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Research Article

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Evaluation of Extenders for the Liquid Storage of Ring-Necked Pheasant (*Phasianuscolchicus*) Semen

Saba Mumtaz[1], Anum Razzaq[1*], Bushra Allah Rakha[1], Mudassar Iqbal[3], Tariq Ahmad[2], Laila Khanum[1], Rida Pervaiz[1], Shamim Akhtar[1], Farha Qayyum[1]

[1]Department of Zoology, Wildlife and Fisheries, PMAS-Arid Agriculture University, Rawalpindi 46300, Pakistan; [2]Northeast Forestry University No. 26, Hexing Road, Xiangfang District, Harbin City, Heilongjiang province, China; [3]Department of Zoology, Hazara University, Mansehra, Pakistan.

ABSTRACT

Introduction: Ring-necked pheasant (*Phasianuscolchicus*) is a famous game bird and has ecological, marketable, aesthetic, sport and nutritive values. It is native to Eastern central Asia, China, Japan and is introduced as game bird to other regions of the world. Population of this species is continuously declining due to hunting pressure, habitat fragmentation, meat purpose and increased urbanization. There is need to conserve the species through techniques of semen banking as may provide a protected net against extinction.

Objective/Aim: The current study was designed to asses range of extenders (Red fowl, Tselutin Poultry, Beltsville Poultry, Chicken semen, Lake and EK extenders) for storage of ring-necked pheasant semen at 5 °C in refrigerator for 48 hours.

Methodology: Short-term semen storage method used for avian semen, as avian sperms are sensitive to higher temperature fluctuations during cryopreservation process. Semen collection was made from trained and mature cocks and diluted in the Beltsville Poultry, Red fowl, Lake, EK, Tselutin Poultry and Chicken semen extenders, stored at 5°C and evaluated for sperm motility (%), plasma membrane integrity (%), viability (%), acrosome integrity (%) and DNA fragmentation at 0h, 3h, 6h, 24h and 48 h of storage.

Results: The spermatozoa motility was higher ($p<0.05$) in RFE (72.50±3.23) and Tselutin poultry extender (53.75±2.39) as compared to other diluents; Beltsville poultry (41.25±1.25) and Lake (30.00 ± 2.04) whereas Membrane integrity was also higher ($P<0.05$) in RFE (80.50±2.1) and Tselutin poultry extender (63.75±2.39). However, RFE (67.00±1.22) maintained sperm viability ($P<0.05$) higher at all storage hours (0, 3, 6, 24 and 48) as compared to TPE (63.75±1.49). Data obtained from current study was analyzed by using ANOVA and LSD.

Conclusion: Hence, it is concluded that ring- necked pheasant semen can be stored at 5ºC for 48 hours in Red fowl extender for captive propagation of the species.

Key Words: Ring-necked pheasant, Liquid storage, Extenders, Semen, Sperm motility, Acrosome integrity

INTRODUCTION

The Ring-necked pheasant (*Phasianuscolchicus*) or common pheasant, also known as Chinese pheasant belongs to family *Phasianidae*and order Galliformes. It is native to Central and Eastern Asia, Japan, China and is introduced as game bird to other regions of the world.[21] The ring-necked pheasant is familiarized to other parts of the world because of its meat and its resistance against different diseases and parasites that are common in most highland game bird species.[10]

Ring-necked pheasant is a medium size bird with oval shaped body, small head and long, tapered tail. They are sexually dimorphic. Males have brilliantly colorful plumage and are larger than females. They have a distinguishing white collar about their neck which gives them the name 'ring-necked'. Pheasants build nests mostly under the dense cover and merely on the ground. They feed on the waste grains, plant materials including seeds, grains, shoots, weeds and insects.[5]

The males are polygynous and are often with harem of many females and show high reproductive potential. Breeding season starts in April and May. Male establishes territories to

