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L-Arginine Co-Administration with Carbamazepine Improves Cognition in Male Sprague-Dawley Rats

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Summary: Cognitive impairment is a common adverse effect associated with carbamazepine use. One of the proposed mechanisms for cognitive impairment may be attributed to the pro-oxidant properties of carbamazepine. This study investigated the effects of L-Arginine supplementation with carbamazepine on cognition in adult male non-epileptic rats. Adult male Sprague-Dawley rats with average weight 200g to 220g were divided into 4 groups; (1) Control group treated with distilled water, (2) L-Arginine group treated with L-Arginine (100mg/kg BW) in distilled water, (3) Carbamazepine group treated with carbamazepine (25mg/kg BW twice daily) in distilled water, and (4) Carbamazepine + L-Arginine group treated with Carbamazepine and L-Arginine as above for two weeks to assess the acute changes in cognition and oxidative stress markers. Following two weeks of treatment, cognition was assessed using the Y-maze, after which the rats were humanely sacrificed with the hippocampus and frontal lobes isolated from the brain and subsequently homogenized for assessment of oxidative stress markers [(Catalase, superoxide dismutase (SOD), malondialdehyde (MDA), and reduced Glutathione (GSH)]. Arm entry and correct alternation were significantly higher ($p < 0.05$) in the L-Arginine and L-Arginine + Carbamazepine groups compared to carbamazepine group. In the frontal lobe, L-Arginine significantly increased ($p < 0.05$) catalase and GSH levels compared to other groups while in the hippocampus**,** it significantly ($p < 0.05$) reduced MDA with no change in other parameters. Likewise, SOD and MDA levels were significantly lower ($p < 0.05$) in the L-Arginine + Carbamazepine group compared to other groups. Oral L-Arginine supplementation with carbamazepine improved cognitive performance on Y maze.

Keywords*:* Carbamazepine, L-Arginine, Cognition, Oxidative stress

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INTRODUCTION

Carbamazepine is one of the most commonly prescribed antiepileptic drugs and also useful in the management of non-epileptic disorders. It is a first line drug in the management of focal seizures however, its negative effect on cognitive function has been well documented (Wesnes *et al.*, 2009; Gulec Suyen *et al.*, 2016). In addition, cognitive impairment is commonly reported in epileptic seizures (Mula and Trimble, 2009). Occurrence of cognitive impairment is usually more pronounced on drug introduction and during upward titration of drug dosage. As a result of this, patients may be poorly adherent or become non-adherent to drug therapy with a consequent suboptimal seizure control (Perucca and Gilliam, 2012). Evaluating the mechanism and interventions for early cognitive changes will help to address this.

Carbamazepine acts by inhibiting voltage gated sodium channels but its effect on cognition may not be related to its mechanism of action because lamotrigine which acts through a similar mechanism is not linked to negative cognitive impacts (Arora *et al.*, 2010). In fact, some studies have reported an improvement in cognitive profile following lamotrigine use (Shinnar *et al.*, 2017; Zhang *et al.*, 2017).

Generation of oxidative stress following carbamazepine use has been reported as the basis for the development of cognitive impairment (Arora *et al.*, 2010; Reeta *et al.*, 2010). Oxidative stress is a common phenomenon which occur due to a failure in the normal balance between production and the mopping up of reactive oxygen (ROS) and nitrogen species (RNS) which leads to the disruption of structural integrity and normal function of the lipid membrane, protein and the DNA (Ullrich and Kissner, 2006). The major source of these ROS is from the mitochondrial respiratory chain and nitric oxide synthase activity. Reactive species responsible for oxidative stress are superoxide O_2^-, hydrogen peroxide H_2O_2, hydroxyl radicals OH^- and other nitrogen containing radicals such as NO^-, NO^+, NO_2^-, ^-OONO (Ullrich and Kissner, 2006). Superoxide (O_2-) and H_2O_2 are generated from mitochondrial respiration, they undergo further enzymatic reactions

to produce other reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Egea *et al*., 2017).

Cellular and tissue defences against ROS and RNS include the enzymes superoxide dismutase catalase, glutathione peroxidase, peroxiredoxins and the nonenzymatic antioxidants, glutathione (GSH), thioredoxin, ascorbate, α-tocopherol and uric acid (Egea *et al.*, 2017). The levels and activities of these are used to determine oxidative stress. The extent of tissue and cellular damage from oxidative stress is obtained by measuring the markers of lipid peroxidation such as malondialdehyde, 4-hydroxy-alkenals, thiobarbituric-reactive substances (TBARS) and DNA damage like 8-Hydroxyguanine (8-OHdG) and 8-Hydroxy-2'-Deoxyguanosine (oH8DG) (Silva *et al*., 2014).

Oxidative stress has been shown to affect the synthesis and release of neurotransmitters which are critical components of cognition (Waldbaum and Patel, 2010). Carbamazepine has been associated with oxidative stress in both animal and human studies (Reeta *et al.*, 2010; Tutanc *et al.*, 2015). This suggest that anti-oxidants may be helpful in ameliorating the effect of carbamazepine on memory. An earlier study has demonstrated the benefit of a natural antioxidant, curcumin in cognitive impairment associated with carbamazepine use (Reeta *et al.*, 2010). It has also been stated that L-Arginine subserves positive cognitive impacts by increasing the levels of glutathione and inhibiting the release of superoxide (Liang *et al*., 2018). This is because of its ability to increase the level of nitric oxide in the prefrontal cortex and hippocampus, both areas of which are important for cognitive improvement (Wei *et al.*, 2013; dos Santos *et al*., 2014).

Similar to the somatotopic organisation of the brain, the hippocampus and amygdala mediate memory formation, while processing speed and executive functions are localized to the prefrontal lobes, although, there is an overlap in the function of various regions (Purves, 2012). A large number of studies have reported the effects of carbamazepine on cognitive function, however there is paucity of data on how to prevent this cognitive adverse effect. Thus, this study investigated the role of L-Arginine supplementation on early cognitive changes, oxidative stress indices, in both the frontal and hippocampus and their relative contribution to cognitive changes.

MATERIALS AND METHODS

Animals

Healthy adult male Sprague-Dawley rats (average weight 200g to 220g) were obtained from the Animal House of the College of Medicine, University of Lagos. The animals were housed in polypropylene cages (30 x 15 x 15cm) in groups of eight rats per cage with free access to pelletized rat chow and water *ad libitum.* Rats were kept under normal light conditions (12 hours light/dark cycle) and normal room temperature (24 ± 2 °C). All experiments were carried out during the light phase between 9:30 a.m. to 12.00noon and performed in accordance with the international regulations to minimise pain on laboratory animals. Our research protocol was also in line with the guidelines of the College of Medicine, University of Lagos, Health Research and Ethics Committee.

Drugs and dosing schedule

Carbamazepine (Tegretol) was obtained from Novartis Pharma AG, Basle, Switzerland while L-Arginine was obtained from Now Foods, Bloomingdale, USA. Carbamazepine was administered at a dose of 25mg/kg twice daily and L-Arginine (100mg/Kg) daily both for 2 weeks. This dose for carbamazepine was used based on reports from previous study (Nissinen and Pitkanen, 2007). The control animals received distilled water daily for the same period of study. On the final day of experiment, both drugs were administered an hour prior to cognitive assessment in the animals.

Assessment of cognition

Cognition was assessed in the rats at 2 weeks following carbamazepine treatment and L-Arginine supplementation. The animals in all groups were pre-exposed to the Y maze twenty-four hours before the experiment and were brought to the laboratory at least two hours before the experiments for acclimatization on the day of the experiment.

Y-Maze

The Y- maze is composed of three equally spaced arms (120°, 41cm long and 15cm high). The floor of each arm is 5cm wide. The Y-maze is used to assess short term spatial memory, general locomotor activity and stereotypic behaviour. The Y-maze is used to assess cognitive function in animals (Ishola *et al.*, 2017). The ability to have correct alternation in the maze is a measure of working memory which includes short term memory, attention and information processing. This requires an unaltered signally pathway between the hippocampus and the prefrontal cortex (Morellini, 2013).

Parameters measured in this study using the Y maze included number of arm entry and percentage correct alternation. Each rat was placed at the centre of the Y-maze and the sequence of arm entries was documented, and percentage correct alternation calculated. A correct alternation is defined as entry into all three arms consecutively. The Y-maze assessment was carried out for 4 minutes. The apparatus was cleaned with 5% alcohol and allowed to dry between sessions.

Brain tissue for oxidative stress assessment

The rats were humanely sacrificed by using a standard cervical dislocation procedure. The brains were quickly isolated with hippocampus and frontal lobe

dissected from the brain, weighed, washed in ice-cold normal saline and homogenized in 10% ice-cold 0.1 M potassium phosphate buffer (pH 7.4). The homogenates were centrifuged at 3000 RPM for 15 minutes in a cold centrifuge with supernatants separated and stored at -70^{o} C for the measurement of oxidative stress markers [(Superoxide dismutase (SOD), Malondialdehyde (MDA) and reduced glutathione (GSH), and Catalase levels)] as described in a previous study (Arora *et al*., 2010).

Glutathione level was estimated using the method described by Ellman (Ellman, 1959). Catalase activity was determined by the colorimetric method as previously described (Clairborne, 1985). Malondialdehyde was estimated using the method described by Ohkawa and co-workers (Ohkawa *et al.*, 1979), and Superoxide dismutase activity was determined by the pyrogallol auto-oxidation method described by Marklund (Marklund, 1985).

Statistical Analysis

All results were expressed as mean ± S.E.M of rats 8 in each group. The data were statistically compared by carrying out One-way Analysis of Variance (ANOVA) using GraphPad Prism 7 followed by post-hoc Tukey's test for inter-group comparisons. A value of $p < 0.05$ was considered statistically significant for comparison.

RESULTS

Assessment of Cognition

The number of arm entry and percentage correct alternation were significantly lower ($p < 0.05$) in the carbamazepine group compared to control. L-Arginine and CBZ + L-Arginine groups had significantly higher arm entry and percentage correct alternation ($p < 0.05$) compared to control and the carbamazepine group (Figures 1 and 2).

Glutathione levels

In the frontal lobe, GSH level was significantly higher ($p < 0.05$) in the L-Arginine group compared to control, carbamazepine and CBZ + L-ARG groups. Likewise, CBZ + L-ARG group had significantly increased GSH level ($p < 0.05$) compared to carbamazepine but no significant difference compared to control. In the hippocampus GSH level was significantly lower ($p < 0.05$) in the carbamazepine group compared to control while it was significantly higher ($p < 0.05$) in the L-Arginine group compared to control and CBZ +L-ARG groups (Figure 3).

Catalase

In the frontal lobe, catalase level was significantly higher ($p < 0.05$) in the L-ARG and CBZ + L-ARG groups compared to control and CBZ groups while there was no significant difference in the levels of catalase in the CBZ group compared to control. In the hippocampus there was no significant difference in catalase levels in all the groups (figure 4).

Superoxide dismutase

In the frontal lobe, SOD concentration was significantly higher ($p < 0.05$) in the CBZ group compared to the others groups. However, in the hippocampus, CBZ + L-ARG group had significantly

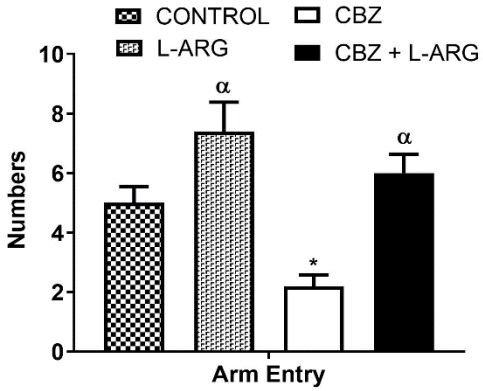

Figure 1. The effect of L-arginine co-administration with carbamazepine on Arm entry in Control, L-ARG, CBZ, and CBZ + L-ARG rats. *P < 0.05 Vs. Control., αP < 0.05 Vs CBZ.

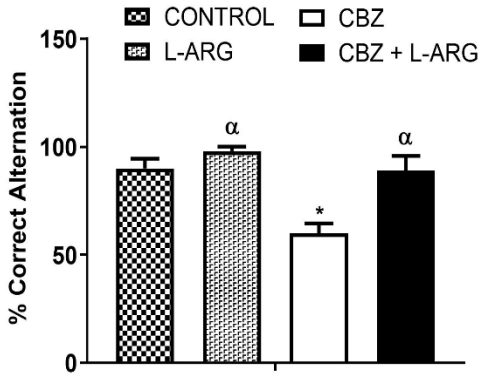

Figure 2. Percentage Correct alternation in Control, L-ARG, CBZ, and CBZ + L-ARG rats following L-Arginine co-administration with carbamazepine.
*P < 0.05 Vs. Control., αP < 0.05 Vs CBZ.

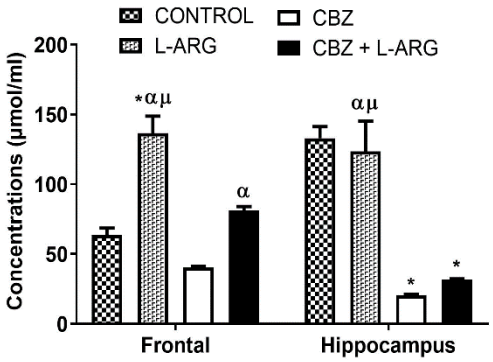

Figure 3. The effect of L-Arginine co-administration with carbamazepine on GSH concentrations in Frontal lobe and Hippocampus in Control, L-ARG, CBZ, and CBZ + L-ARG rats.*P < 0.05 Vs. Control., αP < 0.05 Vs CBZ., µP < 0.05 Vs CBZ + L-ARG

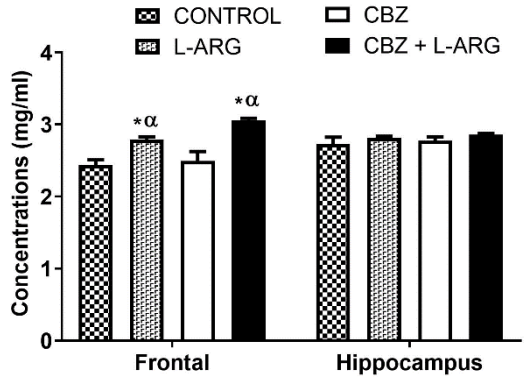

Figure 4. Catalase concentration in Control, L-ARG, CBZ, and CBZ + L-ARG rats in Frontal lobe and Hippocampus following L-Arginine co-administration with carbamazepine. *P < 0.05 Vs. Control., αP < 0.05 Vs CBZ.

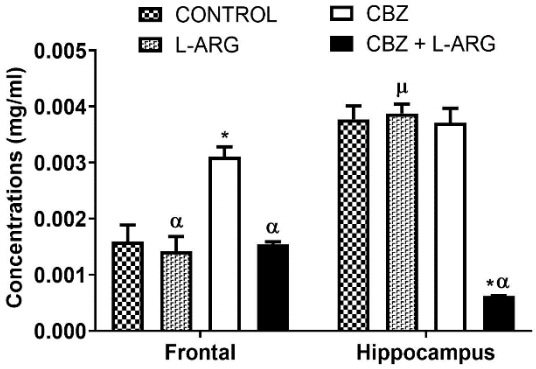

Figure 5. The effect of L-arginine co-administration with carbamazepine on SOD concentrations in Frontal lobe and Hippocampus in Control, L-ARG, CBZ, and CBZ + L-ARG rats. *P < 0.05 Vs. Control., αP < 0.05 Vs CBZ., μP < 0.05 Vs CBZ + L-ARG

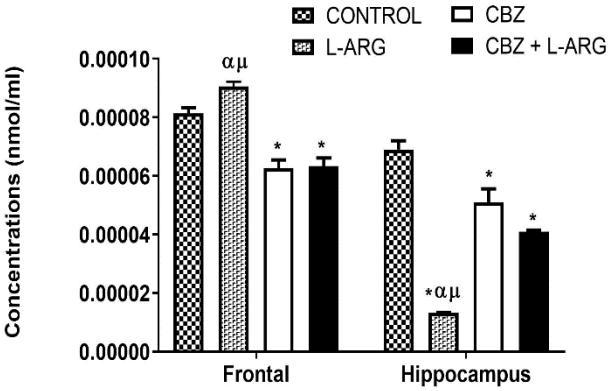

Figure 6. The effect of L-arginine co-administration with carbamazepine on MDA concentrations in Frontal lobe and Hippocampus in Control, L-ARG, CBZ, and CBZ + L-ARG rats.*P < 0.05 Vs. Control., αP < 0.05 Vs CBZ., μP < 0.05 Vs CBZ + L-ARG

lower (p < 0.05) level of SOD compared to the other groups (figure 5).

MDA concentration

In the frontal lobe, CBZ and CBZ + L-ARG groups had significantly lower (p < 0.05) levels of MDA compared to control and L-ARG groups. Likewise, in the hippocampus, MDA was significantly lower (p < 0.05) in the CBZ group compared to controls. While the L-ARG and CBZ + L-ARG groups also had significantly lower (p < 0.05) levels of MDA compared to the control group (Figure 6).

DISCUSSION

The study showed that carbamazepine reduced the total number of arm entry, number of alternation and percentage correct alternation in the Y maze compared to controls. These findings support previous studies demonstrating the negative impact of carbamazepine on different cognitive domains in both human and animal studies (Garcia-Penas *et al*., 2014; Xiao *et al*., 2018; Gulec Suyen *et al*., 2016; Olusanya et al., 2017).

Carbamazepine was shown to impair spatial learning and attention in non-epileptic rats (Shannon and Love, 2004; Shannon and Love, 2005; Shannon and Love, 2007; Barker-Haliski *et al*., 2016). However a few studies have reported improvement in cognitive function or no changes following carbamazepine therapy (Bernardi and Barros, 2004). Most of the study reporting improvement are limited by the fact that studies were carried out in epileptic rats and reduction in seizure frequency may contribute to the improvement in cognition rather than the direct effect of carbamazepine (Bernardi and Barros, 2004).

To the best of our knowledge, this is one of the few studies that have addressed the cognitive dysfunction associated with carbamazepine use. The group administered with L-Arginine alone and carbamazepine + L-Arginine had improved parameters which suggests that L-Arginine improves cognition and its supplementation is protective against cognitive impairment associated with carbamazepine use. Studies have shown the association between improved cognition and the use of L-Arginine.

L-Arginine is a semi-essential amino acid which participates in multiple biochemical processes in the body. It is a substrate for the formation of nitric oxide (NO) which serves as a neurotransmitter mediating the release of glutamate, GABA and dopamine (Sardo and Ferraro, 2007). NO activates guanylyl cyclase to produce cGMP, a mediator of neuronal plasticity and excitability (Flynn *et al.*, 2002; Gornik and Creager, 2004). Reduced nitric oxide has been linked to cognitive impairment (Katusic and Austin, 2014; Morita *et al.*, 2014). L-Arginine has been shown to increase the level of NO in the prefrontal cortex and hippocampus which correlates with cognitive improvement (Wei *et al*., 2013; dos Santos *et al.*, 2014).

Similarly, it has also been associated with increase in the expression of α7 nAChR immunoreactivity and protein expression in the prefrontal cortex and hippocampus which are all associated with improved cognitive function in animal studies (Wei *et al.*, 2013). Alpha 7- nicotinic receptors are located mainly on presynaptic neurones on the hippocampus, amygdala

and prefrontal cortex where they regulate the release of neurotransmitters like GABA and glutamate, and on postsynaptic neurons where they mediate fast excitatory neurotransmission (Sinkus *et al.,* 2015; Kalkman and Feuerbach, 2016). The anti-oxidant and anti-inflammatory properties of L-Arginine may also explain its beneficial effect on cognition (Fonar *et al.,* 2018) since both inflammation and oxidative stress have been implicated in cognitive impairment (Magenta *et al.,* 2014).

One of the possible mechanism for the development of cognitive impairment with carbamazepine use is due to its pro-oxidative capability which has been demonstrated in several studies (Reeta *et al*., 2010; Li *et al*., 2010; Tutanc *et al*., 2015). Oxidative stress is implicated in the disruption of both glutamatergic and cholinergic neurotransmission (Waldbaum and Patel, 2010).

In this study, carbamazepine significantly reduced the levels of glutathione in the hippocampus and this supports the pro-oxidant capacity of carbamazepine as previously documented (Reeta *et al.,* 2010). Other studies have reported no change in the levels of glutathione following the administration of carbamazepine (Yuksel *et al.,* 2000, Arora *et al.,* 2010). Various studies have demonstrated conflicting reports on SOD levels in relation to carbamazepine use. For example, some studies have reported an increase in SOD levels (Liu *et al.,* 1998, Yuksel *et al.,* 2001) following carbamazepine administration, while other studies have reported a reduced or an unchanged SOD levels (Yuksel *et al.,* 2000, Verrotti *et al.,* 2002) following carbamazepine administration. Our study showed a significant increase in SOD levels in frontal lobe in the CBZ group compared to the other groups.

The source of oxidative stress following carbamazepine use is unknown, but may be related to its metabolism via cytochrome P450 system. This needs to be confirmed in further studies. A few studies however did not find any effect on oxidative stress parameter in non-epileptic rats and humans (Arora *et al.,* 2010; Menon *et al.,* 2014). A study on human subjects reported no difference in oxidative stress parameters in patients on antiepileptic drugs which included carbamazepine and drug naïve patients with epilepsy. The study concluded that oxidative stress is secondary to the presence of seizures not the use of antiepileptic drugs (Menon *et al.,* 2014).

This study showed different oxidative stress pattern in the frontal lobe compared to the hippocampus (Figures 3 - 6). This pattern of differential markers of oxidative stress with relation to location and timing has been demonstrated in different studies (Freitas *et al*., 2005; Bellissimo *et al.,* 2001). Freitas *et al.,* (2005) suggested that catalase/and/or GSH as the major radical scavenging system in the hippocampus because their levels were more likely to be reduced following oxidative stress.

The outcome following the use of antioxidants and conditions associated with cognitive dysfunction such as Alzheimer's disease and epilepsy have been conflicting (Skvarc *et al.,* 2017; Farina *et al.,* 2017; Tarantini *et al.,* 2018). A recent study demonstrated that treatment of status epilepticus with N-acetylcysteine and sulforaphane; drugs that increase the levels of GSH, was protective against oxidative stress and cognitive decline (Pauletti *et al*., 2017). Only a few studies addressed the use of antioxidants in drug-induced cognitive dysfunction. Reeta et al. (2010) reported that Curcumin, a compound derived from plants was shown to pevent cognitive dysfunction in rats administered carbamazepine and this was linked to its anti-oxidants potential.

L-Arginine improved oxidative parameters in this study. This suggests that improved cognitive function in the rats on L-Arginine may be related to its anti-oxidative properties, though other mechanisms like increase in nitric oxide synthesis and alpha 7 nicotinic acetylcholine receptors activation have been linked to its positive cognitive effect (Wei *et al.,* 2013; dos Santos *et al.,* 2014).

Our study demonstrated improved cognitive function in the carbamazepine + L-Arginine group compared to the other groups in non-epileptic rats but this is unlikely due to direct effect of oxidative parameters. Thus, further study in epileptic rats with a possibility to explore other mechanisms of actions like the effect on neurotransmission, and anti-oxidant protein activity in this improved cognitive process is suggested.

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Prevalence of Premenstrual Syndrome and Changes in Blood Pressure with Menstrual Cycle Among University Students

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Summary: We aimed to investigate prospectively the prevalence of premenstrual symptoms (PSM), changes in blood pressure with menstrual cycles and to investigate the relative severity of each symptoms and to cluster these symptoms into factors, and the relative contributions of each factor in a sample of undergraduate students of Ahmadu Bello University, Zaria and Bingham University, Karu. A total of 370 female undergraduate students reported on the severity of 23 PMS in a full cycle. Their blood pressures (BP) were measured during follicular and luteal phases. Paired sample student *t*-test was used to investigate difference in mean of blood pressures based on menstrual phases. We fitted PCA to cluster the symptoms. Backache, wish to be alone, joint or muscle pain, fatigue, and pain in the thigh were the five most frequently reported symptoms. About 89% of the participants reported experiencing at least one symptom during each cycle. Mean arterial blood pressure, systolic, and diastolic blood pressures were significantly (P <0.001) higher at the luteal phase than at the follicular phase while, pulse rate was significantly higher (P <0.001) at the follicular phase than the luteal phase. The 23 symptoms were reduced into four clusters; affective, physical, somatic, and GIT/physical symptoms. In conclusion, our findings indicate a high prevalence of PMS with majority indicating that the symptoms as mild to severe. Blood pressure significantly increased at luteal phase than follicular phase. The 23 symptoms were reduced into four clusters.

Keywords*:* Premenstrual syndrome, Blood pressure, Menstruation

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INTRODUCTION

Premenstrual symptoms, commonly referred to as premenstrual syndrome (PMS) is a group of physical, psychological, and behavioural symptoms experienced by premenopausal women during the luteal phase of their menstrual cycle (Usman *et al.*, 2008). Diagnostic criteria for PMS was published by the American College of Obstetrics and Gynaecology (ACOG, 2000), and states as follows: a woman is considered to have PMS if she reports having at least one of the 6 affective symptoms and one of the 4 somatic symptoms 5 days prior to onset of menstruation in 3 previous consecutive cycles and the symptoms should remit within 4 days of menses onset and that the symptoms should not recur until at least day 13 of the next cycle. The cause of PMS is unclear. However, it is believed that serotonin, progesterone, regulation of gonadotrophins secretion by endorphin, psychological and biosocial factors play a key role in its aetiology (Yonkers *et al.*, 2008).

Commonly reported symptoms include headaches, breast tenderness, depression, anxiety, social withdrawal, food cravings, abdominal bloating, fatigue, mood swings, irritability, and oedema. Although affected women don't suffer all these symptoms at the same time, most women however, report suffering from recurrent symptoms of PMS during each cycle (Grosz, 1988). Symptoms of PMS are evident 4-7 days (premenstrual phase) prior to and in some cases, during menstruation (Braverman, 2007). Studies from developed and developing countries have consistently reported the existence of these symptoms and dysmenorrhoea among women of childbearing age and nationalities (Hallow and Campbell, 2004; French, 2005; Tabassum *et al.*, 2005; Latthe *et al.*, 2006; Pinar *et al.*, 2011).

There has not been agreement in the prevalence estimates of PMS among authors (Sammon *et al.*, 2016). It has been shown that about 50-90% of premenopausal women report at least symptom of PMS, 10-30% report symptoms of PMS that interfere

with daily activities, while about 1-8% report at least five psychological symptoms that meet premenstrual dysphoric disorder (PMDD) criteria (Halbreich *et al.*, 2003). A study by Hylan *et al.* (1999) in the UK reported 31% as having severe symptoms of PMS out of which 53% sought medical intervention. Similar studies in the USA and France in 1998, reported 45% and 29% respectively, of women reporting severe symptoms of PMS seeking for medical intervention, while in Switzerland, 41% of women with severe symptoms of PMS reported seeking medical help in a study conducted between 1986 and 1993 (Angst *et al.*, 2001).

Variabilities in blood pressure during menstrual cycle among Nigerian females are not well documented, and available studies have conflicting reports. Earlier study by Greenberg and colleagues (1985) reported a higher systolic blood pressure (SBP) at days 17 – 26 (luteal phase) of the cycle than at other phases of the cycle. Contrary to this cross-sectional study, in a smaller prospective study, this same group found significant decrease in diastolic blood pressure (DBP) at days 17 – 26 than in other phases of the cycle in a sample of 33 women who were visited three times on weekly basis for 8 consecutive weeks. Kelleher and colleagues (1986) performed similar prospective study on 18 women and reported a significant increase in SBP at the premenstrual phase. Consequently, increase in SBP at ovulatory phase has also been reported (Freedman *et al.*, 1974). Hassan *et al.* (1990) found that SBP was similar at the menstrual phase, days 1 – 2; at the late follicular phase, days 12 – 15 and at the late luteal phase, days 21 – 26, while DBP was significantly lower at the luteal phase. However, Dunne *et al.* (1991) reported a significantly higher SBP and DBP at onset of bleeding than at most other phases of the cycle. However, after adjustment, DBP was found to be higher in the follicular than at the luteal phase. Various mechanisms have been suggested to interplay between PMS and increase in blood pressure. Notable is the renin-angiotensin-aldosterone system dysfunction and deficiencies of micronutrients (Bertone-Johnson *et al.*, 2005; Rosenfeld *et al.*, 2008; Chocano-Bedoya *et al.*, 2011; 2013).

To better understand the prevalence of PMS and the changes in blood pressure during follicular and luteal phases of menstrual cycle, we report the findings from 370 undergraduate students. We first sought the prevalence of PMS among the students then investigate the extent to which participants report severity of premenstrual symptoms. We then aimed to explore the changes in arterial and blood pressures during follicular and luteal phases. Finally, we investigated phenotypic factor loadings of PMS.

MATERIALS AND METHODS

The prospective study was conducted in Ahmadu Bello University, Zaria, and Bingham University, Karu, in Northwestern and Northcentral Nigeria respectively. Data were obtained from subjects using structured questionnaires that were randomly administered to them. The sample included 370 undergraduate female students, aged 16-33 years. All subjects have regular menstrual cycles of between 28 – 34 days. No subject reported taking medication known to alter blood pressure and none was on oral contraceptives in the past 6 months prior to the study. Oral contraceptives have been reported to both exacerbate and alleviate PMS (Severino and Moline, 1989). The study was approved by Ahmadu Bello University Research and Ethics Committee. Participants provided informed consent prior to the study. Subjects were also informed that participation was voluntary and that they can withdraw at any time. Data were collected at inception of semester.

Questionnaire

The study was based on a questionnaire distributed to the students in their hostels. Brief description of the study was made to each subject, followed by issuance of consent form and questionnaire. The questionnaire consisted of three sections. A general section concerned with self-reported data of subject and those of her family such as age, menarcheal age, size of sibship, ethnic affiliation, place of residency (urban or rural), parents' level of education. Parental education was categorized into four groups: no formal education, primary, secondary, and tertiary education. Anthropometric and physiological section in which weight, height, SBP and DBP measurements were taken. Finally, symptoms of PMS section which included 23 symptoms of PMS were reported prospectively in one cycle. This section asked the subjects to tick each item according to the degree of severity, graded with a Likert-type scale from 1 to 4 (no pain, mild pain, moderate pain, severe pain) to all 23 symptoms. Hostel number and phone number of each participant was collected in order to track them. Subjects were asked to tick the severity of each of the 23 symptoms of PMS on the questionnaire.

Measurements and Protocol

Anthropometry. Weight and height were measured twice, and the average was calculated. Weight was measured to 0.1 kg using a standard beam balance in minimal clothing and bare feet. Height was determined to the nearest 1 mm with portable stadiometers. The portable scale and stadiometer were calibrated each day. BMI was calculated according to the formula:

$$\text{BMI (kg/m}^2\text{)} = \frac{weight\ (kg)}{height\ (m^2)}$$

All measurements of heights and weights were taken by two of the authors; J.K. and C.E. according to

standardized anthropometric techniques (Lohman *et al.*, 1988).

Blood pressure

Blood pressure of subjects was measured in a systematized approach at different stages of their menstrual cycles as follows: days 1 – 6, days 7 – 14, days 15 – 23 and days 24 – 28, with adjustment for cycles lengths 29 and 34 days (no subject reported having cycle length <28 days). Follicular phase was dated as days 1 – 14 while luteal phase was dated as days 15 – 28. Two consecutive blood pressures were measured each morning of the study per stage and the average calculated as the blood pressure for that stage. The average of blood pressure for days 1 – 6 and 7 – 14 constitutes the follicular phase while the average of blood pressure for days 15 – 23 and 24 – 28 constitutes the luteal phase. To account for variabilities in temperature, all blood pressure measurements were taken in the hostel. The phase of the cycle at point of entry was determined from the date of onset of bleeding.

The indirect (auscultatory) method was employed to measure the SBP and DBP with the use of sphygmomanometer. Briefly, an inflatable cuff containing a pressure gauge was wrapped around the upper arm, and a stethoscope was placed over the brachial artery just below the cuff. The cuff occludes the brachial artery due to compression. The cuff was then inflated with air to a pressure greater than SBP. Systolic blood pressure was recorded following an appearance of tapping sound while DBP was recorded following disappearance of muffling sound. Pulse rate was recorded as the number of pulse per minute at the radial artery with the middle finger. Mean arterial pressure was calculated using the formula:

Mean arterial pressure =

$$\text{diastolic pressure} + \frac{1}{3}(\text{systolic pressure} - \text{diastolic pressure})$$

Statistical Analysis

Descriptive statistics including means and standard deviations were calculated for sociodemographic, anthropological, and physiological variables. Paired sample *t*-test was used to calculate difference in means of physiological variables based on menstrual cycle. Statistical analyses were performed using STATISTICA™ version 7 (StatSoft, Tulsa, OK). *P* values <0.05 (two-tailed) were considered statistically significant.

RESULTS

In total, 370 female students, aged 16-33 (mean age20.66 ± 2.29), were included in the study. The participants' baseline characteristics are presented in Table 1. The participants were undergraduate students of Ahmadu Bello University Zaria and Bingham University Karu. Mean age at menarche was 12.74 ± 1.98 years. Means and standard deviations of their weight, height, and BMI are also presented on the same table. The prevalence of PMS was 89%, that is, 329 subjects reported having at least one symptom of PMS while 11% (41 subjects) did not report any symptom of PMS (Figure 1). Figure 2 presents severity of PMS as reported by the 329 subjects who reported

Table 1: Participant characteristics at baseline (n = 370)

	Mean ± SD	Range
Age (yrs.)	20.66 ± 2.29	16.00 – 33.00
Menarcheal age yrs.)	12.74 ± 1.98	8.00 – 15.00
Weight (kg)	58.78 ± 11.65	39.00 – 120.00
Height (cm)	160.85 ± 7.27	145.00 – 188.00
BMI (kgm^{-2})	22.68 ± 3.91	15.62 – 44.62

BMI = body mass index

Table 2: Comparison of physiological variables during follicular and menstrual cycle

	Follicular phase	Luteal phase
Physiologic variables	Mean ± SD	Mean ± SD
Systolic blood pressure (mm Hg)	111 ± 13	117 ± 10*
Diastolic blood pressure (mm Hg)	73 ± 13	79 ± 9*
Pulse rate (beats/min)	81 ± 13	76 ± 11*
Mean arterial pressure (mm Hg)	100 ± 13	104 ± 10*

*: P<0.001

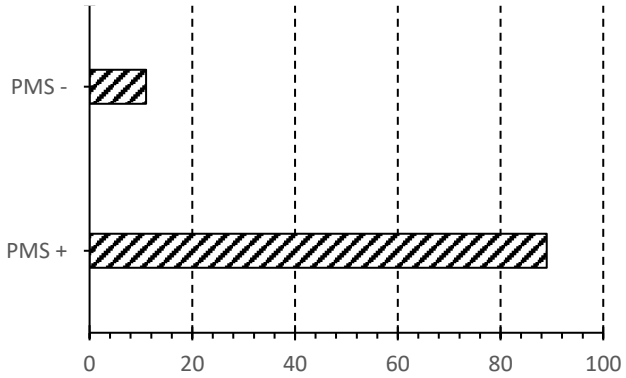

Figure 1. Prevalence of PMS

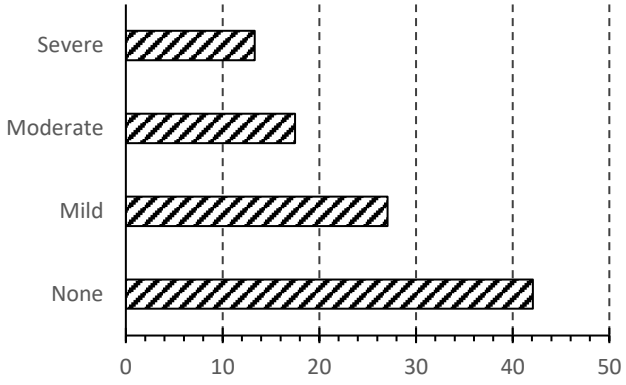

Figure 2. Degree of severity of PMS among the subjects

Table 3: Premenstrual symptom loads experienced by participants in one menstrual cycle

	Degree of severity				
Premenstrual symptoms	**No (%)**	**Mild (%)**	**Moderate (%)**	**Severe (%)**	**Mean ± SD**
Backache	25	21	18	36	1.65 ± 1.21
Wish to be alone	31	20	21	28	1.47 ± 1.20
Joint or muscle pain	34	20	21	25	1.38 ± 1.20
Fatigue	26	26	24	24	1.37 ± 1.02
Pain in the thigh	35	18	25	22	1.35 ± 1.17
Acne/pimples	31	31	18	20	1.19 ± 1.03
Reduced urination	51	13	20	16	1.19 ± 1.03
Depression	35	28	21	16	1.17 ± 1.08
Loss of appetite	37	23	25	15	1.17 ± 1.09
Difficult concentration	40	23	22	15	1.13 ± 1.11
Aggressiveness	43	19	24	14	1.10 ± 1.11
Sleeplessness	44	22	18	16	1.06 ± 1.12
Oily skin	41	26	20	13	1.03 ± 1.06
Dizziness	45	25	17	13	0.97 ± 1.06
Excessive sleep	50	22	15	13	0.90 ± 1.07
Nausea/vomiting	55	16	16	13	0.86 ± 1.10
Crying spells	57	20	13	10	0.78 ± 1.04
Diarrhoea	57	19	16	8	0.76 ± 1.01
Headache	56	20	17	7	0.73 ± 0.96
Overeating	60	18	12	10	0.71 ± 1.01
Sweating	58	21	13	8	0.70 ± 0.97
Confusion	61	16	16	7	0.69 ± 0.98
Constipation	70	12	12	6	0.53 ± 0.91

Table 4: Factor loadings for PMS symptoms from four factor analyses

	Factor Loadings			
Premenstrual symptoms	**One**	**Two**	**Three**	**Four**
Depression	73	-	-	-
Aggressiveness	70	-	-	-
Confusion	70	-	-	-
Difficulty concentrating	64	-	-	-
Crying spell	57	-	-	-
Fatigue	50	-	-	-
Dizziness	39	-	-	-
Wish to be alone	39	-	-	-
Oily skin	-	75	-	-
Overeating	-	55	-	-
Acne	-	51	-	-
Pain in the thigh	-	-	76	-
Joint pain	-	-	73	-
Backache	-	-	69	-
Low appetite	-	-	43	-
Nausea	-	-	-	58
Diarrhoea	-	-	-	57
Sweating	-	-	-	56
Constipation	-	-	-	49
Reduced urine	-	-	-	42
Excessive sleep	-	-	-	36

Correlation determinant = 0.013, Kaiser-Meyer-Olkin Measure of Sampling Adequacy = 0.819, Bartlett's test of sphericity P <0.001. All decimal points are omitted ***Factor 1:*** *Affective symtoms,* ***Factor 2:*** *Physical symptoms* ***Factor 3:*** *Somatic symptoms* ***Factor 4:*** *GIT/Physical symptoms*

having at least one symptom of PMS. From the graph, 42.1% reported that the symptoms are not severe while 27.1%, 17.5% and 13.3% reported having mild, moderate and severe symptoms respectively. Table 2 compares the blood pressures of participants at the follicular and luteal phases. Systolic and diastolic blood pressures and the mean arterial pressure at the luteal phase are significantly higher (P <0.001) than at

the follicular phase, pulse rate at the follicular phase is significantly (P <0.001) higher at the follicular phase than the luteal phase. Backache, wish to be alone, joint or muscle pain, fatigue, pain in the thigh, and acne/pimples are the six most severe and most commonly reported symptoms (Table 3). More than 50% of the subjects experienced the first eleven PMS symptoms. Most of these symptoms were affective symptoms.

Determination of Clusters of PMS

Table 4 showed the factor loadings, estimated separately for the four factors. Affective symptoms have the highest loadings while GIT/physical symptoms have the lowest symptoms. Depression correlated the most. The correlation decreases in the following order by aggressiveness, confusion, difficulty concentrating, crying spell, fatigue, dizziness, and wish to be alone. The Eigenvalues of factors one, two, three, and four are respectively 4.82, 1.59, 1.48, and 1.41. These four factors explained over 58% (not shown). Data reduction technique, Principal Component Analysis (PCA) starting with squared multiple correlations as the prior communality estimates to extract the principal factors from the 23 symptoms of PMS was performed. After performing preliminary Principal Factor Analysis of the symptom data and after rotation to allow for correlation between factors, four factors were identified using the criterion of the proportion of total variance explained. Sequel to rotation, factor 1 was an "affect" factor (high loadings from depression, aggressiveness, confusion, difficulty concentrating, crying spell, fatigue, dizziness, and wish to be alone). Factor 2 "were physical symptoms" (loading from oily skin, overeating, and acne). Factor 3 were "somatic symptoms" (loading from pain in the thigh, joint pain, backache, and low appetite). Factor 4 were "GIT/physical symptoms" (loading from nausea, diarrhoea, sweating, constipation, reduced urine, and excessive sleep). Symptoms of headache and sleeplessness did not load >3.0 on any of the four factors. Factors are extracted if their Eigenvalues are >1.0. The Eigenvalues of the fourth, third, and second factors are all >1 but substantially less than that for the first factor (4.81). Inter-factor correlations ranges from 0.03 to 0.37. Examination of the scree plot confirmed the appropriateness of selecting only one factor to explain the data.

DISCUSSION

Given the efforts made over the years to gain a better sense on the cause and possibly treatment of PMS and how much it affects the social and wellbeing of women that report suffering from it, over 89% (329) out of the 370 undergraduate students in our study reported experiencing at least one or more symptoms of PMS during each cycle. The findings in the present study is in keeping with previous studies in Nigeria (Antai *et al.*, 2004; Udezi and Ochei, 2014). This finding is also similar to that earlier reported by (Obeidat *et al.*, 2012) who reported a prevalence rate of 91.5% among Jordanian college students. Our result is also in keeping with previous findings (Angst *et al.*, 2001, Takeda *et al.*, 2006, Danborno and Oyibo, 2008; Victor *et al*., 2015). Derman and colleagues (2004) reported an incidence rate of 61.4% among Turkish adolescent girls and more recently, Cheng and colleagues (2013) reported an incidence rate of 39.85%.

