INFLUENCE OF BUTYLATED HYDROXYTOLUENE IN THREE EXTENDERS ON OXIDATIVE STRESS PARAMETERS OF CHILLED SEMEN OF RED SOKOTO BUCKS

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ABSTRACT

The aim of this study was to evaluate the influence of butylated hydroxytoluene (BHT) in triscoconut water (TCW), tris egg-yolk (TEY), and citrate egg-yolk (CEY) on oxidative stress of chilled red Sokoto bucks' semen. Semen was collected from 10 bucks weekly using a batteryoperated electro-ejaculator. Ten ejaculates collected once weekly from 10 bucks were pooled together and analyzed. This was repeated for 8 weeks. The semen was extended with TEY, CEY, and TCW each containing BHT at 0, 0.5, 1.0, 1.5, and 2.0 mM/mL BHT concentrations, and transferred into a refrigerator for storage at 4 oC. Malondialdehyde (MDA) concentration and superoxide dismutase (SOD) activities were determined at 0, 24, 48 and 72 hours. After 72 hours, semen extended in CEY, and the 1.0 mM/mL BHT group had the lowest MDA concentration (3.57 – 0.16 nMol/mL). The SOD activity in semen extended with CEY was lowest in the 2.0 mM/mL BHT group. Semen extended in TEY with 0.5 mM/mL BHT group had the lowest activity of SOD after 72hrs. MDA profile for semen extended in TCW with the addition of 0.5 mM/mL BHT after 72 hours was 2.66 – 0.25 nMol/mL, which was significantly ($P < 0.05$) lower than values recorded in other groups except the 1.0 mM/mL BHT group. From this study, Red Sokoto bucks' semen can be extended in CEY + 1.0 mM/mL BHT, TEY + 1.0 mM/ml BHT and TCW + 0.5mM/ml BHT and chilled for 72 hours for reduced MDA concentrations and maintained SOD activity to keep buck semen under cold storage.

Keywords: ***Semen extenders, Bucks, Antioxidant, Reactive Oxygen Species***

INTRODUCTION

Genetic improvement of goats requires the selection of superior breeding stock and the application of semen preservation and insemination techniques (Bitto and Egbunike, 2006). Semen collection, dilution, and preservation are processes needed before artificial insemination (AI) can be performed; when poorly done, it negatively impacts semen quality (Allai *et al.*, 2018).

Extended semen quality can be affected by oxidants like reactive oxygen species (ROS), which are produced in living cells during respiration, abnormal or dead sperm, and phagocytic cells (Asadpour *et al.*, 2010). During the process of semen storage, lipid peroxidation occurs due to oxidative stress (Solihati *et al.*, 2018), which correlates with exposure of spermatozoa to ROS (Alvarez and Storey, 1992).

Seminal plasma contains natural antioxidants like superoxide dismutase (SOD) whose concentration decreases due to semen dilution using an extender and increased oxidative stress during storage affects semen quality (Marzony *et al.*, 2016). Oxidative stress cannot be totally prevented, but certain measures such as the addition of antioxidants

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such as butylated hydroxytoluene (BHT) which is a phenolic analogue of vitamin E can help ameliorate the effect on sperm cells (Thangamani *et al.*, 2018).

The Red Sokoto goat is one of the predominant indigenous goat breeds in Nigeria (Fabusoro *et al.*, 2007). The high amount of polyunsaturated fatty acids in spermatozoa (plasma membrane) makes them very susceptible to oxidative stress, which influences the quality and fertility potential of spermatozoa (Motemani *et al.*, 2017). Various additives are used to provide antioxidant protection during semen extension to improve stored semen quality, which protects the sperm plasma membrane from destabilization during cooling (Nitin *et al.*, 2018).

There is a paucity of information on the effect of BHT and its combination with different extenders on MDA production which is a pointer to lipid peroxidation levels and activities of SOD in semen of Red Sokoto bucks after 3 days of cool storage at 4 °C. Therefore, this study was designed to investigate the effects of various concentrations of butylated hydroxytoluene in three semen extenders (Tris-Coconut water, Tris-egg-yolk, and Citrate egg yolk) on oxidative stress biomarkers in the semen of Red Sokoto Bucks after chilling for.

MATERIALS AND METHODS

Bucks and Management Ten (10) healthy and sexually matured Red Sokoto bucks from the Small Ruminant Research Programme of the National Animal Production Research Institute weighing an average of 25.5 – 2.5 kg and aged 2.0 – 1.5 years were selected for the study. The bucks were screened for diseases and internal parasites before the study commenced. All bucks were maintained under uniform management conditions using a semiintensive system of management, fed ad libitum with hay, and supplemented with commercial small ruminant concentrate at a rate of 2% body weight/head/day.

