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COMPARATIVE EFFECT OF CENTRIFUGATION ON SPERM VIABILITY OF FUNAAB ALPHA CHICKENS DURING SLOW AND RAPID FREEZING PROTOCOLS

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The aim of this study was to compare the effects of centrifuging FUNAAB Alpha chickens during slow and rapid freezing protocols on sperm quality endpoints in cryopreserved chicken sperm. For this experiment, ejaculates from thirty cocks 25-30 weeks old with normal feather, naked neck, and frizzle feather from FUNAAB alpha chickens were pooled and frozen in Tris-extenders before being thawed and evaluated. The semen samples were split into two groups. The first part was washed with regular saline water and centrifuged at 500xg for 5 minutes to extract the seminal plasma, while the second part remained unwashed. Washed and unwashed sperm samples were cryopreserved using slow and rapid cryoprotocols, and then sperm viability was assessed. Two-way ANOVA was used to analyze the data. Results showed higher ($p<0.05$) motility for slow freezing (SF) cryoprotocols compared to samples subjected to rapid freezing (RF) for unwashed and washed spermatozoa. In washed spermatozoa, SF livability was found to be higher ($p<0.05$) than RF. Unwashed spermatozoa subjected to RF had higher membrane integrities ($p<0.05$) than unwashed spermatozoa subjected to SF and washed SF and RF cryoprotocol. Washed sperm subjected to SF cryoprotocol had lower malondialdehyde concentration (MDA) concentrations ($p<0.05$) than washed sperm subjected to RF cryoprotocol, but were equivalent to unwashed sperm subjected to RF cryoprotocol. In both the SF and RF cryoprotocols, seminal leukocytes in washed and unwashed semen samples were comparable. In conclusion, removal of seminal plasma did not increase the viability of the spermatozoa using slow freezing cryoprotocols.

Keywords: Cryoprotocols, Seminal plasma, Spermatozoa, Unwashed, Washed

One of the most important assisted reproductive technologies for improving reproductive efficiency is artificial insemination of cryopreserved sperm (Leboeuf et al., 2000; Martinez et al., 2007). Cryopreservation, on the other hand, decreases sperm viability (Pegg, 2007). According to Ciftci & Aygun (2018), the average fertility of frozen/thawed poultry sperm is between 2-80%. They hypothesized that the issue stemmed from the fact that chicken semen contains more polyunsaturated fatty acids than mammalian semen, necessitating the use of more antioxidant defense.

Due to inadvertent changes during various phases of cooling, thawing, and dilution, a significant amount of permanent membrane damage occurs in poultry spermatozoa (Tarvi, 2013). However, due to the fragility of poultry sperm compared to mammalian sperm, Tarvi (2013) found that exposing sperm to a cryoprotectant for an extended period of time may have negative effects on the sperm. In his

study, spermatozoa cryopreserved in dimethylacetamide had 41% motility and 45% membrane intact sperm, ethylene glycol had 38% motility and 39% membrane intact sperm, and methylformamide had 28% motility and 37% membrane intact sperm. Glycerol-exposed samples, on the other hand, had a higher proportion of motile sperm (54%) and membrane-intact sperm (58%) than control samples. This revealed that using frozen/thawed spermatozoa resulted in an average percentage of fertility potential. As a result, if a cryopreserved semen sample is used, low fertility is likely.

FUNAAB Alpha chicken strains were established at the Directorate of the University Farm, Federal University of Agriculture, Abeokuta, Ogun State, Nigeria's Poultry Breeding Unit. To improve the indigenous chickens, the potential variability that exists among them was exploited. They retained the Nigerian indigenous chicken's different plumage colors and three feathering patterns (Normal feathered, Frizzled feathered, and Naked neck) (Wheto et al., 2017)

The low fertility potential of cryopreserved semen of chicken had limited the use of artificial insemination by individual, thus is accountable for immediate insemination of fresh semen collected without attempts being made for storage for future use in most farms in the developing countries.

The problem also accounted for the absence of semen bank for chicken in Africa as compared to what is obtainable for cattle. This however necessitates this study to look into different cryoprotocols and observe if any of the protocols could be beneficial to the improvement of fertility and viability of chicken sperm. This is expected to assist the farmers in storing semen even after the death of the cock. This will further assist the breeder in genetic improvement of chickens as well as ease of obtaining fertilized eggs.

The study is aimed at providing a lasting solution to the storage and transportation of semen. This will also contribute to the tool of genetic improvement in the poultry industry, as artificial insemination plays a vital role in this regard. Therefore this study was conducted to investigate the effect of rapid and slow freezing with or without centrifugation on different sperm parameters.

MATERIALS AND METHODS

Experimental site

The study was carried out at the Teaching and Research farm, FUNAAB, which falls within $7^{o}10N$ and $3^{o}2E$ and altitude 76m above sea level. It lies in the South-West part of Nigeria with a prevailing tropical climate, a mean annual rainfall of 1037 mm and an average temperature of $34.7^{o}C$. The laboratory analyses were carried out at the Animal Physiology Laboratory of the Department of Animal Physiology, Federal University of Agriculture Abeokuta, Ogun State, Nigeria.

