Journal of Applied Veterinary Sciences, 7 (3): 86-93 (July, 2022).
ISSN: Online: 2090-3308, Print: 1687-4072
Journal homepage : https://javs.journals.ekb.eg

Cysteine enhance Holstein bull's sperm assessment quality and conception rate when added to duck egg yolk low-density lipoproteins

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ABSTRACT

Spermatozoa undergo a significant transformation during the cryopreservation process, including oxidative stressors during dilution, cooling, equilibration, or freezing and thawing stages that postpone their motility and viability. When duck egg yolk is added to low-density lipoproteins (LDL), which has a considerable cryoprotective effect, the sperm is successfully protected from cold shock and the lipid-phase transition during the freeze-thaw process. The amino acid cysteine, on the other hand, functions as a precursor of intracellular glutathione (GSH). The purpose of the current study was to examine the effects of employing 10 percent LDL duck egg yolk, with or without cysteine, for Holstein bull spermatozoa and to use cryopreserved straws in fertility trials to verify the findings. According to experimental findings, 10mM cysteine applied to 10% LDL duck.

Original Article:
DOI:https://dx.doi.org/10.21608/javs.2022.141246.1152
Received :30 May, 2022.
Accepted :02 July, 2022.
Published in July, 2022

J. Appl. Vet. Sci., 7 (3): 86-93.

Keywords: Cysteine, Duck egg Yolk, Holstein's Semen, LDL.

The spermatozoa undergo chemical, toxic, osmotic, thermal, mechanical, and oxidative stressors during dilution, cooling, equilibration, or freezing and thawing stages of semen cryopreservation, which reduces their motility and viability **(Hussein, 2018).** Therefore, biological membranes exhibit a behaviour known as the membrane transition phase, which makes polyunsaturated fatty acids potentially vulnerable to reactive oxygen species (ROS), causes sperm membrane lipid peroxidation (LPO), damage to proteins, DNA fragmentation, and enzyme inactivation, and reduces post-thawing sperm motility and bull semen viability **(Gualtieri *et al*., 2014).**

According to research, egg yolk's favourable involvement in sperm cryopreservation can be ascribed to the phospholipids, cholesterol, and low-density lipoproteins (LDL) content, all of which successfully protect sperm from cold shock.

In this experiment we tried to study the effect of another source of LDL such as duck egg yolk combined with two different levels of cysteine on Holstein bull's spermatozoa.

MATERIALS AND METHODS

Animals and semen collection

A total of five mature Friesian bulls (600-700±50kg) aged between 3-4 years old were used in the present study. They were housed individually under semi-open sheds at the International Livestock Management Training Center (ILMTC), Sakha. Belonging to Animal Production Research Institute, Agricultural Research Center, Egypt. All bulls were healthy, allowed to drink freely all the day. A total of 30 ejaculates more than 70% mass motility were collected twice weekly through the experiment at 8.00 to 10.0 am, for five successive weeks, by using an artificial vagina; then taken immediately to the laboratory for semen evaluation and processing. The collected ejaculates were pooled in order to have sufficient semen for a replicate and to eliminate the bull effect. The semen was given a holding time for 10 min at 37^{o}C in the water bath before dilution.

Experimental procedures

1. Semen evaluation

1.1. Progressive sperm motility (%)

It was estimated by adding one drop of raw semen to a test tube containing 2 ml warm physiological saline (0.9% NaCl) and suspended in 37°C water bath. The mixture was gently shaken and a drop of semen was taken from the test tube with a warm Pasteur pipette and placed on a worm slide. The drop was covered by a wormed cover slide and immediately examined under the 40x objective lens. The samples were graded according to the percentages of spermatozoa moving forward motion across the field of vision with normal vigorous swimming motion **(Hussein, 2018)**.

1.2. Live sperm (%)

Immediately after collection, a smear was made from a drop of freshly ejaculated semen and stained by eosin negrosin mixture prepared as described by **Hancock, (1951)** then dried by warm air. The percentage of live spermatozoa was calculated from total number 100 spermatozoa counted in different microscopic fields under magnification of 400x.