Semen Collection

A total of 10 ejaculates were collected once weekly from 10 bucks using an electroejaculator. The probe of the electroejaculator was lubricated with petroleum jelly for easy insertion into the rectum and pushed forward slowly. A series of short electrical stimulations (5 V and 100 mA) were applied intermittently for approximately 2-5 seconds by push of a button until erection and ejaculation were achieved. Ejaculates were placed in a water bath at 37 °C before microscopic evaluations were conducted. Precautions were taken not to exert long electrical stimulations so as not to hurt the buck. The semen samples collected were kept at 37ºC and taken to the laboratory immediately for analysis. To reconstitute the extenders, buffers were first prepared. The tris (hydroxymethyl aminomethane) and citrate buffers (g/100 mL in distilled water) buffers were prepared to be used for TEY, CEY, and Tris-coconut (TCW) extenders respectively.

100mls of all extenders were prepared and placed in a beaker of warm water (37 ºC).

Coconut water (CW) was obtained from coconut fruits on the day of the experiment to maintain freshness. The coconut water collected from coconut fruits was first filtered through a sieve, and centrifuged for 5 minutes at 3000 rpm and the supernatant obtained was carefully separated into a sterile glass bottle as described by Baldaniya *et al.* (2019) and used as an extender. Considering BHT molarity (220), 3.4g of BHT white crystals were dissolved in 15.6 mL of absolute ethanol (99%) to make 1 molar solution. This solution was kept as stock which was then used subsequently to obtain the desired concentration for every treatment. For more elaboration, 15.60 L of the stock contains 1 mM of BHT. This was dissolved in 2 ml of diluted semen sample to obtain 0.5 mM/mL of BHT concentration.

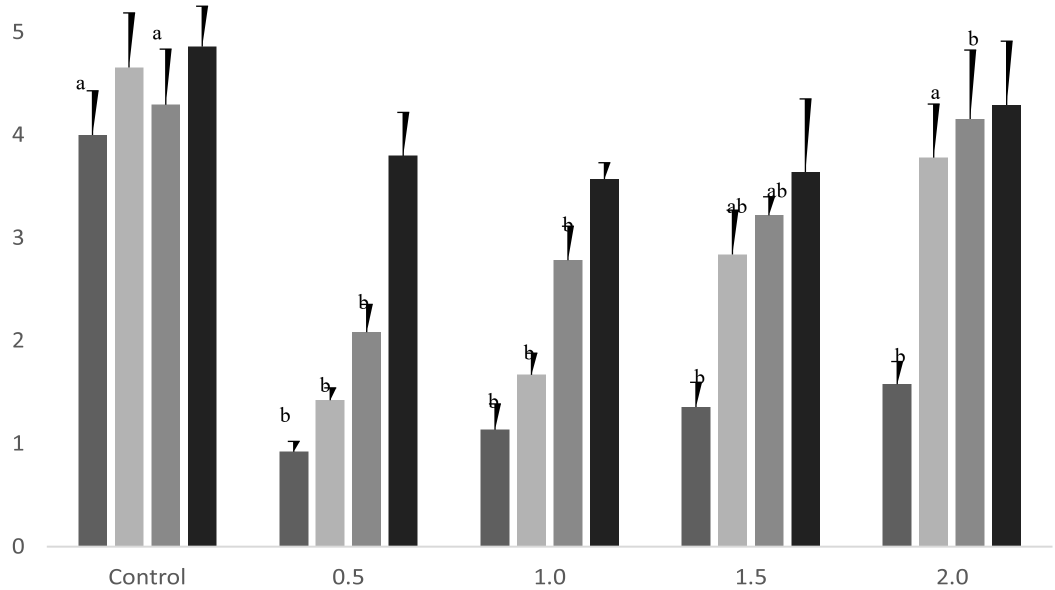
However, the stock solution and diluted sample were not directly mixed together because the ethanol contained in the stock solution is detrimental to spermatozoa. Therefore, the desired quantity of the stock was put into a clean empty cryovials first, left opened at room temperature to allow the ethanol to evaporate leaving the dried BHT crystals to attach to the cryovials before 2mls of diluted semen sample was added to the test tube which was adequate to make up the desired concentration. Semen samples were diluted with all extenders: Tris-egg yolk, Tris coconut water, and Citrate egg-yolk extenders in pre-warmed test tubes, containing BHT antioxidants at 0, 0.5, 1.0, 1.5, and 2.0 mM/mL to obtained 15 extended semen samples. The samples were packed into labeled Bijou bottles placed in a padded flask and then transferred into a refrigerator at 4°C for storage.