The discrepancy in physiological state of subjects at follicular and luteal phase is striking. Their SBP, DBP, and mean arterial pressure at the menstrual phase are significantly higher than at the follicular phase (Table 3). These insights suggest the role of emotional state and endocrinological underpinnings of the subjects. Because the participants to this study were students, stress might also play a role in raising their BP.

Increased adrenaline increases the force of contraction of the heart and cardiac output. DBP decreases with increase in adrenaline by reducing total peripheral resistance. The association between anxiety and PMS has been reported previously (Gold *et al*., 2007; Obeidat *et al.*, 2012; Cheng *et al*., 2013). Both studies reported positive association between premenstrual anxiety and PMS.

However, there are other factors that were believed to cause symptoms of PMS that have also been associated with rise in BP such as excessive intake of salt and dairy products (Abdul-Razzak *et al.*, 2010). In addition to excessive salt intake and dairy products, other dietary intake that could contribute to higher BP are consumption of diets inadequate in vitamin D and calcium. Vitamin D and calcium have also been associated with the severity of PMS. The nexus between vitamin D and depression in women with PMS was unmasked by Bertone-Johnson in 2009. Calcium, a major component of bones and teeth and a micronutrient needed for blood clotting, proper functioning of heart and nervous has been implicated in severe PMS (Penland and Johnson, 1993; Thys-Jacobs *et al.*, 1998). The Dietary Approaches to Stop Hypertension (DASH, 2011) studies found significant reduction in BP of subject who consumed a low-fat diet rich in fruits and vegetables. For instance, Reed *et al*. (2008) found that women with PMS consume meals rich in fat than women without PMS. It is important to point out that BP at the menstrual phase may be influenced by symptoms of PMS such as emotional state or anxiety, and by many possible interacting lifestyle and dietary factors (though not accounted for in the present study).

In the present study, the 23 symptoms were clustered into four PMS factors namely; affective, physical, somatic, and GIT/physical factors. Symptoms, including depression, aggressiveness, confusion, and difficulty concentrating that have previously been

reported among affective symptoms of PMS (Danborno and Oyibo, 2008; Reiber, 2009; Obeidat *et al.*, 2012; Cheng *et al.*, 2013; Victor *et al*., 2015). Although previous studies have reported findings from several factor analyses of PMS, reported clusters or factors extracted are not in tandem according to the symptoms considered. The variance may be due to the symptoms included, study design (retrospective or prospective), sample population, age group, extraction method adopted (PCA, unweighted least squares, generalized least squares, etc.), factor analysis rotation among others (York *et al*., 1989; Boyle, 1992; Rivera-Tovar *et al*., 1992; Bancroft *et al.*, 1993; Condon, 1993; Bancroft, 1995; Mira *et al*., 1995; Freeman *et al*., 1996). Most other studies which findings are in consonant to ours also used prospective daily ratings of severity of symptoms (Gehlert *et al.*, 1999; Woods et al. 1999). Most single factor studies are retrospective in nature, combining an annual symptom experience rather than case-by-case consideration (Kendler *et al.*, 1992; Treloar *et al.*, 2002).

In conclusion, our findings indicate high prevalence rate of PMS. Blood pressure at late luteal phase of menstrual cycle is significantly higher than at the follicular phase. Backache, wish to be alone, joint or muscle pain, fatigue, pain in the thigh and acne/pimples are the six most commonly reported symptoms. Four factors or clusters (affective, physical, somatic and GIT/physical symptoms) explained data on the 23 symptoms of PMS.

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Short Communication

A Comparative Study of Different Body Fat Measuring Instruments

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Summary: There is an increase in the occurrence of obesity worldwide. The purpose of this study was to compare various convenient and affordable body fat measuring instruments in man to percentage body fat calculated using skinfold thickness to ascertain if they can be used as a substitute for more expensive` gold standard instruments used for measuring body fat. Seventy male students (20-30years) of the University of Benin where recruited in this study. Subjects were non-athletes without systemic disease, liposuction and not on routine medication. All measurements were taken between 7am and 10am daily. Subjects came fasting refrained from exercise 12 hours before the study and body weight (kg) was measured with a digital weighing scale. A standiometer, was used to measure height (m). BMI (kg/m^2) was calculated from weight and height. The Waist Circumference (WC) (cm) and Hip Circumference (HC) (m) of each subject were measured using a measuring tape. The Waist-Hip-Ratio (WHR) was calculated by dividing the subject's WC by the HC. Skinfold thickness (mm) of the chest, abdomen and thigh were taken with a calibrated Lange skinfold caliper. Body density (BD**)** values were calculated using the skinfold equation of Jackson and Pollock for men. Body fat percentage (%BF) was calculated from BD using the formula of Siri, with respect to the age of each individual. Results were presented as means ± S.E.M. Microcal origin 8.0 was used to analyze collected data and correlation studies were used to investigate the relationship between groups. *P* values less than 0.05 were considered statistically significant. BMI, WC, HC, skinfold thickness (abdomen, thigh and Chest), weight and estimated lean body mass were positively correlated with %BF in our study population while WHR and height were weakly and negatively correlated with %BF respectively. These alternative means of assessing body fat may be useful when more sophisticated methods are unavailable.

Keywords*:* Percentage Body fat percentage, Obesity, Skinfold thickness, Body Mass Index.

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INTRODUCTION

Obesity is an international health problem for children, adults, and the elderly (Popkin and Doak, 1998). It can lead to the development of type II diabetes, enhance risk factors for cardiovascular and related diseases, and is associated with increased cancer risk and renal failure. Childhood obesity foreshadows its persistence into and through adulthood (Guo *et al*., 2002) and obesity is becoming a common problem among the elderly (Seim and Holtmeier, 1993). Obesity is generally displayed as excess adipose tissue and a high body weight, but in some elderly persons and others with limited mobility it takes the form of sarcopenic obesity, in which a preferential loss of muscle tissue increases the percentage of body fat (Arterburn *et al*., 2004).

By 2025, it has been projected that approximately three billion people will be overweight worldwide; of these, 700 million will be obese. The assessment of obesity does not depend solely on the measurement of an individual's total body mass but also on body composition and fat distribution. Body composition can be measured by various techniques, including highly sophisticated and accurate methods like densitometry, plethysmography, underwater weighing, nuclear magnetic resonance and Dual-energy X-Ray Absorptiometry (DXA). However, these methods are complex and expensive, and their use in clinical practice and large epidemiological studies is limited (Horie *et al.*, 2008).

Measurements of body mass index (BMI), waist-hip ratio (WHR), waist circumference (WC) , and body composition assessments using skinfold thickness (ST) and bioelectrical impedance analysis (BIA) have been widely used due to their convenience and relatively low cost (Rezende *et al*., 2007).

BMI [BMI = weight (kg)/height (m)^2] has been the most widely used index for assessing weight status (WHO, 1995) due to its simplicity, ease of application, reduced demand for training and reliance on less expensive equipment.

The WC is a qualitative measurement of the central distribution of body fat and is considered a strong indicator of visceral fat, thereby predicting metabolic risks and potential chronic disease burden. Like WC,

WHR indicates the type of fat distribution and an individual's risk for developing disease (Huxley *et al*., 2010).

ST is a measure of body composition that allows the indirect estimation of body density and percent body fat (%BF) by means of equations. It is one of the most widely used assessment methods because it is easy to perform in daily practice, is cost effective, and is highly correlated with total body fat.

Few studies in the literature have assessed the ability of these methods to measure body fat among individuals, especially the overweight and obese persons. The assessment of body composition in these individuals can assist in identifying risks of co-morbidities and monitoring their evolution in clinical practice (Horie *et al*., 2008). It is also useful for research purposes.

It is important to know if these various convenient and affordable body composition techniques correlate well with body fat% so they can be used as a substitute for expensive gold standard instruments used for measuring body composition. The purpose of this study therefore was to compare different affordable body fat measuring instruments in man to body fat percentage calculated using the skinfold thickness.

MATERIALS AND METHODS

Subjects

Seventy male (70) students in the University of Benin aged between twenty (20) to thirty (30) years where recruited for this study questionnaires were used to rule out presence of any medical ailment, liposuction and use of routine medication subjects were not athletes. A signed informed consent was obtained from all subjects after explaining to them the objectives and procedures of the study.

Sampling

The study selection was based on the method of random selection sampling which was unbiased.

Procedures

All measurements were taken in the morning hours between 7am and 10am. Subjects were asked to come fasting and to refrain from exercise for at least 12 hours before the study (Chahar, 2014). The following measurements where then taken;

Measurement of Body Weight

The weight was measured with a digital weighing scale calibrated in kilograms and recorded for all subjects.

Measurement of Height

A standiometer, calibrated in meters was used. The measurement was done with subjects standing upright and bare footed.

Body Mass Index (BMI)

The Subjects' body weight in kilograms divided by the square of the height in metres was calculated to determine the BMI for each subject.

Waist Circumference (WC)

The WC (cm) of each subject was measured using a measuring tape.

The measurement was taken while standing tall with feet as close together as possible. It was measured directly against the skin and subjects were asked to breathe out normally. The tape was snug, without compressing the skin. It was measured halfway between the lowest rib and the top of the hipbone, roughly in line with the umbilicus. Multiple measurements were taken until they are within ¼ inch of each other. An average of the results was recorded.

Hip Circumference (HC)

The HC (cm) was also measured with a measuring tape. It was measured at the largest circumference around the hip. Measurements were taken while standing tall and relaxed with the feet together (or as close together as possible). The measuring tape was kept horizontally all the way around the hip and buttock. Multiple measurements were taken until they were within ¼ inch of each other. An average of the results was recorded.

Waist Hip Ratio (WHR)

The WHR was calculated by dividing the subject's WC by the HC.

Skinfold Thickness (ST) Measurement

ST measurements were taken on the right side of the body with a calibrated Lange skinfold caliper to the nearest 1.0 mm. The same instrument was used throughout the study. The measurement was taken on the following sites of the body; chest, abdomen and thigh.

Body density (BD) values were calculated using the skinfold equation of

Jackson and Pollock for men (Jackson and Pollock, 1978). Percent body fat was calculated from BD using the formula of Siri, with respect to the age of each individual.

Body Density = 1.1093800 – (0.0008267 x sum of skinfolds) + 0.0000016 x (sum of skinfolds) 2 - 0.0002574 x age.

% Body Fat= [(4.95/BD) – 4.5] 100.

Statistical Analysis

Results were presented as means ± S.E.M. Microcal origin 8.0 was used to analyze collected data. Pearsons' correlation coefficients (*r*) were calculated to assess the link and the degree of relation between percentage body fat and all the body fat measurements were. *P* values less than 0.05 were considered statistically significant.

RESULTS

The relationship between waist circumference (WC) and Hip Circumference (HC) and percentage body fat (%BF) showed a positive correlation, stronger than that observed in BMI and %BF. A weak correlation was observed between waist-hip ratio (WHR) and percentage body fat (%BF). Strong positive correlations were observed between skinfold thickness (abdomen, Chest and Thigh) and percentage body fat (%BF). With the Abdomen and Thigh strongly more correlated to %BF than that of the Chest (figure 1).

A negative correlation was observed between height and percentage body fat (%BF). Thus, height may not a reliable method in estimating body fat composition. In this study we also observed a positive correlation between weight and percentage body fat (%BF). A weak positive correlation was observed between estimated lean body mass and percentage body fat (%BF), (figure 2).

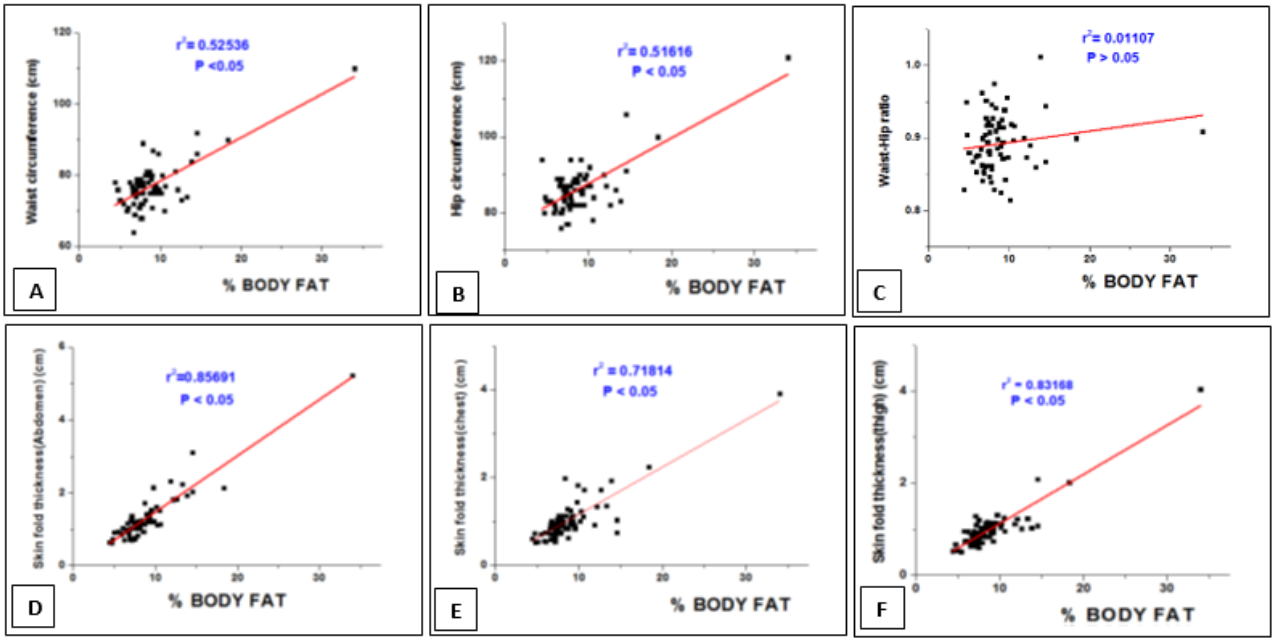

Figure 1. Relationship between percentage body fat and (A) Waist Circumference, (B) Hip Circumference, (C) Waist-Hip Ratio, (D) Abdominal Skin fold thickness, (E) Chest Skin fold thickness and (F) Thigh Skinfold thickness.

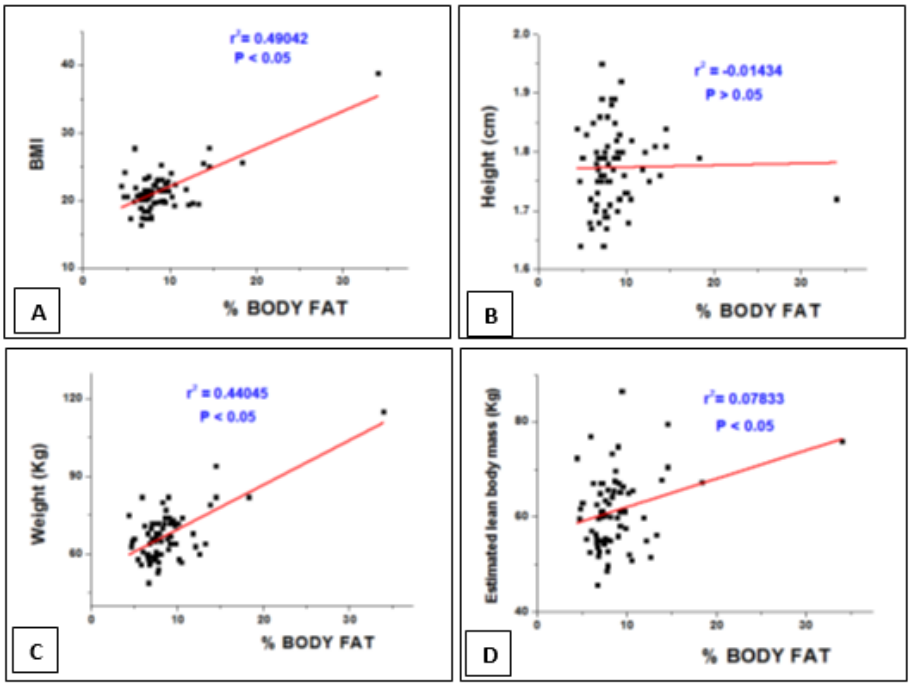

Figure 2. Relationship between percentage body fat and (A) Body Mass Index, (B) Height, (C) Weight and, (D) Estimated lean body mass.

DISCUSSION

Many clinical and epidemiological studies have confirmed the existence of a close relationship between the distribution of body fat, metabolic disorders and increased risk of morbidity and mortality. Thus, the main prognostic problem in obesity is to estimate accurately the quantity and distribution of fat in the body. There however is the problem of most of the gold standard methods for assessing body fat being expensive or even unavailable in this part of the world.

From our study, Body Mass Index (BMI) was positively correlated with percentage body fat (%BF). Ranasinghe *et al*. (2013) reported a strongly positive correlation between BMI and % BF estimated using bioelectric impedance. Meeuwsen (2010) however, documented a not so good relationship between BMI and %BF on a study performed on male UK adults particularly when the BMI is less than 25 kg/m^2. BMI values of most of our participants were between less than 25kg/m^2 this could explain why the correlation observed was not as strong as that observed by Ranasinghe *et al*. (2013). Discrepancies observed in these studies, could be as a result of physiological differences in the characteristics of the study populations and different body composition methodology employed as well.

Our work was in accordance with the work of Giugliano and Melo, (2004) and Freitas *et al*. (2009) who documented significant positive correlations observed between %BF and BMI, WC, HC, skinfold thickness (abdomen), skinfold thickness (thigh), skinfold thickness (chest), weight and estimated lean body mass. WHR and height demonstrated a weak and non-significant correlation with %BF in accordance with WHO, (1995).

Overweight and obesity increase the risk of high blood pressure, reproductive disorders, coronary heart disease, ischemic heart disease, ischemic stroke, type 11 diabetes mellitus and certain cancer Tesfaye *et al*., (2007) hence these cheaper, accurate and available methods of assessing body composition are necessary for screening, diagnosis and research purposes.

In this work, we observed that BMI, WC, HC, skinfold thickness (abdomen), skinfold thickness (thigh), skinfold thickness (chest), weight and estimated lean body mass were positively correlated with %BF in our study population while WHR and height were weakly and negatively correlated with %BF respectively, Our findings support using these alternative means of assessing body fat to predict obesity when more sophisticated methods are unavailable.

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Effects of Honey on Postprandial Hyperlipidemia and Oxidative Stress in Wistar Rats: Role of HMG-CoA Reductase Inhibition and Antioxidant Effect

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Summary: Postprandial hyperlipidemia is associated with oxidative stress and is an important risk factor for atherosclerosis and cardiovascular disease. The aims of this study were to investigate the antihyperlipidemic effect of honey administered 5 or 60 minutes before a high-fat diet (HFD), to explore the role of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase in antihyperlipidemic effect of honey and to investigate the effect of honey on postprandial oxidative stress. Rats were fasted and randomized into 5 groups. Groups 1 and 2 were administered portable water. After 60 minutes, the groups were given portable water and HFD, respectively. Group 3 was administered honey. After 5 minutes, the rats were given HFD. Groups 4 and 5 were administered honey and simvastatin, respectively. After 60 minutes, the rats were given HFD. Four hours after portable water or HFD administration, the rats were sacrificed. Group 2 had significantly ($p < 0.01$) higher total cholesterol (TC), triglycerides (TG), low density lipoprotein (LDL) cholesterol, very low density lipoprotein (VLDL) cholesterol, catalase activity and significantly ($p < 0.05$) lower high density lipoprotein (HDL) cholesterol and HMG-CoA: mevalonate ($p < 0.001$) compared with Group 1. Group 3 had significantly ($p < 0.01$) higher TG and VLDL cholesterol and lower HMG-CoA: mevalonate compared with Group 1. Groups 4 and 5 exhibited significantly ($p < 0.05$ or $p < 0.001$) higher HDL cholesterol and HMG-CoA: mevalonate and lower LDL cholesterol compared with group 2. Honey pretreatment 60 minutes before HFD feeding exerts more significant antihyperlipidemic effect and attenuates more considerably postprandial hyperlipidemia-induced oxidative stress than honey administered 5 minutes before HFD in Wistar rats. This marked antihyperlipidemic effect of honey pretreatment is mediated in part via inhibition of HMG-CoA reductase.

Keywords*:* Honey, Postprandial hyperlipidemia, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase, Oxidative stress

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INTRODUCTION

Hyperlipidemia is a main risk factor that triggers atherosclerosis and coronary heart disease (CHD) (Mensink *et al.*, 2003). Hence, reduction of hyperlipidemia is an important therapeutic strategy aimed at reducing atherosclerosis and preventing other vascular events including CHD and cerebrovascular disease (Collins *et al.*, 2004). Statins remain the most prescribed drugs for the treatment of hyperlipidemia. They competitively inhibit 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase (Goldstein and Brown, 1990). This enzyme is a rate-limiting enzyme which plays an important role in the biosynthesis pathway of cholesterol where it catalyzes the conversion of HMG-CoA to mevalonate (Friesen and Rodwell, 2004). To derive maximal therapeutic benefits, many patients require long-term statin therapy. However, prolonged treatment of statins is associated with serious adverse effects including muscle weakness, rhabdomyolysis, liver damage and cognitive problems (Golomb and Evans, 2008). Consequently, treatment of dyslipidemia and associated disorders with agents that are devoid of adverse effects or have fewer side effects is desirable (Peluso *et al.*, 2014). Besides fasting hyperlipidemia, postprandial hyperlipidemia is an important risk factor for cardiovascular diseases (Pirillo *et al.*, 2014). The lethality of postprandial hyperlipidemia is reinforced

by evidence showing that postprandial hyperlipidemia is a stronger predictor of cardiovascular risk than fasting hyperlipidemia (Mora *et al.*, 2008). Postprandial hyperlipidemia is associated with endothelial dysfunction and inflammation (Lee *et al.*, 2002, van Oostrom *et al.*, 2004). Available evidence suggests that the deleterious effect of postprandial hyperlipidemia is mediated via induction of oxidative stress (Bae *et al.*, 2001). Hence, the importance of dietary interventions aimed at mitigating postprandial hyperlipidemia and the accompanying oxidative stress cannot be overemphasized.

It is therefore not surprising that, in the recent decade, there has been an upsurge in the utilization of complementary and alternative medicines in the treatment of several diseases such as diabetes mellitus and hyperlipidemia (Nies *et al.*, 2006, Erejuwa, 2014). In addition to fewer episodes of side effects, most of the complementary, alternative or traditional medicine agents are of natural origin and cheaper though efficacy and safety remain major concerns (Qidwai *et al.*, 2014). Honey is one of such natural agents with several attributed pleiotropic effects (Erejuwa *et al.*, 2012; Kolawole *et al.*, 2015). Honey supplementation in diabetic rats was associated with amelioration of lipid abnormalities and reductions in coronary and cardiovascular risk indices (Erejuwa *et al.*, 2016). In combination with metformin or glibenclamide, honey administration markedly enhanced the antihyperlipidemic effect of these drugs (Erejuwa *et al.*, 2011, Nasrolahi *et al.*, 2012). Emerging evidence suggests honey has an ameliorative effect on obesity anthropometric parameters in rodent models of obesity (Erejuwa *et al.*, 2017a, Samat *et al.*, 2017). Recently, it was demonstrated that combination of honey with simvastatin resulted in augmented amelioration of body mass index and adiposity in HFD fed Wistar rats (Erejuwa *et al.*, 2017b). Currently, the mechanism of antihyperlipidemic and anti-obesity effects of honey is unknown. Likewise, the mechanism by which honey enhanced the effects of metformin, glibenclamide or simvastatin in ameliorating lipid abnormalities and adiposity is unclear. Therefore, this study was performed in Wistar rats with the following three aims: (i) To investigate and compare the antihyperlipidemic effect of honey administered 5 or 60 minutes before HFD feeding, (ii) To explore the potential role of HMG-CoA reductase in the antihyperlipidemic effect of honey, (iii) To investigate the effect of honey on postprandial hyperlipidemia-induced oxidative stress.

MATERIALS AND METHODS

Chemicals

Sodium arsenate, hydroxylamine hydrochloride and thiobarbituric acid were purchased from Sigma-Aldrich, MO, USA. All other reagents used were of analytical grade.

Animals

Male and female Wistar rats were obtained from animal house unit, Nsukka, Nigeria. The rats were allowed to acclimatize for 10 days. The rats were housed separately in plastic cages in an animal room. The animal house was well ventilated and had a temperature of 26 ± 2 °C and a 12-hour light:dark cycle. The rats were provided rat chow and drinking water *ad libitum*. The study protocol was approved by the Research Ethics Committee of Ebonyi State University (EBSU/DRIC/UREC/Vol. 04/005). The handling of rats strictly followed institutional and international guidelines on the Use and Handling of Laboratory Animals.

Honey

The honey was obtained from a bee farm in Abakaliki, Ebonyi State, Nigeria. The honey had a NAFDAC (National Agency for Food and Drug Administration Control) number. The honey was purchased from a bee farm registered with NAFDAC to ensure the honey used in the study was original, genuine and unadulterated. The honey was dissolved in drinking water (1:1) before administration. It was administered at a dose of 1.0 g/kg body weight (BW). The choice of this dose was based on findings from our previous studies in which 1.0 g/kg BW was demonstrated to be the optimal dose in ameliorating hyperlipidemia, excess weight gain, adiposity and body mass index in diabetic and obese rats (Erejuwa *et al.*, 2016, Erejuwa *et al.*, 2017a).

Treatment

The Wistar rats were fasted for about 36 hours. The fasted rats were randomly divided into 5 groups. Each group consisted of 5 rats. The rats were treated as follows:

Group 1: Fasted rats were administered portable water (1.0 ml/kg BW). After 60 minutes, the rats were given portable water (1.0 ml/kg BW) (control).

Group 2: Fasted rats were administered portable water (1.0 ml/kg BW). After 60 minutes, the rats were given HFD (5.0 ml/kg BW) (HFD only).

Group 3: Fasted rats were administered honey (1.0 g/kg BW). After 5 minutes, the rats were given HFD (5.0 ml/kg BW) (Honey5+HFD)

Group 4: Fasted rats were administered honey (1.0 g/kg BW). After 60 minutes, the rats were given HFD (5.0 ml/kg BW) (Honey60+HFD)

Group 5: Fasted rats were administered simvastatin (20 mg/kg BW). After 60 minutes, the rats were given HFD (5.0 ml/kg BW) (Simvastatin+HFD)

All the various agents (portable water, honey, simvastatin or HFD) were administered orally using a gavage needle. Before administering the agents, body weight was measured using a weight measuring scale.

The administered HFD was a mixture of olive oil and coconut oil (1:1). After 4 hours of HFD administration, the rats were sacrificed under diethyl ether anesthesia. Using plain tubes, blood samples were collected. The plain tubes containing the blood samples were left to stand at room temperature for about 3 hours. The clotted blood samples were centrifuged at 1500 rpm for 20 min. The serum samples were collected and used for analyses of lipid profile. A portion of the serum samples was used for the assay of catalase activity, malondialdehyde, total antioxidant status and total protein. The livers were rapidly harvested and used for the assay of HMG-CoA reductase activity.

Biochemical analysis

The serum samples were used for the estimation of total cholesterol (TC), high density lipoprotein (HDL) cholesterol and triglycerides (TG). The TC, HDL cholesterol and TG were measured using commercially available kits (Agappe Diagnostics, Knonauerstrasse, Cham, Switzerland) on UV-Visible spectrophotometer (752 UV-VIS Spectrophotometer, China) based on the manufacturer's instructions. The LDL and VLDL cholesterol were calculated using the Friedewald equations (Friedewald *et al.*, 1972).

LDL cholesterol $= TC - HDLc - VLDLc$

VLDLc $= {}^{TG}/_{5}$

Estimation of non-HDL cholesterol, atherogenic index, coronary risk index and cardiovascular risk index

Non-HDL cholesterol, atherogenic index, coronary risk index and cardiovascular risk index were estimated from measured lipid profile parameters using the formulae below (Abbott *et al.*, 1988, Alladi *et al.*, 1989).

Non-HDLc $= TC - HDLc$

Atherogenic index $= {}^{LDLc}/_{HDLc}$

Coronary Risk Index $= {}^{TC}/_{HDLc}$

Cardiovascular Risk Index $= {}^{TG}/_{HDLc}$

Assay of HMG-CoA reductase activity

The activity of HMG-CoA reductase was determined in liver homogenate according to the method of Rao and Ramakrishnan, 1975 (Rao and Ramakrishnan, 1975). In this procedure, the concentrations of HMG-CoA and mevalonate in liver homogenates are assessed in terms of absorbances. The ratio of HMG-CoA to mevalonate is considered an index of HMG-CoA reductase activity that catalyzes the conversion of HMG-CoA to mevalonate. Briefly, equal volumes of 10% liver homogenate was mixed with dilute perchloric acid and kept for 5 min. The mixture was centrifuged at 2 000 × g for 10 min. The hepatic HMG-CoA was measured by its reaction with alkaline hydroxylamine reagent. Similarly, the hepatic mevalonate was determined by its reaction with acidic hydroxylamine reagent. After 10 min, the absorbance readings were taken at 540 nm against a similarly treated saline-arsenate blank. The HMG-CoA: mevalonate ratio is inversely proportional to the activity of HMG-CoA reductase. Higher HMG-CoA: mevalonate indicates lower activity of HMG-CoA reductase and vice versa.

Assay of catalase activity

The activity of catalase was determined as described by Goth (Gott, 1991). Briefly, the method entails incubating a test tube containing H_2O_2 (0.5 mL) and serum (0.1 mL). Addition of ammonium molybdate (0.5 mL) terminated the reaction after incubation at 37 °C for 60 seconds. This was followed by the measurement of absorbance of the yellow complex of ammonium molybdate and H_2O_2 at 405 nm using a spectrophotometer. One unit of catalase referred to the quantity of enzyme that catalyzes the breakdown of 1 μmol of H_2O_2/min.

Assay of malondialdehyde

Malondialdehyde (MDA) was assayed as thiobarbituric acid reactive substances (TBARS) using the method of Ohkawa and colleagues (Ohkawa *et al.*, 1979). In brief, the test tubes containing serum (100 μL) or MDA standards (100 μL), glacial acetic acid (pH 3.5; 1.5 mL), sodium dodecyl sulphate (200 μL), thiobarbituric acid (TBA) (1.5 mL) and portable water (700 μL) were incubated at 95 °C for 60 minutes. After incubation, the test tubes were allowed to cool and centrifuged at 3000 × g for 10 minutes. The MDA concentration was measured at 532 nm using a spectrophotometer. TBARS concentration was expressed as nmol of MDA per mg protein.

Assay of total antioxidant status

Total antioxidant status (TAS) was assayed using the method of Koracevic et al. (Koracevic *et al.*, 2000). Briefly, sodium benzoate (0.5 mL), Fe-EDTA complex (0.2 mL), and H_2O_2 (2 mL) were pipetted into a test tube containing serum (0.010 mL) and sodium phosphate buffer (0.49 mL). Control (blank) test was also performed for each sample. The assay tubes were vortexed and then incubated at 37 °C for 60 minutes. Acetic acid (1 mL) and TBA were added. The tubes were placed in a water bath containing boiling water for 10 minutes. The tubes were cooled to room temperature and the absorbance was measured at 532 nm against portable water using a spectrophotometer. Serum TAS was estimated using uric acid as standard.

Assay of total protein

The serum protein concentration was measured using Bradford's method (Bradford, 1976). Briefly, 100 uL of serum or protein standards were added to a tube

containing 5 mL of Coomassie Blue. The mixture was vortexed and incubated for 10 min. The absorbance was read at 595 nm against blank. Bovine serum albumin was used as the standard.

Statistical analysis

Data are expressed as mean ± SEM. The results were analyzed using SPSS 16.0. Data were analyzed using one-way analysis of variance (ANOVA) and Tukey's post hoc test. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of honey on HDL cholesterol

The data on the effect of honey on HDL cholesterol are presented in Figure 1. The HDL cholesterol was significantly ($p < 0.05$) lower in control HFD fed rats than in normal control rats. The levels of HDL cholesterol in rats administered honey or simvastatin 60 minutes before HFD feeding were significantly ($p < 0.05$) higher than the level in control HFD fed rats. The level of HDL cholesterol in rats administered honey 5 minutes before HFD feeding was not significantly ($p > 0.05$) different from that of control HFD fed rats.

Effect of honey on LDL cholesterol

The results on the effect of honey on LDL cholesterol are shown in Figure 2. The control HFD fed rats had significantly ($p < 0.05$) higher LDL cholesterol compared with normal control rats. The levels of LDL cholesterol in rats administered honey or simvastatin 60 minutes before HFD feeding were significantly ($p < 0.05$) lower than the level in control HFD fed rats. The level of LDL cholesterol in rats administered honey 5 minutes before HFD feeding was not significantly ($p > 0.05$) lower than that of control HFD fed rats.

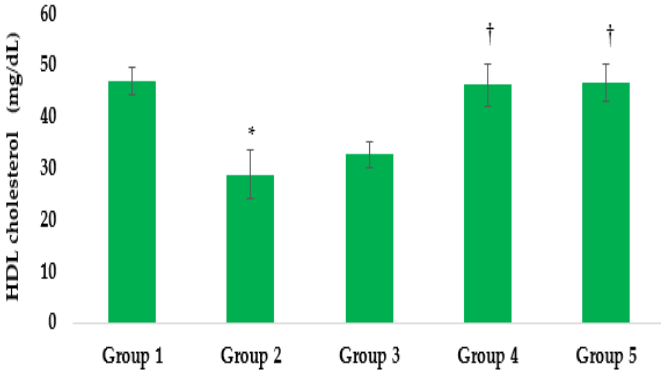

Figure 1. Effect of honey on HDL cholesterol in high fat diet fed rats. Group 1 (control); Group 2 (HFD only); Group 3 (Honey5+HFD); Group 4 (Honey60+HFD); Group 5 (Simvastatin+HFD); * $p < 0.05$ compared with Group 1; † $p < 0.05$ compared with Group 2.

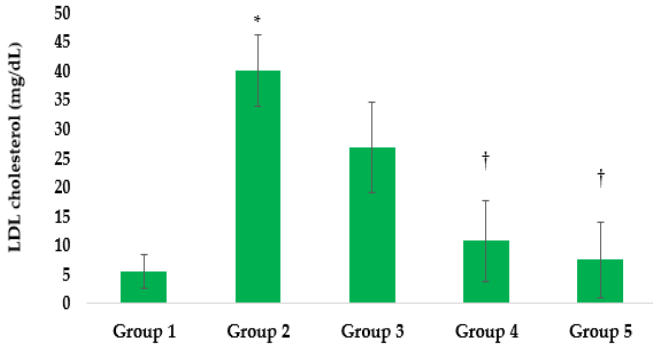

Figure 2. Effect of honey on LDL cholesterol in high fat diet fed rats. Group 1 (control); Group 2 (HFD only); Group 3 (Honey5+HFD); Group 4 (Honey60+HFD); Group 5 (Simvastatin+HFD); * $p < 0.05$ compared with Group 1; † $p < 0.05$ compared with Group 2.

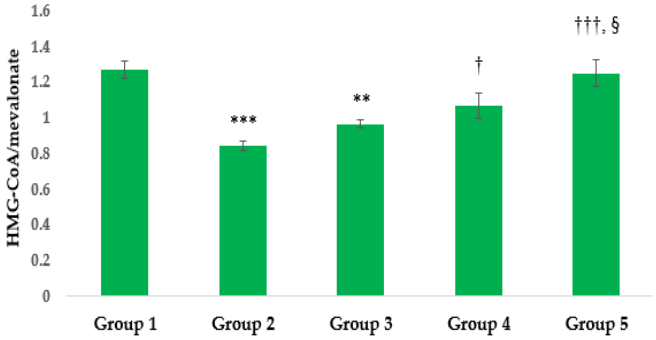

Figure 3. Effect of honey on HMG-CoA/mevalonate in high fat diet fed rats. Group 1 (control); Group 2 (HFD only); Group 3 (Honey5+HFD); Group 4 (Honey60+HFD); Group 5 (Simvastatin+HFD); ** $p < 0.01$ & *** $p < 0.001$ compared with Group 1; † $p < 0.05$ & ††† $p < 0.001$ compared with Group 2; § $p < 0.05$ compared with Group 3.

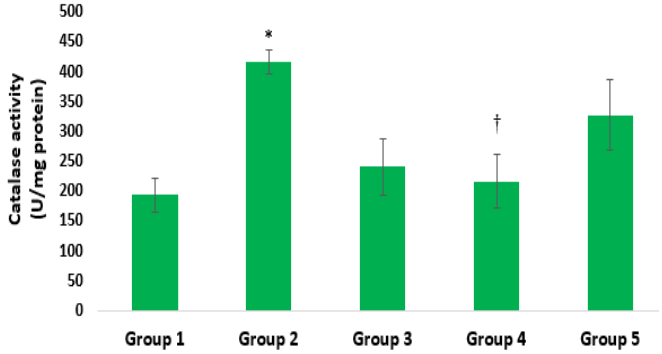

Figure 4. Effect of honey on serum catalase activity in high fat diet fed rats. Group 1 (control); Group 2 (HFD only); Group 3 (Honey5+HFD); Group 4 (Honey60+HFD); Group 5 (Simvastatin+HFD). * $p < 0.05$ compared with Group 1; † $p < 0.05$ compared with Group 2.

Effect of honey on HMG-CoA (3-hydroxy-3-methyl-glutaryl-coenzyme A)/mevalonate

Figure 3 shows the results of the effect of honey on HMG-CoA/mevalonate. The rats administered portable water 60 minutes or honey 5 minutes before HFD feeding showed significantly ($p < 0.01$ or $p < 0.001$) lower HMG-CoA/mevalonate compared with normal control rats. The HMG-CoA/mevalonate in

Table 1. Effects of honey on serum total cholesterol, triglycerides, non-HDL cholesterol and VLDL cholesterol

	Total cholesterol (mg/dL)	Triglycerides (mg/dL)	Non-HDL cholesterol (mg/dL)	VLDL cholesterol (mg/dL)
Group 1	65.2 ± 3.5	65.8 ± 9.7	19.3 ± 3.5	13.2 ± 1.9
Group 2	96.9 ± 3.7 **	155.8 ± 22.5 **	63.4 ± 2.3 ***	31.2 ± 4.5 **
Group 3	85.1 ± 6.1	154.0 ± 15.7 **	56.0 ± 5.2 **	30.8 ± 3.1 **
Group 4	85.9 ± 4.9	110.8 ± 13.4	45.3 ± 8.3 *	20.0 ± 2.0
Group 5	86.7 ± 8.1	104.2 ± 10.4	34.6 ± 6.0 †	20.8 ± 2.1

Group 1 (control); Group 2 (HFD only); Group 3 (Honey5+HFD); Group 4 (Honey60+HFD); Group 5 (Simvastatin+HFD). * $p < 0.05$, ** $p < 0.01$ & *** $p < 0.001$ compared with Group 1; † $p < 0.05$ compared with Group 2

rats administered honey or simvastatin 60 minutes before HFD feeding was significantly ($p < 0.05$ or $p < 0.001$) higher than that in control HFD fed rats. The rats administered simvastatin 60 minutes before HFD feeding had significantly ($p < 0.05$) higher HMG-CoA/mevalonate than the rats administered honey 5 minutes before HFD feeding.

Effect of honey on serum catalase activity

The data on the effect of honey on serum catalase activity are shown in Figure 4. The control HFD fed rats had significantly ($p < 0.05$) higher serum catalase activity compared with normal control rats. The serum catalase activity in rats administered honey 5 minutes before HFD feeding was borderline ($p = 0.054$) lower than that of control HFD fed rats. The serum catalase activity in rats administered honey 60 minutes before HFD feeding was significantly ($p < 0.05$) lower than that of control HFD fed rats.

Effects of honey on serum total cholesterol, triglycerides, non-HDL cholesterol and VLDL cholesterol

The results of the effects of honey on total cholesterol, triglycerides, non-HDL cholesterol and VLDL cholesterol are shown in Table 1. The control HFD fed rats had significantly ($p < 0.01$) higher total cholesterol compared with normal control rats. The levels of total cholesterol in rats administered honey 5 or 60 minutes as well as simvastatin 60 minutes before HFD feeding were statistically non-significantly ($p > 0.05$) different from that of the normal control rats. The control HFD fed rats and rats administered honey 5 minutes before HFD feeding had significantly ($p < 0.01$) higher triglycerides compared with normal control rats. The levels of triglycerides in rats administered honey or simvastatin 60 minutes before HFD feeding were non-significantly ($p > 0.05$) different from that of the normal control rats.

The control rats and rats administered honey 5 or 60 minutes before HFD feeding had significantly ($p < 0.001$, $p < 0.01$ and $p < 0.05$, respectively) higher non-HDL cholesterol compared with normal control rats. The non-HDL cholesterol in rats administered simvastatin 60 minutes before HFD feeding was significantly ($p < 0.05$) lower than that in control HFD fed rats. Rats of control HFD fed rats or rats administered honey 5 minutes before HFD feeding showed significantly ($p < 0.01$) elevated levels of VLDL cholesterol compared with normal control rats. The levels of VLDL cholesterol in rats administered honey or simvastatin 60 minutes before HFD feeding were non-significantly ($p > 0.05$) higher than the level in normal control rats but non-significantly ($p > 0.05$) lower than the level in control HFD fed rats.

