Niger. J. Physiol. Sci. 31(June 2016) 001-010
www.njps.com.ng

Niger. J. Physiol. Sci.

Chemopreventive Effect of Tadalafil in Cisplatin-Induced Nephrotoxicity in Rats

[1*]Adeneye A.A. and [2]Benebo A.S.

[1]Department of Pharmacology, [2]formerly of the Department of Pathology and Forensic Medicine, Faculty of Basic Medical Sciences, Lagos State University College of Medicine, 1-5 Oba Akinjobi Way, G.R.A., Ikeja, Lagos State, Nigeria

Summary: Nephrotoxicity remains a common untoward effect of cisplatin therapy with limited effective chemopreventive options available till date. This study aims to evaluate the possible chemopreventive effect and mechanism(s) of action of 2 $mgkg^{-1}$ and 5 $mgkg^{-1}$ of Tadalafil in cisplatin-induced nephrotoxic rats. In this study, twenty-five male Wistar rats were randomly divided into five groups (n = 5 rats per group) and daily pretreated with oral doses of distilled water (10 $mLkg^{-1}$), ascorbic acid (100 $mgkg^{-1}$), Tadalafil (2 $mgkg^{-1}$ and 5 $mgkg^{-1}$) for 7 days before cisplatin (5 $mgkg^{-1}$, intraperitoneal) was administered. 72 hours post-cisplatin injections, rats were sacrificed humanely and blood samples for serum electrolytes, urea and creatinine and renal tissues for reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and malonialdehyde dehydrogenase (MAD) assays and histopathology were collected. Results showed that cisplatin injection caused significant decreases in the serum sodium (Na^+), potassium (K^+), bicarbonate (HCO_3^-), calcium (Ca^{2+}), phosphate (PO_4^{2-}) and concomitant significant increases in the serum urea and creatinine levels. In addition, there were significant decreases in the renal tissue GSH, SOD, CAT and increased MAD and GSH-Px levels which were corroborated by histopathological features of tubulonephritis. However, these histo-biochemical alterations were significantly attenuated by ascorbic acid and Tadalafil pretreatments. Overall, results of this study showed the chemopreventive potential of Tadalafil against cisplatin-induced nephrotoxicity which was possibly mediated via antioxidant and anti-lipoperoxidation mechanisms.

Keywords*:* Cisplatin-induced nephrotoxicity, Renal function parameters, Oxidative markers, Histopathology

**Address for correspondence:* adejuwon.adeneye@lasucom.edu.ng; adeneye@yahoo.com; +234-802-069-0946

Manuscript Accepted: March, 2016

INTRODUCTION

Cisplatin [cis-diamminechloroplatinum (II)] is a divalent, inorganic, water-soluble platinum-coordination complexes alkylating antineoplastic drugs used for the treatment of solid tumors such as testicular, ovarian, bladder, head and neck, lung and colon tumors (Kintzel, 2001; Morgan *et al.*, 2012; Ozkok and Edelstein, 2014). Its chief dose-limiting extramedullary side-effects include amongst others peripheral motor and sensory neuropathy, ototoxicity and nephrotoxicity with 20% of patients on high-dose cisplatin treatment eventually developing severe renal dysfunction (Go and Adjei, 1999; Tew *et al.*, 2001; Ekborn *et al.*, 2003; Yao *et al.*, 2007).

The mechanism for cisplatin-related renal cell injury has been the focus of intense investigation for many years, and recent studies suggest that the *in vivo* mechanisms of cisplatin nephrotoxicity are complex and involve oxidative stress, apoptosis, inflammation, and fibrogenesis of the renal proximal convoluted tubules (Lieberthal *et al.*, 1996; Li *et al.*, 2014). Reactive oxygen species (ROS) which are produced via the xanthine-xanthine oxidase system, mitochondria, and reduced nicotinamide adenine dinucletide phosphate (NADPH) oxidase in renal cells act directly on the renal cell components, including lipids, proteins, and DNA, and compromise renal cell integrity (Kawai *et al.*, 2006). Cisplatin induces glucose-6-phosphate dehydrogenase and hexokinase activity, which increase free radical production and decrease antioxidant production (Yilmaz *et al.*, 2004). Cisplatin also increases intracytoplasmic calcium level which activates NADPH oxidase and stimulates ROS (such superoxide anion, hydrogen peroxide and hydroxyl radical) production within the damaged mitochondria of the cisplatin-treated kidneys (Shino *et al.*, 2003; Kawai *et al.*, 2006). These free radicals damage the lipid components of the cell membrane by peroxidation and denature proteins, leading to enzymatic inactivation as well as mitochondrial dysfunction (Yilmaz *et al.*, 2004). However, vigorous hydration with saline and simultaneous administration of mannitol before, during, and after cisplatin administration, has been reported to reduce

cisplatin-induced nephrotoxicity. This strategy has been accepted as standard clinical practice in the prevention of cisplatin-related nephrotoxicity (Carnelison and Reed, 1993) although another randomized trial demonstrated that saline alone or with furosemide offers better renal protection than saline plus mannitol (Santoso *et al.*, 2003). Other chemotherapeutic drugs used clinically in ameliorating cisplatin-related nephropathy include amifostine (Capizzi, 1999), procainamide (Viale *et al.*, 2000), interleukin-10 (Deng *et al.*, 2001), N-acetylcysteine (Nisar and Feinfeld, 2002), salicylate (Li *et al.*, 2002), fibrate (Nagothu *et al.*, 2005), allopurinol and ebselen (Lynch et al., 2005), serum thymic factor (Kohda *et al.*, 2005), glutamine (Mora *et al.*, 2003), melatonin (Kilic *et al.*, 2013), captopril (El-Sayed *et al.*, 2008), capsaicin (Shimeda *et al.*, 2005), lipoic acid (Somani *et al.*, 2008) and most recently metformin (Li et al., 2016) and trimetazidine (El-Sherbeeny and Attia, 2016). Despite availability of these therapeutic strategies, they are costly and have limited effectiveness as some patients on these therapies still progress to develop severe cisplatin-related renal dysfunctions and/or other systemic toxicities (Vermeulen *et al.*, 1993). In view of these drawbacks, the current study has been designed primarily at exploring the possible chemopreventive effect and mechanism(s) of action of 2 mg/kg and 5 mg/kg of Tadalafil, a long-acting phosphodiesterase-5 (PDE-5) inhibitor, against cisplatin-induced nephrotoxicity in Wistar rats, outside its current therapeutic uses in the management of erectile dysfunction and pulmonary arterial hypertension. This is with the view of discovering and developing a new effective chemopreventive drug available for cisplatin-related renal toxicities and without significantly altering its antineoplastic activity. Doses of Tadalafil and cisplatin used in the present study were chosen based on results of previous studies (Greggi-Antunes *et al.*, 2000; Ajiboye and Oluwole, 2012) and results of the preliminary study conducted.

MATERIALS AND METHODS

Drugs and Chemicals

Ascorbic acid (Sigma Chemical Co., St. Louis, Missouri, U.S.A.), Tadalafil (Tadalis-20®, Evans Medical Plc, Agbara Industrial Estate, Ogun State, Nigeria), Cisplatin (Cisplatyl® 50 mg, Laboratoire Roger Bellon, France).

Experimental Animals

A total of twenty-five young adult male Wistar rats were procured from the Rat Colony of Animal House, College of Medicine of the University of Lagos, Idi-Araba, Surulere, Lagos State, Nigeria, in the month of August, 2012, after institutional ethical clearance has been obtained for this study. The rats were allowed to acclimatize under standard laboratory conditions for 14 days at the Rat Colony of the Animal House, Lagos State University College of Medicine, Ikeja, Lagos State, Nigeria. Rats were cared for and handled according to existing guidelines on the Use and Care of Experimental Animals as prescribed by United States National Institutes for Health (1985). They were allowed free access to standard rat feed and potable drinking water for 2 weeks before being used for experimentation.

Induction of cisplatin-induced nephrotoxicity and drug treatment

Twenty-five adult male Wistar rats were randomly allotted to five groups (Groups I-V) of five rats per group such that the weight difference between and within groups does not exceed ±20% of the mean sample population. The rats were orally pretreated with distilled water, ascorbic acid and Tadalafil at between 07:00 hour and 09:00 hour once daily for 7 days. Twenty-four hours after the last oral treatment with 10 $mLkg^{-1}$distilled water, 100 $mgkg^{-1}$ of ascorbic acid and 2 $mgkg^{-1}$ and 5 $mgkg^{-1}$ of Tadalafil, rats in Groups II-V were treated with 5 $mgkg^{-1}$ of cisplatin given through the intraperitoneal route as described by Greggi-Antunes *et al*. (2000) and Chen-zhe *et al*. (2012). Details of drug treatments of rats are as follows:

Group I: oral treatment with 10 $mLkg^{-1}$ of distilled water for 7 days + 1 $mLkg^{-1}$ of distilled water *i.p.* on 8^{th} day

Group II: oral treatment with 10 $mLkg^{-1}$ of distilled water for 7 days + 5 $mgkg^{-1}$ of cisplatin *i.p.* on 8^{th} day

Group III: oral treatment with 100 $mgkg^{-1}$ of ascorbic acid for 7 days + 5 $mgkg^{-1}$ of cisplatin *i.p.* on 8^{th} day

Group IV: oral treatment with 2 $mgkg^{-1}$ of Tadalafil in distilled water for 7 days + 5 $mgkg^{-1}$ of cisplatin *i.p.* on 8^{th} day

Group V: oral treatment with 5 $mgkg^{-1}$ of Tadalafil in distilled water for 7 days + 5 $mgkg^{-1}$ of cisplatin *i.p.* on 8^{th} day

Blood collection and measurement of renal function parameters

Seventy-two hours after induction with 5 $mgkg^{-1}$ of cisplatin, rats were sacrificed humanely under inhaled halothane and blood samples were collected directly from the heart chambers using 21 G needles mounted upon a 5 mL syringe (Unique Pharmaceuticals, Sango Otta, Ogun State, Nigeria). Each blood sample obtained for each rat was collected into a well labeled 10 mL capacity plain sample bottle. The blood samples were allowed for complete clotting for about 1-2 hours before they were centrifuged with Uniscope Laboratory Centrifuge (Model SM 112, Surgifriend Medicals, England) at 2000 revolution per minute for 15 minutes. This was aimed at separating the sera from clotted blood cells. The sera were carefully separated into new, well labeled, corresponding plain

sample bottles at room temperature 23-26 ºC. The sera were assayed for serum sodium, potassium, bicarbonate, urea and creatinine. Serum creatinine and blood urea were assayed using Randox Diagnostic kits (Randox Laboratories Ltd., Crumlin, U.K.) by methods of Varley and Alan (1984) and Tietz *et al*. (1994).

Serum levels of sodium, potassium, chloride, calcium, bicarbonate and phosphate were determined using the ISE 6000 BYY SFRI spectrophotometer. The machine when powered on carries out self-calibration for all parameters. When calibration was complete, the serum sample was placed into the probe and the tune button on the machine was pressed on the screen of the machine. The machine aspirated the sample and beeped with a screen display "remove sample". The machine then processed the sample and displayed the test result. The machine then printed out the result of the test showing all the required electrolyte levels namely: sodium, potassium, chloride, bicarbonate, calcium and phosphate.

Collection of kidney for renal tissue oxidative stress markers assay

After blood collection through cardiac puncture, a deep longitudinal incision was made into the ventral surface of the rat abdomen. The kidneys were identified and thereafter, harvested from each animal. One of the kidneys was rinsed in 1.15% potassium chloride (KCl) solution in order to preserve the oxidative enzyme activities of the kidney before being placed in a clean sample bottle which itself was in an ice-pack filled cooler. This was to prevent the breakdown of the enzymes for kidney function enzyme biomarkers.

Determination of renal tissue superoxide dismutase activity

Superoxide dismutase (SOD) activity in renal homogenate was determined according to the method of Sun and Zigma (1978) and Usoh *et al*. (2005). This method is based on the generation of superoxide anions by pyrogallol autoxidation, detection of generated superoxide anions by nitro blue tetrazolium (NBT) formazan color development and measurement of the amount of generated superoxide anions scavenged by SOD (the inhibitory level of formazan color development). The homogenate was centrifuged at 105,000 for 15 minutes at 4 ºC. To 0.25 mL of supernatant, 0.5 mL of tris cacodylic buffer, 0.1 mL of 16% triton X-100 and 0.25 mL NBT were added. The reaction was started by the addition of 0.01 mL diluted pyrogallol. Incubation was maintained for 5 minutes at 37 ºC. The reaction was stopped by the addition of 0.3 mL of 2 M formic acid. The formazan color developed was determined spectrophotometrically (Spectronic 501, Shimadzu). Enzymatic activity was expressed as Umg^{-1} protein.

Determination of renal tissue catalase activity

Serum catalase activity was determined using the method of Gaetani *et al*. (1989) by measuring the decrease in absorbance at 240 nm due to the decomposition of hydrogen peroxidase (H_2O_2) in UV recording spectrophotometer. The reaction mixture (3 mL) contained 0.1 mL of serum in phosphate buffer (50 mM, pH 7.0) and 2.9 mL of 30 mM of H_2O_2 in the phosphate buffer pH 7.0. An extinction coefficient for H_2O_2 at 240 nm of 40.0 M^{-1} cm^{-1} according to Aebi (1984) was used for calculation. The specific activity of catalase was expressed as moles of reduced H_2O_2 per minutes per mg protein (Umg^{-1} protein).

Determination of renal tissue glutathione peroxidase activity

The renal tissue glutathione peroxidase activity was determined using the method described by Gaetani *et al*. (1989). Glutathione peroxidase (GSH-Px) was determined in renal homogenate according to the method of Lawrence and Burk (1976). This method is based on measuring the oxidation of reduced nicotinamide adenine dinucletide phosphate (NADPH) using hydrogen peroxide as the substrate. A reaction mixture of 1 mL contained 50 mM potassium phosphate buffer (pH = 7), 1 mM disodium ethylene diamine tetra acetic acid (EDTA), 1 mM sodium trinite (NaN3), 0.2 mM NADPH, 1 unit/mL oxidized glutathione reductase and 1 mM GSH was prepared. The homogenate was centrifuged at 105,000 for 15 minutes at 4 ºC. 0.1 mL of the supernatant was added to 0.8 mL of the reaction mixture and the solution was incubated for 5 minutes at 25 ºC. 0.1 mL of 0.25 mM hydrogen peroxide solution was added to initiate the reaction. Absorbance was measured at 340 nm for 5 minutes, and an extinction coefficient of 6.22×10^{-3} was used for calculation. The results were expressed as $\mu molmin^{-1}g^{-1}$ tissue. The changes in the absorbance at 340 nm were recorded at 1 min interval for 5 min. The results were expressed as Umg^{-1} protein.

Determination of renal tissue reduced glutathione activity

In the renal homogenate, reduced glutathione (GSH) was determined according to the methods of Ellman (1959) and as adopted by Sedlak and Lindsay (1968). The method is based on the reduction of Ellman's reagent [5,5'-dithio-bs- (2-nitrobenzoic acid)] by SH groups to form 1.0M of 2-nitro-5-mercaptobenzoic acid per mole of SH. The nitro-mercaptobenzoic acid has an intense yellow color and can be determined spectrophotometrically. To 0.5 mL of 10% trichloroacetic acid. 6 mM disodium EDTA, 0.5 mL of homogenate was added and shaken gently for 10-15 minutes. This was followed by centrifugation at 2,000 rpm for 5 minutes. 0.2 mL of the supernatant

was mixed with 1.7 mL of 0.1M potassium phosphate buffer (pH = 8). At least a duplicate was made for each sample. 0.1 mL of Ellman's reagent was added to each tube. After 5 minutes the optical density was measured at 412 nm against a reagent blank. The results were expressed as μmoLmg^{-1} protein.

Determination of renal tissue malondialdehyde activity

Malondialdehyde (MDA) levels in renal tissue homogenates were determined spectrophotometrically using the method of Buege and Aust (1978). 0.5 mL of tissue homogenate was shaken with 2.5 mL of 20% trichloroacetic acid in a 10 mL centrifuge tube. To the mixture, 1 mL of 0.67% thiobarbituric acid was added, shaken and warmed for 30 minutes in a boiling water bath followed by rapid cooling. Then 4 mL of n-butyl-alcohol was added and shaken. The mixture was centrifuged at 3,000 rpm for 10 minutes. The resultant n-butyl-alcohol layer was taken and MDA content was determined from the absorbance at 535 nm. The results were expressed as μmolmg^{-1} protein.

Histopathological studies of rat kidneys

After the animals were sacrificed, postmortem examination was performed. The rat kidneys were identified and carefully dissected out *en bloc* for histopathological examination. After rinsing in normal saline, sections were taken from each harvested kidney. The tissue was fixed in 10% formo-saline, dehydrated with 100% ethanol solution and embedded in paraffin. It was then processed into 4-5 μm thick sections stained with hematoxylin-eosin and observed under a photomicroscope (Model N - 400ME, CEL-TECH Diagnostics, Hamburg, Germany).

Statistical Analysis

Data were expressed as mean ± standard error of the mean (SEM) of five rats and analyzed using One-way Analysis of Variance followed by Newman-Keuls test as post hoc test on statistical software package, GraphPad Prism (Graph Pad Software; version 5.0, Graph Pad Software Inc., La Jolla, California, U.S.A.). Significant levels were considered at $p<0.05$, $p<0.001$, $p<0.0001$.

RESULTS

Effect of daily oral pretreatment with 2 mgkg^{-1} and 5 mgkg^{-1} of Tadalafil on serum electrolytes, urea and creatinine in cisplatin-induced nephrotoxic rats

Single intraperitoneal injection of 5 mgkg^{-1} of cisplatin was associated with significant ($p<0.0001$) decreases in the serum levels of sodium, potassium, chloride, bicarbonate, calcium, phosphate and significant ($p<0.0001$) increases in the serum urea and creatinine concentrations in the untreated model control (Group II) rats when compared with untreated control (Group I) rats (Table 1). However, daily oral pre-treatment with 2 mgkg^{-1} and 5 mgkg^{-1} of Tadalafil for 7 days before cisplatin injection significantly ($p<0.05$, $p<0.0001$) attenuated decreases in the serum sodium, potassium, chloride, bicarbonate, calcium and phosphate concentrations in a dose-dependent fashion when compared to untreated model control (Group II) values (Table 1). In the same pattern, 2 mgkg^{-1} and 5 mgkg^{-1} of Tadalafil also significantly ($p<0.05$, $p<0.0001$) attenuated significant elevation in the serum urea and creatinine concentrations when compared to Group II values (Table 1). In addition, the protective effects of 5 mgkg^{-1} of Tadalafil on serum electrolytes, urea and

Table 1.Effect of 2 mgkg^{-1} and 5 mgkg^{-1} of Tadalafil and 100 mgkg^{-1} of ascorbic acid on the serum electrolytes, urea and creatinine in cisplatin-treated rats

	I	II	III	IV	V
Na^+ (mmol/L)	141.60 ± 1.21	121.60 ± 1.17^b	130.40 ± 1.75^d	126.60 ± 1.57^c	135.60 ± 1.29^e
K^+ (mmol/L)	6.94 ± 0.20	3.90 ± 0.22^b	5.60 ± 0.14^e	5.12 ± 0.09^e	6.54 ± 0.19^e
Cl^- (mmol/L)	100.30 ± 2.25	79.80 ± 2.87^b	96.60 ± 1.83^e	92.80 ± 1.53^e	98.40 ± 0.51^e
HCO_3^- (mmol/L)	14.02 ± 0.53	7.88 ± 0.32^b	9.84 ± 0.17^d	9.02 ± 0.12^c	9.74 ± 0.12^d
Ca^{2+} (mmol/L)	1.73 ± 0.05	0.64 ± 0.09^b	1.32 ± 0.09^e	1.10 ± 0.07^e	1.38 ± 0.07^e
PO_4^{2-} (mmol/L)	1.44 ± 0.06	0.68 ± 0.09^b	1.04 ± 0.05^d	0.90 ± 0.04^c	1.16 ± 0.09^e
Urea (mg/dL)	6.74 ± 0.18	17.28 ± 1.35^a	9.50 ± 0.24^f	7.88 ± 0.25^f	07.00 ± 0.18^f
Creat (mg/dL)	65.40 ± 1.58	102.00 ± 4.69^a	81.70 ± 1.97^f	85.80 ± 2.42^f	74.40 ± 3.42^f

^{a} and ^{b} represent significant increases and decreases at $p<0.0001$, respectively, when compared to Group I values; ^{c,d} and ^{e} represent significant increases at $p<0.05$, $p<0.001$ and $p<0.0001$, respectively and ^{f} represent a significant decreases at $p<0.0001$ when compared to Group II values.Group I: oral treatment with 10 mLkg^{-1} of distilled water + 1 mLkg^{-1} of distilled water *i.p.* Group II: oral treatment with 10 mLkg^{-1} of distilled water + 5 mgkg^{-1} of cisplatin *i.p.* Group III: oral treatment with 100 mgkg^{-1} of ascorbic acid + 5 mgkg^{-1} of cisplatin *i.p.*Group IV: oral treatment with 2 mgkg^{-1} of Tadalafil in distilled water + 5 mgkg^{-1} of cisplatin *i.p.* Group V: oral treatment with 5 mgkg^{-1} of Tadalafil in distilled water + 5 mgkg^{-1} of cisplatin *i.p*

Table 2. Effect of 2 mgkg^{-1} and 5 mgkg^{-1} of Tadalafil and 100 mgkg^{-1} of ascorbic acid on oxidative stress markers in cisplatin-treated kidney tissues.

Group	GSH (μmolmg^{-1} protein)	SOD (Umg^{-1} protein)	CAT (Umg^{-1} protein)	MDA (μmolmg^{-1} tissue)	GSH-Px (Umg^{-1} protein)
I	0.68 ± 0.07	12.92 ± 1.25	54.65 ± 4.39	0.15 ± 0.02	02.42 ± 0.39
II	0.20 ± 0.03^{a}	05.54 ± 0.43^{b}	23.66 ± 1.05^{b}	0.44 ± 0.03^{c}	04.86 ± 0.48^{c}
III	01.02 ± 0.07^{d}	16.35 ± 1.06^{d}	65.78 ± 3.24^{d}	0.27 ± 0.02^{g}	03.56 ± 0.18^{e}
IV	01.37 ± 0.11^{d}	11.90 ± 0.85^{d}	45.30 ± 2.41^{d}	0.29 ± 0.03^{g}	03.62 ± 0.16^{f}
V	02.28 ± 0.15^{d}	19.59 ± 1.11^{d}	50.13 ± 3.24^{d}	0.21 ± 0.02^{g}	03.06 ± 0.25^{f}

^{a} and ^{b} represent significant decreases at p<0.001, p<0.0001, respectively, while ^{c} represents a significant increase at p<0.0001 when compared to Group I values; ^{d} represent a significant increase at p<0.0001 while $^{e, f}$ and ^{g} represent significant decreases at p<0.05, p<0.001 and p<0.0001, respectively, when compared to Group II values.Group I: oral treatment with 10 mLkg^{-1} of distilled water + 1 mLkg^{-1} of distilled water *i.p.*Group II: oral treatment with 10 mLkg^{-1} of distilled water + 5 mgkg^{-1} of cisplatin *i.p.* Group III: oral treatment with 100 mgkg^{-1} of ascorbic acid + 5 mgkg^{-1} of cisplatin *i.p.*Group IV: oral treatment with 2 mgkg^{-1} of Tadalafil in distilled water + 5 mgkg^{-1} of cisplatin *i.p.* Group V: oral treatment with 5 mgkg^{-1} of Tadalafil in distilled water + 5 mgkg^{-1} of cisplatin *i.p.*

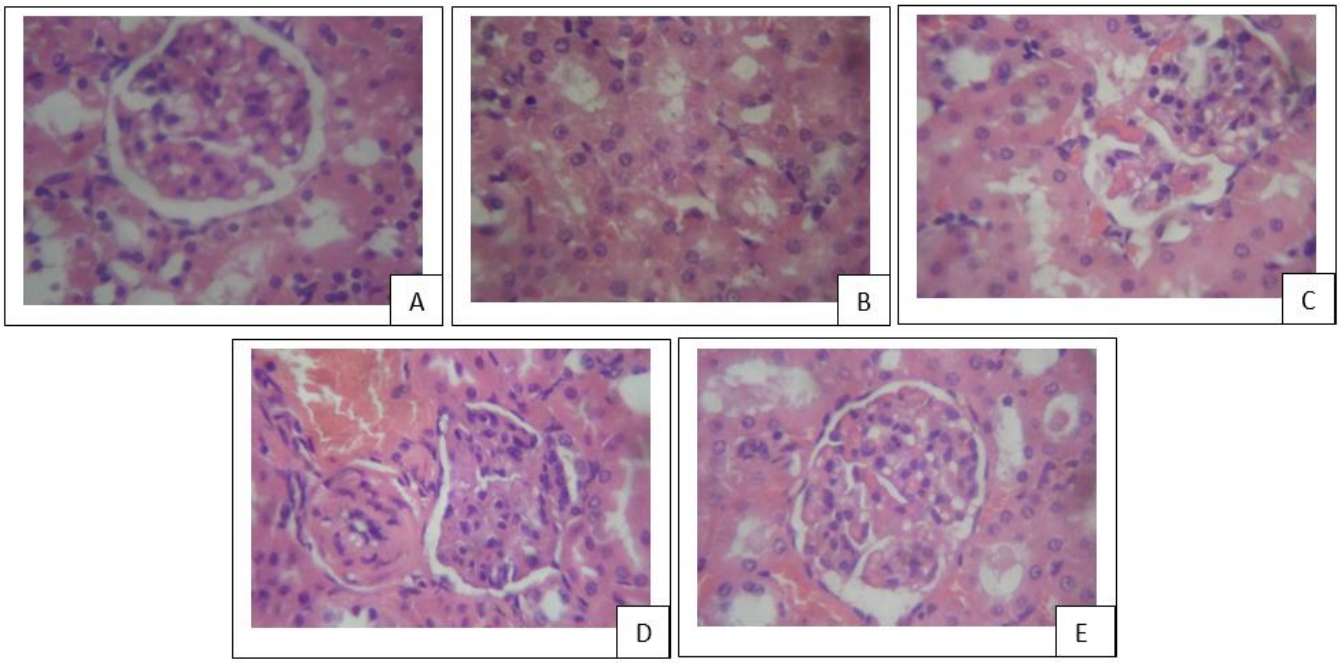


Figure 1. Transverse sections of rat kidney in **(A)** showing normal renal architecture in Normal rats treated with 10 mLkg^{-1} distilled water; **(B)** 5 mgkg^{-1} cisplatin-treated rats showing remarkable proximal tubular swellings and degenerative necrosis of the proximal tubules; **(C)** 100 mgkg^{-1} ascorbic acid-pretreated, cisplatin-treated kidney showing few areas of early tubular necrosis and hemorrhages; **(D)** 2 mgkg^{-1} Tadalafil-pretreated, cisplatin-treated kidney showing few patchy proximal tubular necrosis of the kidney, and; **(E)** 5 mgkg^{-1} Tadalafil showing glomeruli with no remarkable changes but few insignificant, patchy proximal tubular necrosis. (X400, H and E)

creatinine were comparable to that of oral pretreatment with 100 mgkg^{-1} of ascorbic acid (Table 1).

Effect of oral pretreatment with 2 mgkg^{-1} and 5mgkg^{-1} of Tadalafil on renal tissue GSH, SOD, CAT, MDA and GSH-Px in cisplatin-induced nephrotoxic rats

Table 2 shows effect of daily oral pretreatment with 2 mgkg^{-1} and 5 mgkg^{-1} of Tadalafil andsubsequent treatment with 5 mgkg^{-1} of cisplatin on renal tissue levels of GSH, SOD, CAT, MDA and GSH-Px. Intraperitoneal injection with 5 mgkg^{-1} of cisplatin was associated with significant (p<0.001, p<0.0001) decreases in the renal tissue concentrations of GSH, SOD, CAT and significant (p<0.0001) increases in the renal tissue concentrations of MDA and GSH-Px of untreated model control (Group II) (Table 2). With repeated daily oral pretreatment with 2 mgkg^{-1} and 5 mgkg^{-1} of Tadalafil and 100 mgkg^{-1} of ascorbic acid, renal tissue GSH, SOD and CAT concentrations were significantly (p<0.0001) restored when compared to untreated model control (Group II) values (Table 2). Similar significant (p<0.0001) restorative effects were recorded with repeated oral daily pretreatments with 2 mgkg^{-1} and 5 mgkg^{-1} of Tadalafil and 100 mgkg^{-1} of ascorbic acid (Table 2).

Histopathological studies of daily oral pretreatment with 2 mgkg^{-1} and 5 mgkg^{-1} of Tadalafil on cisplatin-treated renal tissue

Figure 1A depicts architecture of normal rat kidney showing intact glomerulus and tubules. However, single intraperitoneal injection of 5 mgkg^{-1} of cisplatin was associated with remarkable proximal tubular swellings and degenerative necrosis of the proximal tubules (Figure 1B) when compared to normal renal architecture of the kidney (Figure 1A). In rat kidneys pretreated with 100 mgkg^{-1} of ascorbic acid, the proximal tubule showed few areas of tubules with early necrosis and hemorrhages (Figure 1C) while rat kidneys pretreated with 2 mgkg^{-1} and 5 mgkg^{-1} of Tidalafil, glomeruli showed no remarkable glomerular changes but few insignificant and patchy proximal tubular necrosis of the kidneys (Figures 1D and 1E, respectively).

DISCUSSION

Drugs are a common source of acute renal injury resulting in drug-induced nephropathy, a clinical condition that is often reversible when detected early since its clinical signs may not be apparent in its early phases until it reaches an advanced stage when acute deterioration of the renal function or chronic renal insufficiency manifest (Modesti *et al.*, 2003). Drugs notorious for causing nephropathies include penicillins and cephalosporins, cimetidine, diuretics, allopurinol, NSAIDs, angiotensin converting enzyme inhibitors, cyclosporine, aminoglycosides, lithium, amphotericin B, radiocontrast agents, cisplatin, quinolones, among others (Paller, 1990; Hoitsma *et al.*, 1991; Naughton, 2008; Kodama *et al*., 2014). Cisplatin as a causative agent for drug-related nephropathy which may manifest as pre-renal azotemia, fluid and electrolyte imbalance, acute tubular necrosis, acute interstitial nephritis, and chronic interstitial nephritis, is well documented (Miller *et al.*, 2010). Drugs involved in causing nephrotoxicity exert their deleterious effects through one or more pathophysiologic mechanisms which include altered intraglomerular haemodynamics, tubular cell toxicity, inflammation, crystal nephropathy, rhabdomyolysis, and thrombotic microangiopathy (Zager, 1997; Perazella, 2003; Schetz *et al.*, 2005; Naughton, 2008; Miller *et al.*, 2010, El-Sherbeeny and Attia, 2016).

Serum electrolytes, uric acid, urea and creatinine are considered reliable indirect markers of renal function test parameters and profound alterations in the serum levels of these markers are diagnostic of nephropathy (Hakim and Lazarus, 1988; Gowda *et al.*, 2010; Saka *et al.*, 2012), although most recently, Cystatin C and β-trace protein are considered more effective markers of glomerular filtration rate and better diagnostic parameters of nephropathy (Gowda *et al.*, 2010). In the present study, nephrotoxicity was reliably established with single intraperitoneal injection of 5 mgkg^{-1} of cisplatin to treated rats and this nephrotoxicity was associated with profound reductions in the serum electrolytes and profound increases in the serum urea and creatinine levels which are consistent with those earlier reported (Greggi-Antunes *et al.*, 2000; Prabhu *et al.*, 2013). However, the significant attenuations of reductions in the serum Na^+, K^+, Cl^-, HCO_3^-, Ca^{2+} and PO_4^{2-} and concomitant elevations in the serum urea and creatinine in rats pretreated with 2 mgkg^{-1} and 5 mgkg^{-1} of Tadalafil are indicative of the possible protective effect of Tadalafil against cisplatin-induced nephrotoxicity.

Another notable finding of this study was the effect of cisplatin on oxidative stress markers. Literature has shown that cisplatin-induced nephrotoxicity to be significantly associated with significant reductions in the renal tissue levels of GSH, CAT and SOD and concomitant elevations in the tissue activities of MAD and GSH-Px (Nisar and Feinfeld, 2002; Kadikoylu *et al.*, 2004; Karimi *et al.*, 2005) and our results are consistent with those previously reported. Cisplatin is known to induce nephrotoxicity via generation of toxic, highly reactive oxygen-free radicals resulting increase in lipid peroxidation and a decrease in the activity of enzymes protecting the body against lipid peroxidation as well as decrease in the body's antioxidant status (Sadzuka *et al.*, 1992a; Sadzuka *et al.*, 1992b; Karimi et al., 2005). In addition, reactive nitrogen species have also been implicated in the mechanism of cisplatin-induced nephrotoxicity resulting in an increase in the renal content of peroxynitrite and nitric oxide (Sadzuka *et al.*, 1994; Yildirim *et al.*, 2003). Peroxynitrite causes changes in protein structure and function, lipid peroxidation, chemical cleavage of DNA, and reduction in cellular defenses by oxidation of thiol pools resulting in cisplatin-induced nitrosative stress and nephrotoxicity (Chirino *et al.*, 2004). However, reactive/oxidative stress is measured by the activities of oxidative enzyme markers such as catalase, superoxide dismutase and glutathione levels while that of lipid peroxidation is measured through malonialdehyde dehydrogenade and glutathione peroxidase activities (Nisar and Feinfeld, 2002; Kadikoylu *et al.*, 2004; Karimi *et al.*, 2005). Also, cisplatin is known to primarily cause tubulo-interstitial lesions affecting the proximal tubules, specifically the S3 segment of the outer medullary stripe (Tanaka *et al.*, 1986; Vickers *et al.*, 2004). Again, the histological features of tubolo-interstitial nephritis induced by cisplatin in this study is also in strong agreement with that previously reported by Tanaka *et al.* (1986) and Vickers *et al.* (2004).

Tadalafil is a potent, highly selective and long-acting type-5 cyclic guanosine monophosphate (cGMP) phosphodiesterase-5 inhibitor which has been widely reported to be highly efficacious and well tolerated by a broad population of men with

erectile dysfunction (Padma-Nathan, 2003; Eardley *et al.*, 2004; Broderick *et al.*, 2006; Porst *et al.*, 2006; La Vignera *et al.*, 2011) and to control symptoms and signs of benign prostatic hypertrophy (Egerdie *et al.*, 2012). Tadalafil is clinically useful for the prevention of erectile dysfunction after radiotherapy for prostate cancer (Pisansky *et al.*, 2014). It inhibits PDE-5 in the corpus cavernosum to help achieve and maintain penile erection (Laties, 2009). It is also approved for World Health Organization group 1 pulmonary arterial hypertension (PAH) to improve exercise ability (Humbert *et al.*, 2004). Sildenafil, another prototype of PDE-5 inhibitor, has been reported to boost human erythrocyte antioxidant status by enhancing activities of erythrocyte superoxide dismutase and catalase (Perk *et al.*, 2008) as well as prevent vascular oxidative stress in insulin resistant rats by increasing NO release and regulating vascular superoxide release (Oudot *et al.*, 2009). Tadalafil has equally been reported to possess antioxidant activity mediated via increased tissue/serum levels of nitric oxide and increased serum activity of SOD (Serarslan *et al.*, 2010; Koka *et al.*, 2010; La Vignera *et al.*, 2012). A recent study has reported the protective effect of 10 $mgkg^{-1}$ of Tadalafil pretreatment against high intra-abdominal pressure (IAP)-induced renal failure in Congestive Heart Failure (Bishara *et al.*, 2012) while another recent study reported the relaxant effect of Tadalafil in the isolated KCl pre-contracted ileum of both normal and diabetic rat which was mediated via NO and PDE mechanisms (Hekmat *et al.*, 2013). Attenuation of profound reductions in the tissue levels of GSH, CAT and SOD as well as concomitant elevations in the renal tissue levels of MAD and GSH-Px strongly indicate the possible antioxidant and anti-lipoperoxidative effects of Tadalafil. However, this protective effect by Tadalafil was corroborated by the unremarkable histological alterations in the tubulo-interstitial architecture in rats pretreated with 2 $mgkg^{-1}$ and 5 $mgkg^{-1}$ of Tadalafil before cisplatin treatment, thus, suggesting protection from cisplatin-induced tubulonephritis.

Overall, the biochemical and histopathological results of this study strongly support the dose-related chemopreventive effect of the daily oral 2 $mgkg^{-1}$ and 5 $mgkg^{-1}$ of Tadalafil in cisplatin-induced nephrotoxic rats and this was mediated via antioxidant and anti-lipoperoxidative mechanisms.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the technical assistance of Mr. Adenekan S.A., a Principal Technologist, in the Department of Biochemistry, College of Medicine of the University of Lagos, Idi-Araba, Lagos State, Nigeria, in the areas of tissue oxidative stress marker assays.

REFERENCES

Aebi, H. (1984) Catalase *In vitro* In: Colowick, S.P., Kaplane, N.O., editors. *Methods in Enzymology* 105: 121-126.

Ajiboye, K.I., Oluwole, F.S. (2012). Possible mechanisms for the anti-ulcer protective activity of Tadalafil, a type V phosphodiesterase inhibitor on indomethacin-induced gastric ulceration in rats. *European Journal of Scientific Research* 89(3): 350-358.

Bishara, B., Abu-Saleh, N., Awad, H., *et al.* (2012). Phosphodiesterase 5 inhibition protects against increased intra-abdominal pressure-induced renal dysfunction in experimental congestive heart failure. *European Journal of Heart Failure* 14(10): 1104-1111.

Broderick, G.A., Donatucci, C.F., Hatzichristou, D., *et al.* (2006). Efficacy of tadalafil in men with erectile dysfunction naïve to phosphodiesterase-5 inhibitor therapy compared with prior responders to sildenafil citrate. *Journal of Sexual Medicine* 3(4): 668-675.

Buege, J.A., Aust, S.D. (1978). Microsomal lipid peroxidation. *Methods in Enzymology* 52: 302-310.

Capizzi, R.L. (1999). Amifostine reduces the incidence of cumulative nephrotoxicity from cisplatin: laboratory and clinical aspects. *Seminars in Oncology* 26(2 Suppl 7): 72-81.

Chen-zhe, J., Lihyun, H., Seyoung, C. (2012). Decursin mediated protection on cisplatin-induced nephrotoxicity in SD rats and BDF1 mice. *Journal of Northeast Agricultural University* 19(1): 50-56.

Chirino, Y.I., Hernández-Pando, R., Pedraza-Chaverrí, J. (2004). Peroxynitrite decomposition catalyst ameliorates renal damage and protein nitration in cisplatin-induced nephrotoxicity in rats. *BMC Pharmacology* 4: 20.

Cornelison, T.L., Reed, E. (1993). Nephrotoxicity and hydration management for cisplatin, carboplatin, and ormaplatin. *Gynecologic Oncology* 50(2): 147-158.

Deng, J., Kohda, Y., Chiao, H., *et al.* (2001). Interleukin-10 inhibits ischemic and cisplatin-induced acute renal injury. *Kidney International* 60(6): 2118-2128.

Eardley, I., Gentile, V., Austoni, E., *et al.* (2004). Efficacy and safety of tadalafil in a Western European population of men with erectile dysfunction. *British Journal of Urology International* 94(6): 871-877.

Egerdie, R.B., Auerbach, S., Roehrborn, C.G., *et al.* (2012). Tadalafil 2.5 or 5 mg administered once daily for 12 weeks in men with both erectile dysfunction and signs and symptoms of benign prostatic hyperplasia: results of a randomized, placebo-controlled, double-blind study. *J Sex Med* 9(1): 271-281.

Ekborn, A., Lindberg, A., Laurell, G., *et al.* (2003). Ototoxicity, nephrotoxicity and pharmacokinetics of cisplatin and its monohydrated complex in the guinea pig. *Cancer Chemotherapy and Pharmacology* 51(1): 36-42.

Ellman, G.L. (1959). Tissue sulfhydryl groups. *Archives of Biochemistry and Biophysics* 17: 214-226.

El-Sayed E-S. M., Abd-Ellah M.F., Attia, S.M. (2008). Protective effect of captopril against cisplatin-induced nephrotoxicity in rats. *Pakistani Journal of Pharmaceutical Sciences* 21(3): 255-261.

El-Sherbeeny, N.A., Attia, G.M. (2016). The protective effect of trimetazidine against cisplatin-induced nephrotoxicity in rats. *Canadian Journal of Physiology and Pharmacology* 94(7): 745-751

Gaetani, G.F., Galiano, S., Canepa, L., *et al.* (1989). Catalase and glutathione peroxidase are equally effective in detoxification of hydrogen peroxide in human erythrocytes. *Blood* 73: 334-339.

Go, R.S., Adjei, A.A. (1999). Review of the comparative pharmacology and clinical activity of cisplatin and carboplatin. *Journal of Clinical Oncology* 17: 409-422.

Gowda, S., Desai, P.B., Kulkarni, S.S., *et al.* (2010). Markers of renal function tests. *North American Journal of Medical Sciences* 2: 170-173.

Greggi-Antunes, L.M., Darin, J.D.C., Bianchi, M.D.P. (2000). Protective effects of vitamin C against cisplatin-induced nephrotoxicity and lipid peroxidation in adult rats: A dose-dependent study. *Pharmacological Research* 41(4): 405-411.

Hakim, R.M., Lazarus, J.M. (1988). Biochemical parameters in chronic renal failure. *American Journal of Kidney Disease* 11(3): 238-247.

Hekmat, S., Ardekani, M.S., Ebrahimi, S.E.S., *et al.* (2013). Tadalafil and gastrointestinal relaxivity effects. *Pharmacologia* 4(1): 60-64.

Hoitsma, A.J., Wetzels, J.F., Koene, R.A. (1991). Drug-induced nephrotoxicity: Aetiology, clinical features and management. *Drug Safety* 6(2): 131-147.

Humbert, M., Sitbon, O., Simonneau, G. (2004). Treatment of pulmonary arterial hypertension. *New England Journal of Medicine* 351: 1425-1436.

Kadikoylu, G., Bolaman, Z., Demir, S., *et al.* (2004). The effects of desferrioxamine on cisplatin-induced lipid peroxidation and the activities of antioxidant enzymes in rat kidneys. *Human and Experimental Toxicology* 23: 29-34.

Karimi, G., Ramezani, M., Tahoonian, Z. (2005). Cisplatin nephrotoxicity and protection by milk thistle extract in rats. *Evidence Based Complementary and Alternative Medicine* 2(3): 383-386.

Kawai, Y., Nakao, T., Kunimura, N., *et al.* (2006). Relationship of intracellular calcium and oxygen radicals to cisplatin-related renal cell injury. *Journal of Pharmacological Sciences* 100(1): 65-72.

Kilic, U., Kilic, E., Tuzcu, Z., *et al.* (2013). Melatonin suppresses cisplatin-induced nephrotoxicity via activation of Nrf-2/HO-1 pathway. *Nutrition & Metabolism* 10: 7.

Kintzel, P.E. (2001). Anticancer drug-induced kidney disorders. *Drug Safety* 24(1): 19-38.

Kodama, A., Watanabe, H., Tanaka, R., *et al.* (2014) Albumin fusion renders thioredoxin an effective anti-oxidative and anti-inflammatory agent for preventing cisplatin-induced nephrotoxicity. *Biochimica et Biophysica Acta* 1840: 1152-1162

Kohda, Y., Kawai, Y., Iwamoto, M., *et al.* (2005). Serum thymic factor, FTS, attenuates cisplatin nephrotoxicity by suppressing cisplatin-induced ERK activation. *Biochemical Pharmacology* 70(9): 1408-1416.

Koka, S., Das, A., Zhu, S.G., *et al.* (2010). Long acting phosphodiesterase-5 inhibitor tadalafil attenuates doxorubicin-induced cardiomyopathy without interfering with chemotherapeutic effect. *Journal of Pharmacology and Experimental Therapeutics* 334(3): 1023-1030.

La Vignera, S., Condorelli, R.A., Vicari, E., *et al.* (2012). Endothelial antioxidant compound prolonged the endothelial anti-apoptotic effects registered after tadalafil treatment in patients with arterial erectile dysfunction. *Journal of Andrology* 33(2): 170-175.

La Vignera, S., Condorelli, R.A., Vicari, E., *et al.* (2011). Endothelial apoptosis decrease following tadalafil administration in patients with arterial ED does not last after its discontinuation. *International Journal of Impotence Research* 23(5): 200-205.

Laties, A.M. (2009). Vision disorders and phosphodiesterase type 5 inhibitors: A review of the evidence to date. *Drug Safety* 32: 1-18.

Lawrence, R.A., Burk, R.F. (1976). Glutathione peroxidase activity in selenium-deficient rat liver. *Biochemical and Biophysical Research Communication* 71: 952-958.

Li, G., Sha, S.H., Zotova, E., *et al.* (2002). Salicylate protects hearing and kidney function from cisplatin toxicity without compromising its oncolytic action. *Laboratory Investigation* 82(5): 585-596.

Li, J., Xu, Z., Jiang, L., *et al.* (2014) Rictor/mTORC2 protects against cisplatin-induced tubular cell death and acute kidney injury. *Kidney International* 86: 86-102.

Li, J., Gui, Y., Ren, J., *et al.* (2016). Metformin protects against cisplatin-induced tubular cell apoptosis and acute kidney injury via AMPKα-regulated autophagy induction. *Scientific Reports* 6: Article number 23975

Lieberthal, W., Triaca, V., Levine, J. (1996). Mechanisms of death induced by cisplatin in proximal tubular epithelial cells: apoptosis vs. necrosis. *American Journal of Physiology* 270: F700-708.

Lynch, E.D., Gu, R., Pierce, C., *et al.* (2005). Reduction of acute cisplatin ototoxicity and nephrotoxicity in rats by oral administration of

allopurinol and ebselen. *Hearing Research* 201(1-2): 81-89.

Miller RP, Tadagavadi RK, Ramesh G, *et al.* (2010). Mechanisms of cisplatin nephrotoxicity. *Toxins* 2: 2490-2518.

Modesti, P.A., Burberi, F., Moroni, F. (2003). Drug-induced nephropathy. *Annali Italiani di Medicina Interna* 18(3): 126-135.

Mora, L.O., Greggi-Antunes, L.M., Francescato, H.D., *et al.* (2003). The effects of oral glutamine on cisplatin-induced nephrotoxicity in rats. *Pharmacological Research* 47(6): 517-522.

Morgan, K.P., Buie, L.W., Savage, S.W. (2012). The role of mannitol as nephroprotectant in patients receiving cisplatin therapy. *The Annals of Pharmacotherapy* 46(2): 276-281.

Nagothu, K.K., Bhatt, R., Kaushal, G.P., *et al.* (2005). Fibrate prevents cisplatin-induced proximal tubule cell death. *Kidney International* 68(6): 2680-2693.

National Institutes for Health (1985). Guide for the Care and Use of Laboratory Animals. NIH Publication no. 85-23, United States National Institutes for Health, Bethesda, U.S.A.

Naughton, C.A. (2008). Drug-induced nephrotoxicity. *American Family Physician* 78(6): 743-750.

Nisar, S., Feinfeld, D.A. (2002). N-acetylcysteine as salvage therapy in cisplatin nephrotoxicity. *Renal Failure* 24: 529-533.

Oudot, A., Behr-Roussel, D., Le Coz, O, *et al.* (2009). How does chronic sildenafil prevent vascular oxidative stress in insulin-resistant rats? *Journal of Sexual Medicine* 7(1 Pt1): 79-88.

Ozkok, A., Edelstein, C.L. (2014). Pathophysiology of cisplatin-induced acute kidney injury. *BioMed Research International* Article ID 967826, 17 pages.

Padma-Nathan, J. (2003). Efficacy and tolerability of tadalafil, a novel phosphodiesterase-5 inhibitor, in treatment of erectile dysfunction. *American Journal of Cardiology* 92(9A): 19M-25M.

Paller, M.S. (1990). Drug-induced nephropathies. *Medical Clinics of North America* 74(4): 909-917.

Perk, H., Armagan, A., Naziroğlu, M., *et al.* (2008) Sildenafil citrate as phosphodiesterase inhibitor has an antioxidant effect in the blood of men. *Journal of Clinical Pharmacology and Therapeutics* 33(6): 635-640.

Pisansky, T.M., Pugh, S.L., Greenberg, R.E., *et al.* (2014). Tadalafil for prevention of erectile dysfunction after radiotherapy for prostate cancer: The Radiation Therapy Oncology Group [0831] Randomized Clinical Trial. *JAMA* 311(13): 1300-1307.

Porst, H., Giuliano, F., Glina, S., *et al.* (2006). Evaluation of the efficacy and safety of once-a-day dosing of tadalafil 5 mg and 10 mg in the treatment of erectile dysfunction: results of a multicenter, randomized, double-blind, placebo-controlled trial. *European Urology* 50(2):351-359.

