**Cytoprotective effects of natural honey on spermatozoa quality of extended breeder cock semen**

**ABSTRACT**

Quality and fertilizing potential of spermatozoa is indispensable for successful artificial insemination in poultry farms. Thus, the present study aimed to investigate the cytoprotective effect of natural honey on spermatozoa quality of extended breeder cock semen. The experiments comprises of three (3) treatments with four (4) replicates at three (3) different hours (0, 3, and 6). T1 (100% normal saline + 0% honey) represented the positive control in the ratio 1:4 semen-extender, T2 (normal saline + antibiotics g/100 mL + 0% honey), served as the negative control, while T3 contained antibiotics g/100 mL + semen at the same ratio but supplemented with 4.0 µg/L of 10% honey and stored at 4°C. The samples were evaluated at 0, 3, and 6 hours for pH, normal spermatozoa (NS) (%), bacterial load, liveability (%), lipid peroxidation (LP), and acrosome integrity (AcI) in a completely randomized design. Data obtained were analyzed and results showed that the pH values of extended semen at 0, 3 and 6 hours were not significantly (p > 0.05) different, except at 0 hour. However, the mean values were within the accepted range. 10% natural honey inclusion in extender for semen at T3 over 6 hours gave significantly (p < 0.05) better spermatozoa liveability and NS, but AcI were statistically similar (p < 0.05). There were no significant differences (p > 0.05) in the bacterial load in all groups at the 6 hours. This study suggested that 10% natural honey extender has significant positive effects on spermatozoa quality in extended breeder cock semen.

**Keywords:** natural honey; spermatozoa; fertilising potential; semen quality; semen extension.

**INTRODUCTION**

“The significance of artificial insemination (AI) in poultry reproduction has led to several studies on ideal conditions for liquid (short-term) and frozen (long-term) semen storage. The poultry industry is one of the agriculture subsector’s fastest expanding sectors in Nigeria. It contributes 9 to10% of the agricultural Gross Domestic Product (GDP)” (Folajinmi *et al*., 2020). However, “one of the problems limiting poultry production in Nigeria is the low reproductive rate” (Udeh *et al.,* 2011). “It is no doubt that artificial insemination in poultry can be more beneficial when compared to natural mating and can be adopted to minimize reproductive problems in poultry. Artificial Insemination (AI) is one of the biotechnologies that play a crucial role in the poultry sector, as the improvement of this sector will support food security” (Bekele *et al.,* 2022). “There is always a need to extend the lifespan of spermatozoa for the purpose of research and experimentation. However, availability and affordability of standard cell freezing reagents and extenders, especially in developing countries, have been very challenging” (Agbor *et al.,* 2022). “Poultry semen has low volume per ejaculation but is very concentrated, containing about 6 billion spermatozoa per milliliter of semen” (Donoghue and Wishart, 2000).

Spermatozoa output and semen characteristics are affected by the accumulation of excess reactive oxygen species (ROS). “Polyunsaturated fatty acids present in poultry spermatozoa are extremely susceptible to lipid peroxidation, generating a lot of ROS, which are harmful to sperm cells” (Ahsan *et al.,* 2014; Surai, 2016). “Spermatozoa physiology requires the presence of ROS in minimal quantity for proper functioning of acrosome reactions, capacitation, and fertilization. However, excessive generation of ROS, such as witnessed under heat stress conditions, portends danger to the membrane of spermatozoa” (Khan *et al.,* 2012). “A good semen extender will provide an energy source for the spermatozoa, like the natural medium of the semen; the seminal plasma will maintain conducive osmolarity and pH levels. Numerous extenders have been recommended for poultry semen; therefore, choosing the right extender is an important prerequisite of processing semen for AI” (Peterson *et* *al.,* 2007; Ogbu *et al.,* 2014). “The difficulty of freezing chicken spermatozoa was due to specific reproductive physiology and high variability from species and breeds/strains” (Rakha *et al.,* 2016). The uses of synthetic antioxidants have been reported to be toxic and contain carcinogens, and this has led to the shift in focus to the use of natural antioxidants (Sanchez-Escalante *et al.,* 2003). Due to increasing interest in the use of artificial insemination as a reproductive technique to meet the high demand of animal protein in the tropics, it is important to evaluate the effectiveness of natural antioxidants that can be sourced from readily available materials such as honey on spermatozoa quality.

