Trimethyl glycine (TMG) influences on blood biochemistry and organ indices of heat stressed growing rabbits

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Abstract

Trimethyl glycine has been supplemented in livestock diets for decades due its role as an essential osmo-protectant, protecting proteins, enzymes, inhibiting cellular apoptosis and reducing energy expenditure, under osmotic stress. A study was conducted to determine the effect of trimethyl glycine (TMG) on blood biochemistry and organ indices of heat-stressed growing rabbits. Thirty rabbits were randomly allotted to three experimental treatments. Each treatment was replicated five times with two rabbits per replicate in a completely randomized design. A one-week adjustment period was given before commencement of the feeding trial. Trimethyl glycine was supplemented at 0, 1.0 and 1.5%. Results indicated that temperature humidity index (THI) in the afternoon was higher than THI in the morning by 27.16%, indicating the absence of heat stress in the morning and the presence of very severe heat stress in the afternoon. There were significant (P<0.05) differences observed in malondialdehyde (MDA) and catalase (CAT) levels. No significant differences (P>0.0) across treatments were observed for superoxide dismutase (SOD) and glutathione peroxidase (GPx) activity. There were significant (P<0.05) differences observed in aspartate transaminase and alanine transaminase. Other parameters such as globulin, albumin, total protein, low-density lipoprotein, high-density lipoprotein, very low-density lipoprotein, total cholesterol, triglycerides and alkaline phosphatase were not significant (P>0.05). There were significant (P<0.05) differences observed in white blood cell and basophils levels. Kidney and liver weights were also significantly (P<0.05) affected. It can be concluded that TMG had positive effects on blood biochemistry and organ indices of growing heat-stressed rabbits and is recommended up to 1.5% in heat-stressed growing rabbit diet.

Keywords: Trimethyl glycine, rabbits, stress, antioxidant, serum biochemical

Influences de la triméthyl glycine (TMG) sur la biochimie sanguine et les indices d’organes de lapins en croissance stressés par la chaleur

Résumé

La triméthyl glycine est supplémentée dans l’alimentation du bétail depuis des décennies en raison de son rôle d’osmo-protecteur essentiel, protégeant les protéines, les enzymes, inhibant l’apoptose cellulaire et réduisant la dépense énergétique, sous stress osmotique. Une étude a été menée pour déterminer l’effet de la triméthyl glycine (TMG) sur la biochimie sanguine et les indices d’organes de lapins en croissance stressés par la chaleur. Trente lapins ont été répartis au hasard dans trois traitements expérimentaux. Chaque traitement a été répété cinq fois avec deux lapins par répétition dans une conception entièrement randomisée. Une période d’ajustement d’une semaine a été accordée avant le début de l’essai d’alimentation. La triméthyl glycine a été complétée à 0, 1,0 et 1,5 %. Les résultats ont indiqué que l’indice d’humidité de la température (IHT) de l’après-midi était supérieur à celui du matin de 27,16 %, indiquant l’absence de stress thermique le matin et la présence d’un stress thermique très sévère l’après-midi. Des différences significatives (P < 0,05) ont été observées dans les niveaux de malondialdéhyde (MDA) et de catalase (CAT). Aucune différence significative (P>0,0) entre les traitements n’a été observée pour l’activité de la *superoxyde dismutase (SOD) et de la glutathion peroxydase (GPx). Il y avait des différences significatives (P<0,05) observées dans l'aspartate transaminase et l'alanine transaminase. D'autres paramètres tels que la globuline, l'albumine, les protéines totales, les lipoprotéines de basse densité, les lipoprotéines de haute densité, les lipoprotéines de très basse densité, le cholestérol total, les triglycérides et la phosphatase alcaline n'étaient pas significatifs (P>0,05). Des différences significatives (P < 0,05) ont été observées dans les taux de globules blancs et de basophiles. Le poids des reins et du foie était également significativement (P<0,05) affecté. On peut conclure que le TMG a eu des effets positifs sur la biochimie sanguine et les indices d'organes des lapins en croissance stressés par la chaleur et est recommandé jusqu'à 1,5 % dans l'alimentation des lapins en croissance stressés par la chaleur.*

