INFLUENCE OF METFORMIN ON GROWTH DYNAMICS, SEMEN QUALITY, AND BLOOD TESTOSTERONE LEVELS OF RABBIT BUCKS REARED IN HOT TROPICAL CONDITION OF SOUTHEASTERN NIGERIA

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Abstract. Heat stress is one of the environmental factors that affect rabbit health and reproduction. Therefore, inexpensive methods to enhance sperm production and functions in heat-stressed rabbits have gained attention. One of these methods involved the use of metformin, that has proven antioxidant properties, to mitigate the negative consequences of heat-induced oxidative stress in rabbits reared in hot tropical conditions of Nigeria was investigated. Thirty-six rabbit bucks aged 10 to 12 months were divided into 3 groups (CD1, CD2, and CD0), with each group having 12 rabbits with three replications in a completely randomized design (CRD). Rabbits in the CD1 group were given metformin at 12.5 mg/kg body weight (BW) once daily for 5 days, while those in the CD2 group received metformin at 12.5 mg/kg BW twice daily for 5 days. Rabbits in the CD0 group served as the control and received normal saline for the same period. Semen and blood samples were collected from one rabbit in each replicate at the end of the experiment and analyzed statistically. Results revealed that feed intake, feed conversion ratio (FCR), and body weight gain (BWG) of rabbits exposed to heat stress were not affected by metformin administration. However, sperm motility and active motile cells were enhanced by metformin in the CD2 group. Likewise, sperm with abnormal morphology (big head, headless, pin head, and bent neck) were significantly lower in the CD1 and CD2 than in the CD0 group. In contrast, rabbits in the CD0 group produced sperm with shorter tails than those in the CD1 and CD2 groups. Results also showed significantly increased serum testosterone in the CD1 and CD2 than in the CD0 group. It is concluded that metformin at 12.5 mg/kg BW twice daily for 5 days improved serum testosterone, sperm motility, and active motile sperm cells and reduced the production of sperm with abnormal morphology in rabbits reared in hot tropical conditions of Nigeria.
Keywords: *rabbits, heat stress, weight gain, sex hormone, sperm motility, abnormal morphology*

Introduction

Rabbits are an important source of quality animal protein in many parts of the world (El-Deep et al., 2020, 2021). Studies have demonstrated that rabbits possess high growth rates and high prolificacy. Several aspects of the physiology of rabbits have been investigated (Abdelnour et al., 2020; Amber et al., 2021). Measurable criteria such as blood indices and semen quality have been evaluated in rabbits reared in hot tropical environments (Iwuji et al., 2020; Jimoh et al., 2021). However, changes in environmental temperatures have been found to have negative impacts on these measurable criteria in rabbits, resulting in poor feed intake, weight gain, immune responses, and semen quality (Rashamol et al., 2020; Amber et al., 2021). Heat stress is an important environmental factor that affects animal health and productivity (El-Deep et al., 2019; Abdelnour et al., 2020). Rabbits are susceptible to heat stress due to their low thermoregulation capacity (El-Ratel et al., 2020; El-Sabrout, 2020), which in turn

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affects nutrient metabolism, resulting in poor growth performance and reproduction (Hassan et al., 2021). According to Marai et al. (2002) and Jimoh et al. (2021), rabbits tend to perform optimally in terms of improved reproduction and physiological traits when the temperature-humidity index (THI) is less than 27.8°C. However, during the summer, the THI rises above 30 (severe heat stress), thereby affecting the ability of rabbits to regulate their body temperature (Abdelnour et al., 2020) and sperm production (Jimoh and Ewuola, 2018). The impaired reproductive functions in rabbits may be due to oxidative damage caused by heat-induced stress. Thus, it is vital to improve the welfare of rabbits under heat-stress conditions as a measure for enhancing reproductive efficiency (Marai et al., 2001; Jimoh et al., 2018).

