Niger. J. Physiol. Sci. 30(2015) 005-009
www.njps.com.ng

Niger. J. Physiol. Sci.

Vitamin C Prevents Sleep Deprivation-induced Elevation in Cortisol and Lipid Peroxidation in the Rat Plasma

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Summary: Sleep deprivation (SD) is biological stressor that alters metabolic parameters, induced oxidative stress and lipid peroxidation. Previous studies have shown that antioxidants substances such as melatonin, tryptophan, vitamin E and vitamin C improved stress tolerance in laboratory animals. In this study, we examined the potential protective effects of administration of vitamin C on acute and chronic sleep deprivation-induced metabolic derangement. In addition, possible processes involved in vitamin C effects on acute and chronic sleep deprivation-induced metabolic derangement were determined. Thirty-five rats (120-250g) were used. The rats were divided into 7 groups of 5 rats each as Control (CTRL), Acute sleep deprived untreated with vitamin C (AC), Acute sleep deprived treated with vitamin C (AWC), Chronic sleep deprived untreated with vitamin C (CC), Chronic sleep deprived treated with vitamin C (CWC), Chronic sleep deprived + Recovery untreated with vitamin C (RC), and Chronic sleep deprived + Recovery treated with vitamin C (RWC). The SD was carried out for 20h for 1 day on the acute groups, and for 20h/day for 5 days on the chronic group, using the Multiple Modified Platforms (MMP) after oral administration of 300mg/kg of vitamin C to all vitamin C-treated groups. The recovery groups were further observed for five days after SD. The control group were treated with vitamin C and without stress in their home cages. At the end of the experiment, the animals were sacrificed and blood was collected for estimation of plasma glucose, insulin, cortisol and malondialdehyde (MDA). The results showed that acute and chronic SDs significantly ($p<0.05$) increased MDA and cortisol levels, while significantly ($p<0.05$) reduced the levels of insulin. Treatment with vitamin C reversed the changes in the MDA, cortisol and plasma insulin levels. Additionally, allowing the rats to recover for 5 days after sleep deprivation corrected the observed changes. Plasma glucose was significantly ($p<0.05$) reduced in all the sleep deprived groups compared to the control. In conclusion, sleep deprivation induced metabolic, hormonal and lipid peroxidation derangement, and treatment with vitamin C prevented these impairments. Thus, the effects of vitamin C could improve stress tolerance in rats.

Keywords*:* Sleep Deprivation, Lipid Peroxidation, Plasma Cortisol, Plasma Insulin, Plasma Glucose.

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Manuscript Accepted: October, 2015

INTRODUCTION

Sleep, defined as unconsciousness from which a person can be aroused by sensory or other stimuli (Hall, 2015), is an essential physiological need that must be satisfied to ensure normal physiological functions (Rechtschaffen and Bergmann, 2002). Sleep deprivation can be defined as the restriction of sleep below the level of basal sleep need (Lim and Dinges, 2007). This can be acute (a single period of extended wakefulness) or chronic (accumulation of sleep debt over multiple nights of sleep restriction) (Lim and Dinges, 2007).

Despite this importance of sleep, people sleep less in our contemporary environment. Sleep disruptions, which may be caused by several factors, have been said to affect daily life negatively (Chasens *et al.*, 2010). Therefore, sleep restriction in humans has been shown to alter multiple behavioural and metabolic pathways leading to increased prevalence of Insulin Resistance, Diabetes Mellitus, Obesity, Hypertension and Hyperlipidaemia (Lucassen *et al.*, 2012).

Since sleep deprivation is a biological stressor that simulates Corticotropin Releasing Hormone (CRH) and cortisol secretion (Leproult *et al.*, 1997); and negatively alters the body oxidant-antioxidant system through its effects on metabolism and endocrine functions (Spiegel *et al.*, 1999), this research studied effect of deprivation on some metabolic parameters (cortisol, insulin and glucose) and an oxidative parameter (malondialdehyde (MDA)). Cortisol was chosen as a stress marker because it has previously been used for the same purpose in rats (Prasad *et al.*, 2006; Jameel *et al.*, 2014; Joshi and Jameel, 2015). While many laboratory studies such as (Tochikubo *et al.*, 1996; Spiegel *et al.*, 1999; Kato *et al.*, 2000; Meier-Ewert *et al.*, 2004; Spiegel *et al.*, 2004) focused on short-term (acute) Sleep Deprivation, such studies for chronic sleep deprivation is limited, although the results from the short-term can give a potential mechanism through which chronic sleep deprivation affects health (Dinges and Banks, 2007). Also, most studies did not study recovery phenomena from the sleep restriction.

Therefore, this research studied the effect of both acute and chronic sleep deprivations on cortisol, insulin, glucose and malondialdehyde (MDA) as well as how vitamin C (an antioxidant) can affect the levels of these indices during sleep deprivation and recovery phenomena.

MATERIALS AND METHODS

Thirty-five rats of weight 120-250g were used in the research. The rats were acclimatised for 2 weeks under ambient temperature of 25^{o}C and standard photoperiod of 12hr light-dark cycle after which they were subjected to Paradoxical sleep deprivation for 20hrs (11:00am-7:00am next morning) for one day in the acute group or 20hrs for 5 days in chronic group with 4hr (7:00am-11:00am) rest each day using Modified Multiple Platform (MMP) method. The MMP has been previously described (Oh *et al.*, 2012; Medeiros *et al.*, 1998). The rats were grouped into 7 groups each containing 5 rats as follows:

A- Control group (CTRL)
B- Acute sleep deprived untreated with vitamin C (AC).
C- Acute sleep deprived treated with vitamin C (AWC).
D- Chronic sleep deprived untreated with vitamin C (CC).
E- Chronic sleep deprived treated with vitamin C (CWC).
F- Chronic sleep deprived + Recovery untreated with vitamin C (RC).
G- Chronic sleep deprived + Recovery treated with vitamin C (RWC)

Oral vitamin C was administered to the vitamin C-treated rats at a dose of 300 mg/kg body weight following previous researches in which higher dosages were used (Wadly and McConell, 2010; Derakhshanfar *et al.*, 2012). All the administrations were done in the morning before subjecting them to SD in the acute group and every morning before SD for 5 days in the chronic group. Recovery groups were kept for additional 5 days without SD for observation of their recovery processes. All the rats were sacrificed between 9:00hrs and noon in all groups at their respective days.

Before sacrifice, the body weights of the rats were taken. The rats were afterwards anaesthetised with intraperitoneal injection of 125mg/kg of ketamine (Youth *et al.*, 1973) and blood samples were collected from them into fluoride oxalate bottles through cardiac puncture. The blood samples were centrifuged at 3000rpm for 10 minutes and their respective plasma were removed and stored in plain bottles below -20^{o}C inside a freezer until the day of analyses.

Biochemical assays from all the groups were done the same day. Estimations of insulin and cortisol levels were done using commercially available kits (Agape Diagnostics, Switzerland Gmbh) according to manufacturers recommended protocols. Absorbance readings were obtained using an automated blood chemistry analyser (URIT-810 Chemistry Analyzer, URIT Medical Electronic Co., Ltd. Guangxi, PR China). Plasma MDA levels were analysed using GOD-PAP and thiobarbituric acid methods respectively. Glucose concentration was determined with AccuCheck (Roche Diagnostics, Indianapolis, IN, USA).

Statistical Analysis

All values are presented as mean ± SEM (Standard Error of Mean). Statistical analyses were done with Statistical Package for Social Sciences (SPSS) using one-way Analysis of Variance (ANOVA) and Least Significant Difference (LSD) multiple comparison analysis taking p-value <0.05 at any stage as significant.

RESULTS

Plasma Cortisol

The results, Fig. 1., showed a significant rise in plasma cortisol levels in both the acute sleep deprived untreated rats (AC) with a mean value of 7.78±0.34pg/dl ($p<0.01$) and the chronic sleep deprived (SD) rats (CC) with the mean value of 5.26±0.13pg/dl ($p<0.05$) compared with the control (3.98±0.14pg/dl). However, chronic sleep deprived rats treated with vitamin C (CWC) showed significant (2.78±0.09 pg/dl, $p<0.05$) reduction plasma cortisol concentration when compared with the control.

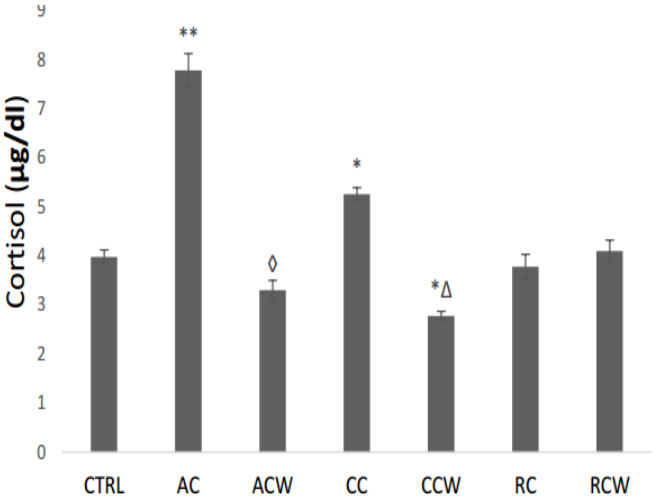

Figure 1. Effects of vitamin C on plasma cortisol level in sleep-deprived rats; *p<0.05 vs Control, **p<0.01 vs Control, ◊p<0.05 vs Acute, Δ p<0.05 vs Chronic. CTRL Control, AC Acute, AWC Acute treated with vitamin C, CC Chronic, CWC Chronic treated with vitamin C, RC Recovery, RWC Recovery treated with vitamin C.

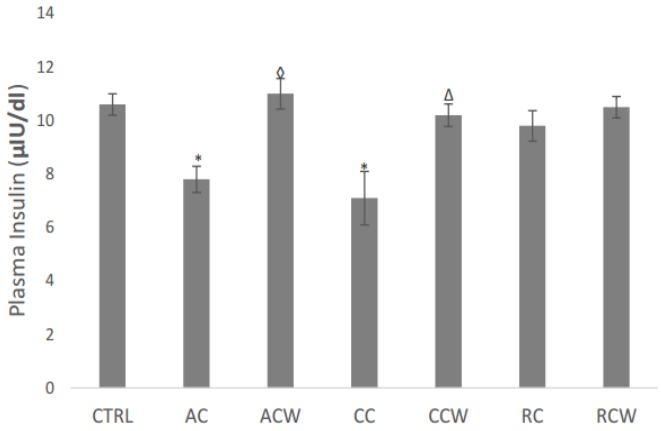

Figure 2. Effects of vitamin C on plasma insulin level in sleep-deprived rats; *p<0.05 vs Control, **p<0.01 vs Control, ◊p<0.05 vs Acute untreated, Δ p<0.05 vs Chronic untreated. CTRL Control, AC Acute, AWC Acute treated with vitamin C, CC Chronic, CWC Chronic treated with vitamin C, RC Recovery, RWC Recovery treated with vitamin C

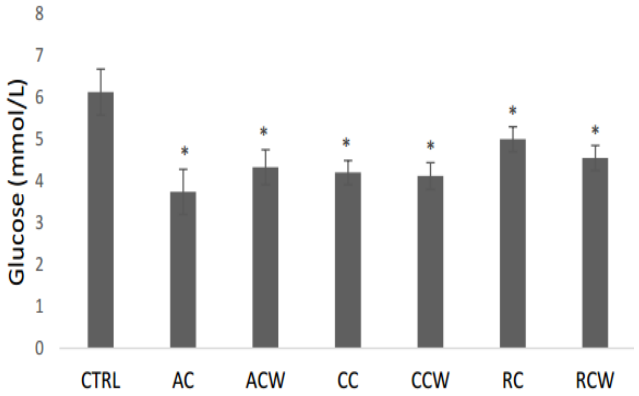

Figure 3. Effects of vitamin C on plasma glucose level in sleep-deprived rats; *p<0.05 vs Control. CTRL Control, AC Acute, AWC Acute treated with vitamin C, CC Chronic, CWC Chronic treated with vitamin C, RC Recovery, RWC Recovery treated with vitamin C.

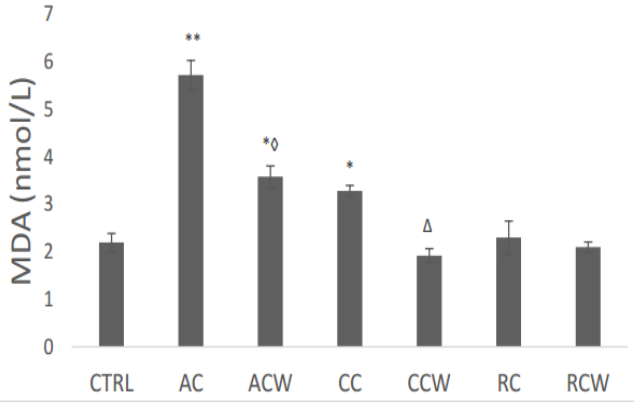

Figure 4. Effects of vitamin C on plasma MDA level in sleep-deprived rats; *p<0.05 vs Control, **p<0.01 vs Control, ◊p<0.05 vs Acute, Δp<0.05 vs Chronic. CTRL Control, AC Acute, AWC Acute treated with vitamin C, CC Chronic, CWC Chronic treated with vitamin C, RC Recovery, RWC Recovery treated with vitamin C.

Treatment with vitamin C in both acute and chronic sleep deprived groups significantly (p<0.05) reduced the levels of cortisol compared to the untreated groups.

Plasma Insulin

The results in Fig. 2, showed significant (p<0.05) decrease in insulin levels of acute (AC) (7.8±0.5pIU/dl, p<0.05), chronic (CC) (7.1±1.0pIU/dl, p<0.05) sleep deprivation compared to control (10.6±0.4pIU/dl). In addition, post-hoc multiple comparisons showed a significant (p<0.05) increase in plasma insulin concentration following vitamin C treatment in both acute and chronic sleep deprivation groups.

Plasma Glucose

The result (Fig. 3) showed a significant reduction (p<0.05) in glucose levels in all the treated groups compared to the control group.

Plasma MDA

The results, Fig. 4, showed significant increase in the levels of MDA of the acute sleep deprived groups (treated or untreated with vitamin C, 5.72±0.31nmol/L, p<0.01 and 3.58±0.23nmol/L, p<0.05 respectively) and chronic untreated with vitamin C (3.28 ± 0.12nmol/L, p<0.05) when compared to the control group (2.20 ± 0.19nmol/L). The results also showed significant reduction (p<0.05) in the levels of MDA in both acute and chronic sleep deprived groups following vitamin C treatment when compared with the untreated groups (5.72±0.31nmol/L vs 3.58±0.23nmol/L and 3.28±0.12nmol/L vs 1.92±0.15nmol/L). There were no significant differences in the levels of MDA of the recovery groups and the control group.

DISCUSSION

The reason for this study was to know the role of vitamin C, an antioxidant, in mitigating stress response in rats. Previous studies have shown that antioxidants such as tryptophan and vitamin E normalise oxidative stress markers due to sleep deprivation in laboratory animals (Alzoubi *et al*, 2012; Hosseini and Hoseini, 2013)

During periods of stress, cortisol levels are increased (Galliot *et al.*, 2007). Therefore, since sleep deprivation is a biological stressor, cortisol level generally increased in sleep deprivation process. In fact, sleep duration has been said to affect cortisol levels in the late afternoon and evening with a graded inverse relationship between sleep duration and cortisol (Spiegel *et al.*, 2004). Other studies such as (Gonzales-Oritz *et al.*, 2000; Schmid *et al.*, 2007 and Nedeltcheva *et al.*, 2009) found no significant changes in the cortisol levels in the morning following periods of sleep deprivation. However, in this study, increase

in cortisol levels was found in acute and chronic sleep deprived rats untreated with vitamin C.

The significant drop in cortisol levels observed in both the vitamin C treated acute and chronic sleep deprived rats in this study might be indirectly due to ability of vitamin C to mitigate stress response. Lower level of cortisol in recovery treated group than the control and untreated recovery group shows ability of vitamin C to hasten recovery from stressful situation of sleep deprivation. Reduction in cortisol levels of chronic vitamin C treated and untreated groups than their acute counterparts might be due to adaptation of the rats to the stress thereby reducing the effect of such stressful situation.

Increased insulin secretion in all vitamin C treated groups in both acute and chronic conditions compared to corresponding untreated groups might be accounted for by ability of vitamin C to stimulate insulin secretion as it has been previously shown (Gokkusu *et al.,* 2001; Shawer *et al.,* 2014). It has been said that the elevation of insulin hormone level with vitamins C and E is explained by effects of vitamins C and E in maintaining residual P-cell function though they act as a free oxygen radical scavenger hence, prevent P-cell cytotoxicity. Also vitamins C and E may improve the functions of P- cells, elevate plasma insulin and C-peptide levels possibly by increasing the antioxidant capacity. In addition, antioxidants may also block the ability of the immune system to recognise P-cells (Gokkusu *et al.,* 2001). The increase might also result from cortisol actions to increase free fatty acids and mobilise proteins or by cortisol actions to cause insulin resistance which can stimulate pancreas further to release more insulin.

Glucose levels in this study were reduced in all groups compare to the control and all the levels in the experimental groups were not significantly different. Generally reduced glucose level might be due to high utilisation of glucose by the body undergoing stress especially the brain as previously described (Galliot *et al.,* 2007). It might also be due to too much insulin level which may stimulate increased glucose uptake (Shashank, 2007) more than cortisol level that stimulates glucose release from tissues such as muscle (Hoehn and Marieb, 2010). Insignificant differences among glucose levels of experimental animals might be due to counteracting effect of both insulin and cortisol.

Wakefulness involves high neuronal metabolism to maintain neuronal electrical potentials, which requires a great amount of oxygen, resulting in a significant production of oxidants. Thus, sleep represent with an increased antioxidant activity which promotes a brain protection against free radicals via a diminution of in oxidant production (Reimund, 1994). Increased MDA (oxidative marker) levels which was significant in both acute and chronic SD groups denotes the effect of sleep deprivation as a stressor to increase tissue oxidation. This is in agreement with the result of a previous research (Thamaraiselvi *et al.,* 2012) which also showed increased MDA. Likewise, De Oliveira and colleagues (De Oliveira *et al.,* 2002) observed a decreased glutathione and an increase thiobarbituric acid reactive substance (an index of lipid peroxidation) levels in SD rats. However, in this study, MDA levels reduced in vitamin C treated groups compared to corresponding untreated groups. The recovery groups showed similar MDA levels as that of the control group.

Conclusion

It is concluded that vitamin C could mitigate stress response in rats, at least in the case of plasma cortisol, which could help to improve stress tolerance.

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Niger. J. Physiol. Sci. 30(2015) 011-016
www.njps.com.ng

Niger. J. Physiol. Sci.

Honey Attenuates the Detrimental Effects of Nicotine on Testicular Functions in Nicotine Treated Wistar Rats

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Summary: Effect of honey on reproductive functions of male rats exposed to nicotine was examined in this study. Thirty-two adult male wistar rats (n=8/Group) were grouped as Control (distilled water), Nicotine (1.0mg/kg bwt), Honey (100mg/kg bwt) and Nicotine with Honey. The animals were orally treated for 35 days consecutively. Epididymis sperm motility, viability, morphology and counts were estimated, serum Follicle Stimulating Hormone (FSH), Leutinizing Hormone (LH) and Testosterone were assayed using ELISA method and testicular histology were also assessed. Significant reduction in percentage sperm motility, viability, morphology and counts were observed in nicotine group *($p<0.05$)* compared to control. Serum FSH, LH and testosterone levels were significantly reduced in nicotine group($p<0.05$) when compared with the control. There was significant improvement in sperm motility, viability, morphology, counts, FSH, LH and Testosterone in group co-treated with nicotine and honey *($p<0.05$)* relative to nicotine group. Also, the degenerative seminiferous tubule architecture due to nicotine was improved by honey. In conclusion, honey may suppress nicotine toxic effect on reproductive functions in male Wistar rats.

Keywords*:* Nicotine, Honey, FSH, LH, Testosterone, Rats.

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Manuscript Accepted: December, 2015

INTRODUCTION

Nicotine is the principal alkaloid contained in tobacco which has been reported to have detrimental effects on male reproductive functions. Cigarette smoking and tobacco chewing are the major means which nicotine is consumed throughout the world (Abel, 1983). Nicotine is a highly toxic substance that can be absorbed quickly through the respiratory tract, oral mucosa and skin.

Cotinine is a nicotine metabolite which has a long half-life, both nicotine and cotinine adversely affected spermatogenesis, epididymal sperm count, motility, and the fertilizing potential of sperms (Aydos *et al.*, 2001). Oral administration of nicotine in male rats have been associated with testicular degeneration, disorganization of the cytoarchitecture and decreased serum testosterone levels, reduced sperm characteristics and fertility (Oyeyipo *et al.*, 2010; Oyeyipo *et* al.,2011).

Honey is a natural product of bees formed from nectar collected from flowering vegetation. It contains moisture, sugars such as glucose and fructose, enzymes such as catalase and glutathione reductase, trace essential elements such as iron, copper, zinc and calcium, vitamins such as vitamin A, C and E as well as some flavonoids and phenolic acids (Gheldof *et al.*, 2002; Al- Waili, 2003; Yao *et al.*, 2004; Michalkiewicz *et al.*, 2008).

Its rich nutrients make it to boost and maintain health. It also possesses antibacterial, antioxidant and wound healing properties (Aljady *et al.*, 2000; Estevinho *et al.*, 2008). In Arab countries honey is considered to increase human male potency. Honey had been reported to increase sperm motility and spermatogenesis in rats and subnormal human (Abdul-Ghani *et al.*, 2008; Abdelhafiz and Muhamad, 2008).

Recently, Mahaneem *et al.*, (2011 and 2012) reported the protective effect of honey on toxic effects of cigarette smoke on testicular structure, antioxidant and spermatogenesis in male rats. Also, Noorhafiza *et al.*, (2013) showed that honey improved testicular structure and testosterone secretion in nicotine treated rats.

Since nicotine is one of the active compounds present in cigarette smoke, it is imperative to investigate the effect of honey on sperm motility,

viability counts, morphology and some reproductive hormones in adult male rats exposed to nicotine.

MATERIALS AND METHODS

Nicotine Preparation

Nicotine hydrogen tartrate was obtained from BDH Chemical Ltd Poole English. Nicotine stock solution was prepared at concentration of 1mg/ml and stored in foil-wrapped glass bottle 4 °C for no longer than ten days.

Honey

The honey used in this study was obtained from Department of Agriculture, University of Ilorin, Nigeria. It was concentrated (20% w/v water) by oven drying at 40 °C.100mg/kg of honey was administered to the rats. This dose was worked out relative to the local human consumption of honey which is 0.2 g/kg body weight daily. Honey at the dose of 1.0 g/kg body weight was freshly diluted with distilled water to prepare 0.5 mL of diluted honey for each rat. Then, 0.5 mL of the diluted honey was immediately administered to each rat by oral gavage.

Experimental Animals

Thirty-two male Wistar rats weighing between 180 to 200g were used. They were kept in the animal house of Madonna University, Elele campus, Nigeria under standard laboratory conditions with 12 hours light and 12 hours dark cycle. They were fed with standard laboratory chow and had access to water *ad libitum.* The animals were acclimatized for one week. The animal grouping is shown in table 1. The administration was through oral gavage for 35 days consecutively

Experimental Procedure

Blood samples were collected from the anesthetized animals through cardiac puncture, serum was obtained and used for FSH, LH and testosterone assays. Caudal epididymis was immediately dissected to obtain spermatozoa which were used for estimation of sperm motility, counts, viability and morphology. The testes were also dissected and fixed in 10% formalin for histology.

Sperm motility

As described previously by Kaur and Bansal (2004), the caudal epididymis was identified and its content squeezed into 1ml of normal saline at room temperature. One drop of the sperm suspension was charged into a Makler counting chamber and the number of motile and non-motile spermatocytes was counted in ten random fields. The number of motile spermatocytes was then expressed as a percentage of the total number of the counted spermatocytes (Mahaneem *et* al.,2011).

Table 1. Animal grouping

S/N	Groups (n =8)	Treatment
1	Control	Normal saline
2	Nicotine	1.0mg/kg of nicotine
3	Honey	100mg/kg of honey
4	Honey and Nicotine	1.0mg/kg of nicotine and 100mg/kg of honey

Sperm count

Sperm count was performed as reported earlier (Narayana *et al*, 2005) with minor modifications. Briefly, caudal epididymis was minced in 2 ml of normal saline to obtain sperm suspension, which was then stained with 2% eosin and sperm heads were counted using a Neubauer haemocytometer counting chamber.The sperm were counted and expressed as million per ml.

Sperm Viability

The caudal epididymis sperm was dropped on the slide and mixed with a drop of 0.5% eosin solution. After 2 minutes, the slide was examined under microscopy with 40X objective lens to count the percentage of viable (unstained) and non-viable sperm (stain red) (Cheesbrough, 2006).

Sperm morphology

This was determined by smearing a drop of the stained sperm suspension obtained during determination of sperm count on a glass slide; the smear was allowed to dry and subsequently examined under the light microscope at X400 magnification. For each sample, 200 spermatocytes were carefully observed and the percentage of total abnormalities of the spermatocyte head and tails were determined as described by Narayana et al (2005).

Reproductive hormones measurement

Testosterone, Luteinizing Hormone (LH) and Follicle-Stimulating Hormone (FSH) in rat serum were measured by enzyme linked immunosorbent assay (ELISA) using commercially available kits form Endocrine Technologies, USA. Samples were run in the same assay to avoid inter-assay variations.

Testicular Histology

The testes of all the rats were fixed in 10% formalin, dehydrated stepwise in the graded ethanol, cleared in xylene and then embedded in paraffin wax. A section of 5pm thickness paraffin section was taken from the mid portion of each testicular tissue and stained with heamatoxylin and eosin, followed by examination under a light microscope with 200X magnification. Photomicrographs were taken as appropriate and analyzed by a pathologist with requisite experience. and their micrographs was taken.

Statistical Analysis

All statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the LSD post hoc tests for pair-wise comparisons were performed using SPSS 17.0 version. All data were expressed as Mean ± Standard Error of mean (SEM) and $p<0.05$ was considered significant.

RESULTS

Table 2 showed the effects of honey on sperm motility, counts, viability and morphology of rats exposed to nicotine. Results obtained indicate that administration of nicotine caused a significant diminution in percentage sperm motility, counts, viability and normal morphology with increased in abnormal heads and tails ($p<0.05$) as compared to control rats. Also, sperm counts and viability significantly reduced in group administered with honey compared to control group *(*$p<0.05$*)* while honey apparently, but insignificantly increased sperm motility and normal sperm morphology ($p<0.05$*).*

Co-administration of nicotine and honey caused a general and significant improvement in all the sperm parameters (sperm motility, counts, viability, and morphology) ($p<0.05$) when compared to nicotine group, but significant reduction *(*$p<0.05$*)* was observed in sperm motility, counts and viability when compared to control (Table 2).

Table 3 showed that FSH, LH and testosterone were significantly reduced in group treated with nicotine relative to the control group *(*$p<0.05$*)*, while the level of luteinizing hormone was significantly increased in honey group when compared with the control *(*$p<0.05$*)*. Furthermore, co-administration of nicotine and honey caused significant increase in the serum levels of FSH, LH and testosterone as compared to nicotine group ($p<0.05$), but significant reduction was observed when compared with control ($p<0.05$).

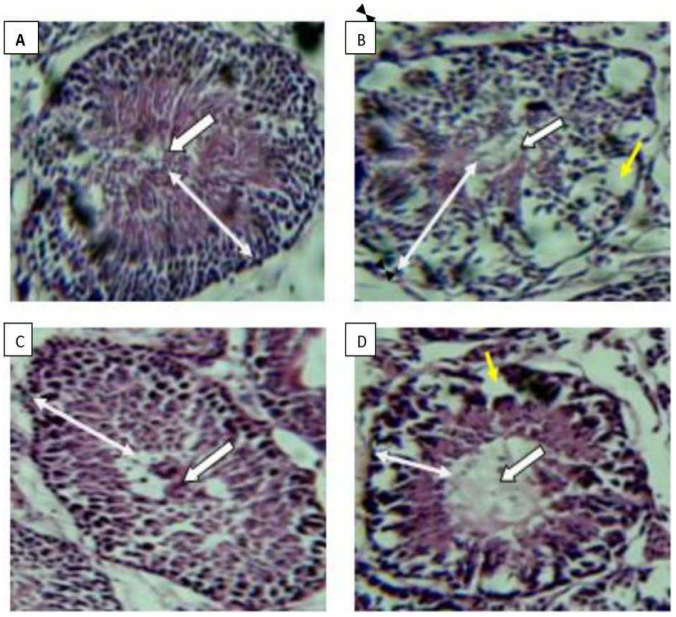

Figure 1: Testicular photomicrograph (H&E, 200X). Control and Honey groups (A &C) shows normal seminiferous tubule with normal germinal cell layer revealing spermatocytes muturation (spanned), the lumen (white arrow) shows spermatid and spermatozoa. Nicotine exposed group (B) showed one of numerous vacuolated (Yellow arrow) seminiferous tubules, the germinal cell layer is disorganized, no production of spermatids (spanned) the lumen (white arrow) is empty without spermatozoa. Nicotine with Honey treated group (D)shows re-organization of germinal cell layer that indicator repair of seminiferous tubule (spanned) with few vacuole (Yellow arrow).

DISCUSSION

This study was undertaken to investigate the effects of honey on reproductive functions in adult male rats exposed to nicotine. The present study showed that nicotine has negative effects on the sperm motility, sperm count, viability, morphology and increased the percentage of abnormal sperms. This finding is in support of the past studies that nicotine has ability to reduce reproductive capacity and has a mutagenic

Table 2: Effects of Honey on Sperm Motility, Counts, Viability and Normal Morphology of Rats Exposed to Nicotine

Groups	Sperm motility (%)	Sperm counts (million/ml)	Sperm viability (%)	Normal Sperm morphology (%)
Control	67.2 ± 1.59	39.0 ± 2.30	78.6 ± 0.93	79.0 ± 2.86
Nicotine	41.2 ± 1.07[a]	22.2 ± 1.16[a]	47.0 ± 0.95[a]	62.4 ± 1.86[a]
Honey	70.6 ± 1.98	33.4 ± 1.63[a]	66.8 ± 1.93[a]	79.4 ± 1.44
Nicotine + honey	55.4 ± 1.66[a,b]	25.4 ± 1.33[a,b]	58.2 ± 1.56[a,b]	73.4 ± 1.29[b]

Data are expressed in Mean ± SEM of 8 rats, [a, b] *Mean significant difference relative to control and nicotine respectively at* $p<0.05$

Table 3. Effects of honey on FSH, LH and Testosterone of Rats Exposed to Nicotine

Groups	FSH (mlU/ml)	LH (mIU/ml)	Testosterone (ng/ml)
Control	9.1 ± 1.24	9.4 ± 0.30	12.9 ± 0.09
Nicotine	0.7 ± 0.01[a]	2.6 ± 0.04[a]	11.9 ± 0.19[a]
Honey	7.6 ± 0.47	21.0 ± 0.24[a]	12.7 ± 0.01
Nicotine + honey	1.35 ± 0.16[a,b]	3.31 ± 0.07[a,b]	12.6 ± 0.04[a,b]

Data are expressed in Mean ± SEM of 8 rats, [a, b] *Mean significant difference relative to control and nicotine respectively at* $p<0.05$

consequences towards the germ cell production and maturation as well as the reproductive organ itself and accessory reproductive organs (Yamamoto *et al.,* 1998; Patil *et al.,* 1999; Oyeyipo *et al.,* 2011; Jana *et al.,* 2010, Seema *et al.,* 2007; Aruldhas *et al.,* 2005).

Oxidative stress through generation of reactive oxygen species (ROS) had been proposed as one of the possible mechanisms of actions of detrimental effects of nicotine on male reproductive functions. It has been proven that nicotine increased the production of ROS by increase generations of testicular H_2O_2 and hydroxyl radicals in experimental rats (Bandopadhyay *et al.*,2008). Oxidative stress has been shown to lead to testicular damage following exposure to nicotine (Rajpurkar *et al.,* 2000).

In this study, it was observed that honey attenuated the detrimental effects of nicotine on the semen parameters. Honey has been reported to have some vitamins and antioxidants such as vitamins A, C and E (Al-Waili, 2003), flavonoids (Yao *et al.,* 2004) and phenols (Mohamed *et al.,* 2010). Therefore, it is plausible to suggest that the effect of honey in attenuating the nicotine induced impaired spermatozoa motility, viability, counts and morphology in this study could be partly mediated by its counteraction on oxidative stress within rat reproductive organs via its antioxidant properties.

The results of this study also showed the adverse effects of nicotine on testosterone, FSH and LH. Nicotine decreased the serum levels of testosterone, LH and FSH. The decreased in serum testosterone concentration observed in this study following nicotine administration may be attributed to disruption of testicular cytoarchitecture which may adversely affect leydig cells number and function, as well as reduction in LH concentration. Testosterone is secreted by the interstitial cells of Leydig cells in the testes but only when they are stimulated by LH from the anterior pituitary gland. Also, the decreased in serum testosterone in nicotine administered rats in this study may be attributed to the cholinergic agonist activity of nicotine which had been reported to inhibit testosterone secretion (Kasson and Hsueh, 1985). Testosterone plays a major role in spermatogenesis by being the main hormone for spermatogonia conversion and spermatids formation. Therefore, a drop in the testosterone level will lead to sterility in males. The decreased in the sperm count may be linked to decrease in testosterone level observed in this study.The findings in this study are in accordance with previous findings where it has been established that nicotine administration decreased serum testosterone and sperm counts in mature male Wistar rats (Yamamoto *et al.,* 1998; Oyeyipo *et al.,* 2010). Inhibition of FSH and LH by nicotine may be as a result of its negative effect on central nervous system that can inhibit the neural stimulus essential for the release of pituitary gonadotrophins (Reddy *et al.,* 1995), which lead to a lack of pituitary gonadotrophins essential for initiating and completing spermatogenesis and steroidogenesis in the testis (Aydos *et al.,* 2001).

There was an improvement in the levels of the reproductive hormones of the nicotine exposed rats when treated with honey. This shows that honey has a positive effect on the hormonal levels. The mechanism by which this is brought about may be due to its antioxidant properties. (Al-Waili, 2003; Gheldof *et al.,* 2002; Yao *et al.,* 2004). Recently, it was reported that honey contained antioxidant such as phenols and possess anti-radical and antioxidant properties (Mohamed *et al.,* 2010). Therefore, it is plausible to suggest that the effect of honey in attenuating the nicotine-induced impaired testicular functions in this study could be partly mediated by its counteraction on oxidative stress within rat reproductive organs via its antioxidant properties.

Mohamed *et al.* (2010) also observed that honey supplementation improved spermatogenesis in normal rats. Due to its antioxidant properties, it has been proved that honey is able to reduce testicular damage. This would suggest that honey has the potential healing properties against the toxic effects of nicotine.

In conclusion, administration of honey significantly attenuated the detrimental effect of nicotine on sperm counts, motility, viability, morphology, testosterone, FSH and LH levels in rats. This study indicates that honey may possess a protective effect against nicotine-induced impaired testicular functions in rats, but further research to elucidate its exact mechanism of action is essential.

Acknowledgements

The authors are grateful to Mr Ezeokafar Emmanuel, chief technologist of the Department of Physiology, Madonna University, Elele campus, Rivers State for his assistance in the sperm parameter analysis.

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Niger. J. Physiol. Sci. 30(2015) 017-023
www.njps.com.ng

Niger. J. Physiol. Sci.

Renal Doppler Indices in Children with Nephrotic Syndrome: Findings from a Tertiary Hospital in Nigeria

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Summary: The resistive and pulsatility indices are known tools for assessing renal function in kidney diseases, especially in proteinuric conditions like Paediatric Nephrotic syndrome (NS) which is a glomerular disease. However, there is a limited knowledge in the use of Doppler Resistive and pulsatility indices in the management of this disease condition. This was a case control study involving 53 cases and 57 controls. The Doppler parameters, resistive index (RI) and pulsatility index (PI) of the renal interlobar arteries were determined for the upper, middle, and lower poles bilaterally for both controls and cases. The mean RI on the right and left were 0.59 ± 0.06 and 0.58 ± 0.06 respectively for the NS cases whereas for the controls it was 0.61 ± 0.05 and 0.60 ± 0.04 on the right and left respectively. The mean PI on the right and left measured 0.96 ± 0.16 and 0.94 ± 0.15 respectively for the NS cases while that for the control cases measured 0.98 ± 0.13.and 0.95 ± 0.12 on the right and left respectively. Although, the interlobar arteries mean RIs were generally less than that for the controls, but only the left middle pole showed statistically significant mean difference (p= 0.004). There was also statistically significant mean difference (p= 0.048) between the cases and controls in the left middle pole PI. However, no correlation was found when the renal RI and PI are compared with the serum albumin and creatinine. Although there was no statistical significance between the mean RI and PI of the NS cases and controls, except in the left middle pole RI, it is recommended that Doppler ultrasound should still be part of management of Nephrotic syndrome patients especially those who have developed end stage renal disease in order to monitor their renal function.

Keywords*:* Paediatric, Nephrotic Syndrome, Renal, Resistive Index, Pulsatility Index

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Manuscript Accepted: July, 2015

INTRODUCTION

Nephrotic syndrome is a major cause of chronic renal disease among the paediatric age group (Gbadegesin & Smoyer, 2008) It is basically a manifestation of many glomerular diseases and a leading cause of significant renal morbidity and mortality in Nigeria (Abdurrahman et al, 1990; Anochie & Eke, 2003; O. M. Ibadin & Ofovwe, 2003; Ocheke et al., 2010) Nephrotic syndrome is a form of chronic kidney disease in which there is massive proteinuria, hypoalbuminaemia, and anasarca. (Gbadegesin & Smoyer, 2008; Lane & Langman, 2011). Nephrotic syndrome, also known as nephrosis is technically defined as the presence of nephrotic- range proteinuria (>40mg/m /hr), or urine protein to creatinine ratio of >2-3mg/mg, hypoalbuminaemia (<2.5g/dl) and oedema. Other clinical conditions like hyperlipidaemia may also be present (Gbadegesin & Smoyer, 2008; Hendrickse & Adeniyi, 1979; Lane & Langman, 2011).

According to Lane and Langman, Nephrotic syndrome can be classified based on the aetiology and response to steroid therapy. (Lane & Langman, 2011). The aetiological classes include idiopathic or primary, congenital and secondary nephrotic syndromes.

The idiopathic nephrotic syndrome is basically of an unknown cause and these include minimal change nephrotic syndrome (MCNS), focal segmental glomerulosclerosis (FSGS), membranous nephropathy (MN), membranoproliferative glomerulonephritis (MPGN), IgA nephropathy, IgM nephropathy, mesangioproliferative glomerulonephritis and idiopathic crescentic glomerulonephritis. They have all been implicated as causes of idiopathic nephrotic syndrome, the diagnosis of which are made at biopsy (Gbadegesin & Smoyer, 2008; Lane & Langman, 2011; Mohammed, Al-badri, Al-latteef, Mohammed, & Abdulhussain, 2009).

The congenital nephrotic syndrome is the type that appears within the first 3months of life, while the secondary is due to known causes (Ademola et al., 2012; Dathan, Heyworth, & MacIver, 1974; Hellier, Webster, & Eisinger, 1972; Hull & Goldsmith, 2008; Novis et al. 1988; Ojo & Akinkugbe, 1967; Orth & Ritz, 1998).

Based on the response to steroid therapy, Nephrotic syndrome can also be classified into: Steroid-sensitive nephrotic syndrome (SSNS), Steroid-resistant

nephrotic syndrome (SRNS), Steroid-dependent nephrotic syndrome (SDNS), and Frequent relapsing nephrotic syndrome (FRNS) (Gbadegesin & Smoyer, 2008).

Renal Resistive index (RI) has been shown as a prognostic instrument in assessing the progression of renal disease, especially in hypertension, as well as proteinuria (Parolini et al., 2009). Studies have demonstrated that high RI, proteinuria and hypertension are known risk factors for the progression to chronic kidney disease (Parolini et al., 2009; Radermacher, Ellis, & Haller, 2002; Sugiura & Wada, 2009). An initially high RI denotes poor prognosis. (Parolini et al., 2009). This eventually leads to more rapid disease progression. Studies have demonstrated that high RI, proteinuria and hypertension are known risk factors for the progression of chronic kidney disease, though an association between RI and specific histological subtype has not been shown to be of statistical significance (Parolini et al., 2009; Radermacher et al., 2002; Sugiura & Wada, 2009).

The generally acceptable normal value of the renal RI is taken as ≤ 0.7 (Mostbek et al., 1991). However, a slightly higher RI value (0.72± 0.03) has been shown in healthy young children of age four and a half years and below. Thus, the RI value of 0.70 is only applicable for older children (Sigirci et al., 2006). It is notable that a significant correlation has been shown between the RI, glomerular sclerosis and focal interstitial fibrosis (Mostbek et al., 1991). Similar to the resistive index, though with less emphasis, the clinical significance of the pulsatility index (PI) has been documented in previous studies. It has been shown to increase in chronic kidney disease and correlates with the severity of renal disease (Petersen et al., 1997; Petersen, et al, 2006). An inverse correlation has been reported between age and PI, which ranges between 0.96 and 1.27 in healthy children (Sigirci et al., 2006). However, there is apparently very limited studies on renal RI and PI in nephrotic syndrome. The aim of this study therefore was to find out if RI and PI can be used to predict the outcome and/ or monitor progression and regression of nephrotic syndrome among peadiatric age group especially in resource poor settings.

MATERIALS AND METHODS

Study settings

The University College Hospital (UCH) Ibadan is the first and foremost premier teaching hospital situated in Western Nigeria with 850 bed spaces. On the average, 30 new Nephrotic syndrome cases are seen annually by the paediatric department.

Ethical approval This was given by the institutional UI/UCH ethical review committee (UI/EC/13/0257).

The subjects

This was a case-control study in which 53 children with Nephrotic syndrome (diagnosed based on clinical findings, and the presence of albuminuria and hypoalbuminaemia) were recruited from the children emergency ward and children outpatient clinic of the University College Hospital over a period of 12 months from July 2013. The content of a consent form was duly explained to the parent/ guardian of the proposed cases and healthy controls in the local languages for those who were not literate. The consent form was filled and signed by those parents/ guardian who were literate and thumb printed by those who were not literate, while a verbal assent was also obtained from each child who was old enough to agree to the study. Age- matched healthy children of members of staff and friends were recruited as controls, after screening by urinalysis as well as B mode ultrasound. Every consenting subject (case and control) was recruited until the minimum sample size was attained.

Ultrasound Technique

The renal Doppler assessment was done in the lateral or lateral oblique position using Mindray M7, 2010 by Shenzhen Mindray bio- medical electronics company limited ultrasound machine with low frequency (2-5 KHz) transducer. The kidneys were scanned with the B mode to check for any gross or incidental abnormalities. The colour Doppler was then turned on to identify the interlobar arteries and the sample volume applied such that almost the entire arterial diameter is covered. The Pulse Repetition Frequency (PRF) and the wall filter were adjusted and optimized so as to avoid aliasing and allow for slow diastolic flow. Doppler angle was also kept below 60^0. (Boote, 2003). Three consecutive Doppler spectral patterns of velocity- time graph, that was representative of the arterial blood flow from early systolic to end of diastolic flow, were obtained before the RI and PI parameters were measured. The upper, middle and lower poles renal interlobar arteries RI and PI were assessed for each kidney and the average of the measurements taken (Figure 1). The mean of the values of these measurements (upper, middle and lower poles) were also taken for each of the two kidneys. Doppler parameters were measured at breath-holds, especially in children that could cooperate with instructions. However, in younger children, measurements were derived during slow respiration. (Sigirci et al., 2006; Zubarev, 2010).

The arterial Resistive Index (RI) was defined as (Peak Systolic velocity - End diastolic velocity) divided by (Peak Systolic Velocity) and Pulsatility Index (PI) was defined as (Peak systolic velocity - End diastolic velocity) / Mean systolic velocity, while their respective values were derived via the computer algorithm in the ultrasound machine.

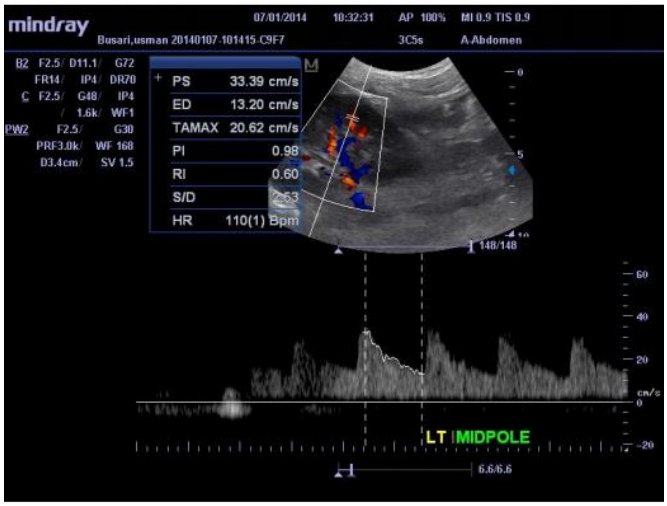

Figure 1: The Renal Doppler ultrasound of the left kidney midpole showing a typical doppler indices measurement.

Laboratory parameters

The results of the serum albumin and serum creatinine with urinary protein (dipstix) were also derived. The serum albumin and creatinine were derived by analyzing the subjects' serum, as they were compared to the locally standardized laboratory values. The urinalysis is however derived by using the qualitative method of dipstix, using the standardized colour code.

Statistical Analysis

The renal RI and PI for the cases and the control group, as well as the comparison between these groups were presented in tables. The association between the renal RI of NS cases and controls as well as renal PI of cases and controls were all determined by independent student t test. Likewise, the degrees of significance of the mean difference of RI and PI among the different levels of proteinuria were determined by analysis of variance (ANOVA). The level of significance was set at $p < .05$.

RESULTS

Sociodemographic Characteristic of the subjects

A total of One hundred and twelve children were recruited into the study, made up of fifty- five patients with nephrotic syndrome and fifty- seven healthy controls. However, two of the recruited NS cases did not complete the study. Hence, these were not included in the final analysis due to incomplete data collection. Thirty- two (60.4%) of the analysed cases were males, while thirty- four (59.6%) of the control group were males (Figure 2). The average age of the NS cases was 126.24 ± 40.11months, with a majority (56.6%) in the 10 - 15years age brackets. The mean age of the control subjects is slightly lower (117.63± 38.11months).

The age of diagnosis of nephrotic syndrome among the cases ranged between 24 and 158months (mean= 105.58 ± 40.09months). The NS cases were also predominantly either overweight or obese, when compared to the healthy control subjects. More than half (50.9%) of the NS cases showed BMI of ≥ 85 percentile, as shown in Table 1.

Table 1: Sociodemographic characteristics of the cases and the control.

	NS CASES		CONTROLS	
Parameters	N	%	N	%
Age (months)				
<60	6	11.3	2	3.5
60-119	15	28.3	25	43.9
120-179	30	56.6	28	49.1
180+	2	3.8	2	3.5
Weight (Kg)				
<25	15	28.3	29	50.9
25- 34.5	11	20.8	15	26.3
35- 44.5	15	28.3	11	19.3
45+	12	22.6	2	3.5
Height (cm)				
<110	6	11.3	2	3.5
110-129.5	14	26.4	23	40.4
130-149.5	16	30.2	20	35.1
150+	17	32.1	12	21.1
Body Mass Index (Kg/m^2)				
<5 Percentile	-	-	2	3.5
5- 84.9 Percentile	26	49.1	46	80.7
85- 94.9 Percentile	12	22.6	7	12.3
>95 Percentile	15	28.3	2	3.5

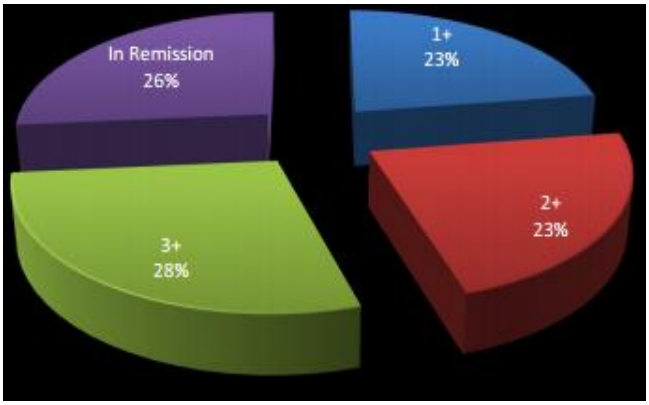

Figure 2. A pie chart of Urine dipstix analysis of Nephrotic syndrome (NS) cases showing the percentage and level of proteinuria of the patients

The serum albumin and creatinine measured 2.2 ± 1.0g/dl and 0.6 ± 0.5mg/dl respectively. The average duration of disease was about 20months. The dipstix urinalysis is depicted in Figure 2.

