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Effect of Caffeine on Serum Tumour Necrosis Factor Alpha and Lactate Dehydrogenase in Wistar Rats Exposed to Cerebral Ischaemia-reperfusion Injury

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Summary: Caffeine is known to confer neuro-protection via A_1 and A_{2A} adenosine receptor antagonism in which adenosine neuro-modulates excitotoxic release of glutamate. Currently, it is unclear whether caffeine modulates inflammation in ischaemic stroke model. The present study examined effects of caffeine following ischaemia-reperfusion injury on neuro-inflammatory tumour necrosis alpha (TNF-α), lactate dehydrogenase (LDH), as well as effect of caffeine against brain ischaemic damage on histology. Thirty three adult male Wistar rats (180-300 g) were used in this study. They were randomly divided into four groups (n=5 each): Group I (Control) that received neither the operation nor any treatment; Group II (Sham/Water) received a *pseudo*-ischaemic-reperfusion and 1ml water for injection; Group III (BCCO/Water) that received complete bilateral common carotid occlusion (BCCO) and 1ml water for injection; Group IV (BCCO/Caffeine) that received complete BCCO and caffeine solution intraperitoneally at a dose of 50% LD_{50} value (144mg/kg); and thirteen rats were used for LD_{50} assessment. Sensory and motor functions significantly ($p<0.05$) decreased in the rat following ischaemia-reperfusion injury when compared to pre-injury state on Garcia neurological score. Caffeine reduced brain ischaemic injury and significantly reduced ($p<0.05$) TNF-α activity. While no significant effects ($p>0.05$) of caffeine was observed on LDH activity. This study has shown neuro-protective roles of caffeine against ischaemia-reperfusion damage to brain tissue, inflammatory TNF-α activity, but not on LDH activity.

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

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INTRODUCTION

Stroke is the world's second-leading cause of death and a major factor in causing disabilities in humans (Kim *et al*., 2015). Stroke incidence as at 2010 worldwide was around 17 million persons per year, with a total of 33 million people being still alive after stroke, with about 70 % of all stroke patients staying with residual symptoms (Douven *et al*., 2016). Brain stroke results from either vessel occlusion (ischemic stroke) or cerebral blood-related neurotoxicity (haemorrhagic stroke). Thus, stroke is classified into ischaemic and haemorrhagic types. While the former type accounts for 85% of all strokes, the latter accounts for 15% of all incidence of strokes (Wang *et al*., 2015). Ischaemia-reperfusion injury is the exacerbation of tissue damage when blood supply is re-established in a previously ischaemic organ (Kalogeris *et al*., 2014). Neuro-inflammation that is mediated by microglia and astrocytes plays a critical role in neuro-degeneration (Melani *et al*., 2009) and is a major factor known to contribute to the expansion of ischaemic lesions following ischaemic stroke (Simats *et al*., 2015). Neuro-inflammation during cerebral ischaemia is associated with a series of cascading cellular events including enhancement of prior glutamate excitotoxicity and production of a plethora of inflammatory mediators that trigger apoptosis and exaggerate neuronal damage (Brothers *et al*., 2010). Biomarkers of neuro-inflammation offer an objective and quantitative data on neurochemical mechanisms of brain inflammation, and a number of potentially neuro-protective strategies are based on mediating inflammatory mediators (Piskunov, 2010). Tumour necrosis factor alpha (TNF-α) in the serum is a sensitive marker of inflammation and tissue injury whose concentration increases rapidly in brain tissue

following hypoxia-ischaemia (Graham *et al*., 2016). Lactate dehydrogenase (LDH) is a cytoplasmic cellular enzyme present in essentially all major organ systems, and in the extracellular space, although of no further metabolic function in this space, are still of benefit because they serve as indicators suggestive of disturbances of the cellular integrity induced by pathological conditions (Kato *et al*., 2006).

Adenosine is an endogenous neuro-modulator, which acts on at least four distinct receptor subtypes: A_1, A_{2A}, A_{2B}, and A_3, all of which are expressed in the brain (Fredholm *et al*., 2001), with A_1 and A_{2A} receptors of critical importance in ischaemic damage after stroke (Chen *et al*., 1999; Yang *et al*., 2013). Adenosine via A_1 and A_{2A} receptors modulates presynaptic release of excitotoxic glutamate. There is concomitant elevation of both adenosine and glutamate in response to cerebral ischaemia (Dai *et al*., 2010) and adenosine neuro-modulation via A_{2A} receptor stimulates glutamate outflow from neurons and glial cells, thereby contributing to glutamate excitotoxicity (Wardas, 2002; Marchi *et al*., 2002; and Pedata, 2008). Caffeine (1, 3, 7-trimethylxanthine) exerts its physiological effects in the central nervous system (CNS) through antagonism of A_1 and A_{2A} adenosine receptors (Nehlig, 2002), and increase in sympathetic nervous system (SNS) activity by stimulating α and β-adrenoreceptors (Belza *et al*., 2009; Joy *et al*., 2016). Caffeine has been well established to offer neuro-protection via A_1 and A_{2A} adenosine receptors antagonism to intervene glutamate excitotoxicity in ischaemic stroke model (Rudolphi et al., 1989; Bona *et al*., 1995; Alexander *et al*., 2013). There is also evidence that adenosine A_{2A} receptors are also involved in regulating factors responsible for the detrimental inflammatory effects in brain ischemia (Melani *et al*., 2009). This evidence seemingly suggested that, post-ischaemic A_{2A} receptor blockade using caffeine might also offer protection against neuro-inflammation, which currently is yet to be investigated. Hence this study examined effects of caffeine following experimentally induced ischaemia-reperfusion injury on neuro-inflammatory tumour necrosis alpha (TNF-α), lactate dehydrogenase (LDH), as well as the effects of caffeine on brain ischaemic damage on histology.

MATERIALS AND METHODS

Chemicals and Equipment: Analytical grade caffeine powder, ketamine hydrochloride and water for injection were sourced commercially from ScP chemical limited. Elisa kit for TNF-α (Catalogue number E-EL-R0019) was sourced from Elabscience Company, China, while Elisa kit for LDH was sourced from Agappe Diagnostics, Switzerland.

Experimental Animals: Thirty three adult male Wistar rats weighing 180-300 g were used for the study. They were housed in plastic cages at room temperature (32^0C), monitored under normal photoperiod and fed with standard animal feed and water *ad libitum*. Animal care and handling were conducted in accordance with ethical provisions of Bayero University Kano.

Acute Toxicity (LD_{50}) Study: An initial acute toxicity study was conducted by the method of Lorke (1983) using 13 adult male Wistar rats to determine the dose range of caffeine to be used for the study. In the first phase, nine rats were divided into three groups of three rats each; and treated with caffeine solution at doses 10, 100, and 1000 mg/kg intraperitoneally, after which they were observed frequently for 24 hours for signs of toxicity. All the animals in the group given 1000 mg/kg developed convulsions and died three hours after caffeine administration. The result obtained from the first phase determined the dose given in the second phase which was 140 mg/kg, 225 mg/kg, 370 mg/kg and 600 mg/kg selected from Lorke's standard table. One animal per group was used in the second phase, and the animal given 370 mg/kg and 600 mg/kg were dead within 24 hours in the second phase. The LD_{50} value was finally calculated to be 228 mg/kg accordingly (Akhila *et al*., 2007).

Experimental Design: Twenty rats were randomly divided into four groups of five rats each (n=5) and treated as follows:

Group I (Control): received neither the operation nor any treatment;
Group II (Sham/Water): received sham operation (i.e. bilateral carotid arteries were exposed but without occluding them) and 1 ml water for injection intraperitoneally (*i.p*);
Group III (BCCO/Water): received complete bilateral common carotid occlusion (BCCO) for 5 minutes and 1 ml water for injection *i.p*;
Group IV (BCCO/Caffeine): received complete BCCO for 5 minutes and caffeine solution intraperitoneally at a dose of 144 mg/kg.

Bilateral common carotid artery occlusion: BCCO was carried out to induce cerebral hypo-perfusion. Rats were food-deprived overnight before surgery in order to stabilize plasma glucose, and surgical procedure was performed between 9.00 am and 12 noon in order to minimize diurnal variations (Aytac *et al*., 2006). Rats were anaesthetised with 50 mg/kg of ketamine hydrochloride *i.p*, and the level of anaesthesia was assessed by the absence of withdrawal reflex, additional anesthesia was administered if necessary. A midline skin incision in the neck was performed and the common carotid arteries were identified lateral to trachea and behind sternomastoid

muscle and carefully isolated from the accompanying nerve. Bilateral carotid arteries were then completely occluded for 5 minutes followed by reperfusion. The wound was then closed with silk sutures and the animals were monitored until consciousness was regained.

All treatments were administered at 1, 3, and 6 hours after rats fully recovered from anaesthesia. After 24-hour following the surgery, rats were sacrificed under anaesthesia and blood was collected by cervical decapitation into plain bottles. The samples were allowed to clot and then centrifuged at 15,000 rpm for 20 minutes and the sera were acquired and used for determination TNF-α and LDH contents.

Neurological Assessment Using Garcia Score

Garcia *et al*., (1995) neurological evaluation of sensory and motor function was carried out in the experimental group that received BCCO prior to and after the BCCO procedure to assess the effect of ischaemia-reperfusion injury. The test consist of six components; spontaneous activity, symmetry in the movement of four limbs, symmetry of fore limb, climbing, body proprioception, and response to vibrissae. Test evaluations were carried out by an observer who was blinded to the experimental procedure and the score assigned to each rat at completion of the evaluation equals to the sum of all six test scores. The highest score possible of Garcia score is 18, while the least is 3.

Laboratory Procedures

Serum level of TNF-α and LDH were analysed using their respective ELISA kits following the manufacturer's manual. The serum level of TNF-α was analysed by method of Sandwich-ELISA which is based on the principle that, optical density (OD) of the standard preparation measured using sphectrophotometer at a wavelength of 450 nm is proportional to the concentration of TNF-α. Briefly, 100 micro litres (µL) of the serum samples were added to micro ELISA plate wells, followed by successive addition of biotinylated detection antibody, avidin-horseradish peroxidase (HRP) and stop solution (sulphuric acid) to each micro ELISA plate well, and OD was then immediately measured at 450 nm wavelength. The LDH kit principle was based on the reversible reaction catalyzed by lactate dehydrogenase enzyme to convert pyruvate to lactate. Accordingly, 24 ml of reagent R1 which composed of Tris buffer (pH 7.4), pyruvate, and sodium chloride, was mixed with 6 ml of reagent R2 which composed of NADH to obtain a working reagent, which was added to each 10 µL of sample and the change in absorbance per minute (ΔOD/min) was measured after 1, 2, and 3 minutes.

Histological Evaluation of the Brain Cortex

One rat brain from each group was randomly selected and used for histological analysis. Immediately after harvesting, the brain was fixed in 10% formalin after which it was allowed to dehydrate in ascending grade of alcohol. Brain tissue was then cleared from alcohol for 2 hours using xylene after which it was embedded in molten wax and allowed to solidify for 2 hrs. Section was cut using microtone at 5 microun after which it was subsequently stained (Chatterjee, 2014).

For staining, each section was de-waxed in xylene for 1 min before dehydrating in descending grades of alcohol starting with absolute alcohol for 30 secs. Slides were then inserted in Harris hematoxylene solution for 10-15 mins after which it was rinsed in distilled water, and then washed in Scotts tap water for 3 min. Slides were then differentiated in acid alcohol and washed in distilled water. They were then counter stained with Eosin, washed with distilled water and then dehydrated in ascending grades of alcohol. Slides were finally cleared in xylene and then mounted using mounting medium DPX (Ravikumar *et al*., 2014).

Statistical Analyses: Obtained data were processed using Statistical Package for Social Science (SPSS), version 20 and expressed as Mean ± Standard Error of Mean (SEM). Mean values of TNF-α and LDH from control and experimental groups were compared for differences using one-way analysis of variance (ANOVA) with Schaffe post hoc test, while mean values of Garcia scores were compared using paired t test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Neurological Evaluation

There was significant neurological deficit following ischaemia-reperfusion injury for the animals in both groups III and IV as indicated by decreased Garcia scores (P = 0.001) (Figure 1).

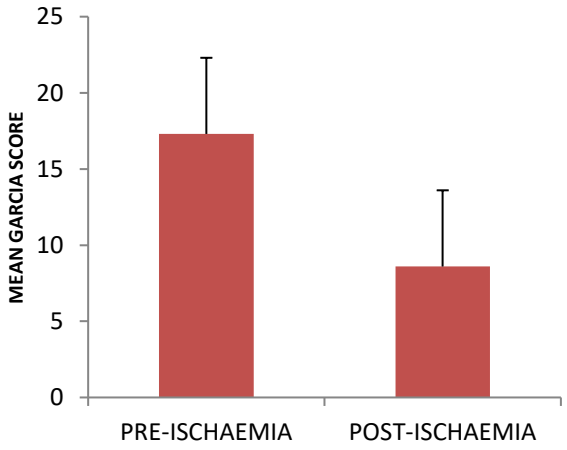

Figure 1: Neurological evaluation on Garcia score following ischaemia-reperfusion injury in rats.Values on each bar represent the means of both group III and IV at pre and post ischaemic reperfusion stage. Mean difference are statistically significant (P = 0.001).

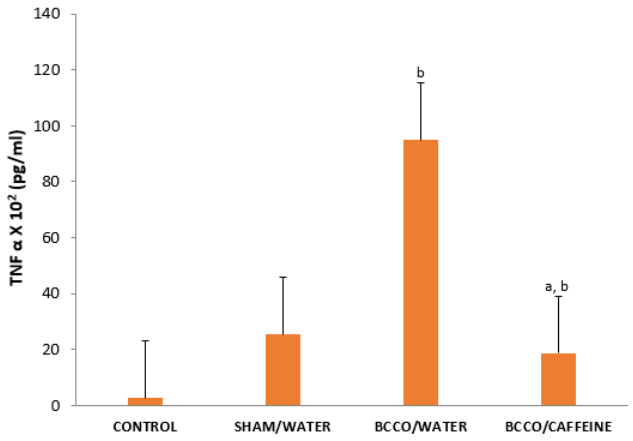

Figure 2: Effect of caffeine (144 mg/kg, i.p) on serum level of TNF-α (pg/ml) following ischaemia-reperfusion injury in rats. [a]Mean differences is statistically significant compared to control (P = 0.001). [b]Mean differences is statistically significant compared to BCCO/WATER (P = 0.001).

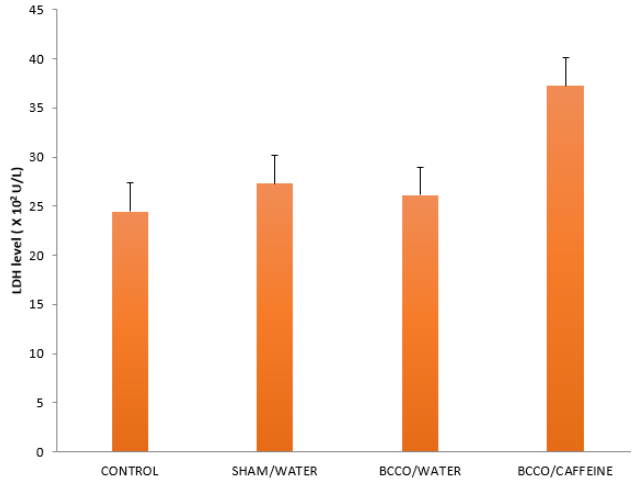

Figure 3: Effect of caffeine (144 mg/kg, i.p) on serum level of LDH following (U/L) ischaemia-reperfusion injury in rats. Mean difference are not statistically significant (P = 0.409).

Tumour Necrosis Factor Alpha Level

TNF-α level was significantly higher in the BCCO/WATER group compared to the control (P = 0.002), indicating that BCCO induced increase in TNF-α. The TNF-α level of BCCO/CAFFEINE group is significantly lower than that of the BCCO/WATER group (P = 0.009). This shows that caffeine treatment has reduced the raised TNF-α value seen in the BOOC/WATER group back to normal (Figure 2).

Lactate Dehydrogenase (LDH) Level

There is no major difference in LDH level of BCCO/WATER group compared to the control (P=0.997). The LDH level of BCCO/CAFFEINE group is non-significantly higher than that of the BCCO/WATER group (P=0.593) (Figure 3).

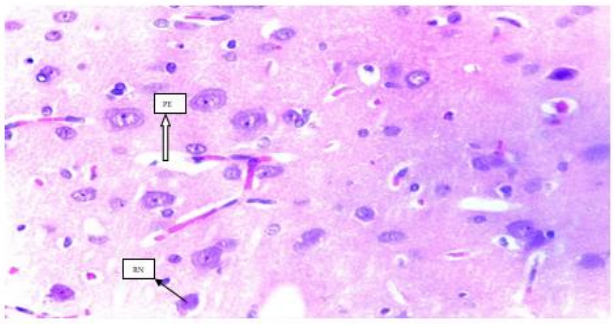

Plate IA: Photomicrograph of cerebral cortex of Control group rat. Note the perivascular edema (PE), red neuron (RN). H & E stain × 400 magnification.

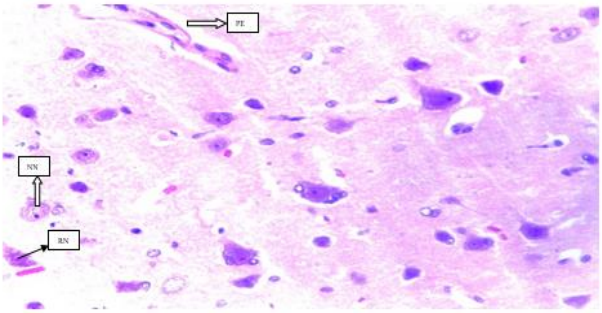

Plate IB: Photomicrograph of cerebral cortex of Sham/Water group rat. Note the perivascular edema (PE), red neuron (RN), normal neuron (NN). H & E stain × 400 magnification.

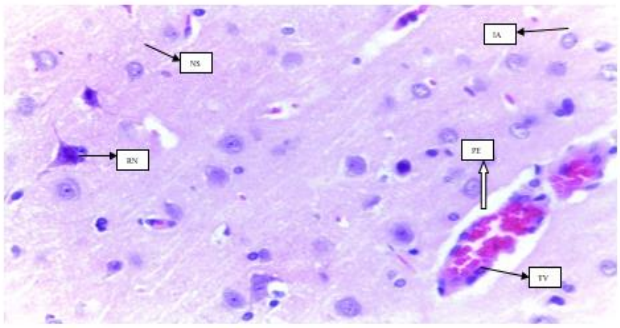

Plate IC: Photomicrograph of cerebral cortex of BCCO/Water group rat. Note the infarct area (IA), perivascular edema (PE), red neuron (RN), thrombosed vessels (TV), neutrophil spongiosis (NS). H & E stain × 400 magnification.

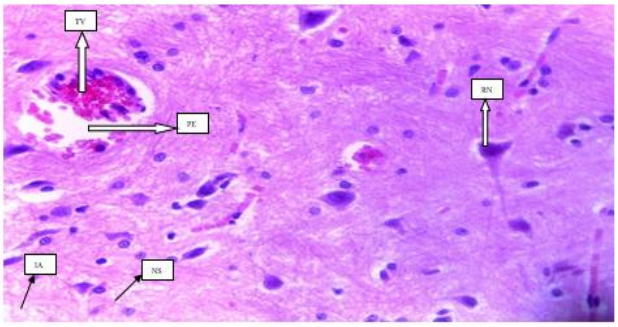

Plate ID: Photomicrograph of cerebral cortex of BCCO/Caffeine group rat. Note the infarct area (IA), perivascular edema (PE), red neuron (RN), thrombosed vessels (TV), neutrophil spongiosis (NS). H & E stain × 400 magnification.

Histological Evaluation of the Brain Cortex:
Plate I shows result of brain cortex histology of rat in control group which received neither the operation nor caffeine (Plate 1A); sham/water group that received sham operation and water (Plate 1B); BCCO/water group that received bilateral common carotid occlusion and water (Plate 1C) and BCCO/Caffeine that received bilateral common carotid occlusion and 144 mg/kg of caffeine (Plate 1E).

DISCUSSION

Pathophysiologic pathways leading to neuronal death during cerebral ischaemia consists of immediate (within minutes) *peri*-infarct depolarization and excitotoxicity. Hours later, inflammation and oxidative stress occurs, while days later, it is followed by apoptosis (Woodruff *et al*., 2011; Muhammad *et al*., 2016). Cerebral ischaemia-reperfusion injury leads to the appearance of different degrees of sensory motor and other impairments, making assessment of neurological symptom in the final outcome of experimental cerebral ischaemic models critical (Zarruk *et al*., 2011; Desland *et al*., 2014). Garcia exam is scored on a scale from 3 to 18 between pre- and post-ischaemic cerebral damage. BCCO ischaemic stroke model has been shown to significantly reduce functional vascular area in the anterior part of the cortex (Gong *et al*., 2013). It has also been reported to moderately decrease cerebral blood flow (CBF) in the forebrain of adult rats into oligaemic range (Schmidt-Kastner *et al*., 2005) and, thus induce sensorimotor deficit. This is in agreement with present finding as Garcia score in post-ischaemic rats was significantly ($p<0.05$) lower in comparison to pre-ischaemic rats scores.

There was significant decrease ($p<0.05$) in serum level of TNF-α at caffeine acute dose of 144 mg/kg administered intraperitoneally, in experimental group compared to control group as revealed by One-way ANOVA (Figure 2). Post-hoc Scheffe test revealed that the significance lies between BCCO/CAFFEINE and BCCO/WATER groups. TNF-α released by microglia is one of pro-inflammatory cytokine that becomes locally expressed in response to acute cerebral ischaemia (Denes *et al*., 2010; Kalogeris *et al*., 2014). TNF-α interacts with two receptors, TNFR1 and TNFR2, to induce extrinsic apoptotic cellular death pathway via Fas associated death domain (FADD) and inflammation via nuclear factor kappa-light- chain enhancer of activated B cells (NFκB), respectively (Sedger and McDermottc, 2014). Numerous neuro-protective agents have shown their neuro-protective functions by attenuating the expression TNF-α in ischaemic stroke models (Mohamed *et al*., 2012; Ewen *et al*., 2013; Yuan *et al*., 2014; Guan *et al*., 2015; Han *et al*., 2016). Caffeine has been reported to provide potentials in modulation of neuro-inflammation by suppressing the generation of pro-inflammatory mediators such as NO, PGE_2 and TNF-α (Kanga *et al*., 2012), and attenuate the number of activated microglia within the hippocampus (Brothers *et al*., 2010) in lipopolysaccharide-induced neuro-inflammation. Hence the finding herein is in conformity with the aforementioned previous findings. The proposed mechanism through which caffeine confers anti-inflammatory effect is through attenuation of glutamate-induced microglia activation (Brothers *et al*., 2010; Kanga *et al*., 2012).