Corresponding Author:
Anum Razzaq, Department of Zoology, Wildlife and Fisheries, PMAS-Arid Agriculture University Rawalpindi 46300, Pakistan.
Email: anumrazzaq001@gmail.com

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attract females and females lay a clutch of 10-12 eggs in each breeding season.[37] Pheasants provide many ecological, marketable, aesthetic, sport, and nutritive values. In many countries, pheasant species are hunted as game and bred to use as food. Nutritionally, they are a rich source of iron, protein, niacin, vitamin B12 and other nutrients.[6]

Ring- necked pheasant status in IUCN red list is least concern.[19]However, its population is declining continuously due to hunting pressure, habitat fragmentation, rearing for recreational and meat purpose, increased urbanization and changed agricultural product.[14]*Ex situ* and *in situ* techniques can be used for Species Conservation. However, preference should be given to *in situ* methods. Hence, ex-situ techniques should be accompanied with in vitro methods of semen banking and artificial insemination.[26]For the semen banking and artificial insemination, we can collect semen from potential males and store for long or short period of time.[29]

Avian sperms are morphologically different from those of mammals; highly concentrated with very minimal seminal plasma, less cytoplasm and nuclear contents, small head and elongated tail and are fragile to higher temperature fluctuations during cryopreservation process.[12] The semen storage, results in the decrease of fertilising capability of sperms with the time even at low temperatures in a species-specific manner. However, the use of liquid storage method of semen is still best option for many avian species.[8]Before its storage the semen should be diluted in a diluent. An ideal diluent should consist of energy source required for metabolism, maintains pH and osmolarity and helps to withstand feasibility and cell functionality.[13,18]

In the available literature, various extenders have been tested/ used for avian species viz; TPE,[38] BPSE,[15] RFE,[30] LE [24]and EKE.[26] Suspending sperms in diluent which can maintain its structural integrity and function is the most commonly used method for liquid storage of semen.[24] Extenders can be prepared or already available prepared extenders can be used for avian species because there is no universal diluent due to difference in composition of seminal plasma in different species, sperm enzyme systems and metabolic supplies of sperm.[40]

It is essential to identify an appropriate extender for the short-term storage of ring-necked pheasant preservation of semen at 5^0C that can maintain cellular integrity and is suitable for its physiology. Therefore, the current study was designed for following objectives;

- To assess range of extenders (Red fowl semen, Beltsville Poultry, EK, Chicken semen, Tselutin Poultry and Lake extender) and different storage hours (0 to 48 hours at 5 °C) on viability sperm motility, Acrosome integrity, plasma membrane integrity and DNA fragmentation of ring-necked pheasant semen.

MATERIALS AND METHODS

Birds management during experiment

Ring-necked pheasant males were managed in avian research center at the main campus of PMAS-Arid Agriculture University Rawalpindi. Fresh water and commercially available Islamabad poultry feed was given to them throughout the experiment. Chicks of ring-necked pheasant were trained for the collection of semen and this training was given to birds until they were capable enough to give semen which was without contamination with feces.

Collection and processing of semen

Abdominal massage was done to individual bird for the collecting semen. Volume of semen was measured by weighing the test tube before collecting and after collecting semen sample. Motile sperms were observed and concentration was measured by diluting 5 µL ring- necked pheasant semen sample in 200 µL of NAR. Ejaculates which hadmotility more than 60 % were processed further for evaluation Ejaculates which had motility > 60 % were diluted in either of the extenders viz;Red fowl, Beltsville Poultry, EK, Lake, Chicken semen and Tselutin poultry extenders. Composition of extenders is given in Table 1.

Semen quality assay

The semen which was already diluted was cooled in two hours to 5 °C and was store for 48 h at 5 °C. Livability (%), spermatozoa motility (%), sperm plasma membrane integrity (%), acrosomal integrity of sperm and DNA fragmentation of ring-necked pheasant spermatozoa was measured at storage hours 0, 3, 6, 24 and 48.

