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Cryopreservation of goat semen using Soy milk as alternative of egg yolk

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Abstract: This study was carried out at Research Station, and laboratory, Animal Production Dept., Agric. College, Al-Muthanna Univ., during /2016 to 2017 to improve the quality of cryopreservation semen goats by identifying the best concentration of soy milk and comparing it to Egg yolk, besides glycerol 7% was used as a check treatment. The results showed a significant superiority(P≤0.05) for the TFCSM (Consists of tristtmen 3.63 g/100 ml, fructose 0.5 g/100 ml and citric acid 1.99 g/100 ml+5% soy milk) and difference in cry protectants results. G7% with TFCSM showed significant superiority(P≤0.05) in the percentage of progressive motility Type A, the non-progressive motility type D, Agglutination, live sperm with averages 18.88, 45.44, 3.86 and 76.50, respectively. The EG5% with TFCSM showed significant superiority(P≤0.05) in the percentage of progressive motility type A and B, the non-progressive motility type C and D and the percentage of normal sperm, with averages 17.76, 20.15, 15.82, 45.64 and 88.51, respectively.

Keywords: Cryopreservation, goat semen, Soy milk.

تجميد السائل المنوي للماعز باستخدام حليب الصويا كبديل لصفار البيض

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المستخلص :

اجريت هذه الدراسة في محطة الابحاث الثانية التابعة لكلية الزراعة/جامعة المثنى ومختبر الدراسات العليا التابع لقسم الانتاج الحيواني /كلية الزراعة/جامعة المثنى للمدة من2\10\2016 ولغاية 2017/7/9 بهدف تحسين نوعية السائل المنوي المجمد للماعز الافغاني، لمقارنة مخفف حليب الصويا بمخفف صفار البيض التقليدي، للتأكد من إمكانية استبدال الجليسيرول بموانع تجميد ذات وزن جزيئي منخفض مثل الاثلين كلاي كول إذ استخدم الجليسيرول كمعاملة مقارنة بتركيز 7% واستخدم ثلاث تراكيز مختلفة من الاثلين كلاي كول (3، 5 و7% على التوالي) ومقارنة تداخل كل واحد من موانع التجميد على مخفف حليب الصويا وصفار البيض. وجد تفوق معنوي (P≤0.05) لمخفف الTFCSM (تكون من الTris 3.63غم/100مل، fructose 0.5غم/100 مل و citric acid 1.99 غم/100مل+5% حليب الصويا) و بتباين نتائج موانع التجميد اذ اظهر الG7% مع ال TFCSM تفوق معنوي(P≤0.05) في النسبة المئوية للحركة التقدمية من النوع A، الحركة غير التقدمية من النوع D، تكتل النطف، النطف الحية اذ كانت المتوسطات 18.88، 45.44، 3.86 و 76.50 على التوالي. أما الEG5% مع الTFCSM فاظهر تفوقاً معنوياً (P≤0.05) في النسبة المئوية للحركة التقدمية من النوع A و B والحركة غير التقدمية من النوع C وD والنسبة المئوية للنطف الطبيعية اذ كانت المتوسطات 17.76، 20.15، 15.82، 45.64 و 88.51 على التوالي.

Introduction

The cryopreservation of sperm is an affective technique for improving the goat breeding program. Cryopreservation causes Biological, functional and vital changes in the sperm, especially it effects on the sperm membranes and reduce its functions (Chelucci et al., 2015). the animal products such as egg yolk and milk are used to protect the sperm during cryopreservation and thawing (the integrity of sperm membranes is affected by cold shock, osmotic stress, lack of sperm motility and low fertilization). But animal products are a source of concern for those concerned with biosecurity in many countries. However, they are routinely used in sperm cryopreservation processes to minimize the Side effects of cryopreservation processes (Papa et al., 2011). Animal products cause some problems because they increase the risk of microbial contamination that leads to the production of endotoxin, which reduces sperm fertility and increases the risk of disease transmission during sperm exchange (Beccaglie et al., 2009). In addition, the problems associated with extenders containing egg yolk in the semen of the goat have been attributed to the egg yolk coagulating enzyme, which is harmful to sperm cells (Purdy, 2006). It also turns out that the fatty globules of egg yolk make the microscopic evaluation of semen difficult (Singh et al., 2012). Therefore, researchers to this day are looking for appropriate alternative to animal products that can be used to cryopreservation sperm. Plant products such as soy milk can be the appropriate alternative to protecting sperm from damage to the cryopreservation process (Chelucci et al., 2015).