1.3. Abnormal sperm (%)

The same smear prepared for live-dead count was also used for estimation the incidence of abnormal spermatozoa. Live and abnormal sperm percentage were counted using hand tally counter. Accounting of 100 spermatozoa were made in each slide **(Vasan, 2011)**.

1.4. Plasma membrane integrity (%)

The plasma membrane integrity of spermatozoa was assessed using the hypotonic swelling test (HOST) as described by **Jeyendran *et al.*, (1984)** after dilution**,** equilibration and freezing and thawing semen. HOS solution was prepared by dissolving 0.735gm of sodium citrate and 1.351gm fructose in 100ml distilled water. The HOS assay was performed by mixing 50µl of the semen sample to 500µl of the pre-warmed (37°C) HOS solution and incubated at 37°C for 30-45 min. After incubation, sperm swelling was assessed by placing 15µl of well-mixed sample on a warm slide (37°C) which was covered with a cover glass before being observed under light microscopy at 400x magnification. At least 200 spermatozoa per slide were observed. The spermatozoa were classified as positive or negative based on the presence or absence of coiled tail.

Normal spermatozoa, when exposed to hypo-osmotic stress due to the influx of water, undergo swelling and subsequent increase in volume. The sperm plasma membrane surrounding the tail fibers appears to be more loosely attached than the membrane surrounding the head, so, the tail regions show the swelling effect more clearly (**Ballester *et al.*, 2007 and Lodhi *et al.*, 2008).**

1.5. Acrosome integrity (%)

Acrosome integrity was determined by using a Giemsa stain procedure as described by **Watson, (1975).**

2. Semen extender

The cryopreservation extenders were prepared including Tris-based extender (control) by dissolving 3.025 gm Tris, 1.675gm citric acid, 0.75 gm glucose, 7ml glycerol, 0.25gm lincomycin, 0.005gm streptomycin and 20% duck egg yolk (DEY) in 100 ml bi-distilled water. The other extenders for the first experiment differed only with extender for the control by addition of 10% DEY Low density Lipoprotein (LDL). In second experiment, Cysteine concentrations were added to Tris-based control extender (10% DEYLDL) at concentrations of 5, 10, 15 and 20 mmol (mM).

3. Extraction of LDL from duck egg yolk

Low Density Lipoproteins were extracted from yolk according to **Moussa *et al.* (2002)**. Fresh duck eggs were manually broken. Yolks were separated from the albumen. Each yolk was carefully rolled on a filter paper (Whatman International Ltd., Maidstone, Kent , UK) to remove chalazes and traces of albumen adhering to the vitelline membrane.

The vitelline membrane was then disrupted with a scalpel blade and yolk was collected in a beaker, which is placed, on ice. Egg yolk was diluted twice with an isotonic saline solution (0.17M NaCl) (w/w) and stirred for 1 h before centrifugation at 10.000xg for 45min at 10°C. The supernatant (plasma) was centrifuged again for complete removal granules. The plasma was mixed with 45 % ammonium sulfate (Sigma-Aldrich, Germany) to precipitate livetins. After 1h of stirring at 4°C, the mixture was centrifuged at 10,000xg for 45 min. The sediment was discarded and the supernatant was dialyzed against distilled water in order to eliminate ammonium sulfate. The solution was again centrifuged (10.000xg for 45min at 4°C) and the floating residue, rich in LDL, was collected.

Experimental Design

Experiment 1:

This experiment was held to evaluate the best two concentrations of cysteine on semen characteristics by using of 5, 10, 15 and 20mM Cysteine to 20% duck egg yolk cryopreservation extender media and examined after dilution, equilibration at 5°C and after thawing. The evaluation was done for progressive motility, live sperm, membrane integrity, abnormality and acrosome integrity.

