture, Zagazig University, Zagazig, Egypt, during the period from October, 2015 to February, 2016.

Experimental Animals and Management

Twelve New Zealand White (NZW) rabbit bucks 6 months age and 3.0-3.20 kg body weight were used in the present study. Animals were healthy and clinically free of external and internal parasites. The animals were raised in flat deck batteries with universal specification, accommodated with feeders for providing animals pelleted ration and automatic fresh water drinkers and were kept under the same managerial, hygienic and environmental conditions. All animals were fed and watered *ad libitum*. All batteries were located in naturally ventilated windowed house. A commercial balanced diet (16.3% crude protein, 13.2% crude fiber and 2600 k/cal digestible energy /kg) was used in this experiment.

Preparation of Extenders

Coconut milk (CME)

The preparation of coconut milk extender followed a simple but aseptic procedure. The meat of freshly harvested coconut (*Cocos nucifera*) was thoroughly blended and collected in a 250 ml conical flask. The water from the coconut was added to the blend and the mixture allowed standing for about 1 hr. Thereafter, the mixture was wrapped in a heat sterilized white cloth and tightly squeezed to express the milk. The milk was filtered through sterilized white clothes thrice to get rid of all residues and the liquid was collected in a sterilized flask. The recovered milk was stored in a refrigerator at 4°C until use (Ogbu *et al.,* 2014).

Tris Yolk-Glucose (TYG) consisted of 0.04 g Citric acid anhydrous, 1.25 g Glucose and 3.208 g Tris (Tris (Hydoxymethyl) amino methane, Aldrich Chemical Co. Lt 64.) and 10 ml Egg yolk. Distilled water was added to these components to have 100 ml final volume.

Lactose Yolk-Citrate (LYC) consisted of 2.9 g Sodium citrate dehydrate, 0.04 Citric acid anhydrous, 1.25 g Lactose, and 10 ml Egg yolk. A 500 I.U. ml penicillin + 500 µg/ml Streptomycin sulphate were added to each extender.

Experimental Design and Procedures

Semen collection

Semen was collected from bucks twice a week throughout the experimental period, by means of an artificial vagina between 08.00 – 10.00 AM. The temperature of the inner rubber sleeve of the artificial vagina was adjusted to 43°C. Lubrication of the inner sleeve was performed using white vasline with sterile rode. The pressure in the lumen of the artificial vagina was adjusted to suit male rabbits. After collection ejaculates with < 70% sperm motility were only used. Each ejaculate was kept in a water bath at 37°C till semen is collected from all bucks.

Semen was pooled and then partitioned into three parts each was extended with Tris Yolk-Glucose, Lactose Yolk-Citrate or Coconut Milk. The extension was carried out by adding the appropriate volume of the extender (1:4 semen to extender) slowly to the semen. Extended semen (in tubes) was kept below of water in a water bath at all times to avoid fluctuations in semen temperature.

Chilling of semen at 5°C

The test tubes containing extended semen were placed in a 500 ml beaker containing water at 30°C. A test tube containing water at 30°C with a thermometer was also placed in the beaker in order to facilitate periodic checking of the temperature during the cooling period. The beakers were then placed in a refrigerator and then gradually cooled till their temperature reached 5°C during a period of 2 hr. At 0, 24 and 48 hours of storage semen samples from each semen treatment were taken to be tested for motility (%), dead and abnormal sperms and acrosome loss (%).

Measurements

Sperm motility (%)

Rabbit sperm motility was subjectively assessed by visual examination (Rosato and Iaffaldano, 2011). A 10 µml drop of semen was transferred to a clean glass slide, pre-warmed to 37°C, and covered with a coverslip. The mounted slides were then observed on a warm-plate 400X magnification microscope and percentage sperm motility (%) was assessed.

