

# Effect of freezing methods on semen quality: A study on FUNAAB Alpha (normal feather), Arbor Acre and Dominant Black chickens

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**ABSTRACT:** Sperm cryopreservation is a useful technique in reproduction with variations in sperm quality among different breeds. This study assessed the spermatozoa cryotolerance among different breeds of chickens using slow and rapid freezing procedures. Ten (10) breeder birds from each breed were selected for this experiment at 30 weeks of age with an average body weight of 3.0 kg. Semen samples collected from FUNAAB Alpha (Normal feather) (NF), Arbor Acre (AB) and Dominant Black (DB) were subjected to cryopreservation in a completely randomised design and analysed for sperm viability, sperm functional integrity and oxidative stress parameters respectively. The quality of sperm cells from Normal feather, Arbor Acre, and Dominant Black chicken breeds in reaction to slow and rapid freezing was assessed. Under slow freezing, the result showed that Arbor Acre has the highest sperm progressive motility of 63.04% and livability of 77.67% with low abnormalities. Dominant Black chickens showed moderate sperm progressive motility of 48.00% and an abnormality rate of 1.07%. Rapid freezing lowered sperm quality in all breeds, although Arbor Acre showed a major reduction in sperm progressive motility. Oxidative stress parameters indicated that Normal feather chickens experienced a significant increase in lipid peroxidation under rapid freezing. The leukocyte values were similar across the breeds. Therefore, the findings of this research proved that slow freezing better preserves chicken semen compared to rapid freezing. Among the breeds examined, Dominant black chicken spermatozoa was better preserved.

**Keywords:** FUNAAB Alpha, Arbor Acre, Dominant Black, semen, cryopreservation, oxidative stress.

## INTRODUCTION

The process of keeping living things (tissues and cells) at below-freezing temperatures for an extended length of time is called cryopreservation. Any cryopreservation process' primary objectives are to lessen the harm that low temperatures could cause to cell membranes and prevent the formation of deadly intracellular ice crystals (Guo and Zhang, 2024). Many advances in cryopreservation technology have led to the development of protocols and techniques that allow the extremely low temperatures maintenance of different cell types, including male and female sex cells, microscopic multicellular organisms, as well as more complex living organisms like embryo

(Murray and Gibson, 2022).

Commercial breeders have found that cryopreservation of avian sperm cells is an effective method for artificial insemination (Mphaphathi *et al.*, 2016). However, because rooster sperm cells are not very motile and viable after freeze-thawing, the fertility effectiveness of thawed sperm cells is suboptimal (Partyka and Nizański, 2022). The primary cause of this setback is a number of injuries sustained during cryopreservation, which include; mechanical, biochemical, and ultrastructural changes to sperm (Fahy and Wowk, 2015). The quality and performance capacity of thawed sperm cells are negatively

impacted by these injuries. Feyzi *et al.* (2018) reported that rooster sperm cells are more vulnerable to cryo-injuries because their plasma membranes contain relatively high percentages of polyunsaturated fatty acids.

In slow freezing, the temperature is gradually reduced in a stepwise manner during the cryopreservation process, allowing cells to adapt gradually to the decreasing temperature and minimizing intracellular ice formation (Chankitisakul *et al.*, 2022). On the other hand, rapid freezing or vitrification involves a rapid rate of temperature reduction, leading to a significant increase in cooling rate. This method can prevent ice crystal formation by quickly solidifying the cells into a glass-like state (Tarig *et al.*, 2017). Thus, this research aimed to identify the most effective freezing method for maintaining sperm quality in these specific chicken breeds. The findings could have significant implications for optimizing breeding programs and improving the efficiency of poultry production.

## MATERIALS AND METHODS

### Experimental site

This experiment was conducted at the PEARL-FUNAAB Poultry Breeding Center at the Federal University of Agriculture in Abeokuta, Nigeria. The university is situated at latitude 70°10'N and longitude 30°2'E, having an elevation of 76 meters above sea level. The average annual rainfall is 1,037 mm, an average annual temperature of 28°C and a relative humidity of 82%. It is situated in the South Western region of Nigeria, which has a tropical climate.