Prabhu, V.V., Kannan, N., Guruvayoorappan, C. (2013). 1,2-diazole prevents cisplatin-induced nephrotoxicity in experimental rats. *Pharmacological Report* 65(4): 980-990.

Perazella, M.A. (2003). Drug-induced renal failure: update on new medications and unique mechanisms of nephropathy. *American Journal of Medical Sciences* 325(6): 349-362.

Sadzuka, Y., Shoji, T., Takino, Y. (1992a) Effect of cisplatin on the activities of enzymes which protect against lipid peroxidation. *Biochemical Pharmacology* 43: 1872-1875.

Sadzuka, Y., Shoji, T., Takino, Y. (1992b). Mechanism of the increase in lipid peroxide induced by cisplatin in the kidneys of rats. *Toxicology Letters* 62: 293-300.

Sadzuka, Y., Shimizu, Y., Takino, Y. (1994) Role of glutathione S-transferase isoenzymes in cisplatin-induced nephrotoxicity in the rat. *Toxicology Letters* 70: 211-222.

Saka, W., Akhigbe, R., Popoola, O., *et al.* (2012). Changes in serum electrolyte, urea and creatinine in *Aloe vera*-treated rats. *Journal of Young Pharmacists* 4(2): 78-81.

Santoso, J.T., Lucci, J.A. III, Coleman, R.L., *et al.* (2003)Saline, mannitol, and furosemide hydration in acute cisplatin nephrotoxicity: a randomized trial. *Cancer Chemotherapy and Pharmacology* 52(1): 13-18.

Schetz, M., Dasta, J., Goldstein, S., *et al.* (2005). Drug-induced acute kidney injury. *Current Opinions in Critical Care* 11(6): 555-565.

Sedlak, J., Lindsay, R.H. (1968). Estimation of total, protein-bound, and non-protein sulfhydryl groups in tissue with Ellman's reagent. *Analytical Biochemistry* 25(1): 192-205.

Serarslan, Y., Yönden, Z., Ozgiray, E., *et al*. (2010) Protective effects of tadalafil on experimental spinal cord injury in rats. *Journal of Clinical Neurosciences* 17: 349-352.

Shimeda, Y., Hirotani, Y., Akimoto, Y., *et al.* (2005). Protective effects of capsaicin against cisplatin-induced nephrotoxicity in rats. *Biological & Pharmaceutical Bulletin* 28(9): 1635-1638.

Shino, Y., Itoh, Y., Kubota, T., *et al.* (2003). Role of poly(ADP-ribose-)polymerase in cisplatin-induced injury in LLC-PK1 cells. *Free Radicals Biology & Medicine* 35(8): 966-977.

Somani, S.M., Husain, K., Withworth, C., *et al.* (2008). Dose-dependent protection of lipoic acid against cisplatin-induced nephrotoxicity in rats: Antioxidant defense system. *Pharmacology & Toxicology* 86(5): 234-241.

Sun, M., Zigma, S. (1978). An improved spectrophotometric assay of superoxide dismutase based on ephinephrine antioxidation. *Analytical Biochemistry* 90: 81-89.

Tanaka, H., Ishikawa, E., Teshima, S., *et al.* (1986). Histopathological study of human cisplatin nephropathy. *Toxicological Pathology* 14(2): 247-257.

Tew, K., Colvin, M., Chabner, B.A. (2001). Alkylating agents. In: Chabner, B.A., Longo, D.L., editors. Cancer Chemotherapy and Biotherapy: Principles and Practice. 3rd ed., Lippincott Williams and Witkins, Philadelphia, pp: 373-414.

Tietz, N.W., Pruden, E.L., Siggaard-Anderson, O. (1994). In: Burtis, C.A., Ashwood, E.R., editors. Tietz Textbook of Clinical Chemistry. W.B. Saunders Company, London, pp. 1354-1374.

Usho, I.F., Akpan, E.J., Etim, E.O., *et al.* (2005). Antioxidant actions of dried flower extracts of *Hibiscus sabdariffa* L. on sodium arsenite-induced oxidative stress in rats. *Pakistani Journal of Nutrition* 4(3): 135-141.

Varley, H., Alan, H.G. (1984). Tests in renal disease. In: Practical Clinical Biochemistry. William Heinemann Medical Book Ltd., London, vol. 1123.

Vermeulen, N.P., Baldew, G.S., Los, G., *et al.* (1993) Reduction of cisplatin nephrotoxicity by sodium selenite. Lack of interaction at the pharmacokinetic level of both compounds. *Drug Metabolism and Disposition* 21(1): 30-36.

Viale, M., Vannozzi, M.O., Pastrone, I., *et al.* (2000). Reduction of cisplatin nephrotoxicity by procainamide: does the formation of a cisplatin-procainamide complex play a role? *Journal of Pharmacology and Experimental Therapeutics* 293(3): 829-836.

Vickers, A.E., Rose, K., Fisher, R., *et al.* (2004). Kidney slices of human and rat to characterize cisplatin-induced injury on cellular pathways and morphology. *Toxicological Pathology* 32(5): 577-590.

Yao, X., Panichpisal, K., Kurtzman, N., *et al.* (2007). Cisplatin nephrotoxicity: A review. *American Journal of Medical Sciences* 334(2): 115-124.

Yildirim, Z., Sogut, S., Odaci, E., *et al***.** (2003). Oral erdosteine administration attenuates cisplatin-induced renal tubular damage in rats. *Pharmacological Research* 47(2): 149-156.

Yilmaz, H.R., Iraz, M., Sogut, S., *et al.* (2004). The effects of erdosteine on the activities of some metabolic enzymes during cisplatin-induced nephrotoxicity in rats. *Pharmacological Research* 50(3): 287-290.

Zager, R.A. (1997). Pathogenetic mechanisms in nephrotoxic acute renal failure. *Seminars in Nephrology* 17(1): 3-14.

Niger. J. Physiol. Sci. 31(June 2016) 011-022
www.njps.com.ng

Niger. J. Physiol. Sci.

The Deterioration Seen in Myelin Related Morphophysiology in Vanadium Exposed Rats is Partially Protected by Concurrent Iron Deficiency

Usende, Ifukibot Levi[1,2]; Leitner,Dominque F[3]; Neely Elizabeth[3]; Connor, James R[3] and Olopade, James O[1]*

[1]Department of Veterinary Anatomy, University of Ibadan, Nigeria, [2]Department of Veterinary Anatomy, University of Abuja, Nigeria, [3]Department of Neurosurgery, Experimental Neurosurgery Division, Penn State College of Medicine, Hershey, USA.

Summary: Oligodendrocyte development and myelination occurs vigorously during the early post natal period which coincides with the period of peak mobilization of iron. Oligodendrocyte progenitor cells (OPCs) are easily disturbed by any agent that affects iron homeostasis and its assimilation into these cells. Environmental exposure to vanadium, a transition metal can disrupt this iron homeostasis. We investigated the interaction of iron deficiency and vanadium exposure on the myelination infrastructure and its related neurobehavioural phenotypes, and neurocellular profiles in developing rat brains. Control group (C) dams were fed normal diet while Group 2 (V) dams were fed normal diet and pups were injected with 3mg/kg body weight of sodium metavanadate daily from postnatal day (PND) 1-21. Group 3 (I+V) dams were fed iron deficient diet after delivery and pups injected with 3mg/kg body weight sodium metavanadate from PND1-21. Body and brain weights deteriorated in I+V relative to C and V while neurobehavioral deficit occurred more in V. Whereas immunohistochemical staining shows more astrogliosis and microgliosis indicative of neuroinflammation in I+V, more intense OPCs depletion and hypomyelination were seen in the V, and this was partially protected in I+V. In *in vitro* studies, vanadium induced glial cells toxicity was partially protected only at the LD 50 dose with the iron chelator, desferroxamine. The data indicate that vanadium promotes myelin damage and iron deficiency in combination with vanadium partially protects this neurotoxicological effects of vanadium.

Keywords*:* Vanadium, iron deficiency, hypomyelination, behavioural deficits, neurotoxicity.

**Address for correspondence:* jkayodeolopade@yahoo.com Tel: +2348023860829

Manuscript Accepted: May, 2016

INTRODUCTION

The increase in environmental pollution due to the recent increased exploitation of minerals (Olopade *et al.*, 2005) has suggested a strong link between environmental pollution and the incidence of neuropathologies of the brain (Calderon-Garciduenas *et al*, 2002; Igado *et al.*, 2008). Vanadium (V) is a metallic transition element widely distributed in the environment (Hope, 1994) with atomic number 23. It exists in oxidation states ranging from -1 to +5, and frequently forms polymers (Clark, 1973). Combustion of fossil fuels provides a significant environmental source of vanadium and particulate emission has been estimated to comprise about 53% of the total atmospheric vanadium (Hope, 1994). Environmental contamination also occurs by spilling and burning of vanadium containing crude oil into relatively confined ecosystems (Bycakowski and Kulkarni, 1996). Aside from the acute environmental and occupational exposure to vanadium which is not uncommon (Shrivastava, 2007) the general population is increasingly exposed to this metal mostly as a result of the increased utilization of vanadium containing petroleum fuel (Bycakowski and Kulkarni, 1996). As a vanadate anion, vanadium penetrates the blood-brain barrier and leads to disruption of the ependymal cellular junctions which in turn loose their cilia (Domingo, 1996) as well as central nervous system disorders (Berman, 1980; Garcia *et al.*, 2005), and has been shown to have neurotoxic effects (Garcia *et* al., 2005; Haider *et al.*, 1998).The inhibitory effect of vanadium on the uptake and release of noradrenaline has been demonstrated (Garcia *et al.*, 2005; Haider *et al.*, 1998) and vanadium inhalation shown to produce a time dependent loss of dendritic spines, necrotic-like cell death, notorious alterations of the hippocampus CA1 neuropile which correlate with spatial memory impairment (evaluated by Morris water maze) and significant dopaminergic neuronal loss in the substantia nigra (and corpus striatum) resulting in morphological alterations of the striatum medium sized spiny neurons (Domingo, 1996). Administration of graded doses of vanadium in the form of sodium metavanadate to rats caused lipid peroxidation leading to the alteration of lipid metabolism and

protein concentrations in different regions of the brain (Sasi *et al.,* 1994). In addition, Soazo and Garcia (2007) have also demonstrated significant reductions in both general activity and learning following oral vanadate administration. Recently, Mustapha *et al.,*(2014) showed a progressive reduction in body weight gain, reduction in locomotor activity, graded reactive astrogliosis and hypomyelination attributable to down regulation of cyclic Nucleotide 3'-Phosphohydrolase (CNPase) and Myelin basic Protein (MBP) in mice pups expose to vanadium through lactation. The data of Mustapha *et al.,* (2014) supports the earlier report of Olopade *et al.,* (2011) that showed vanadium induced behavioural deficits, significant reduction in body weight gain and absolute brain weight, and reactive astrogliosis.

It has been proposed that most of the toxic effects of vanadium on the brain are due to the generation of reactive oxygen species and consequent lipid peroxidation (Olopade and Connor, 2011). Todorich *et al.,* (2011) proposed that developmental exposure to vanadium will cause hypomyelination via destruction of oligodendrocyte progenitors in part by increasing iron release from ferritin. The authors thus suggested that the resulting oxidative stress and apoptosis of the oligodendrocyte progenitor cells (OPCs) by vanadium induces further release of iron from ferritin, and that the consequent iron exposure exacerbates its cytotoxic effects.

The brain normally contains a substantially greater concentration of iron than other metals (Yehuda and Youdim, 1988), usually in the form of ferritin (Octave *et al.,* 1983). Connor *et al.,* (2003) have shown iron to be important in the brain as impaired acquisition leads to neurological problems. The homeostasis of brain iron is thought to be necessary for normal brain function, especially in learning and memory (Youdim, 1990). Thus a high content of brain iron may be essential, particularly during development, but its presence means that injury to brain cells may release iron ions that can lead to oxidative stress via formation of oxygen free radicals.

While it is tempting to believe that lowered iron status may be protective against vanadium exposure, iron deficiency itself also carries a neurological burden of hypomyelination. In biological systems generally but specifically in neuronal cultures, vanadium is bound to transferrin, giving the possibility that vanadium could interfere with the uptake, storage and metabolism of iron (and vice versa) (EVM, 2002; Todorich *et al.,* 2011). Whereas iron deficiency is a nutritional problem, vanadium exposure is an environmental concern and the dual effect could be clinically devastating (Todorich *et al.,* 2011). A sizeable population exposed to crude oil burning in Nigeria Niger Delta experience iron deficiency either through nutritional deficits (Ekwochi *et al.,* 2013), hookworm infestation (Ezem *et al.,* 1977) and teen age pregnancy (Oriji *et al.,* 2011) making the study of the interaction of iron deficiency and vanadium exposure one of epidemiological importance. The aim of this work is to establish the interaction between iron deficiency and vanadium exposure on myelination infrastructure and its related neurobehavioural phenotypes.

MATERIALS AND METHODS

Reagents

Desferroxamine (Sigma) was dissolved in water and frozen in 50mM aliquots of stock solution and were used for individual experiments. Sodium metavanadate (Sigma) was obtained as powder, diluted with Dulbecco's Phosphate Buffered Saline (DPBS) to a stock solution of 2.5mM which was kept in aliquot at -20^{o}C, and was used as needed.

Animals

Experiments on animals were performed in accordance with Ethical Standards and Institutional Animal Care and Use Committee (IACUC, Protocol Number 43269).

Pregnant Sprague-Dawley rats (CRL SD) were purchased from Charles River Laboratories when they were two weeks pregnant. The rats were housed at Pennsylvania State University College of Medicine Animal Core facilities. Diets used for the research were supplied by Harlan Laboratories, Inc.

Three groups of rat pups with four dams per group were used. Control group (C) dams were fed with normal diet and pups injected with Dulbecco's Phosphate Buffered Saline (DPBS) daily from PND1-21. Group 2 (V) dams were fed with normal diet and pup injected intraperitoneally (IP) with 3mg/kg body weight of sodium metavanadate (Garcia *et* al., 2004; Soazo and Gracia, 2007; Todorich et al 2011) daily from PND1-21. Group 3 (I+V) dams were fed with iron deficient diet after delivery and pups injected with 3mg/kg body weight sodium metavanadate IP from PND1- 21. All litters were culled to eight to ensure standard access to nursing across all groups. The pups were housed with their dams with *ad libitum* access to feed and water. Daily body weight was measured using a benchtop scale.

Behavioural test

At PND 15, rotarod testing of motor function, negative geotaxis, a test of motor coordination and open field test of behaviour was performed across all groups.

Rotarod testing

Rotarod test was as described by Todorich *et al.,* (2011). All animals were taken through a training period on the rotarod, and subsequently tested in three independent trials at a speed of 5rpms. The outcome was measured as the time it took the rat

pups to fall off the rotarod. Two investigators blinded to the experimental condition with good concordance scored the rotarod performance. The times of three trials were averaged across each group and evaluated for statistical significance using two way analysis of variance (ANOVA).

Open field test

For the open field test, we used an open field arena of glossy white plywood cage of 64 cm x 64cm with 30cm high walls. A black marker was used to draw lines on the floor of the field. The lines divided the floor into sixteen 16 x 16 cm squares. A central square (16 cm x 16 cm) was drawn in the middle of the open field (Brown, *et al.*, 1999) with a red marker. A video camcorder, Logitech QuickCam Pro 9000 (Logitech International SA; Newark, California, USA) used for video capturing of behaviour was positioned 65 cm above the centre of the open arena floor. The entire arena in the zone of the camera's view was captured from this position. The video camcorder was connected to a Samsung laptop, (Samsung Electronics, South Korea). The animal's behaviour inside the open field arena was tracked using ANY-maze 4.70 (Stoelting Co.; Wood Dale, Illinois, USA) software. Before commencement of the experiment, the software was used for setting up a tracking protocol. Each rat pup was allowed to explore the open field for five minutes after which they were removed. The open field arena was cleaned with 70% ethanol solution and allowed to dry before the next rat pup was tested.

In our study, parameters measured are as described by Brown *et al.*, (1999) and include:

1. *Line Crossing:* the number of times rat pups crossed one of the grid lines with all four paws.
2. *Center Square Entries:* the number of times rat pups crossed one of the red lines with all four paws into the central square.
3. *Center Square Duration:* the length of time the rat pups spent in the central square.
4. *Rearing:* the number of times rat pups stood on their hind legs in the maze.
5. *Grooming:* the length of time the rat pup spent licking or scratching itself while stationary.
6. *Freezing:* the length of time the rat pup was completely stationary.
7. *Urination:* puddles or streaks of urine number.
8. *Defecation:* fecal boli number.

Negative geotaxis

Negative geotaxis, was used to assess motor coordination of the pups when challenged on a sloped surface (Olopade *et al*., 2011). We used a test apparatus consisting of sloped platform of an angle of 30^{o} from horizontal to the desktop for this test. Rat pups were placed on the paper-towel covered sloped platform facing a downward direction. The latency to turn and orient to face up (180^{o}) the slope was recorded. A delay in ability to reorient was indicative of delays in motor, balance, or vestibular function. All performance was scored by two investigators blinded to the experimental condition with good concordance. Data obtained were subjected to statistical analysis using two way ANOVA (Graph pad prism, version 4.0) considering litter from dams in a group as a single unit.

Immediately after the last behavioural test, four pups from each dam in each group were then sacrificed by lethal injection using a combination of xylazine (10mg/kg) and ketamine (100mg/kg) and the brain harvested over dry ice. The brain weight was taken using a benchtop scale. One half of the brain was immersion fixed in 4% paraformaldehyde (in 0.1M PBS) for 48 hrs and then changed to 0.1M PBS for 48hrs. Subsequently, brain slides were prepared from paraffin blocks by routine histological method. The other hemisphere was frozen for quantitative analysis of markers used. At PND 21, the remaining pups from each group went through the same routine behavioural test and sacrificed as in PND15.

Immunohistochemistry

The prepared brain slides were air dried and labelled with pencil, baked for 20-30mins at 60^{o}C to dewax. They were deparaffinzed in two changes of xylene and hydrated in decreasing percentage of ethanol. Antigen retrieval was done in 10mM citrate buffer (pH=6.0) for 25min, with subsequent peroxidase quenching in 3% H_2O_2/methanol for 20 min. Slides were washed in PBS and sections were circled with PAP pen and blocked in 2% PBS milk for 1hr in humidity chamber (200µl/slide). All sections were probed with anti-Myelin basic protein-MBP (abcam, ab22460), anti-GFAP (Dako, z0334), anti-iNOS (calbiochem, cat. No. 482728)), anti-CNPase (abcam, ab6319), anti-Iba-1(Wako, Japan), anti-NG2 (Millipore, MAB5384), anti- ferritin(abcam ab69090) and anti-transferrin receptor antibody (abcam ab8598) diluted in 1% PBS milk overnight in humidity chamber at 4^{o}C. Detection of bound antibody was done using appropriate HRP-conjugated secondary antibodies in VECTA-STAIN kit (Vector Labs) according to manufacturer's protocol. Reaction product was enhanced with DAB (1:25 dilution) for 5-10min, with subsequent dehydration in ethanol. The slides were mounted with permount, coverslipped and allowed to dry. Images were captured using a light microscope (Bio-microscope, YJ-2005 series) connected to a laptop computer (Hp, China) with TSView 1.0 and AmScope ToupView 3.2 softwares.

Primary cell culture

Primary cultures were prepared from newborn rat pups of CRL SD rats according to standard protocols of McCarthy and de Vellis (1980). To purify different types of glia, mixed glial cultures obtained were

subjected to a serial shaking purification protocol (McCarthy and de Vellis 1980). First, the microglia were removed by shaking for 1 hour at 265 rpms. Subsequently, oligodendrocytes progenitor cells were removed by shaking for 18hrs at 265rpm.The remaining astrocytes were allowed to recover for one day, and then collected by trypsinization, counted and plated for experiments in 96 well plates. The oligodendrocytes fraction was subsequently further purified by differential adhesion through incubation in petric dish for 30 mins to remove more adherent astrocytic and microglial contamination (Todorich *et al.*, 2011). These cells were then replated in 96 well plated in N2S or N2B2 media for immature and mature oligodendrocytes respectively and used for experiments.

Cytotoxicity assay

Primary cells (oligodendrocytes and astrocytes) were treated with 0µM, 10µM, 75µM, 100µM and 200µM concentrations of sodium metavanadate with or without the iron chelator, desferroxamine (100qM) for 48 and 96 hrs, unless indicated otherwise. Untreated cells served as control. Cells viability was determined using MTT assay (cell proliferation kit 1, cat. No. 11465007001) according to manufacturer's protocol and absorbance measured on plate reader at 595-650nm.

Western Blot

The frozen sections of the brain were homogenized, sonicated for 30 sec, and centrifuged at 4^{o}C, 8000g for 5mins. Supernatant was collected into a new tube and dilution of 90µl PBS to 10µl sample was done for BCA assay (PierceTM BCA protein Assay Kit No 23225) according to manufacturer's protocol, incubated at 30^{o}C for 30min and read on plate reader at 570nm.

Samples were mixed with sample buffer and boiled for 10min. Samples were transferred to ice and centrifuged for 5 secs then to a TGX 4-20% gel and run at 250V for 25 min using a tris-glycine gel running buffer. It was transferred to a nitrocellulose membrane for 20min at 150 V at 4^{o}C. 5% TBST milk was used to block for 1 hr at room temperature and then probed overnight with anti- GFAP (1:7500) and anti-iBA-1(1:1000) primary antibodies at 4^{o}C. Detection of bound antibody was done using appropriate secondary antibodies for 1 hr at room temperature, washed 3 times for 10min each and ECL for 1min before exposure in Fuji. Multiple replicates of result from a group were presented as a single unit.

Statistical Analysis

All data generated were evaluated for statistical significance using two way ANOVA with Bonferroni's post-test comparison to controls.

RESULTS

The effects of vanadium exposure on the glial cells especially the myelin producing cells in the CNS with iron or iron deficiency was investigated both in vivo and in vitro. Results showed constant daily body weight gain with no difference from PND1-12 across all groups. Differences in body weight gain occurred from PND13-21 in I+V group and PND14-21 in V group with both lower than control. I+V rat pups showed least body weight gain (Fig. Ia). The same pattern was seen in brain weight gain across groups (Fig. Ib) when the pups were sacrificed at PND15 and 21.

The neurobehavioural test detected some impairment of motor function for both of the vanadium treated groups (V and I+V) relative to control at the two time points (PND15 and 21).

Specifically, the rotarod test revealed a significant impairment at PND21 in I+V group (Fig. II a); V group was also significantly impaired relative to control.

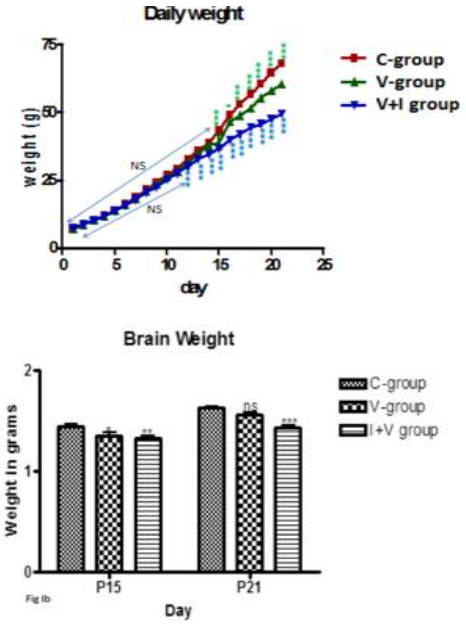


Figure I. Vanadium exposure during early development leads to reduced body and brain weight gain which was aggravated with iron deficiency. Newborn rat pups were injected with 3 mg/kg body weight of Na-metavanadate solution IP once per day for PND1-21 to V and I+V groups. Injection of equal volume of DPBS to littermate siblings served as control. Each day, bodyweights (a) of the pups were measured and at PND15 or 21, the pups were sacrificed and the brain harvested and measured (b) and plotted as means with standard deviation. The mean body and brain weights were evaluated for statistical significance using two way ANOVA with Bonferroni's post-test comparison to control at each time point (ns, not significant; *P<0.05;**P<0.01;***P<0.0001)

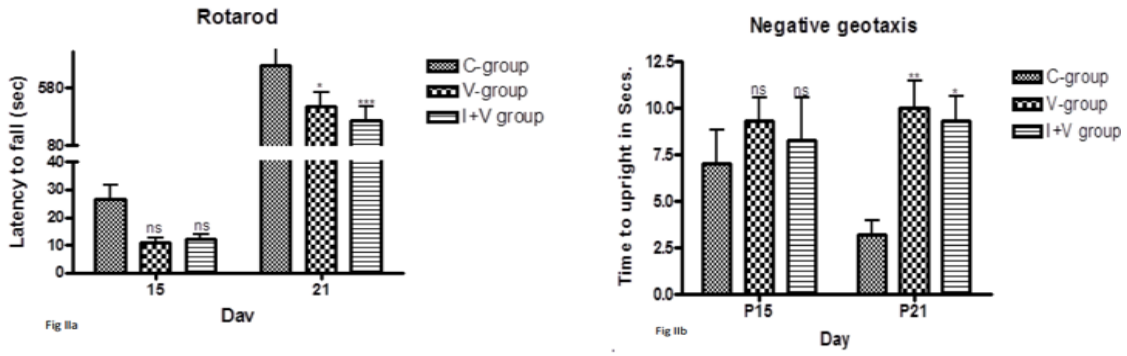


Figure II. Vanadium exposure during early post-natal period leads to impaired motor functioning. At PND15 and 21, groups V, I+V and control rats were subjected to rotarod testing for motor function and negative geotaxis; a test used to assess motor coordination of the pups when challenged on a sloped surface. The time from start of rotarod to the moment that the rat fell of the rotarod (a) and the latency to turn and orient themselves to face up (180^{o}) the slope (b) was recorded by two blinded investigators. The values were averaged and evaluated for significance using two way ANOVA with Bonferroni's post-test comparison to control (ns, not significant; *P<0.05;**P<0.01;***P<0.0001)

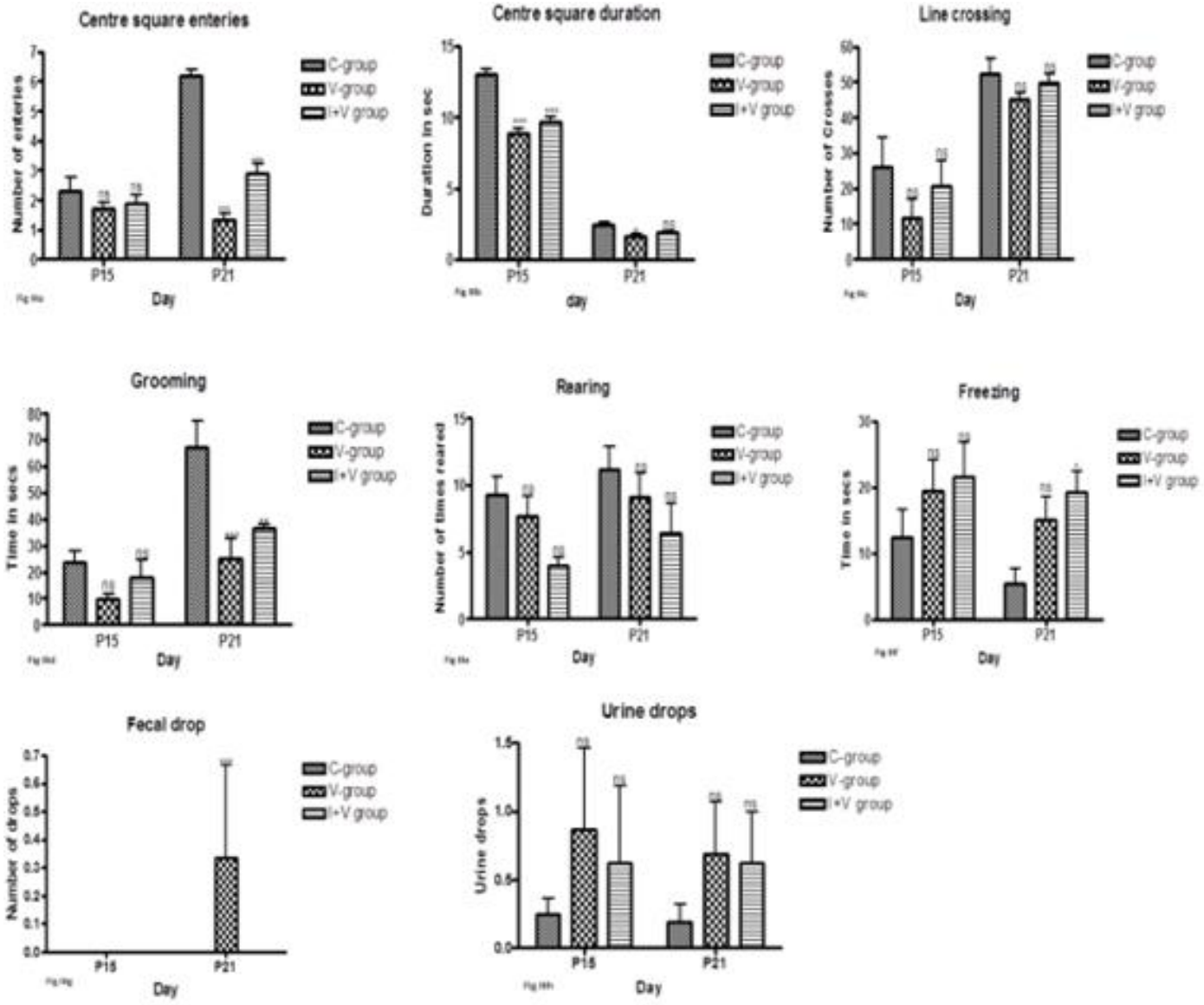


Figure IIIa At P15 AND 21, rat pups from all groups were exposed to open field test of behaviour. Centre square enteries measures the number of times rat pups crossed one of the red lines with all four paws into the central square. Vanadium treated rat pups (V and I+V) crossed less. Although not statistically significant at P15, centre square enteries test of behaviour was significant at P21. The least cross was seen in V group. (ns, not significant; *P<0.05;**P<0.01;***P<0.0001)

Fig IIIb At P15 AND 21, rat pups from all groups were exposed to open field test of behaviour. Centre square duration measures the length of time the rat pups spent in the central square. Vanadium treated rat pups (V and I+V) spent less time both at P15 and 21. The least time was seen in the V group. (ns, not significant; *P<0.05;**P<0.01;***P<0.0001)

Fig IIIc At P15 AND 21, rat pups from all groups were exposed to open field test of behaviour. Line crossing measure the number of times rat pups crossed one of the grid lines with all four paws. Although not statistically, Vanadium treated rat pups (V and I+V) crossed less both at P15 and 21. (ns, not significant; *P<0.05;**P<0.01;***P<0.0001)

Fig IIId At P15 AND 21, rat pups from all groups were exposed to open field test of behaviour. Grooming measures the length of time the rat pup spent licking or scratching itself while stationary. Although not statistically at P15 but significant at P21, Vanadium treated rat pups (V and I+V) spent less time grooming. (ns, not significant; *P<0.05;**P<0.01;***P<0.0001)

Fig IIIe At P15 AND 21, rat pups from all groups were exposed to open field test of behaviour. Rearing measures the number of times rat pups stood on their hind legs in the maze. Although not statistically significant at both time points, Vanadium treated groups (V and I+V) spent less time rearing. (ns, not significant; *P<0.05;**P<0.01;***P<0.0001)

Fig IIIf At P15 AND 21, rat pups from all groups were exposed to open field test of behaviour. Freezing measures the length of time the rat pup was completely stationary. Although not statistically significant at P15, I+V group spent highest time freezing at P21 and this was statistically significant. (ns, not significant; *P<0.05;**P<0.01;***P<0.0001)

Fig IIIg At P15 AND 21, rat pups from all groups were exposed to open field test of behaviour. Defecation measures fecal boli number. No fecal bolus was seen in the field in both C and I+V groups at both time points exposed. Only one pup in V group dropped one bolus in the field at PND21

Fig IIIh At P15 AND 21, rat pups from all groups were exposed to open field test of behaviour. Urination measures puddles or streaks of urine number in the field. Although not statistically significant at both time points, rat pups in V group voided more urine than those in I+V group when compared to control (C) (ns, not significant)

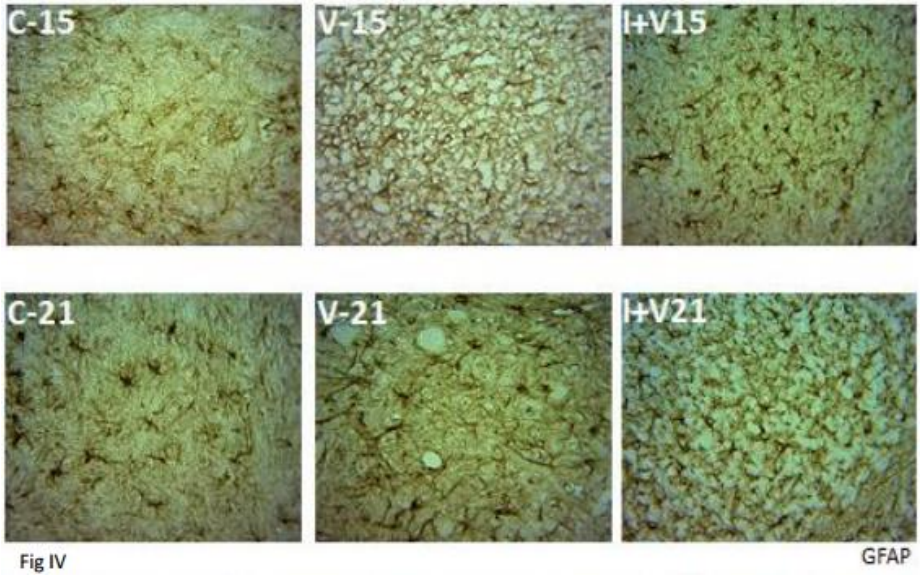


Figure IV. *In vivo* vanadium exposure during early development results in increased GFAP-positive astrocytes in the cerebellum of P15 &21- V (V-15 & V-21)& I+V (I+V15 & I+V21) rat pup when compared with C(C- 15&C-21). Astrogliosis was greater in I+V(I+V15 & I+V21). Cellular vacuolationswere observed both in V and I+V groups but worst in V group.

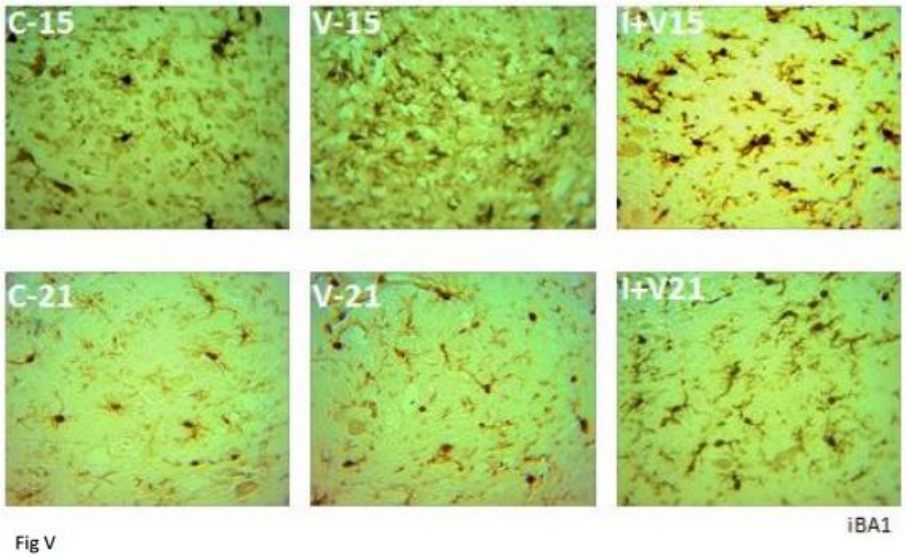


Figure V. *In vivo* vanadium exposure during early development results in increased Iba-1-positive cells in the cerebellum of P15 &21- V (V-15 & V-21)& I+V (I+V15 & I+V21) rat pups when compared with C (C-15 & C-21) group. Microglia activation was more in I+V(I+V15 & I+V21) group

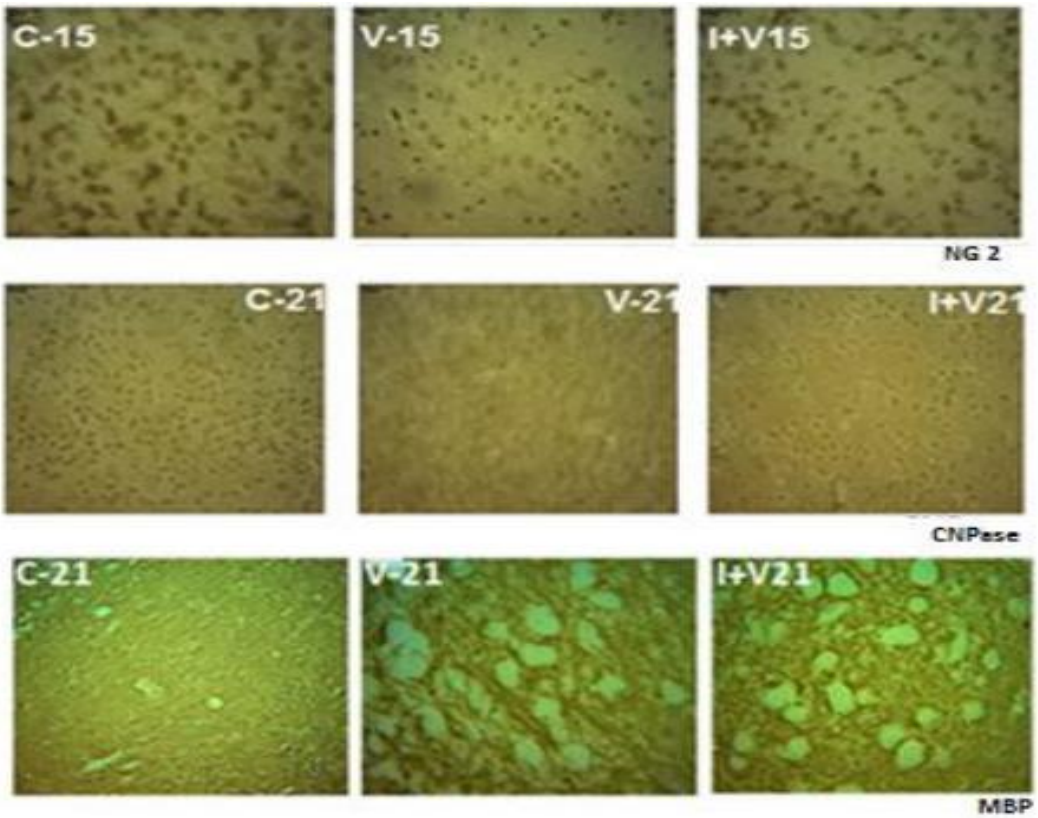


Figure VI. *In vivo* vanadium exposure during early development results in decreased NG-2 positive oligodendrocyte progenitors, P15 (V-15 & I+V15) ,CNPase-positive oligodendrocytes and MBP expression, P21 (V-21 & I+V21) in the cerebellum of rats pups of V & I+V groups. There was a partial protection on pups of I+Vgroup when compared with C (C-15 & C-21). Cellular vacuolations were observed both in V and I+V groups but worst in V group.

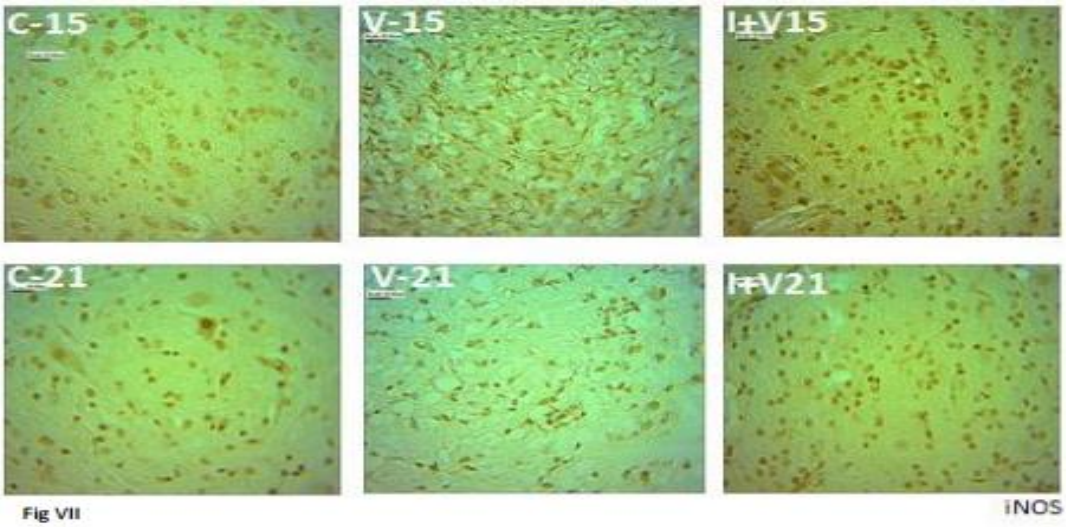


Figure VII. *In vivo* vanadium exposure during early development results in increased iNOS-positive staining in the cerebellum of P15 &21- V (V-15 &V-21) & I+V (I+V15 & I+V21) rat pups when compared with C (C15 & C- 21). Staining was more in I +V (I+V15 & I+V21)

For negative geotaxis, vanadium treated groups (V and I+V) took longer to reorient themselves to face up when challenged with a sloped platform both at PND15 and 21. The longest time was seen in V group (Fig. II b) and this was statistically significant.

When rat pups from all groups were exposed to five minutes exploration of the open field, behavioural deficits occurred in V and I+V rat pups on all parameters taken at the two time points. Deficits on centre square entries, centre square duration, line crossing, and grooming were observed more in V group (Fig. III a, b, c, and d) while rearing and freezing were more in I+V group (Fig. III e and f). No faecal bolus was seen in the field in both C and I+V at both time points exposed. Only one pup in V group dropped one bolus in the field at PND21 (Fig. III g). Although not statistically significant at both time points, rat pups in V group voided more

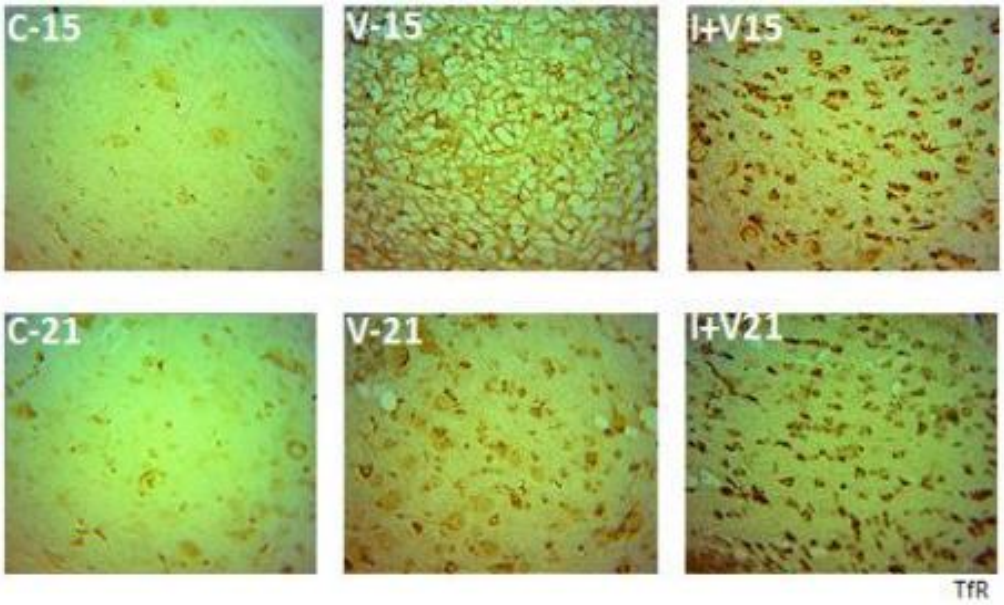


Figure VIII. *In vivo* exposure to iron deficiency to pups through lactation from dams on iron deficient diet and concurrent vanadium intraperitoneal injections during early development results in increased TfR positive staining at P15 and P21(I+V15 & I+V21) in the cerebellum of I+V rat pups when compared with C (C15 & C- 21).

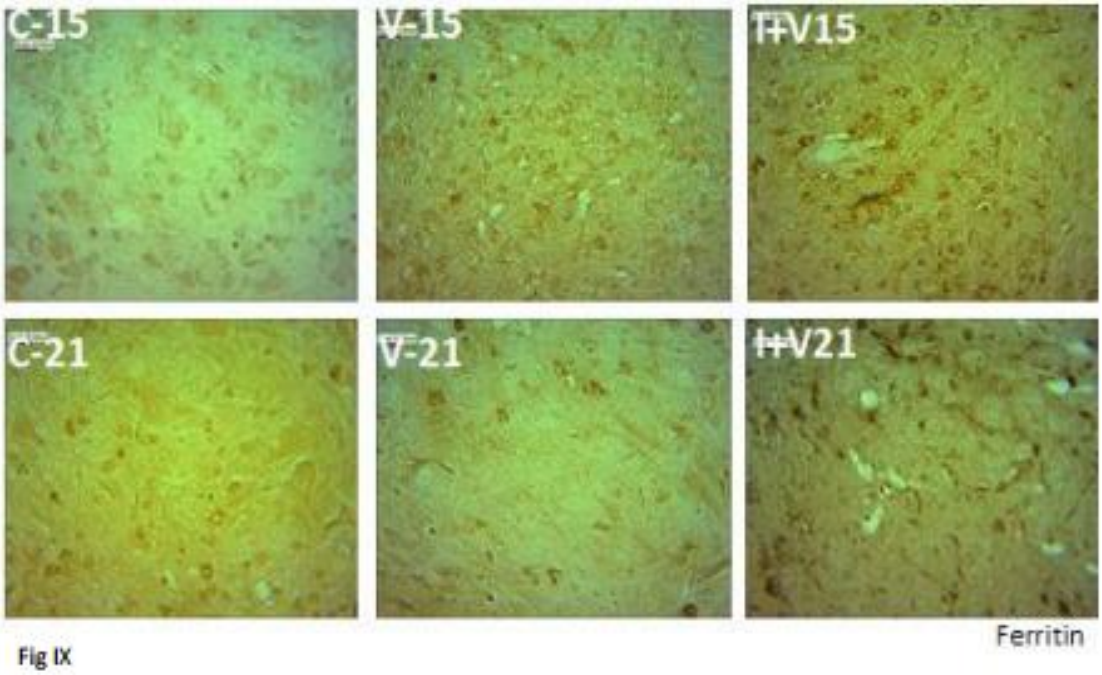


Figure IX. *In vivo* exposure to iron deficiency to pups through lactation from dams on iron deficient diet and concurrent vanadium intraperitoneal injections during early development results in increased ferritin positive staining at P15 and P21 (I+V15 & I+V21) in the cerebellum of I+V rat pups when compared with C (C15 & C- 21).

urine than those in I+V group when compared to control (C) (Fig. III h).

Upon immunohistochemical examination of the brains of the rat pups at PND15 and 21, we examined the genu of the corpus callosum and the arbor vitae of the cerebellum and observed that vanadium exposure was associated with increased number of astrocytes (Fig. IV) and microglia (Fig. V) in both organs. These were observed more in I+V group. Conversely, we observed a decrease in the same regions of the brain in the number of NG-2 positive oligodendrocyte progenitor cells, mature myelinating oligodendrocytes, decreased myelination and cellular and tissue vacuolation (Fig. VI) in V and I+V groups, which were however worst in V group. There was increased staining for iNOS (Fig. VII) positive cells in V and I+V groups but was more intense in I+V group. To determine the pathophysiologic status of iron in the brain, we stained for transferrin receptors nd ferritin (Fig. VIII and Fig. IX) and our result showed an increase in I+V group.

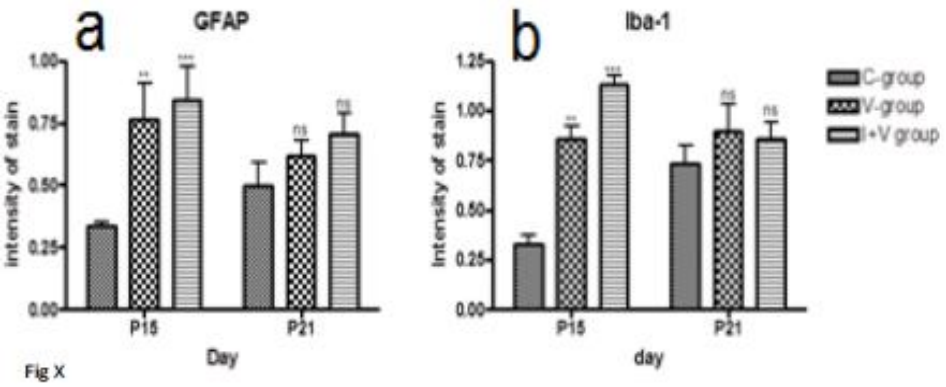


Fig X

F

igure X. *In vivo* vanadium exposure during early development results in increased GFAP and Iba1 -positive expression exacerbated with iron deficiency. At PND15 and 21, brains of V, I+V and control rat pups were harvested after lethal injects and astrogliosis (a) and microgliosis (b) quantify using western blot. Data of multiple experiments were pooled together and means evaluated for significance using two-way ANOVA with Bonferroni's post-test comparison to control (ns, not significant; *P<0.05;**P<0.01;***P<0.0001).