This study was aimed to compare the effect of soya milk extender used in goats semen cryopreservation with the egg yolk extender and the effect of replacing glycerol with low molecular weight cryoprotectants (ethylene glycol) in soy milk and egg yolks extenders.

Materials and Methods

The study was carried out for the period from 2/10/2016 to 9/7/2017 at the second research station in University of Al-Muthanna (8 km south-east of Samawah) and the postgraduate laboratory of the Animal Production Department collage of Agriculture.two types of cryoprotectants were used: Glycerol 7%(control), Ethylene glycol 3, 5 and 7% respectively, and two types of extenders TFCEY (with 20% concentration) and TFCSM (with 5% concentration).

1. Experimental animals

Three adult males of goats, with ages between 1.5-2.5 years and body weight between 87 to 91 kgs. The males were all right healthy, clinically, sick, external and internal parasites. The nutrition was modified to compliance the requirement of animals during the reproduction season (37% barley, 35% wheat bran, 20% yellow corn, 5% soybean meal, 2% limestone and 1% salt). Fresh water was continuously available throughout the experimental period.

2. Preparation of soya milk

Soya milk is prepared by weighing 10 grams of soybean seeds and washed with distilled water and soaked overnight in 100 ml of distilled water. washed again with distilled water and addition 100 ml distilled water and heated at 80-100ºC for 20 minutes. Immediately after heating, it mixed with a high-speed mixer for 5 minutes the mixture be formed it call slurry. After the formation of the slurry, it is heated again for 10 minutes with continuous stirring and maintaining the temperature at 80-100°C to inactive the effects of the enzyme Lipoxygenase (Nelson et al., 1976; Shurtleff and Aoyagi, 1979). Then the slurry was cooled and filtered with a clean gauze cloth. The centrifuge was then rotated at 4000 rpm for 20 minutes to completely dispose of the solids substance, and adjust the pH level to 7 (Kasimanickam et al., 2011).

3. Semen collection

The goat's artificial vagina was used to train goat males on a collected. It was collected once a week, and the artificial vaginal temperature at the collected was 40-42°C. After collecting the semen sample, it was directly diluted in the field with Tris extender and transferred to the laboratory by thermos flask at 35-37ºC. As soon as possible, to performance following tests:

3. 1. Sperm agglutination

The agglutination was measured according to (Who, 2010).

3. 2. Sperm viability

The viability was measured according to (Swanson and Beardon, 1951)

3. 3. Sperm abnormalities

The sperm abnormalities was measured according to (Who, 2010).

4. preparation of semen Extender

The main extender is Tris (TFC) with tris 3.63 g/100 ml, fructose 0.5 g/100 ml and citric acid 1.99 g/100 ml. The TFCEY egg

yolk extender is made of tris 3.63 g/100 ml, fructose 0.5 g/100 ml and acid citric 1.99 g/100 ml+20% egg yolks. The TFCSM soybean milk extender is made from Tris 3.63 g/100 ml, fructose 0.5 g/100 ml and citric acid 1.99 g/100 ml+5% soy milk and the pH is adjust to 7. (Table, 1).

5. Semen Cryopreservation Protocol

After the semen sample is collected it diluted by TFC extender (At 1:10) then divided sample into four sections and add the cryoprotectants (7% Glycerol, 3%, 5%, 7% Ethylene glycol) and cold shock protect (20% EY and 5% SM). The semen was filled in straws with 0.25 ml (10 straws per treatment) and cooled to 4º C in two hours and then transferred to liquid nitrogen vapor (-75 ° C) at a distance of 7 cm above the surface of the nitrogen for 10 minutes Finally, the straws was submerged in liquid nitrogen (-196 ° C) and for a minimum of one month until thawing. A month later, the straws was removed from liquid nitrogen and placed in a water bath (37 ° C in 60 seconds) for thawing it and conducting tests to evaluate the semen after cryopreservation (Jerez., et al 2016).

6. Statistical analysis

The results of the study were statistically analyzed by CRD using a two-factor. The first factor is the concentration of cryoprotectants and the second factor is the extender type as shown in the following mathematical model:

$Yijk = \mu + Ci + Ej + CE(ij) + eijk$

Yijk = the observed value of the studied properties, M = the general average, Ci = the effect of the cryoprotectants concentrations, Ej = the extender type effect, CE (ij) = the overlap between the cryoprotectants concentration and the extender type.

