Remittances Review
September 2024,
Volume: 9, No: S 4, pp. 688-702
ISSN: 2059-6588(Print) | ISSN 2059-6596(Online)

Received : 20 July 2024, Accepted: 25 September 2024
DOI: https://doi.org/10.33282/rr.vx9i2.38

Effect of Ascorbic Acid on Semen Cryopreservation of Black Australorp

Abdul Basit [1], Umer Ali [2], Bushra Allah Rakha[3*] and Abdur Rehman Azam[4]

[1]Department of Zoology, University of Education, Township Campus, Lahore Pakistan
[2]Department of Biological Sciences, Tennessee State University, Nashville, TN 37209, USA
[3]Department of Wildlife, Pir Mehr Ali Shah Arid Agriculture University Rawalpindi, Pakistan
[4]Department of Pathobiology, College of veterinary medicine, University of Illinois at Urbana Champaign, USA
Corresponding author: Bushra Allah Rakha

Abstract:

Black Australorp chicken is an Australian breed and now these are present all over the world. BA is attractive birds that are docile, hardy, and productive layers, making them perfect for beginners to captivity. 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 Black Australorp at different concentrations of ascorbic acid. Bird sperm is much more susceptible to cryo-damage due to its specific shape and physical properties, which lowers the survival of bird sperm. With low birth rates lines of chicken, glycerol is less harmful and most efficient cryoprotectant. Glycerol has also been shown to react with sperms metabolism, modify lipids bilayers, & lead to limited fertilizing capacity through influencing semen acrosome reaction. Semen was cooled from post-dilution stage (37ºC) to post-cooling (20ºC). Then it further cooled gradually to get post-equilibration stage (4ºC) within 24 hours. After that 7% glycerol was added to the sample. Then it was transferred to liquid nitrogen (LN_2) cylinder for 24 hours. The better results were seen at 0.5 mM treatment of ascorbic acid. Sperm showed significantly different ($P < 0.05$) percentage on 0.5 mM treatment on all the stages of cryopreservation rather than other treatments.

Keywords: Black Australorp, Ascorbic acid, Semen Cryopreservation, Liquid Nitrogen

INTRODUCTION

Pakistan has near about 65 bigger size mammal species, 109 small mammal species (Roberts, 2005a, b), only 22 amphibian species, 185 reptile species (Khan, 2006), and greater than 650 species of birds, Pakistan has a great diversity of ecological community that gets their consideration of avian species (Roberts, 1997; Grimmett et al., 2001; Mirza, 1998; Mirza and

Wasiq, 2007; Minton, 1966). Black Australorp (BA) is a breed having multi- purpose. BA is attractive birds that are docile, hardy, and productive layers, making them perfect for beginners to captivity. The American Poultry Association recognized them as a standard breed in 1929, after they were introduced to America in the 1920s (John, 2011; Shahin et al., 2024).

Black Australorp chicken is an Australian breed and now these are present all over the world (Fourie & Grobbelaar 2003). The very important imported chicken which is frequently utilized in South Africa is Black Australorp. Black Australorp hen was produced as a consequence of advancement the English Orpington in Australia. Chickens were known to Black utility Orpington. The color of Australorp hens is black. Male's Australorp weigh is 3.85 kg while female's weight is 2.94 kg (Fourie & Grobbelaar 2003). 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 -1960 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). Sperm cryopreservation is the most efficient technique for storage of germ cells as well as maintains the genetic variety of birds (Blesbois, 2011). Techniques of cryopreservation are better just for the semen, instead of oocyte or embryo (Rakha et al., 2017). Oxidative stress is described as the inability to stabilize redox signaling as a consequence of reactive oxygen species (ROS) overabundance or the debility of regulation antioxidant systems (Peña et al., 2019). The strength and period of oxidative stress could really affect cell fortune, fluctuating from modifying feedback to apoptosis or necrosis (Lushchak, 2014). Because the existence of oxidative stress in cryopreservation, cooling or defrost of a cell generates a larger generation of ROS (Baumber et al., 2005). The consequences of ROS upon germ cells activities can be fatal (Baumber et al., 2000). Moreover, a large concentration of reactive oxygen species (ROS) can disrupt the functioning and physiology of biological large molecules, and also disrupt typical cellular metabolism. Exposure to light and oxygen radiations during the freezing and storage of sperm cells stimulates the production of reactive oxygen species as well as lipid peroxidation of germ plasma membrane (Andrabi et al., 2008). Antioxidants are naturally present in very low concentrations in spermatozoa. However, the proportion of naturally occurring antioxidants was lowered throughout freeze/thaw procedure (Bilodeau et al., 2000).