### Experimental animals

At 30 weeks of age, 10 breeder birds from each breed were selected for this experiment, each with an average body weight of approximately 3.0 kg. These birds were raised under an intensive management system, and fed with growers mash diet to ensure proper nutrition. They were also provided with clean water to maintain their health and well-being throughout the study.

### Semen collection

According to Kanatiyanont *et al.* (2012) abdominal massage method was used to collect semen. This method involved gently massaging and stroking the testes at the dorsum until the cloacae protruded, which allowed the semen to be released. After that, the semen was properly collected into a graduated Eppendorf tube so the volume of each sample could be measured.

Once collected, semen was mixed with a pre-prepared extender and stored in a thermos flask at 37°C for transportation to the laboratory, where it was evaluated for

sperm quality and further processing after cryopreservation at 24 hours.

## Microscopic semen evaluation

### *Sperm progressive motility*

Motility was determined using the method described by Kowalczyk (2022). The cryopreserved semen sample was briefly thawed in a water bath at approximately 37°C. 5 µl of the semen was then placed directly on a pre-warmed microscope slide and covered with a 22 x 22 mm cover slip. Using a Celestron PentaView digital microscope (LCD-44348 by RoHS, China) at 400x magnification, various microscopic fields were examined to assess the percentage of progressively motile spermatozoa, ensuring an accurate evaluation of sperm motility.

### *Sperm plasma membrane integrity*

The hypo-osmotic swelling test (HOST), as reported by Hufana-Duran *et al.* (2015), was used to assess the integrity of the sperm membrane. For 30 minutes, 10 µl of semen was incubated at 37°C in a hypo-osmotic solution (9 grams of fructose and 4.9 grams of sodium citrate per 100 millilitres of distilled water). A 0.1 ml of the mixture was spread over a warm slide and covered with a cover slip. The sample was then observed under an LCD digital microscope at 400x magnification. The percentage of spermatozoa positive to the Hypo-Osmotic Swelling Test (HOST), characterized by swelling with curled tails, was determined. Spermatozoa that did not exhibit swelling and had uncurled tails were classified as having abnormal membrane integrity, indicating compromised cell function.

### *Sperm livability and abnormality*

According to Cecere (2014), sperm abnormalities were evaluated using eosin-nigrosine. A thin layer of the eosin-nigrosine solution and semen mixture was spread on the slide and allowed to dry. Under a 400x magnification LCD microscope, the proportion of spermatozoa with morphologically aberrant defects in the head, midpiece, and tail was measured. Spermatozoa that emerged white were considered live, whereas those that absorbed the stain were considered dead.

### *Acrosome integrity*

The procedure of Ahmad *et al.* (2014) was followed in order to determine the percentage of sperm cells with undamaged acrosomes. 50 µl of semen sample was added to 500 µl of formalin citrate solution. After placing a drop of this mixture on a microscope slide, 200 spermatozoa were recorded using a 400x magnification

microscope. The intactness of the acrosome was determined by the presence of a normal apical ridge on the spermatozoa, indicating healthy and functional sperm cells.

### Malondialdehyde concentrations

According to the procedure outlined by Pipan *et al.* (2017), the thiobarbituric acid reactive substances (TBARS) assay was used to measure the concentration of malondialdehyde (MDA), an indicator of lipid peroxidation in the cryopreserved semen, 0.1 mL of sperm suspension and 0.1 mL of 150 mM Tris-HCl (pH 7.1) were incubated in a water bath for 20 minutes at 37°C for this assay. Following incubation, the mixture was incubated in boiling water for 30 minutes before receiving 1 mL of 10% trichloroacetic acid (TCA) and 2 mL of 0.375% thiobarbituric acid. After that, the mixture was centrifuged in a blank tube for 15 minutes at 3000 rpm. A UV spectrophotometer was used to measure the absorbance of the supernatant at a wavelength of 532 nm in order to quantify the MDA concentration. The absorptivity of MDA was  $1.56 \times 10^5$  molar.

