The Use of Different Low-Density Lipoproteins Concentrations for Developing a New Extender for Awassi Ram's Semen

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ABSTRACT

It is very important to develop a new extender to achieve acceptable fertility rate after artificial insemination with frozen-thawed ram's semen. The study aims to evaluate the efficiency of low-density lipoproteins (LDL) instead of egg yolk in Awassi ram semen extenders. During the breeding season semen from four Awassi rams was collected via artificial vagina and frozen in liquid nitrogen in four extenders: the control extender containing (sodium citrate + 20% egg yolk), and three experimental extenders, egg yolk was replaced by three concentrations of LDL (6%, 8%, 10%), respectively. Motility was evaluated using phase contrast microscope, which also used to define live-dead spermatozoa by eosin-nigrosin staining technique. Motility and other quality parameters of thawed frozen semen was also estimated using CASA system. The results showed that the concentration of 8% LDL was superior to egg yolk 20%, 6% LDL, and 10% LDL (P<0.001) in term of motility. After cooling (equilibration), the motility was 73%, 67.8%, 68.7%, and 68.6% for the extenders 8% LDL, 20% egg yolk, 6% LDL, and 10% LDL respectively. The motility decreased after freezing to 62.66%, 54.18%, 51%, and 56.4%, respectively. Using CASA system, the results ensure the superiority of 8% LDL extender compared to 20% egg yolk extender (P>0.001) in terms of motility (60.67% versus 53.69%), and progressive motility (42.38% versus 39.89%). This study showed that 8% LDL extender has improved spermatozoa motility with better efficiency compared to the 20% egg yolk extender and the other two used extenders.

Key Words: Awassi Ram, Extenders, Low Density Lipoproteins, Semen.

INTRODUCTION

The main idea of semen preservation depends on restraining the movement of spermatozoa and its metabolic activities. Semen extenders play an important role in the success of artificial insemination (AI) at a reasonable cost. The major challenge facing artificial insemination industry for sheep is the lower conception rate with frozen semen compared to fresh semen and natural mating.

Egg yolk is the most common non-penetrating cryoprotectants used in semen extenders (Ritar and Salamon, 1982; Tuli and Holtz, 1994) it protects the spermatozoa against cold shock and confers protection during freezing and thawing (Salamon and Maxwell, 2000). Egg yolk contains cryoprotective components such as phospholipids, cholesterol low-density lipoproteins (LDL) and cephalin (Saack, 1993).

Egg yolk-based extenders are known to be practical and efficient in protecting ram's spermatozoa against cold shock during storage for AI. Furthermore, egg yolk contains cholesterol, phospholipids and low-density lipoproteins which prevent the ice crystal formation thus, protects integrity of sperm plasma membranes against cold shock during the freeze-thaw process (Hu *et al*., 2010).

In recent years, however, there has been a trend against the use of egg yolk in cryoprotective media because of the risks associated with the use of animal products. Egg yolk is an extremely complex product, whose composition may be extremely variable and may differ between batches. Its lipid composition may change with the hens' diet. Moreover, egg yolk contains substances (granules) that prevent the metabolic exchanges of the spermatozoa and

reduce their motility (Kampschmidt *et al*., 1953; Pace and Graham 1974; Watson and Martin, 1975). It also represents a potential risk of microbiological or virological contamination. Furthermore, Bousseau *et al*. (1998) demonstrated that the eggs were often contaminated, to a variable degree depending on their source, by bacteria such as Salmonella or Staphylococci. Due to these risks, many investigators have begun looking at the use of LDL solely in freezing extenders as a replacement to whole egg yolk.

Recent studies have demonstrated that LDL extracted from hen egg yolk could be largely responsible for the protection against cold shock, improvement in sperm motility, acrosome and plasma membrane integrity and more effective protection against sperm DNA fragmentation (Demianowicz and Strzeżek 1996; Moussa *et al*., 2002; Bencharif *et al*., 2008). Amirat *et al*. (2004) demonstrated that the extraction process used to obtain LDL from egg yolk reduces bacterial contamination by 10^7 colony forming units/ml. It would therefore be advantageous to use this complex solution with its component molecules that are responsible for its cryoprotective effect.