“Honey is composed of approximately 82.4% of total carbohydrates (38.5% fructose, 31.0% glucose, and 12.9% from carbohydrates consisting of maltose, sucrose, and other sugars)” (Khan *et al.,* 2007; Vallianou *et al.,* 2014). “It has an increasing effect on the levels of antioxidants, iron, and rare elements in blood” (Theunissen *et al.,* 2001). Abd-El Aal *et al.* (2007) showed that “honey had an inhibitory effect on gram negative bacteria in comparison to commonly used antimicrobial agents”. Honey contains active ingredients that have demonstrated antioxidant properties with the potential to preserve or maintain spermatozoa integrity over a period.

El-Sheshtawy *et al.* (2014) mentioned that “the addition of 10% honey solution to cattle bull semen extender improved sperm motility in chilled and frozen semen and improved the conception rate”. Although there are numerous studies about chicken semen extension (Al-Daraji, 2012a, 2012b; Ogbu *et al.,* 2014; Rashid and Qistina, 2015; Balogun *et al.,* 2016, 2017; Okoro *et al.,* 2016; Oluwatoba *et al.,* 2017; Freitas *et al.,* 2018; Daramola *et al.,* 2019; Ezzat *et al*., 2019). Furthermore, previous works (Malik *et al*., 2019; Arif *et al*., 2023; Hidayat *et al*., 2023) on the effect of the addition of honey to semen extender on the semen quality of breeder’s cock. However, there is paucity of information on the effect of the addition of honey to semen extender on the semen quality of Nigerian local breeder cocks. Therefore, this research was conducted to evaluate the effect of adding natural honey on spermatozoa quality characteristics of extended breeder cock semen.

**MATERIALS AND METHODS**

**Experimental Site**

The study was carried out at the Poultry Unit of the Teaching and Research Farm University of Ibadan, Ibadan, Nigeria. Semen collection was done at the Poultry Unit of the Teaching and Research Farm, University of Ibadan (7° 20’N, 3° 50’E; 200 - 300 above sea level), while the analyses of semen were carried out at the Animal Physiology and Bioclimatology Laboratories of the Department of Animal Science, University of Ibadan, Ibadan, Nigeria.

**Management of Experimental Animals and Semen Collection**

Six (6) Nigerian local breeder cocks of 28 weeks were used for semen collection. The cocks were kept under intensive management and maintained under a uniform feeding regime with concentrate feed and water given *ad libitum*. Cocks were well fed to enhance their response to semen collection using the abdominal massage method.

**Experimental Treatment Layout and Design**

Aliquot portions of diluted semen were allotted to three treatments (T1, T2, and T3) with four replicates (n = 12) per treatment in a completely randomized design (CRD). Evaluations of semen quality, lipid peroxidation, and acrosome integrity were carried out at time intervals of 0, 3, and 6 hours.

**Honey preparation and Treatment Description**

Honey used for the study was purchased from the University of Ibadan farm, Ibadan, Nigeria. 10 mL of honey was measured with the syringe and weighed on a weighing balance. Thereafter, it was transferred to a beaker and diluted with 100 ml of distilled water in a ratio of 1:10 (v:v). Then, 10 % of honey is equal to 10 ml of honey in 100 ml of distilled water. Dextrose saline (5% dextrose in 0.9% normal saline; Unique Pharmaceuticals, Nigeria) base diluent was procured from a reputable pharmacy in Ibadan, Nigeria.