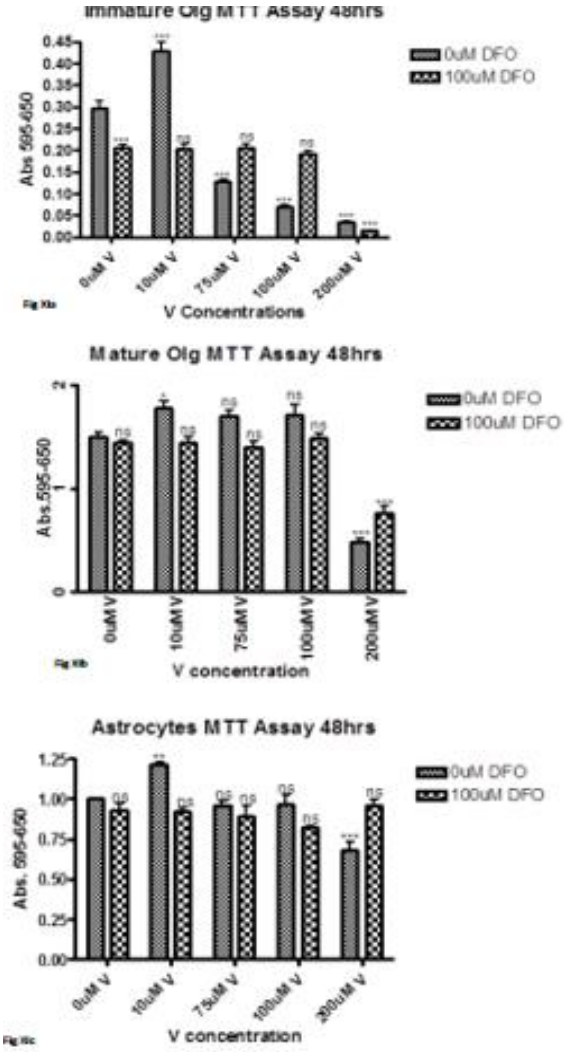


Figure XI. *In vitro,* primary oligodendrocyte progenitors (OPCs) (Fig XIa) are more vulnerable to cytotoxic effects of vanadium than mature oligodendrocytes (Fig XIIb) or astrocytes (Fig XIIc). Primary astrocytes, OPCs and mature oligodendrocytes were exposed to increasing concentrations of Na-metavanadate for 48 h. MTT reagent was added for last 4 h of treatment, cells solubilized and absorbances measured at 595-650 nm. Data of at least four experiments were pooled together and means evaluated for significance using two-way ANOVA with Bonferroni's post-test comparison to control (ns, not significant; *P<0.05;**P<0.01;***P<0.0001).

Since our *in vivo* data showed astrogliosis and microglia activation in the vanadium treated (V and I+V) groups, we quantified these with brain samples of all groups using the same antibodies used for immunostainning. Our results were consistent with the immunostain results with astrogliosis (Fig. X a) and microglia activation (Fig. X b).

Furthermore, because our *in vivo* data showed astrogliosis, and depletion of oligodendrocyte, we investigated if these changes were due to the differences in the relative sensitivity of the glial cells to the cytotoxic effects of vanadium and if iron deficiency can protect the cells from these cytotoxic effects *in vitro.* Enriched cultures of astrocytes, immature and mature oligodendrocytes were raised. We demonstrated that vanadium was cytotoxic to all the cells types. Immature oligodendrocytes were more sensitive to the cytotoxic effects of vanadium with LD50 of approximately 75µM than mature oligodendrocytes and astrocytes which have LD50 of around 200µM. Our results also showed that these cells were protected partially from vanadium toxicity by the iron chelator, desferroxamine only at the LD50 dose for the three cell types (Fig. XI a, b and c).

DISCUSSION

We showed in this study that vanadium exposure caused reduction in body and brain weight gain which was exacerbated with iron deficiency. Our findings are consistent with previous reports of decreased body and brain weights of vanadium exposed animals when compared to controls (Altamirano *et al.,* 1993; Olopade *et al.,* 2011 Sanchez *et al.,* 1991, and Todorich *et al.,* 2011).

Our findings contrast those of Garcia *et al.,* (2004; 2005) who reported that body and brain weight of vanadium exposed rats did not differ from control rats, and physical conditions were almost normal in young adult mice. Thus we can attribute the reason

for the differences in our study to be the age difference in the rats and duration of vanadium exposure used for the experiments. While three month old rats were used for their studies, we exposed our rats from PND1, and noticed weight differences from PND 13-21. Our data in combination with those of Garcia *et al*., (2004; 2005) strongly argue for a developmental effect. Moreover our data on the oligodendrocytes cultures are consistent with the developmental effect concept because the immature oligodendrocytes were more vulnerable than mature. Vanadium is a potent inhibitor of DNA and protein synthesis and affects several metabolic processes (Leonardo and Geber, 1994; Roldan and Altimirano, 1990). Vanadium has more effect on growth when exposed to tissue and animals that are undergoing a high degree of cellular proliferation (Todorich *et al.,* 2011). The reduced brain weight from vanadium exposure is thus most likely the product ofcellular and tissue (Avila-Costa *et al.,* 2004, 2006, Olopade *et al.,* 2011) loss and vacuolation (Igado *et al*.,2012) in the brain.

Our *in vivo* experiments showed that vanadium induced neurobehavioural deficits. Deficits on rotarod, rearing and freezing were worse in I+V group. However, deficits on negative geotaxis, centre square entries, centre square duration, line crossing, grooming, faecal bolus drops and urination was more in V group. In summary, iron deficient and vanadium exposed rat pups fared better in neurobehavioural responses compared to rat pups exposed to vanadium alone. We hypothesize that myelination plays a role in locomotor activities and because in iron deficiency, OPCs and mature oligodendrocytes were partially protected rescuing the myelin, neurobehavioural deficits were relatively less in the iron deficient and vanadium treated (I+V group).

We demonstrated *in vivo* vanadium induced OPCs and mature oligodendrocytes depletion (NG2 and CNPase stains) at PND 15 and 21, similar to the earlier report of Todorich *et al.,* (2011). Also, because iron deficiency confer a partial protection to the OPCs both *in vivo* and *in vitro,* our immunohistochemical staining with MBP at PND 21showed more myelination in I+V group compared to V group suggesting that the iron deficient status minimized the loss of the myelin producing oligodendrocytes. It is likely that though vanadium induced reactive oxygen species affected oligodendrocytes in I+V and V groups, intracellular iron overload which is an additional apoptotic pathway in vanadium induced OPC depletion (Todorich *et al.,* 2011) was relatively spared in I+V group. It had been shown that astrocytes transfected with ferritin constructs rich in intracellular iron were more vulnerable to vanadium induced cell death than those with constructs having minimal iron concentrations (Todorich *et al.,* 2011).

Microglial activation and astrogliosis are commonly observed during the neuroinflammation associated with brain injury, infection, and neurodegenerative diseases (Sunyach *et al.,* 2012). Our results with GFAP, Iba1, and iNOS showed oxidative stress and neuroinflamation induced by vanadium exposure was enhanced by iron deficiency. This enhancing effect of iron deficiency can be attributed to the increased expression of transferrin receptors in I+Vrat pups as a response to increased need for iron in the brains. Incidentally however, this increased transferrin receptors will lead to increased uptake of vanadium through the blood brain barrier (Nagaoka et al., 2004). While iron deficiency induced more astrogliosis and microglia activation after vanadium exposure, it confers a partial protection to OPCs. This consequently protects against hypomyelination in part due to reduced OPC and mature oligodendrocytes depletion resulting from a minimized vanadium induced iron overload in these cells.

In our *in vitro* studies, we manipulated the availability of iron to oligodendrocytes in the presence of vanadium due to the fact that iron is a key component of oligodendrocyte maturation and vanadium's binding to transferrin protein (EVM 2002; Monteiro *et al,* 1991). Interestingly, we show that the partial protection of not only the OPCs but the glia cells in general by the iron chelator, desferroxamine is only at the LD 50 of vanadium. These data are consistent with the earlier report of Todorich *et al.,* (2011). Interestingly, we show that at doses above or below the LD50, iron deficiency exacerbates the toxic effects of the glia cells to vanadium exposure. This will warrant further investigation. In addition however, we observed that vanadium at low doses led to proliferation of astrocytes, mature and immature oligodendrocytes. This was an unexpected effect but may be due to the ability of vanadium to modulate growth-factor-mediated signal transduction pathways and promote cell transformation. This explanation is consistent with vanadium's mitogenic action and its capacity to mimic mitogenic growth factors, and stimulate expression of proto-oncogenes (Stern et al., 1993).

In conclusion, we showed that vanadium induced OPCs depletion, hypomyelination and neurobehavioural deficits were partially protected with iron deficiency. However, vanadium induced reduced body and brain weight gain as well as astrogliosis and microgliosis was exacerbated with iron deficiency. Iron deficiency therefore has a partial protection effect on the myelin profile, and thus suggests that limited iron exposure may be helpful during acute vanadium toxicity.

Acknowledgement

The authors acknowledge the ISN (CAEN) travel grant to UIL.

REFERENCES

Altamirano M, Alvarez L, Roldan E. (1993). Cytogenetic and teratogenic effects of Vanadium Pentoxide on Mice. Med Sci Res 21: 1-3

Avilla-Costa MR, Montiel FE, Colin-Barenque L, Ordonez JL, Gutierrez AL, Nifio-Cabrera HG, Mussali-Galante P, Fortoul TI. (2004). Nigrostriatal Modifications after Vanadium Inhalation: An Immunocytochemical and Cytological Approach. Neurochemical Research 29: 1365-1369

Avilla-Costa MR, Fortoul TI, Nifio-Cabrera HG, Colin-Barenque L, Bizarro-Nevares P, Gutierrez AL, Ordonez JL, Rodriguez-Lara V, Mussali-Galante P, Diaz-Bech P, Anaya-Martinez V. (2006). Hippocampal Cell Alteration induced by inhalation of Vanadium pentoxide (V2O5) promote memory deterioration. Neurotoxical 27: 1007-1012

Berma E. (1980). Toxic Metal and their Analysis. Hayden, Philadelphia

Brown RE, Corey SC, Moore AK. (1999). Differences in measures of exploration and fear in MHC-congenic C57BL/6J and B6-H-2K mice. Behavior Genetics 29: 263-271. doi: 10.1023/A:1021694307672

Bycakowski JZ, Kulkarni AP. (1996). Pro-oxidant Biological effects of Inorganic components of petroleum: vanadium and oxidative stress. Technical Report AL/OE-TR-0126

Calderon-Garciduenas L, Azzarelli B, Acune H, Garcia R, Gambling TM, Osnaya N. (2002). Air pollution and Brain Damage. Toxicol Pathol 30: 373-389

Clark RJH. (1973). Vanadium. In: Trotman-Dickenson AF , ed. Comprehensive Inorganic Chemistry Vol. 3, Pergamon, New York, pp. 519-521

Connor JR, Boyer PJ, Menzies SK, Dellinger B, Allen RP, Ondo WG, Earley CJ. (2003). Brain iron acquisition in restless leg syndrome. Neurology 61: 304-309

Domingo JL. (1996). Vanadium: A Review of The Reproductive and Developmental Toxicity. Rep. Toxicol. 10(3): 175-182.

Ekwochi U,Odetunde O, Maduka I, Obi IE. (2013). Iron deficiency among non anaemic under- five children in Enugu, South-East Nigeria. Ann Med health Sci Res. 3:402-6. Doi 10.4103/2141-9248.117943

EVM.(2002). Review of vanadium. UK Expert Group on Vitamins and Minerals. EVM/00/04, 116

Ezem BU, Fleming AF, Werblinska B. (1977). Treatment of severe iron Deficiency Anaemia of Hookworm Infestation with Ferastral, a new intramuscular iron preparation. Scand J Haematol Suppl.32:326-45

Garcia GB, Biancardi ME, Quiroga AD. (2005). Vanadium (V)-induced Neurotoxicity in the Rat central Nervous System: A Histo-Immunohistochemical Study. Drug and Chemical Toxicology 27: 281-293

Garcia GB, Quiroga AD, Sturtz N, Martinez AI, Biancardi ME. (2004). Morphological alterations of Central Nervous System (CNS) Meylin in Vanadium (V)- exposed Adult Rats. Drug Chemical Toxicology 27: 281-293

Haider SS, Abdel-Gayoum AA, el-Fakhri M, Ghwarsha KM. (1998). Effects of Selenium on Vanadium Toxicity in Different Regions of Rat Brain. Human and Experimental Toxicology 17: 23-18

Hope BK. (1994). A global Biogeochemical Budget for Vanadium. Sci. Total Environment 141: 1-10.

Igado OO, Olopade JO, Onwuka SK, Chukwudi AC, Daramola OA, Ajufo UE. (2008). Evidence of Environmental Pollution in caprine Brains obtained from a Relatively Unindustrialized Area in Nigeria. African Journal of Biomedical Research 11: 305-309

Igado OO, Olopade JO, Adesida A, Aina OO, Farombi EO. (2012). Morphological and Biochemical Investigation into the possible Neuroprotective effects of Kolaviron (Garcinia kola bioflavonoid) on the brains of rats exposed to vanadium. Drug Chem Toxicol. 35 (4):371-80. doi: 10.3109/01480545.2011.630005.

Leonard A, Gerber GB. (1994). Mutagenicity, carcinogenicity and teratogenicity of Vanadium compounds. Mutat Res 317: 81-88

Mc Carthy KD, De Vellis J. (1980). Preparation of separate Astroglial and Oligodendroglial Cell cultures from Rat cerebral tissues. J Cell Biol 85:890-902

Monteriro HP, Winterbourne CC, Stern A. (1991). Tetravalent vanadium releases ferritin iron which stimulates Vanadium-dependent lipid peroxidation. Free Radic Res Commun. 1: 125-129

Mustapha O, Oke BO, Offenc N, Siren A, Olopade JO. (2014). Neurobehavioral and cytotoxic effects of vanadium during oligodendrocyte maturation: A protective role for erythropoietin. Environmental Toxicology and Pharmacology, Volume 38, Issue 1, Pages 98-111.

Nagaoka MH, Akiyama H, Maitani T. (2004). Binding patterns of vanadium to transferrin in healthy human serum studied with HPLC/high resolution ICP-MS Analyst. 129:51-4.

Octave JN, Schneider YJ, Crichton RR, Trouet A. (1983). Iron mobilization from cultured hepatocytes: effect of desferrioxamine B. Biochem Pharmacol 32: 3413-3418.

Olopade JO, Connor JR. (2011). Vanadium and Neurotoxicity: A review. Current topics in Toxicology 7: 33-39

Olopade, JO, Fatola, IO and Olopade,FE. (2011). Vertical Administration of Vanadium through Lactation Induces Behavioural and Neuromorphological Changes: Protective Role of Vitamin E. Nig. J. Physiol. Sci 26: 055-060

Olopade JO, Onwuka SK, Adejumo D, Ladokun AA. (2005). Analysis of some industrial areas of Nigeria: An over-view. Chemistry and Biodiversity 2: 1277-1289.

Oriji VK, Enyindah CE, Nyeche S. (2011). Factors Determining Compliance to iron Supplimentation in Pregnancy at the University of PortHarcourt Teaching Hospital. Niger J Med 20:131-4

Roldan E, Altamirano M. (1990). Chromosomal aberrations, sister chromatid exchanges, cell- cycle kinetics and satellite association in human lymphocytes cultures exposed to vanadium pentoxide. Mutat Res 245(2): 61-65

Sanchez DJ, Ortega A, Domingo JL, Corbella J. (1991). Prevention by sodium 4,5-dihydroxybenzene-1,3-disulfonate (tiron) of vanadium-induced behavioural toxicity in Rats. Biol. Trace Element Res. 69: 249-259

Sasi MM, Haider SS, Fakhri M-EL, Ghwarsha KM. (1994). Microchromatographic Analysis of Lipids, Proteins and Occurance of Lipid Peroxidation in Various brain Areas of Vanadium Exposed Rats: A Possible Mechanism of Vanadium Neurotoxicity. Neurotoxicity 15: 413-420

Shrivastava S, Jado A, Shukla S. (2007). Effect of Tiron and its combination with nutritional supplements against Vanadium intoxication in female albino rats. Journal of Toxicological Science 32: 185-192

Soazo M, Garcia GB. (2007). Vanadium Exposure Through Lactation Produces Behavioural Alterations and CNS Myelin Deficit in Neonatal Rats. Neurotoxicol. Teratol. 29: 503-10

Stern A, Yin X, Tsang S, Davison A, Moon J. (1993).Vanadium as a Modulator of Cellular Regulatory Cascades and Oncogene Expression. Biochemistry and Cell Biology 71(3-4): 103112, 10.1139/o93-018

Sunyach C, Michaud M, Arnoux T, Bernard-Manassal N, Latyszenok V, Gouarne C, Raoul C, Pruss RM, Bordet T, Pettmann B. (2012). Olesoxime delays muscle denervation, astrogliosis. Microglial activation and motoneuron death in an ALS mouse model. Neuropharmacology 62:2346-52 doi 10.1016/J Neuropharm 2012.02.013

Todorich B, Olopade JO, Surguladz N, Zhang X, Neely E, Connor JR. (2011). The Mechanism of Vanadium-mediated Developmental Hypomyelination is related to Destruction of Oligodendrocyte progenitors through a relationship with ferritin and Iron. Neurotoxicity Research 19: 361-373

Yehuda S, Youdim MBH. (1988). Brain iron deficiency: Biochemistry and Behavior. In: Youdim MBH. ed. Brain Iron. Taylor and Francis, London. pp 89-114.

Youdim MBH. (1990). Neuropharmacological and neurobiological aspects of iron deficiency. In: Dobbing J. ed. Brain, Behaviour and Iron in the Infant Diet. Springer-Verlag, London UK. pp. 83-97

Niger. J. Physiol. Sci. 31(June 2016) 023-029
www.njps.com.ng

Niger. J. Physiol. Sci.

Restraint Stress Impairs Glucose Homeostasis Through Altered Insulin Signalling in Sprague-Dawley Rat

*** Ayodele O. Morakinyo[1], Kolawole I. Ajiboye[2], Gabriel O. Oludare[1] and Titilola A. Samuel[3]**

[1]Department of Physiology, College of Medicine of the University of Lagos, Idi-Araba 100254, Lagos, Nigeria. [2]Department of Physiology, Ben Carson (Snr) School of Medicine, Babcock University, Ilisan-Remo, Ogun, Nigeria. [3]Department of Biochemistry, College of Medicine of the University of Lagos, Idi-Araba 100254, Lagos, Nigeria.

Summary: The study investigated the potential alteration in the level of insulin and adiponectin, as well as the expression of insulin receptors (INSR) and glucose transporter 4 GLUT-4 in chronic restraint stress rats. Sprague-Dawley rats were randomly divided into two groups: the control group and stress group in which the rats were exposed to one of the four different restraint stressors; 1 h, twice daily for a period of 7 days (S7D), 14 days (S14D) and 28 days (S28D). Glucose tolerance and insulin sensitivity were evaluated following the final stress exposure. ELISA were performed to assess the level of insulin and adiponectin as well as expression of INSR and GLUT4 protein in skeletal muscle. Plasma corticosterone level was also determined as a marker of stress exposure. Restraint stress for 7 days caused transient glucose intolerance, while S14D rats demonstrated increased glucose intolerance and insulin insensitivity. However, restraint stress for 28 days had no effect on glucose tolerance, but did cause an increase in glucose response to insulin challenge. The serum level of adiponectin was significantly ($p< 0.05$) lower compared with the control value while insulin remained unchanged except at in S28D rats that had a significant ($p<0.05$) increase. The expression of INSR and GLUT4 receptors were significantly ($p< 0.05$) decreased in the skeletal muscle of restraint stress exposed rats. There was a significant ($p< 0.05$) increase in the plasma corticosterone level of the stress rats compared with their control counterparts. Restraint stress caused glucose intolerance and insulin insensitivity in male Sprague-Dawley rats, which becomes accommodated with prolonged exposure and was likely related to the blunted insulin signalling in skeletal muscle.

Keywords*:* Stress, Glucose tolerance, Insulin sensitivity, Glucose transporter-4, Corticosterone.

**Address for correspondence:* morakinyofemi@yahoo.com, aomorakinyo@cmul.edu.ng Tel: +2348055947623

Manuscript Accepted: _____

INTRODUCTION

Stress may be defined as the state in which the brain interprets the quantity of stimulation as excessive or its quality as threatening (Chrousos, 1998). It is any condition that impairs the balance of the organism physiologically or psychologically. Exposure to stressors results in a series of important adaptive responses that enable an organism to cope with a changing environment (Sabban and Kvetnansky, 2001; Carrasco and Van de Kar, 2003). Prominent among the adaptive responses to stress are secretion of catecholamines from the adrenal medulla, corticosteroids from the adrenal cortex, and adrenocorticotropin from the anterior pituitary (Kevetnansky *et al*., 1993; Strommer *et al*., 1998). In fact, the sympato-adrenal and hypothalamic-pituitary-adrenocortical systems have complex interactions to maintain the internal environment during exposure of organism to a wide variety of stressors (Strommer *et al*., 1998; Lay *et al*., 2014).

Stress primarily target the metabolic system and contributes significantly to the development of metabolic diseases (Soop *et al*., 2001; Lay *et al*., 2014). Indeed, a considerable amount of evidence from clinical and animal experiments has shown that stress reliably alter glucose metabolism resulting in hyperglycemia and has a role in the induction of insulin resistance in different tissues (Bonner-Weir *et al*., 1981; Chalkley *et al*., 2002). Elevated glucocorticoid and catecholamine levels antagonize the effects of insulin and also increase blood glucose concentration independent of their effects on insulin (Björntorp, 1997). Thus chronic over-secretion of these stress mediators may therefore contribute to the development of insulin resistance, overweight, and obesity (Vanltallie, 2002; Ozcan *et al*., 2004; Rozanski *et al*., 2005).

Although it has been shown that the expression of insulin receptors (INSR) and glucose transporter-4 (GLUT4) are disrupted in insulin resistant rodents (Pessin and Saltiel, 2000), these changes in rats exposed to restraint stress are scarcely available in literature. This study therefore investigated the effect of restraint stress on glucose homeostasis as well

insulin signalling factors such as INSR and GLUT4 in male Sprague-Dawley rat. Previous research done in our laboratory indicated a deterioration in insulin sensitivity in forced-swimming (physical stress) and water-avoidance (psychological stress) models of physical in rats (Morakinyo *et al*., 2016).

MATERIALS AND METHODS

Animals

Adult male rats (n=16; 12 weeks old) were obtained from Animal House of the College of Medicine, University of Lagos and housed 8 per cage under controlled conditions for the light/dark cycle, temperature, and humidity. The animals were kept in the same animal facility for at least 1 week before the experiments. Rats were fed a standard chow diet and water *ad libitum*. All experiments and procedures were performed in accordance to the Guide for the Care and Use of Laboratory Animals published by the National Research Council, and was approved by the Ethics Committee of the College of Medicine of the University of Lagos.

Stress protocols

To acclimatize the rats to manipulation by humans, all rats (stressed and controls) were handled daily for 1 wk. Rats were divided into two groups, stressed and control (n=6/group). Rats of the stressed group were exposed to one of the four different restraint stressors; 1 h, twice daily for a period of 7 days (S7D), 14 days (S14D) and 28 days (S28D). The first exposure was between 09:00 and 12:00 h, and the second between 14:00 and 17:00 h (Toleikis and Godin, 1995). To minimize habituation, the sequence of the stressors was randomized for both the morning and afternoon sessions of the first week of exposure, and was repeated during the second week with the morning and afternoon sequences exchanged. The same procedure was done for the third and fourth week of the experiment. Control rats were weighed weekly and remained in their home cages throughout the experiment except when blood samples had to be taken. The stressors were as follows: (a) towel wrap secured with tape, (b) restraint in a plexy glass box (15×5 cm) with lid, (c) restraint in a polyvinyl chloride tube (L=15 cm, ID=4.5 cm) closed at either end, (d) immobilization on a board with tape (Vazquez-Vela *et al*., 2008). The animals exposed to stressors were returned to the animal facilities 15 min following stress exposure to minimize disturbance to the control group.

Assessment of food intake and body weight

Food intake was measured daily between 09:00 and 10:00 throughout the experiment by measuring the difference between the amount of feed put in the cage and the remaining amount. The weight of the animals was measured once a week during the experimental period by a digital scale (Ohaus Scout Pro, Pine Brook, New Jersey, USA).

Glucose tolerance and insulin tolerance tests

For the oral glucose tolerance test (OGTT), rats were fasted for 16 hr prior to the time the test commenced. Subsequently, a zero-time (baseline) blood sample was drawn and designated 0 min glucose level. Thereafter, each rat was given an oral glucose load of 2g/kg BW *(*Morakinyo *et al*., 2016*)* of glucose solution (D-Glucose: Sigma Cat. No. G-7528). Blood sample was drawn from tail vein after the glucose load at intervals of 30, 60, 120 and 180 min for measurement of glucose level. The glucose level was measured with a portable Accu-Chek glucose meter (Roche Diagnostics, Germany).

Rats that were used for insulin tolerance test (ITT) were fasted for 4 h. Basal blood glucose levels (0 min) were measured followed by injection of insulin (0.5 U/kg BW; Human Insulatard, Novo Nordisk) into the peritoneum, and blood glucose level was measured at 15, 30, 60, 90 and120 min by portable glucose meter using tail vein blood. Total area under the curves (AUC) in response to glucose or insulin administration was calculated using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA.

Expression of corticosterone, insulin, insulin receptor, adiponectin and GLUT4

After light ether anaesthesia, blood samples were taken following over-night (16 h) fasting, 1ml blood was collected in an Eppendorf tube containing 5 µl heparin (5000 IU/ml) (Chalkley *et al*., 2002), and centrifuged at 3000 rpm for 5 min at 4 °C (Toleikis and Godin, 1995). Plasma was removed and kept at −20 °C for measuring the corticosterone, insulin and adiponectin. The skeletal tissue homogenate was used for the determination of insulin receptor and GLUT4 concentrations. The gastrocnemius muscle was homogenized in 9-volumes of ice-cold 0.1 mM phosphate buffer saline (pH 7.4) to prepare 10% homogenate. The homogenate was then centrifuged for 5 min at 5000×g to get the supernatant used for the measurement. These parameters were determined using enzyme immunoassay (EIA) kit (Elabscience Biotechnology Co., China). The procedure specified in the manufacturer's manual for the kits were followed. A 96-well microtitre plate was used to conduct the analysis.

Statistical analysis

Data are presented as mean ± SEM. One-way analysis of variance (ANOVA) were performed and followed by Tukey test, $p > 0.05$ was considered to be statistically significant. Graph Pad Prism version

5.00 for Windows, GraphPad Software, San Diego California USA was used for all statistical analysis

RESULTS

Food intake, body weight and weight gain

Before evaluating the effect of restraint stress on food intake and mean body weight, we assessed the basal level of these parameters and found no significant difference in the experimental rat within our facility. However, food intake in the restraint-challenged rats were significantly lower than the control rat only in the S7D rats (Table 1). The mean body weight values of all stress groups were not significantly different from the control; however, the mean weight of animals in the S7D, S14D and S28D groups were 3.95, 6.19 and 6.65 % lower than that of the control group even though both stress and control groups of rats consumed the same amount of food (Table 1).

Glucose tolerance and Insulin sensitivity

Before glucose administration (0 min), both groups showed comparable FBG in S7D, S14D and S28D (Figure 1a-c). After glucose administration, blood glucose at 60 min was significantly higher in the S7D group compared with control rats but there was no significant difference at other time-points. The S14D rats exhibited decrease glucose tolerance as indicated by higher blood glucose levels during the glucose tolerance test and a higher AUC_{GTT} (Figure 1b). The glucose response in S28D rats was not significantly different form the control rats (Figure 1c).

The S14D rats showed decreased insulin sensitivity as demonstrated by a significantly lower timed blood-glucose levels (significant at all times post-glucose injection except 120 min) during the ITT as well as a higher AUC_{ITT} (Figure 2b) compared to the control. In S28D rats, insulin sensitivity was higher than control evidenced by lower glucose levels (significant at 60, 90 and 120 min post-insulin injection) and lower AUC_{ITT} (Figure 2c) compared to the control (Figure 2c). However, there was no significant difference in the time blood-glucose level in S7D rats pre- and post- insulin injection compared to the control rats (Figure 2a).

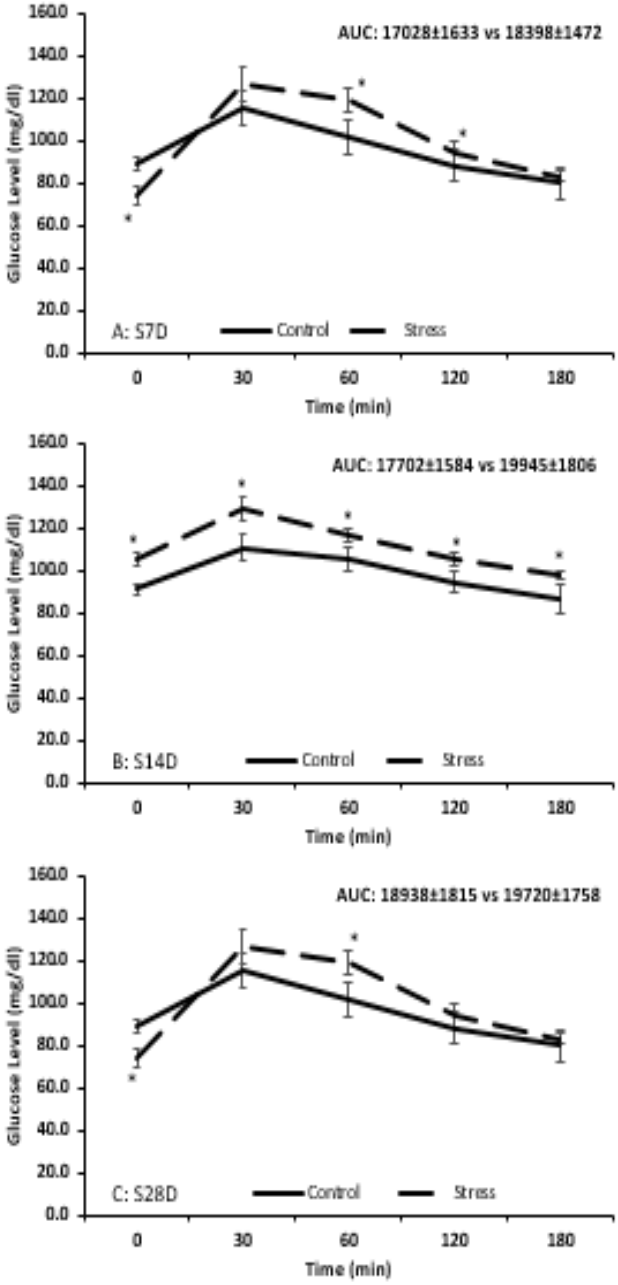


Figure 1. Effect of restraint stress on glucose tolerance at Days 7 (A), 14(B) and 28 (C). Insets data are the corresponding results of the OGTT as analysed by area under the curve (AUC_{GTT}). *p<0.05

Table 1: Effects of restraint stress on food intake, body weight and weight gain

Group	Days	Food Intake	Body Weight		%WG
			Initial	Final	
Control	**S7D**	82.14±4.59	173.67±10.04	182.33±7.31	4.99
	S14D	93.57±0.70	171.83±8.16	194.25±6.28	13.05
	S28D	135.28±5.23	175.50±12.63	211.50±10.04	20.51
Stress	**S7D**	72.85±3.20*	174.36±9.36	176.17±6.01	1.04
	S14D	90.14±1.83	172.81±7.05	184.67±4.81	6.86
	S28D	140.28±2.06	174.13±7.62	198.26±4.76	13.86

Data expressed as mean±SEM (n=6); *P<0.05 vs control; Initial = baseline / pre-stress exposure values; Final = post-stress values; %WG = % weight gain.

Table 2. Effect of restraint stress on corticosterone, insulin and adiponectin

Group	Stress	Corticosterone	Insulin	Adiponectin
S7D	-	711.43±30.64	4.24±1.29	25.64±2.72
	+	1266.31±72.09*	4.57±1.62	17.82±2.13*
S14D	-	698.15±33.27	5.06±1.12	28.27±3.14
	+	1194.56±80.18*	6.18±1.73	16.39±2.01*
S28D	-	676.60±35.03	4.48±1.08	29.73±4.21
	+	962.00±86.1*	6.51 ±1.01*	19.90±3.36

Data are expressed as mean ± SEM. *P < 0.05, compared with the control rats; n = 8. "+" indicates exposure to stress challenge, "–" indicates no exposure to stress challenge.

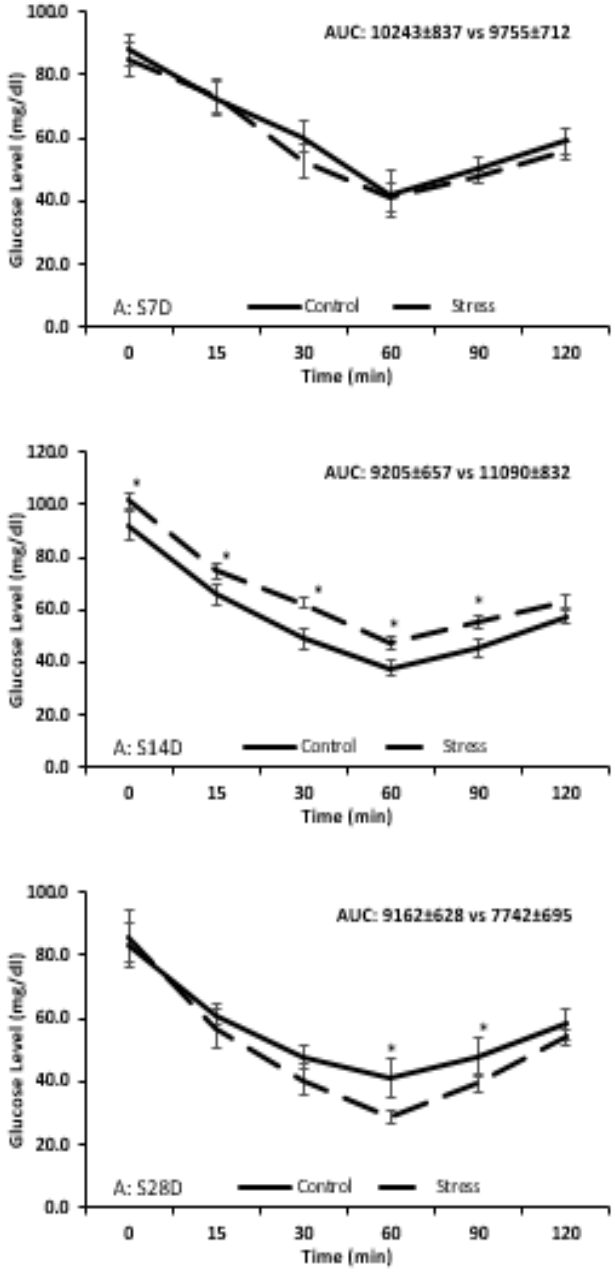


Figure 2. Effect of restraint stress on insulin sensitivity at Days 7 (A), 14(B) and 28 (C). Insets data are the corresponding results of the ITT as analysed by area under the curve (AUC_{ITT}). The values are presented as the means ± SEM of 6 rats per group. *p<0.05.

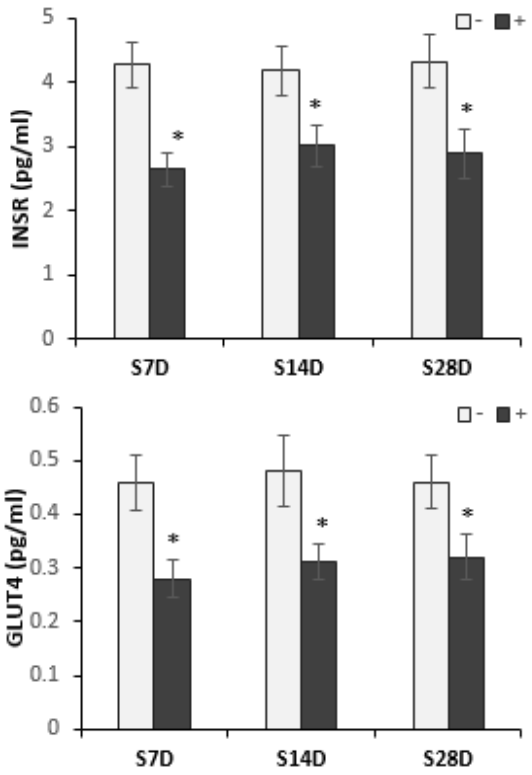


Figure 3: Quantitative analysis of INSR (a) and GLUT4 (b) expression in skeletal muscle. Data are expressed as mean ± SEM. *P < 0.05, compared with the control rats; n = 6

Corticosterone, insulin and adiponectin

The serum concentration of corticosterone, insulin and adiponectin were shown in Table 2. The results show a significant effect of restraint exposure on stress level in S7D, S14D and S28D rats compared with control groups. The corticosterone level in S7D was higher than S14D rats while S28D had a lower level of corticosterone as compared to the S14D rat, thus indicating habituation to the stress procedure. In addition, the expression of adiponectin, a known anti-inflammatory adipokine, was significantly reduced in all stressed groups except the S28D rats compared with their respective control group. However, serum insulin concentrations were not significantly different in all stress groups compared with the control after

repeated stress with the exception of S28D rats that had a significant ($p<0.05$) increase in insulin level.

INSR and GLUT4

We examined the mechanism underlying this effect on insulin tolerance in the stressed rats by measuring the expressions of INSR and GLUT4 in the skeletal muscle. Restraint stress increased the expression of GLUT4 but reduced INSR in S7D, S14D and S28D rats compared with the levels in control rats (Figure 3).

DISCUSSION

Any stimulus (stressor) that endangers the body's integrity or function results in a stress response, an adaptive response to solve stressful situation and determine new coping strategies (Landowski, 2007). For the present study on the potential effect and underlying mechanism of restraint stress on glucose homeostasis, the level of insulin and adiponectin, as well as the expression of INSR and GLUT4 in restraint-stress rats were investigated. The present results showed that the duration of exposure to restraint stress appears to produce different responses on glucose homeostasis. The results suggest that restraint-stress exposure (RSE) for 14-day produced greater glucose intolerance and insulin insensitivity compared with both 7-day and 28-day. In addition, the adiponectin level, as well as the expression of INSR and GLUT4 receptors in the skeletal muscle of the RSE rats were diminished relative to the control. However, prolonged exposure to restraint stress appears to cause a habituating effect in the S28D rats with muted / lower adverse responses in these endpoints.

Restraint stress can induce a complex stress reactions involving the hypothalamo-pituitary-adrenal (HPA) axis (Samson *et al*., 2007). Previous studies reported significant increases in plasma corticosterone levels after restraint-stress exposures (Pitman *et al*., 1988; Malisch *et al*., 2007). Serum corticosterone levels at the end of the exposure were significantly higher in all restraint-stress rats compared with controls, however, the severity appears to be diminished with increasing duration of stress exposure. This suggests that restraint induced stress reactions, and that the rats showed adaptation to the restraint after prolonged exposure.

Glucose homeostasis is critical for normal functioning of the central nervous system and cells which have an obligatory requirement for this metabolic substrate. The present findings indicated that restraint stress caused a significantly increase in the blood glucose level of rats in a time-coursed GTT. The mechanism by which stress raises the levels of glucose in these animals may be related to the possible enhanced activity of hypothalamic-pituitary adrenal axis during stress, resulting in increased secretion of adrenocorticotropic hormone (ACTH) and corticosteroids into the circulation. Release of ACTH in stress stimulates the adrenals to increase the production of catecholamines. These hormones mobilize stored carbohydrate reserves from the tissues which lead to elevated levels of blood glucose *(*Nade *et al*., 2009*)*. Another possible mechanism of hyperglycaemia is the blunted insulin signalling in the skeletal muscle observed in the restraint-stress rats.

Insulin resistance (insensitivity) is defined as an inadequate response by insulin target tissues, such as skeletal muscle, liver, and adipose tissue, to the physiologic effects of circulating insulin. Considering the results of the present study, glucose response to exogenous insulin administration was significantly lower in restraint-stress rats compared with their control counterpart. These findings are probably suggestive of insulin resistance in these animals and are consistent with similar reports of heightened insulin insensitivity under stressful conditions (Ceriello and Motz, 2004; Nakatani *et al*., 2005; Zardooz, 2006; Hentiksen *et al*., 2011). Insulin resistance has been induced by cortisol administration in rodents (van Donkelaar *et al*., 2014). In addition, sleep deprivation, a physiologic stressor has also been shown to increase cortisol concentrations and decrease insulin sensitivity in humans (McEwen, 2006; Donga *et al*., 2010). It is therefore plausible to conclude that stress-induced corticosteroid secretion has a negative consequence on insulin sensitivity. In another vein, decreased adiponectin levels as observed in this study could also impair systemic insulin sensitivity. Adiponectin is a novel adipocyte-specific protein that has been suggested to play a role in the development of insulin resistance. Reduced expression of adiponectin has been associated with some degree of insulin resistance (Wang *et al*., 2007). In order to assess whether defects in insulin-stimulated glucose transport activity was responsible for the stress-induced hyperglycaemia, we measured the expression of INSR and GLUT4 protein receptors in the skeletal muscles. The results showed that the expression of both receptors were significantly decreased compared with control rats. In skeletal muscle, activating the INSR leads to the translocation of GLUT4 from intracellular locations to the plasma membrane, where it facilitates the transport of glucose into the cell (Brewer *et al*., 2014). A major mechanism by which insulin signalling can be negatively regulated is via the alteration of INSR as well as GLUT4 activations. Since insulin-stimulated glucose uptake cannot function independently of the INSR, it is very likely that its reduced expression in this study account for the observed hyperglycaemia and insulin resistance. It thus seems to suggest that stress-induced disorder of glucose control could be

due to inadequate expression of INSR and GLUT4 protein receptors.

In summary, we found that exposure to restraint-stress was associated with glucose intolerance and insulin resistance in SD rats. Multiple insulin signalling receptors such as INSR and GLUT4 appear to be involved in the mechanism of stress-induced diabetogenic effects. However, these effects appear to become blunted with prolonged exposure to the stress factor.

Acknowledgements
The authors wish to thank Sunday Ogunnowo who provided technical assistance during the experiments.

REFERENCES

Björntorp P. Body fat distribution, insulin resistance, and metabolic diseases. Nutrition 1997; 13(9):795-803

Bonner-Weir S, Trent DF, Honey RN, Weir GC. Responses of neonatal rat islets to streptozotocin, limited β cell regeneration and hyperglycemia. Diabetes 1981; 30:64–9.

Brewer PD, Habtemichael EN, Romenskaia I, Corley Mastick C, Coster AC. Insulin-regulated Glut4 translocation: membrane protein trafficking with six distinctive steps. J Biol Chem 2014; 289:17280-17298.

Carrasco GA, Van de Kar LD. Neuroendocrine pharmacology of stress. Eur J Pharmacol 2003; 463:23–72.

Ceriello A, Motz E. Is oxidative stress the pathogenic mechanism underlying insulin resistance, diabetes, and cardiovascular disease? The common soil hypothesis revisited. *Arterioscler Thromb Vasc Biol* 2004; 24: 816-823

Chalkley S.M., Hettiarachchi M., Chisholm D.J., Kraegen E.W. Longterm high-fat feeding leads to severe insulin resistance but not diabetes in Wistar rats. Am J Physiol Endocrinol Metab 2002; 282(6):E1231–E1238.

Chalkley SM, Hettiarachchi M, Chisholm DJ, Kraegen EW. Long term high-fat feeding leads to severe insulin resistance but not diabetes in Wistar rats. Am J Physiol Endocrinol Metab 2002; 282(6): E1231–E1238.

Chrousos GP. Stressors, stress, and neuroendocrine integration of the adaptive response: The 1997 Hans Selye Memorial Lecture. Ann NY Acad Sci 1998; 851:311–35.

Donga E, van Dijk M, van Dijk JG, Biermasz NR, Lammers GJ, van Kralingen KW, *et al*. A single night of partial sleep deprivation induces insulin resistance in multiple metabolic pathways in healthy subjects. J Clin Endocrinol Metab 2010; 95:2963-2968.

Hentiksen EJ., Diamond-Stanic MK, Marchionne EM. Oxidative stress and the etiology of insulin resistance and type 2 diabetes. Free Radic Biol Med 2011; 51 (5): 993–999

Kevetnansky R, Fukuhara K, Pacak K, Cizza G, Goldstein DS, Kopin IJ. Endogenous glucocorticoids restrain catecholamine synthesis and release at rest and during immobilization stress in rats. Endocrinology 1993; 133:1411–9.

Landowski, J. Neurobiology of stress. Neuropsychiatry Neuropsychol 2007; 2: 26–31.

Lay SL, Simard G, Martinez MC, Anriantsitohaina R. Oxidative Stress and Metabolic Pathologies: From an adipocentric point of view. Oxid Med Cell Longev 2014; http://dx.doi.org/10.1155/2014/908539

Malisch JL, Saltzman W, Gomes FR, Rezende EL, Jeske DR, Garland T. Baseline and stress-induced plasma corticosterone concentrations of mice selectively bred for high voluntary wheel running. Physiol Biochem Zool 2007; 80(1):146–156.

McEwen BS. Sleep deprivation as a neurobiologic and physiologic stressor: Allostasis and allostatic load. Metabolism 2006; 55: 20-23.

Morakinyo AO, Iranloye BO, Samuel TA, Mofolorunso AM and Adegoke OA. Insulin receptor and glucose transporter-4 expression in the skeletal muscle of chronically stressed rats. J Afr Ass Physiol Sci 2016; 4 (1): 25-31

Nade VS, Kawale LA, Naik RA, Yadav AV. Adaptogenic effect of Morus alba on chronic footshock-induced stress in rats. Indian J Pharmacol. 2009; 41:246–51.

Nakatani Y, Kaneto H, Kawamori D, Yoshiuchi K, Hatazaki M, Matsuoka T, Ozawa K, Ogawa S, Hori M, Yamasaki Y, Matsushisa M. Involvement of endoplasmic reticulum stress in insulin resistance and diabetes. *J Biol Chem 2005;* 280: 847-851

Ozcan U, Cao Q, Yilmaz E, Lee A, Iwakoshi NN, Ozdelen E, Tuncman G, Gorgun C, Gilmcher LH, Hotamisligi GS. Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. S*cience 2004;* 306 (5695): 457-461

Pessin J.E and Saltiel A.R. Signaling pathways in insulin action: molecular targets of insulin resistance J Clin Invest 2000; 106 (2):165-169

Pitman DL, Ottenweller JE, Natels BH. Plasma corticosterone levels during repeated presentation of two intensities of restraint stress: Chronic stress and habituation. Physiol Behav 1988; 43(1): 47-55

Rozanski A, Blumenthal JA, Davidson KW, Saab PG, Kubzansky L, *et al*. The epidemiology, pathophysiology, and management of psychosocial risk factors in cardiac practice: the emerging field of behavioral cardiology. Brain Beh Imm 2005; 20(2):113-9.

Sabban EL, Kvetnansky R. Stress-triggered activation of gene expression in catecholaminergic systems: dynamics of transcriptional events. Trends Neurosci 2001; 24:91–8.

Samson J, Sheeladevi R, Ravindran R, Senthilvelan M. Stress response in rat brain after different durations of noise exposure. Neurosci Res 2007; 57:143-147.

Soop M, Nygren J, Myrenfors P, Thorel A, Ljungqvist O. Preoperative oral carbohydrate treatment attenuates immediate postoperative insulin resistance. Am J Physiol Endocrinol Metab 2001; 280(4): E576–E583.

Strommer L, Permert J, Arnelo U, Koehler C, Isaksson B, Larsson J, *et al*. Skeletal muscle insulin resistance after trauma: insulin signaling and glucose transport. Am J Physiol 1998; 275: E351–8.

Toleikis PM, Godin DV. Alteration of antioxidant status in diabetic rats by chronic exposure to restraint stressors. Pharmacol Biochem Behav. 1995; 52:355–66.

van Donkelaar EL, Vaessen KR, Pawluski JL, Sierksma AS, Blokland A, Canete R, *et al*(2014). Long- term corticosterone exposure decreases insulin sensitivity and induces depressive-like behaviour in the C57BL/6NCrl mouse. PLoS One 2014; 9: e106960.