The concentration of MDA was calculated as follows:

The concentration of malondialdehyde (MDA) (nmol/mL) =  $AT - AB / 1.56 \times 10^5$

Where: AT = the absorbance of the sample, AB = the absorbance of the blank,  $1.56 \times 10^5$  molar absorptivity of MDA.

### Seminal leukocyte

The peroxidase test as modified by Vujisić *et al.* (2005) and in compliance with WHO (1999) guidelines, was used to calculate the seminal leukocyte count. To get the stock solution for peroxidase ready, 50 mL of distilled water was mixed with 50 mL of 96% ethanol and 125 mg of benzidine. 5 µL of 30% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was added to 4 mL of the peroxidase stock solution in order to get the working solution. 20 µL of the peroxidase working solution was mixed with 20 µL of cryopreserved semen for the assay, and the mixture was kept at room temperature for five minutes. Following incubation, 20 µL of the peroxidase working solution, 20 µL of cryopreserved semen and 20 µL of phosphate-buffered saline were mixed. After that, 10 µL of the final mixture was dropped on a hemocytometer, and leukocytes—which are dark brown, round cells—were counted under a microscope.

### Statistical analysis

Data from the experiment was subjected to analysis of variance (ANOVA). In a completely randomized design,

using SPSS 2000 (SAS, 2000) and the means were separated by Duncan Multiple Range Test (Duncan, 1995). The model is given below.

$$Y_{ijk} = \mu + G_i + T_j + \Sigma_{ijk}$$

Where;  $Y_{ij}$  = the observed value of the dependent variable,  $\mu$  = population mean,  $G_i$  =  $j^{\text{th}}$  effect of genotype (NF, DB and AB),  $T_i$  =  $i^{\text{th}}$  effect due to protocol,  $\Sigma_{ik}$  = Random experimenter error

## RESULTS

Table 1 compares the impacts of slow and rapid freezing on spermatozoa quality across the three chicken breeds (NF, AB, and DB). Under slow freezing, the Arbor Acre breeds showed a significant ( $P < 0.05$ ) higher motility (63.04%) and livability (77.67%), with lower sperm abnormality (1.29%), which indicated superior preservation of sperm quality. The Dominant Black breeds had moderate motility (48.00%) and lowest abnormality (1.07%), compared to the Normal feather (NF) breeds which had lower motility (38.67%) and higher spermatozoa abnormality (2.17%). Under rapid freezing, Dominant Black (DB) breeds exhibited a higher sperm motility (40.09%), compared to other breeds. However, all the breeds showed increased spermatozoa abnormality and decreased sperm cell livability. Arbor acre breeds (AB) had the lowest spermatozoa motility of 26.33% and high sperm abnormality of 1.97%. The differences between breeds and protocols were statistically significant, as indicated by the P-values.

The functional integrity of FUNAAB Alpha (Normal Feather), Arbor Acre and Dominant Black chicken spermatozoa cryopreserved using two different cryoprotocols: slow and rapid freezing is shown in Table 2. The Dominant Black (DB) breed showed higher acrosome integrity in slow freezing (88.67%) and membrane integrity (47.33%), while rapid freezing decrease acrosome integrity across the breeds. The Normal feather (NF) breeds maintained relatively high acrosome and membrane integrity under both protocols. The Arbor Acre (AB) breeds had the lower acrosome integrity in both protocols. There were significant ( $p \leq 0.05$ ) differences across the breeds with respect to Acrosome integrity. However, Plasma membrane integrity was not significantly ( $p \geq 0.05$ ) different across the breeds.

Oxidative stress parameters of FUNAAB Alpha (Normal Feather), Arbor Acre and Dominant Black chicken spermatozoa cryopreserved using slow and rapid freezing cryoprotocols is shown in Table 3. Leukocyte percentages were statistically similar ( $p \geq 0.05$ ) across the breeds and freezing protocols, with no significant differences ( $P > 0.05$ ). Lipid peroxidation values were higher in semen placed under rapid freezing, with the Normal feather (NF) breeds having the highest value of Malondealdehyde (MDA) of  $(6.00 \times 10^{-6})$ .

