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Preliminary Study on Cryopreservation of Local Chicken Spermatozoa in Liquid Nitrogen

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Abstract

This research was designed to investigate the most effective cryoprotectant to be used for spermatozoa of local chicken *Gallus gallus domesticus* in liquid nitrogen. Semen was collected from local chickens through abdominal massaging. The sperm motility and concentration of fresh semen were immediately evaluated, with subsequent weekly evaluation of the cryopreserved semen in liquid nitrogen for a maximum of three weeks. The cryoprotecting facilities used were dimethyl sulfoxide and phosphate buffered saline (DP); dimethyl sulfoxide and Ginzburg fish ringer (DF); glycerol and phosphate buffered saline (GP); glycerol and Ginzburg fish ringer (GF); egg yolk and phosphate buffered saline (EY+P); egg yolk and Ginzburg fish ringer (EY+F); quail Egg yolk and phosphate buffered saline (QEY+P); quail egg yolk and Ginzburg fish ringer (QEY+F). Results showed that the motility and concentration of freshly collected sperm recorded 70% and 1.79×10^9 spermatozoa/ml, respectively, while there was a decrease in sperm motility and concentration over time. Importantly, the yolk-based cryoprotective agents with Ginzburg fish ringer had the most effective cryoprotective properties; however, the quail egg yolk was better and could be employed for long-term cryopreservation.

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Introduction

Cryopreservation of biological samples has been identified as one of the means of preserving animal genetic resources and biodiversity [1]. Cryopreservation could be defined as a technique whereby cells, tissues, extracellular matrix, organelles, organs or any other biological constructs that are vulnerable to damage caused by unregulated chemical kinetics are being preserved by cooling to very low temperatures for a long period of time using extenders and cryoprotectant agents to protect the cells from damage during freezing and thawing of the frozen semen [2]. Globally, protection of biodiversity and conservation of the gene pool are of prime importance, and this is highly necessary to protect species of poultry and livestock that are especially endangered, and to increase the population of improved breeds [3]. Cryopreservation of sperm has allowed for transporting samples of livestock, poultry, and aquatic animals across many regions to produce offspring with superior genetics in order to improve the species, and this has removed the cost of transporting the live animals [4]. The cryopreserved biological sample could be employed to increase genetic diversity and prevent extinction of species [5]. At present, one of the most practicable methods for long-term storage of genetic material is semen cryopreservation [6]. The technique of artificial insemination complements cryopreservation of spermatozoa irrespective of the location and availability of the male animal [7]. Essentially, therefore, in order to protect the biodiversity of indigenous chicken successfully, an optimized protocol for cryopreservation needs to be established through sperm cryopreservation of a particular strain of local chicken, which could be stored in liquid nitrogen. The straw that has been frozen can then be transported to other facilities without the need for shipping live animals that would need quarantine [8].

The series of losses in biodiversity and adaptive local gene pool with pure strain of male stock has necessitated the need to conserve livestock and poultry genetic resources. Cryopreservation has been established to provide an effective and plausible method to harvest sperm cells from male local chicken and consequently cryopreserved for use in artificial insemination programmes [9]. This eliminates the need to keep live male chicken and their transportation burden. Moreover, this study employed the technique of cryopreservation to protect local chicken spermatozoa. Therefore, the objective of this study was to investigate the most effective and efficient cryoprotectant to be employed for long-term cryopreservation of local chicken spermatozoa.

Materials and Methods

Location and samples

The experiment was conducted at the biotechnology and reproductive physiology laboratories of the Department of Animal Sciences, Obafemi Awolowo University, Ile-Ife, Nigeria. Seven mature male cocks of local chicken, *Gallus gallus domesticus*, of a minimum age of one year were selected, and their semen was collected through abdominal massage [5, 10].

Preparation of extender-cryoprotectant

Two extenders of phosphate buffer saline (PBS) and Ginzburg Fish Ringer (GFR) with pH of 7.4 and 7.6, respectively, were utilized. Tables 1 and 2 showed how the extenders were prepared, and thereafter sterilized for 20 minutes.

Sperm cryopreservation

Before cryopreservation of the spermatozoa, the motility of the fresh sperm was evaluated using a light microscope (Olympus Trinocular Microscope XSZ-156T, Ningbo Huasheng Precision Technology Co., Ltd., China) at high power objective (×40) and was diluted with 0.9% saline water using a dilution ratio of 1:20, and the motility was evaluated. The semen was mixed with extender-cryoprotectant at a ratio of 1:7 and stored in cryotubes. The cryotubes were gently shaken to allow semen to properly mix with extender-cryoprotectant and placed in the refrigerator at 4^{o}C for 10 minutes. This was later transferred into a Dewar container containing liquid nitrogen for preservation. 30 µl of cryoprotectant was added to 5 µl of semen for storage in the liquid nitrogen.

