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## COMPARATIVE STUDY OF FREEZING AND CHILLING EFFECTS ON SURVIVAL AND OXIDATIVE CHARACTERISTICS OF FUNAAB ALPHA (NORMAL FEATHER) CHICKEN SPERM

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### ABSTRACT

The impact of freezing and chilling on the semen of FUNAAB Alpha (Normal feather) chickens was examined in this study. Pooled semen collected from ten FUNAAB Alpha (Normal feather) chicken were diluted with Tris –based extender. Diluted semen was divided into two fraction, the first fraction was subjected to chilling at 4°C and the second fraction was subjected to freezing at 1°C for 24hrs. Thereafter semen were evaluated for viability, functional integrity and oxidative stress (**Malondialdehyde concentrations** MDA and seminal leukocytes). The result showed a consistent variation in the viability and functional integrity. However, Sperm motility and livability preserved at 4°C was significantly higher ( $p < 0.05$ ) than those preserved at 1°C. Abnormal sperm (head, mid piece and tail) recorded for those preserved at 4°C was significantly lower ( $p < 0.05$ ) than those preserved at 1°C. The findings indicate that sperm viability, functional integrities and oxidative stress parameters of FUNAAB alpha (Normal feather) were better preserved by chilling compared to freezing method.

**Keyword;** FUNAAB Alpha chicken; freezing; chilling; oxidative stress; sperm viability.

### INTRODUCTION

Poultry production has advanced as a result of assisted reproductive technologies like artificial insemination. The semen quality test performed, allows only high quality semen from a superior males to be use for artificial insemination. Proper semen handling, storage, thawing and evaluation are requisite for implementing a successful artificial insemination program (Dumpala et al., 2006). The fundamental goal of cryopreservation is to maintain the structural and functional integrity of semen over time while providing spermatozoa an environment that allows them to survive the stress of freezing and thawing (Sharma, et al., 2015). In poultry semen however, factors such as variation in thawing temperatures, osmotic pressure, breeds, stress, extender effects impact parameters of semen quality like, motility, livability, abnormality, membrane and acrosome integrity, while the sample is being cryopreserved (Tuncer et al., 2011). Gametes' ability to initiate normal embryonic development necessary for fertilization is impaired due to oxidative stress caused by Reaction Oxygen Species (ROS), depending on its concentration (Aitken, 2017).

Chilling and freezing protocols as a cryopreservation method have helped to lower sperm temperature, dehydrate sperm, and induce intra- and extracellular freezing in order to protect sperm cells from oxidative damage caused by higher concentrations of ROS (Lucio et al., 2016; Pignataro et al., 2020).

The nature of the diluent and the storage temperature are very important to preserve cock semen and prevent a

reduction in sperm quality (Dumpala et al., 2006). It has been established that sperm motility and the fertilizing ability of undiluted cock semen stored *in vitro* usually decrease one hour after collection (Vasicek et al., 2015). In contrast to frozen semen, chilled semen requires fewer special techniques and inexpensive equipment for processing and transportation (Hori et al., 2017). But to preserve the quality of semen, chilled sperm has a limited shelf life, necessitating the use of extenders (Rodenas et al., 2014). According to Bencharif et al. (2013), semen preservation during liquid storage can also be aided by factors like refrigeration speed and storage temperature.

According to research (Hesser et al., 2017), assisted reproduction with liquid stored semen (frozen or chilled) can produce a pregnancy rate comparable to that of fresh semen in other animals, such as bulls, boars, and rams, if done and managed correctly (95% and 94%, respectively). Therefore, the goal of our research was to assess how different storage temperatures, (freezing and chilling), affect the oxidative stress parameters, functional characteristics and viability of sperm in FUNAAB Alpha (Normal feather) cock semen.

### MATERIALS AND METHOD

#### Experimental site

The experiment was carried out at PEARL- FUNAAB Poultry Breeding Center, Federal University of Agriculture, Abeokuta, and the laboratory analysis was conducted at the Department of Animal Physiology Laboratory, College of Animal Science and Livestock Production, Federal University of Agriculture, Abeokuta.