**Treatment description:**

Treatment 1: Normal saline

Treatment 2: Normal saline + Antibiotics (streptomycin+ penicillin ) in standard concentration of 500 µg/mL and 500 IU/mL, respectively

Treatment 3: Antibiotics + semen +10% natural honey

**Semen Collection, Processing, Extension and Evaluation**

Before the commencement of semen sampling, the cocks were trained for a period of two weeks for semen collection. Semen sampling by the abdominal massage method took place when the birds were 28 weeks of age. This was collected three times a week for two weeks consecutively into labeled Eppendorf tubes by 08.00 h. Laboratory examination of the semen was done immediately. Semen samples collected were immediately evaluated for mass activity. The volume of collected ejaculate was estimated by weighing on a top loader balance. Semen aliquots were diluted in each of te experimental extenders (treatments). Diluted semen was preserved in a thermoregulated refrigerator at 4 °C and evaluated for spermatozoa liveability, normal spermatozoa, semen pH, acrosome integrity and bacterial load at 0, 3, and 6 hours.

**Semen Quality Evaluation**

**pH**

The pH of the extended semen treatments was measured using a calibrated digital pH meter (Mettler Toledo IP67®). The unit was calibrated using a 2-point calibration procedure utilizing buffers with pH of 4.0, 7.0, and 9.20 values.

**Spermatozoa Liveability**

A drop of the extended semen was placed on a warm microscope slide with a micropipette, and a drop of eosin-nigrosin stain was added, smeared, immediately air-dried, and viewed under the microscope at magnification of X400. The proportions of live (eosin-impermeable) and dead (eosin-permeable) spermatozoa in a sample were assessed on the basis of 100 cells counted and expressed in percent.

**Morphological Evaluation**

Normal spermatozoa are sperm with an intact head, mid-piece, and tail. It was measured by adding a drop of extended semen sample on a glass slide. Thereafter, a drop of eosin-nigrosin stain was added and mixed gently and smeared on a slide with the edge of another clean slide, air dried, and viewed under the microscope at a magnification of X400. From each smear, a total of 100 spermatozoa was examined. All spermatozoa with intact head, mid-piece, and tail were, tallied and expressed in %. On the other hand, abnormal sperm have head or tail defects, such as a large or misshapen head or a crooked or double tail.

**Bacteria Load Counts**

The agar dilution method described by Olutiola *et al.,* (1991) was employed; the test semen was aseptically streaked on nutrient agar plates in triplicates and incubated aerobically at 37 °C for 24 hours. This was repeated for all treatments. Bacterial colonies were carefully picked and purified by repeated sub-cultures on nutrient agar plates, and the mean count for triplicate culture was recorded as the bacterial count in the sample (Ikuomola and Eniola, 2010). The result was expressed as cfu/mL (APHA, 1992).

**Lipid Peroxidation**

Lipid peroxidation level of seminal plasma was measured by determining the malondialdehyde (MDA) production, using thiobarbituric acid (TBA) as per the method of Buege and Aust (1978) and modified by Suleiman *et al.* (1996). Lipid peroxide levels were measured in seminal plasma after the addition of 2 mL of TBA-TCA reagent (15% w/v TCA, 0.375% w/v TBA, and 0.25 N HCL) to 1 mL of seminal plasma suspension. The mixture was treated in a boiling water bath for 30 minutes. After cooling, the suspension was centrifuged at 3000 rpm for 10 min. The supernatant was then separated, and absorbance was measured at 535 nm using spectrophotometer (Cecil CE 7200 spectrophotometer, Cecil Instrument Limited, Milton Technical Centre, England).

**Acrosome Integrity**

Ejaculate smears of all samples were prepared for confirmation of acrosome integrity condition utilizing eosin-nigrosin stain. All the slides were assessed under oil immersion at x1000 magnification using a bright field microscope. A total of one hundred spermatozoa per slide were assessed, and normal acrosomes (spermatozoa without apical shifts) were expressed as percentages.

**Statistical Analyses**

Results were subjected to statistical analysis using the analysis of variance procedure of statistical analysis software (SAS, 2016). The treatment means are presented with group standard errors of means and, where significant, were compared using the Duncan procedure of the same software.

**RESULTS & DISCUSSION**

**RESULTS**

**Effects of Natural Honey on Spermatozoa Quality of Extended Breeder Cock Semen**

The effects of natural honey on the quality of extended breeder cock semen are presented in Tables 1 - 3. Table 1 showed that there were significant differences (p < 0.05) in the pH, liveability and bacterial load values. At 0 hour, T2 (7.17) and T3 (6.92) are statistically similar, but significantly (P< 0.05) different from other T1 (7.48). T1 having higher value of pH than T2 and T3. The mean values of liveability indicated that T2 recorded higher value (95.25%) than T1 (90.50%) and T3 (91.00%). The mean values for normal sperm morphology were 88.00, 91.25, and 90.00% for T1, T2, and T3, respectively were statistically similar (p > 0.05). There were no significant differences (p > 0.05) in normal sperm morphology values in the three (3) treatment groups.