Dead Spermatozoa (%)

The nigrosin/eosin staining procedure of Hackett and Macpherson (1965) was carried out by dissolving 10.00 g nigrosin and 1.67 g eosin in distilled water up to 100 ml. Seven drops of the stain were placed into a test tube and warmed to 37°C in a water bath before semen dilution. One drop of mixture was removed by pipette and placed at the end of a warm slide. A thin smear was made by drawing the edge of a second slide across mixture. The stained slide was allowed to dry, and then examined under high power magnification (x400) microscope. The live clearly defined and their heads were bright and retractile against the backgrounds spermatozoa. The dead spermatozoa were stained pink, their outlines were not clearly defined and their heads were bright and retractile against the background. The percentage of dead spermatozoa was estimated by counting the number of dead sperms in 100 spermatozoa in different locations in the microscopic field.

Sperm abnormalities (%)

The morphological abnormalities of spermatozoa were determined in the same smears prepared for live/dead ratio using high power magnification (x400) of light microscope. The total abnormalities per hundred spermatozoa were calculated, using the same slide of live/dead spermatozoa. Spermatozoa with abnormal tail and head were evaluated.

Acrosome loss (%)

A drop of the extended semen was smeared on a pre-warmd slide and dried in a current of warm air. The smears were fixed by immersion in dried formal saline (Campbell *et al.,* 1956) in a water bath at 37°C for 15 minutes. The slides were washed in the buffered Giemsa stain for 90 minutes after which it was rainsed briefly in distilled water and dried. One hundred of stained spermatozoa were examined under oil immersion lens at a magnification of (1000x) to find out the percentage of spermatozoa with lost acrosome (Watson, 1975).

Statistical Analysis

Motility of spermatozoa, dead spermatozoa, abnormalities and acrosome loss were compared among the treatments by ANOVA, followed by Duncan's comparison test. Differences were considered statistically significant at ($P< 0.05$). All statistics were calculated by SPSS version 16 (2007).

RESULTS AND DISCUSSION

Results of the effects of using three different extenders (TYG, LYC and CME), during storage at 5°C for 48 hr., on sperm motility, dead and abnormalities spermatozoa and lost acrosome (%) are shown in Tables 1, 2, 3 and 4 and Figs. 1, 2, 3 and 4.

Sperm Motility (%)

Sperm motility is often considered to be the most important parameter when assessing the potential reproductive performance of stored spermatozoa as it is believed to have a high correlation with the *in vivo* fertility of spermatozoa (Castellini, 2008; Hagen *et al.,* 2010).

Sperm motility values were found to be the highest ($P<0.01$) when semen was extended with TYG and stored at 5°C for 24 and 48 hr., compared with other extenders. The worst motility

Table 1. Motility (%) of cooled rabbit's spermatozoa with various extenders during storage at 5°C

Storage time (hour)	Lactose Yolk Citrate (LYC)	Tris Yolk-Glucose (TYG)	Coconut Milk (CME)	Over all mean
Zero	$70.00^{a} \pm 1.80$	$73.00^{a} \pm 0.83$	$69.67^{a} \pm 0.65$	70.83^{A}
24 hr.	$58.33^{b} \pm 2.24$	$66.67^{a} \pm 1.67$	$52.50^{b} \pm 2.48$	59.17^{B}
48 hr.	$43.83^{b} \pm 0.83$	$52.50^{a} \pm 1.96$	$40.50^{b} \pm 1.60$	45.61^{C}
Over all mean	57.38^{B}	65.00^{A}	53.22^{C}	

Means with different letters in the same row (a,b,c) or column (A, B, C) are significantly different ($P<0.05$).

