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Effect of Adding Lyophilized Low-Density Lipoproteins (L-LDL), Methionine and Their Combinations on Semen Quality of Holstein Bulls after Cryopreservation

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ABSTRACT

This experiment was conducted at the Research Station of the College of Agriculture/ University of Al-Muthanna with the aim of knowing the effect of using lyophilized low-density lipoproteins (L-LDL) dried from egg yolk at a concentration (6%) and methionine at a concentration (5 mmol) and their combination to TRIS extender on semen characteristics of the Holstein bulls after cryopreservation for different periods (48 hours, 1 month and 2 months). This experiment was conducted for the period from December 2019 to April 2020 and in three stages, the first was the extraction of low-density lipoproteins from egg yolk in the diagnostic section of the Plant Protection Department, the second stage was lyophilized (liquid LDL) and make it a dry material and packed in sterile cans and then keep it freezing, and the third stage were done in the graduate studies laboratory at the College of Agriculture/ University of Muthanna. The study included semen collection from tow Holstein bulls and its ages ranged between 2-3 years. Artificial vagina were used to collect semen by 1 ejaculate/ bull/ week, and then pooled semen were done to reduce the differences between ejaculations, then semen were divided to four groups as following: (T1) Control group, (T2) 6% L-LDL, (T3) 5 mmol methionine and (T4) 6% L-LDL+5mmol methionine as combination, to study the effect of each of these treatments on percentage of individual motility, viability, plasma membrane and acrosome integrity for different time of preservation at freezing. It was observed through this study that there was superiority in the treatments of methionine, lyophilized LDL and their combinations over the control treatment during preservation periods.

Keywords: Lyophilized low-density lipoproteins, methionine, semen, bull, cryopreservation.

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INTRODUCTION

There are many factors that affect the activity and viability of the sperm, as it was found that the process of preserving the sperm at low temperatures leads to a number of variables that the sperm is exposed to during preservation by freezing, which leads to their exposure to cold shock (McBee and Cotterill, 1979). Thus, this process leads to damage to the sperm cells, resulting in a loss of sperm motility, viability, fertilization ability, reduction in the integrity of plasma membrane and acrosome and a destruction of the genetic material of DNA (Aitken *et al.*, 1985; Medeiros *et al.*, 2002; Vishwanath and Shannon, 2000).

Many factors influence the motility and viability of sperm during cryopreservation (Bag *et al.*, 2002; Nur *et al.*, 2010) represented by the types of preservatives and their concentrations which affect the success and failure of the preservation process (Kemal *et al.*, 2010). Preservation of sperm during the freezing process led to the formation of ice crystals, change in osmotic pressure, elevation of oxidative stress, as well as resulted in a reorganization of proteins in the sperm cell membranes (Bailey *et al.*, 2000; Watson, 1995).

The freezing and thawing process leads to a continuous release of reactive oxygen species (ROS) by immature and abnormal sperms, and this is a result of the continuous decrease in the concentrations of antioxidants in the semen and seminal plasma extenders (Sikka, 2004), in addition to the oxidation of the lipids of the sperm membranes and damage to their genetic material, which negatively affected the fertility (Kemal *et al.*, 2010; Iaffaldano *et al.*, 2014). About 36% of the weight of eggs makes up the egg yolk, while 68% of the low-density lipoproteins (LDL) forms in the yolk, while the high-density lipoproteins (HDL) represent 16% of the components of the egg yolk, while the phosphoproteins called phosphatin make up 4% (Anton, 2007).

From this it was found that part of the egg yolk used had the active role and is responsible for providing protection from cold shock (Manjunnath *et al.*, 2007; Moussa *et al.* 2002). Nevertheless, supplementing of LDL to bull semen extender at 8% lead to significant increase in sperm motility, acrosome and plasma membrane integrity post cryopreservation (Alani *et al.,* 2016). Adding amino acids to the extenders of the semen had an effective role in reducing damage to the sperm cell membrane when treated as an antioxidant through its role in cell membrane integrity and respiratory processes, and the amino acid methionine was added to the liquid extenders of semen which act to reduce damage of the cell and remove toxins and oxidative stress (Reed, 1990).

Therefore, this study aimed to extract and freeze low-density lipoproteins (LDL) from egg yolk and use it with methionine as a substitute for egg yolk in Holstein semen extenders, and its effect on the quality and viability of sperm after freezing.

MATERIALS AND METHODS

Experiment animals

This study was conducted at the research station of the Faculty of Agriculture / Muthanna University for the period from December 2019 to April 2020, in which 2 Holstein bulls were trained to collect semen using an artificial vagina, with a weight ranging between 450-550 kg, and an age of 2-3 years. About 1 ml of semen were collected from each bull per ejaculate, semen pooling was performed to reduce differences between ejaculates, then semen was divided to four groups as following: (T1) Control group, (T2) 6% L-LDL, (T3) 5 mmol methionine and (T4) 6% L-LDL+5mmol methionine as combination. Bulls were vaccinated to black leg, foot and mouth disease. Bulls were exposed to a standardized feeding level which included concentrated diet at a rate of 4-5 kg/ animal, 35% barley, 33% wheat bran, 10% yellow corn, 20% soybean meal, 0.5% limestone, 0.5% salt and 1% vitamins, as the level of crude protein was 18%, the energy was estimated 3323 kilocalories/ kg, while the amount of roughage diet was 6-9 kg/ animal/ day and 50-60 kg green diet. Drinking water was available to the animals *ad libitum*, as well as the animals were housed in semi-open pens.

