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Sperm viability of FUNAAB-alpha chicken at refrigeration and freezing

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Abstract

Artificial insemination is a critical component of animal breeding programs. The effectiveness of artificial insemination, on the other hand, is highly dependent on the consistency of the sperm. As a result, sperm preservation is critical for the storage and protection of animal genetic resources. Several experiments on the cryopreservation of chickens have failed miserably, but there is no evidence of such a research being conducted with FUNAAB alpha chickens. This experiment used 30 FUNAAB-alpha chickens of 25-30 weeks old. Semen samples were diluted in a Tris-based extender and stored in an electric freezer for 24 hours in refrigeration and freezing. The experiment was set up in a 3x2 factorial arrangement. One-way ANOVA was used to analyze the data. As compared to the freezing protocol, the results showed that semen samples subjected to refrigeration protocols had higher ($p<0.05$) percentages in motility, livability, acrosome integrity, membrane integrity, and recovery rates. Refrigeration decreased sperm abnormality ($p<0.05$) as compared to freezing. When compared to refrigeration, freezing resulted in lower leukocyte and MDA levels ($p<0.05$). The study found that refrigerated spermatozoa had higher sperm viability than those frozen.

Keywords: Artificial insemination; Cryopreservation; Freezing; Refrigeration; Semen; Spermatozoa

Viabilité du sperme du poulet alpha de FUNNAB à la réfrigération et au gel

Résumé

L'insémination artificielle est une composante essentielle des programmes d'élevage des animaux. L'efficacité de l'insémination artificielle, d'autre part, dépend fortement de la consistance du sperme. En conséquence, la préservation du sperme est essentielle pour le stockage et la protection des ressources génétiques animales. Plusieurs expériences sur la cryoconservation des poulets ont échoué lamentablement, mais il n'y a aucune preuve d'une telle recherche menée avec des poulets alpha de FUNNAB. Cette expérience a utilisé 30 poulets alpha de FUNAAB de 25-30 semaines. Des échantillons de sperme ont été dilués dans une extension à base de tris et stockés dans un congélateur électrique pendant 24 heures en réfrigération et en gel. L'expérience a été mise en place dans un arrangement 3x2 factoriel. L'ANOVA à sens unique a été utilisée pour analyser les données. Par rapport au protocole de congélation, les résultats ont montré que les échantillons de sperme soumis à des protocoles de réfrigération étaient plus élevés ($p <0,05$) pour les pourcentages de la motilité, de la vie privilégiable, de l'intégrité acrosomère, de l'intégrité de la membrane et des taux de récupération. La réfrigération a diminué d'anomalie de sperme ($p <0,05$) par rapport au gel. Par rapport à la réfrigération, la congélation a abouti à des niveaux de

leucocyte inférieur et de MDA (P <0,05). L'étude a révélé que les spermatozoïdes réfrigérants avaient une viabilité de sperme plus élevée que celles gelées.

Mots-clés: insémination artificielle; Cryoconservation; Gelé; Réfrigération; Sperme; Spermatozoa

Introduction

Nigerian chickens are considered to be dual-purpose birds that are used for meat and egg production in the country's rural and peri-urban areas (Kperegheyi *et al.,* 2009). Under a conventional family scavenging management scheme, they can be found in large numbers across various agro ecological categories (Sonaiya and Olori,1990). Indigenous poultry birds are important tools for livestock production because their genetic diversity enables poultry to be raised in a variety of environments (Sonaiya *et al*., 1999). Nigerian chickens are said to be small-bodied, slow-growing, poor feed converters, and poor meat animals, despite the fact that they are important sources of raw materials for developing a sustainable protein supply within the country (Nwanta *et al*., 2006). In Nigeria, efforts have focused on the creation of indigenous chicken breeds that produce more meat and eggs. These efforts resulted in the National Animal Production Research Institute (NAPRI) developing Shika brown layers (Ikanni and Annatte, 2000) and the Federal University of Agriculture Abeokuta developing FUNAAB– alpha (Adebambo, 2015). The FUNAAB-Alpha was created for improved meat and egg production without sacrificing adaptation to tropical environment and diseases through crossbreeding of indigenous and exotic breeds and intensive selection over several generations. To improve the indigenous chickens, the potential variability that exists among them was exploited. They also have the various plumage colors and three feathering patterns that Nigerian Indigenous chickens have (normal feathered, frizzled feathered, and naked neck) (Wheto *et al.,* 2017). Semen cryopreservation is a valuable tool in the poultry industry because it allows the genetics of poultry resources to be preserved. Cryopreserved sperm also prevents germ plasma from deterioration and aids livestock artificial insemination programs. Akintunde *et al.* (2020) reported that semen output is also influenced by different genotypes of chickens, hence, the need to carry out study on FUNAAB alpha chickens. The success of cryopreservation in sperm cells differs between bird species. Variations in membrane fluidity, which play a role in the restoration of the physiological state after freezing, are one of the possible biological factors responsible for such variations (Blesbois et al., 2005). Furthermore, despite its long-established importance for optimizing male genetic potential in domestic avian species, semen preservation is only occasionally used in breeding practice (Blesbois *et al.,* 2005). One of the key reasons is that freezing procedure performance is highly variable, depending on species and line specificity, among other things (Hammerstedt, 1995; Etches, 1996; Massip *et al*., 2004). As a result, various preservation procedures must be used to assess the viability of FUNAB-chickens, as the future of mating in poultry development is highly dependent on effective artificial insemination.