There was no significant difference ($p>0.05$) in serum level of LDH at caffeine acute dose of 144 mg/kg administered intraperitoneally, in the experimental group compared to control group as revealed by One-way ANOVA (Figure 3). Lactate dehydrogenase (LDH) is a ubiquitous cytoplasmic enzyme that catalyses the reversible conversion of pyruvate to lactate within cells during anaerobic glycolysis. It is known to be retained by viable cells with intact plasma membranes and released from cells with damaged membranes (Seetapun *et al*., 2013), and as such elevation of LDH during cerebral ischaemia is primarily due to glutamate induced excitotoxicity (Tikka *et al*., 2001) or secondary to cytokine mediated neuronal damage (Erez *et al*., 2014). Obtained results demonstrated the highest increase in mean value of LDH in experimental rats given the BCCO/CAFFEINE treatment compared to all other groups (Figure 3). This is in sharp contrast to previous findings of numerous neuro-protective agents that were found to reduce LDH release in ischaemic stroke model (Tikka *et al*., 2001; Kagiyama *et al*., 2004; Hurtado *et al*., 2005; Cimarosti *et al*., 2005; Seetapun *et al*., 2013; Cai *et al*., 2016; Ram *et al*., 2016). The reason behind caffeine effect in increasing LDH might be due to its well established physiological effect of phosphodiestrase inhibition (Astrup *et al*., 1990; Acheson *et al*., 2004; Belza *et al*., 2009). It is known that many intracellular signal pathways depend upon production of cyclic adenosine monophosphate (cAMP), and increased cAMP activity is short-lived because it is terminated by an enzyme known as phosphodiesterase which catalyses the conversion cyclic AMP back to AMP. Therefore, inhibition of phosphodiesterase enzyme results in high tissue concentrations of cyclic AMP (cAMP) which has many physiological and pathophysiological implications (Yu *et al*., 2009). One of such implications is sustained activation of protein kinase that results in cAMP-mediated up-regulation/stabilisation of LDH activity. The mechanism is that cAMP activates protein kinases; both protein kinases A (PKA) and C (PKC), which leads to LDH transcriptional gene regulation through stabilisation of LDH-A mRNA and subsequent increase of intracellular LDH-A mRNA levels (Hong *et al*., 2004; Richard *et al*., 2005; Huang *et al*., 2009).

On histological analysis, cerebral ischaemia-reperfusion damage was assessed based on the degree of infarct area (IA), presence of red neurons (RN), perivascular edema (PE), neurophil spongiosis (NS), and thrombosed blood vessels (TV). These were the key histologic features reported in the phase of acute neuronal injury after cerebral infarct (Mena *et al*., 2004). All rats of both experimental and control groups showed the presence of red (eosinophilic) neurons. Red neurons being a manifestation at the cellular level are earliest histological feature in neuronal injury that consisted of increasing cytoplasm eosinophilia, with the nucleus appearing shrunken and darkly basophilic or developing clumped chromatin condensation (Margaritescu *et al*., 2009). The presence of perivascular edema, neutrophil spongiosis and thrombosed vessels indicated vascular changes in acute ischemic stroke. Perivascular edema is secondary to plasma components extravasation in the area of the cerebral infarct, and closely related is neutrophil spongiosis linked to plasma leakage into the adjacent brain. The presence of thrombosed vessels indicates vascular endothelial damage of small arteries adjacent to necrotic zones (Margaritescu *et al*., 2009). The infarct area is due to the necrosis in the hypoperfused cortical regions. Based on revelation of histological finding, the extent of ischaemia-repefusion injury in BCCO/CAFFEINE group rat is reduced in comparison with BCCO/WATER group rat, while the degree of injury in SHAM/WATER group rat is about the same with that of CONTROL group rat.

In conclusion, caffeine significantly reduced serum levels of inflammatory TNF-α as well as manifested noticeable neuro-protective roles against ischaemia-reperfusion damage to the brain tissue, but conversely did not produce significant effect on serum LDH levels.

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Heparin Enhances the Effects of Mesenchymal Stem Cell Transplantation in a Rabbit Model of Acute Myocardial Infarction

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Summary: Stem cell transplantation in combination with administration of bioactive compounds has shown promising results in treating myocardial infraction (MI). In the current study, we investigated the effect of combining mesenchymal stem cells (MSCs) transplantation with heparin into the infarcted heart rabbits. For this purpose, 35 male New Zealand white rabbits were randomly divided into five groups: sham, MI, MI+ MSCs, MI+ heparin and MI+MSCs+ heparin. MI was induced by 30 min ligation of the left anterior descending coronary artery. The animals of MSCs and MSCs +heparin groups were injected cell culture containing MSCs intramyocardially into the infarct area. Functional parameters of the left ventricle by echocardiography, serum levels of VEGF by enzyme-linked immunosorbent assay, size of fibrotic area by Masson's trichrome staining, evaluation of morphology by Haematoxylin-Eosin and capillary density alkaline phosphatase staining were compared between groups. Ejection fraction, fractional shortening and levels of VEGF significantly improved in MSCs and MSCs + heparin group ($P<0.05$). The fibrotic area was significantly reduced ($p=0.009$) in MSC + heparin treated animals in comparison with MSCs. Number of live cells and angiogenesis were increased significantly in MSCs + heparin groups in comparison with MSCs ($p< 0.05$). Although injection of MSCs significantly restored normal function of fibrotic area, we found that administration of heparin combined with MSCs to infarcted heart of animals could have better effects on LV functional parameters in fibrosis area and resulted in superior therapeutic outcome in enhancing neovascularization and improving cardiac fibrosis.

Keywords*:* Mesenchymal stromal cells, heparin, myocardial infarction.

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INTRODUCTION

A growing body of evidence shows benefits of transplantation of adult bone marrow-derived stem cells as a potential therapy to repair damaged myocardium following an acute infarction (Donndorf *et al.*, 2013, Hass *et al.*, 2011, Hsiao *et al.*, 2013, Li *et al.*, 2017). In MI, myocardial cells are replaced with fibrotic or scar tissue, which result in reducing normal function of the heart (Zamilpa *et al.*, 2014). These stem cells which are able to differentiate into various types of cells in vitro and in vivo, could partly repair damaged tissue and restore normal function of the heart (Donndorf *et al.*, 2013, Hsiao *et al.*, 2013).

Whereof poor retention within the myocardium (6% or less) of transplanted bone marrow-derived cells in concomitant with little functional improvement in myocardium, there is an unmet need whether some soluble protein which are contribute to transplanted cells for restoring and recovering significant function of myocardium simply There is an unmet need to find some soluble protein contributing to transplanted cells to restore within the myocardium and improve function of myocardium significantly (Freyman *et al.*, 2006, Webber *et al.*, 2010).

For this reason paracrine factor linked to transplanted stem cell are considered as facilitators to cardiac protection, repair, and regeneration following infarction, specially, chemokines and growth factors which are secreted from mesenchymal stem cells such as vascular endothelial growth factor (VEGF). VEGF may be involved in cardiovascular signaling and it is a growth factor affecting angiogenesis and improves the outcomes of MI (Gnecchi *et al.*, 2005, Tang *et al.*, 2011, Webber *et al.*, 2010).

These paracrine endogenous factors require some adjuvants to stimulate or augment the impacts of mesenchymal stem cells on repairing damaged

myocardium following an acute infarction (Caplan *et al.,* 2006, Nagaya *et al.,* 2005).

In current study was designed to examine the paracrine effects of heparin as a coordinator with VEGF on increasing angiogenesis and myocardium blood flow. Heparin, which is used as anticoagulant, is able to attach to VEGF and increase the affinity of this protein to its receptor and enhances the mitogenic and angiogenic activity of this protein. VEGF could be considered as promising angiogenic therapy for managing myocardial infarction by promoting angiogenesis and cell survival and also improve mesenchymal stem cell sheet transplantation therapy

It seems that it is a good choice to augment the beneficial effect of stem cell transplantation (Webber *et al.*, 2010).

Little is known about the effect of heparin on increasing the ability of transplanted MSCs in recovery of ischemic heart. In other hand, heparin is widely used for clinical approach as a common anticoagulant in patients with cardiovascular disease. Therefore, in the current study, we aimed to assess the effects of heparin added to MSCs on myocardial damage after acute infarction.

MATERIALS AND METHODS

Animal model

This investigation conformed to the "Guide for the Care and Use of Laboratory Animals" published by the United States National Institutes of Health (NIH Publication, 8th Edition, 2011).

The experimental protocol was approved by ethical committee of Iran University of Medical Sciences. Thirty five New Zealand white rabbits (weight 2500-3000 g) purchased from Razi Vaccine and Serum Research Institute were housed in controlled environment conditions (22 ± 2◦C; light–dark cycle 7 AM–7 PM). Animals were allowed to access water and standard laboratory food ad libitum. Rabbits were randomly divided into 5 groups.

1) Sham operated group (n=7): normal rabbits, which receives injection of cell culture media (150 µl) in both sides of descending anterior artery.

2) Control group (n=7): MI induced animals, which did not receive any treatment.

3) MI+MSCs group (n=7): MI induced animals, which were treated by injecting MSCs intramyocardially into the infarct area.

4) MI+MSCs + heparin group (n=7): MI induced animals, which were treated by MSCs injection followed by subcutaneous administration of heparin (200 U/Kg for 28 days).(Diquélou et al, 2005)

5) MI+ heparin group (n=7): MI induced animals, followed by subcutaneous administration of heparin (200 U/Kg for 28 days)

Induction of myocardial infarctions

Animals underwent general anesthesia (2% sodium pentobarbital; 40 mg/kg i.p.). Next, they were intubated and ventilated by room air using a rodent ventilator (tidal volume2–3 ml, respiratory rate 65–70 per minute, Harvard rodent ventilator model 683, Holliston, MA, USA). Left intercostal thoracotomy (between the two and three costal space) was performed under sterile condition and *left anterior descending* (LAD) was ligated between first and second diagonal branches of coronary artery with a 5-0 silk suture. Successful performance of coronary occlusion was confirmed by observation of the development of a pale color in the distal myocardium after ligation as well as dyskinesia of the anterior wall.

Human mesenchymal stem cell (hMSCs) isolation and culture

Human bone marrow samples were provided by Shariati Hospital (Tehran University of Medical Sciences). Human samples were collected under ethical permissions approved by Iran University of Medical Sciences. After separating mononuclear cells, the amount of live cells were determined using trypan blue staining. The extracts were then cultured in DMEM, 20% FBS, 1% penicillin - streptomycin and incubated at 37ºC, 20% O2 and 5% CO2. The culture medium was removed and replaced with fresh medium two times weekly and adherent cells were retained. At 50% to 60% confluence, the cells were harvested for subculture with 0.25% trypsin containing 0.02% EDTA (Gibco). HMSCs were isolated after changing the media (DMEM, 10% FBS, 1% penicillin-streptomycin) continuously for 3 days.

The animals in MSCs and MSCs +heparin groups were injected with 150 µl of cell culture containing MSCs (1×10^6) intramyocardially into the infarct area, and the sham group was injected with an equal volume of cell culture media.

Flow cytometry

HMSCs grown up to 80% confluency were trypsinized and resuspended in 500 µl of PBS after centrifugation at 900 ×g for 7 min, analyzed with a flow cytometer (Partec, Germany). The antibodies used in this experiment were included : phycoerythrin (PE)-conjugated anti-CD105 (BD Pharmingen™, 562759), FITC-conjugated anti- CD90 (BD Pharmingen ™, 551401), PE-conjugated anti-CD160 (BD Pharmingen™, 550257), FITC-conjugated anti-CD44 (BD Pharmingen TM, 560977) for detecting MSC specific markers and fluorescein isothiocyanate (FITC)-conjugated anti-CD45 (BD Pharmingen™, 345808), FITC-conjugated anti-CD34 (BD Pharmingen™, 340668) as negative markers. Mixture of antibodies and cells were incubated for 1h on ice and in dark place.

Assessment of LV functional activity (echocardiography)

Echocardiography was used to measure functional parameters of LV including ejection fraction (EF), and

fraction shortening (FS). Rabbits were anesthetized and placed in the supine position. M-Mode Echocardiography was performed in 3 axis (short, long and average of axes) using VVIPE3; General Electric instrument before and after MI induction and at 4 and 8 weeks after treatment. All measurements were analyzed by two independent observers who were blinded to the treatment status of the animals.

VEGF measurement
VEGF concentrations were determined in serum by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (ABIN365534). Briefly, 100 µl of plasma were pipetted into an antibody coated, 96-well plate. After growth factor binding and washing, VEGF conjugate (enzyme-linked antibody specific for VEGF) was added to each well. Following more washes, a substrate solution was added to the wells and incubated for 30 minutes at room temperature. The reaction was stopped by adding 50 µl stop solution, and absorption was measured by a microplate reader (Biotech, USA) at 450 nm. The kit detection Range was 1.56-100 pg/mL of VEGF.

Histological examination
One month after all cardiac functional recordings, the animals were sacrificed under deep anesthesia and their hearts were extracted for histological examinations. After washing in normal saline, the hearts were fixed by immersion in 4% paraformaldehyde for 24–48 h and embedded in paraffin. Transverse sections (7 µm) of hearts were prepared using a microtome. Slices were put on gelatin coated slides and stained for following purposes:
Measuring the fibrotic area: After removing paraffin from tissue by series of hydration (using xylol) and alcohol, the prepared slides were stained with Masson's trichrome (Sigma- Aldrich Co., MO, USA) for measurement of fibrosis. Five to ten sections were obtained from the LV free wall of each rabbit. Myocardial fibrosis was calculated the ratio of fibrotic area (blue color) to total cardiac section and expressed as percent fibrosis. Quantitation of fibrous areas was performed with NIS-Elements D software.
Evaluation of morphology: The paraffin-embedded sections were stained by Hematoxylin and eosin (H&E) for 10 min. After washing with water, the slides were treated with eosin solution for 15 min. In the last stage, water is removed from slides according to the protocol.
Capillary Density (alkaline phosphatase staining): The slides were primarily treated with magnesium chloride 1% and then incubated in ALP substrate (100 mU/ml) (Alkaline phosphatase kit, Sigma-Aldrich) for 2h at room temperature. After washing with double distilled water (dH2O), the samples were stained with green nuclear counterstain.
Alkaline phosphatase was applied for determining the rate of angiogenesis in ischemic tissue; moreover, percentage of pyknotic nuclei was detected using trichrome masson and Haematoxylene-Eosin methods, respectively.

Statistical analysis:
ANOVA test (post hoc, Tukey) was used to compare case and control groups. Kruskal-Wallis and Mann Whitney tests were performed to compare MSCs +heparin results with MSCs results. All data were presented as mean ± standard deviation (mean ± SD). Two-tailed $P<0.05$ was considered statistically significant. All study data were analyzed using the SPSS version 15.0 for Windows (SPSS Inc. Chicago, IL, USA.

RESULTS

Characterization of isolated MSCs
After plating human bone marrow aspirates for 24h, adherent MSCs appeared in cell culture plate. Non-adherent hematopoietic cells were further separated from MSCs by culturing and removing media continuously for 3 days. Primarily culture of MSCs took 10-14 days. In order to characterize MSCs extracts, we use flow cytometry assay for specific markers of MSCs vs. hematopoietic cells. Flow cytometry results showed that obtained MSCs were positive for CD44 (96.8%), CD90 (98.8%), CD166 (93%) and CD105 (99.5%). Few amount of cells expressed hematopoietic markers including CD45 (3.8%) and CD34 (2.8%). We also used control isotype antibodies for preventing non-specific antigen-antibody interactions.

Transplanted MSCs and heparin administration for recovery of MI animals
Effects of transplanted *MSCs* alone and in combination with heparin for recovery of MI lesions were assessed by cardiac function, biochemical and histological factors.

Effect of MSCs and heparin on functional parameters
Within each group we compared fractional shortening and ejection fraction before and after MI induction and at 4 and 8 weeks after treatment (Fig1). After LAD ligation, EF and FS were decreased in all groups in comparison with sham group (table 1). MSCs transplantation significantly improved the ejection fraction (EF) in 4 and 8 weeks after treatment (p: 0.01, p: 0.04 respectively) and Fractional Shortening (FS) in 4 and 8 weeks after treatment (p: 0.03, p: 0.01 respectively) (table 1). All these beneficial effects of MSCs were markedly enhanced by heparin treatment. EF in MSCs+ heparin (55±0.009) was higher than MSCs (52±0.001) after 4 weeks (p: 0.08) and also after 8 weeks (59±0.015 in MSCs+ heparin vs. 57±0.003 in MSCs p: 0.07). Although EF had not

Table 1: Comparisons of cardiac function measured by echocardiography between groups after MI induction and at 4 and 8 weeks after treatment

		Sham	control	MSCs	MSCs+heparin	heparin	p-value
EF %	**After MI**	70±0.014	49±0.018	51±0.09	52±0.015	52±0.08	0.001
	4 weeks	70±0.04	46±0.002	52±0.001	55±0.009	53±0.01	0.01
	8 weeks	70±0.01	45±0.02	57±0.003	59±0.015	53±0.005	0.04
FS	**After MI**	45.7±0.02	22.7±0.019	23.3±0.021	23.3±0.023	23±0.009	0.001
	4 weeks	45.9±0.05	20.5±0.002	24.8±0.05	31.7±0.03	22±0.001	0.03
	8 weeks	42.1±0.001	21±0.001	29.1±0.007	33±0.006	22±0.009	0.01

EF: ejection fraction, **FS:** fractional shortening, **MI:** myocardial infarction, **MSCs:** mesenchymal stem cells. One way ANOVA test showed MSCs transplantation significantly improved the ejection fraction (EF) in 4 and 8 weeks after treatment (p: 0.01, p: 0.04 respectively) and Fractional Shortening (FS) in 4 and 8 weeks after treatment (p: 0.03, p: 0.01 respectively). The difference between EF in MSCs+ heparin and MSCs after 4 weeks and also after 8 weeks was not statistically significant (p: 0.08, p: 0.07 respectively). The difference between FS in MSCs+ heparin and MSCs after 4 weeks and also after 8 weeks (p: 0.04, p: 0.04 respectively).

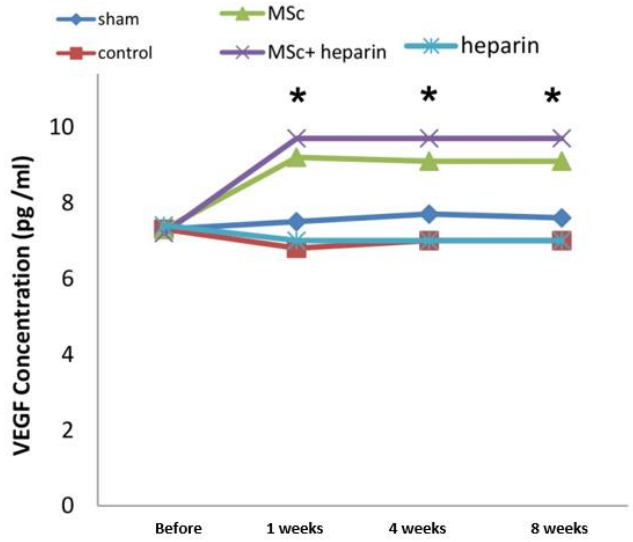

Figure 1: Comparison the amount of VEGF in serum of different study groups. *: significant differences between MSCs (p<0.001 one, four and eight weeks after treatment) and MSC + heparin (p: 0.01 1week and p<0.001 4 and 8 weeks after treatment) treated animals vs. control group. VEGF in MSCs+ heparin had significant differences compared to MSCs after 1 week (p =0.01), after 4 weeks (p = 0.05) and after 8 weeks (p = 0.01).

statistically increased in MSCs+ heparin in comparison with MSCs after 4 and 8 weeks, these increases can be clinically remarkable (table1). Fractional Shortening in MSCs+ heparin (31.7 ±0.03) was improved significantly than MSCs (24.8±0.01) after 4 weeks (p = 0.04) and also after 8 weeks (33 ±0.006in MSCs+ heparin vs. 29.1±0.007 in MSCs p = 0.04) (table1).

Biochemical analysis:

The levels of VEGF in the serum which compared between groups are shown in figure 1. Before MI induction, blood VEGF level was assessed in all animals. The mean of VEGF level before study was 7.10± 0.05 (pg/ml). Level of VEGF in the MSCs transplantation group was significantly higher than control group, 1 week (9.2±0.17 (pg/ml) vs. 6.8±0.1 (pg/ml), P <0.001) and 4 weeks (9.1±0.17 (pg/ml)vs. 7±0.2 (pg/ml), P <0.001) after treatment. Level of VEGF in the MSCs transplantation+ heparin group was also significantly higher than control group, 1 week (9.7±0.22 (pg/ml)vs. 6.8±0.1 (pg/ml), P= 0.001) and 4 weeks after treatment (9.8±0.25 vs. 7±0.2, P<0.001).

The VEGF in MSCs+ heparin (9.85±0.05 (pg/ml)) level was significantly higher than MSCs (9.21± 0.007 (pg/ml)) after 1 week (p: 0.01) and after 4 weeks (9.88±0.02 (pg/ml) in MSCs+ heparin vs. 9.15± 0.02 (pg/ml) in MSCs p: 0.05) and also after 8 weeks (9.90±0.05 (pg/ml)in MSCs+ heparin vs. 9.21± 0.01 (pg/ml) in MSCs p: 0.01).

There were no significant differences between heparin only group and control group, 1 week (7±0.01 (pg/ml) vs. 6.8±0.1 (pg/ml), P: 0.8) and 4 weeks (7±0.1 (pg/ml) vs. 7±0.2 (pg/ml), p: 0.9).

Histological analysis:

Fibrosis in the heart tissue was measured 8 weeks after treatment using trichrome staining. The percent of fibrotic area was significantly reduced (p=0.01) in MSCs and MSCs + heparin treated animals in comparison with control group (MSCs: 15% and MSCs + heparin: 10% vs. control: 29%). The difference was also significant between MSCs and MSC + heparin groups (p= 0.04) (Fig 2 a and b). The histological evaluation using hematoxylin and eosin staining revealed that number of normal cells were increased significantly in MSCs and MSC + heparin groups in comparison with sham and control animals (p= 0.01) (Fig 3). Interestingly, numbers of viable cells in MSCs and MSC + heparin models were significantly more than control group (MSCs: 57.7 and MSCs + heparin: 51 vs. control: 29 p= 0.02).