Vanltallie TB. Stress: a risk factor for serious illness. Metabolism 2002; 1:40-5

Vazquez-Vela ME, Torres N, Tovar AR. White adipose tissue as endocrine organ and its role in obesity. Arch Med Res 2008; 39:715–28.

Wang C, Mao X, Wang L, Liu M, Wetzel MD, Guan KL, Dong LQ, Liu FJ. Adiponectin sensitizes insulin signaling by reducing p70 S6 kinase-mediated serine phosphorylation of IRS-1. Biol Chem 2007; 282(11):7991-6.

Zardooz H, Zahedi Asl S, Gharib Naseri MK, Hedayati M. Effect of chronic restraint stress on carbohydrate metabolism in rat. Physiol Behav 2006; 89:373-378.

Niger. J. Physiol. Sci. 31(June 2016) 031-036
www.njps.com.ng

Niger. J. Physiol. Sci.

Bronchoalveolar Lavage Fluid Cellular and Haematological Changes in Different Types of Caprine Pneumonia

Jarikre T.A. *, Emikpe B.O., Ohore O.G., Akinremi T.A. and Akpavie S.O.
Department of Veterinary Pathology, Faculty of Veterinary Medicine, University of Ibadan, Ibadan, Nigeria.

Summary: Goats in the tropics are often reared under the traditional extensive and semi-intensive management systems. These and other factors influence the pattern of pneumonia complex in goats. We investigated the bronchoalveolar lavage fluid (BALf) cellular changes and haematological response in different types of caprine pneumonia in Nigeria. Haematological indices and BALf cells were analysed from 300 goats randomly selected from 700 goats comprising different breed, age and body scores. The pneumonia status was well characterised using standard pathological tools. Data is summarized as Mean ± SEM and compared using non-parametric statistics at 5% significance. There was leukocytosis in the pneumonic animals. The overall lavage recovery rate was 55.5%. The differences in Haemoglobin concentration, and Lymphocyte-Neutrophil ratio were significant ($p<0.05$). BALf changes in the neutrophil, macrophage and eosinophil counts were significantly different ($p<0.05$). The diagnostic features including increased percentage neutrophils, Macrophage-Neutrophil ratio and eosinophils observed in BAL were reliable and also correlated positively to the pathological findings. BAL should be considered a component of the diagnostic approach to caprine pneumonia complex, as it may accurately aid diagnosis and identification of the causal organisms.

Keywords: Bronchoalveolar lavage, Haematology, Pneumonia, Comparative, Caprine

**Address for correspondence:* get2theo@yahoo.com Tel: +234 8062602408

Manuscript Accepted: June, 2016

INTRODUCTION

Goats constitute 42.3% of Nigerian ruminant livestock population and contributing about 12.7% of the total agricultural gross domestic product (Lawal-Adebowale 2012). Small ruminant production have been limited by myriads of infectious diseases of which respiratory infections are of paramount importance (Emikpe *et al.*, 2013).

Bronchoalveolar lavage (BAL) is a tool in diagnosis of respiratory diseases (Reynolds 2000, Ezeasor *et al*., 2012 and Lee *et al.*, 2015). The cellular changes support clinical diagnosis in the absence of biopsy (Rottoli and Bargagli, 2004, Lee *et al.*, 2015). However, the correlations between BAL fluid (BALf) analysis and corresponding morphological features of the lungs have not been well defined in caprine pneumonia complex.

Previous studies in our laboratory has shown that caprine pneumonia in Nigeria is more of bronchopneumonia with fibrinous (30%) and suppurative (10%) types, others observed were interstitial pneumonia (15%), broncho-interstitial pneumonia with giant cells (40%) and atelectasis (5%) (Emikpe *et al.*, 2013, Jarikre *et al.*, 2016) Furthermore, the cellular response in the respiratory tract after vaccination and safety of non-conventional route of vaccine administration was well assessed using bronchoalveolar lavage celluar changes and haematology respectively in goat (Ezeasor *et al*., 2012; Tenuche *et al*., 2013, Ezeasor et al., 2014) however the use of BAL in evaluating normal and diseased lungs in goats is still scanty in literature especially in pneumonia of ruminants (Mohammad *et al*., 2007), horses (McKane, 2010), companion animals and man (Rottoli and Bargagli, 2004). In addition, due to non-specificity of the clinical signs in respiratory diseases and auscultation, examining the cytological characteristics of the respiratory secretions do provide both aetiology and an indication of treatment response (McKane, 2010), hence in tropical environment where management systems encourages transportation stress (Minka *et al.*, 2009) and other stressors, the health of those animals need to be evaluated routinely using these techniques. With the current focus on caprine respiratory diseases internationally, and the fact that BAL has been explored to determine the cellular and humoral responses to infectious agents' in cattle (Blodörn *et al.*, 2015) and experimentally in goats (Ezeasor *et al*., 2012; Tenuche *et al*., 2013), emphasis should then be on the cellular changes in naturally occurring caprine pneumonia which have not been previously investigated.

This study investigates the BALf cellular and haematological changes in the different pattern of caprine pneumonia in a tropical setting.

MATERIALS AND METHODS

The source of animals and pattern of pneumonia have been previously described (Jarikre *et al.*, 2016). Blood and bronchoalveolar lavage samples from 300 goats out of 700 were randomly collected for haematology and BALf analysis.

Haematology

Packed cell volume (PCV) and haemoglobin (Hb) concentration were determined by microhaematocrit and cyanmethemoglobin methods, respectively (Schalm *et al*. 1975). Red blood cells (RBC), White blood cells (WBC) and Platelets were counted using haemocytometric methods and light microscope (Schalm *et al*. 1975). Differential leucocyte counts were estimated from Romanowsky-stained smear at high dry (40X) and oil magnification (100X) counting 200 cells and classifying by type. Mean corpuscular volume (MCV) and Mean corpuscular haemoglobin concentration (MCHC) were calculated. Plasma protein was determined using the refractometer.

Bronchoalveolar lavage fluid

Lavage was as described by Khin (2009) and modified by Ezeasor *et al* (2012). Briefly, following slaughter, the trachea together with the lungs were resected and lavaged by introducing 40 ml of warm sterile phosphate buffered saline (PBS), pH 6.8 into the lungs. This was followed by gentle massage of the lungs before the fluid was re-collected into a measuring beaker. The colour and consistency of the BALf was noted before centrifuged at 2000 rpm for 15 minutes and the supernatant decanted. The sediment was smeared on clean glass slides, fixed with methanol and stained with Giemsa for cytological details. The BALf cellular differential count was as described by Dawson *et al.* (2005), 400 cells were counted on each of the stained slides with the reader blinded as to the identity of each goat.

Statistics Analysis

Data was presented as Mean ± SEM, and compared using non-parametric statistics at 5% significance.

RESULTS

Distribution of pneumonia

Of the 300 goats, 224 had varying pneumonic lesions bronchopneumonia (148), broncho-interstitial pneumonia (49), interstitial pneumonia (37), granulomatous pneumonia (3) and verminous pneumonia (2) while 61 were normal.

Haematological changes

The haematologies of pneumonic and non-pneumonic goats are presented in table 1. Generally, in pneumonic goats, there were increases in the haemoglobin concentration (HB), white blood cells, platelet, lymphocytes, and neutrophils. And a slight decrease in plasma protein. The leukocytosis, neutrophilia and hypoproteineamia were significant ($p<0.05$). The haematological changes for the different types of pneumonia are shown in Table 2.

There was significant decrease in HB of goats having broncho-interstitial, interstitial and granulomatous pneumonia; leucocytosis in goats with bronchopneumonia and leucopaenia in goats with interstitial and verminous pneumonia; thrombocytosis in goats with granulomatous pneumonia; lymphocytosis and neutrophilia in goats with bronchopneumonia; and increased Lymphocyte-Neutrophil ratio in goats with granulomatous pneumonia ($p<0.05$).

Bronchoalveolar lavage fluid (BALf) cytology

The colour of the lavage fluid recovered was clear to turbid with a top foamy layer, the volume recovered ranged from 15 ml to 25.3 ml, while the overall mean volume recovered was 22.2 ± 3.5 ml. The overall lavage recovery rate was 55.5% of the 40 ml of saline solution instilled into the lungs. Most of the stained smears showed a pinkish background with strands of mucus and fibrin. The BALf differential cell counts are shown in Table 1 and 2.

Generally, BALf changes in the pneumonic goats included significant increases in neutrophils, alveolar macrophages, Macrophage-Neutrophil ratio, lymphocytes and eosinophil counts ($p<0.05$). The cellularity was low to high constituting alveolar

Table 1. Haematological and BALf cellular count changes in normal and pneumonic goats

	Normal (61)	Pneumonic (239)	p-value
Haematology			
PCV %	28.3±0.7	28.1±0.4	0.80
HB g/dl	8.7±0.3	8.5±0.1	0.59
RBC $x10^3/\mu L$	6.1±0.4	8.3±2.1	0.60
WBC $x10^3/\mu L$*	$10.3±0.4^a$	$12.8±0.1^b$	0.03
PLT $x10^3/\mu L$	147±14.6	135±32.7	0.24
LYM $x10^3/\mu L$*	$8.8±0.9^a$	$11.0±2.3^b$	0.04
NEUT $x10^3/\mu L$	0.8±0.1	1.1±0.2	0.64
L:N	12.3±0.4	12.7±0.3	0.44
MON $x10^3/\mu L$	0.3±0.0	0.3±0.1	0.62
EOS $x10^3$ /µL	0.1±0.0	0.1±0.0	0.86
PP (g/dl)	7.8±0.1	7.4±0.3	0.62
MCV fl	56.6±2.9	55.9±1.6	0.85
MCHC g/dl	30.8±0.6	30.6±0.7	0.800
BALf			
NEUT cells $x10^2$/Ml	$7.7±1.5^a$	$127±8.4^b$	0.00
MQ cells $x10^2$/Ml	$47.8±6.4^a$	$175±5.3^b$	0.00
MQ:N	$3.1±0.5^a$	$5.9±0.6^b$	0.00
LYM cells $x10^2$/Ml	$32.0±3.4^a$	$73.2±3.7^b$	0.02
PC cells $x10^2$/Ml	$6.2±2.0^a$	$11.3±1.1^b$	0.08
EOS cells $x10^2$/mL	$0.1±0.1^a$	$7.5±1.8^b$	0.01
MAST cells $x10^2$/Ml	0.2±0.1	0.2±0.1	0.92

MON- monocytes, Neut- neutrophils, LC- lymphocytes/plasma cells, L:N- lymphocyte-neutrophil ratio, MQ- macrophage, MQ-N ratio macrophage:neutrophil ratio, EOS- eosinophil, MAST- Mast cells. Values with different superscript are significantly different across row at 5%

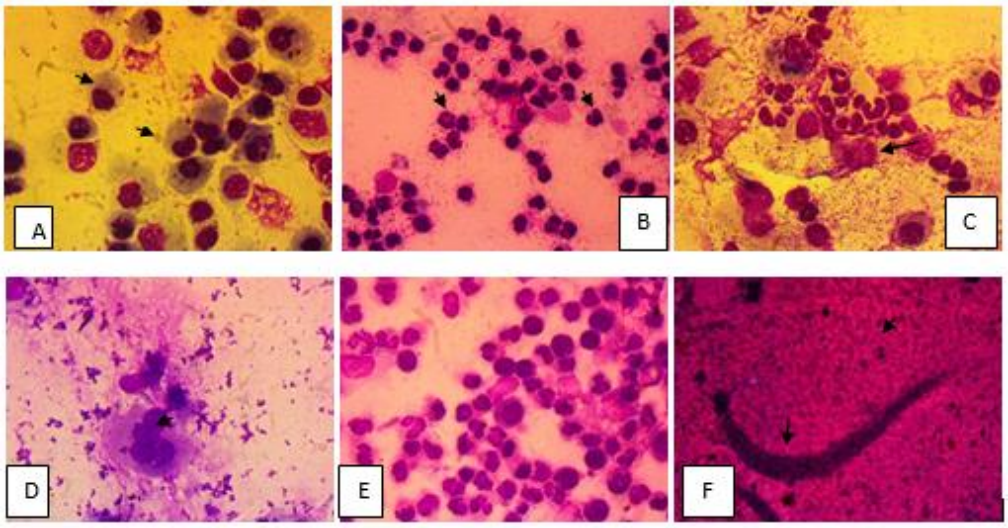
Table 2. Haematological and BALf cellular count changes in the different pattern of pneumonia in caprine goats

	Normal (61)	Broncho Pneumonia (148)	Broncho-interstitial (49)	Interstitial pneumonia (37)	Granulomatous pneumonia (3)	Verminous pneumonia (2)	p-value
Haematology							
PCV	28.4±0.7	28.2±0.5	28.6±1.0	27.1±1.0	24.0±5.7	27.0±0.0	0.72
HB*	8.7±0.3	8.7±0.2^{a}	8.1±0.3^{b}	8.2±0.4^{b}	7.5±2.1^{b}	9.2±0.2	0.04
RBC	6.1±0.5	9.9±3.4	6.1±0.5	5.4±0.5	6.5±2.6	4.5±0.0	0.93
WBC x10^{3*}	9.4±0.4^{a}	14.9±0.2^{b}	9.5±0.4	7.1±0.4^{c}	10.0±2.0	7.4±2.3^{c}	0.03
PLT x10^{3*}	133±6.3^{a}	142±6.5	136±8.3	123±8.7	205±81^{b}	145±0.7	0.05
LYM x10^{3*}	8.4±0.8^{a}	13.2±3.8^{b}	8.5±1.0	6.3±0.6	9.1±1.5	6.5±3.2	0.01
NEUT x10^{3*}	0.8±0.1^{a}	1.3±0.4^{b}	0.8±0.1	0.6±0.1	0.7±0.3	0.6±0.3	0.03
L:N*	12.3±0.4	12.3±0.3^{a}	13.3±0.6	13.3±0.7^{b}	15.7±4.4	11.1±0.3	0.02
MON x10^{3}	0.2±0.1	0.4±0.1	0.2±0.1	0.2±0.0	0.2±0.1	0.2±0.0	0.83
EOS	0.01±0.0	0.01±0.0	0.01±0.0	0.01±0.0	0.0±0.0	0.1±0.0	0.95
PP	7.8±0.9	7.3±0.4	6.9±0.1	8.3±1.5	6.4±0.2	6.3±0.3	0.88
MCV	56.6±2.9	53.2±1.9	60.6±4.4	61.0±4.4	48.4±3.4	60.1±0.1	0.36
MCHC	30.8±0.6	31.2±0.5	29.0±1.0	30.2±0.8	30.6±2.2	33.3±0.7	0.30
BALf cells x10^{2}/Ml							
NEUT*	7.1±8.1^{a}	165±10.1^{b}	72.3±13.0	57.3±15.2^{b}	89.3±85.4	64.0±16.0	0.00
MQ*	45.2±6.0^{a}	155±6.7^{b}	205±10.2	208±10.7^{b}	249±55	192±32.0	0.00
MQ:N	3.1±0.5	4.4±0.6	10.1±1.6	5.9±1.4	11.5±11.2	3.3±1.3	0.00
LYM	30.0±2.9	60.3±4.3	90.5±8.4	109±10.2	42.7±3.5	56.0±16.0	0.00
BPC	5.5±1.8	9.5±1.2	17.1±2.7	11.7±3.2	8.0±8.0	20.0±20.0	0.09
EOS*	0.0±0.0^{a}	8.4±2.4^{a}	5.1±2.5	3.9±3.3^{a}	0.0±0.0^{a}	68.0±52.0^{b}	0.01
MAST	0.2±0.1	0.02±0.02	0.3±0.3	0.9±0.5	0.0±0.0	0.0±0.0	0.06

MON- monocytes, Neut- neutrophils, LC- lymphocytes/plasma cells, L:N- lymphocyte-neutrophil ratio, MQ- macrophage, MQ-N ratio macrophage:neutrophil ratio, EOS- eosinophil, MAST- Mast cells. Values with different superscript are significantly different across row at 5%.

Table 3: Percentage BALf differential counts observed in the different types of pneumonia

Pneumonia type	Neutrophil	Macrophage	Lymphocyte	Plasma cell	Eosinophil	Mast cell	Total
Normal	6.1%	56.5%	32.1%	5.0%	0%	0.3%	100%
Bronchopneumonia	41.1%	39.2%	15.1%	2.4%	2.1%	0%	100%
Broncho-interstitial	18.2%	52.9%	23.2%	4.4%	1.3%	0.1%	100%
Interstitial	14.6%	53.3%	27.9%	3.0%	1.0%	0.2%	100%
Granulomatous	20.5%	66.2%	11.3%	2.0%	0%	0%	100%
Verminous	16.0%	48.0%	14.0%	5.0%	17.0%	0%	100%



Figures 1 A-F: Photomicrographs showing different BAL cellular changes: A-Alveolar macrophages (arrow). B-Neutrophils. C- Degenerate neutrophils (arrow). D- Giant cell (arrow). E- Mixed cellular population. F- parasitic larva (arrow). Giemsa X1000

macrophages (fig A) and other inflammatory cells, with a few phagocytosed bacteria, fungi and larva stages of helminthes present. Inflammatory cells include neutrophils (fig B), macrophages, lymphocytes (figures E), plasma cells, and eosinophils.

Morphologically, some of the neutrophils were degenerate (fig C) in the BALf (76), with numerous

intracellular bacteria rods in phagocytic cells (21). The macrophage-neutrophil (M:N) ratio varied greatly with pattern of pneumonia ($p<0.05$). The percentage BALf differential counts observed in the different types of pneumonia are shown in Table 3. There was significant changes in the percentage neutrophils, macrophages, lymphocytes and eosinophil counts ($p<0.05$).

In the pneumonic goats, 80 of the goats had neutrophil counts within range (<10%), 152 severe neutrophilia (>50%) and 68 moderate neutrophilia (in between). Concurrently, 127 had lymphocyte counts less than 15% (normal) and 173 with lymphocytosis (>15%). Low level of oesinophils (<1-2%), free erythrocytes and erythrophagocytosis (haemorrhage), giant cells (fig D), intracellular bacteria and Curschmann's spirals were also observed in a few of the BALf.

In bronchopneumonia, there was increased cellularity, clear to pinkish background and with a remarkable increase in neutrophils, macrophages and eosinophils ($p<0.05$). Numerous bacteria rods are free in smear and intra-cellular in neutrophils and macrophages. A few of the neutrophils are degranulated and degenerate. In broncho-interstitial pneumonia, there was increase in macrophages, macrophage-neutrophil ratio (M:N), lymphocytes and neutrophils ($p<0.05$). A few syncytial giant cells were present.

Interstitial pneumonia also showed remarkable increases in macrophages and lymphocytes ($p<0.05$). Similar pattern was observed in granulomatous pneumonia with increases in macrophages and M:N ($p<0.05$), while verminous pneumonia showed increases in eosinophils ($p<0.05$).

DISCUSSION

This study has been able to show the haematological and BALf cellular changes in the caprine pneumonia complex. These changes could serve as predictors in the diagnosis of caprine pneumonia in a tropical setting. Possible diagnostic indices to predict pneumonic lesions in these goats includes (macrophage-neutrophil ratio from BALf and blood leucocyte counts as erythrogram indices could be non-specific.

The prevalence, patterns and type of pneumonia in the examined goats has been previously described (Jarikre et al., 2016). Meanwhile, 300 goats with correlating signalment, body condition, haematological, BALf and morphological findings were reported.

The values for the haematological parameters observed were similar to those previously reported in goats by Daramola et al (2005) and Ezeasor *et al* (2015). The leukocytosis and neutrophilia observed are suggestive of tissue injury and or inflammation. Most of the other haematological parameters were within range of this specie. However, the leucogram changes are indicative of stress.

BALf differential cell count has been shown to be an accurate predictor of the cellular changes occurring in the lungs (Lee *et al.,* 2015). In this study, the differential cell count was used to ascertain the presence or absence of inflammatory response in the lungs. It was observed that there was a strong correlation between morphological changes and macrophage-neutrophil ratio (M:N). This was also reported in cattle and experimentally in goats (Tenuche *et al*., 2013; Ezeasor *et al*., 2015).

The amount of mucus in lower airways increases with pulmonary irritation. Specific causes of increased mucus or mucopurulent exudate includes bacterial, fungal or parasitic pneumonia, and chronic bronchitis. However, the significance of mild increases in the amounts of mucus in the airways remains unresolved and the point at which increased mucus is significant has not been defined. This was observed in this as also reported in horses with increased amounts of mucus, but no, or mild increases in the number of neutrophils and many activated macrophages (McKane, 2010). Mild elevations in the proportions of inflammatory cells, often accompanied by a mild increases in mucus, probably represent a normal response to noxious stimuli and in all probability do not contribute to decreased respiratory function (Lee *et al.*, 2015).

Quantification of the total number of cells/ml of sample retrieved may also help to indicate overall cellularity as well as assisting interpretation of relative numbers of individual types of inflammatory cells in BALf however defining cut-off values for normal percentages of inflammatory cells is difficult due to considerable variation between studies. It has been considered that BALf should have < 5% neutrophils, < 2% mast cells and <0.5% eosinophils. Wider ranges in the proportions of lymphocytes (30-60%) and macrophages (40-70%) have been reported (Taniuchi *et al*., 2009). However, from our observation neutrophil counts above 10% was suggestive of pneumonia. The presence of macrophages in all types including normal underscores the importance of alveolar macrophages in pulmonary clearance.

The high neutrophilia and lymphocytosis observed suggests tissue reactions to stress, bacterial and or viral injuries in the lungs. A number of factors may influence the accuracy of these counts, including variable dilution by infused saline and large amounts of mucus which can trap cells. Nonetheless, increase in the number of epithelial cells in samples was relatively rare. On the other hand, a few epithelial cells with degenerative changes were observed. Moreover, viruses and bacterial toxins cause direct damage to the

airway epithelium in cases of respiratory infections (Reynolds, 2000; Emikpe and Akpavie, 2011, 2012).

Pulmonary alveolar macrophages (PAMs) are the most abundant inflammatory cell in the BALf even from normal lung; this was very much evident in our findings. Therefore, increased proportions of these cells are difficult to detect. Occasionally increased amounts of mucus, increased total nucleated cell count (TNCC) and increased numbers of activated macrophages were quite useful. Lymphocytes also occur in higher proportions in BALf. Neutrophils respond to a variety of stimuli, and their numbers may fluctuate rapidly. This, however, reflects the innate response of the pulmonary system. Also, the effectiveness of the defense mechanisms in face of injury or irritants in the airways and gaseous exchange compartments in goat.

The usefulness of BAL as compared to other forms of washings cannot be overemphasized. BAL gives a more accurate representation of lung pathology than upper respiratory tract aspirates, except when lesions are localised to the cranioventral lung lobes (Espinasse, 1991). The volume of saline instilled is quite adequate and volume recovered is in range of those reported from 60–500 ml in large animals and 20- 50ml in small ruminants. The volume of fluid instilled may directly influence recovery rate and some studies have suggested that differential cell counts may be biased by small volume lavages (McKane 2010).

The Indications for BAL are generally not vague including signs of lower respiratory tract (LRT) disease, fever of unknown origin, poor performance, the presence of mucopurulent material in the trachea and possibly dyspnoea (Dawson *et al*. 2005). The colours of the BALf observed from the goats were also important. Normal BAL are clear or mildly turbid, whereas increased turbidity and presence of flocculent material reflects increased mucus, cells and cellular debris suggested pneumonia as was reported by Dawson *et al.*, (2005), while BALf differential cell counts were useful in determining the stage of the inflammatory process. In the initial stages of the caprine pnumonia complex, multinucleated syncytial giant cells were frequently observed. This is the case in broncho-interstitial pneumonia; neutrophils were few and alveolar- macrophages predominated (Allen, 1992, Blodörn *et al.*, 2015). In well advanced stages, neutrophils predominated. Cytological examination can therefore be used to assess stages of the pneumonic process. Occasionally, as when lungworm is involved, findings were diagnostic (eosinophilia). However, the low incidence of verminous pneumonia in this study may not be unconnected to the widespread use of broad spectrum anthelmintic.

The surface area has been the most widely used method for assessing severity of pulmonary consolidation in both experimental and naturally occurring cases of pneumonia. This method also has its shortfalls, especially not picking onset of pneumonic lesions, technicalities and experience involved. Nonetheless, its clinical usefulness cannot be overemphasized. Thus, the combination of gross consolidation, morphological changes and BALf analysis may to a large extent accurately detect pneumonia.

In conclusion, BAL should be considered a component of the diagnostic approach to caprine pneumonia, as it may accurately aid diagnosis and identification of the causal organisms. In Nigeria, where respiratory disease is a perennial problem in goats, it is worthwhile to ensure that investigation is carried out early enough in an outbreak, for effective diagnosis. Given the recognized losses and treatment costs associated with respiratory disease in ruminants, the relative expense of this procedure and laboratory techniques should not preclude its use in routine investigations.

Acknowledgement: We appreciate the management of the University of Ibadan Zoological garden for provision of the research animals.

Funding: This study was funded by the Research Grant (SRG/FVM/2010/6^{A}) of the University of Ibadan given to B.O. Emikpe

REFERENCES

Allen, JW, Viel L, Bateman KG, Rosendal S, Shewen PE (1992) Cytological findings in bronchoalveolar lavage fluid from feedlot calves: associations with pulmonary microbial flora. *Can. J. Vet Res., 56(2)*:122-6.

Blodörn K, Hägglund S, Gavier-Widen D, Eléouët J, Riffault S, Pringle J, Taylor G, Valarcher J (2015). A bovine respiratory syncytial virus model with high clinical expression in calves with specific passive immunity. BMC Vet Res. 11: 76.

Daramola JO, Adeloye AA, Fatoba TA, Soladoye AO (2005). Haematological and biochemical parameters of West African Dwarf goats. Livest Res Rural Dev 17 (95). Retrieved December 18, 2015, from http://www.lrrd.org/lrrd17/8/dara17095.htm

Dawson S, Else RW, Rhind, SM, Collie DD (2005) Diagnostic value of cytology of bronchoalveolar fluid for lung diseases of sheep. *Vet. Rec., 157(15)*:433-6.

Emikpe BO, Akpavie SO (2011) Clinicopathologic effects of Peste Des Petit Ruminant Virus infection in West African dwarf goats. Small Ruminant Research 95 168–173

Emikpe BO and Akpavie SO (2012). Clinicopathological observations in experimental *Peste des petits ruminants* virus and *Mannheimia*

haemolytica A:2 co-infection in goats. Niger. J. Physiol. Sci. 27 (2): 129 –136

Emikpe BO, Jarikre TA, Eyarefe OD (2013) Retrospective Study of Disease Incidence and Type of Pneumonia in Nigerian Small Ruminants in Ibadan, Nigeria. *Afr. J. Biomed. Res. 16; 107 - 113*

Emikpe BO, Tanko PN, Onilude OM, Sabri MY (2013) The influence of dexamethasone treatment and successive road transport stress on the occurrence of caprine pneumonia in a hot humid tropical environment. Vet world 6(8): 497-501,

Espinasse, J, Alzie, JP, Papageorgiou C, Beguin JC, Van Gool F (1991) Use of transtracheal aspiration to identify pathogens in pneumonic calves. Veterinary Record 129, 339

Ezeasor CV, Emikpe BO, Yussof SM, Afusat J, Anosa VO (2013) Cellular responses in the respiratory tract following intranasal administration of Peste des petits ruminant vaccine in goats. *Int. J. Morphol., 31(2)*:687-692.

Ezeasor CK, Emikpe BO, Anosa VO (2015) Haematological changes associated with intranasal and parenteral routes of vaccination against Peste des petits ruminants in West African dwarf goats. Comp Clin Pathol 24:189–192.

Khin, M. N.; Zamri-Saad, M.; Noordin, M. M. and Effendy, A. W. M. (2009) Effect of intranasal attenuated *Pasteurella multocida* B:2 on haemorrhagic septicaemia in calves. *Online J. Vet. Res., 13(2)*:65-72,

Jarikre T.A., Emikpe B.O., Morenikeji O.A., Akpavie S.O. 2016). Pattern and associated risk factors of caprine pneumonia in Nigeria. *Asian Pac J Trop Dis* 6(3): 930-934

Lawal-Adebowale O.A. (2012). Dynamics of Ruminant Livestock Management in the Context of the Nigerian Agricultural System, Livestock Production, Dr. Khalid Javed (Ed.), ISBN: 978-953-51-0814-6, InTech, Available from: http://www.intechopen.com/books/livestock-production/dynamics-of-ruminant-livestock-management-in-the-context-of-the-nigerian-agricultural-system

Lee W, Chung WS, Hong K, Huh J (2015). Clinical Usefulness of Bronchoalveolar Lavage Cellular Analysis and Lymphocyte Subsets in Diffuse Interstitial Lung Diseases. Annals of Laboratory Medicine. 35(2): 220–225.

McKane SA (2010) Tracheal wash and. bronchoalveolar lavage: sampling technique and fluid interpretation. Proceedings of the 49th British Equine Veterinary Association Congress BEVA Sep. 8 – 11, 2010 Birmingham, United Kingdom. Reprinted in IVIS with the permission of BEVA

Minka, N. S., Ayo, J. O., Sackey, A. K. B., Adelaiye, A. B. (2009) Assessment and scoring of stresses imposed on goats during handling, loading, road transportation and unloading, and the effect of pretreatment with ascorbic acid. *Livest. Sci* 125: 275-282.

Mohammadi GR,Nazifi S, Rezakhani A, Esmailnejad Z (2007) Effect of transportation stress on blood and bronchoalveolar lavage fluid components in calves. *Comp. Clin. Pathol., 16*:85-95.

Reynolds HY (2000) Use of bronchoalveolar lavage in humans—past necessity and future imperative. Lung 178:271-293

Rottoli P., and Bargagli E. (2004) Is bronchoalveolar lavage obsolete in the diagnosis of interstitial lung disease? Curr Opin Pulm Med 10:320

Schalm OW, Jain NC, Carol EJ (1975) Veterinary Haematology 3rd Ed. Lea and Febiger, Philadelphia, p 807

Taniuchi N, Ghazizadeh M, Enomoto T, Matsuda K, Sato M, Takizawa Y, Jin E, Egawa S, Azuma A, Gemma A, Kudoh S, Kawanami O. (2009) Evaluation of Fractional Analysis of Bronchoalveolar Lavage Combined with Cellular Morphological Features. *International Journal of Medical Sciences* 6(1):1-8

Tenuche O, Emikpe BO, Sabri MY (2013) Cellular and mucosal immune responses in the respiratory tract of Nigerian goats following intranasal administration of inactivated Recombinant *Mannheimia hemolytica* bacterine. *Niger. J. Physiol. Sci. 28: 121–125*

Zamri-Saad M, Effendy AW, Israf DA, Azmi ML (1999) Cellular and humoral responses in the respiratory tract of goats following intranasal stimulation using formalin-killed *Pasteurella haemolytica* A2. *Vet. Microbiol., 65(3)*:233-40.

Niger. J. Physiol. Sci. 31(June 2016) 037-042
www.njps.com.ng

Niger. J. Physiol. Sci.

Effect of Surgical and Immunological Castration on Haematological Variables, Reproductive Hormones and Ejaculate Characteristics in Mongrel Dogs

*** Temitope A. Ajadi [1] and Oladele S. Gazal[2]**

[1]Department of Veterinary Public Health and Reproduction, Federal University of Agriculture, Abeokuta, Ogun State, Nigeria. [2] Department of Biological Sciences, St. Cloud State University, St. Cloud, Minnesota, USA.

Summary: Welfare concerns are growing regarding surgical castration (SC) in pets, necessitating the need for non-surgical alternatives. Administration of vaccines against gonadotropins releasing hormone (GnRH) have been reported as alternative to SC. This study determined the effect of surgical and immunological castrations (IC) on complete blood counts, plasma testosterone (T), luteinizing hormone (LH) concentrations and ejaculate characteristics in mongrel dogs. Ten intact male dogs were randomly divided into two groups (A & B). Dogs in group A were surgically castrated, while dogs in group B were immunologically castrated with single subcutaneous injection of GnRH vaccine (Improvac®). Blood and semen were collected before SC or IC and fortnightly until sixteen weeks. Blood was analyzed for packed cell volume (PCV), white blood cell count (WBC), haemoglobin concentration (Hb), absolute neutrophil (NEUT) and lymphocyte counts (LYMP), T and LH. Sperm volume (SV), concentration (C), motility (SM), live-dead ratio (LDR) and percentage of abnormal spermatozoa were determined for the semen. Data were presented as mean ± standard deviation and compared using analysis of variance. The PCV and HB of dogs surgically castrated increased progressively up to16th week after castration but only up to10 weeks in dogs immunologically castrated. Both PCV and HB decreased progressively after 10 weeks in dogs immunologically castrated. Similarly, the WBC of dogs surgically castrated steadily increased from 2 weeks up to week 16, while it increased from 6 weeks up to 16 weeks in dogs immunologically castrated. However, PCV, Hb, WBC, NEUT and LYMP did not differ significantly ($p > 0.05$) between SC and IC. In both groups, the SV, SC, SM, LDR and percentage of abnormal spermatozoa did not differ significantly. It was therefore concluded that there is no significant haematological or endocrinological changes between surgical and immunological castration and that immunological castration may provide safer alternative.

Keywords*:* Surgical castration, immunosterilization, dogs, GnRH vaccine, Testosterone, Luteinising hormone

**Address for correspondence:* ayisat_ajadi@yahoo.com Tel: +234-806-956-0868

Manuscript Accepted: .....

INTRODUCTION

Dog overpopulation remains a serious problem in many developing countries including Nigeria despite local efforts to control population growth. In these countries, free roaming dogs are sources of ecological and social problems. They attack other animals and people, result in road accidents, frighten the public and contaminate the environment with urine and faeces (Ortega- Pacheco, 2006). Therefore, the development of effective population control measures has a high priority. Traditionally, surgical sterilization and mass euthanasia campaigns are used in developed countries but the impact has proved to be low in developing countries. Besides, the high cost of mass euthanasia campaigns is prohibitive and the often inhumane handling of the dogs is against international animal welfare regulations.

Surgical castration is the traditional method of gonadectomy in male dogs and cats **(**Reichler, 2008). However, welfare concerns are on the increase regarding surgical method of castration in most domestic animals. Such concerns are that surgery is painful and places the animal at risk because it requires anaesthesia and that surgical removal of the testes is unnatural and objectionable. A study conducted in Brazil showed that the main reasons for the avoidance of surgical castration of adopted shelter dogs included compassion (56.5%), while 11.4% of the respondents believe the procedure is unnecessary (Soto et al., 2005). A study conducted in Nigeria showed that castration is seldomly performed because majority of dog owners believed it is cruelty against the dog (Ajadi et al., 2013). Another study carried out by Canadian Veterinary Private Practitioners found complication rates of 19% following castration in male dogs (Pollari and Bonnet, 1996). Serious complications such as infections, scrotal abscesses, rupture of the surgical wound, and chewed out sutures were reported at a 1-4% frequency, with surgical castration accounting for 10% of these complications (Lund et al., 2006). This

growing concern about surgical castration has necessitated the need for the development of alternative methods of castration which would be effective and acceptable to the animal owners in terms of welfare concerns.

The ideal non-surgical castration technique should produce permanent loss of fertility, permanent loss of sexual behaviour including displays of some forms of aggressive behaviour, requires single injection, safe, with no deleterious side effects for the target and non-target species (including humans) in case of accidental exposure or self-injection, has good efficacy (high success rate in treated animals), technically feasible, stable in formulation, allow for storage and handling under field conditions and should be affordable and cost effective (Kutzler & Wood, 2006). Advantages of non-surgical castration of dogs include less technical administration compared with surgery, lack of a requirement for anesthesia or surgery packs, and avoiding removal of the testes, which is widely viewed as unacceptable in some cultures.

Immunocastration is an immunological castration method which is currently undergoing evaluation for its efficacy and adverse effects. Immunocastration works as a vaccine, stimulating the immune system to produce antibodies against the gonadotropin-releasing hormone (GnRH) (Thompson, 2000). The antibody neutralizes endogenous GnRH resulting in suppression of secretion of luteinizing hormone (LH) and follicle stimulating hormones (FSH) (Walker et al., 2007). This will result in testosterone deprivation and subsequent impairment of spermatogenesis. Vaccine against GnRH also has the advantage of suppressing sexual behaviour in males and females (Kutzler & Wood, 2006). The main concern with immunocastration is the un-quantified side effects and achieving efficacy over long periods of time following single injection. The aim of this study is to compare the effect of surgical and immunological castration on complete blood counts, plasma testosterone and luteinizing hormone concentrations and ejaculate characteristics in mongrel dogs.

MATERIALS AND METHODS

Ten adult male mongrel dogs mean weight of 10.0 ± 1.4kg, and age ranging between one and three years (mean age= 2.4 ± 0.3years) were used. They were sourced from households who used them either for security or hunting. The dogs were housed individually in concrete-floored kennels and allowed moderate exercise three hours daily. They were fed once daily on Indomie waste, supplemented with sufficient amount of proteins (fish) and palm oil, while water was provided ad libitum. They were dewormed with subcutaneous injection of 10% Levamisole hydrochloride at the dose of 10mg/kg, while external parasites were treated by dipping in Diazintol (Animal Care, Nigeria) solution. In addition, the dogs were treated for any blood protozoan parasites with intravenous injection of 5% oxytetracycline (Oxytet®, Topsurf, Vancouver, Canada) at 10mg/kg for five days. The dogs were trained for eight weeks to get them accustomed to semen collection by masturbation method and their semen was analyzed to ensure that they were fertile prior to commencement of study. Also, the dogs were adjudged to be clinically healthy based on results of physical examinations, complete blood counts and faecal examinations before the commencement of the study. Ethical approval for this study was obtained from the Research Ethics Committee, College of Veterinary Medicine, Federal University of Agriculture, Abeokuta, Ogun State, Nigeria.

Experimental Design

This study was a controlled, randomized design involving two groups. Group A comprised of five intact male dogs surgically castrated, while group B comprised of five intact male dogs treated with a single subcutaneous injection of 400 µg of Improvac ((Pfizer laboratories (Pty), Sandton, South Africa).

Experimental procedure

The dogs were weighed using puppy type weighing scale before the commencement of the study Semen was obtained from the dogs before castration and thereafter fortnightly up to sixteen weeks of castration using digital manipulation method. In addition, blood was obtained from the cephalic vein fortnightly up to sixteen weeks for the determination of complete blood counts and plasma concentration of testosterone and luteinizing hormone.

Determination of haematological variables

The Packed Cell Volume (PCV) and haemoglobin (Hb) concentration (g/dl) were determined using Mindray® BC-2800Vet automatic haematology analyser (Beckman Coulter, UK). The machine operated by sampling blood and suckling a standard amount through narrow tubing containing light detectors and electrical impedance, that determined the PCV and Hb concentration.

The total white blood cell count was determined using Mindray® BC-2800Vet automatic haematology analyser (Beckman Coulter, UK). The system made use of 10µL of blood. The red cells were lysed in the microcuvette by a haemolysing agent and the white blood cells stained by methylene blue. The camera in the analyser takes 37 images throughout the cuvette, and the cells were counted by image analysis and classified into each sub group. The total white blood cell, absolute neutrophil and lymphocyte count were then counted in less than five minutes and recorded in the range of $x10^9$/L.

Determination of Plasma testosterone concentration

The plasma concentration of testosterone was determined by radioimmunoassay technique using a fixed quantity of ^{125}I-labelled testosterone. All reagents were equilibrated to room temperature. Duplicate tubes for total counts (T), zero standard (Standard 1 = B0), standards (S2-6), control (C) and samples (Sx) were labelled. All reagents and samples were mixed thoroughly before use avoiding excessive foaming. 50 µl each of standards, control and samples were pipetted into the properly labelled tubes.50µl of tracer solution was pipetted into all tubes.50µl of the antiserum was pipetted into all tubes except T. All tubes except T were thoroughly vortex mix for 2-5 seconds. The tubes were covered with a plastic foil, and allowed to incubate at 37^{0}C for three hours. T tubes were placed on a separate tube rack. The bottle containing magnetic immunosorbent was gently shaken until homogeneity. 500 µl was added to each tube except T. All tubes were thoroughly vortexed and incubated for five minutes at room temperature. The bound fraction was separated by centrifuging all tubes for 15 minutes at 1500 rpm or greater. The supernatant was gently aspirated taking care of not disturbing the precipitate. The radioactivity of each tube was counted for at least 60 seconds or longer in a gamma counter (Cobra II, Auto Gamma, Packard Instrument Company, USA). The concentration of hormone in the sample was calculated automatically by plotting a standard curve

Determination of Plasma luteinizing hormone concentration

The plasma concentration of luteinizing hormone was determined by radioimmunoassay technique using a fixed quantity of ^{125}I-labelled luteinizing hormone. All reagents were equilibrated to room temperature. Disposable tubes (12X75mm) were labelled in duplicate for total count (TC), nonspecific binding (NSB), zero standard (Bo), standards and samples.200µl of assay buffer was pipetted into NSB tubes and 100 µl assay buffer into Bo tubes. Starting with the most dilute, 100 µl of each standard (S1-7) was pipetted into the appropriately labelled tubes.100 µl of unknown sample (Mx) was pipetted directly into appropriately labelled tubes.100 µl antiserum was then added into all tubes except NSB and TC. The TC tubes was stoppered and set aside for counting. All tubes were thoroughly vortex mixed, covered and incubated for four hours at 37^{0}C. 100 µl of tracer was then pipetted into all tubes. All tubes were again vortex mixed thoroughly, covered and incubated overnight at 37^{0}C. 400 µl of the second antibody was added into each tube except the TC. All tubes were vortexed thoroughly and incubated at 37^{0}C.The antibody bound fraction was then separated by decanting off the solution and the tubes were left for 15 minutes. All tubes were then centrifuged at 4°C for 10 minutes at 1500 rpm. After which the tubes were carefully placed into suitable decantation racks, then poured off and supernatant discarded. The tubes were then inverted and place on a pad of absorbent tissues and allow to drain for 5 minutes. The radioactivity of each tube was counted for at least 60 seconds or longer in a gamma counter (Cobra II, Auto Gamma, Packard Instrument Company, USA). The concentration of hormone in the sample was calculated automatically by plotting a standard curve

Determination of ejaculate parameters

Both the volume and the colour of the first and second fractions of the ejaculate were determined. Thereafter the sperm motility, concentration, morphology and abnormalities were then determined. Sperm motility was assessed immediately after collection. A drop of semen was placed on a slide and examined at 100X magnification using light microscope. Both the total motility (% of spermatozoa that are moving) and progressive motility (% of spermatozoa that are moving in a straight line were determined. The concentration of spermatozoa was measured on the collected ejaculate containing F1 and F2 fraction. The WBC Unopette system (Becton Dickinson, Rutherford NJ) was used. Semen was drawn up into the 20 microliter pipette and dispensed it into the 2 ml diluent container according to kit instructions. The solution was then discharge into both chambers of the Haemocytometer. The number of spermatozoa in the central 1 millimeter square (the square that fills the field using the 10X objective) was then counted. Sperm morphology was assessed by staining the semen sample with Giemsa stain (DiffQuik, Baxter Healthcare, Miami FL) and observing the cells under 1000X magnification (oil immersion). The Spermatozoa appeared purple on a clear background. Under oil immersion, 100 spermatozoa were examined and counted. The number of normal spermatozoa in 100 is the percentage morphologically normal spermatozoa. Total normal for the sample was calculated by multiplying total number of spermatozoa in the ejaculate by percentage morphologically normal. Also, the stained slide was examined for sperm abnormality. Abnormal spermatozoa were classified as primary defects (those that occur during spermatogenesis, including defects in head shape, bent midpiece, persistent proximal cytoplasmic droplet, and doubling of any portion of the spermatozoon) or secondary defects (those that occur during epididymal maturation or slide preparation, including detached heads, persistent distal cytoplasmic droplets, and bent tails).

Statistical Analysis

Data were presented as mean ± standard deviation. The packed cell volume, haemoglobin concentration, white blood cell counts, absolute neutrophil and lymphocyte counts, plasma concentration of testosterone and luteinizing hormone, sperm volume, percentage sperm motility and sperm concentration were compared between the different groups using analysis of variance (ANOVA) for repeated measures. Least square difference was used for post-hoc analysis. All statistical analyses were performed using SPSS 17.0 software.

RESULTS

The PCV and Hb of dogs surgically castrated increased progressively up to the 16th week after castration, while the PCV and Hb of dogs immunologically castrated first increased up to 10 weeks of castration and thereafter decreased progressively up to 16 weeks after castration (Fig. 1a & b). There was no significant ($p > 0.05$) difference in the PCV and Hb of surgically or immunologically castrated dogs. Following surgical castration, the WBC and NEUT of the dogs decreased at week 2 and thereafter steadily increased up to week 16 after castration. Similarly, WBC of dogs that were immunologically castrated also decreased up to week 6 after castration and thereafter steadily increased up to week 16 (Fig. 2 & 3). There was no significant difference ($p>0.05$) in the WBC & NEUT between surgically and immunologically castrated dog. However, the LYMP was significantly ($p < 0.05$) lower in surgically castrated dogs than immunologically castrated dogs (Fig. 4).

Following surgical castration, the plasma testosterone of the dogs dropped significantly ($p < 0.05$) from week 2 after castration and remained steady at that value up to week 16 after castration (Fig. 5). The plasma testosterone of dogs that were immunologically castrated also dropped from week 2after castration, reached basal level at week 6 to week 16 after castration (Fig. 5). The plasma LH of both the surgically castrated and immunologically castrated dogs decreased progressively from the base line value up to week 8 post treatment at which point

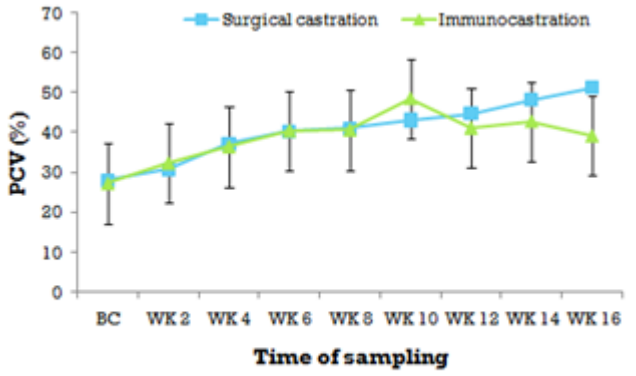


Fig 1a: Changes in packed cell volume following surgical or immunological castration in mongrel dogs

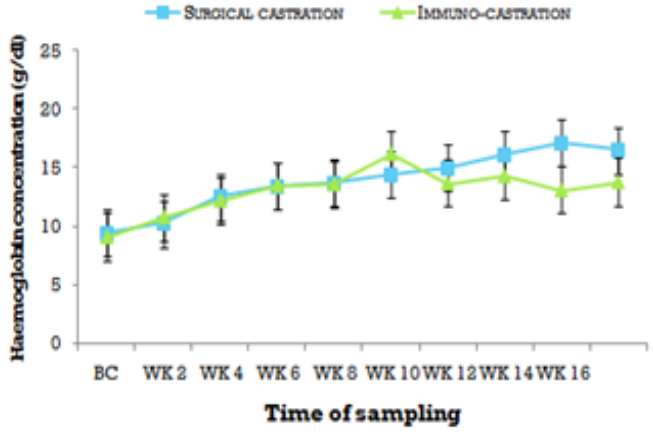


Fig. 1b: Changes in haemoglobin concentration following surgical or immunological castration in mongrel dogs

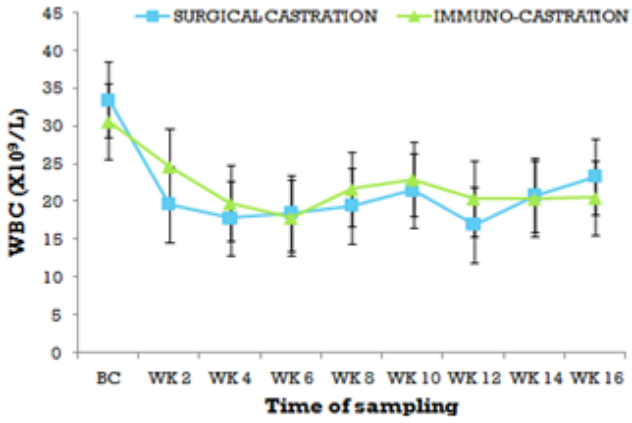


Fig. 2: Changes in total white blood cell count following surgical or immunological castration in mongrel dogs

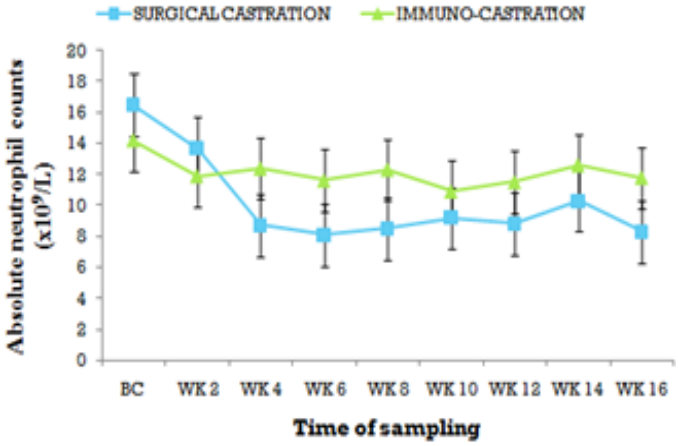


Fig. 3: Changes in absolute neutrophil counts following surgical or immunological castration in mongrel dogs

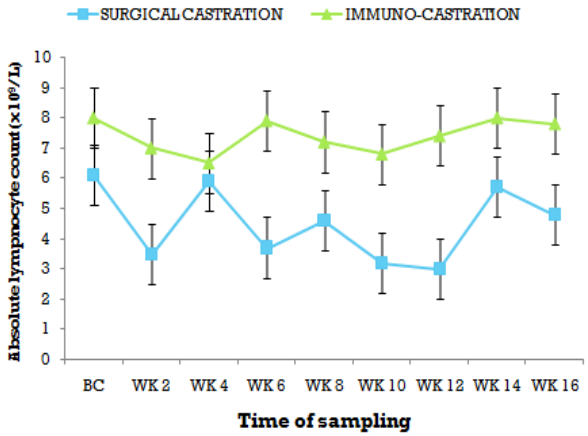


Fig. 4: Changes in plasma absolute lymphocyte counts following surgical or immunological castration in mongrel dogs

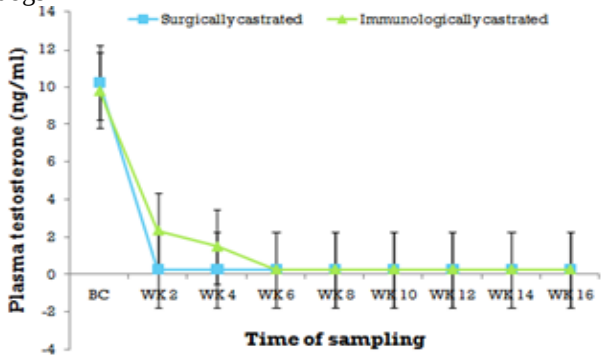


Fig. 5: Changes in plasma testosterone following surgical or immunological castration in mongrel dogs

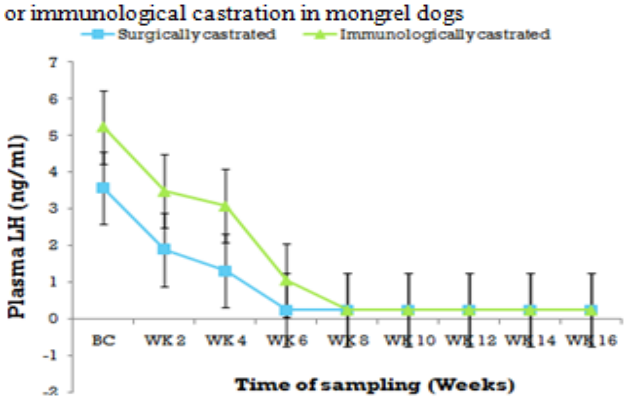


Fig. 6: Changes in plasma luteinizing hormone following surgical or immunological castration in mongrel dogs

it reached the minimum value detectable, and thereafter the plasma LH became constant up to week 16 (Fig. 6). There was no significant difference ($p > 0.05$) in the plasma LH of surgically and immunologically castrated dogs (Fig. 6).