Cryoprotectants are chemical compounds. They protect cells against harm caused by after-freezing (Bhattacharya, 2018). With such a decrease in temperature, the bio-physical features of spermatozoa of a particular species are crucial for very specific association with a specific

extender (Holt, 2000 a, b; Han et al., 2005; Rakha et al., 2013). Germ cells obtained from infertile individuals that have undergone testicular sperm eviction are generally cryopreserved (Di Santo et al., 2012). One very major reason of male sterility is a problem with spermatozoal role, which is hard to treatable (Hull, et al., 1985). Of all the existing cryoprotectants, glycerol and dimethyleacetamide (DMA) are thought to be the most effective (Woelders et al., 2006). With low birth rates lines of chicken, glycerol is the less harmful and most efficient cryoprotectant (Blesbois et al., 2007). In the mechanism of male reproduction, gonads normally create large quantities of reactive oxygen species in comparison to certain other structures; they are more susceptible to oxidative harm, despite the fact that they have a higher antioxidant activity (Simmons et al., 2018). Fowl sperms show strong metabolic reactions, with polyunsaturated fatty acids and deactivated scavenger enzymes abundant inside the membrane; as a result, they're particularly vulnerable towards oxidative stress (Attia, Abou-Shehema, Asmaa, & Abdella, 2020a; Attia et al., 2019; Najafi et al., 2020).

Oxidative stress can affect the integrity of semen membranes, resulting in decreased mobility and acrosome integrity, increased DNA destruction, and decreased male fertility (Najafi et al., 2019). Both mammals &avian, increased reactive oxygen species production is related to low sperm characteristics (Moller, 2014; Agarwal, 2014). Ascorbic acid is a familiar antioxidant which enhances sperm of chickens maintained during extreme heat circumstances, providing 65 percent of seminal plasma's antioxidant potential; it controls body hotness & hormone production (Khan et al., 2012). 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 Black Australorp at different concentrations of ascorbic acid.

MATERIALS AND METHODS

Study Area

The current study was carried out at the Pir Mehr Ali Shah Arid Agriculture University in Pakistan's Avian Research Center.

Sample Size

The study was consisted on 12 adult male Black Australorp. Individual wood cages measuring 3.5 feet x 4 feet were used to populate the birds. According to Burrows and Quinn, (1935) the sperm was obtained using abdominal massage.

Experimental Techniques

Just those samples were used for the assessment of semen quality parameters that have motility greater than 75-80 %. Mobility, plasma membrane integrity, acrosome integrity, and viability

were tested in pooled samples with motility greater than 75–80 percent. This were divided into five halves and filled with falcon tubes.

Extender Preparation

The extender was put into each falcon tubes. After that, we'll put 0 mM of vitamin C in first tube, 0.5 mM in second tube, 1.5 mM in third, 2.5 mM in fourth, then 3.5 mM in fifth. Ascorbic acid is the antioxidant that was investigated in this experiment. The previously diluted sperm was further processed. It gradually cooled from 37 ºC to 20 ºC then further cool down to 4 ºC at the rate of -0.275 ºC /min. After that, it was evenly balanced for 10 minutes at 4 ºC after the addition of 7% glycerol to each extender. The cooled semen was then placed in a cold cabinet unit and placed 5 cm above the level of liquid nitrogen vapors (LN_2) for 10 minutes to freeze from 4 ºC to -80 °C at a rate of -8.4 °C/min. the straws were then immersed in LN_2 for 24 hours at -196 ºC in plastic goblets attached to the canes. After 24 hours, the straws were thawed for 30 seconds in water bath at 37 ºC and then keep it at 37 ºC for four hours to examine acrosomal integrity, viability, plasma membrane integrity and motility.

Sperm Quality Assays

Sperm motility was evaluated as the percentage of motile sperm cells. A droplet of sperm sample was placed on an already warmed (37 ºC) glass slide using a Pasteur pipette under phase contrast microscope at x 400 as described by (Zemjanis, 1970) at different stages of cryopreservation like, pre-dilution, post-dilution, post-equilibration and post-thawing. The percentage motile sperm was subjectively assessed on a scale ranging from 0 to 100 %.

This is a membrane that serves as a barrier between extra-cellular and intra-cellular material. It enhances sperm survival in the female reproductive tract in order to sustain fertilization capacity and osmotic balance (Santiago-Moreno et al., 2009). One gram of $Na_3C_6H_5O_7$ (sodium citrate) was dissolved in 100 milliliters of distilled water to make the hypo-osmotic swelling test solution. Already diluted semen (25 ul) was mixed with 500 ul of HOS solution with an osmotic pressure of 100mOsmol/kg. It was then incubated at 25 ºC for 30 minutes. Furthermore, 2 % eosin was used for fixation. After that, a drop of incubated solution was fixed on a pre-warmed (37 ºC) glass slide. Sperm cells with swollen heads and coiled tails were considered to be normal spermatozoa with undamaged as well as biochemically active cellular membranes. Sperm that did not respond to the HOS solution had a damaged plasma membrane and was considered abnormal or dead.

The addition of lake glutamate solution to the sperm count examined the live sperm count. Lake glutamate solution (Bakst & Cecil., 1997) was prepared by the addition of $C_5H_8NO_4Na$ (sodium glutamate) of 0.01735 g, $C_2H_3NaO_2$ (Sodium acetate) of 0.0085 g, Potassium citrate ($C_6H_5K_3O_7$) of 0.00128 g and Magnesium chloride $MgCl_2$ of 0.000676 g in 100 ml distilled water. One to two

Remittances Review
September 2024,
Volume: 9, No: S 4, pp. 688-702
ISSN: 2059-6588(Print) | ISSN 2059-6596(Online)
drops of stain and one drop of semen sample were mixed together. After that, a smear was prepared on a glass slide, which was then fixed and air dried.