Materials and methods

Experimental site

The research was conducted at the Federal University of Technology, Abeokuta, Nigeria (FUNAAB) Teaching and Research Farm, which is located at an altitude of 76 meters above sea level and lies between the latitudes of 7°10N and 3°2E. It is located in

Nigeria's south-west region, with a tropical climate, 1037 mm of annual rainfall, and an average temperature of 34.7°C. The laboratory tests were conducted at the Animal Physiology Laboratory of the Department of Animal Physiology at the Federal University of Agriculture in Abeokuta, Nigeria.

Experimental birds and management

For this experiment, a total of 30 FUNAAB alpha chickens between the ages of 25 and 30 weeks were used, with 10normal feathers, 10naked-neck feathers, and 10frizzle feathers. Commercial breeder mash containing 17.5 percent crude protein and 2700kcal metabolizable energy was fed ad libitum to the birds. There was no shortage of clean water. Medications and vaccines were administered in accordance with protocol.

Semen collection

Thirty cocks were used to collect sperm samples and10for each chicken strain. The abdominal massage method described by Burrows and Quinn(1937)was used to collect sperm. After selection, the semen was put in a water bath at 37°C for 15 minutes before being transported to the laboratory for examination. Using eosin-nigrosin staining and a haemocytometer, ejaculates were assessed for volume (mL), color, pH, density, mass movement (0-5), sperm motility (percent), sperm abnormality (percent), and sperm viability (percent) and sperm concentration (n x 10^9 sperm/mL). For each of the three strains of FUNAAB-alpha chickens (NN, NF, and FF), fresh semen samples were randomly collected from three cocks. A preliminary examination of sperm samples was performed. To reduce individual variations, semen samples with >80% sperm motility were pooled for each strain (Bucak and Tekin, 2007). At room temperature, the semen sample was diluted with a Tris-based extender containing tris-hydroxymethyl-aminomethane (2.42 g), citric acid (1.35 g), glucose (1 g), and penicillin (0.028 g), as well as egg yolk (20 mL) and distilled water to make up 100 mL. Each diluted semen sample was divided into two equal parts in separate test tubes for each strain (one for refrigeration and the other for freezing), and then each semen sample was further divided into four aliquots with three replicates for each strain. The first aliquot of each strain (NN, NF, FF) was used as a control (fresh semen), while the remaining three aliquots were chilled for 20 minutes in a Haier thermocool refrigerator (Model: HR-170T) with four compartments of varying temperatures of 20°C, 15°C, 10°C, and 5°C. The semen samples for freezing were equilibrated for 10 minutes after the last compartment with 5°C temperature and then kept in the freezer for 24 hours, while the samples for chilling were kept at 5°C in the refrigerator for 24 hours. Following that, the samples were assessed.

Semen evaluation

Sperm motility, recovery rate, acrosome integrity, sperm membrane integrity, live sperm, sperm abnormality, leukocyte counts, and seminal malondialdehyde concentration were all assessed in the sperm.