Pace and Graham (1974) purified egg yolk by ultracentrifugation and found that a fraction of egg yolk known as low-density lipoprotein (LDL) has a cryoprotective effect on the integrity of the plasma membrane. In addition, Moussa *et al*. (2002) and Amirat *et al*. (2004) stated that LDL preserves bull semen and maintains its fertility during storage, freezing, and thawing. Also it affects positively the percentage of canine normal spermatozoa and sperm motility (Bencharif *et al*., 2010).

Low-density lipoprotein has an average density of 0.982g/ml, a spherical format with 17 to 60 nm in diameter, with a lipid layer comprising triglycerides core (Cook and Martin, 1969) and cholesterol, which are surrounded by a film of phospholipids and protein. The phospholipids play a key role in the stability of the LDL structure because the forces of association between molecules are essentially hydrophobic. The LDL contains between 83 - 89% lipids and 11 -17% protein (Anton *et al*., 2006). During the freezing–thawing process, LDL disrupted and the phospholipids liberated into the medium, which could form a protective film at the surface of sperm membranes (Quinn *et al*., 1980; Cookson *et al*., 1984).

Moussa *et al*. (2002), Amirat *et al*. (2004) and Hu *et al*. (2006) demonstrated that LDL was responsible for the freezing process in freezing–thawing process. The first of gelation was the disruption of the LDL structure and this disruption favored the spermatozoa dehydration caused by the freezing–thawing process. Polge (1980), Graham and Foote (1987) and Bergeron *et al*. (2004) suggested that LDL could adhere to cell membranes during the freezing–thawing process and preserve the membrane integrity of sperm. Moussa *et al*. (2002) suggested that adsorption and gelation of apoproteins around the spermatozoa membrane could form a protective film against ice crystals generated during freezing. Furthermore, Moussa *et al*. (2002) revealed that LDL extracted from egg yolk with 97 % purity possessed remarkable cryoprotective properties freeze –thaw bull spermatozoa as higher motility percentage and better movement characteristics than when egg yolk was used.

The objective of this study was to find an optimum concentration of LDL to be used instead of the whole egg yolk in Awassi ram's semen extenders.

MATERIAL AND METHODS

1. Extraction of LDL from egg yolk:

LDL was extracted from egg yolks according to the method described by Moussa *et al*. (2002) as follow:

Ten to fifteen fresh eggs were collected from one flock of hens, which received a standard diet. After the disinfection with 75% ethanol, eggs were manually broken and yolks were separated from the albumen, and carefully rolled on filter paper to remove chalazas and traces of albumen adhering to the vitellin

membrane. The vitellin membrane was then disrupted with a scalpel blade and yolk was collected in beaker cooled in iced water to prevent bacterial growth. The purified egg yolk was diluted with an isotonic saline solution (0.17 M NaCl) (w/w), stirred for 1 h at +4°C and centrifuged at 10.000×g for 45 min at +4°C. The supernatant (plasma) was separated from the sediment (granules). To avoid mixture with granules completely, the plasma was centrifuged again. Plasma was then mixed with 40% ammonium sulfate (Sigma A: 4418; equivalent to 20.5 g per 100 ml of plasma) to precipitate live tins. After 1 h of stirring in refrigerator at 4°C, the mixture was centrifuged at 10.000×g for 45 min to separate a supernatant from sediment. The sediment was discarded and the supernatant was then dialyzed using a dialysis tubing cellulose membrane (D9527 Sigma, Saint Quention Fallavier, France) about 12 h against distilled water in order to eliminate ammonium sulfate. After complete ammonium sulfate elimination, the solution was again centrifuged at 10.000×g for 45 min at 4°C and the floating residue, rich in LDL, was collected.

2. Rams and Semen Collection:

This step was carried out at the Laboratory of Artificial Insemination and Embryo Transfer in Ezra'a Research Station, Arab Center for the Studies of Arid Zones and Dry Lands (ACSAD) during 2012-2013. Semen was collected during the breeding season from four Awassi rams at rate of three collections per week. The rams were three years old with an average body weight of 75±3 kg. Two ejaculates per collection from each ram were obtained using an artificial vagina. Rams used in this experiment were pretested in Ezra'a Research Station and no significant differences in the semen characteristics, therefore ejaculates mixed together after first evaluation.