With regards to viable cells, difference was also significant between MSCs and MSC + heparin groups (p: 0.04) (Fig 4). The amount of angiogenesis was measured by alkaline phosphatase staining. Our data showed that amount of angiogenesis in MSC + heparin and MSCs are significantly higher than control (MSCs: 25 and MSCs + heparin: 20 vs. control: 5 number/mm^2, p<0.001). The results showed

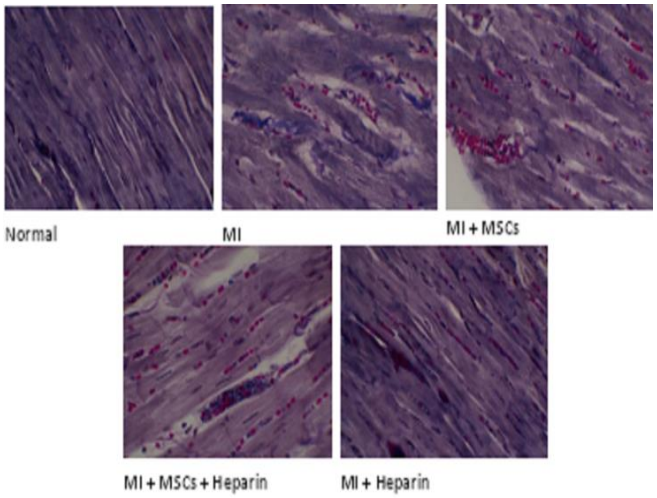

Figure 2a (40× magnification): Evaluation of fibrotic area was performed before and after intramyocardially MSCs delivery with the induction of experimental MI using Masson's trichrome staining.

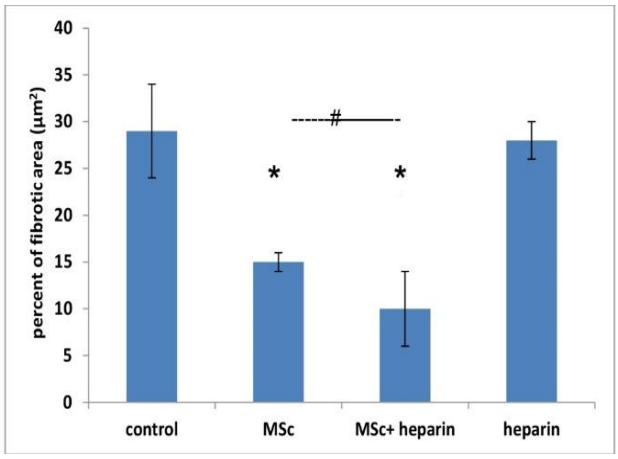

Figure 2b: one way ANOVA test showed significant decrease in area of lesion could be observed in groups treated with MSCs and MSC + heparin in comparison with control group (* p = 0.01).

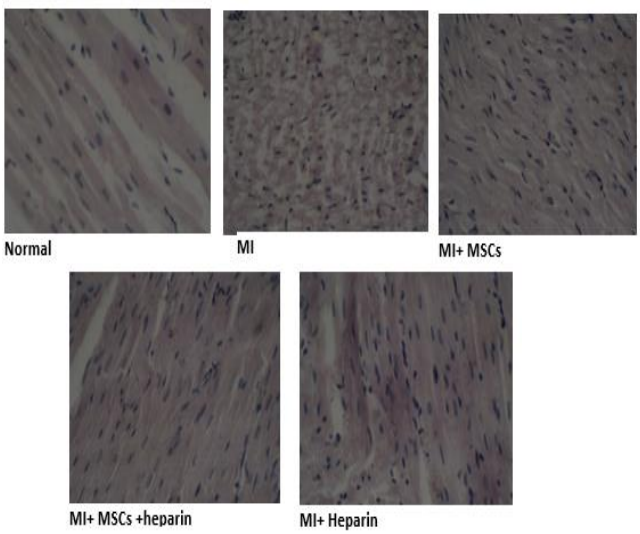

Figure 3: Histological analysis of the lesion areas in heart tissue. H&E showed replacement of damaged tissue by normal cells containing flat normal nucleus and light granules (40 × magnification).

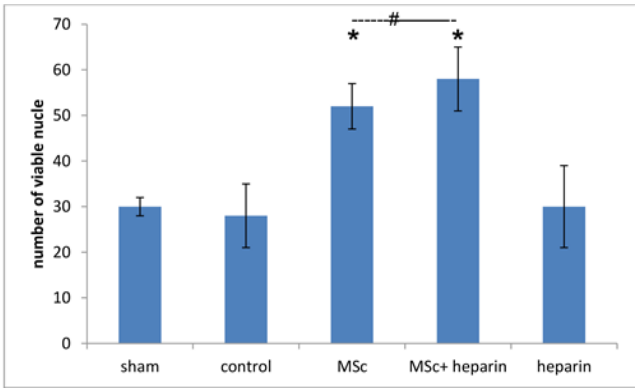

Figure 4: Comparison the number of viable cells between treated and non-treated animals (one way ANOVA test).

a) Amount of viable cells was increased significantly in MSCs and MSCs + heparin group in regard to control (*: p: 0.02). Number of viable cells was increased significantly in MSC + heparin compare to MSCs group. # p: 0.04 by Mann Whitney U test

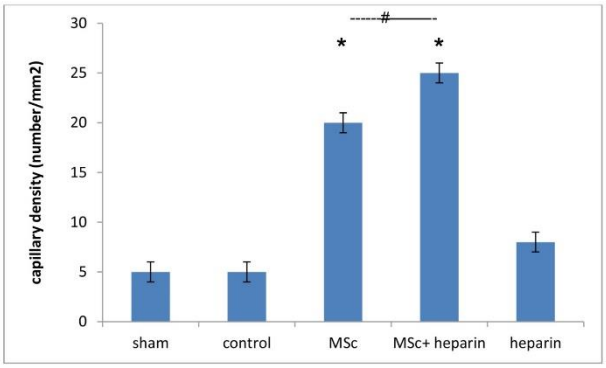

Figure 5: Analysis the amount of angiogenesis in different study groups.

Comparison different groups of study after 8th weeks by one way ANOVA test showed significant increase in amount of angiogenesis for rabbits treated with MSCs and MSC + heparin compared to control(*p <0.001). Capillary density was increased significantly in MSC + heparin compare to MSCs group. # p: 0.01 by Mann Whitney U test.

increase in amount of angiogenesis in MSC + heparin group compared to MSCs after 8 weeks (P: 0.01) (Fig 5).

DISCUSSION

Based on evidence gained from several studies demonstrating the capability of mesenchymal stem cell to partially restore cardiac function after ischemic damage in animal models and clinical results is mandatory to make a step forward in improving cardiac cell therapy as a relevant therapeutic tool (Donndorf *et al.*, 2012). Further efforts will most likely need to be taken to find ways to augment or facilitate stem cell effects on myocardial regeneration. Paracrine action of engrafted MSCs in ischemic myocardium is noteworthy for reduced remodeling and recovery of cardiac performance. The release of VEGF leads to efficient vascular regeneration and resultant blood flow and also attenuates the apoptotic pathway

(Elnakish *et al.*, 2012, Tang *et al.*, 2005, Tang *et al.*, 2011).

VEGF has been known as an endothelial cell mitogen, regulator of various cellular stress responses, as well as survival, proliferation, migration, and differentiation. Generally, VEGF has angiogenenic and nonangiogenic role in cardiovascular system (Augustin *et al.*, 2013, Nichols *et al.*, 2014).

In current study, our aim was to examine the heparin in collaborating with VEGF to emboss its paracrine effects on increasing angiogenesis and resultant blood flow. Heparin, which is used as anticoagulant, is able to attach to VEGF and increase the affinity of this protein to its receptor and seems that it is a good choice to augment the beneficial effect of stem cell transplantation (Gnecchi *et al.*, 2005, Tang *et al.*, 2011).

According to this goal, VEGF level was measured in all groups. Our findings showed that VEGF was significantly increased in MSCs injected group in comparison with control after 1, 4 and 8 weeks. Interestingly, VEGF in MSCs+ heparin was significantly higher than MSCs after 1, 4 and 8 weeks.

Tang YL et.al concluded that the enhancement of cardiac function in MSCs therapy may be attributed to the capacity of self-renewal of MSCs that can maintain the long-lasting effect of angiogenesis and cardiomyocyte recovery by inhibition of apoptosis via MSCs transplantation and VEGF protein secreted by transplanted cells likely are the causes of this advantage. (Elnakish *et al.*, 2012)

In our study, we found angiogenesis was increased in ventricular myocardium of animals who received MSCs and the capillary density was significantly higher in animals who received MSCs+ heparin in comparison with MSCs alone.

Our findings prove that MSCs transplantation, as pivotal contributing factor in making new vessels in one hand and the effect of VEGF in neovascularization in other hand seemingly leads to notable improvement of infarcted heart. It seems that heparin Facilitate this effect of MSCs transplantations. Interestingly, histological analysis confirmed this effect of MSCs transplantation alone or together with heparin in recovery of ischemic heart by increasing angiogenesis.

Similar to these results, histological findings showed highlighted increase in amount of angiogenesis, decrease in fibrotic area and increase in number of viable cells in group with MSCs injection compared to control and also in MSCs+ heparin in comparison with MSCs alone.

Induction of angiogenesis which is usually triggered by growth factors released by cells is an important process required for functional tissue formation can improve myocardium perfusion. Though hypoxia which generated by coronary artery stenosis can induce VEGF and angiogenesis in ischemic myocardium, it is insufficient to allow recover cardiomyocytes (Elnakish *et al.*, 2012). Glycosaminoglycan such as heparan sulfate in the extracellular matrix aid in proper functioning of these growth factors (Arslan *et al.*, 2014, Mammadov *et al.*, 2011). Mammadov et al (2011) designed and synthesized a self-assembling peptide molecule that is functionalized with biologically active groups to mimic heparin. By these peptide nanofibers, angiogenesis was induced without the addition of exogenous growth factors and bioactive interactions between the nanofibers and the growth factors enabled robust vascularization in vivo as well (Mammadov *et al.*, 2011). While, Webber et al (2010) designed and synthesized heparin-binding peptide amphiphilic (HBPA) nanofiber networks which serve as a synthetic extracellular matrix and biomimetic delivery vehicle for paracrine factors had a marked ability to augment function and healing in ischemic tissues (Webber *et al.*, 2010).

Its use in these studies demonstrates the marked biological activity of the paracrine factors released by stem cells coupled with their material to augment function after an MI or enhance vasculature following critical ischemia (Mammadov *et al.*, 2011, Webber *et al.*, 2010, Zhang *et al.*, 2009). But we were looking for to prove that heparin, which is used as an anticoagulant to treat and prevent deep vein thrombosis, pulmonary embolism, and arterial thromboembolism and also used in the treatment of heart attacks and unstable angina and it appears to be relatively safe for use during pregnancy and breastfeeding (Xu *et al.*, 2011) can be used to induce various desired physiological responses of growth factors like VEGF for tissue repair in infarcted cardiac area.

Therefore, our data indicated that cell therapy and to large extent heparin administration could reduce the surface area of injury. In MSCs transplantation and MSCs + heparin groups, the numbers of cells with euchromatin nucleus in ischemic tissue were increased. Interestingly these results indirectly confirmed pervious report about the role of VEGF secreted by MSCs in repairing infracted heart tissue (Tavakoli *et al.*, 2013).

To best of our knowledge, this is the first report on the role of heparin administration combined with stem cell therapy in the restoration of MI heart. However, we suggest that follow ups of our study should be carried out beyond 8 weeks and the status of VEGF receptors should be analysed as well as inflammatory markers such as IL6, TGF α, TNF α and bFGF in association with tested factors on recovery of MI animals.

Our studies to date using exogenous heparin indicate a marked ability to augment function of mesenchymal stem cell to heal the damaged tissue after infarction. The mechanisms seem to be connected with the paracrine effects of VEGF secreted by MSCs as an endothelial cell mitogen, regulator of various cellular

stress responses, as well as survival, proliferation, migration, and differentiation and totally angiogenenic and nonangiogenic effects of VEGF in cardiovascular system.

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Effect of Chronic Caffeine Consumption on Cardiac Tissue Metabolism in the Rabbit

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Summary: Previous studies on the ability of caffeine to enhance endurance and boost performance have focused on the energy substrates that are utilized by the skeletal muscle and the brain but nothing of such has been reported on cardiac tissue. This study was designed to investigate the effect of caffeine on cardiac tissue metabolism in the rabbit. The study was carried out on adult male New Zealand rabbits divided into 3 groups (n=5). Group I rabbits served as control and were given 0.5ml/Kg of normal saline while group II and III rabbits were administered with 2mg/Kg and 6mg/kg of caffeine respectively for 28 days. Blood samples were collected by retro orbital puncture for biochemical analysis. Animals were sacrificed by cervical dislocation and cardiac tissue biopsies were collected for biochemical and immunohistochemical analysis. Cardiac tissue glycogen concentration was determined by anthrone reagent method. Cardiac tissue CPT 1 activity and cAMP concentration were determined by immunohistochemistry and colorimetry techniques respectively, with assay kits obtained from Biovision Inc. The results showed that Caffeine at 2 and 6 mg/kg significantly inhibited MPO activity from 0.72±0.05 to 0.164±0.045 and 0.46±0.12 U/L respectively ($p<0.05$). Caffeine at 2mg/kg had no effect on serum nitric oxide but at 6mg/Kg, it significantly increased serum nitric oxide form 28.01±6.53 to 45.25±3.88µM of nitrite ($p<0.05$). Also, Caffeine at 2 and 6mg/kg increased cardiac tissue glycogen from 15.62±0.73 to 40.69±6.35 and 38.82±6.91mg/100g respectively and carnitine palmytol transferase 1 activity from 18.3 to 20 and 25.2% respectively. In conclusion, the study showed that caffeine consumption increased CPT 1 activity suggesting increased utilization of free fatty acids for energy metabolism and sparing of cardiac tissue glycogen by mechanism(s) which probably involved blockade of A1 adenosine receptors and cAMP signaling pathway.

Keywords: Caffeine, Cardiac tissue metabolism, Rabbit

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INTRODUCTION

Caffeine remains the most commonly consumed stimulant in the world (Yang et al. 2009). It is present in many commercial beverages and medicines (Graham et al 2001; Chukwu et al 2006). In Nigeria, it is present in some common masticatory such as kola nut. Caffeine has been reported to boost performance and enhance endurance (Graham and Spriet 1991; Goldstein et al 2010). Central to caffeine's ergogenic ability is its capacity to alter substrate metabolism. Caffeine has been reported to alter skeletal muscle metabolism by increasing skeletal muscle lipolysis and oxidizing free fatty acids thereby sparing skeletal muscle glycogen usage (Jensen *et al*., 2007; Egawa *et al*., 2009). In the brain, caffeine increases glucose utilization in a number of regions such as the thalamus, hypothalamus, monoaminergic cell groupings and structures belonging to the extrapyramidal motor system (Nehlig *et al*., 1987, 1999). In the liver caffeine was reported to reduce the levels of hepatic lipid content by activation of autophagy in in vitro and in vivo studies (Ray 2013). It has also been reported to alter glucose metabolism in the liver of dogs by increasing net hepatic glucose uptake (Pencek *et al.*, 2004). There is however, no available report on how caffeine alters cardiac tissue substrate metabolism. The cardiac muscle tissue is considered a metabolic omnivore as it uses lipids, glucose, lactate, ketones and amino acids as substrate to generate energy (Saddik *et al.*, 1991; Lopaschuk *et al.*, 1994; Stanley and Chandler 2002). The normal adult heart predominantly utilizes fatty acids to generate energy for its pumping activities. However, the heart has been noted to be flexible in its choice of substrate for energy metabolism, depending on substrate availability and metabolic effectors (Taegtmeyer *et al.*, 1994). The choice of substrate for energy metabolism by the cardiac muscle following caffeine administration is not clear. Thus, this study was designed to investigate

the effect of caffeine on cardiac tissue substrate metabolism in the rabbit.

MATERIALS AND METHODS

Experimental animals

Adult male New Zealand rabbits with body weights ranging from 1.3 - 2.0 Kg were purchased from the University of Ibadan Veterinary Animal House and acclimatised for a period of 2 weeks in the Central Animal House of the University of Ibadan before the experiment was carried out. The animals were housed 1 per cage and were given regular rabbit laboratory chow and water *ad libitum*. Ethical regulations were observed all through experimental periods in accordance with national and institutional guidelines for the protection of the animal's welfare (PHS, 1996). Protocol for animal use was in consonant with the criteria outlined in the Guide for the care and the Use of Laboratory Animals prepared by the National Academy of Science and published by the National Institute of Health.

Experimental design

Adult male New Zealand rabbits were divided into 3 groups (n=5). Group I rabbits served as control and were given 0.5 ml/Kg of normal saline while groups II and III rabbits were administered with 2 mg/Kg and 6 mg/kg of caffeine respectively. Caffeine or normal saline administration in the rabbit was carried out daily through oral gavage for twenty eight (28) days. At the end of 28 days, blood samples were collected by retro orbital puncture for biochemical analysis and then animals were sacrificed by cervical dislocation followed with collection of cardiac tissue biopsies for biochemical and immunohistochemical analysis.

Plasma and Serum Preparation

About 3 mL of blood was collected from the retro-orbital venous plexus of the animals into EDTA sample bottles and plain sample bottles before animals were sacrificed by cervical dislocation. Blood samples were centrifuged at 4000 rpm for 15 minutes to obtain plasma and serum respectively.

Isolation of Post-Mitochondrial Fraction of the rabbit heart

Biopsies of rabbit heart was harvested on dry ice, rinsed in saline, and homogenized in aqueous potassium buffer (0.1 M, pH 7.4) and the homogenate centrifuged at 10,000 rpm (4ºC) for 10 min to obtain the supernatant fraction.

Determination of Cardiac cAMP concentration, Blood glucose level and Cardiac glycogen content.

Direct competitive immunoassay principle was used in the determination of cardiac cAMP concentration in post-mitochondrial fractions of the heart. The post-mitochondrial fractions were diluted with 0.1 M HCL and cAMP assay kit (Biovision Inc.) was used to determine the respective cAMP concentrations with the aid of a microplate reader at optical density of 450 nm. Blood glucose was determined with one touch glucometer using glucose oxidase method. The glucometer was checked against standard glucose solution at regular interval to ensure accuracy. Glycogen content of cardiac muscle tissue was determined by the anthrone reagent method as earlier described by Ishehunwa et al. (2013).

Biochemical Analysis

The serum myeloperoxidase activity was measured spectrophotometrically by a peroxidase-coupled assay system involving O-dianisidine and hydrogen peroxide as previously described by Xia and Zweier (1997). Production of Nitric Oxide was evaluated by measuring the level of nitrite (an indicator of NO) in the serum with the aid of Griess reagent system and assay kit was purchased from Promeg. Serum total cholesterol and triglyceride concentrations were determined by spectrophotometric methods using commercially available Randox Kits.

Immunohistochemistry of Cardiac Carnitine Palmitol Transferase I activity

The whole heart was fixed in 10% buffered formalin. The cardiac tissue was processed and embedded in paraffin wax. Altogether, 5–6 mm thick sections were made. Tissue sections were deparaffinized and hydrated using xylene followed by passage through ethanol of decreasing concentration (100–80%). The manufacturer's protocol for the localization of antigens in tissue sections was followed. Ready-to-use IHC kit for the study was purchased from Biovision Inc. and CPT I antibodies from Bioss USA. The immunoreactive positive expression of CPT I intensive regions were viewed starting from low magnification on each slide then with 100× magnifications using a photo microscope (Olympus) and a digital camera (Toupcam VR, Touptek Photonics, Zhejiang, China). The immune-positive reactions were quantified with Image J software.

Statistical Analysis

All values are expressed as mean±SE. The test of significance between two groups was estimated by Student's t-test. The test of significance amongst all groups was estimated by One way Analysis of Variance (ANOVA) with Tukey's posthoc test using Graph pad prism 5.0 with p-values <0.05 considered statistically significant.

RESULTS

Effect of Caffeine on Serum Myeloperoxidase Activity, Nitrite Concentration, Cholesterol, Triglyceride and Blood Glucose

Table 1 shows the effect of administration of caffeine on serum myeloperoxidase activity, nitrite concentration, total cholesterol level, plasma triglyceride and blood glucose concentrations. Caffeine produced a significant reduction in MPO activity in response to the two doses administered. The lower dose of caffeine produced a more significant reduction in MPO activity than the higher dose. Caffeine, however, caused an increase in

Table 1: Effect of 28 days administration of caffeine on serum myeloperoxidase activity, nitrite concentration, cholesterol, triglyceride and blood glucose concentrations

GROUP	MPO (U/L)	NOx (μM)	Blood glucose (mg/dl)	Total cholesterol (mg/dl)	Triglyceride (mg/dl)
Control	0.72±0.05	28.07±2.53	118.2±4.81	56.34±6.03	103.4±7.27
CAF (2mg/kg)	0.16±0.05***	31.94±2.52	121.2±5.70	120.20±29.89*	142.8±21.10**
CAF (6mg/kg)	0.46±0.07*	45.25±3.88*	191.4±4.82***	117.00±24.85*	144.5±11.52***

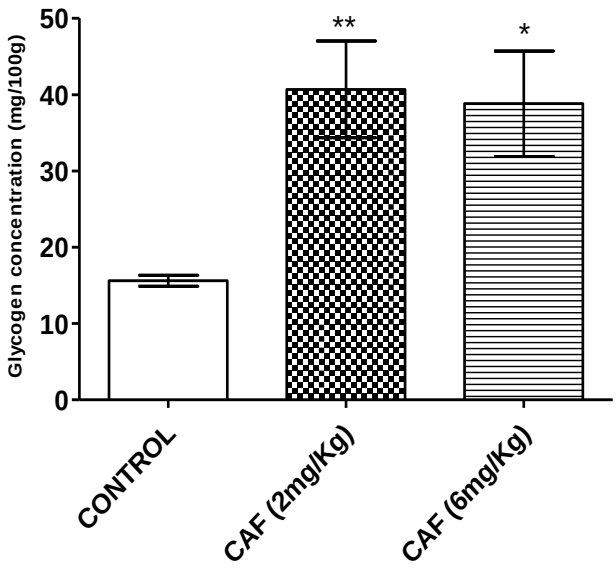

Figure 1: Effect of chronic administration of caffeine on cardiac tissue glycogen

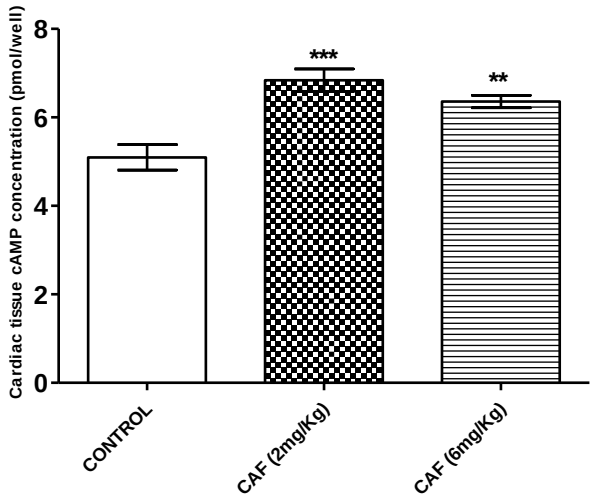

Figure 2: Effect of chronic administration of caffeine on cardiac tissue cAMP concentration

serum nitrite levels and this was significant only at a dose of 6 mg/kg. The two doses of caffeine also significantly increased the blood glucose levels in a dose-dependent manner. Administration of low and high doses of caffeine also significantly increased serum total cholesterol and triglyceride levels. There is no significant difference in the levels of total cholesterol and triglycerides in response to the two doses of caffeine.