Mots-clés : Trimethyl glycine, lapins, stress, antioxydant, sérum biochimique

Introduction

High temperatures have been shown to directly affect the physiological status, growth performance, reproduction, and immune function of animals (He *et al*., 2018; Awad *et al*., 2020; Ahmad *et al*., 2022). In response to high temperature, animals tend to compensate by producing higher levels of reactive oxygen species (ROS) which cause oxidative stress. Oxidative stress refers to the imbalance between free radical production and the ability of the antioxidant defense system of the body to detoxify or impair oxidative damage to DNA, proteins, and lipids (Prasad and Bao, 2019). It is well known that body temperature and respiration rate of growing rabbits are increased following exposure to high (30°C) environmental temperatures (Marai *et al*., 2001). A significant increase in free radical production was reported along with an increase in the expression of antioxidant enzymes during a period of non-damaging exercise (McArdle and Jackson, 2000). These increases in antioxidant enzyme activities have been considered as protective responses to oxidative stress (Altan *et al.,* 2003).

Different physical and physiological methods have been used to alleviate the heat load in heat stressed animals. Antioxidants offer the first line of defense that inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions. They are chemical substance that limit ROS production, scavenges existing free radicals and promote the repair of cell structures damaged by ROS (Agarwal and Allamaneni, 2004). Trimethyl glycine (TMG) or Betaine (BET) is a naturally occurring compound with osmo-protective properties, which is vital in the nervous, immune, renal and cardiovascular systems (Kidd *et al*., 1997). Although oxidative stress studies with TMG using rabbit models is lacking, Wang *et al.* (2004) suggested that dietary betaine supplementation seemed to improve resistance to stress. Previous studies show that trimethyl glycine supplementation could decrease hepatic triglyceride accumulation (Pekkinen *et al.,* 2013; Xu *et al.,* 2015), improve some blood constituents of rabbit bucks raised under high ambient temperature (Hassan *et al.,* 2012) and prevent fatty liver in rats fed high-fat-diets (Zhang *et al.,* 2013; Deminice *et al.,* 2015). The present study investigated the effect of trimethyl glycine on the biochemical, immune and organ indices of heat-stressed growing rabbits.

Materials and Methods

Experimental site, diet and animal management

The experiment was conducted at the Teaching and Research farm, Animal Production and Health Department, Federal University Wukari, Taraba state in the Southern Guinea savannah zone of Nigeria. The experimental diets were standard maize and soyabean-based diets with TMG supplemented at 0, 1.0 and 1.5% (Table 1) with the 0 % diet serving as the control. The diets were formulated to meet the nutritional requirements for weaner rabbits. A total of 30 weaner rabbits were used for the study. The experimental design was a completely randomized design, consisting of three treatments, with five replicates made up of two rabbits per replicate. The animals were kept in cages equipped with feeders and drinkers. The cages had wire screen bottoms; this allowed faeces and urine to pass through.

Table 1: Ingredient composition and calculated analysis of the rabbit diets

	Trimethyl glycine (%)		
Ingredients (%)	0	1.0	1.5
Maize	46.00	46.00	46.00
Soyabean meal	25.10	25.10	25.10
Maize Offal	10.00	10.00	10.00
Rice offal	15.00	15.00	15.00
Bone meal	3.00	3.00	3.00
Table salt	0.30	0.30	0.30
Vitamin-mineral premix*	0.25	0.25	0.25
DL-Methionine	0.20	0.20	0.20
L-Lysine	0.20	0.20	0.20
Total	**100.00**	**100.00**	**100.00**
Calculated Analysis			
Metabolizable Energy, Kcal/Kg	2643	2643	2643
Crude Protein (%)	17.47	17.47	17.47
Ether Extract (%)	4.67	4.67	4.67
Crude Fibre (%)	10.00	10.00	10.00
Ca (%)	0.87	0.87	0.87
Available P (%)	0.52	0.52	0.52

