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Study on Low Density Lipoprotein of Duck Egg Yolk as Cryoprotectants of Holstein Bulls Semen Cryopreservation

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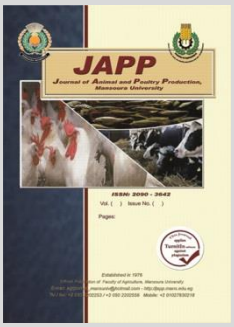

ABSTRACT

The study's goal was to evaluate the sperm qualities and fertility of Holstein bulls' cryopreserved semen in tris-extender that included various quantities of duck low density lipoproteins (DLDL) in place of whole duck egg yolk. Ejaculates with more than 70% motility and normal morphological characteristics before any supplemented. Semen was collected twice weekly from four Holstein bulls utilizing vaginal artificial means.Semen was dived to five groups after being diluted represented as, G1 contains 20% duck egg yolk, G2 contains 4%, G3 contains 6%, G4 contains 8%, and G5 contains 10% duck low density lipoprotein. A diluted sperm was inserted in 0.25 ml straws, which were then cooled and stored at 5 °C for four hours before being frozen in liquid nitrogen and stored at -196 °C. Data revealed that, G5- 10% was improvement significantly ($P \leq 0.05$) in all semen characteristics than other groups.In conclusion the use low density lipoprotein of duck egg yolk at level 10% in semen extender improves sperm characteristics and fertility rate in Holstein bull semen.

Keywords: Holstein, semen, lipoprotein, characteristics, fertility

INTRODUCTION

The efficiency and sustainability of animal production for food must urgently be improved in view of the growing global population. For this problem to be solved, increasing animal fertility is essential, especially in cows. Particularly in dairy farming, where a single sample of male semen is used to induce pregnancy in a number of females, Artificial insemination (AI) contributed significantly to the development of genetic potential. (Perumal, 2018). When performing artificial insemination (AI) on dairy cow, cryopreserved sperm is frequently used.

Bull sperm cryopreservation is currently thought to be acceptable, despite the fact that existing approaches only yield a 50% recovery rate after thawing (Layek *et al*, 2016). An essential component of semen processing for AI is the semen extender. Using frozen semen could lower the danger of breed and population extinctions and cut shipping costs (Stanishevskaya *et al*, 2021). The effectiveness of sperm cryopreservation is determined by a number of parameters, including the type of extender utilized, the concentration of cryoprotectant, the freezing techniques, the length of the equilibration period, and the temperatures used (Purdy, 2006). The effective administration of semen collection, storage, and utilization is essential for an AI program to be successful (Leboeuf *et al*, 2000).

Although egg yolk is required for cryopreservation and functions as a cryoprotectant, egg yolk from other bird species has been shown to improve the quality of cryopreserved semen (Anand *et al*, 2014). The usefulness of cooling, frozen sperm was thought to be maintained by egg yolk, a crucial component. Commonly used as a cryoprotectant substance for sperm preservation in various species is domestic chickens' egg yolk (Sansone *et al*,2000).

By limiting the production of ice crystals during the freeze-thaw process, egg yolk (depending on whether it includes cholesterol, phospholipids, and low-density lipoprotein) safeguards sperm plasma membrane integrity in the face of cold shock (Hu *et al*, 2010). In recent years, there has been a lot of criticism to the use of egg yolk due to the increased risk of microbial contamination and the consequent production of endotoxin, which may reduce the potential fertilizing power of spermatozoa (Aires *et al*, 2003). When Egyptian buffalo bull spermatozoa are frozen and subsequently thawed, the quality and fertility rate are increased by utilizing duck egg yolk (DEY) rather than chicken egg yolk in the extension (El-Sharawy *et al*, 2015).