Effect of Caffeine on Cardiac Tissue Glycogen

The effect of chronic administration of caffeine on cardiac glycogen tissue content is shown in figure 1. Caffeine increased cardiac glycogen content from 15.62±0.73 to 40.69±6.35 and 38.82±6.91 mg/100g of cardiac tissue at doses of 2 and 6 mg/kg respectively.

Effect of Chronic Administration of Caffeine on Cardiac Tissue cAMP Concentration

Figure 2 shows the effect of chronic administration of caffeine on cardiac tissue cAMP concentration in rabbits. Caffeine at doses of 2 and 6 mg/kg significantly increased cardiac tissue cAMP concentrations from 5.097±0.289 to 6.840±0.257 and 6.358±0.139 pmol/well respectively when compared to control.

Effect of Chronic Administration of Caffeine on Cardiac CPT I Activity

The effect of chronic administration of caffeine (2 and 6 mg/kg) on cardiac CPT I percentage activity in the rabbit is shown in plate 1. Chronic administration of caffeine at the two doses of 2mg/kg and 6mg/kg significantly increased the percentage activity of CPT I enzyme in the rabbit cardiac muscle without any significant difference in the responses to the two doses of caffeine.

DISCUSSION

The cardiac muscle tissue has been described as a metabolic omnivore which can use many substrates such as lipids, glucose, lactate, ketones and amino acids as energy substrate to keep the heart functioning (Saddik *et al.*, 1991; Lopaschuk *et al.*, 1994). The purpose of the present study was therefore to determine if caffeine intake alters the choice of cardiac energy substrate and if the alteration is a possible contributory mechanism to the well-known ability of caffeine to boost performance and enhance endurance. Hence caffeine doses within the ranges commonly consumed by man were employed in the present study. The increase in blood glucose levels following caffeine administration has earlier been reported in man (Pizziol et al., 1998, Graham et al., 2001, Dekker et al., 2007), dog (Salahdeen and Alada 2009a), rats

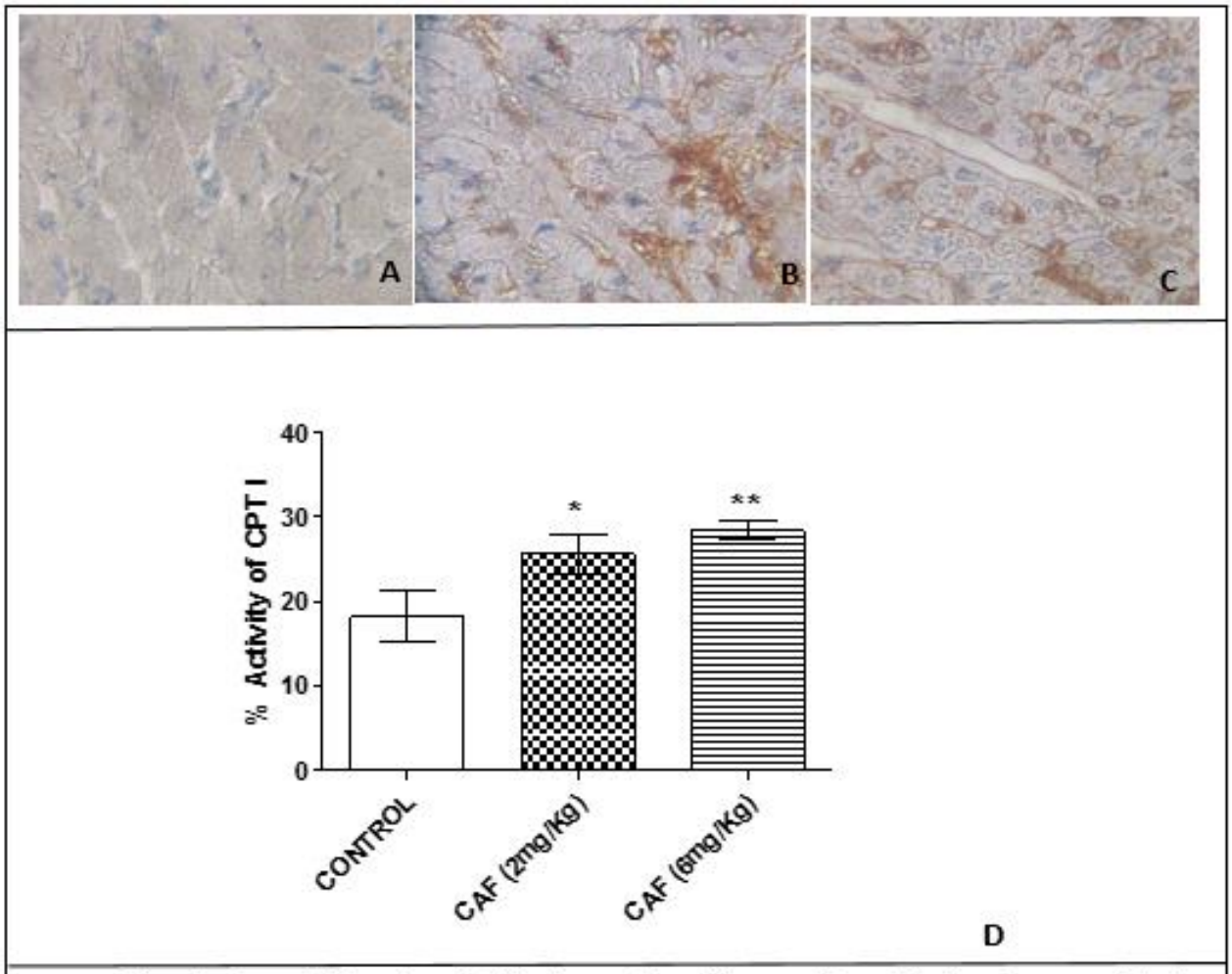

PLATE 1: Effect of 28 days administration of caffeine (2mg and 6mg/kg) on carnitine palmitol transferase I activity in the rabbit cardiac tissue. Photomicrographs A - C are group representative photographs of CPT I activity in rabbit cardiac tissue examined by immunohistochemical staining (× 400 magnification). A represents control. B represent animals treated with caffeine (2mg/Kg) for 28 days .C represent animals treated with caffeine (6mg/Kg) for 28 days. D is the graphical representation of the Effect of caffeine (2mg and 6mg/kg) on CPT I activity in the rabbit cardiac tissue.

(Leblanc et al., 1995) and mouse (Nagasawa et al., 2001). There are conflicting reports on the effect of caffeine administration on plasma lipid levels in rodents. Several studies in rodents have reported an increase (Yokogoshi et al., 1983, Kempf et al., 2010), or a decrease (Muroyama et al., 2003) plasma lipids levels following caffeine administration. However, the present study observed increases in both total cholesterol and triglycerides levels. The reason for the inconsistency observed in the effect of caffeine on plasma lipid levels is obscure. However, most of the previous studies worked with caffeine doses that are not relevant to human consumption. For example, 0.1% of caffeine in drinking water which is commonly reported in most rodent studies is about 100mg/kg which is totally off the range of caffeine consumption in humans (Milanez 2011). The observed increase in the heart carnitine palmitoltransferase-1 (CPT 1) activity and the high levels of cardiac glycogen in this study following caffeine administration suggest that the major substrate being used for energy metabolism in the heart are fatty acids. While caffeine tends to promote the use of fatty acids in the cardiac tissue it stores up glucose which is evident in the observed increase in cardiac tissue glycogen in this study. The glycogen sparing effect of caffeine has earlier been reported in the skeletal muscle (Graham et al., 2001). CPT 1 is a mitochondrial enzyme that transports long-chain fatty acyl carnitine across the outer mitochondrial membrane. The carnitine palmitoyltransferase system is an essential step in the beta-oxidation of long chain fatty acids. This transfer system is necessary because, while fatty acids are activated (in the form of a thioester linkage to coenzyme A) on the outer mitochondrial membrane, the activated fatty acids must be oxidized within the mitochondrial matrix. Long chain fatty acids such as palmitoyl-CoA, unlike short- and medium-chain fatty acids, cannot freely diffuse through the mitochondrial inner membrane, and require a shuttle system to be transported to the mitochondrial matrix (Berg et al., 2007). Carnitine palmitoyltransferase I is the first

component and rate-limiting step of the carnitine palmitoyltransferase system, catalyzing the transfer of the acyl group from coenzyme A to carnitine to form palmitoylcarnitine. A translocase then shuttles the acyl carnitine across the inner mitochondrial membrane where it is converted back into palmitoyl-CoA. A sentinel paper in 1977 described the inhibition of the oxidation of fatty acids by isolated mitochondria by malonyl-CoA in vitro and its correlation with the inhibition of the ‘‘outer’’ CPT activity that could be measured in intact, well-coupled mitochondria (McGarry et al., 1977; McGarry et al., 1978). This explained the ability of some tissues to perform high rates of fatty acid synthesis or oxidation under separate physiological conditions. Malonyl-CoA, an intermediate of fatty acid synthesis, through inhibition of the ‘‘rate limiting’’ step of fatty acid oxidation, ensures that the two processes would not occur simultaneously. The inhibition of CPT 1 by malonyl-CoA is crucial because it is the point at which metabolism of fatty acids and glucose come into the most direct ‘‘contact’’ to influence each other’s metabolism. Other interactions, such as the inhibition of phosphofructokinase by citrate, the provision of glucose-derived glycerol for acylglyceride synthesis, the regulation of pyruvate dehydrogenase activity by fatty acid-derived acetyl-CoA, do not have the same ability to affect the continuous substrate selection that tissues have to make primarily between glucose and fatty acid oxidation. The increase in CPT-1 activity due to caffeine administration in this study therefore corroborates the fact that caffeine spares the use of glucose in active muscle tissues thereby building up tissue glycogen. Thus, the increase in cardiac tissue glycogen due to caffeine administration in the present study shows that caffeine affects the cardiac choice of energy substrate and increases the ability of the heart to use fatty acids as energy substrate. Furthermore, the significant increase in circulating blood glucose and lipids following caffeine administrations in the present study suggest that the mechanism by which caffeine influences the cardiac choice of energy substrate is more dependent on metabolic effectors than on various substrate availability.

The observed increase in cardiac tissue cAMP following caffeine administration in the present study suggests the possibility of blockade of adenosine A1 receptors by caffeine and the role of cAMP in the signaling pathway leading to the chronic action of caffeine in the heart tissue. Stiles (1986) had earlier reported on the rise of tissue cAMP following adenosine A1 receptor blockade in animals chronically administered caffeine.

The reduction of MPO activity by caffeine in this study portends that caffeine could be cardioprotective. Since increase in MPO activity has earlier been reported to precipitate atherogenesis (Schindhelm et al; 2009). Possible mechanisms by which caffeine could reduce MPO activity may be due to its ability to decrease activation of resident macrophages and microglia (Lv et al. 2010; Chavez-Valdez et al. 2009).

Serum nitrite has been reported to be an indicator for NO production *in vivo* (Chaea et al.2004). Hence the increase in serum nitrite in this study indicates an increase in NO production due to caffeine administration. Increase in NO production promotes tissue perfusion which could be a contributory mechanism of caffeine’s endurance enhancing ability (Christopherson and Bredt, 1997).

In conclusion, this study has demonstrated in the rabbit that caffeine consumption within dose ranges often consumed by man increased CPT1 activity suggesting increased utilization of free fatty acids in energy metabolism and sparing of cardiac tissue glycogen by mechanism(s) which probably involve blockade of A1 adenosine receptors and cAMP signaling pathway.

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Ahaptoglobinaemia in a Nigerian Cohort

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Summary: Ahaptoglobinaemia have been indicated in blacks from West Africa. Owing to the clinical and biologic importance of haptoglobin (hpt), this work explores the situation in a Nigerian cohort since there are no published values of haptoglobin levels of individuals in this locality. The study was aimed at determining the amount of haptoglobin in the blood of normal healthy Nigerians. Haptoglobin was quantitatively estimated in one hundred and fifty-two apparently healthy individuals using highly sensitive immunoassay technology. Blood grouping and haemoglobin genotype were assayed for all subjects to know if they influence haptoglobin levels. The association between haptoglobin and blood group was also established. Serum levels of haptoglobin among all subjects analyzed revealed a marked decrease in their haptoglobin levels when compared to other reference intervals. A further association between haptoglobin and gender did not reveal a statistical significant relationship ($p>0.05$). However, there was a significant different when haptoglobin levels of different blood groups and haemoglobin genotype when compared. Our data suggest that serum levels of haptoglobin are significantly lower in healthy Nigerians. The lower limit was remarkably lower than the internationally acceptable Caucasian reference range suggesting a clear necessity for establishing reference African values.

Keywords*:* Haptoglobin, Nigeria, Ahaptoglobinaemia, reference interval

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INTRODUCTION

Haptoglobin (Hpt) is an α2 glycoprotein that irreversibly binds free haemoglobin released from lysed red cells. The free haemoglobin forms a haptoglobin – haemoglobin complex which is removed by the reticulo-endothelial system (Lim *et al.*, 2008). This protein is characterized by a genetic polymorphism of two autosomal co-dominant genes Hpt1 and Hpt2 which results in three phenotypic variants; Hp1-1, Hp 2-1 and Hp 2-2 (Smithes, 1955, Smithes *et al.*, 1955). Hpt is also an acute phase protein synthesized primarily by the liver cells as a response to the production of IL6 and TNF. Apart from its function as a laboratory marker for the diagnosis of haemolytic anaemia, it serves other biologic functions which include; modulation of immune response, formation of new blood vessels in conditions such as wound healing, systemic vasculitis and tumor growth (Langlois et al 1996). It has antioxidant ability and protects against toxic radicals. Free iron and haemoglobin can cause the oxidation of low density lipoprotein by the generation of hydroxyl or superoxide radicals (Grinshtin *et al.*, 2003). Haptoglobin can reduce the damage caused by free radicals to vascular endothelial cells. Furthermore, free haemoglobin released from intravascular haemolysis can cause damage to the renal tubules if haemoglobin is not cleared by hepatocytes after forming a complex with haptoglobin (Sandrzadeh *et al.*, 1999). Haptoglobin is also involved in angiogenesis, bioavailability of nitric oxide and endothelial relaxation and may be involved in the suppression of lymphocyte function (Langlois *et al.*, 1996).

However, functional and structural differences that exist between the different phenotypes may have marked implications in biologic, clinical variations and disease outcomes (Braeckman et al 1999, Woben et al 2008). Evidence has linked frequency of these genes to geographic location (Carter et al 2007, Kasvosve et al 2000). Furthermore, recent studies have associated haptoglobin gene polymorphism with the initiation and development of some disease processes and pathways (Fowkes et al., 2006; Atkinson et al., 2007). Disorders such as atherosclerosis, chronic inflammatory disease and infections have been implicated (Mohieldein et al., 2012; Quaye, 2008). Recent studies have analyzed the effect of haptoglobin polymorphism on serum cholesterol and albumin concentration, high density lipoprotein triglycerides and serum ceruloplasmin. Although the precise reason

is arguable, Hpt 2-2 was found to have more angiogenic property than other phenotypes (De Kleijn, 2002). Furthermore, other works have linked Hpt1 with more protective ability against oxidation and peroxidation (Dobryszycka, 1997). Since increased levels of free haemoglobin will lead to markedly reduced haptoglobin serum levels, finding the reference range of a particular population becomes vital. More especially when studies are trying to find reasons for their increase in some diseases. Some studies have demonstrated that haptoglobin influenced conditions like cancer, some infectious diseases, epileptic seizures, atherosclerosis and diabetes (Delanghe, 1995). Other reports have emphasized the relationship between haptoglobin phenotypes and increased susceptibility to disease development (Levy et al., 2002).

Increasingly, haptoglobin polymorphism is linked to modifications in disease presentation and associated with other systems example blood group and haemoglobin types (Dobryszycka, 1997). Furthermore, a recognized phenotype Hp O characterized by absence or reduced production of haptoglobin gene results primarily in decreased levels of serum concentration called ahaptoglobinaemia (Kirk et al., 1970; Constans, 1981). It is believed that blacks have different levels of haptoglobin when compared to Caucasians (Teye et al., 2003; Mastana et al., 1994). Some studies compared the frequency of haptoglobin gene distribution in various countries (Shim et al., 1964; Allison et al., 1958). More than 30% of blacks in West Africa were reported with increased frequency of ahaptoglobinaemia (Park et al., 2004; Trape et al., 1988). Owing to the importance of haptoglobin in medicine and the paucity of documented scientific information in the case of Nigeria, this study attempts to determine the distribution of haptoglobin levels in serum of normal individuals in Nigeria.

MATERIALS AND METHODS

One hundred and fifty-two apparently healthy Nigerians in Enugu State were enrolled in the cross sectional study between March and August 2016. The subjects were of both sexes and were aged between 25 to 45years. Following approval by the ethics committee and review board of the University of Nigeria Teaching Hospital Ituku- Ozalla, informed consent was obtained from each person using approved protocol. All subjects and protocol were handled according to guidelines as outlined by Helsinki declaration. Exclusion criteria included those with infections; viral (human immunodeficiency virus, hepatitis B surface antigen, hepatitis C virus), bacterial, parasitic infestation, pregnancy, lactating women and history of illness for two to one month. Blood samples were collected from all inclusive subjects at enrollment for laboratory assays: haptoglobin, blood group and haemoglobin typing according to procedure as described by Dacie and Lewis (Dacie et al., 2006). Blood group and haemoglobin types were determined by serologic analysis and haemoglobin electrophoresis.

Collection of blood

Seven mililitres of blood was drawn from each participant and four mililitres was transferred into EDTA for the assessment of blood group and haemoglobin typing. Four milliliters was dispensed into a sterile plain tube, allowed to clot and serum expressed for the estimation of haptoglobin levels.

Measurement of haptoglobin levels

Serum levels of haptoglobin were determined in duplicates using commercially available sandwich Enzyme Linked Immunosorbent Assay (ELISA)kits by My Biosource San Diego CA. A high sensitive well calibrated ELISA technique was used due to availability. ELISA is a technique that may be used in lieu of immunoturbidometry and is commonly available for assays in this part of the world-resource poor country. A high sensitive and specific assay kit with no known cross reactivity or interference between other analogues and Hpt was used. The assay employed microtiter plates coated with polyclonal antibody as capture antibody and biotin conjugated antibody as detecting antibody. The detection range was between 0.312-4000ng/ml while the minimum detectable dose was <0.108ng/ml. the coefficient of variation for between run studies and within run was less 10%.

Methods for blood grouping and haemoglobin genotype

Whole blood was used for the determination of blood groups and haemoglobin type using antigen and antibody agglutination technique. High reactive anti-sera by Lorne laboratories UK was employed for ABO and Rhesus typing using standard tube technique while haemoglobin electrophoresis was used for haemoglobin typing according to techniques by Dacie and Lewis.

Statistical Analysis

Data was analyzed using statistical package for social science -SPSS {version 20} software. Shapiro- Wilk test was used to test for normality to ascertain population distribution. Frequency histogram is shown for population distribution. Statistical significance was calculated using non parametric technique Mann Whitney for sex comparison, Kruskal-Wallis test for blood group comparism. T -test was employed in the comparison with Caucasian value. Relationship between sex/blood group/genotype and haptoglobin was analyzed using Spearman correlation. The haptoglobin levels were measured in µg/ml and the result established with the values at median (50^{th}

percentile), 2.5th and 97.5th percentile. 2.5th and 97.5th percentile values from the population were recorded as the upper and lower reference limit.

RESULTS

The levels of haptoglobin in normal individuals in Nigeria, a country in West Africa are presented in Table 1. The mean haptoglobin level for 152 apparently healthy individuals was 61.59 ± 17.0 µg/ml. The reference interval for haptoglobin level was determined to be 25.90 - 92.29 µg/ml for 2.5 and 97.5 percentiles; median 61.65 µg/ml. The mean haptoglobin level of males (58%) was 63.21(95% Cl= 26.35-94.36 µg/ml) while that for females (42%) was 59.72 (95% Cl= 25.53-91.12 µg/ml).

Table 1 Serum concentration of haptoglobin (µg/ml) in normal adult subjects

	Haptoglobin (µg/ml)	Minimum	Maximum	Median
Total	61.59±1.38	25.04	95.14	61.65
Males (90)	62.3±17.2	25.9	95	69.2
Females (62)	59.6±15.8	25	87	62.9

Table 2 Serum levels of haptoglobin (µg/ml) according to blood group

BLOOD GROUP	Haptoglobin (µg/ml)
A+	70. 39±4.23
B+	57.29±9.09
O+	58.29±1.85
AB+	82.29±4.75

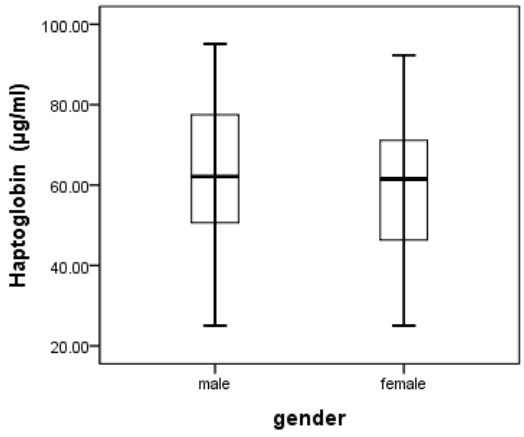

Figure 1 box plot showing relationship between gender and haptoglobin level

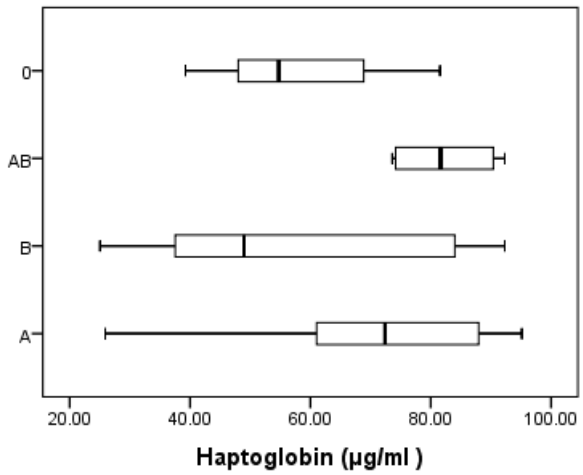

Figure 2 box plot showing relationship between haptoglobin and blood group

The Mann Whitney U test revealed no notable statistical significant difference in haptoglobin levels between sex (p= 0.760). Table 2 shows the haptoglobin levels charted according to blood group of all participants. A Kruskal Wallis technique was used to evaluate the differences between haptoglobin and different blood types(95%Cl). There was a statistically significant difference between blood group and haptoglobin level (X^2 = 12.25, P = 0.007) and between haemoglobin type and haptoglobin (X^2 = 23.60, P = 0.014). Blood group AB (74.6µg/ml) had the highest levels while B blood group had the lowest levels. Hb AA had the highest hpt level and HbSS had the lowest. When the relationship was sought between haptoglobin and sex (r=-0. 081, p>0. 05), there was found to be none.