*0.25 kg of premix will supply the following: Vitamin A 1500 IU, Vitamin D 300 IU, Vitamin E 3.00, Vitamin K 0.25 g, Thiamine 0.2 mg, Riboflavin 0.6 mg, Pantothenic acid 1.00 mg, Pyridoxine 0.4999 mg, Niacin 4.00 mg, Vitamin B_{12} 0.002 mg, Folic acid 0.10 mg, Biotin 0.008 mg, Choline chloride 0.05 g, Antioxidant 0.012 g, Manganese 0.0096 g, Zinc 0.0060 g, Copper 0.0006g, Iodine 0.006 g Iodine 0.00014 g, Selenium 0.024, Cobalt 0.004 mg

The rabbits were allowed a one-week adjustment period before the feeding trial commenced. The initial weights of the animals were taken before the experimental diets were introduced to the animals for 28 days. All routine and management practices were strictly adhered to.

Thermoregulatory measurement

Indoor temperature and relative humidity readings were recorded at 8.00 am and 2.00 pm daily throughout the experimental period using, an electronic digital thermo-hygrometer. Temperature-humidity index (THI) was calculated using the equation given by Marai *et al*. (2001):

$$THI = t - [\left(0.31 - 0.31\ \mathrm{X}\ (\frac{\mathrm{RH}}{100})\right)(t - 14.4)]$$

where,

THI = temperature-humidity index in °C

t = temperature

RH =relative humidity

Heat stress was classified as absence of heat stress (<27.8), moderate heat stress (27.8-28.8), severe heat stress (28.9-29.9) and very severe heat stress (>30.0) according to Marai *et al.* (2001).

Blood analyses

At day 28 of the study blood was obtained from one rabbit from each replicate by severing the jugular vein for full blood count using collection tubes containing EDTA. Whole blood was collected using evacuated tubes containing no anticoagulant and centrifuged for 15 min to harvest the serum. All samples were stored at 20°C until analysis. The serum obtained was used to assay for serum antioxidant indices including glutathione peroxidase (GPx), catalase (CAT), malondialdehyde (MDA), and superoxide dismutase (SOD) which were determined using commercial kits. The serum obtained was also used to assay for triglycerides, total cholesterol, high-density lipoprotein (HDL) concentrations, low-density lipoprotein (LDL), liver function enzymes- alanine transferase (ALT), alkaline phosphatase (ALP) and aspartate transaminase (AST). Values of very low-density lipoprotein (VLDL) were calculated using the following formula (Friedewald *et al*., 1972) as shown:

$$VLDL = \frac{Triglycerides}{5}$$

The haematological and serum analyses were determined at the Haematology Laboratory of the Faculty of Medicine, Ahmadu Bello University, Zaria.

Organ Collection and Examination

Five rabbits per treatment were randomly selected, slaughtered and eviscerated. Body weights, as well as weight of liver and kidneys were measured. Relative weights of each organ expressed as percentage of live weight was determined.

Data analysis

Data collected from the experiment were analyzed in JMP® (2012) software using to analysis of variance (ANOVA) method. Where the result of ANOVA was statistically significant, Tukey's post-hoc test for multiple comparisons was performed to compare means of all groups.

Results and Discussion

Temperature-Humidity Index

The average temperature humidity index (THI) inside the rabbit cage during the experimental period for mornings and afternoons are shown in Figure 1. Temperature humidity index (THI) in the mornings averaged 21.91 while the afternoon THI averaged 30.08. The results obtained indicated that THI in the afternoons were higher by 27.16% than THI in the mornings indicating the absence of heat stress in the mornings and the presence of very severe heat stress in the afternoons (Marai *et al*., 2001). High temperature is associated to heat stress, which can induce molecular and physiological changes in mammals (Emtenan *et al*., 2010). As a consequence of the high THI observed in this study, thus raises the body temperature of the rabbits which leads to increases in reactive oxygen substances in mitochondria leading to oxidative stress (Huang *et al*., 2015).