Using SPSS (2008) For the analysis the results of this study, Duncan (1995) to test the differences between the treatment with a significant level ($P \leq 0.05$).

Table (1) Components of extenders to dilute the semen of goat per 100 ml (Mocé et al., 2010)

Ingredient	TFC	TFCSM	TFCEY
Tris (g)	3.63	3.63	3.63
Fructose (g)	0.5	0.5	0.5
Citric acid (g)	1.99	1.99	1.99
Soybean milk (%)	0	5	0
Egg yolk (%)	0	0	20
Penicillin (IU)	100000	100000	100000

Streptomycin (mg)	100	100	100

Results

1. Effect of overlap between cry protectants and extender type on the agglutination, motility and immobility sperm.

The results in table 2 showed significant superiority (P≤0.05) in the percentage of agglutination due to overlap G7% with TFCEY at an average of 0.44±3.79 compared with overlap EG3% with TFCEY, overlap EG5% with TFCEY and overlap EG7% with TFCEY With an average of 0.51±4.69, 0.45±4.10 and 1.09±6.52 respectively, while there were no significant differences from overlap EG3% with TFCEY and EG5% with TFCSM. The average was 0.51±4.69 and 0.45±4.10 respectively. A significant increase (P≤0.05) was observed in the overlap of G7% with TFCSM with an average of 0.40±3.86 compared to overlap EG3% with TFCSM, overlap EG5% with TFCSM and overlap EG7% with TFCSM. The results were 0.44±5.25 and 0.47±3.96 and 0.60±8.12 Respectively. There was no significant difference between EG3% with TFCSM and overlap EG5% with TFCSM. The results were 0.44±5.25 and 0.47±3.96, respectively. The best results from the overlap EG7% with TFCEY or TFCSM were in favor of TFCEY at an average of 1.09 ± 6.52 and there were no significant differences from the overlap G7%, EG3% or EG7% with TFCEY or TFCSM.

In general, the best result obtained for the percentage of agglutination was from overlap G7% with TFCEY or TFCSM at average 0.44±3.79 and 0.40±3.86 or from overlap EG5% with TFCSM at an average of 0.47±3.96. The results of the study showed a significant superiority (P≤0.05) in the percentage of motility sperm from overlap G7% with TFCEY and overlap EG5% with TFCEY with an average of 54.71±1.22 and 1.96±53.76, respectively Compared to overlap EG3% with TFCEY and overlap EG7% with TFCEY at averages of 49.29±1.15 and 2.09±36.89, respectively. We also note that there was a significant superiority (P≤0.05) in the percentage of sperm motility as a result of the overlap of G7% or EG5% with TFCSM at averages 1.32±54.55 and 1.68±53.76 respectively compared to overlap EG3% or EG7% with TFCSM at 2.02±44.80 and 2.40±35.25 respectively We found that the best results from the overlap G7% with TFCEY or

TFCSM were for TFCEY at an average of 54.71±1.22. The best results from overlap EG3% with TFCEY or TFCSM were for TFCEY with an average of 49.29±1.15 and no significant differences from EG5% with TFCEY or TFCSM. The best results from overlap EG7% with TFCEY or TFCSM were in favor of TFCEY at an average of 2.09±36.89.

Table (2). the Effect of overlap between cry protectants and extender type on the agglutination, motility and immobility sperm (mean ± standard error)

cry protectants	Extender type	Agglutination%	Immobility sperm%	Motility sperm%
(control) G7%	TFCEY	0.44 ± 3.79^{A}	$*1.22\pm54.71^{A}$	$*1.22\pm 45.28^{A}$
	TFCSM	0.40 ± 3.86^{a}	1.32 ± 54.55^{a}	1.32 ± 45.44^{a}
EG3%	TFCEY	0.51 ± 4.69^{AB}	$*1.15\pm49.29^{B}$	$*1.15\pm 50.70^{B}$
	TFCSM	0.44 ± 5.25^{b}	2.02 ± 44.80^{b}	2.02 ± 55.19^{b}
EG5%	TFCEY	0.45 ± 4.10^{A}	1.96 ± 53.76^{AB}	1.96 ± 46.23^{AB}
	TFCSM	$*0.47\pm3.96^{a}$	1.68 ± 53.74^{a}	1.68 ± 46.25^{a}
EG7%	TFCEY	$*1.09\pm6.52^{B}$	$*2.09\pm36.89C$	2.09 ± 63.10^{C}
	TFCSM	0.60 ± 8.12^{c}	2.40 ± 35.25^{c}	2.40 ± 64.74^{c}