DISCUSSION

Haptoglobin is an acid phase protein that does not have only an immune-modulatory function but is a potent antioxidant and a known indicator of haemolysis (Langlois et al., 1996). Some studies have attributed low levels of serum haptoglobin to various populations and pointedly to Africa (Trape et al., 1988). These works have implied that the functional differences between various genetic phenotypes lead to varied influence on human pathology. Other studies suggested that particular haptoglobin phenotype are prevalent in blacks (Constans, 1981). Confounding factors like hemolysis, time of collection of blood samples and duplicate analysis were taken into consideration during the design of this work. This study was done to establish the serum levels of haptoglobin from a Nigerian population; a country in tropical West Africa. We found that serum levels of haptoglobin in the study population were lower than that reported for Caucasians (Allison et al., 1958).

Both the lower limit and the upper limit were significantly decreased when compared to known international reference limit which is reported to be 300 - 2150 µg/ml (Kasvosve et al., 2000). The normal range found in this study was between 25 to 92µg/ml. This means that the levels of serum haptoglobin were quite markedly reduced in the study population. Decreased levels of haptoglobin in blood, hypoglobinaemia and ahaptoglobinaemia may be acquired as a result of association with malaria endemicity or may be congenital due to a deletion in the promoter region of haptoglobin gene (Boreham et al., 1979; Shinton et al., 1995). It has been reported by various works that malaria endemicity may directly correlate with the serum levels of haptoglobin and therefore may be linked to ahaptoglobinaemia (Imrie et al., 2006). It is probable that increased clearance of the haptoglobin- haemoglobin complex during malaria parasitaemia may have resulted in the observed levels. Nigeria is a known malaria endemic country. Also, hpt levels have been reportedly influenced by their phenotypes (Atkinson et al., 2007; Hunt et al., 2001). Researches from other works have linked hpt polymorphism and phenotypic variations to the differences in hpt blood levels and invariably to susceptibility to disease outcomes. We intend to see the implication in the Nigerian situation subsequently in further research.

The reduced serum levels of haptoglobin found in this study agrees with some results from other African countries: Congo, Zaire, East Africa, Gambia and South Africa (Allison et al., 1960; Boreham et al., 1979). Apart from genetic factor which have been attributed to ahaptoglobinaemia in some populations, other authors have linked ethnicity and close geographical proximity as the key factor that may influence levels of hpt (Barnicot et al., 1959). Others have argued that it may be purely genetics (Smithes et al., 1955; Woben et al., 2008).

Furthermore, we did not find any significant variations between the haptoglobin level and sex. However, we found significance difference between haptoglobin and blood group. Blood group AB appears to have the highest level of hpt while B blood group has the lowest. The low levels of hpt found in Hb SS may be as a result of haptoglobin – haemoglobin complex formed as a result of free haemoglobin released during haemolysis.

In summary, these results suggest that there was a considerable difference in the reference range of haptoglobin in the study population when compared to the levels established for Caucasians. Already, haptoglobin is associated with the evolution and modification of disease presentation and pathology (Calderon et al., 2006; Nevo et al., 1986). Therefore, knowing the reference interval in a community becomes indispensable and of paramount importance.

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Haemostatic Indices as Markers for Monitoring Pulmonary Tuberculosis Treatment

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Summary: Tuberculosis (TB) is an infectious disease inducing a state of chronic inflammation which could affect the haemostatic mechanism as part of host defences against infection. Proper diagnosis and monitoring of tuberculosis patients undergoing therapy is still a challenge especially in a poor resource country such as Nigeria. This study aims to assess some haemostatic indices of tuberculosis patients and their possible use as markers in monitoring response to anti-tuberculosis treatment. One hundred and twenty TB patients aged 15-60 years and 120 apparently healthy (control) subjects age and gender-matched were studied. Demographic/bio data was compiled by interview and from patients' case notes. Diagnosis of TB was by sputum smear microscopy, radiography and clinical assessment. Platelet count (PLT), platelet factor 4 (PF4), prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin clotting time (TCT) and fibrinogen (FIB) were determined using standard techniques. The platelet factor 4, prothrombin time, activated partial thromboplastin time and fibrinogen levels of TB patients were significantly higher while the thrombin clotting time was significantly lower ($P<0.05$) when compared with healthy subjects. While PF4, TCT and FIB improved significantly ($P<0.05$) as anti-tuberculosis therapy progressed, PLT, PT and APTT remained the same. It is concluded that abnormal activation of haemostasis occurs in TB condition thus pre-disposing TB patients to bleeding complications**.** Furthermore, platelet factor 4, thrombin clotting time and fibrinogen improved as therapy progressed and therefore may be used as markers for monitoring response to anti-tuberculosis therapy.

Keywords*:* Tuberculosis, Haemostasis, Inflammation, Anti-tuberculosis therapy, Infection

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INTRODUCTION

Haemostasis is an efficient system in humans with the ability to stop bleeding from sites of blood vessel injury through a series of enzymatic reactions. It describes a fragile balance between procoagulant as well as anticoagulant mechanisms involving an intricate series of events (Hoffbrand *et al*., 2011). When there is tissue injury due to infection, the body reacts in a process known as inflammation. This defense reaction attempts to remove or at least limit the spread of the offending agent, and in addition clear necrosed cells and tissues from the affected area (Mohan, 2010). Tuberculosis (TB) as a disease is a state of chronic granulomatous inflammation arising from infection by a family of organisms collectively called the *Mycobacterium tuberculosis* complex (Iseman, 2000). It has been reported that inflammation results in activation of the haemostatic system, with the latter also affecting the activity of the former (Verhamme and Hoylaerts, 2009). Although haemostatic function is geared towards preserving the integrity of the circulatory system, it can become an imbalanced process with morbidity and mortality as possible sequel. Haemostatic activity that is poorly controlled as a consequence of inflammation may be a significant contributor to the development and course of disease as well as its progression. This is clearly expressed in systemic inflammatory response to infection as may be the case in tuberculosis (Margetic, 2012). Tuberculosis (TB) is still a significant public health problem in Nigeria with a prevalence of 590,000 and 170,000 deaths recorded in 2014 (Federal Ministry of Health Nigeria, 2008; World Health Organisation, 2012; WHO, 2015). The high death rate due to TB is unacceptable since diagnosis in good time and correct treatment can ensure that virtually all TB patients are cured of the disease. Proper diagnosis and monitoring of tuberculosis patients is still a challenge especially in a poor resource country such as Nigeria. This has led to continuous spread, non-compliance and development of multi-drug resistant TB. This study therefore aims to assess some haemostatic parameters

of tuberculosis patients and their possible use in monitoring response to anti-tuberculosis therapy.

MATERIALS AND METHODS

Study design/ethical considerations: The study area for this research work is Calabar, located in the southern part of Cross River State in Nigeria's south-south geo-political zone. Case-control experimental study design was used involving TB patients and non-patients of similar age. Approval was obtained from the Health Research Ethics Committee, Ministry of Health, Cross River State. Informed consent was sought and obtained from all participants.

Subject selection: Subjects comprised of 120 male and female tuberculosis patients within the age range of 15-60 years including newly diagnosed patients and those on anti-tuberculosis therapy, attending tuberculosis treatment centres at Dr Lawrence Henshaw Memorial Hospital, General Hospital, Ekpo-Abasi and Adazi health centres, Calabar. Diagnosis of TB was by sputum smear microscopy, radiology or clinical assessment. One hundred and twenty apparently healthy subjects, age and gender matched with a negative tuberculin skin test (mantoux) in the preceding six months and no history of tuberculosis, selected from residents of Calabar metropolis served as controls. Tuberculosis patients with other disease conditions and subjects who objected to participation in the study were excluded.

Demographic data/sample collection: Demographic data were compiled by interview while treatment duration was obtained from patients' case notes. Two sputum samples were collected on consecutive days for the diagnosis of pulmonary TB. Six milliliters of venous blood was collected asceptically; 2ml was dispensed into ethylene diamine tetra acetic acid (EDTA) to a final concentration of 2mg/ml and used for platelet count within 4 hours of collection; 2ml was dispensed into 0.2ml of 3.13% trisodium citrate, centrifuged at 3000rpm for 10 minutes to obtain platelet poor plasma for the determination of prothrombin time, activated partial thromboplastin time, thrombin clotting time and fibrinogen; the remaining 2ml was dispensed into a plain container, allowed to clot to obtain serum which was stored frozen until used for determination of platelet factor 4.

Sample analysis: Sputum samples were mixed and smeared thinly on a microscope slide, the smear was heat fixed and stained using the Ziehl-Neelsen technique (Brooks *et al*., 2013). The slides were examined microscopically using x 100 objective for the presence of acid fast bacilli which appear as red rods (bacilli) on a green background. The bacilli load was determined using the WHO/International Union against tuberculosis and lung disease (IUATLD) Grading Guidelines (1998). Platelet count (PLT) was determined using an automatic cell counter. Prothrombin time (PT) and activated partial thromboplastin time (APTT) were by the Quick's one stage technique (Dacie and Lewis, 2010) using kit purchased from Giesse Diagnostics, Italy. Thrombin clotting time (TCT) and fibrinogen concentration (FIB) were measured by Clauss technique (Clauss, 1999) with kit purchased from Giesse Diagnostics, Italy. Determination of PT, APTT, TCT and FIB was by addition of respective reagents to platelet poor plasma in glass tubes at 37^{o}C in a water bath and recording the time of clot formation using a stop watch. Platelet factor 4 (PF4) was measured based on sandwich enzyme immunoassay (ELISA) using kit purchased from Cloud-Clone Corporation, USA. The manufacturers' instructions for storage and assay were followed strictly.

Statistical analysis: Data obtained from this study are presented as chart and in tables as mean ± standard deviation. Chi-square analysis, one way analysis of variance (ANOVA) and Games-Howell post hoc test and Student's T-test were used to test hypotheses on statistical package for social sciences (SPSS) version 20 software. A P-value ≤ 0.05 was considered to be statistically significant.

RESULTS

Haemostatic and inflammatory parameters of one hundred and twenty tuberculosis (TB) patients and one hundred and twenty apparently healthy subjects (control) were assessed in this research. In table 1, the demographic parameters of TB patients were compared with their controls. Significant differences were observed between the test group (TB patients) and their controls with respect to educational and occupational status ($P<0.001$). No significant difference ($P>0.05$) was found between TB patients and their controls with respect to age, gender and marital status. Some haemostatic parameters of tuberculosis patients and control subjects are presented in table 2. The platelet factor 4, prothrombin time, activated partial thromboplastin time and fibrinogen concentration were significantly higher ($P<0.001$) while the thrombin clotting time was significantly lower ($P<0.001$) than values obtained for control subjects. However, the mean platelet count of TB patients was not different from control.

Figure 1 shows three groups of TB patients based on treatment duration. The first group (23%) consists of newly diagnosed TB patients who were yet to commence treatment (untreated). The second group called the intensive phase (42%), refers to day one of treatment up to two months. The third group called the

TABLE 1 Demographic parameters of tuberculosis patients and control subjects

Parameters	TB Patients (N=120)	Control (N=120)	P-VALUE
Age (Years)	33.4±11.2	29.7±7.2	0.074
Gender			
Males	75 (62.5%)	38 (63.3%)	0.913
Females	45 (37.5%)	22 (36.7%)	
Marital Status			
Single	66 (55.0%)	27 (45.0%)	
Married	47 (39.2%)	32 (53.3%)	0.212
Divorced	3 (2.5%)	0 (0.0%)	
Widowed	4 (3.3%)	1 (1.7%)	
Educational Level			
Nil	14 (11.7%)	0 (0.0%)	
Primary	21 (17.5%)	2 (3.3%)	0.001
Secondary	59 (49.2%)	10 (16.7%)	
Tertiary	26 (21.6%)	48 (80.0%)	
Occupation			
Business/Trading	33 (27.5%)	5 (8.3%)	
Students	23 (19.2%)	8 (13.4%)	
Civil/Public Servant	11 (9.2%)	45 (75.0%)	0.001
Others	39 (32.5%)	2 (3.3%)	
Unemployed	14 (11.6%)	0 (0.0%)	

TABLE 2. Haemostatic indices of tuberculosis patients and control subjects

Parameters	TB PATIENTS (N=120)	CONTROL (N=120)	P-VALUE
Platelet Count ($X10^9$/L)	172.67±59.72	176.85±56.22	0.646
Platelet Factor 4 (pg/ml)	43.87±32.58	28.58±27.67	0.001
Prothrombin Time (s)	20.03±3.77	13.67±1.17	0.001
Activated Partial Thromboplastin Time (s)	46.07±8.81	34.63±4.49	0.001
Thrombin Clotting Time (s)	12.91±5.09	24.13±7.53	0.001
Fibrinogen (mg/dl)	349.96±137.75	150.42±73.18	0.001

Table 3. Haemostatic indices of tuberculosis patients based on duration of anti-tuberculosis therapy

PARAMETERS	New Patients (Untreated) (n=28)	Intensive Phase ≤ 2 Months (n=50)	Continuation Phase > 2-6 Months (n=42)	Significance Level
Platelet Count ($X10^9$/L)	178.75±56.83	178.08±57.37	162.17±64.16	NS
Platelet Factor 4 (pg/ml)	53.84±37.64	46.57±30.94	34.02±28.78	*b
Prothrombin Time (s)	19.75±3.56	19.76±3.91	20.55±3.77	NS
Activated Partial Thromboplastin Time (s)	46.71±7.65	46.00±9.44	45.74±8.93	NS
Thrombin Clotting Time (s)	9.11±1.52	10.78±1.82	17.98±5.30	***a,b,c
Fibrinogen (mg/dl)	484.39±92.81	390.28±90.99	212.33±76.03	***a,b,c

NS= No Significant difference; *= P<0.05; ***P<0.001, a= Difference between new and intensive phases, b= Difference between new and continuation phases c= Difference between intensive and continuation phases

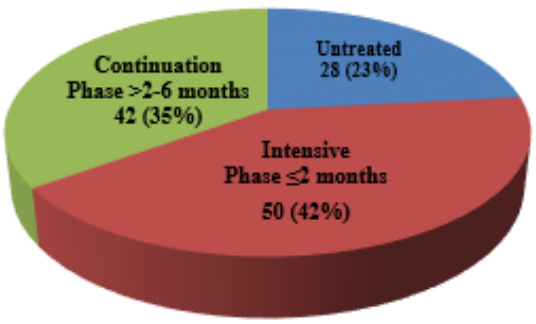

FIG. 1: Grouping of tuberculosis patients based on duration of anti-TB therapy

continuation phase (35%) refers to treatment period greater than two months up to the end of treatment at six months. The effect of duration of anti-tuberculosis therapy on some haematological parameters is presented in table 3. The platelet factor 4 level of the continuation group was significantly lower (P<0.05) than that of the untreated group. While the fibrinogen concentration decreased significantly (P<0.001) the thrombin clotting time increased significantly (P<0.001) from the value obtained for new patients to intensive and continuation phases as therapy progressed. However, no statistical difference (P>0.05) was observed for platelet count, prothrombin time and activated partial thromboplastin time among the three groups.

DISCUSSION

In this study, the TB patients were observed to belong to a lower socio-economic class with respect to educational level and occupation with 11.6% of them being unemployed (table 1). Poverty and poor nutrition are factors which predispose individuals to TB infection if exposed as supported by previous report (Cegielski and McMurray, 2004). The present finding also correlates with the WHO report that most TB cases occur in the economically active age group (15-50 years) with an adult losing about 3-4 months of work due to the disease (WHO, 2012). The platelet factor 4 was significantly higher for TB patients when compared to control (table 2). The increase observed could be attributed to abnormal platelet activation induced by the state of chronic inflammation. In normal physiology, platelets are inactive; protected by inhibitory factors like nitric oxide (NO) and prostacyclin (PGI_2) present in intact endothelium. However, in a state of chronic inflammatory response such as in TB, there is dysfunction of the endothelium resulting in raised synthesis of thromboxane A2 and von Willebrand factor with reduced levels of PGI_2 leading to platelet activation and increased reactivity. Following activation, platelets release bioactive peptides from their alpha and dense granules which include heparin neutralizing factor also called platelet factor 4 thus resulting in the elevation seen in TB patients. Previous reports support this finding (Gawaz *et al*., 2005; Lowenberg *et al*., 2010; Nurden, 2011; Speth *et al.*, 2013). The prothrombin and activated partial thromboplastin times of TB patients were significantly higher than values for control subjects. In addition, the PT and APTT of the TB patients were prolonged beyond the reference ranges of 12-16 seconds and 26-36 seconds respectively suggesting that the clotting factors assessed are depleted. When a state of chronic inflammation is developed; an integral part of the host defense geared towards eradication of the offending pathogen involves systemic activation of the haemostatic system as suggested by earlier reports (Schouten *et al*., 2008; Hoffbrand *et al*., 2011). However, an exaggerated systemic activation of the haemostatic system can result in disseminated intravascular coagulation (DIC) with consumption of clotting factors as a consequence. This explains the prolonged PT and APTT observed for TB patients and implies that both extrinsic and intrinsic pathways of blood coagulation are affected. The inability of blood to clot may pre-dispose these TB patients to bleeding problems in the event of injury. The thrombin clotting time of TB patients was lower than the control values. This is probably due to the fact that thrombin has been used up as a consequence of excessive activation of coagulation induced by the TB infection and inflammation. In inflammatory condition, thrombin suppresses clotting by activating protein C, a native anticoagulant that splits factors V and VIII, thus contrasting the procoagulant activities of thrombin; this is achieved via the binding of thrombin to thrombomodulin. The loss of procoagulant properties by thrombin further explains the reduction in thrombin levels as well as the prolonged PT and APTT observed for tuberculosis patients. The fibrinogen concentration of the TB patients was significantly higher ($p<0.05$) than values for control; this is in line with hyperfibrinogenaemia (increase in fibrinogen levels above the reference value) previously reported in tuberculosis disease (Awodu *et al*., 2007; Akpan *et al*., 2012). The increase in fibrinogen levels observed could be attributed to several factors. First, the interaction between *Mycobacteria* and the monocyte-macrophage system of the host results in the synthesis of large quantities of pro-inflammatory cytokines which in turn bring about hepatic acute-phase reactions that change coagulation proteins' levels; one of which is fibrinogen. Fibrinogen has indeed been known to be a key player in the regulation of immune response and inflammation in infection. Its role involves the increased formation of fibrin clots which serves to trap the offending pathogen (Degen *et al*., 2007; Esmon *et al*., 2011; Davalos, 2012). Again, since thrombin is responsible for clotting fibrinogen, a reduction in thrombin levels will lead to accumulation of fibrinogen as observed in this study.

Platelet factor 4 levels of TB patients decreased significantly while the thrombin clotting time increased significantly as therapy progressed (table 3). The decrease in platelet factor 4 as well as the increase in thrombin clotting time implies that the inflammation-induced activation of haemostasis is resolved with proper treatment. Thus, the platelet factor 4 and thrombin clotting time could be used to monitor response to anti-tuberculosis treatment. Conversely, the prothrombin time and activated partial thromboplastin time were similar for untreated TB patients as well as their counterparts who were on treatment. Although treatment of the underlying infection with appropriate antibiotics is expected to improve haemostatic interactions, it has been observed that abnormalities of the haemostatic system may proceed in some cases (Dellinger *et al*., 2008). This observation correlates with the findings in this study. A possible explanation is that the normal coagulation control systems are impaired by the infection-induced inflammation in tuberculosis. Previous report suggests that the protein C system is significantly affected through decreased synthesis, increased consumption and degradation as well as down-regulation of thrombomodulin and endothelial protein C receptor at the endothelial surface resulting in disturbed regulation of the haemostatic system (Marti-Carvajal *et al*., 2007). This may be responsible for the non-resolution of the prolonged PT and APTT as therapy progressed. It is also possible that it will take some

time for the clotting factors which have been depleted by excessive activation of coagulation, to be restored to normal levels in the plasma thus implying that TB patients may be prone to bleeding complications even after completion of treatment. Fibrinogen concentration of TB patients decreased significantly from the untreated through the intensive to the continuation phases of treatment (table 3). It is suggested that the anti-TB drugs inhibit the binding of thrombin to thrombomodulin thereby resulting in the availability of thrombin to bind fibrinogen. The acute phase response is also suppressed by the clearance of the offending pathogen hence fibrinogen which is an acute phase protein is no longer produced in large quantities by the liver leading to a decrease in its concentration with therapy. This indicates that fibrinogen concentration could be a useful marker in the monitoring of response to therapy in TB management.

In conclusion, this study has revealed abnormal activation of haemostasis in tuberculosis infection which may pre-dispose them to bleeding even after the completion of treatment. However, since platelet factor 4, thrombin clotting time and fibrinogen improved with therapy, these indices could be used as markers to monitor response to treatment in TB management.

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Metabolic Fate of the Glucose Taken up by the Intestine During Induced Hyperglycaemia in Dogs

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Summary: Available data showed that the intestine increases it glucose uptake in response to hyperglycemia induced by any cause. However, what the intestine does with the glucose is not known. This study investigated the metabolic fate of the glucose taken up by the intestine during hyperglycaemia in dogs. Experiments were carried out on fasted, male, anaesthetized mongrel dogs divided into 4 groups. The control (group 1, n=5) received normal saline (0.2 ml/kg) while groups 2-4 (subdivided into two as low or high dose, n=5 each) received adrenaline (1 µg/kg or 5 µg/kg), glucagon (3 ng/kg or 8 ng/kg) and glucose (10 mg/kg/min or 20 mg/kg/min). Through a midline laparatomy, the upper jejunum was cannulated for Intestinal Blood Flow (IBF) measurement. Blood glucose and lactate levels were determined using glucose oxidase and lactate dehydrogenase methods, respectively. Intestinal Glucose/Lactate Uptake (IGU/ILU) was calculated as the product of IBF and arterio-venous glucose /lactate difference [(A-V) $_{glucose/lactate}$]. Jejunal tissue samples were obtained for the determination of Glycogen Content (GC) and activities of Glycogen Synthase (GS), Glycogen Phosphorylase 'a' (GPa), hexokinase and glucose-6-phosphatase. Anthrone method was used to determine GC while activities of GS, GPa, hexokinase and glucose-6-phosphatase were determined spectrophotometrically. Data were subjected to descriptive statistics and analyzed using student's t-test and ANOVA at $\alpha_{0.05}$. Arterial and venous blood glucose and lactate were increased by adrenaline, glucagon and glucose. Venous lactate was higher than arterial lactate in all groups. Intestinal blood flow, (A-V) glucose and (A-V) lactate were increased in all the experimental groups. Intestinal glucose uptake increased by 624% (adrenaline), 705% (glucagon) and 589% (glucose) while intestinal lactate release increased by 422%, 459% and 272% respectively. Intestinal GC increased from 138.72 ± 4.58 mg/100 g to 167.17 ± 4.20 mg/100 g (adrenaline), 229.21 ± 6.25 mg/100 g (glucagon) and 165.17 ± 4.20 mg/100 g (glucose). Adrenaline and glucose had no effect on GS activity but it was increased by glucagon; GPa was decreased while hexokinase activity was increased by adrenaline, glucagon, and glucose. Glucose-6-phosphatase activity was not affected by adrenaline and glucagon but decreased by glucose. The intestine modulates blood glucose levels through lactate formation, glycogen formation and most probably conversion of lactate to glucose through gluconeogenesis.