The acrosomal intactness is described by an organelle that develops over the half anterior part of the head in animal and human spermatozoa. Giemsa stain was used to examine the acrosomal integrity of sperm at various stages (Rakha et al., 2015a, b; Rakha et al., 2016). The stain was prepared by the addition of Giemsa (3gram) and 2 ml of Sorenson phosphate buffer saline at pH 7.0 into 35 ml distilled water. Smear was made by adding a drop of semen sample (5ul) on a clear glass slide and drying it completely. It was then fixed in neutral formal-saline, i-e 5 % formaldehyde, for 30 minutes. After that, the fixed slides were stained with Giemsa for 1.5 hours and air dried it.

RESULT

Sperm motility:

Data on the impact of ascorbic acid on sperm motility of Black Australorp, post dilution of sperm motility shows highest percentage at (0 mM) (92.5 ± 2.89) as well as in post thawing of sperm motility (0.5 mM) shows maximum percentage (40.0 ± 0.00) and (3.5mM) shows lowest percentage (10.0 ± 0.00).

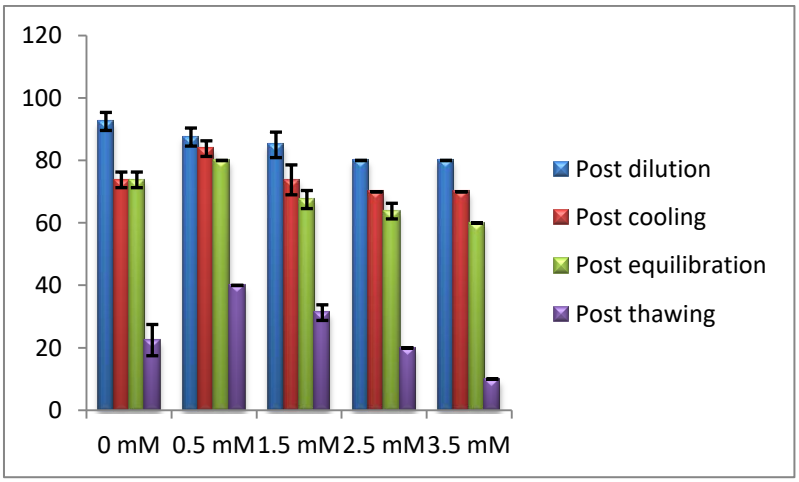

Figure 1. This Figure shows that treatment 0.5 mM of post thawing shows the significant effect and rest of the treatments (0 mM, 1.5 mM, 2.5 mM, 3.5 Mm) shows minimum percentage on sperm acrosomal integrity at the stage of post thawing.

Acrosomal integrity:

Data on the impact of ascorbic acid on sperm acrosomal integrity of Black Australorp, post dilution of sperm acrosomal integrity shows highest percentage at (3.5 mM) (91.8 ± 2.36) as well

as (0 mM and 1.5 mM) shows equal percentage (82.5 ± 2.89). Post equilibration (2.5 mM and 3.5Mm) shows almost similar percentage (70.5 ± 1.73). On the other hand, in post thawing of sperm acrosomal integrity (0.5 mM) shows highest percentage (44.8 ± 6.08) and (0 mM) shows lowest percentage (19.8 ± 1.26).

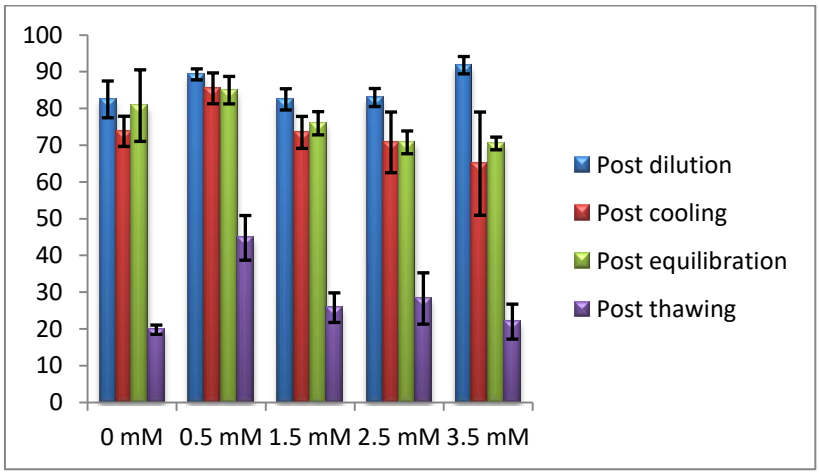

Figure 2. This Figure also shows that treatment 0.5 mM of post thawing shows the significant effect and rest of the treatments (0 mM, 1.5 mM, 2.5 mM, 3.5 Mm) shows minimum percentage on sperm motility at the stage of post thawing.