Experiment 2

To assess the cryoprotective effect of 10% duck's LDL egg yolk on freezability and fertility of Holstein bull's semen. After semen evaluation, the pooled fresh semen was split into four equal fractions; one fraction was diluted with the Tris control extender containing 20% whole duck egg yolk (DEY). In the second fraction the 20% DEY was replaced with 10% LDL-DEY.

Experiment 3

Assessment of the cryoprotective effect of Cysteine added to 10% LDL-DEY on freezability and fertility of Holstein bull's semen to determine its cryoprotective effect. The amino acid, Cysteine, was added to Tris control extender (10% LDL-DEY) at the

concentrations 5 and10mM as a result for the first experiment.

Experiment 4

In this experiment about 120 Friesian cows were used in this study which artificially inseminated with all experimental groups using to confirm the efficiency of semen extenders used in this study. Insemination was done just after heat detection and one more time after 12h for the same female. The pregnancy detection was done by rectal palpation after 40 days of insemination.

Semen Processing and Freezing Procedure

Ejaculates processing more than 70 % visual motility and more than 85% normal sperm morphology were pooled. Semen was diluted at 37°C in experimental extenders in order to contain approximately 80x10^{6} motile spermatozoa/ml. The vial contained the extended semen were placed in a water bath at 37^{o}C for 10 min. After that freshly diluted semen characteristics were evaluated as (Progressive motility, live sperm, abnormal sperm, Acrosome and membrane integrities), then, diluted semen was divided into two parts, the first was evaluated the best two concentrations of cysteine to be added to semen extender, while the second part was placed into a refrigerator at 5°C for 2 h. for semen equilibration then used for filling and freezing in the straws.

At the end of the equilibration period the extended semen was packaged in 0.25 French straws using a semen filling machine. The extended packed semen was transferred into processing container and located horizontally in static nitrogen vapor 4 cm above the surface of liquid nitrogen (-90 - -100°C) for 10 minutes, then the straws were placed vertically in liquid nitrogen container for storage at -196°C. Calculations were made so that each straw contain 20 x 10^{6} motile sperm before freezing.

Thawing the Frozen Semen

After one day of freezing, the frozen semen was thawed by dipping the frozen straws into a water bath at 37^{o} C for 30 seconds, then the percentages of progressive motile spermatozoa, live sperm, abnormal sperm, acrosome integrity and plasma membrane integrity were estimated.

Statistical Analysis

The experimental data were statistically analyzed using the general model program **(SAS, 1999)**. Data were subjected to analysis of variance using one way (for DNA damage data analysis). The difference among means were tested using Duncan's multiple range test **(Duncan, 1955).** Also, the correlation coefficient analysis was done between the different parameters.

RESULTS

The results of the first experiment showed that 5mM and 10mM of cystine added to 20% DEY extender has the positive semen assessments. Progressive motility, live spermatozoa, plasma membrane integrity and acrosome integrity were totally enhanced with low sperm abnormalities just after dilution (Table 1) post equilibratin at 5^{o}C (Table 2) and after thawing (Table 3) for both 5mM and 10mM cystine over the other treated groups.

Table 1: Effect of different concentrations of Cysteine added to Holstein bull spermatozoa's extender, after dilution (Mean ± S.E):

Item	Control DEY 20%	Cysteine treatment				S.E.
		5mM	10mM	15mM	20mM	
Progressive motility (%)	70.00^{b}	76.00^{a}	78.50^{a}	72.00^{b}	71.50^{b}	±1.22
Live spermatozoa (%)	77.20^{b}	82.90^{a}	85.40^{a}	79.30^{b}	76.50^{b}	±0.99
Abnormality (%)	11.40^{ab}	8.70^{c}	6.80^{d}	10.20^{b}	11.80^{a}	±0.49
Membrane integrity (%)	74.30^{b}	80.40^{a}	81.70^{a}	77.20^{b}	74.50^{b}	±1.06
Acrosome integrity (%)	78.80^{b}	83.50^{a}	85.40^{a}	79.80^{b}	77.80^{b}	±0.90

A,B,C: The different superscripts within the same row are significant (P<0.05) among treatments.