3. Cryopreservative Extenders:

Semen was diluted twice at 34°C with the cryoprotective extenders to obtain a concentration of 250×10^6 spermatozoa/ml. The control cryoprotective extender (Menger *et al*., 1989) consists of 3.52g Sodium Citrate, 194 mg Glucose, 20 % (v/v) Egg yolk, 6.4 % (v/v) glycerol, 0.1g Streptomycin, and 1000 IU penicillin G. For the experimental extenders, Egg yolk in the control extender was replaced by the extracted LDL to obtain concentrations of 6%, 8% and 10% (w/v) in order to determine an optimum LDL concentration. All extenders were adjusted to pH 6.6. The osmotic pressure of the cryoprotective media was measured with an automatic Osmometer (Roebling, Berlin, Germany). Semen was apportioned between the different extenders as shown in table (1).

Table (1): Basic components of extenders used in the experiment.

	Control extender	Experimental extenders containing LDL (w/v)		
basic components	3.52 g Sodium Citrate	3.52 g Sodium Citrate		
	20% Egg yolk	6% LDL	8% LDL	10% LDL
	6.4% Glycerol	6.4% Glycerol (v/v)		
	194 mg Glucose	194 mg Glucose		
	Streptomycin 0.1g/ml Penicillin G 1000IU/ml	Streptomycin 0.1g/ml Penicillin G 1000IU/ml		

4. Freezing and post-thawing:

The tubes containing the diluted semen with the cryoprotective extenders were cooled from 34 to 4°C for 2.5h. Then, straws of 0.5 ml (I. M. V, L Aigel, France) were prepared for each of the tubes containing the cryoprotective extenders. The straws were maintained at 4°C for 2.5h before freezing. The straws were transferred to automated

freezing unit (IMV, France) which reduce the temperature from +4 to –140°C within 4 min. The pre-frozen straws were then plunged directly into liquid nitrogen (-196°C) for storage. The frozen semen was thawed by immersing the straws in a water bath at 37°C for 30 Sec and then by incubation at 37°C for 10 min. The thawed samples (5 straws each) were transferred into 1.5 ml plastic tubes to be evaluated. Total number of 180 samples for each extender were examined for motility, live –dead spermatozoa through semen handling (fresh- refrigerated, frozen) using a phase contrast microscope and eosin-nigrosin staining technique.

5. Automated Analysis of Semen Motility:

Sperm motility and other quality parameters was assessed with CASA system (Computer Assisted Sperm Analysis System) (Sperm Vision® 3.5, Minitub, Tiefenbach, Germany) to evaluate the efficiency of the different used extenders after freezing. The analysis was carried out at the Laboratory of Artificial Insemination and Embryo Transfer in the Faculty of Veterinary Medicine, University of Al-Baath, Hama, Syria. Sperm quality parameters were assessed using a 20-µm depth Cell-Vision counting chamber. For each extender, 108 straws were analyzed, three of which were thawed separately. The content of each straw was transferred into a plastic tube and incubated at 37°C for 10 min in a water bath. Then, 2.5µl of each straw and seven fields were examined. The CASA-derived sperm quality characteristics studied were total motility (MOT %), progressive motility (PROG %), Velocity Average Path- µm/sec (VAP): defined as Length of a derived ‘average’ path of sperm head movement per unit time, Curvilinear Line Velocity-µm/sec (VCL): defined as time-averaged velocity of a sperm head along its actual curvilinear path, as perceived in two dimensions in the microscope, Straight Line Velocity-µm/sec (VSL): defined as Net space gain of the sperm head per unit time.

Statistical Analysis:

All values were expressed to the Duncan test (1995). The least squares mean is used to fix the significance of the differences. The obtained data were subjected to statistical analysis using general linear model procedure adapted by Statistical Analysis System Institute (SAS Institute, 2.ed., Cary, NC, USA,2008), according to the following model:

Model $Y_k = \mu + E_k + e_k$

Where: Y_k general studied parameter

μ: mean of studied parameter

E_k: effect of tested extenders, $k=1, 2, 3, 4$.

e_k : standard error.

RESULTS AND DISCUSSION

The comparison of the motility percentages of spermatozoa during the semen processing steps (after diluting, after cooling, after thawing), showed a significant superiority for the extender containing 8% LDL compared to the control and other LDL containing-extenders (6% and 10%). At the end of equilibration period (2.5h by cooling at +4°C) a significantly ($P<0.001$) higher motility percentage (73%) was observed in the semen samples diluted in 8% LDL extenders compared with66.77 , 68.71 and 68.6% for the control, 6% LDL, and 10% LDL, respectively (table 2).