Sperm motility

Motility was evaluated by putting semen sample one drop which was, already extended to 1:5 (v/v) with Red fowl semen extender on glass slide pre-warmed to 37 ºCand motility observed using a phase contrast microscope (400x).[44] Motile sperm percentage was assessed on a scale having range 0 -100%.

Plasma membrane integrity

HOST test reported by Santiago-Moreno et al.[31] was used for the evaluation of integrity of Plasma membrane of ring-necked pheasant spermatozoa. The solution of HOS was made by dissolving sodium citrate (1 g) in 100 ml of distilled water.25 µL sample previously diluted with extender was mixed with HOS solution (500 µL and 100m Osm kg–1) and this solution was incubated for 30 minutes at room temperature (25 ºC). Then added one drop on a warm slide and fixed in 2% glutaraldehyde. The spermatozoa which had puffy and coiled tails and bloated heads were declared as normal spermatozoa they had undamaged plasma membrane. Total sper-

matozoa count was 100. They were counted by using phase-contrast microscope in at least 4 different fields.

Sperm viability

Viability of ring-necked pheasant spermatozoa was assessed by mixing eosin-nigrosine stain in the solution Lake's glutamates. This solution 3 was made by adding potassium citrate (0.00128 g), sodium acetate (0.0085 g) magnesium chloride (0.000676) and sodium glutamate (0.01735 g), in 100 ml water which is distilled. 5g nigrosine and 1g eosin-bluish which are both water soluble were mixed into Lake's glutamate solution. Twelve µl stain and semen sample 1 µl were mixed. On a clear glass slide smear was made, fixed and air dried. Hundred sperms were observed per slide using phase-contrast microscope (1000x with oil immersion). The live sperms were appeared unstained and dead were appeared pinkish stained.

Acrosomal integrity

Giemsa stain as described by Jianzhong and Yiling[20] was used for the assessment of spermatozoa acrosomal integrity of Pheasant. By adding phosphate buffer saline (2 Ml) and (3 g) giemsa at pH 7.0 into distilled water(35 mL). One drop of sample was taken on clear glass slide smear and fixed for thirty minutes in neutral formal-saline (5% formaldehyde). The slides were kept in stain for one and half hour. Spermatozoa which had normal acrosome were evenly stained and sperms having damaged acrosome were not stained. Total one hundred sperms were assessed in at 4 different fields observed using phase-contrast microscope (1000x with oil immersion).

DNA fragmentation

DNA fragmentation was assessed by using aniline blue stain.[13] Smear was prepared, dried in air and incubated in 3% buffered glutaraldehyde and washed in distilled water. Slides were kept for 5 min in 5 % aniline blue stain and 4% acetic acid then washed with distilled water. Then they were kept in 0.5% eosin for 1 min [41] and observed in oil immersion under microscope at 1000 X. Sperms having normal DNA had Light blue heads and dark blue sperm heads showed DNA damage.

Statistical analysis

Data analysis of replicates obtained in experiment was done by ANOVA using (MSTAT-C, Version 1.42 Michigan State University, and East Lansing, MI, USA) and was showed as means (±SE). For the post hoc comparisons between the means LSD test (Fischer's' protected) was used.

RESULTS

Extenders effect on motility of sperm

The data on the effect of extenders (Red fowl, Beltsville poultry semen, EKE, Lake, TPE and CSE) extenders and storage hours ((0, 3, 6, 24 and 48) at5 ºC on sperm motility of ring-necked pheasant are given in Figure 1. There was significant interaction between extenders and storage hours. The spermatozoa motility was found considerably higher ($p<0.05$) in RFE (72.50±3.23) and Tselutin poultry extender (53.75±2.39) as compared to other diluents; Beltsville poultry (41.25±1.25), Lake (30.00 ± 2.04), EK (31.25±1.25) and Chicken semen extender (27.50±1.44)at all storage hours (0,3, 6, 24 and 48).