The colour of collected semen from dogs in both groups was cloudy white and devoid of blood. As shown in table 1, the mean semen volume, sperm motility, sperm concentration and percentage live sperms were significantly ($p < 0.05$) decreased 2 weeks post-surgical castration or administration of GnRH vaccine in immunological castration and the dogs were aspermic thereafter. The percentage abnormal sperm of the dogs ranged between 4.00 ± 1.4% and 5.75 ±1.5%. and were significantly ($p< 0.05$) decreased in similar manner with the sperm characteristics (Table 1).

DISCUSSION

The result of this study showed that both surgical and immunological castration in dogs resulted in increased packed cell volume and leukocyte parameters with a decrease in the plasma concentration of testosterone and luteinizing hormone, as well as the quality of the ejaculate. This effect occurred as early as two weeks following surgical castration or administration of GnRH vaccine with the dogs becoming aspermic thereafter. Also, the effect lasted for up to sixteen weeks after castration by either method. However, there was no significant difference in all these parameters between surgically and immunologically castrated dogs.

The hypothalamic-pituitary gonadal axis has been target for the development of non-surgical castration methods. Under normal condition, GnRH stimulates the release of luteinizing hormone (LH) by binding to its receptors on the anterior pituitary (Donovan et al., 2013). LH secretion is necessary for normal testosterone synthesis in the males. Following immunization with GnRH, antibodies are produced

Table 1. Mean Semen volume, sperm motility, sperm concentration, percentage live sperm, percentage abnormal sperm of mongrel dogs following surgical or immunological castration

Time of sampling (weeks)	Semen volume (ml)		Sperm motility (%)		Sperm concentration ($x10^6$/ml)		Percentage live sperm (%)		Percentage abnormal sperm (%)	
	SC (n=5)	IC (n=5)	SC (n=5)	IC (n=5)	SC (n=5)	IC (n=5)	SC (n=5)	IC (n=5)	SC (n=5)	IC (n=5)
BC	0.68 ± 0.15^a	0.63 ± 0.15^a	73.75 ± 5.5^a	80.00 ± 5.0^a	410.00 ± 37.0^a	484.25 ± 45.0^a	83.50 ± 7.5^a	88.20 ± 7.0^a	5.75 ± 1.5^a	4.50 ± 1.5^a
2	0.25 ± 0.23^b	0.28 ± 0.14^b	26.25 ± 6.5^b	41.50 ± 5.7^b	234.75 ± 53.0^b	248.00 ± 43.0^b	36.50 ± 6.5^b	41.50 ± 5.7^b	2.75 ± 1.3^b	2.50 ± 1.2^b
4	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c
6	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c
8	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c
10	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c
12	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c
14	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c
16	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c	0.00 ± 0.00^c

BC: Before castration, SC: Surgical- castration group, IC: Immuno-castration group. Values with different superscript are significantly different at $p < 0.05$ along the same column.

which block the binding of GnRH to its receptors on the anterior pituitary thus inhibiting the secretion of LH and consequent synthesis of testosterone (Janett et al., 2009). This might have been responsible for the decreases in the plasma concentrations of the hormones beyond the detection limit. Decrease in testosterone secretion has been shown to result in testicular atrophy and disruption of normal spermatogenesis (Ghoneim et al., 2012). This may probably account for the disruption in normal spermatogenesis evidently leading to the progressive drop in the ejaculate parameters of the dogs until the dogs became aspermic.

It has been shown that testosterone is required for the maintenance of the seminiferous tubule epithelium in most mammals (Donovan et al., 2013). Withdrawal of testosterone secretion in the treated dogs might have caused severe reduction of spermatids in the treated dogs.

Sperm motility is an important characteristic in semen assessment. Spermatozoa gain motility during ejaculation as pH and bicarbonate concentration increases during mixing of sperm and seminal plasma (Vyt et al., 2004). The motility of the spermatozoa in the present study decreased progressively until the dogs became azoospermic in both groups. This is contrary to the findings in pigs (Bilskis et al., 2012) and may be related to the inability of the dogs to produce seminal plasma fluid.

There was no significant difference in the percentage of abnormal sperm cells in both groups. This result was expected because vaccination indirectly locks the release of FSH which is required for normal spermatogenesis, while surgically removal of the testis might have send a negative feedback to the hypothalamus to inhibit the secretion of LH and FSH. In conclusion, administration of single injection of GnRH vaccine to dogs resulted in decrease in the size of the testis and destruction of the germinal epithelium of the seminiferous tubules with resultant decrease in the ejaculate parameters until the dogs became azoospermic.

The advantage of the immuno-castration over surgical castration will be its virtual lack of any adverse effects and the ability to suppress semen production as early as two weeks following administration. In addition, relative cheap cost provides an additional advantage over surgical castration. The vaccine can be incorporated into the routine immunization plans for dogs and thus be very useful in developing countries where there is lack of surgical facilities. In conclusion, immuno-castration with GnRH vaccine appears to have advantage over surgical castration because of its lack of adverse effect and the lower cost when compared with surgical castration. However, the reported inability of the vaccine to produce a long lasting sterility may be a limitation especially where permanent sterility is required.

REFERENCES

Bilskis, R., Sutkeviciene, N., Riskeviciene, V., Januskauskas, A., Zilinskas, H. (2012). Effect of active immunization against GnRH on testosterone concentration, libido and sperm quality in mature *AI boars*. Acta Veterinaria Scandinavia 54(1): 33-39

Donovan, C. E., Grossman, J. L., Lamb, S., Bobe, G., Kutzler, M. A. (2013)**.** *Effects of* commercial canine gonadotropin releasing hormone vaccination on intact Ilamas and Alpacas. Journal of Vaccine.

Ghoneim, I. M., Waheed, M. M., Al-Eknah, M. M., El-Bahr, S. M. (2012). Immunization against GnRH in the male camel (Camelus dromedaries): effect on sexual behaviour, testicular volume, semen characteristics and semen testosterone concentration. Theriogenology 78(5): 1102-1109.

Janett, F., Stump, R., Burger, D., Thun, R. (2009)**.** Suppression of testicular function and sexual behaviour by vaccination against GnRH (Equity) in adult stallion. Animal Reproduction Science 115 (1-4): 88-102.

Kutzler, M. and Wood, A. (2006). Non-surgical methods of contraception and sterilization. Theriogenology 66(3): 514- 525.

LaCroix, C. E. (2006). Evaluation of a single intra-testicular injection of zinc gluconate neutralized by Arginine (Neutersol ®) as a chemical sterilant in sexually matured male. International Symposium on Non-Surgical Contraceptive Methods for Pet Population Control Pp 1-7

Levy, J. K., Crawford, P. C., Appel, L. D., Clifford, E. L. (2008). Comparison of intratesticular injection of zinc gluconate versus surgical castration to sterilize male dogs. American Journal of Veterinary Research 69: 140-14

Oliveira, E. C. S., Moura, M. R., Silva, V. A., Peixoto, C. A., Saraiva, K. L. A. (2007). Intra-testicular injection of a zinc-based solution as a contraceptive for dogs, Theriogenology 68: 137-145.

Ortega-Pacheco, A. (2006). Reproduction of dogs in the tropics. Doctoral Thesis. Swedish University of Agricultural Sciences, Uppsala

Thompson, D. L. (2000). Immunization against GnRH in male species (comparative aspects). Animal Reproduction Science 60: 459-469.

Vyt, P., Maes, D., Rijsselaere, E., Dejonckheere, E., Castryck, F., Van Soom A. (2004). Motility assessment of porcine spermatozoa: a comparison of methods. Reproduction in Domestic Animals 39: 447-453.

Walker, J., Ghosh, S., Pagnon, J. (2007). Totally synthetic peptide based immunocontraceptive vaccines show activity in dogs of different breeds. Vaccine 25(41):7111-7119

Wiebe, V. J., Howard, J. P. (2009). Pharmacological advances in canine and feline reproduction. Topics in Companion Animal Medicine 24(2): 71-99

Niger. J. Physiol. Sci. 31(June 2016) 043-047
www.njps.com.ng

Niger. J. Physiol. Sci.

Frequency of Hepatitis B and C Co-Infection in Chronic Liver Disease Patients in Calabar, Cross River State, Nigeria

M. Kooffreh-Ada [1], D. C. Okpokam[2], Z. A. Okaormhe [3], V. U. Nna[4]

'Department of Internal Medicine (Gastroenterology Unit), University of Calabar Teaching Hospital, Calabar, Nigeria,[2] Department of Medical Laboratory Science (Haematology Unit), University of Calabar, Calabar, Nigeria,[3] Department of Medical Laboratory Science (Chemical Pathology Unit), University of Calabar, Calabar, Nigeria, [4]Department of Physiology, Faculty of Basic Medical Sciences, College of Medical Sciences, University of Calabar, Calabar, Cross River State, Nigeria.

Summary: Hepatitis B (HBsAg) and C (HCV) virus are becoming a significant causative factors in the aetiology of chronic liver disease (CLD) worldwide. However, the information on the frequency of HBsAg and HCV virus co-infection in CLD is sparsely reported in Nigeria. In this study, we assessed the frequency of HBsAg and HCV co-infection in CLD. One hundred and eleven subjects aged 19 - 76 years, comprising of 76 CLD patients and 35 apparently healthy subjects without CLD were tested for both HBsAg and HCV virus antibodies using ELISA test kits. Out of the 111 subjects recruited for this study, 76 (68.5%) were CLD patients tested positive for HBsAg and 35 (31.5%) tested negative for HBsAg and served as control. Out of the 76 CLD patients that tested positive for HBsAg, 34 (44.7%) of them also tested positive for HCV, thus, having co-infection with HBV. Incidence of co-infection was highest in those aged 36 - 45 years, and greater in males than females. Among the control group, 4 (11.4%) of the subjects (3 males and 1 female) tested positive for HCV, while 31 (88.6%) subjects (20 males and 11 females) tested negative. This work has shown that the co-infection with HBV and HCV among chronic liver disease patients and the incidence of HCV is high in our locality. Also, some of the supposed apparently healthy subjects in this study tested positive for HCV, hence the need for improving the awareness of this virus. It is therefore necessary to give immunization and test for HBsAg and HCV in both rural and urban areas.

Keywords*:* Chronic liver disease, Co-infection, Hepatitis B, Hepatitis C, Calabar

**Address for correspondence:* oghalove@gmail.com Tel: +2348068896860

Manuscript Accepted: June, 2016

INTRODUCTION

Viral hepatitis is an infection of the liver caused by a group of viruses having affinity for the liver and causing an overlapping pattern disease. These viruses include hepatitis A, B, C, D, E, G (Crawford, 1999). Hepatitis B virus, a major public health problem worldwide is more prevalent in the developing countries (WHO, 2000).

Hepatitis B virus, which causes serious liver damage is one of the World Health Organization's (WHO's) target for global eradication by 2020 (Dusheiko *et al*, 1999). The virus can be passed on through blood transfusion or sexual contact, and has an incubation period ranging from 3 weeks to several years before any symptoms appear (Peter and Tokyo, 2000). It is a resilient virus that can exist on almost any surface for about 1 month. More than 2 billion people are infected with HBsAg worldwide, while some 280 million are chronic carriers harbouring the virus in their liver (Clement *et al*, 1990). Sometimes, one may be co-infected with HDV/HBV. Okpokam *et al*, (2015), reported high rate of HDV/HBV coinfection in Calabar, Nigeria, which was higher in males than females.

Hepatitis C is an infected disease caused by HCV. The infection is often asymptomatic, but chronic infection can lead to scarring of the liver and ultimately to cirrhosis, which is generally apparent after many years. In some cases, those with cirrhosis will go on to develop liver failure, liver cancer, or life threatening esophageal and gastric varies (Ryan and Ray, 2004). Hepatitis C differs from HBsAg because it tends to stay longer in the body not causing any problem (Otegbayo *et al.*, 2012). The primary route of transmission in the developed world is intravenous drug use (IDU), while in the developing world, the main routes are blood transfusion and unsafe health procedures (Maheshwari *et al*, 2008). Hepatitis C virus has become a leading cause of CLD worldwide (Edemariam, 2004). An estimated 130 - 170 million people worldwide are infected with hepatitis C. In Nigeria, about 6 - 20% of the population are infected with Hepatitis C virus (Abiodun and Agumadu, 2012). This study therefore seeks to determine the incidence of HCV in CLD patients tested positive for HBsAg in the University of Calabar Teaching hospital, Calabar,

Cross River State, Nigeria.

MATERIALS AND METHODS

Study place and duration

This study was done in the Medicine out-patient and Haematology Department, University of Calabar Teaching Hospital (UCTH), Calabar, Cross River State, from September 2012 to March 2013.

Subjects

A total of one hundred and eleven (111) subjects were used for this study. Out of the 111 subjects, 76 were CLD patients visiting the University of Calabar Teaching Hospital (UCTH) and 35 were apparently healthy (control) subjects. Biodata and consent from each of the subjects was taken for this study in order to fulfil the ethical guidelines of research conducted on humans. The inclusion criteria for the selection of the 76 CLD patients for this study were presence of jaundice, ascites, hepatomegaly, edema while the laboratory investigations were prothrombin time test and deranged liver function test (alanine amino transferase). The control subjects were also subjected to clinical examination and laboratory investigations and their results showed absence of CLD. Out of the 111 subjects, 69 were males and 42 were females. Moreover, out of the selected 76 CLD patients that were positive for HBsAg using ELISA technique, 46 were males and 30 were females, while out of 35 apparently healthy subjects that were negative for HBsAg, 23 were males and 12 were females. These positive (76 patients) and negative (35 apparently healthy) subjects were then screened using ELISA method for hepatitis C. Both ELISA test is a solid-phase microtiter plate coated with monoclonal antibodies to human IgM which is based on "sandwich principle". ELISA for hepatitis B, HBsAg test kit (Catalog number KAPG4SGE3, DIAsource ImmunoAssays, Belgium) was used. For hepatitis C, Anti-HCV ELISA 4.0 test kit (Catalog number KAPG4NAE3, DIAsource ImmunoAssays, Belgium) was used.

Statistical analysis

PRIMER version 17 was used for statistical analysis of this study. The Chi-square (X) test was performed for quantitative variables to check for relationship of HBV and HCV infection. Percentages were calculated directly for HBV and HCV. P = 0.05 was used as the accepted significance level.

RESULTS

Subject distributions

One hundred and eleven (111) subjects were recruited in this study. Seventy-six (76) were selected patients attending Medicine out-patient Department (MOPD) of the University of Calabar Teaching Hospital (UCTH). These 76 patients were CLD patients and all tested positive for Hepatitis B surface antigen (HBsAg) while thirty-five (35) were apparently healthy control subjects and all tested negative for HBsAg (Figure 1).

Age distribution of subjects

HBsAg and HCV seropositivity were not statistically significant (P > 0.05) when associated with age group as shown in table 1 above. In this study, the seropositivity of 111 subjects was higher in those aged 26 - 35 years than those aged 36 - 45 years. This was similar to HBsAg seropositive subjects. However, seropositivity of HCV subjects was higher in those aged 36 - 45 years than those aged 26 - 35 years (Table 1).

Gender distribution of subjects

According to gender, the seropositivity of 111 subjects in this study was highest in males (62.2%) than in the females (37.8%). The same applied to those in HBsAg and HCV. There was no statistically significant difference (P > 0.05) between gender and seropositivity rate in this study (Table 2).

Co-infection of HBV and HCV

All 76 CLD patients tested positive (100%) for HBsAg and 34 (44.7%) tested positive for HCV, while 42 (55.3%) tested negative for HCV (Figure 2). Out of the 76 CLD patients positive for HBsAg, 46 (60.5%) were males and 30 (39.5%) were females, while in those CLD patients positive for HCV, 22 (64.7%) of the patients were males and 12 (35.3%) were females. However, in those tested negative, 24 (57.1%) were males and 18 (42.9%) were females (Figure 3). Among the 35 apparently healthy subjects, we observed that 4

Table 1: Seropositivity of HCV and HBsAg according to age

Age Group	Subjects Tested (%)	Subjects Tested Positive (%) for HCV	Subjects Tested Positive (%) for HBsAg
19-25	19(17.1)	5(13.2)	16(17.8)
26-35	44(39.6)	12(31.6)	36(40.0)
36-45	28(25.2)	14(36.8)	21(23.3)
46-55	13(11.7)	4(10.5)	10(11.1)
56-65	3(2.7)	1(2.6)	3(3.3)
>76	4(3.6)	2(5.3)	4(4.4)
Total	111 (100)	38(100)	90(100)

Table 2: Seropositivity of HCV and HBsAg according to gender

Sex	Subjects Tested (%)	Subjects tested Positive (%) for HCV	Subjects Tested Positive (%) for HBsAg
Male	69(62.2)	25(65.8)	55(61.1)
Female	42(37.8)	13(34.2)	35(38.9)
Total	111(100)	38(100)	90(100)

P>0.05

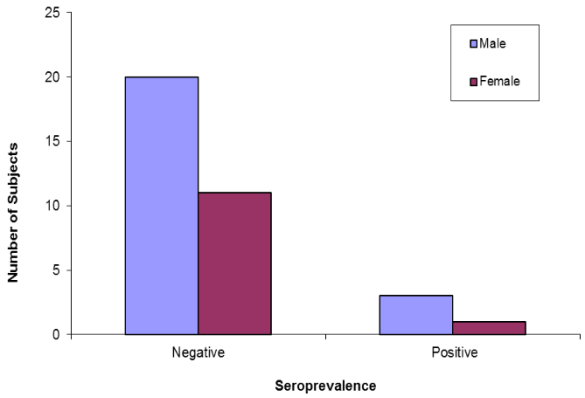


Figure 1: Subject Distribution

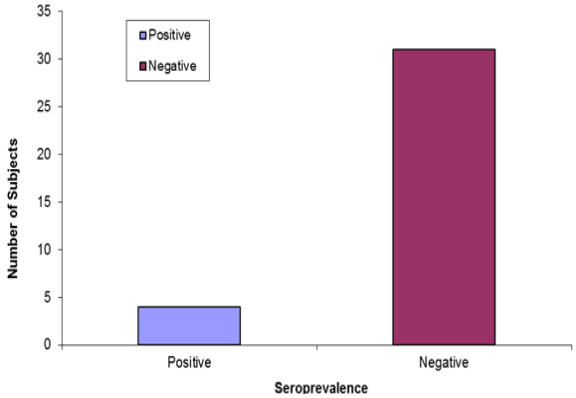


Figure 2: Seropositivity and seronegativity of HBsAg and HCV among CLD patients

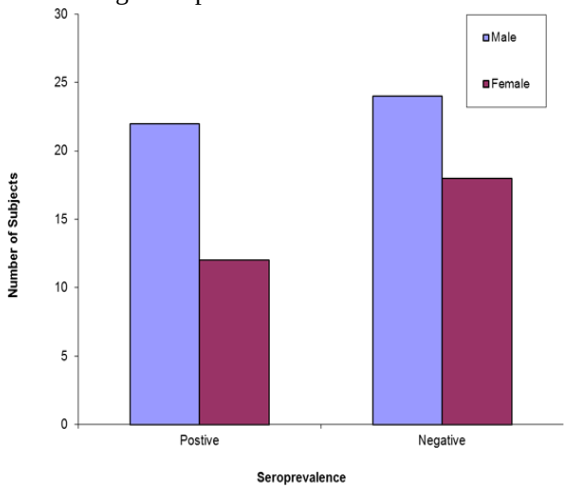


Figure 3: Seropositivity and seronegativity of HCV among CLD patients based on sex

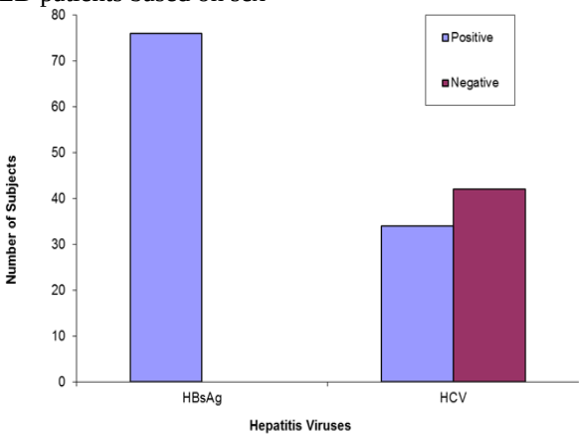


Figure 4: Frequency of HCV in 35 apparently healthy subjects (control)

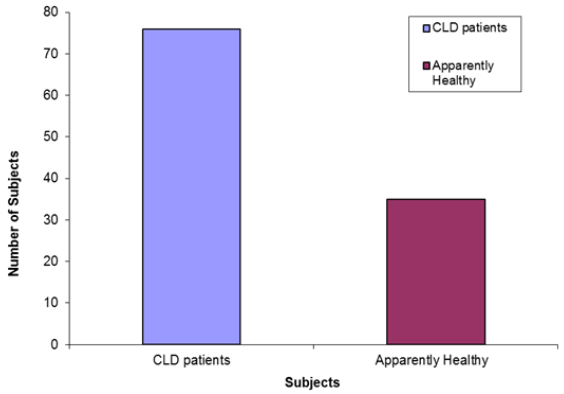


Figure 5: Frequency of seropositive and seronegative HCV among 35 apparently healthy subjects (control) based on sex

(11.4%) tested positive for HCV (3 males and 1 female), while 31 (88.6%) tested negative for HCV (20 males and 11 females) (Figures 4 and 5).

DISCUSSION

Hepatitis B virus and HCV share common transmission pathways. Therefore, coinfection can be anticipated. The percentages of 111 subjects at various age groups were similar with a mean of 36.2 ± 1. 17 and showed no significant difference (P > 0.05). However, we observed that the majority of 111 subjects were in the age group 26 - 35 years which are in the third decade of life. From the results obtained from this study, those in age group 26 - 35 years had the highest number of positive HBsAg (81.8%) while those with HCV (50.0%) had the highest number of positive in age group 36 - 45 years. Inyang-Etoh *et al*, (2014), also reported recently in their study in Calabar that those in age group 31 - 40 years had the highest prevalence rate with HCV infection (7.3%). The percentages of HBsAg and HCV infection according to gender showed that in both infections (HBV and HCV), males (61.1% and 65.8%) were more infected than females (38.9% and 34.2%) respectively, but there was no statistically significant difference (P > 0.05) based on gender in this study. In this study, the results of co-infection with HBsAg and HCV among 76 CLD patients showed that all were positive for HBsAg using ELISA test and 34 (44.7%) of the CLD patients were also positive for HCV, thus, showing co-infection with HBV. Out of the 34 (44.7%) CLD patients that were positive for HCV, 64.7% were males while 35.3% were females. The reason for the co-infection with HBV and HCV could be attributed to the fact that both virus infection presupposes from same source and also have same transmission pathway. Although, the number of samples used in the study is few, it still indicates the interactions between the two viruses which occur in chronic infections like in our study. This study conforms with the findings by Omuemu *et al*, (2012), which assessed 115 subjects with CLD. In their study, the prevalence of HBsAg in the CLD patients was 40.9%, of which 85.1% were

males and 14.9% were females, while prevalence of HCV among CLD patients was 1.7% of which all (100%) were males. In Pakistan, 52 patients with positive HBsAg and anti-HCV antibodies were included in their study, 32 (61.5%) were males and 20 (38.5%) were females. The percentage of HBV infection was 38.85% while percentage of HCV infection was 24.0%. Their mean age in years was 40 ± 10.125 SD (Mazhar *et al*, 2014). Also, in this study, the CLD patients were not classified into their various subtypes (i.e. chronic hepatitis, liver cirrhosis and HCC) so as to know the stage and duration when the infection occurs. A study by Ola *et al*, (2004) in Ibadan, South-Western Nigeria found HCV infection in 20% of their patients with liver cirrhosis and 14% of their patients with HCC. In another study, in Ibadan, Olubuyide *et al* (1997), found HCV infection in 18.7% of their patients with HCC. Furthermore, Shehu, (2002), in his study in Jos, North-Central Nigeria found that 11.8% of their patients with CLD had evidence of HCV infection, which is lower than the percentage (44.7%), observed in our study. It was also observed that 11.4% of our apparently healthy subjects tested positive for HCV, of which 3 were males while 1 was female. They were found not tested positive for HBsAg. The reason for this could also be linked to lack of awareness and unavailability of vaccines for HCV.

In our study and locality, HCV has been observed to be more in males than in females. According to recent work done by Inyang-Etoh *et al*, (2014) in Calabar, it was discovered that males were more infected with HCV (5.6%) than females (2.2%), but there was no statistically significant difference ($P > 0.05$) in the infections. The World Health Organisation (WHO) at the sixty-third World Health Assembly in 2010 had recognised HCV as a growing public health threat (WHO, 2011). The reason for more males in this study than females could be due to high risk behaviour of men having this viruses for example; having multiple sexual partners, scarification, living in a crowded environment, being uneducated, etc. Bwogi *et al*, (2009), reported that rural residence could also be risk factors for HBV and HCV infection. Also, socioeconomic conditions, especially in the rural areas, may contribute to HBV and HCV exposure.

This study has shown that there appear to be a high percentage of CLD co-infection with HBV and HCV in our locality than in other studies mentioned. Therefore, we recommend nonstop public awareness of both infections especially to the grass root level, sticking to one sex partner, using of protective devices like condom, better environmental sanitation,
drinking safe water, complete immunization against HBV and introducing vaccines for HCV. We also advocate that those infected should take adequate nutritionally balanced meals and develop a better attitude towards taking their drugs. Larger sample size using advanced parameters like the molecular biology techniques [polymerase chain reaction (PCR)] should be carried out in our locality to determine dominant HBV and HCV infection.

REFERENCES

Abiodun P. O., Agumadu U. H. (2012). Hepatitis B virus infection in patients with homozygous sickle cell disease, Need for intervention. *Anna Biomed Sci*. 1: 79-87.

Bwogi J., Braka F., Makumbi I., Mishra V., Bakamutumaho B., Nanyunja M. A., Opio A., Downing R., Biryahwaho B., Lewis R. F. (2009). Hepatitis B infection is highly endemic in Uganda: findings from a national serosurvey. *African Health Sciences,* 9(2):98 - 108.

Clement C. J., Sodeinde O., Odeola H. A., Ayoola E. A. (1990). Survey of Hepatitis B vaccine joins fight against Pandemic disease. *World Health Forum.* 11: 165-8.

Crawford J. M. (1999). Viral hepatitis. In Robbins Pathologic basis of disease. 7th ed. India. 701-706.

Dusheiko G. M., Khakoo S. S., Grellier L. (1999). A national approach to the management of Hepatitis C infection. *British Medical Journal.* 312: 351-364.

Edemariam T. (2004). The liver. In: Principles of medicine in Africa. Parry e, Geoffrey R, Mabey D, gill G (eds). 3rd ed. Cambridge University Press; p.991-1007.

Inyang-Etoh P. C., Eyo G. O., Philip-Ephraim E. E. (2014). Occurrence of hepatitis 'B' and 'C' among patients on antiretroviral drug therapy (ART) in a treatment centre in Calabar, Nigeria. IJMMS. 6(6): 158-160.

Maheshwari A., Ray S., Thuluvath P. J. (2008). Acute hepatitis C. *Lancet.* 372(9635): 321332.

Mazhar S. B., Ziauddin S. A., Muhammad I. S. (2013). Frequency and Clinical presentations of hepatitis B and C virus co-infection in tertiary care hospital, *Rawal Medical Journal,* 38(1): 11 - 14.

Okpokam D. C., Kooffreh-Ada M., Okhormhe Z. A., Akpabio E. N., Akpotuzor J. O., Nna V. U. (2015). Hepatitis D Virus in Chronic Liver Disease Patients with Hepatitis B Surface Antigen in University of Calabar Teaching Hospital, Calabar, Nigeria. *British Journal of Medicine and Medical Research.* 6(3): 312-318

Ola S. O., Odaibo G. N., Olaleye O. D. (2004). HCV and HBV infections in Nigerian patients with Liver cirrhosis and Hepatocellular carcinoma. *Nig Q J Hospital Med,* 14: 3 - 4.

Olubuyide I. O., Aliyu B., Olaleye O. A., Ola S. O., Olawuyi F., Malabu U. H. (1997). Hepatitis B and C virus and Hepatocellular carcinoma. *Trans R Soc Trop Med Hyg,* 91(1): 38 - 41.

Omuenu C. E., Ndububa D. A., Nnabuchi V. C. (2012). Prevalence of Hepatitis B Surface Antigen and anti-hepatitis C antibodies in chronic liver

disease patients in a Nigerian Teaching Hospital. *Journal of Medical Research and Practice,* 2: 23- 6

Otegbayo J. A., Olusegun I. A., Anyinwu S. N., OLadejo O. L., Ndububa D. A., Nwsu M. N., Odike M. A., Agbakwuru E. A., Akere A., Ola S. O., Soyemi M. O, Okonkwo U. C. (2012). Characteristics of inflammatory blood disease in three tertiary health centers in Southern Nigeria. *West African Journal of Medicine.* 31(1): 28033.

Peter H., Tokyo O. (2000). Hepatitis B and its incubation period. http/www/scientistsmagTech.org.

Ryan K. J., Ray C. G. (2004). Shenis Medical Microbiology 4th ed. McGraw Hill; 551-552.

Shehu M. Y. (2002). Prevalence of hepatitis C virus antibodies among patients with chronic liver disease at the Jos University Teaching Hospital. A dissertation submitted to the West African College of Physicians in partial fulfilment of the requirements for the award of Fellowship of the college.

World Health Organization (WHO), (2000). Hepatitis B. http://www.none who.inf/inf- fs/en/fact204.html

World Health Organisation (WHO) (2011). "Hepatitis C". June 2011.

Niger. J. Physiol. Sci. 31(June 2016) 049-053
www.njps.com.ng

Niger. J. Physiol. Sci.

Plasma Adenosine Deaminase Enzyme Reduces with Treatment of Pulmonary Tuberculosis in Nigerian Patients: Indication for Diagnosis and Treatment Monitoring

Ige O.[a], Edem V.F.[b] and Arinola O.G.[b,*]

[a]Department of Medicine, University of Ibadan, Ibadan, Nigeria [b] Department of Chemical Pathology, University of Ibadan, Ibadan, Nigeria.

Summary: Tuberculosis(TB)-specific host biomarkers for diagnosis and monitoring of treatment response have been identified as priorities for TB research. Macrophage and T cell lymphocytes play vital roles in *Mycobacterium tuberculosis* immune response and their associated biomarkers could form good candidates for diagnosis and treatment monitoring. The enzyme adenosine deaminase (ADA) is produced mainly by monocytes and macrophages and increase in biological fluids in the course of infection with microorganisms infecting macrophages. This study comprised sixty-eight (68) participants; twenty-four (24) multi-drug-resistant TB(MDR-TB) patients, twenty-four (24) drug-sensitive TB patients(DS-TB) and twenty (20) non-TB apparently healthy individuals. Five (5) milliliters of blood was drawn before commencement of chemotherapy and 6 anti-TB therapy. In DSTB and MDR-TB patients before commencement of chemotherapy and 6 months of anti-TB treatment, the mean plasma levels of ADA were significantly increased compared with control. At 6 months of anti-TB chemotherapy of DSTB or MDR TB patients, ADA level was significantly decreased compared with before chemotherapy. Plasma ADA in DSTB patients before and 6 months of chemotherapy were not significantly different compared MDR TB patients. Plasma ADA level is a promising biomarker for the screening and treatment monitoring of pulmonary tuberculosis but not to differentiate MDR TB from DSTB patients.

Keywords*:* Tuberculosis Patients, Biomarkers, Chemotherapy, Lymphocytes.

**Address for correspondence:* drarinolaog64@yahoo.com; +2348023451520

Manuscript Accepted: June, 2016

INTRODUCTION

Tuberculosis (TB) is a major global health challenge. It causes ill-health among millions of people each year and ranks alongside the human immunodeficiency virus (HIV) as a leading cause of death worldwide (WHO, 2015). Recent estimates indicate an incidence of 9.6 million new TB cases with 1.5 million deaths annually (WHO, 2015). In addition, the global emergence of multidrug-resistant TB, extensively drug-resistant TB, and more recently, totally drug-resistant TB present a formidable challenge to TB control especially in sub-Saharan Africa, Asia and Eastern Europe (Alexander and De, 2007).

Timely diagnosis and proper treatment of TB have been identified as essential factors for successful TB control. It is estimated that availability of a widely used rapid diagnostic test for TB could avert 625,000 TB deaths annually (Keeler et al, 2006). Also, studies have demonstrated delays in TB diagnosis due to drawbacks of the presently available diagnostic tools (Storla et al, 2008; WHO, 2006). Mycobacterium culture that is the gold standard for TB diagnosis takes eight weeks before result is available. Sputum smear microscopy, a quick screening method is not a sensitive method while polymerase chain reaction (PCR) test is expensive, requires sophisticated equipment and cannot be used for monitoring treatment response (Adekambi et al, 2015). Hence, there is need for more biomarkers to monitor treatment and diagnosis of TB.

Adenosine deaminase (ADA) is an enzyme of the purine metabolic pathway (Shore, 1981). Itcatalyses the irreversible conversion of adenosine and 2' deoxyadenosine to inosine and 2' deoxyinosine respectively (Piras et al, 1978). ADA is essential for proliferation and differentiation of lymphoid cells, especially T cells, and is essential in the maturation of monocytes to macrophages. High concentration of adenosine or deoxyadenosine as a result of non-conversion to inosine or deoxyinosine is toxic to lymphocytes and macrophages (Zavialov et al, 2010). Also, ADA deficiency has a direct effect on the lungs, aslung damage and inflammation have been associated with elevated adenosine and deoxyadenosine in lungs of ADA-Severe Combined Immuno-Deficient patients (Blackburn et al, 1998). Both adenosine and 2' deoxy

adenosine have potent physiological effects on cells. Adenosine elicits its actions on cells by engaging G proteins coupled with receptors on the cell surface (Olah and Stiles, 1995) while 2' deoxyadenosine has been associated with disruption of cell growth and development and influence apoptosis (Liu et al, 1996). These pathways have been identified to play important roles in many aspects of lung inflammation and damage (Jacobson and Bai, 1997).

Given the roles T cells and macrophages play in protection against *Mycobacteriam tuberculosis* (Mtb) infection, ADA levels may be reflective of shifts in protection against Mtb infection and treatment response.Data regarding ADA levels in PTB concentrated in pleural, peritoneal and pericardial fluids has been documented (Cimen et al, 2008; Greco et al, 2003). Moreover, few reports regarding blood levels of ADA in PTB are inconsistent, was not determined in treatment follow-up and did not classify TB into DSTB and MDR TB (Boonyagars and Kiertiburanakul 2010; Afrasiabian et al, 2013)..This study determined plasma ADA levels in DSTB and MDR TB pulmonary TB patients before and at 6 months of anti-TB chemotherapy compared with non-TB controls.

MATERIALS AND METHODS

Study participants

Sixty eight (68) participants were recruited for this study which comprised of twenty four (24) MDR-TB patients, twenty four (24) drug-sensitive TB patients and twenty (20) non-TB apparently healthy individuals after obtaining written informed consent. MDR-TB patients had been previously diagnosed as being infected with isoniazid and rifampicin resistant strains of Mtb using clinical history, chest Xray and GENE Xpert test and were admitted into the MDR TB centre, University College Hospital (UCH) Ibadan, Nigeria for treatment. DS-TB patients were recruited from the Medical Out-patient clinic, University College Hospital, Ibadan, Nigeria by a consultant Chest Physician after Zeihl Neelsen staining technique, Sputum culture, chest X-ray and clinical history. The study protocol was reviewed and approved by the University of Ibadan/University College Hospital Institutional Research Ethics Committee.

Five (5) milliliters of blood was drawn from the anti-cubital fossa vein into lithium heparin tubes before commencement of chemotherapy and after 6 months of anti-TB therapy. Blood samples were centrifuged and plasma obtained were analyzed.

PTB Treatment protocol

All bacteriologically confirmed MDR-TB patients received intensive phase for 6-8 months in the hospital followed by 12 months of continuation phase in the community based on World Health Organization (WHO) updated guidelines in 2011 (WHO, 2011). Standardized treatment regimen was used including five drugs: kanamycin/Amikacin, Levofloxacin, Prothionamide, Cycloserine, Pyrazinamide (with Pyridoxine). This present study was conducted during the intensive phase of treatment.

Sputum smear positive DSTB patients received DOTS intensive phase for 2 months and 4 months continuation in the hospital based on WHO updated guidelines in 2011 (WHO, 2011). Standardized treatment regimen with fixed drugs containing; Rifampicin, Isoniazid, Pyrazinamide and Ethambuthol during intensive phase, and Rifampicin and Isoniazid in continuation phase, were used.

Biochemical analysis

Enzyme-linked immunosorbent assay (ELISA) was used for the measurement of adenosine deaminase (Human ADA; Lot: AK0016MAR21058, Elabscience, China). Assay protocol was as specified by the manufacturer and the absorbance was measured at 450nm with an ELISA reader (SpectraMax Plus 384, Molecular Devices LLC, USA).

Statistical analysis

Data obtained were analyzed using statistical package for social sciences (SPSS) version 17.0. Independent Student t-test was used to compare the mean values of PTB patients and controls while paired t-test was used to compare the mean values of PTB patients before commencement of chemotherapy and 6 months of anti-TB chemotherapy. Values were considered significant at $p<0.05$.

RESULTS

In DSTB and MDR TB patients before and at 6 months of anti-TB treatment, the mean plasma levels of ADA were significantly increased when compared with control. (Table 1)

At 6 months of anti-TB chemotherapy of DSTB or MDR TB patients, mean ADA level was significantly decreased when compared to the mean value before

Table 1. Mean comparison of ADA levels in PTB patients with controls

Group	ADA (ng/ml)	t	p
Control	28.09±6.15		
DSTB 0	79.38±10.98	16.508	0.000*
DSTB 6 months	37.56± 6.84	4.222	0.000*
MDR 0	72.04 ± 17.64	9.453	0.000*
MDR 6 months	38.23±6.47	4.666	0.000*

*Significant at $p<0.05$ compared with control

DSTB 0 = Before commencement of chemotherapy in drug sensitive TB, DSTB 6 months= 6 months of chemotherapy in drug sensitive TB, MDR 0 = Before commencement of chemotherapy in multidrug resistant TB, MDR 6 = 6 months of chemotherapy in multidrug resistant TB

Table 2. Mean comparison of ADA level in DSTB and MDR TB patients before and at 6 months of anti-TB chemotherapy

Group	DSTB	MDR	t	p
0 months	79.38±10.98	72.04 ± 17.64	1.498	0.143
6 months	37.56± 6.84	38.23±6.47	-0.300	0.766
t'	14.080	8.543		
p'	0.000*	0.000*		

t,p – DSTB compared with MDR-TB, t',p' – 0 months compared with 6 months

chemotherapy in DSTB or MDR TB patients respectively. But the level of ADA in DSTB patients at 6 months of chemotherapy was not significantly different compared with ADA level of MDR TB patients at 6 months of chemotherapy. (Table 2)

DISCUSSION

TB-specific host biomarkers for diagnosis of active TB and monitoring of treatment response have been identified as priorities for TB research (Wallis et al, 2009). These biomarkers are being explored to reduce disease misdiagnosis, ensure proper prognostication, monitor treatment response, and provide markers for evaluating efficacy of newly developed therapeutic drugs and vaccines (Rozot et al, 2015; Bloom et al, 2013). Macrophages and T cells play important roles in the formation of lung granulomas and eventual cavity formation, which are key features of TB immunopathology in active lesion (Ernst, 2012). ADA is produced mainly by monocytes and macrophages and is increased in biological fluids in the course of infection with microorganisms infecting macrophages (Boonyagars and Kiertiburanakul, 2010).

This present study found increased plasma ADA in both DSTB and MDR TB patients when compared with controls. This shows that ADA level is useful in differentiating Mtb infected patients from uninfected controls. Our finding is supported by previous studies that reported increased ADA in serum of pulmonary TBpatients (Afrasiabian et al, 2013; Srinivasa Rao et al, 2010; Cimen et al, 2008). Our previous study shows slight increases in total white blood cells count, percent leucocyte migration and percent nitroblue tetrazolium index in TB patients at diagnosis compared with controls (Edem and Arinola, 2015). Increased plasma ADA in PTB patients compared to controls in this present study might be due to activation, proliferation and differentiation of monocytes to macrophages which presents Mtb antigen toCD4+ T cells. Full functionality of Cell Mediated Immunity has been associated with normal lymphocyte metabolism regulated partially by the purine salvage enzyme such as ADA (Giblette et al, 1972). Studies have also shown that monocytes undergoing differentiation and macrophages continuously secrete ADA which induces proliferation of CD 4+ T cells (Zavialov et al, 2010). This is therefore indicative of continuous activation of CMI in TB patients, thus supporting clinical usefulness of plasma ADA as a biomarker for PTB diagnosis. Other studies have demonstrated increased ADA in effusion fluids in extra-pulmonary tuberculosis (Mathur et al, 2006; Zaric et al, 2007 and Gupta et al, 2010;).

At 6 months of anti-TB chemotherapy, plasma ADA level decreased significantly in TB patients compared with ADA levels before commencement of chemotherapy. At 6 months of chemotherapy when plasma ADA levels were reduced compared with before chemotherapy, sputum smear microscopy showed that the patients were sputum smear negative for Mtb.This finding is similar to the report of Swami (2016) who reported decrease in pleural fluid ADA following anti-TB treatment. Our previous report showed slight decreases in white blood cell counts, percent leucocyte migration and percent nitroblue tetrazolium index in TB patients at 6 months of anti-TB (Edem and Arinola, 2015). Thus, plasma ADA level may be a useful biomarker to monitor treatment in either DSTB or MDR TB patients. Sputum smear microscopy is currently used to monitor treatment response in TB patients on anti-TB chemotherapy. However, there is need for blood biomarkers of treatment response since sputum production becomes increasingly difficult to obtain during treatment in TB. Reduced ADA level at 6 months of chemotherapy might be explained by reduced Mtb specific CMI (reduced Mtb specific monocyte and T cell activation) as a result of reduced Mtb antigen or other regulatory immune factors. A previous study reported a shift in CMI response during anti-TB chemotherapy (Cardoso et al, 2002) which was attributed to sequestration of Mtb-specific T cells at the site of disease leading to reduced frequency in peripheral blood, the release of anti-inflammatory cytokines by PBMCs and depression of T-cell responsiveness (Wilkinson et al, 1988).

This present study also observed similar levels of plasma ADA in multi-drug resistant TB patients when compared with drug-sensitive TB patients at diagnosis and at 6 months of chemotherapy. This implies that plasma ADA level may not be useful in distinguishing drug sensitive from drug resistant TB patients and indicating that there may be no difference in the nature of CMI response in DSTB and MDR TB patients. Basile et al (2011) however reported that MDR-Mtb strains induce stronger IL-17 than drug susceptible strains in vitro and MDR TB patients showed high IL-17 expression. This might be explained by the fact that ADA is not involved in all aspects of lymphocyte activities. Plasma ADA after 6 months of treatment of both DSTB patients and MDR TB patients were significantly higher than control values. This indicates

that at 6 months of anti-TB chemotherapy all physiological effects of Mtb infection are not completely repressed. Thus studies with longer follow-up period using larger sample size are required to suggest which duration post TB-chemotherapy reflects complete reversal of physiological effects of Mtb infection.

In conclusion, plasma ADA level is a promising biomarker for the screening and treatment monitoring of pulmonary TB but not to differentiate MDR TB from DSTB patients.

REFERENCES

Adekambi T, Ibegbu CC, Cagle S, Kalokhe AS, Wang YF, Hu Y, Day CL, Ray SM, Rengarajan J (2015). Biomarkers on patient T cells diagnose active tuberculosis and monitor treatment response. J Clin Invest 125(5): 1827-1838.

Afrasiabian S, Mohsenpour B, Bagheri KH, Sigari N, Aftabi K (2013). Diagnostic value of serum adenosine deaminase level in pulmonary tuberculosis. J Res Med Sci 18(3):252-254.

Alexander PE and De P (2007). The emergence of extensively drug-resistant tuberculosis (TB): TB/HIV coinfection, multidrug-resistant TB and the resulting public health threat from extensively drug-resistant TB, Globally and in Canada. Can J Infect Dis Med Microbiol 18(5): 289-291.

Basile JI, Geffner LJ, Romero MM, Balboa L, Garcia CS, Ritacco V, Garcia A, Cuffre M et al (2011). Outbreaks of Mycobacterium Tuberculosis MDR strain induce high IL 17 T-cell response in patients with MDR tuberculosis that is closely associated with high antigen burden. J Infect Dis 204(7):1054-1064.

Blackburn MR, Datta SK, Kellems RE (1998). Adenosine deaminase-deficient mice generated using a two-stage genetic engineering strategy exhibit a combined immunodeficiency. J Biol Chem 273: 5093-5100.

Bloom CI, Graham CM, Berry MPR, Rozakeas F, Redford PS et al, (2013). Transcriptional blood signatures distinguish pulmonary tuberculosis, pulmonary sarcoidosis, pneumonias and lung cancers. PLoS One. 8(8): e70630.

Boonyagars L and Kiertiburanakul S (2010). Use of adenosine deaminase for the diagnosis of tuberculosis. A review. J Infect Dis Antimicrob Agents 27:111-118.

Cardoso FLL, Antas PRZ, Milagres AS, Geluk A, Franken KLMC, Oliveira EB, Teixeira HC, Nogueira SA et al (2002). T-cell responses to the Mycobacterium tuberculosis-specific antigen ESAT-6 in Brazilian tuberculosis patients. Infect Immun 70(12):6707-6714.

Çimen F, Çiftçi TU, Berktafl BM, Sipit T, Hoca NT, Dulkar G (2008). The relationship between serum adenosine deaminase level in lung tuberculosis along with drug resistance and the category of tuberculosis. Turk Respir J. 9:20–3.