Motility declined after thawing to 62.66% in the 8% LDL extender, compared with 54.18%, 51% and 56.4% in the control, 6% LDL, and 10% LDL extenders respectively. The minimum percentages of dead spermatozoa, as well as abnormal spermatozoa were observed by the extender with 8% LDL. Whereas the maximum percentage of dead spermatozoa was detected by the extender containing 6% LDL, whereas the highest percentage ($P<0.01$) of abnormal spermatozoa was observed by the control extender containing 20% egg yolk, (table 2).

Table (2): Effect of extenders on motility, dead and abnormal spermatozoa in different handling steps of semen tested using phase contrast microscope.

Handling step	Spermatozoa(%)	Egg Yolk 20%	LDL6%	LDL8%	LDL10%
After diluting	motility	84.22 ± 0.38[a]	85.38 ± 0.40[a]	83.80 ± 0.39[a]	84.55 ± 0.44[a]
	Dead	7.78 ± 0.44	9.62 ± 0.41	12.20 ± 0.46	8.45 ± 0.44
	Abnormal	8.00 ± 0.44[a]	5.00 ± 0.42[b]	4.00 ± 0.42[b]	7.00 ± 0.41[b]
After equilibration	motility	67.77 ± 0.34[b]	68.71 ± 0.40[b]	73.00 ± 0.34[a]	68.60 ± 0.41[b]
	Dead	22.88 ± 0.52[b]	24.71 ± 0.59[a]	22.22 ± 0.52[b]	23.80 ± 0.49[ab]
	Abnormal	9.44 ± 0.29[a]	5.42 ± 0.33[c]	4.70 ± 0.29[c]	7.60 ± 0.27[b]
After thawing	motility	54.18 ± 0.33[a]	51.00 ± 0.41[b]	62.66 ± 0.36[c]	56.60 ± 0.34[b]
	Dead	35.90 ± 0.43[a]	42.00 ± 0.54[c]	31.66 ± 0.48[b]	35.60 ± 0.45[a]
	Abnormal	9.90 ± 0.40[a]	7.00 ± 0.51[b]	5.66 ± 0.45[c]	8.20 ± 0.42[b]

Samples =180, in a same row, values with different superscripts are significantly different.

Results obtained through CASA-system after freeze-thawing process in LDL and control extenders for Spermatozoa motility parameters are shown in the table3.

Table (3): Quality parameters of spermatozoa after freezing-thawing process in the used extenders; determinate using CASA.

Source of variation	DF	MOT %	PROG %	VAP (µm/s)	VCL (µm/s)	VSL (µm/s)
Extenders	3	(217.37)***	(43.052)***	(266.91)*	(216.97)*	(337.40)***
Standard error	35	17.135	5.467	108.92	325.15	85.57

Number of samples =108 (* $p<0.05$, ** $p<0.01$, *** $p<0.001$)

We demonstrated that substitution of whole egg yolk with different concentrations of purified LDL (6%, 8%, and 10%) in ram semen extenders enhances the most quality parameters and caused a significant superior effect compared to the control extenders. Compared with control extender, the best concentration of LDL was found to be 8%, it improved significantly ($P<0.001$) the following quality parameters obtained using CASAsystem: motility (60.67%), progressive motility (42.38%), VSL (40.76 µm/sec), figure (1). Januskauskas *et al*. (2003) found a significant correlation between CASA assessed spermatozoa motility and field fertility, and such parameters are correlated with the fertilizing ability of spermatozoa (Verstegen *et al*., 2002). Furthermore, Amirat *et al*. (2005) revealed that more than 80% of spermatozoa were injured after incubation for 4 h in egg yolk extender as Triladyl, while 3% and 47% were counted in LDL and Biociphos respectively. In addition, VAP and VCL values for LDL 8% extender were statistically higher (51.20, 71.61 µm/sec respectively) in comparison with the three other extenders ($P<0.05$), figure (2). Velocity parameters (VCL, VAP, VSL) measured by CASA system are an indirect indicator of mitochondrial function in spermatozoa, during cryopreservation mitochondria of spermatozoa undergo damages (Gillan *et al.,* 2004; Peris *et al*., 2004) ,this negatively affect the respiratory activity of frozen thawed ram semen (Windsor, 1997). In this study LDL 8% containing extender has significantly improve the means of velocity parameters comparing with control extender.