Sperm motility

Bearden and Fuquay (1997) method of determining sperm motility was used.The cryopreserved samples were thawed in a Clifton Water bath (Model: 74178 by Nickel Electro Ltd, Weston-S-Mare Somerset, England) at 37°C for a few minutes before being examined for sperm motility with a CelestronPentaView microscope (LCD- 44348 by RoHS, China) at 400 magnifications. 5 mL of sample was immediately poured onto a pre-warmed microscope slide, which was then covered with a 22 mm cover slip. Each sample was analyzed in five microscopic fields and viewed twice (2 slides per sample). Three observers counted progressive motile spermatozoa as spermatozoa that moved

forward essentially in a straight line among the spermatozoa from each microscopic region. The average of the five tests was determined, and the percentage progressive motility was calculated as follows:

$$= \frac{\text{(Number of progressive motile spermatozoa counted)}}{\text{(Total number of spermatozoa)}} \times 100.$$

Acrosome integrity

According to Khan and Ijaz (2007) and Ahmad et al (2014), the percentage of spermatozoa with intact acrosomes was calculated. 50 liters of each thawed sample is carefully blended into a 500-liter formalin citrate solution (96 mL 2.9 percent sodium citrate, 4 mL 37 percent formaldehyde). Using a CelestronPentaView LCD microscope (400 magnification), a small drop of the mixture was mounted on a microscope slide, and 200 spermatozoa were counted in at least three separate microscopic fields for each sample. The existence of crescent-shaped acrosomes in an intact acrosome with a typical apical ridge or spermatozoa was considered normal and reported. The following formula was used to measure the percentage of acrosome integrity:

$$\frac{\text{Number of spermatozoa with normal apical ridge}}{\text{Total spermatozoa that was counted}} \times 100.$$

Sperm membrane integrity

The sperm membrane integrity was determined using the Hypo-osmotic swelling test (HOST) assay defined by Zubair *et al.* (2013). This was accomplished by incubating a 10 liter spermatozoa sample in 100 liters of a 100mOsm/L Hypo-osmotic solution (7.35 grams sodium citrate [0.0285M] and 13.5 grams fructose [0.075M]) at 37 degrees Celsius for 30 minutes. A 0.1 mL sample of the mixture was spread on a warmed slide, covered with a cover slip, and examined under a CelestronPentaView LCD-44348 digital microscope (400 magnification). A total of 200 spermatozoa were counted, and the percentage of spermatozoa positive for HOST (curled tails suggesting intact plasma) was calculated.

Sperm abnormality and sperm livability

According to Bearden and Fuquay (1997) procedure, eosin–nigrosin staining was used to test sperm abnormality and livability (Pintado et al., 2000). A thin smear of semen and eosin-nigrosin solution was drawn across the slide and dried in this assay. Under a CelestronPentaView LCD-44348 digital microscope (400 x magnifications), the percentage of morphologically abnormal spermatozoa with defects in the head, midpiece, and tail was observed and registered. Normal spermatozoa had a spindle-shaped head with a well-marked acrosome and visible tail, whereas abnormal spermatozoa had a swollen head, bent neck, defective mid-piece, or other deformity (coiled tail, lack of tail). A CelestronPentaView LCD-44348 optical microscope (400x magnification) was used to record dead/viable spermatozoa. Spermatozoa that appeared white (unstained cells) were considered live (viable), while those that picked up the stain (eosin-nigrosin stained cells) were considered dead. The livability of sperm (percentage) was determined as follows:

$$= \frac{\text{(Total number of live sperm)}}{\text{(Total number of sperm observed)}} \times 100.$$

Malondialdehyde (MDA) Concentration

According to Yagi (1998), the amounts of malondialdehyde (MDA) were calculated in a thiobarbituric acid reactive substance (TBARS) as indices of lipid peroxidation in stored semen. For this test, 0.1mL of sperm suspension was incubated for 20 minutes at 37°C with 0.1mL of 150mM Tris-HCl (pH 7.1). After that, 2mL of 0.375 percent thiobarbituric acid and 1mL of 10% trichloroacetic acid (TCA) were added. After that, the samples were placed in a boiling water bath for 30 minutes. Following that, samples were centrifuged for 15 minutes at 3000 xg in the blank tube, and the absorbance level was measured using a spectrophotometer set to 532nm. The following formula was used to measure the MDA concentration: MDA

concentration (nmol/mL) = AT – AB/1.56 105; where AT is the sample serum absorbance, AB is the blank absorbance, and 1.56 105 is the molar absorptivity of MDA.

Statistical analysis

The experiment was set up in a 3x2 factorial design. The data was subjected to a one-way ANOVA, and the Duncan's Multiple Range Test of SAS 2000 was used to isolate means with large (P<0.05) variations.