Keywords*:* Glucose uptake, Lactate uptake, Glycogen, Dogs.

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INTRODUCTION

Available evidence in the last three decades have shown conclusively that the gastrointestinal tract increases its glucose uptake during hyperglycaemia irrespective of the cause of the increase in blood glucose. Thus, the intestinal glucose uptake increased by 250 – 1000% following hyperglycaemia induced by adrenaline (Grayson and Oyebola, 1983, Alada and Oyebola, 1995, Alada et al; 2000, Oyebola et al; 2007), nicotine (Grayson and Oyebola, 1985, Oyebola et al; 2008), glucagon (Alada and Oyebola, 1995), glucose infusion (Alada and Oyebola, 1995, Salman and Alada, 2014) and diabetes mellitus (Alada et al; 2000) in dogs or rabbits.

When Grayson and Oyebola (1983, 1985) measured both glucose uptake and oxygen consumption in the upper jejunum of dogs in response to adrenaline or nicotine induced hyperglycaemia, they showed that the intestine has a capacity for huge glucose and oxygen consumption but the increase in glucose uptake was far more than the increase in oxygen consumption. Also, the increase in glucose uptake and oxygen consumption did not occur at corresponding times. It was therefore difficult to conclude that the huge amount of glucose taken up by the gut was involved in glucose metabolism. However, available evidence suggests that the glucose taken up by the gastrointestinal tract may not be utilised for oxidative metabolism. Indeed, Windmueller and Spaeth (1978) showed that isolated, vascularly perfused preparations of rat intestine took up from the circulation nearly as much glutamine as glucose, and glutamine accounted for more than 30% of the carbon dioxide produced.

These results and those of earlier workers suggest that the gastrointestinal tract is more involved in glucose homeostasis than in glucose metabolism. The nature of this involvement is however not clear. The

question therefore is, will the gastrointestinal tract take up glucose and convert it to glycogen or oxidise it to lactate/pyruvate as it is done in the liver? The present study was therefore designed to investigate the metabolic fate of the huge amount of glucose taken up by the intestine following concomitant measurements of the plasma lactate and tissue glycogen during hyperglycaemia induced by adrenaline, glucagon or glucose infusion.

MATERIALS AND METHODS

Experiments were carried out on anaesthetized (Sodium Pentobarbitone, 30 mg/kg) adult male mongrel dogs weighing 9 – 11 kg. Each dog was fasted for 18-24h before the start of the experiment. Light anaesthesia was maintained with supplementary doses of i.v. sodium pentobarbitone as necessary. The trachea was intubated using a Y-piece cannula and the dog was allowed to breathe room air (temp. 25ºC) spontaneously. Cannulae were placed in the left femoral artery and vein. The cannula in femoral artery was advanced to the level of the superior mesenteric artery. Through a midline laparotomy, the jejunum was identified and a vein draining the proximal segment of the jejunum was cannulated using a 1.8 mm (i.d) polyethylene tubing (P.E. 260). The jejunal vein cannula was moved into an extra-corporeal position and a non-crushing clamp was applied to its free end. Sodium heparin, 300 unit per kg was administered i.v to prevent intravascular blood clotting. The abdomen was then closed in two layers with interrupted sutures. Each dog was then allowed to stabilize for 60-90 minutes prior to commencement of experimental procedure.

Experimental procedure

After stabilization, basal measurements were done for jejunal blood flow, arterial and venous glucose and lactate levels. Jejunal blood flow was determined by timed collection as described by Alada and Oyebola (1996). Arterial samples for glucose and lactate estimation were collected from the cannula at the femoral artery while venous samplings were collected from the cannula at the jejunal vein. After the basal measurements, a bolus injection of adrenaline (1 µg/kg or 5 µg/kg), glucagon (3 ng/kg or 8 ng/kg) or 10 minutes infusion of glucose (10 mg/kg/min or 20 mg/kg/min) was given intravenously. Five dogs each were used for the low or high doses of adrenaline, glucagon or glucose. Glucose infusion was carried out using an infusion pump (Palmer, England). Measurement of jejunal blood flow and arterial and venous blood sampling for glucose and lactate were carried out at 0, 5, 10, 15, 20, 30, 45, 60, 75 and 90 minutes post- injection of adrenaline or glucagon or during infusion and post-infusion of glucose. Arterial and Venous glucose and lactate levels were determined using glucose oxidase and lactate dehydrogenase methods, respectively; arterio-venous difference (A-V) was determined for glucose [$(A\text{-}V)_{glucose}$] and lactate [$(A\text{-}V)_{lactate}$]. Intestinal Glucose Uptake (IGU) and lactate uptake were calculated as the product of intestinal blood flow and [$(A\text{-}V)_{glucose}$] or [$(A\text{-}V)_{lactate}$]. Jejunal tissue biopsy was done between 10-15 minutes post injection or post infusion for determination of jejunal glycogen content and homogenised in phosphate buffer saline (PBS, pH 7.4) for activities of glycogen synthase, glycogen phosphorylase, hexokinase and glucose-6-phosphatase enzymes. The protein content of tissue supernatant was determined to allow the expression of the enzyme activities per milligram protein.

Another group of 5 dogs was studied for the effect of normal saline (0.2 ml/kg) on intestinal blood flow, arterial and venous glucose and lactate, intestinal glycogen content and enzyme activities as in the other groups. This group served as the control

Determination of jejunal glycogen content

The glycogen content of the jejunum was determined using the method of Seifter *et al.* (1950) as modified by Jermyn (1975). Briefly, 1 g of jejunum was digested in 10ml of 30% KOH over heat. An aliquot of 4 ml of the digested tissue was washed by adding 5 ml of 95% ethanol, centrifuged for 5 minutes and drained. The precipitate was reconstituted with 0.5ml of distilled water and rewashed to obtain a white precipitate which was reconstituted with 2 ml of distilled water. The reconstituted precipitate or distilled water (0.5 ml) was pipetted into a test tube followed by stepwise addition of 0.5 ml concentrated HCl, 0.5 ml 88% formic acid and 4 ml of anthrone reagent (added slowly to minimize frothing). The solution was mixed thoroughly, incubated at 100^0C for 10 minutes and cooled. A standard curve was obtained by treating several double dilutions of 0.2 mg/ml of standard glycogen with HCl, formic acid and anthrone reagent as it was done for the test/blank. Absorbance of the blue colored solution formed was read at 630 nm against a reagent blank. Glycogen concentration (mg/ml) was obtained from the standard curve while glycogen content/100 g tissue was calculated as follows:

Glycogen content/100 g wet tissue

$$= glycogen\ concentration\ X\ \frac{10}{4}\ X\ \frac{2}{0.5}\ X\ \frac{100}{total\ tissue\ weight}$$

Determination of Liver glycogen synthase activity

Jejunal glycogen synthase activity was assayed using the spectrophotometric stop rate (Kinetic) method of Danforth (1965). Briefly, 100 µl of hepatic tissue supernatant was added to a reaction cocktail containing 0.5M Tris HCl Buffer (pH 8.2), $MgCl_2$, EDTA-tetrasodium, β-Mercaptoethanol, UDPG, glycogen and deionized water. It was mixed by

inversion and incubate for 5 minutes at 30° C. The reaction was stopped by heating both the test or blank for 5 minutes at 100^0C then cool over running tap water. The solutions were transferred into eppendorf tubes and centrifuged. Supernatant obtained (100 µl) was added into another reaction cocktail containing 0.2M Tris HCl Buffer (pH 7.5), KCl, $MgSO_4$, Phosphoenol pyruvate, EDTA-tetrasodium, β-NADH, deionized water and PK/LDH enzyme suspension. It was mixed immediately by inversion and the decrease in absorbance was recorded for five minutes. The final absorbance was obtained for both test and blank supernatant.

Glycogen synthase (Units/mg. protein)

$$= \frac{\Delta \text{ AbsorbanceTest} - \Delta \text{ Absorbance Blank } (2.91)}{(5)(6.22)(0.1)(protein\ concentration)}$$

Determination of Liver glycogen phosphorylase 'a' activity

Glycogen phosphorylase 'a' activity was assayed using the spectrophotometric stop rate (Kinetic) method of Fischer *et al*. (1962) and Bergmeyer *et al.* (1974). Briefly, in the absence of 5´-AMP, 100 µl of hepatic tissue supernatant was added to a reaction cocktail containing 0.5M Potassium Phosphate Buffer, Glycogen, $MgCl_2$, EDTA, phosphoglucomutase and NADPH, pH 6.8 at 30 ^{0}C. It was mixed by inversion and increase in absorbance was monitored at 340nm for approximately 10 minutes and the ΔA_{340}/minute was obtained for both the Test and Blank. The Phosphorylase a units/ml enzyme was calculated as:

$$\frac{\left(\Delta\frac{\text{Absorbance}}{\text{min}}\text{Test without 5´AMP} - \Delta\frac{\text{Absorbance}}{\text{min}}\text{Blank without 5´AMP}\right)(2.95)(df)}{(6.22)(0.1)}$$

Where df is the diluting factor

The Phosphorylase a units/mg protein

$$= \frac{\text{Phosphorylase a units/ml enzyme}}{protein\ concentration}$$

Determination of Hexokinase activity

Hexokinase activity in the jejunum was determined by the method described by Branstrup *et al.* (1957) wherein the rate of disappearance of glucose was determined at 38^0C in a buffer solution containing ATP, Magnessium, KCl and Fluoride. Briefly, 2 ml of Glucose buffer [0.0025 M glucose, 0.0025 M $MgCl_2$, 0.01 M K_2HP0_4, 0.077 M KCl, and 0.03 M Tris (Hydroxy-methyl) aminomethane, (Trizma base) pH 8] was pippetted into a test tube followed by 0.1 ml of 0.18 M ATP solution and 0.9 ml of distilled water. The mixture was preheated in water for 5 minutes at 38^0C, 1 ml of liver homogenate was added and 100µl of the homogenate-buffer substrate mixture was taken immediately for initial glucose analysis. The mixture was then incubated at 38^0C for 30 minutes and another 100 µl was taken for final glucose analysis. The difference in the level of glucose was calculated and hexokinase activity was expressed as glucose metabolised/mg. pr/30min. All assays were carried out in duplicates. In this assay, glucose was assayed using a commercially available Glucose GOD-PAP kit (Fortress Diagnostic®, United Kingdom).

Determination of Glucose-6-Phosphatase activity

Glucose-6-phosphatase activity was assayed according to the method of Koide and Oda (1959) based on the principle that the enzyme acts as phosphohydrolase and phosphotransferase. The reaction involves the formation of covalently bound enzyme-inorganic phosphate intermediate that can liberate inorganic phosphate in the presence of an acceptor. The liberated inorganic phosphate can then be quantified using a suitable method. Briefly, into a test tube, 0.3 ml of 0.1 M citrate buffer (pH 6.5), 0.5 ml of 150 mM glucose-6-phosphate solution and 0.2 ml of sucrose buffer extracted hepatic homogenate were mixed and incubated at 37^0C for 1 hour. At the end of the incubation period, 1.0 ml of 10% trichloroacetic acid (TCA) was added to stop the reaction and placed on ice. After 10 minutes on ice, the mixtures were centrifuged. Aliquot (1 ml) of the supernatant was then used for the determination of liberated phosphate by the method of Fiske and Subbarow (1925).

Statistical Analysis

Data were presented as Mean± SEM of the variables measured. Differences in mean values were compared using student' t test and ANOVA. P values of 0.05 or less were taken as statistically significant.

RESULTS

Effects of normal saline

Normal saline had no effect on blood flow, (A-V)glucose, (A-V)lactate, intestinal glucose uptake and intestinal lactate level. The resting intestinal blood flow was 10.4 ± 1.88 ml/min, (A-V) glucose was 2.51± 0.52 mg/dl, (A-V)lactate was -3.11± 0.83 mg/dl, intestinal glucose uptake was 25.96 ± 6.25 mg/min while intestinal lactate uptake was -30.99 ± 6.4 mg/min. The resting value of intestinal glycogen content was 138.72 ± 4.58 mg/100g tissue, glycogen synthase activity was 1.29 ± 0.13 activity/mg.pr, glycogen phosphorylase activity was $1.74 \pm 0.21 \times 10^{-3}$ activity/mg.pr, hexokinase activity was 1.28 ± 0.20 activity/mg.pr and glucose-6-phosphatase activity was 30.71 ± 1.56 activity/mg.pr.

Effects of adrenaline

Intestinal blood flow (IBF) increased significantly in both the low dose and high dose reaching its peak at

15 minutes post-injection. There was no difference in the increased IBF produced by the two doses of adrenaline. The effects of adrenaline on blood glucose levels and arterio-venous (A-V) glucose difference are shown in table 1. The arterial glucose level increased significantly from a basal value of 102.6 ± 6.05 mg/dl to 162.8 ± 5.94 mg/dl at 15 min post-injection, for low dose adrenaline, while the venous glucose also increased from a basal value of 100.4 ± 5.78 mg/dl to 157.0 ± 5.24 mg/dl at the same period. The (A-V) glucose also increased significantly. High dose adrenaline produced a greater increase in arterial and venous glucose and the (A-V) glucose. As shown in figure 1a, low dose adrenaline produced 546% increased intestinal glucose uptake while high dose of adrenaline produced 624% increased uptake. The effect of the high dose of adrenaline on intestinal glucose uptake is significantly higher than the low dose of adrenaline.

The effect of adrenaline on blood lactate levels and arterio-venous (A-V) lactate difference are shown in table 2. The two doses of adrenaline significantly increased arterial and venous blood lactate levels throughout the post-injection observation period. At each dose of adrenaline, the venous blood lactate level was higher than the arterial blood lactate level. The arterio-venous lactate level was negative at rest which indicates that the intestine releases lactate into circulation at rest. Adrenaline at the two doses administered increased the amount of lactate that is released into circulation during the 90 minutes observation period. The maximum lactate release occurred within the first 15 minutes post-injection.

There was however no difference in the increased blood lactate level or arterio-venous lactate difference produced by the two doses of adrenaline. As shown in figure 2a, intestinal lactate uptake was negative throughout the experiments with the two doses of adrenaline. This is indicative of intestinal lactate release. At the peak of the intestinal response to adrenaline, low dose produced 438% while high dose produced 422% increase in intestinal lactate release at 15 minutes post injection There was however no significant difference in the peak of intestinal lactate production in response to low dose adrenaline and high dose adrenaline.

Table 1: Effect of intravenous injection of low (1µg/kg) and high (5µg/kg) doses of adrenaline on arterial glucose level, venous glucose level, and arterio-venous glucose difference [(A-V) glucose in dogs (n=5). (*P<0.05, **P<0.01, ***P<0.001)

	Dose	0min	5min	10min	15min	20min	30min	45min	60min	75min	90min
Arterial Glucose level (mg/dl)	Low dose	102.6 ±6.05	118.4 ±7.57	151.4 ±9.42**	162.2 ±7.56***	162.8 ±5.94***	153.8 ±5.98***	148.2 ±5.26***	133.8 ±5.66**	125.2 ±6.87*	119.8 ±7.12
	High dose	104.9 ±4.50	124 ±5.79	161.6 ±9.47***	171.2 ±6.91***	171 ±4.50***	158.8 ±5.43***	148.8 ±5.26***	135.6 ±6.19**	128.6 ±6.50*	124.2 ±5.70
Venous Glucose level (mg/dl)	Low dose	100.4 ±5.78	115.2 ±7.18	145.4 ±9.21**	154.2 ±7.60***	157 ±5.24***	150.8 ±5.98***	146 ±5.27***	131.8 ±5.53**	123 ±6.82*	117.4 ±6.83
	High Dose	102.4 ±4.24	119.8 ±5.46	153.6 ±8.70***	161.6 ±6.69***	164.8 ±4.96***	154.6 ±4.11***	146 ±5.27***	133.2 ±5.24**	126.2 ±5.84*	122 ±5.42*
A-V glucose (mg/dl)	Low dose	2.2 ±0.33	3.2 ±0.44	6.0 ±0.63**	8.0 ±0.49***	5.8 ±0.71**	3 ±0.63	2.2 ±0.33	2 ±0.28	2.2 ±0.44	2.4 ±0.36
	High Dose	2.29 ±0.58	4.2 ±0.80	8.0 ±1.30**	9.6 ±0.51***	6.2 ±0.73**	4.2 ±1.65	2.8 ±0.20	2.4 ±1.29	2.4 ±1.32	2.2 ±0.58

Table 2: Effect of intravenous injection of low (1µg/kg) and high (5µg/kg) doses of adrenaline on arterial lactate level, venous lactate level and arteriovenous lactate difference [(A-V) lactate] in dogs (n=5). (*P<0.05, **P<0.01, ***P<0.001

	Dose	0min	5min	10min	15min	20min	30min	45min	60min	75min	90min
Arterial lactate level (mg/dl)	Low Dose	17.48 ±1.69	20.18 ±2.81	16.09 ±2.90	19.63 ±2.02	23.36 ±2.84	24.01 ±2.11*	27.97 ±1.87**	27.24 ±1.23**	27.51 ±1.01**	27.96 ±1.66**
	High Dose	17.71 ±0.75	20.75 ±2.83	21.32 ±1.05**	20.47 ±2.03	22.126 ±1.96*	25.646 ±4.11	26.742 ±2.69**	28.468 ±3.58**	27.74 ±3.83*	22.71 ±3.40
Venous lactate level (mg/dl)	Low Dose	20.98 ±1.67	24.92 ±3.87	24.27 ±3.52	24.80 ±1.90	27.75 ±2.52*	30.69 ±2.48**	31.50 ±1.84**	32.30 ±2.02**	29.43 ±1.56**	31.16 ±0.57**
	High Dose	21.37 ±0.96	26.798 ±2.76*	26.774 ±1.56**	28.846 ±2.43**	29.692 ±2.21**	30.312 ±4.45*	32.34 ±4.10*	33.5 ±4.07**	30.942 ±2.62**	29.55 ±3.02*
A-V Lactate level (mg/dl)	Low Dose	-2.89 ±0.65	-4.74 ±1.08	-8.19 ±1.55**	-5.17 ±0.94*	-4.39 ±1.33	-6.68 ±1.98	-3.53 ±1.13	-5.06 ±1.56	-1.92 ±0.88	-3.20 ±1.53
	High Dose	-2.97 ±0.54	-6.05 ±2.10	-5.44 ±1.05*	-8.38 ±1.47**	-7.57 ±1.08**	-4.67 ±1.90	-5.59 ±2.33	-5.03 ±1.75	-3.20 ±2.92	-6.84 ±1.84
Blood Flow (ml/min)	Low dose	10.2 ±0.86	12.2 ±0.97	15 ±0.71**	15.5 ±0.74***	13.1 ±1.25*	11.8 ±1.11	10.4 ±0.81	10.2 ±0.66	10.2 ±1.16	10.4 ±1.36
	High dose	10.3 ±0.87	11.7 ±0.54	14.8 ±0.80**	15.6 ±1.03**	13.6 ±0.51**	12.2 ±0.37	10.2 ±0.37	10.1 ±0.40	9.6 ±0.75	9.2 ±0.86

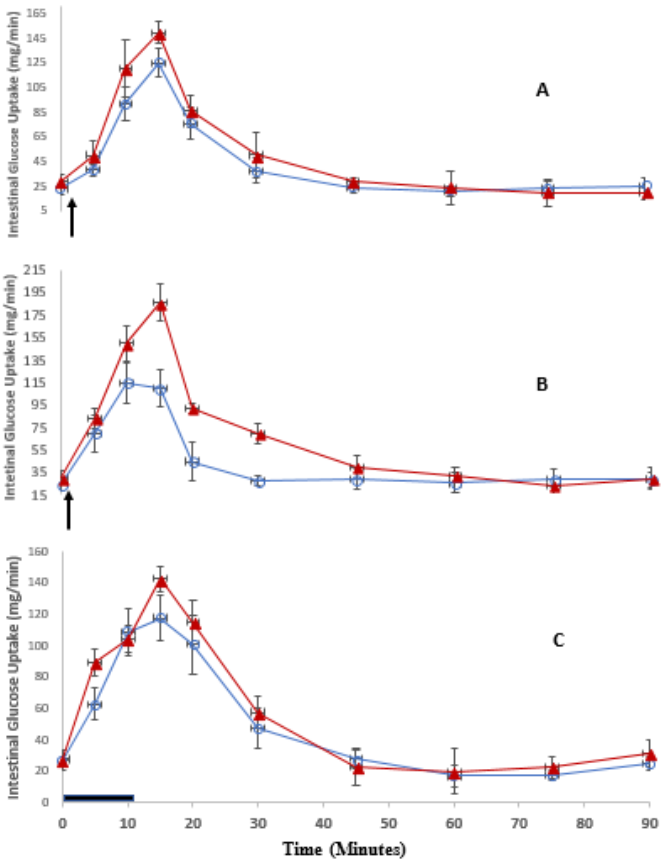

Figure 1. Effect of low(blue) and high (red) doses of adrenaline injection (A), glucagon injection (B) and glucose infusion (C) on intestinal glucose uptake in dogs (n=5). Black arrow indicates point of drug injection. Black bar indicates period of infusion.