Edem VF and Arinola OG (2015). Innate cellular Immunity in newly diagnosed tuberculosis patients and during chemotherapy. Annals of Global Health, 81(5):669-674.

Ernst JD (2012). The immunological life cycle of tuberculosis. Nature Rev Immunol 12:581-591.

Giblett ER, Anderson JE, Cohen F, Pollara B, Meuwissen HJ (1972). Adenosine-deaminase deficiency in two patients with severely impaired cellular immunity. The Lancet. 300(7786):1067-1069.

Greco S, Girardi E, Masciangelo R, Capoccetta GB, Saltini C (2003). Adenosine deaminase and interferon gamma measurements for the diagnosis of tuberculous pleurisy: a meta-analysis. Int J Tuberc Lung Dis 7: 777–786.

Jacobson MA and Bai TR (1997). Purinergic Approaches in Experimental Therapeutics, eds Jacobson KA, Jarvis MF (Wiley-Liss, Inc. Danvers, MA). pp 585–59.

Keeler E, Perkins MD, Small P, Hanson C, Reed S, Cunningham J, et al (2006). Reducing the global burden of tuberculosis: the contribution of improved diagnostics. Nature 444 (Suppl 1): 49-57.

Liu X, Kim CN, Yang J, Jemmerson R, Wang X (1996). Indeuction of apoptotic program in cell-free extracts: Requirement for dATP and cytochrome c. Cell 86 (1): 147-157.

Olah ME and Stiles GL (1995). Adenosine receptor subtypes: characterization and therapeutic regulation. Annu Rev Pharmacol Toxicol. 35:581-606.

Piras MA, Gakis C, Budroni M, Andreoni G (1978). Adenosine deaminase activity in pleural effusions: An aid to differential diagnosis. Br Med J. 2:1751–1752.

Rozot V, Patrizia A, Vigano S, Mazza-Stalder J, Idrizi E, et al (2015). Combined use of mycobacterium tuberculosis-specific CD4 and CD8 T-cell responses is a powerful diagnostic tool of active tuberculosis. Clin Infect Dis 60 (3):432-437.

Shore A, Dosch HM, Gelfand EW (1981). Role of adenosine deaminase in the early stages of precursor T cell maturation. Clin Exp Immunol 44: 152-5.

Srinivasa RK, Kumar HA, Rudresh BM, Srinivas T, Bhat KH. A comparative study and evaluation of serum adenosine deaminase activity in diagnosis of pulmonary tuberculosis. Biomedical Research 2010 (2):189-194.

Storla DG, Yimer S, Bjune GA (2008). A systematic review of delay in the diagnosis and treatment of tuberculosis. BMC Public Health. 8:15.

Swami KK (2016). Diagnostic and prognostic significance of pleural fluid adenosine deaminase estimation in relation to tuberculosis. Ind J Appl Res. 6(1): 232-233.

Wallis RS, Doherty TM, Onyebujoh P, Vahedi M, Laang H, Olesen O, Parida S, Zumla A (2009). Biomarkers for tuberculosis disease activity, cure and relapse. Lancet Infect Dis 37(3):162-172.

Wilkinson RJ, Vordermeier HM, Wilkinson KA, Sjölund A, Moreno C, Pasvol G, Ivanyi J. 1988. Peptide-specific T cell response to *Mycobacterium tuberculosis*: clinical spectrum, compartmentalization, and effect of chemotherapy. J Infect Dis 178:760-768.

World Health Organization (2006). Diagnostic and Treatment Delay in Tuberculosis. World Health Organization, Regional Office for the Eastern Mediterranean. 2006:21.

World Health Organization (2011). Guidelines for the programmatic management of drug-resistant tuberculosis, 2011 Update, WHO, Geneva, Switzerland.

World Health Organization (2015). Global Tuberculosis report 2015. WHO, Geneva, Switzerland.

Zavialov AV, Gracia E, Glaichenhaus N, Franco R, Zavialov AV, Lauvau G (2010). Human adenosine deaminase 2 induces differentiation of monocyte and stimulates proliferation of T helper cells and macrophages. J Leukoc Biol 88:279-290.

Niger. J. Physiol. Sci. 31(June 2016) 055-061
www.njps.com.ng

Niger. J. Physiol. Sci.

Effect of Vitamin C Supplementation on Platelet Aggregation and Serum Electrolytes Levels in Streptozotocin-Induced Diabetes Mellitus in Rats

*** Daniel U. Owu[1]*, Chukwuemeka R. Nwokocha[2], Daniel E. Ikpi[1] and Emmanuel I. Ogar[1]**

[1]Department of Physiology, University of Calabar, Calabar, Nigeria. [2]Department of Basic Medical Sciences, University of West Indies, Mona, Kingston, Jamaica

Summary: Diabetes mellitus (DM) is a disease condition characterised by hyperglycemia; free radical and abnormal haematological indices. Vitamin C can reduce free radical generation and ameliorate adverse conditions of diabetes mellitus. The aim of the present study is to investigate the effect of vitamin C on platelet aggregation and electrolyte levels in Type 1 DM. Male Wistar rats were divided into four groups namely control, DM, DM +Vitamin C and Vitamin C groups. Rats were made diabetic with a single dose of streptozotocin (65 mg/kg) intraperitoneally. Vitamin C was administered orally to diabetic and normal rats at 200 mg/kg body weight for 28 days. Blood samples were analyzed for hematological parameters, platelet aggregation, and serum electrolyte levels. Blood glucose in DM+ Vitamin C group (9.9 ± 1.8 mmol/L) was significantly reduced ($p<0.01$) compared to DM group (32.2 ± 2.1 mmol/L) and significantly higher ($p<0.05$) than control (4.4 ± 0.8 mmol/L). Haemoglobin (Hb) concentration in DM group (12 ± 0.1 g/dL) was significantly reduced ($p<0.01$) when compared with control groups (14 ± 0.24 g/dL) and significantly increased ($p<0.05$) in the DM+vitamin C group (13.5 ± 0.5 g/dL) compared with the diabetic group. The mean corpuscular volume values in DM (68.66 ± 0.5 fL) and DM+vitamin C groups (68.11 ± 0.4 fL) were significantly higher ($p<0.01$) than the control (59.49 ± 0.5fL). Platelet count in DM group (523 ± 8.5 $x10^9$/L) was significantly raised ($p<0.01$) when compared to control (356 ± 6.2 $x10^9$/L) and significantly reduced ($p<0.01$) in DM+ vitamin C-treated group (385 ± 7.8 $x10^9$/L) compared with DM group. Platelet aggregation and serum sodium/potassium ratios was significantly reduced ($p<0.01$) in DM+vitamin C compared with DM group. These results suggest that oral vitamin C administration increases haemoglobin, reduced plasma glucose level, platelet count, serum sodium/potassium ion ratio and inhibits platelet aggregation in streptozotocin-induced DM in rats.

Keywords Diabetes mellitus, electrolytes, Haematological parameters, Platelet aggregation, Red cell indices, Vitamin C

**Address for correspondence:* danielowu@unical.edu.ng; d_owu@yahoo.com

Manuscript Accepted: June 2016

INTRODUCTION

Diabetes mellitus (DM) is one of the most common non-communicable diseases in the world (Ramakrishna and Jailkhani (2007) with a global prevalence of 8.8%. It is a disease that is characterized by vascular smooth muscle and endothelial dysfunction. Endothelial dysfunction plays an important role in the pathophysiology of atherosclerosis, leukocyte adhesion, endothelium platelet aggregation and vascular smooth muscle proliferation (Browne *et al*., 2003; Skrha *et al*., 2007). Haematological complication is a notable feature of diabetes mellitus and consists mainly of abnormalities in the function, morphology and metabolism of various blood cells (Comazzi *et al*., 2004).

Anaemia in DM is associated with erythropoietin deficiency and can occur early in diabetic neuropathy before the onset of advanced renal failure (Bosman *et al*., 2001). Abnormalities in fluid and electrolytes balance are common biochemical findings in DM (Obineche *et al*., 2006) and a reduction in plasma sodium and chloride ions in diabetic patients have been documented (Onwuliri *et al*., 2004). This is probably attributed to either loss, reduced intake/absorption or alterations in metabolism in diabetic condition. Vitamin C intake has many important biological functions such as increasing the white blood cell count and function (Iqbal *et al*., 2004, Hall *et al*., 2011), reducing arterial blood pressure and improving arterial stiffness in patients with type 2 diabetes (Mullan *et al*., 2002). Vitamin C has no adverse effect on serum electrolyte and may protect against atherosclerosis and hypertension (Eteng *et al*., 2006). Ascorbic acid supplementation increases hemoglobin concentration hematocrit level, red blood cell (RBC) count, serum and leucocyte ascorbate concentrations (Jaja *et al*., 2002) and primary defence

mechanisms against oxidative stress in DM (Alsaif, 2009).

DM is also associated with increased ex-vivo platelet aggregation and hypercoagulability of platelets (Skowasch *et al*., 2009), decreased haemoglobin concentration and anaemia (Ritz, 2006; Hasslacher *et al*., 2010). Vitamin C supplementation has been reported to improve erythropoietic activity, ascorbic acid status and electrolyte levels in healthy condition. Common sources of vitamin C are vegetables and fruits. However, data is lacking on the effect of vitamin C on haematological indices and electrolyte balance in diabetic condition. The purpose of this study was to determine the effects of vitamin C on haematological and serum electrolyte parameters in Type 1 diabetic condition. We hypothesized that administration of vitamin C would improve haematological indices, reduce platelet aggregation and restore electrolyte imbalance in Type 1diabetic rats.

MATERIALS AND METHODS

Chemicals

Vitamin C (L-ascorbic acid), streptozotocin, ethylenediamine tetra acetic acid (EDTA), dipotassium monophosphate, monopotassium diphosphate salts were obtained from Sigma Chemical Company, while formalin was purchased from BDH, Poole, UK. All the chemicals used were of pure analytical grade and prepared in deionised distilled water except streptozotocin which was dissolved in citrate buffer.

Experimental animals

A total of 24 male Wistar rats weighing between 170 to 180 g obtained from the animal house of Department of Physiology, College of Medical Sciences, University of Calabar, Calabar, Nigeria, were used for the study. All animal procedures were performed according to the Guide for the Care and Use of Laboratory Animals of the National Institute of Health after ethical approval was obtained from the Faculty of Basic Medical Sciences Animal Research Ethics Committee. The animals were randomly assigned into four groups of six rats namely control, DM, DM + vitamin C and vitamin C groups. They were kept in cages at room temperature of 29 ± 2ºC with a 12 hours light/dark cycle and had free access to water and rat chow.

Induction of diabetes mellitus

Type 1 DM was induced in two groups of experimental rats by intraperitoneal injection of streptozotocin (STZ) dissolved in citrate buffer (pH 4.5) at a single dose of 65 mg/kg body weight. Weight and age-matched control rats were injected with the citrate buffer. Blood glucose level and body weight were measured prior to STZ injection using an automated glucose analyzer (glucometer Acucheck mini plus, Roche, Germany) and weight balance respectively. DM was confirmed 48 hours after STZ injection in animals by the presence of blood glucose level greater than 10 mmol/L and glucosuria using clinistix (Bayer Diagnostics, Mannheim, Germany). Laboratory investigations were carried out in all groups of the animal after four weeks of diabetes mellitus induction. Vitamin C was administered orally to DM + Vit C and Vitamin C groups at a dose of 200 mg/kg body weight (Owu *et al*., 2006, 2012) orally for 28 days while rats in control and diabetic groups received placebo.

Collection of blood samples

Cardiac blood samples from all groups of animals were obtained for haematological analysis. Blood sample from each animal was collected into ethylenediamine tetraacetic acid (EDTA) tube and well-labeled non-heparinized sample tube. The former was used for blood cell count while the latter sample was allowed to stand for 3hours in iced water and centrifuged at 10,000g for 10 minutes. The serum was collected and stored at -20ºC until use. The RBC count, white blood cells count (WBC), packed cell volume (PCV), and hemoglobin concentration were determined using automatic blood cell counter (Hematology analyser KX-21N Sysmex, Deutschland GMBH, Germany).

Determination of platelet aggregation

From the blood sample drawn into the non-heparinized sample tubes, 0.25 ml of each sample was added to the 4.5 ml of buffered EDTA solution and 4.5 ml of buffered EDTA/formalin solution respectively. The blood was thoroughly mixed with each solution to give a volume dilution of 1:20. The samples were centrifuged at 200 g for 10 minutes using MSE centrifuge to obtain platelet-rich plasma (PRP) which was then used for platelet count. Platelets in the platelet rich plasma samples were counted using the light optical microscope and a hemocytometer following standard laboratory procedure. The counting chamber was the improved Neubauer and the power of magnification used was 40x. All counting was done within two hours after centrifuging the samples. Platelet aggregation was determined using the method of Wu and Hoak (1974) by finding the ratio of platelet count in buffered EDTA solution with the platelet count in Buffered EDTA/formalin solution. Serum sodium and potassium were estimated using flame photometer (Corning 410) while chloride ion (Cl^-) was estimated by mercuric thiocynate method using dialab kit. Bicarbonate ion (HCO_3^-) level in serum was determined using the titration methods.

Statistical analysis

The results obtained are expressed as mean ± Standard error of mean (SEM) and analysed using GraphPad Prism software version 5 (GraphPad Software, San

Diego, California, USA). One way analysis of variance (ANOVA) was used to compare means followed by Tukey's multiple comparison tests where F-value was significant. In all cases, *p* value less than 0.05 was considered statistically significant.

RESULTS

Blood glucose and body weight

Type 1 DM rats exhibited a significant ($p<0.01$) increase in blood glucose level compared with control (Table 1). Type 1 DM rats treated with vitamin C for four weeks showed a significant ($p<0.01$) reduction in blood glucose (9.9 ± 1.8 mmol/L) when compared to DM rats (32.2 ± 2.1 mmol/L) though the value was significantly ($p<0.05$) higher than control (4.4 ± 0.8 mmol/L). Vitamin C administration to normal rats did not alter the blood glucose level (4.6 ± 1.2 mmol/L) compared with normal control. There was a significant ($p<0.01$) decrease in final body weight in Type 1 DM rats (185 ± 5 g) when compared to the control (220 ± 8 g). The body weight of animals in DM + Vit C group (210 ± 8 g) was not significantly different from that in control and Vit C group (225 ± 6 g).

Blood cell counts

The haematological indices of the animals in control and DM+Vit C groups are presented Table 1. The mean red blood cell (RBC) count in DM +Vit C group (6.9 ± 0.44 $x10^{12}$/L) was comparable to the control group (7.9 ± 0.22 $x10^{12}$/L). The mean WBC counts in the control was 5.3 ± 0.93 cells/10^9/L and it was 5.9 ± 0.68 cells/10^9/L in DM group. In DM + Vit C group the WBC count was 3.8 ± 0.16 cells/10^9/L while it was 4.8 ± 0.77 $x10^9$/L in Vit C group. There was no significant difference in the WBC counts in DM group when compared to control. The platelet counts in DM group (523 ± 8.5 $x10^9$/L) was significantly ($p<0.05$) raised when compared with control (356 ± 6.2 x 10^9/L) and DM+Vit C group (385 ± 7.8 x 10^9/L). However, Vitamin C administration to diabetic group resulted in a significant ($p<0.05$) reduction in platelet count (385 ± 7.8 $x10^9$/L) when compared with the diabetic group. Administration of vitamin C did not cause any alteration in platelet count in Vit C group (369 ± 10.9 $x10^9$/L) when compared with control.

Haematocrit and haemoglobin concentration

The mean values of haematocrit and haemoglobin are presented in Table 1. The haematocrit values in Type 1 DM groups were similar to the values of the control group. Administration of vitamin C did not alter the haematocrit values when compared to the control group. The mean haemoglobin (Hb) concentration in the Type 1 DM group (12.0 ± 0.1 g/dL) was significantly reduced ($p<0.01$) when compared to control groups (14.0 ± 0.24 g/dL) whereas administration of vitamin C significantly improved ($p<0.05$) the Hb concentrations in the DM + vitamin C-treated groups (13.5 ± 0.5 g/dL) when compared to the DM group. There was a significant decrease ($p<0.01$) in mean corpuscular haemoglobin concentration (MCHC) in DM group when compared to the control group. However, vitamin C produced a significant increase ($p<0.05$) in MCHC value in DM + Vit C diabetic rats. The mean corpuscular volume (MCV) values in both DM and DM + Vit C-treated groups were significantly higher ($p<0.05$) than the control and vitamin C.

Platelet aggregation

In order to determine the extent of platelet aggregability in Type 1 DM, platelet aggregation was determined and the result is shown in Fig. 1. A significant decrease ($p<0.05$) in the platelet aggregation as depicted by high aggregation ratio was observed in DM + Vit C group when compared with control and DM groups. No significant difference was observed between the DM and control groups.

Electrolytes levels

Table 2 shows the mean serum levels of sodium, potassium, chloride and bicarbonate level. A significant increase ($p<0.05$) in serum level of sodium ion was recorded in the two diabetic groups compared to the control. Likewise, a significant increase

Table 1: Mean values of haematological indices and body weights in control and diabetic rats treated with vitamin C

Parameter	Control	DM	DM + Vit C	Vitamin C	F ratio
Body weight (g)	220 ± 8	185 ± 5**	210 ± 8	225 ± 6	6.7
Blood glucose (mmol/L)	4.2 ±0.7	32.2± 2.1**	8.9 ± 1.8†	4.5 ± 1.1	76.1
RBC count (□□10^{12}/L)	7.9 ± 0.22	6.7±0.68	6.9 ±0.44	7.1 ± 0.34	1.35
WBC count (□□10^9/L)	5.3 ±0.93	5.9 ±0.68	3.8 ±0.16	4.8 ± 0.77	1.62
Hb concentration (g/dl)	14 ±0.24	12 ±0.1**	13.5 ± 0.5a	13.8 ± 0.2	9.20
Haematocrit (%)	47 ± 1.1	46 ± 1.3	47 ± 1.6	47 ± 0.7	0.17
Platelet ($x10^9$/L)	356 ± 6.2	523±8.5**	385 ± 7.8†	369 ± 10.9	82.6
MCV (fL)	59.49±0.5	68.66±0.5**	68.11±0.4**	66.19±0.5*	71.1
MCHC (%)	29.78±0.8	26.09±0.5**	28.76±0.7 a	29.36±0.5	6.75

Results are expressed as mean ± standard error of mean, * = $p<0.05$ versus control; **=$p<0.01$ vs. control; † =$p<0.01$ versus DM group; a= $p<0.05$ versus DM; n= 6 in each group.

Table 2: Effect of oral administration of vitamin C on electrolyte levels of diabetic and control rats

Parameter	*Control*	*DM*	*DM + Vit C*	*Vitamin C*
Sodium ion (mmol/L)	127.3 ± 1.9	136.2 ±1.1*	138.4 ± 0.9*†	129.5 ± 2.1
Potassium ion (mmol/L)	4.7 ±0.8	3.5 ± 1.1	7.3 ± 1.1†	5.6 ± 0.4
Chloride ion (mmol/L)	106.0 ± 0.8	106.8 ±0.5	110.3 ± 3.1	102.7 ± 2.2
Bicarbonate (mmol/L)	21.3 ± 1.2	24.8 ± 1.2	16.8 ± 2.2*†	19.7 ± 1.2
Na^+/K^+ ratio	27.1± 1.1	38.9± 1.3	18.9± 0.9*†	23.1± 1.2*†

Results are expressed as mean ± standard error of mean. *=p<0.05 diabetic group compared with control, *=p<0.01 diabetic group compared with control † = p<0.05 DM + Vitamin C- group compared with DM group, n = 6 in each group.

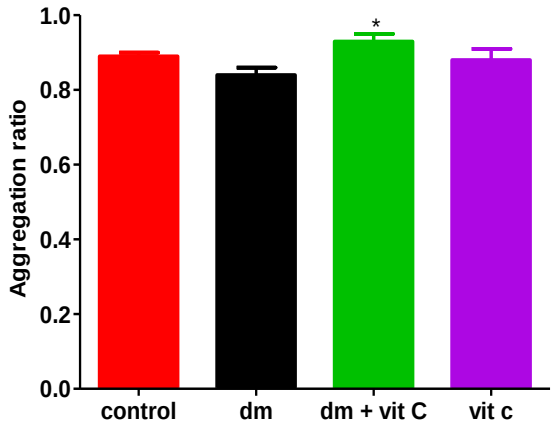


Figure 1: Platelet aggregation ratio in diabetes mellitus group and control treated with vitamin C. * = p< 0.05 compared to DM and control

(p<0.01) in potassium ion level was noted in the DM + Vit C group when compared with DM. The DM + Vit C group had a significantly lower (p<0.05) serum bicarbonate ion level than the DM and control groups. The mean serum chloride (Cl^-) levels were comparable in all groups of animals. The serum sodium/potassium ratio was significantly raised (p<0.05) in DM group when compared with the control. However, administration of vitamin C significantly reduced (p<0.05) this ratio in both groups supplemented with vitamin C when compared with DM and control.

DISCUSSION

The effect of vitamin C-oral administration on some haematological parameters was investigated in Type 1 diabetic male Wistar rats. Streptozotocin was used to induce Type 1 DM, a specific cytotoxic drug that destroys the insulin producing cells in the islets of Langerhans of the pancreas resulting in hyperglycaemia and loss of body weight. The decrease in body weight after diabetic induction is expected since this is one of the effects of Type 1 DM. Blood glucose level was elevated in DM though treatment with vitamin C reduced the noted hyperglycemia in the DM group. This observation confirms previous reports that vitamin C significantly reduced blood glucose level in experimental DM (Owu *et al*., 2006; Al-Shamsi *et al*., 2007).

This study has provided information on the haematological parameters such as RBC and WBC counts, haemoglobin concentration and haematocrit in Type 1 DM. Though there was a decrease in RBC, Hb and WBC in DM + Vit C group, the parameters were within the normal range reported for animals. The PCV was insignificantly higher in DM + Vit C group when compared to the control group.

Although the RBC values were within normal range, the decrease in Hb and MCHC in DM group when taken together reflects anaemia. Morales-Ramirez *et al* (1998) reported that Vitamin C increases the haematocrit through enhanced iron absorption (Atanasova *et al*., 2004) and as such help reverse anaemia. Inadequate production of red cells and some other formed elements have been reported in DM (Rabble *et al*., 1996; Thomas *et al*., 2004). In our results, we observed that the administration of vitamin C to Type 1 DM group significantly increased the haemoglobin and MCHC indices. MCV and MCHC are used to diagnose the types of anaemia (Davidson *et al*., 1981). The present study showed a high value of MCV and low MCHC that reflects macrocytic hypochromic anaemia. Following treatment of Type 1 diabetic rats with vitamin C, the macrocytic hypochromic anaemia as reflected by high MCV and low MCHC values was corrected.

Vitamin C did not significantly alter the WBC count in the treated groups. This is at variance with reports that it increased the total circulating white blood cell count in vitamin C-supplemented animals (Fraser *et al*., 1980; Field *et al*., 2002). Vitamin C significantly decreased platelet count and aggregation in Vitamin C supplemented diabetic group. This result is in agreement with previous studies (Wilkinson *et al*., 1999, Mullan *et al*., 2002) that reported a decreased platelet aggregation and arterial stiffness with vitamin C supplementation. However, Pignatelli *et al.* (2005) reported contrary that vitamin C did not affect platelet aggregation both *in vitro* and *in vivo* in healthy humans. Platelet aggregation, the clumping together of platelets in the blood is one of the underlying events that result in the formation of clot be involved in the genesis of diabetic microangiopathy (Barnett, 1993). Platelet aggregation and thrombosis play key roles in the progression of atherosclerosis and consequent

cardiovascular complications. When there is platelet aggregation, the aggregation ratio is less than one and conversely in the absence of aggregation, the ratio is close to one. The inhibition of platelet aggregation in this study further shows the beneficial role of vitamin C supplementation in its protective role against platelet activation in DM.

An elevated serum level of potassium ion and decrease in levels of sodium and chloride ions has been reported in DM (Onwuliri *et al*, 2004). There are also varied reports of effect of vitamin C on the serum electrolytes. While Al-Shamsi *et al*. (2006) reported significant alterations, Eteng *et al*, (2006) reported no adverse effect of vitamin C on serum electrolytes. We observed a significant variation within physiological limits in the serum electrolytes, with sodium being elevated with vitamin C supplementation. Such elevation had been reported and may be due to intracellular shift occasioned by osmotic diuresis which is a common feature in DM (Rao, 1992).

Various disorders such as renal failures, gastrointestinal diseases, DM and acidosis are characterised by electrolyte disturbances. Sodium-potassium ion (Na^+/K^+) ratio has frequently been used as a diagnostic tool to identify different disease conditions (Pak, 2000). DM has been reported to cause hyperkalemia both through acidosis and the reduced levels of insulin available to promote cellular uptake of potassium (Brink, 1999). Hyperkalemia may result from both a shift of the ion from the intracellular to the extracellular compartment and a decrease in the renal excretion of potassium (Carlotti *et al*., 2013). The hypokalemia reported in this study could arise from a drastic fall arising from the correction of acidosis. However, the elevated serum level of K^+ in DM + Vitamin C could not be easily discerned from this study and is subject to further investigation. The Na^+/K^+ ratio is a measure that compares the level of sodium and potassium ion in the body and a high ratio is associated with specific symptoms including acute stress, DM, heart disease and inflammation (Sjogren *et al*., 1998; Li *et al*, 2009). The high Na^+/K^+ ratio in the DM group in the present study is in agreement with previous studies (Cunningham, 1998; Farvid *et al*., 2004). Vitamin C supplementation in the diabetic group causes a significant reduction of the Na^+/K^+ ratio indicating that vitamin C can cause a beneficial reduction in Na^+/K^+ ratio.

Vitamin C has been reported to participate in mechanism for concerted glucose transport inhibition in cells (Castro *et al.*, 2008). Our result showed that blood glucose level was reduced in diabetic group treated with vitamin C and is in agreement with previously published data that showed improvement in glycaemic control with vitamin C supplementation (Afkhami-Ardekani and Shojaoddiny-Ardekani 2007, Hoffman *et al*., 2012). The beneficial effect of antioxidant on the beta cells, other target tissues and non-oxidative glucose metabolism (Dakhale *et al*., 2011) could be a possible mechanism of reduction of blood glucose by vitamin C in Type 1 diabetic condition. In addition, vitamin C has been reported to have anti-atherosclerotic effects in Type 2 DM by reducing hyper-coagulation of platelets (Gutierrez *et al*., 2013).

There are few limitations in this study. Even though vitamin C is water soluble and expected to be eliminated as such, this study did not quantify the urinary excretion and blood level of this vitamin. In addition, the study was relatively short duration. Despite these limitations, the results of this study showed that there was a reduced haematocrit and an increased platelet count while sodium ion concentration and serum Na^+/K^+ ratio were elevated in Type 1 DM. Oral administration of vitamin C to the Type 1 diabetic group resulted in an increase in haemoglobin concentration a reduction in sodium /potassium ion ratio. Vitamin C could be of immense importance in ameliorating the symptoms and preventing complications of DM considering the readily available dietary sources of vitamin C such as fruits and vegetable.

Conclusion

It is concluded that oral vitamin C administration increased haemoglobin, caused a decrease in plasma glucose level, platelet count, serum sodium/ potassium ion ratio and inhibits platelet aggregation in Type 1 DM in rats.

Acknowledgements

The excellent technical assistance of Mr. Edet Umoh is gratefully acknowledged.

REFERENCES

Afkhami-Ardekani M, and Shojaoddiny-Ardekani A. (2007). Effect of vitamin C on blood glucose, serum lipids & serum insulin in type 2 diabetes patients. *Indian J Med Res.* 126: 471-474.

Alsaif, M. A. (2009). Combined treatment of rutin and vitamin C improves the antioxidant status in streptozotocin-induced diabetic rats. *J Med Sci.* 9: 1-9.

Al-Shamsi, M., Amin, A., and Adeghate, E. (2007). The effect of vitamin C on the metabolic parameters of experimental diabetes mellitus. *Am. J. Pharmacol. Toxicol.* 2: 4-9.

Al-Shamsi, M., Amin, A., Adeghate, E. (2006). Effect of vitamin C on liver and kidney functions in normal and diabetic rats. *Ann. N.Y. Acad. Sci*. 1084: 371-390.

Atanasova, B., Mudway, I. S., Laftah, A. H., Latunde-Dada, G. O., McKie, A. T., Peters, T. J., Tzatchev, K. N., Simpson, R. J. (2004). Duodenal ascorbate

levels are changed in mice with altered iron metabolism. *J. Nutr*. 134: 501-505.

Barnett, A. H. (1993). Origin of the microangiopathic changes in diabetes. *Eye* 7: 218-222.

Bosman, D. R., Winkler, A. S., Marsden, J. T., Macdougall, I. C., Watkins, P. J. (2001). Anemia with erythropoietin deficiency occurs early in diabetic nephropathy. *Diabetes Care*. 24: 495-499.

Brink, S. J. (1999). Diabetic ketoacidosis. *Acta Pediatr*. 88: 14-24.

Browne, D., Meeking, D., Shaw, K., Cummings, M. (2003). Endothelial dysfunction and pre-symptomatic atherosclerosis in type 1 diabetes - pathogenesis and identification. *Br. J. Diabetes Vasc. Dis.* 3: 27-34.

Carlotti, A. P. 1., St George-Hyslop, C., Bohn, D., Halperin, M. L. (2013). Hypokalemia during treatment of diabetic ketoacidosis: clinical evidence for an aldosterone-like action of insulin. *J. Pediatr*.63: 207-212.

Castro, M. A., Angulo, C., Brauchi, S., Nualart, F., Concha, I. I. (2008). Ascorbic acid participates in a general mechanism for concerted glucose transport inhibition and lactate transport stimulation. *Pflugers Arch.* 457: 519-528.

Comazzi, S., Spagnolo, V., Bonfanti, U. (2004). Erythrocyte changes in canine diabetes mellitus: In vitro effects of hyperglycaemia and ketoacidosis. *Comp. Clin. Pathol.* 12: 199-205.

Cunningham, J. J. (1988). Altered vitamin C transport in diabetes mellitus. *Med. Hypotheses* 26: 263-265.

Dakhale, G. N., Chaudhari, H. V., Shrivastava, M. (2011). Supplementation of vitamin C reduces blood glucose and improves glycosylated hemoglobin in type 2 diabetes mellitus: a randomized, double-blind study. *Adv. Pharmacol. Sci*. 2011:195271.

Davidson, R. J. L., Evan-Wong, L. A., Stowers, J. M. (1981).The mean red cell volume in diabetes mellitus. *Diabetologia* 20: 583-584.

Eteng, M. U., Ibekwe, H. A., Amatey, T. E., Bassey, B. J., Uboh, F. U., Owu, D. U. (2006). Effect of oral vitamin C on serum lipid and electrolytes profile of albino Wistar rats. *Niger. J. Physiol. Sci.*21: 15-19.

Farvid, M. S., Jalali, M., Siassi, F., Saadat, N., Hossein, M. (2004). The impact of vitamins and/or mineral supplementation on blood pressure in type 2 diabetes. *J. Am. Col. Nutr.* 23: 272-279.

Field, C. J., Johnson, I. R., Schley, P. D. (2002). Nutrients and their role in host resistance to infection. *J. Leukoc. Biol.*71:16-32.

Fraser, R. C., Pavlovic, S., Kurahara, C. G., Murata, A., Peterson, N. S., Taylor, K. B., Feigen, G. A. (1980). The effect of variations in vitamin C intake on the cellular immune response of guinea pigs. *Am. J. Clin. Nutr*.33: 839-847.

Gutierrez, A. D., Duran-Valdez, E., Robinson, I., de Serna, D. G., Schade, D. S. (2013). Does short-term vitamin C reduce cardiovascular risk in type 2 diabetes? *Endocr. Pract*. 19: 785-791.

Hall, J. A., Chinn, R. M., Vorachek, W. R., Gorman, M. E., Greitl, J. L., Joshi, D. K., Jewell, D. E. (2011). Influence of dietary antioxidants and fatty acids on neutrophil mediated bacterial killing and gene expression in healthy Beagles. *Vet. Immunol. Immunopathol.* 139: 217-228.

Hasslacher, C., Collenberg, E., Mocks, J. (2010). Effect of insulin analogs on the decline of hemoglobin in diabetic patients with nephropathy. *Exp. Clin. Endocrinol. Diabetes*. 18: 341-345.

Iqbal, K., Khan, A., Khattak, M.M.A.K.(2004). Biological significance of ascorbic acid (vitamin c) in human health - A review. *Pak. J. Nutr.* 3: 5-13.

Jaja, S. I., Ikotun, A. R., Gbenebitse, S., Temiye, E. O.(2002). Blood pressure, hematologic and erythrocyte fragility changes in children suffering from sickle cell anemia following ascorbic acid supplementation. *J. Trop. Pediatr.*48: 366-370.

Li, Y., Ma, A., Sun, Y., Liang, H., Wang, Q., Yi, X., Han, X. (2009). Magnesium status and dietary intake of mid-old people in a rural area of China. *Magnes. Res.*22: 66-71.

Morales-Ramirez, P., Mendiola-Cruz, M. T., Cruz-Vallejo, V. (1998). Effect of vitamin C or beta-carotene on SCE induction by gamma rays in radiosensitized murine bone marrow cells *in vivo*. Mutagenesis 13: 139-144.

Mullan, B. A., Young, I. S., Fee, H., McCance, D. R. (2002). Ascorbic acid reduces blood pressure and arterial stiffness in type 2 diabetes. *Hypertension* 40: 804-809.

Obineche, E., Chandranath, I., Adeghate, E., Benedict, S., Fahim, M., Adem, A. (2006). Alterations in atrial natriuretic peptide and its receptor levels in long term, streptozotocin-induced, diabetes in rats. *Ann. N. Y. Acad. Sci.* 1084: 223–234.

Onwuliri, V. A., Bitrus, S., Puppet, F., Maduka, H. C.C. (2004). Blood lipids and electrolyte profiles of male and female diabetics in Plateau State Nigeria. *J. Medical Sci* 4: 221-224.

Owu, D. U., Antai, A. B., Udofia, K. H., Obembe, A. O., Obasi, K. O., Eteng, M. U. (2006). Vitamin C improves basal metabolic rate and lipid profile in alloxan-induced diabetes mellitus in rats. *J. Biosci.* 31: 575-579.

Owu, D. U., Obembe, A. O., Nwokocha, C. R., Edoho, I. E., Osim, E. E. (2012). Gastric ulceration in diabetes mellitus: protective role of vitamin C**.** ***ISRN Gastroenterology*** 2012, Article ID 362805, 7 pages.

Pak, S. I. (2000). The clinical implication of sodium-potassium ratios in dogs. *J. Vet. Sci.* 1: 61-65.

Pignatelli, P., Sanguigni, V., Paola, S., Lococo, E., Lenti, L., Violi, F. (2005). Vitamin C inhibits platelet expression of CD40 ligand. *Free Radical Biol. Med.* 38:1662-1666.

Rabble, G. R., Atherton, J. O., Stuart, H. B., Wong, O. (1996). Anaemia due to erythropoietin concentration in diabetic patient. *Environ Health Perspect* 1996, 104: 229-233.

Ramakrishna, V., Jailkhani, R. (2007). Evaluation of oxidative stress in insulin dependent diabetes mellitus (IDDM) patients. *Diagn. Pathol.*1: 2:22.

Rao, G. M. (1992). Serum electrolytes and osmolality in diabetes mellitus**.** *Indian J. Med. Sci*. 46: 301-303.

Ritz, E. (2006). Anemia and diabetic nephropathy. *Curr. Diabetes Rep.* 6: 469-472.

Sjogren, A., Floren, C. H., Nilsson, A. (1998). Magnesium, potassium and zinc deficiency in subjects with type 2 diabetes mellitus. *Acta Med. Scand.*224: 461-466.

Skowasch, D., Tuleta, I., Viktor, A., Bauriedel, G., Nickenig, G. (2009). Diabetes mellitus is associated with increased *ex vivo*-platelet aggregation and decreased response to aspirin-antithrombotic potential of ACE-inhibitors and AT1-antagonists. *Platelets.* 20: 358-359.

Skrha, J., Prazny, M., Hilgertova, J., Kvasnicka, J.

Kalousova, M., Zima, T. (2007). Oxidative stress and endothelium influenced by metformin in type 2 diabetes mellitus. *Eur. J. Clin. Pharmacol.* 63: 1107-1114.

Thomas, M. C., MacIsaac, R. J., Tsalamandris, C., Molyneaux, L., Goubina, I., Fulcher, G., Yue, D., Jerums, G. (2004). The burden of anaemia in type 2 diabetes and the role of nephropathy: A cross-sectional audit. *Nephrol. Dial. Transplant.*19: 1792-1797.

Wilkinson, I. B., Megson, I. L., MacCallum, H., Sogo, N., Cockcroft, J. R., Webb, D. J. (1999). Oral vitamin C reduces arterial stiffness and platelet aggregation in humans. *J Cardiovasc. Pharmacol.*34: 690-693.

Wu, K. K., Hoak, J. C. (1974). A new method for the quantitative detection of platelet aggregates in patients with arterial insufficiency. *Lancet* 2: 924-926.

Niger. J. Physiol. Sci. 31(June 2016) 063-069
www.njps.com.ng

Niger. J. Physiol. Sci.

Sports Participation, Anthropometric and Physiological Profiles of University Athletes

*** Moses, M.O.[1] and Duduyemi, B.M.[2]**
[1]Department of Sports and Exercise Science, Faculty of Allied Health Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana, [2]Department of Pathology, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana.

Summary: Sports participation has been adjudged to enhance healthy living. This study described anthropometric and physiological (A-P) profiles of university athletes based on types of sports (ToS) and duration (in years) of participation (DoP). One hundred and twenty-nine athletes (69 males, 60 females), aged l5-36, who had played averagely for 5.78±0.29years, from nine games and preparing for Ghana University Sports Association (GUSA) 2014 participated in the study. Ex-post facto research design was adopted. Data on ToS, DoP, age, height, weight, body mass index, waist and hip circumference, body fat and water, blood pressure and heart rate were collected, entered into *SPSS* Data Editor 17.0 and exported to *STATA* 11 where multiple regression analysis and t-test were carried out. ToS has significant effects on anthropometric [$F(_{7,121}) = 2.478$, $p<0.05$] and physiological [$F(_{5,123}) = 5.532$, $p<0.05$] profiles. DoP has significant effects on physiological profiles [$F(_{7,121}) = 5.185$, $p<0.05$] of the athletes. Significant differences existed in age, height, weight, BMI, WHR and SBP ($p<0.05$) based on gender. BMI and HR values were not sufficiently healthy for athletes. Clinical intervention is imperative to determine actual cardiovascular risks of the sample because they might be unfit for national assignment if not properly monitored and trained to be consistent in moderate fitness lifestyles.

Keywords*:* Body mass index; Waist-to- hip ratio; Body fat; Body water; Blood pressure; Heart rate

**Address for correspondence:* moniyl52002@yahoo.com Tel: +233547336905

Manuscript Accepted: May, 2016

INTRODUCTION

Sports participation has always been associated with changes in body systems (Bangsbo et al., 2006). Change in physical appearances is usually noticed after some years of sports participation (Allender et al., 2006). These changes form the basis for training and optimal sports performance (Parsons and Betz, 2001) as well as the state of health for the participants (Bonci et al, 2008). Each sport makes various demands on the structure and functions of athletes' bodies. The type of sports (ToS) and duration of play (DoP) are some of the interwoven components that determine changes in body physiology. Training schedules specifically designed for particular sports have physical and physiological requirement for optimum performance (Olawunmi and Ogunleye, 2010). The degree of commitment to training, actual competition and DoP also prelude healthy and athletic feat (Maron and Pelliccia, 2006). While optimal performance may not be major desirable destiny for many university athletes, good state of health is inevitable. A suitable physique is considered prerequisites for good performance in sports (O'Connor et al, 2007). Appropriate size, shape, body build and composition of athletes are important to success in almost all athletic endeavours and could sometimes be used to determine and qualify physiological characteristics of athletes (Agbonjimi, 1994). BMI is suitable for recognizing trends within sedentary or overweight individuals because there is a smaller margin for errors (Igiri, et al., 2008). Tremendous health benefits such as efficiency of cardiac muscles, decreased risk of coronary heart disease, prevention of excessive weight and obesity, varicose veins, increased hemoglobin and improved cardiorespiratory functional capacity, are accrued to student athletes (Qureshi, 2015). Studies have examined anthropometric and physiological (A-P) characteristics of elite athletes among sports (Da-Cruz-Ferreira, and Ribeiro, 2013; Arazi et al, 2011; Duncan et al., 2006; Jawis et al, 2005; Parsons and Betz, 2001).

Konin and Koike (2008) affirmed that transitioning from university level athletes to professional level requires increased technical skill and understanding of the game. Oftentimes physiological measurements are not considered if an individual can successfully compete at a higher level (Konin and Koike, 2008). However, significant attributes for efficient performance in specific game have been associated with A-P variables like age, height, weight, body mass

index, blood pressure and heart rate of athletes (Da-Cruz-Ferreira and Ribeiro, 2013; O'Connor et al, 2007). Despite over flooding of A-P matters in research, scholarly information on university athletes in Ghana remains unavailable. To contribute to achieving success in sports performance at university level, this study is profiling vital health information about student athletes' A-P characteristics in Ghana. It also presents comparative analysis of A-P based on type of sports (ToS), duration of play (DoP) in years and gender.

MATERIALS AND METHODS

This is an ex-post facto research design study (Barron, 2006). One hundred and twenty-nine (129) Kwame Nkrumah University of Science and Technology (KNUST) athletes (male = 69, 53.5%; female = 60, 46.5%) camped for the 2014 Ghana University Sports Association (GUSA) games participated in the study. Permission of the Head of Sports Directorate and coaches in all the sports as well as written consent of the players obtained. The participants were athletes from nine sports [Football (35, 27.1%), Basketball (13, 10.1%), Volleyball (11, 8.5%), Hockey (27, 20.9%), Athletics (13, 10.1%), Handball (4, 3.1%), Netball (6, 4.7%), Table Tennis (11, 8.5%) and Badminton (9, 7.0%)].

Measurements

Prior discussions on purpose of the study and procedures involved were held with the participants and agreement was reached to assemble at Hale and Hearty center, KNUST, Kumasi, Ghana where anthropometric and physiological profiles were measured.

Anthropometric profiles

Anthropometric profiles of age (yr), height (m), weight (kg), waist and hip circumferences (cm) were assessed to the nearest year, 0.1m, 0.5kg, and 0.5cm respectively. Age of the participants obtained from the university sport council record. Height and body weight were measured with the participants standing bare foot and dressed in short gym clothing using stadiometer (seca 217, Hamburg, Germany). Waist and hip circumferences were assessed with the Png body tape measure. Body mass index (BMI in kg/m) was calculated by dividing weight (kg) with the square of height (m) (Fryar, Gu and Ogden, 2012). Waist-to-hip girth ratio (WHR) computed as abdominal girth (cm) divided by hip girth (cm); waist girth represents the narrowest girth around the natural waist and hip girth reflects the widest girth measured around the buttock (Katch et al., 2011).

Physiological profiles

Physiological profiles were body fat and water, systolic (SBP) and diastolic (DBP) blood pressure as well as heart rate (HR) (Callan et al., 2000; Yoon, 2002; Saad, 2012) measured with aneroid compact sphygmomanometer ce0123 (COD.92310) made in China. Percent body fat and water were measured with TBF-410GS Tanita bioelectrical impedance analyser (BIA) made in Tokyo, Japan, when each of them stepped on the scale in a platform without shoe after inputting weight, height, age and sex. Duration of involvement in competitive sport (DoP) and type of sport (ToS) were obtained via self-reported response from the participants. Measurements took place from 7 to 18 July, 2014.

Statistical Analysis

Data obtained were entered into SPSS Statistics 17.0 Data Editor and exported to STATA 11 where multiple regression and independent t-test analyses were carried out. Statistical significance value of 5% was considered for all analysis.

RESULTS

Descriptive summary of anthropometric and physiological (A-P) profiles of university athletes were presented in table 1. Tables 2-5 showed relative effect of types of sports (ToS) and duration (in years) of participation (DoP) on A-P. Comparison of A-P based on gender is presented in table 6. The participants have DoP range of 1 to 20years (mean: 5.78 ± 0.29years) and age between 15 and 36 (mean: 24.53 ± 0.23years).

Table 1. Descriptive Summary of Participants Profiles

A-P Profiles	Mean ± SD	Skewness
Age (yrs)	24.53± 0.23	0.16
Height (m)	1.61 ± 0.07	0.74
Weight(kg)	66.18 ± 12.75	0.67
BMI(kg/m^2)	25.07 ± 4.42**	0.97
WC (cm)	72.94 ± 12.69	-2.56*
HC (cm)	91.47 ± 13.98	-2.21*
WHR(cm)	0.86 ± 6.26	11.35
Fat (%)	27.72 ± 10.36	0.98
Water (%)	55.13 ± 7.04	-0.14*
SBP(mmHg)	123.20 ± 11.65***	0.11
DBP(mmHg)	76.53 ± 12.89***	0.32
HR (bpm)	74.44 ± 11.32****	0.77

BMI=Body Mass Index, WC=Waist Circumference, HC= Hip Circumference, WHR= Waist-Hip Ratio, SBP=Systolic Blood pressure, DBP= Diastolic Blood Pressure, HR=Heart rate, *Waist, hip and water are negatively skewed. **BMI is within overweight range (CDC). ***BP is within pre-hypertension range (Taplin and Flynn, 2013).****HR indicates below average level (ACSM, 2014)

Table 2. Effect of ToS on Anthropometric Profiles

Model	P	Std. Error	Beta	t	Pvalue
(Constant)	-3.737	8.300		-0.450	0.653
Age	0.216	0.482	0.040	0.448	0.655
Height	6.793	5.082	0.207	1.337	0.184
Weight	-0.024	0.058	-0.117	-0.406	0.686
BMI	-0.102	0.158	-0.174	-0.645	0.520
Waist	-0.014	0.025	-0.078	-0.557	0.578
Hip	0.029	0.021	0.179	1.370	0.173
WHR	-1.507	2.293	-0.065	-0.657	0.512

a. Predictors: (Constant), Age, Height, Weight, BMI, Waist, Hip, WHR b. Dependent Variable: ToS $R=0.354^a$, $R^2=0.125$, Adj $R^2=0.075$, SS =107.874, MS = 15.411, $F_{(7,121)}=2.478$, $P<0.05$

Table 3. Effect of ToS on Physiological Profiles

Model	P	Std. Error	Beta	t	Pvalue
(Constant)	4.961	3.598		1.379	0.170
Fat	-0.066	0.021	-0.266	-3.146	0.002*
Water	0.006	0.031	0.015	0.183	0.855
SBP	-0.035	0.018	-0.157	-1.886	0.062
DBP	0.057	0.017	0.284	3.332	0.001*
HR	0.005	0.019	0.023	0.281	0.779

a. Predictors: (Constant), HR, Water, Fat, SBP, DBP b. Dependent Variable: ToS $R=0.428^a$, $R^2=0.184$, Adj $R^2=0.150$, SS =157.989, MS = 31.598, $F_{(5,123)}=5.532$, $p<0.05$ *Significant at p<005

Table 4. Effect of DoP in sports on Anthropometric Profiles

Model	P	Std. Error	Beta	t	Pvalue
(Constant)	-9.356	8.879		-1.054	0.294
Age	2.489	0.516	0.400	4.823	0.000*
Height	6.011	5.437	0.161	1.106	0.271
Weight	0.023	0.063	0.099	0.366	0.715
BMI	-0.059	0.169	-0.089	-0.352	0.726
Waist	-0.011	0.027	-0.054	-0.409	0.683
Hip	0.020	0.022	0.108	0.882	0.379
WHR	-0.589	2.453	-0.022	-0.240	0.811

a. Predictors: (Constant), WHR, Age, Hip, Height, BMI, Waist, Weight b. Dependent Variable: DoP $R=0.480^a$, $R^2=0.231$, Adj $R^2=0.186$, SS =358.330, MS = 36.904, $F_{(7,121)}=5.182$, $P<0.05$

Table 5. Effect of DoP in sports on Physiological Profiles

Model	P	Std. Error	Beta	t	Pvalue
(Constant)	4.873	4.534		1.075	0.285
Fat	0.015	0.027	0.054	0.581	0.562
Water	0.006	0.038	0.015	0.161	0.872
SBP	0.000	0.023	-0.002	-0.017	0.987
DBP	0.003	0.022	0.012	0.124	0.902
HR	-0.002	0.024	-0.008	-0.088	0.930

a. Predictors: (Constant), WHR, Age, Hip, Height, BMI, Waist, Weight b. Dependent Variable: DoP $R=0.058^a$, $R^2=0.003$, Adj $R^2=-0.037$, SS =3.722, MS = 0.744, $F_{(5,123)}=0.082$, $P>0.05$

Table 6: T-test Gender Comparison of A-P Profiles

A-P Profiles	Gender	Mean±SD	F	P-value	t	Mean Difference
Age	Male	23.00 ± 0.45	9.451	0.003	2.430	0.20*
	Female	17.80 ± 0.48			2.421	
Height	Male	1.64 ± 0.07	6.943	0.009	6.071	0.07*
	Female	1.57 ± 0.06			6.174	
Weight	Male	59.70 ± 11.69	0.063	0.002	6.093	12.11*
	Female	71.81 ± 10.87			6.062	
BMI	Male	23.84 ± 4.33	0.070	0.007	3.033	2.30*
	Female	26.14 ± 4.25			3.038	
Waist	Male	71.81 ± 15.93	1.313	0.254	-.503	-1.27
	Female	73.08 ± 12.07			-.513	
Hip	Male	88.26 ± 16.83	1.936	0.167	-1.955	-5.51
	Female	93.76 ± 14.87			-1.972	
WHR	Male	0.83 ± 0.10	0.513	0.014	3.648	0.07*
	Female	0.76 ± 0.10			3.632	
Fat	Male	26.66 ± 10.45	0.395	0.531	-1.249	-2.28
	Female	28.94 ± 10.21			-1.251	
Water	Male	55.85 ± 6.62	0.148	0.701	1.248	1.55
	Female	54.31 ± 7.46			1.238	
SBP	Male	128.43 ± 11.36	5.040	0.026	6.223	11.25*
	Female	117.18 ± 8.77			6.335	
DBP	Male	75.31 ± 12.36	1.118	0.292	-1.150	-2.61
	Female	77.93 ± 13.45			-1.143	
HR	Male	73.17 ± 11.58	0.001	0.981	-1.377	-2.74
	Female	75.91 ± 10.92			-1.383	

No = (Male 69, Female 60) *Significant difference between male and female athletes in A-P ($p<0.05$)

DISCUSSION

This study described anthropometric and physiological (A-P) profiles of university athletes and examined the effects of ToS and DoP on the profiles. Preference was also given to gender differences in A-P variables. The average age (24.53 ± 0.23 years, table 1) obtained in this study seems to be on the high side for highly vigorous sports peculiar to Africa as the ability to endure aerobically for long will be low. It may place participants, although young adults (Petry, 2002; Rakowski et al, 1990) at disadvantage to compete regularly for long if given shirt in the national team. It may also prone the athletes to potential health/injury risks (Uijtdewilligen et al, 2014; Suliburska et al, 2012) even as athletes. It means that most of the athletes may retire early. This may also account for their inability to win the tournament they were preparing for as reflected in the significant effect of DoP (table 4).