However, the concentration of 10mM of cysteine has the best results over 5mM of cysteine. Current results reveled that after semen dilution with Tris 10%LDL-DEY based extender had better results of Holstein bull's semen parameters over control group as presented in Table (4). However, the combination of 10mM cysteine added to 10% LDL has the best sperm parameters once after dilution (Table 4). The best progressive motility (72.50%) was found in the treatment of 10% LDL+10mM cysteine over the other treatments and control (20%DEY) groups. Similar results were found for live spermatozoa (80.10%), plasma membrane integrity (77.6%) and Acrosome integrity (80.50%) in addition to its low proportion of sperm Abnormalities (10.60%) compared to other groups.

Table 2: Effect of different concentrations of Cysteine added to Holstein bull spermatozoa's extender, after equilibration at 5^0C (Mean ± S.E):

Item	Control DEY 20%	Cysteine treatment				S.E.
		5mM	10mM	15mM	20mM	
Progressive motility (%)	62.50^{b}	69.50^{a}	70.00^{a}	64.50^{b}	62.00^{b}	±1.30
Live spermatozoa (%)	71.40^{b}	77.10^{a}	79.30^{a}	73.30^{b}	71.00^{b}	±1.04
Abnormality (%)	15.50^{a}	12.10^{b}	10.90^{b}	14.00^{a}	15.30^{a}	±0.56
Membrane integrity (%)	67.10^{d}	74.30^{ab}	76.90^{a}	71.60^{bc}	70.00^{cd}	±1.20
Acrosome integrity (%)	72.70^{b}	78.70^{a}	79.40^{a}	74.20^{b}	72.40^{b}	±0.99

A,B,C: The different superscripts within the same row are significant (P<0.05) among treatments.

Table 3: Effect of different concentrations of Cysteine added to Holstein bull spermatozoa's extender, after thawing (Mean ± S.E):

Item	Control DEY 20%	Cysteine treatment				S.E.
		5mM	10mM	15mM	20mM	
Progressive motility (%)	44.00^{b}	53.00^{a}	54.50^{a}	47.00^{b}	46.50^{b}	±1.44
Live spermatozoa (%)	60.40^{b}	70.10^{a}	69.90^{a}	61.30^{b}	60.00^{b}	±1.81
Abnormality (%)	21.50^{a}	17.00^{cd}	15.80^{d}	18.50^{bc}	20.20^{ab}	±0.71
Membrane integrity (%)	58.80^{b}	68.10^{a}	67.50^{a}	62.00^{b}	60.30^{b}	±1.28
Acrosome integrity (%)	64.00^{b}	73.20^{a}	72.20^{a}	64.10^{b}	63.70^{b}	±1.51

A,B,C: The different superscripts within the same row are significant (P<0.05) among treatments.

Table 4: Effects of 10% DEY-LDL and Cysteine based extender on Post-dilution parameters of Holstein bull spermatozoa (Mean±S.E):

Item	Control DEY 20%	10% LDL	10%LDL+ 5mM Cysteine	10%LDL+ 10mM Cysteine	S.E.
Progressive motility (%)	60.74^{c}	63.60^{b}	65.30^{b}	72.50^{a}	±1.27
Live spermatozoa (%)	72.10^{c}	75.82^{b}	77.50^{b}	80.10^{a}	±2.12
Abnormality (%)	13.50^{a}	12.10^{ab}	11.65^{ab}	10.60^{b}	±0.78
Membrane integrity (%)	68.70^{b}	70.82^{ab}	72.64^{ab}	77.60^{a}	±1.64
Acrosome integrity (%)	71.50^{b}	75.82^{ab}	77.40^{a}	80.50^{a}	±1.66

A,B,C: the different superscripts within the same row are significant (P<0.05) among treatments.