LDL Extraction Method

Low-density lipoproteins were extracted from the yolk of unvaccinated chicken eggs according to the method of Moussa *et al.* (2002) which consists of sequential steps until the LDL were purified and packaged in sealed and sterilized cans and dried by freezing. The aim of the drying process is to withdraw moisture and convert it from a rapidly perishable liquid state to a solid state, in order to extend the life of the stored material, and to make it in a solid form in the form of a powder, and then it is packaged in airtight and sterilized boxes and the lyphilization process was done according to Jennings (1997). Low-density fatty acids are weighted and added to TRIS extender and using a magnetic stirrer device for a period of ten minutes to dissolve the dried LDL particles in the extender, and then placed in a water bath at a temperature of 37 ° C at the same temperature as the semen during the dilution.

Semen characteristics

Semen samples were analyzed to calculate the following parameters:

- Individual motility was estimated according to the method of Watson (1995).
- The percentage of sperm viability was estimated according to the method referred by Swanson and Beardon (1951).
- The percentage of sperm plasma membrane integrity was estimated according to the method of Banana (2014).
- Acrosome integrity: Giemsa stain was prepared according to the method described by Hancock (1951) using gentian violet and eosin stain.

Statistical analysis

Data of the present experiment were analyzed using SAS statistical program (2010), while the variation between treatments were analyzed by Duncan's Multiple Range Test Duncan (1955).

RESULTS AND DISCUSSION

The results of the present study showed that there was significant increasing (P≤0.05) in the percentage of sperm individual motility of Holstein bulls after 48 hours, 1 and 2 months of cryopreservation for the treatment T4 compared with other groups (Table 1). Also, the results revealed that's there were a significant decrease (P≤0.05) in the percentage of motility along the periods of preservation for all treatments. These results can be explained that's when adding methionine and lyophilized LDL for the treatment (T4) to the extender, methionine plays an antioxidant role by increasing enzyme activity such as catalase, which is being a protective agent against damage caused by cold shock, in addition to its safety for mitochondrial function and improvement of sperm motility (Bucak *et al.,* 2010). Furthermore, the results indicated that the lyophilized LDL with methionine had the ability to maintain the properties of sperm, and the presence of phospholipids and their role in the stability of sperm structure and molecular of LDL and its hydrophilic property (Eser *et al.,* 2014). These results agreed with the findings of Neves *et al,* (2014) and Moustacas *et al,* (2011) who adding natural and lyophilized LDL to the spermatozoa of canine and rams. This explains that's using of methionine at a concentration of 5 mmol and L-LDL at a concentration of (6%) were given good motility percentage, as well as providing protection from cold shock, in addition to increasing the osmotic pressure, which increased the activity of sperm (Bergeron *et al,* 2004).

Also, the results appeared that the addition of methionine and lyophilized LDL to Tris semen extender had a significant effect (P≤0.05) on the percentage of sperm viability after 48 hours, 1 and 2 months of cryopreservation for the treatments T2, T3 and T4 compared with control group. The progress of the cryopreservation periods showed a significant reduction (P≤0.05) in the values of sperm viability percentage, but the amount of decrease was less in the methionine and LDL treatments compared with the control group (Table 2). The enhancement of sperm viability percentage may be due to the effect of methionine at a concentration (5 mmol) and its synergistic action with lyophilized LDL at a concentration of (6%) which added to the extender and surrounding the sperm cell membranes from outside and preserving it from reactive oxygen species ROS (Çoyan *et al,* 2010) Whereas, the lyophilized LDL works to a fusion with the sperm membranes prior to freezing, which led to an increase in its resistance to cold shocks and thus its able to maintain the prevention of ice crystal formation after freezing (Briand-Amirat *et al.,* 2013).

Furthermore, the results of the present study appeared a significant increasing (P≤0.05) in the sperm plasma membrane integrity of Holstein bulls for the treatment T4 compared with the treatments T1, T2 and T3 for all cryopreservation periods (Table 3). The results also showed that there was significant superiority (P≤0.05) in the percentage of acrosome integrity after 48 hours, 1 and 2 months of preservation at freezing for the treatment (T4) compared with the treatments T1, T2 and T3 (Table 4). The superiority of treatment T4 over other three treatments are due to the role of methionine which may have a protective role from freezing damage, and on the other hand an antioxidant role (Çoyan *et al,* 2010). While the mechanism of action of lyophilized LDL in protecting sperm cells may be due to the association of lyophilized LDL with nuclear plasma proteins in bulls BSPs, which in turn reduced depletion of cholesterol from the plasma membrane (Bergeron *et al.,* 2004).

