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CRYO PRESERVATION OF GOAT EPIDIDYMAL SPERMS UNDER FREEZING CONDITION USING OMEGA 3, 6 AND 9

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Abstract

This study was conducted in the period between 1/10/2016 to 1/9/ 2017 in the laboratory of higher study that due to the Department of Animal Production, College of Agriculture, University of Al–Muthanna to investigate the ability of cryopreservation of epididymal sperms of Iraqi goat. A4 testesof adult bucks were collected from the abattoir immediately after slaughtering of animals and transmitted to the laboratory under low temperature using thermos container, and then cut the cauda epididymis to small pieces and immerged in the extender that prepared before, the mixture of omega 3, 6 and 9 was adding to the recovered goat sperms and diluted by tris extender to concentration (1-10), the epididymal sperm were evaluated prior freezing, than the stock solution was divided into five parts 1ml of each part as following T1 (control), T2 (6% glycerol only), T3 (2% omega+ 4% glycerol), T4 (3% omega + 3% glycerol), T5 (4% omega + 2% glycerol) the diluted semen was loaded into 0.25ML straws and after completion of the freezing process left straws for two months in the liquid nitrogen, the straws were subjected to process of thawing and assessment of goat sperms. The results shows, no significant differences in the progressive motility type A,B, non-progressive motility type c, immotile sperms typed, sperms agglutination and normal sperms in T3,T4 and T5 treatments, while the T1 treatment was significantly surpass ($p<0.05$) compared with another treatments in the live sperms.

Key words : Cryo preservation, goat, epididymal sperms, freezing, omega 3, 6, 9.

Introduction

Goats are closely related to human life through the consumption of meat and milk products, as well as the use of other products such as leather and hair, in a variety of industries, and to provide a percentage of profits to breeders (Mahmoud, 1980 and Ibrahim, 1998). The semen has been frozen for more than half a century for the purposes of artificial insemination, which has become widespread in the world (Calisici, 2010). The production of ROS in most of the laboratory has played a major role in the phospholipids oxidation of sperm membranes, and the destruction of the genetic material which has been linked to conservation by freezing, which is reflected negatively on the decrease in spermsmotility and its potency to fertilization (Chatterjee and Gagnon, 2001; Sarioskan, 2009; Corespilho *et al*., 2014). Oxidative damage occurs naturally on the sperms during the period of freezing, which is a key cause of production of fatty acid peroxide, and basic factor in the reduction of sperms motility and loss of fertilization capacity, especially mammalian sperms (Ball *et al*., 2001). To avoid these problems, adding substances to sperms diluents protect the sperms and increase their storage time and thus have a fundamental role in increasing fertility (Ax *et al*., 2000). Modern sources have shown that the main reasons for the deterioration of semen after freezing is peroxidation (the formation of toxic substances, including hydrogen peroxide) and the best ways to prevent them is the use of antioxidants such as omega acids 3,6 and 9.The sperm plasma membrane in different mammalian species contains 70% phospholipid, 25% neutral fat and approximately 5% glycolipids (Flesch, 2000).

Multi-unsaturated fatty acids such as omega-3 have a large and prominent role in sperms resistance to freezing .In the rabbits, the omega-3 fatty acid group with vitamin E and C improves the quality of semen during storage (Castellini *et al*., 2003). In buffalo, the omega-3 fatty acids, together with vitamin E and C, improve the quality

of sperms during storage (Castellini *et al*., 2003). Feeding on sunflower oil or seeds improved the quality of sperms (Adeel *et al*., 2009). Castellano *et al*. (2010) showed that adding fish oil to the diets of male pigs failed to improve sperms quality. Also adding supplements of Docosahexaenoic acid (DHA) and egg yolks in diluted semen in male pigs did not increase sperms resistance to freezing (Wathes *et al*., 2007), these studies together have revealed us the inconsistent effects of fatty acids on sperms quality when done used in diet or diluted (Abavisani *et al*., 2013). In order to avoid many problems of keeping the sperms frozen, this study came to achieve the following objectives:

1. Identification of the role of omega 3, 6, 9 acids in the process of preservation of epididymal sperm sunderthawing.
2. Determine the best concentration of omega 3, 6, 9 in the preservation of sperms.