Table 1: Compositions of Tris, Citrate egg yolk, and Tris coconut extender (in 100 mL)

Components	TEY	CEY	TCW
Tris (hydroxymethyl amino-methane) (g/100 mL)	2.9	-	2.9
Sodium citrate, dehydrate (g/100 mL)	-	2.9	-
Citric acid monohydrate (g/100 mL)	1.7	-	1.7
Fructose (g/100 mL)	1.25	-	1.25
Egg yolk (% v/v) mL	20	20	-
Coconut water (% v/v) mL	-	-	20
Penicillin 10,000 units, streptomycin 10 g /mL	1000	1000	1000

TEY- Tris egg-yolk; CEY- Citrate egg-yolk; TCW- Tris coconut-water

Malondialdehyde (MDA) and SOD activities were determined at 0, 24, 48 and 72 hours of storage. Seminal MDA levels were analyzed according to the method described by Spirlandeli *et al.* (2014) and SOD was analyzed using the technique of Barranco *et al.* (2019).



Data Analyses
Data collected were expressed as means and standard error of the mean (– SEM). The significance of differences between treatment means was estimated at P < 0.05 with the Tukey-Kramer multiple comparison test of repeated measure analysis of variance (ANOVA). Statistical analysis was conducted using the GraphPad Instat Computer Programme (GraphPad for Windows, Inc., version 8.0.2 of 2019).

RESULTS

Malondialdehyde concentration in semen extended in Tris-egg-yolk with different levels of BHT stored at 4°C for 72 hours. Figure 1 shows the Malondialdehyde (MDA) concentration in semen extended in tris egg yolk with different concentrations of BHT and stored for 72 hours at 4°C. The MDA concentrations in semen after 24 hours of storage had a range of 1.42 – 0.12 to 4.65 – 0.53 nMol/mL (Fig 1). The lowest concentration was observed in semen stored with 0.5 mM/mL of BHT. This value was significantly (P < 0.05) lower than those observed in the control group and semen supplemented with 1.5 and 2.0 mM/mL BHT (Figure 1). Semen stored without BHT supplementation had a significantly (P < 0.05) higher MDA concentration than other groups 4.65 – 0.53 nMol/mL (Fig 1).

There was no significant difference observed in MDA concentrations between semen stored in the 0.5 mM/mL BHT group and the 1.0 mM/mL BHT group. (Fig 1). After 48 hours of chilling, the mean MDA

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concentration for the control group (4.25 – 0.54 nMol/mL), was significantly ($P < 0.05$) higher than that for the 0.5 mM/mL BHT group. At 48 hours of chilling, the mean MDA concentration in the control group was significantly ($P < 0.05$) higher than the values obtained for the 1.0 mM/mL BHT group. After 72 hours of storage, semen with 1.0 mM/mL BHT had the lowest MDA concentration (3.57 – 0.16 nMol/mL). This value was significantly ($P < 0.05$) lower than that of the control group. The MDA concentrations were similar among the groups.

Figure 1: Malondialdehyde concentration in chilled semen extended in Tris-egg-yolk with different concentrations of BHT stored for 72 hours

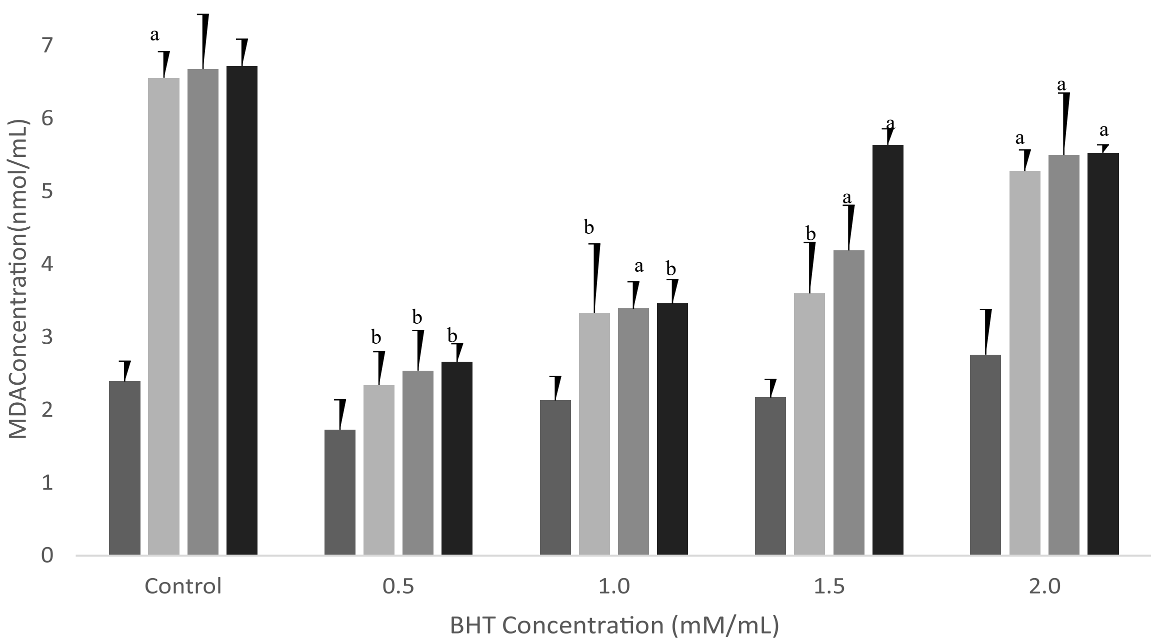
abc = Different superscript between same hours denotes differences ($P < 0.05$), Data expressed as Means – SEM, BHT: Butylated hydroxytoluene

