Scholars Journal of Agriculture and Veterinary Sciences OPEN ACCESS
Abbreviated Key Title: Sch J Agric Vet Sci
ISSN 2348–8883 (Print) | ISSN 2348–1854 (Online)
Journal homepage: https://saspublishers.com

Effects of Different Concentrations of Coconut Water Extended with Glycerol and Ringers Solution Extenders on Cockerel Semen Preservation

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DOI: 10.36347/sjavs.2023.v10i10.002 | **Received:** 30.06.2023 | **Accepted:** 01.08.2023 | **Published:** 20.10.2023

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Abstract **Original Research Article**

The study was conducted at the Poultry Unit of the Teaching and Research Farm, Department of Animal Science, Federal University of Kashere, Gombe State. Nigeria, Kashere is located at an elevation of 431metre above the sea level. Its coordinates lies between latitude 9°46"O"N and longitude 100º57'0" E, Altitude 349m (2006, census). The aim of the study was to determine the effects of cryptoprotectants on cockerel semen preservation. A total of twelve (12) indigenous cockerels with ages ranging from 12 to 15 months, with an unknown previous management background from local markets in Kashere were used. The cockerels were managed intensively and put into pens as experimental units. Cockerels were fed daily with growers mash feed and served water ad libitum; the birds were trained once a day in the morning by abdominal massage for two weeks. The cockerels were randomly allotted into three treatments. Each treatment had 3 replicates. The indigenous cockerels were trained once a day and semen was collected between 10:00am and 11:00am. The cockerels were allowed a period of two week for adaptation, Semen were collected by abdominal massage method described by Burrows and Quinn (1937). The semen concentration were measured using haemocytometer with the direct cell count method. The coconut water was collected from a freshly harvested coconut plant. Five milliliters of the coconut water was placed into different sampling bottles and diluted with different percentages of glycerol. The data on the percentage of sperm progressive motility, live/dead sperm, morphological normal intact sperm, total abnormal head, mid-piece/neck, tail and overall abnormalities were subjected to a one way analysis of variance using statistical Package for Social Sciences (SPSS version 23, 2015). coconut water extended with ringer solution at 2% glycerol at 0-7% concentration showed no significance difference ($P<0.05$). In this study head sperm was significantly high ($P<0.05$) at 2%, 3%, 5% and 6% respectively in Coconut water extended with ringer solution at 2% glycerol than other concentration. The total neck deformed sperm was significantly high ($P<0.05$) at 2% and 3% coconut water. The morphological total intact sperm head were in their acceptable range when preserved with coconut water using glycerol and Ringers solution as extenders but generally had an adverse effect on sperm membrane and possibly leading to low fertility after freezing. Morphological abnormality was most pronounced in the neck than in the head and tail regions. Coconut water preservation of cockerel semen using glycerol and ringers solutionas extenders may not be recommended. Further research using other preservation method /reagents is recommended.

Keywords: Cryptoprotectants, Cockerel, Glycerol, Semen, Morphology.

INTRODUCTION

Indigenous cockerels are widely distributed in the rural areas of the tropics and sub-tropical countries. Indigenous chickens in Africa are generally, adapted to rural environments, survive on little or no inputs and adjust to fluctuations in feed variability (Folasade, 2010). Indigenous chicken constitute 35% of the 120 million poultry birds raised in rural areas in Nigeria (RIM, 1992). They are self-reliant and hardy birds with the capacity to withstand harsh weather conditions and adaptation to adverse environmental Conditions, Folasade (2010). They are known to possess qualities such as the ability to hatch on their own, brood and scavenge for major parts of their feed and poses appropriate immunity to diseases and parasitic infections, Folasade (2010). Their products are preferred by the majority of Nigerians because of the