Table 2. Effects of honey on serum TC/HDL cholesterol, TG/HDL cholesterol and LDL/HDL cholesterol

Group	TC/HDL cholesterol	TG/HDL cholesterol	LDL/HDL cholesterol
1	1.42 ± 0.08	1.82 ± 0.40	0.11 ± 0.06
2	3.27 ± 0.40**	5.21 ± 0.70**	1.34 ± 0.40
3	2.65 ± 0.19*	4.81 ± 0.58**	0.85 ± 0.25
4	2.33 ± 0.32	3.29 ± 0.51	0.54 ± 0.41
5	1.74 ± 0.12 ††	2.34 ± 0.34††,§	0.28 ± 0.16

Group 1 (control); Group 2 (HFD only); Group 3 (Honey5+HFD); Group 4 (Honey60+HFD); Group 5 (Simvastatin+HFD). * $p < 0.05$, ** $p < 0.01$ compared with Group 1; †† $p < 0.01$ compared with Group 2; § $p < 0.05$ compared with Group 3

Effects of honey on TC/HDL cholesterol, TG/HDL cholesterol and LDL/HDL cholesterol

The data on the effects of honey on TC/HDL cholesterol, TG/HDL cholesterol and LDL/HDL cholesterol are presented in Table 2. The control HFD fed rats and rats administered honey 5 minutes before HFD feeding had significantly ($p < 0.01$ and $p < 0.05$, respectively) higher TC/HDL cholesterol compared with normal control rats. The TC/HDL cholesterol was significantly ($p < 0.01$) lower in rats administered simvastatin 60 minutes before HFD feeding than in control HFD fed rats. The control HFD fed rats and rats administered honey 5 minutes before HFD feeding had significantly ($p < 0.01$) higher TG/HDL cholesterol compared with normal control rats. The TG/HDL cholesterol was significantly ($p < 0.01$ or $p < 0.05$) lower in rats administered simvastatin 60 minutes before HFD feeding than in control HFD fed rats or rats administered honey 5 minutes before HFD feeding. The LDL/HDL cholesterol was non-significantly ($p = 0.057$) higher in control HFD fed rats than in normal control rats. Rats administered honey or simvastatin had non-significantly ($p > 0.05$) lower LDL/HDL cholesterol compared with control HFD fed rats.

Table 3. Effects of honey on serum TAS and MDA

	TAS (U/mg protein)	MDA (U/mg protein)
Group 1	3.63 ± 0.41	4.28 ± 0.30
Group 2	4.17 ± 0.32	4.11 ± 0.25
Group 3	3.71 ± 0.38	4.04 ± 0.21
Group 4	3.65 ± 0.49	4.23 ± 0.45
Group 5	4.66 ± 0.39	4.25 ± 0.55

Group 1 (control); Group 2 (HFD only); Group 3 (Honey5+HFD); Group 4 (Honey60+HFD); Group 5 (Simvastatin+HFD).

Effects of honey on serum total antioxidant status (TAS) and malondialdehyde (MDA)

The results on the effects of honey on serum TAS and MDA are presented in Table 3. Though statistically ($p > 0.05$) insignificant, the control HFD fed rats and rats administered simvastatin 60 minutes before HFD feeding had slightly higher TAS compared with rats of other groups. The levels of serum MDA were comparable in all the groups.

DISCUSSION

The control HFD fed rats had marked elevated levels of total cholesterol and triglycerides indicating development of hypercholesterolemia and dyslipidemia in these rats. The moderate hypercholesterolemia in rats administered simvastatin or honey 5 or 60 minutes before HFD feeding suggests that pre-treatment with honey or simvastatin suppressed elevations in total cholesterol and thus confirms the anti-hypercholesterolemic effect of honey (Al-Waili, 2004). The similarity in the levels of triglycerides in control HFD fed rats and rats fed honey 5 minutes before HFD indicates honey administered 5 minutes before HFD did not exert anti-hypertriglyceridemic effect. The considerably higher levels of triglycerides than that of the normal control group further confirms the lack of anti-hypertriglyceridemic effect of honey administered 5 minutes before HFD. In contrast, the levels of triglycerides in rats administered honey or simvastatin 60 minutes before HFD feeding were moderately but insignificantly higher than that of the normal control group. This indicates that honey administered 60 minutes before HFD exerted anti-hypertriglyceridemic effect in rats. Previous studies have demonstrated the anti-hypertriglyceridemic effect of honey (Erejuwa *et al.*, 2016; Samat *et al.*, 2017). Phenolic-rich compounds have been shown to inhibit hyperlipidemia (Tuzcu *et al.*, 2017, Qinna *et al.*, 2012). Honey is rich in phenolic acids and flavonoids (Erejuwa *et al.*, 2012). Therefore, the effect of honey in suppressing hypercholesterolemia and hypertriglyceridemia may be attributed to honey phenolic and flavonoid content.

The levels of HDL and non-HDL cholesterol in rats pretreated with honey 5 minutes before HFD feeding were similar to that of control HFD fed rats. The non-HDL cholesterol reflects the cholesterol in VLDL, IDL (intermediate density lipoprotein) and LDL particles (Millan *et al.*, 2009). These data therefore suggest that honey pretreatment 5 minutes before HFD did not mitigate against HFD-induced reduction of HDL cholesterol and elevation of non-HDL cholesterol. The significantly higher HDL cholesterol level in rats pretreated with honey 60 minutes before HFD feeding indicates that honey pretreatment 60 minutes before HFD ingestion prevented against HFD-induced reduction of HDL cholesterol. The LDL cholesterol is highly susceptible to oxidation and oxidized LDL is atherogenic (Parthasarathy *et al.*, 2010). On the other hand, VLDL cholesterol is converted to LDL cholesterol via the action of lipoprotein lipase and thus further aggravates atherogenicity (Toth, 2016). Hence, the elevated levels of LDL and VLDL cholesterol in control HFD group and rats pretreated with honey 5 minutes before HFD feeding imply that the rats were highly vulnerable to developing atherosclerosis. The findings, as demonstrated by lower levels of LDL and VLDL cholesterol, also suggest that administering honey 60 minutes before HFD feeding suppressed HFD-induced elevations in LDL and VLDL cholesterol and thus may offer protection against cytotoxicity of oxidized LDL cholesterol. The ameliorative effect of honey on hyperlipidemia may be attributed to diverse constituents in honey. Honey is enriched in numerous bioactive substances including phytosterols which have been shown to enhance cholesterol metabolism (Howell *et al.*, 1998). Epidemiological evidence associates high concentrations of HDL cholesterol with several health beneficial effects including antiatherogenic effect, inhibition of LDL oxidation and healthy endothelial function (Chapman *et al.*, 2011). Therefore, the significant increase in HDL cholesterol and marked reduction in LDL cholesterol in rats pretreated with honey 60 minutes before HFD imply these rats were better protected against atherogenicity, LDL oxidation and impaired endothelial function.

In many disorders such as metabolic syndrome, diabetes mellitus and obesity, evaluation of lipid ratios is considered a stronger predictor of disease risks (Gasevic *et al.*, 2014). The TC/HDL cholesterol, for instance, is a coronary risk index which is used to predict risk of CHD (Ingelsson *et al.*, 2007) whereas the TG/HDL cholesterol reflects cardiovascular disease risk (de Giorgis *et al.*, 2014). The markedly elevated levels of TC/HDL cholesterol and TG/HDL cholesterol in control HFD fed rats and rats pretreated with honey 5 minutes before HFD suggest that honey administered 5 minutes before HFD did not prevent against risks of HFD-induced CHD and cardiovascular disease. The lack of significant differences in TC/HDL

cholesterol and TG/HDL cholesterol in rats pretreated with honey 60 minutes before HFD and normal control group imply the protective effect of honey against HFD-induced cardiovascular disease and CHD risks. The effects of honey in ameliorating postprandial hyperlipidemia and lipid ratios (risk indices) corroborate those of previous studies in diabetic and obese rats (Erejuwa *et al.*, 2011, Nasrolahi *et al.*, 2012, Erejuwa *et al.*, 2016, Samat *et al.*, 2017).

To the best of our knowledge, this is the first study in the literature to explore the potential role of HMG-CoA reductase in mediating the antihyperlipidemic effect of honey. In this study, lower HMG-CoA/mevalonate corresponds to a higher HMG-CoA reductase activity and vice versa. The fact that the levels of HMG-CoA/mevalonate in control HFD fed group and rats pretreated with honey 5 minutes before HFD were significantly lower than the level in the normal control rats suggest that honey administered 5 minutes before HFD feeding did not prevent HFD-enhanced HMG-CoA reductase activity. On the other hand, honey or simvastatin administered 60 minutes before HFD feeding was associated with higher levels of HMG-CoA/mevalonate in Wistar rats. Higher HMG-CoA/mevalonate signifies lower HMG-CoA reductase activity and, thus, implies inhibition of HMG-CoA reductase in these rats. Since the literature lacks data on the effect of honey on HMG-CoA reductase, we are unable to compare our findings with those of other researchers. Plant extracts with phytochemicals including phenolic compounds and flavonoids have been shown to inhibit HMG-CoA reductase *ex vivo* (Kwon *et al.*, 2010, Reddy *et al.*, 2014) and *in vivo* (Qinna *et al.*, 2012). Honey consists of numerous bioactive substances such as phenolic compounds which play an important role in its biological effects (Erejuwa *et al.*, 2012). Therefore, the suppression/inhibition of HMG-CoA reductase following honey pretreatment may be attributed to the high phenolic and flavonoid constituents in honey. Our findings, which showed that simvastatin pretreatment caused vast inhibition of HMG-CoA reductase, agrees with data from previous studies demonstrating statins as potent inhibitors of HMG-CoA reductase (Qinna *et al.*, 2012). The findings also revealed that honey pretreatment 60 minutes before HFD was more effective than honey administered 5 minutes before HFD in inhibiting hepatic HMG-CoA reductase. This inference is also substantiated by the serum data which indicated that honey pretreatment 5 minutes before HFD was less effective in ameliorating lipid abnormalities and disease risk indices.

Oxidative stress is associated with several diseases including diabetes mellitus and obesity and is considered a potential therapeutic target in these disorders (Erejuwa, 2012). The role of oxidative stress is implicated in the injurious effects of postprandial hyperlipidemia (Bae *et al.*, 2001). Oxidative stress has been demonstrated in HFD fed rats (Venkateshan *et al.*, 2016). In both healthy and obese subjects, postprandial hyperlipidemia has been shown to generate reactive oxygen species (ROS) and cause oxidative damage (Patel *et al.*, 2007, Stojiljkovic *et al.*, 2002). Some of the commonly generated ROS include superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2). Superoxide dismutase catalyzes the conversion of $O_2^{\bullet-}$ to H_2O_2. Though H_2O_2 is more stable than $O_2^{\bullet}$, it generates a highly reactive hydroxyl radical ($^{\bullet}OH$). H_2O_2 is converted to water and oxygen in the presence of catalase (Haber and Weiss, 1934). The catalysis of H_2O_2 by catalase, therefore, helps to prevent the formation and cellular buildup of $^{\bullet}OH$. The results showed that serum catalase activity was significantly higher in control HFD fed rats than in normal control group. Enhanced activity or expression of certain enzymes including catalase and nitric oxide synthase has been demonstrated following increased levels of H_2O_2 (Tiedge *et al.*, 1999, Cao *et al.*, 2011). Therefore, the higher catalase activity in portable water-pretreated HFD fed rats may imply catalase induction in response to acute production of H_2O_2. The levels of serum catalase activity in rats pretreated with honey 5 or 60 minutes before HFD were similar compared with normal control rats. Hence, it can be inferred that honey pretreatment suppressed postprandial hyperlipidemia-enhanced catalase activity in rats. This is a confirmation that induction of catalase in portable water-pretreated HFD fed rats is an adaptive mechanism in response to increased ROS formation (Crawford and Davies, 1994). This is usually a protective mechanism aimed at preventing or reducing ROS-mediated damage. Compelling evidence indicates honey is a novel antioxidant (Erejuwa *et al.*, 2012). It is therefore not surprising that honey pretreatment prevented HFD-induction of catalase activity or restored catalase activity towards that of normal control group. The antioxidant effect of honey is due to its high phenolic and flavonoid constituents (Erejuwa *et al.*, 2012). These bioactive constituents are free radical scavengers.

In comparison with honey administered 5 minutes before HFD, honey pretreatment 60 minutes before HFD was more effective in preventing postprandial HFD induction of catalase activity in rats. Plant extracts rich in phenolic acids have also been reported to ameliorate hyperlipidemia-induced oxidative stress in HFD fed rats (Sarega *et al.*, 2016). In addition to catalase activity, this study explored the effect of honey on postprandial oxidative stress by assessing TAS and MDA. The study revealed that there was no significant difference in TAS among the groups. The evaluation of TAS reflects the overall contribution of the distinct antioxidants (enzymatic and non-enzymatic) present in a sample (Ghiselli *et al.*, 2000).

A slight induction of TAS in control HFD and simvastatin-pretreated HFD fed rats was however observed. This trend was similar to what was detected in catalase activity. Therefore, this slight induction of TAS in the two groups may be a contributory role of catalase in the assay of TAS. Determination of MDA is usually performed in oxidative stress studies to assess the extent of lipid peroxidation (Halliwell and Gutteridge, 1984). The results indicated that the levels of MDA were comparable among the groups. The lack of significant increase in MDA concentrations in control HFD fed rats, despite considerable postprandial hyperlipidemia, may be a consequence of enhanced catalase activity. Induction of catalase activity, an adaptive or defense mechanism, would result in increased catalysis of accumulated H_2O_2 to water and oxygen. This would invariably prevent or diminish the production of $^{\bullet}OH$. In the absence or low level of $^{\bullet}OH$, there would be no further propagation of ROS nor ROS mediated-lipid peroxidative damage and thus no change in MDA. Therefore, the lack of significant change in MDA in control HFD fed rats may imply that the induced activity of catalase was protective against lipid peroxidation.

In conclusion, honey administered 60 minutes before HFD feeding exerts more profound antihyperlipidemic effect than honey administered 5 minutes before HFD in Wistar rats. This marked antihyperlipidemic effect of honey pretreatment is mediated in part via inhibition of HMG-CoA reductase. Honey pretreatment also attenuates postprandial hyperlipidemia-induced oxidative stress in rats.

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Influence of Nanosilver on Endothelial Function and Vascular Reactivity of Isolated Rabbit Carotid Artery

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Summary: There is paucity of information on the effects and mechanism of action of Nanosilver on vascular tone and endothelial function in spite of the upsurge in nanotechnology application in biomedicine. The present study determined the effect of Nanosilver on vascular reactivity and endothelial function on isolated rabbit carotid artery in standard laboratory 20 mL organ bath procedures containing physiological salt solution (PSS) bubbled with 95% O_2, 5% CO_2. Isometric contractions were recorded electronically with a 4-channel Grass Polygraph and maintained at 37ºC and pH7.4. Cumulative dose response tests to α-receptor agonist phenylephrine (PE) was examined separately, in normal PSS (control) and following 20 minutes exposure to varying concentrations of Nanosilver solution [(NAgs) (1.25 and 2.50)] µg/mL in endothelium intact (+E) (control) and endothelium denuded (-E) rings. Contractile responses were analysed with reference to maximal contractions induced by 8 x 10^{-2} M K^+ in normal PSS. In another experiment, arterial rings were precontracted with EC_{70} M PE, high and /or low (8, 2 x 10^{-2}) M K^+PSS. At stable contractions, cumulative relaxation responses to NAgs was studied. Relaxation responses were analysed with reference to maximal contraction induced by EC_{70} M PE and/or K^+ depolarization in normal PSS. Following 20 minutes exposure to NAgs, dose relaxation response to acetylcholine (ACh) was also examined in normal PSS (control), and pre-incubated L-NAME (NO synthase inhibitor) and indomethacin (cyclooxygenase inhibitor) precontracted arterial rings to further determine mechanisms of action. Data were presented as Means ± SEM. Graphs and statistical analysis were done using GraphPad prism version 7.03 and Student t-test. P-values ($P< 0.05$) were considered statistically significant. The results showed that nanosilver decreased maximum contraction (Emax) and induced attenuated contractile and relaxation responses concentration-dependently in +E and –E carotid arterial rings. Also, Nanosilver-induced relaxation in α- receptor mediated contraction is endothelium-dependent and showed a biphasic dose-dependent response. In conclusion, Nanosilver causes attenuation in carotid arterial smooth muscle reactivity with a biphasic dose-dependent relaxant effect and multiple endothelium-dependent pathways mode of action.

Keywords*:* Nanosilver, Vascular reactivity, Carotid arterial rings, Vascular endothelium.

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INTRODUCTION

Nanoparticles are heterogeneous substances with a size range of between 1 - 100 nm in at least one dimension and characterized by a high surface area-to-mass ratios resulting in better activity (Hector *et al.*, 2009, Tiwarri and Behari, 2011). Nanoscience and nanotechnology have received much significance recently owing to their unique probiotic properties with proven wide range of biomedical health benefits and potential uses in commercial applications (Esenaliev, 2000; Czajka, 2005; Wagner *et al.*, 2006; Emerich and Thanos, 2007;). Nanosilver particles (NAgPs) is one of the fastest-growing nanomaterials consumer products, industrials and biomedical applications owing to their unique properties particularly in catalytic activity and enhanced surface area to mass ratios and in permeating cellular membranes. Consequently, NAgPs have been extensively used for biomedical applications including but not limited to: antiviral, anti-angiogenic, antitumor, biosensors and bioimaging; as well as wound dressing, silver impregnated catheters, vascular prosthetics, clothing undergarments, air filters, laundry detergents, toiletries and water taps (Thote and Gupta, 2005; Holland *et al.*, 2015; Zhang and Kun., 2016).

Regardless of their conventional benefits, current studies have shown conflicting reports on the nanosilver effects on organ function and metabolism as well as very subtle possible tissue adverse effects particularly in cardiovascular toxicity in varied concentrations, size, biological target exposure time and tissue variations (Schrand *et al.*, 2010; Trickler *et al.*, 2010; Kang *et al.*, 2011; Haase *et al.*, 2012; Grosse *et al.*, 2013, Puja *et al.*, 2015). Furthermore, possible mechanisms through which NAgPs may interact with

biological surfaces are only beginning to emerge. Considering the conflicting reports, paucity of information on the effect of nanosilver on cardiovascular health and vascular smooth muscle responses in particular, this present study was designed to evaluate the effects of nanosilver on vascular smooth muscle reactivity and endothelial function in isolated rabbit carotid artery.

MATERIALS AND METHODS

Tissue preparation and Protocol

Arterial segments of the carotid artery were obtained from adult New Zealand rabbits which were sacrificed by stunning and bleeding and placed in physiological salt solution (PSS) of the following composition (mM): NaCl 119, KCl 4.7, $NaHCO_3$ 24.9, NaH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.6, glucose 11.5. The arteries were cleaned of adhering connective tissues and cut into 2-3mm rings. The rings were suspended between 2 L-shaped wire loops in 20 ml organ baths containing PSS. The upper loop was attached to a Grass Model FT03 force transducer connected to a Grass Model 7P polygraph (Grass Instruments Co., Quincy, MA, USA) while the lower loop was fixed to the base of the organ bath. An initial load of 1g was applied. The PSS was bubbled throughout with 95% O_2 - 5% CO_2 gas mixture with the pH and temperature maintained at 7.4 and 37^{o}C respectively. An equilibration period of 60 minutes was allowed; following this, carotid rings were stimulated twice with 8 x 10^{-2}M K^+ PSS, at 20-minute interval. The average of these contractions represented the maximum (100%) agonist/KCl which subsequent contractions to phenylephrine were evaluated (as previously reported Uche and Ebeigbe, 2015).

Concentration-response to agonists

Cumulative concentration-response tests ($1x10^{-9}$ to 2.5 $x10^{-4}$) to the agonist PE were examined in normal PSS (control) (n = 6) (where n is the number of animals from which arterial rings were obtained for each protocol); as well as following 20 minutes exposure to varying concentrations of nanosilver solution (1.25 µg/ml and 2.50 µg/ml) in arterial carotid rings (+E or –E). Contractile responses were analysed with reference to maximal contractions induced by 80 mM K^+ *PSS (n = 6)*

Relaxation-response and mode of action

Arterial rings were precontracted with EC_{70} M PE and /or high (80 mM K^+) PSS. At stable contraction, cumulative relaxation response tests (0.25 to 5.0 µg) to nanosilver were studied in normal PSS (control) (n = 6); in +E and -E rings.

Role of endothelium

To further elucidate the possible mechanisms of action of Nanosilver, the role of vascular endothelium in vascular reactivity by NAgs was studied in intact (+E) and endothelium denuded (-E) HM; PE or high-K^+ precontracted arterial rings. Endothelium removal was effected mechanically (Furchgott and Zawadzki, 1980) by gently rubbing the inner lining surface of the rings with a pair of forceps (Ebeigbe *et al.*, 1990). The effectiveness of de-endothelisation was confirmed by lack of relaxation response to 10^{-5}M Acetylcholine (Ach) and more than 70% relation in phenylephrine - precontracted endothelium-denuded and intact arterial rings respectively (Ebeigbe *et al.*, 1990; Olele et al., 1998). Also, relaxation response tests to acetylcholine ($1x10^{-9}$ to $1x10^{-3}$) was examined in normal PSS (control) and following 20 minutes exposure to NAgs (1.25 µg/ml) only and/or 5 minutes pre-exposure to L-NAME ($1x\ 10^{-5}$M) and indomethacin ($3\ x\ 10^{-6}$ M) or its vehicle Na_2CO_3; final bath concentration less than 0.03%) in arterial rings. Relaxation responses were analysed with reference to maximal contraction induced by EC_{70} M PE in normal PSS.

Chemicals

The following drugs and chemical reagents were used: Phenylephrine hydrochloride (Sigma USA), Nanosilver solution (Mineral for life Ltd, Abuja, Nigeria), L-NAME and Indomethacin (Sigma USA); and prepared fresh by dissolving in distilled water and $NaHCO_3$.

Statistical Analysis

Data are presented as Means ± SEM (standard error of means); n represents the number of rabbits from which arterial rings were obtained. EC_{50} (concentrations producing 50% maximal response) and IC_{50} were determined graphically. Comparison of the means was effected using the Student's t-test, ANOVA and Graph pad prism version 7.03 statistical package. P - Values less than 0.05 ($P<0.05$) were considered statistically significant for two independent variables (test and control).

RESULTS

Effect of Nanosilver on dose-response to agonists

Exposure of arterial rings to varying NAgs concentrations resulted in phenylephrine-induced attenuated contractions. NAgs exposure resulted in significant ($p<0.05$) right-ward shift of the curves and attenuated maximal contraction in both +E and -E rings (fig. 1).

Comparative PE EC_{50} (M) contraction in varying NAgs concentration

Comparative EC_{50} (M) values of α-adrenergic phenylephrine-induced contraction in +E and -E arterial rings exposed to varying concentrations of Nags showed significant decreased Emax contraction ($P<0.05$).

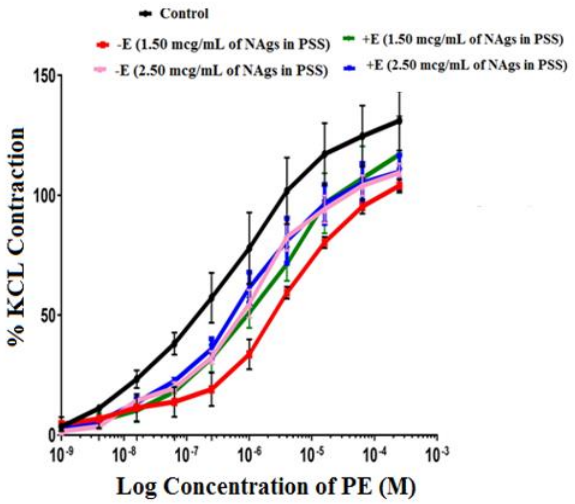

Fig.1. Cumulative concentration responses to phenylephrine following exposures to varying Nanosilver concentration in normal PSS in carotid rings: n = 6; means ± SEM. NAgs exposure resulted in significant ($p<0.05$) right-ward shift of the curves and attenuated maximal contraction in both +E and -E rings.

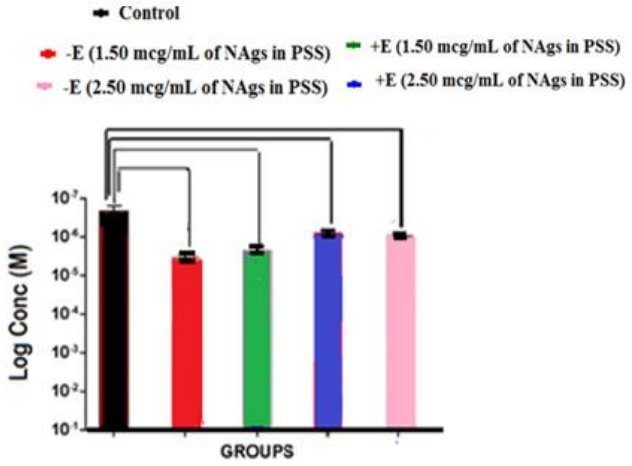

Fig.2. Comparative EC_{50} (M) values of α-adrenergic receptor-PE-induced contractions in +E and -E arterial rings exposed to varying concentrations of NAgs showing significantly decreased Emax contractions ($p<0.05$).

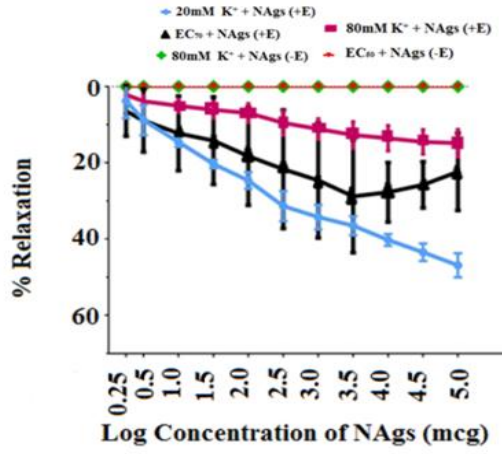

Fig.3. NAgs-induced biphasic relaxation response following EC_{70} M PE precontraction and greater relaxation effect in low (2 x 10^{-2}mM K^+) precontractions whereas PE and 8 x 10^{-2}mM K^+ precontractions were abolished in -E carotid rings.

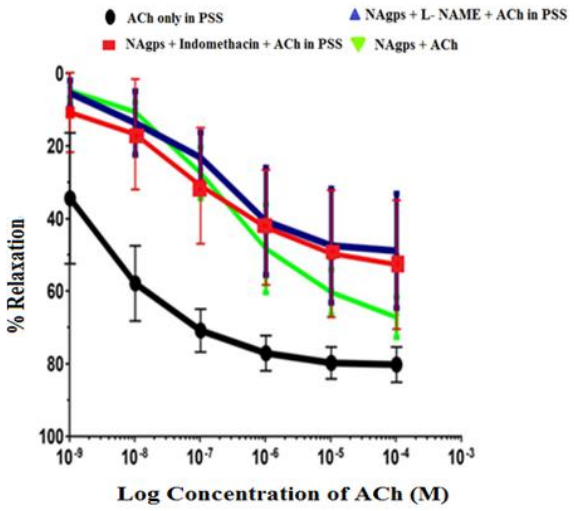

Fig 4. ____endothelium-dependent ACh- induced dose-dependent relaxation response curves to agonist in normal PSS (control) and attenuation in relaxation following exposures to Nanosilver and pre-incubation in L-NAME (NO synthase inhibitor) and indomethacin (cyclooxygenase inhibitor) solution.

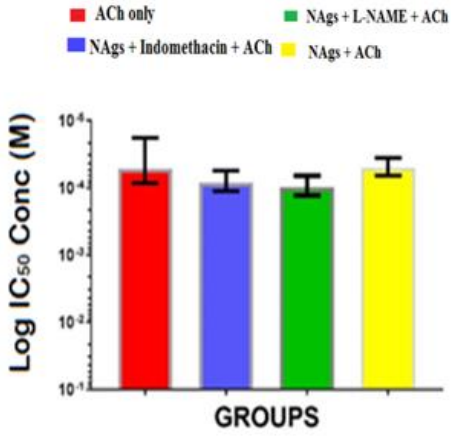

Fig.5. Comparative IC_{50} (M) values of ACh-endothelium dependent induced relaxation in PE-precontracted arterial rings exposed to vasoactive vascular endothelial agents.

Dose-relaxation response to NAgs

Carotid arterial rings were each precontracted with 10^{-7}M PE, high and/or low (8, 2 x 10^{-2}) M K^+. When the contractions were stable, NAgs (0.25 to 5.0) µg/mL was added cumulatively and the effects examined. The resultant relaxation responses were determined and expressed as percentage (%) of the initial tension. Nanosilver induced relaxation is endothelium dependent. (fig. 3).

NAgs in ACh-induced relaxation

Cumulative addition of ACh to 10^{-7}M PE-induced contractions in normal PSS (control) and following 20 minutes exposure to 1.25 µg/mL Nanosilver, and pre-incubation in L-NAME ($1x10^{-3}$) M or indomethacin ($3x10^{-6}$) M carotid arterial rings and maintained throughout the protocol. There was a significant shift of the test response curves to the right of the control

showing attenuated relaxation and Nanosilver endothelium non-specific mode of action (fig. 4).

Acetylcholine IC_{50} (M) relaxation in varying NAgs concentration.

IC_{50} (M) values were graphically determined from the cumulative dose relaxation responses. The IC_{50} (M) values of ACh-endothelium dependent induced relaxation with nanosilver (Nags), L-NAME (nitric oxide synthase inhibitor) and indomethacin (an inhibitor of cyclooxygenase) in phenylephrine-precontracted arterial rings showed no significant relaxant differences suggesting non- specific endothelium mediated responses.

DISCUSSION

This study has identified the effects of nanosilver solution [(NAgs (10ppm)] on endothelial function and vascular reactivity in rabbit isolated carotid artery. The results of the present study indicate that the contractile responses to α-receptor agonist phenylephrine (PE) in isolated rabbit carotid artery in both intact endothelium (+E) and endothelium denuded (-E) rings were attenuated and maximal contraction decreased following exposure to 1.25µg/mL nanosilver solution compared to the contractile response of the control carotid arterial ring in normal PSS. The EC_{50} values of phenylephrine contractions in control and test +E or -E arterial rings were: (7.0×10^{-7}, 5.04×10^{-5}, 2.03×10^{-5}, 8.02×10^{-5} and 8.01×10^{-5}) respectively (figure 2). However, attenuation in contractile response was significantly greater in endothelium denuded than intact endothelium arterial rings suggesting greater direct diffusivity effect of Nanosilver molecules across plasmalemma membrane. This observation is in tandem with previous reports alleging of an emerging risk represented by the wide diffusion of nanoparticles, such as the silver nanoparticles, as well as their worldwide diffusion for industrial processes and treatments (Marzhan *et al.,* 2013); and the ability of nanosilverparticles to cross the capillary wall (Bachler *et al.,* 2013, Holland et al., 2015). Exposure to silver nanoparticles has been associated with inflammatory, oxidative stress, genotoxic, and cytotoxic consequences (Marzhan *et al.,* 2013).

We also demonstrated that exposure to 1.25µg/mL nanosilver solution significantly attenuated endothelium-dependent vascular smooth muscle relaxation induced by ACh in carotid artery in normal PSS dose-dependently; whereas pre-incubation with NO synthase inhibitor [(L-NAME ($1x\ 10^{-5}$M))] and cyclooxygenase inhibitor [(indomethacin (3×10^{-6} M))] followed the same tenet but with no significant change in the response curves compared to ACh-induced relaxation in NAgs incubated carotid rings. The IC_{50} values of acetycholine-induced relaxations in pre-incubated L-NAME (endothelium nitric oxide synthase inhibitor) and indomethacin (an inhibitor of cyclooxygenase) arterial rings showed no significant difference comparatively (figure.4) Therefore mechanisms of Nanosilver modulatory relaxant effect may be mediated through endothelium-related-multiple pathways. Acetycholine-induced vascular relaxation is well known to be endothelium-dependent (Furchgott and Zawadzki, 1980, Ebeigbe and Aloamaka, 1985; Obiefuna et al., 1991). Several other vasorelaxant agents are equally known to mediate their effects via interaction with vascular endothelium in both receptor operated channels and voltage-sensitive channels- mediated contractions (Bolton,1979; Van de Voorde and Leusen, 1983; Ebeigbe and Aloamaka, 1985). Previous reports on vascular reactivity have shown that vascular endothelium modulates VSM responses, regulates and maintain vascular tone and homeostasis, as well as vascular resistance (Ajay et al., 2007); possibly by secretion of vast array of endothelium-derived factors including: Nitric oxide (NO), prostacyclin, endothelium-derived hyperpolarization factor (EDHF) (Furchgott and Zawadzki, 1980, Mori et al., 2006, and Kroetsch and Bolz, 2013). The exact nature of EDHF has not been fully characterised however, the following constituents have been identified including epoxyeicosatrienoic acids (EETs), K+, gap junction, and hydrogen peroxide (Mori et al., 2006). Hence different EDHF might exist in different forms and the contribution of each component to endothelium-dependent relaxation might vary, depending on the species tested and the vessel type used (Mori et al., 2006)

Nonetheless, these three different endothelium-derived vasodilators, nitric oxide, prostacyclin and EDHF play a vital role in the regulation of vascular tone. Vascular tone is a key determinant of local organ blood flow and peripheral resistance (Mori *et al.,* 2006). Additionally to elucidate further mode of action, following precontractions by EC_{70}M PE and/or $8{,}2 \times 10^{-2}$M K^+ PSS with no significant difference in Emax, cumulative addition of NAgs elicited a greater biphasic concentration-dependent relaxation with receptor-mediated contraction compared to high- K^+ in intact endothelium arterial rings; suggesting a greater effect of NAgs on mechanism associated with Ca^{2+} influx and that nanosilver may elicit contractile responses in higher concentrations (figure 3). NAgs-induced relaxant effects were however abolished in endothelium denuded arterial rings in both receptor and non-receptor mediated contractions in normal PSS whereas there was greater NAgs relaxation effect in low potassium depolarization compared to high K+ and &-receptor mediated phenylephrine contractions (figure 3). It is suggestive that different EDRFs-related multipathways probably mediate the vasorelaxant effect of NAgs. However, we observed no effect of Nanosilver on basal tone. To our knowledge, literature is scarce on the effects of nanosilver particles and silver ion on vascular reactivity in vitro and vascular

endothelial modulators effects on endothelial function. Therefore, these observations are quite novel in nanomaterial vascular reactivity. In conclusion, the results of this study show that nanosilver solution via a direct action on vascular smooth muscles, inhibits contractile responses and attenuates ACh-induced endothelium dependent relaxation in a dose-dependent manner in isolated rabbit carotid arterial rings. The inhibitory effect of nanosilver is probably not unconnected with the release of endothelium-derived relaxation factors.

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Evaluation of Tramadol-Midazolam-Ketamine Anaesthesia in Rabbits

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Summary: Selected anaesthetic indices of, and the heart rate, respiratory rate and rectal temperature responses of 6 healthy rabbits to the intramuscular administration of 2mg/kg midazolam and 25 mg/kg ketamine alone (MK) and combined with 4mg/kg tramadol (MKT) were evaluated over a 60-min observation period. Time to loss of righting reflex with MKT (1.7±0.3min) was significantly ($p<0.05$) shorter than with MK (4.2±1.5min). Duration of recumbency with MKT (76.8±5.1min) and MK (77.8±3.6min) were similar. Time to standing with MKT (9.3±1.1min) was shorter than with MK (15.2±2.4 min). Mean heart rates ranged from 204.7±13.0 to 257.5±3 beats/min with MK, and from 207.3±4.6 to 238.8±8.7 beats/min with MKT. Mean respiratory rates ranged from 33.8±6.2 to 64.3±15.0 breaths/min with MK; from 36.2±2.5 to 54.0±8.6 breaths/min with MKT. Mean temperature ranged between 38.0±0.2 and 38.9±0.2^{o}C with MK and between 37.9±0.3 and 39.1±0.1^{o}C with MKT. Neither MK nor MKT produced analgesia. It was concluded that although the inclusion of tramadol did not produce analgesia, it produced a faster onset of action than midazolam-ketamine alone. Midazolam-ketamine-tramadol will be useful for non-painful procedures where rapid drug action is needed.

Keywords*:* Analgesia, Anaesthesia, Ketamine, Midazolam, Rabbits, Tramadol.

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INTRODUCTION

Injectable anaesthesia involving the use of ketamine drug combinations are currently popular in rabbit anaesthesia because of ease of administration, low cost and relative safety of ketamine (Henke *et al.*, 2005; Orr *et al.*, 2005; Grint and Murison, 2008) and the practical problems associated with inhalation anaesthesia in this species (Flecknell, 2009). The combination of an alpha 2 adrenergic agonist with ketamine is a commonly used anaesthetic protocol for rabbit surgery (Longley, 2008) with xylazine being the most combined agent with ketamine (Lipman, 2008). Xylazine-ketamine combination produces surgical anaesthesia (White and Holmes, 1976; Lipman *et al*., 1990) but it is accompanied by significant side effects, including cardiovascular and respiratory depression (Flecknell, 1984; Longley, 2008). Diazepam-ketamine is another popular ketamine combination (Flecknell, 1998; Kilic, 2004; Oguntoye and Oke, 2014). Diazepam-ketamine combination produces good muscle relaxation and complete immobilization (Longley, 2008; Flecknell, 2009). In addition, diazepam decreases only respiratory rates but not heart rates while ketamine stimulates the cardiovascular system thereby making this combination relatively safer than ketamine combinations with alpha 2 agonists (Longley, 2008). However, many studies have found little or no analgesia with the diazepam-ketamine combination (Kazemi, 2002; Redah, 2011; Oguntoye and Oke, 2014) making it unsuitable for surgery or any painful procedure. Current anaesthetic techniques entail polypharmacy to ensure balanced anaesthesia which is surgical anaesthesia produced by a combination of two or more drugs or techniques with each contributing its own pharmacologic effects including tranquilizers, opioids, nitrous oxide, muscle relaxants and inhalants (Hall *et al.*, 2001; Muir *et al.*, 2013). Thus, the addition of an analgesic to the diazepam-ketamine combination may make it useful for painful procedures. Indeed, many anaesthetic protocols involving the rabbit include the opioids for analgesia (Longley, 2008). The addition of butorphanol to both xylazine-ketamine and medetomidine- ketamine (Marini *et al*., 1992; Longley, 2008) increases duration of anaesthesia and produces analgesia. Another opioid, fentanyl in combination with fluanisone combined with diazepam is a good injectable anaesthetic in rabbits but its usefulness is limited by the associated prolonged recovery (Harcourt-Brown, 2002). However, the traditional opioids are associated with certain side effects including respiratory depression, mental depression, hypothermia, bradycardia and sometimes reduced GIT motility in rabbits (Longley, 2008). Tramadol is a relatively new analgesic with mixed opioid and non-opioid activities (Garrido *et al*., 2000). The pharmacokinetics of tramadol has been

well studied in humans and is reported to cause less respiratory depression compared with morphine or other opioid analgesics (Bhattacharya *et al*., 2005; Natalini *et al.,* 2007). Tramadol may be a good alternative to the traditional opioids with diazepam-ketamine for anaesthesia in rabbits. In addition, tramadol is not under strict regulation and control, is cheap and readily available in developing countries (Ajadi *et al.,* 2009). Midazolam, another benzodiazepine, has some advantages over diazepam. It causes minimal haemodynamic and respiratory changes, is water soluble and therefore can be mixed with other water-soluble substances in a single syringe (Henke *et al*., 2005). Its water solubility property also makes it non-irritating to tissues making it more suitable for intramuscular administration. In addition, it is reportedly to be twice as potent as diazepam (Muir *et al*., 2013). The addition of tramadol to the midazolam-ketamine combination may be able to produce analgesia and longer duration of anaesthesia than midazolam-ketamine alone. The aim of this study, therefore, was to evaluate the anaesthesia produced by midazolam-ketamine-tramadol in rabbits.

MATERIALS AND METHODS

Experimental Animals

The animals used were adult New Zealand x Chinchilla rabbits consisting of three (3) bucks and three (3) does with weight range between 1.2kg to 1.9kg. The rabbits were acquired from a local breeder and were housed singly in cages that provided ample space for movement. They were fed ad libitum with grower's mash which was supplemented with *Tridax procumbens*. Water was also supplied *ad libitum* in their cages. They were stabilized for two weeks, to enable them become familiar with their new home, human restraint, feeding regime and to observe any possible health problem. At the first week of their arrival they were dewormed with ivomec® and given multivitamins (multinon®). The rabbits were judged to be healthy based on complete physical examination done before the commencement of the experiments.

Drugs Used

1. Midazolam (Dormicum®, Hoffmann-La Roche Ltd., Basel, Switzerland) supplied as 5 mg/ml in 2ml ampoule for injection.
2. Ketamine hydrochloride (ROTEXMEDICA, Trittau, Germany) supplied as 5mg/ml solution in 10ml vial for injection.
3. Tramadol hydrochloride (Tramaden®, Laborate pharmaceutical, India) supplied as 100mg in 2ml vial for injection.

Study Design

Two series of randomized experiments were carried out on each rabbit with an interval of one week allowed between both experiments to allow for complete metabolism and excretion of the drugs used.

In the first series of experiments each of the six rabbits was pre-medicated with midazolam 10 minutes before the injection of ketamine then in the second series, each of the six rabbits was pre-medicated with midazolam, followed by the concurrent injection of ketamine and tramadol 10 minutes later.

Experimental Procedures

The rabbits were allowed free access to feed and water until the time of drug administration. In the first series of the experiment, 2mg/kg midazolam was administered intramuscularly followed 10 minutes later by intramuscular injection of 25mg/kg ketamine hydrochloride.

For the second series, 2mg/kg midazolam was intramuscularly administered 10 minutes before the concurrent administration of 25mg/kg ketamine hydrochloride and 4mg/kg tramadol hydrochloride.

Following loss of righting reflex, rabbits were placed in right lateral recumbency and attached to a multiparameter patient monitor (Grady Vet 9200, China).

Measurements

The heart rate (HR), respiratory rate (RR), and rectal temperature (RT) (baseline) data were recorded and subsequently at 5 minutes interval for a period of 60 minutes. Haemostatic forceps closed to the first ratchet was applied on the inter-digital space of the hindlimb to test for analgesia every two minutes throughout the course of the experiments.

HR (beats/min) and RR (breaths/min) were determined using the patient monitor while rectal temperature (degrees Celsius) was determined using digital clinical thermometer. The anaesthetic indices calculated in the course of the trials were:

a) Time to loss of righting reflex: The time interval (in minutes) between the injection of ketamine and loss of righting reflex by the rabbit.
b) Duration of recumbency: Time interval (in minutes) between the loss of righting reflex and the assumption of sternal recumbency by anaesthetized rabbit.
c) Time to stand: Time interval (in minutes) between the assumption of sternal recumbency and standing posture by the anaesthetized rabbit.