Table 2. Percentage of dead sperms of the cooled rabbit's spermatozoa with various extenders during storage at 5°C

Storage time (hour)	Lactose Yolk Citrate (LYC)	Tris Yolk-Glucose (TYG)	Coconut Milk (CME)	Over all mean
Zero	$15.33^{a} \pm 0.98$	$15.33^{a} \pm 0.60$	$16.83^{a} \pm 0.66$	15.83^{C}
24 hr.	$21.50^{a} \pm 1.02$	$18.50^{b} \pm 0.67$	$21.66^{a} \pm 0.76$	20.56^{B}
48 hr.	$26.33^{a} \pm 2.07$	$22.50^{a} \pm 0.88$	$26.16^{a} \pm 1.75$	25.00^{A}
Over all mean	20.88^{A}	18.61^{B}	21.89^{A}	

Means with different letters in the same row (a,b,c) or column (A, B, C) are significantly different ($P<0.05$)

Table 3. Abnormal spermatozoa (%) of cooled rabbit's semen with various extenders during storage at 5°C

Storage time (hour)	Lactose Yolk Citrate (LYC)	Tris Yolk-Glucose (TYG)	Coconut Milk (CME)	Over all mean
Zero	$13.83^{a} \pm 0.74$	$13.00^{a} \pm 0.85$	$14.83^{a} \pm 1.52$	13.89^{C}
24 hr.	$20.50^{a} \pm 1.08$	$17.50^{b} \pm 1.02$	$21.66^{a} \pm 1.66$	19.89^{B}
48 hr.	$26.33^{b} \pm 0.88$	$23.16^{a} \pm 0.74$	$29.16^{c} \pm 0.54$	26.22^{A}
Over all mean	19.88^{B}	17.55^{C}	22.5^{A}	

Means with different letters in the same row (a, b, c) or column (A, B, C) are significantly different ($P<0.05$)

Table 4. Percentage of acrosome loss of the cooled rabbit's spermatozoa with various extenders during storage at 5°C

Storage time (hour)	Lactose Yolk Citrate (LYC)	Tris Yolk-Glucose (TYG)	Coconut Milk (CME)	Over all mean
Zero time	$10.83^{a} \pm 0.74$	$10.66^{a} \pm 1.49$	$11.83^{a} \pm 0.74$	11.11^{C}
24 hr.	$15.50^{a} \pm 1.82$	$13.66^{ab} \pm 1.42$	$19.16^{a} \pm 0.98$	16.11^{B}
48 hr.	$21.50^{b} \pm 2.17$	$21.16^{b} \pm 1.99$	$27.00^{a} \pm 2.32$	23.22^{A}
Over all mean	15.94^{B}	15.16^{B}	19.33^{A}	

Means with different letters in the same row (a,b,c) or column (A, B, C) are significantly different ($P<0.05$)

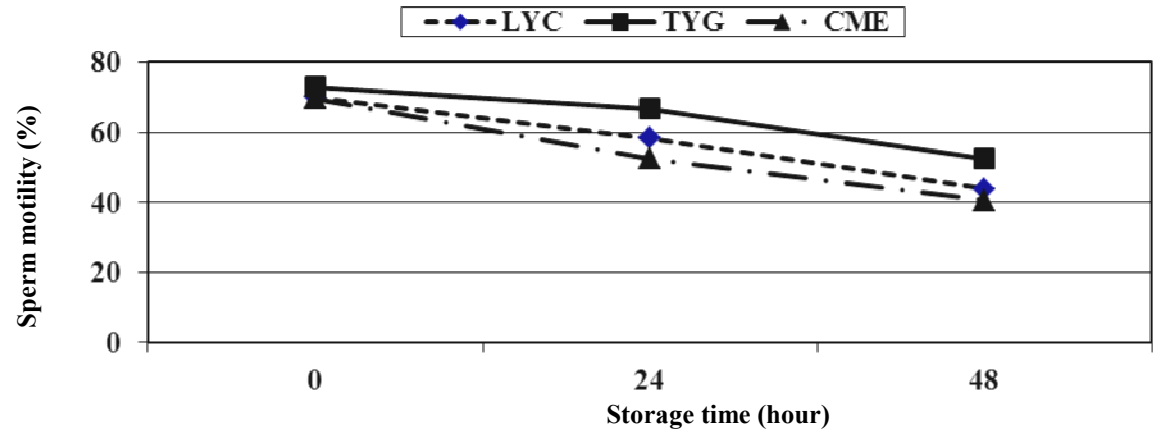

Fig. 1. Effect of different extenders on sperm motility (%) of rabbit semen ((Means ± SEM) after 0, 24 and 48 hr., of storage at 5 °C