RI and PI findings

The mean RI value was 0.59, 0.59 and 0.58 as well as 0.59, 0.58 and 0.57 on the right and left respectively for each of upper, middle, and lower pole interlobar renal arteries in NS cases. These interlobar renal artery values were seen to be higher (yet <0.70) in controls, measuring 0.61, 0.61, and 0.60 on the right upper, middle and lower poles respectively, while on the left

Table 2. Resistive Indices (RI) of the Kidneys of Nephrotic Syndrome Cases and Control

	Cases	Controls	
Parameters	Mean ± SD	Mean ± SD	p- Value
Right Kidney			
Upper Pole RI	0.59 ± O.08	0.61 ± 0.05	0.065
Mid Pole RI	0.59 ± 0.08	0.61 ± 0.06	0.108
Lower Pole RI	0.58 ± 0.07	0.60 ±0.06	0.185
Average RI	0.59 ± 0.06	0.61 ± 0.05	0.059
Left Kidney			
Upper Pole RI	0.59 ± 0.07	0.60 ± 0.05	0.585
Mid Pole RI	0.58 ± 0.07	0.62 ± 0.05	0.004*
Lower Pole RI	0.57 ± 0.07	0.58 ± 0.05	0.638
Average RI	0.58 ± 0.06	0.60 ± 0.04	0.121

*p<0.05

Table 3. Pulsatility Indices (PI) of the Kidneys for the Nephrotic Syndrome (NS) Cases and Controls

	Cases	Controls	
Parameters	Mean ±SD	Mean ± SD	p- Value
Right Kidney			
Upper Pole PI	0.96 ±0.20	0.98 ± 0.15	0.582
Mid Pole PI	0.98 ± 0.18	1.00 ± 0.17	0.566
Lower Pole PI	0.94 ± 0.18	0.96 ± 0.15	0.404
Average PI	0.96 ± 0.16	0.98 ± 0.13	0.444
Left Kidney			
Upper Pole PI	0.96 ± 0.17	0.94 ± 0.15	0.638
Mid Pole PI	0.93 ± 0.19	1.00 ± 0.15	0.048*
Lower Pole PI	0.93 ± 0.17	0.92 ± 0.14	0.703
Average PI	0.94 ± 0.15	0.95 ± 0.12	0.623

*p<0.05

Table 4. Comparison of the Mean Resistive Index of the Different Degree of Proteinuria among the Nephrotic Syndrome children

	Right Kidney			Left Kidney		
Degree of Proteinuria	RI	f	P-value	RI	f	P-value
Upper Pole						
1+	0.564	0.301	0.742	0.631	4.819	0.014
2+	0.578			0.550		
3+	0.586			0.599		
Middle Pole						
1+	0.628	3.168	0.054	0.602	4.060	0.026
2+	0.554			0.558		
3+	0.586			0.599		
Lower Pole						
1+	0.603	2.606	0.088	0.608	2.211	0.124
2+	0.554			0.558		
3+	0.606			0.565		
Average of Poles						
1+	0.599	1.542	0.228	0.614	4.275	0.022
2+	0.560			0.550		
3+	0.596			0.592		

f is the ANOVA value; p value < 0.05 is significant. RI means Resistive index

Table 5. Comparison of the Mean Pulsatility Index of the Different Degree of Proteinuria among the Nephrotic Syndrome children

	Right Kidney			Left Kidney		
Degree of Proteinuria	PI	f	P-value	RI	f	P-value
Upper Pole						
1+	0.904	0.462	0.634	1.027	2.633	0.086
2+	0.929			0.877		
3+	0.977			0.991		
Middle Pole						
1+	1.073	3.511	0.040	0.971	3.050	0.060
2+	0.879			0.840		
3+	0.996			1.001		
Lower Pole						
1+	0.978	2.833	0.073	0.988	0.839	0.440
2+	0.852			0.900		
3+	1.005			0.919		
Average of Poles						
1+	0.985	1.764	1.186	0.995	2.420	0.103
2+	0.887			0.872		
3+	0.993			0.970		

f is the ANOVA value; p value < 0.05 is significant. PI means Pulsatility Index

upper, middle and lower poles measured 0.60, 0.62 and 0.58 respectively. The only statistically significant difference in mean was seen in the left middle pole which showed a mean RI of 0.58 in the NS cases and 0.62 in the control group (p= 0.004), as contained in Table 2.

The mean PI value was 0.96, 0.98 and 0.94 as well as 0.96, 0.93 and 0.93 on the right and left respectively for each of upper, middle, and lower pole interlobar renal arteries in NS cases. These interlobar renal artery values were seen to be higher in controls, measuring 0.98, 1.00, and 0.96 on the right upper, middle and lower poles respectively, while on the left the upper, middle and lower poles measured 0.94, 1.00, and 0.92 respectively. The only statistically significant difference in mean, even though marginal, was also seen in the left middle pole which showed a mean PI of 0.93 in the NS cases and 1.00 in the control group (p= 0.048), as depicted in Table 3.

Tables 4 and 5 showed the relationship between the mean RIs and PIs of the different degrees of proteinuria in the NS cases. There was no statistical difference in the mean values of RI except in the upper and middle poles of the left kidney (p= 0.014 and 0.026 respectively). Also the only statistically significant mean difference in PI was found on the right kidney middle pole (p= 0.040).

The duration of the disease, the serum albumin and creatinine did not affect the mean values of RI and PI of NS patients. Likewise, comparison of the RI and PI of the nephrotic cases with proteinuria and those in remission showed no statistical significance. There is also no statistical significance the serum parameters when correlated with the RI and PI of both kidneys in the cases.

DISCUSSION

Nephrotic syndrome has been reported as a major cause of childhood morbidity and mortality (Abdurrahman et al., 1990; Anochie & Eke, 2003; M. Ibadin & Abiodun, 1998; Ocheke et al., 2010), proteinuria has also been shown as a co- factor for progression into chronic renal failure. Nephrotic syndrome as a cause of renal parenchymal disease gives various sonographic patterns which include changes in parenchymal echogenicity, corticomedullary differentiation and renal size. In addition, renal resistive index has been shown to be of high prognostic value in chronic kidney disease, especially in proteinuric states to which Nephrotic syndrome belongs.

This study was able to document the normal parameters for the kidney dimensions, as well as renal interlobar arteries RI and PI as a reference for comparison with the parameters in the children with nephrotic syndrome.

The mean age of 126.24 months in children with NS in this study is higher than that from other regions where mean ages of 60.3 months and 36 months in Kuwait and Saudi Arabia were reported respectively (Kari, 2002; Zaki, Helin, Manandhar, Hunt, & Khalil, 1989). This may be due to demographic characteristic of the cases. The children are predominantly male in this study as seen in other studies (Asinobi et al., 1999; Chijioke & Adeniyi, 2003; M. Ibadin & Abiodun, 1998; Okoro, Okafor, & Nnoli, 2000; Zaki et al., 1989).

Majority of the children 92.7% showed normal serum creatinine, which is in agreement with a previous study done in Finland, where about 82.5% demonstrated normal serum creatinine (Koskimies et al, 1982).

The mean RI value is similar to the findings among the Americans who reported a 0.58 ± 0.05 as the mean RI among the patients with purely glomerular disease (Platt, Ellis, Rubin, DiPietro, & Sedman, 1990). In contrast, a recent study by Calabia et al in Spain reported a higher value, 0.69 ± 0.08 for renal RI among adults with diabetic nephropathy (Calabia et al., 2014). A previous study on vesicoureteric reflux in Austrian children also demonstrated a higher RI value of 0.77 ± 0.07 (Radmayr, Klauser, Maneschg, Bartsch, & Frauscher, 1999). This further buttresses the minimal or no affectation of the renal RI by glomerular disease (Platt et al., 1990).

A similar study from Taiwan among adolescents and young adults is in contrast to this study. Tsai et al reported no statistical significance between renal interlobar artery and albuminuria in non-diabetic patients. This is likely due to a different mode of classification of the 3 groups in the aforementioned study. The index study however classified the NS cases based on qualitative degrees of proteinuria.

This study is in concordance with findings among Turkish children, as demonstrated by Sigirci et al (Sigirci et al., 2006). There is similarity in the value of the renal interlobar artery value. This may be due to similarity in the age distribution of the subjects in both studies.

Not many studies have been carried out on the Doppler indices in childhood nephrotic syndrome hence no further comparison could be made.

In conclusion, this study has shown that there is significant difference in the left midpole RI and PI of the NS cases and controls.

Comparison of the mean renal RI among the 3 levels of proteinuria showed significant differences, however weak in the left kidney upper pole, midpole and average.

In contrast, there was no correlation between the serum parameters (serum albumin and creatinine) and the renal RI and PI.

Although there is weak correlation between renal Doppler indices in children with nephrotic syndrome,

it is recommended that renal ultrasound including Doppler scan should be included in the routine management especially those that have developed chronic renal disease.

Acknowledgement
We appreciate all the children that took part in this study and their care givers for their cooperation.

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Niger. J. Physiol. Sci. 30 (2015) 025 – 032
www.njps.com.ng

Niger. J. Physiol. Sci.

Spinal Cord Studies in the African Giant Rat *(Cricetomys gambianus*, Waterhouse)

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Summary: The African giant rat, AGR, is known for advantageous behavioural patterns among which are cognition and dexterous locomotion. This study investigated the morphological, morphometric and possible functional aspects of the AGR spinal cord (SC) anatomy. Ten adult (5 males and 5 females) AGR were used to determine the gross and histological features of the SC which were typically of rodent features. The mean SC weight and length given as 2.50±0.24g and 15.87±0.24cm respectively for the male and 2.32±0.16g and 15.40±0.61cm for the female showed no sexual dimorphism ($p<.05$). A positive linear relationship between the tail length and SC weight were found in both sexes ($r=0.81$ males; $r=0.95$ females) suggesting significant contribution of the filum terminale to SC weight. Forty-three internal structures including nuclear aggregations and tracts were traced. Eight nuclear aggregations of neurons involved in nociception and limb coordination were observed to be prominent and larger than in laboratory rats. Same was noted for the dorsal, ventral and lateral funicular tracts which control the limbic system. This study provides morphometric baseline research information and delineates the functional aspects of the AGR SC anatomy. The information provided further strengthens the drive proposing the AGR as an indigenous research model for regional anaesthesia and locomotor disease.

Keywords*:* African giant rat; spinal cord; spinal tract; nuclei; spinal segment; morphometry.

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Manuscript Accepted: 2015

INTRODUCTION

Rodents are the largest mammalian order with about fifty percent of the species of mammals being rodents (Sheet, 1989).The African giant rat (AGR) also known as giant pouched rat, is by size one of Africa's largest rodents and is arguably becoming Africa's most intriguing rodent because of its scientific attributes such as the detection of landmines (Verhagen *et al*., 2003) and also in the medical diagnosis of pulmonary tuberculosis (Weetjens *et al.,* 2009), disease vectors (Durnez *et al.,* 2008), potential pest species status (Peterson *et al.,* 2006) among many others. Earlier investigations on the AGR were centered on reproduction and sustenance in captivity (Oke and Oke, 1999; Akinloye, 2009) while recent studies have focused on interpreting function from morphology (Olude *et al.,* 2009; Ibe *et al.*, 2014). Information on the CNS however, is sparse with a brain bias (Ibe *et al.,* 2010). The SC anatomy has received much less attention (Vera and Meyer-Siegler, 2003). This study therefore, was undertaken to add to the meagre research data on the gross and histological anatomy of the SC and interpret functional behaviour from the anatomical knowledge of the AGR SC.

MATERIALS AND METHODS

Animals

Ten adult (5 males and 5 females) African giant rats *(C. gambianus)* were obtained from the wild, stabilized in holding cages designed with dark and light compartments to regulate the sleep and wake cycles of the rats. The average body weight of African giant rats was 0.96±0.05kg for males and 0.91± 0.09kg for females.

Animal Handling

Experimental procedures conformed to the rules and guidelines issued by the University of Ibadan, Ibadan, on health guide for the care and Use of Animals in Experiments.
Animals were anaesthetized by chloroform inhalation. Gross morphometric parameters (body weight, head length, trunk length and tail length) were immediately measured before animals were perfused transcardially

with 4% paraformaldehyde in 0.1M phosphate-buffered saline (PBS), pH 7.4 and post-fixed in 10% formalin for 1 week. The SC was exposed by dissections and laminectomy, harvested and measured using metric instruments. Sections from cervical, thoracic, lumbar, sacral and coccygeal segments were then taken for histology at 5 pm, routinely stained with Thionin stain. Slides were viewed under light microscope (Leica Model DME Microscope, Model: 13595XXX, Leica Microsystems) and images captured with Canon© Power shot S70 camera (PC 1087, No. 033102132). Photomicrographs obtained were traced onto a tracing paper using an HB graded pencil.

Statistical Analysis

All data were analyzed and expressed as mean and standard error of mean using Graph pad prism 5. Statistical significance was determined using t-test and linear regression ($P \leq 0.05$).

RESULTS

Morphometry

The mean SC weight and length were recorded as 2.41±0.14g and 15.63±0.32cm respectively. The mean body measurements and SC measurements were greater in the males than in the females but were all statistically insignificant (Table 1). There was positive relationship between the trunk length and the SC length which was significant for females (p =0.0141) but not for males (p= 0.1999). The strength of relationship between the tail length and SC weight were significant in both sexes (p= 0.037 males, p= 0.0044 females) and were both positively correlated (Figure 1). There was positive relationship between the trunk length [TKL] and the SC length [SCL] which was significant for females (p =0.0141) but not for males (p= 0.1999). The strength of relationship between the tail length [TL] and SC weight [SCW] were significant in both sexes (p= 0.037 males, p= 0.0044 females) and were both positively correlated.

Table 1. Body and Spinal Cord measurements of the AGR ***(C. gambianus)***, Mean ±SEM

PARAMETERS	MALE	FEMALE	OVERALL
Body weight (kg)	0.96 ± 0.05	0. 91± 0.091	0.93± 0.05
Trunk length (cm)	24.70±1.30	24.10 ±1.23	24.40 ±0.85
Tail length (cm)	33.80 ±1.56	33.20 ±0.98	33.5± 0.88
Head length (cm)	6.94±0.31	6.78± 0.34	6.86 ±0.22
SC weight (g)	2.50±0.24	2.32± 0.16	2.41± 0.14
SC length (cm)	15.87 0.24	15.40 ± 0.61	15.63± 0.32

P≤0.05

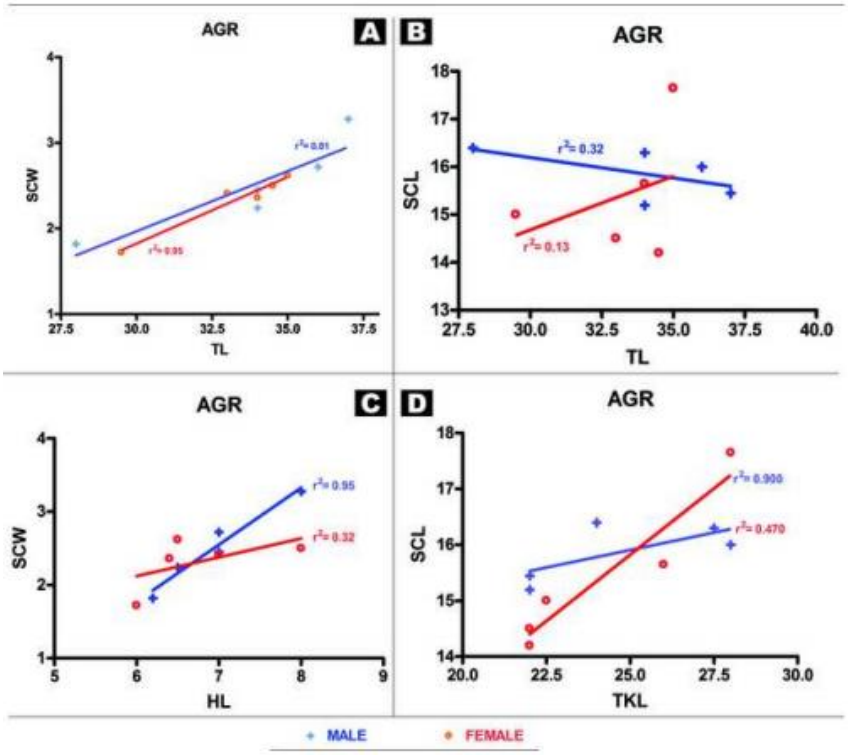

Figure 1: Graphical Representation of the Linear Regression of: (A): Spinal Cord Weight [SCW] versus Tail Length [TL]; (B): Spinal Cord Length [SCL] versus Tail Length [TL]; (C): Spinal Cord Weight [SCW] versus Head Length [HL] and (D): Spinal Cord Length [SCL] versus Trunk Length [TKL]

Figure 2: Dorsal view of the SC of the AGR (arrows) after laminectomy

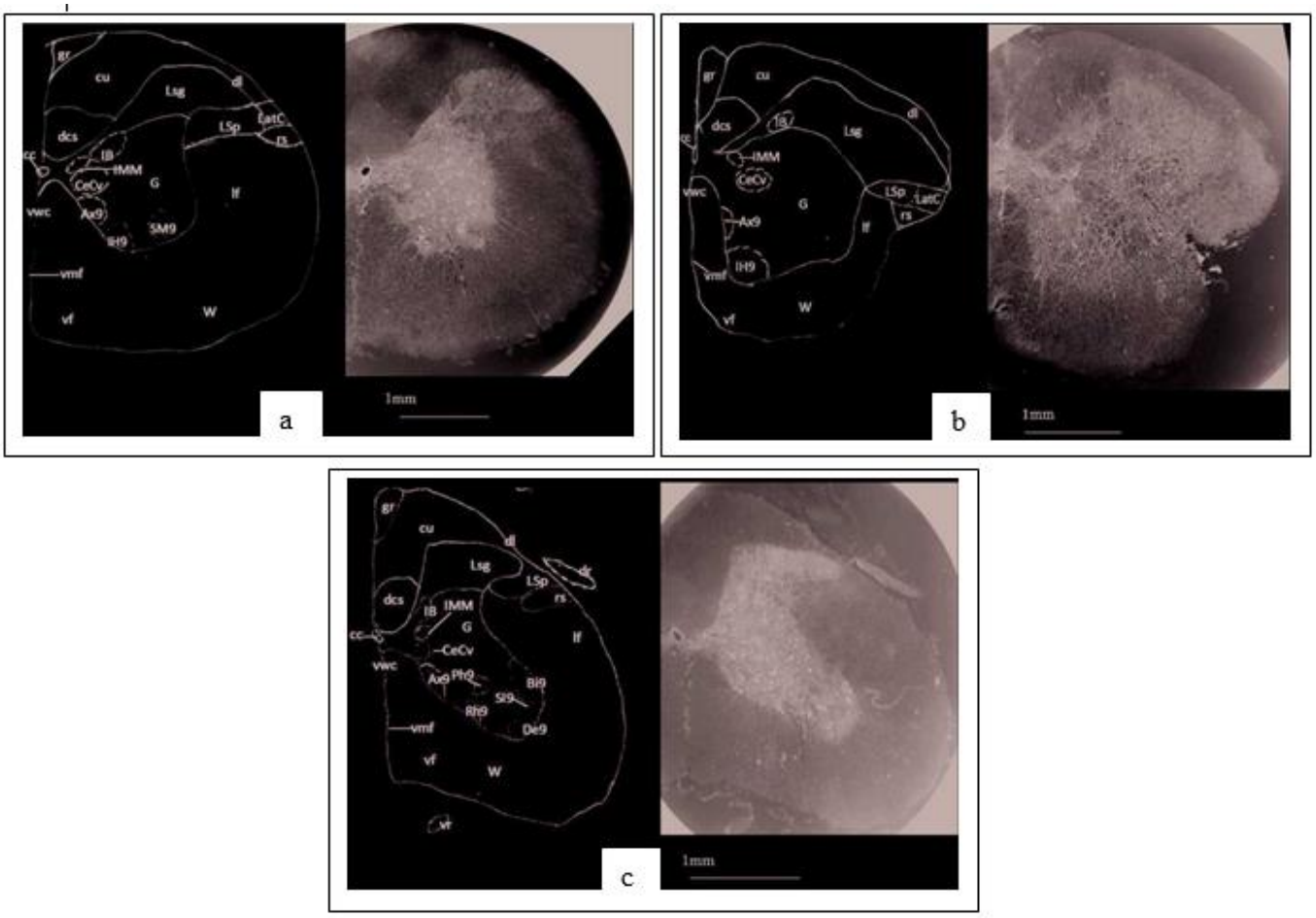

Figure 3. **(a)** The first cervical spinal segments highlighting the nuclei and tracts. *Left panel:* Pencil tracing; *Right panel:* photomicrograph. **(b)** The second cervical spinal segments highlighting the nuclei and tracts. *Left panel*: Pencil tracing; *Right panel:* photomicrograph. **(c)** The fifth cervical spinal segments highlighting the nuclei and tracts. *Left panel:* Pencil tracing; *Right panel:* photomicrograph

Gross morphology

The SC appeared as a whitish cylindrical, tube-like structure situated in the vertebral canal extending from the foramen magnum and continuing as the conus medullaris at vertebra L_4 (n=9) and L_5 (n=1) before terminating as the filum terminale in the coccygeal vertebrae (Figure 2). The SC was covered by the dura matter, which traversed its entire length while the spinal nerves emerged from the SC and exited the vertebral canal through the intervertebral foramina. The cervical enlargement, which contributed to the brachial plexus, spanned from C_4 to T_1 SC segments and was within the nominally corresponding vertebrae (C_4 to T_1) in all animals. The lumbosacral enlargement, which contributed to the lumbosacral plexus that innervates the hind limb, extended from SC segments L_2 to S_3 and was found about the vertebral levels of T_9 to T_{12}.

Histomorphology

Transverse sections of the SC revealed the typical central canal, gray and white matter. The gray matter, stained deep purple with Thionin, had the typical "H" or "butterfly" shape with a reticular formation complex at the lateral area of the dorsal horn. The ventral horn of the gray matter was larger than the

dorsal horn being widest at the lumbosacral segments followed by the cervical segments and then the thoracic; the coccygeal segments being the narrowest.

Shape variations in the central canal were observed across the SC segments. The central canal of the first cervical segment appeared as a vertical slit (Figure 3a), the second to the sixth cervical segments were horizontally oval (Figures 3b-c) while the seventh and the eighth cervical segment appeared circular in shape. In the thoracic segments, the first and second segments were vertical slits while the others were vertically oval in shape (Figures 4a-c). The first and second lumbar segments were circular in shape (Figure 5a) while the other lumbar segments, sacral and coccygeal segments were vertically oval in shape (Figures 5b, 6a-b, 7).

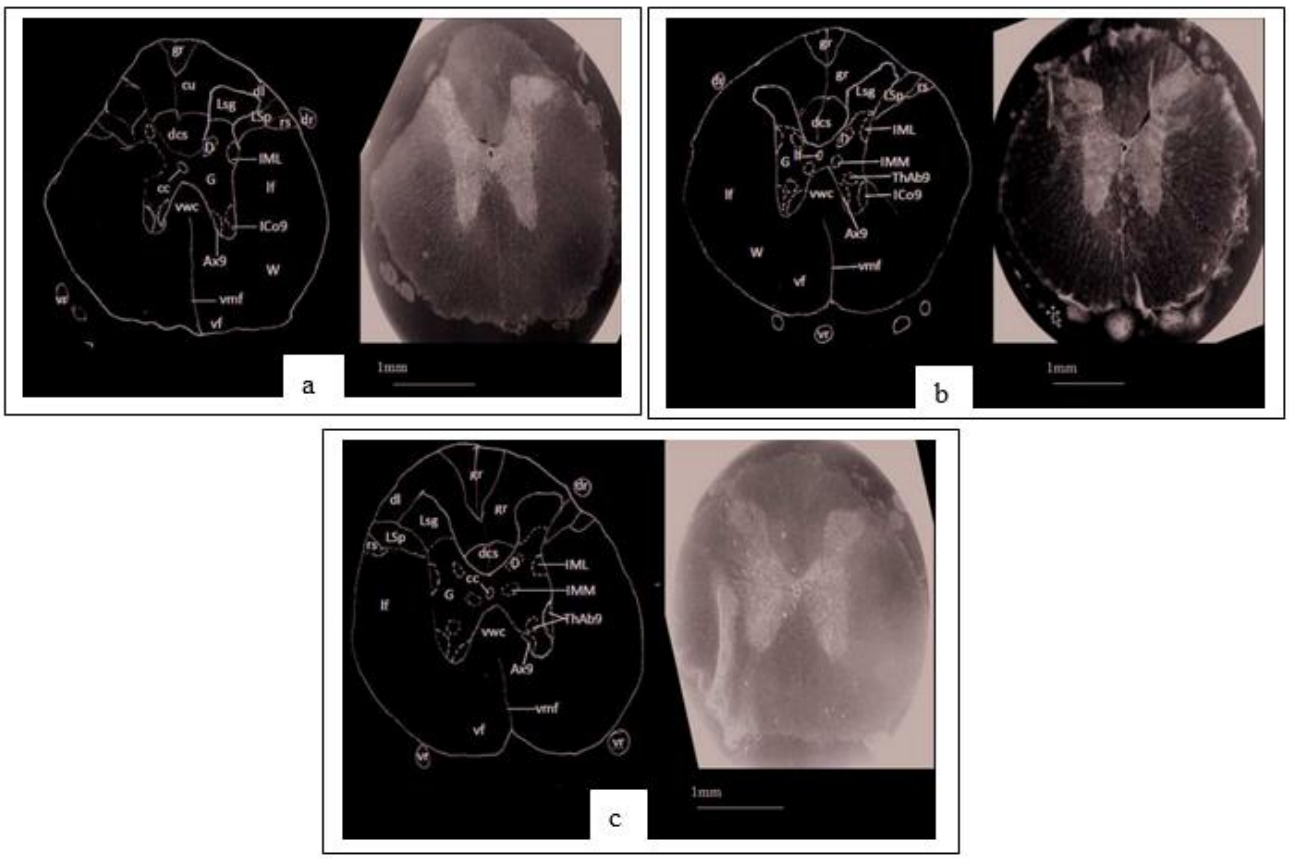

Figure 4. **(a)**The fourth thoracic spinal segments highlighting the nuclei and tracts. *Left panel:* Pencil tracing; *Right panel:* photomicrograph. **(b)**The eighth thoracic spinal segments highlighting the nuclei and tracts. *Left panel:* Pencil tracing; *Right panel:* photomicrograph. **(c)** The twelfth thoracic spinal segments highlighting the nuclei and tracts. *Left panel:* Pencil tracing; *Right panel:* photomicrograph.

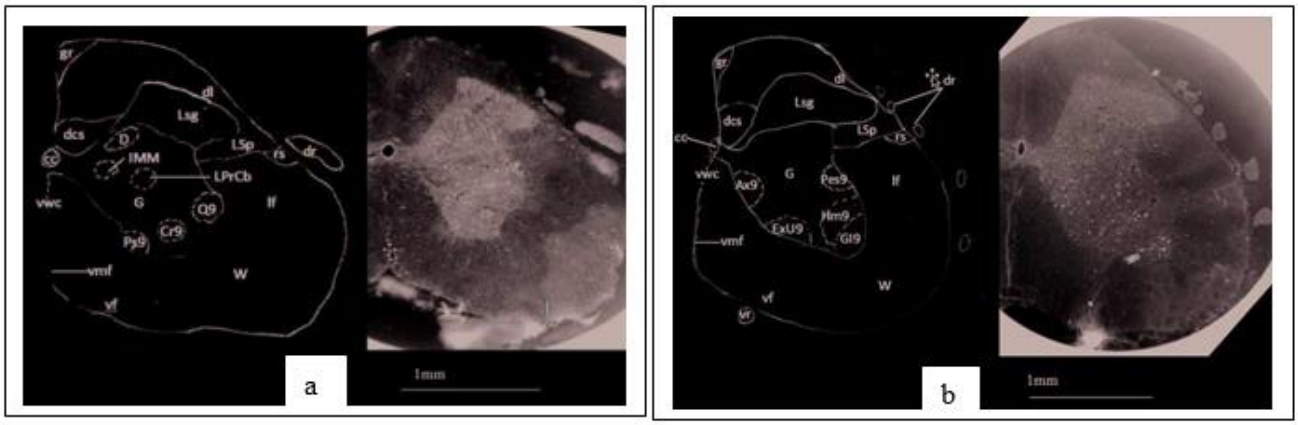

Figure 5. **(a)**The second lumbar spinal segment highlighting the nuclei and tracts. Left *panel:* Pencil tracing; *Right panel:* photomicrograph. **(b)** The sixth lumbar spinal segment highlighting the nuclei and tracts. *Left panel:* Pencil tracing; *Right panel:* photomicrograph.

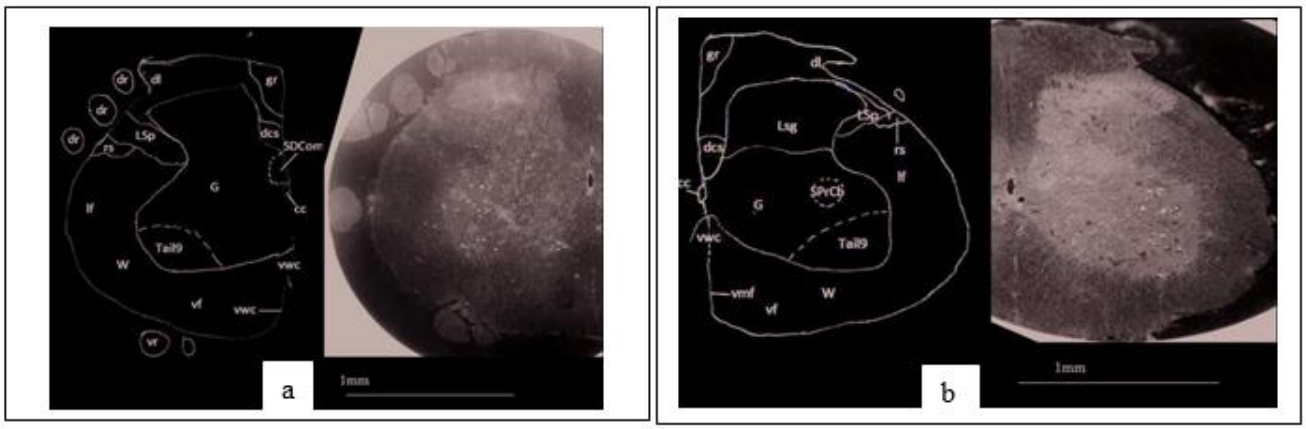

Figure 6. **(a)**The second sacral spinal segment highlighting the nuclei and tracts. *Left panel:* Pencil tracing; *Right panel:* photomicrograph. **(b)** The fourth sacral spinal segment highlighting the nuclei and tracts. *Left panel:* Pencil tracing; *Right panel:* photomicrograph.

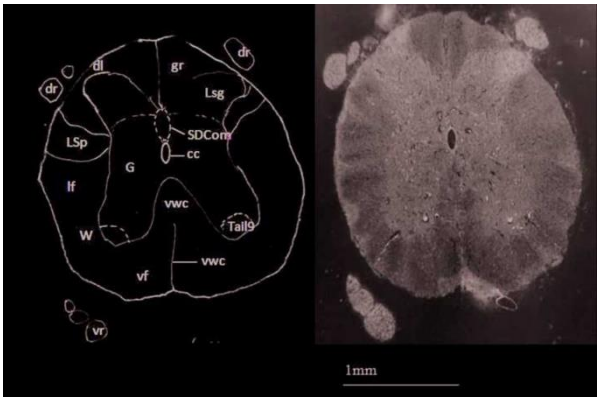

Figure7. The second coccygeal spinal segment highlighting the nuclei and tracts. *Left panel:* Pencil tracing; *Right panel:* photomicrograph.

Table 2. Abbreviations and definitions of structures of the SC traced

Abbreviation	Definition
Ax9	Axial muscle motor neuron of lamina 9
Bi9	Bicep motor neuron of lamina 9
Cc	Central canal
CeCv	Central cervical nucleus
Cr9	Cremaster motor neuron of lamina 9
Cu	Cuneate fasciculus
D	Dorsal nucleus (Clarke)
Dcs	Dorsal corticospinal tract
De9	Deltoid motoneuron of lamina 9
Dl	Dorsolateral fasciculus
Dr	Dorsal root
ExU9	External urethral sphincter motor neuron of lamina 9
G	Gray matter
GL9	Gluteal motor neuron of lamina 9
Gr	Gracile fasciculus
Hm9	Hamstring motor neuron of lamina 9
IB	Internal basilar nucleus
ICo9	Intercostals muscle motor neuron of lamina 9
IH9	Infrahyoid muscle motor neuron of lamina 9
IML	Intermediolateral column
IMM	Intermediomedial column
Lsg	Laminae of spinal gray matter
LatC	Lateral cervical nucleus
Lf	Lateral funiculus
LPrCb	Lumbar paracerebellar nucleus
Lsp	Lateral spinal nucleus
Pes9	Pesmotor neuron of lamina 9
Ph9	Phrenic motor neuron of lamina 9
Ps9	Psoas motor neuron of lamina 9
Q9	Quadriceps motor neuron of lamina 9
Rh9	Rhomboid muscle motor neuron of lamina 9
Rs	Rubrospinal tract
SDCom	Sacral dorsal commissural nucleus
SI9	Supraspinatus and infraspinatus motor neurons of lamina 9
SM9	Sternomastoid motor neurons of lamina 9
SPrCb	Sacral precerebellar nucleus
Tail9	Tail muscle motor neuron of lamina 9
ThAb9	Thoracoabdominal wall muscle motor neuron of lamina 9
Vf	Ventral funiculus
Vmf	Ventral median fissure
Vr	Ventral root
Vwc	Ventral white commissure
W	White matter

Nuclear tracing

Forty-three (43) anatomical structures including nuclear aggregations and tracts were traced (Table 2). It describes the abbreviations and definitions of structures of the SC traced out, which represents the list of structures in the histological pictures.Tracts of the dorsal, ventral and lateral funiculi were observed in the white matter. The dorsal funiculus consisted of the gracilis fasciculus, cuneate fasciculus and also a descending tract - the dorsal corticospinal tract. The gracilis fasciculus extended all through the SC segments, the cuneate fasciculus were indistinct at the caudal spinal segments (lumbar to the coccygeal segments) while the dorsal corticospinal tracts ran through the cervical to the sacral spinal segments and became indistinct at the coccygeal spinal segments (Figures 3 - 7). The lateral funiculus also consisted of a descending tract - rubrospinal tract (Figures 3b, 3c). Eight nuclei namely central cervical, dorsal (Clarke's), internal basilar, lateral cervical, lumbar paracerebellar,

lateral spinal, sacral dorsal commissural and sacral precerebellar nuclei were identified and traced (Figures 3a-b, 4a-c, 5a-b, 6a-b, 7).

The SC appeared as a whitish cylindrical, tube-like structure situated in the vertebral canal extending from the foramen magnum and continuing as the conus medullaris at vertebra L_4 (n=9) and L_5 (n=1) before terminating as the filum terminale in the coccygeal vertebrae (Figure 2). The SC was covered by the dura matter, which traversed its entire length while the spinal nerves emerged from the SC and exited the vertebral canal through the intervertebral foramina. The cervical enlargement, which contributed to the brachial plexus, spanned from C_4 to T_1 SC segments and was within the nominally corresponding vertebrae (C_4 to T_1) in all animals. The lumbosacral enlargement, which contributed to the lumbosacral plexus that innervates the hind limb, extended from SC segments L_2 to S_3 and was found about the vertebral levels of T_9 to T_{12}.

DISCUSSION

The basic features of the AGR SC were typical of rodents (Hebel and Stromberg, 1976; Bjugn *et al.*, 1989). The SC accounts for about 0.26% of body weight of the AGR. It is smaller than the rabbit which weighs 5-7g (about 0.5% body weight) (Farag *et al.*, 2012) but greater than that of the horse which weighed 250-300g (about 0.06% body weight) (Nickel *et al.*, 2004).

Bjugn *et al.* (1989) reported the SC length of mice as 4.4cm (55.7% body length) which is smaller than that of the AGR 15.63cm (64.2% body length). SC length has also been described in several animals amongst which are: 34.7 cm in Wistar rats (70.4% body length) (Hebel and Stromberg, 1976; Aguh *et al.*, 2013); 34.7 cm in rabbits (99.1% body length) (Farag *et al.*, 2012); 53.8 cm in goats (61.6% body length) (Kahvecioglu *et al*., 1995); 167.2 cm in horses (68.6% body length) (Sadullah *et al.*, 2013); 106.8 cm in donkeys (53.4% body length) (Ocal and Haziroglu, 1988); 61.5 cm in brockets (64.7% body length) (Lima *etal.*, 2010).

The positive linear relationship ($p \leq 0.05$) between the tail length and SC weight of males and females established that the variability observed in the SC weight may be explained by the tail length in both sexes, indicating that the bulk of the filum terminale contributed significantly to the weight of the SC. This was further substantiated by a relatively higher tail: body length ratio of 1.07 in the AGR compared to 1.00 in mice, (Brian and William, 2000) 0.47 in fox squirrel, 0.87 in red squirrel, 0.50 in California ground squirrel (Virginia, 2008) and 0.43 in greater cane rats (Fitzinger, 1995).

The anatomical location of the cervical and lumbosacral enlargements - between C_4 and T_1 and between L_2 and S_3 respectively - were typical with laboratory rats (Bjugn *et al.*, 1989). These anatomical positions seem characteristic of rodents as several authors have reported slightly different positions in most domestic animals. The cervical enlargement is found between C_7 and C_8 in pigs (Dellmann and McClure, 1975), C_5 and T_1 in the rabbits (Farag *et al.*, 2012), C_6 and T_1 in the dogs (Miller *et al.*, 1964), C_6 and T_2 in buffalo and camels (Abu-zaid, 1982; Mansour, 1983) and C_5 - T_2 in Indian sheep and donkeys (Mansour, 1980; Rao, 1990). While the lumbosacral enlargement lies between L_2 and S_3 spinal segments in AGR, the following positions have been documented in domestic animals: between L_2 and S_1 in the donkeys (Mansour, 1980), L_6 and S_1 in camels (Mansour, 1983), L_4 and S_3 in rabbits (Farag *et al.*, 2012), the last three lumbar and first two sacral in buffalo (Abu-zaid, 1982), L_4 and S_1 in sheep (Rao, 1990), L_6 and L_7 in the pigs and L_4 and S_1 in dogs (Dellmann and McClure, 1975).

The enlargements at the cervical and lumbosacral segments provide innervations to the fore and hind limbs respectively; contributing to the brachial and lumbosacral plexuses (Bjugn *et al.*, 1989; Rahmanifar *et al.*, 2008). Worthy of note is that the extents of the enlargements, which began earlier in spinal segments, are more extensive in rodents and may characteristically add to limb efficiency than other mammals. Thus; regional anaesthesia for surgical maneuvers in the AGR can be readily achieved based on the knowledge of the extent and anatomical locations of these enlargements (Jonathan and Gerbrand, 2005).

The ventral horn of gray matter coordinates the motor neuron; this explains its relative bigger size compared to the dorsal horn (Gruener and Biller, 2008). The ventral horn appears wider at the cervical region and the lumbosacral region than other segments. This corresponds to the cervical and lumbosacral enlargements (Gruener and Biller, 2008). The AGR uses its tail to dig, defend itself and has been reported to stand on it (personal observation). It is also known to burrow more with the forelimbs and shows high locomotor dexterity (Ajayi, 1977). This probably accounts for the ventral horns of cervical and lumbosacral regions being more developed than other segments.

The dorsal (Clarke's) and central cervical nuclei are particularly essential for the coordination of movement and balance (Gruener and Biller, 2008). These nuclei appeared well developed in the AGR and might explain the dexterous limb movements and balance shown by the AGR. The AGR also has been documented as a fast running, burrowing and shovelling rodent (Olude *et al*, 2010). The lateral cervical, lateral spinal, sacral dorsal commissural nuclei are responsible for nociception (Rea, 2009) and are therefore important for their defence. The internal basilar nuclei are responsible for voluntary motor control and procedural learning relating to routine behaviours and habits (Weyhenmeyer and Gallman,

2007). The AGR has been shown to adapt well to training to detect landmines and diagnose Tuberculosis (Weetjens, 2010). The sacral pre-cerebellar nucleus also relays unconscious proprioception motor (lower extremities and trunk) feedback to the cerebellum.

Shape variation of central canal and tracts observed in the white matter. The central canal of each segment and the tracts observed in the white matter of the SC of the AGR were similar to that of rats (Watson *et al*., 2008).

Conclusion

This study documents baseline data on the morphometric and morphologic features of the SC of AGR, thus contributing to the knowledge of anatomy of the AGR and providing useful information on its regional anaesthesia. It also strengthens the drive in adopting the AGR as a convenient indigenous research model and could assist further researches especially in the study of SC diseases/injuries within the African context.

Acknowledgement

Special thanks to Mr. Anise for helping with the preparation of the histological slides.

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Niger. J. Physiol. Sci. 30(2015) 033-037
www.njps.com.ng

Niger. J. Physiol. Sci.

Anti-Gastric Ulcer Effect of Betulinic Acid in Male Albino Rats

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Summary: Betulinic acid (BA) is a lupane-type triterpene that has been identified and isolated from various plant species used in ethnomedicine in various cultures across the world. This study was undertaken to elucidate the mechanisms underlying the anti-ulcer effect of Betulinic acid. The effect of BA on indomethacin-induced ulcer, gastric mucus secretion, gastric mucus cells count, basal and histamine-induced gastric acid secretion and levels of malondialdehyde formation were studied using dose of 0.5, 1.5, and 3.0 mg/kg. The results showed that BA reduced indomethacin-induced ulceration significantly and significantly increased ($p < 0.05$) gastric mucus secretion in the 1.5 mg/kg and 3.0 mg/kg BA treated rats compared to the control rats. There was a significant increase ($p < 0.05$) in the mucus cells count in all the treated groups which is in a dose- dependent manner compared to the control group. There was significant decrease ($p < 0.05$) in gastric acid secretion in each of the BA treated groups compared to the control. Malondialdehyde concentration significantly decrease ($P < 0.05$) in all the treated groups compared to the control. The anti-gastric ulcer effect of BA may be mediated via decreasing gastric acid secretion, increasing gastric mucus secretions, increasing the number of gastric mucus cells and also by reducing the level of MDA concentration.

Keywords*:* Betulinic acid, Gastric acid, Mucus secretion, Malondialdehyde.

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Manuscript Accepted: September, 2015

INTRODUCTION

Natural products have been used for combating human diseases for thousands of years since they exhibit a wide range of biological properties that can be exploited for medical application such as pentacyclic triterpenes that have been investigated for anti-inflammatory properties (Safayhi and Sailer, 1997). Among these are the lupane-type triterpenes betulinic acid and betulin tested in a number of in vitro and in vivo model systems. Betulinic acid has been isolated from various parts of plants species used in ethnomedicine (Takeoka *et al*, 2000) and in particular, those reported to have anti-secretory and anti-ulcer properties (Adesanwo *et al*, 2003; Pisha *et al*,1995). Animal studies reveal that BA lack toxicity even at high concentrations (Pisha *et al*, 1995). Other reported biological effects of betulinic acid include anti-spasmodic, anti-retroviral (Mayaux *et al*, 1994; Evers *et al*, 1996), anti-tumor (Pisha *et al*, 1995; Schmidt *et al*, 2007) and antimicrobial (Adekunle *et al*, 2003; Setzer *et al*, 2000). The imbalance between the gastroduodenal mucosal defensive factors such as bicarbonate, mucus and aggressive factors such as acid, pepsin (Sostres and Lanas, 2011) has been associated with gastric ulcer and many of the anti-ulcer drugs in use have been found to have adverse effects and recurrent infection after a few weeks (Chan and Leung, 2002). There is dearth of information on the mechanisms of the anti-ulcer effect of betulinic acid. In this study, the effect of betulinic acid on indomethacin-induced ulcer, gastric mucus secretion, gastric mucus cell count, basal and histamine stimulated gastric acid secretion and malondialdehyde concentration were evaluated as means of elucidating the mechanisms of the anti-ulcer effects.

MATERIALS AND METHODS

Animals

Adult male albino rats of Wistar strains weighting 210-240 grams and divided into five studies groups of thirty-two animals each were used for this study. The animals were purchased from the Central Animal House, College of Medicine, University of Ibadan, Nigeria and were kept four per cage in a clean, well-ventilated room maintained under standard condition (12 hours light and 12 hours darkness). The animals were allowed to acclimatize before the studies commences. They were fed with commercial rat chow obtained from Ladokun Livestock Feeds Limited, Ibadan Oyo State Nigeria and water was provided *ad libitum*. The studies were conducted in accordance with the Organization for Economic Development (OECD) guidelines on good laboratory practice (OECD, 2001).

Chemicals

Betulinic acid (Aldrich Sigma) was dissolved in Dimethyl sulphuroxide (DMSO), Sodium acetate (May & Baker, England), Indomethacin (Merck, Sharp & Dohme, Canada) was dissolved in distilled water

with a pinch of sodium carbonate (Na2CO3) added. All other chemicals and reagents were of analytical grade and were products of Aldrich Sigma Chemical limited, Poole, England or Sigma Chemical Company, St Louis, MO, USA.

Experimental Design

The animals for the studies were further divided into five groups of eight animals each. Group 1 is the control and was treated with DMSO in normal saline. Groups 2, 3, 4 and 5 were treated with 0.5mg/kg, 1.5mg/kg, and 3.0mg/kg of BA dissolved with DMSO respectively for seven (7) days. The studies were on effects of betulinic acid on indomethacin-induced ulcer, gastric mucus 4 secretion, gastric mucus cell count, basal and histamine-stimulated gastric acid secretion and possible anti-oxidant effect of betulinic acid.

Effect of betulinic acid on indomethacin induced gastric ulceration

A total of thirty-two (32) rats were used for this study divided into four groups, each with eight rats. Group 1 was the control, treated with DMSO in normal saline. Groups 2, 3 and 4 were treated with BA doses of 0.5mg/kg, 1.5 mg/kg and 3.0 mg/kg orally respectively (Durst *et al*, 2002). The animals were fasted for 24 hours only but allowed free access to water. The method of indomethacin-induced gastric ulceration adopted was that described in previous works (Njar *et al*, 1995; Oluwole *et al*, 2008). One hour after the administration of betulinic acid and DMSO in normal saline, indomethacin at 40mg/kg BW (Merck, Sharp & Dohme, Canada) was administered subcutaneously to all the animals in all the groups. After 4 hours, the animals were sacrificed by cervical dislocation. Their stomachs were removed, opened by cutting along the whole length of the greater curvature, turned inside out and then pinned to a cork mat. This was moistened with normal saline to prevent autolysis. The method used for assessment of the degree of gastric ulceration was that of Alphin and Ward (1967) as modified by Elegbe and Bamgbose (1976). Macroscopic examinations of the washed stomachs were carried out with a magnifying hand lens.

$$Mean\ Ulcer\ Score = \frac{\text{Total Ulcer Score}}{n}$$

Where n = number of rats

Effect of Betulinic acid on Gastric Mucus Secretion

Each glandular portion of the stomach in sacrificed rats was opened along the lesser curvature, everted and soaked for two hours in 0.1% Alcian blue dissolved in 0.16M sucrose buffered with 5 0.05M sodium acetate, adjusted to pH 5.8 with hydrochloric acid. Uncomplexed dye was removed with two successive washes at 15 and 45 minutes in 0.25M sucrose. Dye complexed with mucus was diluted by immersion in 10ml aliquots of 0.5M Magnesium Chloride for 2 hours. The resulting blue solutions were shaken briefly with equal volume of diethyl ether and absorbance of the aqueous phase was measured at 605nm using spectrophotometer (Corney *et al*., 1974). The absorbance of each solution was used to calculate the various concentrations of dye. The weight of dye (expressed in mg) was deduced using a standard curve. The weight of the dye was expressed over the weight of the stomach to give the weight of the mucus.

Thus, gastric mucus secretion (mg/g tissue)

$$= \frac{\text{Weight of dye (mg)}}{\text{Weight of stomach (g)}}$$

Effect of Betulinic acid on Gastric Mucus Cell Count

The rats were sacrificed by cervical dislocation, the stomachs removed and weighed. The glandular portion of each stomach was opened along the lesser curvature and histological slides prepared using Haematoxylin and Eosin as stain. Gastric mucus cell count was done by counting the number of gastric mucus cells that stain with Haematoxylin and Eosin under calibrated light microscope. These are indicated as blue patches. The gastric mucus cells were counted in five randomly selected area of the gastric mucosal tissue. Five cubic boxes each with an area of $1mm^2$ were assessed. This method is an improvement over the earlier described approach for counting by Li *et al* (2002).

Effect of Betulinic acid on basal and histamine-stimulated gastric acid secretion.

Basal Secretion

Each rat was anaesthetized with urethane according to their body weights (0.6 ml/100g body weight), abdomen dissected open and stomach exposed. The femoral vein was exposed by blunt dissection and later cannulated for intravenous administration of histamine. Esophageal cannula was passed down into the stomach. This was used to perfuse the stomach with normal saline. The perfusion was regulated at a rate of 1.0 ml/min using a modified Langardoff apparatus and 10ml of gastric effluent was collected from the stomach cannula. This perfusion preparation is the modified method of Ghosh and Schild (1958).

Histamine-stimulated Secretion

A dose of 0.1mg/g body weight histamine was injected intravenously (i.v) through the femoral vein into the rats. Four samples of gastric contents were collected at 10 minutes intervals. The total acidity of the gastric contents was determined by using 0.0025N NaOH with an initial drop of 1% phenolphthalein added and titrated to end-point.

Effect of Betulinic acid on malonialdehyde (MDA) concentration.

Lipid peroxidation which is also a marker of oxidation was assessed by measuring Thiobarbituric acid

reactive substances (TBARS) produced according to the method of Gutteridge and Wilkins (1982). This method is based on the reaction between 2-thiobarbituric acid (TBA) and malonialdehyde (MDA) which is an end-product of lipid peroxides during lipid peroxidation. On heating in acidic solution, a pink coloured complex was produced that absorbs maximally at 532 nm on the spectrophotometer. 0.1ml of the test sample was mixed with 0.5ml of 10% TCA and 0.5ml of 75% TBA was then added. The mixture was placed in water bath at 80°C for 45 minutes. The absorbance of the resulting pink colour solution was measured against a reference blank of distilled water at 532nm. The test sample was calibrated using the MDA as standard and the result was expressed as the amount of free MDA produced or MDA quantified by using the molar extinction coefficient, C of 1.56×10^5 $M^{-1}cm^{-1}$ according to the expression of Adam Vizi and Seregi (1982).

$$\text{MDA (units/g tissue)} = \frac{\text{Absorbance of sample}}{\text{Molar extinction coefficient,C}}$$

Statistical Analysis

Data are expressed as the mean ± Standard Error of Mean (SEM). Data were analysed using one-way Analysis of Variance (ANOVA) and Graph Pad prism 4. The differences in mean were considered significant at $p \leq 0.05$.

RESULTS

Effect of betulinic acid (BA) on indomethacin-induced gastric ulceration

The mean ulcer score decreases with increasing doses of BA when compared with the control (Table 1). Each administered dose of 1.5 mg/kg BA (0.8± 0.10) and 3.0 mg/kg BA (0.3± 0.09) compared to the control rats (7.0± 0.27) showed significant reduction in mean ulcer score ($p < 0.05$). However, the difference in mean ulcer score for 0.5 mg/kg dose of BA (6.3 ± 0.42) and the control (7.0± 0.27) was not significant ($p > 0.05$). Also it was noticed that, as the dose of betulinic acid increases in the treated animals, percentage inhibition tends toward 100%. These showed a lower incidence of ulceration. The inhibition of gastric ulceration by betulinic acid is therefore dose-dependent.

Gastric Mucus Secretion

The results in figure 1 showed the gastric mucus secretion in the 1.5 mg/kg (4.9 ± 0.22) and 3.0 mg/kg BA treated rats (5.2 ± 0.09) being significantly different ($p < 0.05$) compared to the control rats (4.4± 0.20). Whereas, no significant difference ($p>0.05$) was observed in mucus secretion in the 0.5 mg/kg BA treated rats (4.6 ± 0.16) compared to the control rats (4.4 ± 0.20).

Table 1: The effect of betulinic acid (BA) on indomethacin induced gastric ulceration

Treatment	Mean Ulcer score [a]	Inhibition of ulceration (%)[b]
Control	7.0 ± 0.27	-
BA (0.5mgkg^{-1})	6.3 ± 0.42	10.7
BA (1.5mgkg^{-1})	0.8 ± 0.10*	89.3*
BA (3.0mgkg^{-1})	0.3 ± 0.09*	96.4*

[a]Values are Mean ± SEM for 8 animals per group. [b]Percentage inhibition as described by Raji et al, 2000. *$p < 0.05$ significantly lower compared with control.