Statistical Analysis

All values of data (heart rate, respiratory rate, and rectal temperature) at each time interval in the two series of experiments were expressed for the six (6) rabbits as mean ± standard error of mean. The means of anaesthetic indices (time to loss of righting reflex, onset of analgesia, duration of recumbency and time to standing) were compared using student T test for paired data. The mean values of the measured physiological parameters were compared using

analysis of variance (ANOVA) for repeated measures followed as appropriate by Dunnett's test when a significant difference was indicated. A value of $P < 0.05$ was considered statistically significant for all the comparisons (Dawson & Trapps, 2004).

RESULTS

Observation

No side effects were observed in any of the treated rabbits but they all reacted to pain on application of haemostatic forceps pressure to their hindlimbs throughout the anaesthetic period.

Anaesthetic Indices

The anaesthetic indices calculated for rabbits administered with Midazolam/Ketamine (MK) and Midazolam/Ketamine/Tramadol (MKT) are shown in table 1. Time to loss of righting reflex by the rabbits given MKT (1.7±0.3min) was significantly ($p<0.05$) different from those given MK (4.2±1.5min). The duration of recumbency with MKT (77.8±3.6min) was similar to that of MK (76.8±5.1min). Time to standing with MKT (9.3±1.1min) was not significantly ($p>0.05$) different from that of MK (15.2±2.4min).

Physiological Variables

Mean heart rates ranged from 207.3±4.6 to 238.8±8.7 beats/min with MKT and 204.7±13.0 to 257.5±15.3 beats/min with MK. The mean heart rate values at the 30th, 35th and 40th minutes were significantly different ($p<0.05$) between the MKT and MK treated rabbits (Fig 1).

Table 1. Selected anaesthetic indices of the intramuscular administration of Midazolam/Ketamine alone and with Tramadol in 6 rabbits.

Anaesthetic Indices	TREATMENT	
	MK	MKT
Time to loss of righting reflex	4.2±1.5	1.7±0.3*
Duration of recumbency (min)	76.8±5.1	77.8±3.6
Time to standing(min)	15.2±2.4	9.3±1.1

Data are expressed as means ± standard error of mean (SEM) of 6 rabbits. * $p<0.05$

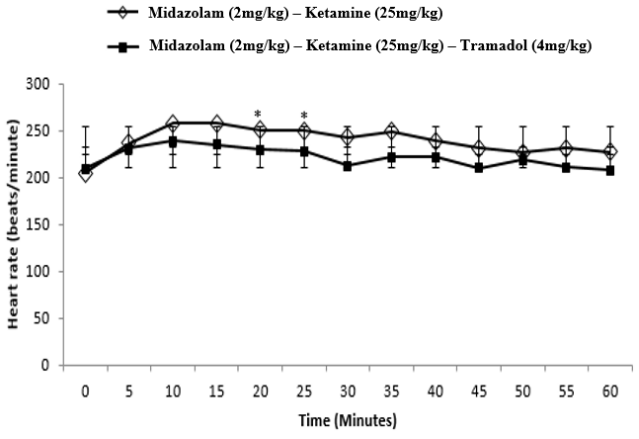

Figure 1: Mean heart rates of 6 rabbits administered with Midazolam/Ketamine (MK) alone or combined with Tramadol (MKT). * $p<0.05$

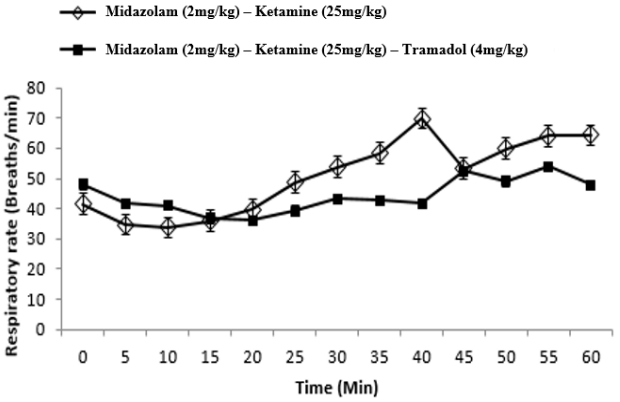

Figure 2: Mean respiratory rates of 6 rabbits administered with Midazolam/Ketamine (MK) alone or combined with Tramadol (MKT).

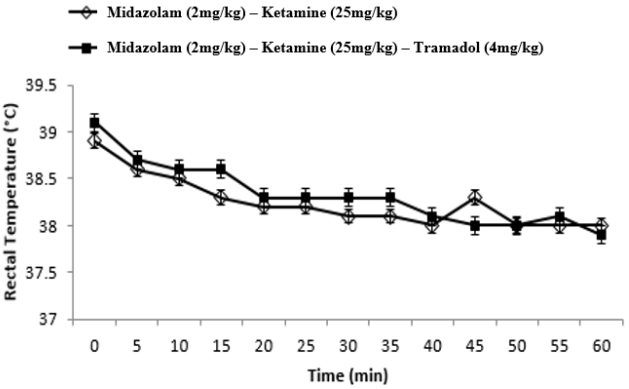

Figure 3: Mean rectal temperatures of 6 rabbits administered with Midazolam/Ketamine alone (MK) or combined with Tramadol (MKT).

DISCUSSION

The results of this study showed that Midazolam-ketamine-tramadol did not produce analgesia in the studied rabbits. It is surprising that the inclusion of tramadol did not confer analgesia on the rabbits in this study since a similar study with diazepam/ketamine and pentazocine reportedly produced analgesia (Adetunji *et al*., 2009). However, a clinical trial involving laparotomy in rabbits to evaluate the use of pentazocine in combination with diazepam and ketamine for surgical anaesthesia reported no analgesia (Udegbunam *et al*., 2017). It may be that a higher dosage of ketamine would be needed in the combination or just a function of strain variation in drug response because Adetunji and others (2009) employed 60mg/kg ketamine whereas Udegbunam and others (2017) used ketamine at a dosage of 15mg/kg and 25mg/kg body weight was used in the present study. Some studies have also shown that there is difference in response to various anaesthetic agents between different rabbit strains (Avsaroglu *et al*., 2003) and individual rabbits (Aeschbacher, 2001). Nonetheless, tramadol inclusion resulted in a faster onset of drug action evidenced by a significantly ($P<0.05$) shorter time to loss of righting reflex by the rabbits in the MKT group. The duration of recumbency

and time to standing were similar in both the MK and MKT treated groups of rabbits. Time to stand was shorter in the MKT but not statistically so (Table 1). The longer duration of recumbency of 76.8±5.1 obtained in the MK rabbits in this study (Table 1) is higher than previous similar studies of 27 min (Bellini *et al*., 2014) and 42min (Dupras, 2001). This observation may be due to the higher dosage of 2mg/kg used in this study compared with 1mg/kg for midazolam in the other studies. However, the lack of analgesia with MK is consistent with results of other similar studies (Dupras, 2001; Bellini *et al*., 2014).

Although the mean heart rates of the MKT treated rabbits were generally lower than those of the MK group, this difference was only significant from the 30th to 40th minute of anaesthesia. Nonetheless, heart rates of the rabbits in the two groups which ranged from 204.7±13.0 to 257.5±15.3 beats per minutes in the MK group and from 207.3±4.6 to 238.8±8.7 beats/min in the MKT group (Fig 1) fell within the normal range of 130 to 325 beats per minutes accepted for awake rabbits, (Harkness and Wagner, 1989; Harcourt brown., 2002).

There was no statistically significant difference in the mean respiratory rates between the MK and MKT treated rabbits. MK group had lower respiratory rates only in the first 15minutes of anaesthesia but subsequently, the MKT rabbits had lower respiratory rates which may be attributed to tramadol. Opioid's major side effect is respiratory depression (Borer-Weir, 2014) but tramadol does not cause significant respiratory depression if normal doses are not exceeded (Vickers *et al*., 1992).

Mean rectal temperature range of 37.9±0.3 and 39.1±0.1 for the MK group and 38.0±0.2 and 38.9±0.2 for the MKT group all fell within the normal range of 38 to 40 (°C) described for awake rabbits, (Harkness and Wagner, 1989; Harcourt – Brown, 2002).

It was concluded that although MKT produced a faster onset of action than MK, it did not produce analgesia. However, both MKT and MK appear safe as they were not associated with any significant cardiorespiratory depression in healthy rabbits not undergoing any clinical procedure. MKT will be useful for non-painful procedures where a rapid drug action is needed.

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A Study of Pain Threshold, Interleukins and NLR in Diabetic Polyneuropathy in a Selected Nigerian Population

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Summary: Pain serves a protective function and is often lost in chronic conditions such as painful diabetic neuropathy (PDN). This has been reported to be associated with ongoing inflammation. This study aims to investigate an association between body immune responses, neutrophil-lymphocyte ratio (NLR) and pain perception in DPN patients. Sixty volunteers were recruited for the study. 30 control and 30 diagnosed DPN patients (used Biothesiometer). All subjects were trained and informed consents were obtained. The pain threshold was significantly ($p<0.05$) lower in DPN (23.48±1.19 sec) compared to control group (30.38±1.9 sec), there was significant lower NLR in DPN (1.27±0.09) compared to control group (1.93±0.1) and the serum level of IL6 (15.31±0.85 pg/ml) in DPN was significantly higher compared to control group (11.9±0.15 pg/ml), likewise the serum level of IL10 (13.26±2.78 pg/ml) in DPN is significantly higher compared to control group (6.59±1.07 pg/ml). This study showed that hyperalgesia seen in patients with DPN was independent of increased NLR, and increased IL6 & IL10 seen in this group of patients indicates need to further explore the role of immunological response in the pathogenesis and progression of DPN.

Keywords*:* Diabetes, inflammation, interleukins, pain threshold, polyneuropathy

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INTRODUCTION

Diabetes Mellitus (DM) is a group of metabolic diseases in which there are high blood sugar levels over a prolonged period. Fasting plasma glucose ≥ 7.0mmol/l (126mg/dl), 2hour plasma glucose≥ 11.1mmol/l (200mg/dl) after a 75g oral glucose load in a glucose tolerance test, or glycated haemoglobin (HbA1C) ≥ 4.8% (WHO, 2014). The World Health Organization estimates that 422 million (8.5%) adults globally were living with diabetes in 2013 and around 80% of these people live in developing countries. Shaw *et al* in 2010 reported that the prevalence of people living with Diabetes in Nigeria is 2,819,000 (3.9 %) of the over 174 million which is expected to hit 4.3% by 2030. Diabetic polyneuropathy (DPN) is the most common complication of diabetes mellitus, affecting up to 50% of patients "20% of them are presenting with pain" which usually present with one or more of these symptoms "tingling, shooting, numbness or burning pain sensation especially in the limbs" more frequently in the lower limbs (Dyck *et al.*,2011). Even though, DPN affect all peripheral nerves including pain fibres, motor neurons and the autonomic nervous system (Vinik and Mehrabyan, 2004), typically sensory neuropathy is the first stage of DPN, and if not treated appropriately and strict blood glucose control is not implemented, it will precipitate to motor and autonomic disorders. Motor neuropathy will lead to loss of intrinsic muscle innervation causing different foot deformities (Cancelliere, 2016) like foot ulceration, Charcot neuroarthropathy, and lower-extremity amputation (Boulton *et al.*, 2005). This is responsible for over 60% of non-traumatic amputations annually (Holzer *et al.*, 1998). The relationship between diabetes mellitus and the common micro-vascular complications "Diabetic Retinopathy (DR), Diabetic polyneuropathy (DPN) and Diabetic nephropathy (DN)" has been studied to have more knowledge about the likely pathogenesis of these micro-vascular complications (Hewapathirana and Page, 2012). However, there are two main suppositions of the proposed mechanism of painful diabetic neuropathy: vascular and metabolic (Czyzyk, 1987), but the current hypothesis suggests that neuroimmune interactions actively contribute to the onset and persistence of pain in diabetes (Vinik *et al.*, 2006; Bishnoi *et al.*, 2011).

Total white blood cell count (TWC) is a basic, simple, cheap and readily available procedure. It is a good indicator of on-going inflammation in the body (Zahorec, 2001). On the other hand, the use of pro-inflammatory markers like c-reactive protein, IL 2, IL 6, TNF are expensive, time consuming and requires a lot of man power and expert techniques. Studies have shown a link between absolute neutrophil count (ANC) and the development and progression of DN (Virginia *et al.,* 2012). Recently, the neutrophil-lymphocyte ratio (NLR), a novel potential marker to determine inflammation, has been demonstrated to be a higher sensitive factor than TWC, ANC or absolute lymphocyte count (ALC) in the prognostic outcomes in various medical conditions like cancer and cardiovascular diseases (Lee *et al.,* 2012; Uthamalingam *et al.,* 2011; Jie *et al.,* 2015).

However little is known about the relationship between ANC, ALC and NLR, and DPN especially in this part of the world (Nigeria) and most importantly the role of immunological response if any in the pathogenesis of this disease.

In this study, the objectives were to investigate an association between systemic ANC, ALC, NLR, IL6, IL10 and pain perception of DPN patients in diabetic clinic of Ekiti-State University Teaching Hospital, Ado-Ekiti, Ekiti-State, South-West, Nigeria.

MATERIALS AND METHODS

Human Subjects

Sixty (60) volunteers were recruited for the study based on the recommendation of Voorhis and Morgan (2001). Thirty (30) healthy volunteers were randomly selected in the community and 30 volunteer patients with presence of symptoms or signs of peripheral nerve dysfunction in people with diabetes type 2 after other possible causes have been excluded (Zeng *et al.,*2017) were selected consequentially from diabetic clinic in Ekiti State University Teaching Hospital.

Protocol: These individuals were older than 20 years, known diabetic neuropathic patient presenting with minimum of two symptoms (pain plus any other) and Vibration Perception Threshold (VPT) was also measured, using a biothesiometer, (Model: Vibrometer-VPT; Serial No: V117093366; Made by Diabetic Foot Care India PVT Limited, India) which was applied to six assigned locations at the dorsum of the foot and the average value of these measurements calculated to define the presence of diabetic neuropathy on each foot with a cut off VPT of more than 25 volts for the diagnosis of loss of protective sensation, this was carried out at diabetic clinic in Ekiti-State University Teaching Hospital, Ado-Ekiti. They were recruited, trained on what they should expect during the study and informed consent was obtained. Demographic features of the subjects are shown in Table 1

Subjects were excluded if they had other neurological disorders (such as shingles and fibromyalgia), infection, psychiatric illness, myocardial infarction, cancer, HIV, blood diseases that affect neutrophils and lymphocytes counts (e.g. leukaemia) or if they were unable to give written informed consent.

All subjects underwent the following procedures: history taking, physical examination, sub-maximal effort tourniquet test, biochemical analysis. All procedures were performed in the morning after an overnight fast. Approval (Protocol number: EKSUTH/A67/2016/12/005) was obtained from the Research and Ethical Review Committee of the Ekiti State University Teaching Hospital, Ado Ekiti, Ekiti State, Nigeria.

Sub-maximal effort tourniquet test: The ischemic pain testing (sub-maximal effort tourniquet test) was based on the method described by Plesan *et al.* (2000). A blood pressure cuff was placed around the non-dominant upper arm of the subject's (on the brachia artery}. The cuff pressure was increased to 20mmHg above the subject's systolic blood pressure. With the pressure maintained, subject performed a hand grip exercise on an elastic ball. The subject closes his/her eyes for the entire procedure to minimize distraction and time cues. Subjects were then asked to indicate when they first detected (feel) the pain and when they could no longer tolerate the pain (to a maximum of 300 seconds). Once pain tolerance was reached, the pressure curve was immediately deflated and end-points were measured in seconds with the process performed 3 times and average of the readings documented (Plesan *et al.,* 2000).

Pain threshold assessment: The pain threshold is defined as the point between being "about to be painful" and "just became painful" and the time taken for this to occur is recorded in seconds. The process is performed 3 times and the average is documented

BIOCHEMICAL ANALYSIS

Determination of glycated heamoglobin level: This assay employs the chromatography technique. The non-glycasylated haemoglobin, which consists of the bulk of haemoglobin, has been designated HbAo. A haemolysed preparation of whole blood is mixed continuously for 5 minutes with a weakly binding cation-exchange resin. The labile fraction is eliminated during the haemolysate preparation and during the binding. During this mixing, HbAo binds to the ion exchange resin leaving GHb free in the supernatant. After the mixing period, a filter separator is used to remove the resin from the supernatant. The percentage glycasylated haemoglobin is determined by measuring absorbances of the ratio of the absorbances of the glycosylated haemoglobin (Ghb) and the Total haemoglobin fraction (THb). The ratio

of the absorbances of GHb and THb of the control and test is used to calculate the percentage GHb of the sample (Jeppsson *et al.*, 1986).

Determination of full blood count (FBC) and differential white blood cell count: Blood samples were collected at the cubital vein, separated in two different sample bottles. EDTA bottle was used for the full blood count analysis and the remaining blood sample collected in a plane bottle was centrifuged at 1000 rev/min for 5 minutes, serum separated into another plane bottle then store at -70ºC in the refrigerator for the analysis of IL10, IL6 and CRP. All values of full blood count (FBC) and its differential white blood cell counts (ANC,ALC) were measured by an automated haematology analyzer (Sysmex kx-2in).

Determination of neutrophil lymphocyte ratio (NLR): NLR was calculated as the ratio between the ANC and the ALC both obtained from the same blood sample of each subject.

Determination of serum interleukin 6 (IL6): This assay employs the competitive inhibition enzyme immunoassay technique. A monoclonal antibody specific to Interleukin 6 (IL6) has been pre-coated onto a microplate. A competitive inhibition reaction was launched between biotin labelled IL6 and unlabelled IL6 (Standards or samples) with the pre-coated antibody specific to IL6. After incubation the unbound conjugate was washed off. Next, avidin conjugated to Horseradish Peroxidase (HRP) was added to each microplate well and incubated. The amount of bound HRP conjugate was reverse proportional to the concentration of IL6 in the sample. After addition of the substrate solution, the intensity of colour developed was reversed proportional to the concentration of IL6 in the sample.

Determination of serum interleukin 10 (IL10): This assay employs the competitive inhibition enzyme immunoassay technique. A monoclonal antibody specific to Interleukin 10 (IL10) has been pre-coated onto a microplate. A competitive inhibition reaction was launched between biotin labelled IL10 and unlabelled IL10 (Standards or samples) with the pre-coated antibody specific to IL10. After incubation the unbound conjugate was washed off. Next, avidin conjugated to Horseradish Peroxidase (HRP) was added to each microplate well and incubated. The amount of bound HRP conjugate was reverse proportional to the concentration of IL10 in the sample. After addition of the substrate solution, the intensity of colour developed was reversed proportional to the concentration of IL10 in the sample.

STATISTICAL ANALYSIS

All data were expressed as the Mean ± SEM; the effects of the varied intervention of each of the groups were tested for homogeneity using Independent-Samples T test using SPSS version 20 software with the level of significance set at $p \leq 0.05$. A difference between two means was considered to be statistically significant when $p < 0.05$.

RESULTS

Effect of diabetic polyneuropathy on glycated haemoglobin

The results showed that the diabetes neuropathic group (6.60±0.26 %) has significantly higher HbA1C level when compared to the control (4.32±0.68 %) with the $p < 0.01$ (figure 1).

Effect of diabetic polyneuropathy on pain threshold

The pain threshold was significantly ($p<0.05$) lower in DPN (23.48±1.19 seconds) compared to control group (30.38±1.9 seconds) (figure 2).

Effect of diabetic polyneuropathy on serum interleukin 6 (IL6)

The IL6 level (15.31±0.85 pg/ml) in DPN is significantly ($p<0.01$) higher compared to normal group (11.94±0.15 pg/ml) (figure 3).

Table 1: Demographic features of subjects

	CONTROL	DPN
Number of subjects	30 healthy	30 diagnosed
Gender	Female = 18	Female = 20
	Male = 12	Male = 10
Mean age (years)	51.7±1.72	58.17±1.6

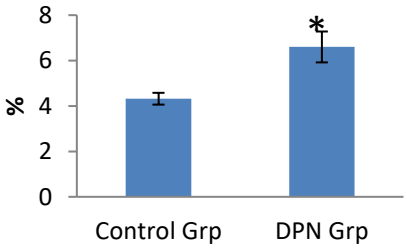

Figure 1: HbA1C level among the Control group and Diabetic neuropathy (DPN) patients. Values are expressed in Mean± SEM, *p < 0.01

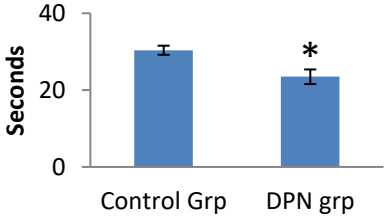

Figure 2: Pain Threshold among the Control group and Diabetic neuropathy (DPN) patients using ischaemic-induced pain test. Values are expressed in Mean± SEM, *p < 0.01

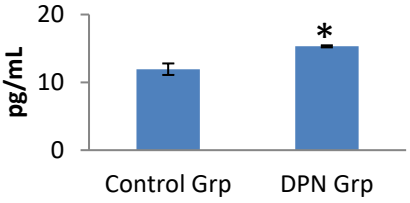

Figure 3: Serum level of interleukin 6 among the control group and diabetic neuropathy (DPN) patients. Values are expressed in Mean± SEM, *p < 0.01

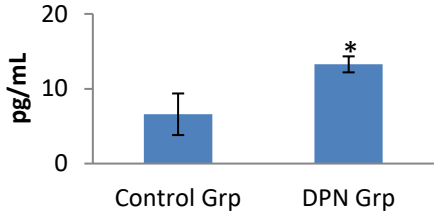

Figure 4: Serum level of interleukin 10 among the control group and diabetic neuropathy (DPN) patients. Values are expressed in Mean± SEM, *p < 0.05.

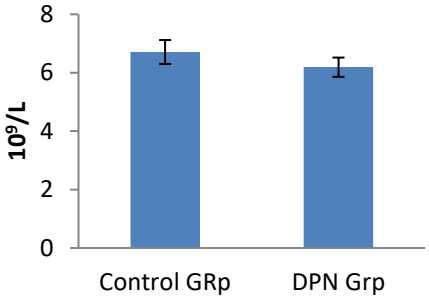

Figure 5: Total white blood count among the control group and Diabetic neuropathy (DPN) Patients. Values are expressed in Mean± SEM

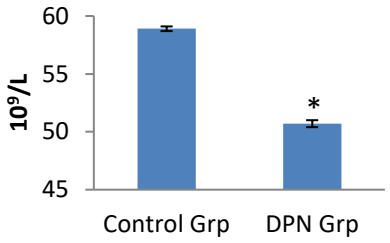

Figure 6: The absolute neutrophil count of the control group and diabetic neuropathy (DPN) patients. Values are expressed in Mean± SEM, *p < 0.01.

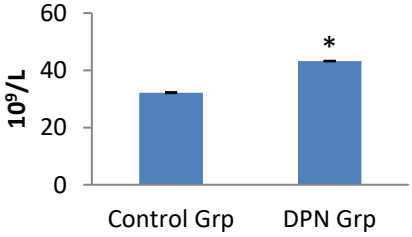

Figure 7: The absolute lymphocyte count of the control group and diabetic neuropathy (DPN) patients. Values are expressed in Mean± SEM, *p < 0.01.

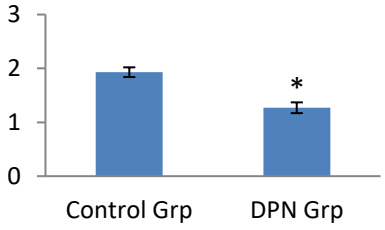

Figure 8: The NLR of the control group and diabetic neuropathy (DPN) patients. Values are expressed in Mean± SEM, *p < 0.01.

Effect of diabetic neuropathy on serum interleukin 10 (IL10)

The level of IL10 (13.26±2.78 pg/ml) in DPN is significantly higher compared to control group (6.59±1.07 pg/ml) with the p<0.03 (figure 4).

Effect of diabetic polyneuropathy on total white blood count (TWBC)

There was no significant difference in the TWBC in control group (6.71±0.41 10^9/l) and DPN group (6.19±0.33 10^9/l) with the p>0.05, (figure 5).

Effect of diabetic polyneuropathy on absolute neutrophil count

There was significant reduction in the neutrophil count in DPN (3.1±0.2 10^9/l) when compared to normal group (4.02±0.3 10^9/l) with the p<0.01 (figure 6).

Effect of diabetic polyneuropathy on absolute lymphocyte count

There was significantly higher lymphocyte count in DPN (2.7±0.19 10^9/l) when compared to normal group (2.12±0.12 10^9/l) with the p<0.01 (figure 7).

Effect of diabetic polyneuropathy on neutrophil lymphocyte ratio (NLR)

NLR was significant (p<0.01) lower in DPN (1.27±0.09) when compared to normal group (1.93±0.1) (figure 8).

DISCUSSION

The present study investigated the association between systemic ANC, ALC, NLR, IL-6, IL-10 and pain perception of DPN patients, with a view to understanding the likely role of adaptive immune system as well as inflammatory responses in the pathogenesis of diabetic neuropathy.

The result showed that, there is significantly lower pain threshold in DPN group compared to the control group indicating hyperalgesia which is consistent with previous **study** (Farmer *et al.*, 2012). This finding is accompanied by significantly higher glycated haemoglobin (HbA1c) level in DPN group. HbA1c is a clinical parameter used to measure three months average plasma glucose concentration which makes it a better clinical tool for monitoring of the glucose control of these patients when compared to their fasting blood sugar or random blood sugar level. The

higher glycated haemoglobin seen in DPN group indicates prolong hyperglycemia is associated with increase in intracellular glucose in nerves cells and its supporting tissues in the central nervous system, it is reported that hyperglycemia leads to hyper-saturation of normal glycolytic pathway with extra glucose shunted to polyol pathway and then converted to sorbitol and fructose by enzymes aldose reductase and sorbitol dehydrogenase with resultant increase in nerve cell damage and gene dysregulation (Arikawa, 2007).

It is also suggested that nerve injury may be due to immune responses as shown by significantly higher serum level of IL-6 and IL-10 in DPN group when compared to the normal subjects. The relationship between the pain behaviour noticed in these patients and their serum level of IL-6 is best explained in the context that IL-6 influences transduction, conduction, and transmission of the nociceptive signal, resulting in prolonged or permanent signalling to the brain's cognitive centres in the absence of a painful noxious or non-noxious stimulus. This cytokine is synthesized by the neurons, microglia and the astrocyte cells after nerve injury in the peripheral nerves, in dorsal root ganglia (DRG) and in the spinal cord (Olivera *et al.,* 2011).

According to Hirota *et al*. (1996) spinal IL-6 mRNA, spinal IL-6, microglial and astrocyte activation, and pain behaviour did not differ in rats that sustained an injury at L5 either proximally or distally to the DRG. They also noted that a significant amount of IL-6 receptors (IL6R and glycoprotein 130 (gp130)) on cell membranes increases under this condition, suggesting a physiological role of IL-6 in pain behaviour in experimental rats. The findings in this study is also in agreement with that of Muller *et al*. (2002) which reported that with the development of diabetic complications, a substantial rise of systemic IL-6 was found.

IL-10, produced by helper T (Th2) cells, is a well-known immune regulatory cytokine, which regulates T cells and monocytes/macrophages (D'Andrea *et al.,* 1993). The results from this study showed tremendous increase serum level of IL-10 in patients with DPN when compared to the control hereby suggesting the role of adaptive immune system. This supports the hypothesis that DPN is a chronic pathology that has a strong correlation between the body immune system and the pain noted in these patients (Vinik *et al.,* 2006). IL-10 inhibits pro-inflammatory cytokines, especially TNF, IL-1, and IL-6, produced by activated macrophages and monocytes, stimulating endogenous production of anti-inflammatory cytokines. Besides, IL-10 suppresses the pro-inflammatory functions of antigen presenting cells (APCs) by antagonizing expression of co-stimulatory molecules, the release of pro-inflammatory cytokines and, in general, APC maturation (Langenkamp *et al*., 2000). This then suggest a positive relationship between the serum level of IL-10 and the pain behaviour seen in PDN. This relationship seen in the result (figure 4) suggests that the body attempt to contain the effects of the pro-inflammation markers (e.g. IL-2, IL-6 and others) by the production and release of IL-10 which function is part of the general effort of the body to self-contain the extent of the inflammatory process in situations when the exposure to a given insult is in continuum as seen in DPN patients.

To further support the hypothesis that DPN may be due to an immunological and not just inflammatory response, our results showed that, there were significantly reduced Neutrophil count and higher Lymphocyte count in DPN group compared to the control group although both results are within the normal acceptable range according to Dacie and Lewis (2011). These range are 2.0–7.0 x 10^9/l (40–80%) for neutrophil and lymphocytes 1.0–3.0 x 10^9/l (20–40%) of Total White Blood Count (TWBC). Even though our result suggested that the neutrophil count is close to the lower margin of normal and lymphocytes count is also close to the upper limit of the normal control range. However, worthy of note is their relative high level of lymphocyte count supporting the role of the immune system in the later stage of this pathology.

Clinicians are looking for less expensive, less elaborate and accurate techniques that can be used in the assessment and as projective tool in clinical managements of various medical illnesses especially in the developing and under-developed world (Guo *et al.,* 2015). NLR is one of these techniques that is relatively cheap, and readily assessable in most clinical institutions. NLR has been used to screen out many patients that have sensorineural hearing loss (Sukhija *et al.,* 2007), diabetic retinopathy (Ulu *et al.,* 2013), adverse cardiac events (Azab *et al.,* 2013) and diabetic nephropathy (Azab *et al.,*2012) resulting from the vascular complications of diabetes via inflammation pathways. The fact that the present study demonstrate hyperalgesia with significantly lower NLR in DPN group compared to the control group suggests that DPN may occur with or without ongoing inflammatory process.

This study has shown that though patients with DPN are more susceptible to pain as shown by their pain threshold, NLR may not be elevated but reduced in them and focus should shift to the immunological roles and not just inflammation in the pathogenesis and progression of DPN, knowing that sensory neuropathy (e.g with abnormal pain sensation) is the first stage of DPN which if well managed will halt the progression of DPN. Further studies will be required to fully understand and if possible isolate the likely antibody resulting in the pathogenesis and the progression of DPN and other micro-vascular complications of diabetes mellitus, as this will aid more efficient clinical management of DPN.

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Thyroid Hormone Profile in a Population of Nigerian Patients with Traumatic Brain Injury

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Summary: Traumatic brain injury (T.B.I.) has an annual incidence of 200/100000. There is little or no information on neuroendocrine sequelae following T.B.I. in the Nigerian population. The purpose of the study is to evaluate the effect of T.B.I on the thyroid axis and relate it to outcome by outlining the change in thyroid axis of head injured patients. One hundred and fifteen patients were recruited with 85% male and 15% female. Of these patients, 71.7% presented within 24 hours of injury. The head injury was mild in 53%, moderate in 16% and severe in 31% of the patients. Serum T3 was high in 52.2%, low in 7.8% and normal in 40%. Serum T4 was high in 4.3%, low in 68.7%, and normal in 27%. Serum TSH was high in 16.5% TSH levels, low in 6.1% and normal in 77.4%. There was no correlation between the severity of head injury measured by the Glasgow coma score and patterns observed in the thyroid function test. With respect to outcome of head injury, serum T4 was low in patients who died or had persistent vegetative state (p=0.012). Traumatic brain injury in Nigerian patients is associated with an elevation of T3, low levels of T4 and normal TSH values. Death and persistent vegetative state were associated with low T4.

Keywords*:* Caffeine, ischaemia-reperfusion injury, cerebral ischaemia, neuro-inflammation, stroke.

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INTRODUCTION

Traumatic brain injury is a major public health problem, with an annual incidence of 200/100000 (Bruns and Hauser 2003) in Africa. Passenger motor vehicular road traffic accident causes over 65% of traumatic brain injury in the African population(Adeolu *et al.*, 2005), and is a major cause of death(Hodgson, Stewart, and Girotti 2000), as well as physical, psychological, cognitive, behavioral and social morbidity(Salazar *et al.*, 2000).

Neuroendocrine sequelae have been described in traumatic brain injury. Studies have been carried out in increasing numbers which have shed light and have improved the understanding of traumatic brain injury induced hypopituitarism, low levels of cortisol has been identified predominantly as well as low levels of thyroid hormones (Olivecrona, Dahlqvist, and Koskinen 2013; Malekpour, Mehrafshan, and Saki 2012). These hormones are important in coping with stress of disease and injury, like traumatic brain injury. The prevalence of anterior pituitary dysfunction following traumatic brain injury ranges from 8% to 68%(Schneider et al. 2006) with that of thyroid hormone deficiencies ranging between 0 and 29% (Fernandez-Rodriguez and Bernabeu 2011)

Hypopituitarism secondary to traumatic brain injury has remained under-diagnosed(Ghigo et al., 2005; Gasco et al. 2012). This can be explained by the fact that the patients with traumatic brain injury can have the signs and symptoms of hypopituitarism masked by the head injury(Czirják, Rácz, and Góth 2012) and some of the hormone disorders are not life threatening, hence the index of suspicion is low.

Traumatic brain injury is of great importance in low- and middle-income countries which make up 70% of the global population(Krug et al. 2002; Rubiano et al. 2013; "Centers for Disease Control and Prevention. National Center for Injury Prevention and Control. Web-Based Injury Statistics Query and Reporting System" 2004) because trauma accounts for the largest percentage of potentially productive year of life lost. Proper management of head injury and recognition of prognostic factors is important for the quality of life of survivors.

The exact mechanism and the pathophysiology of hypopituitarism and derangement of the thyroid hormones following traumatic brain injury is not well understood and several mechanisms have been

postulated (Maiya et al. 2008). A head injured patient may have other confounding factors that can mimic pituitary dysfunction for example the body's metabolic response to trauma. The drugs used in the early phases of trauma or acute illness may alter the metabolism of protein binding hormones resulting in false deficiencies. Thyroid hormone dysfunction has been described in severe trauma including burns and major surgery(Luo, Yu, and Li 2017; Sofianos et al. 2017), which reflected a decrease in the T4, T3 and free T3 and an increase in the TSH suggesting that the dysfunction is from the hypothalamic-pituitary axis. There is paucity of data on the effect of head injury on the thyroid hormone, with relation to severity of the head injury and relation of this to outcome of the injury.

Low levels of T3 and T4 have been described in severe head injury with no change observed in TSH and TBG. A poor outcome of heads injury was also observed with reduction in the levels of T4 (Malekpour, Mehrafshan, and Saki 2012; Shamaeva et al. 2012).

Whereas post-traumatic anterior pituitary dysfunction is a well-recognized entity, its frequency and potential impact on the outcome of head injured Nigerians has not been previously explored. A distinct feature of this population is that it consists mainly of the young population and middle-aged group that are still active with about 93.2% of its population below 54years (25-54years-30.1%, 15-24years 19.3%)("The World Factbook — Central Intelligence Agency" 2015).

MATERIALS AND METHODS

The study was a cross-sectional one carried out at the Department of Neurological Surgery, University College Hospital, Ibadan, Oyo State, Nigeria.

Study Population:
All patients with traumatic brain injury mild, moderate and severe, presenting at the Emergency Department of the University College Hospital, Ibadan and the Accident and Emergency Unit of the Ladoke Akintola University Teaching Hospital, Osogbo, during the study period (September 1st, 2014 – April 15th, 2015) were recruited into the study.

The patients were assessed: duration of trauma was noted, signs of head injury (altered sensorium, loss of consciousness, memory impairment, headaches, irrational talk and behavior, focal neurological deficits) were noted at presentation at the Emergency Department for eligibility to participate in the study without interrupting the planned care for the patient. Traumatic brain injury was classified using the post resuscitation Glasgow Coma Scale (GCS) score.

Mild: GCS score 13-15
Moderate: GCS score 9-12
Severe: GCS score <8.

Pregnant and breast-feeding women, patients on steroid therapy, patients on hormonal supplements, anti-androgen and anti-oestrogen, patients on thyroid supplements, patients with features of sepsis or burns and patients that had received any form of intervention prior to presentation were excluded from the study.
A sample size of 113 was calculated using the formula**:**
n = Z2 x. **(p)** x **(1-p)** / **C2**
where **n** =sample size, **Z** = Z value which is 1.96 for a 95% confidence level, **p** is percentage of patients with thyroid dysfunction following traumatic brain injury 8% (0.08), **C** is confidence interval in decimals 0.05), which is ideal for a descriptive study. A total number of 115 patients were recruited.

Blood Samples Collection and Handling
A large caliber vein was identified, usually at the cubital fossa, and the overlying skin was prepared with an ethanol-soaked cotton swab. The arm containing the infusion cannula was avoided. 5mls of venous blood was drawn with a 21G needle and a 5ml syringe. Samples were stored in a sterile plain bottle and kept in a refrigerator at 40C until serum was separated, usually within 48hours.

Serum was obtained by spinning the blood samples at 3600RPM in a centrifuge for 5 minutes and the supernatant removed and kept in a new sterile universal bottle. Serum samples were kept frozen at -20^0C while samples were being pooled, before analysis.

Sample Analysis:
Samples were analyzed in batches of 50 using Enzyme linked immunosorbent assay (ELISA). Dialab GmbH Elisa (Austria) for T3, T4 and TSH were used for analysis. According to the test kit used, hormone values were described T3: high > 2.0ng/ml, normal 0.8- 2.0 ng/ml, low < 0.8ng/ml; T4: high > 161nmol/l, normal 58 – 161nmol/l, low < 58nmol; TSH: high > 4.7mIU/L, normal 0.5 – 4.7 mIU/L, low < 0.5mIU/L.

Statistical Analyses:
Categorical data were presented as frequencies and proportions while continuous data were represented as means and standard deviations. mean values were compared using Students T test. Point of significance was set as $P < 0.05$. The Data obtained were analyzed using IBM Statistical Package for Social Sciences version 22.

RESULTS

Most of the patients were between the ages of 21 and 40 years which was 51.3% of the total population sampled (*Table 1)* and the least represented were the elderly (10.4%). About fifty-three percent of the patients had mild head injury while 15.8% and 30.7% of the patients had moderate and severe head injury respectively. About fifty-two percent of the patients

had a high total T3 while only 7.8% had low T3. Conversely, only 4.3% of the patients had high T4 values while 68.7% of the patients had low T4. Most of the patients (77.4%) had normal TSH values (Table 2). The Glasgow Outcome Scale Extended shows that 46.5% of the patients fell into normal range while 24.2% died.

Table 1: Age distribution of the patients

Age in years	Frequency	Percentage
0-20	24	20.9
21 – 40	59	51.3
41 – 60	20	17.4
> 60	12	10.4

Table 2. Duration of trauma in hours prior to presentation.

Duration of trauma before presentation (Hours).	Frequency	Percentage
< 24	81	71.7
24 – 48	12	10.6
> 48	20	17.7

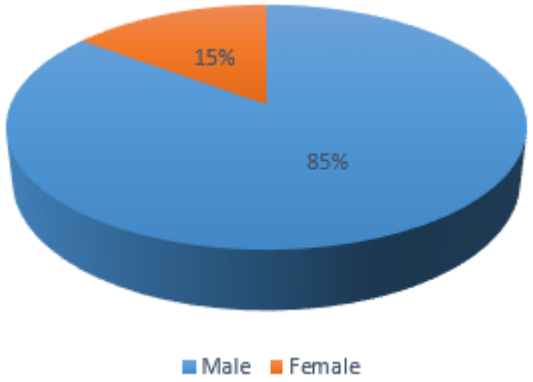

Fig 1: Sex distribution of the patients

Table 3 shows the result of cross tabulation of thyroid function test (in three variables: high, normal and low) with Glasgow Coma Scale (in three variables: mild, moderate and severe) of the patients. Total T3 was elevated in general amongst the sampled population 60%. Total T3 values was highest amongst patients with mild head injury (high T3 = 51.7%), and the decreases in severely head injured patients were noted (high T3 = 31.7%). Lowest values were however noticed in moderate head injury (high T3 = 16.7%). This observed difference was however not statistically significant (p = 0.945)

There was a general decrease in T4 levels with 78% of the sampled population having low T4 levels. The pattern noticed with Total T4 values showed high values in mild head injury (high T4 = 80%) and decreases in severe head injury (high T4 = 20%) while dipping to the lowest value in moderate head injury (high T4 = 0%), however all groups of head injured patients had a higher percentage of people in that group having low T4 whether mild, moderate or severe. This observed difference was not statistically significant (p = 0.664).

A higher percentage of the sampled population had normal TSH values across the different severity of head injured patients 80%. Amongst the patients with high TSH, those with severe head injury formed the bulk of the population.

There was a consistent decline in the percentages of patient with high T3, from normal patients with high T3 65.3% of that population, patients with moderate disability had high T3 in 6.1% of that population and patients with severe disability had high T3 in 2.0% of that population. Persistent vegetative state and death were the worst outcomes and were put together, only 2 patients in the total population were in persistent vegetative state and they eventually died. Fifty-two percent of the patient that died had high T3 while 48% of these patients had normal T3.