These findings are in accordance with

previous studies affirming the importance of LDL as a cryoprotectant agent instead of whole egg yolk during freeze thaw process of the sperm of many animal species such as bull sperm (Moussa *et al*., 2002; Amirat *et al*., 2004), buck sperm (ALI-Alahmad *et al*., 2008), rainbow trout and gilthead sea bream sperm (Perez-cerezalez *et al*., 2010; Cabrita *et al*., 2005), boar sperm (Hu *et al*., 2006; Jiang *et al*., 2007), canine sperm (Bencharif *et al*., 2008), buffalo sperm (Akhtar *et al*., 2011) and stallion sperm (Pillet *et al*., 2011). Those studies show positive effect to use LDL and post thaw results.

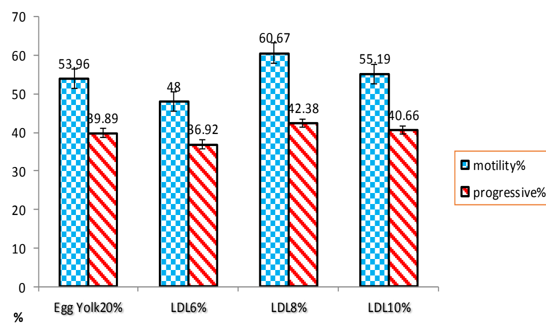

Fig. (1) Means of motility and progressive motility after freezing thawing process using extenders with different concentrations of LDL or egg yolk.

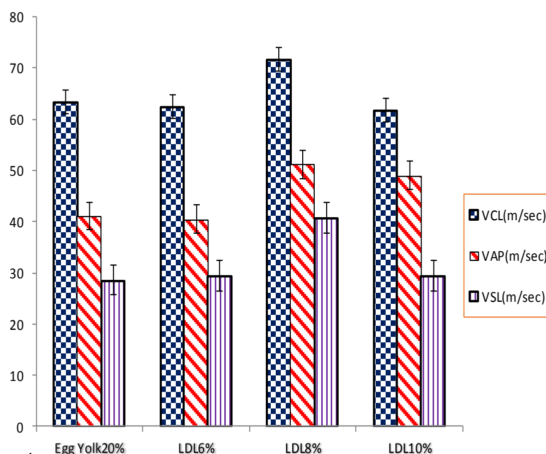

Fig (2) Means of VAP, VCL and VSL after freezing thawing process using extenders with different concentrations of LDL or egg yolk.

Several studies try to explain the mechanism behind the protection action of LDL during freeze –thawing process, Bergeron *et al*. (2004) revealed that low density lipoproteins adhere to the sperm cell membrane and form an interfacial film during the freezing-thawing process. This explanation is nearly similar to that previously cited by Moussa *et al*. (2002) who demonstrate that distruption of LDL structure during freezing –thawing process causes liberation of phospholipids and triglycerides and suggest that adsorption and gelation of LDL apporoteins around the spermatozoa membrane could form a protective film against ice crystals generated during freezing. Moussa *et al*. (2002) and Bergeron *et al*. (2004) also reported that LDL reduced the alternation of spermatozoa plasma membrane structure during freezing-thawing process. Furthermore, low-density lipoproteins provided a protective mechanism either through the stabilization of membrane or by the replacement of membrane phospholipids that are lost during the cryopreservation process. In addition, Quinn *et al*. (1980) reported that phospholipids could form a protective film at the surface of spermatozoa membranes.

Another hypothesis is that LDL prevents the loss of membrane phospholipids, which increases the resistance of spermatozoa to cold shock (Parks and Graham 1992), saturated fatty acids, crystallize in a more regular and ordered form in comparison to PUFA as temperatures decrease. Therefore, the fatty acid composition of the phospholipids is important because the ratio of saturated fatty acid (SFA)/polyunsaturated fatty acid (PUFA) tends to render sperm membrane less susceptible to cold shock. The egg yolk presented in control extender contains generally 50% dry matter, 66% of which are LDL, consequently it means that control extender naturally contains 6.6 % (w/v) LDL, this concentration is slight lower than in our optimal LDL extenders, which achieve superior motility parameters, this suggests that egg yolk could contain some deleterious components which are potent to reduce semen motility (Moussa *et al*., 2002), such as HDLs (high-density lipoproteins) (Hu *et al*., 2011). Pace and Graham (1974) also reported, that granules of yolk had a harmful effect on bulls spermatozoa post-thaw motility. Watson and Martin (1975), in another study with ram spermatozoa also

corroborated this assessment. The same results about harmful effect of HDL on boar spermatozoa were cited by (Demianowicz and Strezek, 1996). Similarly Vera-munoz *et al*. (2009) revealed that LDL extender is less complex in chemical composition than the standard egg yolk extender, which could explain the protective effect of low-density lipoproteins, especially on the plasma membranes of spermatozoa that have been subjected to cryopreservation. Another explanation about the protective mechanism of LDL during freeze–thaw process was cited by Manjunath *et al*. (2002), they showed that the low-density fraction (LDF) once isolated from the egg yolk interacts with the bovine seminal plasma (BSP) proteins, which may prevent their detrimental effect on sperm membrane.