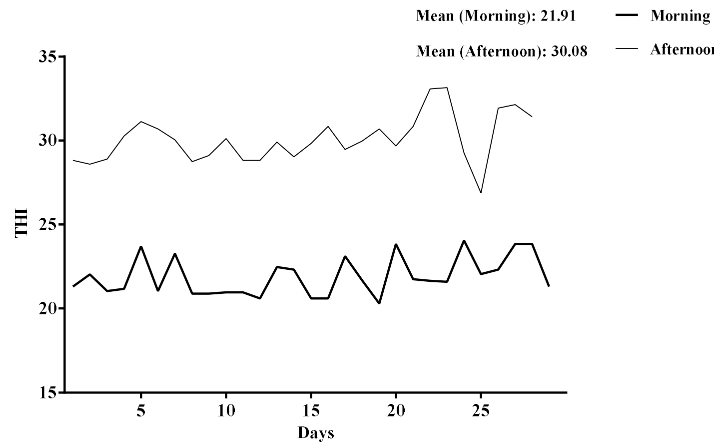

Figure 1: Daily Temperature Humidity Index

Antioxidant indices

Table 2 shows the effect of TMG on antioxidant indices of heat-stress on growing rabbits. Malondialdehyde levels ranged from 14.20 to 19.48 µmol/mg and was significant ($P<0.05$). Both diets containing TMG were similar ($P>0.05$), with rabbits receiving 1.5 % TMG having the highest ($P<0.05$) MDA levels. Numerous studies have reported that high temperature commonly increases oxidative stress by down-regulating the production of antioxidant enzymes (Jana *et al*., 2010; Tusell *et al*., 2014). Trimethyl glycine exerts its antioxidant properties by increasing the activity of antioxidases (Hoque *et al*., 2007; Alirezaei *et al*., 2015). Changes in MDA concentration have been considered as heat stress response (Altan *et al*., 2000). Malondialdehyde is one of the major aldehyde derivatives of lipid peroxidation and it is a by-product of the lipid peroxidation process (Smith *et al.,* 2005; Alirezaei *et al*., 2011). El-Moniem *et al.* (2016) reported a reduction in the MDA levels in the control rabbits as a result of heat stress induced oxidative stress which supports our findings.

Table 2: Effect of TMG on antioxidant indices of heat-stressed growing rabbits

	Trimethyl glycine, %				
Indices	0	1.0	1.5	SEM	P -Value
MDA, µmol/mg	14.20^{b}	16.82^{ab}	19.48^{a}	1.07	0.0219*
SOD, U/ml	18.34	21.25	22.86	2.70	0.5134
CAT, U/mg	37.17^{a}	35.097^{a}	19.43^{b}	1.43	0.001*
GPx, µg/ml	29.45	27.02	19.65	2.74	0.0766*

$^{a, b}$ Means with different superscript on the same row differ significantly (P < 0.05), SEM: Standard error of mean, TMG: Trimethylglycine; MDA: malondialdehyde, SOD: superoxide dismutase, CAT: catalase, GPx: glutathione peroxidase

It is widely accepted that CAT and SOD, which are responsible for the destruction of peroxides, have a very specific role in protecting tissues against oxidative damage (Ganesan *et al*., 2007; Alirezaei *et al*., 2011). Previous reports indicated that TMG increases only the activity of SOD and does not affect the activity of other antioxidases (Alirezaei *et al*., 2012). This was not observed in this study where differences for SOD (18.34-22.86 U/ml) across treatments were not significant (P>0.05). Catalase levels ranged from 19.43 to 37.17 U/mg and was significant (P<0.05). Catalase was observed to decrease (input the figure) with rising levels of TMG with 1.5% TMG having the least catalase level and differing (P<0.05) from the rest of the treatments including the control. Our findings are contrary with the report of Elham *et al.* (2016) who reported increased CAT activity in betaine supplemented diets fed to rabbits compared with the control. Catalase (CAT) catalyzes the conversion of hydrogen peroxide to water and molecular oxygen and thus plays an important role in dealing with hydrogen peroxide-producing reactions, including that of the SODs (Ighodaro and Akinloye, 2019). Our findings on GPx (19.65-29.45 µg/ml) agrees with the report of Zhang *et al*. (2016), who reported that GPx levels remained unaffected among betaine-containing groups in relation to the control. The concentration of gluthathione in cells is regulated by a balance between its synthesis and utilization. Trimethyl glycine is a precursor of S-adenosylmethionine (SAM), which contributes to an increase in the supply of substrate needed for the synthesis of GPx which functions in conjunction with catalase to reduce hydrogen peroxide levels in the cell. Gluthathione peroxidase is located in the cytoplasm and mitochondria, while catalase is mainly in peroxisomes (Baud *et al.*, 2004; Ighodaro and Akinloye, 2019).