Table 1 Composition of extenders (g/l) tested for cryopreservation of the sperm of local chicken in liquid nitrogen.

Composition	Phosphate buffer saline	Ginzburg fish ringer
NaCl	0.8	0.7
KCl	0.002	0.028
CaCl	-	0.033
$NaHCO_3$	0.023	-
Na_2HPO_4	0.115	-
KH_2PO_4	0.02	-

Table 2 Composition (%) of the cryoprotective agents used.

Cryoprotective agent	DMSO	Glycerol	PBS	GFR	Glucose/sucrose
DP	10	-	90	-	-
DF	10	-	-	90	-
GP	-	10	90	-	-
GF	-	10	-	90	-
EY+P	-	-	90	-	-
EY+F	-	-	-	90	-
QEY+P	-	-	90	-	-
QEY+F	-	-	-	90	-

DP (Dimethyl sulfoxide and Phosphate buffered saline); DF (Dimethyl sulfoxide and Ginzburg fish ringer); GP (Glycerol and Phosphate buffered saline); GF (Glycerol and Ginzburg fish ringer); EY+P (Egg yolk and Phosphate buffered saline); EY+F (Egg yolk and Ginzburg fish ringer); QEY+P (Quail Egg yolk and Phosphate buffered saline); QEY+F (Quail Egg yolk and Ginzburg fish ringer)

Motility evaluation

After thawing, the motility and sperm count were evaluated using a haemocytometer and viewed under a light microscope (Olympus Trinocular Microscope XSZ-156T, Ningbo Huasheng Precision Technology Co., Ltd., China) at a high-power objective (×40) but with magnification of 400. Sperm motility = (number of motile sperm cells × 100)/ total no. of sperm cells. The sperm count was also evaluated using the haemocytometer after proper dilution with 0.9% saline solution. The sperm count was carried out in five replicates. The total sperm count was calculated as thus: total sperm count = sperm count × dilution factor / volume of sperm.

Data analysis

The data recorded for sperm motility and sperm count per week for the respective cryoprotective agent were subjected to statistical analysis using the General Linear Model (GLM) procedure of software SAS, 2002 (SAS Institute, 2002).

Results and Discussion

The motility and concentration of freshly collected sperm were respectively recorded as 70% and 1.79 × 10^9 spermatozoa/ml. Table 3 shows the weekly effect of cryopreservation on sperm motility and concentration stored in liquid nitrogen. The sperm motility of the semen deteriorated after the first week of preservation, depending on the type of cryoprotective agent used for the preservation of the fresh semen, and this agrees with the result obtained by other researchers that the motility of sperm reduces after one hour of semen collection if stored *in vitro* [11,12]. After freeze-thawing, the motility of the sperm has been identified as one of the good indicators of semen quality in poultry [6]. During cryopreservation, the spermatozoa lose the required energy being supplied by mitochondria as ATPs for their motility [13]; therefore, a good cryoprotective agent protects and preserves the spermatozoa by minimizing the effect of energy loss. Moreover, the decrease in motility could be due to metabolic activities, such as pH resulting from the cryopreserved sperm that ultimately affects the biological environment [14].

For the first week, for the entire cryoprotective agent period between the weeks, the motilities of the semen were statistically different (p<0.05) and decreased progressively. This followed the same pattern for all the cryoprotective agents in which the motility decreased progressively [6]. However, within each cryoprotective agent, and the week, the yolk-based cryoprotective agent, EY+F (63.33%), gave the best result (p<0.05), followed by QEY+F (57.33%) (p<0.05); however, both of the Ginzburg fish ringer as extender [15, 16]. Both cryoprotective agents maintained their motility values for the second and third weeks at their respective significant levels. Importantly, the phosphate-buffered saline-based cryoprotective agent in combination with the yolk did not give a good result in comparison to the Ginzburg fish ringer. This could be explained by the report of other researchers that egg yolk has the most effective cryoprotective agent of freezing extender that protects sperm against cold shock [17, 18]. Egg yolk has been in use for cryopreservation for decades because of its protective properties for biological materials [19]. It is reported to contain protective components such as cholesterol, phospholipids, low-density lipoprotein, fatty acids, etc. [20, 21]. Sperm concentration was maintained in most of the cryoprotective agents, and there was

Table 3 Weekly evaluation of sperm motility and concentration stored in liquid nitrogen.