In general, the best percentage of motility sperm was from overlap G7% with TFCEY as well as from overlap EG5% with TFCEY or TFCSM. We also note from the results of the study shown in table.1 that there was a significant superiority ($P\leq0.05$) in the percentage of immobility sperm due to overlap G7% with TFCEY and overlap EG5% with TFCEY at average of 45.28±1.22 and 1.96±46.23 respectively compared with overlap EG3 With TFCEY and overlap EG7% with TFCEY. The averages were 1.15±50.70 and 63.10±2.09 respectively, There was also a significant superiority ($P\leq0.05$) of overlap EG5% with TFCSM with an average of 1.68±46.25 compared to overlap G7% with TFCSM, overlap EG3% with TFCSM and overlap EG7% with TFCSM with average 45.44±1.32, 2.02±55.19 and 2.40±64.74 respectively. The best results from the overlap G7 with TFCEY or TFCSM were in favor of TFCSM. The results showed that the best percentage of immobility sperm was from overlap G7% with TFCEY with an average of 45.28±1.22 and EG5% with TFCEY or TFCSM. The average was 1.96±46.23 and 1.68±46.25, respectively. TFCSM with an average of 1.74±88.51 and

the best percentage of abnormal sperm is from overlap EG5% with TFCSM averaging 1.74±11.48.

2. Effect of overlap between cryoprotectants and extender type on the normal and abnormal sperm of goats.

The results of the statistical analysis shown in table 3 obtained from the cryopreservation of the semen samples of goats revealed that there were significant differences (P≤0.05) due to the overlap of the cryoprotectants with the extender type in percentage of the normal and abnormal sperm of goats after cryopreservation. We observed a significant superiority (P≤0.05) of overlap G7% with TFCEY and overlap EG5% with TFCEY where the averages were 1.59±80.88 and 1.16±79.99 respectively and There was no significant difference between overlap EG3% with TFCEY and overlap EG7% with TFCEY where the averages was 2.83±77.99 and 1.34±79.71, respectively.

There was also a significant difference (P≤0.05) from the overlap of EG5% with TFCSM with an average of 1.74±88.51. There were no significant differences in the overlap G7 with TFCSM, overlap EG3% with TFCSM and overlap EG7% with TFCSM with averages 1.98±82.92, 2.63 82.91 and 1.12±82.02 respectively. The percentage of abnormal sperm was significantly superiority (P≤0.05) from overlap G7% with TFCEY at average 1.59 ± 19.11 and no significant differences from overlap EG5% with TFCEY and overlap EG7% with TFCEY where averages 1.16 ± 20.01 and 1.34±20.28 Respectively. The highest percentage of abnormal sperm is from overlap EG3% with TFCEY at an average of 2.38±22.00. There was also a significant difference (P≤0.05) from the overlap of EG5% with TFCSM with an average of 1.74±11.48 and no significant differences from the overlap G7% with TFCSM, overlap EG3% with TFCSM and overlap EG7% with TFCSM with averages of 1.98±17.07, 2.63±17.08 and 1.12±17.97, respectively.

In general, the best percentage of normal sperm is from overlap EG5% with

Table (3). The Effect of overlap between cry protectants and extender type on the normal and abnormal sperm of goats (mean ±standard error)

Cry protectants	Extender type	Normal sperm %	Abnormal sperm %
(Control) G7%	TFCEY	1.59 ± 80.88^{A}	$*1.59\pm19.11^{A}$

	TFCSM	*1.98±82.92[b]	1.98±17.07[b]
	TFCEY	2.83±77.99[B]	*2.38±22.00[B]
EG3%	TFCSM	*2.63±82.91[b]	2.63±17.08[b]
	TFCEY	1.16±79.99[A]	*1.16±20.01[A]
EG5%	TFCSM	*1.74±88.51[a]	1.74±11.48[a]
	TFCEY	1.34±79.71[A]	*1.34±20.28[A]
EG7%	TFCSM	*1.12±82.02[b]	1.12±17.97[b]