Lipid profile

Result showing the effect of TMG on lipid profile of heat-stressed growing rabbits is shown in Table 3. Lipid indices measured (Total Cholesterol, Triglycerides, HDL, LDL and VLDL) were not affected (P>0.05) by the inclusion of TMG. The plasma total cholesterol level results agree with previous studies that reported no change when betaine was administered to mice (Du *et al.,* 2018). While Singh *et al*. (2015) reported that the addition of betaine resulted in lowered blood serum triglycerides, this was not the case in this study. Our findings agree with the report of Overland *et al*. (1999), who observed that betaine has no impact on plasma triglycerides. It also agrees with the findings of Jewell and Jackson (2022) who reported no significant changes in triglyceride and cholesterol concentrations between the control and betaine containing diets fed to dogs. He *et al*. (2015) who fed 0.1 % betaine to broiler chickens under heat stress reported significant reductions in triglycerides, LDL and HDL. Lipinski *et al.* (2012) reported that betaine addition increased the concentration of serum VLDL (very low-density lipoproteins)

Table 3: Effect of TMG on lipid profiles of heat-stressed growing rabbits

Biochemical Indices	Trimethyl glycine, %			SEM	P -Value
	0	1.0	1.5		
Total Cholesterol, mmol/L	2.73	2.93	3.43	0.22	0.1241
Triglycerides, mmol/L	0.30	0.25	0.30	0.04	0.5694
HDL, mmol/L	1.58	1.85	1.28	0.31	0.4536
LDL, mmol/l	1.00	0.95	1.70	0.45	0.4520
VLDL, mmol/l	0.06	0.05	0.06	0.01	0.5694

SEM: Standard error of the mean, TMG: Trimethylglycine, HDL: High-density lipoproteins, LDL: Low-density lipoproteins, VLDL: Very low-density lipoproteins

which is involved in lipid transport in the circulation system of rats.

Organ indices

Result showing the effect of TMG on organ indices of heat-stressed growing rabbits is shown in Table 4. Aspartate transaminase levels ranged from 35.10-72.64 U/L and was significant (P<0.05). AST levels increased with rising levels of TMG, with both TMG levels (1.0 % and 1.5 %) different (P<0.05) from the control. ALT levels ranged from 113.27-153.41 U/L. Rabbits fed 1% TMG showed the highest ALT levels (P<0.05) and differed from the rabbits in the control which also had lowest ALT levels. This is contrary to the findings of Konca *et al.* (2008), who demonstrated that betaine decreased the serum ALT activity of broilers exposed to heat stress. Betaine has been demonstrated to ameliorate liver injury in mice under stress conditions (Kim and Kim, 2002; Ji and Kaplowitz, 2003). Kidney weights, though were similar (P>0.05) across the experimental groups. Inclusion of TMG in the diet of growing rabbit affected (P<0.05) the liver weight (8.54-34.91%) which increased with rising levels of TMG. Increased liver weight is always regarded as one index of stress condition (Puvadolpirod and Thaxton, 2000a). This is probably due to an increase in liver lipid due to the process of gluconeogenesis, because liver lipids have been shown to increase significantly in broilers treated with adrenocorticotropic hormone (ACTH) (Puvadolpirod and Thaxton, 2000 a, b). This may also be partially attributable to liver tissue enlargement because corticosteroids have been shown to stimulate protein synthesis in the liver (Baxter and Rousseau, 1979*).* Neto *et al*. (2000) however reported that liver weight was not affected by TMG.