**Table** **1: Effect of Natural Honey on Spermatozoa Quality of Extended Breeder Cock Semen (0 Hour)**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Parameters** | **T1** | **T2** | **T3** | **SEM** | **P-Value** |
| pH | 7.48a | 7.17b | 6.92c | 0.04 | 0.002 |
| Liveability (%) | 90.50b | 95.25a | 91.00b | 0.97 | 0.013 |
| Normal Spermatozoa (%) | 88.00 | 91.25 | 90.00 | 0.95 | 0.103 |
| Bacterial Load (× 10-6 cfu/mL) | 0.65b | 0.38b | 2.45a | 0.48 | 0.026 |

abc Mean values in the same row with different superscript are significantly different (p < 0.05 SEM - standard error of the mean; P - probability.

At 3 hours, T1 (6.67), T2 (6.69), and T3 (6.67) are not significantly (P> 0.05) different from each other, with T2 presenting the highest pH while the least pH was obtained in T1 and T3 (Table 2). The mean spermatozoa liveability was ranged from 86.75% to 91.25%, however, the recorded difference was not significant among the treatment groups (p > 0.05). There were no significant differences (p > 0.05) in normal sperm morphology values in the three (3) treatment groups. However, the mean values for bacteria load show significant difference (p < 0.05) among the treatment groups where T3 recorded highest bacterial load (4.03× 10-6 cfu/mL)) and lowest (0.85× 10-6 cfu/mL)) by T2.

**Table 2: Effect of Natural Honey on Spermatozoa Quality of Extended Breeder Cock Semen (3 Hours)**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Parameters** | **T1** | **T2** | **T3** | **SEM** | **P-Value** |
| pH | 6.67 | 6.69 | 6.67 | 0.05 | 0.967 |
| Liveability (%) | 89.75 | 91.25 | 86.75 | 1.14 | 0.060 |
| Normal Spermatozoa (%) | 89.25 | 90.00 | 90.50 | 1.22 | 0.773 |
| Bacterial Load (× 10-6 cfu/mL) | 1.60b | 0.85c | 4.03a | 0.64 | 0.010 |

abcMean values in the same row with different superscript are significantly different (p < 0.05). SEM - standard error of the mean; P – probability.

In Table 3, results show that there were no significant differences (P > 0.05) in the mean values of pH and bacteria load at 6 hours. However, normal spermatozoa morphology and spermatozoa liveability indicated significant differences (P < 0.05) among the treatment groups. T3 recorded highest spermatozoa liveability (86.75%) compared to T1 and T2 with 63.50% and 73.50% liveability, respectively. Similarly, T3 recorded highest normal spermatozoa morphology (88.50%) in contrast with 72.25% and 77.75% normal spermatozoa morphology, respectively. The same pattern was observed at 0 and 3 hours of extension, for bacterial load.

**Table 3: Effect of Natural Honey on Spermatozoa Quality of Extended Breeder Cock Semen (6 Hours)**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Parameters** | **T1** | **T2** | **T3** | **SEM** | **P-Value** |
| pH | 6.53 | 6.56 | 6.65 | 0.05 | 0.183 |
| Liveability (%) | 63.50c | 73.50b | 86.75a | 1.14 | 0.004 |
| Normal Spermatozoa (%) | 72.25c | 77.75b | 88.50a | 2.00 | 0.001 |
| Bacterial Load (× 10-6 cfu/mL) | 0.35 | 0.18 | 0.45 | 0.11 | 0.262 |

abcMean values in the same row with different superscript are significantly different (p < 0.05). SEM - standard error of the mean; P - probability.