During the freezing and thawing processes, reactive oxygen species (ROS) are continually released process by immature and damaged sperm. Losses of cholesterol from the plasma membrane (PM) are one of the key alterations that occur during cryopreservation (Srivastava *et al*, 2013). The structure of the extreme cryopreservation conditions has the greatest influence on the plasma membrane (PM). (Tapia *et al*, 2012). Additionally, the proper physiological operation of other sperm components including the acrosome and mitochondria depends on PM state (Breitbart *et al* 2005 and Amaral *et al*, 2013). One element that reduces the quality of semen is high density lipoprotein HDL in egg yolk, which leads to the outflow of cholesterol from the sperm plasma membrane and changes the fluidity of the sperm, increasing its sensitivity to cold shock (Amirat *et al*, 2005).

After freezing, low density lipoprotein (LDL) maintains the functional characteristics of bull spermatozoa at higher levels (Simonk *et al*., 2019). The main factor causing the semen quality to decline in comparison to fresh semen during the freeze-thaw process has been linked to an excess production of reactive oxygen species (Shiva *et al*, 2010). Naturally, cholesterol plays a significant part in the physiological processes of the plasma membrane (Horokhovatskyi *et al*, 2016).

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The goal of this study was to demonstrate the cryoprotective effects of different Duck low density lipoproteins (DLDL) for improved cryopreserved semen quality (motility, livability, plasma membrane integrity, and intact acrosome spermatozoa) and fertility in Holstein semen bulls.

MATERIALS AND METHODS

This study was conducted at the International Livestock Management Training Center (ILMTC) in Sakha, which is affiliated with the Animal Production Research Institute, the Agricultural Research Center, and the Ministry of Agriculture.

Animals used in experiments:

The present study used four sexually mature, healthy Holstein bulls that were 3–4 years old at the beginning of the trial and weighed an average of 550–600 kg.

They were kept separately in semi-open sheds and fed the Animal Production Research Institute's (1997) recommended ration, with access to water all day. All of the bulls were in good health and parasite-free both internally and externally.

Semen collection:

Throughout the experiment, semen ejaculates were taken twice a week between 8 to 10 am. Every bull served as a teaser for mounting, and an artificial vagina was used to remove one ejaculate from each bull once a week. Collected ejaculates were pooled in order to have enough semen for a duplicate and eliminate the bull effect. Prior to dilution, the sperm was placed in a 37 °C water bath for 10 minutes. Following that, characteristics of newly diluted sperm were evaluated as (Progressive motility, live sperm, abnormal sperm, membrane and acrosome integrities).

Experiment extender preparation:

3.025 gm Tris (hydroxymethyl amino methane), 1.675 gm citric acid, 0.75 gm glucose, 0.25 gm lincomycin, 7 mL glycerol, 0.005 gm streptomycin, and 20% duck egg yolk in 7 ml glycerol, all of the components were fully dissolved in 100 mL of bi-distilled water. Tris-extender control was diluted in lieu of various concentrations of duck low density lipoprotein (DLDL) at levels of 4, 6, 8, and 10% as (G2, G3, G4, and G5), respectively, and entire duck egg yolk in accordance with Moussa *et al* (2002) approach. Semen was diluted with all extenders at rate of 1:20. All of the substances used in this study were purchased from (Sigma-Aldrich, Germany).

Filling the straws and freezing them:

Extended sperm was always stored in an ice bath to maintain a temperature of 5 oC while sperm packed in straws was kept in a chilled ice chest. A semen filling machine was used to package the prolonged semen in (.025ml, 20×106 motile sperm) French straws after the equilibration phase. The expanded packed semen was put horizontally in static nitrogen vapor 4 cm above the surface of liquid nitrogen (-90 : -100C) for 10 minutes in a processing container. After that, the straws were vertically put in a metal canister and totally immersed in liquid nitrogen for storage at -196°C.

Semen evaluation:

To thaw the frozen semen, the frozen straws were put in a 37° C water bath for 30 seconds.

