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Article

Ascorbic Acid Effect on Frozen and Thawed on Sperm Motility, Plasma Membrane Integrity, Livability and Acrosome Integrity of Ring-Necked Pheasant (Phasianus colchicus) Semen

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Abstract: Ring necked-pheasant (*Phasianus colchicus*) is bird of order Galliformes. Ring necked-pheasant is also known as gallinaceous bird and game bird. It looks like chicken bird. It is national bird of South Dakota. It is mostly found in wild areas but in Pakistan it exists as Domestic bird. It has high proteins and low fat in its meat. The present study was conducted to check the effect of ascorbic acid on sperm Motility, sperm cytoplasmic membrane integrity, sperm livability and acrosome integrity of Ring-necked pheasant at different concentrations of ascorbic acid (0mM, 1mM, 2mM, 3mM and 4mM) were used. Semen was collected by abdominal massage technique. Motility of sperm was greater than 70%.Then it was processed further. The cryopreservation of semen was checked at various stages like Post Dilution, Post Cooling, Post Equilibration and Post Thawing. Semen was cooled from post-dilution stage (37^0 C) to post-cooling (20^0C).Then it further cooled gradually to get post-equilibration stage (4^0 C) within 24 hours. After that 10% glycerol was added to the sample. Then it was transferred to liquid nitrogen (LN_2) cylinder for 24 hours. After thawing stage sample was removed from LN_2 cylinders and placed in water bath for 4 hours to achieve post-thawing. Then performed sperm quality assays at each stage of cryopreservation. Sperm motility was assessed by Neubauer Chamber Hemocytometer. Plasma Membrane Integrity was checked by using Hypo Osmotic Swelling Test with the help to 2% Eosin. Sperm livability was assessed by Lake's glutamate solution and sperm Acrosome Integrity was checked by dual staining technique by using Formal citrate solution and Giemsa stain. The better results were seen at 3mM treatment of ascorbic acid. Sperm motility percentage was significantly different ($P < 0.05$) on 3mM treatment on all the stages of cryopreservation rather than other treatments. Plasma membrane integrity assay also showed good results at same concentration ($P < 0.05$) on all the stages of cryopreservation rather than other treatments. At the sperm livability stage 3mM treatment showed significantly difference ($P < 0.05$) on all the stages of cryopreservation rather than other treatments. The sperm acrosome integrity showed highest percentage on 3mM treatment ($P < 0.05$) on all stages of cryopreservation rather than other treatments. Effect of ascorbic acid on all the quality parameters (sperm motility, plasma membrane integrity, livability and acrosome integrity) was showed significant difference ($P < 0.05$) at 3mM treatment of ascorbic acid as compare to control as well as remaining treatments. It was seen that by increasing the concentration of ascorbic acid from 3mM concentration to onward it shows negative results on its cryopreservation. Now this cryopreserved semen can be transfer from one place to another place to obtain good varieties as well as better genetic characteristics of this species.

Keywords: ring necked-pheasant; ascorbic acid; semen cryopreservation; semen quality

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1. Introduction

Common Pheasant *(Phasianus colchicus)* is a bird of order Galliformes (Jobling, 2010). The word "Galliforme" means chicken like bird. The first population of pheasant was observed in 1881 in Willamette valley in North America (Johnson & Knue, 2011). This bird is also known as Game bird. All the pheasants are place in this order. Ring necked-pheasant is indicated as Gallinaceous and non-migratory bird. Large numbers of families are kept in this order. Every family has a specific bird with special features. This order is very developed (Farris et al., 2011).

Pheasant family is recognized by their bright red head with wattles and iridescent green colour is present on the neck. The male of this family is identified by white ring which is present around the neck and female is identified by their light brown and black flecking present on their feathers.

Colchicus is the name of country of black sea, that's why Ring necked- pheasant are known to Europeans (Bird Life International, 2012). There is also a great resemblance between domestic chicken and pheasant (Farris et al., 2011).Ring necked pheasant is the most hunted bird in the World (Robertson, 1997).

Ring necked pheasant is the natural species of the Europe (Sibley 2000, p. 141). Firstly, this specie was systematically identified by Carl Linnaeus in his Naturae Systema book in 1758. In US the pheasant is called as Ring Neck Pheasant, and chink is known in North America or Montana, (Phezzens) (Proper, 1990). In china it is known as" pheasant fowl" and English name is" Common Pheasant"(Lin-Liu et al., 2006).