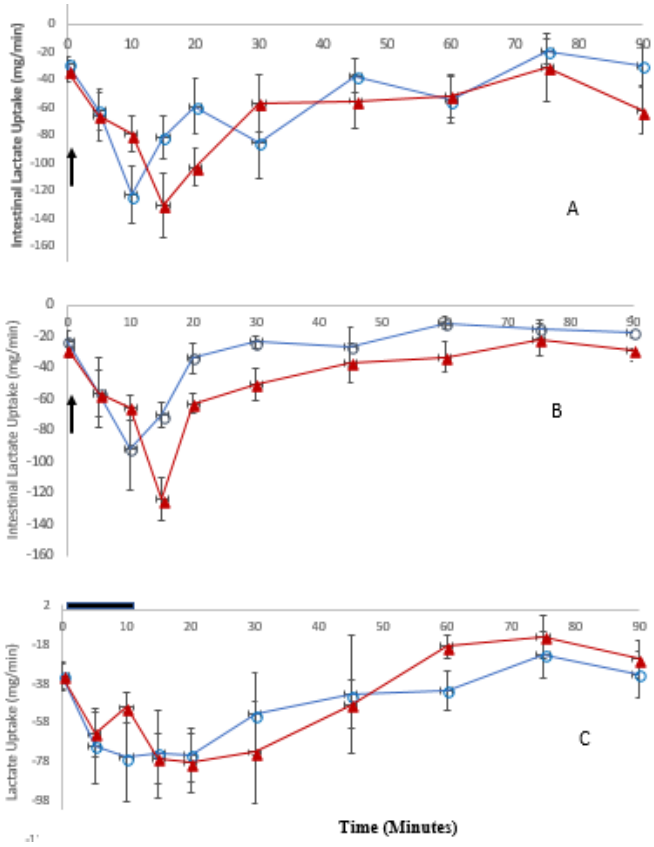

Figure 2. Effect of low(blue) and high (red) doses of adrenaline injection (A), glucagon injection (B) and glucose infusion (C) on intestinal lactate uptake in dogs (n=5). Black arrow indicates point of drug injection. Black bar indicates period of infusion.

Table 3. Effect of intravenous injection of low (1µg/kg) and high (5µg/kg) doses of adrenaline on glycogen contents and activities of glycogen synthase, glycogen phosphorylase, hexokinase and glucose-6-phosphatase enzymes in jejunum of dogs (n=5). (*P<0.05, **P<0.01, #P<0.0.05 low vs high dose)

	Control	Low Dose	High Dose
Intestinal Glycogen Content (mg/100g tissue)	138.72 ± 4.58	141.84 ± 4.13	167.17 ± 4.20**
Glycogen Synthase (Activity/mg.pr)	1.29 ± 0.13	1.35 ± 0.11	1.17 ± 0.03
Glycogen Phosphorylase a ($x\ 10^{-3}$ Activity/mg.pr)	1.74 ± 0.21	1.02 ± 0.12**	1.05 ± 0.11**
Hexokinase (Activity/mg.pr)	1.28 ± 0.20	4.26 ± 0.25**	3.21 ± 0.17** #
Glucose 6-Phosphatase (Activity/mg.pr)	30.71 ± 1.56	28.52 ± 1.18	31.41 ± 1.00

Table 3 shows the effects of adrenaline on intestinal glycogen content and activities of glycogen synthase, glycogen phosphorylase, hexokinase and glucose-6-phosphatase. Adrenaline at low dose had no effect on glycogen content when compared with the control. However, high dose adrenaline significantly increased intestinal glycogen contents. The two doses of adrenaline significantly decreased activity of glycogen phosphorylase activity and increased hexokinase activity while they had no effect on glycogen synthase and glucose-6-phosphatase activities.

Effects of glucagon

Glucagon injection produced a transient but significant increase in blood flow which occurred within the first twenty minutes post-injection of the two doses and declined towards basal value in the remaining part of the 90 minutes observation period. Low dose glucagon caused intestinal blood flow to increase from a basal value of 10.6 ± 0.86 ml/min to peak value of 12.5 ± 0.40 ml/min at 10 minutes while high dose of glucagon increased intestinal blood flow from 10.4 ± 0.51 ml/min to a peak value of 12.4 ± 0.51 ml/min at 15 minutes. There was no difference in the increased intestinal blood flow produced by the two doses of glucagon. glucagon on blood glucose and arterio-venous (A-V) glucose difference is shown in table 4.

Table 4. Effect of intravenous injection of low (3ng/kg) and high (8ng/kg) doses of glucagon on arterial glucose level, venous glucose level, and arterio-venous glucose difference [(A-V) glucose in dogs (n=5). (*P<0.05, **P<0.01, ***P<0.001)

	Dose	0min	5min	10min	15min	20min	30min	45min	60min	75min	90min
Arterial Glucose level (mg/dl)	Low dose	104.6 ±8.22	126.2 ±10.37	141.0 ±8.73*	146.8 ±7.19**	143 ±6.71**	133.6 ±8.06*	131.8 ±10.16	121 ±6.43	115.2 ±6.41	110.6 ±5.77
	High dose	106.1 ±5.63	128.6 ±5.51*	150.8 ±6.32**	159.4 ±5.37***	158 ±5.40***	144.6 ±5.45**	134.8 ±7.69*	125.6 ±5.25	119.2 ±5.03	115.4 ±3.77
Venous Glucose level (mg/dl)	Low dose	102.2 ±8.11	120.4 ±9.54	132.2 ±7.90*	138 ±8.11*	139 ±7.62**	131 ±7.93*	129 ±9.59	118.6 ±6.71	112.4 ±6.57	107.8 ±5.50
	High Dose	103.4 ±5.06	120.8 ±5.24	138.4 ±4.88**	144.4 ±5.89**	149.8 ±5.69**	138.2 ±5.97**	130.8 ±6.85*	122.4 ±5.36	117 ±5.25	112.4 ±3.50
A-V glucose (mg/dl)	Low dose	2.2 ±0.33	5.8 ±0.99**	8.8 ±0.87***	8.8 ±1.07***	4.0 ±1.26	2.6 ±0.22	2.8 ±0.66	2.4 ±0.61	2.8 ±0.59	2.8 ±0.82
	High Dose	2.6 ±0.66	7.8 ±1.02**	12.4 ±1.53***	15.0 ±1.18***	8.2 ±0.37***	6.4 ±0.68**	4.0 ±1.05	3.2 ±0.58	2.2 ±0.58	3.0 ±0.63

Table 5. Effect of intravenous injection of low (3ng/kg) and high (8ng/kg) doses of glucagon on arterial lactate level, venous lactate level and arteriovenous lactate difference [(A-V) lactate] in dogs (n=5). (*P<0.05, **P<0.01, ***P<0.001)

	Dose	0min	5min	10min	15min	20min	30min	45min	60min	75min	90min
Arterial lactate level (mg/dl)	Low dose	19.87 ±2.18	23.78 ±2.82	19.98 ±2.34	21.10 ±2.92	24.73 ±2.82	24.30 ±1.67*	26.74 ±2.94*	29.14 ±3.28*	25.86 ±3.55*	26.98 ±2.35*
	High dose	18.85 ±1.08	23.15 ±2.78	21.15 ±1.23**	22.31 ±2.70*	24.70 ±2.06**	25.38 ±3.85*	30.09 ±2.16**	30.72 ±2.59**	27.17 ±3.10**	22.79 ±2.82**
Venous lactate level (mg/dl)	Low dose	21.64 ±1.23	27.86 ±2.15*	28.25 ±2.12*	27.14 ±3.04	28.11 ±3.02*	26.68 ±1.88*	29.25 ±3.22*	30.32 ±3.59*	28.07 ±3.47	28.49 ±2.74*
	High Dose	21.73 ±0.96	27.97 ±3.19*	26.48 ±1.75**	32.26 ±2.38**	30.34 ±2.34**	30.15 ±3.32**	33.90 ±3.65**	34.05 ±2.51**	29.38 ±2.47**	26.16 ±3.33*
A-V lactate (mg/dl)	Low dose	-2.33 ±0.80	-4.28 ±1.76	-6.87 ±1.90*	-5.65 ±0.73**	-2.98 ±0.74	-2.38 ±0.56	-2.52 ±1.11	-1.19 ±0.45	-1.61 ±0.64	-1.50 ±0.98
	High dose	-2.64 ±0.51	-5.36 ±1.67	-5.34 ±0.70	-9.95 ±1.13**	-5.63 ±0.64*	-4.77 ±1.15	-3.81 ±1.51	-3.33 ±1.04	-2.21 ±1.27	-3.37 ±1.29
Blood Flow (ml/min)	Low dose	10.4 ±0.35	11.5 ±0.29	11.8 ±0.37*	11.6 ±0.28	11.4 ±0.47	10.3 ±0.51	10.6 ±0.50	10.4 ±0.39	10.2 ±0.48	10.2 ±0.19
	High dose	10.6 ±0.24	11.2 ±0.24	12.5 ±0.43**	12.1 ±0.19**	11.5 ±0.71	10.4 ±0.68	10.6 ±0.39	10.2 ±0.19	10.1 ±0.32	10.1 ±0.37

Table 6. Effect of intravenous injection of low (3 ng/kg) and high (8 ng/kg) doses of glucagon on glycogen contents and activities of glycogen synthase, glycogen phosphorylase, hexokinase and glucose-6-phosphatase enzymes in jejunum of dogs (n=5). (*P<0.05, **P<0.01, #P<0.0.05 low vs high dose)

	Control	**Low Dose**	**High Dose**
Intestinal Glycogen Content (mg/100g tissue)	138.72 ± 4.58	138.30 ± 4.14	229.21 ± 6.25**
Glycogen Synthase (Activity/mg.pr)	1.29 ± 0.13	2.67 ± 0.13**	2.72 ± 0.19**
Glycogen Phosphorylase a (x 10^{-3}Activity/mg.pr)	1.74 ± 0.21	2.17 ± 0.11*	1.01 ± 0.11** #
Hexokinase (Activity/mg.pr)	1.28 ± 0.20	3.39 ± 0.28**	3.22 ± 0.22**
Glucose 6-Phosphatase (Activity/mg.pr)	30.71 ± 1.56	34.56 ± 1.99	32.31 ± 1.47

Glucagon injection caused significant increase in arterial and venous blood glucose level. The maximum increase in arterial and venous blood glucose level was achieved at 15 minutes post-injection of glucagon and the glucose levels declined thereafter throughout the remaining observation period. Arterio-venous glucose difference was significantly increased by the two doses of adrenaline with the peak values attained at 15 minutes post-injection. High dose glucagon produced a more significant effect on arterial and venous blood glucose. As shown in figure 1b, intestinal glucose uptake in response to glucagon is 458% and 705% increases for low and high doses of glucagon respectively. The latter dose had a significantly higher effect than the former dose.

Blood lactate level and arterio-venous lactate difference in response to glucagon injection are shown in table 5. Glucagon produced an immediate rise in both arterial and venous lactate level. The increased in arterial and venous lactate levels were sustained throughout the 90 minutes post-injection observation period. The venous lactate levels produced by the two doses of glucagon were higher than their corresponding arterial lactate level throughout the experiment. At the peak, arterio-venous lactate difference increased from -2.33 ± 0.51 mg/dl to -6.87

±1.33 mg/dl and -2.64 ± 0.51 mg/dl to -9.95 ±1.13 mg/dl in response to low dose and high dose of glucagon respectively. Figure 2b shows the effect of glucagon on intestinal lactate uptake. Intestinal lactate uptake was negative throughout the experiments with the two doses of glucagon. This is indicative of intestinal lactate release. Intestinal lactate release increased from basal value of 24.35 ± 6.44 mg/min to a peak value of 108.22 ± 20.14 mg/min at 10 minutes post injection of low dose glucagon. Following high dose injection, intestinal lactate release increased from 27.92 ± 5.51 mg/min to 124.08 ± 13.58 mg/min at 15 minutes post injection. There was no difference in the peak response produced by the two doses of glucagon on intestinal lactate release.

The effects of glucagon on intestinal glycogen content and activities of glycogen synthase, glycogen phosphorylase, hexokinase and glucose-6-phosphatase are shown in table 6. While low dose of glucagon had no effect on intestinal glycogen content, high dose of glucagon produced a 65% increase intestinal glycogen content. Glycogen synthase activity was increased by the two doses of glucagon while glycogen phosphorylase activity was increased by low dose of glucagon but decreased by high dose of glucagon. The two doses of glucagon increased hexokinase activity with no effect on glucose-6-phosphatase activity. There was no difference in the increases in glycogen synthase and hexokinase activity produced by the high dose of glucagon compared with the low dose.

Effects of glucose

Intestinal blood flow increased from basal value of 10.2±0.37 ml/min to a peak value of 11.8±0.37 ml/min and 10.2±0.58 ml/min to 12.4±0.68 ml/min in response to low dose and high dose of glucose respectively. As shown in table 7, arterial blood glucose increased from 103.4 ± 4.90 mg/dl to 170.4 ± 12.81 mg/dl and venous blood glucose increased from 101.1 ± 5.26 mg/dl to 160.0 ± 12.49 mg/dl for the low dose at 5 minutes post-infusion while; arterial blood glucose increased from 104.8 ± 6.54 mg/dl to 205.6 ± 6.67 mg/dl and the venous blood glucose increased from 102.2 ± 6.33 mg/dl to 194.8 ± 5.66 mg/dl for the high dose at 10 minutes post-infusion. Following infusion of glucose, arterio- venous glucose difference

Table 7. Effect of intravenous infusion of low (10 mg/kg/min) and high (20 mg/kg/min) doses of glucose on arterial glucose level, venous glucose level, and arterio-venous glucose difference [(A-V) glucose in dogs (n=5). (*P<0.05, **P<0.01, ***P<0.001)

	Dose	0min	5min	10min	15min	20min	30min	45min	60min	75min	90min
Arterial Glucose level (mg/dl)	Low Dose	103.40 ±4.90	138.00 ±10.09*	153.40 ±11.38**	170.40 ±12.81**	163.00 ±8.15***	141.60 ±8.54**	130.80 ±6.57**	121.40 ±4.38*	117.40 ±6.39*	113.60 ±5.81
	High Dose	104.8 ±6.54	142.8 ±4.76**	169.2 ±7.56***	185.4 ±5.62***	205.6 ±6.67***	172.2 ±5.80***	150.6 ±6.14**	135.8 ±6.10*	125 ±5.08*	118 ±3.22*
Venous Glucose level (mg/dl)	Low Dose	101.10 ±5.26	132.20 ±10.25*	143.20 ±10.57**	160.00 ±12.49**	153.20 ±6.50***	137.20 ±8.40**	128.20 ±6.29**	119.80 ±4.07*	115.60 ±6.25	111.00 ±6.12
	High Dose	102.2 ±6.33	134.6 ±4.16**	159.6 ±7.87***	171.8 ±5.06***	194.8 ±5.66***	166.4 ±5.33***	148.2 ±4.97***	133.8 ±5.73**	122.8 ±5.53	114.8 ±3.42
A-V Glucose (mg/dl)	Low dose	2.40 ±0.51	5.80 ±0.73**	10.20 ±1.49***	10.40 ±0.93***	9.80 ±1.71**	4.40 ±1.03	2.60 ±0.51	1.60 ±0.68	1.80 ±0.37	2.60 ±0.51
	High Dose	2.8 ±0.37	8.2 ±0.97***	9.6 ±0.60***	13.6 ±1.16***	10.8 ±1.24***	5.8 ±0.97*	2.4 ±1.29	2.0 ±1.45	2.2 ±0.58	3.2 ±0.86

Table 8. Effect of intravenous infusion of low (10 mg/kg/min) and high (20 mg/kg/min) doses of glucose on arterial lactate level, venous lactate level and arteriovenous lactate difference [(A-V) lactate] in dogs (n=5). (*P<0.05, **P<0.01, ***P<0.001)

	Dose	0min	5min	10min	15min	20min	30min	45min	60min	75min	90min
Arterial Lactate level (mg/dl)	Low Dose	20.49 ±2.17	24.05 ±1.49	22.49 ±2.40	22.36 ±2.16	21.02 ±2.09	20.84 ±1.69	25.39 ±2.95	24.98 ±3.57	26.27 ±4.24	24.33 ±2.34
	High Dose	19.40 ±1.44	23.20 ±1.62*	25.86 ±1.93*	24.14 ±2.36*	25.94 ±2.03**	26.53 ±1.81**	31.60 ±2.64**	30.59 ±2.79**	26.08 ±2.80**	24.89 ±4.59
Venous lactate level (mg/dl)	Low Dose	21.778 ±2.04	30.67 ±2.06*	29.256 ±1.78*	28.784 ±3.378	28.29 ±2.43*	26.12 ±2.40*	29.354 ±4.17*	29.248 ±3.42*	28.588 ±4.24*	27.8 ±3.64
	High Dose	22.74 ±1.19	29.15 ±2.36*	29.43 ±1.56**	31.396 ±2.31***	33.28 ±1.99***	33.91 ±2.00***	36.38 ±3.31***	32.60 ±2.98**	27.75 ±2.66*	27.50 ±5.33
A-V lactate (mg/dl)	Low Dose	-2.88 ±0.95	-6.62 ±2.08	-6.76 ±2.04	-6.43 ±2.03	-7.27 ±1.57*	-5.28 ±2.38	-3.97 ±3.15	-4.27 ±1.29	-2.32 ±1.39	-3.47 ±1.49
	High Dose	-2.79 ±0.70	-5.95 ±1.44	-4.57 ±0.72	-7.25 ±1.52*	-7.34 ±1.53*	-7.38 ±2.91*	-4.78 ±1.32	-2.01 ±0.65	-1.67 ±1.36	-2.61 ±1.08
Blood flow (ml/min)	Low dose	10.2 ±0.37	10.6 ±0.51	11 ±0.45	11.8 ±0.37*	11 ±0.77	10.6 ±0.60	10.4 ±0.51	10 ±0.32	10 ±0.32	10.2 ±0.37
	High dose	10.2 ±0.58	11.2 ±0.37	11.8 ±0.20*	12.4 ±0.68*	10.6 ±0.24	10.4 ±0.51	10.2 ±0.49	9.6 ±0.24	9.8 ±0.37	10.2 ±0.37

Table 9. Effect of intravenous infusion of low (10 mg/kg/min) and high (20 mg/kg/min) doses of glucose on glycogen contents and activities of glycogen synthase, glycogen phosphorylase, hexokinase and glucose-6-phosphatase enzymes in jejunum of dogs (n=5). (*P<0.05, **P<0.01, #P<0.0.05 low vs high dose)

	Control	Low Dose	High Dose
Intestinal Glycogen Content (mg/100g tissue)	138.72 ± 4.58	155.26 ± 7.17**	165.45 ± 10.59**
Glycogen Synthase (Activity/mg.pr)	1.29 ± 0.13	1.40 ± 0.12	1.32 ± 0.11
Glycogen Phosphorylase a ($x 10^{-3}$ Activity/mg.pr)	1.74 ± 0.21	1.29 ± 0.17*	1.33 ± 0.08*
Hexokinase (Activity/mg.pr)	1.28 ± 0.20	2.13 ± 0.31*	3.01 ± 0.26**#
Glucose 6-Phosphatase (Activity/mg.pr)	30.71 ± 1.56	16.93 ± 1.21**	16.52 ± 0.68**

immediately increased reaching its peak 20 minutes post-infusion and thereafter returned gradually to the basal level. The low dose of glucose increased arterio-venous glucose difference from a basal value of 2.40 ± 0.51 mg/dl to a peak value of 10.4 ± 0.93 mg/dl while at high dose of glucose, arterio-venous glucose difference increased from 2.8 ± 0.37 mg/dl to 13.6 ± 1.16 mg/dl. High dose of glucose produced a more profound effect on blood glucose and arterio-venous glucose difference. Intestinal glucose uptake response to glucose infusion also followed the same pattern as that of arterio-venous glucose difference. While low dose of glucose produced 456% increase in intestinal glucose uptake, high dose of glucose increased intestinal glucose uptake by 589% (figure 1c).

The effects of glucose infusion on blood lactate levels and arterio-venous lactate difference are shown in table 8. Low dose of glucose has no effect on the arterial lactate level throughout the observation period while it caused an immediate increase in venous lactate level that was sustained throughout the post-infusion observation period. High dose of glucose on the other hand caused significant increase in both the arterial and venous lactate within 5 minutes of infusion and these increases were sustained throughout the post-infusion observation period. The venous lactate level was consistently higher than arterial lactate level produced by infusion of the two doses of glucose. Arterio-venous lactate difference increased from -2.88 ± 0.95 mg/dl to -7.27 ±1.57 mg/dl and -2.79 ± 0.70 mg/dl to -7.34 ±1.13 mg/dl in response to low dose and high dose of glucose respectively. As shown in figure 2c, intestinal lactate uptake was negative at basal and this was increased by glucose infusion up to the 45th minutes of post-infusion observation period. In other words, within the first 45 minutes post-infusion observation period, glucose significantly increase lactate release into the circulation. Intestinal lactate release increased from basal value of 29.53 ± 8.88 mg/min to a peak value of 79.22 ± 15.14 mg/min in response to infusion of low dose of glucose while, it increased from 28.64 ± 3.56 mg/min to 86.51 ± 13.58 mg/min in response to infusion of high dose of glucose. There was however, no difference in response produced by the two doses of glucose on intestinal lactate release.

The two doses of glucose infused significantly increased intestinal glycogen content. While low dose of glucose caused a 12% increase in intestinal glycogen content, high dose of glucose caused a 19% increased glycogen content. Glycogen synthase activity was unchanged while glycogen phosphorylase activity was significantly reduced during infusion of the two doses of glucose. Hexokinase activity increased in a dose-dependent manner in response to glucose infusion. Low dose of glucose increased intestinal hexokinase activity from 1.28 ± 0.20 activity/mg.pr to 2.13 ± 0.31 activity/mg.pr while at high dose, hexokinase activity increased from 1.28 ± 0.20 activity/mg.pr to 3.04 ± 0.22 activity/mg.pr. Glucose-6-phosphatase activity was significantly reduced by the two doses of glucose (table 9).

DISCUSSION

The resting blood flow of 10.4 ± 1.88 ml/min observed in this study is similar to the resting jejunal blood flow observed in previous studies on dogs (Alada and Oyebola, 1996; Alada et al., 2005 and Salman et al., 2014). The blood flow results obtained in this study suggest that the jejunal territory drained in this study is approximately equal to the territory of the upper jejunum drained in previous studies using dogs (Alada and Oyebola, 1996; Alada et al., 2005 and Salman et al., 2014).

The significant increase in the intestinal glucose uptake following intravenous injection of adrenaline is consistent with earlier findings in previous experiments in dogs (Grayson and Oyebola, 1983; Alada and Oyebola, 1996) and rabbits (Oyebola et al., 2009). Following administration of adrenaline, both arterial and venous blood glucose levels significantly increased. The increase in arterial blood glucose was however greater than the venous increase. The hyperglycemic response to adrenaline is well-known and has been extensively studied. The response is highly complex and incompletely understood (Oyebola and Alada, 1991). Basically, adrenaline has been reported to induce hyperglycemia by increasing glucose production, decreasing glucose clearance and increase glycogenolysis in muscle producing lactic

acid which is subsequently converted in the liver to glucose (Rizza et al., 1979;1980; Steiner et al., 1991; Stevenson, 1991).