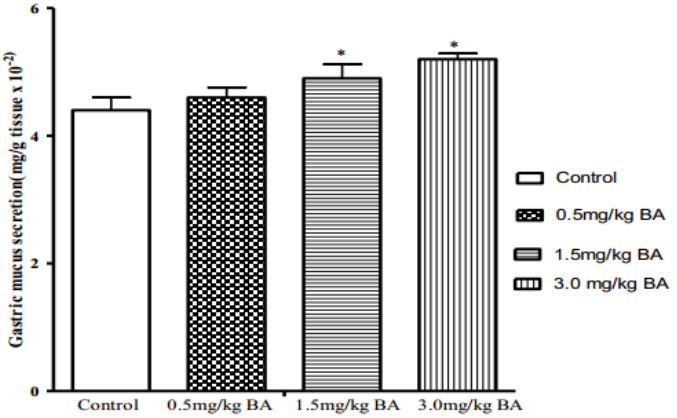

Figure 1: Effect of Betulinic Acid on Gastric Mucus Secretion

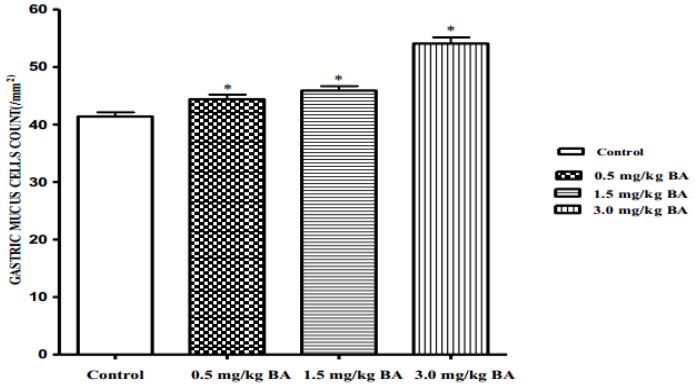

Figure 2. Effect of Betulinic Acid on Gastric Mucus cells count (mm^2)

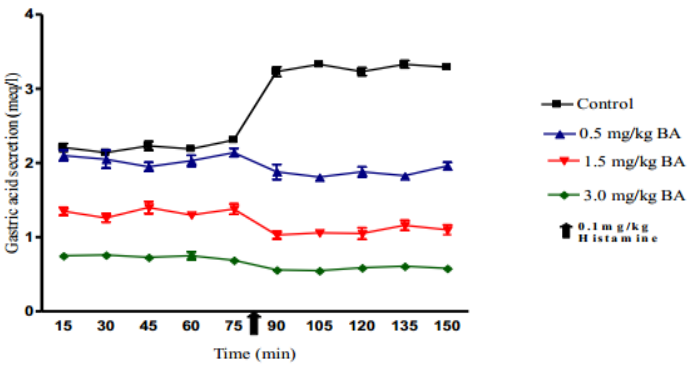

Figure 3: Effect of Betulinic acid on Gastric acid secretion

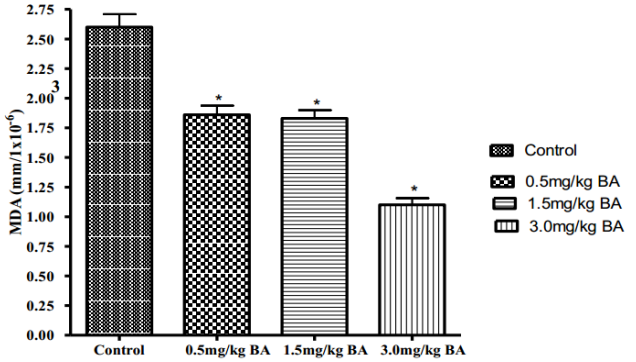

Figure 4: Effect of Betulinic acid on Malonialdehyde (MDA) Concentration

Gastric acid secretion

From fig. 3, there were decreases in basal gastric acid secretion between 0-50 minutes in all the treated rat groups. After the 50th minutes, histamine a known secretagogue was administered intravenously through the femoral vein. After 10 minutes there was a sharp increase in gastric acid secretion in the control rats, while the BA treated groups showed a fall in gastric acid secretion that were all significantly different ($P < 0.05$), compared to those of the control group. Analysis of the 50th - 70th minutes interval also showed a significant decrease in the pretreated rats compared to the control rats.

Malonialdehyde (MDA) Concentration

The results obtained shows that there is a significant decrease ($P < 0.05$) in MDA concentration between the treated rats groups; 0.5 mg/kg BA (1.86 ± 0.076), 1.5 mg/kg BA (1.83 ± 0.069) and 3.0 mg/kg BA (1.10 ± 0.056) compared to the controls group (2.60 ± 0.110).

DISCUSSION

The results obtained showed that BA has anti-ulcer effect (Table 1) and a stimulatory effect on gastric mucus secretion (Fig. 1) and gastric mucus cells (Fig. 2). These effects are similar to that of known drugs such as sucralfate and misoprostol reported to increase gastric mucus production in vivo through increasing inositol triphosphate (IP_3) content by activating phospholipase C (Slomiany et al, 1991). The resulting IP_3 elicited Ca^{2+} mobilization is then involved in the stimulatory effect of sucralfate.

BA in the present study significantly suppressed gastric acid secretion when compared with the control group ($p < 0.05$). The result (Fig. 3) clearly suggests that there is a good relationship between reduction of gastric acid secretion by BA and its anti-ulcer effect. Several workers have reported that prostaglandins of the A, E and F types are potent anti-secretory agents. BA being a triterpene exhibits similar effect to that of related triterpenoid, sodium carbenoxolone which has been reported to protect gastric mucosa from acid effect by selectively inhibiting prostaglandin F_2 (Aguwa and Okunji, 1986). Also BA had been reported to inhibit prostaglandin synthesis in vitro (Carter, 1980). BA shares the same five membered ring structures of H2 receptor antagonists with Cimetidine, Ranitidine and Famotidine that are known to relieve or heal peptic ulcers (Yamada, 1996). In this regard, BA may be acting as antihistaminic agent while the stimulation of gastric acid secretion might be inhibited competitively by selective H_2 -receptor antagonists (Hirschowitz and Molina, 1983).

The pretreatment of the animals with BA significantly decrease ($p < 0.05$) the MDA concentration compared to the control (Fig. 4). This reduction of MDA concentration may be due to the ability of BA to increase anti-oxidant activity. This supports other studies that had demonstrated a reduction in lipid peroxidation of the gastric mucosa (DelaLastra and Motilva, 1999). Other studies have also shown that the protective activity of gastric mucus is due to the anti-oxidant activity conferred on it by its rich glycoprotein content (Oluwole and Saka, 2001). The results of these studies showed that the anti-gastric ulcer effect of BA may be mediated via decreasing gastric acid secretion, increasing gastric mucus secretions, increasing the number of gastric mucus cells and also by reducing the level of MDA concentration. Thus BA could be used as a promising drug against peptic ulcer. More studies are needed to shed more light in all these areas.

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Antinociceptive and anti-arthritic properties of hydroethanolic leaf extract of *Clausena anisata* (Willd.) Hook. f. ex Benth (Rutaceae) in Rodents: possible mechanism of actions

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Summary: The leaves of Clausena anisata (Willd.) Hook. f. ex Benth (Rutaceae) is used in Traditional African medicine for the treatment of various ailments including arthritis. The present study sought to investigate the antinociceptive and anti-arthritic properties of hydroethanolic leaf extract of Clausena anisata (HeCA). HeCA (100, 200 or 400 mg/kg, p.o.) was administered 1 h before intraplantar injection of formalin 1%v/v in saline to evaluate antinociceptive effect. Moreover, its possible mechanism of antinociceptive action was investigated through pretreatment of mice with antagonists of receptors implicated in nociception. Anti¬inflammatory effect of the extract was investigated using the carrageenan-induced paw oedema and complete Freund's adjuvant (CFA)-induced arthritis models in rats. HeCA (400 mg/kg) treatment significantly reduced the duration of paw licking/biting during both in the early (42.12%) and late (75.79%) phases of formalin-induced nociception. However, the antinociceptive effect elicited by HeCA was reverse by pretreatment of mice with naloxone, prazosin, yohimbine, ketanserin, L-arginine, and parachlorophenylalanine (PCPA). HeCA produced dose-dependent and time course decrease in carrageenan-induced paw oedema. Pre- and post-treatment of rats with HeCA ameliorated CFA-induced arthritis evidenced in the significant decrease in arthritic index comparatively similar to the effect of celecoxib. CFA- induced oxidative and nitrosative stress were attenuated by subchronic treatment with HeCA. Findings from this study shows that C. anisata possesses antinociceptive activity through possible interaction with opioidergic, noradrenergic, L-arginine-nitric oxide and serotonergic pathways as well as anti-arthritic property which could be attributed to its ability to prevent the release of inflammatory mediators and oxidative stress.

Keywords*:* Complete Freund's adjuvant; L-arginine-nitric oxide; nociception; antioxidant; rheumatoid arthritis; serotonergic.

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Manuscript Accepted: September, 2015

INTRODUCTION

Clausena anisata (Wild) Hook .F. ex Benth (Rutaceae) is a tropical shrub or tree up to 10 meters high growing in and on evergreen forests. It is commonly known as "mbiet ekpene'' (Ibibios, Niger Delta region) and "Agbasa'' (Yoruba, Southwest) of Nigeria. The plant is traditionally used as effective remedies for worm infections, respiratory ailments, hypertension, malaria, fever, rheumatism, and other inflammatory conditions, headaches, pains, toothaches, convulsions and others (Hutchings et al., 1996). A mixture of Clausena anisata, Afraegle paniculata and Azadirachtha indica is taken against gut disturbance and a concoction of the latter called "Agbo" (Yoruba, Southwest, Nigeria) is used as an antimalarial medicine. The Ibibios use the plant to treat measles (Ajibesin et al., 2005), malaria, pains and inflammations. In the Temeke district (Tanzania) traditional healers employ C. anisata against epilepsy and as an anticonvulsant. All members of the genus contain different bioactive compounds from different chemical classes (Arbab et al., 2012). The plant has been reported to contain coumarins, limonoids, carbazole alkaloids, monoterpenoids furanocoumarin lactones and essential oils (Usman et al., 2010). Reports of antimicrobial (Gundidza et al., 1994), antibacterial (Senthikumar and Venkatesalu, 2009), antidiabetic (Ojewole, 2002), anticonvulsant (Makanju, 1983), and antineoplastic (Ito et al., 2009) have been published. Thus, this study was carried out to investigate the antinociceptive and anti-arthritic activities of the hydroethanolic leaf extract of Clausena anisata in rodents

MATERIALS AND METHODS

Plant material

The fresh leaves of Clausena anisata were collected from Abatadu Village, Ikire, Osun state. Botanical Identification and authentication was done by Mr. T.K Odewo a forestry expert of the herbarium section, Department of Botany, University of Lagos, Akoka, Lagos State, Nigeria. A voucher specimen number

LUH 5703 was deposited in the herbarium for reference.

Preparation of the extract: The leaves of Clausena anisata were washed and air dried. Dried leaves were then grinded and weighed. Eight hundred and twenty grams of the powdered leaves were soaked with 2 L of 70% ethanol in distilled water for 72 h after which the preparation was decanted using the Whatman No.1 filter paper (size 9 cm). The filtrate was concentrated on Rotavapor and the concentrate was oven dried at 40°C. The percentage yield was 2.29% of dark-brownish extract.

Preliminary phytochemical analysis: The preliminary qualitative phytochemical analysis was done using the method of Harbone (1973). Quantitative estimations of total phenolic, tannins, alkaloid, saponins and cardiac glycosides contents were carried out as described by El-Olemy et al. (1994) and Senguttuvan et al. (2014).

Experiment animals: Male Sprague-Dawley rats (90-110 g) and albino mice (15-19 g) were obtained from the laboratory Animal Centre, College of Medicine, University of Lagos. The animals were maintained under standard environmental conditions (12 h/12 h light/dark cycle) and had free access to standard rodent pellet diet (Livestock Feed Plc, Lagos, Nigeria) and water. The animals were acclimatized in the laboratory conditions for a week before the commencement of the study. The experimental procedures adopted in this study were in strict compliance with the ethical standards of the Research Grant and Animal Experimentation Committee of the College of Medicine, University of Lagos, Nigeria and in accordance with the United States National Institutes of Health Guidelines for Care and Use of Laboratory Animals in Biomedical Research (1985).

Drugs and chemicals: Complete Freund's adjuvant (CFA), ethanol, prazosin, formalin, carrageenan, ketanserin, yohimbine, L-arginine, reserpine, naloxone, glibenclamide (Sigma Aldrich, Louis, MO, USA), celecoxib (Pfizer manufacturing Deutschland GmbH, Illertissen, German), morphine (Martindale Pharma, Essex, United Kingdom).

Acute toxicity test: Female albino mice were fasted for 12 h before the study but had access to water. Five groups of mice (n = 5) received; normal saline 10 ml/kg, C. anisata (250, 500, 1000 and 5000 mg/kg,p.o.). They were observed for toxic symptoms and behavioral changes (sedation, hyperactivity, diarrheal, writhing, piloerection, restlessness etc.) for 2 h post-administration and 14 days for signs of delayed mortality (Ishola et al., 2014a).

PHARMACOLOGICAL STUDIES

Antinociceptive activity

Formalin induced nociception test: Mice (17-19 g) were fasted for 12 h before the study and randomly assigned into five groups of six mice each. The treatment include; Group I: normal saline (10 ml/kg, p.o), Group II: morphine (3 mg/kg, p.o), Group III-V: C. anisata (100, 200, and 400 mg/kg, p.o., respectively). One hour after oral administration, 20pl of formalin (1%v/v in saline) was injected subcutaneously into the right hand paw of each mouse. The time (in seconds) spent in licking or biting the injected paw, indicative of pain, was recorded for each animal. The responses of the mice were observed for the first 5 min and 15-30 min post-formalin injection. The values were recorded and percentage inhibition calculated (Ishola et al., 2011).

Elucidation of possible mechanism of antinociception in mice: To investigate the roles of opioid system in the antinociceptive effect of HeCA, mice (n =6) were pretreated with naloxone (5 mg/kg, s.c, non-selective opioid receptor antagonist) (Rajendran et al., 2000) or vehicle (10 ml/kg), 15 min after pretreatment, HeCA (400 mg/kg, p.o.) or vehicle (10 ml/kg, p.o.), 1 h later, formalin test was carried out. The effect of serotonin synthesis on HeCA-induced antinociception was also investigated. Mice were pretreated with pCPA (100 mg/kg, i.p., tryptophan hydroxylase inhibitor) (Ishola et al., 2014b) or vehicle, for 4 consecutive days, 1 h after last treatment, HECA (400 mg/kg) was administered. Similarly, the effect of monoamine neurotransmitter depletion was also assayed. Mice were pretreated with reserpine (2 mg/kg, i.p.; vesicular monoamine transporter inhibitor) (Ishola et al., 2014b), 24 h prior to oral administration of HeCA (400 mg/kg). One hour later, the formalin test was carried out.

The specific role of serotonin receptor subtypes was also investigated, mice were pretreated with ketanserin (5 mg/kg, i.p.; non-selective 5HT2A/C receptor antagonist) (Alchaider, 1991) or vehicle, 15 min later, HECA (400 mg/kg) was given, 1 h post treatment, the formalin test was carried out.

The contribution of L-arginine-nitric oxide pathway in HECA-induced antinociception was assayed. Mice were pretreated with L-arginine (750 mg/kg, i.p., nitric oxide precursor) (Morgan et al., 1992) or vehicle, and 15 min later, they received HECA (400 mg/kg, p.o.) or vehicle (10 ml/kg, p.o.), 1 h later, formalin test was carried out. The possible participation of adrenergic system in the antinociceptive effect of HeCA was also evaluated, mice were pretreated with prazosin (1 mg/kg, i.p.; a1-adrenoceptor antagonist) or yohimbine (1 mg/kg, i.p.; a2-adrenoceptor antagonist) (Kaur et al., 2005; Ishola et al., 2014b) or vehicle (10 ml/kg,

p.o.), 15 min later, HECA (400 mg/kg, p.o.) or vehicle (10 ml/kg, p.o.) was administered. One hour after treatment, effect in formalin test was done.

The effect of ATP-sensitive potassium channels pathway on HeCA-induced antinociception was also examined. Mice were pretreated with glibenclamide (10 mg/kg, i.p; ATP-sensitive potassium channels blocker) (Alves et al., 2004), 15 min later, HECA (400 mg/kg, p.o.) was administered and 1 h later, formalin test was carried out.

Anti-inflammatory activity

Acute inflammation (Carrageenan-induced paw oedema model): Male Sprague-Dawley rats were fasted for 12 h before the study and randomly assigned into five groups of five rats each. The animals were treated 1 h before intraplantar injection of 100 pl of carrageenan (1%w/v in normal saline) into the right hind paw as follows: Group I: Normal saline (10 ml/kg, p.o), Group II: celecoxib (3 mg/kg, p.o), Group III-V: C. anisata (100, 200, and 400 mg/kg, p.o., respectively). The circumference of the injected paw was measured before and at 1, 2, 3, 4, 5 and 6 h post-carrageenan injection using the cotton thread method of Bamgbose and Noamesi, (1980).

Chronic inflammation (Complete Freund's adjuvant-induced arthritis model): Male Sprague-Dawley rats (90-110 g) were fasted for 12 h before the study and randomly assigned into seven groups of seven rats each. Arthritis was induced by intradermal injection of 100 pl of Complete Freund's adjuvant (CFA) into the right hind paw (Ahmad et al., 2006). The adjuvant contained Mycobacterium tuberculosis (10 mg) in 10 ml of paraffin oil. Treatments were given orally, 1 h before injection of CFA as follows: Group I: Control untreated group (Normal saline 10 ml/kg), Group II: Normal saline (10 ml/kg) + CFA, Group III: Celecoxib (3 mg/kg) + CFA, Group IV- VI: HeCA (100, 200 and 400 mg/kg p.o., respectively) + CFA, Group VII: HeCA (400 mg/kg; treatment commenced 4 days post CFA injection). The treatments were given for 28 and 24 days, respectively, for pre- and post-treatment groups, respectively. Arthritis was assessed by means of physical and biochemical measurements (Anburajan et al., 2012). The paw diameter and body weight were measured on day 0, i.e. before the injection of CFA and thereafter every 4th day for 28 days using cotton thread method of Bamgbose and Noamesi, (1980). Arthritic index was calculated using the method described by Kokkola et al. (2003) using ordinal scales as follows: 0 = unaffected; 1 = 1 type of joint affected; 2 = 2 types of joints affected; 3 = 3 types of joints affected; 4 = 3 types of joints affected and maximal erythema and swelling (maximum obtainable score per rat was set at 8 (4 points x 2 hind paws). On day 28 post-arthritic induction, the animals were anaesthetized with chloral hydrate (400 mg/kg, i.p.). Blood samples were collected for heamatological analysis through ocular puncture. The animals were euthanized by cervical decapitation.

Haematological analysis

The fully automated clinical haematological analyzer (Pentra-XL 80, Horiba ABX, USA) was used to evaluate the haematological parameters including total white blood cell count (leucocyte count), red blood cell count (RBC), haemoglobin (Hb), platelet count (PLT), neutrophil, mean cell haemoglobin concentration (MCHC), mean red cell volume (MCV) and mean cell haemoglobin, haematocrit (PCV).

Measurement of in vivo antioxidants and MDA levels

The determination of serum catalase (CAT), superoxide dismutase (SOD), reduced glutathione (GSH), nitrite, and malondialdehyde (MDA) were carried out using the protocol of Awodele et al. (2013).

Statistical Analysis

The results obtained were expressed as mean ± SEM and statistical level of significance between treatment groups was analyzed by one- or two-way Analysis of Variance (ANOVA) followed by Tukey's or Bonferroni post hoc multiple comparison tests, respectively (whichever is applicable), using Graphpad prism 6 (Graphpad software Inc., CA, USA).

RESULTS

Acute toxicity test

The extract up to 5000 mg/kg, p.o. did not induce mortality up to the 14th day of observation but the following toxicity behaviours were observed; writhing, hyperactivity, tachypnea and stooling.

Phytochemical Analysis of HeCA

The preliminary phytochemical screening of the hydroethanolic leaf extract revealed the presence of alkaloids, flavonoids, tannins, saponins, steroids and cardiac glycosides. Also, the quantitative analysis of the extract indicates; total phenolic 162.50±0.31 mg GAE/g, tannins 95.26±0.14 mg CE, alkaloids 47.31±0.27 mg/100g, saponins 16.39±0.43 mg DE/ 100g, and cardiac glycosides contents 57.48±0.13 mg DE/100 g. (GAE, gallic acid equivalent; CE, catechins equivalent, DE, dried extract).

Effect of HeCA on formalin-induced nociception in mice:

Intraplantar injection of formalin produced flinching or biting behaviour indicative of pain; the observed

Table 1: Effect of *Clausena anisata* leaf extract on duration of paw licking in formalin test

Treatment (mg/kg)	0-5 min	Inhibition (%)	15-30 min	Inhibition (%)
Vehicle 10ml/kg	54.39±4.00		106.20±16.65	
Morphine 3	15.63±2.24[c]	71.26	19.55±6.45[c]	81.59
HeCA 100	37.20±1.12[a]	31.61	115.00±13.85	-8.30
HeCA 200	33.39±5.25[a]	38.61	54.08±15.12[c]	49.08
HeCA 400	31.48±3.17[a]	42.12	25.71±1.01[c]	75.79

Values are expressed as Mean ± S.E.M. (n=6) [a]p<0.05, [c]p<0.001 versus vehicle 10 ml/kg treated group. Statistical level of significance using one way ANOVA followed by Tukey *post hoc* multiple comparison tests.

duration of paw licking in vehicle treated control are; 54.39±4.00 s and 106.20±16.65 s in the early and late phases, respectively (Table 1). However, the formalin-induced nociception in early and late phases in mice were significantly reduced by HeCA (100-400 mg/kg) treatment, to 31.48±3.17s (42.12% inhibition) and 25.71±1.01 s (75.79% inhibition) in the early and late phases, respectively, at 400 mg/kg (peak effect). In addition, morphine (3 mg/kg) treatment, significantly reduced the duration of nociception by 71.26% and 81.59% in the early and late phases, respectively (Table 1).

Mechanism of antinociceptive action of HeCA:

As shown in table 2, pretreatment of mice with naloxone (non-selective opioid receptor antagonist) prevented the anti-nociceptive effect elicited by HeCA in formalin test. Post hoc analysis revealed that naloxone pretreatment significantly (P<0.0001) reversed the effect of the extract in early and late phases. However, monoamine vesicular storage depletor, reserpine, failed to block the anti-nociceptive effect elicited by HeCA in murine formalin-induced nociceptive model (Table 2).

Four consecutive days pretreatment of mice with pCPA (serotonin synthesis inhibitor) blocked the effect of the extract. Pretreatment of mice with selective 5HT2A/2C receptor antagonist, ketanserin completely reversed the antinociceptive effect elicited by HeCA (P<0.001). pretreatment of mice with L-arginine (precursor of nitric oxide synthesis) abolished the antinociceptive effect of the extract in both phases of formalin test. In another series of experiment, the pretreatment of mice with prazosin (an a1 adrenoceptor antagonist) (P<0.001) or yohimbine (a2 adrenoceptor antagonist) (P<0.0001) prevents the antinociceptive effect elicited by HeCA (Table 2). In contrast, pretreatment of mice with glibenclamide (ATP-sensitive K+ channels blocker) failed to prevent the HeCA-induced antinociceptive effect in mice.

Effect of HeCA on carrageenan-induced paw oedema in rats: The intraplantar injection of carrageenan induced a time course increase in paw circumference (indicative of oedema), that peaked 3 h post injection (0.70±0.05 cm). However, pretreatment of rats with celecoxib (3mg/kg), produced time course and significant reduction of early and late phases of carrageenan-induced oedema, with peak effect 84.14% inhibition of oedema at 6h. Although, low doses of HeCA (100 and 200 mg/kg) could not attenuate the early stage oedema (1-2 h) but they effectively inhibit late phase of oedema. Moreover, HeCA (400 mg/kg) produced time course and significant reduction of oedema in both phases with peak effect 98.57% inhibition of oedema at the 5th h. In addition, two way ANOVA revealed significant effect of HeCA and celecoxib treatment [F(5,150)=48.43,P<0.0001] (Table 3).

Effect of HeCA on CFA-induced arthritis in rats

As shown in the table 4, intraplantar injection of complete Freund's adjuvant (CFA) into the right hind paw of rats produced time course and significant (p<0.001) increase in paw diameter which peaked on day 4 and thereafter gradually reduced by day 28 but still significant increase when compared to normal untreated rats. However, two way ANOVA revealed significant effect of subchronic treatment with HeCA (100-400 mg/kg) [F(6,245)=10.83,P<0.001], time course and significant decrease in paw oedema induced by CFA were observed. Moreover, the peak arthritogenic effect of CFA on day 8 was significantly reduced by HeCA 400 mg/kg (35.71%)

but the standard reference drug, celecoxib failed to produce a significant reduction. Moreover, the anti-arthritic effect of celecoxib was evident by day 12 of treatment (27.08% inhibition; P<0.05), with peak anti-arthritic effect on day 28 (55.07% inhibition; p<0.001).

In addition, post treatment with HeCA (400 mg/kg, p.o.) from day 4 post-induction, produced time course and significant (p<0.01, p<0.001) decrease in paw oedema-induced by CFA from day 12 to day 28 (44.93% inhibition on day 28), but in pretreatment group, peak anti-arthritic effect recorded in HeCA 200 mg/kg, treatment (57.97% inhibition, p<0.001) (Table 4). The results presented in Table 5 depict changes in body weight of the rats from week 1 to week 3 of the study. Intraplantar injection of CFA induced reduction of the body weight of vehicle- treated, control when compared to untreated control group. However, the decrease in body weight observed in vehicle-treated arthritic rats were

Table 2: Possible mechanism of antinociceptive action of HeCA in mice

Treatment	Dose (mg\kg)	0-5 min	Inhibition (%)	15-30 min	Inhibition (%)
Vehicle	10ml/kg	117.60±8.66		106.70±7.66	
HeCA	400	31.48±3.17***	73.18	25.71±1.01***	75.90
NAL + Veh	5	110.32±12.56		99.76±18.67	
NAL + HeCA	5 + 400	83.48±11.77[d]	29.01	95.18±12.79[d]	10.80
Reserp + Veh	2	17.60±8.66		106.70±7.66	
Reserp+ HeCA	2 + 400	58.55±4.01	50.21	15.40±3.04	85.57
PCPA + Veh	100*4d	96.17±16.52		98.80±16.54	
PCPA+ HeCA	100*4d +400	72.27±4.29[c]	38.54	111.30±7.20[d]	-4.31
KET + Veh	5	109.60±12.18		98.06±19.27	
KET + HeCA	5 + 400	97.28±12.85[d]	17.27	120.20±10.34[d]	-12.65
L-ARG + Veh	750	122.33±18.77		106.70±16.32	
L-ARG + HeCA	750 + 400	100.10±8.78[d]	14.88	69.63±6.72[d]	34.74
Prazosin + Veh	1	119.21±11.08		97.15±7.66	
Prazosin +	1 + 400	89.74±4.10[d]	23.69	92.91±7.19[d]	12.92
YOH + Veh	1	102.60±10.78		91.06±16.76	
YOH + HeCA	1 + 400	89.62±13.29[d]	23.79	48.39±2.91	54.65
GLIB + Veh	10	112.23±12.87		89.45±12.09	
GLIB+ HeCA	10 + 400	52.71±5.28	55.18	4.42±1.55	95.86

Values are expressed as mean ±S.E.M. (n=6), ***p<0.001 versus vehicle-treated control; cp<0.001, dp<0.0001 versus HeCA 400 mg/kg treated. (Veh-vehicle, NALnaloxone, KET-ketamine, GLIB-glibenclamide; reserp-reserpine; YOH-yohimbine PCPA, para-chlorophenylalanine for 4 consecutive days).

Table 3: Effect of HeCA on carrageenan-induced paw oedema in rats

Treatment (mg/kg)	Time course of paw edema (cm)					
	1h	2h	3h	4h	5h	6h
Vehicle	0.21±0.05	0.64±0.05	0.70±0.05	0.70±0.07	0.70±0.05	0.70±0.05
Celecoxib 3	0.13±0.0[a] (38.10)	0.37±0.04[a] (42.19)	0.37±0.05[a] (47.14)	0.29±0.0[c] (58.57)	0.11±0.0[c] (84.29)	0.09±0.03[c] (87.14)
HeCA 100	0.17±0.04 (19.05)	0.63±0.05 (1.56)	0.60±0.09 (1.43)	0.47±0.0[a] (32.86)	0.33±0.0[b] (52.86)	0.23±0.06[b] (67.14)
HeCA 200	0.19±0.04 (9.52)	0.49±0.04[a] (23.44)	0.41±0.01[a] (41.43)	0.29±0.0[b] (58.57)	0.14±0.0[c] (80.00)	0.03±0.02[c] (95.71)
HeCA 400	0.11±0.0[b] (47.62)	0.53±0.03(17.19)	0.39±0.03[a] (44.29)	0.23±0.0[c] (67.14)	0.10±0.0[c] (98.57)	0.01±0.01[c] (98.57)

Values are expressed as mean ±S.E.M. (n=6) ap<0.05, bp<0.01, cp<0.001, versus vehicle 10 ml/kg control treated group; Values in parenthesis represent % inhibition of oedema.

Table 4: time course **e***ffect of HeCA on CFA-induced arthritis in rats*

Treatment (mg/kg)	Change in paw circumference (cm)						
	Day4	Day8	Day12	Day16	Day20	Day24	Day28
Vehicle 10 ml/kg	0.02±0.02	0.05±0.02	0.10±0.02	0.13±0.02	0.15±0.02	0.15±0.02	0.15±0.02
Vehicle 10 ml/kg + CFA	1.12±0.04	0.98±0.07	0.96±0.10	0.94±0.07	0.89±0.04	0.77±0.05	0.69±0.07
Celecoxib 3 + CFA	0.80±0.06 (28.57)	0.73±0.03 (25.51)	0.70±0.04[a] (27.08)	0.70±0.05[a] (25.53)	0.63±0.05[a] (29.21)	0.38±0.06[b] (50.65)	0.31±0.07[c] (55.07)
HeCA 100 + CFA	0.91±0.03 (18.75)	0.77±0.04 (21.43)	0.71±0.04[a] (26.04)	0.66±0.04[a] (29.79)	0.60±0.05[a] (32.58)	0.56±0.06[a] (27.27)	0.33±0.05[b] (52.17)
HeCA 200 + CFA	0.90±0.06 (19.64)	0.97±0.06 (1.02)	0.84±0.08 (12.50)	0.69±0.06[a] (29.79)	0.60±0.04[a] (32.58)	0.53±0.05[a] (31.17)	0.29±0.06[c] (57.97)
HeCA 400 + CFA	0.94±0.03 (16.07)	0.63±0.12[a] (35.71)	0.69±0.08[a] (28.13)	0.67±0.07[a] (28.72)	0.51±0.09[b] (42.70)	0.41±0.09[b] (46.75)	0.37±0.09[b] (46.38)
HeCA 400 PT +CFA	- -	0.90±0.04 (8.16)	0.74±0.06[a] (22.92)	0.73±0.05[a] (22.34)	0.56±0.09[a] (37.08)	0.56±0.07[a] (27.27)	0.38±0.08[a] (44.93)

Values are expressed as mean ±S.E.M. (n=6), ***p<0.001 versus normal untreated rats; ap<0.05, bp<0.01, cp<0.001 versus vehicle 10 ml\kg + CFA treated group. CFA complete Freund's adjuvant; PT, post treatment from day 4; Values in parenthesis indicate % inhibition of oedema.

Table 5: Effect of HeCA on body weight of CFA-induced arthritic rats

Treatment (mg/kg)	**Change in body weight (g)**		
	Week 1	**Week 2**	**Week 3**
Vehicle 10 ml/kg	8.88±2.06	15.88±3.81	21.38±4.41
Vehicle 10 ml/kg + CFA	7.00±2.16	15.50±4.76	18.88±6.24
Celecoxib 3 + CFA	13.13±1.17	17.50±1.97	26.75±2.53[a]
HeCA 100 + CFA	10.71±2.61	14.57±2.10	23.29±3.32
HeCA 200 + CFA	11.38±2.42	15.88±2.90	28.38±12.47[b]
HeCA 400 + CFA	13.25±2.24	20.75±2.52	22.75±3.14
HeCA 400 PT + CFA	14.83±1.01	20.67±0.80	25.33±1.02

Values are expressed as mean ±S.E.M. (n=6); ap<0.05, bp<0.01 in comparison with vehicle treated CFA-induced arthritis control group. CFA-complete Freund's adjuvant; PT, post treatment from day 4.

Table 6: Effect of HeCA on arthritic index in rats

Treatment	Day	Day 4	Day 8	Day 12	Day 16	Day 20	Day 24	Day 28
Vehicle+CFA	4.00±0.	6.67±0.4	7.30±1.27	7.50±2.47	7.00±2.42	6.67±1.84	6.45±1.61	6.00±1.44
Celecoxib 3+CFA	4.00±0.	5.86±0.5	5.71±0.52[a]	4.86±0.40[b]	4.71±0.47[c]	4.00±0.38[a]	3.00±0.38[c]	3.00±0.38
HeCA 100+CFA	4.00±0.	7.00±0.4	7.00±1.13	5.50±0.62[b]	4.50±0.50[c]	3.67±0.56	3.17±0.31[c]	3.17±0.31
HeCA 200 + CFA	4.00±0.	6.50±0.3	6.33±0.33	4.83±0.40[b]	3.67±0.49[c]	3.33±0.33	2.83±0.16	2.83±0.17
HeCA 400 + CFA	4.00±0.	6.50±0.3	6.33±0.33	4.83±0.40[b]	3.67±0.49[c]	3.33±0.33[c]	2.83±0.16	2.83±0.17
HeCA 400 PT +	-	-	4.67±0.49[b]	4.17±0.65[c]	3.50±0.43[c]	3.50±0.43[c]	3.50±0.43[c]	3.50±0.43

Values are expressed as mean ±S.E.M. (n=6); ap<0.05; bp<0.01, cp<0.001 versus vehicle treated CFA-induced arthritic group. CFA-complete Freund's adjuvant; PT, post treatment from day 4.

Table 7: Effect of HeCA on haematological parameters.

Group	WBC	RBC	Plat	Neut	Hb	PCV	MCV	MCH	MCHC
1	9.40±1.90	7.42±0.20	681.00±36.86	3.23±0.18	13.60±0.32	41.30±1.01	56.20±1.42	18.37±0.15	33.23±0.09
2	6.67±1.51	7.33±0.32	739.30±53.99	3.62±0.25	13.63±0.24	42.87±1.18	55.60±2.94	19.27±0.12	33.70±0.26
3	12.53±4.52	7.49±0.12	614.30±28.05	4.02±0.26	14.47±0.15	45.07±0.69	57.47±0.35	19.30±0.23	31.33±0.54
4	9.47±3.03	7.74±0.15	718.70±14.31	4.45±0.39	14.53±0.23	45.53±0.90	76.60±4.22	18.97±0.24	31.97±0.58
5	7.77±3.43	7.73±0.14	784.00±41.48	4.04±0.46	14.1±0.23	45.67±0.32	57.53±1.09	19.17±0.12	31.17±0.24
6	13.63±3.36	7.68±0.11	716.00±57.30	4.51±0.23	14.2±0.26	45.87±1.41	58.73±2.28	19.10±0.20	31.47±0.33
7	14.5±4.03[a]	7.55±0.24	666.00±30.92	4.70±0.19	14.57±0.18	46.20±0.32	58.00±0.87	19.07±0.41	30.97±0.41

Values are expressed as mean ±S.E.M. (n=6) aP<0.05 versus vehicle 10 ml\kg + CFA or vehicle only treated. WBC (103/µL), white blood cells; RBC ((103/µL), red blood cells; Plat (104/µL), platelet count; Neut (103/µL) neutrophil count, Hb (g/dl), Haemoglobin; PCV, Packed Cell Volume; MCV (fl), Mean Corpuscular Volume; MCH (pg), Mean Corpuscular Haemoglobin; MCHC (g/dl), Mean Corpuscular Haemoglobin Concentration. 1= Vehicle 10 ml/kg, 2= Vehicle 10 ml/kg + CFA, 3= Celecoxib 3 + CFA,4= HeCA 100 + CFA, 5=HeCA 200 + CFA, 6=HeCA 400 + CFA, 7=HeCA 400 PT+ CFA

Table 8: Effect of HeCA treatment on CFA-induced oxidative and nitrosative stress in rats

Treatment (mg/kg)	GSH(U/mg)	SOD(U/mg)	CAT(U/mg)	MDA(U/mg)	Nitrite
Vehicle 10 ml/kg	1.08±0.10	3.73±0.01	31.52±1.37	0.09±0.01	8.13±1.50
Vehicle 10 ml/kg + CFA	0.63±0.21**	3.87±0.18	31.57±1.64	0.20±0.01***	12.10±0.12*
Celecoxib 3 + CFA	0.95±0.07	3.96±0.12[a]	36.96±0.25[a]	0.10±0.04[b]	10.10±0.70[a]
HeCA 100 + CFA	0.79±0.12	3.49±0.08	31.19±0.58	0.11±0.01[b]	9.75±1.47[b]
HeCA 200 + CFA	1.57±0.36[a]	4.80±0.84[b]	39.89±6.92[a]	0.24±0.10	10.90±0.40[a]
HeCA 400 + CFA	1.59±0.05	2.87±0.19	25.29±2.19	0.13±0.03[a]	8.90±0.12[b]
HeCA 400 PT + CFA	0.99±0.07	2.87±0.19	25.29±2.19	0.13±0.03[a]	8.60±0.12[b]

Values are expressed as mean ±S.E.M. (n=6), *p<0.05; **p<0.01; ***p<0.001 versus vehicle 10 ml/kg only treated group; ap<0.05, bp<0.01 versus vehicle 10 ml/kg + CFA treated group. Statistical level of significance analyzed using one way ANOVA followed by Tukey *post hoc* multiple comparison test; CFA-complete Freund's adjuvant; PT, post treatment from day 4. GSH, reduced glutathione; SOD, superoxide dismutase; CAT, catalase; MDA, malondialdehyde.

significantly reversed by HeCA (200 and 400 mg/kg) and celecoxib treatments [$F(2,120) = 32.31, P<0.001$], compared with the control group (vehicle 10 mg/kg + CFA) by the 3 week.

Effect of HeCA on Arthritic Index

Intraplantar injection of CFA produced a time course and significant increase in arthritic index that peaked on day 12 (7.50±2.47), as shown in table 6. However, the increase in arthritic index induced by CFA was significantly reduced by pretreatment of rats with HeCA (100-400 mg/kg) and post-treatment with HeCA (400 mg/kg). Similar observations were recorded in celecoxib pretreated rats (Table 6).

Effect of HeCA on haematological parameters in arthritic rats.

In this study, one way ANOVA revealed no significant change in haematological parameters except, a significant increase in level of WBC in HeCA 400 mg/kg (pre and post treatment) following 28 days subchronic administration of the extract when compared to control (vehicle 10 ml/kg + CFA) as shown in table 7. But no significant change was recorded in celecoxib treatment group.

Intraplantar injection of CFA significantly increased the level of lipid peroxidation and nitrite generation with concomitant decrease in the level of glutathione, catalase and superoxide dismutase. However, subchronic oral administration of HeCA (100 - 400 mg/kg) significantly attenuated the increase in the level of lipid peroxidation and nitrite generation with concomitant increase in the level of glutathione, catalase and superoxide dismutase following deficit induced by CFA injection (Table 8). Similarly, celecoxib pretreatment also reversed the increase in MDA and nitrite levels with increase in the activities of catalase and superoxide dismutase.

DISCUSSION

The results of this study demonstrate antinociceptive and anti-arthritic properties of the hydroethanolic leaf extract of Clausena anisata. Clausena anisata inhibited both early and late phases of formalin induced nociception test. This test which represents a model of persistent pain can also be used to determine the ability of new compounds to affect peripheral or central nociceptive pathways due to its biphasic nociceptive characteristics, known as the early and late phase, resulting from formalin administration (Malmberg et al, 1992). The early phase classified as the neurogenic pain, is an acute response observed immediately after the administration of formalin which lasts for 0-5 mins. The late phase, classified as an inflammatory pain is a tonic response resulting from inflammatory processes generated by inflammatory mediators such as histamine, serotonin, PGE and bradykinin (Verma et al., 2005). Centrally acting drugs (e.g. opioids) inhibit both phases, while peripherally acting drugs (e.g. NSAIDS) inhibit only the late phase. The results also suggest that Clausena anisata possesses central antinociceptive effect.

Moreover, the ability of the extract to attenuate the late phase of formalin test suggests not only anti-nociceptive effect but also anti-inflammatory activity (Zakaria et al., 2007).

To elucidate possible mechanism of antinociceptive effect of the extract, various pathways implicated in pain signaling mechanisms were evaluated. Most of the drugs used in managing chronic pain targets opioidergic pathway and results obtained from this study suggest central antinociceptive effect of the extract (Zakaria et al., 2007). Hence, possible participation of opioid receptors in the antinociceptive effect of the extract was investigated through subcutaneous injection of naloxone (non-selective opioid receptors antagonist). Findings from the study showed that the pretreatment of mice with naloxone reversed the antinociceptive effect elicited by the extract in formalin-induced nociceptive test, suggesting possible interaction of the extract with opioid receptors (Knights et al., 2004). Serotonin is known to play complex modulatory roles in pain signaling mechanisms, serotonergic descending pathway from rostral ventromedial medulla to the dorsal horn is crucial to spinal nociceptive processing (Millan, 2002; Suzuki et al., 2004). Conversely, pretreatment of mice with reserpine (vesicular monoamine depleter) failed to reverse the antinociceptive effect of the extract. However, 4 days pretreatment of mice with parachlorophenylalanine (serotonin synthesis inhibitor) prevented the antinociceptive effect of the extract. Moreover, pretreatment of mice with ketanserin (5-HT2A/C receptor antagonist; Alchaider, 1991; Knights et al., 2004) prevented the antinociceptive effect elicited by the extract, suggesting participation of serotonergic systems in the antinociceptive action of C. anisata.

Several lines of evidence have indicated the role of nitric oxide (NO) in the modulation of pain and analgesia (Cury et al., 2011). The reports of Moore et al. (1991) showed the involvement of brain NO system in the mechanisms underlying pain perception based on the fact that L-NG-nitro arginine methyl ester (L-NAME), a selective inhibitor of nitric oxide synthase, produces a potent, long lasting and centrally mediated antinociception in mice. Finding from this study showed that the pretreatment of mice with L-arginine prevented the antinociceptive effect elicited by C. anisata in formalin-induced paw licking model which suggest possible involvement of the L-arginine/nitric oxide/cyclic guanosine monophosphate pathway in the antinociceptive effect of the extract. Moreso, Morgan et al. (1992) have reported antinociceptive effect of L-NAME in formalin-induced paw licking and acetic

acid-induced abdominal constriction test. In addition, NO produced from L-arginine activates soluble guanylate cyclase which produces an increase in cyclic GMP (cGMP) levels (Meller and Gebhart, 1993).

In another series of experiment, the involvement of noradrenergic systems was also investigated, to have more robust understanding of the mechanisms of antinociceptive effect of the extract. Noradrenaline, through its action on ai- and a2-adrenoceptors, is involved in intrinsic control of pain. Peripheral noradrenaline that is mainly released by the sympathetic nervous system has little influence on healthy tissues, whereas in injured or inflamed tissues it has varying effects, including aggravation of pain in neuropathy. The peripheral pronociceptive effect has been associated with injury-induced expression of novel noradrenergic receptors, sprouting of sympathetic nerve fibers, and pronociceptive changes in the ion channel properties on primary afferent nociceptors, whereas an interaction with the immune system may contribute to peripheral antinociceptive effect of noradrenaline. At supraspinal levels, the effect of noradrenergic system on pain varied depending on many factors such as the type of the adrenoceptor, pathophysiological condition, and the brain area (Pertovaara, 2013). In the current study, the antinociceptive effect of the extract was blocked by prazosin (ai-adrenoceptor antagonist) and yohimbine (a2-adrenoceptor antagonist) pretreatment. The ability of the extract to increase noradrenergic activity or receptors may provide an effective way for suppressing pain, particularly in pathophysiological conditions. This synergistic pain inhibitory interaction of the extract with many neurotransmitter systems provide a possibility to maximize pain inhibition while minimizing side effects (Pertovaara, 2013). In another series of experiment, the involvement of ATP-sensitive K+ channels was investigated. In contrary, the K+ATp channels blocker, glibenclamide (Edwards and Weston, 1993) failed to inhibit the antinociceptive effect of the extract. Thus ruling out the involvement of K+ channels in its antinociceptive effect.

In carrageenan induced paw oedema model, the extract of Clausena anisata exerted pronounced effect at early stage of inflammation indicating possible inhibition of histamine, serotonin and kinnins that are involved in the early stage of carrageenan-induced oedema (Vane and Booting, 1987). The extract also reduced later stage of the oedema, an activity that may be due to its ability to inhibit prostaglandin which is known to mediate the second phase of carrageenan induced inflammation. Celecoxib a selective cyclooxygenase-2 inhibitor (COX-2- inducible upon inflammation) whose mechanism of action involves inhibition of prostaglandin inhibited significantly the paw swelling due to carrageenan injection indicating acute anti-inflammatory effect of the extract.

In chronic models of inflammation induced by CFA, rats developed arthritis-like symptoms on day 4 after intraplantar injection of CFA into the right hind paw. There was rapid development of a localized inflammatory response characterized by increased vascular leakage and subsequent swelling of the affected paw. However, oral administration of Clausena anisata extract reduced the inflammation associated with the arthritis as well as arthritic index. The anti-arthritic effect of Clausena anisata was similar to that of celecoxib, the findings corroborated the report of the in vitro assay by Amoo et al. (2013) that dried material of C. anisata effectively inhibited cyclooxygenase activity better than fresh material.

Intraplantar injection of CFA not only induced inflammation but also induced lipid peroxidation and nitrite generation with concomitant deficit in glutathione, catalase and superoxide dismutase activities. The extract showed potent antioxidant effect by attenuating the levels of malondialdehyde and nitrite as well as increase in glutathione, catalase and superoxide dismutase activities indicating antioxidant property of the extract. Interestingly, the deficit in catalase and superoxide dismutase as well as increase MDA and nitrite generations were reversed by subchronic treatment with celecoxib. Also, the decrease in body weight induced by CFA was reversed by the 3 week. Since a change in body weight is a useful index to assess the disease course and response to therapy of the drugs under investigation.

The results of phytochemical screening revealed the presence of alkaloids, flavonoids, tannins, saponins, reducing sugars, and cardiac glycosides which could be attributed to the observed antinociceptive and anti-inflammatory activities. Flavonoids particularly have been shown to possess anti-inflammatory activity through inhibition of the COX pathway (Liang et al., 1999). Studies of the constituents of plants of the genus Clausena have resulted in the isolation of some carbazole alkaloids (Ito et al., 2009). It has been reported that carbazole alkaloids possess various biological activities such as antitumor, antioxidative, antimutagenic, and anti¬inflammatory activities (Nakahara et al., 2002).

Conclusion

The results obtained from this study showed that Clausena anisata leaves extract possesses antinociceptive activity through interaction with opioidergic, noradrenergic, serotonergic and L-arginine-nitric oxide pathways, and anti-arthritic properties which could be attributed to its ability to prevent the release of inflammatory mediators as well as attenuation of oxidative and nitrosative stress. The results of this study support the ethnobotanical use of the plant in the treatment of painful and inflammatory conditions.

Conflict of interest

The authors declare that there are no conflicts of interest in respect of this study.

Acknowledgements

The authors are grateful to Mr. S.O. Adenekan (Department of Biochemistry), and Mr M.C Chijioke of the Department of Pharmacology, Toxicology and Therapeutics, Faculty of Basic Medical Sciences, College of Medicine, University of Lagos, Lagos, Nigeria, for technical assistance provided.

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Effects of Oral Maternal Administration of Caffeine on Reproductive Functions of Male Offspring of Wistar Rats

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Summary: Caffeine was investigated for its possible fetal programming effects on reproductive function of male offspring. Sixty-five pregnant Wistar rats were grouped into four. Group 1 was control and received distilled water. Groups 2, 3 and 4 were treated orally with 1.14, 3.42 and 5.70 mg/kg body weight of caffeine respectively. Each group was subdivided into four based on gestation days (GD) 1-7, 8-14, 15-21 and 1-21. The day of parturition was taken as postnatal day zero (0). Male offspring were sacrificed on postnatal day 70. Parameters determined were: weight at birth, body weight at postnatal day 21 and 70, anogenital distance (AGD) index, sperm parameters, reproductive organ weight, histology and hormonal profile (testosterone, FSH and LH). Data were analyzed using Analysis of Variance. Level of significance was taken at $P<0.05$. Male offspring belonging to caffeine treated dams showed dose dependent significant decreases in birth weight. Male offspring from dams treated with caffeine during GD 1-7 and GD 1-21 had a significant increase in their AGD index. Also, male offspring from dams treated with 1.14 and 5.70 mg/kg body weight of caffeine during GD 8-14 had a significant increase in AGD index. Dams treated with 3.42 mg/kg body weight of caffeine during GD 15-21, had a significant increase in the AGD index of their male offspring. The sperm motility of offspring from dams treated with 5.70 mg/kg body weight of caffeine during GD 1-7 and GD 1-21 were significantly increased. Offspring of GD 8-14 and GD 15-21 dams treated with 3.42 and 5.70 mg/kg body weight of caffeine respectively, showed significantly reduced serum testosterone level. There was a significant decrease in the weight of testes of offspring from dams treated with caffeine during GD 8-14. Histological sections of testes of offspring from caffeine treated dams showed interstitial congestions, edema, reduced germinal epithelial height and detached basal membrane. Maternal caffeine exposure during different gestational periods adversely affected birth weight and some reproductive indices in male offspring of Wistar rats.

Keywords*:* Caffeine, Anogenital distance index, Wistar rat, Sperm motility, Testosterone.

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Manuscript Accepted: 2015

INTRODUCTION

The rise in occurrence of reproductive disorders has raised concerns regarding the impact of endocrine disrupting chemicals on reproductive health especially when such exposures occur during fetal life (Savabieasfahani *et al.*, 2006). Exposure to environmental and occupational toxicants and progressive changes in many aspects of lifestyle, including dietary habits, has been shown to deteriorate reproductive health, thus, affecting the ability of couples to conceive and maintain a healthy pregnancy. Among dietary factors affecting reproduction is caffeine consumption (Dlugosz and Bracken 1992; Kumar *et al.*, 2009). Data from *in vitro* studies suggested that caffeine has variable, dose-related effects on human sperm motility, number and structure (Dlugosz and Bracken, 1992).