Destruction of habitat is the major threats for Ring necked-pheasants. Loss of habitat has many reasons which are describe as below forests are temporary and permanently cut by human for getting timber and for many other purposes. Destruction of trees like agricultural and urban invasion, development of roads and buildings are also the cause of habitat loss (Fuller & Garson, 2004).

Hunting is also a pronounced risk for pheasant species decline. Because, the harvesting of pheasants is being done at large scale for the purpose of food, game and trade. During hunting the destruction of habitat is very common. Habitat loss is the primary and major cause of decline in European bird species and they are also hunted widely (Fuller & Garson, 2004).

Human disturbance also greatly effects the pheasant's population. Human disturbance has most effect on the pheasant population as compare to other birds and animals. Pheasants are mainly dependent on ground's stuff and are nesting birds (Fuller and Garson, 2004). A rare case has been reported which requires investigation. It has been reported that one of the species of ring-necked pheasant which is inherent to Japan (*Phasianus colchicus versicolor/ robustipes/ tanensis*), have been crossed with the Korean species (P. c. karpowi). It is basically to rear more fowls in custody. That in return will reduce hunting (Maru, 1980).

1.1.. Aims and objective:

Present study was designed to check the effects of Ascorbic acids (Vitamin C) on the semen of Ring-necked pheasant (*Phasianus colchicus*) to determine

- Sperm Motility
- Sperm Plasma Membrane Integrity
- Live Sperm Count (Livability)
- Sperm Acrosome Integrity

1.2. Hypothesis:

It is hypothesized that Ascorbic acid may improve the quality of Ring-necked pheasant semen.

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Ring-necked pheasant are very beneficial bird for country because they are game birds and have rich protein content in their meat. They are present in Pakistan in captivity. Their meat is full of proteins and low in fats (Randy, 2011).

1.3. Cryopreservation:

Cryopreservation is a technique to preserve natural cells and tissues at extremely low temperature which stops biochemical reactions. They are not protected for a long time at the same temperature (Woodruff & Snyder, 2007). It is a technique in which tissues and some body organs are frozen and stored at very low temperature such as -196^0 C. This process is used to improve the quality of meat, egg and to make hatchability better. Cryopreservation of semen is very important tool for storing reproductive cells and managing genetic diversity of birds (Blesbois, 2011).

Cryopreservation is basically a non-physiological technology that used to preserve genetic material i.e. semen, spermatozoa cells, embryos, oocytes etc at very low temperature. The process of freezing of water is done by using biological effect of cooling. This is followed by process of concentration of solute-part of the cell which is dissolved in liquid part. However, there are many damages which may occur. Cryoprotectants help the cells to gain protection from several cryodamages such as osmotic and thermal shock during the cryopreservation process at cooling, freezing and thawing. (Blesbois, 2007). In order to decrease cellular damage, chemicals are added to the solution. Major damage is cause by freezing process (Partyka & Niz, 2010). Cryoprotectant when penetrates into sperm cell, reduce crystal formation (Herrera et al., 2009).

There are some critical points that mainly affect the cell's structure and metabolism during the process of cryopreservation are describes below (Blesbois, 2007).

- The relation between sperm cell and cryoprotectant
- Temperature curve
- The form of semen packaging

Ascorbic acid ($C_6H_8O_6$) is also known as Vitamin C. It is a first vitamin which was chemically synthesized. This was discovered in 1912 and isolated in 1929 and 1933 (Victor & Squires, 2011).Vitamin C plays an important role in the human body. It also works as antioxidant (Antioxidant is a substance that inhibits the oxidation that caused by free radicals such as ROS molecules). It founds in the citrus fruits and vegetables. It is necessary for maintaining the health of connective tissues. It enhances the immune system. It is also available in the form of tablets, capsules and in powder form (Micronutrient information center, Oregon State of university).

It is water soluble vitamin. Its deficiency may cause the scurvy (ASOHP, Original on Dec 30, 2016). Initially it was utilized basically in poultry to increase seminal plasma or spermatozoa quality (Surai et al., 2001). The better method to increase the quality of semen of poultry is the food supplement of vitamin C (McDaniel et al., 1998; Elansary et al., 1999; Khan et al., 2012).

The main function of vitamin C in the semen is finite. This study plan was made to check the effect of Ascorbic acid on semen of Ring necked-pheasant. Present study gives information about the use of Vitamin C as a cryoprotectant to increase the sperm quality and hatchability in the Ring necked-pheasant.

2. Materials and methods:

2.1. Study design:

The experiment was designed by following Complete Randomize Design.

2.2. Place of study:

The experimental work was done at "Avian's Research Centre", Pir Mehr Ali Shah Arid Agriculture University Rawalpindi, Pakistan.