The percentages of progressive motile spermatozoa, living sperm, defective sperm, plasma membrane, and acrosome integrity were estimated using a hot microscope stage for post-dilution, equilibrated, and thawed semen (37oC). The percentage of sperm progressive motility was measured by introducing one drop of raw sperm to a test tube containing 2 ml warm physiological saline (0.9% NaCl) and suspended in a 37°C water bath.

After gently agitating the mixture, a drop of semen was taken from the test tube using a warm Pasteur pipette and deposited on a worm slide. The drop was immediately examined with a 40x objective while being protected by a wormed cover slide.

Amman and Hammersted (1980), the percentage of spermatozoa travelling forward across the field of vision with a normal, vigorous swimming motion was used to grade the samples, according to the claim.

A smear was created using a drop of freshly ejaculated sperm, which was subsequently stained with an Eosin Nigrosine solution produced in accordance with Hackett and Macpherson (1965) instructions and dried in warm air.

In order to determine the proportion of living spermatozoa at a magnification of x10, 100 spermatozoa were counted in numerous tiny regions (400x), the anomaly discovered by a manual tally counter during a live-dead count. Each slide contained 100 spermatozoa, which were counted. After dilution, equilibration, freezing, and thawing semen, the hypotonic swelling test (HOST), introduced by Jeyendran *et al*., (1984), was used to assess spermatozoa plasma membrane integrity.

In one hundred milliliters of distilled water, The HOST solution was made by dissolving 0.735 g of sodium citrate with 1.351 g of fructose. 500 µl of the pre-warmed (37 C) HOST solution was combined with 50 µl of the semen sample. to perform the HOST experiment, which was then incubated at that temperature for 30-45 min. After incubation, by distributing 15µ l of well mixed material over a heated slide (37°C) and covering it with a cover glass, sperm swelling was monitored. The slide was then inspected under a 400x magnification light microscope before being recorded. On average 200 spermatozoa were seen on each slide. The presence or absence of a coiled tail determined whether the spermatozoa were positive or negative.

Normal spermatozoa swell and then expand in volume while under hypo-osmotic stress brought on by water incursion. The tail regions displayed the swelling impact more visibly because it appeared that the membrane enveloping the tail fibers in sperm was less tightly linked than the membrane surrounding the head. (Ballester *et al*., 2007); (Foote and Kaproth, 1997) and (Lodhi *et al*., 2008). However, sperm acrosomal integrity percentages were calculated using Watson's (1975) Giemsa stain method and the microscope examination was done making use of an oil immersion lens (x-1000).

Fertility trail:

250 Holstein cows in total in heat were divided into 5 groups. Each group underwent insemination using frozen/thawed semen extended with various types of extenders (G1 control 20% Duck egg yolk (DEY) but G2 is 4%, G3 is 6%, G4 is 8%, and G5 is 10%. Duck low density lipoprotein DLDL), all of which were administered in random doses. Before insemination, the frozen sperm was thawed at 37° C for 30 seconds. The sperm was implanted in the uterine body towards the anterior end of the cervix using the recto-vaginal technique and the universal insemination pistol. 8–14 hours

after the start of the estrous behavior stage, a single insemination dosage (0.25 ml French straw) was administered to each female. 45–60 days after insemination, insemination was detected by rectal palpation. Based on diagnoses of pregnancies 50 days after insemination made by rectal palpation, the conception rate was estimated.

Statistical evaluation:

SPSS (2008) changed the general linear model's technique for the user's manual, was used to statistically evaluate the data along with one-way ANOVA. The Duncan test in the SPSS software was used to discover significant differences between means (Duncan, 1955).

RESULTS AND DISCUSSION

Results:

Sperm characteristics post-diluted semen:

The percentages of progressive motility, livability, abnormalities, and acrosome integrity of spermatozoa were considerably improved (P≤0.05) in G5 (10% DLDL) compared to G1 (control) and other groups, according to the results shown in Table 1. While membrane integrity was considerably enhanced (P≤0.05) in G5 (10% DLDL), G4 (8% DLDL), and other groups, progressive motility was not.