### **Experimental Animals**

Ten (10) parent birds (Normal feathered FUNAAB Alpha) of 52 weeks old were used for the experiment. The animals were reared in an intensive battery cage management system and housed individually. Fresh clean water was given *ad-libitum* along with breeder mash concentrate feed was made available to them.

### **Semen collection and dilution**

Semen samples were collected early in the morning from the cock by abdominal massage procedure, according to Burrows and Quinn (1937), using graduated Eppendorf tube. The volume of each semen sample were recorded. The semen samples collected from each cock were pooled together for uniformity, the pooled semen were diluted with Tris egg yolk extender and then maintained in the dark by covering the holding tube with aluminum foil. The pooled semen were subjected to chilling at 4°C and freezing at 1°C for 24 hours in a refrigerator. Thereafter, semen were evaluated for viability and oxidative stress (MDA and seminal leukocytes) characteristics.

### **Evaluation of Sperm Viability Parameters**

#### **Motility**

The method developed by Bearden and Fuquay (1984) was used to determine sperm motility. After the semen was exposed to light for different hours, the sperm motility was evaluated using a Celestron Penta View digital microscope (LCD-44348 by RoHS, China) at 400x magnification. A 5µL sample of semen was dropped on a microscope slide and covered with a 22 × 22 mm cover slip. The final motility score was determined by taking the mean of the ten consecutive evaluations of each semen sample. Ten microscopic fields were examined to observe progressive motility, which is defined as spermatozoa that move forward in essentially a straight line.

#### **Livability**

Using eosin-nigrosine smears, sperm livability was evaluated as reported by Bearden and Fuquay (1984). On a slide, a thin smear of semen and eosin-nigrosine solution was applied, then dried. The samples were examined for living and dead spermatozoa using a Celestron Penta View LCD microscope with a ×400 magnification. White spermatozoa were identified as live spermatozoa, whereas stain-picking spermatozoa were identified as dead spermatozoa.

#### **Sperm abnormalities**

Eosin-nigrosine smears were used to assess sperm abnormalities in accordance with Bearden and Fuquay's (1997) methodology. A thin layer of semen and eosin-nigrosine solution was applied to the slide, allowed to dry, and then retracted. Using a Celestron Penta View LCD

microscope (×400 magnification), the percentage of morphologically aberrant spermatozoa with abnormalities in the head, midpiece, and tail was counted.

#### **Sperm acrosome integrity**

Alhmadi et al. (2014) reported the percentage of spermatozoa with intact acrosomes. A 500 µL formalin citrate solution (96 mL 2.9% sodium citrate with 4 mL 37% formaldehyde) was properly mixed with 50 µL of each semen sample. Using a Celestron Penta View microscope (× 400 magnification), a little drop of the combination was placed on a microscope slide, and 200 spermatozoa were counted in at least three separate microscopic fields for each sample. The spermatozoa's normal apical ridge, which indicates the intactness of the acrosome, was evaluated.

#### **Sperm membrane integrity**

This was examined using the Hypo-osmotic swelling test (HOST) assay, which was published by Ndubuisi-ogbonna et al. (2022). To achieve this, 10 µL of semen were incubated for 30 minutes at 37 °C in a 100 µL hypo-osmotic solution (9 g fructose plus 4.9 g sodium citrate combined with 1000 mL of distilled water). 0.1 mL of the mixture was then spread out on a heated slide, covered with a cover slip, and examined under a Celestron Penta View LCD digital microscope (× 400 macro magnification). After counting 200 spermatozoa, the percentage of spermatozoa that tested positive for HOST (shown by a coiled tail, which indicates an intact plasma membrane) was calculated. Spermatozoa without swelling (shown by uncoiled tails) were categorized as having abnormal membrane integrity.