Caffeine is a naturally occurring alkaloid found in the seeds, leaves and fruits of more than 60 plants such as coffee and cocoa beans, kola nuts, and tea leaves. It is also present in a wide variety of foods and beverages including coffee, tea, cocoa, chocolate, colas and energy drinks also in a number of prescription drugs including cold and flu remedies, headache treatments, diet pills, diuretics and stimulants (Al-Shoshan, 2007). Average caffeine consumption by adult human varies among different cultures and nations from 80 to 400 mg/70kg person per day (Knight *et al.*, 2004; Mitchell *et al.*, 2014). Caffeine is the most widely consumed xenobiotic in pregnancy, with a potential to adversely affect the developing fetus (James, 1991). About 98% of women in reproductive age regularly consume caffeine in the form of caffeinated beverages or in caffeine containing medications while about 75% of them continue to do so during pregnancy (Sengpiel *et al.*, 2013). Caffeine has been reported to be capable of crossing the placenta barrier (Dlugosz and Bracken,

1992). If consumption of caffeine continues during pregnancy, the developing fetus is exposed to caffeine and its metabolites during the critical periods of development and this may alter growth and development which could affect physiology during adult life. Epidemiological studies suggested that caffeine consumption may affect skeletal growth (Bakker *et al.,* 2010) and cause low birth weight (Bracken *et al.,* 2003). Experimental studies also showed that caffeine consumption affected fetal growth and altered reproductive functions (Dorostghoal, 2012). However, these experimental studies used very high doses of caffeine which are not obtainable in humans. Therefore, the aim of the present study was to determine the effects of maternal caffeine consumption during different gestation periods on reproductive functions of male Wistar rats. It is worth noting that the dosages used here are within the average human caffeine consumption.

MATERIALS AND METHODS

Experimental Design

Sixty-five female Wistar rats weighing 180-200g obtained from the animal house, College of Medicine, University of Ibadan, were used for this study. The animals were housed in well ventilated wire mesh cages with constant 12-h light 12-h dark cycle. They were fed standard rat feed and clean water *ad libitum* and were allowed to acclimatize for two weeks, before the commencement of the study. The study was performed according to the recommendations from declaration of Helsinki on guiding principles for the care and use of laboratory animals (World Medical Association, American physiological society, 2002).

Female Wistar rats were mated with proven male breeders in the ratio 2:1 (female to male) during proestrous phase. The presence of spermatozoa in vaginal lavage confirmed mating on the morning after pairing and the day of confirmation of mating was taken as gestation day (GD) one. The pregnant rats were then divided into four major groups; the control group consisting of 5 animals, groups 2, 3 and 4 consisted of 20 animals each. The groups 2, 3 and 4 were then subdivided into four equal groups according to gestation days (GD), a) 1-7, b) 8-14, c) 15-21 and d) 1-21. Group one (control) received distilled water while groups 2, 3 and 4 were orally administered with caffeine at 1.14, 3.42 and 5.70 mg/kg body weight respectively. Subgroups a, b, c and d received caffeine on gestation days 1-7, 8-14, 15-21 and 1-21 respectively. Caffeine (Aesar Johnson Matthew Company, USA) was freshly prepared by dissolving it in distilled water daily before use. The day of parturition was taken as day zero of life. On postnatal day 1, the birth weight of the male pups was recorded. Also, the anogenital distances were measured according to method used by Sathyanarayana *et al.,* (2010). At postnatal day (PND) 21 (weaning) and 70 (just before sacrifice) the body weight of the offspring was also measured. Five male offspring were randomly selected from each subgroup and sacrificed at PND 70, bringing the total number of male offspring used to 65.

Blood Collection

Blood was collected from the medial canthus vein into plain sample bottles and centrifuged at 3500rmp for 10 minutes. Animals were thereafter sacrificed via cervical dislocation.

Hormonal Assay

Serum concentrations of luteinizing hormone, follicle stimulating hormone and testosterone were determined using ELISA kits (Inteco Diagnostics, UK).

Histology of the testes

The testes, epididymes and seminal vesicles were collected and weighed. Testes were fixed in Bouin fliud in preparation for histological sections. Tissue processing included tissue embedding, microtomy, trimming and nicking to expose the embedded tissue and orientate the tissue block. Staining was done using haematoxylin and eosin. The slides were then viewed with the microscope.

Sperm Analysis

Caudal epididymal fluid was used for sperm analysis. Sperm count was determined using the microscope with the aid of the improved Neubauer hemocytometer. Count was done in five large Thoma square as described by Raji and Bolarinwa (1997). The result was expressed as count $X10^6$ ml^{-1}. Sperm motility was done as described by Zemjanis (1970).

Statistical analysis

Data were expressed as mean ± standard error of the mean (Mean ± SEM). Analysis of variance was used in comparison of data followed by Tukey post-hoc test using SPSS (Version 17.0). $P<0.05$ was considered significant.

RESULTS

Birth weight and anogenital distance Index (AGD)

The birth weight and anogenital distance index (AGD) of offspring are shown in table 1 and figure 1 respectively. There was a significant decrease ($P < 0.05$) in the birth weight of all pups from dams that received caffeine throughout gestation period (GD 1-21). Also, birth weight of pups from dams treated with 3.42 mg/kg body weight of caffeine during GD 1-7 and GD 15-21 decreased significantly. Furthermore, birth weight of pups from all dams treated with 5.70 mg/kg body weight of caffeine, irrespective of the gestation day significantly reduced (Table 1). All pups from GD

1-7 and GD 1-21 treated dams showed significant increase AGD index (P<0.05) (Figure 1). Pups from dams treated during GD 8-14 with 1.14 and 5.70 mg/kg body weight of caffeine had increased AGD index (Figure 1). There was also a significant increase in AGD index of pups from GD 15-21 treated dams that had 3.42 mg/kg body weight of caffeine (Figure 1).

Table 1. Effect of maternal caffeine exposure on the body weight of offspring.

Dosage of Caffeine	Gestation Day (GD)	Birth Weight(g)	Weight(g) at PND 21	Weight(g) at PND 70
Control	-	5.50±.13	27.24±3.3	108±6.7
1.14 mg/kg	1-7	5.44±.51	27.61±.73	122±6.0
	8-14	4.98±.17	24.63±.63	116±3.26
	15-21	5.39±.05	17.20±.73*	109.7±4.4
	1-21	4.56±.04*	26.70±1.8	123±8.2
3.42 mg/kg	1-7	4.26±.10*	21.27±.73	135±6.8*
	8-14	5.30±.26	27.00±00	120±.34
	15-21	4.33±.12*	20.20±2.0	114±7.1
	1-21	3.18±.15*	25.15±.26	108±.40
5.70 mg/kg	1-7	3.32±.09*	20.93±.97	116.8±9.6
	8-14	3.16±.09*	20.68±.72	110±1.6
	15-21	3.04±.05*	21.02±.48	100±2.0
	1-21	3.03±.08*	27.24±.28	111.5±.22

Values expressed as mean ± standard error of mean, n=5, *significantly different from control at P<0.05

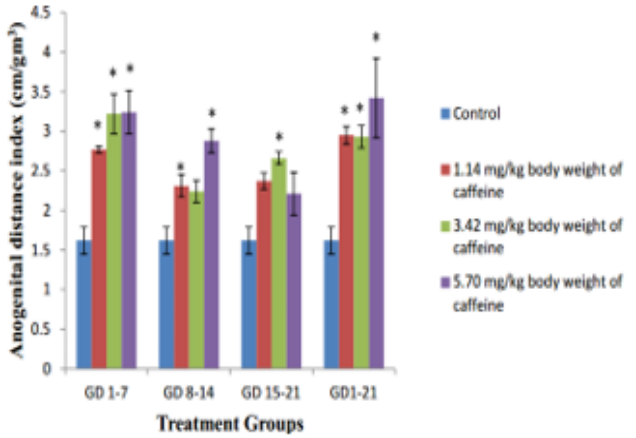

Fig 1. Effect of maternal caffeine exposure on anogenital distance index of male pups at birth. Values expressed as mean ± standard error of mean, n=5, *P<0.05

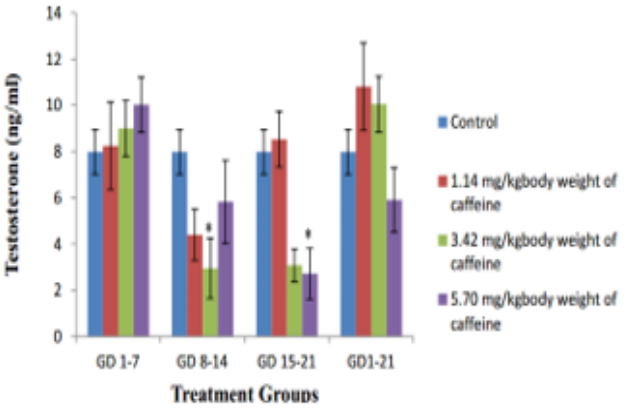

Fig 2. Effect of maternal caffeine exposure on serum level of testosterone. Values expressed as mean ± standard error of mean, n=5, *P<0.05

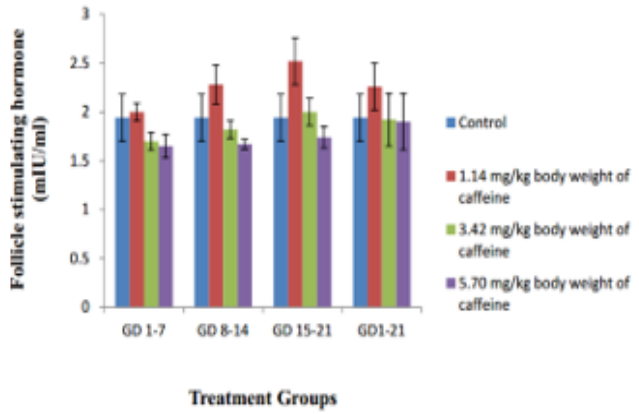

Fig 3. Effect of maternal caffeine exposure on serum level of follicle stimulating hormone. Values expressed as mean ± standard error of mean, n=5

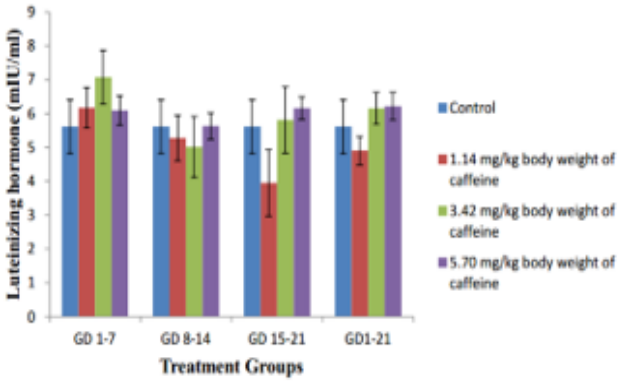

Fig 4. Effect of maternal caffeine exposure on serum level of Leutenizing hormone. Values expressed as mean ± standard error of mean, n=5

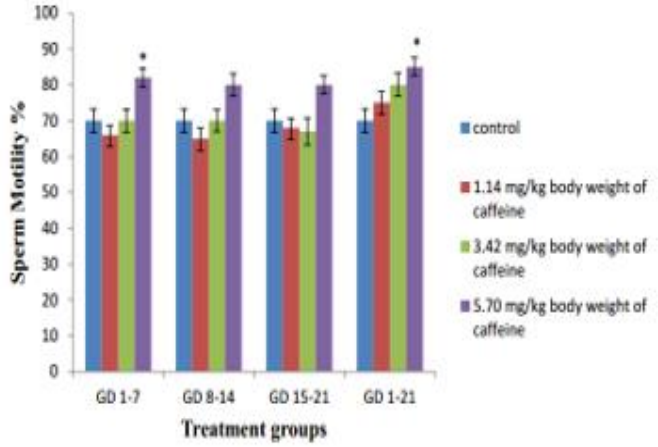

Fig 5. Effect of maternal caffeine exposure on caudal epididymal sperm motility. Values expressed as mean ± standard error of mean, n=5, *P<0.05

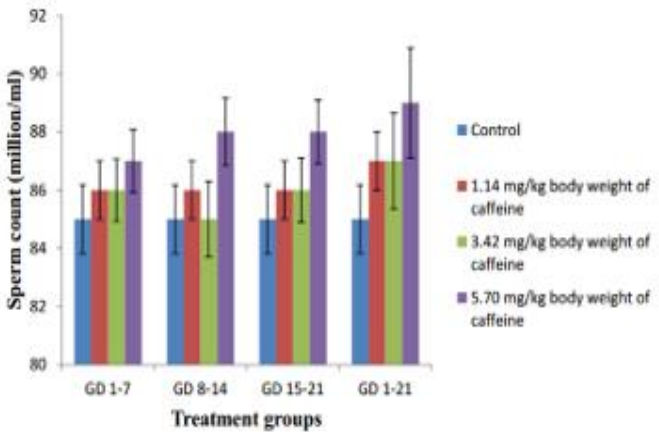

Fig 6. Effect of maternal caffeine exposure on caudal epididymal sperm counts. Values expressed as mean ± standard error of mean, n=5, *P<0.05

Table 2. Effect of maternal caffeine exposure on weight of organs of offspring

Dosage of Caffeine	Gestation Day	Testis (g)	Epididymis (g)	Seminal Vesicle (g)
Control	-	1.08±.01	0.37±.04	0.33±.01
1.14 mg/kg	1-7	0.78±.16	0.24±.05	0.23±.04
	8-14	0.67±.07*	0.27±.04	0.17±.04*
	15-21	0.60±.14	0.16±.04*	0.17±.04*
	1-21	1.00±.17	0.27±.04	0.30±.07
3.42 mg/kg	1-7	0.85±.15	0.30±.07	0.40±.07
	8-14	0.73±.09*	0.24±.03	0.20±.00
	15-21	0.68±.02*	0.24±.04	0.27±.04
	1-21	0.83±.12	0.22±.05	0.23±.04
5.70 mg/kg	1-7	0.77±.11	0.22±.09	0.24±.04
	8-14	0.68±.04*	0.24±.04	0.27±.04
	15-21	0.38±.09*	0.27±.04	0.23±.08
	1-21	0.72±.25	0.20±.07	0.17±.04*

Values are express as mean ± standard error of mean, n=5, *significantly different from control at P<0.05

Hormonal Study

Offspring of dams treated during GD 8-14 and GD 15-21 with 3.42 and 5.70 mg/kg body weight of caffeine respectively showed significant reduction (P<0.05) in serum testosterone level (Figure 2). However, there was no significant difference in the serum concentrations of follicle stimulating hormone and luteinizing hormone of adult offspring when compared with that of the control offspring (Figure 3 and 4).

Sperm Characteristics

The sperm motility of offspring from dams treated with 5.70 mg/kg body weight of caffeine during GD 1-7 and GD 1-21 was significantly increased (P<0.05) (figure 5). However, sperm count showed no significant difference across all groups (Figure 6).

Body weight and weight of organs

There was a significant decrease (P<0.05) in the body weight of offspring from dams treated with 1.14 mg/kg body weight of caffeine during GD 15-21 at PND 21 (Table 1). There was a significant increase (P<0.05) in weight of offspring from dams treated with 3.42 mg/kg body weight of caffeine during GD 1-7 at PND 70 (Table 1). However, there was a significant decrease (P<0.05) in the weight of the testes of offspring from all dams treated during GD 8-14 irrespective of the dose of caffeine administered. Offspring from dams treated during GD 15-21 with 3.42 and 5.70 mg/kg body weight of caffeine, showed significant decrease (P<0.05) in weight of testes (Table 2). The epididymal weight of offspring from dams treated during GD 15-21 with 1.14 mg/kg body weight of caffeine significantly decreased (P<0.05) (Table 2). Similarly, there was a significant decrease (P<0.05) in the weight of the seminal vesicle of offspring from dams treated with 1.14 mg/kg body weight of caffeine during GD 8-14, GD 15-21 and 5.70 mg/kg body weight of caffeine during GD 1-21 (Table 2).

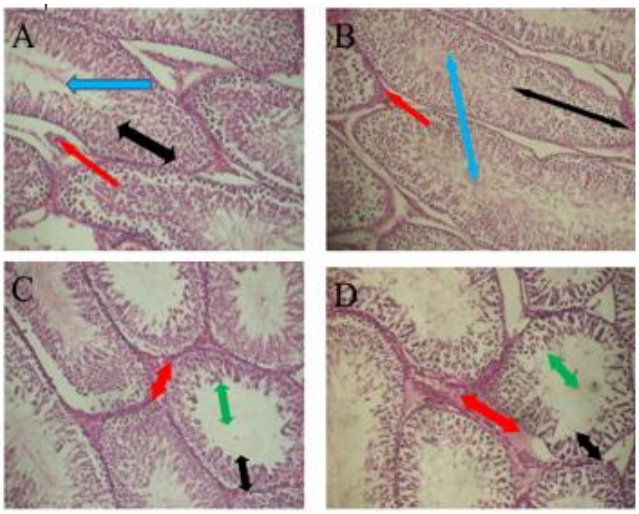

Plate 1: Transverse section through the testes of offspring from control (***A***), 1.14 (***B***), 3.42 (***C***) and 5.70 mg/kg body weight (***D***) caffeine treated dams during GD 1-7. Note the normal architectural layout with no visible lesion (***A*** and ***B***) and reduced germinal epithelial height (black arrow), empty lumen (green arrow), congestion and edema in parts of the interstitium (red arrow) in ***C*** and ***D***. Stain: H&E. Magnification: X100

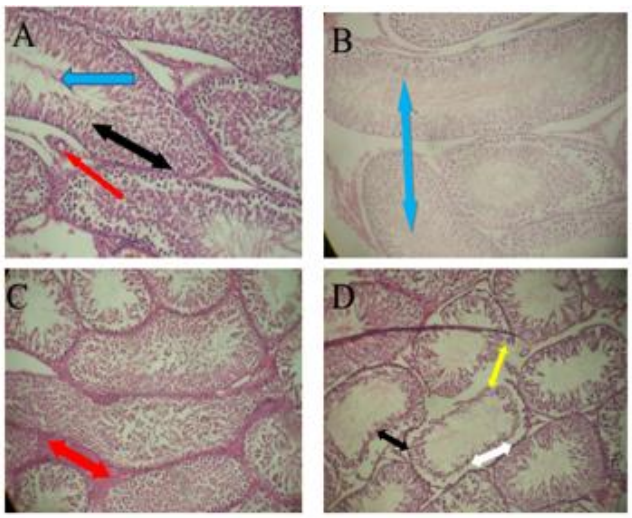

Plate 2: Transverse section through the testes of offspring from control (***A***), 1.14 (***B***), 3.42 (***C***) and 5.70 mg/kg body weight (***D***) caffeine treated dams during GD 8-14. Note the normal architectural layout with no visible lesion (***A*** and ***B***), Interstitial congestion (red arrow) in ***C***, Epithelial cells detached from basal membrane (white arrow), absence of basal lamina (yellow arrow) and reduced germinal epithelium (black arrow) in ***D***. Stain: H&E. Magnification: X100.

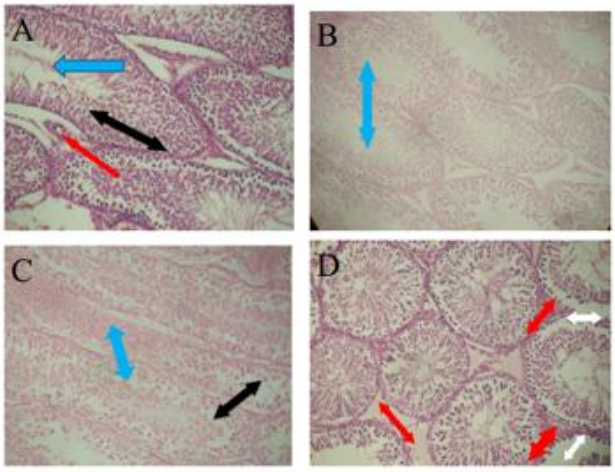

Plate 3: Transverse section through the testes of offspring from control (***A***), 1.14 (***B***), 3.42 (***C***) and 5.70 mg/kg body weight (***D***) caffeine treated dams during GD 15-21. Note the normal architectural layout with no visible lesion (***A*** and ***B***), Scanty germ cell (black arrow) and disorganized cytoarchitecture of the seminiferous tubules (blue arrow) in ***C*** and Interstitial edema (red arrows) and detached germinal cells (white arrows) in ***D***. Stain: H&E. Magnification: X100

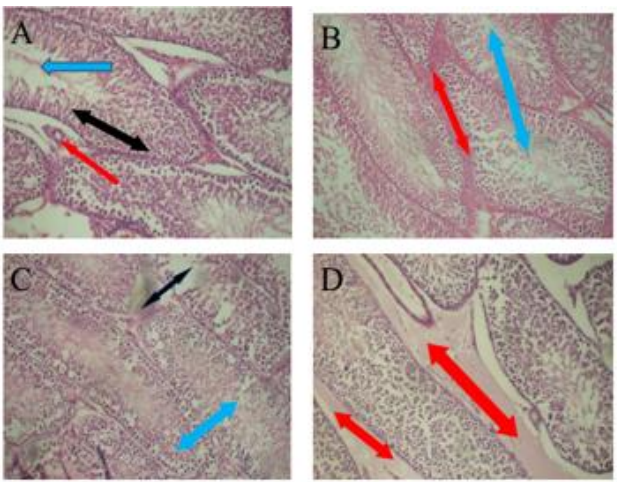

Plate 4: Transverse section through the testes of offspring from control (**A**), 1.14 (**B**), 3.42 (**C**) and 5.70 mg/kg body weight (**D**) caffeine treated dams during GD 1-21. Note the normal architectural layout with no visible lesion (**A**), congestion in parts of the interstitium (red arrow) in **B**, detached germinal cells (black arrow) in **C** and interstitial edema fluid accumulation (red arrows) in **D**. Stain: H&E. Magnification: X100

Histology of testes of offspring from control and caffeine treated dams

There were visible lesions in the testes of offspring from caffeine treated dams when compared with their control counterparts. Offspring from dams that received 3.42 and 5.70 mg/kg body weight of caffeine at the different gestation days and throughout gestation had visible lesions. These were characterized by disorganized cytoarchitecture of the seminiferous tubules, empty lumen, vascular congestion, interstitial

edema and scanty germ cells with reduced germinal epithelial height. Offspring from dams that received 1.14 mg/kg body weight of caffeine throughout gestation showed congestion in parts of the interstitium. However, those from dams that received caffeine at the same dose but at the different gestation days had no visible lesion (Plates 1-4).

DISCUSSION

The results from this study showed that maternal caffeine exposure caused reduction in birth weight of offspring from dams that were treated with the highest dose of caffeine and offspring of dams treated with caffeine throughout gestation period. Low birth weight has been reported to be a marker of intrauterine growth restriction (Harding and Johnson, 1995). The results also showed that birth weight was affected by the highest dose as well as by the duration of administration. This was inferred from the fact that only those that received the highest doses and those that received caffeine all through pregnancy gave birth to pups with low birth weight.

Gestation day (GD) 1-7 in rats encompasses the preimplantation period and beginning of implantation (Witschi, 1962) and the developmental changes arising before implantation are likely to affect cell lineages (Fowden and Forhead, 2001). GD 8-14 in rats is the period of organogenesis and maximum fetal growth during which environmental insults may cause discrete structural defects that permanently reduce the functional capacity of the organ (Rhind *et al.,* 2001). During the phase of rapid growth, insults which alter the supply, uptake and utilization of nutrients will influence tissue growth and may switch the cell cycle from proliferation to differentiation with adverse consequences for total cell number (Fowden *et al.*, 1998).

Aldridge *et al.,* (1981) noted that caffeine enters the intracellular tissue water and is found in all body fluids and plasma. Caffeine increases metabolic rate and also speeds the rate of oxidative phosphorylation in the mitochondria so that the energy rich compound ATP is formed. It may cause a rise in the absorption of Iron and Calcium from food by a mother during pregnancy thereby depriving the fetus. These may affect fetal nutrition, causing a negative effect on growth and hence intrauterine growth restriction. Therefore, the low birth weight of the pups in this study may be attributed to the aforementioned factors, which correlate with the observation of Bracken *et al.,* (2003). However, there was an eventual catch up growth of the male offspring of caffeine treated dams at adulthood suggesting that decrease in birth weight in early postnatal life were transient. The association between low birth weights to adult disproportionate phenotype has been linked to poor nutrition and oxygenation during early life because of placental compromise (Harding and Johnson, 1995).

Anogenital distance is the distance from the upper rim of the anus to the caudal rim of the genitalia, i.e base of the penis or vagina. The anogenital distance has been used to determine the sex of animals, since males have longer lengths than females (Hsieh *et al.,* 2008). Moreover, human studies in infants have also established that boys have longer perineal length than girls (Thankamony *et al.,* 2009). Measuring the anogenital distance in neonatal humans has been suggested as a noninvasive method to determine male feminization and thereby predict neonatal and adult reproductive disorders (Welsh *et al.,* 2008). If insult occurs during gametogenesis, reproductive potential of the next generation may be impaired (Rhind *et al.,* 2001). In the present study, all male pups of dams treated during GD 1-7 and GD 1-21 showed significantly increased AGD index. Also male pups of dams treated with 1.14 mg/kg and 5.70 mg/kg body weight of caffeine during GD 8-14 showed a significant increase in AGD index and pups from dams treated with 3.42 mg/kg body weight of caffeine during GD 15-21 showed significant increase in AGD index. Anogenital distance index shows a relationship between the AGD and body size. It is a ratio of anogenital distance and cube root of body weight which gives a normal index (Vandenberg and Huggett, 1995). Michael *et al.,* (2011) reported that AGD varies based on the integrity of androgen pathways. Sertoli cells contain aromatase or *CYP19* which is the key enzyme responsible for the conversion of androgens to estrogens. The action of caffeine may be by inhibiting this *CYP19 or aromatase,* preventing the conversion of testosterone to estradiol. Hence it is suggested that this may be the reason for the longer AGD that was noted in this study which supports the findings of Clark *et al.,* (1990) who reported that a longer AGD is controlled by dihydrotestosterone, which is responsible for masculinization of external genitalia.

There were no significant differences in the serum concentrations of follicle stimulating hormone and luteinizing hormone of offspring from all treated dams as compared with that of the control offspring. Offspring of dams treated during GD 8-14 and GD 15-21 with 3.42 mg/kg and 5.70 mg/kg body weight of caffeine respectively, showed significantly reduced serum testosterone level. Testosterone is required in the normal development of the male reproductive organs. Its decrease in this study corresponds with the significant decrease in weight of reproductive organ of the offspring from the caffeine treated dams that were observed particularly the testes, epididymis and seminal vesicles as compared to the control offspring.

There was a significant increase in sperm motility of offspring from dams treated with 5.70 mg/kg body weight of caffeine during GD 1-7 and GD 1-21, while

sperm count showed no significant difference in the offspring from all treated dams compared to control offspring. As the spermatozoa leave the testes they are not fully motile, they continue their maturation and acquire progressive motility during their passage through the epidydimis. Caffeine is a cyclic nucleotide phosphodiesterase inhibitor, which may act directly by affecting cellular metabolism, though its effect depends on the concentration of calcium ion. Meanwhile sperm maturation process in the epididimis involves activation of a unique protein called CatSper which is localized in the principal piece of the sperm tail. This protein is a calcium ion channel and it permits the cyclic AMP-generalized calcium ion influx. El-menorfy *et al.,* (1986) stated that the effect of inhibiting phosphodiesterase as a result of caffeine activity may result in an increase in intracellular cyclic AMP thereby potentiating the permission of the cyclic AMP-generalized calcium ion influx. Cyclic AMP initiates the changes which takes place during the process of sperm capacitation which includes increase in sperm motility among other changes. The increased sperm motility that was shown in the present study can be related to the findings of El-Gaafary (1994), where he noted that caffeine markedly increased and maintained the respiration and motility of ejaculated bovine spermatozoa.

Histology results showed that administration of caffeine to the dams throughout the period of pregnancy caused dose dependent alterations in the cytoarchitecture of the testes of the offspring. Vigezzi *et al.,* (2006) reported that the extent of testicular damage is closely related to the duration of drug consumption. The offspring of dams treated with 3.42 and 5.70 mg/kg body weight of caffeine throughout gestation and during the different gestation periods showed varying degrees of testicular abnormalities; GD 8-14 showed the most severe alteration especially in the basal lamina, germinal epithelium and interstitium. Rhind *et al.,* (2001) showed that GD 8-14 in rats is the period of organogenesis and maximum fetal growth during which environmental insults may cause discrete structural defects that can permanently reduce the functional capacity of the organ. This may be a cause of the alteration in functional capacity of the cells of the testes to secrete the male reproductive hormones as observed in this study. Also, the accumulation of fluid in the interstitium which is often an indication of other health problems may have impaired the normal Leydig cell function, thus affecting the amount of testosterone secreted. Alteration in basement membrane could impair testicular metabolism, thus causing germinal cell hypoplasia and tubular atrophy (Reuhla *et al.,* 2001) which may have resulted in reduced germinal epithelial height thereby affecting the number of spermatogenic cells.

CONCLUSION

This study has shown that maternal exposure to caffeine had adverse effects on the birth weight, cytoarchitecture of the testes and serum testosterone level of male offspring of Wistar rats. Maternal administration of caffeine during gestation day 8-14 caused the most severe alterations.

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Niger. J. Physiol. Sci. 30(2015) 059-064
www.njps.com.ng

Niger. J. Physiol. Sci.

Effects of Hypothyroidism and Exogenous Thyroxine on Gastrointestinal Organs of Rat

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Summary: Thyroxine (T4) is important in gut development and maturation, and its use in treating hypothyroidism is becoming more popular. This study was conducted to evaluate the effect of thyroidectomy and thyroxine replacement on some gastrointestinal organs. Ten out of 20 thyroidectomised rats received 100pg/kgbw of T4 for five weeks to become euthyroid while the rest were left to become hypothyroid. Ten sham operated rats were made hyperthyroid by giving 100pg/kg.bw of T4 for five weeks, while the other ten sham operated rats served as control. 10mg/kg.bw intraperitoneal injection of ketamine was given as anesthesia for thyroidectomy and sham operation. At the end of the fifth week, the animals were sacrificed. Liver, stomach and small intestine were harvested and their morphological dimensions measured. Everted sacs were made from the small intestine for glucose transfer studies and slides for histomorphometry. There was no significant difference in the weights of the liver and stomach of the groups when compared with the control group. There was significant ($p<0.05$) increase in length and diameter but reduced wall thickness in the hyperthyroid small intestine; unlike that of hypothyroid which had significant ($p<0.05$) shorter length, decreased diameter but increased wall thickness. Villi length and crypt depth was higher in hyperthyroid ($p<0.01$) but smallest in the hypothyroid ($p<0.05$). Glucose transfer was lesser in the hypothyroid but greater in the hyperthyroid intestine. These findings show that hypothyroidism diminishes the morphological variables of absorption in the small intestine as a mechanism to reducing its transfer capacity, while thyroxine replacement increases these variables as mechanism to increasing intestinal transfer capacity.

Keywords*:* Thyroidectomy, Thyroxine, Gastrointestinal organs, Everted sac, Glucose transfer.

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Manuscript Accepted: December, 2015

INTRODUCTION

Thyroxine (T4) is important in the development of the gut (Hodin, et al., 1992) and postnatal intestinal maturation (Hodin, et al., 1996). The potential role of the intestine, in the interactions of the gut with thyroid hormone, is to serve both as a reservoir for thyroid hormones and as a regulator of the hormone activity. Excess of or deficiency in thyroid hormone alters the function and metabolism of the gastrointestinal tract with hypothyroidism appearing to affect the gastrointestinal (GI) tract more profoundly than hyperthyroidism. Thyroxine affects the transportation of substances, including glucose, across the small intestine. A good number of researchers have reported an increased glucose transport after thyroxine treatment (Adeniyi and Oloowookurun 1987; Olaleye and Elegbe 2005), while some have reported an inhibition of glucose transport in the small intestine upon thyroxine ingestion (Halliday, et al., 1962; Matty and Seshadri 1965). Khoja and Kellett (1993), for instance, have reported normal glucose absorption and an increased net transmural transport of glucose. Thus, animal studies have demonstrated complex and conflicting effects of thyroxine on active, electrogenic transfer of glucose in the small intestine, even though it was shown, in a more recent study that glucose transport across the small intestine was increased (Fabiyi, et al., 2014). GI tract disorders common to hypothyroidism include: intestinal pseudo-obstruction, intestinal myxedema, dilatation of the GI tract and decreased intestinal motility among others. Thyroxine (T4) is the first hormone replacement therapy, first initiated over a century ago. Shafer, et al., (1994) has been able to show that gastrointestinal motility was corrected in hypothyroid patients upon T4 replacement. It is, however, observed that there is paucity of comprehensive research on how chronic administration of oral T4 alters the morphology of the gastrointestinal tract and the influence of such alteration on absorption of substances in the small intestine. Again, considering the fact that exogenous Thyroxine (T4) orally administered is absorbed from the lumen of the jejunum and ileum within hours of ingestion (Hays 1991); this study seeks to investigate

how exogenous thyroid hormone, upon chronic administration, affects gastrointestinal function and morphology, especially those concerned with absorption. And the extent to which the structural gastrointestinal alterations seen in hypothyroidism can be reversed by T4 replacement.

MATERIALS AND METHODS

Experimental animals

Forty albino Sprague Dawley rats weighing 150-200g were obtained from the animal house in the Department of Anatomy, University of Ibadan. They were kept in a 12-hour light and dark cycle animal house with standard animal housing conditions and were fed with standard rat pellets and clean water ad libitum until the experiment was carried out (Guide for the Care and Use of Laboratory Animals, NIH publication 86-23 revised 1985). Experimental protocols complied with the 'Principle of Laboratory Animal Care' (NIH publication No. 85-23) guidelines (PHS, 1996).

Animal grouping

Forty (40) animals were used for this study. Twenty (20) of them were sham operated (SO), while the remaining twenty (20) were thyroidectomised (TX), after anesthesia with an intraperitoneal injection of 10mg/kgbw ketamine hydrochloride. Ten SO rats were given 100pg/kgbw of levothyroxine (Forley Generics Ltd, UK), which was administered orally for 35 days to make them hyperthyroid; while the other ten SO rats served as control. Again, ten TX rats orally received 100pg/kgbw levothyroxine orally for 35 days to serve as the Euthyroid group, while the other ten TX rats served as the hypothyroid group.

Thyroxine (T4) Assay

On the 35th day, blood was collected from the animals through cardiac puncture. The blood was centrifuged at 3,000rpm for 5 minutes, and the serum was separated. Serum T4 was quantified using immuochemiluminiscence.

Animal sacrifice

The rats were sacrificed on the 35th day post-surgery via cervical dislocation. Their stomachs, small intestines and liver were harvested and weighed.

Morphological measurements

Length of the small intestine of the rats was measured using thread and a standard meter ruler. Intestinal thickness and diameter were measured by means of a digital venire caliper.

Glucose transport studies

Everted sacs were made from jejunum and ileum (- as described in an earlier study- Fabiyi, et al., 2014). 2mls of Krebs solution containing 522mg/dl concentration of glucose was injected into the sacs (serosal fluid) before tying its ends. The sacs were incubated in 15mls of Krebs bicarbonate solution containing 522mg/dl (mucosal fluid) for 30minutes. Glucose concentration in the sac and in the suspending fluid was measured using a glucometer. Disappearance of glucose from the outer mucosal fluid was termed mucosal glucose transfer (MGT) while increase in glucose concentration in the inner serosal fluid was termed serosal glucose transfer (SGT).

Preparation of Tissues for Microscopic examination

Tissue preparation for microscopic examination was done in line with the method of Drury and Wallington (1994). The small intestine was cut open and its contents emptied and the intestine rinsed in normal saline. Tissue blocks from the small intestine was fixed in 10% neutral formation after which they were dehydrated using alcohol and then cleared in xylene. They were embedded in paraffin wax and thin sections cut at 5 microns. The sections were stained with hematoxylin for 15 minutes, differentiated with 1% acid alcohol counter-stained in eosin for 2 minutes and mounted with dextrene polystyrene xylene (DPX). The sections were viewed under a microscope, after which photomicrographs were taken.

Determination of Villus Dimension (Histomorphometry)

The dimension of villi was measured in sections of the intestine examined under a dissecting microscope. Images of jejunum and ileum were captured using a digital camera, and were displayed on a computer connected to the microscope. Five villi each were selected for macroscopic analysis using x40 magnifications. The villus height was taken as the distance from the crypt opening to the tip of the villus and the crypt depth measured from the base of the crypt to the level of the crypt opening (Obembe et al., 2011). Motic image plus 2.0ML software was used to measure the villus height and crypt depth.

Statistical Analysis

Data was expressed as mean ± SEM and analysed using GraphPad Prism version 4.00 (GraphPad Software, San Diego California USA). Statistical significance was tested using One-way ANOVA with Newman Kaul's post-test. P value of 0.05 was considered significant.

RESULTS

Hyperthyroid group had significantly ($p<0.001$) high thyroxine level, while hypothyroid T4 level was markedly ($p<0.05$) lower than that of the control (Figure 1).

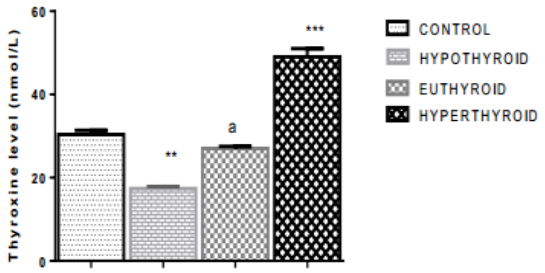

Figure 1: T4 level in the rats after the treatment period. *The Values are expressed as Mean ± S.E.M. (n=10 rats). **p<0.01, ***p<0.001, ᵃP>0.05*

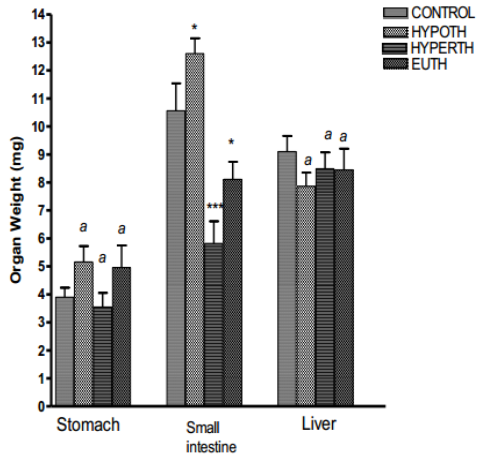

Figure 2: Gastrointestinal organ weights of the rats after the treatment period. *Values are expressed as Mean ± S.E.M. (n=10 rats). **p<0.01, ***p<0.001, ᵃ P>0.05*

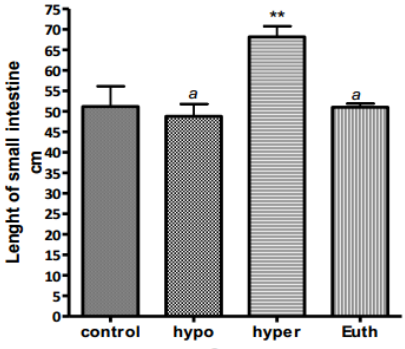

Figure 3: The length of the small intestine after the treatment period. *Values are expressed as mean ± S.E.M. (n = 10 rats). **p<0.01; aP> 0.05*

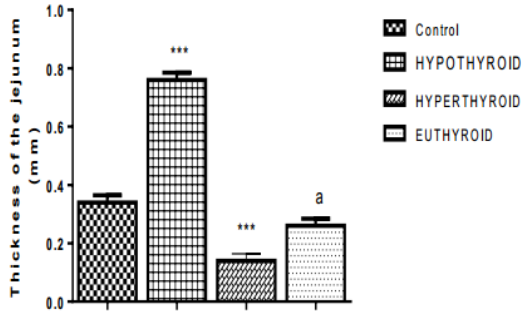

Figure 4: Thickness of the small intestine of thyroxine treated and thyroidectomised rats. *Values are expressed as mean ± S.E.M. (n = 10 rats). **p<0.01 significantly different from control; aP>0.05 no significant difference from control.*

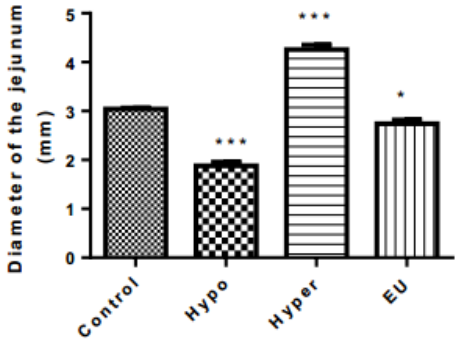

Figure 5: Luminal diameter of the small intestine of thyroidectomised and thyroxine treated rats. *Values are expressed as mean ± S.E.M. (n = 10 rats). ***p<0.001; *p<0.05*

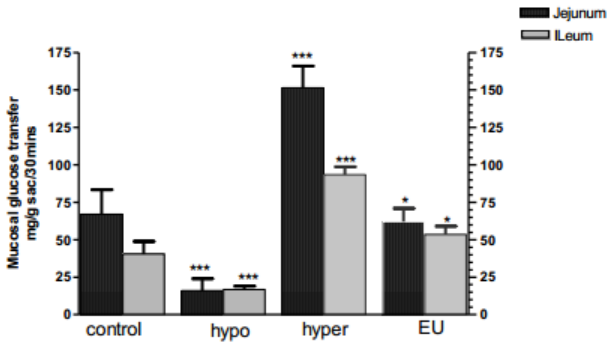

Figure 6: Mucosal glucose transfer capacity in the small intestine of TX and T4 treated rats. *Values are expressed as mean ± S.E.M. (n = 6). *p<0.05, ***p<0.001 significantly different*

The weights of the stomach and liver were significantly unaffected by either TX or T4. However, the hypothyroid small intestine showed a significant (p<0.05) decrease in weight, while the hyperthyroid and euthyroid small intestines showed significant (p<0.5) increase in weight (Figure 2). In addition, the hyperthyroid small intestine was significantly (p<0.01) longer than the control (Figure 3). Hypothyroid small intestine showed significant (p<0.001) increase in thickness and decrease in luminal diameter, while hyperthyroid small intestine was greatly (p<0.001) reduced in thickness but increased in luminal diameter (figures 4 and 5). Mucosal glucose transfer (MGT) is an index of Glucose transfer capacity in the small intestine. Hyperthyroid small intestine had a greater (p<0.001) MGT while hypothyroid small intestine had the least MGT (figure 6).

Histomorphometry

Villi length was highest (p<0.05) in hyperthyroid jejunum and ileum, but was smallest (p<0.05) in the hypothyroid jejunum and ileum. Euthyroid jejunum villi lenght was greater (p<0.05) than the control. Crypt dept was lower (p<0.01) in hypothyroid but higher (p<0.05) in hyperthyroid than control (table 1). The villi of the hypothyroid jejunum appeared shorter

Table 1: Villus length and crypt depth in the small intestine of hypothyroid and thyroxine treated rats

	Villus Length		**Crypt depth**	
	Jejunum (µm)	Ileum (µm)	Jejunum (µm)	Ileum (µm)
CONTROL	911.16 ±33	636.53±52.6	257±15	126.77±17.6
HYPO	608.29±29.5*	491.3±60.5+	122.6±19.4*	148.6±23.4[a]
HYPER	2548.63±115**	945.6±54.7*	316±11.7+	224.73±7.4*
EUTH	1052.97±130+	501.8±35.8[a]	237±11.4[a]	127.17±12.6[a]

Values are mean ± SEM with n=6. **p< 0.001, *p<0.01, +p<0.05 vs control, aP>0.05 not significant

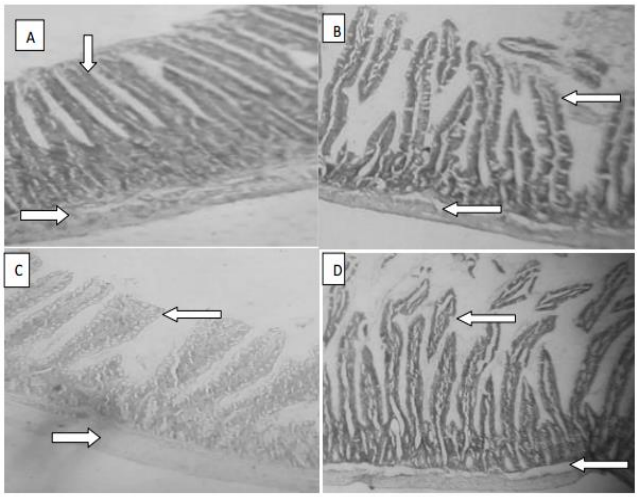

Figure 7: Histology of the small intestine (jejunum) in different thyroid states. A-Control; B- Euthyroid; C-Hypothyroid; D-Hyperthyroid

and wider (thicker), and the wall was thicker than that of other groups, as well as control. However, thinning of the intestinal wall was observed in the hyperthyroid jejunum (Figure 7).

DISCUSSION

It has previously been shown by Tata et al (1962) that the basal metabolic rate of rats is raised by the T4 administration of 10-25ug/100g body weight every fourth day. As a result of this, thyroxine dose of 100mg/kgbw/day was used in this study to induce hyperthyroidism and euthyroidism. Treatment efficacy was confirmed by determination of serum total T4 level to ascertain the establishment of the altered thyroid states in the rats. The hypothyroid group has a significantly lower serum thyroxine level than the control after the removal of the thyroid gland, thus showing a progressive fall in extrathyroidal store of thyroxine overtime.

Thyroid hormones act on almost all organs throughout the body and regulate basal metabolism. The gut and viscera are not excluded, and disturbances in thyroid function have numerous gastrointestinal manifestations (Maser et al., 2006). The seemingly reduced stomach size in the hyperthyroid and increased stomach weight in the hypothyroid may be due to increased rate of gastric emptying and muscle edema respectively. The stomach empties rapidly in hyperthyroidism, whilst in hypothyroidism gastric emptying is prolonged (Holdsworth and Besser 1968; Gunsar et al., 2003). Gastric dysmotility seen in hypothyroidism, may be as a result of altered myoelectrical activity and muscle edema (Greenspan and Rapaport 1992). The hypothyroid group had a slightly reduced liver size which may be due to decreased hepatic glucose output and activities (Liverini 1992).

The Significant increase in weight gain and intestinal wall thickness observed in the hypothyroid small intestine may be due to myxoedematous infiltration or fluid retention in the intestinal wall. According to Shafer et al., (1984), the effect of hypothyroidism on the gastrointestinal tract seems to be multifactorial and caused by infiltration of the intestinal wall with a reduction in peristalsis, being the main pathophysiologic process. The hyperthyroid group had significant decrease in the weight of their small intestines. This may be due the thinning or reduction in the thickness of the intestinal wall observed in this study, such thinning of the intestine had also been observed in dietary restricted (semi starved) rats by Neale and Wiseman (1969). The decrease in the weight of the hyperthyroid intestine observed in this study is at variance with the findings reported by Liberman et al., (1979), who observed that there was no significant change in intestinal weight of euthyroid rats made hyperthyroid, and Levin and Smyth (1963) who observed an increase in intestinal weight in hyperthyroid rats. The disparity in these studies may be due to differences in dose, duration and methods of thyroxine administration. This study, in particular, has shown that chronic administration of thyroxine to euthyroid rats results in a decrease in intestinal weight and wall thickness. Hypersecretory state within the hyperthyroid intestinal mucosa has been reported (Tenore et al., 1996, Kim and Ryan 2002), and this could be a reason for the thinning or decrease in the thickness of the intestinal wall. This may also be due to tissue wasting associated with hyperthyroidism resulting from increased metabolism. However, it was also observed, though not reported, that some of the hyperthyroid intestine had very thin

wall which bleed, resulting, suggestively, in excessive erosion of intestinal wall.

Again, unlike the reduction in the intestinal weight, there was a marked increase in the length of the hyperthyroid small intestine, contrary to the shortening of the small intestinal length of rats reported by Hindmarsh et al., (1967). Hindmarsh actually reported thinning of the intestinal wall in rats subjected to semistarvation. The semistarved rats had a weight loss of 18 -28% of their initial body weight and about 24 -29% loss in intestinal dry weight. This was accompanied by a slight shortening in the length of the intestine. Hyperthyroidism in this study mimicked this weight loss and thinning of intestine, but not the shortening of intestinal length. Our current study observed that thyrotoxic rats eat more, have increased lipogenesis and predominantly burn fat, just as Oppenheimer et al., (1991) observed in his study. This implies that although the rats' food intake was voracious, it was almost immediately burned out, and never enough to compensate for the increased metabolism induced by excess thyroxine. Thus, some conditions seen in starvation may appear. Intestinal hypermotility and increased myoelectrical activity may have led to the stretching or lengthening of the intestine in hyperthyroid rats (Wegener et al., 1992). Glucose may leave the intestine at a faster rate in the hyperthyroid state, and in the presence of intestinal hurry, may be distributed to a greater length and mucosal surface area. This could be a mechanism to make up for the short transit time and highly increased absorption seen in hyperthyroidism.

Diameter of the small intestine was significantly reduced in hypothyroid rats but increased in hyperthyroid rats. This is in agreement with the report of Fraichard et al., (1997) that the diameter of the jejunum and ileum were reduced in thyroxine receptor knockout mice.

The Initial glucose concentration was set at 522md/dl which was close to 500mg/dl used by Beryl et al (1961). The changes in concentration in mucosal and serosal solutions depended on the relative movements of fluid and glucose. Across the groups, the concentration of glucose in the mucosal fluid at the end of the everted sac experiment was lower than the initial glucose concentration. The lower mucosal glucose concentration at the end of the experiment shows a positive glucose transfer from the outer mucosal fluid to the inner serosal fluid in the sacs. The greatest mucosal transfer was observed in the hyperthyroid and the least transfer was seen in hypothyroid intestine. There was also a significant decrease in glucose concentration in the serosal fluid of hyperthyroid intestine. This means that glucose transport was impaired in hypothyroid intestine but increased in euthyroid and hyperthyroid intestines. In this study, histomorphometry showed an increase in height of the villi of hyperthyroid rat indicating hypertrophy of the microvillus border (Wall, et al., 1970). The decreased villus height and crypt depth in the hypothyroid intestine and generally in the ileum suggest reduced absorptive and secretive activity (Kelly, et al., 1998). In addition, the infiltration of the hypothyroid intestine and thickening of the wall may slow down the rate of glucose transport, thus, contributing to impaired glucose absorption. Increased villi length and crypt depth in the hyperthyroid jejunum and ileum, compared to the other groups, evidently support, the increased glucose absorption observed in hyperthyroidism, while increase in Euthyroid jejunum suggests that thyroxine replacement ameliorates the effect of thyroidectomy on the villi size.