**Table 1.** Sperm viability of FUNAAB Alpha (Normal Feather), Arbor Acre and Dominant Black chicken spermatozoa cryopreserved using slow and rapid freezing cryoprotocols.

| Cryoprotocol   | Breeds | Motility (%)              | Livability (%)             | Abnormality (%)           |
|----------------|--------|---------------------------|----------------------------|---------------------------|
| Slow freezing  | NF     | 38.67 ± 1.16 <sup>c</sup> | 69.80 ± 6.27 <sup>ab</sup> | 2.17 ± 4.79 <sup>a</sup>  |
|                | AB     | 63.04 ± 1.16 <sup>a</sup> | 77.67 ± 6.27 <sup>a</sup>  | 1.29 ± 4.79 <sup>b</sup>  |
|                | DB     | 48.00 ± 1.16 <sup>b</sup> | 66.80 ± 6.27 <sup>b</sup>  | 1.07 ± 4.79 <sup>c</sup>  |
| Rapid freezing | NF     | 33.00 ± 1.16 <sup>b</sup> | 57.93 ± 6.27 <sup>c</sup>  | 1.70 ± 4.79 <sup>ab</sup> |
|                | AB     | 26.33 ± 1.16 <sup>c</sup> | 73.67 ± 6.27 <sup>a</sup>  | 1.97 ± 4.79 <sup>a</sup>  |
|                | DB     | 40.09 ± 1.16 <sup>a</sup> | 65.20 ± 6.37 <sup>b</sup>  | 1.91 ± 4.79 <sup>a</sup>  |
| P value        |        | 0.001                     | 0.017                      | 0.003                     |

Means with different superscript on the same row differ significantly ( $P < 0.05$ ), Where NF = Normal feather, AB = Arbor Acre and DB= Dominant black.

**Table 2.** Functional integrities of FUNAAB Alpha (Normal Feather), Arbor Acre and Dominant Black chicken spermatozoa cryopreserved using slow and rapid freezing cryoprotocols.

| Cryoprotocol   | Breeds | Acrosome %                | Membrane %   |
|----------------|--------|---------------------------|--------------|
| Slow freezing  | NF     | 78.17 ± 2.71 <sup>b</sup> | 44.67 ± 2.31 |
|                | AB     | 67.67 ± 2.71 <sup>c</sup> | 42.33 ± 2.31 |
|                | DB     | 88.67 ± 2.71 <sup>a</sup> | 47.33 ± 2.31 |
| Rapid freezing | NF     | 70.00 ± 2.71 <sup>b</sup> | 48.67 ± 2.31 |
|                | AB     | 64.00 ± 2.71 <sup>c</sup> | 46.33 ± 2.31 |
|                | DB     | 68.33 ± 2.71 <sup>c</sup> | 46.00 ± 2.31 |
| P value        |        | 0.007                     | 0.413        |

Means with different superscript on the same row differ significantly ( $P < 0.05$ ), Where NF = Normal feather, AB = Arbor Acre and DB= Dominant black.

**Table 3.** Oxidative stress parameters of FUNAAB Alpha (Normal Feather), Arbor Acre and Dominant Black chicken spermatozoa cryopreserved using slow and rapid freezing techniques.

| Cryoprotocol   | Breeds | Leukocyte % | Mda( $\times 10^{-6}$ ) |
|----------------|--------|-------------|-------------------------|
| Slow freezing  | NF     | 1.00 ± 0.18 | 3.33 ± 1.84             |
|                | AB     | 1.13 ± 0.18 | 3.30 ± 1.84             |
|                | DB     | 0.93 ± 0.18 | 5.67 ± 1.84             |
| Rapid freezing | NF     | 1.20 ± 0.18 | 6.00 ± 1.84             |
|                | AB     | 0.67 ± 0.18 | 3.67 ± 1.84             |
|                | DB     | 0.60 ± 0.18 | 3.67 ± 1.84             |
| P value        |        | 0.147       | 0.469                   |

Where NF = Normal feather, AB = Arbor Acre and DB= Dominant black.