Therefore, continuous exposure of sperm to seminal plasma that contains BSP (BSP proteins: BSP-A1/A2, BSPA3 and BSP-30-kDa) proteins may damage the sperm membrane by removing lipids and rendering the membrane very sensitive to storage in the liquid or frozen states or considering that the major proteins of BSP bind to the sperm surface at ejaculation and stimulate cholesterol and phospholipids efflux from the sperm membrane. The interaction between BSP and LDL would prevent lipid efflux from the sperm membrane moreover, this interaction is rapid, specific and saturable. The present results indicate that LDL extracted from hen egg yolk can replace whole egg yolk successfully in Awassi ram semen extenders. The optimal concentration of LDL for cryopreservation of ram's semen would be 8 %. LDL could be a suitable component in ram's extender; however, field studies should carried out to investigate its effect on fertility and lambing rates. Ultimately, other researches are needed to evaluate and understand the respective roles of LDL in freezing semen of other animal species in Syria.

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استخدام تراكيز مختلفة من الليبوبروتينات منخفضة الكثافة لتطوير محلول تمديد جديد للسائل المنوي عند كباش العواس

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الملخص

نظرا لأهمية تطوير محاليل تمديد السائل المنوي لكباش العواس لتحسين معدلات الخصوبة الناتجة عن التلقيح الاصطناعي بالسائل المنوي المجمد، فقد هدفت الدراسة الدراسة إلى تقييم فاعلية استخدام الليبوبروتينات منخفضة الكثافة (LDL) بديلا عن صفار البيض الكامل في محلول تمديد السائل المنوي لذكور أغنام العواس.

استخدمت أربعة كباش عواس خلال الموسم التناسلي لجمع السائل المنوي بواسطة المهبل الاصطناعي، واستخدمت أربعة محاليل لتمديد السائل المنوي: الأول سترات الصوديوم و20 % صفار بيض كامل واعتمد كمحلول شاهد، واستبدل صفار البيض الكامل بثلاثة تراكيز (6 % و8 % و10 %) من جزيئات LDL في المحاليل الثلاثة الأخرى.

قيمت حيوية الحيوانات المنوية باستخدام مجهر ضوئي متغاير الطور وقدرت نسبة الحيوانات المنوية الحية والميتة باستخدام صبغة إيوزين – نيكروزين، كما استخدم جهاز (CASA) لتقييم معايير الحركية بعد الإذابة.

أظهرت النتائج تفوقا معنويا ($P<0.001$) لمحلول التمديد LDL 8 % في صفة الحركية، مقارنة مع بقية المحاليل المختبرة حيث بلغ متوسط الحركية فيه بعد التبريد 73 % مقابل 67.8 % و68.71 % و 68.6 % في محاليل الشاهد، LDL 6 %، LDL 10 % على التوالي، كما كانت الحركية 62.66 % في محلول LDL 8 % متفوقة معنويا بعد التجميد مقارنة مع 54.18 % في محلول الشاهد و51 % و56.4 % في محلولي LDL 6 % و LDL 10 %.

كما أظهرت نتائج التحليل بجهاز CASA بعد إذابة التجميد تفوقا معنويا ($P<0.001$) لمحلول LDL 8 % مقارنة مع محلول صفار البيض الكامل 20 % في مؤشرات الحركية (60.67 % مقابل 53.69 %) والحركة التقدمية الأمامية (42.38 % مقابل 39.89 %).

يستنتج من الدراسة أن استخدام جزيئات LDL المستخلص من صفار البيض بتركيز 8 % في محلول التمديد قد حسن من حركية الحيوانات المنوية بشكل أفضل من استخدام صفار البيض الكامل وبالمقارنة مع بقية التراكيز الأخرى.

الكلمات المفتاحية: السائل المنوي، كباش العواس، الليبوبروتينات منخفضة الكثافة، محاليل التمديد.