Retrograde decline in sperm motility was noticed from 0-3 hours of storage at 5 ºC in all extenders but Red fowl (61.25±4.37) and Tselutin poultry (47.50±4.33)diluents maintained the higher sperm motility ($p< 0.05$) than Beltsville poultry (33.75±2.39), Lake (25.00±2.04), EK (25.00±2.04) and Chicken semen extender (22.50±1.44). Similarly, at 6 hours of storage, higher sperm motility ($P<0.05$)was recorded inRed fowl extender (53.75±3.75)and Tselutin poultry extender (42.50±4.33) as than all other extenders used; BPSE (28.75±2.39), LE (20.00 ± 2.04), EKE (20.00±2.04)and CSE (17.50±1.44).

Extenders effect on plasma membrane integrity

The data on the effect of extenders used and storage hours (0, 3, 6, 24 and 48) on integrity of plasma membrane of ring-necked pheasant sperm are given in Figure 2. Membrane integrity was found higher ($P<0.05$) in Red fowl extender (80.50±2.1) and Tselutin poultry extender (63.75±2.39) as compared to Beltsville Poultry (51.75±0.85), Lake (40.50±1.85), EK (41.25±1.31) and Chicken semen extender (37.00±1.47) at all storage hours.

Although significant decline was observed in plasma membrane integrity of sperm from 0 to 48 hours of storage at 5 ºC but Red fowl extender (45.75±1.31) and Tselutin Poultry extender (43.00±3.76) maintained higher plasma membrane integrity ($P< 0.05$) at all storage hours compared to all other extenders used in experiment {Tselutin poultry (43.00±3.76), Beltsville poultry (24.75±2.14), Chicken semen (17.75±1.25), Lake (18.75±1.89) and EK (19.00±1.87).

There is strong interaction between storage hours and extenders used in experiment and this proposed that various extenders have their own abilities different to each other in maintaining the integrity of plasma membrane for the period of different hours of storage at 5ºC

Extenders effect on sperm viability

The data on the effect of diluents and hours of storage on sperm viability is given in Figure 3. The viability was noticed higher ($P<0.05$) in RFE (76.50±1.32) and TPE extenders (73.50±1.32) as compared to other extenders (BPSE (50.75±0.65), LE (46.25±2.02), EKE (46.25±2.02) and CSE (38.75±1.38) used in experiment at all storage hours (0, 3, 6, 24 and 48).

The number of viable sperms significantly decreased from (0 to 48 h) of storage. However, RFE (67.00±1.22) maintained the higher sperm viability (P<0.05) at all storage hours (0, 3, 6, 24 and 48) as compared to TPE (63.75±1.49), BPSE (29.00±0.91), LE (28.00± 1.47), EKE (29.00±1.08) and CSE (25.50±1.44).

Extenders effect on acrosomal integrity

The data on the effect of extenders and hours of storage on integrity of acrosome sperm are given in Figure 4. It was found significantly higher (P<0.05) in RFE (71.75 ±1.18) and TPE extenders compared to BPSE (53.50 ±1.85), LE (40.50 ±0.87), CSE (36.00 ± 0.7) and EKE (37.50 ± 1.04) at all storage hours (0, 3,6,24 and 48 hours).

From 3 to 6 hours of storage the acrosomal integrity reduced significantly however, RFE (68.25 ±1.18)and TPE (63.75 ±1.75) still maintained the higher acrosomal integrity (P<0.05) of sperm than BPSE (41.75±2.81), LE (37.00±1.08), EKE (35.25±1.25)and CSE (30.75±0.48). At 48 hours of storage integrity of acrosome was found greater (P<0.05) in RFE (61.75±1.18)and TPE (53.25±1.25)as compared to BPSE (31.50±1.19), LE (31.25±1.70), EKE(26.50±1.55) and CSE (22.25±0.85). However, RFE maintained the higher sperm acrosomal integrity at all storage hours compared to TPE, LE, BPSE, CSE and EKE.