Viability:

Data on the impact of ascorbic acid on sperm viability of Black Australorp, post dilution of sperm viability shows highest percentage at (0 mM) (96.3 ± 2.22) as well as (1.5 mM) shows less percentage (87.5 ± 1.91).Post cooling shows maximum percentage at (0.5 mM) (92.5 ± 2.52). Post equilibration and post thawing at (0.5 mM) shows highest percentage (83.8 ± 6.08) (40.5±11.79) respectively.

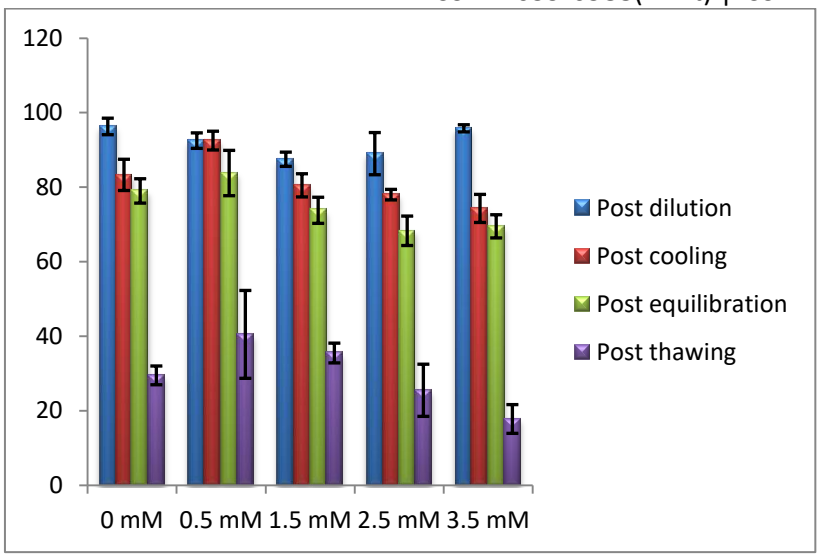

Figure 3. This Figure also shows that treatment 0.5 mM of post thawing shows the significant effect as compared to the other treatments (0 mM, 1.5 mM, 2.5 mM, and 3.5 mM).

Plasma membrane integrity:

Data on the impact of ascorbic acid on sperm plasma membrane integrity of Black Australorp, post dilution of sperm plasma membrane integrity shows highest percentage at (0 mM) (96.5 ± 4.36) as well as (3.5 mM) shows less percentage (77.8 ± 2.06). Post thawing at (0.5 mM) shows highest percentage (42.5 ± 2.89) and minimum at (3.5 mM) (15.0 ± 4.69).

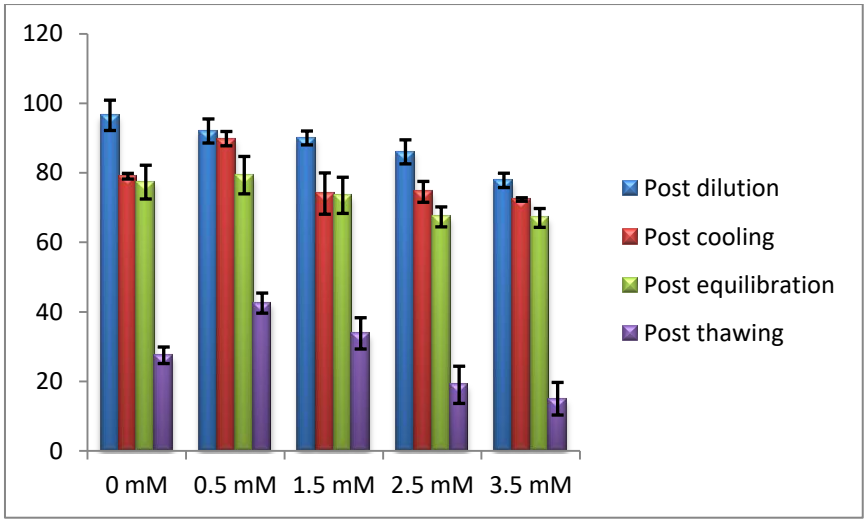

Figure 4. Like other Figures this Figure also shows that treatment 0.5 mM of post thawing shows the significant effect as compared to the other treatments (0 mM, 1.5 mM, 2.5 mM, and 3.5 mM).