Therefore, there is a need to enhance reproductive efficiency in rabbits using inexpensive preparations with proven antioxidant properties. Metformin, one of such inexpensive preparations, is a biguanide-derivative antidiabetic drug demonstrated to have antioxidant activity against oxidative damage caused by reactive oxygen species (ROS), as reported by Cahova et al. (2015) and Buczyńska et al. (2024). Despite its antioxidant properties, the use of metformin to enhance spermatogenesis and function may also be linked to its positive effect on the 5' adenosine monophosphate (AMP)-activated protein kinase (AMPK) pathway (Ferrannini, 2014; Bertoldo et al., 2015). Currently, there is a scarcity of published data on the impact of metformin on the productivity of rabbits reared in hot tropical conditions. This study, therefore assessed the positive effect of metformin administration on growth performance and reproductive outcomes of rabbit bucks reared in hot tropical conditions of southeastern Nigeria.

Materials and methods

The mean temperature, relative humidity (RH), and THI inside the rabbit house were 28.2°C ± 3.2°C, 75.4 ± 2.6%, and 30.4, respectively. The THI was calculated using the method of Marai et al. (2002). The THI of 28.2°C ± 3.2°C in the present study suggests that rabbits were exposed to moderate heat stress (Marai et al., 2002; Jimoh et al., 2021). Rabbit handling and treatments adhered to the institution guidelines for the care and use of animal models. Metformin (SKG, Pharm Limited Nigeria) was procured from a reputable pharmaceutical store in Owerri, Imo State, Nigeria.

Thirty-six (36) matured male rabbits aged 10-12 months were used for the experiment. The rabbits were divided into three treatment groups (CD1, CD2, and CD0), having 12 rabbits with three replications each. The rabbits were randomly assigned to three treatment groups in a completely randomized design (CRD). Metformin solution was obtained by dissolving 500 mg of the tablet in 100 mL of distilled water to obtain 5 mg/ml. Rabbits in the CD1 treatment group received 2.5 mL of the solution containing 12.5 mg metformin/kg BW once daily for 5 days, whereas those in the CD2 treatment group received 2.5 mL of the solution containing 12.5 mg metformin/kg BW twice daily (morning and night) for 5 days. The CD0 treatment group served as the control and received distilled water for 5 days. The decision of split dosage of metformin was informed by the fact that the best daily aqueous volume of metformin rabbits can tolerate is yet to be determined. In addition, the choice of metformin dose in this study was based on the value earlier reported for rabbits (Yasin et al., 2022).

The rabbits were individually housed in standard cages fitted with drinkers and feeders under standard husbandry practices. The rabbits were maintained in a pelleted

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diet (*Table 1*) and clean water ad libitum. The rabbits were acclimatized for 7 days before administering the metformin. The fact that the experiment lasted for eight weeks, even though the animals received metformin for 5 days, was to allow the rabbits to complete one spermatogenic cycle so as to ascertain the actual effect of spermatozoa production and quality. All experimental protocols were observed under strict supervision and metformin administration was carried out orally.

Rabbits were weighed at the onset of the experiment and weekly thereafter using a digital scale (Model: iScale i-01); values were recorded in grams. The BWG was determined by subtracting the initial live weights from the final live weights. Feed intake was recorded as the quantity of feed consumed by each rabbit after deducting the leftover feed from the amount served the previous day. The FCR was computed by dividing the feed intake by the BWG of the rabbit.

Semen was collected from one rabbit buck per replicate at the end of the 8th week of the study using the artificial vagina (AV) and a teaser doe as described by Iwuji et al. (2020) into calibrated collection tubes. The volume of the ejaculate was measured by reading the semen level off the calibrated collection tubes and recorded in millimeters. The color of the ejaculate was observed visually immediately after collection as described by Obafemi et al. (2021). The pH of the semen was measured immediately after collection using pH paper (Bbr Chemocraft, India). One each of the paper was inserted into the semen for 5 s and removed to air dry. The color change observed after drying was matched to the color chart on the pH paper.