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2.3. Study duration:

The present study was done from January 2020 to September 2020. It was started after the approval of synopsis.

2.4. Sample size:

Experimental Birds

Eight males of Ring-necked pheasant were used in this research. The birds were kept in captivity under natural photoperiod, individually in the pens of 3.5feet x 4 feet at bird exploration center at PMAS-Arid Agriculture University Rawalpindi, Pakistan. Commercial food was given to birds 100g/per day that was approved from Islamabad poultry handler feed center. Fresh water *ad libitum* was also provided during experimental time period.

2.5. Sampling techniques:

Semen collection and evaluation

Sample was collected from birds in a graduated plastic tube by abdominal massage of Ring-necked pheasant as described by (Burrows and Quinn, 1935). The quantity of sample was measured in microliters through micropipette. Initial motility of the each ejaculate was identified by stirring 10 microliters of sample in 500 µl in PBS (phosphate buffer saline) having PH of 7.2 and 300 mOsmol/kg (Zemjanis, 1970).

The percentage of spermatozoa was determined by Phase Contrast Microscope (PCM) at 400x (Olympus Bx20, Japan) by adding a drop of sample on a pre-warmed glass slide at 37^0C. Sperm value was checked by taking 1µL of sample and 200µL formal citrate solutions (1ml of 30% formaldehyde in 99ml of 2.9% w/v Sodium citrate) with Neubauer hemocytometer chamber (Marienfeld, Germany) Under Phase Contrast microscope (400x, Olympus Bx20, Japan). Experiment was repeated three times.

2.6. Extender preparation and processing:

Semen sample that have motility greater than 75-80% was pooled and processed. Evaluated for motility, Plasma membrane integrity, Growth (livability) and acrosome integrity and divided into 5 allots (A, B, C, D, E) for dilution in 1:5 ratios in modified ''Red fowl'' extender (D-Fructose of 1.15g, Sodium Glutamate of 2.1g, Polyvinylpyrrolidone of 0.6g, Glycine of 0.2g, Potassium acetate of 0.5g, Distilled water of 100ml and pH 7.0 with Osmotic pressure of 380 mOsmol/kg) and different Ascorbic acid (vitamin C) treatments like (0mM) will be controlled group and 1 mM, 2 mM,3 mM and 4mM were examined. After that each of them labeled as A, B, C, D and E aliquots (Jabbar et al & J. Anim., 2015).

2.7. Freezing and thawing process:

The previously diluted semen was (final unit at 1.72 x 10/ml/mg) cooled at 37^0C to 20^0C then 4^0C in 2 hours at -0.275^{0}C $min^{-1}$and then it was equilibrated for 10 min after the addition of 10 % glycerol to each extender at 4^0C. Cooled semen was placed in cooled cabinet unit about 15 minutes and kept over Liquid Nitrogen vapors (5cm above level of LN_2) for 10 minute to freeze from 4 to -80^0C at -8.4^{0}C/min. After that they were placed into Liquid Nitrogen for storage (-196^0C). After 24 hours the straw was thawed for 30s in water bath at 37^0C and were placed over there for 4h to access Motility, Cytoplasmic membrane integrity, Livability and Acrosomal integrity.

2.8. Sperm quality assays:

2.8.1. Sperm Motility:

Sperm motility is describes as the movement of sperm trough the female reproductive track or may be assessed with the help of water to combine with egg.

The post- thawed sperm motility was assessed by putting of drop of sample on the prepared slide at 37^0C down Phase contrast Microscope (PCM) at 400x (Zemjanis, 1970) at

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different stages of cryopreservation. Percentage of motile sperm was calculated on the scale that ranges from 0-100%.

2.8.2. Sperm Plasma membrane integrity:

It is a membrane that acts as a barrier between extra and intra-cellular material. It helps the sperm to survive in female reproductive tract to maintain fertilization capability and osmotic equilibrium.

Plasma membrane integrity was assessed by hyper-osmotic swelling test (HOST) suggested by (Moreno et al., 2009; Rakha et al., 2015a, b). The Hypo-osmotic swelling test solution was prepared by the putting of 1gram of $Na_3C_6H_5O_7$ in 100 milliliter of distilled water. Previously prepared 25µl was stirred in 500µl of Hypo-osmotic swelling solution (100 mOsmol/ kg) and was incubated at 37^0C for 30 minute.

A drop of incubated solution was putted on the prepared slide at 37^0C and added in Eosin 2% cold blooded and glass slide was placed on the sample. The spermatozoa head that showed as swollen or coiled was live and straight tail sperm was dead and having intact cytoplasmic membrane. Count all number of sperm at four separate fields under Phase Contrast Microscope (1000x with oil immersion).