Effects of extenders on DNA fragmentation

The data on the effect of extenders and hours of storage on sperm acrosomal integrity are given in Figure 5. At 0 hour of storage DNA fragmentation was found least in RFE extender (11.00±1.68) as compared to TPE (17.00±1.22), BPSE (31.50±1.44), LE (38.50±2.22), EKE (35.75±0.85) and CSE (35.75±0.85) extender. However, at 3 hours of storage increase in DNA fragmentation was observed in all diluents but it was recorded least in RFE (16.00±1.83) as compared to TPE (16.00±1.83), BPSE (39.50±1.55), LE (41.25±1.97), EKE (41.00±1.35) and CSE (37.50±0.65). Similarly, at 6 hours of storage further increase in DNA fragmentation was noticed.DNA fragmentation was decreased significantly from 24-48 hours of storage however RFE (34.00±1.47) showed least DNA damage as compared TPE (36.25±1.31), BPSE (51.50±1.71) LE (57.25±1.25), EKE (55.00±1.08) and CSE (52.25±1.11).

DISCUSSION

Sperm motility is considered to be one of the most significant and fundamental parameters used in assessing the fertilizing ability of sperm as it defines the sperms potential to reach the egg to fertilize it by passing well through the reproductive tract of female.[33] In the present study spermatozoa mobility found greater in Red fowl extender than BPSE, TPE, CS, EKE and LE. Though, time dependent reduction in motility of spermatozoa has been noticed at all storage hours 0, 3, 6 24 and 48 in all extenders and RFE has better maintained the sperm motility in red jungle fowl semen as studied by Rakha et al.[29]

Siudzinska and Lukaszewicz[35] reported the similar results that motility of sperms reduces at all hours of storage in all diluents at 5° C. They reported that EK extender was found to be the most appropriate in four chicken breeds. Schneider et al.[32] found that lake extender has maintained higher motility in cockatiel semen as compared to other diluents. In another study Blesbois& Raviers[7] proved that both Lake and BPSE has similar effects on motility of sperm at 4 ºC. The reason for the different results in all the studies may be that different extenders used in experiment have different effects on the motility of sperm in different species.

There is a crucial role of sperm plasma membrane in recognition of ovum, sperm– egg binding and fertilization. Sperm outer membrane integrity is not only important for the sperm survival but also enables it to fertilize ovum and maintain osmotic equilibrium.[28] The loss of sperm plasma membrane integrity is often linked with infertility in spite of normal semen parameters.[4] Physical integrity of plasma membrane of sperm is used to differentiate dead cells from live ones.[9]

In the current study sperm PMI was noticed greater in Red fowl extender than other diluents Tselutin poultry, lake, EK, Beltsville poultry and chicken semen extender at all hours (0, 3, 6, 24 and 48) at 5 ºC. Clarke[10] reported that Beltsville poultry semen extender was better able to maintain integrity of plasma membrane than other extenders in chicken and turkey semen stored at 5° C. Amount of fructose in diluents increases the fertilizing ability of spermatozoa as compared to the diluent having no fructose.[24] The Presence of higher amount fructose in turkey semen extender is responsible for maintaining better plasma membrane integrity in sperm.[29]

Sperms viability has a positive relationship with motility and fertilizing potential of sperm[25] because sperm which is alive is able to fertilize the oocyte only Livability test is performed when sperm motility is fewer than 25 %. All non-motile sperms are not dead and some may be alive but they are unable to swim up and reach to the egg due to some structural or biochemical problems.[42]

A detailed assessment of semen and sperm livability is necessary and can be done by using techniques of differential staining.[17] In another study by Han et al.[16] reported that EK Extender have showed higher livability as compared to Turkey semen extender and Lake extender for duck. In current study found that time dependent decrease in sperm livability at all storage hours (0, 3, 6, 24 and 48) in all diluents which is similar to the previous study conducted by Mohan et al.[27] on guinea fowl semen and found reduction in viability after 24 hours. On contrary to this Siudzinska & Lukaszewicz[35]

found that sperm livability did not reduce in EK extender as much as in semen of fowl which is extended in Lake and Tselutin extender. The reason of different results may be that different semen diluents are suitable for maintaining sperm livability of different species and breeds.