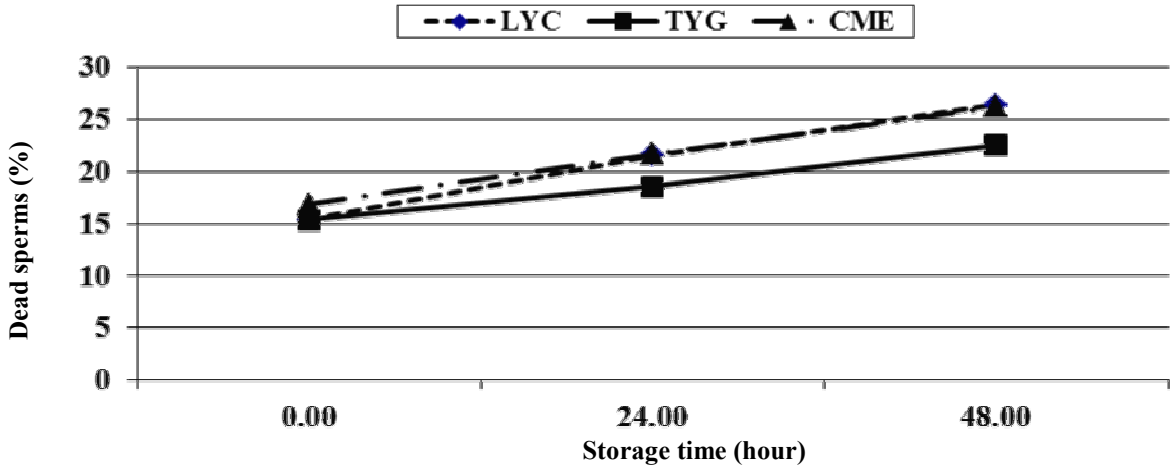

Fig. 2. Effect of different extenders on dead sperms (%) of rabbit semen (Means±SEM) after 0, 24 and 48 hr., of storage at 5°C

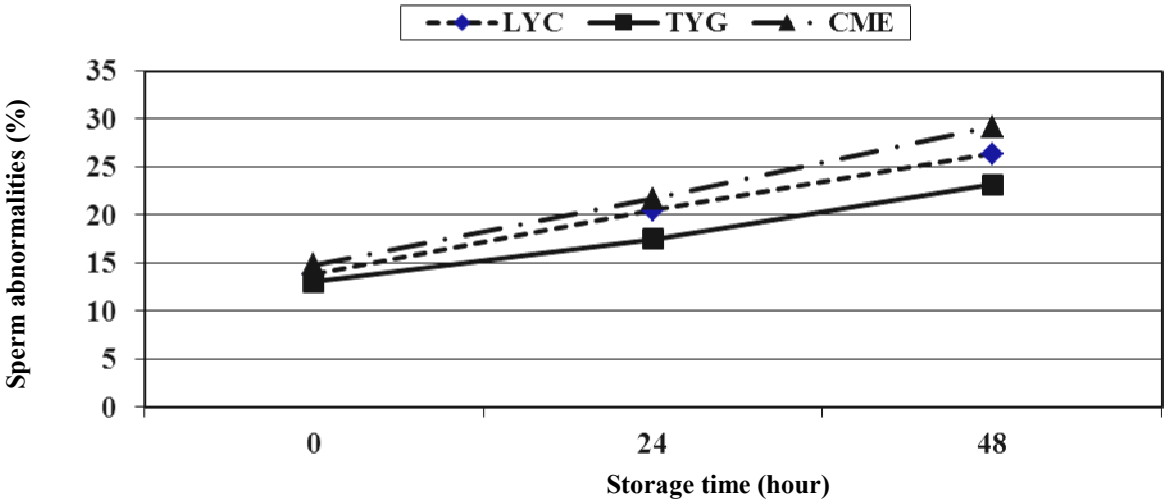

Fig. 3. Effect of different extenders on sperm abnormalities of rabbit semen (Means ± SEM) after 0, 24 and 48 hr., of storage at 5°C