Malondialdehyde concentration in chilled semen extended in Tris-coconut with different concentrations of BHT stored for 72 hours

Mean MDA concentration for semen extended in tris coconut ranged from 1.73 – 0.46 – 2.66 – 0.11 nMol/mL, with the semen extended with 0.5 mM/mL of BHT having the lowest mean concentration at 24 hours of chilling (Fig. 2). The MDA concentration in control group was significantly (P < 0.05) higher than other groups, except the 2.0 mM/mL BHT group (Fig. 2). There were no

addition of 1.5 and 2.0 mM/mL of BHT did

Figure 2: Malondialdehyde concentration in chilled semen extended in Tris-coconut with different concentrations of BHT stored for 72 hours



significant differences in mean MDA concentrations between semen extended with

addition of 0.5, 1.0 and 1.5 mM/mL BHT after 24 hours of chilling (Fig 2). The MDA concentration semen extended with the

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not significantly differ (Fig 2). At 48 hours of chilling, mean MDA concentration increased ranging from 2.54 – 0.75 – 6.66 – 0.62 nMol/mL. MDA values for the 0.5mM/mL BHT group were significantly (P < 0.05) lower compared with the control and 2.0 mM/mL BHT group. Semen extended in triscoconut and chilled for 72 hours revealed mean MDA concentration with the highest

■ 0 hrs ■ 24 hrs ■ 48 hrs ■ 72 hrs

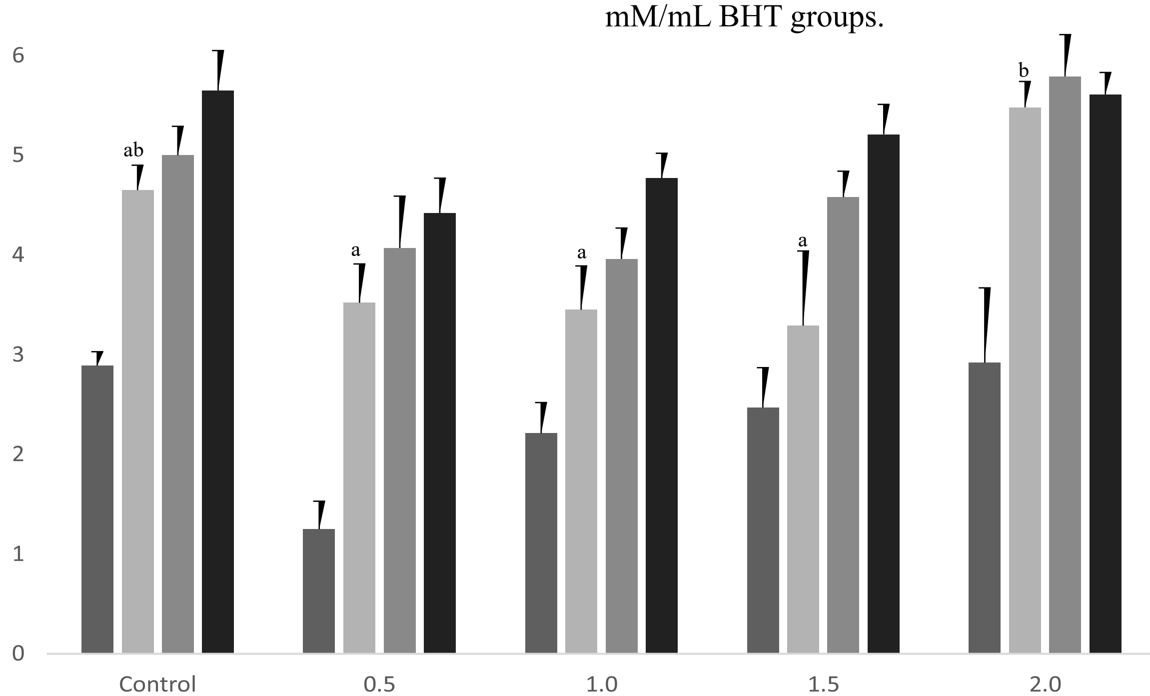
mM/mL BHT group (Fig 2).

abc = Different superscript between same hours denotes differences (P < 0.05), Data expressed as Means – SEM

value in the control group (6.72 – 0.37 nMol/mL), which was significantly (P < 0.05) higher than those observed in 0.5 and 1.0 mM/mL BHT groups (Fig 2). The semen extended with the addition of 0.5 mM/mL BHT had a mean concentration of MDA (2.66 – 0.25 nMol/mL) which was significantly (P < 0.05) lower than that recorded in every other group, except the 1.0