Table 3: Thyroid function test /Glasgow coma scale

Variable	Glasgow Coma Scale			Total	Chi square	P value
	Mild	**Moderate**	**Severe**			
Total T3						
High	31 (51.7%)	10(16.7%)	19(31.7%)	60 (100%)	0.749	0.945
Normal	25(55.6%)	6 (13.3%)	14 (31.1%)	45 (100%)		
Low	5(55.6%)	2 (22.2%)	2 (22.2%)	9 (100%)		
Total T4						
High	4 (80%)	0 (0%)	1 (20%)	5 (100%)	2.390	0.664
Normal	17 (54.8%)	6(19.4%)	8 (25.8%)	31(100%)		
Low	40 (51.3%)	12(15.4%)	26 (33.3%)	78 (100%)		
TSH						
High	6 (31.6%)	4 (21.1%)	9 (47.4%)	19(100%)	5.814	0.214
Normal	50 (56.8%)	14(15.9%)	24 (27.3%)	88 (100%)		
Low	5 (71.4%)	0 (0%)	2 (28.6%)	7 (100%)		

Table 4. The relationship between thyroid function test values with Glasgow Outcome Score

Variable	Glasgow Outcome Score				Total	Chi square	P value
	Normal	**Moderate Disability**	**Severe Disability**	**PVS - Death**			
Total T3							
High	32(65.3%)	3(6.1%)	1(2.0%)	13(26.5%)	49(100%)		
Normal	25(59.5%)	3(7.1%)	2(4.8%)	12(28.6%)	42(100%)	5.578	0.427
Low	7(87.5%)	0(0%)	1(12.5%)	0(0%)	8 (100%)		
Total T4							
High	4(80.0%)	1 (20.0%)	0 (0.0%)	0 (0%)	5(100%)		
Normal	15(55.6%)	2 (7.4%)	2 (7.4%)	8 (29.6%)	27(100%)	5.166	0.523
Low	45(67.2%)	3 (4.5%)	2 (3.6%)	17 (25.4%)	67(100%)		
TSH							
High	8 (57.1%)	1(7.1%)	0(0%)	5(35.7%)	14(100%)		
Normal	52 (65.8%)	5(6.3%)	4(5.1%)	18 (22.8%)	79(100%)	2.508	0.868
Low	4 (66.7%)	0(0%)	0(0%)	2 (33.3%)	6(100%)		

Table 5: Thyroid function test amongst patients that died

Variable	Glasgow Coma Scale			Total	Chi square	P value
	Mild	**Moderate**	**Severe**			
Total T3						
High	1 (8.3)	2(16.7%)	9 (75.0%)	12 (100%)	2.294	0.318
Normal	2(18.2%)	0 (0%)	9 (81.8%)	11 (100%)		
Low	0(0%)	0 (0%)	0(0%)	0 (0%)		
Total T4						
High	0 (0%)	0 (0%)	0 (0%)	0 (0%)	8.830	0.012
Normal	3 (42.9%)	1(14.3%)	3 (42.9%)	7(100%)		
Low	0 (0%)	1(6.3%)	15(93.8%)	16 (100%)		
TSH						
High	0 (0%)	1 (20.0%)	4 (80.0%)	5(100%)	2.476	0.649
Normal	3 (18.8%)	1(6.3%)	12 (75.0%)	16 (100%)		
Low	0 (0%)	0 (0%)	2 (100%)	2 (100%)		

Similarly, Total T4 values showed a consistent decline in percentages from normal patients (high T4 = 40%) to patients with moderate disability having high T4 in 20% of the population and none of the patients with severe disability and death had a high Total T4. Amongst the patient that died (worst outcome), 77% of them had low T4 while 23% had normal T4. While 70% of those with the best outcome also had normal T4. These observed differences were however not statistically significant (p = 0.527). In the same vein, Total TSH values showed a consistent decline in percentages from upper normal (high TSH = 42.9%) to upper severe (high TSH = 0%) and low values in lower severe (high TSH = 0%) and persistent vegetative state (high TSH = 0%). Total TSH in relation to death was distinct with high TSH values of 35.7%, normal TSH values of 21.5% and low T4 values of 33.3%. These observed differences were however not statistically significant (p = 0.963)

Based on the pattern observed that patients who died had deviation from the trend that was observed for other patients, the outcome severity of injury of patients that died was crossed with the thyroid function tests and T3 was either high or normal with no statistical significance (p= 0.318) T4 was low in 84% of patients with severe head injury and low in 50 % of patients with moderate head injury (p=0.012). The patients with mild head injury had normal T4. None of the patients that died had an elevation in T4 (Table 5).

DISCUSSION

Traumatic brain injury is a major source of economic burden as majority of people affected by this problem are in their active years as shown in the results with majority of affected patients being between the ages 21 – 40 years making up more than half of the respondents (Table 1). More males are affected than females since they engage in risky activities and take on jobs that expose them to the hazards of the road. This data is like what Benvenga et al observed in their series(Benvenga et al., 2000). In this series 24.2% of patients died. These deaths are economic losses to the relatives of the patients and the country.

A rise in the levels of T3 and a reduction in the levels of T4 with TSH being within normal levels was observed in this study. This is at variance with that of Malekpour et al (2012), in their series observed a reduction in T3 and T4 with T3 being more significant, however their study was on severely head injured patients (Malekpour, Mehrafshan, and Saki 2012), while this study focused on head injury in general, whether mild, moderate or severe. The pattern

observed with TSH being within normal limit is similar to the findings Malekpour et al (2012 and Shamaeva et al. (2012) When this pattern was compared with Glasgow coma score, in each group of head injured patients i.e. mild, moderate or severe, the percentage of patients with T3 higher than normal levels in each category remained the majority, while those with T4 lower than normal levels were also the majority across board in each group of head injured patients. Lieberman et al described a global reduction in freeT4 and TSH in a population group who had head injury and they were already past the acute phase, on rehabilitation however, in their series, they did not assay for T3(Lieberman, Oberoi, and Gilkison 2001). The observed elevation of T3 in these group of patients is like the finding by Cernak et al.(1999) who observed a rise in T3 within the first 5 days of head injury in patients with mild head injury however, a reduction in T3 was observed in severely head injured patients.

The changes in the levels of the thyroid hormone levels in the different spectra of head injury did not show statistical significance. Most studies reported a low T3, T4, and TSH especially in severely head injured patients. Chiolero et al (1988) reported such a finding in 35 severely head injured patients, this study also had a similar number of severely head injured patients presenting within 24 – 48 hours post trauma; however, the findings are different in these population.

Most studies compared the thyroid hormones with the severity of head injury. This study showed a pattern with outcome of head injury. Elevated T3 values was associated with upper normal Glasgow outcome score in 44.9% of respondents without statistical significance. The percentages reduced as the scale moves towards the poor outcomes up to persistent vegetative state.

T4 was reduced in most of the patients with head injury as noted earlier however the pattern was that a higher percentage of people with low T4 had an upper normal and lower normal Glasgow outcome score. These findings can also be explained by most of the patients had mild head injury, and they are expected to make significant recovery and have good outcomes at recovery. However, death in the Glasgow Outcome Score also showed a different pattern on evaluation of patients T4. This is in concordance with other studies previously carried out. The observed pattern of T3 which is different from what is previously documented is still a subject for investigation. The metabolic response to trauma may explain the elevated T3 and normal TSH (Desborough, 2000), but this would not explain the reduced T4 in this group of patients. Total T3 and Total T4 was assayed, however the active form of the thyroid hormones are the free unbound molecules. Hence assaying for both the bound and the free hormones may not give the exact picture of the response of the thyroid axis to the stress of head injury

In conclusion, traumatic brain injury in Nigerian patients is associated with an elevation of T3, Low levels of T4 and normal TSH values. The levels of the hormones assayed T3, T4, and TSH observed is not significant when compared with the severity of head injury. Low T4 is observed in patients with the worst outcomes of traumatic brain injury (death), using the Glasgow outcome score extended, while high and normal T3 levels is observed in this group of patients.

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Short Communication

High Reticulocyte Count with Abnormal Red Blood Cell Morphology in Normal Wistar Rats after Garlic Administration

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Summary: Despite the high acceptability of *Allium sativa* (Garlic) as a remedy for many diseases as earlier stated by many researchers, previous studies have shown that chronic and unregulated consumption of garlic may result to intra vascular haemolytic anaemia in rats. The present study was conducted to examine the effect of crude extract of garlic on microscopic status of red blood cells and some other haematological indices of normal albino rats. The animals were grouped into two; group 1 were normal animals treated with water while group 2 were normal animals administered 150mg/kg body weight of crude extract of garlic on alternate days for three weeks. At the end of three weeks treatment, blood samples obtained from the tail vein of the rats were used for haematological indices and erythrocyte morphology. The values obtained were expressed as Mean± SEM and compared using student t test. The results showed that there was no significant difference in the PCV which was 43.20 ± 0.80% and 45.00 ± 0.36% in both control and experimental groups respectively. However, the RBCs were significantly decreased ($P< 0.05$) from 166.80 ± 3.44 $x10^6$ μL^{-1} in the control group to 87.80 ± 9.34 $x10^6$ μL^{-1} in the treatment group. The percentage reticulocyte counts on the other hand significantly increased from 2.60±4.25% in control group to 11.20± 16.4% in treated group. Fragmented RBCs with a lot of schistocytes with adequate platelets were seen on peripheral blood film of crude garlic treated rats as compared to control. Our results suggested intravascular haemolysis and numerous reticulocytes on blood film confirmed our view on bone marrow response. The presence of schistocytes and acanthocytes may be an indication that the liver is involved in the observed effect.

Keywords*:* Garlic, Red blood cells, Reticulocytes, Rat.

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INTRODUCTION

Garlic (*Allium sativum),* an important bulb vegetable, is used as a spice and flavouring agent of food and medicinal plants throughout the globe. The traditional use of garlic cannot be over emphasised and, in most cases, the plant is consumed in raw form. The effectiveness of garlic in disease amelioration has been scientifically validated (Amagase *et al* 2001; Hussein *et al* 2007) and its anti-glycaemic (Eidi *et al.*, 2006), antioxidant (Jaisuwal and Rizvi, 2011) and antihypertensive (Singh and Singh, 2008) effects have been authenticated through scientific manipulation. Phytochemical study revealed that *Allium sativum* contained organic compounds such as steroids, saponins and flavonoids, it is also characterised by a high content of organo sulphur compound (Singh and Singh, 2008).

Despite high acceptability of *Allium sativa* (Garlic) as a remedy for amelioration of many diseases as earlier stated by many researches (Eidi *et al*; 2006; Hussein *et al*, 2007; Rizvi, 2011), Some Studies have demonstrated that chronic and unregulated consumption of garlic may cause intra vascular haemolytic anaemia in rats (Shashikanth *et al*, 1986; Umar *et al*, 1996) and in dogs (Yamoto and Maeds, 1992). Intra-vascular haemolysis have been attributed to the presence of peroxidisable poly-unsaturated fatty acid and malondialdehyde (MDA) that impair erythrocyte membrane stability (Mansour and Mansour, 2009), this might be the cause of an increased erythrocyte osmotic fragility in rats fed with allium (Salami *et al*, 2012). There are also contrasting views that consumption of aged garlic extracts suppressed deformity and haemolysis rate induced by peroxidation and non-peroxidation, with improved microcirculation (Nasr, 2014).

Some reports have shown that the effectiveness of erythropoiesis can be assessed by quantitative determination of reticulocyte count which are clinical markers of haemolysis (Choi, 2001) while morphology of RBC can also be used as a diagnostic feature of sickle cell anaemia (Olanrewaju, 2002). In view of these reports on garlic consumption, an experiment

was designed to demonstrate the microscopic picture of red blood cell in an attempt to visualise red blood cell morphology and reticulocyte count, since peripheral blood smear is a critical step in the evaluation of any anaemia.

MATERIALS AND METHODS

Plant Materials: Garlic was purchased from the Monday market in Maiduguri, Borno state and authenticated by plant taxonomist in the department of biological sciences, University of Maiduguri, Nigeria.

Animals: Ten (10) adult albino rats weighing between 150g and 250g were obtained from physiology department animal house. The animals were kept in plastic cages at special room in the animal house at room temperature of 30±2 ˚C and less than 30% relative humidity under a 12hours light-dark cycle. They were fed standard pallet food (sanders SPEEC feed Plc. Jos, Nigeria) and had access to water *ad libitum.*

Preparation of Crude Garlic Extract: Five (5) gram of garlic was weighed and then pulverised and mixed with 50ml of distilled water. The preparation served as stock and stored at 4˚C.

Study Design: The rats were grouped into two groups of five (5) rats each. The first group served as control and received 0.2ml of distilled water on alternate days. Group two were treated with crude extract of *Allium sativa* (150mg/kg/day) on alternate days too. The choice of dose was based on previous studies (Umar *et al.,* 1996; Salami *et al.,* 2012). The experiment lasted for 3 weeks; a week corresponds with occurrence of maximal anaemia in rats fed with garlic (Umar *et al*, 1996). At the end of three weeks blood samples were obtained through rat tail after they were anesthetised with ether.

At the end of three weeks blood samples were obtained through rat tail after they were anesthetised with ether.

Determination of Haematological Indices: The blood samples collected were immediately used for haematological indices. The haematocrit or packed cell volume was determined according to the haematocrit method described Alexander and Griffiths (1993). Using a Hawksley microhematocrit reader, the values obtained were expressed in % volume of the blood count occupied by the red blood cell.

Red Blood Cell (RBC) and Reticulocyte Count: The principle was based on counting the number of RBC in a known small volume of accurately diluted blood. Blood was diluted 1:200 with Hayem's solution (an isotonic red blood cells diluting fluid) in a red cell pipette. A small volume of the diluted blood was counted using the improved Neubauer Hemocytometer. The reticulocytes count was carried out using Miller's disc method, and percentage reticulocytes for each rat was calculated using the following formula;

$$\%\ Reticulocyte = \frac{Total\ Reticulocyte}{Total\ RBC}\ X\ 100\ \%$$

Erythrocyte Morphology: A blood film was made by gently touch of fresh drop blood onto one end of a clean grease-free glass slide. Using another glass slide (spreader) that touches the edges of drop blood at an angle of 45^{o}, the spreader was pushed along the slide, drawing the blood behind it, until the whole blood was smeared. The blood smeared was allowed to dry at room temperature and stained using Leishman's for RBC morphology. Reticulocytes are visualized by staining with supravital stain (Brilliant cresyl blue) that precipitate the RNA and organelles. The immature cells (Reticulocytes) appeared microscopically visible as dark-blue clusters and filaments.

Statistical Analyses: The data obtained from the haematological parameters were subjected to statistical analysis using Instat graphpad version 3.05. The values obtained were expressed as Mean± SEM and compared using student t test. P value < 0.05 were considered significant.

RESULTS

Table 1 presents the PCV, RBC and reticulocyte count after 3 weeks of garlic extract administration. There was no significant difference in the PCV between the rats that received garlic (45.00± 0.36%) and control (43.20±0.80%). The mean RBC count of garlic treated rats (87.80±9.34x10^{6} µL^{-1}) was significantly low P<0.001 compared to that of control rats (166.80± 3.44x10^{6} µL^{-1}), while opposite effect was recorded in percentage reticulocyte count with the means 11.20± 16.4% in treated group and 2.60±4.25% recorded in control rats, the difference in percentage reticulocyte count was statistically significant (P<0.005)

Figure 1A shows the blood film of one of the rats fed with garlic showing features of haemolysis i.e fragmented red cells/ schistocytes and red cell agglutination while 1B shows peripheral blood film of red cell from control having normal microscopic blood with adequate white blood cell and platelets.

Figure 2 shows peripheral blood film of another rat fed with garlic showing fragmented red cells/ schistocytes, red cell agglutination, and acanthocytes which infer liver involvement.

Table1. Effect of oral Administration of Garlic Extract on Mean Packed Cell Volume, Red Blood Cell and percentage Reticulocyte count in rats.

	Control	Garlic Treated
RBC (10^{6} µL^{-1})	166.80±3.44	87.80±9.34*
PCV%	43.20±0.80	45.00±0.36
Reticulocytes%	2.60±4.25	11.20±6.43*

*P<0.005

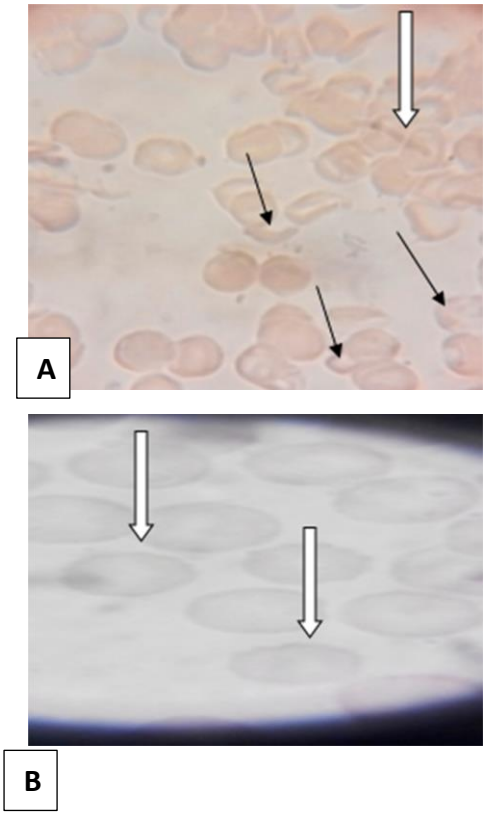

Fig. 1: (A) Shows the blood film of one of the rats fed with garlic showing features of haemolysis i.e fragmented red cells/ schistocytes (slim arrows) and red cell agglutination (bold arrows) MgX40. **(B)** Peripheral blood film of control animals showing normal RBC (bold arrow) X100.

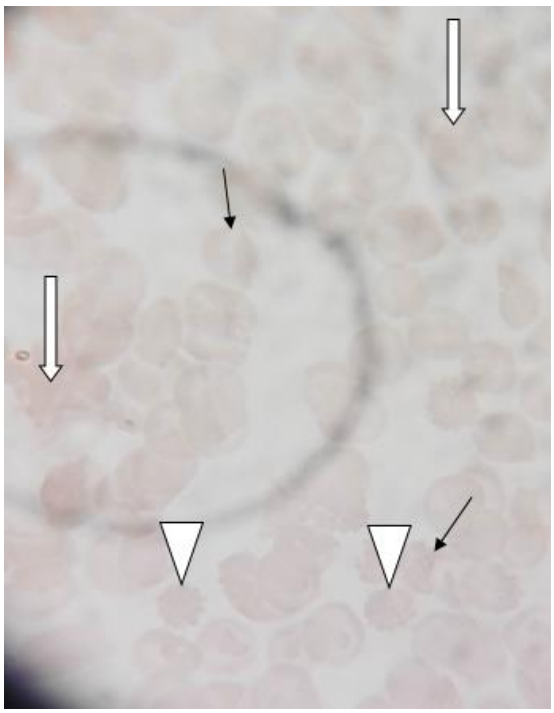

Fig. 2: Shows peripheral blood film of another rat fed with garlic showing fragmented red cells/ schistocyte (slim arrow), red cell agglutination (bold arrow) and acanthocyte (arrow head) MgX40

DISCUSSION

Peripheral blood film was considered in this study because several reports showed that chronic and unregulated intake of garlic causes anaemia (Meleola and Edwin, 1979) of haemolytic type and the degree of heamolysis induced by garlic correlate well with decrease in erythrocyte glutathione (GSH) (Umar *et al*., 1996). This was also confirmed by an increased erythrocyte osmotic fragility of rats fed with garlic (Salami *et al.*, 2012).

In the present study, we observed a remarkable change in morphology of red blood cell both in terms of number and shape. Garlic supplementation showed a feature of intravascular heamolysis (Fragmented cell) and reticulocytosis (the normal response of bone marrow to peripheral loss of red blood cells). The high reticulocyte count recorded in this study agrees with previous study that haemolytic anaemia and other haemopoetic conditions can be monitored by assessing reticulocyte count (Luczynski *et al.*, 2006). Agglutination with a significantly decreased in number of cells both by counting and appearance on peripheral blood film was also seen. This is in support of previous studies that showed peroxides and free radicals caused oxidative damage of membrane; whose stimulation is caused by Garlic (Kashinath, 1990).

Appearance of schistocytes on blood film is suggestive of liver involvement. This is a significant finding because previous studies showed amelioration of garlic on liver damage caused by alcohol consumption (Hussein *et al*., 2007; Shankaran *et al.*, 2010). The lack of unobserved liver damage may probably be due to low quantity of garlic consumed, because study on different concentration of garlic in diet revealed a negative effect at high concentration (Aka *et al*., 2010).

The observed intravascular haemolysis might have resulted into release of haemoglobin and Haem; these two substances have been implicated by some researchers for their involvement in liver cell damage. (Kumar and Bandyopadhayay, 2005). Acute haemolytic anaemia was also reported to induce massive iron accumulation in tissue as one of its characteristic metabolic features (Dhaliwal *et al*, 2004) that can lead to fibrosis of the liver (Brune *et al* 2001).

The data obtained in this study also showed no change in PCV. This is not surprising taking into consideration the high concentration of reticulocytes, a characteristic feature of heamolysis and represent normal response of bone marrow to peripheral blood loss (Gurpreet *et al*,2004).

Thus, our results suggest that oral administration of raw garlic should be monitored especially the liver, if the quantity and duration is mean to be high and long respectively.

Garlic afford a compensatory response by increasing reticulocyte count to counter haemolytic effect. We also observed that garlic attack only old RBCs, this observation is consistent with previous studies that supplementation of high dose of garlic could predispose an animal or human to water loss and metabolic disorder (Aka *et al.*, 2010).

In conclusion, Garlic stimulates erythropoiesis in response to haemolytic effect on RBC membrane as evident by increased reticulocyte circulation and the presence of schistocytes and acanthocytes may be seen as an indication of liver involvement. Thus, our results suggest that oral administration of raw garlic should be monitored, if the quantity and duration is meant to be high and long respectively.

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Effects of *Telfairia Occidentalis* Leaf Extract on Plasma Lactate and Liver Glycogen in Rats

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Summary: *Telfairia occidentalis* is a green vegetable popularly consumed among the native of Africa and it is generally believed to be of medicinal and nutritional value. Studies have reported its hypoglycaemic and hyperglycaemic effects in rats. In addition to these conflicting reports, the mechanisms for its effects on blood glucose remain inconclusive. The objective of this study was to investigate the mechanism involved in the increased blood glucose following treatment with *T. occidentalis*. Twenty five (25) male albino rats (200-250g) were randomly divided into 5 groups (n=5/group). Rats in the control group received normal saline while rats in other groups were orally treated with 100 or 200 mg/kg body weight of the extract for either 1 or 2 weeks. At the end of the treatment, the rats were anaesthetized and blood samples were collected for the estimation of some biochemical parameters. The results showed significant decreases in plasma glucose after 1 week of treatment with 100 mg/kg and 200 mg/kg. However, after 2 weeks of treatment with both doses, plasma glucose levels increased significantly and were higher than those of the control and the rats treated for 1 week with both doses. There were also dose- and duration-dependent decreases in glycogen concentration in the treated rats, especially those treated for two weeks. Glucose-6-phosphatase activity and liver glycogen concentration were lower in rats treated for 2 weeks when compared with those treated for 1 week with both doses. Moreover, plasma lactate concentration was lower in the treated groups when compared with control. The results suggest that *Telfairia occidentalis*-induced lowering of plasma glucose after one week of treatment probably favoured lactate oxidation/gluconeogenesis and elicited breakdown of liver glycogen which resulted in increased plasma glucose after two weeks of treatment.

Keywords*:* Blood glucose, Lactate, Liver glycogen, Glucose-6-phosphatase, *Telfairia occidentalis*

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INTRODUCTION

Telfairia occidentalis is a tropical vine grown in West Africa as a leaf vegetable and for its edible seeds. Common names for the plant include fluted gourd, fluted pumpkin, and Ugwu. *Telfairia occidentalis* is a member of the Curcurbitaceae family and is indigenous to southern Nigeria (Akoroda, 1990). There is paucity of information on the phytochemical constituents of the leaf of *T. occidentalis*. However, it has been reported that the leaf contains tannins, flavonoids, alkaloids, saponins, steroids, anthraquinones, and glycosides (Eseyin *et al*., 2000; Oboh, 2006). The presence of long chain n-3-unsaturated fatty acid in the leaf has also been reported. Palmitoleic acid (16.62%) and elaidic acid (0.85%) are the predominant omega 9 fatty acids present in the leaf (Inuwa *et al*., 2012). The carbohydrate content of the leaf is 25% (Oyenuga, 1968; Akwaowo *et al*., 2000). The amino acid profile of *Telfairia occidentalis* had also been shown to be very rich and includes alanine, aspartate, leucine, glycine, glutamine, histidine, lysine, methionine, tryptophan, arginine, cystine, serine, threonine, phenylalanine, isoleucine, valine and tyrosine (Tindall, 1968; Fasuyi, 2006).

Medicinal and Nutritional values of *Telfairia occidentalis* have also been reported; its shoots contain high levels of potassium and iron, while seeds are composed of 27% crude proteins and 53% fats (Aiyelaagbe and Kintomo, 2002). Its anxiolytic and sedatives properties (Akindele and Ajao, 2013), blood coagulation (Nubila *et al*., 2013), immunomodulatory (Egba *et al*., 2013a; Egba *et al*., 2013b), phytoextraction (Iyagba and Offor, 2013), testiculoprotective (Akang *et al*., 2010; Saalu *et al*., 2010), amelioration of radiation-induced testicular injury (Adejuwon *et al*., 2014), cancer chemopreventive (Iweala and Obidoa, 2009), anti-oxidant and anti-microbial properties (Oboh *et al*., 2006; Iweala and Obidoa, 2009), hepatoprotective (Ekpenyong *et al*., 2012), anti-anaemic (Alada, 2000; Dina *et al*., 2000; Oboh, 2004) anti-convulsant (Gbile, 1986), anti-inflammatory (Oluwole *et al*., 2003) and

purgative (Dina *et al*., 2001) properties have also been reported.

Until recently, the effects of *T. occidentalis* on blood glucose has been controversial with some studies reporting a reduction (Aderibigbe *et al*., 1999; Eseyin *et al*., 2000; Emudianughe and Aderibigbe, 2002; Nwozo *et al*., 2004; Salman *et al*., 2008; Eseyin *et al*., 2010; Eseyin *et al*., 2014) while others reported an increase in blood glucose (Adisa *et al*., 2012) following short-term and long-term treatment respectively. Salman *et al.* (2013) recently reported a decrease in blood glucose after one week of treatment with *T. occidentalis* and an increase in blood glucose after two weeks of treatment. While Salman *et al*. (2013) reported that the blood glucose-lowering effect of *T. occidentalis* observed after one week of treatment could be insulin-dependent as the authors observed an increase in plasma insulin while plasma insulin returned to normal after two weeks of treatment following an increase in blood glucose, the mechanism(s) involved in the increased blood glucose following two weeks of treatment with *T. occidentalis* remains poorly understood

It is hoped that the present study aimed at investigating the effects of *T. occidentalis* on plasma lactate and liver glycogen will throw more light on the probable biphasic effects of *T. Occidentalis* with respect to its role in glucose metabolism.

MATERIALS AND METHODS

Animals and Grouping

Twenty-five (25) male albino rats (200-250g) from the Central Animal House of the Faculty of Basic Medical Sciences, College of Health Sciences, University of Ilorin, Nigeria were used for this study. The animals were housed in a well-ventilated room at a temperature of 23-27ºC on a 12hour light/dark cycle. The principles of laboratory animal care (NIH publication No. 85-23, revised in 1985) were followed. All experiments were examined and approved by the University of Ilorin ethics committee. All necessary protocols were followed to ensure the humane treatment of the animal.

The rats were divided into five groups (A-E, n=5/group) as follows:

Group A- control: received only standard feeds and 0.2ml normal saline for 2 weeks.

Group B- lower dose 1 (LD1): received 100 mg/kg of *Telfairia occidentalis* for 1 week.

Group C- lower dose 2 (LD2): received 100 mg/kg of *Telfairia occidentalis* for 2 weeks.

Group D- higher dose 1 (HD1): received 200 mg/kg of *Telfairia occidentalis* for 1 week.

Group E- higher dose 2 (HD2): received 200 mg/kg of *Telfairia occidentalis* for 2 weeks.

A day after the last treatment in each group, blood sample from each rat was collected by cardiac puncture into lithium heparinised capillary tubes. Plasma was collected from each sample and preserved at -20ºC.

Plant Materials, Preparation and Extraction

Fresh leaves of *Telfairia occidentalis* were bought at Oja Tuntun, Ilorin, Kwara State, Nigeria and authenticated by Mr Bolu Ajayi of the Department of Plant Biology, University of Ilorin, Ilorin, Nigeria. Its voucher number (UIH1063) was deposited in the departmental herbarium. The leaves were washed with water to remove sand and other debris, chopped into smaller bits, air-dried and ground with a mortar and pestle. The leaf material weighing 220g was macerated in 2.5 litres of distilled water for 72 hours and stirred at intervals. The extracts obtained were filtered and the filtrate was dried at 40ºC to obtain a solid extract of 50g.

Measurement of Plasma Biochemical Parameters:

Plasma glucose

Plasma glucose was measured by standard laboratory procedure using the glucose oxidase method (Trinder, 1969).

Plasma lactate

Plasma lactate was assayed by an enzyme linked immunosorbent method using a clinical kit supplied by Monobind Inc., Lake Forest, CA, USA. The protocol was carried out using the manufacturer's instructions. Spectrophotometric readings were taken on a microplate reader at 550 nm wavelength.

Glycogen

The glycogen was extracted from rats' crude liver homogenate and made to react with Anthrone reagent to form a blue-colour solution that was compared spectrophotometrically with that formed by a known amount of glycogen (Seifter *et al*., 1950).

Glucose-6-phosphatase activity

Glucose-6-phosphatase activity was determined using kit from Elabscience Biotechnology Co. Ltd, Wuhan, Hubei Province, China. The ELISA kit uses Sandwich-ELISA as the method. The micro ELISA plate provided in the kit was pre-coated with an antibody specific to Glucose-6-phosphatase. Standards or samples were added to the appropriate micro-ELISA plate wells and combined to the specific antibody. Then a biotinylated detection antibody specific for Glucose-6-phosphatase and Avidin-Horseradish Peroxidase (HRP) conjugate were added to each microplate well and incubated. Free components were washed away. The substrate was added to each well. Only those wells that contain Glucose-6-phosphatase, biotinylated detection antibody and Avidin-HRP conjugate appeared blue in colour and were read spectrophotometrically at 450 nm.

Statistical Analysis

Data were analyzed using SPSS version 20.0 for windows (IBM Corporation, Armonk, NY, USA). All

values given were the Mean ± S.E.M. of the variables measured. Significance was assessed by the one-way analysis of variance (ANOVA), followed by a post-hoc Least Significance Difference (LSD) test for multiple comparisons. *p*-Values of 0.05 or less were taken as statistically significant.

RESULTS

Effects of *Telfairia occidentalis* on plasma glucose level.

There were significant decreases in plasma glucose levels following treatment with 100 mg/kg ($p<0.01$) and 200 mg/kg ($p<0.05$) of *Telfairia occidentalis* for 1 week when compared with the control. However, after 2 weeks of treatment with both doses, plasma glucose was significantly higher than the control and the groups treated with 100- and 200 mg/kg for 1 week (Figure 1).

Effects of *Telfairia occidentalis* on lactate concentration

The plasma lactate concentration decreased significantly ($p<0.05$) in the group of rats treated with 200 mg/kg for 1 week while there was no change in those treated with 100 mg/kg for 1 week when compared with the control. However, plasma lactate decreased significantly after treatment with both 100- and 200 mg/kg for two weeks (Figure 2).

Effects of *Telfairia occidentalis* on liver glycogen concentration

The *T. occidentalis* treatments caused significant reductions in glycogen concentration in all the treated groups except LD1 compared to the control. However, glycogen concentration was significantly lower in rats treated for 2 weeks when compared with those treated for 1 week with the same doses. Similarly, glycogen concentration was also higher in rats treated with 100 mg/kg for 1 week compared with those treated with 200 mg/kg for 1 week. That is, the effect of *T. occidentalis* on glycogen is both dose- and duration-dependent (Figure 3).

Effects of *Telfairia occidentalis* on Glucose-6-phosphatase (G6Pase) activity

There were significant reductions in G6Pase activity in all the rats treated for two weeks while there was no significant change in those treated for one week when compared with the control. Moreover, the two doses of *T. occidentalis* caused significant reductions in G6Pase activity after two weeks of treatment compared to one week of treatment. Furthermore, G6Pase activity was significantly higher in rats treated with 200 mg/kg for 2 weeks when compared with those treated with 100 mg/kg for 2 weeks (Figure 4).

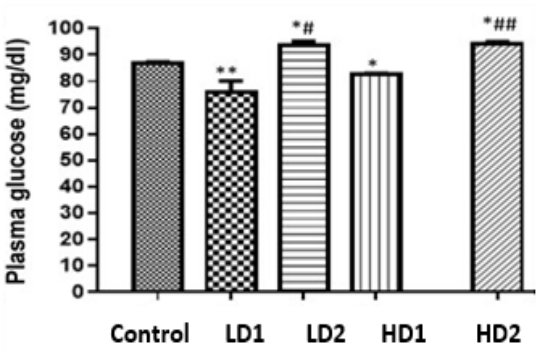

Figure 1: Effects of *Telfairia occidentalis* on blood glucose**.** *$p<0.05$ vs control, **$p<0.01$ vs control; #$p<0.05$ vs 1 week of the same dose, ##$p<0.01$ vs one week of the same dose.

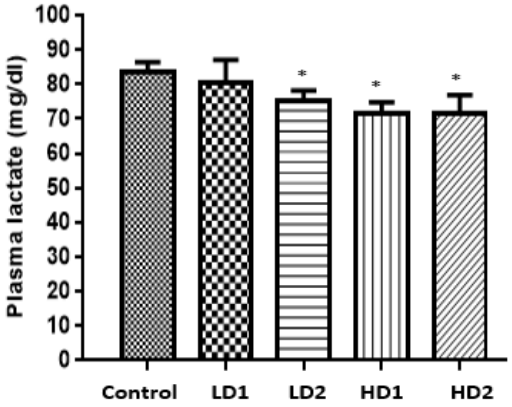

Figure 2: Effects of *Telfairia occidentalis* on plasma lactate concentration. *$p<0.05$ vs control.

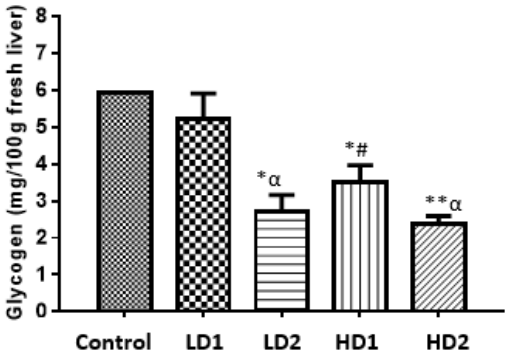

Figure 3: Effects of *Telfairia occidentalis* on liver glycogen concentration. α$p<0.01$ compared to the respective 1 week treatment. *$p<0.01$ vs control, **$p<0.0001$ vs control, #= $p<0.05$ vs LD1.

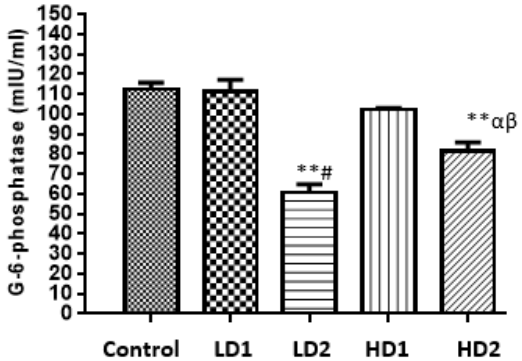

Figure 4: Effects of *Telfairia occidentalis* on Glucose-6-phosphatase activity**.** **$p<0.001$ vs control, #$p<0.01$ vs LD1, α$p<0.01$ vs HD1, β$p<0.01$ vs LD2.

DISCUSSION

The present study showed that *Telfairia occidentalis* caused significant reduction in plasma glucose levels after one week of treatment and significant increase after two weeks of treatment. These findings are consistent with the previously reported hypoglycaemic (Emudianughe and Aderibigbe, 2002; Nwozo *et al*., 2004; Salman *et al*., 2008; Eseyin *et al*., 2010; Salman *et al*., 2013) and hyperglycaemic effects (Adisa *et al*., 2012; Salman *et al*., 2013) of *T. occidentalis*.

The decrease in blood glucose after one week of treatment could be as a result of increase in plasma insulin following treatment with *T. occidentalis* as previously reported (Salman *et al*., 2013). Insulin is a well-known hypoglycaemic agent which regulates glucose metabolism by direct and indirect actions. Through binding to its receptors in the liver, kidney, muscle, and adipose tissue, insulin activates its signaling pathway which involves a complex cascade of protein kinases and regulatory proteins of which insulin receptor substrate 1 (IRS-1) and insulin receptor substrate 2 (IRS-2) are the most important. This causes (1) suppression of glucose release from liver and kidney, (Meyer*et al.*, 1998), (2) translocation of glucose transporters in muscle and adipose tissue to increase their glucose uptake (Oster-Jorgensen *et al*., 1990).

Of great interest is the dose- and duration-dependent decrease in liver glycogen concentration in the treated animals. A cursory look at the data on liver glycogen concentration showed that there was a decrease of about 50% and 60% respectively in liver glycogen concentration in the rats treated with 100- and 200 mg/kg for two weeks when compared with the control. In the liver, glycogen can be degraded by a hydrolytic as well as a phosphorolytic pathway (Vandebroeck *et al*., 1985). However, the hydrolysis of glycogen by α-glucosidase in the lysosomes is only a manifestation of autophagocytosis and is quantitatively unimportant in the overall process of glycogen mobilization. The regular phosphorolytic pathway of glycogenolysis is catalysed by phosphorylase-α and results in the release of glucose-1-phosphate, which is in equilibrium with glucose 6-phosphate by the action of phosphoglucomutase. In the periportal zone, which is rich in glucose 6-phosphatase, glucose-6-phosphate is mainly converted into glucose when the circulating glucose concentrations are falling. In the perivenous zone, on the other hand, glucose 6-phosphate is mainly converted into lactate in the post-absorptive phase (Jungermann and Kietzmann, 1996). Thus, the breakdown of liver glycogen can either lead to the production of glucose or lactate. However, since plasma glucose increased significantly while lactate decreased, it is tempting to suggest that the glycogen was probably converted to glucose.

A question that readily comes to mind is, what was the fate of the glucose removed from the blood after one week of treatment. Was it phosphorylated or converted to glycogen? Liver glycogen was measured because the liver is the main organ involved in glucose homeostasis which has the capacity to store excess glucose as glycogen. Interestingly, there was no any increase in liver glycogen in any of the treated groups in this study. However, since the fate of glucose taken up by cells is either to be oxidized and converted to pyruvate/lactate or stored as glycogen, it will not be unreasonable to speculate that *T. occidentalis* could have the potential of increasing glycolysis since there was no increase in glycogen or lactate concentration after one week of treatment. This is reasonable because glycogenesis can only be favoured when there is hyperglycaemia but on the contrary, *T. occidentalis* actually caused a reduction in plasma glucose after one week of treatment.

The physiological significance of the reduction in lactate concentration observed in this study and the probability of lactate contributing to the increased blood glucose seen after two weeks of treatment with *T. occidentalis* is not clear. However, in resting mammals, oxidation accounts for approximately half lactate disposal and gluconeogenesis approximately 20% (Stanley *et al*., 1986; Brooks *et al*., 1991). Thus, the significant reduction in plasma lactate in this study suggests that the lactate was probably used for oxidation, gluconeogenesis or glycogenesis via the indirect pathway.

For much of the 20th century, lactate was largely considered a dead-end product of glycolysis due to hypoxia, the primary cause of the oxygen debt following exercise, a major cause of muscle fatigue, and a key factor in acidosis-induced tissue damage. Since the 1970s, a lactate revolution has occurred. At present, we are in the midst of a lactate shuttle era; the lactate paradigm has shifted. It now appears that increased lactate production and concentration as a result of anoxia or dysoxia are often the exception rather than the rule (Gladden, 2004). What is now known as the cell-to-cell lactate shuttle was introduced by Brooks (1985) simply as the lactate shuttle. Since its introduction, this hypothesis has been repeatedly supported by studies using a wide variety of experimental approaches. It posits that lactate formation and its subsequent distribution throughout the body is a major mechanism whereby the coordination of intermediary metabolism in different tissues, and cells within those tissues, can be accomplished.

The importance of lactate as a carbohydrate fuel source is underscored by the fact that during moderate intensity exercise, blood lactate flux may exceed glucose flux (Brooks, 2000). Because of its large mass and metabolic capacity, skeletal muscle is probably the major component of the lactate shuttle, not only in

terms of lactate production but also in terms of net lactate uptake and utilization as well. At rest, muscles slowly release lactate

into the blood on a net basis, although at times they may show a small net uptake. During

exercise, particularly short-term, high-intensity exercise, muscles produce lactate rapidly while lactate clearance is slowed. This results in an increased intramuscular lactate concentration and an increased net output of lactate from muscles into the blood. Later, during recovery from short-term exercise, or even during continued, prolonged exercise, there is net lactate uptake from the blood by resting muscles or by other muscles that are exercising at a low to moderate intensity (Richter et al. 1988; Brooks, 2000; Gladden, 2000).

Recently, Miller et al. (2002a,b) investigated subjects exercising at a moderate exercise intensity with lactate infusion to maintain lactate concentration at approximately 4mM. Overall, they (Miller et al. 2002a) found a significant increase in lactate oxidation accompanied by a decrease in glucose oxidation; the interpretation is that lactate competes successfully with glucose as a carbohydrate fuel source, thus sparing blood glucose for use by other tissues. For instance, lactate, which is a substrate for lactate dehydrogenase, has been shown to maintain synaptic formation and sustain synaptic adaptation during hypoglycaemia in the mammal hippocampus (Schurr *et al*., 1988; Sakurai *et al*., 2002). Since the fate of lactate taken up is to be oxidized or converted to glycogen or glucose and there is no increase in liver glycogen, it probably means the reduction in plasma lactate observed in this study was due to its oxidation or conversion to glucose. However, lack of data on plasma pyruvate is a major limitation of this study which makes it difficult to conclude that the reduction in plasma lactate was actually due to its oxidation. Further studies (acute and chronic) involving simultaneous measurement of lactate and pyruvate, glycolytic enzymes, hormones and enzymes of glycogen and lactate metabolism will shed more light into the mechanism of *T. occidentalis*-induced reduction in plasma lactate and liver glycogen and the resultant increase in plasma glucose.