## DISCUSSION

Semen cryopreservation is essential for preserving sperm cells, enabling their thawing and use in artificial insemination, this has aided livestock improvement by

allowing breeders to utilize genetically superior animals (Mphaphathi *et al.*, 2023). This study revealed that slow freezing positively affected the semen of FUNAAB Alpha (Normal Feather), Dominant Black, and Arbor Acre chickens. Sperm cells exposed to slow freezing demon-

strated better motility, livability, and acrosome integrity compared to those subjected to rapid freezing. This is attributed to the gradual temperature reduction during the slow freezing process, which minimizes cellular damage (Comizzoli and Holt 2022), as against the fast cooling rate in rapid freezing. This result aligns with the findings of Daramola and Adekunle (2017), who reported significantly better outcomes for semen subjected to slow freezing compared to rapid freezing. Ndubuisi-Ogbonna *et al.* (2021), reported that slow freezing preserved chicken semen more effectively than rapid freezing.

Acrosome and membrane integrity provide crucial insights into sperm functionality after thawing, which is vital since the acrosome reaction is essential for fertilization. Although, controlled slow freezing has been shown to better preserve acrosome integrity, than rapid freezing, where ice crystal formation can disrupt the acrosome (Santos *et al.*, 2023). These findings with respect to acrosome and membrane integrity after freezing were in line with the study of Batista *et al.* (2012) who reported that more spermatozoa with acrosome and membrane integrity in samples processed by slow freezing.

Interestingly, membrane integrity did not show significant differences between the two freezing protocols, although slight variations existed among the breeds. The Normal Feather breeds exhibited marginally higher membrane integrity during rapid freezing compared to slow freezing. Despite the expectation that slow freezing would better preserve membrane integrity, this result indicated that sperm cells from certain breeds, such as Normal Feather (NF), may possess greater resilience to rapid freezing. However, membrane integrity is often correlated with sperm viability and overall fertilization potential (Grötter *et al.*, 2019).

Cryopreservation can lead to cellular damage, resulting in sperm abnormalities (Agossou and Koluman, 2018). This study indicated that sperm abnormalities were lower in the slow-freezing group compared to the rapid-freezing group. Sperm cells subjected to rapid freezing experienced cold shock due to the abrupt temperature drop, leading to structural and biochemical damage. However, according to Kumar *et al.* (2019) reported that slow freezing gradually reduces the temperature as well as mitigates these damages.

Decreased sperm cell viability observed during rapid freezing may be linked to factors such as dilution procedures, cryoprotectant used, thawing methods and differences in temperature, which can cause greater damage to sperm cells (Stuart *et al.*, 2019; Peris-Frau *et al.*, 2020).

According to Roychoudhury *et al.* (2016), oxidative stress parameters are effective and sensitive markers of seminal oxidative stress. The decreased MDA concentrations in slow freezing as opposed to rapid freezing demonstrated the positive impact of slow freezing on spermatozoa viability. MDA, a byproduct of lipid peroxidation, is one of the major indicators of oxidative stress (Agarwal *et al.*, 2014). Oxidative damage is more

severe when the MDA content is higher. Seminal MDA and sperm viability were found to be negatively correlated, which supports the harmful effects of excessive lipid peroxidation (Sengupta *et al.*, 2024).

## Conclusion

The outcome of this investigation proved that the slow-freezing method of cryopreserving chicken sperm cells consistently improved sperm cell viability and performance while lowering oxidative stress unlike the rapid-freezing method.

Spermatozoa progressive motility and livability of Arbor Acre chickens was higher under slow freezing conditions while Dominant black exhibited superior acrosome integrity and plasma membrane integrity with lower sperm cell abnormality. Rapid freezing had the greatest negative impact on Normal Feather, indicating a higher sensitivity to oxidative stress and decreased viability. This suggests that slow freezing conditions could preserve chicken spermatozoa better than the rapid freezing method.

## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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## REFERENCES

- Agarwal, A., Mulgund, A., Sharma, R., & Sabanegh, E. (2014). Mechanisms of oligozoospermia: an oxidative stress perspective. *Systems biology in Reproductive Medicine*, 60(4), 206-216.
- Agossou, D. J., & Koluman, N. (2018). An objective analysis of factors affecting buck semen quality attributes during cryopreservation: A mini review. *Annual Research & Review in Biology*, 27(3), 1-7.
- Ahmad, M., Nasrullah, R., Riaz, H., Sattar, A. & Ahmad, N., 2014. Changes in motility, morphology, plasma membrane and acrosome integrity during stages of cryopreservation of buck sperm. *Journal of the South African Veterinary Association*, 85(1), 1-4.
- Batista, M., Santana, M., Alamo, D., González, F., Niño, T., Cabrera, F., & Gracia, A. (2012). Effects of incubation temperature and semen pooling on the viability of fresh, chilled and freeze-thawed canine semen samples. *Reproduction in Domestic Animals*, 47(6), 1049-1055.
- Cecere, J. T. (2014). Eosin-Nigrosin staining in the evaluation of sperm. In: Dascanio, J. J., & McCue PM. *Equine reproductive procedures* (pp. 373-376). Wiley Online Library.

- Chankitisakul, V., Boonkum, W., Kaewkanha, T., Pimprasert, M., Ratchamak, R., Authaida, S., & Thananurak, P. (2022). Fertilizing ability and survivability of rooster sperm diluted with a novel semen extender supplemented with serine for practical use on smallholder farms. *Poultry Science*, *101*(12), 102188.
- Comizzoli, P., & Holt, W. V. (2022). Recent progress in spermatology contributing to the knowledge and conservation of rare and endangered species. *Annual Review of Animal Biosciences*, *10*(1), 469-490.
- Daramola, J. O. & Adekunle, E. O., 2017. Effects of Washing Protocols on Cryosurvival of Spermatozoa from West African Dwarf Goat Bucks. *Cryo Letters*, *38*(3), 210-215.
- Duncan, D. B. (1955). Multiple range and multiple F tests. *Biometrics*, *11*(1), 1-42.
- Fahy, G. M., & Wowk, B. (2015). Principles of cryopreservation by vitrification. *Cryopreservation and freeze-drying protocols* (pp. 21-82). Springer Nature.
- Feyzi, S., Sharafi, M., & Rahimi, S. (2018). Stress preconditioning of rooster semen before cryopreservation improves fertility potential of thawed sperm. *Poultry science*, *97*(7), 2582-2590.
- Grötter, L. G., Cattaneo, L., Marini, P. E., Kjelland, M. E., & Ferré, L. B. (2019). Recent advances in bovine sperm cryopreservation techniques with a focus on sperm post-thaw quality optimization. *Reproduction in Domestic Animals*, *54*(4), 655-665.
- Guo, S., & Zhang, A. (2024). Review of different temperatures for biopreservation. *International Journal of Refrigeration*, *157*, 53-59.
- Hufana-Duran, D., Mallari, R. P., Suba, D. P., Duran, P. G., Abella, E. A., & Mamuad, F. V. (2015). Hypo-osmotic swelling test for membrane integrity evaluation of frozen-thawed water buffalo (*Bubalus bubalis* Linn.) spermatozoa. *Philippine Journal of Science*, *144*(2), 209-219.
- Kanatiyanont, N., Kornkaewrat, K., Suthanmapinunt, P., & Pinyopummin, A. (2012). Effect of semen collection techniques on semen quality and sperm motility parameters in Siamese fighting cock (*Gallus gallus*). *The Thai Journal of Veterinary Medicine*, *42*(4), 439-445.
- Kowalczyk, A., Gałęska, E., & Bubel, A. (2022). The concentration of ProAKAP4 and other indicators of cryopotential of spermatozoa cryopreserved in extender with Holothuroidea extract addition. *Animals*, *12*(4), 521.