Table 1: Complete

Sperm Motility	Cryopreservation stages	0 mM	0.5 mM	1.5 mM	2.5 mM	3.5 mM
	Post dilution	92.5 ± 2.89^{c}	87.5 ± 2.89^{b}	85.0 ± 4.08^{b}	80.0 ± 0.00^{a}	80.0 ± 0.00^{a}
	Post cooling	73.8 ± 2.50^{a}	83.8 ± 2.50^{b}	73.8 ± 4.79^{a}	70.0 ± 0.00^{a}	70.0 ± 0.00^{a}
	Post equilibration	73.8 ± 2.50^{a}	80.0 ± 0.00^{b}	67.5 ± 2.89^{c}	63.8 ± 2.50^{d}	60.0 ± 0.00^{e}
	Post thawing	22.5 ± 5.00^{b}	40.0 ± 0.00^{a}	31.3 ± 2.50^{c}	20.0 ± 0.00^{b}	10.0 ± 0.00^{d}
Acrosomal integrity	Cryopreservation stages	0 mM	0.5 mM	1.5 mM	2.5 mM	3.5 mM
	Post dilution	82.5 ± 5.00^{a}	89.3 ± 1.5^{b}	82.5 ± 2.89^{a}	83.0 ± 2.45^{a}	91.8 ± 2.36^{b}
	Post cooling	73.8 ± 4.11^{ac}	85.5 ± 4.20^{c}	73.5 ± 4.36^{ac}	70.8 ± 8.26^{a}	65.0 ± 14.05^{a}
	Post equilibration	80.8 ± 9.74^{bc}	85.0 ± 3.74^{b}	76.0 ± 3.16^{ac}	70.8 ± 3.10^{a}	70.5 ± 1.73^{a}
	Post thawing	19.8 ± 1.26^{a}	44.8 ± 6.08^{c}	25.8 ± 4.03^{ab}	28.3 ± 6.99^{b}	22.0 ± 4.76^{ab}
Viability	Cryopreservation stages	0 mM	0.5 mM	1.5 mM	2.5 mM	3.5 mM
	Post dilution	96.3 ± 2.22^{c}	92.5 ± 2.08^{bc}	87.5 ± 1.91^{a}	89.0 ± 5.66^{ab}	95.8 ± 0.96^{c}
	Post cooling	83.3 ± 4.19^{c}	92.5 ± 2.52^{d}	80.5 ± 3.11^{bc}	78.0 ± 1.41^{ab}	74.3 ± 3.77^{a}
	Post equilibration	79.0 ± 3.27^{bc}	83.8 ± 6.08^{c}	73.8 ± 3.50^{ab}	68.3 ± 3.95^{a}	69.5 ± 3.11^{a}
	Post thawing	29.5 ± 2.52^{bc}	40.5 ± 11.79^{d}	35.5 ± 2.65^{cd}	25.5 ± 7.00^{ab}	17.8 ± 3.86^{a}
Plasma membrane	Cryopreservation stages	0 mM	0.5 mM	1.5 mM	2.5 mM	3.5 mM
	Post dilution	96.5 ± 4.36^{d}	$92.0 \pm$	$90.0 \pm$	86.0 ± 3.46^{b}	$77.8 \pm$

integrity			3.46[cd]	2.00[bc]		2.06[a]
	Post cooling	79.0 ± 0.82[b]	89.8 ± 2.06[c]	74.0 ± 5.94[a]	74.5 ± 3.00[ab]	72.3 ± 0.50[a]
	Post equilibration	77.3 ± 4.86[b]	79.3 ± 5.38[b]	73.5 ± 5.20[ab]	67.3 ± 2.87[a]	67.0 ± 2.71[a]
	Post thawing	27.5 ± 2.38[d]	42.5 ± 2.89[c]	33.8 ± 4.50[b]	19.0 ± 5.35[a]	15.0 ± 4.69[a]

DISCUSSION:

Solubility in water is a characteristic of ascorbic acid, also referred to as vitamin C. This has anti-oxidant properties as well as defends against bacterial infection and other micro-organisms. Moreover, it functions as a detoxifying agent as well as assists in the synthesis of the collagen fiber found in tissue, teeth as well as other body organs. Citrus fruits & leafy vegetables both contain it.

Ascorbic acid was added to Aseel chicken's sperm in earlier research on sperm plasma membrane integrity to measure sperm acrosomal integrity as well as other parameters. This was shows that a 1 percent concentration of ascorbic acid produces excellent outcomes to 0 percent, 0.5 percent 2 percent and 4 percent against reactive oxygen species storage in liquid nitrogen on the sperm acrosomal integrity, sperm livability, fertility issue as well as morphometric issues.

According to Tabatabaei, (2012) the same findings from a research of Turkey sperm, that was assessed before the research of chicken sperm, show excellent outcomes on livability, motility and morphologically deformities. After the addition of Vitamin C to a Turkey sperm assessment, sperm viability & motility weren't improved, and outcomes were totally separate due to various species tested (Donoghue, 1997; Bilal[a,b] et al., 2021). According to the findings of earlier research, Turkey semen & Aseel semen both benefit from Vitamin C in comparison to each other in terms of plasma membrane integrity, and livability.

However, in this research on Black Australorp semen, ascorbic acid is really efficient anti-oxidant for reactive oxygen species. It has a strong anti-oxidant effect against peroxides discovered in soluble phase (Donoghue, 1997). Comparatively advantageous in the seminal plasma to pheasants, Vitamin C makes up around 65 percent of the body's anti-oxidants in rabbits. For the production of male hormones such as testosterone, it is crucial to examine reproductive characteristics (Sonmez et al., 2005; Shah et al., 2022). However, prior studies indicated that 0.5 Mm treatment had adverse effects on the sperm of Aseel chickens.

In this study, 0.5 mM had a beneficial impact on Black Australorp sperm. Vitamin C treatment in Black Australorp exhibits poor outcomes as concentrations are increased or decreased starting at

0.5 Mm. Ascorbic acid was utilized as an antioxidant in the semen of Nili-Ravi Buffalo in prior-research (Rakha et al., 2011). This study found that freezing procedures increased the growth of reactive oxygen species and decreased anti-oxidant activity that decreased sperm livability, acrosomal integrity, plasma membrane integrity and their motility in bull spermatozoa (Sissy et al., 2007).