Malondialdehyde concentration in chilled semen extended in Citrate egg yolk with different concentrations of BHT stored for 72 hours

Figure 3 shows the mean MDA concentrations in semen extended in egg yolk citrate with different concentrations of BHT. After 24 hours of chilling, the 2.0 mM/mL BHT group had the highest mean concentration of MDA with a value of 5.48 – 0.26 nmol/mL (Fig 3). This was significantly (P < 0.05) higher than the concentrations of MDA in all other groups, except the control group. The 1.5 mM/mL BHT group had the lowest mean MDA value of 3.29 – 0.75 nmol/mL (Fig 3). No significant differences in MDA values were observed in all groups, but after 72 hours of chilling, the control group had the highest mean MDA concentration (5.65 – 0.40 nmol/mL) (Fig 3). The lowest mean MDA concentration (4.42 – 0.26 nmol/mL) was obtained in the 0.5 mM/mL BHT group (Fig 3). There was no significant difference in the MDA concentrations recorded for 0.5 and 1.0 mM/mL BHT groups.



BHT Concentration (mM/mL) 0

hrs 24 hrs 48 hrs 72 hrs

Figure 3: Malondialdehyde concentration in chilled semen extended in Citrate egg yolk with different concentrations of BHT stored for 72 hours

abc = Different superscript between same hours denotes differences ($P < 0.05$), Data expressed as Means – SEM

Superoxide Dismutase concentration in chilled semen extended in Tris egg-yolk with different concentrations of BHT stored for 72 hours

Fig 4 shows mean SOD activity in semen extended in Tris egg yolk. After 24 hours of chilling, the highest mean activity of SOD was observed in the 0.5 mM/mL BHT group (4.38 – 1.79 U/mL) (Fig 4). The lowest mean activity was recorded in the 2.0 mM/ml BHT group, with a mean value of 3.63 – 1.07 U/mL (Fig 4). No Significant differences were recorded between groups. After 72 hours of storage, the 0.5 mM/mL BHT group had the lowest mean activity of SOD with a value of 1.81 – 0.47 U/mL (Fig 4). The highest mean SOD activity was recorded in the 1.0 mM/mL BHT group (3.56 – 0.66 U/mL) (Fig 4).

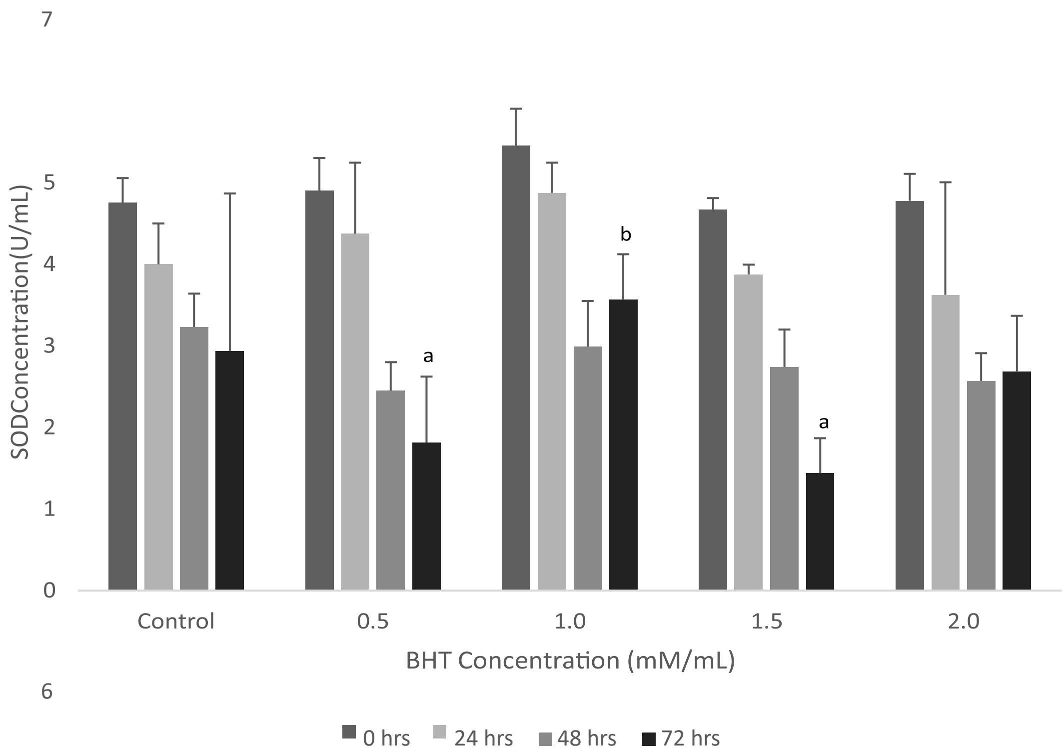


Figure 4: Superoxide dismutase concentration in chilled semen extended in Tris egg-yolk with different concentrations of Butylated hydroxytoluene stored for 72 hours