After equilibration at 5^0C for two hours similar enhancement of semen parameters was continued for the 10%LDL-DEY over the control group. Whereas the treatment group of 10%LDL+10mM Cysteine has the best results of Holstein semen assessments over all treated groups (Table 5). After thawing the treatment of 10% LDL-DEY has the same respectable parameters over the control group. On the other hand as shown in table 6, the 10% LDL+10mM cysteine has the best progressive motility (54.60%), live spermatozoa (67.82%), plasma membrane integrity (63.80%), Acrosome integrity (66.82%) and low present of sperm abnormality (16.12%) over other treated and control groups.

The insemination study was presented in Table (7), the highest conception rate was performed by 10%LDL+10mM cysteine meanwhile the lowest conception rate was found for control group (66.7%).

Table 5: Effects of LDL and Cysteine based extender on Post-equilibration at 5°C parameters of Holstein bull spermatozoa (Mean ± S.E):

Item	Control DEY 20%	10% LDL	10%LDL+ 5mM Cysteine	10%LDL+ 10mM Cysteine	S.E.
Progressive motility (%)	52.50^{b}	53.45^{b}	54.50^{b}	59.00^{a}	±1.18
Live spermatozoa (%)	65.80^{c}	69.46^{b}	71.20^{b}	74.30^{a}	±1.56
Abnormality (%)	19.00^{a}	16.00^{ab}	14.30^{b}	13.70^{b}	±1.60
Membrane integrity (%)	62.10^{c}	63.36^{b}	65.60^{b}	70.30^{a}	±1.25
Acrosome integrity (%)	67.80^{bc}	70.36^{b}	72.20^{ab}	74.80^{a}	±1.74

A,B,C: the different superscripts within the same row are significant (P<0.05) among treatments.

Table 6: Effects of LDL and Cysteine based extender on Post-thawing parameters of Holstein bull spermatozoa (Mean ± S.E):

Item	Control DEY 20%	10% LDL	10%LDL+ 5mM Cysteine	10%LDL+ 10mM Cysteine	S.E.
Progressive motility (%)	47.43^{c}	49.50^{b}	51.30^{b}	54.60^{a}	±1.04
Live spermatozoa (%)	52.70^{c}	62.50^{b}	63.60^{b}	67.82^{a}	±1.41
Abnormalit+y (%)	23.90^{a}	20.36^{b}	18.60^{b}	16.12^{c}	±1.01
Membrane integrity (%)	56.00^{b}	58.36^{ab}	59.70^{ab}	63.80^{a}	±1.44
Acrosome integrity (%)	58.64^{b}	61.36^{ab}	63.09^{ab}	66.82^{a}	±1.75

A,B,C: the different superscripts within the same row are significant (P<0.05) among treatments.

Table 7: Conception rate of Friesian cows inseminated with frozen semen cryopreserved in different concentrations of Duck egg yolk LDL and Cysteine:

Item	No. of inseminated females	No. of conceived females	Conception rate (%)
Control (20%DEY)	30	20	66.7
10% LDL-	25	17	68
10% LDL+5mM CY	35	24	68.6
10% LDL+10mM CY	30	22	73.3

DISCUSSION

According to reports, several amino acids, particularly cysteine, shield a variety of animal cells, including sperm, from the damaging effects of freezing **(El-Sheshtawy *et al.*, 2008). AL-Ameri *et al.*, (2020)** discovered that cysteine (5Mm) had a larger proportion of individual motility relative to groups (cysteine 10Mm) and tryptophan (5 and 10 mM) at different durations, with similar cryopreservation outcomes. Individual motility was improved for 72 hours by the addition of 10mM cysteine to extender semen. On the other hand, according to **Khalili *et al.* (2010)**, Moghani ram semen combined with 10 and 15 mM cysteine has the best assessment parameters when compared to other groups. Progressive motility, viability, and plasma membrane integrity were all increased by adding 1.0 mM cysteine to the tris-citric acid extender.