Cryoprotective agent	Motility per week (Mean±SD) (%)			Sperm count/week (Mean±SD) spermatozoa/ml		
	1	2	3	1	2	3
DP	52.67 ± 2.52^{a}	34.33 ± 1.15^{b}	16.00 ± 1.00^{c}	$8.01\times10^{8}\pm5.79\times10^{8a}$	$7.83\times10^{8}\pm 5.65\times10^{8a}$	$7.74\times10^{8}\pm5.73\times10^{8a}$
GP	45.00 ± 2.00^{a}	21.00 ± 1.00^{b}	15.33 ± 1.53^{c}	$7.70\times10^{8}\pm5.70\times10^{8a}$	$7.37\times10^{8}\pm5.44\times10^{8a}$	$7.03\times10^{8}\pm5.14\times10^{8a}$
DF	45.33 ± 1.53^{a}	22.00 ± 1.00^{b}	15.67 ± 2.08^{c}	$8.84\times10^{8}\pm1.50\times10^{7a}$	$8.58\times10^{8}\pm8.89\times10^{6a}$	$7.81\times10^{8}\pm2.60\times10^{7a}$
GF	40.33 ± 0.58^{a}	16.00 ± 1.00^{b}	13.67 ± 0.58^{c}	$4.89\times10^{8}\pm1.00\times10^{8a}$	$4.72\times10^{8}\pm1.66\times10^{7a}$	$3.24\times10^{8}\pm2.91\times10^{7a}$
EY+F	63.33 ± 1.53^{a}	41.33 ± 1.53^{b}	27.00 ± 1.00^{c}	$9.74\times10^{8}\pm1.70\times10^{7a}$	$8.62\times10^{8}\pm 3.25\times10^{7a}$	$8.15\times10^{8}\pm8.30\times10^{7a}$
EY+P	48.67 ± 1.53^{a}	31.33 ± 1.53^{b}	20.00 ± 1.00^{c}	$7.90\times10^{8}\pm\times5.57\times10^{6a}$	$5.87\times10^{8}\pm 1.99\times10^{7b}$	$3.67\times10^{8}\pm7.37\times10^{6c}$
QEY+F	57.33 ± 2.52^{a}	52.67 ± 2.52^{b}	41.00 ± 1.73^{c}	$1.50\times10^{9}\pm 3.26\times10^{8a}$	$8.42\times10^{8}\pm1.26\times10^{7b}$	$8.11\times10^{8}\pm1.26\times10^{7b}$
QEY+P	41.33 ± 1.53^{a}	20.00 ± 1.00^{b}	17.33 ± 0.58^{c}	$1.43\times10^{9}\pm1.05\times10^{8a}$	$6.79\times10^{8}\pm1.40\times10^{7b}$	$4.54\times10^{8}\pm7.02\times10^{6c}$

[abc]Means within a row with different superscripts are significantly different (P<0.05). DP (Dimethyl sulfoxide and Phosphate buffered saline); DF (Dimethyl sulfoxide and Ginzburg fish ringer); GP (Glycerol and Phosphate buffered saline); GF (Glycerol and Ginzburg fish ringer); EY+P (Egg yolk and Phosphate buffered saline); EY+F (Egg yolk and Ginzburg fish ringer); QEY+P (Quail Egg yolk and Phosphate buffered saline); QEY+F (Quail Egg yolk and Ginzburg fish ringer)

weeks, except in the cryoprotective agent based on phosphate-buffered saline in combination with the yolk. This study revealed that the yolk-based cryoprotective agents (QEY+F and EY+F) preserved the motility of the semen better for the first week (p<0.05); and there was also a significant difference between both (p<0.05). At the third week, the motility within the cryoprotective agent revealed QEY+F (41.00%) as the best, though followed by EY+F (27.00%) (p<0.05). From the results obtained from this study, it was shown that egg yolk has the most effective cryoprotective properties, and notably, quail egg yolk was better.

Conclusion

In conclusion, cryopreservation has a great effect on the motility of spermatozoa, especially when the duration of preservation is long. The longer the duration of cryopreservation, the more the sperm loses its energy of motility. However, long-term cryopreservation of sperm is of importance in poultry production; therefore, with a good cryoprotectant agent, as demonstrated, this study showed that the yolk-based cryoprotective agent with Ginzburg fish ringer has the most effective cryoprotective properties. Importantly, quail egg yolk was better for long-term preservation.

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Conflict of interest

The authors declare no conflict of interest.

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