2.8.3. Sperm Livability:

Live sperm count is the checking of sperm growth and movement. Live sperm count of sperm was examined by addition of lake's glutamate solution. Lake's glutamate solution (Cecil & Bakst,.1997) was developed by addition of $C_5H_8NO_4Na$ (sodium glutamate) of 0.01735g, $C_2H_3NaO_2$ (Sodium acetate) of 0.0085g, Potassium citrate ($C_6H_5K_3O_7$) of 0.00128g and Magnesium chloride $MgCl_2$ of 0.000676g in 100ml distilled water. One to two drops of stain were stirred with 1 drop of sample.

Then smear was prepared on glass slide, fixed and air dried. Calculate all number of sperm under the Phase Contrast Microscope (1000x with oil immersion). The solution was given a clear backdrop against blur to enhance the Contrast of white unstained ''motile'' sperms and pinkish blur was 'non motile'' sperms.

2.8.4. Sperm Acrosome integrity:

It is an organelle that develops over the half anterior portion of the head in spermatozoa of animals and humans that describes the acrosomal intactness.

Sperm acrosomal integrity at different stages was checked by Giemsa stain (Zhang & Jianzhong, 2006; Rakha et al., 2015a, b; Rakha et al., 2016). The stain was prepared by the addition of Giemsa (3gram) and 2mL of Sorensen phosphate buffer saline at pH of 7.0 into 35ml distilled water. Smear was prepared by taking a drop of sample on a clean dried glass slide and fixed in neutral formal-saline solution (5% formaldehyde) for 30 min. Then adjusted slides were kept in Giemsa stain for 1.5 h.

Sperm with normal acrosome was assessed by even color but abnormal spermatozoa that were unevenly colored while spermatozoa had ruptured acrosome were remained clean. Count total number of spermatozoa was checked at least at four different fields under Phase Contrast Microscope (400x, Olympus Bx20., Japan) at magnitude 1000x with oil immersion.

2.9. Data analysis (statistical analysis):

Result of this study was presented as mean ± SEM. Obtained data was analyzed by ANOVA (one factor ANOVA)to check the effect of Ascorbic acid on motility, cytoplasmic membrane integrity, Livability and sperm acrosome integrity by ($P < 0.05$).

3. Results

3.1. Sperm motility:

The data on the effect of Ascorbic acid on the sperm motility of Ring-necked pheasant at various steps of cryopreservation on post dilution, post cooling, post equilibration and

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post thawing are shown in Figure 1. The concentrations of Ascorbic acid were used (0mM, 1mM, 2mM, 3mM and 4mM) treatment.

3.1.1. Post dilution:

The data on the effect of ascorbic acid on sperm motility of Ring-necked pheasant at the stage of post dilution shows that (3mM) treatment of Ascorbic acid has highest (90.0±0.0)percentage of sperm motility as compare to control in sperm extender with significant value ($P < 0.05$) but (0, 1, and 2mM) treatments has no significant difference on sperm motility. 4mM treatment has significant difference ($P < 0.05$) of sperm motility from all other treatments with minimum percentage (73.3±2.8).

3.1.2. Post Cooling:

The data on the effect of ascorbic acid on sperm motility of Ring-necked pheasant .At the stage of post cooling (3mM) treatment of ascorbic acid shows highest percentage (80.0±0.0) of sperm motility in experimental extender from control (0 mM), (1 & 2mM) treatments of ascorbic acid does not show significant difference ($P < 0.05$) in sperm motility and (4mM) treatment show minimum percentage (56.6±2.8) of sperm motility from all treatments.

3.1.3. Post Equilibration:

The data on the effect of ascorbic acid on sperm motility of Ring-necked pheasant at the stage of post equilibration show significantly difference ($P < 0.05$) (3mM) treatment of ascorbic acid has highest percentage (76.6±2.8) as compare to control. Whereas 4mM treatments shows minimum percentage (51.6±5.0).

3.1.4. Post Thawing

The data on the effect of ascorbic acid on sperm motility of Ring-necked pheasant at the stage of post thawing shows that (3mM) treatment of ascorbic acid has highest percentage (68.3±7.6) ($P < 0.05$) sperm motility in experimental extender.0mM, 1mM and 2mM shows slight difference from each other but 4mM treatment of ascorbic acid shows minimum percentage (45.0±5.0) of sperm motility in experimental extender.

So, in sperm motility over all (3mM) treatment of ascorbic acid shows highest percentage as compared to all other treatments.