#### **Malondialdehyde concentrations**

According to Pippneger et al. (1998), the concentration of malondialdehyde (MDA) was evaluated in a thiobarbituric acid reactive substances (TBARS) as 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 for 20 minutes at 37°C. After that, 2 mL of 0.375 % thiobarbituric acid and 1 mL of 10% trichloroacetic acid (TCA) were added, and the mixture was heated to boiling for 30 minutes. Then, using a blank tube, it was centrifuged for 15 minutes at 3000 rpm. Using a UV spectrophotometer (SW7504 model by Surgifriend Medicals, England) set to 532 nm, the absorbance was recorded. The concentration of MDA was calculated as follows: The concentration of MDA (nmol/mL) =  $\frac{AT - AB}{1.56} \times 10^5$ ; where AT = absorbance of the semen sample, AB = absorbance of the blank,  $1.56 \times 10^5$  molar absorptivity of MDA.

#### **Seminal Leukocytes**

50 milliliters of distilled water, 50 milliliters of 96% alcohol, and 125 milligrams of benzidine were combined to create a stock solution. 4 milliliters of stock solution, 5µl of 30% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was added to create the working solution. Subsequently 20µL of working solution and 20µL of semen samples were mixed together and the mixture was incubated for a period of ten minutes. After that, 20µL of Phosphate Buffered Saline (PBS) and 20µL of working solution were added to it. Thereafter, 5 µL of the mixture was dropped on hemocytometer, peroxidase-positive cells—that is, round, dark brown cells—were identified as leukocytes and recorded.

**Statistical analysis**

SPSS 2000 was used to perform a two-way analysis of variance (ANOVA) on the collected data. While significantly different means were separated using Tukey HSD. Below is a description of the model:

$$Y_{ijk} = \mu + C_i + F_j + \Sigma_{ijk}$$

Where:

Y<sub>ijk</sub> = Dependent Variable

μ = Population mean

C<sub>i</sub> = i<sup>th</sup> effect due to chilling (i= chilling)

F<sub>j</sub> = j<sup>th</sup> effect due to freezing (j = freezing)

Σ<sub>ijk</sub> = Experimental Error

**RESULT**

Table 1 presents the viability parameters of sperm from FUNAAB Alpha Normal Feather chickens that were frozen and chilled. Sperm motility and livability were consistently higher (p<0.05) in chilled semen as opposed to frozen semen. In chilled semen, the number of abnormal cells (head, midpiece, and tail) was significantly less (p<0.05) than in frozen semen.

The functionality parameters are presented in Table 2. The acrosome and sperm membrane integrity for the chilled and frozen semen was observed to be in consistency with the fresh semen, but was higher (p<0.05) in chilled semen compared to the frozen semen. There is no discernible difference (p>0.05) in the oxidative stress parameters seen in spermatozoa exposed to freezing and chilling, as shown in Table 3. Oxidative stress was found to be lower in chilled semen than in frozen semen.

**Table 1; Effect of Freezing and Chilling on Sperm Viability of FUNAAB ALPHA (Normal Feather) Chicken**

Protocol	Motility (%)	Livability (%)	Abnormal head (%)	Abnormal mid piece (%)	Abnormal tail (%)
Fresh	98.67 ± 0.63 <sup>a</sup>	99.73 ± 4.08 <sup>c</sup>	0.44±0.11 <sup>c</sup>	0.52±0.12 <sup>c</sup>	1.53±0.19 <sup>a</sup>
Chilled (4°C)	92.00 ± 2.11 <sup>b</sup>	94.00 ± 2.73 <sup>a</sup>	0.80±0.24 <sup>b</sup>	0.73±0.36 <sup>ab</sup>	0.19±0.40 <sup>c</sup>
Freezing (1°C)	35.33 ± 1.57 <sup>c</sup>	73.00±12.48 <sup>ab</sup>	1.90±0.36 <sup>a</sup>	2.54 ± 0.56 <sup>a</sup>	0.68±0.24 <sup>b</sup>
P value	0.000	0.005	0.000	0.004	0.000

*a,b,c Values within column with different superscript differs significantly (p<0.05)*