pigmentation, taste and leanness of their meat. The discovery of cryoprotectants, such as ringer solution, glycerol and dimethylsulphoxide, has facilitated the preservation of cockerel semen. Cryoprotectants used for preservation of sperm cells, provide protection from cold shock and other damages during storage. Ringer solution and glycerol at various concentration levels has been extensively used as a cryoprotectant (Purdy, 2006). Ringer solution and glycerol will be used in this research because there are a good number of researches conducted elsewhere though with different concentration levels. The percentage of glycerol and ringer solution added to the diluent is very important for survival and sperm cell viability, the chicken fowl sperm metabolize quickly and have the ability to become reversibly immobile under a variety of conditions during cryopreservation such as lack of oxygen and reducing the sugar level in the diluent Tijjani *et al.*, (2014) reported that glycerol is beneficial to sperm preservation they reported that glycerol would preserve the motility of frozen chicken fowl spermatozoa; much work has been conducted in the development of techniques for the preservation of poultry semen.

In order to achieve maximum success not only proper diluents and sperm dilution rates are required but also a thorough knowledge of the sperm and its physiology (Purdy, 2006). The major constraint in poultry production is inadequate good breeding stock which can lead to poor fertility and hatchability of birds. Since reproduction is very important for continuity of life in living organisms and continuous availability of animal protein, adequate semen evaluation, processing, preservation and artificial insemination will help to improve poultry production by providing good quality semen, adequate semen extenders which will help in breeding and genetic resources conservation. Semen evaluation and preservation programs of farm animals will help to improve indigenous cockerel production by ensuring the use of good quality semen. Additionally, the optimal level of glycerol and ringer solution concentration will be used as a guide for poultry semen cryopreservation, thus increasing poultry production in Nigeria, and solving the problem of protein deficiency in the country Folasade, (2010).

MATERIALS AND METHODS

Study Area

The study was conducted at Poultry Unit, Department of Animal Science Teaching and Research Farm of Federal University of Kashere, Gombe State. Kashere is located at an elevation of 431metre above the sea level. Its coordinates lies between latitude 9°46"O"N and longitude 100º57'0" E, Altitude 349m (2006, census). The annual rainfall of Kashere ranges between 800mm-900mm per annum and it's characterized by distinct dry season (October - May) and rainy season (June - September). The annual temperature ranges from 30-32° C, and it experiences a relative humidity of 70-90%.

Experimental Birds Management and Training Period

A total of twelve (12) indigenous cockerels with ages ranging from 12 to 15 months, with an unknown previous management background from local markets in Kashere were used. The cockerels were managed intensively and put into pens as experimental units. Cockerels were fed daily with grower's mash feed and served water *ad libitum*; the birds were trained once a day in the morning by abdominal massage for two weeks.

Experimental Design

The cockerels were randomly allotted into three treatments. Each treatment had 3 replicates.

Experimental Equipment

The experimental equipment's included: microscope, pH test strip, micropipettes, collection tubes, haemocytometer, slide and slide cover, syringe, beaker, foil paper, thermometer, etc.

Data Collection

Semen Collection

The indigenous cockerels were trained once a day and semen was collected between 10:00am and 11:00am. The cockerels were allowed for adaptation for a period of two weeks, which served as an adaptation period in order to make the cocks familiar with the semen collection process and to improve the effectiveness of collection; likewise they were trained to respond to the abdominal massage technique prior to the onset of semen collection. Semen were collected by abdominal massage method described by Burrows and Quinn (1937), after being given two weeks period of adaptation. The technique involved restraining the male and gently stroking the back of the male from behind the wings toward the tail with firm rapid strokes. The male responds with tumescence (erection) of the papillae by which the handler will gently squeezes the cloacae expelling semen through external papillae of the ductus deferens collecting the semen into a container, thereafter, the semen were pooled into the same sampling bottle and later examinde for semen characteristics.

Semen Evaluation Technique

1. Semen Volume, Colour and pH

The ejaculate volume from pool cockerel semen were measured with the use of 1ml syringe. Semen colour were visually evaluated immediately after collection and graded on a scale of 1— 4 (where, 1 = watery, 2 = slightly creamy, 3 creamy, 4= milky) (Peters *et al.*, 2008). Semen pH were measured using pH test strip.