Although, there may be no direct evidence yet, the present study has provided an insight into the possibility of lactate gluconeogenesis/oxidation contributing to the increased blood glucose observed after two weeks of treatment. However, overall contribution of gluconeogenesis to the increase in blood glucose seen following *T. occidentalis*-induced lowering of blood glucose could not be ascertained or quantified in this study since there are many other gluconeogenic substrates apart from lactate such as alanine and glutamine that might have been used for gluconeogenesis. It should be noted that *T. occidentalis* itself is rich in gluconeogenic amino acids such as alanine and glutamine. Thus, the role of amino acid constituents of *T. occidentalis* in the increased blood glucose after two weeks of treatment may also need further investigation in order to be able to quantify the overall contribution of gluconeogenesis to the increased blood glucose after two weeks of treatment.

It is also noteworthy that blood glucose in rats treated for two weeks rose to values above the control in response to *T. occidentalis* -induced lowering of blood glucose. This probably suggests there was overactivation of the counterregulatory response. For instance, insulin hypoglycaemia has been shown to accelerate the release of blood-sugar-raising hormones from the adrenal-pituitary system, setting off a running contest between the latter and excessive insulin action. Under the recurrent stress of hypoglycaemia, the insulin-opposing factors can gain ascendency over insulin action and thus can produce hyperglycaemia despite hyperinsulinism (Somogyi *et al*., 1959). Further investigation on the role of counterregulatory hormones such as glucagon and adrenaline in the response to the blood-glucose-lowering effect of *T. occidentalis* will shed more light into the mechanisms involved in the increased blood glucose levels after two weeks of treatment.

Therefore, the increase in blood glucose to values above the control in rats treated for two weeks could have resulted in the reduction in the glucose-6-phosphatase activity presumably to shut down glycogenolytic and gluconeogenic activities in order to maintain glucose homeostasis. However, since glucose-6-phosphatase catalyzes the final step of both glycogenolysis and gluconeogenesis which are the counterregulatory responses to hypoglycaemia, it will not be unreasonable to suggest that an increase in the activity of the enzyme could have probably occurred much earlier (i.e. between the 8^{th} and 14^{th} day of treatment) at a time when the activity of the enzyme was not determined in this study. The increase could not be observed probably because the estimation of glucose-6-phosphatase activity was done 24 hours after the last day of treatment with *T. occidentalis* when blood glucose had risen to values above that of the control rats; a situation that could lead to a reduction in glucose-6-phosphatase activity and consequently a reduction in glycogenolytic and gluconeogenic activities in order to prevent further rise in blood glucose and maintain glucose homeostasis. Therefore, estimation of the activity of this enzyme between the 8^{th} and 14^{th} day of treatment will shed more light into the activities of the enzyme during *T. occidentalis*-induced lowering of the plasma glucose.

In conclusion, the present study has shown that *T. occidentalis*-induced reduction in plasma glucose after one week of treatment elicited dose- and duration-dependent reduction in liver glycogen and plasma

lactate which resulted in the increased plasma glucose after two weeks of treatment.

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Salivary Electrolytes, Total Protein and Immunoglobulin A in Patients with Chronic Kidney Disease: A Case Control Study

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Summary: The objectives of this study were to compare levels salivary electrolytes, total protein and immunoglobulin A (IgA) in patients with chronic kidney disease (CKD) and healthy individuals; and to determine the relationship between the salivary and blood levels of these factors between the two groups. Ninety-eight participants consisting of 48 patients with CKD and 50 healthy individuals (age and gender matched) were included. Whole saliva and blood samples were collected and analyzed for concentrations of electrolytes (K^+, Na^+, Ca^{2+}, Cl^-, and HCO_3^{2-}), total protein and IgA. Data were analyzed using Independent-Samples t-test and Pearson correlation test. Concentrations of salivary K^+, Ca^{2+}, Cl^-, and total protein were higher; while concentrations of salivary Na^+, HCO_3^{2-} were lower in patients with CKD compared with healthy individuals. There was no difference in the salivary IgA levels in patients with CKD compared with healthy individuals. Salivary calcium level showed linear correlation with the plasma calcium level while salivary chloride level showed negative correlation with plasma chloride level among patients with CKD. These findings indicate that saliva and plasma from patients with CKD are characterized by higher potassium, chloride, and lower sodium concentrations than their levels in healthy individuals; thus, suggesting a possible increased adrenal-cortical activity in patients with CKD.

Keywords*:* Saliva, electrolytes, total protein, correlation, chronic kidney disease

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INTRODUCTION

Chronic kidney disease (CKD), a progressive disorder marked by a loss of kidney function over time has become major problem worldwide (Meguid and Bello, 2005; Brosnahan and Fraer, 2010). The early stages of CKD are characterized by kidney damage and are generally asymptomatic. As the kidney disease aggravates, kidney function begins to worsen, leading to end-stage kidney disease, which requires kidney transplantation or hemodialysis (Levey et al., 2003). Individuals with CKD manifest diverse systemic alterations including oral complications and variations in the flow and composition of the saliva (Nandan et al., 2005; De la Rossa-Garcia et al., 2006; Davidovich et al., 2011; Fregoneze et al., 2013; Oyetola et al., 2015).

Saliva is a unique fluid that can be used to monitor oral and systemic diseases. It has many advantages over serum because its collection is non-invasive, simple, and requires minimal skill. Saliva sampling is appropriate for all age groups and can be repeated more frequently. It also offers a cost-effective method for the screening of large populations (Mandel, 1987; Samaranayake, 2007; Liu and Duan, 2012).

Saliva plays an essential role in the maintenance of oral homeostasis. Its constituents are the important factors responsible for its physiological functions (Mandel, 1987; Tabak, 2001; De Almeida *et al.*, 2008). Various salivary functions, including lubrication, cleansing action, antibacterial activity, buffering action, maintenance of tooth integrity, taste sensation and digestion may be disturbed by changes in salivary flow and biochemical properties. Parameters in saliva can be affected by many factors including diet and genetics; hence its usage as a biomarker or diagnostic fluid is still subject to continuous research.

Few articles in the literature have specifically analysed salivary biochemical changes in patients with CKD, and their findings are contradictory (Earlbaum and Quinton, 1981; Davidovich et al., 2011; Anuradha et al., 2015; Rodrigues, 2016). This study was therefore designed to compare levels of salivary electrolytes, total protein and IgA in patients with CKD and healthy individuals; and to determine the relationship between salivary and plasma levels of these factors.

MATERIALS AND METHODS

Design: A cross sectional case control study of patients with CKD and healthy individuals as controls (age and gender matched).

Study population: The study received ethical approval from the institution Research Ethics Committee (UI/UCH EC/13/0099). Patients with CKD were individuals attending the University College Hospital, Ibadan, Nigeria, diagnosed of the disease having estimated GFR of < 60 ml/min/1.73 m2 and stages 4 and 5 of National Kidney Foundation – Kidney Disease Outcome Quality Initiative (NKF-KDOQI) staging. The etiologies of CKD in the patients were hypertension, chronic glomerulonephritis, obstructive uropathy and diabetes mellitus. Most of the patients with CKD were undergoing dialysis treatment because of their late stage presentation. Also included in the study were the healthy individuals who were volunteers within the community and had no history of kidney disease, systemic or oral disease. Participants were provided information regarding risks and benefit of the study and verbal consent was taken. Participants had oral examination before saliva collection.

Saliva and blood sampling: This was performed according to the method previously described (Lasisi et al., 2016). Saliva collection was between 8.00 am and 10.00 am and participants had not taken meal for at least 2 hours before saliva collection. Whole saliva was collected by spitting method. Participants were asked to spit (after rinsing the mouth with distilled water) into calibrated universal plastic bottles until about 3 mls of saliva was collected. Simultaneously, 5mls of blood samples were taken from the participants by venipuncture into lithium heparin bottles and the plasma was used for the analysis. Saliva and plasma samples were stored at -20 °C until laboratory analysis. Saliva samples were defrosted at room temperature and then centrifuged at 3000 rpm for 10 min in order to remove contaminants before being used for the analysis.

Determination of total protein and electrolytes concentrations: Estimation of total protein was done using Biuret method (Jenzano et al., 1986). Sodium and potassium levels were determined using spectrophotometry, while estimation of calcium was done using indirect colorimetric method (De Loureiro, 1944). Concentrations of chloride and bicarbonate were determined by Schales method using mercuric nitrate (Shales and Shales, 1941).

Determination of salivary IgA levels: Immunoglobulin A level in the saliva samples was quantified using the Enzyme Linked Immunosobent Assay (ELISA) method (Salimetrics®, UK) according to the kits manufacturers' instructions. Briefly, each test sample was diluted 1:10,000 and 100 µL of test or standard was dispensed in duplicate with pipette into pre-designated wells. The micro titre plate was incubated at room temperature for thirty minutes and the contents of the wells were aspirated. Each well was filled with diluted Wash Solution and aspirated. This was repeated three times. The wells were filled again with undiluted wash buffer, drained by inversion and blotted (striking the wells on absorbent paper). This was repeated 3 times. Enzyme-Antibody Conjugate (100 µL) was pipetted to each well. The plate was covered and incubated in darkness for thirty minutes. The wells were thereafter, washed and blotted. TMB Substrate Solution (100 µL) was pipetted into each well, incubated in the dark at room temperature for 10 minutes after which 100 µL of Stop Solution was added. The plate reader was calibrated and the absorbance of the content of each well was determined at 450 nm.

Statistical analysis: Data are presented as mean ± standard deviation (SD) and compared using Independent student t-test. Correlation between plasma and salivary biochemical parameters was determined using Pearson correlation test. All analyses were done using IBM SPSS Statistics (version 22) at 5% level of significance.

RESULTS

The demographic data of the patients with CKD and the healthy controls are shown in table 1. Salivary levels of sodium and bicarbonate were significantly lower, whereas level of potassium was higher in patients with CKD compared to healthy individuals (table 2).

Similarly, plasma levels of sodium and bicarbonate were significantly lower, whereas level of potassium was higher in patients with CKD compared to healthy individuals (table 3).

Salivary levels of total protein, chloride, and calcium were significantly higher in patients with CKD compared to their levels in healthy individuals (table 2). There was no significant difference comparing levels of salivary IgA between patients with CKD and healthy individuals.

There was positive correlation between saliva and plasma calcium levels (figure 1); whereas, saliva and plasma chloride levels showed negative correlation in patients with CKD (figure 2).

Table 1: Demographic distribution of participants

	Healthy control (N = 50)	Patients with CKD (N = 48)
Age (yrs)	39.1 ± 7.34	39 .82 ± 11.07
Male	20	18
Female	30	30

Table 2: Salivary levels of electrolytes, total protein and IgA in patients with CKD and healthy control

	Healthy control	Patients with CKD	P value
Sodium (mmol/L)	15.79 ± 3.5	5.79 ± 4.41	< 0.001
Potassium (mmol/L)	18.50 ± 3.94	21.88 ± 8.63	0.02
Chloride (mmol/L)	3.72 ± 1.85	12.83 ± 8.51	< 0.001
Bicarbonate (mmol/L)	22.69 ± 2.42	15.61 ± 8.03	< 0.001
Calcium (mg/dL)	1.68 ± 1.09	6.82 ± 3.23	< 0.001
Total protein (mg/dL)	6.64 ± 4.71	17.81 ± 13.33	< 0.001
IgA (µg/mL)	491.40 ± 26.21	490.87 ± 43.52	0.96

Table 3: Plasma levels of electrolytes and total protein in patients with CKD and healthy control

	Healthy control	Patients with CKD	P value
Sodium (mmol/L)	137.87 ± 9.39	126.14 ± 12.45	< 0.001
Potassium (mmol/L)	3.89 ± 0.55	6.51 ± 4.09	< 0.001
Chloride (mmol/L)	105.17 ± 7.71	96.50 ± 10.56	< 0.001
Bicarbonate (mmol/L)	20 ± 1.78	17.38 ± 3.45	< 0.001
Calcium (mg/dL)	6.21 ± 2.16	7.57 ± 2.65	0.01
Total protein (mg/dL)	81.72 ± 7.88	57.26 ± 21.42	< 0.001

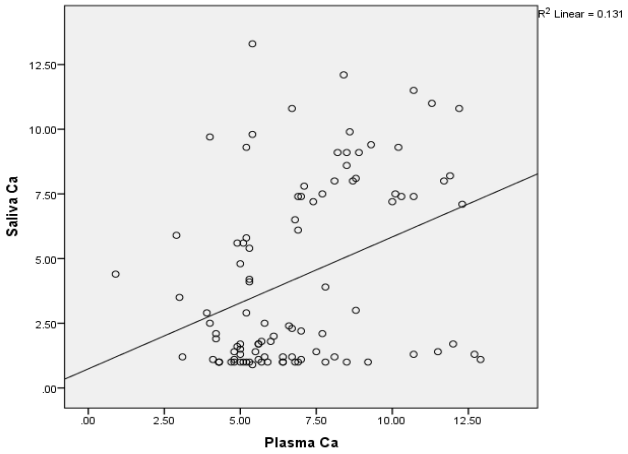

Figure 1: Correlation between salivary and plasma calcium concentrations in patients with CKD

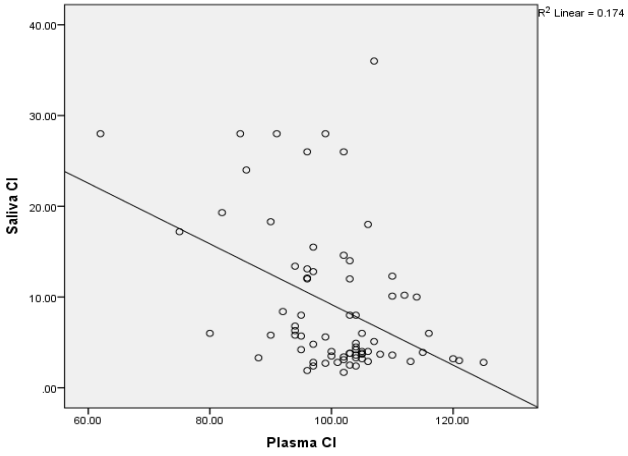

Figure 2: Correlation between salivary and plasma chloride concentrations in patients with CKD

There were no correlations between saliva and plasma sodium, potassium and bicarbonate in patients with CKD as well as the healthy controls (table 4 and 5).

Table 4: Relationship between plasma and salivary factors in patients with CKD

	Pearson correlation coefficient (r)	P value
Sodium	-0.20	0.19
Potassium	0.12	0.45
Bicarbonate	-0.14	0.36
Total protein	0.16	0.28

Table 5: Relationship between plasma and salivary factors in healthy controls

	Pearson correlation coefficient (r)	P value
Sodium	0.12	0.52
Potassium	-0.04	0.84
Chloride	0.16	0.40
Bicarbonate	-0.08	0.69
Calcium	-0.31	0.03
Total protein	-0.14	0.34

DISCUSSION

The comparatively lower concentration of salivary sodium in patients with CKD observed in the present study might have been related to the lower plasma level. Hyponatremia is a common complication of CKD due to many factors. Patients with CKD are at additional risk of hyponatremia due to compromised capacity to dilute or concentrate urine (Berl, 2008). Furthermore, diet restriction and use of multiple drugs are common and can contribute to the sodium derangements (Dhondup and Qian, 2017). However, our finding is contrary to the reports of higher salivary sodium concentrations (Anuradha et al., 2015; Bagalad et al., 2017) and lack of difference in the concentration of salivary sodium (Davidovich et al.,

2011) in patients with CKD compared with healthy controls. The differences in the findings are probably due to the occurrence of both hypo and hypernatremia in patients with CKD. Bagalad et al., (2017) also reported elevated serum levels of sodium with positive correlation with salivary levels in patients with CKD.

In this study, the higher concentration of salivary potassium in the patients with CKD is in agreement with the previous reports (Thomas et al., 2008, Davidovich et al., 2011; Bagalad et al., 2017). Similar to the report of Bagalad et al., (2017), the elevated salivary potassium level in the present study reflected the blood level. Hyperkalaemia is one of the complications of chronic CKD, usually developing when glomerular filtration rate (GFR) falls below 20% of normal (Gennari and Segal, 2002; Muso, 2004a; 2004b). The hyperkalaemic state in patients with CKD occurs for several reasons that include high dietary potassium intake relative to reduced renal function; extracellular shift of potassium caused by metabolic acidosis and treatment with renin–angiotensin–aldosterone system blockers that inhibit renal potassium excretion (Einhorn et al., 2009).

Low level of sodium, corresponding to high level of potassium, in the saliva relative to the plasma is a reflection of the active transport mechanism that has been reported in the literature (Poulsen, 1998; Turner and Sugiya, 2002). Furthermore, the lower salivary sodium concentration with higher potassium concentration in patients with CKD might be related to the reduced salivary flow rate previously reported in individuals with CKD (Hong-Seop et al., 1999; Thomas et al., 2008; Oyetola et al., 2015). The salivary electrolytes vary with the flow, such that a decrease in flow rate could explain, for example, a decrease in the concentration of sodium and an increase in the concentration of potassium. It has been reported that the reduced salivary flow rate in patients with CKD could be attributed to the underlying mechanisms that were suggested as direct glandular damage and/or the inadequacy in fluid intake (Epstein et al., 1980; Gavalda, 1999).

In the present study, despite reduction in the salivary concentration of sodium, salivary concentration of chloride was higher in patients with CKD. Dissociation between changes in salivary sodium and chloride has been reported previously by McCance, (1938) who observed that acute salt deficiency in five normal subjects produced a consistent fall in the concentrations of salivary sodium while the corresponding changes in chloride were inconsistent. Also, similar to our finding Davidovich et al., (2011) reported elevated salivary chloride in patients with CKD. Changes in salivary chloride levels were similar to changes in potassium levels, probably reflecting an increase in corresponding anion secretion, due to the active secretion of cation (in this case, potassium).

The salivary concentration of calcium in the patients with CKD in the present study was higher than in the control which is in contrast to previous reports of lower concentration of salivary calcium in patients with CKD (Anuradha et al., 2015; Bagalad et al., 2017). The plasma concentration of calcium was also higher in the patients with CKD compared with control and this may account for the elevated level in saliva. The higher level of salivary calcium in patients with CKD may suggest increased protection against dental caries in these individuals. A systematic review by Andrede et al., (2014) has documented lower dental caries prevalence in individuals with CKD. Similarly, Sewon et al., (1998) reported that high salivary calcium was associated with low DMF scores (caries index).

In the present study, despite lower plasma protein, salivary total protein was significantly higher in patients with CKD compared with healthy control. This finding corroborates the recent report of higher salivary total protein in experimental CKD in rat by Romero et al., (2017). It was suggested that the higher level of salivary total protein in chronic kidney disease could be attributed to amylase level. Amylase accounts for approximately 50% of the total protein produced by the salivary glands (Schenkels et al., 1995). In the experimental study by Romero et al., (2017), a significant increase in salivary amylase activity was observed in CKD group in response to isoproterenol stimulation. The higher concentration of salivary total protein in patients with CKD in this study might explain the increased buffering as well as reduced caries prevalence in these patients.

Lack of difference in the salivary IgA levels in patients with CKD compared with healthy control in the present study may suggest that components of the local humoral immune response (of which salivary IgA predominates) are not impaired in these individuals. Secretory immunoglobulin A (sIgA) is the most frequently found immunoglobulin in mixed saliva and is considered to be a secretory factor for acquired immunity in the oral cavity. Through restriction of microbial adhesion, salivary IgA forms part of the first line of defense and also participates in the preservation of the integrity of oral tissues (Bokor-Bratic, 2000; Dodds, 2005). Thus, sIgA plays an important role in oral homeostasis and is an important indicator of the defensive status of the oral cavity (Bernimoulin, 2003).

In conclusion, the findings from the present study indicate that the electrolyte content of whole saliva from patients with CKD is characterized by higher potassium, chloride, and lower sodium concentrations than saliva of healthy individuals. These findings are similar to the changes observed in the blood electrolyte concentrations; thus, suggesting a possible increased adrenal-cortical activity in patients with CKD. In addition, the finding of higher levels of salivary total

protein and calcium in patients with CKD in this study suggest their role in the reduced caries prevalence in these individuals.

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Modulatory Role of Vitamins A and E on Memory and Motor Functions of Cyanide Induced Neurotoxicity in Adult Swiss Mice

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Summary: Cyanide is a potent neurotoxic substance that can initiate series of intracellular reactions leading to oxidative stress. To evaluate effect of sublethal administration of potassium cyanide (KCN) on sensorimotor functions and long term visuo-spatial learning and memory in adult Swiss mice and possible ameliorative role of vitamins A and E. These vitamins A and E (dietary) are antioxidants that have scavenging properties against free radicals and reactive oxygen species as a result of oxidative stress induced by cyanide. Thirty-five mice weighing between 18-22 g were used for the study. The animals were randomly divided into five groups (n = 7) and exposed to sublethal concentration of potassium cyanide (10% LD_{50}; 1.5 mg/kg). KCN was administered orally while vitamin A (25 mg/kg) and vitamin E (50 mg/kg) were administered intra-peritoneal (IP) once daily for 28 days. Potassium cyanide (KCN) was first administered and after 10 minutes intervals, followed by vitamin A and then E after 5 minutes, vitamin E were administered across the different treatment groups. Mice were examined for signs of toxicity. Vitamins pre-treatment ameliorated toxic signs. In the dynamics of wire grid, coat hanger and stationary beam test, the latency to fall in weeks 2 and 4 were statistically significant. In acquisition and retention, using elevated plus maze (EPM), KCN treated group recorded high transfer latencies in seconds (50.40±1.72 secs) and (57.60±0.93 secs) as compared to group IV (29.40±0.68 secs; 5.60±0.60 secs). Cyanide is a neurotoxin that affects motor functions with progressive decline in motor strength and coordination. KCN affects acquisition and retention memory while pre-treatment with antioxidant vitamins A and E ameliorated these deficits.

Keywords*:* Cyanide; Neurotoxicity; Memory; Motor function

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INTRODUCTION

Cyanide is a rapidly acting, potentially deadly chemical that can exist in various forms which is toxic to humans and animals; exposure can occur in various ways. Many substances are potential sources of cyanide exposure, including edible and non-edible plants (e.g., cassava), industrial operations (e.g., plastics processing), fires, and cigarette smoke (Ogundele and Olu, 2012). Although the primary natural source of cyanide poisoning is from plants, other natural sources include volcanoes, bacteria, and fungi. Its toxicity is well known but it is still used in the surgical dressing, metal-plating, mining, chemical and agricultural industries (Al-Ghanim and Mahboob, 2012). The toxic effects of cyanide (HCN) have traditionally been attributed to inhibition of cytochrome C oxidase, the terminal enzyme of respiratory chain, which compromises oxidative phosphorylation leading to cytotoxic hypoxia (Caxton *et al.*, 2010).

Neurological disturbances have been reported from parts of Africa with protein-deficient populations and attributed to cyanide (CN^-) exposure from prolonged dietary use of cassava (Mathangi *et al.*, 1999; Osuntokun, 1981). Acute cyanide intoxication causes confusion, agitation, disorientation and cognition deficits while, chronic cyanide exposure that results from consumption of improperly processed cassava leads to neurodegenerative disorders (Konzo and Tropical Ataxic Neuropathy) Cardosso *et al*., 2002. One form of cassava related disease is a slowly developing ataxic myeloneuropathy. It is a local Zairian term for a disease first described in 1938, in the democratic republic of Congo (formally Zaire), but has also been observed in Mozambique, Tanzania,

Central African Republic Cameroon and Nigeria (Ernesto *et al.,* 2000; Mathangi and Namasivayan, 2000).

The syndromes grouped as TAN can differ widely in clinical presentation, natural history and response to treatment. It is an upper-motor-neuron disease characterized by irreversible but non-progressive symmetric spastic paraparesis that has an abrupt onset (Oluwole *et al.,* 2003). The main clinical features of some of the syndromes have included sore-tongue, angular stomatitis, skin desquamations, optical atrophy, neurosensory deafness and sensory gait ataxia (Link *et al.,* 2000). The development of these syndromes is hypothesized to depend on 1) Amount and duration of exposure to dietary cyanide and 2) The ability of the body to detoxify cyanide, a function that may vary with nutritional status (Mathangi *et al.,* 1999).

Oxidative stress arises from an imbalance between the production of free radicals and physiological antioxidant capability (Kapur *et al.,* 2004). However, during the oxidative stress as previously reported in cyanide poisoning, the body's antioxidant systems are overwhelmed by the oxidants. Under these circumstances, a provision of exogenous antioxidants becomes imperative to neutralize the damaging effect of reactive oxygen species. Vitamin A and its derivatives (retinoids) play important roles in many physiological processes. It is important for both development and maintenance of adult brain homeostasis (Bailey and Lane, 2005). Throughout adulthood, vitamin A remains important in other central nervous system (CNS) related functions, for instance learning and memory (Carta *et al.,* 2002). Furthermore, vitamin A and its related retinoids easily penetrate into blood-brain-barrier and mammalian CNS contains the molecular apparatus responsible for the production and maintenance of all-trans-retinoic acid in neurons, through retinal dehydrogenases and cellular retinoid proteins action (Deuster, 2000). Thus, the CNS is able to transport and metabolize retinoid molecules and may rapidly increase their concentrations. Vitamin E is an essential nutrient in humans and well known antioxidant substance that reduces free radicals and reactive oxygen species activity (Asonye and Okolie, 2004). Like other antioxidants, vitamin E slows or prevents memory impairments that accompany several conditions such as mental stress, diabetes, cerebral ischemic injury, Alzheimer's disease, stroke and aging (Mohammed *et al.,* 2 014). The aim of the present study is to investigate the effect of vitamins A and E on KCN-induced neurotoxicity.

MATERIALS AND METHODS

Drugs and Chemicals

Potassium cyanide was purchased from sigma Aldrich USA, It was dissolved in di-ionised water. Vitamin A (1000IU) and E (100 mg DL alpha tocopherol) were purchased from Patterson zoochonis Ltd, Nigeria) and were reconstituted in soy oil to 1% solution and 20 mg/ml respectively.

Animals

Thirty-five adult Swiss male mice (weighing between 18-22 g) were obtained from the animal house of Department of Physiology, Faculty of Medicine Ahmadu Bello University Zaria, Nigeria. All animals were housed in a steel cage, at room temperature and had free access to drinking water and their diets. The animals were acclimatized for 7 days to their environment and diet prior to commencement of the study.

Treatment of Animals

The animals were randomly grouped into five groups (n = 7) as follows: Group I (control, received deionised water), group II (1.5 mg/kg KCN only), group III (1.5 mg/kg KCN + 25 mg/kg vitamin A), group IV (1.5 mg/kg KCN + 50 mg/kg vitamin E), and group V (1.5 mg/kg KCN + 25 mg/kg vitamin A + 50 mg/kg vitamin E). KCN was administered orally while vitamins (A and E) were administered via intra-peritoneal (IP) route once daily for 28 days. Potassium cyanide (KCN) was first administered and after 10 minutes intervals, followed by vitamin A and then vitamin E after 5 minutes were administered across the different treatment groups. The animals were observed for toxic signs and possible deaths throughout the study period. The experiment was performed according to the guidelines on animal research of the Animal Research Ethic Committee of the Ahmadu Bello University, Zaria.

NEUROBEHAVIORAL ASSESSMENTS

All behavioural parameters were assessed by two trained observers blind to the animals' treatment status in order to eliminate bias.

Wire Grid Test

Motor strength was evaluated using wire grid test. Strength suspension (four paws) was assessed on a grid bordered with masking tape to prevent the mouse from walking off the edge (Delatour *et al.,* 2008). The mouse was placed on the centre of the grid that was slowly turned upside down at a 20 cm height above the floor. Latency to fall was recorded (cut off: 1 min).

Coat Hanger Test

The assessment of motor coordination was evaluated using coat hanger test as described by Delatour *et al*., (2008). An iron horizontal bar (1.3 mm diameter) was placed at a height of 30 cm from the floor; a cardboard wall was inserted at each end of the bar in order to prevent mice from escaping sideways. Mice were placed in the middle part of the horizontal bar. Each trial began only when the forepaws gripped the bar at the moment of release. Three trials were recorded

(inter-trial interval: 5 min), and after each trial, mice were re-isolated. The latencies of the traction reflex (i.e. time taken by the animal to catch the bar by one of its hind paws) and alternatively the latencies before falling (cut off: 30 s) were measured.

Stationary Beam Test

Beam walk was conducted in order to detect any neurological deficits in sensory, balance, or motor performance. The materials used were Methylated spirit, cotton wool, and the Beam walk apparatus which is made up of 75 cm strips of two smooth woods. One is flat like a ruler of 25 mm width by 10 mm thickness. The other is round and a diameter of 20 mm. Narrow supports stand to hold up the start section of the raised beam (15 mm cross-section, 40 cm high) was also used, together with the Goal box (20 cm x 15 cm, with a 4 × 5 cm entrance hole) secured on top of a support stand.

Elevated plus-maze test

An elevated plus maze was conducted as described by Komada *et al.,* (2008). Briefly, the elevated plus maze consisted of two open arms (25 x 5 cm) and two closed arms of the same size with 15 cm high wooden walls. The arms and central square were made of wood, elevated 55 cm above the floor. Each mouse was placed at the distal part of the open arm maze (5 x 5 cm), facing away from the closed arm. On the training day (first day), each animal was placed at the end of one open arm, facing away from the central platform. The latency of the mouse to move from the open arm to the enclosed arms was recorded within 90 seconds. Following entry into the arm, the animals were allowed to explore the apparatus for 20 seconds. Twenty-four hours later, the second trial (retention test) was performed and the animals were observed for 90 seconds. Reduction in latencies between day one (acquisition) and day two (retention) indicates memory of the learned task. After each trial, the maze was wiped with a cloth dipped in 70% ethanol, and allowed to dry to remove any olfactory cue. An overhead camera video camera recorded movement of the mice for later quantification.

Statistical analysis

Results were expressed as the mean ± standard error of the mean (SEM). Data for multiple variable comparisons were analyzed by one-way analysis of variance (ANOVA). For the comparison of significance between groups, Duncan's test was used as a *post hoc* test according to the statistical package program (SPSS version 17.0). All values $p < 0.05$ was considered as significant for all statistical analysis in this study.

RESULTS

Signs of toxicity

Toxic signs observed in the potassium cyanide treated group included huddling, asphyxiation, dyspnoea, mild tremors, piloerection and soft faecal bolus (diarrhoea). With progressive weeks, one death was recorded in group II. The vitamin pre-treated groups (III, IV and V) showed milder toxic signs compared to the KCN-treated group and these include piloerection, soft faecal bolus (diarrhoea) and dyspnoea.

Effect of treatments on wire grid test

As shown in figure 1, analysis of neurological performance in the WGT showed a significant decrease ($p < 0.05$) of motor strength in the KCN-treated group as compared with control and vitamins pre-treated group. A global decline of performance was noted with progressive weeks 2 and 4 respectively (12.60±0.40 secs) and (9.20±0.58 secs) in group II as compared to group V (27.60±0.93 secs) and (38.00±1.05 sec) $p < 0.05$.

Effect of treatments on coat hanger test

The latency to fall from the coat hanger was also measured as an alternative index for motor strength. Analysis of variance of this variable indicated a global decline of performance in KCN-treated group with progressive increase in weeks 2 and 4 respectively (11.00±0.45 secs; 8.20±0.58 secs) when compared to control (24.40±0.68 secs; 26.60±0.51 secs). Pretreatment with vitamins showed increase in motor performance as seen in group IV in contrast to group II (8.20±0.58 secs and 24.60±0.68 secs) $p < 0.05$.

Effect of treatments on stationary beam test

Motor coordination was evaluated with a stationary beam test. Analysis of the number of crossed segments in group II showed decreased activity in KCN treated group (19.60±0.51 secs; 15.80±0.58 secs) when compared with group IV (6.40±0.51 secs; 4.60±0.68 secs), $p < 0.05$. A decline in motor performance as the week progresses, as it was recorded in their transfer latencies.

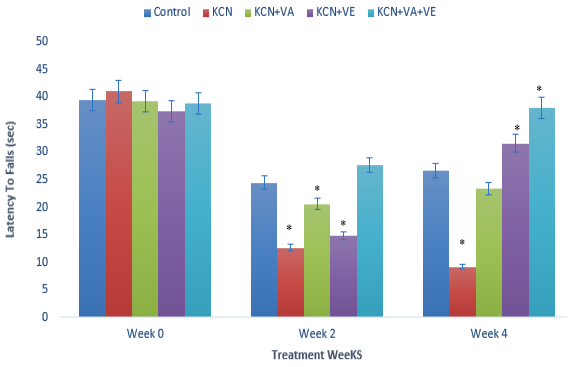

Fig. 1: Effect of Sublethal Administration of Potassium Cyanide and Administration of Vitamins A and E on wire grid Test in Swiss Mice. *$p < 0.05$.

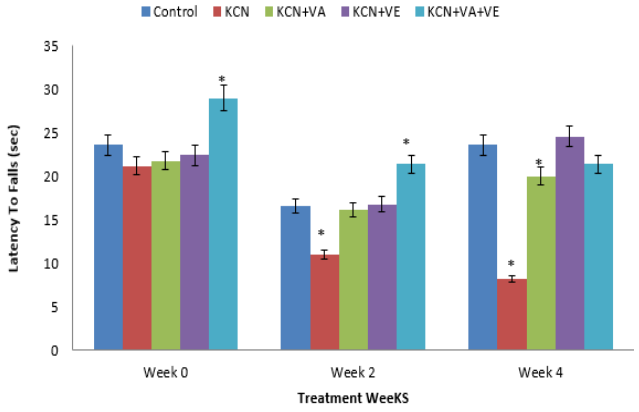

Fig. 2: Effect of Sublethal Administration of Potassium Cyanide and Administration of Vitamins A and E on Dynamics of Coat Hanger Test in Swiss Mice. *p < 0.05.

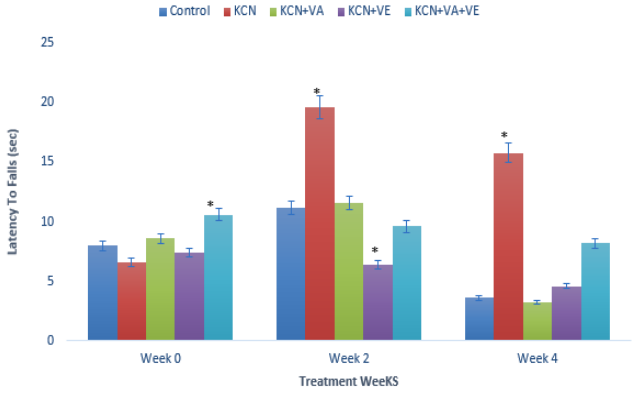

Fig. 3: Effect of Sublethal Administration of Potassium Cyanide and Administration of Vitamins A and E on Dynamics of Beam Walk Performance Test in Swiss Mice. *p < 0.05.

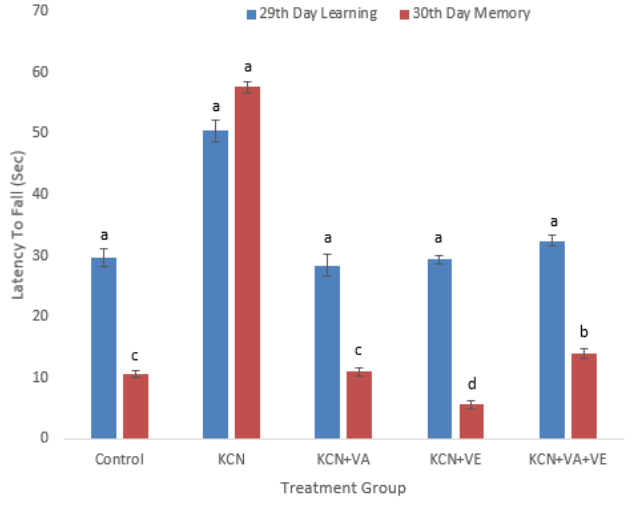

Fig. 4: Effect of Sublethal Administration of Potassium Cyanide and Administration of Vitamins A and E on Learning and Memory in Swiss Mice. *p < 0.05.

Effect of treatments on elevated plus-maze test

In the EPM test, this was assayed for long-term visuo-spatial memory acquisition and retention latencies. Acquisition latencies (seconds) for KCN treated group and control (50.40±1.72 secs and 29.60±1.43 secs) while retention transfer latencies were (57.60±0.93 secs and 10.60±0.51 secs) for both groups. In vitamins pre-treated groups III and V acquisition latencies (28.40±1.81 secs and 32.40±0.93 secs) while retention transfer latencies were (11.00±0.71 secs and 14.00±0.70 secs), figure 4. The KCN-treated group mice spent more time in open arms than in the closed arms in contrast to the vitamin groups underlining avoidance of the anxiogenic area of the apparatus.

DISCUSSION

The toxic signs observed in the KCN-treated group which included tremors, convulsion and salivation in the KCN-treated group were consistent with symptoms observed in cyanide poisoning. The mitigation of toxic signs and mortality in mice pretreated with vitamins A and E demonstrated the protective role of vitamins (A and E) on cyanide induced toxic signs and deaths. This shows the role of oxidative stress in toxic signs evoked by cyanide.

Analysis of neurological performance in the wire grid test showed a significant reduction of all paws grip, reflecting deficit in all paws motor strength following sublethal exposure to cyanide treated group in Swiss mice. The impairment can be as a result of lesion in cerebellar cortex, motor area or maybe explained by the triggering of region specific toxic pathways in which oxidative stress maybe a common activator (Borowitz *et al.*, 1999; Hahm *et al.*, 2010). This may be due to partly reduced brain and perhaps muscle oxidative damage. The antioxidant pre-treated groups motor deficits were less compared to potassium cyanide treated groups. The group that received vitamin A and E had better grip and longer latency in comparison to group that received KCN + vitamin A, and KCN + vitamin E. The vitamins group (A and E) was able to mitigate the effects of cyanide induced oxidative damage via their antioxidant properties.

The latency to fall from the coat hanger was also measured as an index of motor-strength. Analysis of this variable indicates a decrease of performance as week progresses, reflecting deficits in forepaw motor strength following sublethal exposure to KCN in Swiss mice. The impairment of motor strength by potassium cyanide may have been due to oxidative damage to muscles resulting in muscular rigidity (Calabressi *et al.*, 2008). Similarly, reports have also shown that reduced hand strength and loss of muscle strength following acute cyanide poisoning (Messing, 1991). Antioxidant vitamins treated groups ameliorated the effects of cyanide induced neurotoxicity on the muscle mass.

Motor co-ordination was evaluated using the stationary beam walk. It assayed for muscular coordination and balance. It is an integrated form of behaviour requiring pertinent level of consciousness, memory, sensorimotor and cortical functions mediated by the cortical areas (De-Latour *et al.*, 2008). The loss

of equilibrium followed by erratic movements was observed together with imbalanced body activity and muscular incoordination was seen in group pretreated with KCN. This correlates with the work of Ogundele *et al.,* 2014. The progressive increase in which the mice slipped off the beam indicates impairment of motor co-ordination. This impairment can be as a result of either due to cortical damage (Ayuba, 2014), cerebellar damage (Ogundele *et al*., 2014) and or pyramidal motor system damage, which controls all of the voluntary movements (Hamakawa *et al*., 2011). Furthermore, cyanide has been shown to induce cell death in a varying mode of different brain areas (Hahm et al., 2010). This might have been responsible for the deficit in beam walk performance in KCN treated group. The impairment of motor strength by cyanide may have also been due to the reduced motor-strength which has been observed in humans following prolonged cyanide exposure as seen in endemic exposed areas (Osuntokun, 1981). It is well documented in the literature that the brain is a critical site for anoxia, which may be due to changes in electrical activity which cause damage to the region of the brain associated with the maintenance of equilibrium (De-Latour *et al*., 2008, 2012) as was observed in the present study. However, groups pre-treated with antioxidant vitamins (A and E) had improved motor performance and decreased number of slipping. When these groups were compared with control, the transfer latency was increased, which shows that antioxidant vitamins may not be the only mechanism involved in motor coordination deficits induced by cyanide exposure.

Deficits in behaviour and performance in visuo-spatial memory and learning tasks was observed in KCN treated mice. The underlying thread is that cyanide impairs performance in memory and learning. Mice spent more time in the open arms, less explorative to find the enclosed arms, showing less aversion to open space and exhibiting avoidance behavior. The present study also corroborates with the work of Bukachi *et al.,* 2014, which showed memory deficits is associated with sublethal cyanide poisoning relative to cyanate toxicity in rodents. The impairment can be as a result of different hippocampal neurotransmitters (Ayuba, 2014) or cholinergic deficiency; leading to reduced acetylcholine synthesis, or combination of both parameters.

On the other hand, antioxidant vitamin administered was able to ameliorate these acquisition and retention decline through their antioxidant properties. Vitamin E administration from the results showed better performance as compared to vitamin A, or co-administered vitamins. This is in agreement with the work of Abe *et al.,* 2005 that oxidative stress induced by hyperoxia significantly impaired cognitive performance of 3 month old mice, effects that were partially attenuated by dietary vitamin E supplementation. Furthermore, the brain is rich in polyunsaturated fatty acids and relies heavily on the antioxidant properties of the lipid-soluble vitamin E (α-tocopherol) (Rashid *et al*., 2011).

The present study revealed that (i) potassium cyanide significantly decreased motor performance (motor strength and coordination) in Swiss mice and also a decline in cognitive functions; (ii) the antioxidant vitamins (A and E) significantly ameliorated the deficits in motor performance and also improved learning and memory functions of the brain in Swiss mice.

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Gross Description and Osteometrics of the Axial Skeleton (Ribs and Vertebrae) of *Eidolon Helvum* (African Fruit Bat)

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Summary: The *Eidolon helvum* is a straw coloured fruit bat, also known as the African fruit bat. This study details the anatomical peculiarities, gross description and morphometrics of the ribs. Fifteen adult bats were used for this study. The vertebral formula was $C_7T_{13\text{-}14}L_{3\text{-}4}S_7Cd_{3\text{-}4}$. Spinous process was rudimentary on thoracic vertebrae 1 to 7. The numbers of the ribs ranged from 13 to 14, with the longest being rib 7, and the shortest being rib 14. Twenty percent of the males and 50% of the females had 14 ribs; the male and one female had the 14th rib being unilateral (present on the right side of male and left side of female). The first rib did not articulate directly with the sternum, but through a connecting triangular-shaped bone. The thoracic inlet and outlet were larger in males, relative to the females (inlet height – males 21.82 ± 2.68 mm, females 20.44 ± 4.91 mm; outlet height – males 36.46 ± 3.76 mm, females 33.23 ± 4.33 mm). The sternum was segmented, five in number and had a ventral elongation like the avian keel. Data obtained may find application in comparative and applied anatomy, and forensic medicine.