In conclusion, thyroxine caused the thinning of the intestinal wall to reduce transfer barrier; caused the lengthening of the small intestine; increased luminal diameter of the small intestine; increased villi length and crypt depth to increase absorption; and increased glucose transport in the small intestine. But the reverse of these is obtained by thyroidectomy. These findings show that thyroxine increases morphological absorption variables in the small intestine to match the increase in absorption of nutrient such as glucose induced by thyroxine, while these variables, as well as glucose transport activities, are reduced by thyroidectomy.

Acknowledgements

The authors acknowledge Addax petroleum for the post-graduate research fund awarded for this work. Also, the authors appreciate Dr O.S Oyedun and Dr Aina of Anatomy and Veterinary departments respectively of the University of Ibadan, Ibadan, for their assistance with the histological studies in this work

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Niger. J. Physiol. Sci. 30(2015) 065-072
www.njps.com.ng

Niger. J. Physiol. Sci.

Acute Administration of Methionine Affects Performance of Swiss Mice in Learning and Memory Paradigms

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Summary: Methionine, an essential amino acid, plays an essential role in the central nervous system CNS development. It serves as a crucial intermediate in the methylation, trans-sulfuration and amino- phosphorylation pathways, necessary for the synthesis of nucleic acids, phospholipids, hormones, neurotransmitters, antioxidants, polyamines, catecholamines and other biogenic amines. The effect of methionine on learning and memory in mice was investigated using Morris water maze (MWM), Elevated plus maze(EPM) and Y maze (YM). Animals were administered with distilled water (control), methionine (1,700mg/kg); folate (3mg/kg) or methionine (1700mg/kg) plus folate (3mg/kg) for 14 days. Escape latency and time spent in target quadrants; transfer latency and percentage spontaneous alternations were measured in the MWM, EPM and YM respectively. The animals were anaesthetized with inhalational chloroform and their brains subsequently harvested, homogenized and assayed for acetylcholinesterase24 hours after the experiment. Folate significantly($p<0.05$) increased transfer latency (53.33 ± 12.62) as compared to control (20.1 ± 5.01) and reduced spontaneous alternations significantly (25.0 ± 8.9) when compared to control (44.33 ± 3.07). When folate was combined with methionine there was also a significant increase in transfer latency (43.0 ± 14.39) when compared with control (20.1 ± 5.01). Folate-methionine combination also significantly ($p<0.05$) reduced spontaneous alternations (20.4 ± 8.4) as compared to the control (44.33 ± 3.07) much more than folate alone. Acetylcholinesterase activities in all groups were not statistically significant. It can be concluded that acute methionine administration has some benefits in memory enhancement. However, a short course folate supplementation impairslearning and working memory especially when combined with methionine which may be as a result of sudden overwhelming of the methylation cycle, leading to homocysteinemia which is pro-dementia.

Keywords*:* Methionine, Memory, Learning, MWM, EPM, Y-Maze

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Manuscript Accepted: September, 2015

INTRODUCTION

Memory is one of the earliest cognitive functions to show decline during the aging process. Due to growing stress and competition in our world, memory related problems are multiplying (Parle and Vasudevan, 2007; Sunita et *al.*, 2010). It is projected that by 2050, people aged 60 and over will account for 22% of the world's population with four-fifths living in Asia, Latin America or Africa. This translates to more cases of either pathologic (e.g Alzheimer's disease) or physiologic (e.g Age associated memory impairment) memory losses (WHO, 2013). It is pertinent to note that myriads of supplements including—caffeine, *Ginkgo biloba*, *Bacopamonniera* etc have been employed to reverse memory loss, no significant outcome has been achieved (Sunita et *al.*, 2010).

Methionine, an essential amino acid, is an important substrate in the brain's methylation cycle. Interference with its metabolism has been found to result in hyperhomocysteinemea which is pro-dementia (Miler, 2003). It is an indispensable dietary amino acid required for normal growth and development of humans; a substrate for protein synthesis and the main methyl group donor to both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) intermediates (Mohammad *et al.*, 2006; Deborah *et al.*, 2007). Methionine gets activated by reacting with ATP to form S-adenosylmethionine (SAMe), which when decarboxylated, results in biosynthesis of polyamines necessary for cell proliferation and growth (Robert *et al.*, 2009). Also, methionine residues constitute an important antioxidant defense mechanism via formation of methionine sulfoxide with free radicals (Rodney, 1996) and production of potent antioxidants like glutathione and cystathione necessary for preventing cellular damage (Tor-Agbidye, 1998; Tor-Agbidye, 1999; Nkabyo, 2006). Chronic administration of methionine has been found to cause memory deficit in the Morris water maze task by the elevation of blood homocysteine levels in experimental animals. High homocysteine levels have

the potentials of causing vascular dementia either by direct cerebral vascular damage, over-activation of N-methyl-D-aspartate receptors or enhanced vulnerability of hippocampal neurons to excitotoxic insults (Rajeshkumar et *al.*, 2008; Rajeshkumar et *al.*, 2009). Similarly, chronic blood homocysteine (HCY) levels have also been shown to cause adult neurological disorders by excitotoxicity and generation of reactive oxygen species (ROS) (Louisa, 2004).

Central cholinergic system plays a major role in regulation of cognitive functions and inhibition of acetylcholinesterase leads to increased levels of brain acetylcholine (Dinesh and Kumar, 2012). Dementia illnesses (e.g Huntington's disease) has been proven to result from loss of acetylcholine-secreting neurons in the thinking areas of the cerebral cortex. Measuring levels of brain acetylcholinesterase would be a good way of knowing the brain acetylcholine levels which is a good parameter for assessing memory functions (Guyton and Hall, 2011).

To the best of our knowledge, there are paucity of data on the effect of acute administration of methionine on learning and memory in normal healthy subjects. We employed multiple mazes for a more reliable result, considering the fact that it is unsafe to rely on any one assay in making a claim regarding the psychological basis of a behavioral phenotype (Laurence, 2004).

The present study aimed at investigating the effect of methionine and folate supplementation on learning and memory in rodents. It simply seeks to know the effect of an essential amino acid like methionine (known to mediate various cellular reactions) on the learning and memory functions of an apparently normal brain. This is in a bid to proffer long-term prophylactic solutions to memory impairment that may occur in later life.

MATERIALS AND METHODS

Chemicals and Drugs

Methionine (Neros pharmaceuticals {Vietnam}Batch number:11007CX, Registration number: VD5202-08, NAFDAC number: A4-0546). Folic acid (Pharmacy of Saint Luke's Anglican Hospital Wusasa, Zaria, NAFDAC number 040169)

Animals

A total of 20 Male Swiss mice weighing between 15-25g(6-8weeks) were obtained from the Animal House, Federal College of Animal Health Facility, National Veterinary Research Institute, Vom, Plateau State, Nigeria. They were housed in standard polypropylene cages in the Animal House Facility, of the Department of Human Physiology, Faculty of Medicine, Ahmadu Bello University Zaria, Nigeria under normal environmental temperature and were fed with standard pellet diet and water, *ad libitum.* They were allowed to acclimatize to the laboratory environment for one week before the commencement of the experiment. They were randomly divided into four groups of 5 animals each (Normal control, methionine treated, methionine plus folate and folate only group). Methionine was administered orally at a dose of 1700mg/kg (Rajeshkumar, 2009) and folate was at 3mg/kg (Shin *et al.,* 1999) for 14 days. The animals were tested in the various mazes between day 11 and 14 and were euthanized on day 15 with subsequent craniotomy for acetylcholinesterase assessment.

MAZES

14 days Supplementation of Methionine and Folate in the Morris Water Maze (MWM) experiment

MWM was first described by Morris (Morris, 1981). It was used to assess visuo-spatial memory, reference memory and learning which involves using extra maze cues to find location of a hidden escape platform (Villarreala et *al.*, 2002). The maze constructed out of a circular plastic tank was filled with water at room temperature to a depth of 14 cm to enable the animal swim unhindered. A sealed cylindrical plastic container submerged 1cm below the water surface in the escape quadrant served as the escape platform. The animals were subjected to a 3-day acquisition training phase with starting points changed sequentially as shown in Table 1.

Table 1. Sequence table for acquisition training in MWM

DAY 1	Q1	Q2	Q3	Q4
DAY 2	Q2	Q3	Q4	Q1
DAY 3	Q3	Q4	Q1	Q2

The time (escape latency) to locate the hidden platform after 120seconds of exploration on each day was noted. Reduction in escape with training is a positive index of learning and memory recall. In case an animal fails to locate the hidden platform it was assisted to the platform and allowed to stay there for 30 seconds to build cohesive visuospatial memory and appropriate representation of the pool. The fourth day being the last day (the probe phase), each animal was given a single trial. They were released into the pool from Q4 (where the hidden platform has always been) but this time without the hidden platform to explore for 120 seconds. The total time spent in Q4 in search of the removed platform was measured. Prolonged time spent in the target quadrant in search of the absent platform was an index of positive memory recall.

14 Days Supplementation of Methionine and Folate in the Elevated Plus Maze (EPM) Experiment

The Elevated plus maze for mice (Lister, 1987) which have also been employed in studying learning and retention in experimental animals was also used. The maze consisted of two perpendicular open arms and

closed arms. The open and closed arms were connected by a central platform and raised 45 cm above the floor. The entire experiment lasted two days as described by Jiro (1990) and Dinesh *et al* (2012). On the first day (after completing the day-3 acquisition trial on the MWM) each animal was placed at the end of an open arm facing away from the central platform. The time taken (transfer latency) for the animal to move from the open arm to the enclosed arm within the space of 90seconds was measured. This process was repeated the next day (1 hour after completing probe trial on the MWM experiment). If the animal fails to enter the closed arm on the first day, it was gently assisted into one of the closed arms and allowed to remain there for 20seconds to enable it integrate memory. After each trial the maze was wiped with a cloth dipped in 70% ethyl alcohol and allowed to dry, to prevent subsequent animals from getting any clue. The transfer latency for Day 1 was designated T1 and that of Day 2 T2. A short transfer latency on Day 2 was taken as a measure of good memory recall.

14 days Supplementation of Methionine and Folate in the Y- Maze experiment

The Y-maze as described by Reddy (Reddy, 1998) was also used to assess working and spatial memory. It consisted of three identical arms A, B and C at 120° to each other. This test was based on the innate preference of animals to explore an arm that has not been previously explored (Swonger and Rech, 1972; Drew, 1973; Hughes, 2004). Approximately 1 hour after completing the day-2 EPM experiment, trial 1 for the Y-maze experiment was commenced. One of the arms of the maze was blocked with a shutter, allowing for a 5-min exploration of only two arms. After a 30 min delay, trial 2 was commenced in similar manner, this time with all three arms open for another 5-minutes exploration. Trial 2 took advantage of the innate tendency of mice to explore novel unexplored areas (the previously blocked arm). After each trial, the maze was wiped with a cloth dipped in 70% ethyl alcohol and allowed to dry, to remove any olfactory clue. The number of maximum spontaneous alternations (total number of arms entered minus two) and actual alternations (i.e ABC, ACB, BAC, BCA, CAB, OR CBA). Percentage alternation was therefore calculated thus:

$$\text{percentage alternations} = \frac{\text{actual alternations}}{\text{maximum alternations}} \times 100$$

High percentage alternations were considered as positive index of memory recall.

Brain preparation and Acetylcholinesterase (Ache) activity

The animals were sacrificed 24 hours after the last experiment by cervical dislocation under light anaesthesia with chloroform vapour. Following craniotomy, brain homogenization was done according to Luigi *et.al* (1968) with slight modifications. The brains of the animals were gently homogenized with a small pestle and mortar using 0.1 M sodium phosphate buffer at pH of 8. The tissue concentration was approximately 20mg of tissue per ml of buffer. The homogenates were cold centrifuged.

Samples were transferred into plain sterilized bottles and taken to Chemical Pathology Department, Ahmadu Bello University Teaching Hospital, Shika, Zaria-Nigeria for biochemical analysis. Acetylcholinesterase activities were determined spectrophotometrically using the Ellman method (Ellman et *al.*, 1961). The Ellman method for assaying enzymes is based on the reaction between thiols and chromogenic 5'5'-dithiobis-2-nitrobenzoic acid (DTNB) as it measures the formation of the yellow ion of 5'-thio-2-nitrobenzoic acid (TNB). Cholinesterase activity is measured indirectly by quantifying the concentration of TNB ion formed (i.e a product of substrate hydrolysis by cholinesterase). Absorbance was measured at a wavelength of 412mp using a spectrophotometer. Rates were calculated as follows:

$$R = \frac{(5.74 \times 10^4)AA}{C}$$

R = Rate, in moles of substrate hydrolyzed per minute per gram of tissue, AA= Change in absorbance per minute, *C*= Original concentration of tissue (mg/ml)

Statistical Analysis

Data obtained from the study were expressed as mean ± SEM. The differences between the groups were analyzed by One-way analysis of variance (ANOVA) followed by Dunnett post hoc test for multiple comparisons using SPSS statistical tool version 22. Values of $p < 0.05$ were considered significant.

RESULTS

Escape latency in MWM

There was found no statistically significant difference across the groups as seen in Fig 1. The average escape latency decreased consistently in all groups.

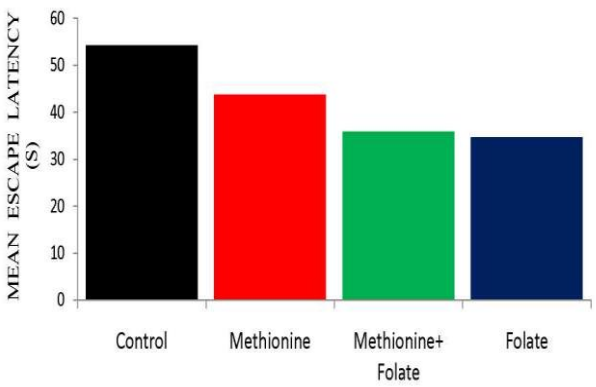

Fig 1. Effect of 14 days supplementation with methionine and folate on mean escape latency in MWM.

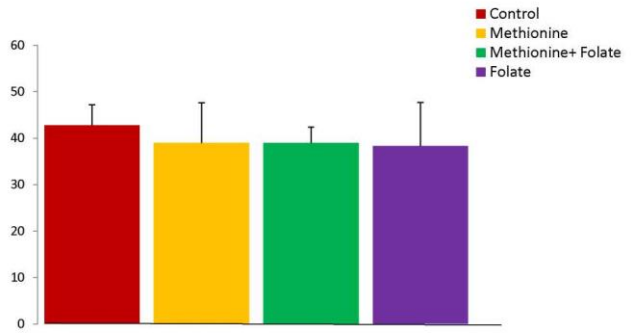

Fig 2. Effect of 14 days supplementation with methionine and folate on mean time spent in target quadrant in MWM.

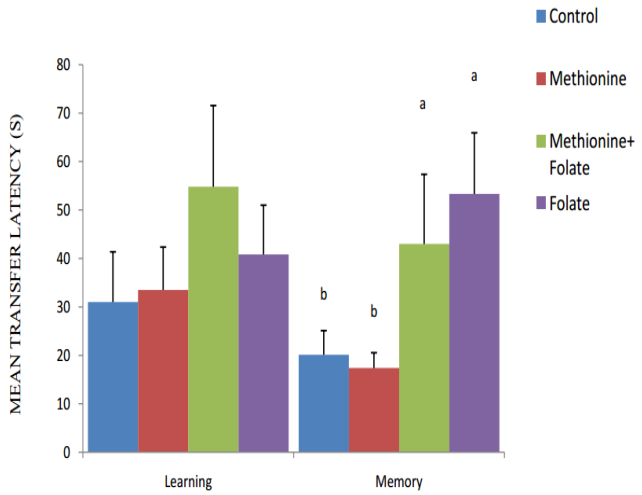

Fig 3. Effect of 14 days supplementation with methionine and folate on mean transfer latency in EPM. [a]significant increase in transfer latency, [b]significant decrease in transfer latency.

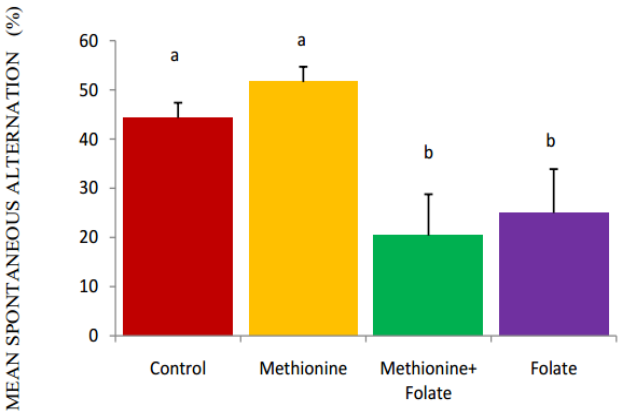

Fig 4. Effect of 14 days supplementation with methionine and folate on mean spontaneous alternation in Y-Maze. [a]significant increase in mean spontaneous alternations, [b]significant decrease in mean spontaneous alternations

Mean time spent in target quadrant in MWM

The mean time spent in target quadrant was similar across groups. There was no statistical difference between the methionine groups compared with the control. There was also no difference between the other groups and the control group as seen in Fig 2.

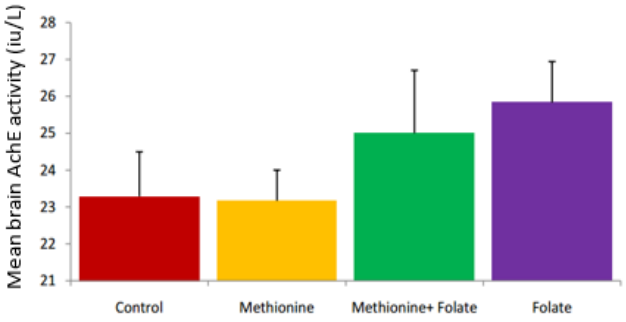

Fig 5. Effect of 14 days supplementation with methionine and folate on mean brain acetylcholinesterase activity.

Mean transfer latency in EPM

The group with methionine plus folate and that with folate alone had a significant reduction in memory recall when compared to the methionine and control group. The methionine-only group had a better performance in both learning and memory as evidenced by their shorter transfer latencies as shown in fig 3.

Percentage spontaneous alternations in Y-Maze

The methionine plus folate and the folate-only groups showed a statistically significant decrease in spontaneous alternations compared with the control as shown in Fig 5. The methionine group had the highest mean percentage spontaneous alternation but not statistically significant when compared with the control.

Acetylcholinesterase activity

There is no statistically significant difference in the brain acetylcholinesterase activity in all groups as shown in Fig 5.

DISCUSSION

The present study investigated the effect of 14 days methionine and folate supplementation on learning and memory paradigms in Swiss mice. Morris Water Maze, Elevated Plus Maze and Y Maze tests employed in this study, are widely accepted models for evaluating learning and memory in experimental animals (Rajeshkumar et *al.*, 2008; Brian *et al.*, 2009; Robert, 2004; Dinesh *et al.*, 2012).

The other groups had a reduction in escape latency compared to control but not statistically significant. Time spent in target quadrant increased proportionately across groups indicating good memory retrieval in all groups. This agrees with the work of Rajeshkumar et al (2008) where a similar downward trend in escape latency time (ELT) was seen in methionine-treated rats during the acquisition trials (Rajeshkumar et *al.*, 2008). A study by Cao *et* al (2008), agrees to some extent with this work, where they demonstrated both electrophysiologically and by Morris water maze test that S-adenosyl methionine

administered for a period of 20-22 days improved impaired learning ability induced by lead (Cao *et al.,* 2008). Similar results could be seen in the work of Sheryl et al (2008) where short-term administration of S-adenosyl methionine improved working memory in transgenic mice (Sheryl *et al.,* 2008). However, on the contrary, the work of Rajeshkumar et *al* showed that methionine administered at a similar dose of 1.7 g/kg/p.o significantly reduced time spent in target quadrant during the probe trial indicating memory impairment. This could possibly be due to the fact that they administered methionine for 32 days which was more than twice the time of exposure to methionine in this experiment. So, such chronic exposure may have resulted in the vascular dementia they found in their animals resulting from high homocysteine following chronic levels of methionine that overwhelms the brain methylation cycle (Rajeshkumar et *al.,* 2009). A similar but remote study by Wikken et *al* (1998) also revealed that individuals at risk of coronary artery disease have reduced ability to metabolize homocysteine when their methylation pathway is stressed by administering loading dose of methionine (Wikkenet *al.,* 1998). The age of the animals used in this study may also support the contrasting result, seeing that previous studies involved administration of methionine to adult and aged rats resulted in memory impairment. Still in support of the effect of age variation, Dasarathy et al (2010) propounded that young human subjects (neonates) have more efficient transsulfuration and transmethylation processes due to their high demand of glutathione from methionine metabolism (Dasarathyet *al.,* 2010).

The folate-treated mice and the folate plus methionine-treated group in the EPM experiment had significantly prolonged transfer latency when compared with the methionine and the control groups. This is an indication of suppressed short term/working memory. No study could be found that employed methionine in assessing learning and memory using specifically the elevated plus maze. Even though the results of this study tend to contradict some research findings that associate folate with improved cognitive function especially memory (Matteet *al.,* 2009; Jane *et al.,* 2007; Linda *et al.,* 1999; Huang *et al.,* 2007). Some other studies however have shown that folic acid supplementation does not affect cognitive function. In a human trial, where 195 people aged 70years and older with no or moderate cognitive impairment received either of folic acid, folic acid plus Vitamin B12 or a placebo for 24 weeks; there was no improvement in cognitive function (Eussenet *al.,* 2006). Similarly, another long term study found greater cognitive decline in people with a high intake of folic acid. The researchers therefore remarked that these findings where "unexpected" and called for further studies (Morris *et al.,* 2005). In support of the homocysteine pathway for memory impairment in folate deficiency, some studies claim that folic acid reduces high homocysteine levels but other studies propose the reverse (Malinow et *al.,* 1999). Possibly, folate is being converted to more methionine within the methylation cycle, thus resulting in hypermethioninemea and ultimately homocysteinemea, which may have resulted in the poor performance noticed in both the folate and folate-methionine groups.

The Y-maze gave a similar result with the EPM experiment. The folate only and folate- methionine groups performed poorly (reduced percentage spontaneous alternations) at p value <0.05 when compared with the methionine-only and control groups, signifying memory impairment by these supplementations. They methionine-only group had a comparatively better performance when compared to the control group though not statistically significant. This contradicts the work of Zhang *et al* (2012) where folate prevented impairment in Y-maze performance in animals demented from middle cerebral artery occlusion. It however tend to agree with the work of Carla *et al* (2007) where they discovered that folate supplementation had only very minimal benefit in apolipoproteinE deficient mice lacking B-vitamins when tested on a T-maze; with no benefit at all in a water maze experiment. Again to the best of our knowledge no work has been found which investigated the effect of methionine on working memory using the Y-maze. Similar explanation as in the EPM experiment can be proffered i.e. more folate results in hypermethioninemea leading to homocysteinemea and subsequently poor memory in the folate and folate-methionine groups.

The minimal positive effect of methionine could possibly be attributed to its sulfur component which has similar activity as other sulfur-containing amino acids like cysteine and glutathione which has been well established as potent antioxidants; protecting neural cells not only from oxidant damage and apoptosis but also form progression of the pathology of dementia illnesses (Andrew, 2006; Andrew *et al.,* 2010).

Acetylcholine is considered to be one of the important neurotransmitter involved in the regulation of cognitive functions. Cognitive dysfunction has been shown to be associated with impaired cholinergic transmission and the facilitation of central cholinergic transmission resulting in improved memory. Moreover, selective loss of cholinergic neurons in certain brain parts appeared to be a characteristic feature of senile dementia (Dinesh *et al.,* 2012). Brain acetylcholinesterase activity in this study remained stable in all groups. This contradicts the finding of Rajeshkumar et *al* in which chronic administration of methionine at 1.7g/kg p.o led to a rise in brain

acetylcholinesterase activity. The possible explanation of this discrepancy could be as a result of the differences in the duration of exposure to methionine, where in this study, it was more a less an acute exposure. To support this assertion, Franciella et al (2007) in their work showed that acute administration of methionine did not alter cerebral cortex AChE activity, rather only chronic experimental hypermethioninemia caused cognitive dysfunction and an increase of AChE activity that might be related, at least in part, to the neurological problems presented by hypermethioninemic patients (Franciellaet *al.*, 2007).

In conclusion, this study employed the use of multiple neurobehavioural models in assessing the possible effect of acute methionine administration on learning and memory paradigms in Swiss mice. Methionine was found to possess minimal benefits on learning and memory which may be attributable to its sulfur component which is neuroprotective. Acute folate supplementation proved counterproductive when the EPM and Y-maze were employed as test models; more so when combined with methionine. Possible overwhelming of the brain methylation cycle, leading to increased homocysteine (which is pro-dementia) could be an explanation. Further work will involve investigating the influence of graded doses of methionine on learning and memory. Also, assaying other brain neurotransmitters like dopamine, glutamate as well as neurotrophic factors such asbrain derived neurotrophic factor (BDNF) in a bid to establishing possible mechanism(s) of action.

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Niger. J. Physiol. Sci.

Genistein Precipitated Hypothyroidism, Altered Leptin and C-Reactive Protein Synthesis in Pregnant Rats

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Summary: Genistein is an isoflavone constituent of soya. This study examined the mechanism by which genistein produced adverse effects in pregnant laboratory rats. Pregnant rats were divided into control (Con) and genistein (Gen) force fed (2 mg/kg) groups. At terminal gestation day (GD) ranging from 0-20, the rats were sacrificed, and blood samples and amniotic fluids were collected. Thyroid hormone, C-reactive protein (CRP) and leptin assay was carried using the blood samples. Leptin was also assayed in the placenta and amniotic fluid supernatant. Oral exposure of pregnant rats to genistein significantly altered maternal T3, (GD18; Con 1.65 ± 0.01, Gen 1.03 ± 0.04 nmol/L), T4 (GD6; Con 29.60 ± 0.00, Gen 36.04 ± 1.29 nmol/L), Leptin (Placenta GD20; Con 0.08 ± 0.01, Gen 0.31 ± 0.02 ng/ml, amniotic fluid ;GD 20; Con 0.02 ± 0.00, Gen 0.35 ± 0.05 ng/ml) in genistein group. These changes were accompanied with loss of embryonic implants and a decrease in fetal and placental weights. The CRP level was significantly decreased and increased at the onset and toward late pregnancy respectively. Oral exposure of pregnant rats to genistein precipitated hypothyroidism, altered some metabolic hormones with a reduction in fetal and placental growth and increased resorption of embryonic implants.

Keywords*:* Genistein, embryonic implants, pregnancy, thyroid hormone, leptin, C - reactive protein.

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Manuscript Accepted: October, 2015

INTRODUCTION

Genistein is a non-steroidal phytoestrogen (4, 5, 7-trihydroxyisoflavone) derived from soya and soya products. Although it is also present in other legumes, the highest concentration is found in soya and soya products (Price and Fenwick 1998). Infant soya formulae contain approximately 65% of genistein (Chen and Donovan 2004). Genistein exerts estrogenic effects, having a higher affinity for the beta than for alpha estrogenic receptors (Santell et al. 1997). It is a known tyrosine kinase inhibitor (Akiyama et al. 1987) and has the potential to inhibit several intracellular enzymes (Degen 1990).

Early exposure of female rats to genistein before sexual maturity resulted in marked structural and functional changes in their reproductive organs before the normal sexual maturity age (Awoniyi et al. 1998; Awobajo et al. 2013). Genistein has also been shown to exert anti-obesity effects with prompt weight reduction and reduced fat deposit during exposure in ovariectomized mice and non-pregnant female rats (Naaz et al. 2003, Kim et al. 2006, Nwicka-Stanczk et al. 2012). This effect has been adduced to its ability to down regulate lipoprotein lipase gene expression in adipose tissue with a possible decrease in blood lipid profile (Yousef et al. 2004). Genistein has also been reported to influence two body hormones, i.e. insulin and leptin, closely linked to body metabolism and food consumption. Exposure to genistein precipitated a reduction in insulin and leptin level in sexually immature female rats (Nogowski et al. 2007) and at higher doses in mature non pregnant rats (Nwicka-Stanczk et al 2012).

Establishment of pregnancy is dependent upon successful completion of fertilization leading to blastocyst implantation in the endometrium. Several bioactive agents produced within the maternal environment and those found to have originated from the environment can influence implantation, uterine receptivity and embryonic development. Several substances, including leukocyte inhibitory factor, heparin-binding epidermal growth factor (EGF), colony stimulating factor-1, interleukin-1 (Pampfer et al. 1991; Stewart et al. 1994; Simon et al. 1994), calcitonin (Ding et al 1995), and thyroid hormones (Zhang et al. 1997; Ashkar et al. 2009) have been observed to actively participate in the regulation of the implantation process. Reduced fetal and placental weight was reported as part of the consequences of genistein exposure in pregnant rats at a dose of 2 mg/kg body weight (Santell et al. 1997). It is well established that thyroid hormones play a significant role during in-utero development and even during neonatal and infant stages of development (Legrand 1986). They also have a long term impact on the behavior, locomotors ability, speech, hearing, and cognition of the offspring (Legrand 1986; Biondi and

Cooper 2008). The thyroid hormones; thyroxine (T4), and triiodothronine (T3), are tyrosine based hormones that are involved in the regulation of cellular metabolism. Their deficiency during pregnancy has been linked to reduction in placental and fetal growth (Evers 2012). To the best of our knowledge, most experimental and clinical trials on genistein and thyroid hormone to date have not included a pregnant model. There have been reports on healthy adult male and female subjects (Hampl et al. 2008), prostate cancer patients (Lazarevic et al. 2011), oophorectomized women (Mittal et al. 2011), and osteopenia postmenopausal women (Bitto et al. 2010). There have also been experimental reports in mature healthy male and female rats (Nwicka-Stanczk et al. 2012).

Therefore, we have attempted to determine the thyroid hormone profile, and leptin level in amniotic fluid, placental tissue, and plasma C-reactive protein (CRP) levels during pregnancy in rats orally exposed to genistein. Embryo implantation sites were also counted while fetal, and placenta growth were quantified with the respective weights.

MATERIALS AND METHODS

Chemical: Genistein (purity 98.2% was purchase from Chengdu Biopurify Phytochemicals ltd. China

Animals grouping and drug administration: Fifty-four adult regularly cycling female Sprague-Dawley rats, weighing between 150-160 g were used for this study. At proestrous stage, they were cohabited overnight with male rats (ratio; 1 male: 2 females). The presence of sperm cells in the vaginal smear confirmed successful mating and the day this was observed was recorded as day zero of pregnancy (Marcondes et al. 2002). The pregnant rats were divided into the following two groups; control (Cont) and genistein treated (Gen). Each group was further sub-divided into the following five sub-groups; pregnancy day 0, 6, 12, 18 and 20 of six rats per sub-group (the two groups shared day zero as there was no treatment done on this day). Rats in the Gen group were orally treated with genistein suspended in dissolved in distilled water at a dose of 2 mg/kg body weight per day. Our earlier studies using 1 mg and 2 mg/kg body weight showed significant pregnancy outcome impairment at 2 mg/kg body weight (Awobajo et al. 2013) throughout the gestational period. There are several reports of usage of wide dosages of genistein ranging from 0.2 mg to 100 mg/kg body weight in rodents (Elsa and Wendy 2010). The control group received equal volume of distilled water; the vehicle for genistein administration. Clean water and phytoestrogen-free rat chow was provided ad libitum throughout the experimental period. All protocols used including animal welfare, dissection, and humane euthanasia were approved by the Research and Ethics Committee of the Institution and they conformed to the Guidelines for Care and Use of Laboratory Animals in Biomedical Research (National Institute for Health 1985).

Blood and internal organs collection: Six pregnant rats from each sub-group were stunned and sacrificed by cervical dislocation at GDI, 6, 12, 18, and 20 respectively. Blood samples were collected into heparinized sample bottles via cardiac puncture, and centrifuged at 3000 rpm for 15 min to separate plasma. After bilateral ovariectomy, amniotic fluid was carefully syphoned out of the amniotic sac using a 13-gauge needle into sterile sample bottles, spun and filtered. Uterine horns containing conceptuses were removed and immediately placed on ice while number of embryonic implants and resorption sites were recorded for each rat. Placental were separated and placental tissues from each litter were pooled (four placental tissue from each of the six rats to make 21 placental tissues per sub-group). The ovaries were dissected out and the number of corpus luteum counted and recorded. Placental homogenates (10.0% w/v) were prepared in cold phosphate buffer solution (PBS), using a mechanically driven homogenizer, immersed in an ice pack, and then centrifuged at 3000 rpm for 20 min to obtain the homogenate. The homogenate was subsequently filtered with 40 gauge filter and used for the leptin assay. All the samples for assays were stored at -80^{o}C temperature until used.

Hormonal analysis: Thyroid hormones (T3, T4) were assayed in the plasma using an Enzyme-linked Immunosorbent assay kit (ELISA) while TSH was assayed using Enzyme Immunoassay (EIA) kit (ELISA) according to the manufacturer specifications. Leptin was assayed in the plasma, amniotic fluid and placental tissue homogenate using rat leptin ELISA kit (Crystal Chem Inc, USA) while rat CRP ELISA kit (Immunology Consultants Laboratory Inc. USA) was used for the CRP assay.

Statistical Analysis: Results are presented as mean ± standard error of mean (SEM), differences between each group of rats were statistically evaluated using two-way analysis of variance and post hoc Duncan's multiple range tests. Differences were considered significant at $P \leq 0.05$. A line chart was also used for graphical representation.

RESULTS

Number of embryonic implants, fetal and placenta weights obtained from genistein exposed rats at different gestational days compared to control rats (Table 1):

There was a significant decrease in the number of implanted fetuses or developing embryo at GD 12, 18

and 20 in genistein exposed rats as compared with the number recorded in control rats. There was however, no significant difference in the within group results of number of implanted fetuses or developing embryo between GD 6, 12, 18 and 20 either in the genistein exposed or control group. There was also a significant reduction in the weights of all fetuses and placentas harvested at GD 6, 12, 18 and 20 in the genistein exposed rats compared with those of the control rats.

Pattern of thyroid hormones at different days of pregnancy in genistein treated compared with control rats (Table 2):

TSH concentration pattern in the control group within the gestational period measured showed an initial decrease from GD 0 to 6, followed by an increase towards GD 12 and a subsequent reduction in the plasma concentration towards GD 20. In genistein

Table 1: Number of embryonic/fetal implantation sites recorded at different stages of pregnancy along with the weights of the embryo/fetus and their placenta in pregnant rats orally treated with genistein at a concentration of 2mg/kg body weight compared with unexposed control pregnant rats. Results presented as mean ± SEM, $p \leq 0.05$, (n=6)

Gestation day	Embryo implantation		Fetal weight (g)		Placental weight (g)	
	Control	**Genistein**	**Control**	**Genistein**	**Control**	**Genistein**
0	NA	NA	NA	NA	NA	NA
6	9.20 ± 1.11	8.40 ± 1.03	0.78	NA	NA	NA
12	10.00 ± 0.71	7.80 ± 0.37[b]	1.35	0.24 ± 0.02[b]	1.67 ± 0.25	1.22 ± 0.01 [b]
18	9.80 ± 0.58	7.01 ± 0.45 [b]	1.42	0.84 ± 0.06 [b]	2.03 ± 0.11	1.50 ± 0.01 [b]
20	9.20 ± 0.66	7.02 ± 0.71 [b]	1.89	1.25 ± 0.04 [b]	2.20 ± 0.14	1.53 ± 0.15 [b]

b Significant decrease NA=Data not available

Table 2: Pattern of plasma thyroid hormones and the T3/T4 ratio at different stages of pregnancy in pregnant rats orally treated with genistein at a concentration of 2mg/kg body weight compared with unexposed control pregnant rats. Results presented as mean ± SEM, $p \leq 0.05$, (n=6)

Gestation day	Group (n=6)	TSH (pIU/L)(x 10^{-3})	T3 (nmol/L)	T4(nmol/ L)	%A in TSH within group(x10"^{3}) (after-before)	%A in T3 within group (x10^{-3})	%A in T4 within group (x10^{-3})	T3/T4 ratio
0	Control Genistein	2.15 ± 0.15	1.17 ± 0.03	57.27 ± 3.22				0.0204 ± 0.0006
6	Control	0.30 ± 0.00	1.10 ± 0.02	29.60 ± 0.00	-616.67 ± 50.00	-8.06 ± 3.34	-97.22 ± 7.31	0.0371 ± 0.0008
	Genistein	3.35 ± 0.15 [a]	2.10 ± 0.11[a]	36.04 ± 1.29	35.89 ± 1.61	69.45 ± 10.42	-63.44 ± 9.34	0.0584 ± 0.0009[a]
12	Control	4.75 ± 0.75	1.24 ± 0.04	30.89 ± 2.57	93.53 ± 1.02	12.90 ± 3.26	6.62 ± 5.60	0.0402 ± 0.0021
	Genistein	4.00 ± 0.00 [b]	1.27 ± 0.01[a]	36.04 ± 2.57[a]	16.25 ± 3.75	-73.65 ± 0.08	3.06 ± 7.30	0.0354 ± 0.0027 [b]
18	Control	0.55 ± 0.25	1.65 ± 0.01	30.25 ± 0.64	-1066.67 ± 666.67	24.37 ± 1.71	-6.01 ± 7.17	0.0546 ± 0.0009
	Genistein	2.15 ± 0.05[a]	1.04 ± 0.04 [b]	25.74 ± 0.00	-86.15 ± 4.33	-23.60 ± 2.51	-41.82 ± 6.05	0.0403 ± 0.0015[b]
20	Control	0.00 ± 0.00	1.96 ± 0.04	64.06 ± 3.86	0.00 ± 0.00	15.27 ± 0.83	50.53 ± 1.78	0.0311 ± 0.0013
	Genistein	3.95 ± 0.25[a]	1.77 ± 0.08 [b]	21.88 ± 0.00[b]	45.27 ± 4.73	68.44 ± 16.04	-18.01 ± 0.37	0.0807 ± 0.0035[a]

a Significant increase b Significant decrease

Table 3: Leptin concentration in the maternal plasma, placenta tissue homogenate and amniotic fluid at different stages of pregnancy in pregnant rats orally treated with genistein at a concentration of 2mg/kg body weight compared with unexposed control pregnant rats. Results presented as mean ± SEM, $p \leq 0.05$, (n = 6)

Pregnancy Day	Leptin level (ng/ml)					
	Maternal plasma		Placenta homogenate		Amniotic fluid	
	Control (n=6)	Genistein (n=6)	Control (n=6)	Genistein (n=6)	Control (n=6)	Genistein (n=6)
0	0.32 ± 0.06		SNA		**SNA**	
6	0.24 ± 0.06	1.84 ± 0.00[a]	SNA	SNA	SNA	**SNA**
12	0.52 ± 0.02	1.78 ± 0.17 [a]	0.03 ± 0.01	0.16 ± 00.01[a]	0.01± 0.00	**0.16 ± 0.04 [a]**
18	0.29 ± 0.04	0.70 ± 0.10 [a]	0.06± 0.01	0.26 ± 0.03 [a]	0.01± 0.00	**0.24 ± 0.12 [a]**
20	0.31 ± 0.03	1.86 ± 0.13 [a]	0.08 ± 0.01	0.31 ± 0.02 [a]	0.02 ± 0.00	**0.35 ± 0.05 [a]**

[a] Significant increase at $P \leq 0.05$ [b] SNA= Sample not available

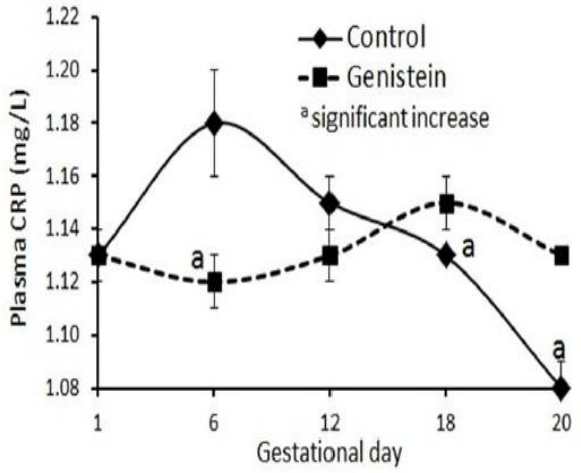

Fig. 1: Plasma C-reactive protein level at different days of pregnancy in rats exposed orally to genistein (2mg/kg body weight)

exposed, the pattern was an increase from GD 0 onward GD 6 and 12. This was followed by a reduction in the plasma TSH concentration towards GD 18, and with another increase towards GD 20 in genistein exposed rats. Plasma TSH level was significantly increased at GD 6, 18, and 20 respectively in genistein exposed rats compared to the control group. The plasma T3 and T4 level also recorded a significant increase at GD 6, 12, while the levels of the two thyroid hormones were significantly decreased at GD 18 and 20 in genistein exposed rats as compared with the plasma levels of the two hormones in control rats. T3/T4 ratio was only significantly increase at GD 6 and 20, while the ratio was significantly decreased at GD 12 and 18 in genistein exposed rats.

Leptin concentration in the maternal plasma, placenta homogenate and amniotic fluid along with plasma concentration of CRP at different stages of pregnancy in genistein treated along with control rats (Table 3):

Plasma leptin level was significantly increased at GD 6, 12, 18 and 20 in all genistein exposed rats compared with control rats. Leptin level in placenta homogenates and amniotic fluids were also significantly increased at GD 12, 18 and 20 in genistein exposed rats compared to control rats. The highest concentration of leptin hormone recorded was in the plasma throughout the gestational period monitored compared with the concentration recorded in the placenta homogenate and amniotic fluid at any of the gestational days monitored.

Plasma C-reactive protein level at different days of pregnancy in rats exposed orally to genistein (Fig 1)

Plasma C-reactive protein level was significantly reduced at gestational day 6, while its level was significantly increased at GD 18 and 20 in genistein exposed rats compared with control rats. The highest and the lowest plasma concentration of CRP were recorded in the control rats at GD 6 and GD 20 respectively. In genistein exposed group of pregnant rats, the highest and the lowest concentration of CRP was recorded at GD 18 and GD 6 respectively.

DISCUSSION

The current study has shown that the administration of genistein to pregnant rats resulted in increased resorption of embryonic implants, and a decrease in placental and fetal weights. These adverse effects which have previously been reported by Ikegami et al. (2006) and Awobajo et al. (2013), indicated the possibilities of genistein adversely influencing some of the mechanisms that control placental and fetal metabolic processes. In addition, T4, a major metabolic hormone produced in the thyroid gland, is known to influence cell differentiation and growth during fetal life (Yen 2001). Therefore, the results of the thyroid hormonal analysis from day 1 to 20 of pregnancy revealed a significant decrease in the maternal secretion of T4, and T3 from mid-gestation onward (tab 2.). This also necessitated the changes recorded in the TSH secretion pattern via negative feedback mechanism. The T3/T4 ratio was significantly decreased most importantly between days 12-18 of pregnancy, indicating impairment of T4 to T3 conversion. Considering the observed genistein-induced hypothyroidism at the dose used, this has adversely affected normal growth of the fetus which may partly explain the reason for the observed reduction in fetal and litter weight. Genistein has been shown to inhibit thyroid-peroxidase, the enzyme responsible for catalyzing iodination of thyroglobulin and oxidative coupling of di-iodothyronine during the synthesis of thyroid hormones (Doerge and Sheehan 2002). Genistein has also been reported to interfere with iodide reutilization by inhibiting sulfotransferase enzymes (Ebmeier and Anderson 2004). This may partly explain the significant alteration recorded in thyroid hormone synthesis in the genistein exposed pregnant rats.

Genistein may also influence the metabolic hormone leptin, a peptide secreted by placental and white adipose tissue (Barr et al. 1997; Hoggard et al. 2000). This study revealed a significant increase in maternal plasma leptin concentration in the amniotic fluid and placental homogenate throughout pregnancy (tab 3). Linnemann et al. (2000) reported that about 98% of the leptin secreted by the placenta are released

into the maternal blood. This report corroborated our findings in which maternal plasma leptin level was increased along with the increase recorded in placenta leptin level (tab 3), throughout the period of gestation. Based on the wide spread expression of the leptin receptor gene in the fetus and placental tissues, it has been strongly suggested that leptin may play an important role in regulating fetal growth (Forhead and Fowden 2009). This was confirmed by the establishment of a correlation between umbilical cord leptin level and fetal birth weight (Tamura et al. 1998). However, contrary to previous reports on leptin and fetal growth (Valuniene et al. 2007), a significant decrease in placental and fetal weight was recorded in pregnant rats orally exposed to genistein from day 6 to 20 of pregnancy (tab 1.), despite the significant increase in plasma and placental leptin level. Other authors have reported a similar decrease in fetal weight when pregnant rats were exposed to genistein (Ikegami et al. 2006; Awobajo et al. 2013). Thyroid hormone is known to exert a negative feedback on leptin synthesis with increase in the synthesis of the latter during hypothyroidism (O'Connor et al. 2007). Therefore, the observed genistein-induced hypothyroidism may have silenced the negative feedback control of thyroid hormone on leptin with resultant increase secretion as observed in this study. Placental growth signified by increase weight as it strives to cope with the increased demand for gaseous, hormonal and nutrient supply to the developing fetus is a pre requisite for normal development of the fetus (Ishikawa et al. 2006). The persistent significant reduction in placental weight from gestational day 18-20 (a critical period in fetal weight gain) as recorded in the genistein exposed rats (tab 1.), will partly explain the reason for the reduction in fetal weights. However, further studies will be required to unravel other mechanism by which increased leptin secretion failed to promote fetal weight gain in genistein exposed rats.

Although, inflammatory activities are usually increased towards term with increased CRP (Mendelson 2009), genistein works in synergy with the process of parturition to significantly increase the plasma CRP level onward the day 20 of pregnancy. CRP is an example of the first acute phase response usually with increased production after any inflammatory reaction (Mackiewicz 1997). The significant decrease recorded in the level of CPR at the early stage of pregnancy (fig 2.), may be connected with the increased resorption rate of embryos reported by some authors (Awobajo et al. 2013). Implantation and placentation stages of pregnancy have the characteristics of an acute inflammatory response and therefore, it is referred to as a pro-inflammatory phase (Mor et al. 2011). It involves break down of the epithelial lining of the uterus by the blastocyst and the invasion of the uterine myometrium by giant trophoblast cells to initiate development of the placentation and angiogenesis. Therefore, the reduction in CPR within the first six days of pregnancy; (the period of implantation) may be an indication in the reduction in this necessary inflammatory process. Genistein has been reported to have anti-inflammatory property (Verdrengh et al. 2003). In summary, the evidence presented here, shows that genistein at a dose of 2 mg/kg body weight precipitated hypothyroidism in the pregnant rats. It also adversely affected the synthesis and leptin levels in maternal plasma, amniotic fluid and placental tissue. These along with a reduced inflammatory process at the period of implantation as indicated by reduced plasma CRP level precipitated increased resorption of embryonic implants, reduced growth of the placenta and development of the fetus.

Conflict of interest

The authors report no conflict of interest.

Acknowledgement

The authors will like to appreciate the technical assistance rendered by Mr. Dada and, Mrs. Ogunsola who are both postgraduate students of the Department of Physiology, University of Lagos, Nigeria. The authors thank Enago for the English language review

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Niger. J. Physiol. Sci. 30(2015) 087-093
www.njps.com.ng

Niger. J. Physiol. Sci.

Effect of Ethanolic Leaf Extract of *Senna Fistula* on some Haematological Parameters, Lipid Profile and Oxidative Stress in Alloxan-induced Diabetic Rats

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Summary: Increasing evidence in both experimental and clinical studies suggests that oxidative stress plays a major role in the pathogenesis of both types of diabetes mellitus. The disease is also known to adversely affect some haematological parameters and cause dyslipidemia. This study was designed to investigate the effect of chronic administration of ethanolic leave extract of Senna fistula on haematological values, oxidative stress and dyslipidemia in experimental diabetic rats. Twenty-four albino rats weighing 120-150 g were divided into 4 experimental groups of six rats each; control, diabetic untreated, diabetic treated with glibenclamide and diabetic treated with 100 mg/kg b.w of Senna fistula. Diabetes was induced by 100 mg/kg b.w. of alloxan monohydrates. The control and diabetic groups received normal saline while the diabetic treated groups were administered with 5mg/kg and 100mg/kg body weight of glibenclamide and ethanolic leaves extract of Senna fistula respectively for 28 days. At the end of experimental period blood samples were taken from the animals for the determination of Red blood cells (RBC), packed cell volume (PCV), Haemoglobin concentration (Hb), total cholesterol, triglycerides (TG), high density lipoprotein (HDL), low density lipoprotein (LDL) and malondialdehyde (MDA), marker of lipid peroxidation. The result showed that in diabetic rats, PCV, RBC and Hb were decreased but the application of the extract increased the parameters ($P<0.05$, $n=6$). Similarly, the result showed a significant increase in total cholesterol, TG and LDL level of the diabetic group when compared with the control, glibenclamide and extract treated diabetic groups, however, there was no significant difference in HDL level in all the groups. The result also showed a significant decrease in elevated MDA ($P<0.05$, $n=6$) of diabetic treated rats. These findings suggest that ethanolic leaves extract of Senna fistula might improve the diabetic induced disturbances of some haematological parameters, reduces the plasma lipid imbalances and decreases the production of free radicals associated with diabetes.

Keywords*:* Glibenclamide, Senna Fistula, Diabetes Mellitus, Packed Cell Volume, Malondialdehyde

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Manuscript Accepted: October, 2015

INTRODUCTION

Diabetes is a disorder in the metabolism of protein, carbohydrates, and fat resulting from absolute or relative deficiency in insulin secretion without/with varying level of insulin resistance (Devlin, 1997; Barar, 2000). It may also be defined as a disease where the body either produces little insulin/cease to produce insulin or progressively resistant to its action (Ranjan, 2002).

Patients with diabetes experience significant morbidity and mortality from macrovascular and microvascular complications. The macrovascular complications are due to accelerated atherosclerosis resulting from increased plasma low density lipoprotein- cholesterol (LDL-C), which leads to increased incidence of stroke and myocardiac infarction. The microvascular abnormalities include renal disease (diabetic nephropathy) leading to anaemia and renal failure, proliferative scaring of the retinal (diabetic retinopathy) resulting into blindness (Halder *et al.*, 2003; Merlin *et al.*, 2005). Diabetic neuropathy involves abnormalities in the autonomic nervous system and peripheral nerves. The neuropathy and atherosclerotic circulatory insufficiency in addition to reduced resistance to infection in the extremities especially in the feet can lead to chronic ulceration and gangrene. The cost of treating diabetes and associated complications exceed $100 billion per year worldwide (Jarald *et al.*, 2008). The complications are less common and less severe in people who have well controlled blood glucose level (Andrew, 2000)

Increased oxidative stress is a well-known factor in the development and progression of diabetes and its complications. (Baynes, 1991; Baynes and Thorpe, 1999; Ceriello, 2000). The disease is usually associated with increased production of free radicals (Baynes and Thorpe, 1999; Baynes, 1991; Chang *et al.*, 1993; Young *et al.*, 1995) or reduced antioxidant defenses (Halliwell and Gutteridge, 1990; Saxena *et*

al., 1993; McLennan *et al.,* 1991). The end result is oxidative stress.