2. Sperm Concentration

The semen concentration were measured using haemocytometer with the direct cell count method.

Haemocytometer is a specially designed slide that contains two counting chambers and two dilution pipettes. The counting chambers are 0.1mm in depth and have a ruled area on the bottom of the chambers that is 1.0mm2 of width. The square is subdivided into *25* smaller squares. In this study, 10 µI of semen were mixed with 990 µl of distilled water at the dilution rate of 1:100. One drop of the diluted semen will then be dropped in one end of the hemocytometer and also on the other end and this was done to allow the diluted semen to settle. The loaded hemocytometer was then placed on the microscope at x40 magnification. The sperm's head that fell within the sub-divided smaller squares at the four edges and center of the hemocytometer were counted and the average per cockerel was found based on the judgment of the individuals making the determination. The concentration of sperm/semen was calculated using the formula as below:

$$\text{Concentration} = \frac{\text{Sperm Counted x Dilution Rate x Depth of Hemocytometer}}{\text{Number of Squares Counted}}$$

3. Semen Mass Motility

A of drop of pool semen was placed on a microscope slide and covered with a glass covered slip to spread the semen in order to have a uniform thickness and to prevent drying. It was then placed on a microscope for examination at x10 magnification and given scores of 0—9 according to Blesbois *et al.* (2008). A scale of mass motility as shown in Table 3.1 will be used for semen mass motility evaluation.

Table 1: Mass motility scale.

Range	Sperm movement
0	Total lack of movement and agglutinates may cover the entire slide area.
1	Sperm moving on the spot without forward movement, and agglutinates may be total.
2	Most of the sperm moving on the spot and up to 10% of them presenting forward motility with agglutinates covering up to 80% of the observation area.
3	Small forward movements for 20-40% of cells and *30-50%* of sperm not in agglutinates.
4	40-60% of cells showing forward motility, but agglutinates covering 30-40% of the area.
5	Up to 80% of cells having forward motility and starting to form waves, but 10- 20% agglutinates still present.
6	Generalized forward motility (90% of sperm and no more than 5% agglutinates).
7	Rapid waves and no agglutinates visible.
8	Very rapid waves and whirlwinds visible but covering no more than 20% of the slide.
9	Whirlwinds covering 30-60% of the area.

Source: Blesbois *et al.,* (2018).

4. Individual Progressive Motility

The collected pooled semen was diluted in a ratio of 1:100 (semen extender) using matured coconut water. The individual cell motility was estimated by placing a drop of the diluted semen for the pooled semen and that of preserved semen during preservation on the slide and cover with glass cover slip. Sperm motility was assessed by microscopic observation at x40 magnification. Motility were expressed as the percentage of motile cell with moderate to rapid progressive movement. At least 15 microscopic fields will be examined and 150 sperm were counted for each sample.

5. Live/Dead Sperm Ratio

The eosin-nigrosin stain was used to determine the percentage of live and dead sperm. The stain were prepared by dissolving 1g of eosin, *5*g nigrosin and 3% sodium citrate in 100 ml of distilled water, which was later pre-warmed to body temperature for about 30 minutes and filtered before use. Briefly l0µl of fresh pooled semen were mixed with coconut water and glycerol. The 10µp1 of eosin-nigrosin stain were dropped into a clean glass slide and mixed with 1Op1 of diluted semen were placed on hourly observations, 5 µl of eosin-nigrosin stain were dropped on the glass slide and mixed with 5µl of preserved semen. The second glass slide were used to swipe quickly and form a thin layer and then air dried. The sperm were examined at l0x magnification under light microscope. At least 200 sperm were counted to determine the percentage of live and dead sperm. The sperm which appear with pink colour (stained with eosin) was regarded as dead and the colorless, the live sperms.