Sperm acrosome integrity is considered to be effective parameter to determine semen viability and fertility. Low acrosomal integrity of sperm has been seen in many infertile cases and may be attributed due to low level of acrosin activty.[39] An intact acrosome is necessary for the sperm to fertilize an egg. Before fertilization, acrosome reaction takes place in sperm cell and enzymes present in acrosome becomes activated making a hole in egg zona pellucida allowing sperm to penetrate and to bind with plasma membrane of oocyte.[43] In present study acrosomal integrity was observed greater in Red fowl extender as compared to other diluents; Tselutin poultry, Beltsville poultry, EK, Lake, and Chicken semen extender stored at 5^{0}C. These results are similar to results of Rakha et al.[29] in a study conducted on red jungle fowl and found that Red fowl semen extender has maintained highest the acrosomal integrity than other diluents.

DNA integrity of sperm is a most important factor of sperm quality for the prediction of infertility.[34] Sperms having high DNA damages are associated with increased risk of genetic abnormalities in embryo, poor development of embryo and it also affects the post-partum development.[45] Sperm damaged DNA may be combined to the embryo's DNA and may results in errors during DNA replication, transcription and translation resulting different diseases.[22]

The main causes of Sperm DNA fragmentation may be irregular/abnormal packaging of chromatin[34] unsuccessful apoptosis and presence of increased amount of reactive oxygen species (ROS).[1] In the current study the DNA fragmentation of sperm was found least in Redfowl extender as compared to Beltsville poultry, EK, Tselutin poultry and chicken semen extender. In many studies it is reported that oxidative stress is one of the causes of DNA damage because it causes the production of ROS.[2]Antioxidants reduce the reactive oxygen species thus results in reduction of DNA damage.[23]

CONCLUSION

In the current study Red fowl extender was able to maintain semen quality; sperm livability, sperm motility, acrosome integrity, plasma membrane integrity, and reduces DNA fragmentation compared to other diluents (Tselutin poultry, Chicken semen, Beltsville poultry, EK and Lake, extenders) examined for liquid storage (5ºC) of ring-necked pheasant semen at all hours of storage. Hence, it is concluded that ring- necked pheasant semen can be stored at 5ºC for 48 hours in Red fowl extender for captive propagation of the species.

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Authors Contribution

Saba Mumtaz and Laila Khanumcollected experimental materials, performed lab work and interpreted results. Bushra Allah Rakha and Shamim Akhtar designed the study, supervised and assisted in manuscript writing. Anum Razzaq wrote up the manuscript draft, formatted and review. Tariq Ahmad, Rida Pervaiz, Farha Qayyum and Mudassar Iqbal participated in statistically analysis. All of the authors read and approved the manuscript.

Conflicts of interest

Authors declared no conflicts of interest.

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Table 1: Extenders composition used for liquid storage of ring- necked pheasant semen at 5°C for 48 hours. The values in table are in g/100 ml of double distilled water.

Components	Diluents					
	BPSE	RFE	LE	EKE	TPE	CSE
Fructose	0.3	1.15		0.2	0.3	0.3
Potassium citrate	0.0384		0.128	0.14	1.92	
Sodium glutamate	0.5202	2.1	1.35	1.4	1.92	
Magnesium chloride	0.0204					

Table 1: ***(Continued)***

Components	Diluents					
	BPSE	RFE	LE	EKE	TPE	CSE
Di- potassium hydrogen phosphate	0.7620					
TES	0.3170					
Potassium hydrogen phosphate	0.039					
Sodium acetate	0.258		0.51			
PVP		0.6		0.1	0.3	0.38
Glycine		0.2				
Potassium acetate		0.5			0.5	
Magnesium acetate			0.08			
Glucose			0.8	0.35		
Inisitol				0.7		
Protaminsulphate				0.02	0.32	0.5
Anhydrous sodium hydrogen phosphate				0.98		
Anhydrous sodium di hydrogen phosphate				0.21		
p^H	7.3	7.0	7.2	7.5	7.05	6.85
Osmotic pressure (mosmol kg–1)	330	371	310	390	320	310