**Effects of Natural Honey on lipid peroxidation and acrosome integrity of Extended Breeder Cock Semen**

Tables 4 to 6 show the results of the effects of natural honey’s effect on lipid peroxidation and acrosome integrity in extended breeder cock semen. The highest mean value of 7.47 was observed in the T2 group for lipid peroxidation, which is significantly (p < 0.05) higher than the values of 4.20 and 2.32 observed in the T1 and T3 groups, respectively, at 0 hours (Table 4). However, similar (p > 0.05) mean values were observed for acrosome integrity across treatments. The mean values for acrosome integrity ranged from 92.50 to 95.00%.

**Table 4:** **Effect of Natural Honey on Lipid Peroxidation and Acrosome Integrity of Extended Breeder Cock Semen (0 Hour)**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Parameters** | **T1** | **T2** | **T3** | **SEM** | **P-Value** |
| Lipid peroxidation (mM MDA/l0-4) | 2.23c | 7.47a | 4.02 b | 0.51 | 0.001 |
| Acrosome integrity (%) | 92.50 | 95.00 | 94.50 | 1.16 | 0.316 |

abcMean values in the same row with different superscript are significantly different (p < 0.05). SEM - standard error of the mean; P - probability.

Results of lipid peroxidation at 3 hours of extension shows that there were significant differences (p < 0.05) among treatments (Table 5). Similar trends were observed as at 0 hours. Mean acrosome integrity values presented in percent ranged from 92.50 to 94.00. However, there were no significant differences (p > 0.05) in acrosome integrity across treatments.

**Table 5: Effect of Natural Honey on Lipid Peroxidation and Acrosome Integrity of Extended Breeder Cock Semen (3 Hours)**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Parameters** | **T1** | **T2** | **T3** | **SEM** | **P-Value** |
| Lipid peroxidation (mM MDA/l0-4) | 1.76c | 2.75b | 3.22a | 0.15 | 0.001 |
| Acrosome integrity (%) | 92.50 | 93.25 | 94.00 | 1.13 | 0.656 |

abcMean values in the same row with different superscript are significantly different (p < 0.05). SEM - standard error of the mean; P - probability.

Table 6 revealed that the highest lipid peroxidation value of 6.46 was obtained in the T3 group, which is significantly (p < 0.05) higher than the values of 3.91 and 2.05 observed in the T2 and T1 treatment groups, respectively, at 6 hours. In addition, the differences in mean values for acrosome integrity were significant (p < 0.05), where the highest value of 91.00% was observed in the T3 group, followed by 87.00% and 83.75% in the T2 and T3 groups, respectively.

**Table 6: Effect of Natural Honey on** **Lipid Peroxidation and Acrosome Integrity of Extended Breeder Cock Semen (6 Hours)**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Parameters** | **T1** | **T2** | **T3** | **SEM** | **P-Value** |
| Lipid peroxidation (mM MDA/l0-4) | 2.05c | 3.91b | 6.46a | 0.19 | 0.001 |
| Acrosome integrity (%) | 83.75c | 87.00b | 91.00a | 1.50 | 0.023 |

abcMean values in the same row with different superscript are significantly different (p < 0.05). SEM - standard error of the mean; P - probability.

**DISCUSSION**

**Cytoprotective Effects of Natural Honey on Spermatozoa Quality of Extended Breeder Cock Semen**

There were fluctuations in the mean values of spermatozoa liveability across the treatments. Spermatozoa liveability was lower in T1, but all the mean values obtained fall within the normal range of quality semen. Hayanti *et al.* (2022) and Badwy *et al*. (2024) reported that the inclusion of an antioxidant (alpha-tocopherol) into the diluent prevents the significant decline in viability of chicken spermatozoa, and this could explain the observation of high mean values of live cells, compared to dead cells. The result from this study indicates natura honey had no effect on the liveability of extended breeder cock semen. Lipid peroxidation results indicate that natural honey did not improve the antioxidant status of extended breeder cock semen. The findings of this study are not consistent with the results of Aljady *et al.* (2000), who reported the antioxidant and antibacterial effects of honey. Shipley *et al.* (1999) reported that percentage normal spermatozoa should be at least 70%. Except for T1 and T2 at 6 hours, other mean values obtained gave abnormal spermatozoa of less than 15%. This observation agrees with the findings of Johnson *et al.* (2000) and John *et al.* (2003), that “high quality semen contains a minimal number (5 to 15%) of abnormal spermatozoa, whereas low quality semen frequently contains a large number (30% or more) of abnormal spermatozoa”. “Morphological abnormalities of spermatozoa can have an adverse effect on fertilization and embryonic development” (Saccke, 2008). Results observed suggested that natural honey could potentially maintain intact spermatozoa morphology at 6 hours of storage and this could be attributed to the presence of the phenolic, and other bioactive properties of honey (Yaman *et al*., 2022). A high percentage of normal spermatozoa above 70% is also an indication of reduced microbial activities on the sperm cells. However, there was no significant effect during 0 and 4 hours of storage.