Table 1. *Ingredient and nutrient composition of experimental diet*

Ingredient	%	Nutrient analysis	%
Maize	34.7	Dry matter	88.54
Brewer dried grain	50.8	Protein	18.85
Soybean meal (44% CP)	7.82	Fiber	13.02
Fish meal	2.93	Fat	2.86
Limestone	1.75	Lysine $^{++}$	0.81
Bone meal	1.00	Methionine^{++}	0.43
Premix^{+}	0.25	Ash	6.59
Sodium chloride	0.50	Calcium $^{++}$	0.64
DL-Methionine	0.25	Phosphorus^{++}	0.81
Total	100.00		

$^{+}$Each 3 kg of the premix contains: cholecalciferol 2200000 IU, retinol 12000000 IU, choline chloride 500 gm, tocopherol 10000 mg, thiamine 1000 mg, niacin 20000 mg, phylloquinone 2000 mg, biotin 50 mg, riboflavin 4000 mg, cobalamin10 mg, selenium 100 mg, pyridoxine 1500 mg, vitamin B5 10000 mg, folate 1000 mg, manganese 55000 mg, iodine 1000 mg, and zinc 50000 mg. $^{++}$Computed via the method of Blas and Mateos (1998)

The corresponding pH number for the color on the chart was recorded as the pH of the sample. Sperm motility was determined by using a micropipette to place a drop of semen on a pre-warmed microscopic slide kept at 37°C with a cover slide and viewed under a microscope at x400 magnification. Sperm motility and morphology were estimated as percentages according to the procedures of Iyeghe-Erakpotobor et al. (2023). Spermatozoa concentrations expressed as the number of sperm cells in 1 ml of semen counts were determined on semen diluted with physiological saline solutions

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(PSS) using the improved Neubauer hemocytometer slide following the procedures of Iyeghe-Erakpotobor et al. (2023).

At the end of the 8th week of the experiment, three rabbit bucks (one from each replicate) were randomly selected. About 2 mL of blood samples were drawn from the marginal ear vein of the selected rabbits into plain tubes without ethylenediaminetetraacetic acid (EDTA) as described by Iwuji et al. (2018). The blood samples were analyzed for testosterone concentration as described by an enzyme-linked immunosorbent assay (ELISA) kit Monobind Inc.

Data obtained from the experiment was subjected to analysis of variance (SPSS, version 25) procedures. Results were expressed as means, standard deviation (SD), and standard of error of the mean (SEM). Significant means were separated using the Tukey test. Differences were considered significant at $p < 0.05$. The statistical model is $Y_{ijk} = \mu + Ti + e_{ijk}$, where Y_{ijk} = growth performance, serum testosterone, and semen quality traits in the kth rabbit; μ = overall mean for each variable; Ti is the fixed effect of ith metformin at 2 doses and normal saline as a control; and e_{ijk} is the random residual effect.

Results

The data on the performance indices of heat-stressed rabbits administered metformin are presented in *Table 2*. There were no significant differences in initial and final live weights among the treatment groups ($p > 0.05$). However, the control rabbits (CD0) had a numerically ($p > 0.05$) higher BWG than the CDI and CD2 rabbits. Results also showed that metformin administration did not affect feed intake and FCR in heat-stressed rabbits.

Table 2. *Growth performance characteristics of heat-stressed rabbits on metformin administration*

Parameters (g/rabbit)	Metformin (mg/kg BW)			SD	SEM	p-val
	CD0	CD1	CD2			
Initial weight	2333.30	2366.70	2363.30	83.7	51.2	0.246
Final weight	3326.70	3300.00	3300.00	45.9	82.0	0.102
Body weight gain	993.40	933.30	936.70	31.0	25.0.	0.053
Feed intake	3622.00	3612.00	3610.00	89.0	59.0	0.082
Feed conversion ratio	3.65	3.64	3.84	1.30	0.43	0.074

[a,b]Means within a row with different letter superscripts differ significantly ($p < 0.05$). *SD* standard deviation, *SEM* standard error mean; *CD1* received 12.5 mg of metformin/kg BW once daily for 5 days; *CD2* received 12.5 mg of metformin/kg BW twice daily for 5 days; *CD0* received normal saline during the same period and served as the control group

The data on the semen quality outcomes of rabbits on metformin administration as presented in *Table 3* suggest that semen color was whitish across the treatment groups. Semen volume and non-motile sperm were numerically higher in the CD1 and CD2 groups than in the CD0, however, the observed differences were not significant ($p > 0.05$). In contrast, semen pH, sperm concentration, and sluggish motile sperm were numerically higher in the CD0 group than in the CD1 and CD2 groups, although the recorded differences were not statistically significant ($p > 0.05$). The results suggest a

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significant increase in sperm motility ($p < 0.05$) and active motile sperm ($p < 0.05$) in the CD2 group compared to the CD0 group. However, the results showed that rabbits in CD0 and CD1 had similar ($p > 0.05$) sperm motility and active motile sperm.