Materials and Methods

This study was conducted in the laboratory of post graduate studies of the Department of Animal Production, Faculty of Agriculture, Al-Muthanna University for the period from 1/10/2016 to 1/9/2017 for the purpose of studying the effect of the mixture of omega 3, 6, 9 acids in improving the qualities of goats frozen semen, used 4 samples of the testis for adult goat males, the samples were brought from the slaughter after slaughtering the animals directly and transferred to the laboratory to complete the epididymal sperms test. This experiment was conducted after obtained the epididymal sperms, diluted with Tris and egg yolk diluent (20%),(1-10 ml) (seminal fluid: diluent), which divided into five treatments: control treatment T1 (pre-freezing) was formed from 1 ml of (semen and diluent) mixture for the purpose of initial evaluation, T2 : 6% of glycerol , T3: 4% glycerol and 2% of omega acids mixture (6,3,9), T4 : 3% omega acids mixture, T5: 2% glycerol and 4% omega acids mixture. After which the treatments were filled in bars and closed from the upper side using a closed electric shutter machine and placed on the rack horizontally in the refrigerator at 5^0C for 3 hours, then transferred to freezer basin, and placed horizontally on a metal rack inside the freezer above the surface of the liquid nitrogen about 10 cm, for ten minutes. Then bars were directly stored within liquid nitrogen and were stored in the nitrogen bottle for two months. The samples were then dissolved after two months of freezing, by taking three bars from each treatment and cutting them from the upper and lower ends, then was put in eppendorf tube which then incubated at 37°C for 30 seconds, then examined by microscope at (400 X).

Studied characteristics

The characteristics of semen were evaluated, including the proportion of dead sperms: according to Swanson and Beardon (1951). The percentage of dead sperms was assessed by taking a drop of semen and placed on a clean glass slide at a temperature of 37°C. A drop of Eosin-Nigrosin dye was added (5%, 10%) and mixed well, then gently smeared on a glass slide by another slide in angle of 45°, examined under the microscope with a magnifying force (400 X). Dead sperms appeared in pink while living one were transparent color due to the pigment didn't penetrate the cell membrane, 200 sperms was calculated in different fields of the slide, then the percentage of dead to malformed sperms was estimated, the percentage of deformed sperms was calculated according to the Hancock method (1951) and on the same slide where the dead one were counted, they were examined under the microscope with a magnification force (400 X). Out of 200 sperms, the percentage of deformed sperms were determined.

The movement and activity of the epididymal sperms : a drop of epididymal sperms was placed on the slide, after a minute they counted at 35-30°C, the microscopic field was regularly examined according to WHO (2010) and the movement of sperm was divided into four varieties, A : was characterized by a high progressive movement, B: circular movement only, C: rotating or tail movement only and D is not moving as indicated by Fakhrildin (2014).

Total motile sperms ratio was calculated on the same slide, which measured the movement and activity of the epididymal sperms, microscopic field is regularly examined under a microscope with a magnification force of 400 X.

Sperms aggregation: The epididymal sperms aggregation was calculated according to WHO (2010). Some sperms that are unable to move due to their adherance to one another, or the tail is attached to the head or head with the head, they were counted by the same slide to measure sperms activity.

Statistical analysis

Statistical data were analyzed statistically using the SAS-Statistical Analysis System (2012) to study the effect of different concentrations of omega and glycerol in the studied traits. Significant differences were compared between the averages using the Duncan (1955) multidimensional test using CRD, at a probability level of 0.05.

Results

The results of the study showed that table 1 the addition of different concentrations of omega and glycerol to the diluted goats' semen. There were no significant differences (P<0.05) in the progressive motility type A between the second treatment T2 (Glycerol 6%) and the third treatment T3 (Glycerol 4%, Omega 2%) and T4 (Glycerol 3%, but significantly lower (P <0.05) than the control treatment T1 (before freezing). The averages of progressive movement type A (17.43, 4.88, 3.30, 3.42, 3.02) for all treatments : T1(before freezing), T2, T3, T4 and T5, respectively.