Keywords*: Eidolon helvum*, Axial skeleton, Osteology, Osteometrics, Triangular bone.

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INTRODUCTION

Bats are the only known mammals that can fly (Balthazary *et al*., 2007; Igado *et al*., 2015). The African straw coloured fruit bat belongs to the Order Chiroptera, Family Pteropodidae, Genus Eidolon, Species *Eidolon helvum* **(**Kerr, 1792**).** It is the second largest and most widely distributed bat in Africa (DeFrees and Wilson, 1988; Juste *et al*, 2000, Igado *et al*, 2012). Bats are of economic importance, for example, they have been reported to cause damage to plants when feeding on them, thereby resulting in a loss to farmers, and they have also been implicated in viral disease transmission e.g. rabies (Crawford *et al*., 2002; Igado *et al.*, 2012).

The skeleton of animals is an essential structure that serves as a landmark for identifying or obtaining access into the other structures or cavities of the animal system. For example, the intercostal space is used to identify the location of the heart, while the lumbar vertebrae serves as a useful landmark for the location of the kidneys. This usefulness of the skeleton makes it imperative to have detailed information about some skeletal structures of the *Eidolon helvum*. Currently, no literature documents the anatomical morphometrics of this particular bat nor of any other bat species. Careful electronic search using www.scholar.google.com and www.pubmed.com (July, 2017 – January, 2018) did not reveal any previous documentation of the morphometrics and detailed description of the osteology of the *Eidolon helvum* nor of other bat species.

This study aims to give a detailed morphological and morphometric description of parts of the axial skeleton (ribs and vertebrae) of the *Eidolon helvum*.

MATERIALS AND METHODS

Ethical approval for this work was obtained from the Ethical Committee, Faculty of Veterinary Medicine, ethical code no 12/13/03.

Experimental animals used for this study were 15 adult bats consisting 10 females and 5 males. Age was determined based on weight, according to DeFrees and Wilson (1988) and Richter and Cumming (2006), who estimated the average weight of adult bats to be 250 – 310 grams. The bats were captured at roosting and foraging sites with the use of mist nets and were transported to the Department of Veterinary Anatomy, University of Ibadan in metal cages and were thereafter euthanized using the chloroform chamber. All procedures for handling the animals complied with the Guidelines for the Care and Use of Experimental Animals (National Institute of Health – NIH, USA).

Bones were obtained by cold water maceration by soaking the specimens individually in plastic containers filled with 1% sodium hydroxide (NaOH) solution for about 5 – 7 days, until muscles and other

attachments had been removed. Pictures were taken with the aid of a digital camera (Sony® Cyber-shot, DSC-HX400V, 50x optical zoom). All linear measurements were done with a digital vernier calliper. Except otherwise indicated, linear measurements were recorded in millimetres, and all measurements obtained from the right side in the case of paired bones. Parameters obtained (highlighted in Figures 1 – 9) included:

1. **Total number of ribs (Rn)** – counted from the first rib to the last floating rib.
2. **Total number of true ribs (Trn)** – number of all the ribs articulating directly with the sternum.
3. **Number of false ribs (Fsn)** – number of all ribs indirectly articulating with the sternum by the costal cartilage.
4. **Number of floating ribs (Fln)** – number of all ribs with no direct or indirect attachment to the sternum. The distal ends were unattached.
5. **Length of each rib (RL) -** measured from the head of the ribs where it articulates with the thoracic vertebra to the point of the sternum. Measured with the aid of a twine, the length of which was measured with a digital vernier calliper.
6. **Maximum rib width (MaRW)** – measurement of the widest part of each rib, close to the head.
7. **Minimum rib width (MnRW)** – measurement of the narrowest part of each rib, close to the sternum.
8. **Length of sternum (SL) -** measured from the cranial tip of the manubrium to the distal point of the xiphoid process.
9. **Thoracic inlet transverse diameter (TiTD) -** measured as the distance between the ventral rim of the first thoracic vertebra to the dorsal aspect of the manubrium.
10. **Thoracic outlet transverse diameter (ToTD) -** measured as the distance between the ventral rim of the last thoracic vertebra to the dorsal aspect of the xyphoid.
11. **Total number of vertebrae (VCn)** – counted from the first vertebral bone (atlas) to the last caudal (tail) bone.
12. **Number of cervical vertebrae (Cvn)** – counted from the first vertebral bone (atlas), to the vertebra preceding that which articulates with the first rib.
13. **Number of thoracic vertebrae (Txn)** – counted from the vertebra with the first rib attachment, to the vertebra with the last rib attachment.
14. **Number of lumbar vertebrae (Lmn) -** counted from the vertebra after the last thoracic, to the vertebra immediately preceding those that articulate with the pelvic and sacral bones.
15. **Number of sacral vertebral bones (Scn)** – counted from the first fused vertebra to the end of the pelvic bones where the vertebrae are no longer fused.
16. **Number of coccygeal vertebral bones (Cxn)** – all vertebrae after the sacrum/sacral vertebrae.

Statistical Analysis

This is calculated using Student 't' test (Graphpad prism statistical software, Version 5, La Jolla, CA, USA). Level of significance was $\alpha_{0.05}$.

RESULTS

The Vertebral Column

The vertebral formula was $C_7T_{13\text{-}14}L_{3\text{-}4}S_7Cd_{3\text{-}4}$. The atlas (Figure 1 A-D) was observed as the typical dorso-ventrally flattened mammalian atlas. The neural canal presented a roughly oval shape. The wings of the atlas as observed in this study appeared to be more pronounced, and projected more caudally than reported in cattle, equine and porcine, but similar to that observed in the dog by Getty (1975). Dorso-cranially (alar foramen), caudally, ventrally, and also on the dorso-cranial aspect of the neural canal, the body of the atlas presented bilateral foramina. The ventral and caudal foramina were connected, while the dorsal (alar foramen) and neural canal foramina were also connected, implying that these are entrance and exits for cervical spinal nerves. The central foramen showed a variation in size, due to the difference in shape of the bony ventral border (Figure 1C and D).

The axis (Figure 2) presented a large spinous process, which was directed dorso-caudally. Cranially, the dens/odontiod process was obvious; caudally, the

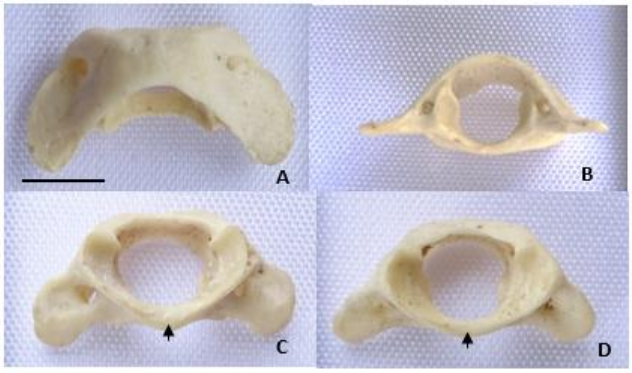

Figure 1: The atlas of the *Eidolon helvum*. A is the dorsal view; B – caudal view; C and D are the rostral view. Note the difference in the shape of the central foramen of C and D, due to the difference in shape of the ventral border of the foramen (arrow heads). Scale bar – 0.5 cm

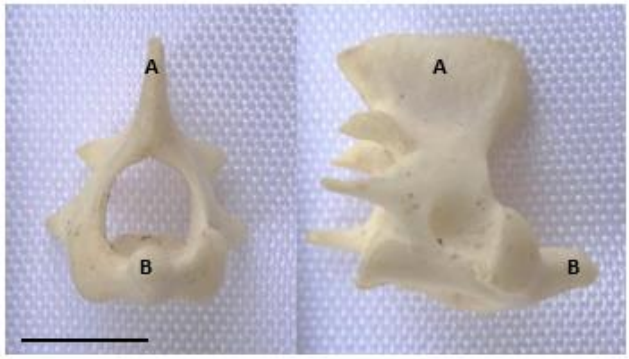

Figure 2: The axis of the *Eidolon helvum*. Left panel shows the cranial view, while the right panel shows the lateral view. Scale bar – 0.5 cm

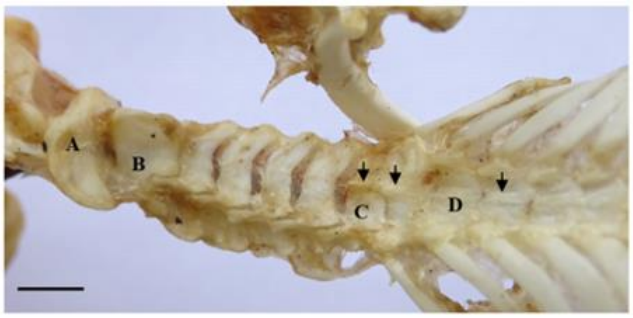

Figure 3: The cervical bones and some thoracic vertebral bones of the *Eidolon helvum*. Note the atlas (A), axis (B), the 7th cervical bone (C), the 2nd thoracic vertebra (D) and the rudimentary spinous processes (arrow heads). **Scale bar – 1 cm.**

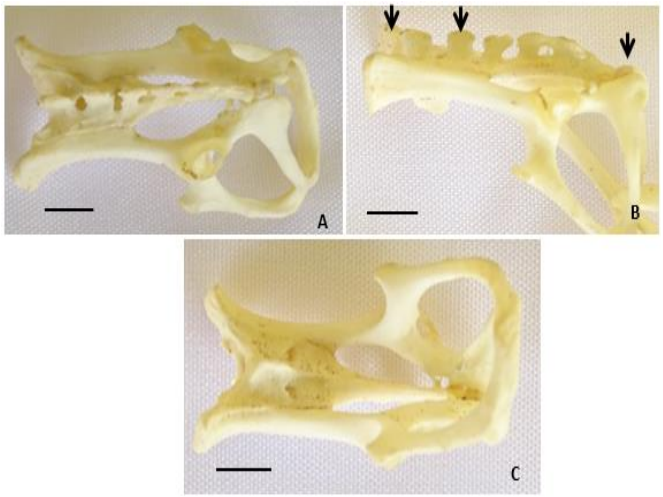

Figure 4: Pelvic girdle of the *Eidolon helvum*. Dorsal view (A), lateral view (B) and the ventral view (C). Note the spinous processes of the sacral bone (arrows). Scale bar – 0.5 cm.

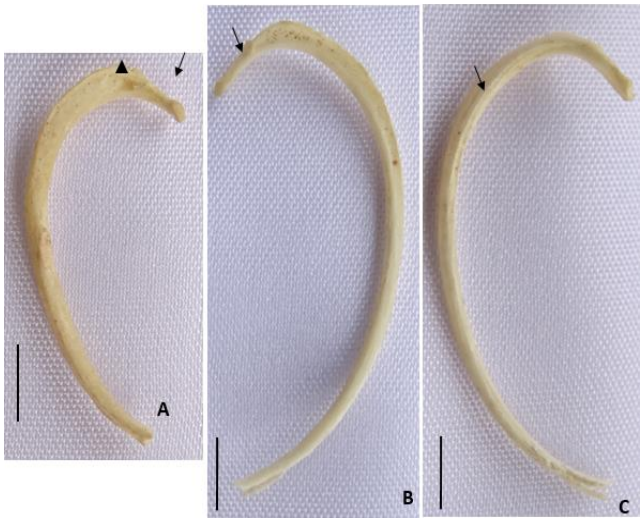

Figure 5: Ribs 2 (A) and 3 (B & C) of the *Eidolon helvum*. Panels 'A' and 'B' are the medial surfaces, while 'C' is the lateral surface. Arrow on 'A' indicates the head of the rib, on 'B' indicates the articular tubercle, while on 'C' indicates the ridge-like elevation on the lateral surface. Note the groove distal to the head on rib 2 (arrow head on 'A'). Scale bar 0.5 cm.

articular and transverse processes and laterally, the bilaterally placed vertebral foramen. The remaining cervical and 1st to 7th thoracic vertebral bones showed rudimentary spinous processes (Figure 3), until about the 15th vertebral bone (8th thoracic), where the spinous processes became more pronounced and became directed dorso-cranially till the last sacral bone.

In addition to the less prominent spinous processes of the thoracic vertebrae, the body appeared stouter, and constricted, relative to the cervical vertebrae. The transverse processes projected caudally and decreased in length as the bones progressed caudally.

The lumbar vertebrae had prominent dorso-cranially directed spinous processes and a body resembling the thoracic vertebrae, although the body appeared slightly longer (cranio-caudally) relative to the thoracic bones. The transverse processes of the lumbar vertebrae projected cranially and were observed to be less prominent.

The sacral bones were consistently 7 in number and were fused. They were fused laterally to the pelvic girdle, had no transverse processes but very prominent spinous processes. They extended almost the whole length of the pelvic girdle. They also possessed a prominent ventral process, along the midline. This ventral process was continuous, unlike the spinous processes which were separate (Figure 4).

The caudal bones were 3 to 4 in number, very small in dimensions relative to the other bones, decreased in size as they progressed caudad. Transverse processes were rudimentary, while spinous processes were not observed. The shape of the bodies was generally similar to the lumbar vertebrae

The Ribs

The ribs possessed a distinctly sickle-shaped appearance and were dorsoventrally flattened in the typical mammalian rib appearance. They had two extremities (proximal and distal), two surfaces (lateral and medial), and two borders (cranial and caudal).

The head of the rib was very distinct and projected cranially. The head was dorsoventrally flattened without any distinct tubercle. A very slight narrowing distal to the head resulted in what could be described as a 'neck'. This neck was not a consistent feature in all the ribs, appearing more distinct in the cranial ribs than the caudal ribs. Caudal to this neck and a little more medial was an articular surface, which articulated with the bodies of the thoracic vertebrae. The shaft was roughly cylindrical in shape, tapering to a thinner distal extremity to articulate with the cartilage at the costo-chondral junction (Figure 5).

The medial surface possessed a groove at the proximal part immediately distal to the head of the rib. The lateral surface was smooth, except for ribs 2 and 3, which had a ridge-like elevation (Figure 5A-C).

The first rib was very straight, lacking any curvature. Ribs 2 to 10 displayed the curvature

Figure 6: Dorsal view of vertebral column of female *E helvum*. Arrows indicate 1st & 14th ribs. Note the straightness of ribs 1, and 11 to 14. Scale bar – 1 cm.

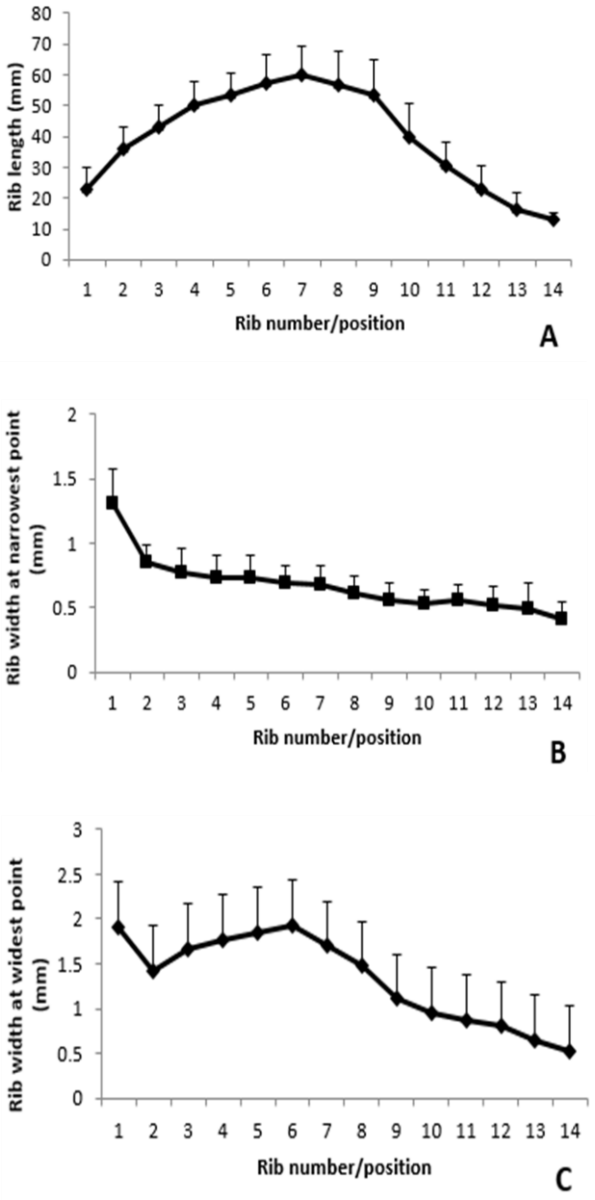

Figure 7: Graphs showing the progression of the lengths (A), width at narrowest point distally (B) and width of ribs at widest point proximally (C) of the ribs of the *Eidolon helvum*. Note that in 'A', highest length was recorded at rib 7; rib 1 was the thickest rib, while rib 14 was the thinnest and shortest rib (A-C).

aforementioned, while 11 to 14 were straight and projected more latero-caudally than latero-ventrally (Figure 6).

The numbers of the ribs ranged from 13 to 14. Of the five males sampled, only 1 (20%) had 14 ribs (unilateral, present on the right; left side had 13 ribs); of the females, 5 (50%) had 14 ribs, one was unilateral, present on the left. The true ribs, articulating with the sternum were consistently 7 in the males, while in the females, only 1 (10%) had 6 true ribs, 9 (90%) had 7 ribs. The false ribs had a common costal cartilage, joined together in the typical mammalian fashion. The length of the ribs increased until about the 7th to 9th rib, when the length gradually reduced. The lengths of the ribs varied greatly, ranging from 59.81 ± 9.54 mm (7th rib) to 13.21 ± 2.24 mm (14th rib). The values for the males (65.39 ± 4.42 mm – 7th rib) were higher than the

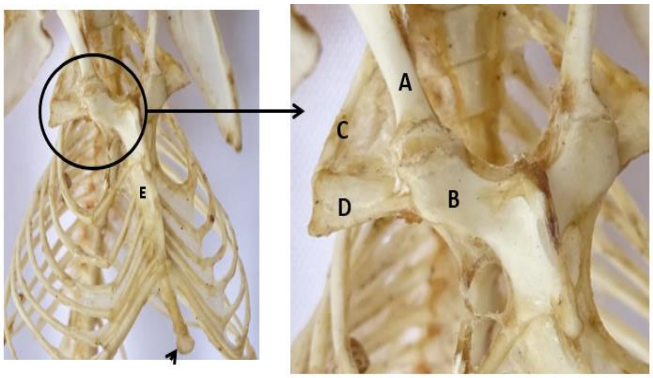

Figure 8: Ventral view of bony thorax of *E helvum*. Right panel is a magnification of highlighted region in the left panel. A – clavicle; B – sternum; C – first rib (R1); D – Triangular bone. Note the keel-like appearance of the sternum and its segmentation (E on left panel). Arrow head is the xyphoid. Note the presence of the costochondral cartilage joining the true ribs to the sternum.

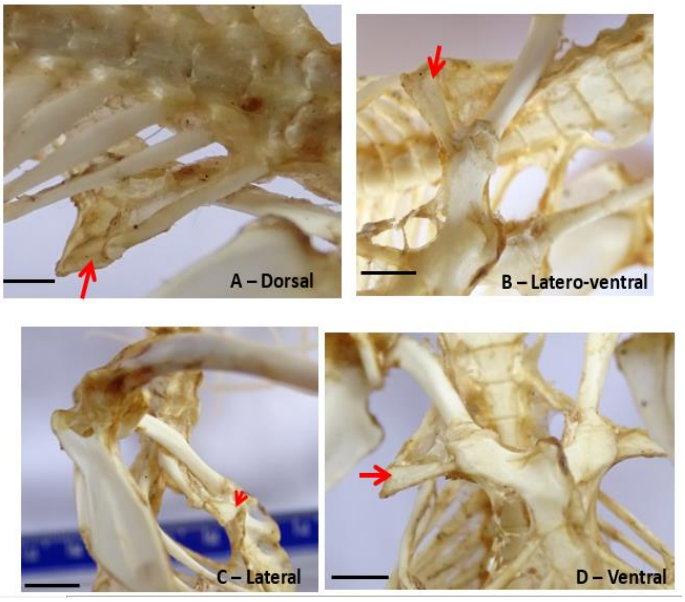

Figure 9: Views of bony thorax of the *E helvum*. The red arrows show the triangular bone from different views. Scale bar – 1 cm

females (57.03 ± 10.34 mm – 7th rib). (Figure 7A–C). The first rib did not articulate directly with the sternum. A small triangular-shaped bone acted as the connection between the first rib and the arm of the Y-shaped manubrium (Figures 8 and 9).

The Sternum

The sternum possessed a roughly keel-like shape, as observed in birds (Figure 8).

The manubrium was distinctly Y-shaped, due to the extensive jugular notch. The ventral surface (or tail of the 'Y') was bilaterally flattened and shaped like the keel-bone of the avian species. The two arms of the 'Y' were dorsoventrally flattened; and attached cranially to each arm was the clavicle; laterally was the small flat triangular bone, the narrow end of which articulated with the lateral aspect of the 'Y' (Figures 8 and 9). This triangular bone served as a link between the first rib and the sternum. The manubrium and the body of the sternum showed divisions at the keel region, which were consistently 5 in number in all the animals. The xyphoid was a thin slender, rod-like bone, pointed caudally (Figure 8). The mean length of the sternum was 31.32±3.90 mm, with the males having statistically significantly ($p > 0.05$) longer sternum (33.82±1.08 mm), relative to the females (30.07±4.24 mm).

The Thoracic Inlet and Outlet

The inlet displayed a roughly conical shape, was bounded dorsally by the thoracic vertebra, ventrally by the manubrium and laterally by the clavicle and the first rib. The outlet was roughly "heart-shaped", bounded dorsally by the last thoracic vertebrae, ventrally by the xyphoid and laterally by the ribs (11th ribs).

The transverse diameter of the thoracic inlet was 20.93 ± 4.19 mm, males – 21.82 ± 2.68 mm, females – 20.44 ± 4.91 mm. The transverse diameter of the thoracic outlet was 34.38 ± 4.30 mm, males – 36.46 ± 3.76 mm, females - 33.23 ± 4.33 mm. The inlet did not show statistically significant differences between the sexes, while the outlet showed statistically significant difference between the sexes.

DISCUSSION

The number of the vertebral bones, and therefore the vertebral formula varies from animal to animal. The vertebral formula of the *Eidolon helvum* observed in the current study was $C_7T_{13\text{-}14}L_{3\text{-}4}S_7Cd_{3\text{-}4}$. The number of cervical vertebrae observed in this study is consistent with what was previously reported in other mammals; the number of thoracic vertebrae was similar to that of ruminants (13); but the lumbar, sacral and caudal bones were dissimilar to previous reports in domestic mammals (Getty, 1975; Dyce *et al*., 2002). The lumbar, sacral and caudal vertebrae were reported to be respectively 6 -7, 4 and 20 – 23 in the porcine, 7, 3 and 20 – 23 in the carnivores, 6, 5 and 15 – 21 in the equine and 6, 5 and 18 and 20 in ruminants (Getty, 1975). However, previous reports in the mole rat showed a slight similarity in the number of the caudal bones (5 in number) (Özkan, 2007).

Characteristically, some of the identifying features of the mammalian thoracic vertebrae include the more pronounced spinous process and the stouter body (Getty, 1975; Rohen *et al*., 1998; Dyce *et al*., 2002; Netter, 2006). Although the thoracic vertebrae observed in this study had a stout body, the spinous processes were rudimentary, except for the last few. The reason for this is not clear, as the spinous processes serve as origin and insertion for muscles that incline the neck, arch the back and assist in extending the head and neck (Getty, 1975). In contrast to mammals, pictorial assessment of the thoracic vertebrae of avians from texts showed the lack of spinous processes (Getty, 1975; Dyce *et al*., 2002). It is therefore possible that since bats are mammals of flight and do not spend a long time walking or swimming like other mammals, the need for the pronounced spinous process was not necessary. This could also account for the more pronounced transverse and spinous processes in the lumbar vertebrae. Muscles attaching to these processes may provide additional strength and support when the bats hang upside down, as the lumbar vertebrae is closer to the pelvic girdle.

In mammals, the number of ribs varies with species – 13 pairs in ruminants and carnivores, and 18 to 19 pairs in equine (Getty, 1975). In the current study of bats, the numbers of the ribs ranged from 13 to 14 pairs in number. Both genders recorded 14 ribs, but the females had a higher incidence. The unilateral extra rib is similar to what is observed sometimes in the equine (Getty, 1975; Dyce *et al*., 2002). The widest point of the thoracic cavity could be said to occur around ribs 7 – 9, as these ribs had the longest values.

The shape of the rib is similar to previous reports in other mammals, although in this species of bats, the curve of the rib is more pronounced at the proximal end than at the shaft. The ribs serve as attachment for muscles of respiration; they protect the thoracic viscera and also define the lateral limits of the bony thoracic cavity (Getty, 1975; Dyce *et al*., 2002). The ridge-like elevation observed on the lateral surface of ribs 2 and 3 is similar to that observed in the mole rat, same rib number, even though these are not flying animals (Özkan, 2007). These ridges may possibly serve as an extra attachment for muscles, to aid in respiration while the animal is in flight.

Generally, the first rib is unique, being relatively straighter and stouter than the other ribs. In this study, it lacked any curvature, probably because the distance between the dorsal and ventral limits of the bony thorax at this point (the thoracic inlet) is the smallest region or point of the thorax. The curvature of the ribs

allows for a greater space in the thoracic cavity. However, unlike other mammals (Getty, 1975; Popesko, 1977; Dyce *et al*., 2002; Netter, 2006; Panyutina *et al*., 2015), the first rib did not articulate directly with the sternum. A small triangular-shaped bone acted as the connection between the first rib and the arm of the Y-shaped manubrium. In view of the fact that careful electronic search did not reveal earlier documentation of this triangular bone connecting the sternum to the first rib, the authors proffer the name *Triangular bone of Igado*.

The jugular notch of the manubrium is observed in humans (Netter, 2006). However, the bat showed an extensive jugular notch, similar to the indentation of the furcular in the avians. The more extensive jugular notch probably provides a greater surface area for muscle attachment, as visual observation during dissection revealed an extensive pectoralis muscle (the major muscle of flight). The sternum was segmented as observed in mammals (Getty, 1975; Popesko, 1977; Netter, 2006), although the ventral aspect was avian-like, having a keel-like appearance.

In conclusion, this study highlights the features and peculiarities of the *Eidolon helvum* axial skeleton previously undocumented, and shows its similarities to the avian skeleton (the keel and reduced spinous processes of the thoracic vertebrae). Results obtained from this study may find application in the fields of applied wildlife medicine, archaeology and forensic medicine, while also providing basic anatomical data.

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Sub-Acute Toxicity Study of Ethylene Glycol Monomethyl Ether on the Antioxidant Defense System of the Testes and Epididymes of Wistar Rats

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Summary: Ethylene glycol monomethyl ether is a toxicant with wide industrial applications. This study is aimed at investigating its effect on the antioxidant system of the reproductive organs of male rats. Fifty male Wistar rats were distributed into five groups. Group I received distilled water, Groups II-V received EGME at 100, 200, 300 and 400 mg/kg body weight respectively. All administrations were done orally for fourteen days and the weight was monitored weekly. On day fifteen, the animals were sacrificed and reproductive organs were collected and weighed. The testes and epididymes were processed for the biochemical estimations, histopathology and spermatozoa analysis. The percentage body weight gained weekly and the relative weight of the testes reduced significantly *($p < 0.05$)* in the treatment groups. The spermatozoa analysis showed decreases in the treatment groups. In the testis and epididymis, various antioxidant parameters such as superoxide dismutase and glutathione-S-transferase were affected. The histopathology results confirmed the biochemical findings. The study suggests that EGME exerts deleterious effects on the testes and epididymes by increasing the oxidative load in rats.

Keywords*:* Ethylene glycol monomethyl ether, Antioxidant defense, Spermatozoa, epididymes, testes.

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INTRODUCTION

Ethylene glycol monomethyl ether (EGME) is also known as methyl cellosolve (commercially), 2-methoxy ethanol, monomethyl ether, methyl glycol, monomethyl glycol, monomethyl ethylene glycol ether or methyl oxitol. Due to its hydrophilic and lipophilic properties, it has wide consumer and industrial applications. EGME is used as an anti-freeze additive in hydraulic fluids and jet fuel. It is also used in paints, stains, inks and surface coating, lacquers, photographic and photo lithographic processes, production of food-contact plastics, textile and leather finishing, and silk-screen printing as well as in the semi-conductor industry (Johanson, 2000, Takei *et al.*, 2010).

In humans and several other species, exposure to EGME either by inhalation, ingestion and/or dermal absorption has been reported to cause reproductive, hematopoietic and developmental toxicities with emphasis on testicular damage (Bagchi and Waxman, 2008). The evidences of reproductive toxicities as a result of exposure to EGME or the active oxidation product include gene expression changes in germ cells and mouse Leydig cells i*n-vitro* (Bagchi *et al.*, 2010); activation of caspases leading to apoptosis triggered by oxidative stress in spermatocytes (Bagchi and Waxman, 2008); prolonged estrus cycle, hypertrophy of corpora lutea evidenced by the presence of round to polygonal luteal cells with abundant vacuolated cytoplasm and ovulatory inhibition in rats (Dodo *et al.*, 2009, Taketa *et al.*, 2011); altered androgen-dependent processes in mouse Leydig cells *in-vitro* (Bagchi *et al.*, 2011); affects microRNAs expression in the testes of rats (Fukushima *et al.*, 2011); caused spermatocyte toxicity correlated with decreased expression of spermatocyte-specific genes (Matsuyama *et al.*, 2018); affects the antioxidant system and increase lipid peroxidation in the rat testes when treated dermally (Malik and Gupta, 2013). However, there is dearth of knowledge on the effect of EGME toxicity on the antioxidant defence system of the epididymes and testes, and report of the effect on the morphology of the spermatozoa is scarce.

According to IIRT (2017), the essence of a sub-acute toxicity study is to unravel the toxic effect of a drug/substance as a result of constant exposure and taking into consideration doses of EGME that have been worked with in previous studies, the duration of administration, mode of administration and the doses used for the study were determined.

This study was therefore designed to investigate the toxic effects of EGME on antioxidant components of the male reproductive organ of Wistar rats.

MATERIALS AND METHODS

Reagents

EGME and trichloro acetic acid were products of LobaChemie (Mumbai, India). All other reagents were either SureChem, U.K. or Sigma Aldrich. St. Louis, MO, U.S.A. products.

Experimental Animals and Care

The protocol for the animal study was approved by the Animal Care and Use Research Committee of the University of Ibadan, Nigeria and the number UI-ACUREC/ APP/ 10/2016 /003 was assigned. Fifty (50) nine weeks old male Wistar rats weighing 140-190 g were obtained from the Primate Colony of the Department of Biochemistry, University of Ibadan and randomly distributed into five groups of ten animals each. They were kept in appropriate laboratory cages and given feed (Ladokun Feed, Nigeria) and laboratory water *ad libitum* in the Animal house of the same Department where they were acclimatized for a week. The 12 hour light/dark cycle was maintained.

Experimental Design

All mandatory laboratory health and safety procedures where adhered to while conducting the experiment. Administrations were carried out orally daily at the same time for fourteen consecutive days and the weight was monitored weekly. Stock solution of EGME was prepared using distilled water as the vehicle. The protocol for administration is as follows:

Group I received distilled water only (Control)
Group II received EGME at 100 mg/kg body weight dosage
Group III received EGME at 200 mg/kg body weight dosage
Group IV received EGME at 300 mg/kg body weight dosage
Group V received EGME at 400 mg/kg body weight dosage

On day 15, the animals were euthanized by cervical dislocation after an overnight fast. The animals were then dissected and the testes, epididymes, prostate gland and seminal vesicles were removed into ice- cold 1.15 % potassium chloride, blotted with Whatman no.2 filter paper and weighed using a Mettler balance. The right testes and epididymes samples were used for spermatozoa analysis and the ones for histopathology were fixed in Bouin's solution, processed, sectioned, mounted on slides and stained for histological examination. The left testis and epididymis were used for the biochemical analyses of antioxidant markers.

Spermatozoa analysis

(a) *Assessment of the characteristics of epididymal sperm*

The method of Zemjanis (1970) was employed to evaluate the motility of the sperm. The improved Neubauer chamber haemocytometer (LABART, Munich, Germany) was used to count the sperm according to Pant and Srivastava (2003). Employing the method of Wells and Awa (1970), a total of 400 sperm/ rat were used for examination of the morphology and application of nigrosin-eosin-sodium citrate dehydrate solution was used for assessing viability.

(b)Testicular sperm number (TSN) and Daily sperm production (DSP) determination

The method of Blazak *et al.*(1993) was employed. To calculate the DSP, the number of spermatids at stage 19 was divided by 6.1 (6.1 is the period of seminiferous cycle in which the spermatids are present in the seminiferous epithelium).

Biochemical analysis

The left testis and epididymis of each animal were homogenized in 4 and 20 volumes respectively of tris-HCl/KCl buffer (pH 7.4) and the homogenates were centrifuged at 4 ^{o}C for 10 mins at 10,000 g. The supernatant was then decanted and used for the antioxidant assays as follows: Protein content was determined using the method of Lowry *et al.*(1951) and was used to normalize the antioxidant indices; malondialdehyde formed was estimated using the method of Varshney and Kale (1990) for the determination of lipid peroxidation (LPO); the method of Claiborne (1985) was used to determine catalase (CAT) activity; superoxide dismutase (SOD) activity was evaluated using the Misra and Fridovich (1972) method; glutathione-S-transferase (GST) activity was determined using the method of Habig *et al.*(1974); vitamin C concentration was assessed using the Jakota and Dani (1982) method; the method of Beutler *et al.* (1963) was employed in determining the concentration of reduced glutathione (GSH); glutathione peroxidase (GPx) was assessed using the method of Rotruck *et al.* (1973) and lactate dehydrogenase (LDH) activity was determined using the method of Vassault (1983). It is important to note that in the assays for GPx, LPO and vitamin C, the trichloroacetic acid was weighed into glass beakers because it is very corrosive.

Statistical analysis

All data, as appropriate, were expressed as mean ± standard error of mean (S.E.M). Statistical analyses were carried out using one- way analysis of variance (ANOVA). Values of $p < 0.05$ were considered to be significant and post- hoc tests were carried out using the least significant difference.

RESULTS

Figure 1 shows the percentage weight gained weekly over the fourteen day treatment period. The groups of the control, 100, 200 and 300 mg/kg doses showed an increase in weight weekly over the period of study while the 400 mg/kg dose group decreased from 12% to 10% between day 7 and day 14.

Table 1 reveals that the weight of the testis decreased significantly ($p < 0.05$) in the 200, 300 and 400 mg/kg

dosage groups compared to the control. This is also presented in the relative weight.

The spermatozoa analysis (Table 2) shows that the daily spermatozoa production, testicular spermatozoa number, epididymal spermatozoa number, sperm motility and sperm viability decreased significantly ($p < 0.05$), especially at the 200, 300 and 400 mg/kg doses compared to the control. The percentage sperm abnormalities increased significantly ($p < 0.05$) in the 200, 300 and 400 mg/kg doses (Table 2).

The details of the abnormalities observed in the spermatozoa are displayed in Table 3. There was significantly ($p<0.05$) increased prevalence of curved- and bent- mid-pieces, curved- and bent- tails in the spermatozoa at the 200 and 400 mg/kg dose of EGME.

Table 4 reveals the effect of EGME on testicular antioxidant parameters. The activities of CAT, GST and GPx increased significantly ($p<0.05$) at the 200, 300 and 400 mg/kg doses compared to the control. The

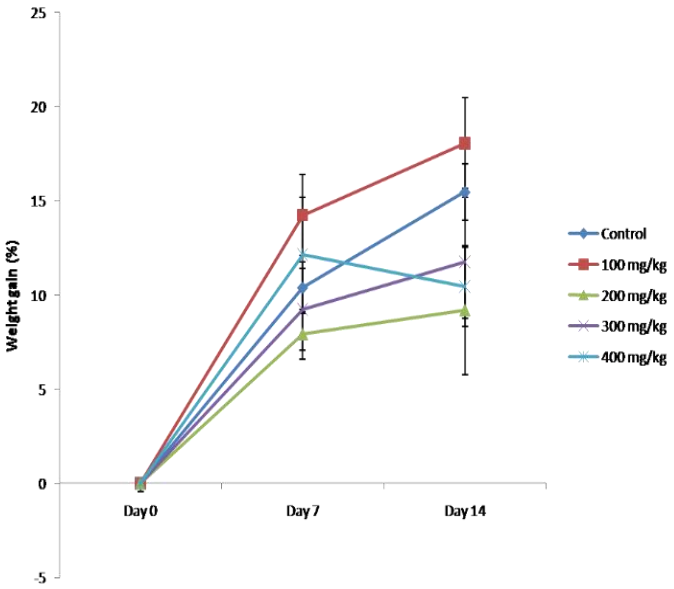

Figure 1: Percentage weight gain of male Wistar rats over fourteen day treatment of ethylene glycol monomethyl ether

Table 1: Effect of EGME on weight and relative weight of organs in male Wistar rats after a fourteen day treatment

	Weight of organs (g)				Relative weight of organs (%)			
Group	**Testes**	**Epididymes**	**Seminal Vesicles**	**Prostate Gland**	**Testes**	**Epididymes**	**Seminal Vesicles**	**Prostate Gland**
I	2.10± 0.06	0.16± 0.01	0.75± 0.10	0.28± 0.03	1.07± 0.02	0.08± 0.01	0.38± 0.05	0.14± 0.01
II	1.94± 0.11	0.16± 0.01	0.66± 0.05	0.26± 0.02	1.02± 0.05*	0.09± 0.00	0.35± 0.02	0.14± 0.01
III	1.28± 0.06*	0.16± 0.00	0.66± 0.13	0.23± 0.03	0.69± 0.03*	0.08± 0.01	0.35± 0.07	0.12± 0.02
IV	1.13± 0.04*	0.14± 0.01	0.49± 0.12	0.21± 0.04	0.63± 0.02*	0.08± 0.00	0.27± 0.05	0.10± 0.02
V	1.12± 0.05*	0.12± 0.01	0.35± 0.03	0.22± 0.04	0.65± 0.07*	0.07± 0.01	0.19± 0.02	0.12± 0.02

Note: n = 10; *- significant at $p < 0.05$; values are mean±standard error of mean

Table 2: Effect of fourteen day treatment of EGME on the spermiogram in Wistar rats

	Group				
Parameter	**I**	**II**	**III**	**IV**	**V**
Daily Spermatozoa Production (x 10^6 / gm testis)	19.31± 0.61	15.06± 1.68	15.05± 0.96	16.54± 2.16	14.80± 1.14
Testicular Spermatozoa Number (x 10^6/ gm testis)	46.00± 3.61	33.33± 3.53	26.67± 3.28*	24.67±2.40*	21.00± 1.73*
Epididymal Spermatozoa Number (x 10^6/ml)	132.33± 3.62	123.00±3.14	107.57± 6.93*	102.57±5.98*	107.14±5.75*
Motility (%)	92.22± 0.88	80.00± 2.11*	71.25± 2.95*	70.00± 3.78*	65.00± 3.42*
Viability (%)	97.33± 0.44	96.50± 0.50	93.78± 1.72	95.25± 1.56	86.57± 7.94*
Abnormalities (%)	10.60± 0.46	11.53± 0.27	12.28± 0.60*	12.05± 0.59*	12.52± 0.40*

Note: n = 10; *- significant at $p < 0.05$; values are mean±standard error of mean

Table 3: Constituents of spermatozoa abnormalities

Group	**Head (%)**	**Mid piece (%)**		**Tail (%)**				
	Tailless	**Curved**	**Bent**	**Headless**	**Rudimentary**	**Bent**	**Curved**	**Looped**
I	1.14± 0.11	2.03± 0.13	2.10± 0.13	1.02± 0.07	0.47± 0.08	1.98± 0.08	1.98± 0.08	0.47± 0.07
II	1.23± 0.09	2.21± 0.11	2.26± 0.08	1.14± 0.08	0.42± 0.07	2.24± 0.09	2.19± 0.09	0.42± 0.07
III	1.32± 0.06	2.28± 0.12	2.37± 0.12	1.07± 0.10	0.47± 0.09	2.34± 0.14*	2.29± 0.11*	0.41± 0.07
IV	1.24± 0.09	2.29± 0.15	2.16± 0.13	1.24± 0.07*	0.43± 0.08	2.29± 0.15	2.19± 0.14	0.37± 0.06
V	1.28± 0.10	2.62± 0.11*	2.41± 0.11*	1.10± 0.11	0.50± 0.09	2.45± 0.08*	2.55± 0.11*	0.39± 0.07

Note: n = 10; *- significant at $p < 0.05$; values are mean±standard error of mean

Table 4: Effect of EGME on testicular antioxidant parameters in Wistar rats

Group	**LPO**	**CAT**	**SOD**	**GST**	**VIT.C**	**GSH**	**GPx**	**LDH**
I	4.22± 0.13	49.33± 1.02	8.83± 0.61	6.39± 0.25	34.50± 2.01	4.84± 0.12	238.01± 7.51	23.05± 1.00
II	4.50± 0.12	49.32± 1.82	3.44± 0.57*	7.13± 0.51	41.83± 3.17	4.50± 0.08	260.32± 14.25	16.27± 1.43*
III	6.86± 0.30*	79.96± 3.23*	3.74± 0.75*	9.97± 0.60*	41.00± 3.71	4.49± 0.25	395.00± 18.69*	14.06± 1.54*
IV	6.18± 0.39*	105.79±6.57*	4.69± 0.70*	11.83± 0.28*	40.25± 3.12	4.85± 0.16	439.77± 15.21*	8.24± 0.17*
V	7.14± 0.60*	101.75± 14.43*	2.85± 0.16*	12.38± 0.92*	35.67± 1.20	4.57± 0.10	464.22± 22.65*	6.24± 0.45*

Note: n = 10; *- significant at $p < 0.05$; values are mean±standard error of mean; KEY: Parameter (unit):- LPO- Lipid peroxidation (µmol malondialdehyde formed/ mg protein); CAT- Catalase (mmoles H_2O_2 consumed/min/mg protein); SOD- Superoxide dismutase (unit/mg protein); GST- Glutathione-S-Transferase (µmol/min/mg protein); VIT. C- Ascorbic acid (µg ml-1); GSH- reduced glutathione (µmol/ g tissue); GPx- Glutathione peroxidase (µg/ml/mg protein); LDH- Lactate dehydrogenase (unit/ mg protein)

Table 5: Effect of EGME on epididymal antioxidant parameters in Wistar rats

Group	LPO	CAT	SOD	GST	VIT.C	GSH	GPx	LDH
I	17.62± 0.17	127.48± 12.14	16.67± 2.38	9.18±0.75	3.50± 0.50	12.64± 1.26	3.05± 0.20	30.92± 3.75
II	17.79±1.68	120.32± 13.20	19.05± 2.38	14.24±1.99*	4.67± 0.67	12.84± 0.77	3.15± 0.09	2.66± 0.12*
III	17.40±1.28	135.97± 15.08	21.43± 5.05	13.09±1.05	3.50± 0.22	10.80± 1.17	3.16± 0.10	3.37± 0.56*
IV	24.60± 3.21*	140.27±18.89	28.57± 7.14	20.36± 2.84*	7.20± 0.58*	15.89± 1.24	3.68± 0.26*	2.99± 0.92*
V	24.42± 2.18*	135.06± 7.14	30.95± 2.38*	21.27± 3.44*	6.00± 0.41*	16.25±1.37*	4.02± 0.28*	0.97± 0.10*

Note: n = 10; *- significant at $p < 0.05$; values are mean±standard error of mean; KEY: Parameter (unit):- LPO- Lipid peroxidation (µmol malondialdehyde formed/ mg protein); CAT- Catalase (mmoles H_2O_2 consumed/min/mg protein); SOD- Superoxide dismutase (unit/mg protein); GST- Glutathione-S-Transferase (µmol/min/ mg protein), VIT. C- Ascorbic acid (µg mol-1); GSH- reduced glutathione (µmol/ g tissue); GPx- Glutathione peroxidase (µg/ml/mg protein); LDH- Lactate dehydrogenase (unit/ mg protein)

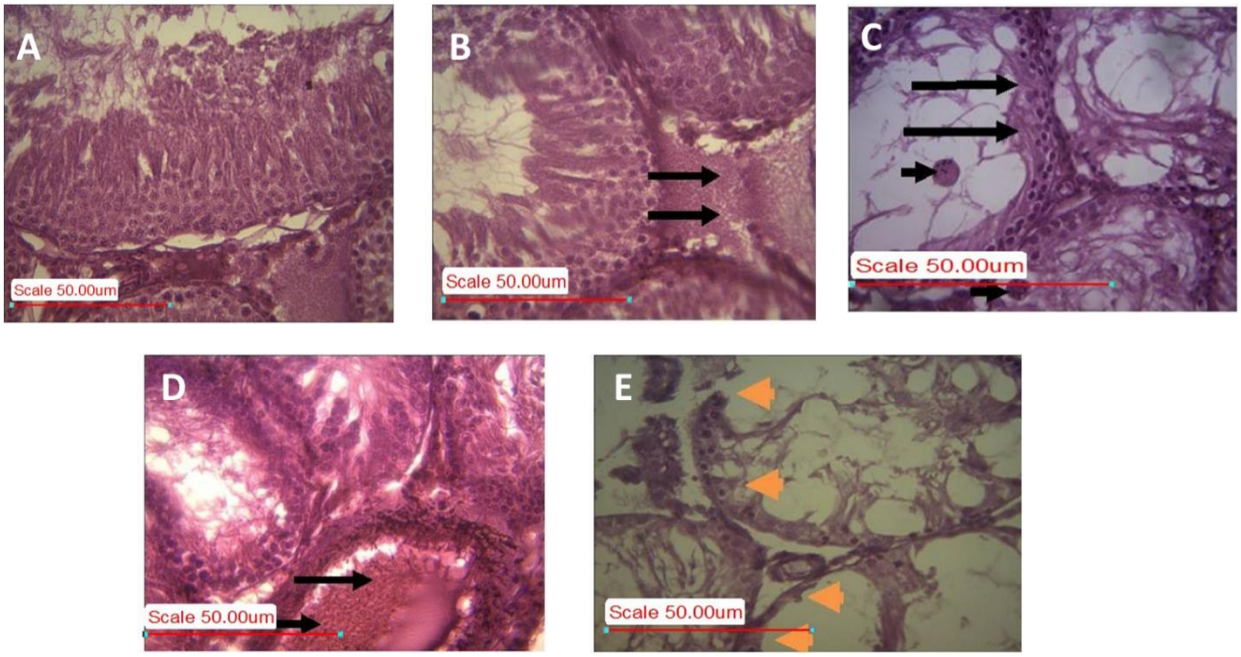

Figure 2: Photomicrographs of Testis in the control and treatment groups after 14 day treatment of EGME. H&E, Mag. x400. A: Control- No lesions; B: 100mg/kg- Mild quantity of pink staining (oedema) fluid in the interstitium; C: 200 mg/kg- Some sections of the seminiferous tubules have a greatly reduced germinal epithelial height (*long arrows*). Late maturing stages appear absent and there are few cellular clumps in the lumina (*short arrows*); D: 300 mg/kg- Mild congestion of the interstitial vessels; E: 400 mg/kg- Severe epithelial erosion.