**Table 2; Effect of Freezing and Chilling on Sperm Functionality of FUNAAB ALPHA (Normal Feather) Chicken**

Protocol	Acrosome integrity (%)	Sperm membrane (%)
Fresh	95.67 ± 0.92	98.33 ± 0.55a
Chilled (4°C)	96.33 ± 0.86	97.33 ± 0.62ab
Freezing (1°C)	94.67 ± 1.07	96.00 ± 0.84c
P value	0.465	0.054

*a,ab,c Values within column with different superscript differs significantly (p<0.05)*

**Table 3; Effect of Freezing and Chilling on Oxidative stress parameters of FUNAAB ALPHA (Normal Feather) Chicken semen.**

Protocol	Leukocytes (%)	MDA (n/mol/10 <sup>-5</sup> )
Fresh	0.40 ± 0.13	5.96 ± 2.55
Chilled (4°C)	0.20 ± 0.11	1.84 ± 0.50
Freezing (1°C)	0.47 ± 0.19	2.81 ± 1.71
P value	0.421	0.309

## DISCUSSION

A reversible reduction of metabolic activity is strategic in extending the life of spermatozoa, therefore, hypothermia reduces metabolic activity of cells by decelerating enzymatic reactions (Viswanathan *et al.*, 2000; Freitas-Ribeiro *et al.*, 2019). In storage of chicken spermatozoa irrespective of the method employed, aerobic condition is needed (Long *et al.*, 2003). Following ejaculation and exposure to air, at 40 to 41°C, the quality of cock spermatozoa begins to undergo a gradual degradation, but with a lower temperature of about 2 to 5°C lipid peroxidation is considerably reduced (Zhu *et al.*, 2017).

Improved sperm viability attributes (motility, abnormality and livability), functional integrity (acrosome integrity and membrane integrity) and oxidative stress (MDA concentration and seminal leukocytes counts) parameters observed in spermatozoa subjected to chilling at 4°C indicated the efficacy of this protocol to maintain quality characteristics of stored spermatozoa over the frozen at 1°C. This is in line with the work of (Dumpala *et al.*, 2006) who stored semen at different temperature (4°C, 21°C and 41°C) respectively and obtained the best result at 4°C. The difference could be as a result of less production of toxic end products such as 3- carbon glycolytic intermediates which subsequently form toxic by-product (Lu and Imlay, 2021), breed as reported by Ndubuisi-ogbonna (2022). Also in the study of (Vasicek and Chrenek, 2021) no negative effect of low temperature (4-8 °C) on storage of rooster spermatozoa was observed. The improvement in the sperm quality indices revealed the preservative effect of storing spermatozoa at a lower temperature of about 4°C than freezing at about 1°C, could be attributed to the changes in the osmotic rate and mitochondria activities (Kumar *et al.*, 2019). Cold shock causes structural and biochemical damages in sperm as a result from sudden reduction in temperature (Bucak *et al.*, 2009). Such damages can be forestalled by cooling semen slowly in the presence of protective agents. Slow dilution helps to reduce the metabolic rate of sperm and minimizes damage due to cold shock (Curry, 2007).

An effective and sensitive technique to identify seminal oxidative stress is through oxidative stress parameters (Das *et al.*, 2009). Oxidative stress is one of the most important factors contributing to poor quality semen (Tuncer *et al.*, 2010). Sperm functions are negatively impacted by high leukocyte concentrations, especially those of activated leukocytes (Henkel, 2011), and MDA, a byproduct of lipid peroxidation, is also one of the key indicators of oxidative stress. Increased oxidative damage is reflected in higher MDA content and leukocyte concentrations (Rowe *et al.*, 2000). Therefore, the reduced MDA concentrations and leukocyte count in chilling at 4°C compared to freezing at 1°C indicated the beneficial effect of chilling on the viability of spermatozoa (Das *et al.*, 2009). Which was attributed in this study to a sudden drop in temperature from chilling to freezing.

## CONCLUSION AND RECOMMENDATION

Chilling is a better preservation method for cock semen when using liquid storage cryopreservation techniques. This will reduce oxidative stress, resulting in high productivity and successful artificial insemination post thawed.

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