Discussion

In the past few years, there has been considerable incentive to develop suitable alternatives to the traditional egg yolk to cryopreservation the semen. The experiment was conducted to investigate the effect of overlap between two types of extenders TFCEY and TFCSM and two types of cryoprotectants glycerol 7% (control) 3, 5 and 7% ethylene glycol on some semen characteristics of goat after cryopreservation

In the results of the experiment, we found that there was a significant difference in the progressive motility A and B, the non-progressive motility D, the motility sperm and the immobile sperm due to the overlap G7% with TFCSM and the overlap EG5% with TFCSM compared with the overlap G7 with TFCEY, overlap EG3% with TFCEY or TFCSM, overlap EG5% with TFCEY and overlap EG7% with TFCEY or TFCSM. Therefore, in this study, we found that the TFCSM extender superiority the most important criteria for semen evaluation for artificial insemination, Progressive motility A and B, for their significant contribution arriving sperm to the fertilization site. The reason for the superiority of TFCSM extender is that soy milk contains a high proportion of proteins, The proteins are the basic structure of all cell life and are essential for cell function, such as transport and storage nutrient element, cell structure development, etc. (Medic et al., 2014). Soybeans contain a high proportion of fat (8.1 to 24%), mostly unsaturated fatty acids (Zaman et al., 2010). Fat plays an important role in energy storage, cell membrane synthesis, defense against pathogens, and temperature regulation (Karasula., et al 2011). In addition, soybeans contain energy-rich sources of approximately

35% carbohydrates, most of which are soluble sugars such as sucrose, glucose, fructose, and raffinoz (Middelbos, 2008). Sucrose and glucose are desirable sugars because of their sweet taste (Wang et al., 2014) Sugars play an important role in withdrawing part of the water inside the cell, which reduces the chances of forming ice crystals inside the cells during rapid chilling (Aisen and Medin, 2002). It is important to note that raffinoz and other sugars play an important role as antioxidants in plant tissues, same Role in cells sperm (Van den, 2013). Antioxidants inhibit the production of free radicals or break down hydrogen peroxide (H2O2), thereby preventing the formation of ROS, which is toxic to the sperms (Mcdowell et al., 2007).

The EG5% cryoprotectant is superior to the G7% (control) because of the EG characteristics of its high permeability due to low molecular weight, but its increased concentration in the middle of the cryopreservation causes sperm poisoning (Li et al., 2015). In this study, the use of EG resulted in similar results (G7%) during cryopreservation and other times the use of high or low EG concentrations resulted in weaken the results. In addition, Guthrie et al., 2005 found that EG had less adverse effects on sperm motility and vitality compared to Glycerol, and this suggests the possibility of using it as a substitute for glycerol. In the results, we found that significant superiority ($P \leq 0.05$) in the percentage of normal and abnormal sperm due to the overlap of EG5% with TFCSM and the significant difference ($P \leq 0.05$) in the percentage of normal and abnormal sperm due to overlap G7% with TFCSM, phospholipid is the main component of cell membranes and therefore plays a major role in important physiological functions that reduce the point of freezing and regulate temperature and thus avoid the formation of large ice crystals (Giraud et al., 2000; Waterhouse et al., 2006) This is why the TFCSM extender is more important than the fact that the phospholipids contained in soybean lecithin replace the phospholipids in the outer membrane of the sperm and this contributes to maintaining the structure and functions of the plasma membrane. Another possibility is that the phospholipids in soybean soils do not enter the cell membrane to change Concentrate of phospholipids but may form a protective membrane around the cell to prevent the formation of ice crystals within the cell and protect the sperm membrane from damaging

the cryopreservation process (Zang et al., 2009). TFCSM reduces the number of

abnormal and die sperm to contain soybeans on antioxidants such as vitamin E , One molecule of vitamin E is capable of protecting 1000 molecules of unsaturated fatty acids found in large quantities In the plasma and acrosome membrane of the sperm (Kontush et al., 1996; Tramer et al., 1998). Vitamin E also protects sperm from damage caused by free radical oxidation, as the high level of free radicals leads to dysfunction in the central region of the sperm, To produce ATP energy this reduces the energy source Prepare for the sperm thus decreases the movement and vitality of sperm (Williams and Ford, 2001). Vitamin E also plays an important role in preventing deformity of sperm and maintaining the integrity of the sperm and its metabolic functions (Burk., 2007).

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