Data expressed as Means – SEM, ab = Different superscript between same hours denotes differences ($P < 0.05$)

Superoxide dismutase concentration in chilled semen extended in Tris-coconut with different concentrations of BHT stored for 72 hours

The lowest mean SOD activity recorded for semen chilled in a tris coconut extender for 24 hours was observed in the 2.0 mM/mL BHT group with a value of 2.37 – 1.96 U/mL (Fig 5). The 0.5 mM/mL BHT group had the highest activity with a value of 4.53 – 1.04 U/mL (Fig 5). After 72 hours of chilling, the mean SOD activity in semen stored in tris coconut was highest in the 0.5 mM/mL BHT group with a value of 3.77 – 1.15 U/mL (Fig 5). The difference between the 3 groups was not significant.

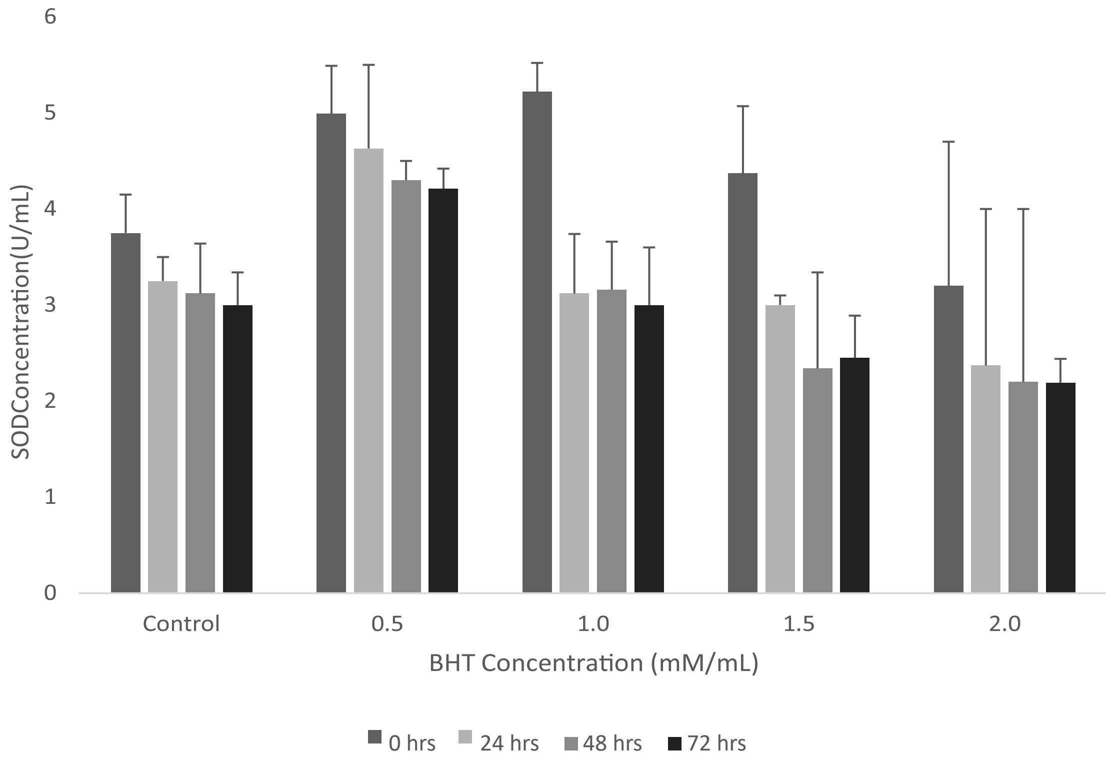


Figure 5: Superoxide dismutase concentration in chilled semen extended in Tris-coconut with different concentrations of Butylated hydroxytoluene stored for 72 hours

Data expressed as Means – SEM

Superoxide dismutase concentration in chilled semen extended in citrate egg-yolk with different concentrations of BHT stored for 72 hours

Figure 6 shows the mean SOD activity in semen chilled in citrate egg yolk for 72 hours. The activity was highest in the 0.5 mM/ml BHT group with a value of 5.29 – 1.41 U/mL at 24 hours. The lowest mean SOD activity at 24 hours was recorded in the 2.0 mM/mL BHT group with a value of 2.97 – 0.87 U/mL (Fig 6). No significant differences were recorded in mean SOD concentrations across all groups, although there were relative differences in numerical values. At 72 hours of chilling, the lowest mean SOD activity was recorded in the 2.0 mM/mL BHT group. The highest mean activity of SOD was recorded in the 1.5 mM/mL BHT group (4.05 – 1.29 U/mL) (Fig 6), there were no significant differences (p > 0.05).