The results of the study for progressive movement type B showed there were no significant differences between T1 and T2, which increased significantly (P <0.05) compared to T3 and T 5. At the same time, no significant difference was observed between (T2 and T4) and (T3, T4 and T5). The averages of progressive movement type B (17.98, 22.18, 17.93) for all treatments :T1, T2, T3, T4 and T5, respectively. The non-progressive movement of type C for the goat's sperms showed no significant differences between T1, T2, T3, T4 and T5. The means of the non-progressive movement type C were (33.93, 32.72, 38.80, 35.32, 35.85) for T1, T2, T3, T4 and T5, respectively. In the progressive movement type D, the study showed that there were no significant differences between T2, T3, T4 and T5, which in turn exceeded the control T1. Progressive movement type D averages were (18.91, 36.00, 39.88, 38.79, 43.66) for T1, T2, T3, T4 and T5, respectively. For the motile sperms, the results of the study showed that there is a significant increase (P > 0.05) in T1 compared to T2, T3, T4 and T5 (with no significant difference between them). The means of motile sperms were (81.08, 63.98, 60.11, 61.19, 56.32) for T1, T2, T3, T4 and T5, respectively. In the non-motile sperms, we observed that there were no significant differences between T2, T3, T4 and T5, which in turn surpassed the control T1. Progressive movement type D averages were (18.91, 36.00, 39.88, 38.79, 43.66) for T1, T2 , T3, T4 and T5, respectively. In table 2, we found that the sperms aggregation in T2, T3, T4 and T5 did not display any significant difference, at the same time, it was significantly higher (P <0.05) than T1. The averages of the sperms aggregation were (1.51, 2.48, 2.75, 3.51, 2.91) for T1, T2, T3, T4 and T5, respectively. The normal sperms, there was a significant increase (P <0.05) inT1 compared to T2, T3 and T5, but there was no significant difference observed between T1 and T4 and between, T2, T3, T4 and T5. The normal sperms averages were (92.06, 88.00, 86.97, 89.23, 88.82) for T1, T2, T3, T4 and T5, respectively. In the malformed sperms, the results of the study showed a significant increase (P <0.05) in T2, T3, T4 and T5 compared tocontrol treatment T1, which in turn no significant difference was observed between them, and between control treatments T1 and T4. The averages of mutated spermswere (7.93, 11.99, 13.01, 10.75, 11.16) for T1, T2, T3, T4 and T5, respectively.The results of the study of the dead sperms showed that there was no significant difference between T3, T4 and T5, which were significantly higher (P <0.05) than T1 and T2, with means of dead sperms (11.08, 22.55, 33.30, 33.10 and 38.41) for T1, T2, T3, T4 and T5, respectively. According to live sperms, the results showed that there were no significant differences between T3, T4 and T5, which in turn decreased significantly (P <0.05) compared to T1 and T2. The means of live sperms were (88.90, 77.43, 66.69, 66.88 and 61.64) for T1, T2, T3, T4 and T5, respectively.

Discussion

This experiment was conducted to determine the effect of omega acids mixture and glycerol at different concentrations T2 (glycerol 6%), T3(omega 2% and glycerol 4%), T4 (omega3% and glycerol 3%), T5 (omega 4% and glycerol 2%), respectively, on the characteristics of goat frozen epididymal sperms : (progressive movement A, B, non-progressive movement type C, non-motile sperms D, sperms aggregation, sperms morphology and vitality. Table 1 results showed no significant differences between T2, T3, T4 and T5 in progressive movement type A, which in turn decreased significantly compared to control treatment T1(before freezing) and in progressive movement type B, we did not notice any significant differences between treatment T2 and T1, while decreased in T1, T4 and T5 compared to T1. The non-progressive movement type C, there were no significant differences between (T2, T3, T4 and T5) compared to T1. We also observed increase in non-motile sperms ratio of type D for all treatments compared to T1, this may be due to the fact that freezing leads to severe stress of sperms and has a negative effect on sperms characteristics (Andrabi, 2007; Mahfouz, 2010; Watson, 2000 and Lemma, 2011) and keeping the sperms in a deep freezing (-196°C) using liquid nitrogen causes structural, biochemical and functional damage to the sperms (Leboeuf *et al*., 2000). Studies have showed that there is an obvious difference between animals sperms in freezing resistance, these differences due to their sperms differ in plasma membrane phospholipids content. The plasma membrane with a high elasticity sperms characterized by tolerating the shock of cold, the most important characteristic of flexibility of sperms are

Table 1 : Effect of different concentrations of omega on progressive and non-progressive motion and total sperm movement at 1-10 during freezing (mean ± standard error).