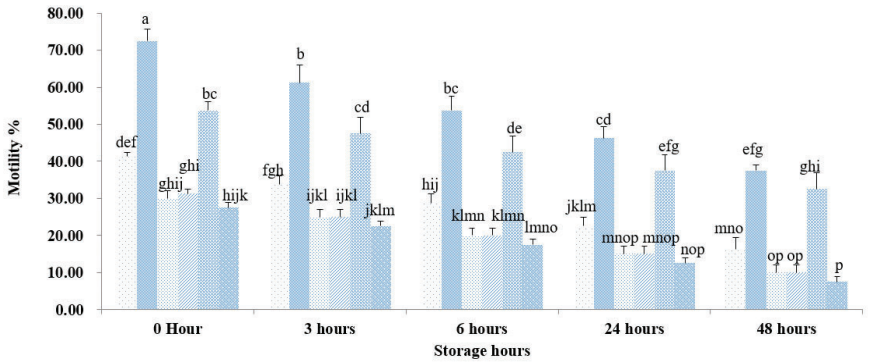

Figure 1: Effect of different extenders (Red fowl, Tselutin poultry, Beltsville poultry semen, Lake and EK extender) on the sperm motility of ring-necked pheasant semen stored at 5°C. The Bars written with different letters above them vary significantly (P < 0.05) within a given period of storage.

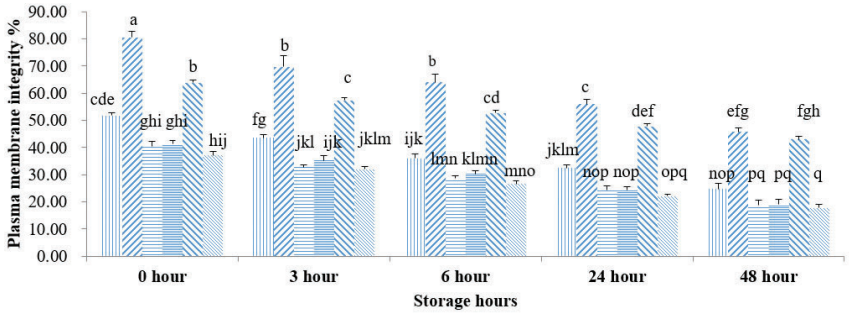

Figure 2: Effect of different extenders (Red fowl, Tselutin poultry, Beltsville poultry semen, Lake and EK extender) on the integrity of plasma membrane of ring-necked pheasant sperm stored at 5°C. The bars written with different letters above them vary significantly (P < 0.05) within a given period of storage.