Experimental Birds and Management

A total number of thirty (30) cocks between 25-30 weeks old of three strains of FUNAAB alpha chickens consisting of 10 Normal feathers, 10 Naked-neck, and10 Frizzle feather was used for this experiment. The birds were fed *ad-libitum* with commercial breeder mash containing 17.5% crude protein and 2700kcal metabolizable energy. Clean water was supplied *ad-libitum.* Medications and vaccinations were done as required.

Semen collection

Semen samples were collected from thirty cocks, ten for each strain of chicken (NF, NN, and FF). The abdominal massage method described by Burrows and Quinn was used to collect sperm (1937). The technique entails restraining the male and softly stroking the back of the bird with firm quick strokes from behind the wings to the tail. The handler gently squeezes the cloaca, extracting semen through the external papillae of the ductus us deferens (vas deferens), and gathering the semen into a jar while the male responds with tumescence erection of the phallus. According to Burrows and Quinn (1937), AI techniques begin even before the operation. It involves separating the male poultry from the hens in order to increase the amount of usable sperm. Since the phallus and anus of the bird are in the same duct, eliminating food 12 hours before collection will help avoid faecal contamination of the semen. Breeder cocks

must be regularly prepared for semen collection for several days before the AI procedure to ensure that each bird is fertile, as determined by a microscopic analysis of the sperm.

According to Burrows and Quinn (1937), the bird's sexual organ must be stimulated to stretch outside of his body, much like semen collection in other farm animals. Small birds, such as chickens or quail, can be handled by one person; larger birds, such as turkeys or geese, usually need two people. After collection, semen was placed in a water bath at 37°C and transferred to the laboratory for semen evaluation in 15 minutes. Semen sample was split into 2 equal aliquots and washed with non-culture medium (normal saline) by centrifuging at 500xg once for 5 minutes each in order to remove semen plasma while the control group was not washed. Following washing and removal of supernatant, the sediments were then diluted at 32^{o}C and cryopreserved using slow and rapid freezing protocols.

Ejaculates were evaluated for volume (ml), colour, pH, density, mass movement (0-5), sperm motility (%), sperm abnormality (%) and sperm viability (%) using eosin-nigrosin staining and sperm concentration (n x 10^9 sperm/ml) by haemocytometer.

Statistical Analysis

The experiment was laid out in 3x2x2 factorial arrangements. Data obtained was subjected to a two-way ANOVA and significantly ($P<0.05$) different means were separated by Duncan Multiple Range Test using SAS 2000.

RESULTS AND DISCUSSION

Means Interactions of different washing protocols and cryoprotocols on the sperm functional attribute of FUNAAB alpha chickens are presented on Table 1. The result showed variations ($p<0.05$) in motility and livability among the unwashed and washed semen subjected to SF and RF cryoprotocols. However, no variations ($p>0.05$) observed in sperm abnormalities among the unwashed and washed semen samples subjected to slow and rapid freezing cryoprotocols, hence the values ranges from 46.87-67.84%.

Interactions of different protocols and cryoprotocols on the sperm functional integrities of FUNAAB alpha chickens are presented in Table 2. The result showed significant variations ($p<0.05$) in membrane integrity among the unwashed and washed semen subjected to SF and RF cryoprotocols. However no variations ($p>0.05$) observed in acrosome integrity among the unwashed and washed semen samples subjected to slow and rapid freezing cryoprotocols, hence the values ranges from 40.89-46.11%.

The interactions of different protocols and cryoprotocols on the seminal oxidative stress parameters of FUNAAB alpha chickens are presented in Table 3. The results showed variations ($P<0.05$) among the protocols and cryoprotocols for MDA concentration. However no variations ($P>0.05$) observed in leukocyte among the washed and unwashed spermatozoa subjected to slow and rapid freezing, hence the values ranges from 0.30-0.84 x10^3/mL

Among unwashed and washed semen samples, spermatozoa subjected to SF had higher ($p<0.05$) motility compared to samples subjected to RF. However, sperm motilities were similar in semen samples subjected to SF in both unwashed and washed spermatozoa. The results showed that livabilities were comparable among the unwashed and washed semen samples, spermatozoa subjected to SF and RF except higher livability observed in SF compared to RF in washed spermatozoa. In addition, sperm abnormalities were comparable among the unwashed and washed spermatozoa subjected SF and RF.