Treatments		Traits					
		Progressive movement of sperm		Non-progressive movement of sperm	Non-moving sperm	Total movement of sperm	
		A	B	C	D	Non-moving sperm	Animated sperm
Before freezing		17.43 A±1.73	29.70 A±0.98	33.93 A±1.57	18.91 B±3.65	18.91 B±3.65	81.08 A±3.65
After freezing with the addition of %	Glecerol 6	4.88 B ±0.59	26.36 AB±2.67	32.72 A±1.87	36.00 A±1.81	36.00 A±1.81	63.98B±1.81
	Glecerol 4 Omega2	3.30 B±0.42	17.98 C±1.83	38.80 A ±4.08	39.88 A±2.27	39.88 A±2.27	60.11B±2.27
	Glecerol 3 Omega3	3.42 B±0.37	22.18 BC±1.76	35.32 A±2.65	38.79 A±1.60	38.79A±1.60	61.19B±1.60
	Glecerol 2 Omega4	3.02 B±0.32	17.93 C±2.04	35.85 A±1.87	43.66 A±3.04	43.66 A±3.04	56.32B±3.04

The different letters within the same column indicate significant differences at the probability level (P <0.05).

Table 2 : Effect of different concentrations of Omega on the agglutination, morphological and viability of the sperm at the rate of dilution (1-10) during freezing (mean ± standard error).

Treatments		Traits				
			The sperm Morphological		Sperm viability	
		Agglutination sperm	Normal sperm	Distorted sperm	Dead sperm	Life sperm
Before freezing		1.51 B±0.36	92.06 A±0.77	7.93 B±0.77	11.08 C±1.89	88.90A±1.89
After freezing with the addition of %	Glecerol 6	2.48 A±0.32	88.00 B±0.42	11.99 A±0.42	22.55 B±0.70	77.43 B±0.70
	Glecerol 4 Omega2	2.75 A±0.36	86.97 B±0.47	13.01 A±0.47	33.30 A±2.40	66.69 C±2.40
	Glecerol 3 Omega3	3.51 A±0.60	89.23AB±1.01	10.75AB±1.01	33.10 A±2.08	66.88 C±2.08
	Glecerol 2 Omega4	2.91 A±0.22	88.82 B±1.56	11.16 A±1.56	38.41 A±2.97	61.64 C±3.01

The different letters within the same column indicate significant differences at the probability level (P <0.05).

phospholipid and cholesterol, higher cholesterol, increase the flexibility of the sperm plasma membrane (Gillan *et al*., 2004). During freezing and melting, ROS production increases and antioxidant levels decrease, causing enormous changes in the volume of cellular water (Bansal and Bilaspari, 2011). The hypothesis of working in this study is the addition of unsaturated fatty acids omega 3, 6 and 9 to improve the properties of goats frozen epididymal sperms, since the unsaturated fatty acids omega 6, 3 and 9 are antioxidants, used in our study as an antioxidant or a protective substance for the sperms during freezing, in order to reduce the percentage of dead and deformed sperms, the results showed that the omega acids mixture did not give any significant differences when replacing it with glycerol compared to T2. For the sperms aggregation characteristic, table 2, increased significantly in all treatments compared to T1, with notice no significant differences between T2, T3, T4 and T5 in malformed sperms ratio. For dead sperms, there was no significant difference observed between T3, T4 and T5, which in turn increased significantly compared to T2, this agreed with Abavisani *et al*. (2013), Kandelousi *et al*. (2013) when adding different concentrations of omega 3 to diluted semen resulting in reduced motility, vitality and morphological form in the freezing and thawing of semen of the bulls. In addition, glycerol is one of the most popular freezing protective agents and most commonly used in semen freezing of various animal species (Foote, 2002) this is in agreement with Londhe (2005), noting that the level of glycerol (5-7%) was added to a dilute material to be useful in the maintenance of the sperms. Glycerol has the ability to penetrate the sperms plasma membrane

(Bashwat, 2015) as it binds to water molecules by hydrogen bonds (Fuller and Paynter, 2004), it works to conserve cellular water within the sperm, which is necessary to maintain the size of the cells and prevent them from rupturing when water freezes and also interact with the ions and large molecules in the cellular medium and works to reduce the degree of freezing water, which leads to the reduction of electrolyte concentration in the remaining non-freezing part of water, thus reducing the ice crystals that will form inside the sperm (Medeiros *et al*., 2002; Holt, 2000). So, this harmful effect in the motility and vitality of sperms may be attributed to the different levels of the omega acids mixture.

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