The participants' average height (1.61±0.07m, table 1) falls within those found in earlier studies (Igiri et al, 2008; Ransone and Hughes, 2004). As athletes, stamina is an asset to good performance. The average weight of 66.18kg (table 1) in this study was less than 95.25kg expected in a cluster of sports (Manfred, 2014). Literature acknowledged importance of body weight to health and athletic performance that it should be assessed frequently because of increased risk of dehydration and heat-related illness (Turocy et al, 2011). Monitoring of body weight has responsibility of attenuating body mass index to ensure required sustainable lean body mass for health (Suliburska et al, 2012; Ransone and Hughes, 2004). There was significance difference in body weight based on gender (table 6) where female athletes have higher value. This finding may be associated with eating disorders as documented by Milligan and Pritchard (2006) in the study of Johnson, Powers and Dick that out of 1445 Division 1 college athletes, 9% of the female athletes needed treatment for their eating disorders with additional 58% that were at high risk for developing eating disorder behaviours.

The average BMI of 25.07±4.42 kg/m^2 (table 1) obtained was on the high side (overweight) not only for athletes but also for general good health (CDC; Konin and Koike, 2008). Although, there was significant effect of ToS and DoP on the general anthropometric profiles of the participants, there was no significant effect specifically on BMI (table 2 & 4) which may reflect their level of commitment during and to training activities. Gender comparison of BMI

shows significant difference in favour of male (table 6). Despite the finding on BMI, the value of average WHR (0.86cm) in table 1 appears to be within a healthy range of university athletes (Karimfar et al., 2013). The average body fat value (27.72 ± 10.36%, table 1) in the study sample was not only above the expected range in general population but also above that of athletic population (Jeukendrup and Gleeson, 2010) without significant difference in gender (table 6). This value is considered health risk and detrimental to good athletic performance. The average body water (55.13 ±7.04%) for the study sample was just optimal for resting normal individuals but rather less than expected amount for athletes. Study has shown that more than 2 or 3% dehydration of body mass can potentially compromise or disturb physiological function, increase athletes' risk of developing heat cramps, heat exhaustion or heat stroke and influence sports performance negatively (Turocy et al, 2011; ACSM, 2014). This value may be accounted for by the relatively high body fat in the subjects studied.

For athletes to perform optimally, a lower resting blood pressure of 115/75mmHg has been recommended (Hellesvig-Gaskell, 2013). Our study population has an average resting blood pressure of 123/76mHg which is above the recommended average. Gender comparison in table 6 shows significant difference ($p<0.05$) with male having higher systolic blood pressure. This suggests that athletic performance will be suboptimal as blood pressure tends to increase during exercise. This increase will put stress on cardiac efficiency and therefore limit maximal athletic performance aerobically (Bangsbo et al., 2010). Likewise, the observed average resting heart rate value of 74.44±11.32bpm was seen to be high for good athletic performance since studies have shown that a well trained athlete can have normal resting heart rate closer to 40bpm (Baggish and Wood, 2011; Laskowski, 2012). Based on ToS and DoP in tables 3 and 5, the mean heart rate for the group was not significant ($p>0.05$). On the basis of general health and wellbeing, the resting heart rate value was below average in the age range chart (Ostchega et al., 2011) which indicates sedentary. The sample studied need to work on their weight to reduce BMI, body fat, WHR and resting heart rate.

Sports participation fails to yield expected wellness in the population studied. Findings present these student athletes as having inactive health condition when participation in sports uphold major determinant of vigorous active healthy lifestyle. More clinically inclined interventional studies using this population with attention on the observed variables will vividly complement the findings of this study. A comprehensive educational and clinically inclined intervention is imperative to determine the actual state of physiological profiles of university athletes across sports and duration of participation.

Acknowledgements

Authors gratefully acknowledge the cooperation of the Head of Sports Directorate, coaches and athletes of Kwame Nkrumah University of Science and Technology, Kumasi. The efforts of Segede John Divine, Ezekwem Blaise Pascal, Darko Adu, Obour Agnes and Ogunmoriyele Tobi during the conduct of the study are commendable.

REFERENCES

Agbonjimi, A.P. (1994). Physical Characteristics of Male Athletes Profile Relation Performance. Journal of Nigeria Association of Sports Science and Medicine, 3: 96-100

Allender, S., Cowburn, G. and Foster, C. (2006). Understanding participation in sport and physical activity among children and adults: a review of qualitative studies. Health Education Research, 21 (6): 826-835.

American College of Sports Medicine (ACSM, 2014). ACSM's Guidelines for Exercise Testing and Prescription. Ninth Edition. Philadelphia: Wolters Kluwer/Lippincott Williams & Wilkins Health

Arazi, H., Faraji, H., and Mohammadi, S.M. (2011). Anthropometric and Physiological Profiles of Elite Iranian Junior Rowers. Middle-East Journal of Scientific Research, 9 (2): 162-166

Baggish, A.L. and Wood, M.J. (2011). Athlete's Heart and Cardiovascular Care of the Athlete: Scientific and Clinical Update. Circulation, Jun 14;123(23):2723-35.

Bangsbo, J., Mohr, M. and Krustrup, P. (2006). Physical and metabolic demands of training and match-play in the elite football player. Journal of Sports Sciences, 24(7), 665-674.

Barron, L. (2006). Exploratory research, intellectual craftsmanship, Internet research, research design, writing research. In: V. Jupp (ed.). *The SAGE Dictionary of Social Research Methods*. SAGE Publication Ltd. London EC1Y 1SP. pp.163–164.

Bonci, C.M., Bonci, L.J., Granger, L.R., Johnson, C.L., Malina, R.M., Milne, L.W., Ryan, R.R., and Vanderbunt, E.M. (2008). National Athletic Trainers' Association Position Statement: Preventing, Detecting and managing Disorder Eating in Athletes. Journal of Athletic Training, 43(1):80-108

Callan, S. D., Brunner, D. M., Devolve, K. L., Mulligan, S. E., Hesson, J., Wilber, R. L., and Kearney, J. A. Y. T. (2000). Physiological Profiles of Elite Freestyle Wrestlers. Journal of Stregth and Conditioning Research, 14(2): 162-169.

Centers for Disease Control and Prevention (CDC). Defining Overweight and Obesity. Accessed

16/02/2015 Available @ http://www.cdc.gov/obesitv/adult/defming.html

Centers for Disease Control and Prevention (CDC). Health Weight- It's not a Diet, it's a Lifestyle. Accessed 26/03/2015 Available @ http://www.cdc.gov/healthyweight/assessing/bmi/adult_bmi/

Da-Cruz-Ferreira, A.M. and Ribeiro, C.A.F. (2013). Anthropometric and Physiological Profile of Portuguese Rugby Players - Part I: Comparison between Athletes of Different Position Groups. Rev Bras MedEsporte; 19(1): 48-51

Duncan, M.J., Woodfield, L. and al-Nakeeb, Y. (2006) Anthropometric and physiological characteristics of junior elite volleyball players. Br J Sports Med; 40:649-651. doi: 10.1136/bjsm.2005.021998

Fryar, C.D., Gu, Q. and Ogden, C.L. (2012). Anthropometric reference data for children and adults: United States, 2007-2010. National Center for Health Statistics. Vital Health Stat 11(252).

Hellesvig-Gaskell, K. (2013). Blood Pressure Range for Athletes. August 16, Available @ http://www.livestrong.com/article/524559-blood-pressure-range-for-athletes/. Accessed 13/03/2015

Igiri, A., Ekong, M., Ogan, C. and Odey, P. (2008). Body Mass Index Measure Of Young Adult Nigerians Residents in the Calabar Metropolis. The Internet Journal of Biological Anthropology, 2(2):1-4 Available @ https://ispub.com/IJBA/2/2/12890. Accessed 02/03/2015

Jawis, M.N., Singh, R., Singh, H.J. and Yassin, M.N. (2005). Anthropometric and physiological profiles of sepak takraw players. British Journal of Sports Medicine, 39: 825-829. doi: 10.1136/bjsm.2004.016915

Jeukendrup, A and Gleeson, M. (2010). Sports Nutrition: An Introduction to Energy Production and Performance. Second Edition. Human Kinetics, UK ISBN-13: 9780736079624

Karimfar, M. H., Shokri, S., Bayat, A., Afzali, M. (2013). Long Time Exercise Break off Can Affect Body Mass Index and Waist to Height Ratio of Female Athlete Students Participating in the Athletic Race of Health Ministry of Iran. Shiraz E-Medical Journal, July, 14(3):189-198. Available @ http://semi.sums.ac.ir/vol14/iul2013/92009.htm

Katch,V.L., McArdle, W.D. and Katch, F.I. (2011). Essentials of exercise physiology. (4th ed.).Lippincott Williams & Wilkins, a Wolters Kluwer business.

Konin, J. G. and Koike, K. (2008). Body Mass Index in Men's Collegiate Athletics Compared between University and Conference Teams. Presented at University of South Florida Health Research Day held in Tampa, Florida, USA. February, 2008. Available @ http://www.health.usf.edu/nocms/medicine/orthopaedic/docs/BMI_Big_East_Konin_Koike.pdf

Laskowski ER (2012). What's a Normal Resting Heart Rate? (Expert opinion). Mayo Clinic, Rochester, Minn. September 28, 2012.Available @ http://www.mayoclinic.org/healthy-lifestyle/fitness/expert-answers/heart-rate/faq-20057979. Accessed 16/03/2015

Manfred, T. (2014). Here's the Ideal Body Type for Every Sport. Aug. 25, Available @ http://www.businessinsider.com/average-height-weight-nfl-nba-players-2014-8

Maron, B.J. and Pelliccia, A. (2006). The Heart of Trained Athletes: Cardiac Remodeling and the Risk of Sports, including Sudden Death. Circulation, 114(15):1633-1644

Milligan, B and Pritchard, M. (2006). The Relationship between Gender, Type of Sport, Body Dissatisfaction, Self Esteem and Disordered Eating Behaviors in Division I Athletes. Athletic Insight: The Online Journal of Sports Psychology, March, 8(1): 32-46

O'Connor, H., Olds, T. and Maughan, R.J. (2007). Physique and Performance for Track and Field Events. Journal of Sports Sciences. 25 Suppl.,1:S49-60. Available @ http://www.ncbi.nlm.nih.gov/pubmed/18049983.

Olawunmi, S.A and Ogunleye, V.A. (2010). A Comparative Study of Selected Physical Physiological Characteristics of Handball and Basketball Players. In: B.O. Ogundele, J.F. Babalola, O.A. Moronkola & O.A. Abass (eds). Optimal Health Performance: The Basis of Human Movement Education in the 21st Century. Department of Human Kinetics and Health Education, University of Ibadan, Ibadan, Nigeria. Pg 131-137.

Ostchega, Y., Porter, K.S, Hughes, J., Dillion, C.F., Nwankwo, T. (2011). Resting Pulse Rate Reference Data for Children, Adolescents, and Adults: United States, 1999-2008. National health statistics reports; no 41. Hyattsville, MD: National Center for Health Statistics. Available @ http://www.cdc.gov/nchs/govdelivery.htm Parsons. Accessed 22/04/2015

Parsons, E. M., & Betz, N. E. (2001). The Relationship of Participation in Sports and Physical Activity to Body Objectification, Instrumentality, and Locus of Control Among Young Women. Psychology of Women Quarterly, 25 (3), 209-222.

Petry, N.M. (2002). A Comparison of young, middle-aged and older adult treatment-seeking pathological gamblers. Gerontologist, 42 (1): 92-99, [Abstract]. Available @ http://www.ncbi.nlm.nih.gov/pubmed/11815703. Accessed 02/02/2015

Qureshi, W.T., Alirhayim, Z., Blaha, M.J., Juraschek, S.P., Ketevian, S.J., Brawner, C.A. and Al-Mallah, M.H. (2015). Cardiorespiratory Fitness and Risk if Incident Atrial Fibrillation: Results from The Henry Ford Exercise Testing (FIT) Project. Circulation, May 26;131(21):1827-34.

Rakowski, W., Lefebvre, R. C., Assaf, A. R., Lasater, T. M. and Carleton, R. A. (1990). Health practice correlates in three adult age groups: results from two community surveys. Public Health Reports, 105(5): 481-491.

Ransone, J. and Hughes, B. (2004). Body-Weight Fluctuation in Collegiate Wrestlers: Implications of the National Collegiate Athletic Association Weight-Certification Program. Journal of Athletic Training, 39(2), 162-165.

Saad, A. H. (2012). Physiological Profile of the Young Egyptian Wrestlers, World Journal of Sports Sciences, 6(1): 45-50.

Suliburska, J., Bogdanski, P., Pupek-Musialik, D., Glod-Nawrocka, M., Krauss, H, and Pi^tek, J. (2012). Analysis of lifestyle of young adults in the rural and urban areas. Ann Agric Environ Med., 19(1): 135-139.

Turocy, P. S., DePalma, B. F., Horswill, C. A.,Laquale, K. M., Martin, T. J., Perry, A. C., SomovaM.J., Utter, A. C. (2011). National Athletic Trainers' Association Position Statement: Safe Weight Loss and Maintenance Practices in Sport and Exercise. Journal of Athletic Training, 46(3), 322-336.

Uijtdewilligen, L., Twisk, J. W., Singh, A. S., Chinapaw, M. J., van Mechelen, W. and Brown, W. J. (2014). Biological, socio-demographic, work and lifestyle determinants of sitting in young adult women: a prospective cohort study. The International Journal of Behavioral Nutrition and Physical Activity, 11, 7.

Yoon, J. (2002). Physiological Profiles of Elite Senior Wrestlers. Sports Medicine, 32(4): 225-233.

Niger. J. Physiol. Sci. 31(June 2016) 071-075
www.njps.com.ng

Niger. J. Physiol. Sci.

Resveratrol Protects Rabbits Against Cholesterol Diet-Induced Hyperlipidaemia

*** Tanko, Y[1]., Jimoh, A[1]., Ahmed, A[2]., Mohammed, A[1]., Ayo, J. O[3].**

[1]Department of Human Physiology, Ahmadu Bello University, Zaria, Nigeria, [2]Department of Pharmacognosy and drug development, Ahmadu Bello University, Zaria, Nigeria, [3]Department of Veterinary Physiology and Pharmacology, Ahmadu Bello University, Zaria, Nigeria

Summary: The excessive consumption of high cholesterol diet has been associated with an increased incidence of lipidaemia. Lipidaemia is enhanced by formation of oxidative stress, lipid peroxidation and hyperglycaemia. The aim of these experiments was to investigate the protective effect of resveratrol co-administered with cholesterol diet induced hyperlipidaemia in rabbits. Thirty rabbits divided into six groups of five animal (group= 5) each: group 1 = normal control, group 2 = cholesterol diet/high fat diet group only (HFD), group 3 = resveratrol 200 mg/kg (R200), group 4 = resveratrol 400 mg/kg (R400), group 5 = HFD + R200 and group 6 = HFD + R400. The normal group was fed with standard animal feeds only; while the HFD groups were fed with standard animal feeds + cholesterol diet (10% Groundnut oil, 20% Groundnut mill and 2% cholesterol). Resveratrol-treated rabbits received resveratrol suspended in 10 g/L carboxymethylcellulose (CMC) and the control group received the vehicle only, CMC. The preparations were administered for 8 weeks of experimental protocol. At the end of the study period, the animals were sacrificed. Blood and plasma samples were collected. Serum evaluation of lipid profile such as total cholesterol (TC), triacylglycerol (Tg), low density lipoprotein cholesterol (LDP-c) and high density lipoprotein cholesterol (HDL-c) were also assessed. The results obtained show significant ($P < 0.05$) decrease in total cholesterol (TC), Low density lipoprotein cholesterol (LDP-c), total triacylglycerol and an increase in high density lipoprotein cholesterol (HDL-c) in resveratrol treated groups compared to HFD group only. In conclusion, the findings indicated that Resveratrol may contain polar products able to lower plasma lipid concentrations and might be beneficial in treatment of hyperlipidemia and atherosclerosis.

Keywords*:* Cholesterol diet, Lipidaemia, Rabbit; Resveratrol, LDL-c, HDL-c, TC, TG

**Address for correspondence:* yusuftanko@abu.edu.ng

Manuscript Accepted: June, 2016

INTRODUCTION

Regular consumption of food rich in antioxidant are associated with numerous health benefits rooted in their various physiological effects as a result of nutritional constituent (Hunter and fletcher, 2002). Series of clinical trials have demonstrated the therapeutic benefits of treatment with various antioxidants. Antioxidants showed major improvement in patients with impaired glucose tolerance and lipid metabolisms (Beckman *et al*., 2003; Liu *et al.,* 2010; Singh *et al.,* 2011). Resveratrol (3, 5, 4'- trihydroxystilbene) is a polyphenol that occurs naturally in foods and drinks made from grapes and peanuts, and also in a number of herbal remedies. The discovery of resveratrol in wine implicated a role for this compound in the ''French Paradox'', the observation that the French exhibit a relatively low rate of cardiovascular disease although their diet is high in saturated fats (Siemann and Creasy, 1992). Since then, studies have shown that resveratrol is a member of a class of compound called phytoalaxins, which plants use as a defense mechanism against pathogens, and it has also shown that it prevents or slows the progression of a wide variety of illnesses, including treatment of diabetes complications (Soufi *et al*., 2012), cancer, cardiovascular disease (Vigtdeux *et al*., 2010), ischemic injuries and myocardial infarction (Sinha *et al.,* 2002; Lamont *et al*., 2011),

Elevated cholesterol levels have long been recognized as having an association with lipidaemia. Lipidaemia tends to result in an elevation in total cholesterol and triglycerides and a reduction in high-density cholesterol (HDL) (Khaodhiar *et al.,* 2002). Resveratrol has been found to exert a number of potentially cardioprotective effects in vitro, including inhibition of platelet aggregation (Wang *et al.,* 2002), promotion of vasodilatation by enhancing the production of nitric oxide (NO) (Wallerath *et al*., 2002). Experiments have demonstrated that both melatonin and resveratrol, as found in red wine, protect the heart from myocardial infarction (Lamont *et al.,* 2011). The cardioprotective effect of resveratrol was also attributed to its ability to upregulate the activity of catalase, an antioxidant enzyme in the myocardium (Shigematsu *et al*., 2003). Increased adipocyte mass is associated with obesity and impaired

lipid metabolism. Consequently, the high levels of circulating free fatty acids (FFA) and glucose are potent inducers of cellular reactive oxygen species (ROS) (Dunmore and Brown, 2013). The aim of this study is to investigate the protective effect of resveratrol co-administered with cholesterol diet induced hyperlipidaemia in rabbits.

MATERIALS AND METHODS

MATERIALS AND METHODS

Chemicals

All chemicals were obtained commercially and were of analytical grade: Cholesterol (Mumbai India, M. W 386.67, CAS No. 57-88-5, LoT No. 100413) and Mega resveratrol: 99 % pure trans-resveratrol Batch Number: MR 131120, Average particle size: 2.5µm Sigma USA).

Materials and instruments

Electronic weighing scale Model: EK 3052 balance, Spectrophotometer, dissecting set, syringes and niddle (Sologuard Medical Device P.V.T Ltd., Chema-600 096, India, ML No. 750).

Resveratrol preparation and administration

Trans-resveratrol, due to its low solubility in water, was suspended in 10 g/L of carboxymethylcellulose (CMC), and administered orally according to the method of Juan *et al*. (2005).

Experimental animals

Seven weeks old male rabbits of different crossbreeds (New Zealand and local breed), weighing between 300 - 350 g, raised in the Animal House, Department of Human Physiology, Ahmadu Bello University, Zaria were used for the study. The animals were kept in well-aerated laboratory cages in the Departmental Animal House, and were allowed to adjust to the laboratory conditions for a period of three weeks before the commencement of the experiment. They were fed with growers' and starters' mash from (Vital Feeds Company Kaduna, Nigeria), and given access to water during the stabilizing period *ad libitum.*

Induction of lipidaemia

Lipidaemia was induced by feeding the animals with cholesterol diet for eight weeks. The normal groups were fed with standard animal feeds only, while the high fat-diet groups were fed with standard animal feeds + high fat diet (10 % Groundnut oil, 20 % Groundnut mill and 2 % cholesterol).

Experimental Animal Groupings

In the study, 30 rabbits weighing between 300 and 350 g were used, each group comprised five rabbits (n = 5). The animals were grouped according to the method of Joanne *et al.* (2008) as follows:

Group 1: The animals were allowed to free access to a normal diet and received 10 g/L CMC each orally as negative control group

Group 2: The animals were allowed to cholesterol diet/high fat diet (HFD) as feed only and served as positive control group.

Group 3: The animals were allowed to free access to a normal feed and received 200 mg/kg body weight of resveratrol orally (R200).

Group 4: The animals were allowed to free access to a normal feed and received 400 mg/kg body weight of resveratrol orally (R400).

Group 5: The animals were allowed to cholesterol-diet/high fat diet (HFD) as feed only and received 200 mg/kg body weight of resveratrol (R200) orally.

Group 6: The animals were allowed to cholesterol-diet/high fat diet (HFD) as feed only and received 400 mg/kg body weight of resveratrol (R400) orally.

Collection and preparation of serum samples for analysis

Eight weeks after the treatment period, all rabbits were subjected to light anaesthesia by exposing them to chloroform soaked in cotton wool placed in anaesthetic box, covered with lid. Blood samples of about 5 ml were drawn from the heart of each sacrificed animal from all groups by cardiac puncture. The samples were collected in Eppendrof tubes and allowed to clot. Thereafter, the serum was separated by centrifugation, using Denley BS400 centrifuge (England) at 3000 *g* for 10 minutes. The supernatant collected was used for the following analyses:

Determination of serum total cholesterol

Total cholesterol (TC) was determined spectrophotometrically, using enzymatic colometric assay kits (Randox Laboratories Limited Kits, United Kingdom). Briefly,the serum level of total cholesterol was quantified after enzymatic hydrolysis and oxidation of the sample according to the method of Stein (1987). Briefly, 1000 µL of the reagent were added to each of the sample and standard. This was incubated for 10 minutes at 20-25 ^{0}C after mixing, and the absorbance of the sample (A_{sample}) and standard ($A_{standard}$) were measured spectrophotometrically against the reagent blank within 30 minutes at 546 nm. The value of total cholesterol present in the serum was expressed in mg/dL. Total cholesterol concentration = A *sample* /A *standard x* 196.86 mg/dL.

Determination of serum triglyceride

The serum triglyceride level was determined after enzymatic hydrolysis of the sample with lipases as described by Tietz (1990). Briefly 1000 µl of the reagent were added to each sample and standard. This was incubated for 10 minutes at room temperature (20-

25 ^{0}C) after mixing, and the absorbance of the sample (A_{sample}) and standard ($A_{standard}$) were measured against the reagent blank within 30 minutes at 546 nm. The values of triglyceride present in the serum were expressed in mg/dl. Triglyceride concentration = A *sample*/A *standard x* 194.0 mg/dL.

Determination of serum high-density lipoprotein cholesterol

The serum level of HDL-C was measured using the method of Wacnic and Albers (1978). Low-density lipoproteins (LDL), very low density lipoproteins (VLDL) and chylomicron fractions in the sample were precipitated quantitatively by addition of phosphotungstic acid in the presence of magnesium ions. The mixture was allowed to stand for 10 minutes at room temperature (20-25 ^{0}C), and centrifuged for 10 minutes at 1200 *g.* The supernatant represented the HDL-C fraction. The cholesterol concentration in the HDL fraction, which remained in the supernatant, was determined. The values of HDL-C were expressed mg/dL.

Determination of serum low-density lipoprotein cholesterol

The serum level of LDL-C was measured according to the protocol of Friedewald *et al*. (1972) using the equation below: LDL-C = TC - (HDL-C - TGL/2.2). The values obtained were expressed in mg/dL.

STATISTICAL ANALYSIS

Blood glucose levels were expressed in mg/dL and body weight in kg as mean ± SEM. The data were analyzed using ANOVA followed by Dunett's *post-hoc* test to show multiple comparisons versus control group. Data analysis was evaluated using SPSS version 17.0 software and Microsoft Excel (2007). Values of $P \leq 0.05$ were considered as significant (Duncan *et al.,* 1977)

RESULTS

Total cholesterol assay

Figure 1 shows the results of the effects of resveratrol (200 mg/kg and 400 mg/kg) co-administered with high fat diet-fed rabbits. Serum total cholesterol significantly ($P < 0.05$) decreased R200 + HFD and R400 + HFD with values of 1.56 ± 0.12 g/L and 1.54 ± 0.14 g/L when compared to the value recorded for high-fat diet group only with a value of 2.38 ± 0.11 g/L respectively.

Total triacylglyceride assay

Figure 2 represents the results of the effects of resveratrol (200 mg/kg and 400 mg/kg) co-administered with high fat diet-fed rabbits. Serum total triacylglycerides showed significant ($P < 0.05$) decrease in values recorded for R200 + HFD and R400 + HFD with values of 1.28 ± 0.09 g/L and 1.24 ± 0.07 g/L when compared to high fat diet group only with a value of 1.64 ± 0.81 g/L.

High-density lipoprotein cholesterol assay

Figure 3 shows the results of the effects of 200 mg/kg and 400 mg/kg of resveratrol co-administered with high fat diet-fed rabbits. Serum high density lipoprotein cholesterol showed significant ($P < 0.05$) increase in resveratrol co-administered with values of 0.54 ± 0.02 g/L for HFD + R200 and 0.52 ± 0.08 g/L for HFD + R400 when compared to high fat diet group only with a value of 0.38 ± 0.04 g/L.

Low density lipoprotein cholesterol assay

Figure 4 shows the results of the effects of resveratrol (200 mg/kg and 400 mg/kg) co-administered with high fat diet in rabbits. Serum low density lipoprotein cholesterol showed significant ($P < 0.05$) increase in R200 + HFD and R400 + HFD with values of 0.32 ± 0.15 g/L and 0.41 ± 0.16 g/L when compared to high fat diet group only with a value of 1.25 ± 0.09 g/L.

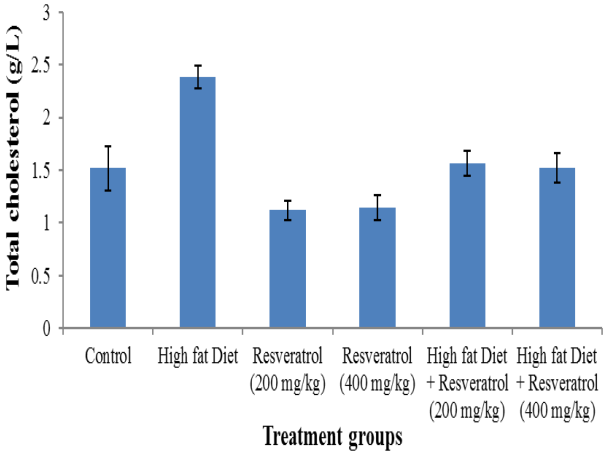


Figure 1: Effect of co-administration of resveratrol and high-fat diet on total cholesterol in rabbits as compared with fed and unfed control group. Values are expressed as mean ± SEM; n = 5 Values with error bars having different superscripts letters are significant ^{a,b,c} = $p < 0.05$ significant.

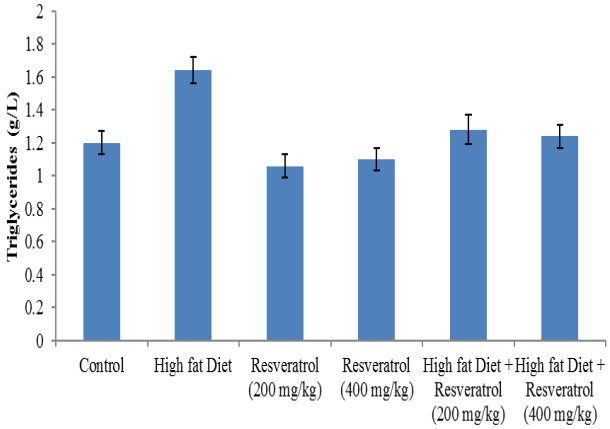


Figure 2: Effect of co-administration of resveratrol and high-fat diet on total triacyglyceride in rabbits as compared with fed and unfed control group. Values are expressed as mean ± SEM; n = 5.

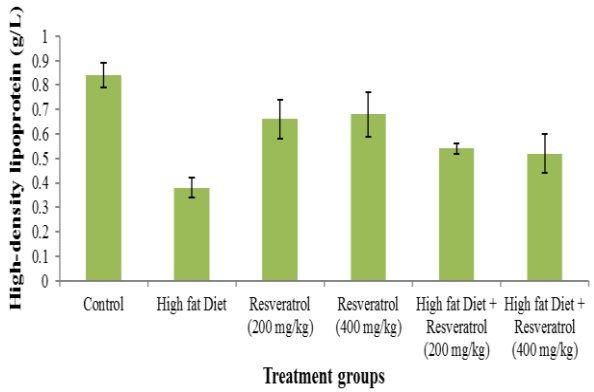


Figure 3: Effect of co-administration of resveratrol and high-fat diet on high density lipoprotein in rabbits as compared with fed and unfed control group. Values are expressed as mean ± SEM; n = 5.

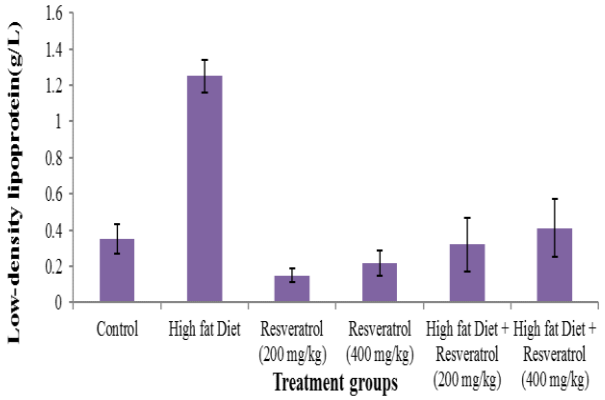


Figure 4: Effect of co-administration of resveratrol and high-fat diet on low density lipoprotein in rabbits as compared with fed and unfed control group. Values are expressed as mean ± SEM; n = 5.

DISCUSSION

The lipid profile obtained in the present study showed a significant decrease in total cholesterol, total triglyceride, low-density lipoprotein and an increase in high-density lipoprotein cholesterol levels in resveratrol groups' treated with cholesterol diet compared to cholesterol diet group only. Lipidaemia observed in cholesterol diet group may be as a result of increase in visceral adipose mass (Brown and Dunmore, 2013), which causes impaired lipid metabolism and consequently result in high levels of circulating free fatty acids and glucose which are potent inducer of cellular reactive oxygen species (Youn *et al.,* 2014). High level of free fatty acid causes elevated cholesterol levels which have long been recognized as having an association with hyperlipidaemia (Buettner *et al,* 2006). The observed decrease in total cholesterol, total triacyglycerol, low density lipoprotein and increase in high density lipoprotein in groups supplemented with resveratrol may be due to low activity of cholesterol biosynthesis enzymes, low level of lipolysis and inhibition of dysregulation of lipid metabolism which hindered mobilization of excess cholesterol into the body system. These findings suggest that one of the possible mechanisms of anti-lipidaemic action of resveratrol supplement is by modulating one or more of the aforementioned mechanisms. The decrease in lipid profile after consumption of cholesterol diet with resveratrol may have demonstrated why resveratrol, as found in red wine, protects the heart from myocardial infarction (Lamont *et al.*, 2011). This cardio-protective effect of resveratrol may also be attributed to its ability to upregulate the activity of catalase, an antioxidant enzyme in the myocardium (Shigematsu *et al*., 2003). Hyperlipidaemia itself usually causes no symptoms, but may lead to symptomatic vascular diseases, including coronary artery disease and peripheral arterial disease (Rohilla *et al*., 2011). Jeong *et al.* (2011), demonstrated that administration of grape skin extract rich in resveratrol to animal fed on high fat diet significantly recorded a decrease in total cholesterol, triglyceride, low density lipoprotein and increase in high density lipoprotein which agrees with our findings. The decrease in serum lipid profile may be as a result of resveratrol inhibiting fat accumulation and fatty acid synthesis by activation of fatty acid oxidation, demonstrated by Sahar and Abdel, (2012).

In conclusion, the findings indicated that Resveratrol may contain polar products able to lower plasma lipid concentrations and might be beneficial in treatment of hyperlipidemia and atherosclerosis.

REFERENCES

Beckman, J. A., creager, M. A. and Libby, P. (2003). Diabetes and atherosclerosis: epidiomology, pathophysiology and management. *Journal of American Medical Association*, 287: 2570-2581.

Buettner, R., Parhofer, K. G., Woenckhaus, M., Wrede, C. E., Kunz-Schughart, L. A., Scholmerich, J. and Bollheimer, L. C. (2006). Definition high fat-diet rat models: metabolic and molecular effects of different fat types. *Journal of Molecular Endocrinology*, 36: 485-501

Duncan, R. C., Knapp, R. G. and Miller, M. C. (1977). Test of hypothesis in population means. In: *Introductory Biostatistics for the health sciences*, John Wiley and Sons Inc. NY: 71-96

Dunmore, S. J. and Brown, J. E. P. (2013). The roles of adipokines in β-cell failure of type 2 diabetes. *Journal of Endocrinology*, 216: 37-45.

Friedewald, W. T., Levy, R. and Fradrickson, D. S. (1972). Estimation ofconcentration of low density lipoprotein. *Fundamentals of Clinical Chemistry.* W. B Sauders Philadelphia, 3: 470-479.

Jeong, Y. S., Jung, H. K., Cho, K. H., Youn, K. S. and Hong, J. H. (2011). Anti-obesity effect of grape

skin extract in 3T3-L1 adipocytes. *Food Science Biotechnology*, 20: 635-642.

Huntler, K. J. and Fletcher, J. M. (2002). The antioxidant activity and composition of fresh, frozen, jarred and canned vegetable. *Innov Food Sci. Emerg. Technol*, 3: 399-406.

Joanne, M. A., Xiaomei, L., Christopher, Q. R., Brandi, P. and Min, Y. (2008). Resveratrol alleviates alcohol fatty liver in mice. *American Journal of Physiology*, 295(4): 833-842.

Juan, M. E., Gonzalez-pons, E., Munuera, T., Ballester, J., Ridnguez-Gil, J. E and Planas, J. M. (2005). Trans- resveratrol, a natural antioxidant from grapes, increases sperm output in healthy rats. *Journal of Nutrition*, 135: 757-760.

Khaodhiar, L., McCowen, K. C. and Blackburn, G. L. (2002). Obesity and its comorbid conditions. *Clinical Cornerstone*, 2: 17-31.

Lamont, K. T., Somers, S., Lacerda, L., Opic, L. H. and Lecour, S. (2011). Is red wine a safe sip away from cardioprotection? Mechanisms involved in resveratrol-and melatonin-induced cardioprotection. *Journal of Pineal Research*, 50(4): 374-380.

Liu, F., Hung, L., Wu, W., Chang, D., Huang, C., Lai, J. and Ho, L. (2010). Chondroprotective effects and mechanisms of resveratrol in advanced glycation end products-stimulated chondrocytes. *Arthritis Research Therapy*, 12(5): R167

Rohilla, A., Rohilla, S., Singh, G., Kumar, A. and Khan, M. U. (2011). Atorvastatin pleiotropism role in cardioprotection. *International Journal of Pharmacute Biology Achieves*, 2(3): 813-818

Sahar, S. and Abdel, M. S. (2012). The effects of varieties sources of omega-3 fatty acids on diabetes in rats. *Food and Nutrition Sciences*, 3: 1404-1412.

Sinha, K., Chaudhary, G. and Gupta, Y. K. (2002). Protective effect of resveratrol against oxidative stress in middle cerebral artery occlusion model of stroke in rats. *Life Science*, 71: 655-665.

Shigematsu, S., Ishida, S., Hara, M., Takashi, N., Yoshimatsu, H., Sakata, T. and Korthuis, R. J. (2003). Resveratrol, red wine constituent of polyphenol, prevents superoxide-dependent inflammatory responses induced by ischemia/reperfusion, platelet-activating factor or oxidants. *Free Radical Biology and Medicine*, 34: 810-817.

Singh, G., Gupta, V., Sharma, A. K. and Gupta, N. (2011). Frequently of thyroid dysfunction among diabetes in punjabi population. *Advance in Bioresearch*, 2: 3-9.

Siemann, E. H. and Creasy, L. L. (1992). Concentration of phytoalexin resveratrol in wine. *American Journal of Enology and Viticulture*, 43: 49-52.

Soufi, F. G., Mohammed-Nejad., D. and Ahmadieh, H. (2012). Resveratrol improves diabetic retinopathy possibly through stress-nuclear factor KB-apoptosis pathway. *Pharmacological Report*, 64: 1504-1514.

Stein, E. A. (1987). Lipids, lipoproteins and Apolipoproteins. In: Treitz, N. W. (Ed). *Fundamentals of Clinical Chemistry*, 3rd Edn., W. B Sauders Philadelphia, pp; 470-479.

Tietz, N.W. (1990). Clinical Guide to Laboratory Test, Second Edition W.B. Saunders Company, Philadelphia, U.S.A. 554-556.

Vingtdeux, V., Giliberto, L., Zhao, H., Chandakkar, P., Wu, Q., Simon, J. E., Janle, E.M., Labo, J., Ferruzzi, M. G., Daries, P. and Marambaud, P. (2010). Amp-activated protein kinase signaling activity by resviratrol modulates amyloid-beta peptide metabolism. *Journal of Biological Chemistry*, 285(12): 9100-9101.

Wacnic, R. G. and Alber, J. J. (1978). A comprehensive evaluation of the heparin manganese precipitation procedure for estimating high density lipoprotein cholestsrol. *Journal of Lipid Research*, 19: 65-76.

Wang, J., Obici, S., Morgan, K., Barzilai, N., Feng, Z. and Rossetti, L. (2002). Overfeeding rapidly induces leptin and insulin resistance. *Diabetes*, 50(12): 2786-2791.

Wallerath, T., Deckert, G., Ternes, T., Anderson, H., Li, H., Witte, K. and Forstermann, U. (2002). Resveratrol, a polyphenolic phyto alexin present in red wine enhances expression and activity of endothelial nitric oxide synthase. *Circulation*, 106(13): 1652 - 1658.

Youn, J. Y., Siu, K. L., Lob, H. E., Itani, H., Harrison, D. G. and Cai, H. (2014). Role of vascular oxidative stress in obesity and metabolic syndrome. *Diabetes*, 63: 2344-2355.

Niger. J. Physiol. Sci. 31(June 2016) 077-081
www.njps.com.ng

Niger. J. Physiol. Sci.

Evaluation of Body Mass Index and Plasma Lipid Profile in Boerboel Dogs

*** Ajadi Rasheed Adetola, Ashogbon Rashidat Oluwatobiloba, Adeniyi Abisola Abosede**

Department of Veterinary Medicine and Surgery, Federal University of Agriculture, Abeokuta, Nigeria

Summary: This study evaluated the body mass index (BMI) and plasma lipid profile in Boerboel dogs. Body weights (BW), height (H) at shoulder and waist circumference (WC) were obtained from fifty-three Boerboels to determine the BMI while, body condition score (BCS) was determined subjectively. Also 5mls of blood was obtained from the dogs for determination of total cholesterol (TC), triglycerides (TRIG), low density lipoproteins (LDL) and high density lipoproteins (HDL). Data were presented as means ± standard deviation and results were compared using analysis of variance. Relationship between BW, H and WC was determined using regression analysis. Value was accepted significant at $p < 0.05$. There were no significant differences ($P > 0.05$) in BW, WC, BMI and GAS between male and female Boerboels, however, H was significantly ($P < 0.05$) higher in male (62.0 ± 1.6 cm) than female Boerboels (57.0 ± 1.5 cm). BMI and HDL were significantly ($P < 0.05$) lower in Boerboels <23 months (112.4 ± 2.8 Kg/m^2; 36.0 ± 2.4mg/dl) compared with those 24 – 47 months (133.4 ± 1.8Kg/m^2; 40.1 ± 2.2mg/dl) and >48 months (137.9 ± 1.6kg/m^2; 45.8 ± 2.6mg/dl) respectively. However, there were no significant differences ($P > 0.05$) in TC, TRIG, HDL and LDL between Boerboels with BCS > 5 compared to those with BCS < 5. BMI linearly increased with decreasing H and WC in Boerboel dogs. It was concluded that BMI did not differ between sexes of Boerboel but differ between age categories.

Keywords*:* Body mass index, Lipid profile, obesity, osteoarthritis, Boerboel

**Address for correspondence:* ade_vsr@hotmail.com Telephone: 234-703-380-0326, 234-802-257-2535

Manuscript Accepted: June, 2016

INTRODUCTION

The Boerboel is a big, strong and intelligent working dog. It is well balanced with good muscle development and buoyant in movement. The dog is impressive and imposing. Boerboel are believed to have originated from South African and was thought to be a crossbreed between Bullmastiff and local South African dog breeds like Bullenbijter. Boerboels are generally known for their good health. However, Boerboels can suffer from hip or elbow dysplasia, cervical spondylomyelopathy, vaginal hyperplasia, ectropion, and entropion (Gray, 2003).

Vascular diseases and cardiovascular risk factors are high amongst people with osteoarthritis (Plumb and Aspden, 2004). Emerging evidence suggests that osteoarthritis may share similar risk factors with cardiovascular diseases (Conaghan *et al.*, 2005). Hypercholesterolemia and hypertriglyceridemia, the risk factors for cardiovascular disease, have been related to risk of osteoarthritis and its progression (Findlay, 2007). It has been reported that humans with osteoarthritis had altered lipid profiles characterized by increased concentration of total cholesterol (Borman *et al.*, 1999). It was demonstrated that serum cholesterol and triglyceride levels were associated with incidence of bone marrow lesions in humans (Davies-Tuck *et al.*, 2009). On the contrary, there were no associations between the progression of experimental knee osteoarthritis and plasma lipid profile of dogs (Alam *et al*., 2006; Ajadi *et al.*, 2012).

Obesity is an escalating global health problem both in humans and domestic animals. It is a very important risk factor in the development of musculoskeletal diseases. The incidence of obesity in dogs and cats has been estimated to be between 20 and 40%, and there is an increasing trend in the incidence of obesity in the pet population (McGreevy *et al.*, 2005). The clinical problem of obesity in animals lies in the fact that it is frequently associated with several metabolic abnormalities such as dyslipidemia, insulin resistance and the development of orthopaedic disorders such as osteoarthritis and intervertebral disc disease (German, 2006). Therefore, early recognition of the pathophysiology of obesity with its metabolic alterations and correlates is essential in its control in large breed of dogs.

It has been shown that apart from food intake, sex hormones plays an important role in weight gain (Kanchuk *et al.*, 2003). For instance, gonadectomised dogs have been shown to have greater risk of growing

obese compared with intact dog (German, 2006). In humans, post-menopausal women are at greater risks of developing osteoarthritis (OA) compared with men of the same age due to low secretion of oestrogen (Srikanth *et al.*, 2005). In spite of the recorded high prevalence of obesity in pet population and the implication of obesity in the pathophysiology of a number of health disorders among pet population, very little is known about the prevalence of obesity amongst Boerboel population in Nigeria.

In humans, the National Institute of Health developed a body mass index (BMI) which measures body fat based on height and weight (Tobias *et al.*, 2014). These indexes are considered the most accurate assessment of body condition available. However, no such index exists for dogs, so it is exceedingly difficult to objectively evaluate dogs' body condition. A visual nine-step chart was developed for subjective assessment of the body condition system (BCS) in dogs (Laflamme, 1997). However, this body condition scoring (BCS) system fails to take into account the wide range of sizes among dogs of a particular breed hence leading to inaccurate judging of the body condition. The method of estimating body mass index (BMI) in humans is currently being evaluated for its accuracy in dogs by comparing the results with body condition scoring system. Till now there is no record of the plasma lipid profile in Boerboel dogs in spite of their high risk for obesity and musculoskeletal diseases. This study compared the accuracy of the body mass index system in Boerboel dogs using the methods described in humans. It also determined the prevalence of obesity among Boerboel population and evaluated the association between body mass index and plasma lipid profile.

MATERIALS AND METHODS

Fifty three client-owned Boerboel dogs of both sexes were used. The dogs were adjudged to be in good health condition based on physical examination findings and complete blood profile. Informed owner's consents were obtained and documented. Also, the diet histories of the dogs were noted. The breed, sex and age of the dogs were also noted. In addition, information on whether the dogs have been neutered or not were obtained.

Body weights (BW) of the dogs were obtained with bathroom scale, while height (H) at shoulder and the waist circumference (WC) were determined using a meter rule. These indices were used to determine the body mass index (BMI). The BMI was calculated as the ratio of the body weight (Kg) to the square of the height (m) i.e. BMI= BW (Kg)/H^2 (m^2). In addition, the body condition score (BCS) was determined subjectively using a scale from 1-9 as described by Laflamme *et al.*, 1999.

About10 mls of blood was obtained from the cephalic vein of the dogs into lithium heparin bottle for the determination of total cholesterol (TC), triglycerides (TRIG), low density lipoproteins (LDL) and high density lipoproteins (HDL) and were determined using Randox Laboratory kit reagents as described by Akpanabiatu *et al.*, 2005.

Statistical Analysis

Data were presented as means ± standard deviation (SD) and results were compared using analysis of variance (ANOVA), while relationship between BW, H and WC was determined using regression analysis. P value was accepted significant at values less or equal to 0.05

RESULTS

The fifty-three Boerboel dogs used in this study comprised of twenty-six (26) males and twenty-seven (27) females. Using the body condition scoring (BCS) twenty (20) of the dogs were classified as obese giving an obesity prevalence of thirty-eight per cent (38%).

There were no significant differences ($P > 0.05$) in body weight, waist circumference and body mass index between the male and female dogs (Table 1). However, the height at shoulder was significantly ($P < 0.05$) higher in the male dogs than the female dogs. Similarly, there were no significant ($P > 0.05$) differences in the plasma concentration of triglycerides, total cholesterol, low density lipoprotein and high density lipoprotein (Table 2).

The body weight, waist circumference and height at shoulder did not differ significantly between the three age categories (Table 3). However, the body mass index was significantly ($P < 0.05$) lower in dogs under the 0 – 23 months age category when compared with other age categories. Similarly, the high density lipoprotein was significantly ($P < 0.05$) lower in dogs under the 0 – 23 months age category (Table 4).