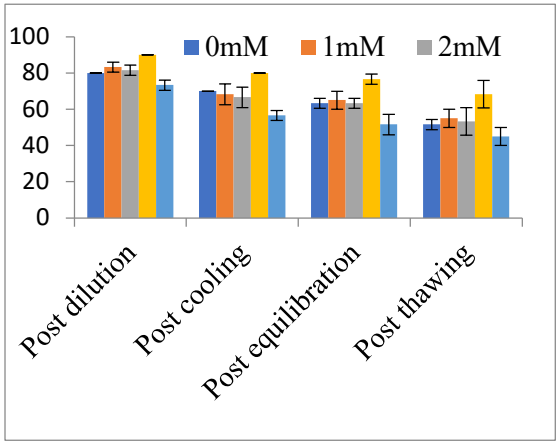

Figure 1. Shows that treatment 3mM shows the significant effect rather than remaining treatments (0, 1, 2, and 4mM) on sperm motility at the stages of post-dilution, post-cooling, post-equilibration and post-thawing respectively.

3.2. Sperm Plasma Cytoplasmic membrane Integrity

The data on the effect of Ascorbic acid on sperm cytoplasmic membrane integrity of Ring-necked pheasant at various steps of cryopreservation on post dilution, post cooling,

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post equilibration and post thawing are shown in Figure 2. The concentrations of Ascorbic acid were used (0mM, 1mM, 2mM, 3mM and 4mM). At the different extender concentration integrity shows different percentage from control.

3.2.1. Post dilution:

Data on the effect of ascorbic acid on cytoplasmic membrane integrity of sperm at the stage of post dilution shows that when 1mM and 4mM treatment of ascorbic acid was added in the sperm extender it does not show any significant difference from control 0mM. But (3mM) treatment of ascorbic acid in the sperm extender shows highest integrity (90.3±2.5) percentage and (2mM) treatment shows minimum percentage (80.3±1.1) of cytoplasmic membrane integrity.

3.2.2. Post Cooling:

The data on the effect of ascorbic acid on cytoplasmic membrane integrity of sperm at the stage of post cooling shows that (3mM) treatment has highest percentage (86.3±2.5) on sperm cytoplasmic membrane integrity ($P < 0.05$).Whereas 1mM & 2mM treatment shows slightly difference from control (0mM) but (4Mm) treatment in sperm extender has very low percentage (72.6±1.5) from all other treatments.

3.2.3. Post Equilibration:

The data on the effect of ascorbic acid on cytoplasmic membrane integrity of sperm at the stage of post equilibration with treatment of ascorbic acid (0mM, 1mM, 2mM, 3mM and 4mM) was added. (3mM) treatment shows highest (85.3±4.5) percentage of cytoplasmic membrane integrity ($P < 0.05$)as compare to control in the sperm extender but the other treatments 1 and 2mM has no significant difference to control (0mM) and (4mM) treatment has minimum percentage (70.0±1.0) of integrity of cytoplasmic membrane.

3.2.4. Post Thawing:

The data on the effect of ascorbic acid on cytoplasmic membrane integrity on sperm at the stage of post thawing (0mM, 1mM, 2mM, 3mM and 4mM) treatments were added. At this stage (3mM) treatment shows highest percentage (79.3±0.5) of integrity of membrane as compare to control (0mM). 1 & 2mM treatment has no difference but (4mM) treatment shows low percentage (65.3±4.7) on cytoplasmic membrane integrity.

This assay also shows that 3mM treatment of ascorbic acid has highest percentage (90.3±2.5) of integrity and 4mM treatment of ascorbic acid has low percentage (65.3±4.7) of cytoplasmic membrane integrity.

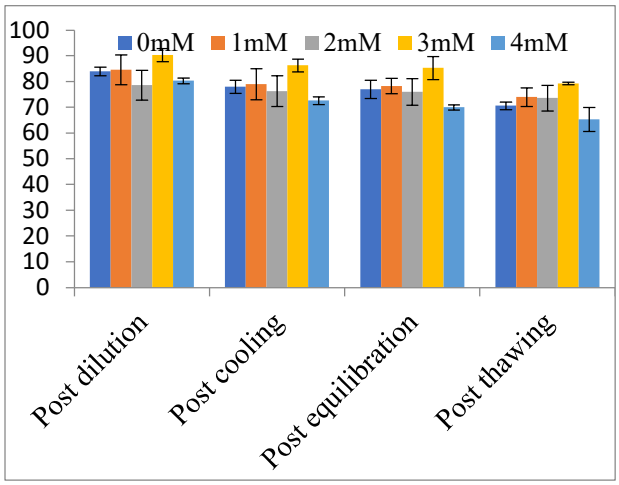

Figure 2. Shows that treatment 3mM shows the significant effect rather than remaining treatments (0, 1, 2, and 4mM) on sperm Cytoplasmic membrane integrity at the stages of post-dilution, post-cooling, post-equilibration and post-thawing respectively.