Table 4: Effect of TMG on organ indices of heat-stressed growing rabbits

Indices	Trimethyl glycine (%)			SEM	P -Value
	0	1.0	1.5		
AST, UL	35.10^{b}	61.61^{a}	72.64^{a}	4.25	0.0004*
ALT, U/L	113.27^{b}	153.41^{a}	122.63^{ab}	9.33	0.0336*
ALP, U/L	1573.85	1426.38	1754.35	145.55	0.3258
Kidney, % LW	74.98	109.87	106.18	17.73	0.3539
Liver, % LW	8.54^{b}	16.47^{ab}	34.91^{a}	6.57	0.0503*

$^{a, b}$ Means with different superscript on the same row differ significantly (P < 0.05), SEM: Standard error of mean, TMG: Trimethylglycine; (AST), alanine transferase (ALT), and alkaline phosphatase (ALP).

Immune indices

Result showing the effect of TMG on immune indices of heat-stressed growing rabbits is shown in Table 5. Results on total protein, albumins and globulins agrees with the findings of Daudu *et al.* (2020) who fed betaine HCl up to 4.5 mg/kg to broiler chicks and reported no significant (P>0.05) effect. Serum total protein is an index for immune status (White *et al*., 2002) and globulin is an index for immunity in animals. Albumin and globulin concentrations remained similar (P>0.05) all treatments. Rao *et al*. (2011) observed that broiler chicks supplemented with betaine supplementation (800 mg/kg) had significantly increased albumin concentration by about 5.1% compared to control group. A high globulin count is an indication of a high immune response (El-Kholy *et al*., 2012). WBC count ranged from 2.48 to 6.93 10^3/µL and was significant (P<0.05). WBC levels increased with increasing levels of TMG, with 1.5% TMG being higher (P<0.05) than the control. The report of El-Moniem *et al.* (2016) who fed betaine and vitamins C and E to growing rabbits, agrees with our findings which shows significant improvement in WBC count. Basophils levels ranged from 0.33 to 0.63 %. Rabbits fed 1.5% TMG had the highest (P<0.05) basophil levels but was however similar to the control. Other haematological parameters measured were not significant (P>0.05). Mendoza *et al.* (2017) reported that, pigs fed 0.10% betaine showed the highest values for WBC, lymphocytes, monocytes, eosinophils, and basophils, regardless of the environment or period.

Table 5: Effect of TMG on immune indices of heat-stressed growing rabbits

Indices	Trimethyl glycine, %			SEM	P -Value
	0	1.0	1.5		
Total Protein, g/Dl	6.43	5.49	4.83	0.55	0.1823
Albumins, g/dL	3.68	2.85	2.39	0.45	0.1742
Globulins, g/dL	2.74	2.65	2.45	0.22	0.6627
White blood cell, 10^3/µL	2.48^b	5.30^{ab}	6.93^a	1.04	0.0405*
Lymphocytes, %	59.85	46.70	44.80	6.28	0.2363
Granulocytes, %	16.50	20.57	31.93	6.27	0.2498
Platelet, 10^3/Ml	186.00	263.00	186.50	59.66	0.5943
Eosinophils, %	10.45	10.10	8.60	1.48	0.6579
Monocytes, %	9.83	11.07	13.80	1.53	0.2251
Basophils, %	0.33^{ab}	0.133^b	0.63^a	0.11	0.0463*
Heterophil-lymphocyte ratio	0.33	0.49	0.77	0.19	0.2939

$^{a, b}$ Means with different superscript on the same row differ significantly (P < 0.05), SEM: Standard error of mean

The major functions of the white blood cell and its differentials are to fight infections, defend the body by phagocytosis against invasion by foreign organisms and to produce or at least transport and distribute antibodies in immune response.

Our findings on lymphocytes agrees with the report of Abdelsattar *et al.* (2019), who showed that betaine supplemented diets in growing lambs receiving saline water did not cause a change in lymphocyte numbers. Our findings on hetrophils and lymphocyte percentages are contrary to Nofal *et al.* (2015) who reported significant reductions and increases in heterophil and lymphocyte percentages in broiler chickens under heat stress. Other studies reported no significant dietary effects on blood hematological parameters, including heterophil-lymphocyte and hematocrit ratios (Nasiroleslami *et al.,* 2018).

Conclusion

Trimethyl glycine (TMG) had a positive effect on some blood biochemistry and organ indices of heat-stressed growing rabbits. From a performance standpoint, supplementing rabbit diets with antioxidants like TMG up to 1.5% will help ameliorate the adverse effects of oxidative stress.

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