Table 1. Effect of Duck egg low density lipoprotein (DLDL) extender on sperm characteristics in Holstein bull semen post-diluted.

Sperm parameters (%)	DEY (G_1) 20%	Levels of DLDL 4% (G_2)	6% (G_3)	8% (G_4)	10% (G_5)	S.E
Progressive motility	65.30^{B}	65.41^{B}	66.25^{B}	68.75^{AB}	72.08^{A}	±1.22
Live spermatozoa	78.70^{C}	78.75^{C}	79.25^{C}	82.20^{B}	85.25^{A}	±0.81
Abnormality	11.60^{A}	11.66^{A}	11.50^{A}	10.00^{AB}	9.00^{B}	±0.55
Membrane integrity	75.00^{B}	75.08^{B}	76.83^{B}	78.75^{A}	81.33^{A}	±0.99
Acrosome integrity	78.60^{C}	79.08^{C}	79.50^{C}	82.75^{B}	85.70^{A}	±0.92

A, B, and C: Significant mean differences in the same row at (P≤0.05). G1-G5 = Extender 1-5.DEY= Duck egg yolk. DLDL= Duck egg low density lipoprotein.

Sperm characteristics after equilibration semen:

Table 2 shows that progressive motility, viability, abnormalities, acrosome, and membrane integrity were considerably (P0.05) improved in G5-extended sperm compared to control and other groups. Simultaneously, viable spermatozoa dropped considerably (P0.05) in G2 compared to other groups, whereas progressive motility was maintained in G5as compared to other groups.

Table 2. Effect of Duck egg low density lipoprotein (DLDL) extender on sperm characteristics in Holstein bull semen post- equilibrated.

Sperm parameters (%)	DEY (G_1) 20%	Levels of DLDL 4% (G_2)	6% (G_3)	8% (G_4)	10% (G_5)	S.E
Progressive motility	56.16^{B}	54.91^{B}	56.60^{B}	59.25^{AB}	62.83^{A}	±1.34
Live spermatozoa	75.42^{BC}	74.50^{C}	76.33^{BC}	77.41^{B}	81.10^{A}	±0.87
Abnormality	13.66^{AB}	14.00^{A}	12.50^{BC}	11.50^{CD}	10.75^{D}	±0.46
Membrane integrity	68.16^{C}	67.25^{C}	68.92^{BC}	72.83^{AB}	76.92^{A}	±1.44
Acrosome integrity	74.16^{C}	73.70^{C}	76.00^{B}	77.70^{AB}	80.92^{A}	±1.28

A, B, and C: (P≤0.05) significant mean differences in the same row. G1-G5 =Extender 1-5 DEY= Duck egg yolk. DLDL= Duck egg low density lipoprotein.

Sperm characteristics after thawing semen:

Table 3 shows that the percentages of progressive motility, viability, membrane and acrosome integrity of spermatozoa after freezing semen were substantially (P≤0.05) higher in G5, although the abnormality was significantly (P≤0.05) lower in G5.

Table 3. Effect of Duck low density lipoprotein (DLDL) extender on sperm characteristics in Holstein bull semen post- thawed.

Sperm parameters (%)	DEY (G_1) 20%	Levels of DLDL 4% (G_2)	6% (G_3)	8% (G_4)	10% (G_5)	S.E
Progressive motility	45.90^{B}	45.08^{B}	46.50^{B}	50.25^{AB}	53.58^{A}	±1.60
Live spermatozoa	64.20^{BC}	61.58^{C}	63.25^{BC}	67.25^{AB}	70.25^{A}	±1.67
Abnormality	19.58^{A}	17.00^{AB}	15.41^{B}	15.33^{B}	13.16^{C}	±0.75
Membrane integrity	57.60^{C}	56.33^{C}	58.50^{C}	62.08^{B}	66.16^{A}	±1.62
Acrosome integrity	66.70^{BC}	65.25^{BC}	67.25^{B}	69.58^{AB}	74.16^{A}	±1.67

A, B and C: Significant mean differences in the same row at (P≤0.05). G1-G5=Extender 1-5. DEY= Duck egg yolk. DLDL= Duck egg low density lipoprotein.