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3.3. Sperm Livability:

To check the data on the effect of Ascorbic acid on sperm Livability of Ring-necked pheasant at different steps of cryopreservation on the Post Dilution, Post Cooling, Post Equilibration and Post Thawing are shown in figure no.4.3.1-4.3.4. The treatments of ascorbic acid (0mM, 1mM, 2mM, 3mM and 4mM) were added in the sperm extender.

3.3.1. Post dilution:

Check the data on the effect of ascorbic acid on sperm Livability. At the stage of post dilution (3mM) treatment has highest percentage (90.6±1.5) ($P < 0.05$) as compare to control (0mM) treatment. In sperm extender and treatment 1 & 2mM shows similar percentages (85.6±6.6, 84.0±2.6) with but 4mM treatments shows no difference (82.3±0.5, 81.6±2.8).

3.3.2. Post Cooling:

Data on the effect of ascorbic acid on sperm livability at the stage of post cooling (3mM) treatment of ascorbic acid shows highest percentage (90.6±1.5) of sperm livability as compare to control in sperm extender. On the other hand (0mM & 4mM) treatments show similar difference ($P < 0.05$) respectively. (4mM) treatment has minimum percentage (81.6±2.8) of sperm livability in sperm extender as compare to other all treatment.

3.3.3. Post Equilibration:

Data on the effect of ascorbic acid on sperm livability at the stage of post equilibration (3mM) treatment of ascorbic acid has highest percentage (82.0±1.7) of sperm livability as compared to control. But 0,1 and 2mM treatments shows similar percentage ($P < 0.05$) of sperm livability but (4mM) treatment shows minimum percentage (70.3±1.5) of sperm livability in experimental extender as compare to all other treatment of ascorbic acid.

3.3.4. Post Thawing:

Data on the effect of ascorbic acid on sperm livability at the stage of post thawing (3mM)treatment of ascorbic acid shows highest percentage (79.3±2.0) ($P < 0.05$) on sperm livability as compared to control (0mM) treatment in experimental extender and (4mM) treatment show minimum percentage (69.0±1.0) on sperm livability in sperm extender. The 0mM, 1mM and 2mM shows no significant difference.

The effect of ascorbic acid on sperm livability of Ring-necked pheasant shows that (3mM) treatment of ascorbic acid shows better results as compare to control in experimental extender.

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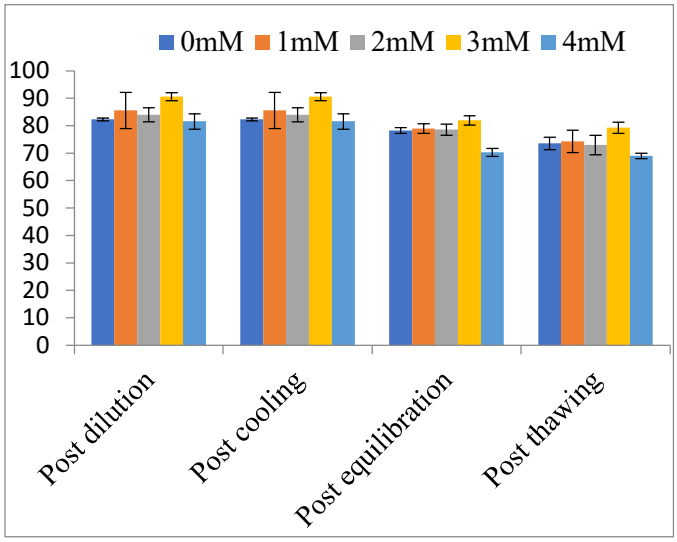

Figure 3. Shows that treatment 3mM shows the significant effect rather than remaining treatments (0, 1, 2, and 4mM) on sperm Livability at the stages of post-dilution, post-cooling, post-equilibration and post-thawing respectively.

3.4. Sperm Acrosome Integrity:

The effect of Ascorbic acid on the sperm acrosome integrity of Ring-necked pheasant at various steps of cryopreservation on the Post Dilution, Post Cooling, Post Equilibration and Post Thawing are shown in figure no. 4.4.1-4.4.4. The treatments of ascorbic acid (0mM, 1mM, 2mM, 3mM and 4mM) were added in the experimental extender.