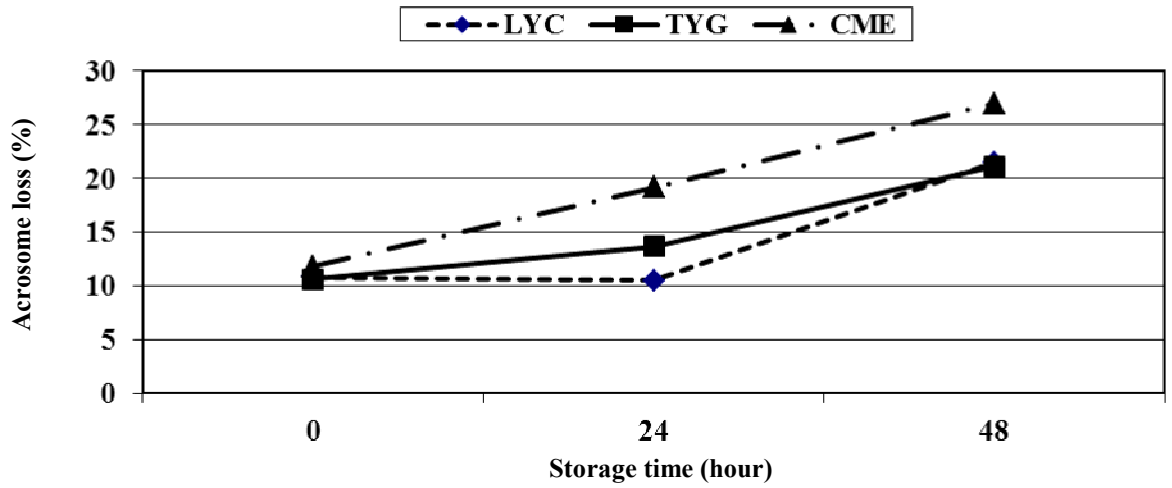

Fig. 4. Effect of different extenders on acrosome loss of rabbit semen ((Means ± SEM) after 0, 24 and 48 hr., of storage at 5°C

values (52.5 and 40.5%) ($P<0.01$) were found with CME after 24 and 48 hr., storage, respectively. While LYC showed insignificantly higher values (58.3 and 43.8%) than CME at 24 and 48 hr., of storage, respectively. But the LYC extender motility values were significantly lower than TYG extender. Similar trends with above results were reported by El-Gaafary (1994) and Roca *et al.* (2000) using a TYG extender.

Regarding the used of coconut milk as an extender for cock semen, Oghu *et al.* (2014) demonstrated that there was no significant difference between sodium citrate, heated coconut milk and saline in sperm progressive motility and total motile sperms. It has been reported that heating coconut milk, a procedure that has not been applied in the present study, has been shown to improve its shelf life and increase the amount of lauric acid which has antimicrobial effects as well as antioxidant properties (Raghavendra and Raghavaroa, 2010; Oghu *et al.,* 2014).

To date, there have been few studies investigating the metabolism of glucose by rabbit spermatozoa during chilled storage. However, in both human and canine spermatozoa it has been observed that motility was improved when higher levels of glucose were used as an energy substrate (Iguer-ouada and Verstegen, 2001). In this study, as a good source energy was not added to CME, the lower percentage of sperm motility reported for rabbit spermatozoa diluted in CME compared to other studies may be due to more rapid depletion of the energy source necessary for preserving sperm motility over time (Johinke *et al.,* 2014). The low percentages of sperm motility obtained with coconut milk may be related to also, a decrease in mitochondrial membrane potential and with a low production of ATP started by the inability of the mitochondria to utilize sugars present in the diluents, due to the blockage caused by the presence of a high content of Ca_2+ in the coconut milk (Bottini-Luzardo *et al.,* 2013).