Fertility trail:

Table 4 data revealed that conception rate was considerably (P0.05) higher for Holstein cows inseminated with semen prolonged by G5 (DLDL 10%) (74.5%) than for those inseminated with other DLDL-extenders (4, 6, and 8%) and control (20% DEY), which were 62%, 66%, 71.1%, and 63.6%, respectively.

Table 4. Effect of levels of duck low density lipoprotein (DLDL) on conception rate of Holstein cow.

Item	DEY (G_1) 20%	Levels of DLDL 4% (G_2)	6% (G_3)	8% (G_4)	10% (G_5)
Inseminated cows	55	45	50	45	55
Non-conceived cows	20	17	17	13	14
Conceived cows	35	28	33	32	41
Conception rate (%)	63.6	62	66	71.1	74.5

Discussion:

The purpose of this study was to assess the cryoprotective effects on Holstein bull sperm of various concentrations of LDL isolated from Duck egg yolk source. This study identified the ideal levels of duck low density lipoprotein (DLDL) for preserving bull sperm under various

situations (thawing, equilibration, and dilution). The current study's data unequivocally show that DLDL 10% concentration greatly boosted sperm motility, sperm life percentage, sperm plasma membrane, acrosomal integrity, and sperm motility, and reduced sperm abnormalities (coiled tail). Comparing the 10% DLDL to the DEY 20% control, 4, 6, and 8% DLDL, it was found to be more suited for the preservation of Holstein bull semen.

The progressive sperm motility revealed that, of the three concentrations tested, 15% DEY in extender offered the highest cryoprotective effect for Egyptian buffalo sperm (El-Sharawy *et al*, 2015). Clulow *et al*. (2007) showed that freezing stallion and Nili-Ravi buffalo sperm in extenders containing duck egg yolk rather than chicken egg yolk increased sperm motility metrics. Waheed *et al*., (2012) discovered that storing sperm in extenders containing duck egg yolk increased sperm motility characteristics.

According to Bathgate *et al.,* (2006), the fundamental components of Chicken egg yolk (CEY) and Duck egg yolk (DEY) did not alter, but the ratios of fatty acids and phospholipid differed. In comparison to chicken egg yolks, duck egg yolks contained higher monounsaturated fatty acids. In addition, phosphatidylinositol was more abundant in duck egg yolk than in CEY. Moussa *et al*., (2002), on the other hand, revealed that 8% DLDL was the optimal concentration for freezing bovine sperm. Additionally, according to Tonieto *et al*. (2010), extenders containing 100 mM trehalose with 8% LDL may preserve the post-thawing motility and membrane integrity of frozen ram sperm exactly as successfully as cryoprotectant extenders like EY and glycerol. According to Silva *et al*., (2014), 8% LDL is preferable. for cryopreserving ram sperm and that LDLs have cryoprotectant capabilities for ram sperm. Canine spermatozoa cryopreserved in LDL maintained flagellar plasma membrane integrity better than those cryopreserved in EY, according to Bencharif *et al*., (2008).

LDL is integrated, according to Bergeron *et al*., (2004) and Moustacas *et al*., (2011) or attached to the plasma membrane, replenishing that lost through normal outflow and, as a result, improving membrane stability and safeguarding the sperm against cold shock, may help to explain the phenomenon. In comparison to chicken egg yolks, duck egg yolks contained higher monounsaturated fatty acids. In addition, Duck egg yolk had a greater phosphatidylinositol concentration than chicken egg yolk (Rawash *et al*., 2020). Furthermore, it has been proven in bovine that LDL protects sperm membranes by interacting with seminal plasma proteins, preventing them from promoting cholesterol efflux of the membrane and therefore causing capacitation, which is undesirable during cryopreservation (Bergeron *et al*., 2004). According to Briand-Amirat *et al*., (2013), LDL fuses with sperm membranes before freezing boosting its resistance to cold shocks and allowing it to continue to inhibit ice crystal formation after freezing