Diabetes has now become an epidemic with a worldwide incidence of about 9% in the general population (WHO, 2012), making it one of the most common non-communicable diseases (Jarald *et al.,* 2008). In the year 2012, about 1.5 million deaths were caused by diabetes directly and 80% of these deaths occur in low and middle income countries (WHO, 2014). Diabetes is projected to be the 7th cause of death by 2030 (Mathers and Loncar, 2006).

Several oral anti-diabetic agents are presently available to reduce hyperglycaemia including sulfonylurea and biguanides. Unfortunately, even with intensive use of current antidiabetic agents more than 50% of diabetic patients still suffer poor glycemic control and some even develop serious complication within six years of diagnosis (Jarald *et al.,* 2008). Moreover, none of the glucose lowering agents control hyperlipidemia adequately and oxidative stress that is frequently associated with the disease (Derek, 2001). Clearly, there is a need for new anti-diabetic agents which will be more effective, cheap, readily available and of natural (plant) origin. One plant claimed to be used in the management of diabetes mellitus in folk medicine of Nigeria is *Senna fistula*

Senna fistula Linn. (Senna) belongs to the family leguminosae, is otherwise referred to as "Golden shower", (English) and *Aidantoro* (Yoruba), is a deciduous and mixed- monsoon forests tree that originates from India and Sri-Lanka but is now cultivated in tropical countries of the World from West indies to Indian, South Africa, East Africa and West Africa especially Nigeria (South-West), (Trease and Evans, 1985). The plant grows to about 15m tall with greenish grey bark, compound leaves with 3-7 pairs of leaflets each 5-12 cm long (Gupta, 2010)

Senna fistula plant has been claimed to be used as a laxative and antibiotic (pods, fruit) (Akanmu *et al., 2004;* Kasugo and Nagaye, 1951), methanolic extract of buds of *S. fistula* has been shown to have antipyretic, analgelsic and anti-inflammatory effect (buds) (Bhakta *et al.,* 1999b; Ilavarasan *et al.,* 2005), the leaf, stem bark, pulp and flower of *Senna fistula* have been shown to have antioxidant activity (Siddhuraju *et* al.,2002), antitumor (seed) (Gupta *et al.,* 2000), also the petroleum ether extract of seeds of *Senna fistula* has been shown to have antifertility effect (Yadav and Jain, 1999) others include antidiabetic, antihypercholesterolemic effects (Nirmala *et al.,* 2008) and also ethanolic leave extract of *Senna fistula* has hepatoprotective effects in diethyl nitrosamine (DEN) induced hepatic injury (Kannampalli, *et al,* 2007; Bhakta *et al.,* 2001).

Studies have shown that diabetes is associated with increased oxidative stress, alteration of some haematological parameters, lipid abnormality and most importantly increased blood glucose. There is dearth of information on studies that evaluated the effect of ethanolic leaf extract on diabetes, therefore the present study was undertaken to evaluate the effect of repeated oral administration of ethanolic leaf extract of *Senna fistula on* hyperglycaemia, hyperlipidemia, haematological and oxidative disturbances associated with diabetes.

MATERIALS AND METHODS

Plant materials and authentication

The plant material was obtained from the herb sellers at Oja-Tuntun market, Ilorin, Nigeria, and was authenticated at the Plant Biology Department of University of Ilorin. It was identified with a voucher specimen UIH 1020 earlier deposited in the herbarium.

Glucometer and Assay Kit

One touch ultra® glucometer was a product of lifeScan, Inc. Milpitas, USA. Assay kits for cholesterol, triglyceride were product of Randox Laboratories, Co-Antrim, UK.

Drugs and chemicals

Glibenclamide was a product of HOVID Bhd, Ipoh, Malaysia, alloxan monohydrate and all other chemicals were products of Sigma-Aldrich Cheme GmbH, Steinheim, Germany.

Laboratory animals

Male albino rats *(Rattus norvergicus)* of Wistar strain, weighing between 120- 150 g were obtained from the animal holding unit of the Department of Biochemistry, University of Ilorin. The animals were fed on rat pellet (Premier feed Ltd Ibadan) and water *ad libitum,* they were maintained under standard laboratory conditions and were subjected to natural photoperiod of 12h light; dark cycle; temperature:28-31^{0}C; humidity: 50-55%

Preparation of the extract.

Fresh leaves of *Senna fistula* were air dried at room temperature for about two weeks. The dried materials were pulverized using an electric blender. A known weight of the powder (158.7g) was extracted in 2 litres of ethanol for 24 hours. The extract was then filtered. The filtrate was evaporated to dryness using water bath which yielded 31.42 g. The calculated amount of the extract was weighed and dissolved in normal saline to give the required dose of 100 mg/kg body weight. 24 albino male rats were randomly assigned into four groups of 6 rats each:

- Group A- (control) normal and received 0.5ml of normal saline
- Group B-(untreated) diabetic, received 0.5ml of normal saline

- Group C-(treated) diabetic, received glibenclamide (5 mg/kg b.w.)
- Group D-(treated) diabetic, received 100 mg/kg b.w. ethanolic extract of *S. fistula*

Diabetes mellitus was induced by single intraperitoneal injection of freshly prepared alloxan monohydrate (100 mg/kg b.w) in sterile physiological saline. 1 hour after alloxan injection the animals were given their pellet and 5% dextrose saline to overcome initial hypoglycemic phase (Sikarwar and Patil, 2010). Diabetes was confirmed by glucose oxidase method using one touch ultra glucometer, 72 hours after alloxan injection. Only animals with blood glucose level higher than or equal to 200 mg/dl were used for the study. (Yakubu *et al.*, 2010; Meral *et al.*, 2004). The blood glucose levels of the animals were also determined before the administration of alloxan using the blood samples that were drawn from the tail vein.

Acute toxicity study

The method described by Lorke (1983) was used for this study. Nine rats were used and were divided into three groups of three rats each, the animals were administered 1000, 2000 and 5000 mg/kg b. w. of ethanolic extract of *Senna fistula* leaves respectively. The animals were observed/monitored closely for 72 hours for symptoms of tiredness and death.

The number of deaths in each group was recorded as percentage of mortality and the LD50 was calculated as the geometric mean of the lowest dose showing death and the highest dose showing no death.

Determination of biochemical parameters

Plasma triglyceride and total cholesterol levels were measured using enzymatic colorimetric diagnostic kits obtained from Randox Laboratories in which the GPO-PAP method of Trinder (1969) was employed. Absorbance was read at 500nm. The phosphotungstate precipitation method of Richmond (1973) as applied in Randox kit was used for the determination of HDL-Cholesterol. The LDL-Cholesterol was estimated using Friedewald (1972) formula:

LDLc= total cholesterol - HDLc - TG/5

Where LDLc = LDL-cholesterol, HDLc= HDL-cholesterol and TG= triglycerides

Determination of haematological parameters

All haematological parameters were determined by an automated haematological analyser, Symex KY-21 (Symex Corporation, Japan) using whole blood sample.

Determination of Malondialdehyde

Plasma MDA was measured by a thiobarbituric acid assay procedure (Albero *et al.*, 1986) which was calibrated using 1,1,3,3 tetraetoxypropane (Sigma chemical, St Louis, Mo USA) as a standard. Results were expressed as nanomoles of MDA per millitre of serum.

Statistical Analysis

The mean value and standard error of mean (SEM) were calculated. The test for significance was carried out using ANOVA, and Duncan new multiple range test (DMRT). All the results were expressed as mean ± SEM, differences were considered statistically significant at $P< 0.05$.

RESULTS

Table 1 shows the result for the acute toxicity test (LD_{50}). There were no records of death within the first 48 hours in all the groups after oral administration of ethanolic extract of *Senna fistula* leaves. However, there was a record of death in the group given 5000 mg/kg b.w. of the extract 72 hours after administration, which represent about 33.33% mortality. The oral LD_{50} was estimated to be 3.162.28 mg/kg in rats accordingly:

$LD_{50} = (5000x2000)^{1/2} = 3162.28$ mg/kg.

Table 2 shows the effect of ethanolic extract of *Senna fistula* leaves on blood glucose level of diabetic rats. The diabetic untreated animal had increasing fasting blood glucose (FBG) throughout the period of the experiment rising from 233.80 mg/dl on the first day of the experiment to 262.20 mg/dl on the 28th day. The FBG of all the groups were significantly different from the control on the first day ($P< 0.05$), however, the animals treated with ethanolic extract of *Senna fistula* leaves and glibenclamide treated diabetic rats had reduced FBG value during the experiment and are significantly different from diabetic untreated group ($P<0.05$).

Table 1: LD_{50} of ethanolic extract of *Senna fistula* leaves in rat

	number of deaths			% mortality		
Dose	24hrs	48hrs	72hrs	24hrs	48hrs	72hrs
1000mg/kg	0	0	0	0	0	0
2000mg/kg	0	0	0	0	0	0
5000mg/kg	0	0	0	0	0	33.3

Table 2. Effect of administration of ethanolic leaf extract of *Senna fistula* on the blood glucose level (mg/dl) of alloxan-induced diabetic rats

Group	Initial	After 28 days
A	77.40 ± 7.97^{a}	61.80 ± 11.35^{a}
B	233.80 ± 26.09^{b}	262.20 ± 10.41^{c}
C	258.40 ± 10.36^{c}	103.40 ± 10.68^{b}
D	362.40 ± 50.78^{d}	93.20 ± 5.63^{b}

Values with different superscript are significantly different, $p<0.05$. A=Normal saline (0.5 ml), B=Normal saline (0.5 ml), C=Glibenclamide (5 mg/kg), D=*Senna fistula* extract (100 mg/kg)

Table 3: Effect of administration of ethanolic leaf extract of *Senna fistula* on haematological parameters, lipid profile, and serum malondehydehyde level in alloxan induced diabetic rats.

Groups/parameters	A	B	C	D
MDA	3.08±0.86[a]	5.00±0.12 [c]	3.44± 0.05[b]	3.58±0.66 [b]
Cholesterol	2.76±0.11 [a]	5.14± 0.21 [d]	3.84±0.21 [c]	3.54±0.19 [b]
TG	0.96±0.09 [a]	2.14±0.16 [c]	1.08±0.12 [a]	1.96±0.51 [b]
HDL-C	1.24±0.15 [a]	1.09±0.15 [a]	1.28 ±0.08[a]	1.12 ±0.06[a]
LDL-C	1.92±0.39 [a]	3.56±0.18 [d]	2.00±0.16 [b]	2.84±0.24 [c]
PCV (%)	37.4±0.2 [b]	27.4±0.2 [a]	41.2±0.5 [c]	42.4±1.0 [c]
Hb (g/dl)	12±0.1 [b]	8.5±0.1 [a]	11.9±0.1 [b]	12.8±0.4 [b]
R BC (^{x} 10^{6})	4.4±0.1 [b]	3.4±0.1 [a]	4.7±0.1 [b]	5.0±0.1 [c]

Values with different superscript are significantly different, p<0.05. A=Normal saline (0.5 ml), B=Normal saline (0.5 ml), C=Glibenclamide (5 mg/kg), D=*Senna fistula* extract (100 mg/kg)

In Table 3 there was a significant decrease in Red blood cell count (RBC), packed cell volume (PCV) and haemoglobin (Hb), values in diabetic (untreated) rats, but administration of the ethanolic extract of *Senna fistula* leaves increased significantly the reduced PCV, RBC and Hb values in diabetic rats. However, there was no significantly different in all these parameters between the control, glibenclamide and the extract treated groups (P<0.05).

As shown in Table 3, the mean value of serum malondialdehyde (MDA) was significantlyelevated in alloxan-induced diabetic rats. Treatment with ethanolic extract of *Senna fistula* leaves causes a significant decrease (P<0.05) in MDA level of diabetic rats which compared favourably with the control and and glibenclamide treated rats. The mean value of cholesterol, triglyceride (TG) and low density lipoprotein (LDL) (Table 3) were significantly increased in diabetic untreated group. Administration of the extract causes a significant reduction in total cholesterol, TG and LDL in diabetic rats in a manner similar to control and glibenclamide treated rats, however, there was no significant difference in high density lipoprotein (HDL) level in all the groups.

DISCUSSION

Plants have been a source of medicinal agents for many years, and a remarkable number of modern drugs have been isolated from plants, many of which are based on their use in traditional medicine." These plant-based traditional medicine systems have continued to play a significant role in health care, with about 80% of the world's populations mainly depending on traditional medicines for their primary health care (Owolabi *et al.*, 2007). Plant products also have an important role in the health care systems of the remaining 20%, who live in developed countries like America, Europe. Many studies have revealed that many plants extract effectively lowered blood glucose level in alloxan induced diabetic animals (Owoyele *et al* 2005; Yakubu *et al* 2010). In this study, ethanolic leaf extract of *Senna fistula* significantly reduced the blood glucose level and effectively restored some biochemical and haematological parameters in alloxan induced diabetic rats.

Study on the acute toxicity suggests that the extract could be toxic at a high dose on acute exposure. However, the dose used in this study is less than the calculated LD50 which demonstrated that the extract could be safely consumed at the dose used in this study.

The use of alloxan to induce diabetes in rats represents a well-established animal models of type I insulin dependent diabetes mellitus characteristically similar to type I diabetes in human (Szkudelski, 2001). Glibenclamide was used as a reference drug mimicking several insulin actions in vivo which include suppressing hepatic glucose production, increasing insulin sensitivity of extrapancreatic tissue, stimulation of lipogenesis and inhibition of lipolysis, enhancing peripheral glucose uptake, decreasing hepatic glycogenolysis and gluconeogenesis and as well as absorption of glucose from the gastrointestinal tract (Wadkar *et al*., 2008; Zeggwagh *et al.*, 2007).

In this present study, intraperitoneal injection of alloxan caused a significant increase in the level of blood glucose indicating establishment of a diabetic state. However, treatment of diabetic animals with 100 mg/kg b.w. of ethanolic leaf extract of *Senna fistula* produced a significant decrease in plasma glucose

level at the end of the experiment (28 days), the decrease in blood glucose level recorded in this study is comparable with that of the reference group (glibenclamide group). The mechanism of hypoglycaemic effect of ethanolic extract of *Senna fistula* leaves is not known yet, but it could be that the extract facilitates glucose utilization by peripheral tissues or by decreasing hepatic glycogenolysis and gluconeogenesis or it could also stimulate increase insulin production from possibly regenerating pancreatic beta cells, however, further study could be carried out to measure insulin level and liver glycogen in order to know its mechanisms of action.

The phytochemical and mineral composition screening of the leaf extract carried out also revealed the presence of flavonoids, alkaloids, tannins, saponins, Ca, K, Zn, Mn, Mg and vitamin C, this may also be partly responsible for its hypoglycaemic property, because studies have documented that medicinal plants with hypoglycaemic property usually contain Alkaloids, Flavonoids, Tannins and Terpenoids (Oladele *et al.,* 1995; Ojewole, 2005).

Diabetes has been found to adversely affect haematological parameters by decreasing the life span of red blood cells and white blood cell count. (Kamenov *et al.,* 1997). In the present study, some haematological parameters were analysed and the result showed that red blood cell (RBC), packed cell volume (PCV), and haemoglobin (Hb) concentration values were decreased in diabetic rats. This is in line with previous work where occurrence of anaemia in diabetes has been documented (Merlin *et al.,* 2005). Treatment with ethanolic leaf extract of *Senna fistula* increased the reduced RBC, PCV, and Hb concentration values in diabetic rats. The increased blood indices could be related to the mineral composition of the leaf extract of *Senna fistula* which include protein, Zn, Ca, K, Mn, Fe, P, Mg and vitamin C most of these mineral components are well known haematological factors that have influence on the production of blood from the bone marrow (Ganong, 2006).

Diabetes hyperglycemia results in an increase in free radical production through mechanisms involving glucose oxidation, protein glycation and oxidative degeneration (Hunt *et al.,* 1990) which may play a role in increased lipid peroxidation in diabetes mellitus. In this study, the increased levels of MDA (marker of lipid peroxidation) in diabetic rats clearly showed that diabetic rats were exposed to an increased oxidative stress via lipid peroxidation, (Velazquez *et al* 1991, Nacitarhan *et al* 1995, Losada and Alio 1996, Mahboob *et al.,* 2005, Kaji *et al.,* 1985). This is in agreement with previous studies documenting elevated serum lipid peroxide level in diabetic subjects (Sato and Hotta-Nsoka 1979; Oberley 1988; Halliwell and Gutteridge, 1990). Administration of ethanolic leaf extract of *Senna fistula* decreased the elevated lipid peroxide in alloxan induced diabetic rats in a manner similar to glibenclamide treated group. The decreased level of lipid peroxide in *Senna fistula* treated rats may be due to decreased oxidative load which the extract might have caused by either directly scavenging the reactive oxygen metabolites due to the presence of many antioxidant compounds like flavonoids or by increasing the synthesis of antioxidant molecules. Furthermore, increased production of lipid peroxide in diabetic rats may also explain the reduction in some haematological parameters (RBC, PCV, Hb) found in diabetic rats. Occurrence of anaemia in diabetes mellitus has been linked to increased non-enzymatic glycosylation of red blood cell membrane proteins. Oxidation of the glycosylated membrane protein and hyperglycemia in diabetes causes an increase in the production of lipid peroxide which in turn leads to haemolysis of red blood cells. Thus increased RBC count, packed cell volume and haemoglobin concentration of ethanolic leaf extract of *Senna fistula* treated rats could be due to reduction of lipid peroxide level in RBC membrane, hence a decreased susceptibility of RBC to haemolysis.

In diabetes, there is hyperlipidemia which may be linked to insulin deficiency; in this state (hyperglycemia) fatty acids are mobilized from adipose tissue causing accumulation of excess fatty acids in the liver which are then converted to triglyceride (Velazquez *et al.,* 1991). Administration of ethanolic leaf extract of *Senna fistula* reduced total plasma cholesterol, triglycerides and low density lipoprotein level in diabetic rats. This is in line with previous studies where hypolipidemic activity of some medicinal plants in alloxan induced diabetic rats have been documented (Nirmala *et al.,* 2008; Ayinla *et al.,* 2011). The observed hypolipidemic effect recorded in this study could be due to the presence of some phytochemical compounds which include alkaloids, flavonoids, saponins and tannins. All these compounds are known to reduce serum lipid level in animal especially alkaloid which is known to normalize lipogenesis due to its insulinogenic effect on lipid metabolism while flavonoids cause decrease in the activity of HMG-CoA reductase in the liver.

In conclusion, the significant reduction of the high blood glucose in diabetic extract treated group to the values of the control and reference treated group indicates anti-hyperglycaemic activity of ethanolic leaf extract of *Senna fistula*. This study also revealed that ethanolic leaf extract of *Senna fistula* can

effectively correct dyslipidemia, oxidative stress and some haematological disturbances associated with alloxan induced diabetic rats.

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Niger. J. Physiol. Sci. 30(2015) 095-101
www.njps.com.ng

Niger. J. Physiol. Sci.

Effects of *Telfairia Occidentalis* Seed Oil on Female Reproductive Functions in Wistar Rats

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Summary: The effects of *T. occidentalis* seed oil on some female reproductive indices were investigated in Wistar rats. The study was divided into two phases: (estrous cycle and pregnancy). Animals were grouped into four: group A received distilled water (control), groups B, C and D received 400, 600 and 800 mg/kg bw of *T. occidentalis* seed oil respectively. The pattern of estrous cycle was determined for three weeks before and during the treatment. Thereafter, each group was sub- divided into two. The sub-group-1 rats were mated with male breeders, the litter size and birth weight of their offsprings was determined. Sub-group-2 rats were sacrificed and histology of organs and serum levels of LH, FSH and estrogen were assayed. There was no significant difference between the pre-treatment and post-treatment estrous cycle length. However, there was a significant decrease in the frequency of diestrus phase during treatment in all the experimental groups when compared with pre-treatment period ($p < 0.05$) but there was no significant difference in the diestrus phase when compared with the control group. Serum estrogen concentration was significantly reduced ($p < 0.05$) in the group that was treated with 800 mg/kg bw of *T. occidentalis* seed oil. Histology of the ovary and uterus in the experimental groups were similar to that of the control group. Birth weight of pups was significantly increased in the group treated with 600 mg/kg bw of *T. occidentalis* seed oil when compared with the control group ($p < 0.05$). The results of this study suggest that *T. occidentalis* seed oil does not alter estrous cycle in Wistar rats.

Keywords*: T. occidentalis*, Wistar rats, Estrous cycle, diestrus, Estrogen.

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Manuscript Accepted: December, 2015

INTRODUCTION

Infertility is a problem of global proportions. The World Health Organization estimates that 1 in every 4 couples in developing countries is infertile (Mascarenhas, 2012). It is also estimated that one in three couples is affected in countries within Central and West Africa (Inhorn, 2003). In sub-sahara African countries, report showed that 20 - 46 % of couples are infertile (Idrisa, 2001). The consequences of infertility in these countries range from economic hardship to social isolation, violence and denial of proper death rites (Abdallah and Daar, 2001).

In oriental cultures reproduction is one of the highest valued factors (Qui, 2001). The way in which people try to solve the problem of infertility is at least partly affected by the values and socio-cultural norms of the community in which they live (Inhorn, 2003). Herbal plants are used in the cure of infertility in Africa. Many of these plants are common vegetables and have been proven scientifically, to actually have fertility effects as well as other health benefit rooted in their physiological effect as a result of their phytochemical and nutritional constituents (Hunter and Fletcher, 2002).

T. occidentalis is commonly known as fluted pumpkin. It belongs to the family of *Curcumbitiacea.* It is found along the fringes of the closed forest in Africa and particularly cultivated in south-eastern part of Nigeria and Seiria leone (Burkett, 1968). It is a creeping vegetable shrub that creeps low across the ground with large leaves and long twisting tendrils (Horsfall and Spiff, 2005). The leaves and the young shoot of the plant are frequently eaten as potherb (Tindal, 1968; Okigbo, 1977; Okoli and Mgbeogun, 1983). In Ibo, it is called ugu, in Yoruba, the seed is called egusi iroko, and in Benin, it is called uwmenkhen. There is hardly any home in Nigeria where ugu is not consumed in daily meal due to its health restoration (Ehiagbonare, 2008).

The seed of *T. occidentalis* is located in its fruits that may weigh up to 13kg. It is dark red in color. It is used as soup condiment in certain communities in rural region of south-eastern Nigeria (Agatemor, 2006). It is also cooked and taken as a meal. Physiochemical analysis of fluted pumpkin seed oil showed that it is rich in iodine, linoleic acid, tannins and poly unsaturated free fatty acids (Egbhekun *et al.*, 1998). It also has an acidic value that indicates that it is edible

and can be used in marmalade production (Akubugwo *et al.*, 2007).

Experiments have shown that *T. occidentalis* seed oil has beneficial effects on male fertility in rats (Akang *et al.*, 2010). It has also been shown that its addition to rat feed led to varying degree of pathology in the heart, liver, kidney and skin (Ajayi *et al.*, 2004). The aqueous extract of *T. occidentalis* has a haematinic effect (Alada, 2000). Research carried out by Salman *et al.*, (2008) suggested that the aqueous extract of the *T. occidentalis* leaf improved male reproductive functions by increasing sperm count, sperm motility and sperm viability. However, there is no scientific report on its effects on the female reproductive functions. The present study was therefore designed to investigate the effects of *T. occidentalis* seed oil on female reproductive functions in Wistar rats.

MATERIALS AND METHODS

Plant Material and Oil Preparation

Healthy fruits of *T. occidentalis* were obtained from Ojo market, Ibadan, Oyo state. The plant and its seed were identified at Forestry Research institute of Nigeria (FRIN), Ibadan, Oyo state, with the identification number, 108846. The fruits were broken and the seeds were collected, decocted and the naked seeds were air-dried for 2 days. The seeds were thereafter oven-dried at 45^0 C for 1 week during which consistent dry weight was obtained. The seeds were blended into powdery form using super master blender and preserved for oil extraction.

The oil extraction was done at the National Institute of Science and Technology (NIST), Samonda, Ibadan, Oyo state, Nigeria. The oil extract was obtained using petroleum ether in continuous extraction with a Soxhlet reflux apparatus as described in earlier works (Reinhold, 1992; Ojaiko and Nwajo, 2006; Akang *et al.*, 2010) at a temperature of 60^0C to 80^0C. On completion of extraction, the petroleum ether in the extracted oil was completely evaporated at 40^0C (Reinhold, 1992). The oil left after the evaporation of petroleum ether was the desired sample.

The desired oil sample had a yellow color and an agreeable smell, though different from that of other known oil. It was liquid at room temperature.

EXPERIMENTAL ANIMALS

Forty female Wistar rats (120-150g) and ten male Wistar rats (150-180g) were purchased from the Central animal house, University of Ibadan, Ibadan, Nigeria. They were maintained under standard laboratory condition and fed with rat feed and they had access to drinking water *ad libitum*. All animals were acclimatized for two weeks.

The female animals were randomly divided into four groups of ten animals each. Group A was the control group and was administered distilled water, Group B was administered a dose of 400 mg/kg bw of *T. occidentalis* seed oil, group C was administered 600 mg/kg bw of *T. occidentalis* seed oil and group D was administered 800 mg/kg bw of *T. occidentalis* seed oil. The dosage regime was adopted from Akang *et al.*, (2010). The treatment was done orally for a period of 21 days. The duration of administration was based on the fact that the mean estrous cycle length of rat is 4 days (Long and Evans, 1922). This provided the opportunity to study the effect of *T. occidentalis* seed oil on rat in 5 consecutive estrous cycles. The weight of the animals was recorded every week during treatment.

EXPERIMENTAL PROCEDURE

Estrous Cycle

The estrous cycle of each rat was established for three weeks without treatment. Thereafter, each experimental group was treated with its designated dosage while the control was treated with distilled water for another three weeks and estrous cycle was also carried out throughout the period. The outcome of both period of estrous cycle was later compared.

Determination of estrous cycle was done using the technique described by Marcondes *et al.*, (2002). This was done between the hours of 7:00- 8:00am every morning. The slides used for preparing vaginal smear for each rat were thereafter stained using Papanicolaou's technique. The morphology of the cells was observed under the microscope using x40 magnification lens and photograph was taken.

Mating of the Animals

At the end of estrous cycle determination, each group was sub-divided into two. Sub-group 1 was cohabited with male breeders during proestrus phase at the ratio of 2:1 (female: male). The day spermatozoa were seen in the vagina smear of rats was taken as day one of pregnancy. Birth weight of offspring and litter size in each group were determined on the day of parturition.

Hormone Assay and Histology

Animals in sub-group 2 were used for this purpose. During proestrus, blood sample was collected through the orbital sinus of each rat into sterile plain bottles using heparinized capillary tube. This was used to determine serum concentration of luteinizing hormone, follicle stimulating hormone and estrogen using the Enzyme-linked immunosorbent assay kits (Inteco®, UK). The animals were then immediately sacrificed by cervical dislocation. Each animal was opened through the linea alba. The uterus and the

ovaries of each animal were harvested, freed of any adherent tissues and weighed immediately. All organs were then fixed in 10% formalin for at least 5 hours. Samples were dehydrated using ascending grades of alcohol, cleared with two changes of xylene, embedded in paraffin wax, trimmed, nicked and sectioned using a microtome and stained with haematoxylin and eosin (H&E) for the purpose of determining the general morphology.

Statistical Analysis

The data from each group were analyzed using Student's paired T- test and one-way analysis of variance (ANOVA) followed by Waller-Duncan's post hoc test. The results were expressed as mean + standard error of the mean (Mean + SEM) and p-value < 0.05 was considered significant.

RESULTS

Effect of T. occidentalis Seed oil on Body Weight gain and Relative Organ Weight

There was no significant difference in the body weight gain (Table 1) and relative organ weight
(Table 2) of the experimental groups when compared with control.

Effect of T. occidentalis Seed Oil on Estrus Cycle

The length of estrous cycle before and during the treatment period was not significantly different. The length of estrus cycle was also not significantly different in all the experimental groups when compared with the control group (Table 3). There was no significant change in the frequency of proestrus phase and estrus phase before and during the treatment in all the experimental groups. There was also no significant difference in the proestrus phase and the estrus phase of the experimental groups when compared with the control group (Figure 1 and figure 2). However, there was a significant decrease in the frequency of diestrus phase in all experimental groups during the treatment period when compared with the pre-treatment period ($p < 0.05$) but there was no significant difference in the diestrus phase when compared with the control group (Figure 3).

Table 1: Effects of *T. occidentalis* seed oil on Body Weight gain

Dosage	Body weight gain (g)		
	Week 1	Week 2	Week 3
Control	13 ± 1.53	12± 1.33	10 ± 1.50
400 mg/kg	11 ± 1.00	12 ± 1.33	9 ± 1.80
600 mg/kg	14 ± 1.67	15 ± 1.34	8 ± 1.00
800 mg/kg	11 ± 1.67	10 ± 2.00	7 ± 2.36

Values expressed as Mean ± SEM. n=5

Table 2: Effect *T. occidentalis* seed oil on Relative Organ Weight

Dosage	Relative ovary weight (%)	Relative uterus weight (%)
Control	0.02 ± 0.00	0.28 ± 0.08
400 mg/kg	0.02±0.00	0.21 ± 0.03
600 mg/kg	0.02± 0.00	0.19 ± 0.05
800 mg/kg	0.02± 0.00	0.28 ± 0.04

Values expressed as Mean ± SEM. n=5

Table 3: Effect of *T. occidentalis* seed oil on estrous cycle length

Dosage	Estrous cycle length (days)	
	Before	After
Control	4.68 ± 0.19	4.67 ± 0.26
400 mg/kg	5.01±0.27	5.09 ± 0.30
600 mg/kg	5.21± 0.14	5.02 ± 0.14
800 mg/kg	4.90 ± 0.13	4.77 ± 0.15

Values expressed as Mean ± SEM. n=5

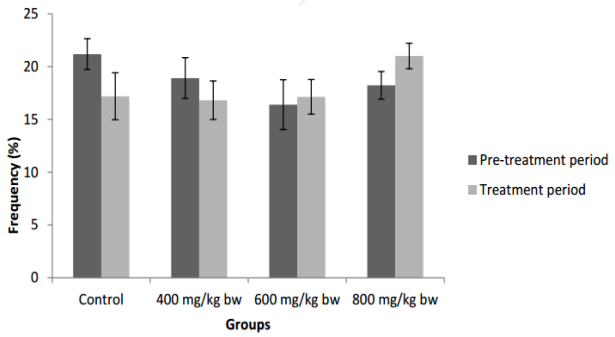

Figure 1: Effect of *T. occidentalis* seed oil on frequency of proestrus phase before and during treatment. n=5.

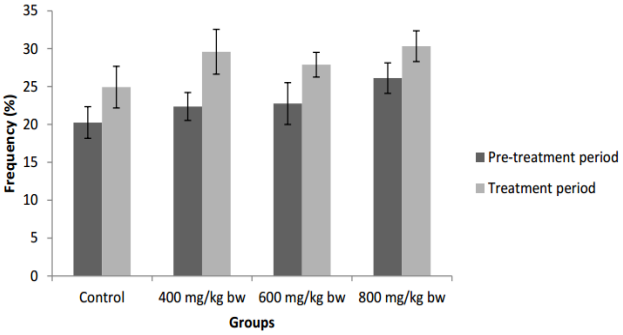

Figure 2: Effect of *T. occidentalis* seed oil on frequency of estrus phase before and during treatment. n=5.

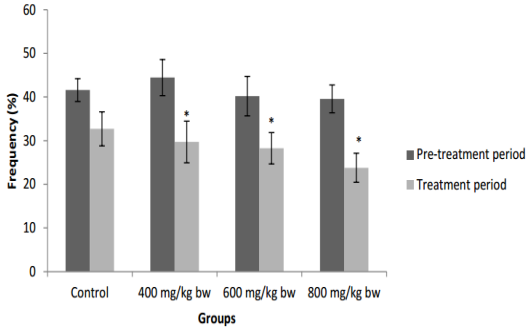

Figure 3: Effect of *T. occidentalis* seed oil on fequency of diestrus phase before and during treatment. n=5. *$p < 0.05$

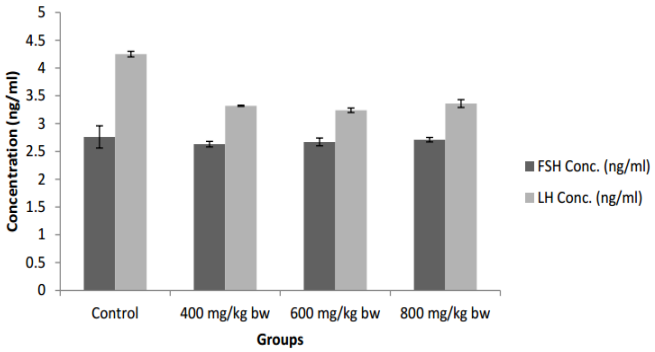

Figure 4: Effect of *T. occidentalis* seed oil on serum concentration of FSH and LH. n=5.

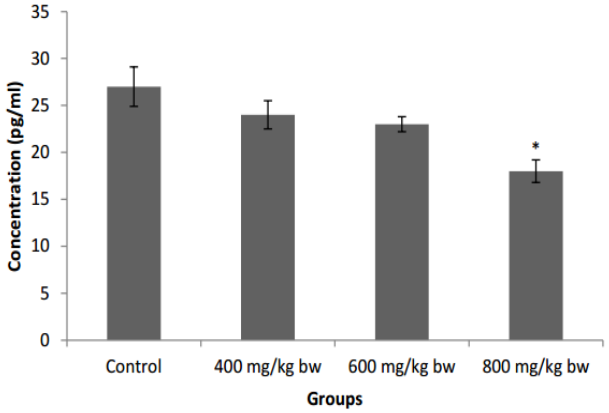

Figure 5. Effect of *T. occidentalis* seed oil on serum concentration of estrogen. n=5. *$p < 0.05$

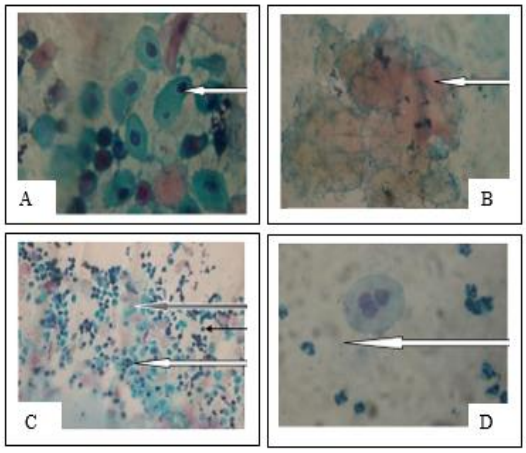

Figure. 6: **(A)** Nucleated epithelial cells in the proestrus phase of the estrous cycle (white arrow). **(B)** Estrus phase of the estrous cycle showing non-nucleated epithelial cells (white arrow). (**C)**Metestrus phase of the estrous cycle: the photomicrograph showing the presence of multinucleated cells (white arrow) single nucleated cells (slender arrow) and non-nucleated epithelial cells (black arrow) **(D)** Diestrus phase of the estrous cycle showing predominant leucocytes cells (white arrow). H&E X400.

Effect of T. occidentalis Seed Oil on Serum Levels of Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH) and Estrogen

There was no significant difference in the serum levels of FSH and LH in all the experimental groups when compared with the control group (Figure 4). However,

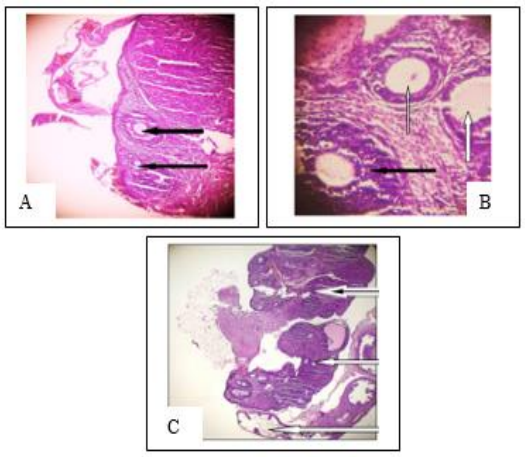

Figure 7:(A): Arrows (black) showing secondary follicles with normal cytoarchitecture, The cortex and inner medullar showed normal histology **(B)** Arrows showing secondary follicle with multilayered epithelium (black arrow) and primary follicle (white arrows) with normal component **(C)** Arrow (white) showing several follicle with normal cytoarchitecture at different stages of development. The cortex and inner medullar (black) shows normal histology.

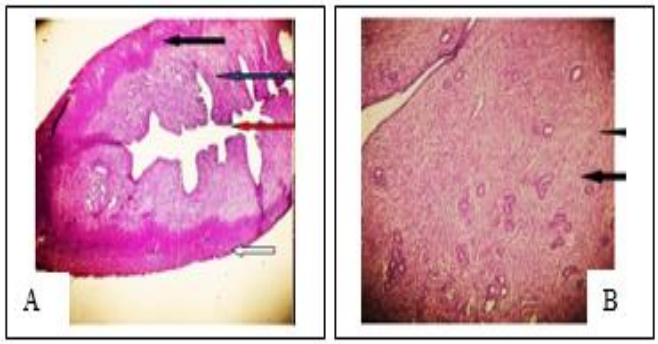

Figure 8: (**A**): Arrows showing myometrium (black arrow), endometrium (blue arrow), uterine cavity (red arrow), and perimetrium (white arrow) (X100). (**B**): Arrows showing normal endometrium and secretary tubular glands (black arrow) (X100).

the serum concentration of estrogen in the group treated with 800 mg/kg bw decreased significantly when compared with the control group ($p < 0.05$) (Figure 5)

Effect of T. occidentalis Seed Oil on Ovarian and Uterine Histology

The histological section of the ovaries in the experimental groups showed no visible lesion with intact cortex and inner medullar. The surface of the germinal epithelium and tunica albuginia showed normal histological appearance with normal follicular growth. The germinal discs seen in some sections appeared normal. The animals in all the experimental groups showed normal features of the uterus. The uterine cavity, perimetrium, myometrium and endometrium all appeared normal. The endometrium

laminae propria mucosa and secretory tubular glands are normal in all the groups.

Table 4: Effect of *T. occidentalis* seed oil on Birth Weight of pups and Litter Size

Dosage	Birth Weight	Litter Size
Control	5.38±0.14	7.2±0.04
400 mg/kg	6.17±0.22	9.66±0.23
600 mg/kg	10.00±0.14*	8.0±0.20
800 mg/kg	6.66±0.23	10.25±0.10

Values are expressed as Mean ± SEM. n=5*p < 0.05

Effect of T. occidentalis Seed Oil on the Vaginal Epithelial Cells

The vagina epithelial cells that characterized each phase of estrous cycle in all groups appeared normal as was observed in the Papanicolaou's staining (Figure 6).

Effects of T. occidentalis Seed Oil on Birth Weight and Litter Size of Pups.

There was a significant increase in birth weight of pups in the group treated with 600 mg/kg bw of *T. occidentalis* seed oil when compared with the control group (Table 4). There was no significant increase in litter size in all the experimental groups when compared with the control group (Table 4).

DISCUSSION

This study was conducted to determine the effects of *T. occidentalis* seed oil on body weight gain, relative organ weight, estrous cycle, serum concentration of luteinizing hormone (LH), follicle stimulating hormone (FSH) and estrogen, histology of the ovary, birth weight and litter size of pups.

It has been reported that *T. occidentalis* seed oil is used for cooking and in the production of cookies (Giami and Barber, 2004). The body weight gain, relative ovary weight and relative uterus weight of groups treated with *T. occidentalis* seed oil was not disrupted. This showed that the oil has no adverse effect on the body and organs. It also showed that the oil is well tolerated by the animals and is indicative of its safety for consumption.

Furthermore, the length of estrous cycle was not affected. However, the occurrence of the diestrus phase reduced in a dose dependent manner. This reduction in frequency of diestrus caused a shift in estrous cycle to favor estrus phase more than it did for proestrus. Although, the reduction of diestrus phase in this study is similar to the report of Kage *et al*, (2009) in which the estrogenic nature of *Trichosanthes cucumerina* was responsible for the reduction of diestrus phase. However, in this study, the reduction in diestrus phase of the estrous cycle in all the experimental groups is suggestive of the fact that *T. occidentalis* seed oil may be beneficial to the development and maturation of follicles because prominent among the events of diestrus phase are rising estrogen level (Shaikh, 1971; Yoshinaga et al., 1969) and low FSH level, which at some point causes growth of preovulatory follicle (Satue and Gardon, 2013). This eventually leads to the next phase (proestrus) during which follicles enlarges and estrogen increases thereby preparing the dominant follicle for ovulation.

The significant reduction in estrogen concentration caused by the administration of 800 mg/kg bw of *T. occidentalis* seed oil is similar to the report of Akang *et al.*, (2010) in which 800 mg/kg bw of the *T. occidentalis* seed oil caused a decrease in serum testosterone concentration. Estrogen production by the ovary or corpus luteum occurs as a result of interplay of different endocrine glands and enzymes. It is possible that *T. occidentalis* seed oil was able to reduce estrogen concentration by interfering with one of the steps that leads to its production.

The histology of ovaries in the experimental groups was similar to that of the control group. Lovejoy, (2002) suggested that linoleic acid and oleic acid both of which are present in *T. occidentalis* seed oil increased membrane fluidity, allows for osmosis, intracellular and extracellular gaseous exchange and caused an increase of lipid storage in lipid droplet and thus, an improvement of oocyte developmental competence respectively.

The increased birth weight in the group treated with 600 mg/kg bw of *T. occidentalis* seed oil is similar to the study of Kowalska, (2008) in which fish oil constituting 3% of the feed fed to rabbits resulted in increased birth weight. Like fish oil, *T. occidentalis* oil contains poly unsaturated fatty acid which is made up of linoleic acid and oleic acid (Nworgu *et al.*, 2007; Bello *et al.*, 2011). It has been reported that polyunsaturated fatty acid maintains the development of mammalian body before and after birth (Kowalska, 2008).

The result of this study showed that oral administration of *T. occidentalis* seed oil did not alter body weight, organ weight and estrus cycle. It also did not have any adverse effect on ovarian and uterine histology. Birth weight however increased in the 600 mg/kg bw. It caused a decrease in frequency of diestrus in all the experimental groups which is of course beneficial. The decreased estrogen concentration that was observed in the 800 mg/kg bw group did not have any effect on the other parameters measured. The results of this study suggest that *T. occidentalis* seed oil does not alter estrous cycle in Wistar rats.

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Niger. J. Physiol. Sci. 30(2015) 103-109
www.njps.com.ng

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Safety Evaluation of Osun River Water Containing Heavy Metals and Volatile Organic Compounds (VOCs) in Rats

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Summary: This study evaluated the pH, heavy metals and volatile organic compounds (VOCs) in Osun river water. It also evaluated its safety in rats. Heavy metals were determined by atomic absorption spectrophotometry (AAS) while VOCs were determined by gas chromatography coupled with flame ionization detector (GC-FID). Male and female rats were exposed to Osun river water for three weeks and then sacrificed. The abundance of heavy metals in Osun river followed the trend Pb > Cd > Zn > Fe > Cr > Cu while VOCs followed the trend benzene < ethylbenzene < toluene < xylene. The concentrations of Pb, Cd and benzene were higher than the permissible limits of Standards Organization of Nigeria (SON) and World Health Organization (WHO) respectively. Rats exposed to Osun river water for three weeks had increased WBC, thiobarbituric acid reactive substances (TBARS), serum proteins and serum aminotransferases. There were also significant decreases ($P < 0.05$) in HCT, PLT, liver aminotransferases and liver glutathione compared to the control. These results show that the pollutants in Osun river water are capable of inducing hematological imbalance and liver cell injury. The toxicity induced in blood was sex-dependent affecting female rats more than male rats.

Keywords*:* Osun river, Haematology, Biochemical parameters, Heavy metals, Volatile Organic Compounds.

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Manuscript Accepted: December, 2015

INTRODUCTION

Water is essential for the continuity of life and surface water such as river is the cheapest source of water supply for drinking, laundry, recreational, agricultural and religious activities. Meeting the water quality for these purposes poses great concerns because of volumes of wastes received by river (Olajire and Imeokpara, 2001; Elleta, 2012). Wastes generated from industries, dumpsites and agricultural activities are washed into the river from uncontrolled management while some people also defecate into the river (Eletta, 2012). These wastes which are sources of heavy metals, poly aromatic hydrocarbons (PAHs), volatile organic compounds (VOCs) and many more are detrimental to humans and aquatic lives abound in river. Some of these pollutants are confirmed carcinogens and can induce oxidative stress which causes damage to DNA, protein and other cellular constituents (Rechenmacher et al., 2010). Illnesses such as diarrhea, cholera, dysentery, typhoid, poliomyelitis, morbidity, mortality and hematological disorders have been attributed to poor quality of water (Wahab et al., 2012). Cancers, respiratory diseases, organ failures, mental retardation of the intellect, gastrointestinal disorders, tremor, ataxia, paralysis, vomiting and convulsion, depression, irritation and pneumonia are some of the diseases that have been traced to these pollutants (Nkolika and Benedict, 2009; Rechenmacher et al., 2010; Eletta, 2012).

Studies conducted by Olajire and Imeokparia (2000; 2001) on heavy metal concentrations and physico-chemical properties of Osun river reported high pollution with Pb, Cd, Ni, Cr, Zn, cyanide ion and ammonia which were mainly from farming, industrial activities and domestic discharges into this river. Equally, Wahab et al. (2012) reported high occurrence of pathogenic organisms in the river which could be as a result of unregulated activities such as defecating into the river. Due to the significance of this river as an international cultural heritage as well as national monument and its usefulness for both recreational and religious activities, there is need to assess its quality for the aforementioned applicable activities. However, no study has reported the concentrations of VOCs which have deleterious effects on both human and aquatic lives in the river. Though, studies have assessed the concentrations of heavy metals in Osun river, the most recent was in 2001 by Olajire and Imeokpara. Since wastes are continuously discharged into this river, there is every tendency that the concentrations of heavy metals might have changed, thus the need to determine the concentrations of heavy

metals. Also, no study has evaluated the toxicological implications of drinking Osun river water on health. Therefore, this study determined the concentrations of VOCs and heavy metals in Osun river and evaluated their effects on hematological and biochemical parameters in wistar rats.

MATERIALS AND METHODS

Study location: Osun river is housed by Osun groove within which there is Osun shrine. Osun worshipers, traditionalists and tourists from all walks of life and different countries gather to celebrate the Osun festival annually. It is one of the rivers ascribed to mythology and there is belief that it cures worshippers of various diseases when taken. It is located on latitude of 6º33'35"N and longitude of 4º03'47"E.

Sampling and analyses: Water samples were collected between November, 2014 and January, 2015 at 5m away from the shrine. Part of it was preserved with concentrated hydrochloric acid (HCl) and stored at room temperature for the determination of heavy metals while another part was used for the determination of VOCs. pH was determined in-situ with Jenway 3505 pH meter (USA).

Heavy metals analysis: Water samples were digested according to the method of Sallah et al., (2011). 50 cm^3 of each sample was treated with 5cm^3 of conc. HNO_3 and heated on a hot plate with gradual addition of conc. HNO_3 as necessary until the solution boiled. It was then evaporated to about 20cm^3; 5cm^3 of conc. HNO_3 was finally added, covered, and allowed to cool, and then filtered. The filtrate was poured into a 50cm^3 standard volumetric flask and made up to the mark with distilled-deionized water. Portion of the solution was used for metal analysis with atomic absorption spectrophotometer (AAS) S series 711047v1.22. Triplicate digestions and analyses were run and average values reported in the results.

VOCs analysis: VOCs in Osun river were determined using gas chromatography coupled with flame ionization detector (GC-FID Hewlett-Packard Model, 501, USA). 10 ml of water samples was emptied into pre-cleaned 20 ml vial. The vial with content was placed in the vial holder of headspace sampler for GC analysis programming. Vial temperature for headspace sampler was set at 40 °C, looping at 100 °C with the transfer line temperature of 100 °C. Vial was shaken for 1 min equilibrating for 0.15 min and pressurized for 1 min. An HP 6890 CP-Sil 5CBcolumn (25 m x 0.32 µm i.d x 0.12 µm film thickness) was used with the initial oven temperature set at 35ºC for 2 min., increasing at a rate of 5ºC/min. to 80ºC and then holding for 10 min. Detector and injector temperatures were maintained at 300°C and 150°C respectively. The target VOC species were identified by their individual retention time. Dechlorinated-distilled water was used as blank and was analyzed just as the Osun river sample. Standards of VOC mixtures containing all investigated constituents were prepared and calibration curves gave significant correlation coefficients (r^2) between 0.9996 and 0.9998. Triplicate analyses were done.

- **Animal groupings and water administration:** 20 male and female albino wistar rats weighing between 141± 29 g were bought from animal breeding unit of Department of Biochemistry, College of Medicine, University of Ibadan. The animals were randomly divided into four groups having five animals each: CM; control males, EM; experimental males, CF; control female, EF; experimental female. They were acclimatized at 25±6ºC with day and night cycle of 12 h each for two weeks on commercial feed and tap water ad libitum. The rats in EM and EF were thereafter exposed to Osun river water *ad libitum* for another three weeks. Groups CM and CF received dechlorinated tap water during this period. Good hygiene was maintained by cleaning the cages of faeces and spilled-off food every day. The animals were used according to the NIH Guide for the Care and Use of Laboratory Animals (NIH, 1985) in accordance with the principles of Good Laboratory Procedure (GLP) (WHO, 1998).

- **Collection of blood and preparation of serum and tissue homogenates:** The procedure described by Yakubu et al., (2005) was used in the preparation of serum and tissue homogenates.

Haematological Indices Analysis: White Blood cells (WBC), red blood cells (RBC), haemoglobin (Hb), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH) and platelet (PLT) were all determined according to the manufacturer's procedure using automated haematological analyzer X model KX-2IW.

Biochemical Assays: Biochemical parameters were determined using standard methods for protein (Gornall et al., 1949) and alanine aminotranstranfers (ALT) and aspartate aminotransferase (AST) (Reitman and Frankel, 1957). Reduced glutathione (GSH) and thiobarbituric acid reactive substances (TBARS) were assayed as detailed below:

Reduced glutathione (GSH) was determined by the method of Ellman (1959). Briefly, 0.5 ml of the liver supernatants was precipitated with 2 ml of 5% TCA and then centrifuged at 604 x g for 20 minutes. A known volume (1 ml) of the resulting supernatant was mixed with 0.5 ml of Ellman's reagent (0.0198% DTNB in 1% sodium citrate) and 3 ml of phosphate buffer (pH 8.0). Glutathione was used as standard. The

absorbance was read at 412 nm and the concentration determined from a prepared standard.