Results

Table 1 shows the effect of various preservation protocols on the functional attributes of FUNAAB alpha chicken sperm. For all of the parameters studied, the findings showed differences (P<0.05). Table 2 shows the impact of various preservation protocols on the functional integrity of FUNAAB alpha chicken sperm. For acrosome and membrane integrities, there were differences (P<0.05) among the protocols. Table 3 shows the effect of various preservation protocols on the oxidative stress parameter of FUNAAB-alpha chicken semen. For MDA concentration, there were differences (P<0.05) among the protocols. However, no differences (P>0.05) were found in the leukocyte protocol, with values ranging from 0.44-0.47x103mL. Table 1 shows that fresh semen (control) had a higher percentage of sperm motility (P<0.05) than semen subjected to refrigeration and freezing protocols. In contrast to the freezing procedure, sperm motility was higher (P<0.05) in semen subjected to refrigeration. In comparison to the freezing protocol and the control, livability was higher (P<0.05) in semen subjected to refrigeration. When compared to semen subjected to refrigeration and the control, frozen sperm had a higher (P<0.05) percentage of sperm abnormality.

Table 1: Sperm functional attributes of FUNAAB alpha chickens subjected to different preservation protocol

PROTOCOL	MOT. (%)	LIV. (%)	ABN. (%)
Fresh	97.2±0.61^{a}	63.1±2.53^{b}	21.2±1.94^{c}
Refrigeration	89.7±1.22^{b}	93.8±1.74^{a}	34.9±3.04^{b}
Freezing	32.8±0.92^{c}	70.5±2.24^{b}	59.6±3.92^{a}

^{a,b,c}Values within columns with different superscripts differ (P<0.05); MOT = Motility, LIV. = Livability, ABN = Abnormality SEM= Standard error mean

Table 2: Sperm functional integrities of FUNAAB alpha chickens subjected to different preservation protocols

PROTOCOL	ACI. (%)	MI. (%)
Fresh	96.8±0.46^{a}	97.6±0.39^{a}
Refrigeration	96.4±0.49^{a}	97.7±0.35^{a}
Freezing	94.9±0.59^{b}	94.6±0.63^{b}

$^{a,\ b,}$ Values within columns with different superscripts differ (P<0.05); ACI= acrosome integrity, MI= membrane integrity SEM= Standard error mean

Table 3: Means (±SEM) seminal oxidative stress parameters of FUNAAB alpha chickens subjected to different preservation protocols

PROTOCOL	LEU(x10^{3}/mL)	MDA (×10^{6})
Fresh	0.47±0.10	1.31±0.15^{a}
Refrigeration	0.49±0.01	1.97±0.15^{a}
Freezing	0.44±0.09	0.97±0.11^{b}

^{a,b}Values within columns with different superscripts differ (P <0.05); MDA= malondialdehyde concentration LEU= seminal leukocyte, SEM= Standard error of mean