There was a reduction in the pH values obtained across the treatments, though the values obtained still fall within the recommended range. Intracellular pH reduction obviously reduced spermatozoa metabolism and increased spermatozoa survival during storage (Johnson *et al.,* 2000). The importance of pH on survival of sperm cells cannot be undermined because there are some sources of biochemical alterations on spermatozoa, which include increased concentration of solute, dehydration, and changes in temperature (Johnson *et al.,* 2000). The results obtained in this study indicate that natural honey does not have negative effects except at 0 hour on the pH of extended cock semen, refrigerated at 4 °C.

**Cytoprotective Effect of Natural Honey on Quality of Extended Breeder Cock Semen**

Acrosome integrity is an important parameter useful in predicting the fertilizing capacity of spermatozoa *in vitro.* From this study, it was observed that acrosome integrity was intact across the treatments from 0 to 6 hours of observation, this is an indication that natural honey has the capacity to maintain the integrity of the acrosome. However, acrosome integrity declines with storage time at 4 °C. The beneficial effects of reduced glutathione were evaluated in different studies; however, the results were positive and negative (Masoudi *et al*., 2019). Our finding reported negative effects of reduced gluthathione which could lower acrosome integrity, impairing the function and fertilizing capacity of chicken spermatozoa. However, other studies (Masoudi *et al*., 2019; Zuha *et al*., 2024) have reported positive effects of reduced gluthathione.

These results were in line with the findings of Waberski *et al.* (2011), who reported that storage of semen for a longer period causes ultrastructural, biochemical, and function damage to the spermatozoa, resulting in a decline in motility, viability, fertility, and impaired acrosome integrity and transport. Also, reduction in acrosome integrity will reduce acrosome reaction of greater a portion of spermatozoa before reaching the site of fertilization (Waberski *et al.,* 2011). This finding indicates that honey inclusion at 10 % competes favourably in preservation of the acrosome integrity.

The improvement in acrosome integrity can be attributed to the enhanced sperm quality resulting from honey supplementation. The increased motility, viability, and reduced oxidative stress in the treatment group contribute to improved sperm functionality and increased chances of successful fertilization. The findings of this study have significant implications for the optimization of artificial insemination protocols in poultry breeding. Sperm viability is crucial for successful fertilization, as only live and functional sperm cells have the capacity to penetrate the egg and fertilize it. This can lead to improvements in the efficiency and effectiveness of poultry breeding programmes, ultimately benefiting the poultry industry.

The preservation of sperm viability can be attributed to the osmotic properties of honey. Additionally, the high osmolality of honey helps maintain the osmotic balance, preventing cellular swelling or shrinkage and preserving sperm cell morphology and functionality. Main cause of sperm chromatin damage is oxidative stress, leading to lipid peroxidation, which can damage sperm cells (Partyka *et al*., 2012; 2023). Antioxidants play a crucial role in mitigating this damage by scavenging free radicals and reducing lipid peroxidation. This study has shown that adding natural antioxidants like honey to the semen as extender can improve sperm quality and viability by minimizing lipid peroxidation as well as enhancing the antioxidant capacity of the semen.

**CONCLUSION**

The results of this study showed that 10 % natural honey extender has significant positive effects on spermatozoa quality in extended breeder cock semen. It is therefore recommended that further studies be conducted to ascertain the mechanism by which natural honey protects the spermatozoa quality in extended breeder cock semen.

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