Table 3. *Semen quality characteristics of heat stressed rabbits on metformin administration*

Variables (%)	Metformin (mg/kg BW)			SD	SEM	p-val
	CD0	CD1	CD2			
Semen color	Milky	Milky	Milky	-	-	-
Semen volume (ml)	0.46	0.65	0.55	0.48	0.06	0.450
Semen pH	7.67	7.50	7.50	1.46	0.16	0.910
Sperm concentration ($\times 10^6$/ml)	1.86	1.88	1.91	0.80	1.27	0.530
Motility sperm (%)	75.00^{b}	88.00^{ab}	95.33^{a}	12.35	4.12	0.012
Active motile (%)	63.33^{b}	76.00^{ab}	84.00^{a}	12.86	4.29	0.036
Sluggish motile sperm (%)	12.67	12.00	11.33	4.03	1.34	0.825
Non-motile sperm (%)	15.23	17.67	16.97	10.99	3.66	0.215

^{a,b}Means within a row with different superscripts differ significantly ($p < 0.05$). *SD* standard deviation, *SEM* standard error mean; *CD1* received 12.5 mg of metformin/kg BW once daily for 5 days; *CD2* received 12.5 mg of metformin/kg BW twice daily for 5 days; *CD0* received normal saline during the same period and served as the control group

The abnormal sperm morphology of heat-stressed rabbits on metformin is displayed in *Table 4*. Metformin reduced the production of sperm with big head ($p < 0.05$), headless ($p < 0.05$), pin head ($p < 0.05$), and bent neck ($p < 0.05$), in the CD1 and CD2 groups compared to the CD0 group. In contrast, metformin did not affect headless sperm, double head sperm, sperm with coiled tail, and pus cells. Rabbit bucks exposed to heat stress on metformin-treated groups (CD1 and CD2) presented sperm with significantly higher ($p < 0.05$) long tails than the CD0 group. Results also indicate that metformin increased ($p < 0.05$) serum testosterone levels in rabbits exposed to heat stress when compared to the control group (CD0), as presented in *Figure 1*.

Table 4. *Abnormal sperm morphology and serum testosterone level of heat stressed rabbits on metformin*

Variables (%)	Metformin (mg/kg BW)			SD	SEM	P value
	CD0	CD1	CD2			
Big head	5.67^{a}	3.00^{b}	3.50^{b}	1.29	0.43	0.021
Headless	4.52^{a}	3.30^{b}	3.32^{b}	0.45	0.15	0.018
Double head	2.33	2.17	2.13	0.53	0.18	0.730
Pin head	6.33^{a}	5.10^{b}	5.20^{b}	0.86	0.12	0.031
Bent neck	5.33^{a}	4.54^{b}	4.20^{b}	0.46	0.16	0.015
Coiled tail	3.67	3.50	3.00	0.53	0.18	0.513
Long tail	4.00^{b}	6.50^{a}	6.00^{a}	1.73	0.58	0.001
Pus cells	3.57	3.18	3.48	0.88	0.29	0.168

^{a,b}Means within a row with different letter superscripts differ significantly ($p < 0.05$). *SD* standard deviation, *SEM* standard error mean; *CD1* received 12.5 mg of metformin/kg BW once daily for 5 days; *CD2* received 12.5 mg of metformin/kg BW twice daily for 5 days; *CD0* received normal saline during the same period and served as the control group