3.4.1. Post Dilution:

Data on the effect of Ascorbic acid on the sperm Acrosomal integrity of Ring-necked pheasant at the stage of post dilution (3mM)treatment of ascorbic acid has highest percentage (89.0±2.0) on sperm acrosome integrity in experimental extender as compare to control (0mM). In this step of cryopreservation (3mM) treatment of ascorbic acid showed highest percentage ($P < 0.05$) on sperm acrosome integrity.

3.4.2. Post Cooling:

Data on the effect of Ascorbic acid on the Sperm Acrosomal Integrity of Ring-necked pheasant at the stage of post cooling (3mM) treatment of ascorbic acid has highest percentage (84.3±3.0) ($P < 0.05$) on sperm acrosome integrity in the sperm extender as compare to control but at (4mM) treatment has minimum percentage (70.0±1.0) of sperm acrosome integrity in sperm extender.

3.4.3. Post Equilibration:

Data on the effect of Ascorbic acid on the sperm Acrosomal Integrity of Ring-necked pheasant at the stage of post equilibration (3mM) treatment of ascorbic acid has highest percentage (83.3±5.6) ($P < 0.05$) on sperm acrosome integrity in the sperm extender as compare to control. But (4mM) treatment of ascorbic acid show minimum percentage (69.6±1.1) on sperm acrosome integrity in experimental extender.

3.4.4. Post Thawing:

Data on the effect of Ascorbic acid at sperm Acrosomal integrity of Ring-necked pheasant at the stage of post thawing (3mM) treatment and 1mM treatment of ascorbic acid show highest percentage (75.3±3.2, 75.3±2.5) on sperm acrosome integrity in the sperm extender ($P < 0.05$) as compared to control. (0Mm & 2mM) treatments of ascorbic acid also show similarity in percentage (70.6±0.5, 71.0±3.6) ($P < 0.05$).Whereas (4mM)

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treatment shows minimum percentage (66.0±2.6) on sperm acrosome integrity in sperm extender.

Hence we can say that in all the stages of Cryo-preservation viz; Post Dilution, Cooling, Equilibration and Post thawing on Sperm motility, cytoplasmic integrity, livability and acrosome integrity (3mM) treatment of ascorbic acid has positive result on the sperm of Ring-necked pheasant.

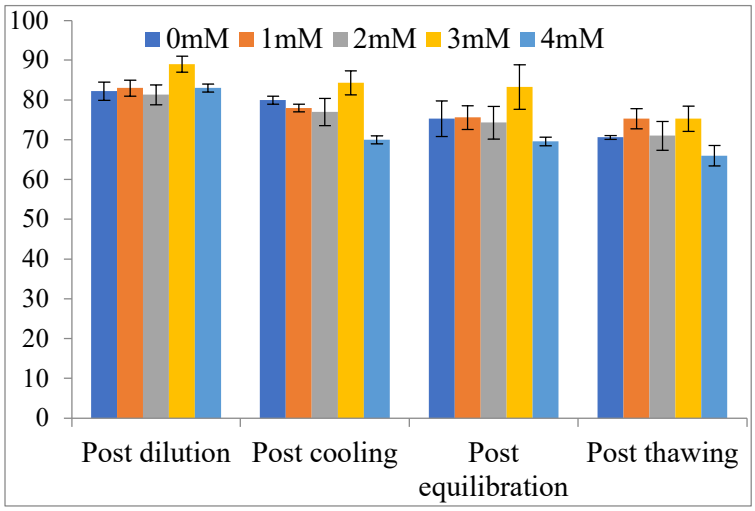

Figure 4. Shows that treatment 3mM shows the significant effect rather than remaining treatments (0, 1, 2, and 4mM) on sperm Acrosome integrity at the stages of post-dilution, post-cooling, post-equilibration and post-thawing respectively.

4. Discussions

Ascorbic acid (Vitamin C) has a property of water solubility and also known as vitamin C. It has antioxidant property and performs fighting function against microbes and bacterial infection or attack. It also acts as detoxifying agent and helps in the formation of collagen fibrous in tissue, skin, teeth and other body organs. It is found in green vegetables and citrus fruits.

In past study of Aseel chicken on sperm motility, Sperm livability and other parameters were assessed by adding ascorbic acid in the semen of Aseel chicken. It was observed that 1% concentration of vitamin C has better results from (0, 0.5, 2, and 4%) against ROS storage in LN_2 on the sperm motility and sperm viability and morphological problems and fertility condition.