Coconut water-based extender, plus 20% egg yolk and 3% glycerol, was found to be effective for cryopreservation of semen from collared peccaries (Silva *et al.,* 2012). Moreover, the inclusion of 80% citrate buffer in CME might be a suitable as buck semen extender for about 3 hours after extension of semen in room temperature (Sule *et al.,* 2007).

Dead Spermatozoa (%)

The results of dead spermatozoa percentage of cooled rabbit semen using three different extenders (CME, TYG and LYC), during storage at 5°C for 48 hr., are shown in Table 2 and Fig. 2. There were significant differences between treatments at 24 hr., of storage at 5°C with respect to the percentage of dead spermatozoa. The lowest ($P<0.05$) percentage of dead sperms was recorded in semen stored with TYG, while with CME, the dead sperms showed

highest values. At 48 hr., storage time of semen, the (%) of dead sperms did not differ significantly amongst different extenders. These results are in parallel with those of El-Gaafary (1990), Zeidan (1994), in Friesian bulls spermatozoa; Roca *et al.* (2000), in rabbit semen and Ogbu *et al.* (2014) in cock semen.

Spermatozoa Abnormalities (%)

As shown in Table 3 and Fig. 3, the effect of type of extender on the percentage of sperms abnormalities of the cooled rabbit semen was highly significant ($P<0.01$), being the highest for CME than TYG and LYC at 24 and 48 hr., of storage. The highest percentages of abnormalities were obtained with coconut milk. These results may be considered as an additional proof for the superiority of TYG in protecting spermatozoa during cool preservation in comparison with other extenders; LYC and CME.

Lost Spermatozoa Acrosome (%)

As shown in Table 4 and Fig. 4, the effect of different extenders on loss acrosome percentage (%) of the cooled rabbit semen was highly significant ($P<0.01$). At 48 hr., of storage, higher acrosome loss values were found with CME (27%) at 48 hr., of storage compared with TYG and LYC extenders (21.16 and 21.50%), respectively. The lowest values for the acrosome loss (21.16%), during the 48 hr., storage were obtained with TYG. Acrosome integrity is of vital importance for embryo development as it determines the paternal genetic contribution to the female oocyte following fertilization (Partyka *et al.,* 2010).

A previous study was in agreement with the above results (El-Gaafary, 1994) who showed that the best normal acrosomes were obtained with Tris-based diluents, (Amer, 1999; Daader and Zeidan, 2008) in rabbits and Ahmadi (2001) in camel spermatozoa. Damage to the sperm membrane is generally caused by the *in vitro* processing of semen during which spermatozoa are often cooled to low temperatures and stored for extended periods of time. During this chilled storage, oxidative stress plays an important role in the decline of sperm quality (Bucak and Tekin, 2007). While a small amount of reactive oxygen species are necessary for the initiation of many critical sperm functions, the high levels of reactive oxygen species generated by spermatozoa during chilling and storage can significantly reduce both viability and fertility over time (Castellini *et al.,* 2003; Awda *et al.,* 2009). There are no studies so far that have reported the effect of coconut milk on the membrane integrity of chilled rabbit semen.

The experiments that have been done to investigate the use of Tris-based extenders for preserving rabbit sperm viability are limited, (Sariozkan *et al.,* 2012; Johinke *et al.,* 2014) and suggesting that a higher storage temperature (15°C) may reduce the adverse effects of cold-shock during short-term storage. Perhaps the detrimental effects associated with reduced temperatures could be alleviated with the addition of egg yolk or other protective substrates to the extender and thus permit successful storage at 5°C.