- Kumar, A., Prasad, J. K., Srivastava, N., & Ghosh, S. K. (2019). Strategies to minimize various stress-related freeze-thaw damages during conventional cryopreservation of mammalian spermatozoa. *Biopreservation and biobanking*, *17*(6), 603-612.
- Mphaphathi, M. L., Seshoka, M. M., Luseba, D., Sutherland, B., & Nedambale, T. L. (2016). The characterisation and cryopreservation of Venda chicken semen. *Asian Pacific Journal of Reproduction*, *5*(2), pp.132-139.
- Mphaphathi, M. L., Thema, M. A., Ledwaba, M. R., Sebopela, D., & Magopa, L. (2023). Conservation of Gametes and Use during Assisted Reproductive Technologies in Equine. In *Equine Science-Applications and Implications of New Technologies*. IntechOpen.
- Murray, K. A., & Gibson, M. I. (2022). Chemical approaches to cryopreservation. *Nature Reviews Chemistry*, *6*(8), 579-593.
- Ndubuisi-Ogbonna, L. C., Daramola, J. O., Akintunde, A. O., Abdullah, A. R., Wheto, M., Ojo, S. T., & Adighibe, B. K. (2021). Comparative Effect of Centrifugation on Sperm Viability of FUNAAB Alpha Chickens During Slow and Rapid Freezing Protocols. *Wayamba Journal of Animal Science*, *13*, 1874-1879.
- Partyka, A., & Nizański, W. (2022). Advances in storage of poultry semen. *Animal Reproduction Science*, *246*, 106921.
- Peris-Frau, P., Soler, A. J., Iniesta-Cuerda, M., Martín-Maestro, A., Sánchez-Ajofrín, I., Medina-Chávez, D. A., & Garde, J. J. (2020). Sperm cryodamage in ruminants: understanding the molecular changes induced by the cryopreservation process to optimize sperm quality. *International Journal of Molecular Sciences*, *21*(8), 2781.
- Pipan, M. Z., Mrkun, J., Strajn, B. J., Vrtač, K. P., Kos, J., Pišlar, A., & Zrimšek, P. (2017). The influence of macro-and microelements in seminal plasma on diluted boar sperm quality. *Acta Veterinaria Scandinavica*, *59*(1), 1-9.
- Roychoudhury, S., Sharma, R., Sikka, S., & Agarwal, A. (2016). Diagnostic application of total antioxidant capacity in seminal plasma to assess oxidative stress in male factor infertility. *Journal of Assisted Reproduction and Genetics*, *33*, 627-635.
- Santos, M. V., Silva, A. M., Aquino, L. V., Oliveira, L. R., Moreira, S. S., Oliveira, M. F., Silva, A. R., & Pereira, A. F. (2023). Different methods for seminal plasma removal and sperm selection on the quality and fertility of collared peccary sperm. *Animals*, *13*(12), 1955.
- SAS (2000).. SAS User's Guide: Statistics Inc., Cary, N.C. 923 p.
- Sengupta, P., Dutta, S., & Irez, T. (2024). Oxidants and antioxidants in male reproduction: roles of oxidative and reductive stress. *Journal of Integrated Science and Technology*, *12*(3), 753-753.
- Stuart, C. C., Vaughan, J. L., Kershaw, C. M., De Graaf, S. P., & Bathgate, R. (2019). Effect of diluent type, cryoprotectant concentration, storage method and freeze/thaw rates on the post-thaw quality and fertility of cryopreserved alpaca spermatozoa. *Scientific Reports*, *9*(1), 12826.
- Tarig, A. A., Wahid, H., Rosnina, Y., Yimer, N., Goh, Y. M., Baiee, F. H., & Ebrahimi, M. (2017). Effect of different concentrations of soybean lecithin and virgin coconut oil in Tris-based extender on the quality of chilled and frozen-thawed bull semen. *Veterinary World*, *10*(6), 672.
- Vujisić, S., Lepej, S. Ž., Jerković, L., Emedi, I., & Sokolić, B. (2005). Antisperm antibodies in semen, sera and follicular fluids of infertile patients: relation to reproductive outcome after in vitro fertilization. *American Journal of Reproductive Immunology*, *54*(1), 13-20.
- World Health Organisation (WHO). (1999). *WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction*. Cambridge University Press.