MDA in liver supernatants was determined colorimetrically by evaluating thiobarbituric acid reactive substances (TBARS) using the procedure described by Buege and Aust (1978). MDA reacts with thiobarbituric acid (TBA) forming a 1:2 adduct (MDA-TBA_2) which produces a complex aromatic structure that strongly absorbs at 532 nm. Briefly, 0.1 ml of the liver supernatants was added to 2 ml of TBA-TCA-HCl reagent in the ratio of 1:1:1 (0.3% TBA, 0.25 N HCl and 15% trichloroacetic acid, TCA). The mixture was then placed in boiling water bath for 15 minutes, cooled and centrifuged at 1073 x g for 30 minutes. The absorbance of the clear supernatant was read at 535 nm against distilled water blank. The values were expressed as nmol MDA/mg protein using the following expression:

$$\text{MDA} = \frac{\text{Absorbance x sample volume x dilution factor}}{1.56 \times 10^5 \times \text{total volume x mg protein/ml}}$$

STATISTICAL ANALYSIS

Data were expressed as mean ± standard deviation of three replicates. They were subjected to one-way ANOVA followed by Duncan Multiple Test. SPSS 15 version was used for the statistical analysis. Significant differences were tested at $p < 0.05$.

RESULTS

pH, heavy metals and VOCs: Table 1 presents pH, concentrations of heavy metals and VOCs in Osun river water with their standards. The values obtained for pH of Osun river water showed that it was slightly acidic. The trend of abundance of these metals followed Pb > Cd > Zn > Fe > Cr > Cu. The concentrations of Pb and Cd were higher than acceptable limits of Standards Organization of Nigeria (SON) while the concentrations of others were lower. These concentrations were higher than what were obtained by Olajire and Imeokparia, (2000). The abundance of VOCs in Osun river followed the trend benzene < ethylbenzene < toluene < xylene. Except benzene whose concentration was higher than World Health Organization (WHO) limit for drinking water, others were below.

Haematological parameters: Table 2 presents the hematological indices of male and female rats exposed to Osun river water and control. There were significant ($p < 0.05$) elevations in WBC and significant ($p < 0.05$) reductions in HCT and PLT in all rats exposed to Osun river water compared to the control. These increases and decreases were sex-dependent with higher elevations in WBC recorded for female rats (88.1%) than male rats (33.58%), higher reductions in HCT of female rats (23.65%) than male rats (12.5%) and PLT of female rats (43.48%) than male rats (19.95%). No significant ($p > 0.05$) changes were obtained in RBC, Hb, MCV, MCH and MCHC.

Biochemical and toxicological parameters: Table 3 presents biochemical parameters of male and female rats exposed to Osun river water and control. There were significant increases ($p < 0.05$) in the levels of liver TBARS, serum total protein and activities of AST and ALT in serum of rats exposed to Osun river water. Significant reductions ($p < 0.05$) were obtained in the levels of liver GSH and activities of AST and ALT in

Table 1: pH, heavy metals and VOCs concentrations in Osun river water

Pollutants	Measured concentration	Standard value
Fe (mg/l)	0.07 ± 0.0016	0.3*
Cd (mg/l)	0.15 ± 0.0011	0.003*
Zn (mg/l)	0.14 ± 0.0002	3.0*
Cu (mg/l)	0.0012 ± 0.0001	1.0*
Cr (mg/l)	0.004 ± 0.0001	0.05*
Pb (mg/l)	1.69 ± 0.0040	0.01*
pH	6.02 ± 0.02	6.5-7.5*
Benzene (ppb)	11.42 ± 2.03	10**
Toluene (ppb)	72.98 ± 13.06	700**
Ethylbenzene (ppb)	66.33 ± 5.92	300**
Xylene (ppb)	237.68 ± 24.07	500**

Data are mean ± standard deviation of three values. Fe – Iron, Cd – Cadmium, Zn – Zinc, Cu – Copper, Cr – Chromium, Pb – Lead, * Standard Organization of Nigeria values, **World Health Organization drinking water guidelines, 2008.

Table 2: Haematological indices of rats exposed to Osun river water and control

Parameter	CM	EM	CF	EF
WBC (10^3/µl)	15.87±2.36	21.20±0.60[a]	12.77±1.03	24.02±2.07[b]
RBC (10^6/ µl)	8.60± 0.67	7.05±0.04	8.47±0.43	6.32±0.27
Hb (g/dl)	13.53± 0.31	12.93±0.42	14.93±0.72	11.43±0.21
HCT (%)	48.23±0.99	42.2±1.80[a]	44.70±3.06	34.13±2.64[b]
MCV (fl)	58.77±1.40	56.73±0.60	59.97±2.21	59.80±0.70
MCH (pg)	17.4±0.36	16.73±0.11	18.70±0.96	17.10±0.36
MCHC (g/dl)	29.63±0.51	29.57±0.15	30.17±0.55	29.04±0.98
PLT (µl)	932.33±13.05	746.33±15.82[a]	1292.67±108.58	730.67±95.52[b]

Data are mean ± standard deviation of five values. CM – control male rat; CF – control female rats; EM – experimental male rats; EF – experimental female rats; a significantly different at $p < 0.05$ from Corresponding CM across the row; b significantly different at $p < 0.05$ from corresponding CF; WBC - white blood cell; RBC - red blood cell; Hb - hemoglobin; HCT - hematocrit; MCV - mean corpuscular volume; MCH - mean corpuscular hemoglobin; MCHC - mean corpuscular hemoglobin concentration; PLT – platelet Count.

Table 3: Biochemical parameters in rats exposed to Osun river water and control

Parameter	CM	EM	CF	EF
Liver TBARS (nmol MDA/mg protein)	1.27±0.21	4.32± 0.24[a]	0.97± 0.02	3.32± 0.43[b]
Serum TP (g/L)	0.11±0.01	0.16± 0.02	0.17± 0.04	0.32± 0.02[b]
Liver GSH (mmol/dl)	66.27±4.02	45.62± 2.43[a]	43.73 ± 2.24	27.06± 5.27 [b]
Liver AST (nmol min^{-1} mg^{-1} protein)	27.23±2.16	14.20±0.53[a]	45.35±1.42	38.57±7.77[b]
Serum AST (nmol min^{-1} mg^{-1} protein)	48.22±5.26	70.86±2.70[a]	26.64±4.04	39.05±3.39[b]
Liver ALT (nmol min^{-1} mg^{-1} protein)	73.09±3.90	33.94±4.56[a]	72.44±15.48	31.92±2.82[b]
Serum ALT (nmol min^{-1} mg^{-1} protein)	35.92±7.57	70.88±10.59[a]	29.73±3.57	56.40±10.36[b]

Data are mean ± standard deviation of five values. CM – control male; EM – experimental male; CF – control female; EF – experimental female; a significantly different at $p < 0.05$ from corresponding CM across the row; b significantly different at $p < 0.05$ from Corresponding CF across the row; TBARS - thiobarbituric acid reactive substances; MDA – malondialdehyde; TP – total protein; GSH – glutathione; AST – aspartate aminotransferase ALT – alanine aminotransferase.

the liver of male and female rats exposed to Osun river water compared to control. There were higher elevations in the levels of serum total protein of male rats than female while no sex-depedence was obtained in TBARS levels. There were higher reductions in the levels of GSH in liver of female rats than males. There were higher elevations of AST activity in serum of female rats than male rats while higher elevations of ALT activity in serum in male rats than female rats were obtained.

DISCUSSION

Toxicological evaluation of wastes washed into the river in rats are necessary due to their deleterious effects. This is so because humans, aquatic life and other living organisms are constantly exposed to them. Therefore, assessment of the effects of contaminated water on the liver and blood of rats is of great importance for a possible prediction of such effects on humans. Human exposure to contaminated water, at high levels, may result in damage to several tissues, leading to death at excessively high levels (Pari and Amali, 2005; Rechenmacher et al., 2010). Given the priority Osun river is accorded because of its mythology, this study assessed the possible health effects of drinking its water.

Heavy metals have been reported to adversely affect health due to their toxicity and bioaccumulative tendencies (Kori-Siakpere and Ikomi, 2011). Exposure to these heavy metals has been attributed to oxidative stress and which has been shown to produce a variety of alterations in tissues which underlie the etiology of many diseases (Jadhav et al., 2007). The high concentrations of heavy metals such as Cd and Pb in Osun river water indicate that it is highly polluted beyond the allowable limits of SON. This could be as a result of washed off torrents from dumpsites, local automobile repair workshops, welders and farming activities around this river (Olajire and Imeokpara, 2000, 2001). VOCs in water constitute health risks even at low concentrations because of their carcinogenicity and mutagenicity (Nikolaou et al., 2002). Therefore, the high concentrations of these compounds especially benzene whose concentrations exceeded permissible limit of WHO in Osun river water could significantly affect health when the river water is consumed untreated. Their presence in the river could have been from pharmaceutical waste materials, dumpsites, spent engine oil and paint wastes washed into the river (Azeez et al., 2013).

Useful information is obtained about diseases and their pathologies when hematologic variables are measured (Saadat et al., 2004; Mansour et al., 2007). Elevations in white blood cell (WBC) count are attributable to acute infections (Jee et al., 2005). This increase could be from counteraction of WBC to fight off deleterious effects of toxic pollutants (Elkind et al., 2001; Jassim and Hassan, 2011). The Osun river water mediated increase in WBC suggests that rats could be reacting to the presence of toxic agents in the water or even infectious agents since the contaminated water might contain microorganisms. Such ability of Osun river to cause increase in WBC might not be unconnected to the presence and concentrations of heavy metals and VOCs in it. These pollutants especially benzene is known to cause leukemia which is the proliferation of white blood cells, thereby increasing their number (Uboh et al., 2008b, Olajire and Azeez, 2012). WBCs are also elevated in response to inflammation by toxic agents. Our results show that exposed female rats were more susceptible to immune system disturbances than exposed male rats. This was also observed by Uboh et al., (2008b) that increase in WBC was sex-dependent. RBC, Hb, HCT, MCV, MCH and MCHC are red blood cell indices and their reductions could result in anemia. Decreases in RBC, Hb and HCT are indicative of anemia, hemorrhage, disturbance in heme biosynthesis and cancer (Eyoung et al., 2004; Mansour, 2007; Kori-Siakpere and Ikomi, 2011). It is not immediately clear why significant reductions were observed for HCT alone. However, increase in blood volume may lead to decrease in the number of cells per unit volume, which is the HCT. This reduction might be from the exposure of rats to Osun river water determined to contain pollutants such as benzene, ethylbenzene, xylenes and Pb which have been reported to destroy red blood cells (Uboh et al., 2008b; Olajire and Azeez, 2012). This observation was

also sex-dependent. Decreases in PLT indicate blood clotting problems (Yilmazet al., 2004). The significant reductions in exposed rats may have been the result of response to the presence of pollutants in Osun river water. Pollutants such as xylene have been linked with the reductions of PLT (Olajire and Azeez, 2012a). Our results show that exposed female rats were more susceptible to PLT reduction than exposed male rats.

The measurement of TBARS which are products of lipid peroxidation is a convenient method to monitor oxidative damage induced by pollutants (Pari and Amali, 2005). Heavy metals and VOCs have been reported to induce free radicals which cause lipid peroxidation (El-Gendy et al., 2010; Olajire and Azeez, 2012). The significant increase in the TBARS in rats treated with Osun river water is indicative of the carcinogenic and mutagenic effects of the pollutants in Osun river because the polyunsaturated membrane lipids succumb easily to deleterious actions of reactive oxygen species (ROS). Increased ROS can be generated from the metabolism of toxicants. The presence of toxicants in the water could have resulted in an increase in ROS production, which has overwhelmed the natural antioxidant system, resulting in oxidative stress. This oxidative stress could have led to oxidative attack on membrane lipids leading to the observed increase in the amount of TBARS. These findings are consistent with reports by Salau et al. (2016) that a toxic agent; diethylnitrosamine caused an increase in concentration of lipid peroxidation products.

GSH plays an important role in preventing the oxidation of cellular macromolecules especially in the lung. It keeps up the cellular levels of active forms of vitamins C and E in blood (Yilmaz et al., 2004; Pari and Amali, 2005). The observed decrease in liver glutathione is indicative of oxidative stress which further corroborates the observed increase in lipid peroxidation. Increased lipid peroxidation and decreased GSH, a natural cellular antioxidant have been observed previously in rats treated with aflatoxin B1 and diethylnitrosamine; two potent carcinogenic agents (Ajiboye et al., 2014; Salau et al., 2016).

Activities of transferases increase in the serum when there is damage to vital organs in the body especially the liver. This can be used as a tool to assess the toxicity of pollutants. ALT and AST are markers of liver cell injury, with ALT being a more sensitive biomarker for the liver than AST (Pramyothin et al., 2006). Increase in the activities of ALT and AST in serum with corresponding decrease in liver observed in this study show that the enzymes have probably leaked from the liver to the extracellular fluid. This may be due to the ealier observed disruption of the ordered bilayer of the liver cell membrane caused by peroxidation of the polyunsaturated phospholipids of the plasma membrane induced by the pollutants in the water. Since AST and ALT are cytosolic and mitochondrial enzymes, their leakage is indicative of the compromise of plasma membrane integrity and possibly mitochondrial membrane. Heavy metals such as Cd and Pb have been well documented to increase the activities of these enzymes in serum (Edewor et al., 2007; Uboh et al., 2008a). The decrease in the activities of these enzymes in the liver would adversely affect carbohydrate and amino acid metabolism and subsequently energy production.

The amount of serum total protein is an index of the synthetic ability of the liver since the plasma proteins; majorly albumin and globulin are synthesized in the liver. It is also a reflection of the balance between protein anabolism and catabolism (Yakubu and Musa, 2012). Increased serum protein has been observed in cases of liver cirrhosis (Mayne, 2005). The increased serum protein in the present study could have resulted from the increased amount of AST and ALT in the blood or imbalance in protein synthesis and degradation. The synthetic ability of the liver might also have been increased as a response to the presence of toxic agents in the water which the rats were exposed to. Increased serum protein was also reported in previous studies where rats were treated with diethylnitrosamine, extracts of *Crateva adansonii*, aqueous leaf extract of *Ficus exasperata* and aqueous root bark extracts of *Anogeissus leiocapus* and *Terminalia avicenniodes* (Salau, 2013; Salau et al., 2012; Akanji et al., 2013)

CONCLUSION

This study has reported the concentrations of pollutants such as heavy metals and volatile organic compounds (VOCs), some of which were higher than the allowable limits of SON and WHO. These pollutants have led to liver cell injury as indicated by alterations in some hematological parameters, increased liver lipid peroxidation, decreased liver GSH, decreased liver cellular enzymes, increased serum protein and increased serum enzyme activities. This means that Osun river water is not safe for drinking despite the religious beliefs and mythology ascribed to it.

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www.njps.com.ng

Niger. J. Physiol. Sci.

Baseline Haematology and Erythrocyte Morphological Changes of Apparently Normal Dogs Raised in Ibadan, Oyo State

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Summary: This study evaluates the haematological parameters and the observed erythrocytes morphological changes in dogs raised in Ibadan, Oyo State in the south western part of Nigeria. Blood samples were collected from sixty-four apparently healthy dogs. The haematological parameters of the blood samples collected were evaluated with the quantification of the percentage erythrocyte morphological abnormalities. The result of the quantitative count of the erythrocyte morphological abnormalities were also converted using reference guide to give the numerical/descriptive clinical grade of the associated morphological abnormalities. There was a significant difference between the haematological parameters reported in this study and the commonly used dog haematological reference ranges from temperate regions used in laboratories in Nigeria. Some of the morphological abnormalities observed in this study include echinocyte (4.12±0.35%), macrocyte (2.61±0.22%) and spherocyte (2.17±0.29%) and eccentrocyte (0.39±0.06%) while other such as acanthocyte (1.39±0.19), leptocyte (0.71±0.13), schizocyte (0.703±0.104) and codocyte (0.50±0.11) were also seen. These study findings show that the associated morphological changes were all not significant and fall within the acceptable range using the reference guide for erythrocyte morphological abnormalities even when the haematological values differ significantly from the reference value. This study provides baseline information on the haematological parameters and the novel correlation of the associated erythrocyte abnormalities seen as a corresponding proof of the apparently healthy status of the dogs raised in Ibadan used for this study. The study while serving as an important means of verification of the reference range of haematological parameters also shows that clinical case interpretation using haematological baseline data from the temperate region should be used with caution in our tropical environment. This thus necessitates the need for an advocacy to build a reference range of haematological parameters that can be used as a working baseline value for the tropical environment.

Keywords*:* Haematological parameters, Erythrocyte morphological changes, South Western Nigeria

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Manuscript Accepted: September, 2015

INTRODUCTION

The use of the quantitative and qualitative evaluation of the haematological parameters of animals and humans has been a vital indicator of the physiological and general well-being for clinical and research purposes. The vital role of the changes in the haematological parameters from the normal values associated with the changes in the internal milieu of the animal or man has thus been a strong basis for its use (Kumar *et al.*, 2005). In clinical practice, the information derived from both the quantitative and qualitative evaluation of the haematological parameters are often used to corroborate the physical examination and the medical history to provide excellent basis for medical diagnosis (Harvey 2012). This evaluation when done to encompass the haematological, haematochemical parameters of the blood metabolites and other component of the body serves as an important investigative tool in the clinical assessment of the physiological and pathological status (Aderemi, 2004; Doyle, 2006). This along with its relatively minimal invasiveness makes it a good means of measuring potential biomarkers (Ginsbury and Haga, 2006), evaluation of the physiological status of the animal (Khan and Zafar, 2005), evaluation of the physiological and pathological responsiveness of animals to pathological and managemental factors arising from changes in the internal milieu and other environmental factors (Khan and Zafar, 2005; Weiss et al., 2010)

Due to the effect of several managemental factors such as stress, nutrition, housing and management system on the haematological parameters, changes in haematological parameters can thus be used to evaluate the effect of stressors and the deviation of an animal from the normal to the stress state (Aderemi, 2004), the evaluation of the nutritional state and the welfare of animals (Jain *et al.*, 1975; Khan *et al.*, 2005; Etim *et al.*, 2014).

A review of literature has shown a plethora of information indicating that researchers have reported data on the haematological parameters (Mshelia *et al.*, 2005; Olayemi *et al.*, 2009) and haematochemical parameters (Awah and Nottidge, 1998; Piccione *et al.*,

2010). Some dog breeds that have been used for these studies include the Nigerian local dogs (Omamegbe and Uche, 1985; Awah and Nottidge, 1998 and Ariyibi, 2002) and the exotic dog breeds also raised in the environment (Awah and Nottidge, 1998; Ariyibi *et al.,* 2002). However, very little information is available on the associated morphological abnormalities of canine blood cells and their role in evaluation and adjudging the normality of haematological parameters and in clinical diagnosis in Nigeria. Disease, infection, genetic disorders, and variations in blood chemistry can alter the RBC shape, reducing its ability to bend and deform (Chien 1987). Abnormal RBC morphologies can impede or even obstruct the circulation, causing tissue necrosis in severe cases (Harvey, 2012). The examination of the blood film for the qualitative assessment of the blood should include blood smear examination for digression from normal of the cell size, shape, distribution, haemoglobin concentration, colour, and intracellular inclusions (Jones, 2009). The detection of these abnormal morphologies and its resultant delineation as either artifactual or pathological finding thus serves as an important means of ascertaining the clinical significance of such abnormal changes. The detected abnormal morphology can thus be correlated with the quantitative values of the haematological parameters for arriving at a more holistic clinical diagnosis (Jones, 2009 and Barger, 2010). As part of measures in the assessment of the blood smear, there is usually a need for a documented protocol for the examination of blood smear for erythrocyte morphological abnormalities. This is important as it serves to provide additional information to the clinician to aid diagnosis and in the institution of the right treatment course. The evaluation of the erythrocyte morphological abnormalities in different laboratories are usually done using either qualitative remarks (few or marked) or a numerical grading (1+ to 4+) based on percentage of variation. There is also a need to describe the type of cell or cells that have caused the variation from the normal (Walton, 1973 and Weiss, 1984). This aids in offering a visual assessment of the blood smear to the clinician to facilitate a more concise diagnosis based on the haematological parameters. However, due to the manual method used in the assessment of the erythrocyte abnormalities, the evaluation is essentially subjective and as such it is important that laboratories establish guidelines based on their patient population (Kaplan, 2003 and Jones, 2009). There is also an important correlation between pathologic processes and the presence of erythrocyte morphological abnormalities (Kaplan, 2003; Jones, 2009 and Barger, 2010).

More so, many laboratories and veterinary clinics in Nigeria uses the standard reference values from foreign laboratories and those obtained from animals raised in temperate regions. These values are not always reliable since the physiological and haematological parameters of apparently healthy dogs are subject to considerable variations due to factors such as physiological state (e.g. lactation, pregnancy), age, sex, breed, nutrition, seasonal variations, sub clinical diseases and climate (Awah and Nottidge 1998). The adoption of such foreign haematological parameters reference intervals for the interpretation of the haematological data for indigenous and exotic breeds raised in our environment may therefore not give an accurate and precise representation of the animal's health status.

This therefore necessitates the need for feasible baseline and verification of the existing foreign haematological values for dogs of all breeds raised in our tropical environment and a measure of the correlation with other erythrocyte morphological indices in assessing the health status of the animals. Therefore, this study evaluates the baseline haematological values for dogs and correlates this with the erythrocyte morphological abnormalities observed in the blood collected from apparently healthy dogs raised in Ibadan, Oyo State, Nigeria

MATERIALS AND METHODS

Study area and sampling procedure

The study was carried out in Ibadan, Oyo State in the South Western part of Nigeria. The area is located in the humid and tropical wet and dry climate with a lengthy wet season and relatively constant temperatures throughout the course of the year. The study was conducted in the month of August - September at the peak of the raining season.

Animals and Selection Criteria

The animals used for this study were apparently healthy large breeds of dogs presented at various clinics (the University of Ibadan Veterinary Teaching Hospital, Mokola Veterinary Hospital, Mokola, and Magma Veterinary Clinics, Bodija) in Ibadan for routine clinical health assessment and/or vaccination. In all the 64 dogs selected include the Nigeria Local Indigenous dog breeds (15) while the exotic dog breeds selected were the large dog breed size and they include: Alsatian (25), Boerboel (9) and Rottweiler (8), and others [Pitbull (3), Neopolitan mastiff (2), Caucasian (1), and Doberman (1)].

All the dogs selected were on a good plan of nutrition with adequate protein supplementation. The physiological parameters (respiratory rate, heart rate, temperature and capillary refill time) of the dogs were also taken for the assessment of the health status and only those with values within normal health range were selected for the study. Blood was collected when the animals were at rest and minimal effort was used in the restraint of the dogs to prevent stressing and agitating the dogs.

Excited and nervous dogs were excluded from the study. Dogs with ectoparasites (such as ticks, fleas) and cutaneous lesions during the physical examination were also excluded from the study. The blood smears and buffy coat smears were also screened for haemoparasites and haemoparasitaemic dogs were excluded from the study.

Sample Collection Procedure

Blood was obtained by venipuncture of the cephalic vein from the subject dogs to obtain whole blood using a 5 mls syringe and 23 gauge needles. About 3 mls of blood were collected and dispensed into heparinized bottles and rocked gentle to allow mixing of the blood with the lithium heparin anticoagulant for haematological analysis. Sampling was done between 8am and 11am to avoid the effect of diurnal variation on the haematological parameters. All the blood samples collected were transported using ice pack and analyzed within 3 hours of collection to reduce preanalytical artifactual errors while standard procedures were also observed in the analysis in order to reduce analytical artifactual errors (Lippi *et al.*, 2005; Harvey, 2012).

Sample/ Blood Analyses Procedures (Haematology)

Analysis of the collected blood samples was divided into the quantitative and qualitative quantification of the red cell, white cells and platelet parameters. The packed cell volume (PCV) was carried out using the microhaematocrit method while the haemoglobin concentration was carried out using the cyanmethaemoglobin method (Cork and Halliwell, 2002). The erythrocyte count was estimated by using the haematocytometer method while the erythrocyte indices (mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration) were calculated using the methods described by Jain (1993). The quantification of the morphological abnormalities was carried using the technique by Weiss 1984. The assessment of the peripheral blood smear for the erythrocyte morphology was done by viewing the thin portion of the smear. The thicker portion of the blood smear was avoided due to the overlap of cells while the edges of the smear where the cells could be artifactually distorted in shape, size and colour. red cells were using the light microscope immersion lens with 1000x magnification (Olympus BX 41) and the morphological abnormalities seen were counted using the tally counter. The values obtained were then converted to % abnormalities by using the formula below as in the case of Echinocytes:

$$\%\textbf{Echinocytes} = \frac{\boldsymbol{Echinocytes\ counted\ out\ of\ 200\ red\ cells\ in\ single}}{\mathbf{200}} \boldsymbol{X100}$$

This is repeated for all the observed morphological abnormalities to obtain the percentage count of each abnormality. The percentage morphological abnormalities obtained was there after scored using the reference guide by Weiss (1984) and Constantino (2015) to determine the severity and to adjudge normality of the animals.

The leucocyte (total white blood cell and differential leucocyte count) and platelet parameters were determined using standard procedures while the absolute leucocyte counts were calculated as described by Sastry (1985) and Coles (1986).

Statistical Analysis

The data collected was analyzed using SPSS v20 statistical package. All data were expressed as Means ± Standard Error of Means (S.E.M) for the measure of central dispersion while One - Way ANOVA was used for the comparison of more than two means at the 5% significance level. Statistical significance was assumed at the p - value of ($p < 0.05$).

RESULTS

Haematological Parameters

Table 1 shows the reference range of the haematological parameters obtained from the dogs used for this study compared with the reference range of the haematological parameters (obtained from dogs raised in the temperate) used in the clinical interpretation of haematological results.

Table1. Heamatological parameter in the studied dogs

Haematological Parameters	Mean±S.E.M	Standard Reference Range*
HB	11.12±0.33 [a]	12.00 - 18.00
RBC	5.61±0.18 [a]	5.50 - 8.50
PCV	34.72±1.04 [a]	37.00 - 55.00
MCV	62.21±0.55 [a]	60 - 77
MCH	20.24±0.65 [a]	19.50 - 24.50
MCHC	32.63±1.07 [1]	32.00 - 36.00
Platelet	1.87±0.10 [a]	2.00 - 5.00
Lymph	2.60±0.21 [1]	1.00 - 4.80
Neutr	6.12±0.47 [a]	3.00 - 11.50
Mono	0.28±0.04 [a]	1.50 - 1.35
Eosin	0.41±0.07 [a]	1.00 - 1.25
Basophil	0.01±0.00 [a]	0 - 140
Total WBC	9.42±0.54 [a]	6.00 - 17.00

Data from Weiss et al., (2010) PCV (%): Packed Cell Volume, Hb (g/dl): Haemoglobin Concentration, RBC (10^3/pL): Red Blood Cell, MCV (fl): Mean Cell Volume, MCH (%): Mean Cell Haemoglobin, MCHC (pg): Mean Cell Haemoglobin Concentration; WBC (*10^3/pL): White Blood Cell, PLT (*10^5/pL): Platelet Count, Lympho (*10^3/pL): Lymphocytes, Neutro (*10^3/pL): Neutrophils, Mono (*10^3/pL): Monocytes, Eosino (*10^3/pL): Eosinophils, Baso (*10^3/pL): Basophils *Where* $^a p < 0.01$ $^b p < 0.02$ $^c p < 0.05$**;** indicates not significant (P>0.05)

Table 2. Erythrocytic Series in the different study dog breeds

Source of Variation		RBC	PCV	HB	MCV	MCH	MCHC
BREED	Alsatian, n = 25	5.71±0.36	35.56±2.19	11.08±0.74	62.87±0.69	20.38±0.36	31.87±0.59
	Rottweiler, n =8	4.94±0.41	31.38±2.29	11.09±0.73	63.99±1.42	20.56±0.38	32.16±0.31
	Boerboel, n =9	5.34±0.39	32.22±2.15	10.19±0.67	60.55±0.64	19.16±0.29	31.66±0.39
	Local, n =15	5.53±0.39	33.67±2.30	11.43±0.70	61.13±0.88	22.18±2.70	36.29±4.37
	Others, n =7	5.70±0.47	36.00±2.26	11.37±0.68	64.10±2.13	20.29±0.77	31.64±0.29
Sex	Male, n =41	5.21±0.22	32.44±1.32	10.35±0.42	62.72±0.61	20.45±1.02	32.68±1.66
	Female, n =23	6.07±0.33	37.26±1.90	12.00±0.63	62.85±0.72	20.90±0.26	32.17±0.20
Age Group (years)	< 1 year, n=18	5.44±0.33	34.06±1.94	10.79±0.61	62.44±0.79	21.79±2.25	35.49±3.65
	1 - 5 years, n =37	5.67±0.26	35.19±1.52	11.02±0.51	62.71±0.63	20.52±0.29	35.49±3.65
	> 5 years, n =9	5.46±0.52	34.22±3.02	10.92±0.94	62.12±1.37	20.17±0.39	31.98±0.28

NS, indicates not significant (P>0.05); PCV (%): Packed Cell Volume, Hb (g/dl): Haemoglobin Concentration, RBC (10^3/pL): Red Blood Cell, MCV (fl): Mean Cell Volume, MCH (%): Mean Cell Haemoglobin, MCHC (pg): Mean Cell Haemoglobin Concentration [Others=Pitbull (3), Neopolitan mastiff (2), Caucasian (1), and Doberman (1)]

Table 3. Leucocytic series and Platelet parameters in the different study dog breed

Source of Variation		WBC	Neutro	Eosino	Baso	Mono	Lympho	Platelet
BREED	Alsatian, n = 25	9.27±0.98	5.76±0.84	0.40±0.10	0.00±0.00	0.27±0.07	2.83±0.41	1.85±0.18
	Rottweiler, n =8	9.76±1.56	6.52±1.45	0.79±0.34	0.00±0.00	0.29±0.10	2.16±0.65	1.80±0.29
	Boerboel, n =9	10.17±1.81	7.32±1.36	0.32±0.14	0.00±0.00	0.27±0.06	2.25±0.43	2.15±0.28
	Local, n =15	9.75±0.95	6.20±0.81	0.37±0.11	0.03±0.02	0.30±0.06	2.85±0.43	1.85±0.15
	Others, n =7	7.12±1.03	4.64±1.17	0.20±0.06	0.00±0.00	0.27±0.08	2.01±0.21	1.45±0.23
Sex	Male, n =41	8.89±0.65	5.82±0.57	0.37±0.08	0.01±0.01	0.25±0.04	2.44±0.28	1.74±0.11
	Female, n =23	10.13±1.00	6.47±0.84	0.49±0.12	0.01±0.00	0.32±0.08	2.84±0.32	2.02±0.18
Age Group (years)	< 1 year, n=18	8.94±0.78	5.69±0.70	0.46±0.16	0.01±0.01	0.27±0.05	2.51±0.33	1.81±0.16
	1 - 5 years, n =37	9.11±0.70	5.76±0.55	0.38±0.08	0.01±0.01	0.28±0.05	2.68±0.32	179±0.12
	> 5 years, n =9	11.04±2.23	8.02±2.06	0.42±0.15	0.00±0.00	0.29±0.11	2.31±0.43	2.14±0.36

WBC (*10^3/pL): White Blood Cell, PLT (*10^5/pL): Platelet Count, Lympho (*10^3/pL): Lymphocytes, Neutro (*10^3/pL): Neutrophils, Mono (*10^3/pL): Monocytes, Eosino (*10^3/gL): Eosinophils, Baso (*10^3/pL): Basophils

The haematological parameters obtained from the study dogs except the lymphocyte and the MCHC value were significantly different and lower than the standard reference range obtained from the foreign studies, however the reference range of the MCHC and the lymphocyte count were higher than those of the temperate region studies.

Table 2 shows and compares the values of the erythrocyte parameters (Red cell count, Haemoglobin concentration and packed cell volume) and the erythrocytic indices (MCV, MCH, MCHC) across the different breed groups, sex and the age group of the study animals. The values of the red cell, packed cell volume and the haemoglobin concentration was higher in the study female animals than in the male dogs used for this study thus showing a significant difference in the erythrocyte parameters between the sexes. There was also a significant difference in the erythrocyte parameters when compared across the different breeds even when they were raised in the same environment. An age group based difference was also seen in the erythrocyte parameters.

As shown below, Figure 3 gives the graphical representation of the observed erythrocyte morphological abnormalities in male and female dogs while Figure 4 gives the graphical representation of the observed morphological abnormalities across the different age groups. The erythrocyte morphological abnormalities of the dogs in the different age group and sex are both not clinically significant and do not indicate any pathological state when evaluated using the reference guide by Weiss (1984).

Table 4. shows the Mean ± S.E.M of the percentage of the erythrocyte morphological abnormalities seen in the study animals. These values were evaluated using the reference range for the adjudging the clinical descriptive and numerical values of the erythrocyte morphological abnormalities for clinical haematology case interpretation as described by Weiss (1984). The values of the dogs used for this study were found to be non-significant and within the normal range for healthy dogs. Figure 1 and Figure 2 shows some of the observed erythrocyte morphological abnormalities.

Table 4. Percentage Erythrocyte Morphological Abnormalities

Parameters	Mean±S.E.M
Eccentrocyte	0.391±0.064
Microcyte	1.063±0.136
Macrocyte	2.609±0.224
Spherocyte	2.172±0.285
Leptocyte	0.711±0.125
Polychromasia	0.891±0.112
Codocyte	0.500±0.111
Elliptocyte	1.258±0.120
Dacryocyte	0.227±0.057
Drepanocyte	0.078±0.032
Keratocyte	0.430±0.063
Stomatocyte	1.094±0.181
Acanthocyte	1.391±0.193
Echinocyte	4.117±0.350
Schizocyte	0.703±0.104

*The Mean value of each of the erythrocyte morphological abnormalities was compared with the scale in the reference guide given by Weiss (1984) and Constantino (2015) used for the evaluation of descriptive value and the clinical significance of the observed erythrocyte abnormalities

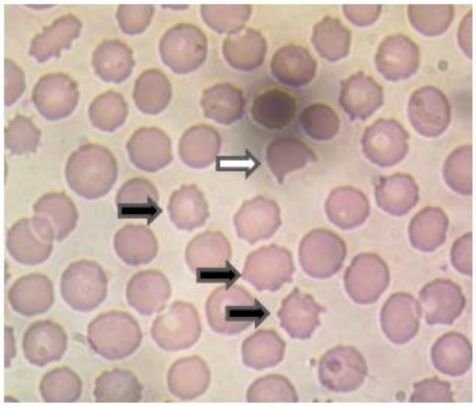

Figure 1: Giemsa stained dog red blood cell showing ecchinocyte (black arrow) and schizocyte (white arrow) (×100)

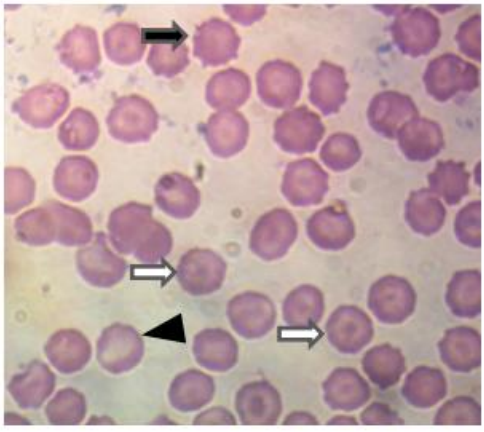

Figure 2: Giemsa stained dog red blood cells showing some ecchinocyte (black arrow), codocytes (white arrow) and stomatocyte (black arrowhead) (×100)

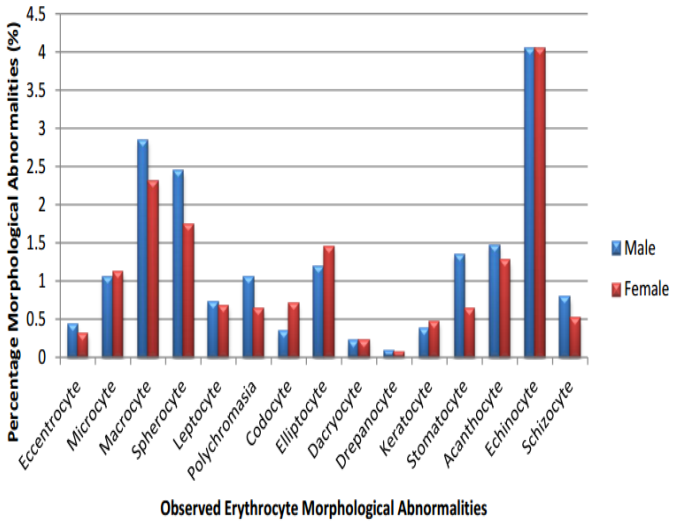

Figure 3. Graphical Representation of the Observed Erythrocyte Morphological Abnormalities in Male and Female Dogs.

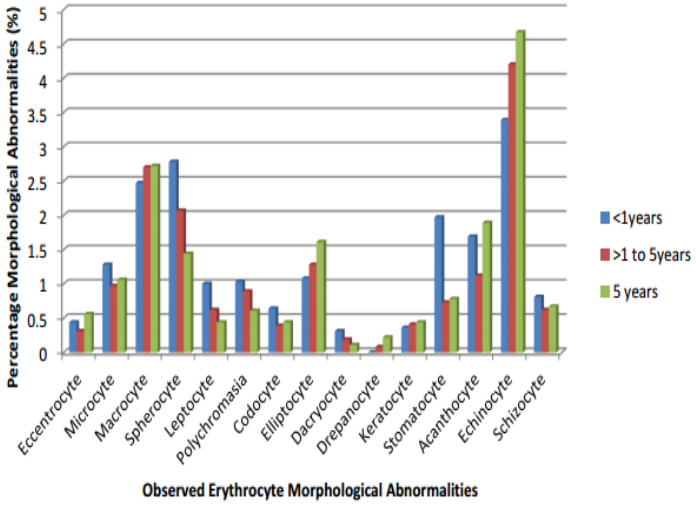

Figure 4. Graphical Representation of the Observed Morphological Abnormalities in the Different Age Groups.

As shown above, Figure 3 gives the graphical representation of the observed erythrocyte morphological abnormalities in male and female dogs while Figure 4 gives the graphical representation of the observed morphological abnormalities across the different age groups. The erythrocyte morphological abnormalities of the dogs in the different age group and sex are both not clinically significant and do not indicate any pathological state when evaluated using the reference guide by Weiss (1984).

As shown in table 5, the percentage erythrocyte morphological abnormalities across the different breed s were expressed in Mean ± S.E.M. compared to the scale by Weiss (1984), all the values were within the normal range for healthy dogs. The table also shows the different in the percentage of the erythrocyte morphological abnormalities across the different breeds.

Table 5. The Numerical Quantification of the Percentage Observed Erythrocyte Morphological Abnormalities in The Different Study Dog Breeds

Source of Variations	Breed				
	Als (n=25)	Rott (n = 8)	Boer (n = 9)	Local (n =15)	Others (n =7)
Eccentrocyte	0.30±0.09	0.63±0.18	0.39±0.18	0.43±0.15	0.29±0.29
Microcyte	1.04±0.23	1.25±0.35	1.50±0.51	0.97±0.26	0.64±0.32
Macrocyte	2.94±0.41	3.56±0.57	2.72±0.53	1.67±0.36	2.50±0.42
Spherocyte	1.78±0.28	1.56±0.68	2.67±0.51	2.37±0.41	3.29±2.16
Leptocyte	0.56±0.16	0.63±0.26	0.61±0.34	1.10±0.36	0.57±0.38
Polychromasia	0.84±0.20	0.44±0.22	1.39±0.33	0.83±0.22	1.07±0.38
Codocyte	0.26±0.10	0.81±0.39	0.61±0.36	0.53±0.30	0.50±0.36
Elliptocyte	1.48±0.20	1.44±0.32	0.78±0.33	1.27±0.25	1.00±0.38
Dacryocyte	0.22±0.10	0.13±0.08	0.00±0.00	0.43±0.16	0.07±0.07
Drepanocyte	0.06±0.04	0.00±0.00	0.17±0.12	0.07±0.07	0.14±0.14
Keratocyte	0.50±0.11	0.13±0.08	0.11±0.07	0.57±0.17	0.36±0.24
Stomatocyte	0.70±0.21	1.00±0.47	1.44±0.48	1.93±0.54	0.29±0.15
Acanthocyte	1.16±0.23	0.94±0.42	2.11±0.90	1.77±0.43	1.00±0.29
Echinocyte	4.40±0.57	3.13±0.63	4.44±1.29	3.77±0.61	3.86±1.02
Schizocyte	0.68±0.19	0.56±0.29	0.50±0.19	0.87±0.25	0.64±0.28

DISCUSSION

The present study has shown a significant difference in some haematological values (RBC, MCV, MCH, WBC, HB, PCV, Platelet count, Neutrophil count, Monocyte count, Eosinophil count and Basophil count) when compared with the reference values from laboratories in temperate regions that are commonly used for evaluation of haematological parameters in our laboratory. This is similar to earlier reports in which the haematological parameters of dog raised in the tropics were comparatively lower than those raised in the temperate region (Bush, 1991; Awah and Nottidge, 1998; Ariyibi et al., 2002). The disparity in the values of the haematological parameters of this animal can be adduced to the significant influence of the environment in the determination of the physiological state and parameters of animals (Etim et al., 2014). The significant influence of the environmental factors (which encompasses factors such as altitude, climate, nutrition etc.) has been studied extensively in human and animals and serves as an important basis for the observed differences (Etim et al., 2014).

The comparative difference seen in the erythrocyte parameters of the male and female dogs is also similar to previous reports in dogs (Ariyibi 2002) and goats (Tibbo et al., 2004). The relatively higher lecucocytic parameter values also seen in the female compared to the male is similar to reports in other studies (Ariyibi 2002, Tibbo et al., 2004) and this has been adduced to different physiological factors associated with the oestrus cycle in females (Tibbo et al., 2004; Mshelia et al., 2005).

From this study, the most common erythrocyte morphological abnormality was echinocytes (Figure 1 and Figure 2). This erythrocyte morphological abnormality has been associated with different factors such as artifactual changes thus buttressing the importance of proper sample collection, prompt and proper sample analysis in order to achieve good smear production devoid of artifacts (Harvey, 2012). This study finding cannot however be completely attributed to be due to artifactual changes since lithium heparin were used for the sample collection thus reducing the possibility of the crenation inducing effects of EDTA (ethylene diamine tetra-acetate) anticoagulant which is known to cause significant preanalytical crenation and echinocyte formation (Lippi et al., 2005; Gorrol and Mulley, 2009) and proper precautions were taken in the smear preparation.

Spherocytes and macrocytes were the next most commonly observed morphological changes which may be due to the need for a rapid turnover of the red cell as well as need for prompt regeneration of red cell possibly associated with haemoprotozoan (both subclinical and clinical manifestation) which are enzootic in this area (FAO, 1983). Other observed abnormalities include polychromasia, and schisocytes which could also be associated with the enzootic haemoprotozoan diseases (Telford et al., 1997). While however these erythrocyte morphological abnormalities were seen prominently, the relative count of these abnormalities when converted using the numerical reference guide were all not significant and

as such cannot be adduced to any pathological condition or disease state (Jones.2009; Constantino, 2015). This is important as it complements the clinical evaluation of the apparently clinically healthy status of the dogs used for this study and the credibility of the fact that the deviation of the haematological parameters seen are not due to associated pathologies.

This study was able to establish a haematological baseline and the associated morphological changes observed in blood of apparently healthy dogs raised in Ibadan, South-western Nigeria. The discrepancies in the range of parameters may be due to the influence of environmental factors on the physiological parameters. Therefore, the baseline obtained from these dogs could be used for interpretation of laboratory data in dogs raised in Ibadan and the significant difference between the haematological parameters of animals gives more credence to the importance of developing a working reference range for dogs raised in our tropical region.

More so, the qualitative examination of the morphological changes when compared with the quantitative evaluation of the haematological parameters can also be employed as an important diagnostic tool in canine practice.

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Niger. J. Physiol. Sci. 30 (2015) 119 –123
www.njps.com.ng

Niger. J. Physiol. Sci.

Salivary Secretion and Composition in Malaria: A Case-control Study

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Summary: No previous studies have documented changes in salivary secretion in patients with malaria. This study aimed to compare salivary secretion and composition in malaria positive and malaria negative individuals. Ninety participants composed of 40 malaria parasite positive and 50 malaria parasite negative individuals (age and gender matched) were included. Malaria diagnosis was achieved by microscopic examination of Giemsa stained thick and thin film of blood smears. A self-administered questionnaire was used to assess presence or absence of oral symptoms in the malaria parasite positive individuals. Whole saliva samples were collected and analyzed for flow rate, pH, total protein and concentrations of electrolytes (K^+, Na^+, Ca^{2+}, Cl^-, $PO4^{2-}$ and HCO_3^{2-}). Data were analysed using Independent-Samples t-test and Spearman's correlation test. The salivary flow rate was significantly reduced in malaria parasite positive individuals (P = 0.001). Oral symptoms were present in 82.5% of the malaria parasite positive individuals. There was no significant difference in the salivary pH, total protein and electrolyte ion concentrations between the two groups. Also, Spearman's correlation test showed no significant relationship between the presence of oral symptom and the salivary parameters. Salivary flow rates are reduced in the individuals with malaria. However, presence of oral symptoms in these individuals may not be attributed to the reduced salivary flow rate. Further studies are needed to validate our findings and elucidate mechanisms involved.
Keywords*:* Saliva, Malaria, Salivary flow rate, Salivary electrolytes, Salivary pH, Salivary total protein.

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Manuscript Accepted: December, 2015

INTRODUCTION

Saliva is critical for preserving and maintaining the health of oral tissues and has been used as a source of non-invasive investigation of metabolism and the elimination of many drugs (De Almeida *et al.,* 2008). However, it receives little attention until its quantity diminishes or its quality becomes altered. At present, saliva represents an increasingly useful auxiliary means of diagnosis (Tabak, 2001; Malamud, 2010). Many researchers have made use of sialometry and sialochemistry to diagnose systemic illnesses, monitoring general health, and as an indicator of risk for diseases creating a close relation between oral and systemic health (Khalili and Biloklytska 2008; Lasisi and Fasanmade, 2012; Bertl *et al.,* 2013; Rathnayake *et al.,* 2013).

Several infectious diseases have been reported to produce marked and identifiable oral and salivary changes (Shahar *et al.,* 2008; Fung *et al,* 2012; Lazarevic *et al.,* 2013; Li *et al.,* 2014), and malaria being one of these diseases can affect the oral cavity, with salivary gland involvement. Since many oral and systemic conditions manifest themselves as changes in the flow and composition of saliva (King *et al.,* 1994; Dodds *et al.,* 2000), malaria may cause alteration in salivary gland function and saliva composition. More so, many metabolic and hematologic complications develop in patients with malaria infection (Bartoloni and Zammarchi, 2012) which can lead to changes in the oral cavity and salivary composition and secretion. Assessment of changes in salivary secretion and composition in malaria infected individuals can serve as indicators of their oral health condition and probably, biological markers of the disease in these individuals. Some oral symptoms of malaria include conditions such as taste impairment, altered sensation, oral dryness, metallic taste and oral ulcers (Owotade and Greenspan, 2008; Scully, 2008) which may be associated with changes in salivary secretion. However, comparative analysis of salivary secretion and composition in malaria positive and negative individuals has not been previously documented to the best of our knowledge. This study was therefore designed to compare salivary secretion and composition in malaria negative and malaria positive individuals.

MATERIALS AND METHODS

Study Population

The study included 90 human subjects (40 malaria

parasite positive and 50 malaria parasite negative individuals; age and sex matched). The participants were consecutive patients attending the General Out Patients Department, University College Hospital, Ibadan. The study received ethical clearance and approval by the University of Ibadan/University College Hospital Research Ethics Committee (UI/EC/13/0099). Participants were provided information regarding risks and benefit of the study and consent was taken. Individuals with severe malaria, other systemic diseases like diabetes mellitus and hypertension and those on medications were excluded. Biodata and oral symptoms (associated with malaria) of the participants were assessed using self-administered proforma.

Malaria Diagnosis

Malaria diagnosis was achieved by microscopic examination of Giemsa stained thick and thin film of blood smears by two independent microbiologists. Thick-film smears were prepared from blood (venipuncture) at the time of presentation, dried and stained with 10% Giemsa. The smears were inspected for parasites by microscopy under 200 x magnifications by the microbiologists. At least 100 malaria parasites and 200 white blood cells were counted. The density of parasites per microliter of blood was calculated with reference to 8,000 white blood cells per microliters according to the Guidelines for the laboratory diagnosis of malaria (Bailey *et al.*, 2013).

Saliva Collection

Saliva collection was undertaken between 8 a.m. and 10 a.m. and participants had not had meal for at least 2 hours. Unstimulated saliva was collected by spitting method. Participants were asked to spit (after rinsing the mouth with distilled water) into calibrated universal plastic bottles for a period of 10 minutes. Rate of resting saliva secretions were expressed in mls/mins and the pH of saliva samples were determined using a calibrated pH meter (PH-012 Portable pH Meter, China). Volumes of the secretions were recorded and stored at -20^{o}C until laboratory analysis. Saliva samples were defrosted at room temperature and then centrifuged at 3000 rpm for 10 minutes before being used for laboratory analysis in order to remove extrinsic contaminants such as oral epithelial cells, micro-organisms and food debris.

Analysis of Salivary Ions

Saliva collected was analyzed for the concentrations of K^+, Na^+, Ca^{2+}, Cl^-, PO_4^{2-} and HCO_3^{2-}. For the determination of salivary ions, saliva was diluted at 1/100 and K^+, Na^+ and Ca^{2+} concentrations were determined using flame emission spectrophotometry. Concentrations of Cl^- and HCO_3^- were determined by Schales method using mercuric nitrate (Schales and schales, 1941) while concentrations of PO_4^- was determined using Cyrus Fiske and Subbarow's method (Fiske and Subbarow, 1925).

Analysis of salivary total protein

Saliva samples were defrosted at room temperature and then centrifuged at 3000 rpm for 10 minutes before use. Total protein concentration expressed as mg/dl was determined using established colorimetric methods with the use of Helios spectrophotometer by reading samples at 720nm (Spectrumlab 23A, Techmel and Techmel, Texas, USA). Bovine serum albumin was used for calibration purposes.