Unfortunately, storage of rabbit semen for longer than 24 to 48 hr., causes deterioration of semen quality with a decrease in fertility (Roca *et al.,* 2000; Lopez-Gatius *et al.,* 2005; Akosy *et al.,* 2008). Therefore, extending the interval of liquid semen storage beyond 48 hr., or freezing the semen, remains one of the major goals in rabbit industry (Di Iorio *et al.,* 2014). This requires non-stop investigations on extenders that help realizing this goal.

The present results revealed the superiority of TYG to other extenders; LYC and CME for maintaining spermatozoa progressive motility, viability and acrosome integrity during cooled storage at 5°C up to 48 hr.

Although the CME gave appreciable results when used as semen extender for other species, the present study was the worst in maintaining sperms motility, viability and acrosome integrity during 48 hr., cooled storage (Figs. 1, 2, 3 and 4). Several factors that might be responsible for the observed inferiority of CME as an extender were the non-addition of source of energy and good buffer. Non heating and high viscosity of coconut milk are also responsible factors for the nonsatisfactory results with extender. It is worth mentioning that when these factors are eliminated, it is possible to obtain promising results with this extender.

It is worth mentioning that although the values of spermatozoa percentages obtained under this experiment seemed to be low, they still acceptable to obtain reasonable fertilizing ability when used for insemination. This assumption is assured by the reasonable fertility results obtained when frozen thawed semen of cattle was used in insemination trials although sperm motility does not exceed 30-40%.

Conclusion

Although the extenders used in this study showed somehow low values of semen parameters, they still reasonable. More investigations are needed to provide more suitable extenders for rabbit spermatozoa storage at 5°C. The development of new assays to evaluate sperm function in different extenders after storage, will allow for the development of new alternative extender for long-term storage that leads to improved efficiency of storage systems for rabbits semen.

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تأثيـــر بعـــض المخففـــات علـــى الســـائل المنـــوي للأرانب المخـــزن على ٥°م لمدة ٤٨ ساعة

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هدفت الدراسة إلى مقارنة لبن جوز الهند كمخفف للسائل المنوي للأرانب مع مخفف الترس (الترس+ صفار البيض + الجلوكوز) ومخفف اللاكتوز (اللاكتوز + صفار البيض + السترات) علي قابلية الحيوانات المنوية للتبريد على ٥°م لمدة ٤٨ ساعة، استُخدم ١٢ ذكر أرنب نيوزيلاندي وتم جمع السائل المنوي وعمل pool semen وتم تخفيف السائل المنوي بالمخففات الترس، واللاكتوز، وجوز الهند وكان معدل التخفيف ١: ٤ (١سائل منوي : ٤ مخفف) لمدة ٨ أسابيع، وتم تقييم السائل المنوي، أظهرت النتائج أن أعلي قيمة لحركة الحيوانات المنوية ٥٢,٥% عند التخزين لمدة ٤٨ ساعة علي ٥°م مع مخفف الترس ولا توجد فروق معنوية بين كلا من مخفف لبن جوز الهند واللاكتوز، أما الحيوانات المنوية الميتة لا تُظهر فروق معنوية بين الأنواع الثلاثة من المخففات بعد التبريد لمدة ٤٨ ساعة علي ٥°م، فيما يتعلق بالحيوانات المنوية الشاذة سجلت النتائج ٢٣,١٦%، ٢٦,٣٣%، ٢٩,١٩% مع مخفف الترس واللاكتوز، ولبن جوز الهند علي التوالي، مع وجود فروق معنوية بين المخففات الثلاثة عند ٤٨ ساعة من التبريد علي ٥°م، سلامة الاكروسوم: ظهرت فروق معنوية لمخفف لبن جوز الهند وباقي المخففات عند التخزين لمدة ٤٨ ساعة وكانت أعلى قيمة لفقد الاكروسوم (٢٧%) مع مخفف لبن جوز الهند، وقد خلصت الدراسة إلى أمكانية تخفيف السائل المنوي للأرانب بمخفف لبن جوز الهند بنسبة تخفيف (١ سائل منوي : ٤ مخفف) وذلك عند التبريد عند ٥°م لمدة ٤٨ ساعة.

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