(Tabatabaei, 2012) concluded the same results that was seen in chicken 1 % of ascorbic acid gives better result on motility, viability and morphological defects as compare to study of Turkey semen which was evaluated before the chicken semen. In Turkey semen test sperm motility, livability was not in better condition after the addition of ascorbic acid and the results was quiet different due to different species(Donoghue, 1997). The summary of previous study is that ascorbic acid in 1% has better results in motility, viability and intactness of membrane in Aseel chicken as compere to turkey semen.

But in the present study on the semen of Ring-necked pheasant vitamin C is very effective antioxidant for ROS molecules. It is very active antioxidant against peroxyl radical that were found in aqueous stage (Donoghue & Donoghue, 1997). In rabbits about 65% of body antioxidant consists of ascorbic acid and very beneficial in the seminal plasma as compare to pheasants (Yousef et al., 2003). It is very important for the formation of male hormones like testosterone to checking of reproductive characteristic (Sonmez et al., 2005). In this research 3mM has positive effect on semen of ring-necked pheasant but previous research told 3mM treatment has negative results on semen of Aseel chicken. In the Ring-necked pheasant as we increase or decrease concentration of ascorbic acid from 3mM treatment it shows negative result.

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In previous study Vitamin C was used as an antioxidant in the semen of Nili-Ravi Buffalo (Rakha et al., 2011). According to this research freezing techniques increased the Reactive oxidative species (ROS) production and decrease the antioxidant property which results decrease of sperm motility, intactness of membrane, livability and acrosome integrity of buffalo /bull spermatozoa (Sissy et al., 2007). It was observed that storage of sperm in liquid nitrogen by adding ascorbic acid on post thawing stage in the semen of buffalo/bull that didn't increase motility in control treatment but in the study of Nili-Ravi buffalo it was observed that addition of ascorbic acid in semen gives high motility of sperm and intactness of membrane (Andrabi et al., 2007). Both results were different from each other (Akhter et al., 2007).

The present study was conducted to check the effect of Ascorbic acid on semen of Ring-necked pheasant. The result was different from all above research. Sperm Motility, Sperm Cytoplasmic Membrane Integrity, Sperm Livability and Sperm Acrosome Integrity was checked by adding ascorbic acid in different treatments (0, 1, 2, 3 and 4mM). In the cryopreservation, effect of treatments of vitamin C was conducted at Post dilution, Post cooling, Post equilibration and Post thawing stages.

In all the stages of sperm motility, treatment of 3mM shows highest percentage (90.0± 0.0) ($P < 0.05$) on semen of Ring-necked pheasant as compared to control (0 mM) treatment. All the treatments show different result ($P < 0.05$) from 3mM. Treatment of 4mM has negative effect on the sperm motility having low percentage (45.0± 5.0) of sperm motility.

On sperm cytoplasmic membrane integrity stage, it was seen that 3mM treatment of ascorbic acid sperm shows high intactness (90.3± 2.5%) ($P < 0.05$) of membrane as compared to control and all others treatment (0, 1, 2, 3 and 4mM) of ascorbic acid. 0, 1 and 2mM shows no significant difference ($P < 0.05$). 4mM treatment shows less percentage (65.3± 4.7) on sperm cytoplasmic membrane intactness.

Effect of ascorbic acid on sperm livability in current study is different as compared to previous study. 3mM treatment shows highest percentage (82.0± 1.7) ($P < 0.05$) on sperm livability and 4mM treatment showed low percentage (69.0± 1.0) on sperm livability.

At the stage of sperm acrosome integrity, 3mM treatment shows highest (89.0± 2.0 %) ($P < 0.05$) and good results of acrosome intactness instead of 1 % concentration in previous study 4mM treatment shows low percentage (66.0± 2.6) of acrosome integrity.

In all the parameters 3mM treatment shows good results as compare to all previous studies due to the availability of different species. Results of Ascorbic acid vary from specie to specie on semen of avian and animal species.

5. Conclusion

The present study stated that different treatments of Ascorbic acid were used (0, 1, 2, 3 and 4 mM) to check the four parameters of Ring-necked pheasant semen. Treatment (3mM) of Ascorbic acid showed positive effect ($P < 0.05$) on semen of Ring-necked pheasant. 3mM treatment showed good results on all parameters such as Sperm Motility, Sperm Cytoplasmic Membrane Integrity, Sperm livability and Sperm Acrosome Integrity of sperm as compared to control. As we increased the concentration of Ascorbic acid from 3mM to 4mM then it showed negative effects on the all sperm parameters of Ring-necked pheasant. It showed different results from previous study due to different species and different environment.

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