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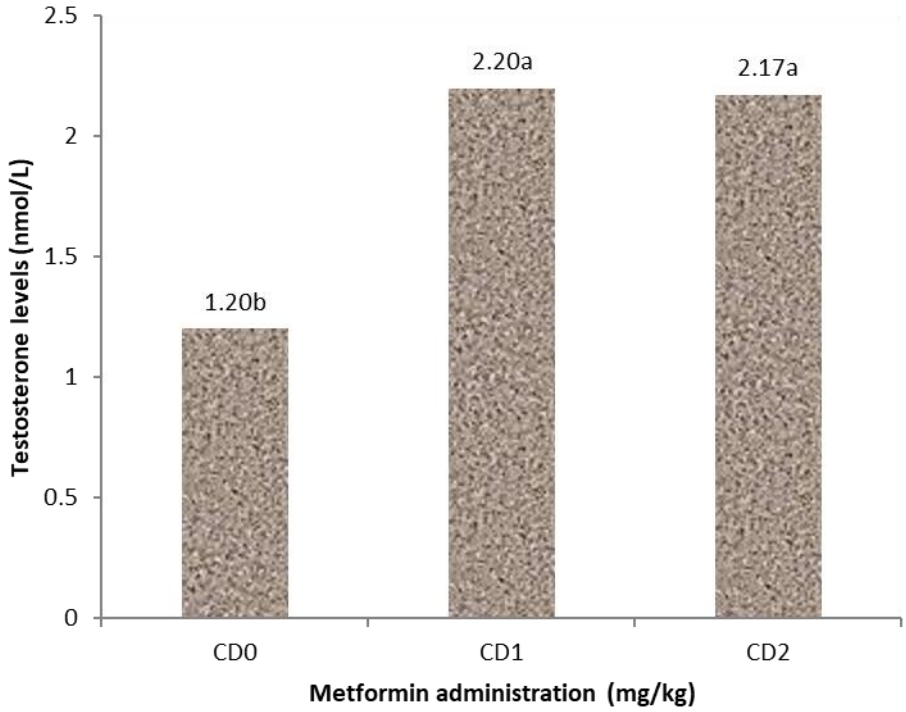

Figure 1. *Impact of metformin on serum testosterone levels of rabbits exposed to heat stress. Bars with different superscripts are significant at $p < 0.05$*

Discussion

The negative consequence of heat stress on the health and physiological functions of rabbits is primarily due to oxidative stress (El-Ratel et al., 2020; Jimoh et al., 2021). In the current investigation, the THI of 28.2°C ± 3.2°C is deemed a moderate heat stress condition for rabbits (Marai et al., 2002; Jimoh et al., 2021). This study demonstrated that metformin had no negative impacts on the growth performance indices of heat-stressed rabbits. However, the numerically reduced feed intake and BWG of rabbits in metformin-treated groups could be related to the appetite-lowering effect of metformin by enhancing the levels of the appetite-suppressing metabolite, Lac-Phe (N-lactoyl-phenylalanine) and improving insulin sensitivity (Scott et al., 2024). The inability of rabbits in metformin-treated groups to gain weight in the present study is of good development, since overweight animals tend to produce semen with impaired motility, reduced sperm concentration, and a high incidence of abnormal morphology (Ameratunga et al., 2023).

Several parameters are employed to determine semen quality and functions in farm animals (Iwuji et al., 2020; Jimoh et al., 2021; Eyeghre et al., 2023). The current results showed that metformin has a positive effect on aspects of semen quality outcomes of heat-stressed rabbits. The improvement effects of metformin on semen quality traits in animals other than rabbits have also been reported (Bertoldo et al., 2014; Eyeghre et al., 2023). The lack of differences in semen color among the treatment groups implies that metformin did not affect sperm concentration in heat-stressed rabbits (Bertoldo et al., 2014). This study revealed that semen volume of heat-stressed rabbits was not affected by metformin. However, the semen volume obtained in the present study falls within the range of 0.42–0.70 mL reported for rabbits by Jimoh et al. (2021). Likewise, the

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similarity in semen pH, and other morphological parameters across the groups suggests that metformin's influence may be selective toward certain semen traits, rather than a broad-spectrum enhancement of all sperm quality metrics. This observation aligns with prior studies indicating that while certain treatments can significantly alter sperm parameters, others remain stable under varying conditions (Iwuji et al., 2020).