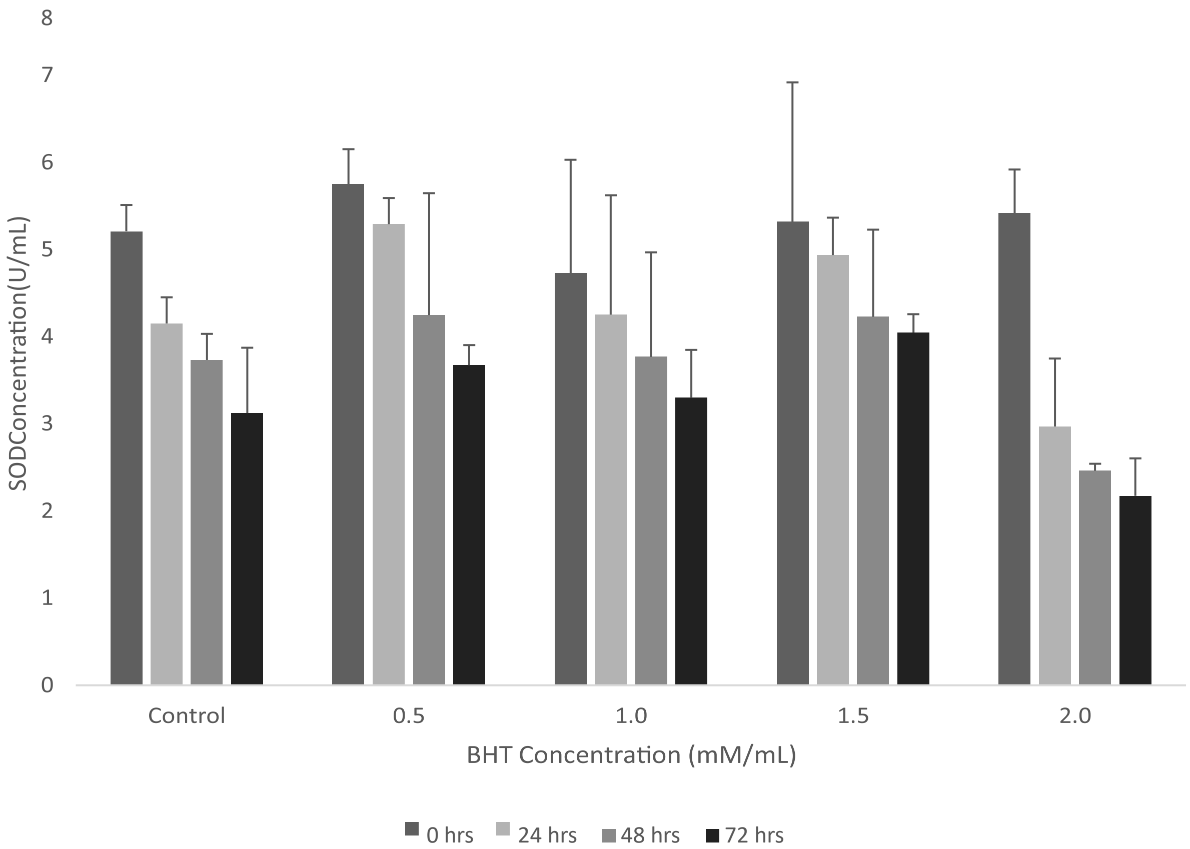


Figure 6: Superoxide dismutase concentration in chilled semen extended in Citrate egg-yolk with different concentrations of Butylated hydroxytoluene stored for 72 hours

Data expressed as Means – SEM

DISCUSSION

Reactive oxygen species (ROS) generated at homeostatic levels are significant in regulating sperm physiological functions such as sperm capacitation, acrosome reaction, hyperactivation, and sperm-oocyte fusion (Patel *et al.*, 2015). However, not all the ROS are beneficial to the sperms, when they are exposed to higher levels of ROS, which induce oxidative stress in the sperms. As a consequence, substantial damage to the sperms, involving plasma membrane damage, leakage, and increased permeability, leads to reduced sperm motility, metabolic activity, longevity, and viability (Patel *et al.*, 2015).

The findings in this study also lay credence to the fact that damage to sperm by ROS generated in storage and cryopreservation processes may be minimized by the addition of antioxidants in extender, which reduces oxidative stress, improves sperm motility and membrane integrity, and decreases lipid peroxidation of sperm (Merino *et al.*, 2020).

Values of Malondialdehyde (MDA) recorded in this study after 72 hours at 4°C revealed higher values than findings reported by Susilowati *et al.* (2019) in Etawa goats. This difference could be a result of variations in breeds and semen extenders used for semen storage (Fathi *et al.*, 2019).