6. Sperm Morphology

The slides of live and dead staining was used to check abnormalities of the sperm. Sperm head (pear head, double head, elongated head, detached head), midpiece (swollen midpiece, coiled mid-piece), and tail (coiled tail, double tail, bent tail). About 200 sperm were examined for each sample under microscope at 10x magnification.

Preparation of extender containing 0%, 2% 3% 4% 5% 6% and 7% coconut water concentrations

Preparation of coconut water

Coconut water was collected from local fruit stall around. Coconut water was filtered using filter paper. Before use the coconut water was filtered using

0.22 micron micro-filter. Sodium bicarbonate was added into the filtered coconut water.

Streptomycin (antibiotic) was added into the coconut water.

Preparation of Extenders

Table 2: Reagents, control and experimental groups for different coconut water containing glycerol concentrations

Treatment	Reagent
1.	Coconut water (control) 0% glycerol plus ringers solution.
2.	2% Coconut water plus 2% glycerol. plus ringers solution.
3.	3% Coconut water plus 2% glycerol. plus ringers solution.
4.	4% Coconut water plus 2% glycerol. plus ringers solution.
5.	5% Coconut water plus 2% glycerol. plus ringers solution.
6.	6% Coconut water plus 2% glycerol. plus ringers solution.
7.	7% Coconut water plus 2% glycerol. plus ringers solution.

The coconut water was collected from a freshly harvested coconut plant. Five milliliters of the coconut water was placed into different sampling bottles and diluted with different percentages of glycerol.

Statistical Analysis

The data on the percentage of sperm progressive motility, live/dead sperm, morphological normal intact sperm, total abnormal head, mid-piece/neck, tail and overall abnormalities were subjected to a one way analysis of variance using statistical Package for Social Sciences (SPSS version 23, 2015).

RESULTS AND DISCUSSION

Effects of Semen Preservation on Sperm Motility, Live, Dead and Normal Sperm

The effects of preservation on sperm motility, live, dead and normal sperm is presented in Table 3. Sperm motility is the most often used criterion for semen evaluation both before and after preservation (Gill *et al.*, 1996; Barram *et al*, 2004). In the present study, coconut water extended with ringer solution at 2% glycerol at 0-7% concentration showed no significance difference ($P<0.05$). These results are consistent with the finding of Kulakaiz *et al.,* (2013) who reported that sperm preservation with 5% glycerol concentration showed better sperm motility than those preserved with 0%, 3%, 7% and 9% glycerol concentration. On the other hand, 4% glycerol concentration with coconut water showed a decrease in motility over time. This is consistent with the report that preservation results in some adverse effects on sperm motility (Watson 2000). The percentage rate of life sper m in the present study were significant ($P<0.05$) at 0%, 4%, 6% and 7% coconut water extended with ringer solution at 2% glycerol than other concentration of coconut water in the experiment. These finding was in agreement with the finding of sonmez and Semirei (2004) who reported that higher concentration of glycerol negatively affect semen quality in chickens. In this study dead sperm was significantly higher ($p<0.05$) at 2%, 3%, and 5% coconut water concentration than other level of concentration. Total normal sperm was significantly high ($P<0.05$) at 0% coconut water concentration extended with ringer solution at 2% glycerol than other level of coconut water concentration. This is an indication that coconut water extender does not have the ability to keep sperm cells alive for a long time probably because the energy might have been lost while lake (1999). Also turkey spermatozoa rapidly lost viability and fertilizing capacity when stored with an extender at physiological temperature Leighton *et al* 1982. Viability of spermatozoa is based on the type of extender and the concentration of extender used Yamashiro *et al* (2010). Ringer"s lactate egg yolk extender maintained the viability of spermatozoa better than the coconut water extender with Ringer solution. Ringer Solution contains physiologic concentration of sodium chloride, potassium, Calcium and lactate Albert *et al* (2009). Ringer lactate solution has potassium and calcium at concentrations that are similar to the ionized concentration found in normal blood plasma das Neves *et al* (2019). According to fujita *et al* (2020) the ions in Ringer's solution prevent cell death, and helps in maintaining cell viability. Rashid and Qistina (2015) reported the same as this study that Ringer extender was the best compared with coconut water evaluated in both percentage of live sperm and abnormality of sperm.