The significantly reduced sperm motility in control rabbits when compared to rabbits in the CD2 group, supports the findings of Attia et al. (2020) that heat-induced stress alters sperm motility in animals. Sperm motility and active motile sperm were increased in heat-stressed rabbits administered metformin at 12.5 mg/kg BW twice daily for 5 days. The increased sperm motility in this experiment agrees with the earlier findings of Eyeghre et al. (2023) that metformin (30 mg/kg) enhances sperm motility and viability in animals by increasing the activities of AMPK, an enzyme that helps maintain cellular energy balance. These results agree with the findings of Ogbuewu et al. (2020), who discovered that as sperm motility increased, the number of sperm with abnormal cells decreased. Research has also shown that oxidative stress has adverse impacts on sperm production in rabbits (Jimoh, 2020; Amber et al., 2021) and may result in alterations of sperm genetic materials and fertilization ability (Liang et al., 2022). The production of sperm with active motile cells in metformin-treated rabbits indicates the potential of metformin to protect sperm membranes against oxidative stress caused by the excessive production of ROS (Jimoh and Ewuola, 2018; Jimoh et al., 2021). These findings agreed with the results of Cahova et al. (2015) and Buczyńska et al. (2024) that metformin has antioxidant activity against oxidative damage caused by ROS. The findings of this study corroborate the results of Jimoh and Ayedun (2020) that products with antioxidant properties could provide antioxidant protection to mitigate oxidative stress induced by heat stress.

There are negative correlations between sperm abnormalities and fertility in farm animals (Bosman et al., 2014). In the present study, rabbits administered metformin at 12.5 mg/kg BW once or twice daily for 5 days produced sperm with reduced big head, headless, pin head, and bent neck compared to the control group. This suggests that metformin has a protective role in sperm production in heat-stressed rabbits. Furthermore, the higher proportion of sperm with big head, headless, pin head, and bent neck in the control group indicates impaired spermatogenesis or altered sperm maturation processes induced by oxidative damage caused by heat stress (Jimoh et al., 2018). Differences were also observed in sperm tail in rabbits, indicating the mitigating potential of metformin on heat-induced oxidative stress in rabbits. The findings regarding tail morphology are particularly critical, as the rabbits in the control group demonstrated significantly shorter tail lengths than those in the metformin-treated groups. Tail length is a crucial determinant of sperm motility and overall fertility potential. Shorter tails are often associated with impaired motility, which could detrimentally affect the sperm's ability to reach the egg and fertilization potential (Nguyen et al., 2018).

Eyeghre et al. (2023) found that metformin (30 mg/kg) administration for 4 weeks increased serum testosterone in animal models. This observation is consistent with the results of this study, which showed that metformin administration at 12.5 mg/kg BW once or twice daily for 5 days increased serum testosterone levels in rabbits. The increase in serum testosterone levels of rabbits in metformin-treated groups could be related to stimulatory effect of metformin on 3-β dehydrogenase type 2 (3βHSD2), cytochrome P450 17A1 (CYP17A1), and hydroxysteroid 17-β dehydrogenase 4 (HSD17B4), the key enzymes that regulate testosterone production in the testis.

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Conclusion

It is concluded that metformin at 12.5 mg/kg BW twice daily for 5 days improved serum testosterone, sperm motility, and active motile sperm cells and reduced the production of sperm with abnormal morphology in rabbits reared in hot tropical conditions of Nigeria. The administration of metformin 12.5 mg/kg once daily for 5 days in heat-stressed rabbits reduced the number of sperm with big head, pin head, without head, and bent neck. However, further research should be channeled toward identifying the dose levels of metformin and duration of administration that optimize growth indices, serum testosterone, and semen quality traits in rabbits as such information is missing in the literature.

Author contributions. IPO, MM, and CAM participated in the study conception and design. Data collection and analysis were performed by the CAM and IPO. The first draft of the manuscript was written by IPO and MM. In addition, CAM edited the manuscript, and all the authors read and approved the final manuscript for publication.

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