In West African Dwarf goats, Daramola *et al.* (2016) reported that the levels of tocopherol had no beneficial effect on the sperm cells and oxidative stress parameters, which is in contrast with findings from our study as BHT is an analogue of tocopherol. This could imply that a phenolic antioxidant (such as tocopherol) in excessive concentration will lose its effectiveness as an antioxidant and may even form a pro-oxidant (Wahjuningsih and Rachmawati, 2012). Therefore, this change from antioxidant to pro-oxidant function or conversion to free radicals causes more unsaturated fatty acids as a result of lipid degradation (Wahjuningsih and Rachmawati, 2012). This process further accelerates and expands the incidence of lipid peroxidation of sperm plasma membrane damage due to the loss of some essential unsaturated fatty acids making up the membrane (Nur *et al.*, 2005).

The MDA production in the present study increased in all extenders as the preservation hours increased. This result is similar to a report by Perumal (2014), where semen chilled for 30 hours had an MDA concentration of 2.5 mmol/108 compared to 0.8 mmol/108 recorded after 1 hour. This could be because as the storage time progressed, lipid peroxidation occurred as a result of oxidation of the membrane lipid. This may ultimately give rise to an increase in the MDA values, observed as storage hours increased.

The MDA values across the extenders revealed the lowest concentration in the TCW extender. This might result as other extenders contained egg yolk, which has a higher amount of low-density lipoprotein that allows sperm to be susceptible to lipid peroxidation (Kothari *et al.*, 2010). This might contribute to greater MDA production observed in tris egg yolk and egg yolk citrate extenders (Tarig *et al.*, 2017). In the present study, apart from the control group, MDA production was highest in all semen extenders in samples with 1.5 and 2.0 mM/mL BHT, corroborating the report by Khumran *et al.* (2015) in bulls that MDA production was highest in samples with 2 and 3 mM/mL BHT. This may occur because excess antioxidant concentration increases the fluidity of the sperm membrane beyond the normal level becoming more toxic to plasma membrane integrity (Khumran *et al.*, 2015). The result is an increase in injury to sperm cells due to toxicity and loss of the physiologic roles of ROS; thus, the increase in MDA production adversely affects semen quality (Khumran *et al.*, 2015).

The result of this study is different from the finding of Pagl *et al.*, (2005) where there was no increase in MDA values in semen of stallion stored for 24 hours. This difference might be because of the different preservation methods employed and the species involved. The trend of increase in MDA production was reported by Nair *et al.* (2006) in the semen of cattle and buffaloes.

The semen samples with 0.5-1.0 mM/mL BHT had lower production of MDA compared to the control group regardless of the extender used and this corroborates the report by Atessahin *et al.* (2008) that MDA levels decline in goat's semen preserved with taurine as an antioxidant. The result is also similar to a report by Esalami *et al.* (2016), which showed a decrease in the concentration of MDA in rooster semen extended with oleic acid as an antioxidant.

The decrease in SOD activities in this study as the storage period progressed was similar to the report by Partyka *et al.* (2012) that the enzyme activity reduced during storage of Minorca rooster's semen. A similar pattern was reported by Bilodeau *et al.* (2000) in bovine semen, which obtained a decrease in SOD activity. During semen storage, the decrease in enzyme activity can be attributed to the leakage of intracellular enzymes due to membrane damage. In the present study, membrane damage might have caused the very low intracellular enzyme activity observed in some groups (Ancuelo *et al.*, 2021).

The enzymatic activity observed in this study revealed higher activities in groups with 0.5 to 1.5 mM/mL BHT, irrespective of the extender type used, and is in consonance with a report where rooster semen as preserved with an antioxidant as palmitoleate (Rad *et al.*, 2015). This could mean BHT at these concentrations sustained SOD activity which has been positively related to total and progressive motile spermatozoa, which is in turn related to in vivo fertility (Barranco *et al.*, 2019). It has also been reported that semen showing the highest SOD activity could also exhibit higher fertility (Barranco *et al.*, 2019). In another study by Papas *et al.* (2019) in equine, semen with a higher specific activity of SOD is positively correlated with sperm ability to withstand cryopreservation.

SOD activity in this present study, decreased across all extenders as preservation time increased, and this might be due to the fact that excessive generation of ROS molecules can compromise the antioxidant system in seminal plasma. The activities of SOD which revealed a decrease as storage time increased is similar to the finding of Nair *et al.* (2006) in semen of bulls and buffaloes. This could occur because the levels of antioxidants decrease during the preservation process, which is due to the excessive generation of ROS molecules (Perumal *et al.*, 2013).

From this study, Red Sokoto bucks' semen can be extended in CEY + 1.0 mM/ml BHT, TEY + 1.0 mM/ml BHT and TCW + 0.5mM/ml BHT and chilled for 72 hours for reduced MDA concentrations and maintained SOD activity.

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