The findings Bencharif *et al*. (2008) revealed that the medium containing 10% LDL was effective, protects acrosome integrity the best. This protection may be caused directly by an exchange or repair of the phospholipids that make up the acrosomal membrane, or because of the filtering action of membrane dialysis, the medium may contain less progesterone than egg yolk.

The progesterone found in egg yolks aids in the capping of cow spermatozoa. (Witte and Schafer- Somi, 2007), 83%–89% of low-density protein is made up of lipids, while 11%–17% of it is protein. The connection between proteins and lipids is disrupted and protein interaction is boosted throughout the cryopreservation process. Ultimately, the freezing and thawing processes have changed the composition of LDL. About 69% of the lipids in LDL are triglycerides, 26% are phospholipids, and 5% are cholesterol.

To shield the sperm from the potentially dangerous ice crystals, these triglycerides and phospholipids are released into the extender, resulting in the formation of an apoprotein gel. Furthermore, phospholipids are produced and create a barrier over the sperm's surface to protect it from cryoinjury or cryodamage (Hu *et al*, 2006).

CONCLUSION

Based on the findings, 10% Duck low density lipoprotein (DLDL) was added to the semen extender, which increased the quality of the features of the semen. (Such as sperm membrane livability, motility, and acrosomal integrity), decreased abnormality during diluted, equilibration and after thawing, and increased conception rates. It was determined that more research must be done to determine the precise functions of low-density lipoprotein (LDL) proteins and lipids as well as to detect and isolate the harmful components found in the entire egg yolk (EY).

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دراسة على كفاءة الليبوبروتينات منخفضة الكثافة لصفار بيض البط كمادة مضافة لمخفف السائل المنوى فى الحالة التجميدية والاخصابية لطلائق الهولشتين

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الملخص

تهدف الدراسة إلى تقييم جودة خصائص السائل المنوي والقدرة الاخصابية لطلائق الهولشتين باستخدام مستويات محتلفة من البروتينات الدهنية منخفضة الكثافة لصفار بيض البط بدلا من صفار البيض الكامل للبط. حيث تم جمع السائل المنوي مرتين أسبوعيا من عدد 4 طلائق هولشتين باستخدام المهبل الصناعى. تؤخذ القذفة ذات خصائص أعلى من 70 ٪ للخصائص المورفولوجية الطبيعية قبل أي اضافة . تم تقسيم السائل المنوي إلى خمس مجموعات بعد تخفيفه متمثلة فى المجموعة الاولى (الكنترول) تركيز 20 % من صفار بيض البط ، أما كل من المجموعة الثانية تركيز 4 ٪ ، المجموعة الثالثة تركيز 6 ٪ ، المجموعة الرابعة 8 ٪ و المجموعة الخامسة تركيز 10 ٪ من البروتين الدهني منخفض الكثافة . تم وضع السائل المنوى المخفف فى الثلاجة على درجة حرارة 5 درجة مئوية لمدة اربع ساعات ثم تعبئتة في اقصيبات بلاستيكية بقطر 0.25 ملم ثم يتم تجميدها فى النيتروجين السائل عند درجة -196 درجة مئوية. أظهرت النتائج أن المجموعة الخامسة تركيز 10 ٪ قد أدت الى تحسين خصائص السائل المنوى عند مستوى ($P\leq0.05$) عن باقى المجموعات.الخلاصة: استخدام البروتين الدهني منخفض الكثافة لصفار بيض البط عند تركيز 10 ٪ في مخفف السائل المنوي أدى الى تحسين صفات الحيوانات المنوية ومعدلات الخصوبة لطلائق الهولشتين.