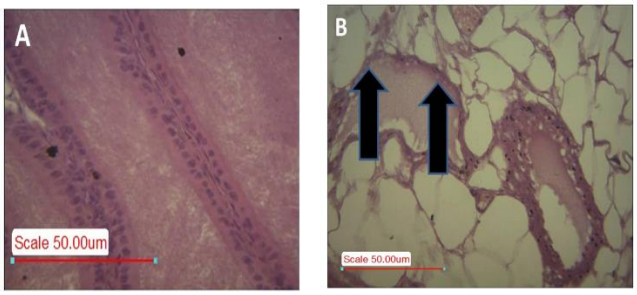

Figure 3: Photomicrographs of Epididymis in the control and 400 mg/kg dose group after 14 day treatment of EGME. H&E, Mag. x400. A: Control- No lesion; B: 400 mg/kg- Severe diffuse germinal cell erosion

activities of SOD and LDH decreased significantly ($p < 0.05$) and LPO increased significantly ($p > 0.05$) in the 200, 300 and 400 mg/kg dosage groups compared to the control. The levels of vitamin C and GSH were not affected (Table 4).

The antioxidant analysis in the epididymis (Table 5) showed that there were increases in LPO; in the activities of CAT, SOD, GST and GPx; and in the levels of vitamin C and GSH compared to the control. These increases were significant ($p < 0.05$) at the 300 and 400 mg/kg doses. There was significant ($p < 0.05$) decrease in the activity of LDH in all doses compared to the control. Figure 2 shows the histopathology of the testis in the control and treatment groups. The control group (A) had no lesions and the 100 mg/kg group (B) showed oedema in the interstitium. The 200 mg/kg dose group (C) showed that some of the sections of the seminiferous tubules have a greatly reduced germinal epithelial height, late maturing stages were absent and there were few cellular clumps in the lumina. The 300 mg/kg dose group (D) showed mild congestion of the interstitial vessels while the 400 mg/kg dose group (E) showed severe epithelial erosion. Figure 3 shows the histopathology of the epididymis in the control and treatment groups. No lesions were observed in the control group (A) and the treatment groups, but the 400 mg/kg dose group (B) showed severe diffuse germinal cell erosion.

DISCUSSION

EGME elicits gonadotoxicity by inducing oxidative stress in the testes and epididymes, as shown by the effect on the enzymatic and non-enzymatic antioxidants, and alterations in the morphology of spermatozoa.

The decrease in body weight in the 400 mg/ kg dose of EGME is a sign of toxicity. The decrease in weight and relative weight of testis in the treatment groups especially 200, 300 and 400 mg/kg doses of EGME is similar to the observations of Welsch (2005) and Malik and Gupta (2013) in rats and humans.

Daily spermatozoa production (DSP) decreased insignificantly in all the treatment groups compared to the control. The testes are the sites for spermatogenesis and androgen production. The gradual significant decrease in testicular spermatozoa number shows a disturbance in spermatogenesis. The epididymis is the site for sperm maturation as they traverse the different regions of the epididymis from the testis. Significant decrease in epididymal spermatozoa number is therefore expected since the testicular spermatozoa number is decreased. The epididymis is also the site where sperm acquires significant motility. Significant decrease in sperm motility in the treatment groups suggests that the integrity of internal milieu of the epididymis is compromised.

The spermatozoa viability decreased gradually in a dose-dependent manner in the treatment groups and was significant at the 400 mg/kg dose of EGME. This implies that there is a larger number of dead spermatozoa compared to live spermatozoa with increased dosage of EGME. Thus, showing that EGME had adverse effect on the spermatozoa. Moreover, the percentage abnormalities which increased significantly in the treatment groups showed that there was increased presence of deformed sperms in the treatment groups. These deformities included tailess- head, curved- and bent- mid-pieces, and headless-, bent- and curved- tails which were significantly present at the 400 mg/kg dose.

Oxidative stress is a condition where the enzymatic and/or non-enzymatic scavengers of reactive oxygen species (ROS) are overwhelmed by the level of ROS in the system (Willcox *et al.*, 2004). Catalase (CAT), glutathione-S-transferase (GST), superoxide dismutase (SOD) and glutathione peroxidase (GPx) are enzymatic ROS scavengers while reduced glutathione and vitamin C are non-enzymatic ROS scavengers. In the testes, the activities of CAT, GST and GPx were increased significantly in the treatment groups especially at 200, 300 and 400 mg/kg doses of EGME. This is a sign of adaptive response. However, the activity of SOD was significantly reduced in the treatment groups thus confirming oxidative stress. Lactate dehydrogenase is an enzyme of the glycolytic pathway and is used to identify the location, and severity of tissue damage. The significant dose-dependent decreased activity of this enzyme in the treatment groups may be as a result of its inhibition by the metabolite of EGME. These observations are at variance with the findings of Malik and Gupta (2013) who administered EGME dermally to rats for 28 days at a dose of 2g/ kg body weight. This may be because the route of administration was different as well as the duration was longer and the dose higher.

Lipid peroxidation, which is an indication of oxidative degradation of lipids by free radicals, was significantly elevated in a dose-dependent manner in the testes of the treatment groups. This shows damage to the membrane of the cells of the testis. The histopathology confirmed the results showing varying degrees of lesions in the treatment groups with the 400 mg/kg dose of EGME group reflecting severe epithelial erosion.

In the epididymis, the activities of GPx, GSH, CAT, SOD, GST and vitamin C level were elevated and were significant at the 400 mg/kg dose of EGME. Again, this shows adaptive response. LPO was also increased significantly at the 300 and 400 mg/kg dose while the activity of LDH was decreased significantly in a dose-dependent manner in the treatment groups. This again suggests that LDH may be inhibited by the metabolite of EGME. The histopathology showed that there was severe diffuse germinal cell erosion only at the 400 mg/kg dose of EGME thus buttressing the elevated lipid peroxidation.

All these findings support the results of the spermatozoa analysis. The toxicity of EGME was further made obvious by the survival rate which was observed to be 90% at 200 mg/kg, 80% at 300 mg/kg and 70 % at 400 mg/kg.

The data show that Ethylene glycol monomethyl ether effects its gonadotoxic potential in the epididymes and testes by inducing oxidative stress, as shown by the effect on the antioxidant defence system. It also shows the effects on the morphology of spermatozoa.

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Disturbances in Calcium and Zinc Homeostasis During Testicular Damage Induced by *Citrus aurantifolia* Juice in Wistar Rats

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Summary: Infertility rate is high globally and in Nigeria. The reported spermicidal activity of *Citrus aurantifolia* juice (CAJ) and its popular consumption may be a contributing factor to the rise in male infertility. This study examined the effects of CAJ on testis and evaluated the role of calcium and zinc in these effects. Twenty-eight male rats (200-220g) were grouped into four (n=7). Group I (control) received 0.5ml normal saline, while groups II, III and IV received 600mg/kg, 900mg/kg and 1200mg/kg of CAJ, respectively, orally for 35 days. Sperm analysis, testicular histology, testicular zinc and calcium concentrations were evaluated. The results showed a significant decrease ($P < 0.001$) in body weight and gonad-somatic index (GSI) of the rats in group IV. No sperm cells were found in the sperm samples of all the treatment groups in contrast to control. There was a significant decrease ($P < 0.001$) in zinc concentration of group III and IV animals and a significant increase ($P < 0.001$) in testicular calcium content of group III and IV animals. Derangement of testicular cyto-architecture, shrinkage or complete destruction of seminiferous tubules as well as absence of spermatogenic cells were observed in the treatment groups. It was concluded that CAJ induced a destructive effect on testes of rats as evidenced by damaged testicular tissue, reduced gonado-somatic index, azospermia and disruption in testicular electrolyte homeostasis. It was concluded that CAJ caused hypercalcaemia and hypozincaemia in the testicular tissue of the treated rats. Concurrently, CAJ also caused damage to testicular histology, azospermia and decreased GSI. *Citrus aurantifolia* juice should be consumed with caution due to its potential to cause infertility in males.

Keywords*: Citrus aurantifolia* juice, Sperm quality, Infertility, Gonado-somatic index, Testicular histology

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INTRODUCTION

Infertility rate is high in Nigeria and the male factor may account for about 40-50% of all the infertility cases (Uadia and Emokpae, 2015). A critical factor causing infertility in males is low sperm quality. Falling sperm count and the rise in male infertility has led to an increase interest in the nutritional factors that influence the development and quality of sperm. Recent researches have shown *Citrus aurantifolia* juice (CAJ) to have spermicidal activity (Okon *et al.*, 2014). The widespread usage of CAJ as food and traditional medicine (Aprioku and Briggs, 2018) and its spermicidal activity calls for concern because this situation may contribute to the development of male infertility among the consumers.

Lime (*Citrus aurantifolia*), a polyembryonic plant belonging to the family Rutaceae, is widely grown in sub-tropic and tropical region of the world (Niththep *et al.*, 2016), and forms an important part of diet as a component of commonly used beverages and for medicinal purposes (Aprioku and Briggs, 2018). The fruits are globose to ovoid berry of about 3 - 6 cm in diameter and sometimes have apical papilla. It is highly acidic and fragrant (Patil *et al.* 2009), yellow when ripe but usually picked green commercially (Enejoh *et al*., 2015). Lime fruit contains an array of bioactive and nutritional constituents which include flavonoid, limonoid, alkaloid, ascorbic acid, tannins, saponin, reducing sugars, cardiac glycosides, citric acid and amino acid (Bakare *et al*., 2009).

Many useful properties of CAJ have been reported, including anti-proliferative and immuno-modulatory effect on activated human lymphocytes (Gharagazloo *et al.*, 2001), antimicrobial activity against respiratory tract infections and cholera (Adeleye 2003), anti-oxidant activity (Boshtam *et al.*, 2011). In contrast, other studies reported negative effects of CAJ. *Citrus aurantifolia* juice reduces the number of ova shaded

and causes irregularity in the histology of the ovaries and uterus in female rats (Bakare *et al.,* 2012). The juice also exhibits anti-fertility effect by disrupting the estrous cycle in Wistar rats (Aprioku and Briggs, 2018). Intra-vaginal douching with CAJ showed a destructive effect on fetal development and female reproductive histology (Solomon *et al*., 2014).

Despite research reports on the biological effects of CAJ, including the aforementioned, there is paucity of literature on its effect on testicular biology. In particular, it is not known how CAJ affects testicular calcium and zinc concentrations. Given the diverse nature of its phytochemical content, it is conceivable that CAJ may affect the homeostasis of some important electrolytes such as calcium and zinc in the testicular tissue, as one of the mechanisms of its effects. Calcium is abundant in seminal fluid and plays an important role in sperm activities such as hyper-activation, chemotaxis, capacitation, and the acrosomal reaction, all of which are essential for successful fertilization and normal male fertility (Polina *et al*., 2014; Qi *et al.,* 2007; Kwon *et al*., 2013; Kwon and Park, 2013; Shukla *et al.,* 2013). Zinc is a micronutrient required for the action of more than 200 metallo-enzymes (Jinxiang *et al.,* 2014). It is a very important mineral for male fertility. Zinc is found in high concentration in male sex organs and sperm (Oliveira *et al*., 2004). It is also necessary for making the outer membrane and tail of the sperm (Awadallah *et al.,* 2003). Deficiency of zinc can impair spermatogenesis and decrease serum testosterone level (Wong *et al.,* 2002). It conserves genomic integrity in the sperm head and tail (Tuerk and Fazel, 2009).

This study examined the effects of CAJ on testicular calcium and zinc concentrations and some testicular and sperm parameters.

MATERIALS AND METHODS

Preparation of crude *Citrus aurantifolia* juice and acute toxicity study

Fresh *Citrus aurantifolia* (lime) fruits were obtained during the rainy season from a local farm in the outskirts of Kano city and authenticated by a botanist and given herbarium accession number- BUKHAN 0028. The fruits were properly washed and sliced after removing the rind. Juice was extracted using a juicer and filtered through a filter paper and pH was determined using a pH meter. Fresh CAJ was prepared everyday of administration.

To obtain the weight of solute in a given volume of CAJ, 120ml of ultra-filtered fresh CAJ was collected into a clean pre-weighed container, dried in an oven at a temperature of 40 ^{o}C. Actual weight of solute was obtained by subtracting weight of container from total weight. Concentration of solutes present in 120ml of CAJ was calculated thus:

Concentration = mass/volume

Median lethal dose (LD_{50}) was estimated in the rats using Lorke's method (1983). This method has two phases. Phase 1 involved nine adult male rats, which were divided into three groups (n=3). Each group of animals were administered different doses (10, 100, 1000mg/kg) of CAJ and observed for 24 hours for signs of toxicity or death. In phase 2 three animals were administered higher doses (1600, 2900 and 5000mg/kg) of CAJ and similarly observed.

Phytochemical screening of secondary metabolites in *Citrus aurantifolia* juice

The extracted CAJ was screened for alkaloids, flavonoids, saponins, steroids, anthraquinones, combined reducing sugars (Sofowora, 1993), tannins (Trease and Evans, 1989) and cardiac glycosides (Parekh and Chanda, 2007) using previously described methods.

Experimental animals and study design

Animals were housed in plastic cages and fed on standard feed and water *ad libitum.* They were maintained under standard conditions. Animal house was well ventilated at room temperature under natural day/night photoperiodicity.

A total of twenty eight adult male Wistar rats weighing between 200 - 220g were subdivided into four groups (n=7). The animals received either normal saline (control), 600mg/kg body weight, 900 mg/kg body weight or 1200mg/kg body weight of CAJ juice for 35 days. Administration was carried out by oral gavage daily using metal cannula.

At the end of the treatment, rats were weighed and then sacrificed after anaesthesia by intraperitoneal injection of 40mg/kg ketamine. The abdominal cavity was opened up through a midline abdominal incision to expose the reproductive organs. Both testes and epididymis were carefully removed and weighed using an electronic analytical and precision balance (BA 210S, d=0.0001- Sartoriusen GA, Goettingen, Germany). Left testis of each rat was homogenized in 2 ml of physiologic saline using a homogenizer. The homogenate was then centrifuge at 10000g for 15 minutes. The supernatant was used for determination of zinc and calcium concentrations.

Sperm count determination

Spermatozoa in the left epididymis were counted by a modified method of Yokoi and Mayi (2003). Briefly, the epididymis was minced with scissors in 5 ml physiologic saline, placed in a rocker for 10 minutes, and allowed to incubate at room temperature for 2 minutes. The supernatant was then diluted 1:100 with solution containing 5 g sodium bicarbonate and 1 ml formalin (35%). Total sperm number was determined by using the new improved Neuber counting chamber (haemocytometer). Approximately 10 µL of the diluted sperm suspension was transferred to each counting chamber of the haemocytometer and allowed

to stand for 5 minutes. This chamber was then placed under a binocular light microscope using an adjustable light source. The ruled part of the chamber was focused and the number of spermatozoa counted in five 16-small squares. The sperm concentration was calculated then multiplied by 5 and expressed as [X] x 10^6/ml, where [X] is the number of spermatozoa in a 16-small square.

Determination of sperm progressive motility

Sperm progressive motility was determined by the method of Sonmez *et al.* (2005) and slightly modified. Accordingly, the fluid obtained from the left cauda epididymis with a pipette was diluted to 0.5 ml with Tris buffer solution. A drop of the solution was placed on a pre-warmed microscopic slide. A cover slip was lowered on to the sample avoiding formation of air bubble. The slide was examined using light microscope at a magnification of x 400. In evaluating motility, sperm cells were classified as non-motile and progressively motile. A progressively motile sperm swims forward in an essentially straight line, whereas a non-progressively motile swims but in an abnormal path, such as in tight circles. Motility estimates were performed from three different fields in each sample. The mean of the three estimations was used as the final motility score.

$$\% \text{ of motile sperm} = \frac{\text{Number of motile sperm}}{\text{Total number of sperm}} \text{X100}$$

Determination of sperm morphology

The sperm cells morphology was determined with the aid of light microscope at x 400 magnification as described by Sonmez *et al*., 2005. The fluid obtained from the left cauda epididymis was pipetted and diluted to 0.5 ml with Tris buffer solution. It was further diluted 1:20 with 10% neutral buffered formalin. Five hundred spermatozoa were examined for morphological abnormalities such as rudimentary tail, round head and detached head (Atessahin *et al.*, 2006). The result was expressed as percentage of abnormal spermatozoa to morphologically normal spermatozoa.

Measurement of testicular zinc concentration

Zinc concentration was determined in testicular tissue homogenate as described by Eliason (2003). 1000µl of reagent was mixed with 50µl of sample and standard, respectively. The mixture was allowed to incubate for 8 minutes at 28 ºC. Standard was taken against the reagent blank. Sample absorbance was measured at a wavelength of 560nm using colorimeter.

Measurement of testicular calcium concentration

The reagent provided in the kit were prepared and then stabilized for three days at 25ºC. 25µl of reagent blank, standard, testes homogenate were pipetted into three test tubes, 1ml of working reagent was then added into each of the test tubes. It was allowed to stand for 50 minutes after which absorbent of the sample and standard was taken against the reagent blank at a wave length of 570nm using colorimeter.

Histological study of testicular tissue

The testicular biopsies were fixed with 10% formol-saline, dehydrated with ascending grade of alcohol (70%, 90% and 95%) cleared with toluene, infiltrated with molten paraffin wax at a melting point of 56 ºC. Sections of 5µ thickness were cut on a rotary microtone. Sections were floated out on clean microscopic slides, to prevent detachment from slides during staining procedure. They were then dried for 2 hours at 37 ºC. Slides were stained with haematoxylin and eosin and then passed through ascending concentrations of alcohol (20-100%). A permanent mounting medium was put on the tissue section. A thin cover slip was placed on the covering mounting medium and underlying tissue sections were allowed to dry and later observed using DIALUX Research microscope. Photomicrographs were taken in bright field at x 400 (Awioro, 2010).

Determination of gonado-somatic index

Gonado-somatic index (GSI) was calculated as described by Caldeira *et al*. (2010) and Silva *et al*. (2014) by dividing the average of the weight of the (right and left) testicles by the live weight of the rat before sacrifice. The result was then multiplied by one hundred.

$$\text{GSI} = \frac{\text{Average weight of testes}}{\text{Live weight of rat}} X\ 100$$

Statistical Analysis

Obtained data were expressed as mean ± SEM and compared using one-way analysis of variance (ANOVA) and Scheffe *post hoc* test on SPSS Statistics version 20.0 (SPSS Inc., Il, U.S.A). Values of $P < 0.05$ were considered statistically significant.

RESULTS

Preliminary phytochemical screening and acute toxicity study

The result of the preliminary phytochemical screening of CAJ revealed the presence of alkaloids, flavonoids, tannins, saponins, anthraquinones, reducing sugars, steroids and cardiac glycosides. There were no signs of toxicity or death in both phase I and II of the acute toxicity study.

Effect of *Citrus aurantifolia* juice on body weight and gonado-somatic index of rats

Table 1 shows the result of the body and organs weight respectively, the weights were recorded after the period of the treatment. There was a significant decrease ($P<0.05$) in body weight and GSI of the group that receive the highest dose (1200mg/Kg) of CAJ in comparison with the control, a similar decline was observed (though not statistically significant) in the moderate (900mg/Kg) and the low (600mg/Kg) doses administered CAJ, respectively.

Table 1: Effect of *Citrus aurantifolia* juice administration on bodyweight (g) and gonado-somatic index of rats.

Treatments	LBW (g)	LT (g)	RT (g)	AWT(g)	GSI(%)
Normal saline	265.57±4.11[a]	1.48±0.01[a,b,c]	1.48±0.01[a,b,c]	1.49	0.55
600 mg/kg	243.00±8.26	1.15±03.05[a]	1.15±0.03[a]	1.13	0.46
900 mg/kg	237.04±7.31	1.27±0.03[b]	1.23±0.03[b]	1.32	0.46
1200mg/kg	232.53±5.73[a]	0.18±0.03[c]	0.24±0.02[c]	0.19	0.28

Values with similar superscripts in the same column are significantly different. Mean ±S.E.M, n=7. BW= live bodyweights, LT=left testis, RT=right testis, AWT=average weight of testes sand GSI=gonado-somatic index.

Table 2: Effect of ***Citrus aurantifolia*** **juice** administration on the sperm count, morphology and motility in rats

Treatments	Sperm count (x 10^6/ml)	% normal morphology	% motile cells
Normal saline	77.14 ± 4.00	52.29 ± 11.5	60.14 ± 4.98
600 mg/kg bw	0.00	NA	NA
900 mg/kg bw	0.00	NA	NA
1200 mg/kg bw	0.00	NA	NA

Mean ±S.E.M, n=7. NA = not available (no sperm cells were present in the sample). CAJ = *Citrus aurantifolia* juice

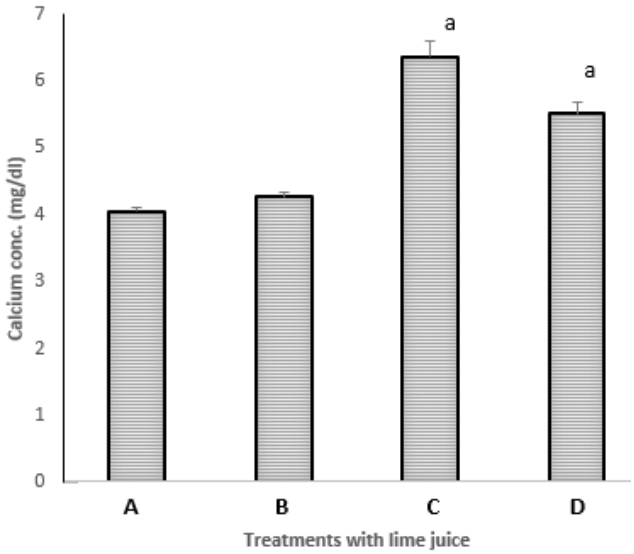

Figure 1: Effect of *Citrus aurantifolia* juice administration on testicular calcium concentration in rats. A= Control, B= 600 mg/kg bw, C= 900 mg/kg bw, D= 1200 mg/kg bw. a = statistically significant (0.001) compared to normal saline group.

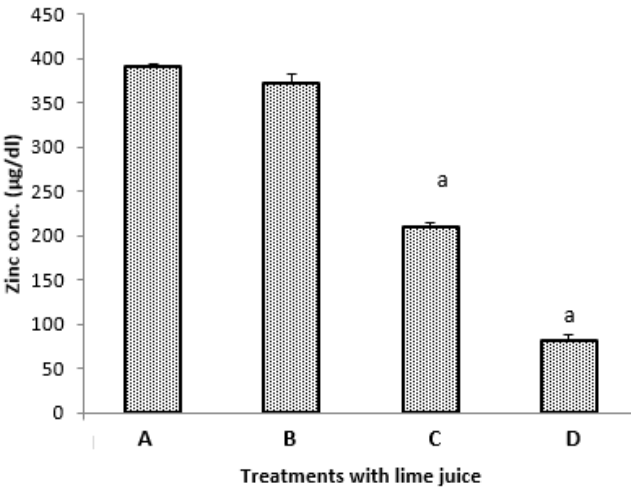

Figure 2: Effect of *Citrus aurantifolia* juice administration on testicular Zinc concentration in rats. A= Control, B= 600 mg/kg bw, C= 900 mg/kg bw, D= 1200 mg/kg bw. a = statistically significant (0.001) compared to normal saline group

Effect of *Citrus aurantifolia* juice on testicular calcium and zinc concentrations in rats

The result of testicular levels of calcium and zinc were presented on figure 1 and 2, respectively. There was a significant decrease ($P<0.05$) in the level of testicular zinc concentration in the 900 mg/kg bw and 1200 mg/kg bw administered groups when compared with the control, while testicular calcium concentration increased significantly ($P<0.05$) in these groups compared to control. There was no statistical significance ($p > 0.05$) difference in lower dose administered group in both testicular calcium and zinc concentrations.

Effect of fruit extract of lime on testicular histology of rats

Results of histological studies of control and treated rats are illustrated using photomicrographs on plate I (A – D). Examination of the slides from testicular tissue of control rats (A) showed normal histological findings- essentially normal and undisturbed pattern and shape of seminiferous tubules, with spermatozoan seen at different stages of development. The testicular tissue of the animals that received 600mg/kg of CAJ (B) revealed slight changes in tubular shapes with decrease in diameter and an increase in the length of seminiferous tubules. There was also disorganized testicular tissue architecture with no spermatogenic cells seen. For the rats that received 900mg/kg of CAJ (C), testicular tissue showed degeneration and shrinkage of the seminiferous tubules with no sperm cells seen in all stages of development. The shape of tubules changed from normal round to an irregular elongated shape. Examination of testicular tissue of rats that received 1200mg/kg of CAJ (D) showed total destruction of the seminiferous tubules and disappearance of testicular architecture with no spermatogenic cell seen.

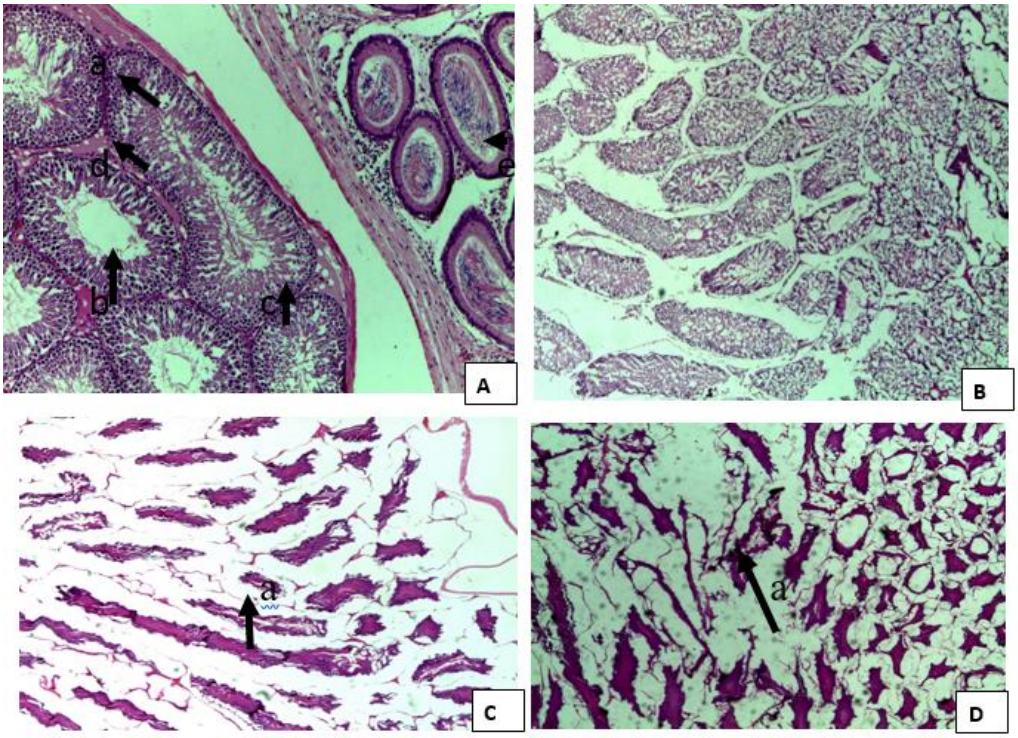

Plate I: Photomicrograph of testicular tissue of rats treated with CAJ (H&E ×400). Note the essentially normal and undisturbed pattern and shape of seminiferous tubules, with spermatozoa seen at different stages of development (A); mild changes in tubular shapes with decreased diameter and an increased in the length of seminiferous tubules (B); distortion of testicular architecture, degeneration and shrinkage of the seminiferous tubules with no sperm cells seen in all stages of development (C); total destruction of the seminiferous tubules and disappearance of testicular architecture with no spermatogenic cells seen (D). a = seminiferous tubules, b = lumen of seminiferous tubules, c = spermatocytes, d = Leydig cells, e= released spermatozoa.

DISCUSSION

Preliminary phytochemical screening of crude lime juice revealed the presence of different phytochemical - non-nutrient bioactive compounds that are produced by plants. Phytochemicals especially polyphenols constitute a major group of compounds that act as primary antioxidants (Giuseppe *et al*., 2007). Flavonoids, which cannot be synthesized by humans, are important components of most plants (McCullough *et al*., 2012; Shashank and Abhay 2013) and are known to possess anti-hypertensive, antioxidant, anti-anxiolytic, anti-inflammatory, anti-cholesteronemic and antimicrobial activities (Liu *et al*., 2014).

The findings of this study is in conformity with the work of Enojoh *et al*. (2015) who, using high-performance lipid chromatography (HPLC) and gas chromatography mass spectrometry, demonstrated that *Citrus aurantifolia* contained flavonoids identified from the plant. A similar study carried out by Nwankwo *et al.* (2015) showed presence of tannins, alkaloids, saponins and flavonoids, with tannins and saponins well known as important plant metabolites (Nwanko *et al.* (2015).

Acute toxicity is the adverse biological effects occurring after oral or dermal administration of a single dose of a substance, or multiple doses given within a short period of time. The toxicity studies in animals are commonly used to assess the potential health risk in humans by intrinsic adverse effect of phytochemicals in the extracts (Oyedemi *et al* 2010). These adverse effects may cause significant alterations in the levels of biomolecules metabolites derangement of histomorphology of tissues and organs (Yakubu *et al* 2009). Changes in general behaviour, weights of body and internal organs are critical parameters for the evaluation of the effect of a compound, such changes are often the first signs of toxicity (Carol 1995).

In this present acute toxicity study, lime juice at a doses of 10mg/kg, 100mg/kg, 1000mg/kg, 1600mg/kg, 2900mg/kg and 5000mg/kg orally given to the rats showed neither sign of toxicity nor death. This result is in line with that of Chunlarathanaphom *et al*. (2007) who showed that acute and sub-chronic toxicities of the water extracts from the roots of *Citrus aurantifolia* in both male female rats did not produce any sign of toxicity or mortality. Another study by Akhtar (2013) showed that the doses above 3.5 g/kg were toxic to rats, so also was the methanol extract of the peels in mice.

The present study demonstrated that *Citrus aurantifolia* juice decreased GSI in a dose-dependent manner, with the highest dose (of 1200mg/kg) having a significant effect. This finding is in line with the reports of Aseth *et al* (1995). Gonadosomatic index indicates calculation of gonads mass relative to body weight and is an important parameter in reproduction (Franca *et al*., 1998). Gonadosomatic index predicts the rates of sperm production as well as sperm function

in a given specie (Gomendio *et al.*, 2006; Adebayo *et al*., 2009).

There is a direct correlation between the testes weight and sperm production. Testis weight primarily reflects the total volume of the seminiferous tubules and its main components. Heavy loss of testicular cells was reported to be a major cause of testicular weight loss in rats (Naganatura *et al.,* 2008). This detrimental effect predisposes the animals to reduction in sperm count which may lead to infertility. In this study, lime juice may have had a toxic effect on the testicular tissue leading to decreased testicular weight relative to the body weight. Similar result was reported previously by Aseth *et al*. (1995). This effect may be due to disturbance in normal regulation of spermatogenesis through a fall in testosterone concentration following reduction in density of Leydig cells (Komili *et al*., 2015). The observed reduction in testes weight tallies with the zero sperm count described in this study.

The loss of body weight observed in this study might be as a result of interruption of metabolism of essential nutrients for health and normal body growth as reported by others (Marija *et al* 2008), and is in line with previous studies (Bakare *et al.,* 2012; Dosephine *et al.,* 2015).

Administration of CAJ induced damage on the testicular tissue, which increased with the increase in dose from absence of spermatozoa, mild distortion of seminiferous tubules to complete destruction of seminiferous tubules and testicular architecture. This effect will predispose the animal to infertility. These changes could be the result of oxidative damage induced by some constituents of the juice such as flavonoids, tannins and alkaloids as determined by the phytochemical screening of the juice in this study. Oxidative stress induced by these substances could result in damage to the cell membranes of the spermatozoa, seminiferous tubules and other testicular cells (Bahorun *et al*., 2006; Halliwell and Gutteridge, 2007; Azza *et al.,* 2010). As reported in this study, CAJ also induced disturbances in calcium and zinc homeostasis. This could cause disturbances in the fluid and electrolyte milieu of the testicular tissue with the resultant destructive histological changes.

Citrus aurantifolia juice abolished sperm cells in the rats in all the treatment doses, in contrast to what was observed in the control animals. No cells were seen to evaluate morphological defects and motility. Testicular function is assessed in parts by analysis of spermatic indices including sperm count, motility and morphology (Zinaman *et al* 2000, Eliason *et al* 2003). These parameters indicate the quality and functionality of sperm, thus, very vital for male fertility. The observed azospermia caused by CAJ in this study gives a clear indicator of the potential of CAJ to induce male infertility.

Several plant extracts and their active constituents have been reported to enhance reproductive process whereas some others act to antagonize the process by adversely affecting the hormonal, testicular and spermatogenic functions. The spermicidal effect of juices of natural products especially lime could be due to one of their characteristics which is acidity (Bakare *et al*., 2009). Lime juice contain high amount of organic acids like citric and coumaric acid (Patil *et al.* 2009) and as testicular milieu is highly sensitive to most chemicals, the destructive effect of CAJ in this study may be attributed partly to the acid constituent of the juice.

The fact that the juice contain high level of pro-oxidants like flavonoids, saponins, anthraquinones, alkaloids, tannins suggest that prolonged administration of the crude lime juice for the period of thirty five consecutive days may lead to oxidative damage due to free radicals (FR) and reactive oxygen species (ROS) generated by the metabolites, presumably by destroying testicular germ cells either due to membrane damage or macromolecular degradation, which resulted in significant decrease in the sperm count and testicular weight (Bahorun *et al*., 2006; Halliwell and Gutteridge, 2007). Alkaloids (such as nicotine) were previously reported to cause testicular degeneration (Jorsarrau *et al* 2008; Azza *et al.,* 2010). The alkaloid content of lime juice as reported in this study may have contributed to the observed destructive effects. Further studies will provide further insight.

Administration of CAJ for 5 consecutive weeks in this study has simultaneously increased calcium and decreased zinc concentrations. Zinc plays an important role in the process of cell growth as a co-factor for both DNA-RNA polymerase activities. Zinc is important for maintenance & regulation spermatogenesis and sperm motility (Sonoko *et al.,* 2009). Lack of zinc causes a decrease in ribonucleic acid (RNA), deoxynobneclric acid DNA and protein activity in the testes of rats (Chealth *et al.,* 1995). Previous studies have reported that high concentration of Zn is detectable in testes and that Zn deficiency inhibited spermatogenesis and caused sperm abnormality (Hidiroglou *et al*., 1984; Merker *et al.,* 1997). Zinc is also essential for the maintenance of germ cells, the progression of spermatogenesis, stabilization of the cell membrane and regulation of capacitation, acrosome reaction and sperm motility (Chandel Chand, 2014). Its deficiency leads to gonadal dysfunction, decreases testicular weight, and causes shrinkage of seminiferous tubules (Zeng *et al.,* 2013). The zinc deficiency state reported in this study may explain the mechanism of azospermia and histological changes here reported following lime juice administration. This is in support of the role of zinc in the destructive effect caused by lime juice on the testis.

The increase in calcium concentration reported in this study is in line with previous findings (Xia *et al.,* 2007; Marquez *et al.,* 2008). Calcium triggers multiple physiological events in spermatozoa, such as hyper-activation, chemotaxis, capacitation, and the acrosomal reaction, all of which are essential for successful fertilization (Ren *et al.,* 2001; Bohmer *et al.,* 2005; Krichok *et al.,* 2006; Qi *et al.,* 2006; Marquz *et al.,* 2008) and some of which are pH-dependent (Ho and Suarez, 2001). It was reported that a potential functional interaction exists between the sperm proteins and Ca^{2+} permeable channel proteins, thus modulating the Ca^{2+} influx mechanism (Kwon *et al.,* 2013; Kwon and Park, 2013; Shukla *et al.,* 2013) and playing a vital role in adjusting male fertility. It is evident that optimum calcium concentration is essential for normal sperm function and male fertility; and a state of increased calcium concentration as induced by lime juice in this study has provided evidence to support the role of calcium in the normal function and dysfunction of spermatozoa. Testicular hypercalcaemia is, therefore, suggested as one of the mechanisms of lime juice-induced testicular damage.

Our data indicate that CAJ caused hypercalcaemia and hypozincaemia in the testicular tissue of the treated rats. Concurrently, CAJ also caused damage to testicular histology, azospermia and decreased GSI. *Citrus aurantifolia* juice should be consumed with caution due to its potential to cause infertility in males.

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