It was found that adding ascorbic acid to the buffalo/bull semen after it had been frozen in liquid nitrogen did not increase sperm motility in the control condition, but that doing so in the Nili-Ravi buffalo increased sperm motility as well as preserved membrane integrity (Andrabi et al., 2007). The two outcomes were distinct from one another (S. Akhter et al., 2007; Jawad et al, 2023). The goal of the current research was to determine how Vitamin C affected the sperm of Black Australorp. The findings were distinct from those of the earlier studies. Vitamin C was added in various treatments to assess sperm viability, sperm plasma membrane integrity, sperm motility, sperm acrosomal integrity (0 mM, 0.5 mM, 1.5 mM, 2.5 mM and 3.5 mM). At the post dilution, post cooling, post equilibration as well as post thawing phases of cryopreservation, ascorbic acid treatment effects were evaluated.

Treatment with 0.5 mM results with maximum percentage (87.5 ± 2.89) ($P < 0.05$) across all sperm motility phases in Black Australorp sperm, compared to control (0 mM) treatment. Every treatment shows a different outcome ($P < 0.05$) from 0.5 mM. Sperm motility is negatively impacted by treatment with 3.5 mM because it has reduced sperm motility (10.0 ± 0.00) percentage.

When it comes to sperm acrosomal integrity, 0.5 mM treatment shows the maximum level of acrosomal integrity (89.3 ± 1.5) ($P < 0.05$) instead of using a 1 percent concentration in the earlier study, 3.5mM treatment shows lowest percentage (22.0 ±4.76) of acrosome integrity. The present study's impact of Vitamin C on sperm viability differs from that of the earlier research. 0.5 mM treatment shows maximum (92.5 ± 2.08) percentage ($P < 0.05$) on sperm viability and 3.5 mM treatment showed least percentage (17.8 ± 3.86) on sperm viability.

When compared to control as well as all other treatments (0, 0.5, 1.5, 2.5 and 3.5mM of Vitamin C), sperm treated with 0.5mM of Vitamin C shows highest levels of plasma membrane integrity (92.0 ± 3.46) ($P < 0.05$) and 3.5 mM treatment showed least percentage (15.0 ± 4.69) on sperm plasma membrane integrity.

Depending on the availability of various species, 0.5 mM treatment shows positive outcomes across all parameters when compared to all earlier research. The effects of ascorbic acid on the sperm of various animal & birds species differ depending on the species.

Conclusion:

According to the current study, four parameters of Black Australorp sperm were examined using various Ascorbic acid treatments (0, 0.5, and 1.5, 2.5 and 3.5 mM). Black Australorp semen responded effectively to treatment with ascorbic acid (0.5 mM) ($P < 0.05$). When compared to the control group, the 0.5 mM treatment had positive effects on all sperm parameters, including livability, motility and sperm acrosome integrity. The Black Australorp sperm parameters were negatively impacted as soon as the Vitamin C concentration was enhanced from 0.5 mM to 1.5 mM. Because of the various species as well as environments, the results were different from those of the earlier study.

REFERENCES:

Agarwal, A., Virk, G., Ong, C., & Du Plessis, S. S. (2014). Effect of oxidative stress on male reproduction. *The world journal of men's health*, *32*(1), 1-17.

Ali, U., Bilal, A., Iqbal, A., Ansari, M. S., Rakha, B. A., & Akhter, S. (2022). Ascorbic acid effect on frozen and thawed on sperm motility, plasma membrane integrity, livability and acrosome integrity of ring-necked pheasant (Phasianus colchicus) semen.

Andrabi, S. M. H., Ansari, M. S., Ullah, N., & Afzal, M. (2008). Effect of nonenzymatic antioxidants in extender on post-thaw quality of buffalo (Bubalus bubalis) bull spermatozoa. *Pakistan veterinary journal*, *28*(4).

Attia, Y. A., Abou-Shehema, B. M., Abdellah, A. A., Aly, O. M., & El-Naggar, A. S.(2020). Effect of ascorbic acid and/or alpha-tocopherol fortification on semen quality, metabolic profile, antioxidants status, and DNA of roosters exposed to heat stress. *The Journal of Animal & Plant Sciences*, *30*(2), 325-335.

Attia, Y. A., El-Naggar, A. S., Abou-Shehema, B. M., & Abdella, A. A. (2019). Effect of supplementation with trimethylglycine (betaine) and/or vitamins on semen quality, fertility, antioxidant status, DNA repair and welfare of roosters exposed to chronic heat stress. *Animals*, *9*(8), 547.

Ali, U., Vungarala, S., & Tiriveedhi, V. (2024). Genomic Features of Homologous Recombination Deficiency in Breast Cancer: Impact on Testing and Immunotherapy. *Genes*, *15*(2), 162. https://doi.org/10.3390/genes15020162

Bakst, M. R., & Cecil, H. C. (1997). *Techniques for semen evaluation, semen storage, and fertility determination*. Champaign, IL, USA: Poultry Science Association.