Table 1: Effect of sex on body weight, height at shoulder, waist circumference, body mass index and gait assessment score of dogs

Sex	BW (Kg)	H (cm)	WC (cm)	BMI (Kg/m^2)
Male N=26	46.5±0.9	62.0±1.6*	76.5 ±2.4	127.4 ± 7.4
Female N-27	45.4±1.8	57.0±1.5*	74.0±2.8	127.2 ± 5.6

N: Number of dogs, **BW**: Body weight, **H**: Height to the shoulder, **BMI**: Body mass index, **WC**: Waist circumference * P = 0.026

Table 2: Effect of sex on plasma triglycerides, total plasma cholesterol, low density lipoproteins and high density lipoproteins

Sex	TRIG (mg/dl)	TC (mg/dl)	LDL (mg/dl)	HDL (mg/dl)
Male N=26	94.3±7.9	159.9 ± 9.8	101.4 ± 8.0	42.8 ± 2.6
Female N-27	96.4±5.7	159.9 ± 7.8	101.2 ± 6.4	38.5 ± 2.5

N: Number of dogs **TRIG**: Plasma triglycerides**, TC**: Total plasma cholesterol, **HDL**: High density lipoproteins**, LDL**: Low density lipoproteins

Table 3: Effect of Age categories on body weight, height at shoulder, waist circumference, body mass index and gait assessment score of dogs

Age categories	BW (Kg)	H (cm)	WC (cm)	BMI (Kg/m^2)
0–23 months	47.1±1.5	64.0±1.9	75.1± 1.8	112.4 ± 2.8*
24-47 months	44.7±1.7	57.7±1.2	75.2± 1.5	133.4 ± 1.8*
> 47 months	45.8±1.4	57.9±1.6	76.8± 1.9	137.9 ± 1.6*

N: Number of dogs, **BW**: Body weight, **H**: Height to the shoulder,**BMI**: Body mass index, **WC**: Waist circumference, ***:** P = 0.003

Table 4: Effect of age categories on plasma triglycerides, total plasma cholesterol, low density lipoproteins and high density lipoproteins

Age categories	TRIG (mg/dl)	TC (mg/dl)	LDL (mg/dl)	HDL (mg/dl)
0–23 months	91.1 ±6.7	155.7±8.3	102.1± 7.4	36.0 ± 2.4*
24-47 months	93.2 ± 7.8	162.2±7.9	101.9 ± 7.8	40.1 ± 2.2*
> 47 months	103.9±5.9	164.2±6.9	101.3 ± 8.2	45.8 ± 2.6*

N: Number of dogs **TRIG**: Plasma triglycerides**, TC**: Total plasma cholesterol, **HDL**: High density lipoproteins**, LDL**: Low density lipoproteins ***:** P = 0.02

Table 5: Effect of body condition scores on plasma triglycerides, total plasma cholesterol, low density lipoproteins and high density lipoproteins

Obesity Status	TRIG (mg/dl)	TC (mg/dl)	LDL (mg/dl)	HDL (mg/dl)
Non-obese N=33	95.1 ± 7.2	161.7 ± 7.5	103.7 ± 6.3	40.9 ± 2.5
Obese N=20	95.9 ± 6.8	157.1 ± 8.4	97.2 ± 5.7	40.2 ± 2.3

N: Number of dogs **TRIG**: Plasma triglycerides**, TC**: Total plasma cholesterol, **HDL**: High density lipoproteins**, LDL**: Low density lipoproteins

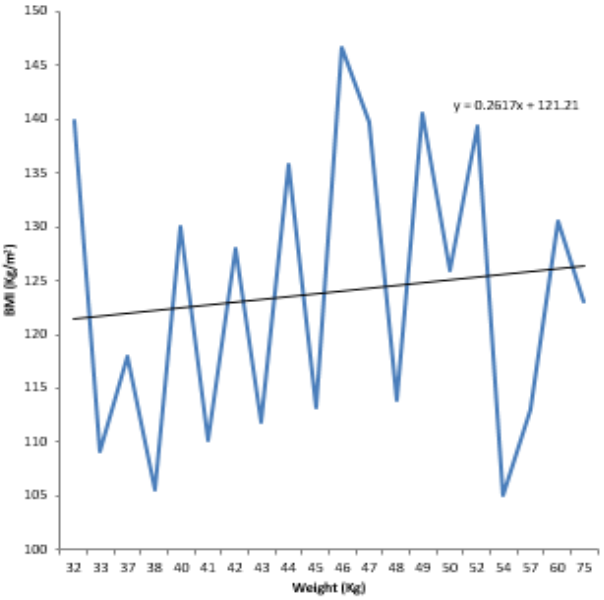


Fig. 1: Changes in BMI with increasing body weight

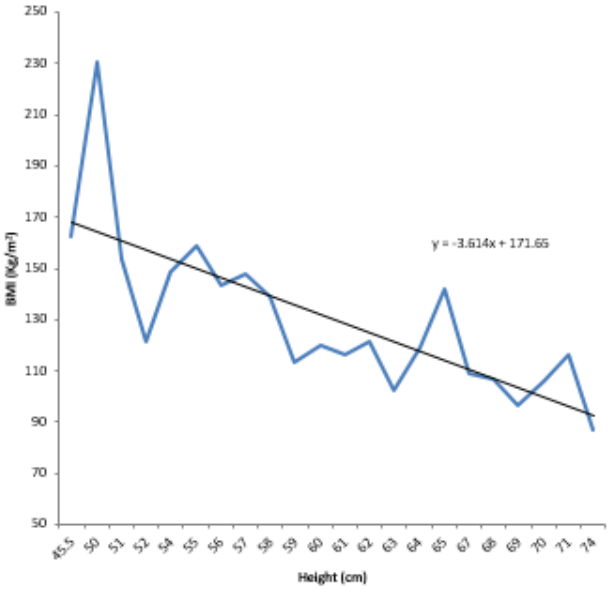


Fig. 2: Changes in BMI with decreasing height at shoulder

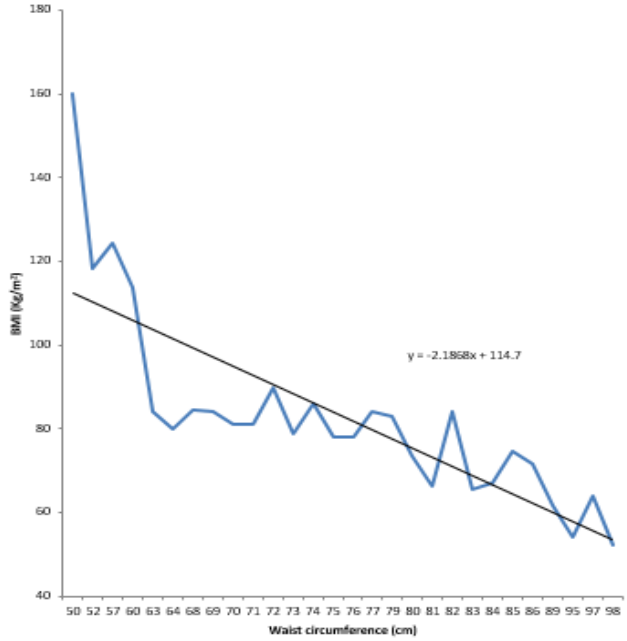


Fig. 3: Changes in BMI with decreasing waist circumference

There were no significant differences in the plasma concentrations of total plasma triglycerides, total cholesterol, low density lipoprotein and high density lipoprotein between dogs with BCS greater than 5 compared to those with BCS less than 5 (Table 5). The body mass index tended not to change with increasing body weight (Fig. 1) but linearly increased with decreasing height at shoulder (Fig. 2) and waist circumference (Fig. 3) in the Boerboel dogs.

DISCUSSION

The body mass index system in humans is universally applicable for all ages and both sexes. However there is no such index for dogs, making it difficult to objectively evaluate the body conditions score (Vogel, 2004). In this study, an attempt was made to estimate the body mass index in dogs using the system

established for Humans. The aim was to see if the method established in humans can be used in dogs to determine the body condition. The result of this study showed that the estimated body mass index did not differ between the sexes of dogs but differ significantly between the different age categories of the Boerboel dogs. This finding implied that the method of estimating body mass index in humans can be used to objectively determine the obesity status of Boerboel dogs instead of the body condition scoring (BCS) method.

The result of this study also showed that the body mass index tended not to change with body weight but increased with decreasing height of the dogs. This finding implied that the height of the Boerboel dogs is a major determinant of whether they will be classified as obese or not. This is similar to the findings in humans (Tobias *et al.,* 2014).

Currently, dogs body condition is subjectively evaluated using the body condition score system (Vogel, 2004). This technique is reported to be difficult for dogs' owners to use because it leads to inaccurate judgment of the dogs' body conditions by their owners. This has necessitated the need to develop a technique which is more objective and is dependent on the weight and height of the dog. In this study, the body condition system chart developed by Laflamme and others (Laflamme, 2006) was used to classify the dogs as obese or non- obese. Thirty-eight per cent of the Boerboel dogs in this study were classified as moderately obese. This result is within the reported prevalence of obesity in dogs in general (McGreevy *et al.,* 2005).

Lipid and lipoprotein alterations have been reported in dogs with obesity (Jeusette *et al.,* 2005; Riveria *et al.,* 2011). These included significant increases in total plasma cholesterol and triglycerides concentrations (Jeusette *et al.,* 2005). In this study, the plasma concentrations of triglycerides and high density lipoproteins did not differ between the different sexes of dogs, but the plasma fractions of the high density lipoproteins increased with increasing age. However, there was no significant difference in the plasma lipid profile between Boerboel dogs with BCS less than 5 and those with BCS of 5 and above. The lack of significant difference in the plasma lipid profile between dogs with BCS less than 5 and those with BCS of 5 and above may be as a result of the closeness in the body condition scores and the BMI of the dogs. This implies that the body condition score system might not be a good index of obesity in the Boerboel dogs.

In conclusion, the body mass index did not differ between the sexes of the dogs but differ between the different age categories of the Boerboel dogs suggesting that it might be a useful quantitative assessment of body condition status. However, there is no significant difference in the lipid profile between the different body condition statuses of dogs evaluated probably due to the age of the dogs and closeness in their body condition scores. This is the first study evaluating the plasma lipid profile in Boerboel dogs and relating it to the body condition to assess the risk of obesity.

It is thus recommended that the current technique of BMI determination in humans can be used to quantitatively evaluate the body condition status and predict the risk of obesity in Boerboel dogs. However, it is yet to be determined, the different BMI ranges for each body condition status in Boerboel dogs. It is also recommended that continual evaluation of the plasma lipid profile in large breed dogs be done routinely to determine the risk of the development of cardiovascular and musculoskeletal diseases.

REFERENCES

Ajadi, R. A., Otesile, E. B., Kasali, O. B. (2012). Short term changes in lipid profile following experimental osteoarthritis in dogs. Bulgarian Journal of Veterinary Medicine 15(3): 166-171

Akpanabiatu, M. I., Umoh, I. B., Udosen, E. O., Udoh, A. E., Edet, E. E. (2005). Rat serum electrolytes, lipid profile and cardiovascular activity on *Nauclea latifolia* leaf extract administration. Indian Journal of Clinical Biochemistry 20(2): 29-34.

Borman, P., Seckin, U., Yucel, M**.** (1999). Dyslipidemia in patients with rheumatoid arthritis and osteoarthritis. Physical Medicine, 2:5-9

Conaghan, P. G., Vanharanta, H., Dieppe, P. A. (2005). Is progressive osteoarthritis an atheromatous vascular disease? Annals of Rheumatic Diseases 64**:**1539-1541

Davies-Tuck, M. L., Hanna, F., Davis, S. R., Bell, R. J., Davison, S. L., Wluka, A. E., Adams, J. and Cicuttini, F. M. (2009). Total cholesterol and triglycerides are associated with the development of new bone marrow lesions in asymptomatic middle-age women- a prospective cohort study. Arthritis Research & Therapy 11(6): 1-7

Findlay, D. M. (2007). Vascular pathology and osteoarthritis. Rheumatology 46:1763-8

German, A. J. (2006). The growing problem of obesity in dogs and cats. Journal of Nutrition 136:1940S-1946S

Gray, M. J., Kirberger, R. M., Spotswood, T. C. (2003). Cervical spondylomyelopathy (wobbler syndrome) in the Boerboel. Journal of South African Veterinary Association 74(4): 104-110

Jeusette, I. C., Lhoest, E. T., Istasse, L. P., Diez, M. O**.** (2005). Influence of obesity on plasma lipid and lipoprotein concentrations in dogs. American Journal of Veterinary Research 66: 81–86

Kanchuk, M. L., Backus R.C. and Calvert, C.C. (2003). Weight gain in gonadectomised normal and lipoprotein lipase-deficient male domestic cats result from increased food intake and not decreased energy expenditure. Journal of Nutrition 133:1866–74.

Laflamme, D. P. (1997). Development and validation of a body condition score system for dogs: a clinical tool. Canine Practice 22:10–5

Laflamme, D. P. (2006). Understanding and managing obesity in dogs and cats. Veterinary Clinics of North America Small Animal Practice 36: 1283-1295

McGreevy, PD, Thomson, PC, Pride, C, Fawcett, A, Grassi, T, Jones, B. (2005). Prevalence of obesity in dogs examined by Australian veterinary practices and the risk factors involved. Veterinary Record 156: 695–702

Plumb, M. S. and Aspden, R. M. (2004). High levels of fats and (N-6) fatty acids in cancellous bone in osteoarthritis. Lipids in Health and Disease 3(12): 1476- 1511

Riveria, N. L., Felix, P. A., Ferreira, F. M., Fisher da Silva, A. V., Maiorka, A. (2011). Body measurements and serum lipid profile of overweight adult dogs fed diet containing conjugated linoleic acid. Cienca Rural 41(11): 2020-2025

Srikanth, V.K., Fryer, J.L., Zhai, G., Winzenberg, T.M., Hosmer, D. and Jones, G. (2005). A meta-analysis of sex differences prevalence, incidence and severity of osteoarthritis. Osteoarthritis & Cartilage13(9), 769-781

Tobias, D. K., Pan, A., Jackson, C. L., O'Reilly, E. J. and Ding, E. L. (2014). Body mass index and mortality and adults with incident type -2- diabetes. New England Journal of Medicine 370: 233-244.

Vogel, C. (2004). Developing a breed specific body mass index: A cooperative project. Perspective Pp 4

Niger. J. Physiol. Sci. 31(June 2016) 093-099
www.njps.com.ng

Niger. J. Physiol. Sci.

Antidepressant-like Potentials of *Buchholzia Coriacea* Seed Extract: Involvement of Monoaminergic and Cholinergic Systems, and Neuronal Density in the Hippocampus of Adult Mice

Onasanwo S.A.*, Faborode S.O., Ilenre K.O.
Neurosciences and Oral Physiology Unit, Department of Physiology, University of Ibadan, Ibadan, Nigeria.

Summary: *Buchholzia coriacea*, taken by elderly, has phytochemicals that have neuro-active metabolites, and the folklore documented its use in neuro-behavioral despairs. Previous study in our laboratory shows that methanol extracts of *Buchholzia coriacea* (MEBC) seeds possess antidepressant-like potentials in laboratory rodents. This present study was conducted to investigate the probable mechanism(s) of action by which MEBC potentiates its effects using laboratory rodents. Involvements of serotonergic, cholinergic and adrenergic systems were studied using Forced Swimming Test (FST) and Tail Suspension Test (TST) models of behavioral despair. Antagonists which including: Prazosin, an alpha-1-adrenergic receptor blocker (62.5 µg/kg, i.p.), metergoline, a 5HT2 receptor blocker (4 mg/kg, i.p.) and atropine, a -muscarinic cholinergic receptor blocker (1mg/kg i.p.) were administered before effective dose of MEBC (50mg/kg). Also, the hippocampi of the animals were studied for changes in neuronal density using Nissl Staining. Our findings showed that mobility was reversed in animals pre-treated with atropine, prazosin, and metergoline significantly ($P<0.05$), showing a possible involvement of the corresponding systems. However, there was a significant reduction in immobility time ($P<0.001$) during FST after chronic administration of the MEBC. The hippocampus showed no significant changes ($P<0.05$) in neuronal density. In conclusion, MEBC probably potentiates its antidepressant-like potentials via the cholinergic, adrenergic and partly by serotonergic systems.

Keywords*: Buchholzia coriacea*, Serotonergic system, Cholinergic system, Adrenergic system

**Address for correspondence:* samphil2002@yahoo.com,sa.onasanwo@gmail.ui.edu.ng Tel: +2348060711936

Manuscript Accepted: May, 2016

INTRODUCTION

Mood disorders like major depression and bipolar disorders are mostly common psychiatric disorders in modern society. About 1% and 16% of the population are estimated to be affected by bipolar disorder and major depression one or more times during their life time, respectively (Kessler *et al*., 2005). In Nigeria alone, about 3.1% of the population has been estimated to be having depression (Gureje *et al.*, 2010). The presence of the common symptoms of these disorders are collectively called 'depressive syndrome' and includes a long-lasting depressed mood, feelings of guilt, anxiety, and recurrent thoughts of death and suicide (Nestler *et al*., 2002). It has been estimated that the genetic contribution to the manifestation of depression is between 40-50% (Fava *et al*., 2000). Further studies carried out extensively have led to a variety of hypotheses for the molecular mechanism of depression, but no definite or specific pathogenic mechanism has been reported.

Many years ago, before the advent of the modern drugs for treating psychiatric conditions, it was discovered that some patients could receive significant relief from severe psychotic depression through severing the neuronal connections between the prefrontal areas of the brain by a method called prefrontal lobectomy. About two-thirds of the depressed patients respond to the currently available therapies but the magnitude of improvement is still disappointing. Therefore, the need for newer, better-tolerated and more efficacious treatments remains the major pharmacological interest and pursuit. Therapists from different regions of the world have used herbal medicines to alleviate affective disorders for many decades. In addition, the search for novel pharmacotherapy from medicinal plants for psychiatric illnesses has progressed significantly in the past years (Zhang, 2004). An increasing number of herbal products have been introduced into psychiatric practice, as complementary or alternative medicines, and also there are a large number of herbal medicines whose therapeutic potentials have been assessed in a variety of experimental animal models (Zhang, 2004). In fact, these models have contributed to the screening of new psycho pharmacological tools and to the understanding of their biological activities (Buller and Legrand, 2001). The plant *Buchholzia coriacea*

(*Capparidaceae*), known as wonder plant (musk tree) of which the seeds are called wonderful kola, has won for itself some folkloric importance as local abortifacients, ecbolic (promoting labor by increasing uterine contractions), vermifages, wound and snake bite treatments. Scientifically, it is known for its therapeutic effects as antimicrobial and antihelminthic potentials (Ajaiyeoba *et al*., 2003), hypoglycemic activities (Adisa *et al*., 2010), antibacterial activities (Mbata *et al*., 2009), anti-inflammatory, analgesic and antipyretic activities (Enechi *et al*., 2006; Onasanwo *et al.,* 2013); anthelminthic activities (Nweze *et al*., 2006). Onasanwo and co-workers showed that methanol extract of *Buchholzia coriacea* has antidepressant-like properties in laboratory mice (Onasanwo *et al*., 2013). However, the mechanisms of potentiation are not yet elucidated. The 'monoamine hypothesis,' which suggests a deficiency or imbalances in the monoamine neurotransmitters, such as serotonin, dopamine and norepinephrine, as the cause of depression, has been the central topic of depression research for the last five decades. This hypothesis has been initiated and supported by the fact that early versions of antidepressants including monoamine oxidase and tricyclics inhibitors have the common effect of acutely enhancing monoamine function (Ressler *et al*., 2000; Manji *et al*., 2001; Morilak *et al*., 2004). Recently, research update has shown that the mode of action of antidepressants is through hippocampal neurogenesis, which is referred to as the production of new neurons in the brain. The process of adult neurogenesis is located in two discrete brain regions: the sub-ventricular zone (SVZ) and sub-granular zone (SGZ) of dentate gyrus of the hippocampus of the brain. The heterogeneous nature of depression suggests an involvement of multiple distinct brain regions, which may be responsible for the diverse symptoms. This hypothesis human imaging and post-mortem studies of the brain have supported, implicating brain areas including the prefrontal and cingulate cortex, hippocampus, ventral striatum, amygdala, and thalamus (Drevets *et al*., 2002). Together, these brain regions operate a series of highly interacting circuits that forms a neural circuitry involved in depression (Nestler *et al*., 2002). The hippocampus is one of several limbic structures that have been extensively studied in individuals with psychiatric and neurologic disorders in the last decade (Nestler *et al*., 2002; Eisch *et al*., 2008). Apart from its critical role in learning and memory, the hippocampus is one of the only two areas in mammalian brain where adult neurogenesis is evidenced (Eisch *et al*., 2008). Therefore, the main objective of this research work is to evaluate the possible mechanisms by which *Buchholzia coriacea* seeds potentiate its antidepressant activities using laboratory rodents, with focus on the monoaminergic system and hippocampal neuronal density.

MATERIALS AND METHODS

Animals

Female mice (20g - 23g) obtained from Central Animal House, University of Ibadan, Ibadan,
Nigeria were used in the study. Each mouse was used once in the study. The animals were caged at room temperature, with 12-hour light-dark cycle. The animals were allowed free access to water, and standard diet was given *ad libitum*.

Plant material and extraction procedure

The *Buchholzia coriacea* seeds were purchased locally from Oje market, Ibadan, Nigeria, and identified at the Botany Department, University of Ibadan, Ibadan, Nigeria. Although, the plant had been authenticated at the herbarium of Forestry Research Institute of Nigeria (FRIN), Ibadan, Oyo State, Nigeria and a voucher specimen was already there. The outer coats of the seeds were peeled off until a purple color was left. They were washed clean with distilled water to remove adhering particles after which they were sliced and properly shade- dried, then pulverized. The powdered seeds were macerated in aqueous methanol (80%v/v) for a total of 15days (solvent was replaced every five days) with daily shaking. The extract obtained was concentrated to a dark-brown residue on a rotary evaporator at 40oC and weighed. The methanol extract of *Buchholzia coriacea* seeds (MEBC) obtained was concentrated to dryness by lyophilization and stored in the refrigerator until needed for analysis.

Drugs and treatment regimen

To assess the involvement of the monoaminergic systems in the antidepressant-like effect of
Methanol Extract of *Buchholzia Coriacea* (MEBC); mice were pre-treated with metergoline (4mg/kg, i.p.), a non-selective 5-HT2 receptor antagonist, at a dose effective in blocking the *in vivo* effect induced by 5-HT2 receptor agonists in mice, prazosin (62.5µg/kg, *i.p*.), an alpha-1-adrenoceptor antagonist, atropine (1mg/kg, *i.p*.), a muscarinic cholinergic receptor antagonist. The test for elucidation of mechanisms made use of 6 groups of 6 mice per group. Group 1 was control and received vehicle only. Group 2 was treated with 50mg/kg MEBC only while Groups 3 to 5 were pretreated with above named antagonist 15minutes before administration of 50mg/kg MEBC, after which they were subjected to the behavioral tests of either forced swimming test or tail suspension test. Group 6 received imipramine (60mg/kg) only, which serves as the standard group.

Neuro-behavioral study-depression models

i. Forced Swimming Test (FST): The forced swim test (FST) has been considered to be the most widely used

pharmacological *in-vivo* model for assessing antidepressant activity (Porsolt *et al.*, 1978). The set-up consists of a clear plexiglass cylinder (20cm high by 12cm diameter) filled with water to a 15cm depth. Water used during the experiment was kept at a temperature (34 ± 1^{o}C).

ii. Tail Suspension Test (TST): The tail suspension test (TST) was performed as earlier described (Steru *et al*, 1985). The mice were individually suspended at 60cm above the surface of the floor with an adhesive tape placed 1cm away from the tip of the tail. Immobility duration was recorded for the last 5minutes after 1 minute of acclimatization during the 6minutes test. Mice were considered immobile when they hung passively and were completely motionless.

Evaluation of the Involvement of Adult Hippocampal Neuronal Density

In each group, animals were classified into two (2) subgroups: one for behavioral studies and other for histological studies. Forced swimming test (FST) was used to assess the behavioural activity. Animals were pretreated with either vehicle or MEBC for 7, 14 or 21days. At the end of their respective days of pre-treatment, animals either undergo forced swimming test or was sacrificed and brain harvested for nissl staining.

Brain harvest and staining: Animals were anaesthetized using ketamine (80mg/kg).
Immediately after the animal lost its righting reflexes, mouse was laid on its back and the thorax was opened carefully to avoid bleeding. Animals were perfused intracardially with normal saline followed by 4% Phosphate-Buffered Formalin (PBF). After proper fixation, the animal skull was cut-open; brain removed and preserved in a glass tube containing 4% PBF for 72hours before slide preparation. Brain tissues were further processed into paraffin blocks and 40µm section were cut and made into slides for Nissl staining. Tissues were stained in 0.1% Cresyl violet.
Number of neuronal cells in the granular zones of the hippocampi was counted per square millimeter, to calculate the neuronal densities.

Statistical Analysis

The results obtained were expressed as mean ± S.E.M. Variance was analyzed using One-way Analysis of Variance (ANOVA), followed by Newman–Keuls' multiple comparisons test. P<0.05 was considered to be statistically significant.

RESULTS

Involvement of the monoaminergic system in antidepressant-like activities of Methanol Extract of *Buchholzia coriacea* (MEBC) in Forced Swimming Test.

On Immobility**:** As shown in figure 1a, 50mg/kg of MEBC significantly reduced immobility (P<0.05) in comparison with control. However, pre-treatment with antagonists Prazosin, an alpha1-adrenergic receptor blocker (62.5 µg/kg, i.p.), metergoline, a 5HT2 receptor blocker (4mg/kg, i.p.) and atropine, a muscarinic cholinergic receptor blocker (1mg/kg, i.p.) reversed decreased immobility as seen with group treated with 50mg/kg of MEBC. Imipramine (60mg/kg) significantly reduced immobility (P< 0.001) when compared to the control group.

On swimming: As shown in figure 1b, MEBC (50mg/kg) and metergoline (pre-treated) groups also show a significant increase (P<0.001) when compared with control. Animals pre-treated with atropine and prazosin showed a significant decrease in swimming activity (P<0.01 and P<0.05 respectively) when compared to the animals that were treated with MEBC (50mg/kg) only. However, there was no significant decrease in animal pre-treated with metergoline when compared to the group that received MEBC (50mg/kg) only.

On Climbing: as shown in figure 1c, MEBC (50mg/kg) and imipramine treated groups showed increase in climbing time (P<0.001) when compared with animals treated with vehicle. Also there was a significant increase in climbing time atropine and Prazosin (P<0.001 and P<0.05, respectively) when compared to groups treated with MEBC (50mg/kg) only. In contrast, there was a significant reduction in climbing time in animals pre-treated with metergoline when compared to animals that received MEBC (50mg/kg) only

Involvement of the monoaminergic and cholinergic systems in antidepressant-like activities of Methanol Extract of *Buchholzia coriacea* (MEBC) in Tail Suspension Test.

As shown in figure 2, imipramine significantly reduced immobility (***P<0.001) when compared to control. There is also a significant reduction in immobility (*P<0.05) in animals treated with MEBC (50mg/kg). However, there was a reversal in mobility in animals pre-treated with antagonist when compared with animals treated with MEBC (50mg/kg) only.

Involvement of chronic administration of Methanol Extract of *Buchholzia coriacea* (MEBC) on immobility and Adult Hippocampal Neuronal Density.

On immobility: As shown in figure 3, all groups pre-treated for 7, 14 or 21 days shows a significant reduction in immobility (P<0.001) when compared with their respective control (vehicle) groups.

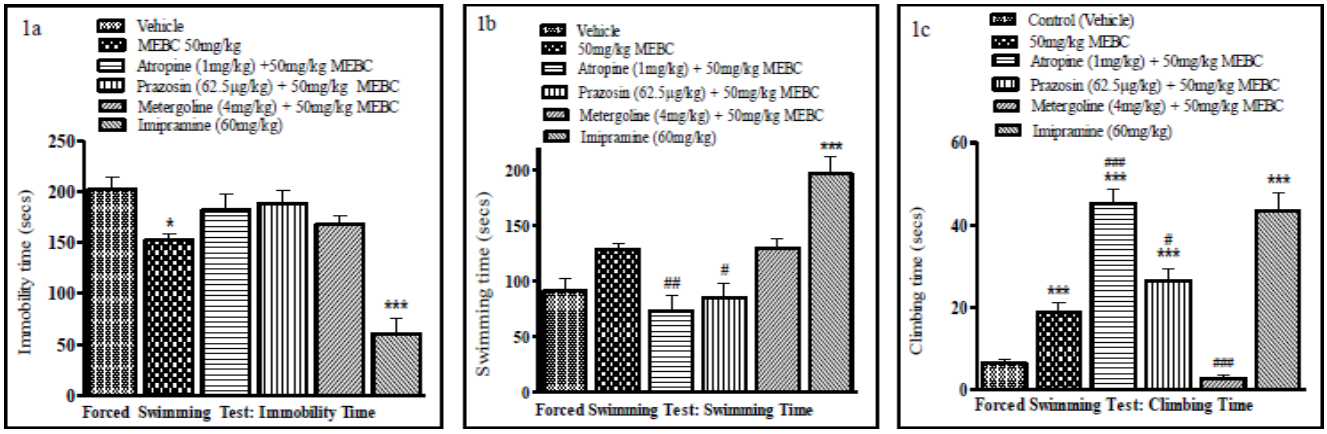


Figure 1. Effect of MEBC, monoamine antagonist, atropine and imipramine on (a)immobility, (b)swimming and (c)climbing times in forced swimming test. Values were expressed as mean ± S.E.M, ***P<0.001, *P<0.05 versus control; (Fig. 1b&1c) ###P<0.001,##P<0.01,#P<0.05 when compared with50mg/kg MEBC treated group, using one way ANOVA followed by Newman-Keuls post-hoc multiple comparison test. (n=6).

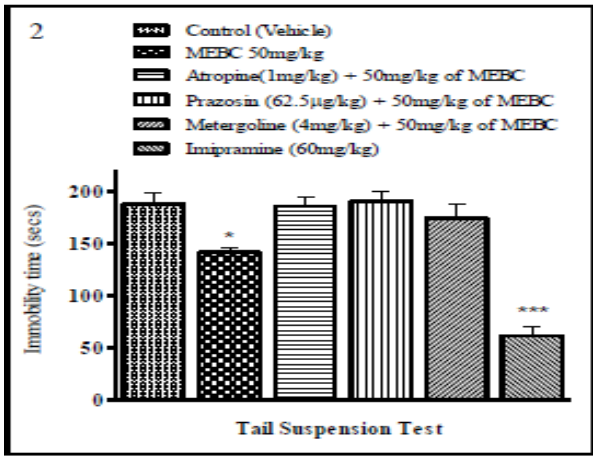


Figure 2**.** Effect of MEBC, monoamine antagonist, atropine and imipramine on immobility time in tail suspension test. Values were expressed as mean ± S.E.M, ***P<0.001, *P<0.05 versus control; using one way ANOVA followed by Newman-Keuls post-hoc multiple comparison test. (n=6).

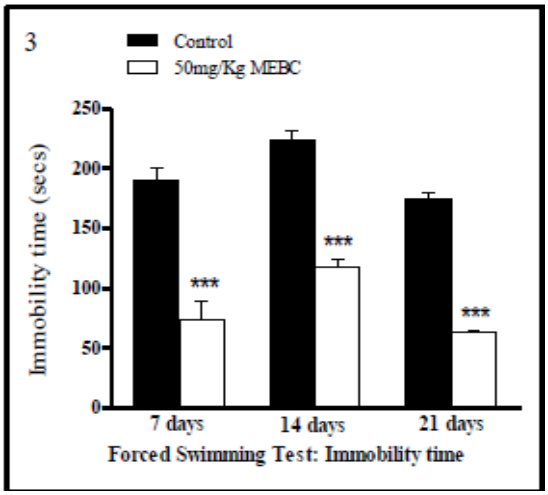


Figure 3. Effect of Methanol Extract of *Buchholzia coriacea* on immobility time in forced swimming test. Values were expressed as mean ± S.E.M, significant decrease ***P<0.001 versus controls; using one way ANOVA followed by Newman-Keuls post-hoc multiple comparison test. (n=6).

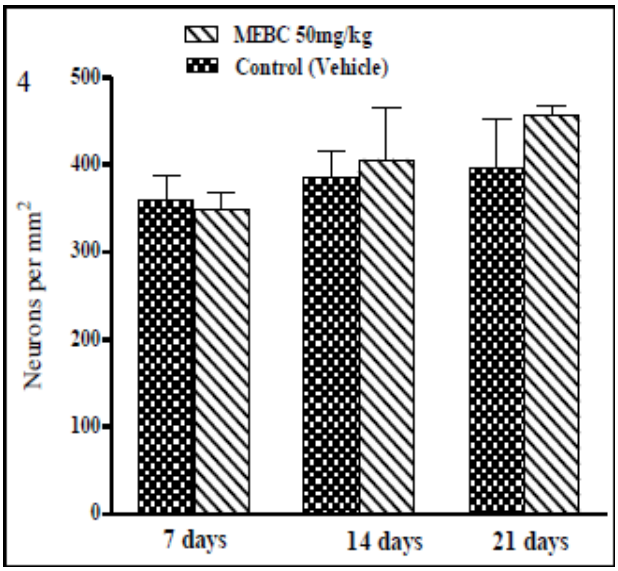


Figure 4. Number of Nissl stained granule neurons per mm2 in granular zone of the dentate gyri of the hippocampi of animals pretreated with either vehicle (10ml/kg) or MEBC (50mg/kg). Values were expressed as mean ± S.E.M.

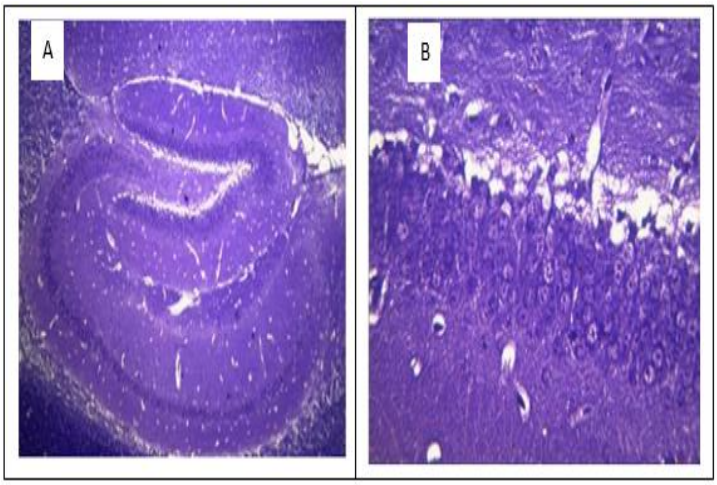


Plate1. Photomicrographs of hippocampus of animals showing positive staining with nissl. (A. Mag. X40, B. Mag. X400)

On Adult Hippocampal Neuronal Density: As shown in figure 4, there was no significant difference ($P<0.05$) in hippocampal neuronal density within the groups treated with either vehicle (10ml/kg) or MEBC (50mg/kg) for 7, 14 or 21 days

DISCUSSION

The pathophysiology of depression is linked to the deficiency of one or more monoamines in affected persons. Antidepressants demonstrated their effects by regulating synaptic levels of one or more monoamines (Elhwuegi, 2004). Hence, this study explored the impact of monoaminergic and cholinergic antagonists on the antidepressant-like effect of the methanol extract of *Buchholzia coriacea* (MEBC). Forced Swimming Test (FST) and Tail Suspension Test (TST) were used as the tests in mechanistic studies due to its increased sensitivity (Cryan *et al*., 2002; 2005a; 2005b). The modified Porsolt forced swimming test has been suggested to have good sensitivity for detecting the effects of antidepressants in rodents and other laboratory animals (Cryan *et al*., 2005). This test involves measuring the immobility, swimming behavior and climbing behavior of rodents upon subsequent exposure to swimming.

On serotoninergic system**,** several studies had established the role of serotonin in depression (Kennett *et al.,* 1987; Elhwuegi, 2004; Tatarczynska *et al*., 2004). Our earlier study showed that MEBC (50mg/kg) reduced immobility in laboratory mice (Onasanwo *et al.,* 2013) which suggests its antidepressant activities. MEBC increased swimming and reduced immobility behavior suggesting an involvement of 5-hydroxytryptaminergic (5-HT) neurotransmission in its antidepressant-like activity. However, this immobility was reversed when metergoline, a nonselective 5HT2 receptor antagonist was administered to the animals before treatment with MEBC. Also, there was a significant increase in swimming activities after treatment with metergoline, Therefore, it may be possible that MEBC produces its antidepressant-like effect through interaction with other serotonergic receptor sub-types.

Analyses of antidepressant drugs in the forced swimming test allow discrimination in between serotonergic drugs (e.g., fluoxetine) that reduced immobility by increasing swimming (Detke *et al*., 1995; Rénéric *et al*., 1998). The enhancement of swimming activity and reduction of immobility in mice that was comparable to that observed after the acute administration of imipramine suggest an antidepressant-like effect of the extract.

On adrenergic system**,** the alpha-1-adrenoceptor has been shown to underlie some of the antidepressant-like responses of drugs in behavioural models of depression (Danysz *et al*., 1986; Cardoso *et al*., 2009). Imipramine increased the climbing activity in this experiment, suggesting a mechanism that involves noradrenergic neurotransmission (Detke *et al*., 1995). Pre-treatment with Prazosin (62.5μg/kg), an alpha-1-adrenergic receptor antagonist reversed the immobility that was observed when MEBC (50mg/kg) was administered alone; both in forced swimming test and tail suspension test paradigms. This result indicates that MEBC may exert its effect in the FST and TST paradigms, by interacting with alpha1-adrenoceptors. Moreover, the blockade of α-1-adrenoceptors mimics depressive states, which, like chronic stress, are associated with α1-adrenoceptor desensitization (Stone *et al*., 2003). In contrast, chronic antidepressants and electroconvulsive therapy enhance the density and functional activity of α-1-adrenoceptors in structures such as frontal cortex and hippocampus (Stone *et al*., 2003; Cardoso *et al*., 2009).

On cholinergic system, pre-treatment with atropine reduced swimming activity which signifies a possible inhibition of cholinergic pathways in mood regulating centers. The hippocampus which is rich in cholinergic neurons and other trajectories to other part of the CNS e.g. prefrontal cortex, suprachiasmatic nucleus and amygdala is also very important in mood regulation. So, the blockade of muscarinic cholinergic pathway may produce possible depressive behaviors in animals, as increased swimming activity is corresponding to anti-depressant activities.

The involvement of hippocampal neuronal density was also studied here. Efficacy of antidepressants has been suggested to be linked to production of new neurons especially in the hippocampus of the brain (Malberg *et al*., 2000; Santarelli *et al*., 2003). Chronic administration of MEBC significantly reduced immobility after 7, 14 and 21 days of pre-treatment when tested using FST, which may suggest the strengthening of neuronal circuitry involved in mood regulation.

In this study, we observed that there was no significant increase in neuronal density in the different pre-treated groups of animals. This could be as a result of the time required for the action of antidepressants, which generally take weeks. However there may be presence of proliferating cells in the hippocampus, but have not differentiated into mature neurons, specific technique like immunohistochemistry targeting these proliferating cells would help in identifying them. (Malberg *et al*., 2000; Santarelli *et al*., 2003). Different animals were used for the behavioral and histological studies, because it has been shown that both acute and chronic stress suppressed neurogenesis of dentate gyrus granule neurons (Baune *et al.,* 2006; Hihn *et al.,* 2006).

This allow for proper physiological examination of the therapeutic effect of the MEBC without any prior influence of stress-induced neuronal loss, which they

would have been exposed to if the same animals were used for histology after successive swimming tests.

In the brain, many circuitries are involved in mood regulation and this information is transferred by synapses between neuronal cells. Depression has been suggested to be associated with loss of neurons especially in the hippocampus which account for low hippocampal volume. Also, it has been suggested that antidepressants stimulate the expression of neurotrophic factors, increase the density, the length and the arborization of the dendrites (Magarinos *et al.*, 1999) and enhance the synaptic plasticity (Magarinos *et al.*, 1999; Manji *et al.*, 2001; Hayley *et al.*, 2005).

In conclusion, antidepressant-like potential of methanol extract of *Buchholzia coriacea* may be mediated by cholinergic, adrenergic and partly by 5-HT2 systems.

Further studies will be to investigate the involvement of other monoaminergic systems like dopaminergic system. It will also be interesting to look into the involvement of the subtypes of the various monoaminergic receptors. Specific compound(s) will be isolated from the seeds of *Buchholzia coriacea*, in order to know which metabolite in the seeds of *Buchholzia coriacea* is responsible for the antidepressant-like activities. The role of Brain Derived Neurotrophic Factor (BDNF) will also be studied. Immunohistochemistry of hippocampal proliferating cells will be carried out, so as to be specific on the role of adult hippocampal neurogenesis in its potentiation of the antidepressant-like activities of MEBC

Acknowledgement: The authors are grateful to Dr. Aina, Reproductive Biology Unit, Faculty of Veterinary Medicine, University of Ibadan for his assistance in the histological studies of this work.

REFERENCES

Adisa, R. A, Choudharyb, M. I and Olorunsogo, O. O. (2011). Hypoglycemic activity of *Buchholzia coriacea* (Capparaceae) seeds in streptozotocin-induced diabetic rats and mice. Exp Toxicol Pathol. 63: 619 – 625.

Ajaiyeoba, E. O, Onocha, P. A, Nwozo, S. O, Sama, W. (2003). Antimicrobial and cytotoxicity evaluation of *Buchholzia coriacea* stem bark. Fitoterapia 74 (7-8): 706-709.

Baune, B. T, Suslow, T, Engelien, A, Arolt, V, Berger, K. (2006). The association between depressive mood and cognitive performance in an elderly general population – the MEMO Study. Dement Geriatr Cogn. Disord; 22(2):142-149.

Buller. R. and Legrand, V. (2001). Novel treatment for anxiety and depression hurdles in bringing them to the market. Drug discovery today 6; 1220 1236.

Cardoso, C.C, Lobato, K. R, Binfaré, R. W, Ferreira P. K, Rosa O. A, Santos, A. R. S, et al.(2009) Evidence for the involvement of the monoaminergic system in the antidepressant-like effect of magnesium. Prog Neuropsychopharmacol Biol Psychiatry; 33: 235–42.

Cryan, J. F, Markou, A, Lucki I. (2002). Review Assessing antidepressant activity in rodents: recent developments and future needs. Trends Pharmacol Sci. 2002 May; 23(5):238- 45.

Cryan, J. F, Mombereau, C, Vassout, A. (2005). The tail suspension test as a model for assessing antidepressant activity: Review of pharmacological and genetic studies in mice. Neurosci Biobehav Rev 2005; 29: 571-625a

Cryan, J. F, Valentino, R. J, Lucki I. (2005). Review Assessing substrates underlying the behavioral effects of antidepressants using the modified rat forced swimming test. Neurosci Biobehav Rev. 2005; 29(4-5):547-69b

Danysz, W, Kostowski, W, Kozak, W, Hauptmann, M. (1986). On the role of noradrenergic neurotransmission in the action of desipramine and amitriptylline in animal models of depression. Pol. J. Pharmacol. Pharm. 1986; 38: 285–98.

Detke, M. J, Rickels, M, Lucki I. (1995). Active behaviours in the rat forced swimming test differentially produced by serotonergic and noradrenergic antidepressants. Psychopharmacol; 121: 66–72.

Drevets, W. C, Bogers, W, Raichle, M. E (2002). Functional anatomical correlates of antidepressant drug treatment assessed using PET measures of regional glucose metabolism. Eur. Neuropsychopharmacol. 2002; 12 (6):527—44.

Eisch, A. J, Cameron, H. A, Encinas, J. M, Meltzer, L. A, Ming, G. L, Overstreet-Wadiche LS. (2008). Adult neurogenesis, mental health, and mental illness: hope or hype? J Neurosci;28(46):11785—91.

Elhwuegi, A. S. (2004). Central monoamines and their role in major depression. Prog Neuropsychopharmacol Biol Psychiatry 2004; 28: 435-451.

Enechi, F. C Ibeafu, K. Ledee, O. F. C Nwodo, (2006). Anti-Inflammatory, analgesic and antipyretic activities of the ethanol extract of the seeds of *Buchholzia coriacea* in experimental animals.

Fava M., Kendler, K. S (2000). Major depressive disorder. Neuron, 28:335-341.

Gureje, O., Uwakwe, R., Oladeji, B., Makanjuola, V. O, Esan, O. (2010). Depression in adult Nigerians: results from the Nigerian Survey of Mental Health and Well-being. J Affect Disord. 2010 Jan;120(1-3):158-64.

Hayley, S, Poulter, M. O, Merali, Z, Anisman, H. (2005). The pathogenesis of clinical depression:

stressor- and cytokine - induced alterations of neuroplasticity. Neuroscience;135(3): 659-678.

Hihn, H., Baune, B. T, Michael, N., Markowitsch, H., Arolt, V., Pfleiderer, B. (2006). Memory performance in severely depressed patients treated by electroconvulsive therapy. J Ect; 22(3):189-195.

Kennett, G. A, Dourish, C. T, Curzon, G. (1987). "Antidepressant-like action of 5-HT1A agonists and conventional antidepressants in an animal model of depression". Eur J Pharmacol. 134 (3): 265–74.

Kessler, R. C, Berglund, P, Demler, O., Jin, R., Merikangas, K. R, Walters, E. E (2005): Lifetime prevalence and age-of-onset distributions of DSM-IV disorders in the National Comorbidity Survey Replication. Arch Gen Psychiatry, 62:593-602.

Magarinos, A. M, Deslandes, A, McEwen, B. S. (1999). Effects of antidepressants and benzodiazepine treatments on the dendritic structure of CA3 pyramidal neurons after chronic stress. Eur J Pharmacol; 371:113-122.

Malberg, J. E, Eisch, A. J, Nestler, E. J, Duman, R. S (2000). Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. J Neurosci 2000;20(24):9104—10.

Manji, H. K, Drevets, W. C, Charney, D. S (2001): The cellular neurobiology of depression. Nat Med, 7:541-547.

Manji, H. K, Duman, R. S. (2001) Impairments of neuroplasticity and cellular resilience in severe mood disorders: implications for the development of novel therapeutics. Psychopharmacol Bull; 35(2):5-49.

Mbata, T. I, Duru, C. M and Owumelu, H. A. (2009). Antibacterial activity of crude seed ectract of *Buchholzia coriacea* E. on some pathogeniccbacteria. Journal of Developmental Biology and Tissue Engineering 1(1): 001-005.

Morilak, D. A, and Frazer, A, (2004): Antidepressants and brain monoaminergic systems: a dimensional approach to understanding their behavioural effects in depression and anxiety disorders. Int J Neuropsychopharmacol , 7:193-218.

Nestler, E. J, Barrot, M, DiLeone, R. J, Eisch, A. J, Gold, S. J, Monteggia, L. M (2002): Neurobiology of depression. Neuron, 34:13-25.

Nweze, N .E. and Asuzu, I. U (2006). The Antihelmintic Effect of *Buhholzia coriacea* seeds. *Nigerian Vetirinary Journal 27(2), 60-65*

Onasanwo, S. A, Obembe, O. O, Faborode, S. O, Elufioye, T. O and Adisa, R. A (2013). Neuropharmacological potentials of *Buchholzia coriacea* (Engl.) seeds in laboratory rodents; Afr. J. Med. med. Sci. 42,131-142.

Porsolt, R. D., Bertin, A., and Jalfre, M. (1978). ‘‘Behavioural despair’’ in rats and mice: Strain differences and the effects of imipramine. Eur. J. Pharmacol. 51(3), 291–294.

Rénéric, J. P, Lucki, I. (1998). Antidepressant behavioral effects by dual inhibition of monoamine reuptake in the rat forced swimming test. Psychopharmacol (Berl).;136(2):190-7

Ressler, K. J and Nemeroff, C. B (2000): Role of serotonergic and noradrenergic systems in the pathophysiology of depression and anxiety disorders. Depress Anxiety, 12(Suppl 1):2-19.

Santarelli, L., Saxe, M., Gross, C, Surget, A., Battaglia, F., Dulawa, S, et al. (2003). Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. Science; 301(5634):805—9.

Steru L., Chermat, R., Thierry, B. and Simon, P. (1985). The tail suspension test: a new method for screening antidepressants in mice. Psychopharmacology 85,367-370.

Stone, E. A, Grunewald, G. L, Lin, Y, Ahsan, R, Rosengarten, H, Kramer, H. K, Quartermain, D.
(2003). Role of epinephrine stimulation of CNS alpha1-adrenoceptors in motor activity in mice. Synapse. 2003; 49(1):67-76.

Tatarczynska, E., Klodzinska, A., Stachowicz, K., Chojnacka-Wójcik, E. (2004). "Effects of a selective 5-HT1B receptor agonist and antagonists in animal models of anxiety and depression". *Behav Pharmacol.* 15 (8): 523–34.

Zhang, Z. J (2004). Therapeutic effects of herbal extracts of constituents in animal models of psychiatric disorders. Life Science 75, 1659-1699.