The superiority of the treatment T4 in the acrosome integrity on all treatments and for all preservation periods may be due to the role of methionine in inducing

effective fusion (Active Infusion) of dried LDL with sperm membranes (Briand-Amirat *et al.,* 2013). It was also found that the lyophilized LDL concentration (6%) increases in the proportion of cholesterol and this component is one of the main substances that work in harmony with phospholipids and thus ensure the fluidity of the cell membrane and play an important role in the functions of sperm cells and thus to protect the cell membranes (Salamon and Maxwell, 2000; Ricker *et al.,* 2006).

Table 1: Effect of adding methionine, L-LDL and its combination to TRIS extender on percentage of sperm individual motility cryopreserved for different periods of Holstein bulls (mean ± standard error)

Treatment	Cryopreservation time			Significance
	48 hours	1 month	2 months	
T1 Control	42.01 ∓3.02 Ca	35.20 ∓ 3.03 Cb	33.46 ∓ 3.05 Cb	*
T2 L-LDL (6%)	55.13 ∓ 3.23 Ba	47.28 ∓ 3.13 Bb	41.81 ∓ 3.11 Bc	*
T3 Methionine (5 mmol)	53.17 ∓ 2.80 Ba	46.33 ∓3.20 Bb	40.51∓3.41 Bc	*
T4 Methionine (5 mmol) + L-LDL(%6)	69.16 ∓ 4.09 Aa	64.70 ∓ 2.97 Ab	58.04 ∓ 2.66 Ac	*
Significance	*	*	*	

* ($p<0.05$).
Different small letters within each row mean significant between periods.
Different large letters within each column mean significant between treatments.

Table 2: Effect of adding methionine, L-LDL and its combination to TRIS extender on percentage of sperm viability cryopreserved for different periods of Holstein bulls (mean ± standard error)

Treatment	Cryopreservation time			Significance
	48 hours	1 month	2 months	
T1 Control	59.43 ∓2.18 Ca	51.09 ∓ 2.31 Cb	49.22 ∓ 2.28 Cb	*
T2 L-LDL (6%)	63.17 ∓ 2.58 Ba	57.31 ∓ 1.81 Bb	54.81 ∓1.91 Bb	*
T3 Methionine (5 mmol)	62.88 ∓ 1.60 Ba	59.37 ∓ 1.65 Bb	56.05∓ 3.37 Bb	*
T4 Methionine (5 mmol) + L-LDL(%6)	74.04 ∓ 2.53 Aa	68.98 ∓ 1.86 Ab	67.14 ∓ 1.92 Ab	*
Significance	*	*	*	

* ($p<0.05$).
Different small letters within each row mean significant between periods.
Different large letters within each column mean significant between treatments.

Table 3: Effect of adding methionine, L-LDL and its combination to TRIS extender on percentage of sperm plasma membrane integrity cryopreserved for different periods of Holstein bulls (mean ± standard error)

Treatment	Cryopreservation time			Significance
	48 hours	1 month	2 months	
T1 Control	51.77 ∓1.70 Da	48.62 ∓ 1.52 Ca	45.04 ∓ 2.82 Ca	*
T2 L-LDL (6%)	62.13 ∓ 1.63 Ba	55.28 ∓ 1.65 Bb	47.42 ∓1.42 Cc	*

T3 Methionine (5 mmol)	57.73 ∓ 1.83 Ca	54.02 ∓ 2.16 Ba	52.18∓ 2.71 Ba	*
T4 Methionine (5 mmol) + L-LDL(%6)	72.13 ∓ 2.41 Aa	67.11 ∓ 1.50 Ab	66.21 ∓ 1.91 Ab	*
Significance	*	*	*	

* ($p<0.05$).
Different small letters within each row mean significant between periods.
Different large letters within each column mean significant between treatments.

Table 4: Effect of adding methionine, L-LDL and its combination to TRIS extender on percentage of acrosome Integrity cryopreserved for different periods of Holstein bulls (mean ± standard error)

Treatment	**Cryopreservation time**			**Significance**
	48 hours	**1 month**	**2 months**	
T1 Control	53.44 ∓ 1.73 Da	50.72 ∓ 1.56 Ca	49.31 ∓ 2.79 Ba	*
T2 L-LDL (6%)	64.16 ∓ 1.68 Ba	58.36 ∓ 1.59 Bb	53.32 ∓1.66 Bc	*
T3 Methionine (5 mmol)	58.37 ∓ 1.85 Ca	55.19 ∓ 2.16 Ba	52.03∓ 2.80 Ba	*
T4 Methionine (5 mmol) + L-LDL(%6)	72.91 ∓ 2.41 Aa	67.52 ∓ 1.65 Ab	65.31 ∓ 1.59 Ab	*
Significance	*	*	*	

* ($p<0.05$).
Different small letters within each row mean significant between periods.
Different large letters within each column mean significant between treatments.

CONCLUSION

It concludes from the results of this experiment that the mixture of methionine + lyophilized LDL (T4) led to a significant increase in the studied traits which exhibit a positive role in improving the properties of the sperm after cryopreservation due to essential role of methionine and lyophilized LDL in improving the semen characteristics through a protective agent to reduce damage from cold shock and their effective role as antioxidants.

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