Baumber, J., Ball, B. A., & Linfor, J. J. (2005). Assessment of the cryopreservation of equine spermatozoa in the presence of enzyme scavengers and antioxidants. *American journal of veterinary research*, *66*(5), 772-779.

Baumber, J., Ball, B. A., GRAVANCE, C. G., Medina, V., & DAVIES-MOREL, M. C. (2000). The effect of reactive oxygen species on equine sperm motility, viability, acrosomal

integrity, mitochondrial membrane potential, and membrane lipid peroxidation. *Journal of andrology*, *21*(6), 895-902.

Bhattacharya, S. (2018). Cryoprotectants and their usage in cryopreservation process. *Cryopreservation Biotechnology in Biomedical and Biological Sciences*, 7-18

Bilodeau, J. F., Chatterjee, S., Sirard, M. A., & Gagnon, C. (2000). Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. *Molecular Reproduction and Development: Incorporating Gamete Research*, *55*(3), 282-288.

Jawad, M., Bilal, A., Khan, S., Rizwan, M., & Arshad, M. (2023). Prevalence and awareness survey of tuberculosis in the suspected population of Bajaur Agency in Fata, Pakistan: Prevalence and awareness survey of tuberculosis. Pakistan Journal of Health Sciences, 56-61.

Blesbois, E. (2011). Freezing avian semen. *Avian biology research*, *4*(2), 52-58.

Blesbois, E., & Brillard, J. P. (2007). Specific features of in vivo and in vitro sperm storage in birds. *Animal*, *1*(10), 1472-1481.

Burrows, W. H., & Quinn, J. P. (1935). A method of obtaining spermatozoa from the domestic fowl. *Poultry Science*, *14*(4), 251-253.

Di Santo, M., Tarozzi, N., Nadalini, M., & Borini, A. (2011). Human sperm cryopreservation: update on techniques, effect on DNA integrity, and implications for ART. *Advances in urology*, *2012*.

Donoghue, A. M., & Donoghue, D. J. (1997). Effects of water-and lipid-soluble antioxidants on turkey sperm viability, membrane integrity, and motility during liquid storage. *Poultry Science*, *76*(10), 1440-1445.

Fourie, C., & Grobbelaar, J. A. N. (2003). Indigenous poultry breeds. *Krugersdorp, Germany, Wing Nut Publications*, 20-21.

Grimmett, R. C., & Inskipp, T. (2001). Birds of Indian Subcontinent, Christopher Helm, London.

Hamouda, F., El-Sissy, A. H., Radwan, A. K., Hussein, H., Gadallah, F. H., Al-Sharkawy, N. A. H. L. A., ... & KAMEL, A. M. (2007). Correlation of karyotype and immunophenotype in childhood acute lymphoblastic leukemia; experience at the National Cancer Institute, Cairo University, Egypt. *J Egypt Natl Canc Inst*, *19*(2), 87-95.

Shah, S. A. H., Bilal, A., Ahmad, M. M., & Bukhari, S. S. (2022). Deforestation is causing a great loss in avian diversity in Pakistan. American Journal of Zoology, 5(3), 24-29.

Han, X. F., Niu, Z. Y., Liu, F. Z., & Yang, C. S. (2005). Effects of diluents, cryoprotectants, equilibration time and thawing temperature on cryopreservation of duck semen. *Int. J. Poult. Sci*, *4*(4), 197-201.

Holt, W. V. 2000a. Basic aspects of frozen storage of semen. Anim. Reprod. Sci., 62:3-22.

Hull, M. G., Glazener, C. M., Kelly, N. J., Conway, D. I., Foster, P. A., Hinton, R. A.,... & Desai, K. M. (1985). Population study of causes, treatment, and outcome of infertility. *Br Med J (Clin Res Ed), 291*(6510), 1693-1697.

John, L. S. (2011). Chicken Breeds and Varieties (A2880). *University of Wisconsin*.

Khan, M. S. (2006). *Amphibians and reptiles of Pakistan* (pp. 1-311). Malabar, Florida, USA: Krieger Publishing Company.

Khan, R. U., Naz, S., Nikousefat, Z., Selvaggi, M., Laudadio, V., & Tufarelli, V. (2012). Effect of ascorbic acid in heat-stressed poultry. *World's Poultry Science Journal*, *68*(3), 477-490.

Bilal, A. (2021). Rabies is a zoonotic disease: a literature review. Occup. Med. Health Aff, 9(2).

Lushchak, V. I. (2014). Free radicals, reactive oxygen species, oxidative stress and its classification. *Chemico-biological interactions*, *224*, 164-175.

Minton, S. A., (1966). A contribution to the herpetology of West Pakistan. Bull. Amer. Mus. Hist. 134(2): 31-184.

Mirza, Z. B., (1998). Illustrated handbook of animal biodiversity of Pakistan. Centre for Environmental Research and Conservation, Islamabad, pp. 99.

Bilal, A., Iftikhar, A., Ali, U., Naveed, N., Anjum, M. I., Fatima, U., ... & Sajjad, M. K. (2021). Comparison of different Covid-19 vaccines globally: An overview. J Gynecol Women's Health, 21, 556071.