When compared to the control group of buffalo bull spermatozoa, the motility obtained with L-Cysteine demonstrated improved post-thaw sperm motility, integrity of the plasma membrane (HOS +ve), and acrosome of extender containing 10% turkey or hen LDLs. The protection offered by LDL may be due to its interaction with the cell membrane, which may have been directly affected by an exchange or repair that increased the stability of the cell membrane during cryopreservation **(El-Badry *et al.*, 2015).** By generating layers that are too thin to shield the spermatozoa from contact with big ice crystals, the LDL extender primarily ensures improved cell viability with fewer physical damages **(Amirat *et al.*, 2005)**.

Numerous hypotheses explain how LDL acts as a cryoprotectant. By stabilising the membrane, LDL

cling to the sperm membrane and protect the sperm (**Peruma, 2018**). According to a second theory, phospholipids in LDL shield sperm by establishing a barrier on their surface or by replenishing phospholipids lost or destroyed during the cryopreservation process **(Anand *et al.,* 2014)**. According to a third way of protection, LDL allegedly captures the harmful proteins found in seminal plasma, enhancing the spermatozoa's ability to freeze (**Bergeron and Manjunath, 2006**). The fourth theory contends that egg yolk lipoproteins compete with harmful seminal plasma cationic peptides for attaching to the sperm membrane, protecting the sperm by preventing membrane lipid loss **(Manjunath, 2012)**.

Our findings of 10% DYLDL+10mM cysteine concur with those of **Khan *et al.* (2021)**, who observed that crossbred bulls had stronger freeze-thawed motility metrics than controls. The DNA integrity and sperm membrane functioning were improved when 4.8mmol of vitamin E and 7.5mmol of L-cysteine were added to freezing diluents. One of the amino acids, cysteine, functions as a precursor to intracellular glutathione (GSH). Due to its indirect radical scavenging abilities, it can easily pass-through cellular membranes, boosting Glutathione manufacture and defending the membrane's lipids and proteins (**Memon *et al*., 2015**).

Cysteine has cryoprotective effect on the functional integrity of axosome and mitochondria improving post thawed sperm motility in many species like ram (**Uysaland and Bucak, 2007**) goat semen (**Bucak and Uysal, 2008**) and bull semen (**Bilodeau, *et al*., 2001**). The data of **Memon *et al*., (2015)** established that cysteine supplementation resulted in higher overall efficiency of cooled and frozen boar goat semen. Supplementation with 5mM cysteine produces better results as compared to 10mM and 20mM concentration and control group. Slight improvements were observed in morphology and acrosome integrity of spermatozoa with the supplementation but no significant difference has been detected in both parameters between the groups.

Those results were confirmed by **Tuncer *et al*., (2021)** who found that addition of 2.5mM cysteine to the freezing medium played a protective role in sperm quality parameters against structural and functional damage of the freezing-thawing process. Finally our results of the fertility trail is the best way to confirm the efficiency of 10%LDL+10mM cysteine with the highest conception rate (73.3%) over other groups.

CONCLUSION

In conclusion, current data results revealed the promising use of 10% LDL-DEY which need more research specially when combined with 10mM of cysteine. This combination is the best of which enhance Holstein bull's spermatozoa assessment parameters that have been confirmed by fertility trails.

ACKNOWLEDGMENTS

We appreciate the financial support provided by FAO during the entire investigation period. Also, we thank S. Gborie, A. Leno, and A. Kamara for their support. All residents in the Koinadugu district who participated in this study are also recognized.

Declaration of Conflicting Interests

The authors declare that they have no conflict of interest.

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How to cite this article:
Y. S. Hussein*, S. H. Soliman, M.F. Saad and Entesar Z. Eid, 2022. Cysteine enhance Holstein bull's sperm assessment quality and conception rate when added to duck egg yolk low-density lipoproteins. Journal of Applied Veterinary Sciences, 7 (3): 86– 93.
DOI: **https://dx.doi.org/10.21608/javs.2022.141246.1152**