Discussion

In the current study, it is found that the refrigeration protocol improved sperm motility as compared to the freezing protocol for 24 hours. This is in line with the findings of Wambeke (1972), who found that semen stored in vitro for 24 hours at 2-5°C had no loss in fertilization potential. However, Latif *et al.* (2005) found a 58.6% sperm motility rate in Hubbard broiler sperm after 24 hours of storage at 5°C. Tabatbaei and Aghaei (2010) found that Ross-308 broilers stored for 24 hours at 4°C had a 45.6 percent lower sperm motility score. During storage, Karunakaran *et al*.(2007) observed a decrease in progressive sperm motility. Similarly, Ndubuisi-Ogbonna *et al*. (2021a) reported that sperm viability of refrigerated spermatozoa wasbetter than the frozen spermatozoa of Normal Feather (NF), Naked Neck (NN) and Frizzle Feather (NF). Also, the results also supported the conclusion of Ndubuisi-Ogbonna *et al*. (2021b) that slow and rapid freezing cryoprotocols had a deleterious effect on the spermatozoa of NF, NN, FF and removal of seminal plasma through centrifugation did not improve the viability of the spermatozoa. Slanina *et al.* (2011) found that storing turkey spermatozoa at a low temperature of 4-8°C for long periods of time maintained greater motility than storing them at a higher temperature. This may be due to a number of factors, including a slow metabolic rate. Consumption and depletion of the limited sperm bioenergetics resources (ATP) is reduced at low metabolic rates. When semen is stored at 5°C instead of 40°C, ATP consumption is decreased by 75 to 80 percent (Wishart, 1984). The number of dead sperm cells in cock semen stored at 41°C was higher than those stored at 4°C or 21°C, according to Dumpala *et al.* (2006). In vitro sperm storage at 5°C decreased cell metabolism and sustained sperm motility for an extended period of time (Karunakaran *et al.,* 2007). The present study found that sperm livability was highest in the refrigeration protocol (93.82%) and lowest in the fresh and freezing protocols (63.09 percent and 70.54 percent, respectively). As compared to the refrigeration protocol and the control, the acrosome and membrane integrities of semen subjected to the freezing protocol were lower ($P<0.05$) (fresh). The acrosome and membrane integrities of semen that had been frozen and those that had not were comparable. Sperm's ability to fertilize depends on its practical competence. Fertilizing ability is dependent on the integrity of the sperm plasma membrane and acrosome. As a result, a problem with sperm membrane integrity may have a significant impact on male infertility. Within 24 hours, both acrosome and membrane integrity were intact for new, refrigerated, and frozen spermatozoa, according to the findings of this report. Daramola *et al.* (2013) found that the acrosome of West African Dwarf buck semen remained intact after 24, 48, 72, and 96 hours of storage. The acrosome reaction needed at the proper time to promote fertilization requires acrosomal intactness (Thomas *et al*., 1997). The acrosomal cap changes mostly due to sperm aging or cryoinjury (Ansari *et al*., 2010). The seminal leukocytes in the protocols and the control were identical. However, when comparing the freezing protocol to the refrigeration protocol and the power, the results showed that MDA concentrations were lower ($P<0.05$) in the freezing protocol. Malondialdehyde (MDA) is a by-product of polyunsaturated fatty acid (PUFA) oxidation and is a metabolic breakdown product. Phospholipids, which contain polyunsaturated fatty acid residues, are abundant in spermatozoa from birds (Surai *et al.,* 2000). They are particularly vulnerable to lipid peroxidation, which can

lead to male infertility. A high proportion of PUFA in poultry spermatozoa is essential for maintaining membrane fluidity and flexibility, which is an important property during the fertilization process (Gwara and Jedrzejczak, 2001). Peroxidation occurs when sperm are exposed to oxygen during storage, according to Fujihara and Howarth (1984). The paradox, according to Long and Kramer (2003), is that the same oxygen that is needed for sperm metabolism is also used in lipid peroxidation and the development of free radicals during the storage of bird sperm. Information of malondialdehyde development during sequential steps of the semen conservation process is useful because it can help protect spermatozoa from damage and maintain their fertilizing capacity during this process. In this analysis, lipid peroxidation was measured using MDA concentrations from freshly collected semen (1.31nmol/10^6), refrigeration at 5^{o}C (1.97nmol/10^6), and freezing protocol (0.97nmol/106) over 24 hours from three FUNAAB alpha chicken strains (NN, NF, and FF). Blesbois *et al.* (1993) found a significantly lower level of peroxidation in rooster sperm (0.02–0.04 nmol/10^9spermatozoa), in contrast to Danikowski *et al.* (2002), who found 3.37 nmol/ml MDA output in freshly pooled rooster semen. It's possible that the difference is due to the different incubation conditions. Meanwhile, the seminal leukocyte values for new, refrigeration, and freezing protocols were all within the same range of 0.47x10^6, 0.49x10^6, and 0.44x10^6. Oxidative stress (the production of reactive oxygen species, or ROS) and the secretion of cytotoxic cytokines were induced by leukocytes in the sperm (lymphokines and monokines). These products can affect the morphology and DNA integrity of sperm. However, recent research suggests that leukocytes have a negative effect on sperm quality due to the presence of reactive oxygen species (ROS), which are mainly released by leukocytes. ROS are thought to be toxic to spermatozoa. According to Agarwal *et al*. (2008), leukocytospermia is linked to sperm tail defects, acrosomal injury, and a high sperm deformity index score.

Conclusion

The findings revealed that refrigerated semen of FUNAAB-alpha chicken strains maintained sperm viability and fertilizing capacity compared with the frozen semen samples. Recovery rates of refrigerated semen samples of FUNAAB-alpha chicken strains were higher than the frozen semen samples.

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