Statistical Analysis

The main outcome variables were mean values of salivary flow rate, pH, total proteins, sodium, potassium, calcium, chloride, bicarbonate and phosphate in malaria positive and malaria negative individuals. Data are presented as median with interquartile range. Data were compared using Independent-Samples Mann-Withney U test. Spearman's correlation test was used to assess the relationship between oral symptoms in malaria parasite positive individuals and the salivary parameters. The level of statistical significance was set at $p < 0.05$.

RESULTS

There were 90 participants comprising 64 females and 26 males with a mean age of 29 ± 7.5 years (range: 18 to 45 years). Malaria parasite positive and malaria parasite negative participants were age and gender matched (Table 1).

Among the malaria parasite positive individuals, the reported oral symptoms are shown in table 2. There were no significant correlations between the presence of oral symptoms and the salivary parameters (Table 3).

The salivary flow rate was significantly reduced in malaria parasite positive) individuals compared to malaria parasite negative individuals (P = 0.001). The salivary pH and total protein levels were not significantly different comparing malaria parasite positive individuals and malaria parasite negative

Table 1: Demographic characteristics of the participants

	Malaria Parasite Positive Individuals	Malaria Parasite Negative Individuals
Males	13	15
Females	27	35
M:F	1:2.1	1:2.3
Age (yrs)	29 ± 7.73	29.76 ± 7.51

M:F= Male to female ratio

Table 2**.** Oral symptoms in malaria parasite positive individuals (N=40)

Oral symptom	Frequency	Percentage
Mouth bitterness	10	25
Dry mouth	2	5
Altered taste	8	20
Mouth bitterness + dry mouth	7	17.5
Mouth bitterness + altered taste	4	10
Mouth bitterness, dry mouth + altered taste	2	5
No symptom	7	17.5

Table 3**.** Salivary flow rate (SFR), pH and Total protein levels between the groups

	Malaria Parasite Positive N = 40	Malaria Parasite Negative N = 50	P value
Flow rate (mls/min)	1.65 (1.03)	2.70 (1.83)	0.001
pH	7.13 (6.91)	7.20 (7.04)	0.79
Total protein (mg/dl)	0.5 (0.3)	0.5 (0.3)	0.90

Table 4. Salivary electrolyte concentrations in malaria parasite positive and negative participants (Data are presented as median (interquartile range)

Salivary Electrolytes	Malaria parasite positive N = 40	Malaria parasite negative N = 50	P value
Na^+ (mmol/L)	15.45 (11.00)	16.00 (13.75)	0.53
K^+ (mmol/L)	25.10 (20.83)	24.60 (20.48)	0.89
Cl^- (mmol/L)	39.00 (29.78)	39.50 (31.75)	0.79
HCO_3^- (mmol/L)	4.00 (3.00)	4.10 (3.03)	0.93
Ca^{2+} (mmol/L)	3.55 (2.03)	4.4 (2.45)	0.39
PO_4^{2-} (mmol/L)	17.80 (15.15)	18.60 (13.68)	0.86

Table 5: Correlation between presence or absence of oral symptoms and salivary parameters

Salivary parameter	Correlation coefficient	P value
Flow rate	0.13	0.43
Total protein	0.03	0.86
pH	0.04	0.81
Sodium	0.19	0.23
Potassium	-0.15	0.35
Chloride	0.14	0.40
Bicarbonate	0.06	0.74
Calcium	-0.13	0.44
Phosphate	-0.14	0.38

individuals (Table 4). Although, there were changes in the salivary electrolytes (K^+, Na^+, Ca^{2+}, Cl^-, PO_4^{2-} and HCO_3^{2-}) concentrations in malaria parasite positive individuals compared to their concentrations in malaria parasite negative individuals, there was no significant difference in the electrolyte ion concentrations between the two groups (Table 5).

DISCUSSION

Saliva plays an essential role in the maintenance of oral health. It is a unique fluid that can be used to monitor both oral and systemic diseases. Its secretion and composition are the major factors responsible for its physiological functions (Tabak, 2001; De Almeida *et al.*, 2008). Alterations in these factors have been implicated in various oral and systemic diseases (Khalili and Biloklytska 2010; Lasisi and Fasanmade, 2012; Bertl *et al.*, 2013; Rathnayake *et al.*, 2013).

In this study, the decrease in salivary flow rate observed could be due pyrexia which usually accompanies malaria infection. Pyrexia is common clinical feature of malaria which may result in hyperthermia leading to dehydration. The hypothalamic control of temperature involves water loss as a compensatory mechanism to lower the raised body temperature (Vybiral *et al.*, 2000). This may explain the reduction in salivary flow rate of malaria positive individuals because dehydration has been associated with reduced salivary flow rate (Falcao *et al.*, 2013). In addition, malaria is commonly associated with nausea, vomiting, diarrhoea and abdominal cramps especially in children (Bartoloni and Zammarchi, 2012) which may also contribute to dehydration resulting in reduced salivary flow rate.

Although some studies have shown the presence of protein biomarkers in malaria positive individuals (Poinsignon *et al.*, 2008; Huang *et al.*, 2012), this did

not manifest as changes in the total protein concentration levels observed in this study. Lack of difference in the salivary total protein in malaria positive individuals compared to malaria negative individuals may be due to its generally low salivary level that is independent of blood protein level. However, there has been no notable report about decrease or increase of salivary total protein in malaria positive individuals. Also in this study, the salivary pH levels were within the normal range and also indicated that malaria infection did not affect the salivary pH level. This may imply that malaria infection does not affect the buffering capacity of saliva hence malaria positive individuals may not be more susceptible to dental caries.

In this study, there was no change in the electrolyte ion concentrations between the two groups. This could be due to the fact that the secretory mechanisms for the various electrolytes in saliva of malaria positive individuals are not affected. Generally, it is expected that the concentrations of the electrolytes would be affected due to the reduced flow rate observed in malaria positive individuals but the lack of significance difference might be due to whole saliva sample used in this study. Gland specific saliva samples are more appropriate for the assessment of secretory mechanisms of saliva (Lofgren *et al.*, 2012) which is one of the limitations of this study.

Similar to the report of previous studies (Scully, 2008; Owotade and Greenspan, 2008), malaria parasite positive individuals reported oral symptoms like dry mouth, altered or metallic taste, as well as bitter taste. However, these symptoms did not show correlation with the salivary factors assessed. Generally, it has been observed that feeling of dry mouth or altered taste may not indicate altered biochemical composition of saliva and more importantly that of the electrolytes. Dry mouth has been described as subjective sensations of dryness of the oral mucosa which may not be associated with salivary glands hypofunction in some individuals. Hence, lack of significant change in the salivary biochemical composition of malaria parasite positive individuals as well as the lack of association between these factors and oral symptoms reported support the subjective nature of the conditions. Also presence of these symptoms in the malaria parasite positive individuals may be attributed to other components of saliva that were not assessed in this study.

Malaria diagnosis in this study was made using the microscopic examination of giemsa stained thick film for malaria parasites which is the gold standard. The detection limit by thick film microscopy is claimed to be in the 100 to 5 parasites per microliter (pl) range on a microscopic slide (Wongsrichanalai *et al.*, 2007). Quality of the data obtained through microscopy can significantly vary, because microscopic accuracy largely relies on the experience and training of the assessor. Thus, in low parasitemia cases, microscopy is ineffective and unreliable in malaria detection (Harris *et al.*, 2010) which is another limitation of this study. Moreover, studies have shown that microscopy underestimates true parasite counts as parasites are likely to be washed off or lysed during Giemsa staining (Bejon *et al.*, 2006).

CONCLUSION

Findings from this study suggest that salivary flow rate is reduced in malaria parasite positive individuals. However, no significant change in salivary pH, total protein and electrolytes was observed in malaria positive individuals. Also there was no association between oral symptoms reported by malaria parasite positive individual and the salivary factors. Further studies including longitudinal follow up and experimental studies are needed in this area.

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Niger. J. Physiol. Sci. 30(2015) 125-129
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A Survey of ABO, Rhesus (D) Antigen and Haemoglobin Genes Variants in Oyo State, Nigeria

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Summary: A survey of ABO and Rhesus (Rh D) antigens and variants of haemoglobin genes (HbGen) in Oyo state was carried out. This longitudinal study involved the determination of ABO and Rh(D) antigens in 3241 and HbGen in 2622 male and female adults (aged 26-65years) respectively using standard methods. 94.5% of the subjects were Rh(D) positive while 5.5% were Rh(D) negative respectively based on the detection (Positive) or absence (Negative) of Rh(D) antigen. 22.8% of the subjects had ABO blood group A, 26.4% were group B, 4.1% were group AB while 46.7% were group O. Further analysis revealed that 695 (21.4%) of the group A were Apositive while 44 (1.4%) were Anegative. 800 of these subjects (24.7%) were Bpositive while 56 (1.7%) were group Bnegative. 133 (4.1%) showed group AB out of which 125 (3.8%) were ABpositive and 8 (0.3%) were ABnegative. 1513 (46.7%) were group O out of which 1444 (44.6%) were Opositive while 69 (2.1%) were Onegative. HbGen determination showed that 1933 of the subjects (73.7%) had HbGen AA; 553 (21.1%) were AS; 119 (4.5%) were AC; 11 (0.4%) were SC while 3 subjects representing 0.1% and 0.2% each had HbGen SS and CC respectively. Although the results were similar to earlier ones; however, the need for sustained counselling towards eradication of SS genes and increased research towards identifying artificial blood substitutes was highlighted in this work. The increasing need for blood transfusion especially with the increase in various politically/communally motivated emergency situations underscores this fact.

Keywords*:* ABO antigen, Rhesus D, Blood group, Haemoglobin genotype, Blood substitutes

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Manuscript Accepted: July, 2015

INTRODUCTION

Oyo state is one of the 36 states in Nigeria with a population figure of 5,591,589 (NPC, 2006); It could be described as the rallying place for all Yorubas in Nigeria. Economically, it is largely an agrarian community but politically remains one of the most sophisticated and volatile in Nigeria. Although it is largely populated by the Yorubas, the hospitality, culture and friendly weather make the place highly cosmopolitan; thus serving as home to people from all the major tribes in the country as well. The clinical significance of the ABO and Rh(D) antigens determination in the management of both acute and chronic diseases is not in doubt (Reid and Bird, 1990); this is underscored by the fact that blood transfusion services remain one of the backbone of emergency services in standard hospitals. Although there are several blood group systems, the ABO and Rhesus blood group systems remain the most important clinically; they are the most immunogenic of all the blood group antigens and constitute the commonest cause of death in blood transfusion incompatibility reactions. Aside from the clinical importance of ABO and Rh(D) blood groups in transfusion medicine, ABO and Rh(D) blood types could be used by lawyers in paternity suits, by police in forensic science, and by anthropologists in the study of different populations.

Historically, an Austrian scientist, Karl Landsteiner, was said to have discovered the first blood group system in the 20th century (Landsteiner, 1940). He noted that red blood cells (RBC) of some individuals were agglutinated by the serum from other individuals. He made a note of the patterns of agglutination and showed that blood could be divided into groups. The ABO blood group of an individual is genetically determined. The ABO blood group antigens are encoded by one genetic locus, the ABO locus, which has three alternative (allelic) forms A, B, and O (Reid and Lomas-Francis, 2004). A child receives one of the three alleles from each parent, giving rise to six possible genotypes and four possible blood types (phenotypes); which are A, B, AB and O respectively (Reid and Lomas-Francis, 2004). The immune system forms antibodies against whichever ABO blood group antigens are not found on the individual's RBCs. Thus, a group A individual will have anti-B antibodies and a group B individual will have anti-A antibodies. Blood group O is common, and individuals with this blood type will have both anti-A and anti-B in their serum. Blood group AB is the least common, and these individuals will have neither anti-A nor anti-B in their serum. Like the ABO antigens, the haemoglobin gene is also genetically inherited; Adult hemoglobin is a

tetrameric hemeprotein [α(2):β(2)] [α(2):β(2)] [α(2):β(2)]found in erythrocytes where it is responsible for binding oxygen in the lung and transporting the bound oxygen throughout the body where it is used in aerobic metabolic pathways. The haemoglobin molecule is made up of two main units: the globin- which is the proteinous part- and the haem prosthetic group which is the oxygen carrying arm of the molecule. Each subunit of a hemoglobin tetramer has a heme prosthetic group while the globin fraction has peptide subunits designated as α, β, γ and δ which are arranged into the most commonly occurring functional hemoglobins. Although the secondary and tertiary structure of various hemoglobin subunits are similar, reflecting extensive homology in amino acid composition, the variations in amino acid composition that do exist impart marked differences in hemoglobin's oxygen carrying properties. Mutations in the globin genes that alter the protein composition but not necessarily the amount of expression are referred to as qualitative mutations. Of the mutations leading to qualitative alterations in hemoglobin, the missense mutation in the β - globin gene that causes sickle cell anemia is the most common. Mutations in the various peptide subtypes during the developmental stages in-utero allow for the formation of six possible types of the globin moiety of the molecule. Based on the amino acid sequence, it is thus possible to have haemoglobin molecules with gene types AA, AS, AC, SS, SC, and CC. The mutation causing sickle cell anemia is a single nucleotide substitution (A to T) in the codon for amino acid 6. The change converts a glutamic acid codon (GAG) to a valine codon (GTG). This form of hemoglobin in persons with sickle cell anemia is referred to as HbS while the nomenclature for normal adult hemoglobin protein is HbA. In addition, the quaternary structure of hemoglobin leads to physiologically important allosteric interactions between the subunits leading to differences in the mass density, net charges and therefore electrophoretic mobility of the different subunits. Electrophoresis of hemoglobin proteins from individuals is an effective diagnostic tool because the variant hemoglobins have different charges. These differences made separation of variants of haemoglobin into the six possible genotypes as stated above. The inherited disorders of haemoglobin are the most common gene disorders with 7% of the world's population being carriers. It is on record that about 300,000 children are born with sickle cell disease (SCD) worldwide every year. Since the peptide subtype of the haemoglobin molecule determines the oxygen carrying capacity of blood, presence of abnormal amino acid sequence as is the case in people with sickling disorders affect the oxygen carrying capacity and hence the rate of aerobic metabolic activity in the cell. Sickling disorders are found very frequently in the Afro-Caribbean populations and sporadically throughout the Mediterranean region, India and the Middle East. These sickling disorders include the heterozygous state for haemoglobin S or the sickle cell trait (AS), the homozygous state for HbS or sickle cell anaemia (SS) and the compound heterozygous state for HbS. The social-economic impact of this disorder coupled with its attendant health problems largely underscores the need for its knowledge. The relevance of haemoglobin genotype especially for medico-social needs is equally obvious as this has largely contributed to the correction and eradication of the superstitious death due to complications of haemoglobinopathy especially in early childhood of people in this part of the world in the past. This work was thus undertaken to determine the frequency of the various phenotypes of the ABO and Rh(D) antigens and the haemoglobin genotypes in Oyo state with a view to providing relevant information for medical planning and statistics in the state.

MATERIALS AND METHODS

This work was part of a medical screening exercise for residents in the 33 local councils of Oyo State in the years 2004, 2006, 2008 and 2010. Ethical Approval was obtained from Oyo State Ministry of Health for the purpose of the Survey.

Figure1. Map of Nigeria showing Oyo state along with other thirty-five states

For this exercise, adult males and females (aged 24-65years) from the 33 local government areas of Oyo state were screened; they gave their informed consent to participate in the exercise. The survey was conducted at five designated centres where participants from adjoining councils were assembled. These were (1) Ibadan (consisting of 11 local councils), (2) Tapa representing Ibarapa (with 3 local

councils), (3) Ogbomoso (with 5 local councils), (4) Saki representing Oke-ogun (with 10 local councils) and (5) Oyo (with 4 local councils). A total of 3241 participants pooled together from the above centres were surveyed. About 5ml of blood was collected from each of the subjects through venepuncture into K_2EDTA bottles. The blood was analysed for the ABO and Rh(D) antigens on the red cell membrane using standard test-tube and plate agglutination reaction techniques (Dacie and Lewis, 1995) and for their haemoglobin genotype using their electrophoretic mobility (Schneider, 1978).

The results obtained were collated and sorted into tables.

RESULTS

Data obtained from this survey are presented in table 1-3 and compared with other studies in tables 3 and 4.

The frequency of ABO and Rh(D)genes and their various phenotypes showed that phenotype group O still constituted the largest population with a cumulative frequency of 1513 subjects (46.7%) out of a total of 3241 subjects surveyed. Out of this total for group O phenotype, blood group O Rh(D) positive constituted 44.6% (1444 subjects) while blood group O negative constituted 2.1% (69 subjects). Blood group B phenotype had the second highest cumulative frequency of 856 subjects (26.4%) out of the total subjects surveyed; this group also occurred as group B Rh (D) positive in (800 subjects) constituting 24.7% and group B Rh (D) negative constituting 1.7% (56 subjects). ABO blood group A was next in frequency of occurrence with a total of 739 subjects (22.8%); this group also occurred as 695 subjects (21.4%) constituting group A Rh (D) positive and 44 subjects (1.4%) constituting group A Rh (D) negative respectively. ABO blood group AB was observed in 133 subjects (4.1%) out of the total population surveyed; this group also consisted of 125 subjects (3.8%) occurring as AB Rh (D) positive and 8 subjects (0.3%) occurring as blood group B Rh(D) negative (Table 2). Although the population surveyed annually looked skewed, the blood group distribution in all the years followed the normal Hardy-Weinberg distribution pattern.

The pattern of haemoglobin genotype results obtained showed that out of the 2622 subjects screened, 1933 of the subjects (73.7%) had HbGen AA; 553 (21.1%) were AS; 119 (4.5%) were AC; 11 (0.4%) were SC while 3 subjects representing 0.1% and 0.2% each had HbGen SS and CC respectively (Table 3). There was a slight drop in the population of HbGen SS and CC from the population studied relative to data from previous works. This slight drop was seen as a slight increase in the percentage of HbGen AA obtained from the population.

DISCUSSION

As stated earlier, the aim of the study was to obtain data on the prevalence of ABO, Rh(D) and the various genes of HbGen in Oyo state for the purpose of health planning and management in the state. The population surveyed was a true representation of the state from all the local councils constituting Oyo state. As expected, blood group O continued to maintain its dominance which earned carriers of the group the title of "Universal Donors". This group was followed in preponderance by blood group B and A in that order with blood group AB constituting the least in terms of %distribution. Thus, the old appellation of carriers of this group as "Universal Recipients" was still confirmed in this study. Comparatively, although blood group O was also predominant in other regions (Akinnuga et al, 2011; Egesie et al 2008; Jeremiah, 2006), incidence of blood group A and then B (in that order) genetic inheritance was next in distribution as against what was observed amongst the Yorubas in Oyo state. This trend (i.e. blood group A coming next in distribution to that of blood group O) was also observed from results obtained in other parts of the world including in black Afro-Americans in the USA (Table IV). Whether this was a coincidence or has a

Table 1: Summary of Frequency (percentage frequency) of Phenotype ABO and Rh(D) antigen Distribution in Oyo state, South-west Nigeria

Year	A^+	B^+	AB^+	O^+	A^-	B^-	AB^-	O^-
2004, N=502	96	126	20	219	11	8	1	21
	(19.1)	(25.1)	(4.0)	(43.6)	(2.2)	(1.6)	(0.2)	(4.2)
2006, N=602	116	150	19	282	8	7	3	17
	(19.3)	(24.9)	(3.2)	(46.8)	(1.3)	(1.2)	(0.5)	(2.8)
2008, N=1206	266	291	60	507	10	29	2	41
	(22.1)	(24.1)	(3.2)	(42.0)	(0.8)	(2.4)	(0.2)	(3.4)
2010, N=981	217	233	26	436	15	12	2	40
	(22.1)	(23.8)	(2.7)	(44.4)	(1.5)	(1.2)	(8)	(4.1)
Total, N=3241	695	800	125	1444	44	56	8	69
	(21.4)	(24.7)	(3.8)	(44.6)	(1.4)	(1.7)	(0.3)	(2.1)
% Rh (D)	**Positive 94.5**				**Negative 5.5**			

Table 2. Summary of ABO phenotype in Oyo State over the 4year period of investigation.

	%	N
A	22.8	739
B	26.4	856
AB	4.1	133
O	46.7	1513
Total	100	3241

Table 3. Incidence of the various Haemoglobin variants in Oyo State

Hb Variants	AA	AS	AC	SC	CC	SS
% Distribution	73.4	21.6	4.4	0.4	0.25	0.3
N	1933	553	119	11	3	3
Total			2622			

Table 4. Comparative distribution of ABO Gene pattern in some parts of Nigeria, Europe and USA

Location	**A**	**B**	**AB**	**O**
		%		
Oyo	22.8	26.4	4.1	46
Ibadan (Falusi et al 2000)	22.0	23.9	4.2	49.9
Ogbomoso (Bakare et al., 2005)	22.9	21.3	5.9	50
Niger/Delta (Egesie et al 2008)	23.72	20.09	2.97	53.22
P/Harcourt (Jeremiah, 2006)	22.9	17.10	4.84	55.16
Nigeria (Akinnuga et al, 2011)	26.9	16.1	4.2	52.9
African/American (Adeyemo and Soboyejo, 2006)	27	20	7	46
Caucasians (Pramanik and Pramanik,2000)	41	9	3	47
Europe (Pramanik and Pramanik,2000)	42	9	3	46

predefined geneological objective remains an issue especially since the pattern has remained constant in virtually all the epidemiological studies carried out over the years in the Southwest area of Nigeria (Falusi et al, 2000; Bakare et al 2011). Data obtained from Europe, India, and USA were clearly different from those obtained from Nigeria and Africa; this further confirms the genetic differences between Africans and these other races. Data obtained for the Rh(D) gene distribution (Table 5) also showed that distribution of Rh (D) genes was similar amongst the people of Ibadan and Ogbomoso in comparison to other zones in Oyo state. Although, the difference in distribution of

Table 5. Rh Gene distribution in some parts of Nigeria and other parts of the world

Location	**Rh(D)$^+$**	**Rh (D)$^-$**
Oyo	94.5.	5.5
Ibadan (Falusi et al, 2000)	94.1	5.9
Ogbomoso (Bakare et al.,2005)	96.7	3.3
Elele, Anambra state (Akinnuga et al, 2011)	91.7	8.3
Benin, Niger-delta (Egesie et al, 2008)	98	2
P/Harcourt (Jeremiah, 2006)	96.8	3.2
Nairobi	95	5
Lahore	92.7	7.3
South India	94.55	5.5
USA- Afro-Americans	95	5

the Rh (D) genes between the Yorubas and Niger Delta tribes could not be said to be significantly different, the frequency was higher amongst the Niger-Deltas compared to what obtained in Yoruba speaking areas (Table 5). These figures were different from the Rh(D) gene distribution and frequency amongst the Europeans, the Caucasians and the African-Americans in the USA (Table 5). The figures obtained for Nigerians were also similar to figures from other African countries. All these underscore the differences in genetic differences in Africans in comparison to those of Caucasians.

Similarly, the HbGen pattern obtained in Oyo state was found to be similar to what was reported for Ibadan and Ogbomoso by Falusi et al (2000) and Bakare et al, (2011) respectively. The observed frequency of HbAA (73.4%) is within the normal range of 55 - 75% earlier reported for Blacks (Fleming and Lehman, 1982). The frequency of HbAS (21.6%) in this study is within the predicted values of, 20 - 30% quoted for Nigeria and 20 - 40% in Africa in general (Fleming and Lehman, 1982; Reid and Famodu, 1988; Sinuo,2003). The % distribution of 0.4, 0.25 and 0.3 for the AS, CC and SS genes respectively were all lower than the predicted % distribution for Nigeria and Africans generally. The disturbance in Hardy-Weinberg equilibrium as depicted by the reduction in the % distribution of the HbSS and the traits and also by the increase in the % distribution of HbAA could be attributed to an increase in awareness probably due to health counselling. It could also be due to an improvement in socio-economic condition on the part of the populace.

Above notwithstanding, the need for sustained counselling towards eradication of SS genes and increased research towards identifying artificial blood substitutes was highlighted in this work. The need for scientists in the area of blood transfusion science to intensify efforts in the search for appropriate blood substitutes has been further highlighted as there seems to be a natural limitation to an increase in the various subtypes of the blood groups. Therefore, tackling the challenge of the upsurge in request for blood transfusion purposes possibly by the use of synthetic blood should be the focus of scientists in this area of medicine. The increasing need for blood transfusion especially with the increase in various politically/communally motivated emergency situations underscores this fact.

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Niger. J. Physiol. Sci. 30(2015) 131-137
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Effects of Maternal Dexamethasone Exposure During Lactation on Metabolic Imbalance and Oxidative Stress in the Liver of Male Offsprings of Wistar Rats

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Summary: It has been reported in human and animal studies that early exposure to glucocorticoids could retard growth and subsequent development of cardio metabolic diseases. Chronic exposure to glucocorticoids induced oxidative stress. Therefore, the role of oxidative stress in some of the observed metabolic imbalance needs to be elucidated. This study examined the effects of lactational dexamethasone exposure on metabolic imbalance and oxidative stress marker in the liver of male offspring of exposed mother. Twenty lactating dams were divided into 4 groups of 5 animals each. Group 1 was administered 0.02 ml/100gbwt/day normal saline through lactation days 1-21. Group 2, 3, and 4 were administered 100 µg/kgbwt/day dexamethasone for lactation days 1-7, 1-14, and 1-21 respectively. The male offspring were thereafter separated and sacrificed at 12weeks of age for evaluation of lipid profile and oxidative stress marker in the liver.

Results from this study indicate that Total Cholesterol (TC), Triglycerides (TAG) and LDL- cholesterol (LDL-C) were significantly ($p<0.001$) higher in the Dex 1-7, Dex 1-14 and Dex 1-21 groups when compared with the control. HDL-Cholesterol (HDL-C) was significantly ($p<0.001$) reduced in the Dex 1-7, Dex 1-14 and Dex 1-21 groups relative to the control. Basal Fasting Blood Sugar (FBS) was also significantly ($p<0.001$) higher in the Dex 1-14 and Dex 1-21 groups when compared with the control. Liver malondialdehyde was significantly ($p<0.001$) higher in the Dex1-14 and Dex1-21 group compared to the control. However, liver catalase and SOD activity were all significantly ($p<0.001$) lower in Dex 1-7, Dex 1-14 and Dex 1-21 groups relative to control. Liver protein was significantly ($p<0.001$) lower in the Dex1-14 and Dex1-21 treatment groups when compared with the control. Findings from this study suggest that there is possible increase in metabolic imbalance in the offspring of mother exposed to dexamethasone during lactation and these effects may be secondary to increase oxidative stress in the liver.

Keywords*:* Dexamethasone; Lactation; Oxidative Stress; Liver; offspring.

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Manuscript Accepted: August, 2015

INTRODUCTION

Exposure to stress and glucocorticoids hormone mediator exert influences on organ growth, developments and subsequent offspring physiology and pathophysiology (Drake et al., 2005). Sources of exposure to synthetic glucocorticoids during development can occur if the mother has a medical condition requiring glucocorticoids treatments, such as asthma (Wang et al., 2010). Prevention or lessen the morbidity of chronic lung disease in preterm infants (Wang et al., 2010) also required glucocorticoids treatment. The treatment regimen for chronic lung diseases typically consists of high doses of dexamethasone for several weeks (Singh et al., 2012).

Evidence from epidemiological studies and animal experiment suggest a strong link between weight during early neonatal life and subsequent development of cardio-metabolic diseases, (Hales et al, 1991; Barker et al., 1993; Lithell et al., 1996). These developments appear to be independent of classical adult lifestyle risk factors. In rodent, glucocorticoids exposure during neonatal life causes reduce weight as well as retard growth (Wang et al., 2010). In explaining these observations, it has been proposed that a stimulus or insult acting during critical periods of growth and development permanently alters tissue structure and function, a phenomenon termed "Fetal Programming" (Drake et al., 2005).

The programming effect of synthetic glucocorticoids is mediated through programming of HPA axis (Kapoor et al., 2006). The hallmark of this programming is an increase in the circulating corticosterone. However excess circulating corticosterone in prenatal life has been shown to induce oxidative stress in cerebellar granule (Ahlbom et al., 2000). In addition, chronic administration of glucocorticoids in rats causes increased lipid peroxidation and decreased antioxidant activity (Orzechowski et al., 2002; Mcintosh et al., 1998).

The role of oxidative stress in the pathogenesis of metabolic imbalance such as insulin resistance, diabetes mellitus and hyperlipidaemia has also been well reported in literature' (Collins, 2005). Numerous studies dealing with maternal dexamethasone exposure and programming of metabolic diseases have focused on exposure during prenatal life. The neonatal effect of dexamethasone exposure have only been observed through direct administration of dexamethasone in pups (Wang et al., 2010), but if administration of dexamethasone in mother during lactation will affects the pup is not known. Tilbrook et al (2006) reported that maternal stress during lactation suppresses HPA activities in the mother. The clinical use of the synthetic glucocorticoids as anti-inflammatory agents called for the understanding of the possible role of maternal exposure through lactation to dexamethasone in the programming of metabolic effects and oxidative stress in the offspring. It is therefore hypothesize that exposure to dexamethasone during lactation may induce increase metabolic imbalance in the offspring and this may be associated with increase in oxidative stress in the liver, since liver is the major metabolic organ.

MATERIALS AND METHODS

Drug: Dexamethasone 21- Phosphate disodium salt was purchased from Sigma Aldrich Chemical, UK. A dose of 100 pg dexamethasone /Kg/day was administered to the treated groups (Drake et al., 2005).

Experimental Animal: Twenty female Wistar rats weighing (150-180 g) were purchased from Central Animal House of University of Ibadan, Ibadan, Nigeria. The animals were housed in the Department of Physiology Animal House and they had free access to water and food. After two weeks of acclimatization, animals in proestrous were exposed to matured male overnight and the presence of sperm in their vaginal in the next morning mark gestation day 1(GD1). After pregnancy has been established, animals were randomly divided into four groups of 5 animals each and they were treated accordingly during lactation (Table 1). Administration was between 09.00am and 10.00am daily. 100 pg/kgbwt/day of dexamethasone was administered for the drug treated groups and 0.02 ml/100gbwt/day normal saline was administered in the control. Administration was done subcutaneously in the dams. The litter size was standardized to 5pups/litter. Biochemical analysis was done on the male offspring at 12weeks of Age. All animal experiments were conducted in accordance with ethical norms acceptable at the University of Ibadan. The male offspring were allowed to grow to adulthood (12wk of age). Blood were thereafter collected from the ocular sinus into plane tube. This was centrifuge at 3000rpm for 10minutes for the preparation of serum for subsequent evaluation of biochemical parameters.

Table 1: Treatment of animals and number of offspring collected.

Group	Treatment	Number of dams (No of male Offspring)
Control	0.02ml/100gbw/day Normal saline	5 (6)
Dex 1-7	100µg/kgbw/day Dexamethasone (PND 1-7)	5 (6)
Dex 1-14	100µg/kgbw/day Dexamethasone (PND 1-14)	5 (6)
Dex1-21	100µg/kgbw/day Dexamethasone (PND 1-21)	5 (6)

Dex (Dexamethasone), PND (postnatal days)

Rats were sacrifice through cervical dislocation. During dissection, the liver was carefully collected and rinsed with ice cold 1.15% KCl solution. Dry weight of the tissue was recorded. They were thereafter placed in 0.1M Potassium phosphate buffer pH 6.5 and homogenize using homogenizer, after which the sample was centrifuge in cold centrifuge at 10,000rpm for 10minutes. The homogenate was removed and stored in a refrigerator for analysis of oxidative stress. Biochemical analysis was done within 48 hours of sample collection.

BIOCHEMICAL ANALYSIS

Determination of Tissues lipid peroxidation: Level of lipid peroxidation (MDA) was evaluated by method of Buege and Aust (1978). Malondialdehyde, formed from the breakdown of polyunsaturated fatty acids, serves as a convenient index for determining the extent of the peroxidation reaction. Malondialdehyde (MDA) has been identified as the product of lipid peroxidation that reacts with thiobarbituric acid to give a red species absorbing at 535nm.

Determination of Tissues Catalase activities: Catalase activity was evaluated by method of Sinha (1971). This method is based on the fact that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of H_2O_2 with the formation of perchromic acid as an unstable intermediate. The chromate acetate then produced is measured colorimetrically at 570-610 nm. Since dichromate has no absorbance in this region, the presence of the compound in the assay mixture does not interfere at all with the colorimetric determination of chromic acetate. The catalase preparation is allowed to split H_2O_2 for different periods of time. The reaction is stopped at a particular time by the addition of dichromate acetic acid mixture and the remaining H_2O_2 is determined by measuring chromic acetate colorimetrically after heating the reaction mixture.

Determination of Tissues Superoxide Dismutase (SOD) Activities: SOD activity was evaluated by method of Mistra and Fridovich (1972). The ability of superoxide dismutase to inhibit the auto oxidation of adrenaline at pH 10.2 makes this reaction a basis for the SOD assay. Superoxide anion (O_2) generated by the xanthine oxidase reaction is known to cause the oxidation of adrenaline to adrenochrome. The yield of adrenochrome produced per superoxide anion increased with increasing pH and also with increasing concentration of adrenaline. These led to the proposal that auto oxidation of adrenaline proceeds by at least two distinct pathways, one of which is a free radical chain reaction involving superoxide radical and hence could be inhibited by SOD.

Determination of Tissues protein activities: Protein estimation was done by method of Lowrey et al (1951). The Folin-Ciocalteau reagent was used in the quantification of proteins by Lowry (1951). In its simplest form the reagent detects tyrosine residues due to their phenolic nature. The reaction of a protein in solution with the Folin reagent occurs in two stages: Reaction with Cu++ in alkaline medium and Reduction of the phosphomolybdic-phosphotungstic reagent by the Cu++ - protein complex. The reduced complex gives a blue solution with an absorption in the red portion of the visible spectrum (600¬800 nm).

Determination of Fasting blood glucose, Lipid profile and serum corticosterone level: Total cholesterol(TC), HDL-Cholesterol, LDL-cholesterol and Triglyceraldehyde (TAG) were evaluated using Randox Kits (Randox laboratory, United Kingdom). Fasting Blood glucose (FBS) was determined (after animals have been fasted for 12hours overnight) using Accu-Check Active glucometer (Roche diagnostics Germany). Serum corticosterone level was assessed using Cloud Clone corticosterone Kit (United State). Serum was collected between 8.00am and 9.00am in the morning to accommodate for the diurnal peak in serum corticosterone level.

Statistical Analysis

Data are expressed as mean ± standard error of mean (SEM) n= 6 Statistical comparisons were performed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test to compare the means of the different treatment groups. Differences between the treatment groups with a p- value < 0.05 were considered significant. Data were analysed with the use of Graphpad Prism Version 5.0 for Windows (GraphPad® Software, San Diego, CA, USA.

RESULTS

Effects of Lactational exposure to dexamethasone on growth pattern

The birth weight and weight at postnatal day 7 were not significantly different in all the treatment groups when compared with the control (Table 2). The mean weight at postnatal day (PND) 14 was significantly reduced ($p<0.001$) in the Dex 1-7, Dex 1-14 and Dex 1-21when compared with the control. In addition, the mean weight at PND 21 was significantly reduced ($p<0.001$) in Dex 1-7, Dex 1-14 and Dex 1-21when compared with the control (Table 2). The mean weight at necropsy (PND 12 weeks) was also significantly reduced in the Dex 1-7 ($p<0.05$), Dex 1-14 ($p<0.05$) and Dex 1-21($p<0.001$) when compared with the control (Table 2).

Effects of Lactational exposure to dexamethasone on serum corticosterone: The mean serum corticosterone concentration was significantly increased in the Dex 1-7 ($p<0.05$), Dex 1-14 ($p<0.01$) and Dex 1-21($p<0.01$) when compared with control (Fig. 1).

Table 2: Effects of maternal dexamethasone treatment during lactation on body weight

	Weights (g)				
	Birth	PND 7	PND 14	PND 21	PND 12weeks (g)
Control	4.98±0.05	7.77±0.44	17.5±0.65	25.39±1.08	203.84±8.4
Dex 1-7	5.47±0.26	8.41±0.57	10.3±0.26**	16.27±0.35**	167.5±7.5*
Dex 1-14	5.14±0.08	9.99±1.21	12.7±0.19**	16.95±0.06**	167.5±7.5*
Dex 1-21	5.26±0.17	9.46±1.39	12.7±0.17**	17.99±0.31**	143.75±5.3**

*p<0.01, **p<0.001; *, shows significant different between the group and the control. PND (Postnatal days)

Table 3: Comparison of lipid profile level in the male offspring at 12 weeks of age following maternal treatment with dexamethasone during lactation in Wistar rats.

	Total Cholesterol (mg/dl)	Triglyceride (mg/dl)	HDL-Cholesterol (mg/dl)	LDL- Cholesterol (mg/dl)
Control	65.67±1.34	71.28±4.45	32.41±2.30	18.95±2.35
Dex 1-7	118.00±1.01***	185.25±16.5***	27.33±1.50***	53.62±4.21***
Dex 1-14	122.00±1.10***	293.39±11.00***##	9.79±0.46***###	53. 59±3.64***
Dex 1-21	87.50±1.50***###	210.84±1.38***	3.20±0.65***###	42.30±2.19***

Values are expressed as mean ± SEM, n=6. *p< 0.05, **p<0.01, ***p<0.001; # p< 0.05, ## p<0.01, ### p<0.001; *, shows significant different between the group and the control; #, shows significant different between the group and Dex 1-7.

Effects of Lactational exposure to dexamethasone on Fasting blood sugar (FBS) and lipid profile: FBS showed a significant ($p<0.001$) increase in the treatment groups' Dex 1-14 days and Dex 1¬21 days when compared with control. FBS in Dex 1-21days and Dex 1-14 days were all significantly ($p<0.001$) higher than Dex 1-7 (Fig. 2). The total cholesterol (TC) increased significantly ($p<0.001$) in all the test groups administered with dexamethasone when compared with the control. TC was however significantly reduced in Dex 1-21 group ($p< 0.001$) when compared with Dex 1-7 and Dex 1-14 (Table 3).

Table 4: Relative liver weight following lactational exposure to dexamethasone

Treatments	Relative liver weight (g tissue/kg bwt)
Control	19.06±1.326
Dex 1-7	20.07±1.621
Dex 1-14	27.21±1.13 **#
Dex 1-21	31.32± 2.1*** ##

**p<0.01, ***p<0.001; # p< 0.05, ##p<0.01,*, shows significant different between the group and the control; #, shows significant different between the group and Dex 1-7

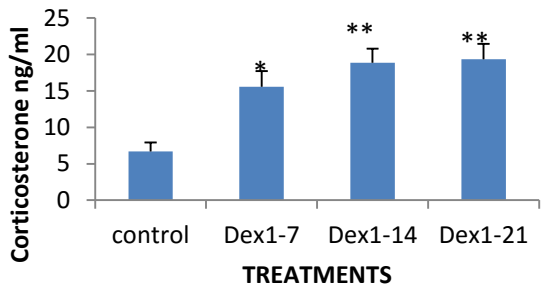

Fig. 1: Serum corticosterone level in the male offspring following maternal dexamethasone treatment during lactation. *p<0.05, **p<0.01, ***p<0.001; N=6, *, shows significant different between the group and the control.

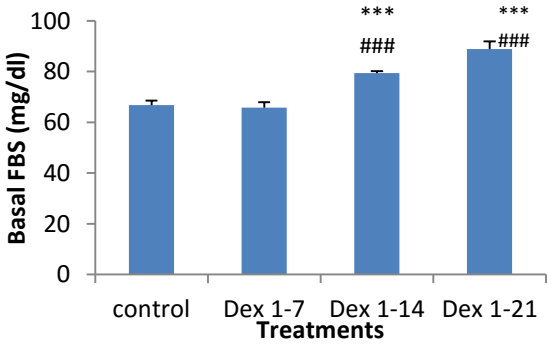

Fig. 2: Comparison of Fasting Blood Sugar (FBS) level in the male offspring at 12 weeks of age following maternal treatment with dexamethasone during lactation in Wistar rats. Values are expressed as mean ± SEM, N=6. *p< 0.05,**p<0.01, ***p<0.001; #p< 0.05, ##p<0.01, ### p<0.001, *, shows significant different between the group and the control; #, shows significant different between the group and Dex 1-7

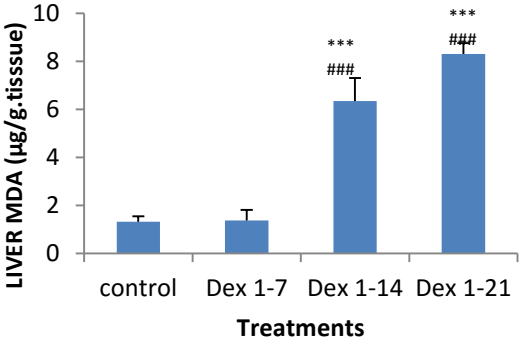

Fig. 3: Comparison of Liver MDA level in the male offspring at 12 weeks of age following maternal treatment with dexamethasone during lactation in Wistar rats. Values are expressed as mean ± SEM, n=6. *p< 0.05, **p<0.01, ***p<0.001; # p< 0.05, ## p<0.01, ### p<0.001; *, shows significant different between the group and the control; #, shows significant different between the group and Dex 1-7.

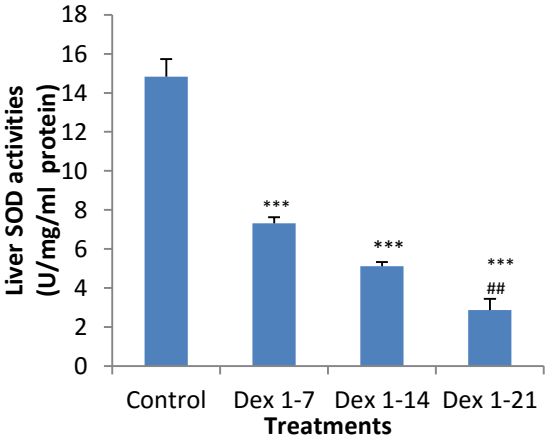

Fig. 4: Comparison of liver SOD activities in the male offspring at 12 weeks of age following maternal treatment with dexamethasone during lactation in Wistar rats. Values are expressed as mean ± SEM, n=6. *p< 0.05, **p<0.01, ***p<0.001; # p< 0.05, ## p<0.01, ### p<0.001; *, shows significant different between the group and the control; #, shows significant different between the group and Dex 1-7

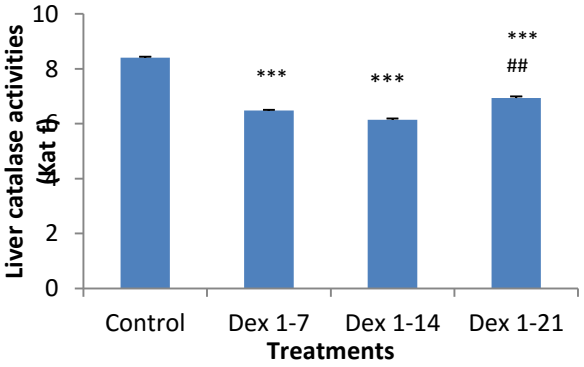

Fig. 5: Comparison of liver catalase activities in the male offspring at 12 weeks of age following maternal treatment with dexamethasone during lactation in Wistar rats. Values are expressed as mean ± SEM, n=6. *p< 0.05, **p<0.01, ***p<0.001; # p< 0.05, ## p<0.01, ### p<0.001; *, shows significant different between the group and the control; #, shows significant different between the group and Dex 1-7.

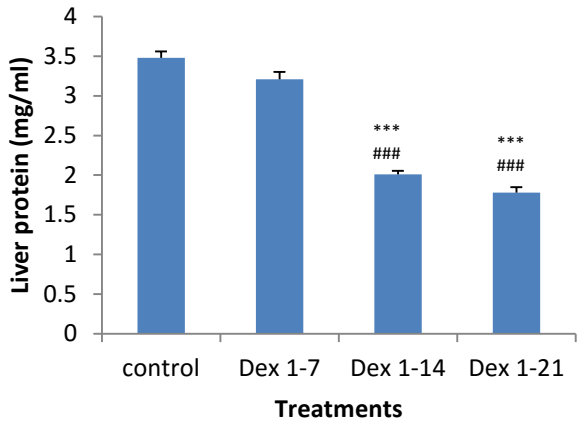

Fig. 6: Comparison of liver total protein level in the male offspring at 12 weeks of age following maternal treatment with dexamethasone during lactation in Wistar rats. Values are expressed as mean ± SEM, n=6. *p< 0.05, **p<0.01, ***p<0.001; # p< 0.05, ## p<0.01, ### p<0.001; *, shows significant different between the group and the control; #, shows significant different between the group and Dex 1-7

The triglyceride concentration levels showed a significant (p<0.001) in all treatment groups when compared with the control. TAG in Dex 1-14 group was however significantly higher than Dex 1-7 and Dex 1-21 (p<0.01) (Table 3). The HDL-cholesterol levels reveal a significant (p<0.001) decrease in all treatment groups when compared with the control. HDL-cholesterol was also significantly (p < 0.001) reduced in Dex 1-21 and Dex 1-14 when compared with the Dex 1-7 (Table 3).

The LDL-Cholesterol levels showed a significant (p<0.001) increase in all treatment groups when compared with the control. It was however, significantly (p<0.01) lower in Dex 1-21 group when compared with Dex 1-7 (Table 3).

Effects of Lactational exposure to dexamethasone on relative liver weight

The relative liver weight was significantly increased in the Dex 1-14 (p<0.01) and Dex 1- 21(p<0.001) group when compared with control (Table 4).

Effects of Lactational exposure to dexamethasone on makers of oxidative stress in the liver: The Liver MDA levels shows a significant (p<0.001) increase in the treatment groups' 1-14 days and 1-21 days, compared with the control Dex 1-14 and Dex 1-21 liver MDA were also significantly (p <0.001) higher than Dex 1-7 group (Fig. 3).

The liver of SOD activities showed a significant (p<0.001) decrease in all treatment groups when compared with the control. Also, Dex 1-21 group liver SOD activities was significantly (p<0.01) lower than Dex 1-7 group (Fig. 4).

The liver catalase activities showed a significant (P<0.001) decrease in all test groups when compared with the control. However, Dex 1-7 and Dex 1-14 groups showed a significant (p<0.01) decrease in liver catalase activities when compared with Dex 1-21. (Fig. 5) The liver protein level was significantly (p<0.001) decrease in Dex 1-14 and Dex 1-21 groups when compared with the control and Dex1-7. (Fig. 6).

DISCUSSION

The study examined the effect of maternal exposure to dexamethasone during lactation on metabolic imbalance and oxidative stress marker in the liver of male offspring in Wistar rats.

The results from this study demonstrated that maternal exposure to 100 pg/Kgbwt/day dexamethasone during lactation could leads to dyslipidaemia in the offspring. The total cholesterol (TC), Triglycerides (TAG) and LDL-cholesterol (LDL-C) levels were raised by the treatments compared to control. However, serum HDL-Cholesterol (HDL-C) was significantly reduced by the treatment. Associated with this observation is the increased serum basal corticosterone level in the treatment groups. Contrary to the finding of this study, exposure to glucocorticoids have direct effects on the circulating lipids and lipoproteins; increasing LDL-C, TAG and HDL-C and total cholesrterol (Ettinger and Hazzard, 1988). Glucocorticoids decrease concentration of LDL-C receptors on hepatocyte (Rainey et al., 1992) leading to higher LDL-C level. It also increases VLDL production and secretion from the liver thereby causing hypertriglyceridemia (Brindley, 1995). Increase in the liver VLDL level may leads to low HDL-C level (Masharani and German, 2007). Another possible explanation is increase circulating glucocorticoids as seen in chusing syndrome leads to insulin resistance (Aron et al., 2007). Insulin resistance could lead to visceral obesity that is presented with this kind of lipid profile. Basal fasting blood glucose (FBS) was also significantly increased in the offspring of the treated mother. However, the increase in FBS is within normal range. According to Sapolsky et al., (2000) chronic exposure to glucocorticoids could lead to muscle and hepatic insulin resistance and precipitate hyperglycemia. Maternal treatment with dexamethasone during first week, second or throughout lactation significantly reduces the body weight of the offspring at PND 7, PND 14 and PND 21. This growth retardation was sustained till adulthood. Evidence is accumulating from epidemiological study in human that early life dexamethasone exposure negatively affects the somatic growth (Wang et al., 2010). Normal somatic growth is the result of the proper interactions between genetic, nutritional, metabolic and endocrine factors (Lin et al., 2006; Huang et al., 2007; Berry et al., 1997; Shrivastava et al.,2000). Although the cause of the lasting growth retardation observed in Dex treated groups offspring remain unclear, this adverse effect may be linked, at least in part to alteration in the maternal HPA activity as a result of the Dex treatment.

Tilbrook et al., (2006) has reported similar alteration in HPA activity due to maternal stress during lactation. Since the neuroendocrine system in the pulps are still undergoing maturation7, altered HPA activity in the dam may programme stress related glucocorticoids secretion in the offspring. Chronic increase in the glucocorticoids level may raise tissue catabolism or protein breakdown (Leitch et al., 1999; Neal et al., 2004). This may lead to increase muscle waste and reduce muscle mass.

It was also found from this study that neonatal dexamethasone administration significantly reduced the antioxidant enzyme Catalase and SOD in the liver and raises the level of lipid peroxidation. Increase lipid peroxidation with reduced antioxidant enzyme level is indicators of oxidative stress (Shcafer and Buettner, 2001). In humans, oxidative stress is thought to be involved in the development of many diseases or may exacerbate their symptoms (Whitworth et al., 2000). Consistent with this finding, is the report that oxidative stress may be especially pronounced with prolonged glucocorticoids exposure (Iuchi et al.,2003). Patients with Cushing syndrome may have increased nitrotyrosine levels (a measure of increased oxidative stress) in vascular tissue and decreased brachial artery reactivity (Celsi and Aperia, 1999). Human umbilical vein endothelial cells exposed to dexamethasone also generate reactive oxygen species via stimulation of NADPH oxidase and xanthine oxidase (Celsi and Aperia, 1999). Increase oxidative stress leads to DNA damage and may also result in cell death. These two effects of oxidative stress on the liver may impaired the liver metabolic functions.

Numerous studies both in human population and animals have reported that prenatal exposure to glucocorticoids can leads to subsequent development of hyperlipidamea, glucose intolerance, insulin resistance and other cardio-metabolic diseases (Drake et al.,2005). This study extends the observation of metabolic imbalance in offspring to maternal Dex treatment during lactation. Indeed, glucocorticoids hormones modulate tissue development during both the prenatal and the weaning period regulating organ maturation at different times in a synchronized and orderly fashion (Celsi and Aperia, 1999). Rats are born relatively immature, with several organs maturation during the weaning period (Kapoor et al., 2006). Therefore, our data suggest a possible increase metabolic imbalance in the offspring of mother xposed to Dex during lactation and these effects may be secondary to increase oxidative stress in the liver.

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