Mirza, Z. B., and H. Wasiq. (2007). A field guide to birds of Pakistan. Published by WWF Pakistan, Bookland Lahore.

Møller, A. P., Bonisoli-Alquati, A., Mousseau, T. A., & Rudolfsen, G. (2014). Aspermy, sperm quality and radiation in Chernobyl birds. *PLoS One*, *9*(6), e100296.

Najafi, A., Kia, H. D., Mehdipour, M., Shamsollahi, M., & Miller, D. J. (2019). Does fennel extract ameliorate oxidative stress frozen-thawed ram sperm?. *Cryobiology*, *87*, 47-51.

Najafi, D., Taheri, R. A., Najafi, A., Shamsollahi, M., & Alvarez-Rodriguez, M. (2020). Effect of astaxanthin nanoparticles in protecting the post-thawing quality of rooster sperm challenged by cadmium administration. *Poultry Science*, *99*(3), 1678-1686.

Peña, F. J., O'Flaherty, C., Ortiz Rodríguez, J. M., Martín Cano, F. E., Gaitskell- Phillips, G. L., Gil, M. C., & Ortega Ferrusola, C. (2019). Redox regulation and oxidative stress: The particular case of the stallion spermatozoa. *Antioxidants*, *8*(11), 567.

Qadeer, S., Khan, M.A., Ansari, M.S., Rakha, B.A., Ejaz, R., Iqbal, R. and Akhter, S. (2015) Efficiency of antifreeze glycoproteins for cryopreservation of Nili-Ravi (*Bubalus bubalis*) buffalo bull sperm. *Anim. Reprod. Sci.*, 157, 56–62.

Shahin, F., Ishfaq, A., Asif, I., Bilal, A., Masih, S., Ashraf, T., ... & Ishfaq, R. (2024). CRISPR-Cas Innovative Strategies for Combating Viral Infections and Enhancing Diagnostic Technologies: CRISPR-Cas in Viral Diagnostics and Therapeutics. Journal of Health and Rehabilitation Research, 4(3), 1-4.

Rakha, B. A., Ansari, M. S., Akhter, S., Zafar, Z., Hussain, I., Santiago-Moreno, J., & Blesbois, E. (2017). Cryopreservation of Indian red jungle fowl (Gallus gallus murghi) semen with polyvinylpyrrolidone. *Cryobiology*, *78*, 27-33.

Rakha, B. A., M. S. Ansari, H. Hussain, and E. Blesbois. 2016. Cryopreservation of Indian red jungle fowl (Gallus gallus murghi) semen. Anim. Reprod. Sci. 174:45–55.

Rakha, B.A., I. Hussain, S. Akhter, N. Ullah, S. M. H. Andrabi and M. S. Ansari. 2013. Evaluation of Tris-citric acid, skim milk and sodium citrate extenders for liquid storage of Punjab Urial (*Ovis vignei punjabiensis*) spermatozoa. Reprod. Biol., 13: 238-242.

Roberts, T. J. (2005). *Field guide to the large and medium-sized mammals of Pakistan*. Oxford University Press.

Roberts, T. J. (2005). *Field guide to the small mammals of Pakistan*. Oxford University Press.

Roberts, T. J., (1997). *The mammals of Pakistan*. Revised Edition. Karachi: Oxford University Press, pp. 525.

Santiago-Moreno, J., Castaño, C., Toledano-Díaz, A., Coloma, M. A., López- Sebastián, A., Prieto, M. T., & Campo, J. L. (2012). Cryoprotective and contraceptive properties of egg yolk as an additive in rooster sperm diluents. *Cryobiology*, *65*(3), 230-234.

Simmons, L. W., Lovegrove, M., & Lymbery, S. J. (2018). Dietary antioxidants, but not courtship effort, affect oxidative balance in the testes and muscles of crickets. *Journal of Experimental Biology*, *221*(20), jeb184770.

Sönmez, V. (2005). *Hayat ve sosyal bilgiler öğretimi ve öğretmen kılavuzu*. Anı Yayıncılık.

Tabatabaei, S., & Aghaei, A. (2012). Effect of L-carnitine on sperm quality during liquid storage of chicken semen. *Comparative Clinical Pathology*, *21*(5), 711- 717.

Tucker, L., Ali, U., Zent, R., Lannigan, D. A., Rathmell, J. C., & Tiriveedhi, V. (2024). Chronic High-Salt Diet Activates Tumor-Initiating Stem Cells Leading to Breast Cancer Proliferation. *Cells*, *13*(11), 912. https://doi.org/10.3390/cells13110912

Woelders, H., C. A. Zuidberg and S. J. Hiemstra. 2006. Animal genetic resources conservation in the Netherlands and Europe: poultry perspective. Poult. Sci., 85: 216-222.

Woodruff, T., & Snyder, K. A. (Eds.). (2007). *Oncofertility: fertility preservation for cancer survivors* (Vol. 138). Springer Science & Business Media.

Zemjanis, R. (1970). Diagnostic and therapeutic techniques in animal reproduction. *Diagnostic and therapeutic techniques in animal reproduction.*, (2nd edn).