Table 3: Motility, Live, Dead, Normal Sperm Count Assessments

Concentration of coconut water (%)	Motility	Live	Dead	Normal
0	140.67	286.33	13.67	275.00
2	132.67	272.33	27.67	263.00
3	138.33	283.00	17.00	263.00
4	139.00	289.00	11.00	273.00
5	139.00	285.00	15.00	264.00
6	140.67	289.33	10.67	266.67

Concentration of coconut water (%)	Motility	Live	Dead	Normal
7	140.67	292.00	8.00	269.00
Mean	138.80	285.29	14.71	267.85
P-Value	0.797	0.0003	0.0003	0.0020
Least significant Difference (LSD)	N/S	S	S	S
	12.258	6.45	6.45	5.74

Effects of Cockerel Semen Preservation on Morphology of Head, Tail, Neck and Total Abnormal Sperm

The effects of Cockerel Semen Preservation on Morphology of Head, Tail, Neck and Total Abnormal Sperm is summarized in Table 4. In this study head sperm was significantly high (P<0.05) at 2%, 3%, 5% and 6% respectively in Coconut water extended with ringer solution at 2% glycerol than other concentration of coconut water in the experiment carried out. The total neck deformed sperm was significantly high (P<0.05) at 2% and 3% coconut water. The morphological total normal intact sperm head were in their acceptable range when preserved with Ringer solution than coconut water as reported by Hafez (2000). This result corroborated the findings of other investigators which reported that during the time storage of there was decrease in live and morphologically deformed sperm and increase in neck deformed spermatozoa. (Lukaszewiez 1988, Blesbois *et al* 1999) these results showed that, coconut water, Ringer Solution and glycerol extender had adverse effect on sperm viability. In this study tail of sperm was significantly high (P<0.05) at 2%, 3%, 5% and 6% respectively for one hour storage. (Tijjani *et al*, 2015) and cardose *et al*, 2003) reported that there was a significant increase in the abnormality at the sperm tail in the 4% glycerol concentration group rather than 2% or other percent groups at 12 hours storage than Using glycerol at 2% which does not have more effect on sperm storage than using 4% glycerol which had a more adverse effect for 12 hours storage.

The total abnormal sperm was significantly high (P<0.05) at 2%, 3%, 5%, 6% and 7% respectively with coconut water extended with ringer solution at 2% glycerol than other level of coconut water concentration. In the present study the overall morphologicaly l abnormal Sperm was lower in the diluents concentration of 0% and 4% the morphological l abnormality was more pronounced in the sperm neck than both the tail and the head region of all groups. This results were consistent with previous finding reported by Baran (2004) and Tijjani *et al* (2015). This indicate that preservation has effect on cockreal sperm membrane damage and possibly leading to low fertility after freezing.

Table 4: Sperm Morphology Assessments

Concentration of coconut water (%)	Head	Neck	Tail	Total AB
0	2.333	15.333	17.333	25.000
2	5.000	23.667	27.667	37.000
3	4.667	20.667	24.667	36.667
4	4.667	18.333	21.333	26.667
5	5.333	19.333	26.667	35.333
6	5.333	19.333	24.333	34.000
7	5.333	19.333	20.000	31.667
Mean	4.380	19.428	23.142	32.333
P-Value	0.0181	0.0380	0.0044	0.0028
Least significant Difference (LSD)	S	S	S	S
	1.751	4.306	4.863	5.969

CONCLUSION

Cockerel semen preservation with coconut water using glycerol and Ringers solution as extenders generally had an adverse effect on sperm membrane and possibly leading to low fertility after freezing. Morphological abnormality was most pronounced in the neck than in the head and tail regions

RECOMMENDATIONS

Coconut water preservation of cockerel semen using glycerol and ringer solution may not be recommended. Further research using other preservation methods /reagents is recommended

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