The current *in vitro* study found that removing seminal plasma via centrifugation or washing at 500xg once for 5 minutes in order to remove semen plasma was able to maintain sperm motility and livability for slow freezing when compared to the control (unwashed), indicating that washed rapid freezing has a negative effect on sperm motility. The reduced motility could result from the cold shock from semen freezing, likewise damages caused by centrifugation. This result contradicted

Table 1: Mean (±SEM) Interactions of different washing protocols and cryoprotocols on the sperm functional attributes of FUNAAB alpha chickens

PROTOCOL	CRYOPROTOCOL	MOT	LIV	ABN
UNWASHED	SF	44.89±2.43[a]	66.64±4.06[ab]	52.63±2.97
	RF	22.56±2.43[b]	68.89±3.03[ab]	46.87±2.22
WASHED	SF	37.89±2.43[ab]	76.38±3.03[a]	67.84±2.22
	RF	2.44±2.43[c]	55.63±3.71[b]	67.12±2.72

[a, b, c] Values within columns with different superscripts differ (P<0.05); SF= slow freezing, RF= rapid freezing, MOT = Motility, LIV. = Livability, ABN = Abnormalities

Table 2: Means (±SEM) Interactions of different protocols and cryoprotocols on the sperm functional integrities of FUNAAB alpha chickens

PROTOCOL	CRYOPROTOCOL	ACI	MI
UNWASHED	SF	43.78±1.35	69.22±1.84[b]
	RF	46.11±1.35	75.78±1.84[a]
WASHED	SF	40.89±1.35	65.89±1.84[b]
	RF	42.56±1.35	37.33±1.84[c]

[a, b, c] Values within columns with different superscripts differ (P<0.05); SF= slow freezing, RF= rapid freezing, ACI= acrosome integrity, MI= membrane integrity.

TABLE 3: Means (±SEM) Interactions of different protocols and cryoprotocols on the seminal oxidative stress parameters of FUNAAB alpha chickens

Protocol	Cryoprotocol	LEU(x10^3/mL)	MDA (×10^6)
Unwashed	SF	0.84±0.11	1.24±0.19[a]
	RF	0.62±0.09	0.47±0.19[b]
Washed	SF	0.49±0.09	0.46±0.19[b]
	RF	0.30±0.10	1.33±0.20[a]

[a, b,.] Values within columns with different superscripts differ (P<0.05); SF= slow freezing, RF= rapid freezing, MDA= malondialdehyde concentration LEU= seminal leukocyte

Morrell et al. (2005) findings, which stated that centrifugation improved Tom's sperm survival during cryopreservation. While unwashed and washed semen abnormalities are comparable in the present study, the result showed an increased morphological defect, this could be due to cold shock which is triggered by the freezing protocols. After freezing and thawing behavior on the semen of Norduz goats during breeding season, Cebi-Sen et al (2015) found that centrifugation decreases sperm motility and increases morphological defects. Despite the fact that seminal plasma protects sperm from oxidative stress, (Saleh and Agarwal, 2002) ejaculated semen containing aging spermatozoa, sperm defects, leukocytes and particle debris can reduce

sperm survivability. Several studies have shown that centrifugation of mammalian spermatozoa from seminal plasma increases motility and fertility after a freeze-thaw process (Kozdrowski et al., 2007).

The results showed that acrosome integrities in washed and unwashed spermatozoa were comparable in both SF and RF cryoprotocols. However, higher ($P<0.05$) membrane integrities were observed in unwashed spermatozoa subjected to RF compared to unwashed spermatozoa subjected to SF and washed SF and RF cryoprotocol. Though membrane integrities of unwashed and washed protocol subjected to SF cryoprotocol were comparable ($P>0.05$), washed sperm subjected to RF showed lower ($P<0.05$) membrane integrity.

Washing of spermatozoa in the present study did not have effect on acrosome integrity compared to the unwashed counterpart. Unwashed sperm had higher membrane integrity when subjected to rapid freezing cryoprotocol, while washed sperm had lower membrane integrity when subjected to rapid freezing cryoprotocol. The results of this study agree with those of Martinez-Fresneda et al. (2019), who found that centrifugation reduced the membrane integrity of frozen-thawed chicken spermatozoa. According to Aurich (2005), sperm washing had no impact on stallion sperm viability, motility, or chromatin integrity.

Reduced ($P<0.05$) MDA concentration was observed when unwashed sperm was subjected to RF cryoprotocol compared to unwashed sperm cryopreserved using SF cryoprotocol. However, reduced ($p<0.05$) MDA concentration was observed in washed sperm subjected to SF cryoprotocol compared to washed sperm subjected to RF cryoprotocol though comparable to unwashed sperm subjected to RF cryoprotocol. The results showed that seminal leukocyte in washed and unwashed semen samples were comparable in both SF and RF cryoprotocols.

The leukocytes of unwashed and washed protocols were comparable. This means that removal of seminal plasma did not increase the seminal leukocyte. Meanwhile the MDA concentration of unwashed semen subjected to slow freezing and washed semen subjected to rapid freezing appeared higher when compared to unwashed semen subjected to slow freezing and washed semen subjected to rapid freezing. Lower values obtained in washed semen subjected to slow freezing indicated that washed protocol can reduce the oxidative stress during slow freezing cryopreservation. MDA concentration within cells is a tension measure (Sorongbe et al., 2019) In this analysis, an abrupt shift in freezing temperature during rapid freezing was blamed for the lower MDA concentration obtained in slow freezing for washed semen compared to rapid freezing.

CONCLUSION

It can be concluded from this study that sperm viability were maintained in washed spermatozoa using slow freezing cryprotocol for the strains.

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