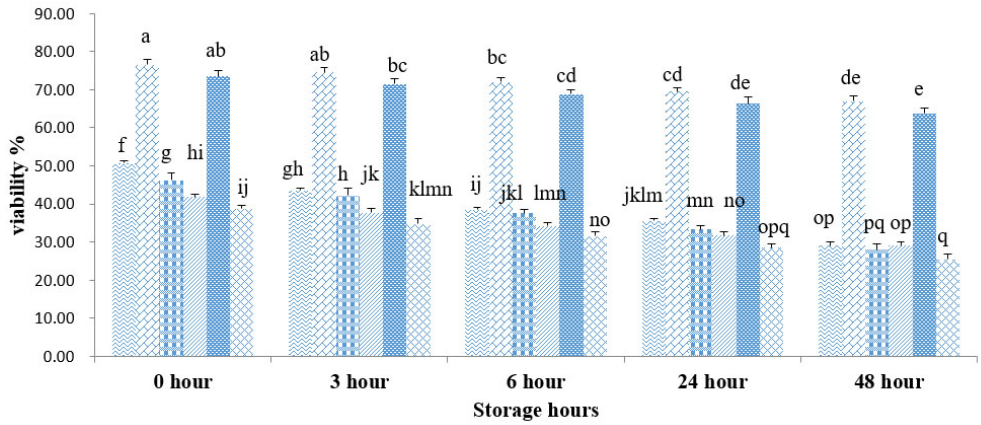

Figure 3: Effect of different extenders (Red fowl, Tselutin poultry, Beltsville poultry semen, Lake and EK extenders) on the viability of ring-necked pheasant sperm stored at 5°C. The Bars written with different letters above them vary significantly ($P < 0.05$) within a given period of storage.

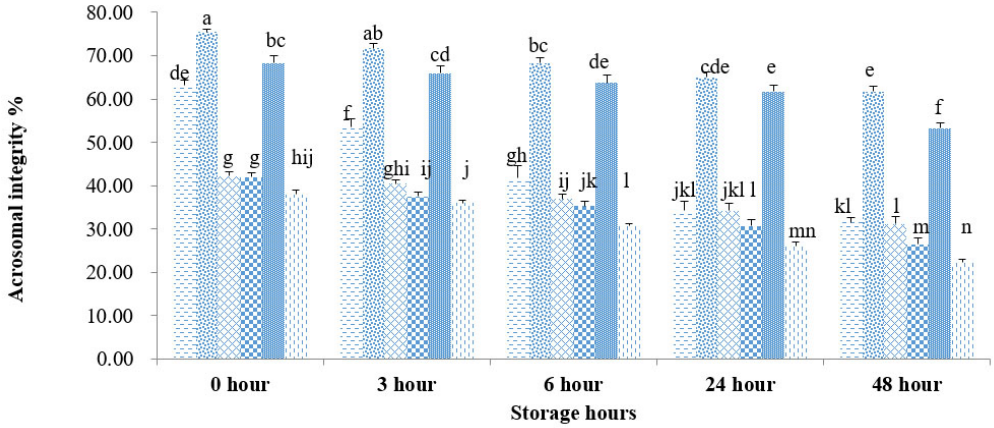

Figure 4: Effect of different extenders (Red fowl, Tselutin poultry, Beltsville poultry semen, Lake and EK extender) on the integrity of acrosome of ring-necked pheasant sperm stored at 5°C. The Bars written with different letters above them vary significantly ($P < 0.05$) within a given period of storage.

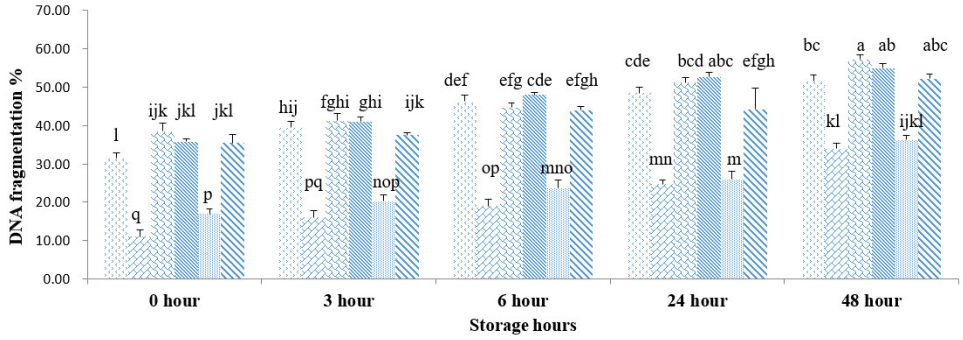

Figure 5: Effect of different extenders (Red fowl, Tselutin poultry, Beltsville poultry semen, Lake and EK extender) on DNA fragmentation of ring-necked pheasant sperm stored at 5°C. The Bars written with different letters above them vary significantly ($P < 0.05$) within a given period of storage.