Administration of adrenaline also significantly increase both the arterial and venous blood lactate levels. However, the venous lactate level was greater than the arterial level. This observation shows that the intestinal tissue was releasing lactate into the blood circulation. The increase in the quantity of lactate that is released into circulation following adrenaline is also consistent with the hyperlactatemic effect of adrenaline as reported in many animals (Issekutz, 1985; Laurent et al., 1998; Gjedsted et al., 2011). Adrenaline caused significant increase in plasma lactate levels in dogs during rest and exercise (Issekutz, 1985). Pronounced hyperlactatemia is associated with conditions characterized by elevated plasma adrenaline level such as pheochromocytoma (Bornemann et al., 1986) or patient treated with adrenaline after cardiopulmonary bypass (Totaro and Raper, 1997). *In vitro* studies using intestinal smooth muscles (Hanson and Parson, 1976; Ishida and Takagi-Ohta, 1996), vascular smooth muscles (Paul et al., 1979) and urinary bladder smooth muscles (Haugaard et al., 1987; Waring and Wendt, 2000) showed significant increases in the utilization of glucose and production of lactate following administration of adrenaline. The utilization of glucose and increase production of lactate in response to adrenaline was irrespective of the level of oxygenation in the intestinal smooth muscle (Paul, 1980).

The increase of about 400% in (A-V) glucose at the peak of response to adrenaline injection in this study shows that adrenaline caused a marked increase in glucose extraction. Interestingly, (A-V) lactate at the peak of response to adrenaline injection was about 300%. Studies using rabbit intestinal smooth muscle have reported that adrenaline relaxant effect on smooth muscle is through production of lactate (Mohme-Lundholm, 1953; 1957). Lundholm and Mohme-Lundholm (1956) also demonstrated that in the presence of high concentration of glucose, adrenaline stimulates huge glucose utilization and lactate production by intestinal smooth muscle of rats.

When the intestinal glucose uptake and lactate uptake were calculated from the product of (A-V) glucose and blood flow and (A-V) lactate and blood flow respectively, it was found that the large increases in intestinal glucose uptake after adrenaline injection corresponded in timing with the increases in intestinal lactate production. The increase in intestinal lactate production is most probably a metabolic response by this segment of the gut to the huge amount of glucose taken up by the small intestine. The response is similar to that reported by Hanson and Parson (1976) in *in vitro* preparations of rat small intestine whereby vascular perfusion of small intestinal tissue resulted in increased utilization of glucose and production of lactate. Also, the uptake of glucose and appearance of lactate were linearly related to time.

However, in the present study, the magnitude of glucose taken up by the small intestine is not the same as the amount of lactate that is released. While intestinal glucose uptake is about 600%, the intestinal lactate production is about 400%. Haugaard et al (1987) had earlier reported on this observation. From an *in vitro* incubation of rabbit bladder, the latter (Haugaard et al., 1987) reported that 11% of the glucose is oxidized to CO_2, 81% is utilized for lactate production while 4.7% was incorporated into glycogen. This partitioning of glucose utilization by the smooth muscle is in contrast to the 52.1% used for lactate production while the remaining was incorporated into glycogen in rat's diaphragm, an example of skeletal muscle (Haugaard et al.,1976).

The observed increase in intestinal glycogen in this study is in agreement with the earlier observation of Haugaard et al (1987) whereby adrenaline injection resulted in increased glycogen level in rabbit urinary bladder. In actual fact, the results of the present study showed significant reduction in the activity of intestinal phosphorylase and a significant increase in the activity of glycogen synthase. In other words, adrenaline injection resulted in significant increases in intestinal glucose uptake which is most probably the effect of induced hyperglycemia. Also, the adrenaline-induced increase in intestinal glucose uptake resulted in significant increase in the production lactate into the blood stream and a significant increase in the intestinal glycogen content. Although some studies (Axelsson et al., 1961; Andersson and Mohme, 1969, 1970; Andersson, 1971) have reported that the relaxing effect of adrenaline on smooth muscle is a result of activation of phosphorylase, glycogenolysis and production of lactic acids, Kolnes et al (2015) have recently shown that the effect of adrenaline on glycogen breakdown depends on the initial level of glycogen in a skeletal muscle. Such that the muscle with high glycogen content resulted in activation of phosporylase enzyme and glycogenolysis while a tissue with low glycogen content resulted in stimulation of glycogen synthase and glycogenesis. This observation led the authors (Kolnes et al., 2015) to the conclusion that glycogen content autoregulates glycogen synthase.

Based on the results of the present study, it could be reasonably concluded that adrenaline administration produced a huge increase in intestinal glucose uptake which is most probably converted largely to produce lactate (through oxidation) and to a little extent form glycogen in the intestinal tissue.

Following administration of glucagon, both the arterial and venous blood glucose levels significantly increased. The increase in arterial blood glucose was however greater than the venous increase. The hyperglycaemia induced by glucagon is consistent

with earlier observations on the effect of glucagon in animals (Myers et al., 1991; Young et al., 1993; Alada and Oyebola, 1996; Oyebola et al., 1998) and humans (Lins et al., 1983; Freychet et al., 1988; Hvidberg et al., 1994). Glucagon stimulates hepatic output of glucose (Beuers and Jungermann, 1990; Doi et al., 2001) by binding its G-coupled receptor which stimulates adenylate cyclase via the Gsα sub-unit to increase intracellular cAMP levels and activates protein kinase A (PKA) while it also stimulates phospholipase C via Gq sub-unit to increase production of inositol 1,4,5-triphosphate and mobilize intracellular calcium (Burcelin et al., 1994; Christophe, 1995). The net effect of glucagon on glucose homeostasis is to increase hepatic glucose production by potentiating glycogenolysis and gluconeogenesis via the activation of Gsα while inhibiting glycolysis and glycogenesis via activation of Gq signal transduction (Jiang and Zhang, 2003).

The direct effect of glucagon on tissue lactate production is obscure. However, the immediate rise in arterial and venous blood lactate level following glucagon administration in this study is most probably a secondary response by the intestine to the glucagon-induced hyperglycaemia. Since both the increase in blood glucose and lactate occurred at corresponding times. Previous studies have shown that during hyperglycaemia, there is active increase in tissue lactate release (Hangström et al., 1990; Henry et al., 1996). It has also been reported that glucose at high concentration can stimulate hexokinase activity hitherto inhibited by glucose-6-phophate in human erythrocyte (Fujii and Beutler, 1985). The increase in hexokinase activity following glucagon induced hyperglycaemia observed in this study is most probably a consequence of the increased glucose availability. Thus, the increase in lactate release is a consequence of the increase in blood glucose, since a linear relationship exist between hexokinase activity and lactate release (Crabtree and Newsholme, 1972). Also, studies showed that glucagon has a relaxant effect on gastrointestinal smooth muscle which is mediated through lactic acid. It has been shown that glucagon inhibits intestinal smooth muscle in the jejunum of man (Dotevall and Koch, 1963) as well as the stomach and duodenum of dog (Necheles et al., 1966). The inhibition of intestinal smooth muscle by glucagon could occur through its stimulation of the adrenal medulla to produce catecholamine which inhibits the activity of the intestinal smooth muscles (Fasth and Hultén, 1971). The relaxant effect of adrenaline is associated with lactate production (Mohme-Lundholm, 1953; 1957). It could therefore be suggested that the increased intestinal lactate production following glucagon injection in this study is most probably a secondary effect of glucagon on adrenal medulla to release catecholamines.

The huge increase in intestinal glucose uptake following glucagon injection in this study agrees with an earlier report in dog experiment (Alada and Oyebola, 1996). The observed increase of about 580% in (A-V) glucose at the peak of response to glucagon injection in this study also, is consistent with previous study of Alada and Oyebola (1996). It also shows that glucagon caused a marked increase in glucose extraction as evident from the observed increase in the activity of hexokinase in the present study. The lactate production from the intestine following glucagon injection was also huge. The increase in intestinal glucose uptake is most probably a metabolic response by the intestine to the hyperglycemia induced by glucagon. In other words, as a result of a significant rise in blood glucose following glucagon injection, the intestine also increased its glucose uptake from bloods circulation. A similar observation has been reported in previous studies (Alada and Oyebola, 1996) and in the present study following adrenaline-induced hyperglycemia. In the present study, administration of glucagon did not just increase intestinal glucose uptake but also produced a significant increase in the amount of lactate released into circulation. While the intestinal glucose uptake increased by about 700%, intestinal lactate release increased by about 400% in response to glucagon-induced hyperglycaemia. Again, the intestinal glucose uptake and lactate release are linearly related with time. Based on the above observation, it would therefore not be unreasonable to conclude that the increase in lactate production is metabolically related to the glucagon-induced hyperglycemia.

The increase in intestinal glycogen in this study is of great interest. Glucagon is a well-known glycogenolytic hormone. However, the observed increased activity of glycogen synthase and reduction in the activity of glycogen phosphorylase in this study seems to show that during glucagon-induced hyperglycemia, the intestinal tissue is actually synthesizing glycogen; thus, the observed increase in intestinal glycogen content following glucagon administration. This observation is consistent with the earlier postulate of Kolnes et al (2015) which shows that glycogen content autoregulates glycogen synthesis.

The observed increases in arterial and venous blood glucose following glucose infusion in this study is consistent in magnitude and pattern to those reported in previous studies (Grayson and Oyebola, 1983; Alada and Oyebola, 1996; Salman et al., 2014). Also, the glucose extraction by the intestine is dependent on the level of blood glucose. In other words, the increase in (A-V) glucose is a result of the increase in blood glucose levels following increase in the dose of glucose infused into the animal. Similar observations have been reported by Alada and Oyebola (1996) and Salman et al (2014). The observed increase in

intestinal glucose uptake in response to glucose-induced hyperglycaemia is similar to the result of previous studies (Alada and Oyebola 1996; Salman et al., 2014) where different doses of glucose infusions produced different magnitude of intestinal glucose uptake. These findings were further strengthened by the observed increase in the activity of hexokinase in the present study. This observation therefore provides further evidence to show that the increase in the intestinal glucose uptake is a metabolic response to the increase in blood glucose.

The observed increases in both arterial and venous blood lactate levels in response to glucose infusion in this study is consistent with the report that intravenous infusion or oral load of glucose increases plasma lactate concentration in dog (Cianciaruso et al., 1991; Youn and Bergman, 1991) and rat (Vine et al., 1995). Furthermore, the present results show that the increases in blood lactate and glucose are linearly related with time. Generally, lactate formation in any tissue is a by-product of anaerobic glycolysis whereby there is insufficient amount of oxygen for the tissue. Ordinarily, glucose is converted to pyruvate which is incorporated in the formation of ATP. However, in the absence of sufficient oxygen, the tissue converts pyruvate to lactate with the help of lactate dehydrogenase. Although lactate dehydrogenase activity has not been determined in this study, the presence of large amount of lactate in blood provided evidence of strong anaerobic glycolysis, the observed increase of about 600% in intestinal glucose uptake followed by an increase of about 300% in intestinal lactate release in the present study provides evidence to show that the intestinal lactate release is most possibly a metabolic response to the remarkable increase in intestinal glucose uptake. Again, both increases in intestinal glucose uptake and lactate release occur at corresponding times. The significant difference in the magnitude of glucose taken up by the gut and the amount of lactate being released shows that the lactate output from the intestine may not be the only by-product of the intestinal glucose taken up.

Indeed, the results of the present study showed a significant increase in intestinal glycogen following infusion of glucose. The observed increases in the activity of intestinal glycogen synthase and inhibition of intestinal glycogen phosphorylase 'a' provides strong evidence to show that glycogenesis probably occurred in response to glucose-induced hyperglycaemia. Similar observations have been reported in this study following hyperglycaemia induced by adrenaline and glucagon.

In conclusion, the present results demonstrated that the fate of the large amount of glucose that is taken up by the intestine following a rise in blood glucose induced by adrenaline, glucagon or glucose is through formation and release of lactate and synthesis of glycogen.

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Serum Levels of Inflammatory Cytokines in Helminth Infested Pregnant Women and Cord Blood of their Babies in Relation to Pregnancy Outcome

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Summary: Pregnancy places a very high demand on physical, physiological and immunological responses of females, especially when aggravated by parasitic infestation. There is strong evidence that maternal infestations with helminth have profound effects on immunity to helminths and other pathogens. This case-control study involved 245 pregnant women aged 18-40 years (>30 weeks of gestation) recruited from three secondary level hospitals in Ibadan, Nigeria. Morning stool samples collected from pregnant women were examine for intestinal helminths using formol-ether concentration method. A total of 38 participants comprising 17 Helminth Positive (HP) and 21 Helminth Negative (HN) pregnant women were purposely selected for the study. Sera from these women (38) and their babies' cord (38) were analysed for immune factors [interleukins 6 and 8 (IL-6, IL-8), tumor necrosis factor-alpha (TNF-α) and immunoglobulin E (IgE)] were analyzed using ELISA. Anthropometric indices [weight and height in mothers and babies and Chest Circumference (CC) in babies] were measured using standard methods. Data were subjected to descriptive statistics and analysed using Student t-test and Pearson correlation at $\alpha_{0.05}$. Only *Ascaris lumbricoides* was found in the 17 (6.9%) infested pregnant women. The mean levels of IL-6 (57.8 ± 32.8 vs 52.8±39.6 pg/mL), IL-8 (24.3±3.5 vs 22.0±7.1 pg/mL) and IgE (333.3±96.6 vs 242.3±96.8 IU/mL) were similar in HP when compared with HN. In cord sera, IL-8 level was significantly higher in babies of HP (23.7±3.9 pg/mL) compared with babies of HN (20.1±5.9 pg/mL). The levels of IL-6, TNF-α and nutritional indices in HP had significant positive correlation with corresponding levels in babies of HP mothers. Only CC was significantly lowered in babies of HP compared with HN mothers. Other anthropometric indices were not significantly different. Therefore, this present study suggests that helminth infestation may lead to strong Th2 immune responses as is reflected by the cytokine levels of mothers and babies as well as anthropometric measurements of babies of infested mothers. The outcomes of this study provide basis to deworm pregnant women during pregnancy.

Keywords*:* Helminths, Pregnancy, Cytokines, Cord-Blood, Pregnancy-Outcomes, IgE

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INTRODUCTION

Pregnancy places a very high demand on physical, physiological and immunological responses of females (Tulman and Fawcett, 2003; Warning et al., 2011; Mpairwe et al, 2014). These changes are aggravated by parasite infection (Mpairwe et al, 2014). Intestinal parasitic infections in pregnancy have been associated with serious adverse outcomes both for the mother and the unborn baby. Exposure of the foetus to helminth antigens and maternal antibodies may modulate the infant's immunity against these infections at a later stage (Gwegweni and Ntombela, 2014).

T-helper (Th) 1 cytokines (interleukin (IL)-8, tumour necrosis factor (TNF) -α, TNF-β and interferon-γ) and Th2 cytokines (IL-4, IL-5, IL-6, IL-10) were detected at various levels in the sera of women at different stages of normal pregnancy (Makhseed *et al.*, 2000; Szarka *et al.*, 2010). Although, there is no systemic inflammation during pregnancy, circulating cytokines (such as IL-6, IL-8) are found to be elevated in maternal plasma (Vitoratos *et al.*, 2010). During pregnancy, the immune system is biased towards Th 2 cytokine immune response rather than towards Th 1 cytokine immune responses which is fundamental for fetal well-being (Saito *et al.*, 2006; Szarka et al, 2010; Saito *et al.*, 2010; Mor *et al.*, 2011). However, Szarka *et al.* (2010) noted that the third trimester of normal pregnancy seems to be a controlled state of systemic inflammation.

Soil-transmitted helminths (STH) are a group of common parasites that infect more than a billion people worldwide (Mulu *et al.,* 2013). Immune responses in helminthiasis are generally associated with a Th2 response (Jackson *et al*., 2009). The triggering of the Th2 responses by helminths through activities of cytokines leads to a stereotyped cascade of effector mechanisms are typically associated with hyper-eosinophilia and considerable IgE production (Moreau and Chauvin, 2010). *In utero* exposure to pathogen derived antigens from helminths have been associated with prenatal immune priming that generates cytokine responses similar to adults and such sensitization is not skewed towards a dominant Th1 or Th2 profile (Polderman and Sobolay, 2008). Stimulation of B and T cell *in utero* by helminth infection during pregnancy is also a factor explaining IgE levels in neonates (King *et al.,* 1998).

There is strong evidence that maternal infestations with helminth have profound effects on immunity during pregnancy. Therefore, this present study suggests that helminth infestation may have effects on the anthropometric measurements of the babies, the cytokine levels in mothers and their babies.

MATERIALS AND METHODS

Study participants: The study was conducted among 245 pregnant women attending antenatal clinics at Our Lady of Apostle Catholic Hospital, Oluyoro, St Mary's Catholic Hospital, Eleta and Adeoyo Maternity Hospital, Yemetu in Ibadan, Oyo State. Nigeria. Maternal gestational age was based on calculated last menstrual period (LMP) and ultrasound assessment by Consultant Gynaecologist.

Ethical clearance: The ethical clearance for the human study was obtained from Institutional Health Research Ethics Committee of University of Ibadan/University College Hospital, Ibadan, Nigeria (UI/EC/14/0234) as well as the various collaborating hospitals and Oyo State Government, Nigeria (AD 13/479).

Inclusion criteria included pregnant women at third trimester who gave consents. Exclusion criteria included pregnant women who did not give consent or those with complicated pregnancies and infectious disease. More so, pregnant women who had diabetes mellitus, renal diseases and chronic hypertension predating pregnancy were also excluded.

Anthropometric measurement: The maternal height (m), weight (kg) and upper arm circumference (cm) were determined using portable stadiometer, digital weighing scale and tape rule respectively. At delivery, baby's birth outcomes such as weight and head to heel length as well as placental weight at delivery were recorded. Birth weight was determined using electronic weighing balance and recorded to the nearest 0.05kg while birth length was determined by measuring tape the nearest 0.1cm. Baby was considered underweight if less than 2.5kg (<2.kg) and preterm if less than 37weeks (<37weeks) gestational age.

Stool Specimen Collection and Processing: A sample of fresh stool specimen was collected from all the participants. Subjects were provided with a labeled leak-proof stool container (polypots), toilet paper, and applicator stick. Approximately 5gm of stool specimens was collected into polypots using applicator sticks. The stool specimens were examined microscopically within 24 hours of collection using the Kato-Katz concentration technique (Arinola *et al.,* 2003). The magnifications of ×10 and ×40 were used respectively to visualize and identify intestinal geohelminth ova.

Collection of blood samples: Five milliliter (5ml) of blood from the mother and cord blood of their babies was collected into plain vacutainer bottles for cytokine assay. Blood in plain bottles was spun at 2000g for 15 minutes to obtain serum which was frozen until analyses. Blood samples of 21 HN babies and their mothers were randomly collected for analysis.

Cytokines and IgE analyses: The serum levels of IL-6, IL-8, TNFα, and IgE were carried out based on kits (Assaypro, MO, USA) as previously carried out (Arinola *et al.,* 2012; Arinola *et al.,* 2014). Sample/standard was added to individual wells of microtiter plates provided by the manufacturer as part of the kit. The samples were run in duplicate. The mixture was incubated for at room temperature and washed four times with a plate washer (Tecan, Mannedorf, Switzerland). Specific biotin-conjugated secondary antibody was added to each well and incubated further at room temperature. Plates were washed four times and incubated for 30 minutes with streptavidin-HRP. Reaction was stopped by adding 50µl stop solution. The absorbance was read at 450 nm by using a microplate reader (Biotek , ELX 800, USA).

Statistical analysis: Statistical analysis was performed using SPSS 17.0 analysis package. The mean (±Standard Deviation) was determined; Student t- test was used to determine the level of significance. Pearson's analysis was used to determine correlations between the data of mothers and neonates. Result was considered significant at $p<0.05$.

RESULTS

Sampling collection

Two hundred and forty five (245) consecutive pregnant women who submitted stool samples and in their third trimesters (30-41 weeks) of pregnancy, attending antenatal clinics namely: Adeoyo Maternity Hospital, Yemetu, Ibadan, St Marys Catholic Hospital, Eleta, Ibadan, and Our Lady of Apostle Catholic

Hospital, Oluyoro, Ibadan were recruited for the study. They were followed to childbirth when cord blood samples were collected from their babies immediately after delivery. Seventeen (6.9%) pregnant mothers were found to have ova of helminths (*Ascaris lumbricoides*) in their stool after examination. No other helminth was detected. See Table 1.

Antropometric measurement

The anthropometric indices of pregnant women with helminthiasis (HP) were compared with non-infected women (HN) in Table 2. The mean value for Weight, Height and Upper Arm Circumference were 1.56 ± 0.084 m, 59.33 ± 17.46kg, 15.8 ± 6.8cm and 1.55 ± 0.22m, 63.25 ± 17.93kg, 13.8 ± 3.5cm for helminth infested and non-infested pregnant women

Table 1 Prevalence of Helminth infestation (%) among pregnant women

	Ascaris lumbricoides	Others	Total
HP	17 (6.9%)	None (0%)	17 (6.9%)
H N	228 (93.1%)	None (0%)	228 (93.1%)
Total	245 (100%)	0	0

HP = Helminth Infested Pregnant women.
HN =Helminth Non-infested Pregnant women.

Table 2: Mean Height, Weight and Upper Arm Circumference (±SD) in helminth infested mothers compared with non-infested mothers at the third trimester.

	HP (n=17)	HN (n=21)	p
Height (m)	1.56 ± 0.084	1.55 ± 0.22	0.787
Weight (kg)	59.33 ± 17.46	63.25 ± 17.93	0.567
UAC (cm)	15.84 ± 6.84	13.78 ± 3.52	0.464

UAC = Upper Arm Circumference

Table 3: Mean Anthropometric parameters between babies from helminth infested mothers compared with babies from non-infested mother.

	Babies of HP (n=17)	Babies of HN (n=21)	p
BW (kg)	2. 3± 0.3	3.04 ± 0.4	0.8
PW (kg)	0.4 ±0.4	0.86 ± 0.2	0.0
CHL(cm)	42.9 ± 11.0	47. 94 ± 2.9	0.1
HC (cm)	32.89 ± 7.37	34.65 ± 3.4	0.4
CC(cm)	15.00 ± 1.00	33.33 ± 1.2	*0.01
GA(<37wks)	11 (73%)	14(68%)	0.2

*Significant at p=0.05
BW = Birth Weight; PW = Placental Weight; CHL =Crown to Heel Length; HC = Head Circumference; CC = Chest Circumference; GA =Gestation Age.

Table 4: Mean Cytokines and IgE levels (±SD) of Helminth infested (HP) mother compared with non-infested (HN) mothers

	HP (n=17)	HN (n=21)	p
IL-6 (pg/ml)	57.8 ± 32.8	52.8 ± 39.6	0. 67
IL-8 (pg/ml)	24.3 ± 3.5	22.0 ± 7.1	0.19
TNFα (ng/ml)	0.09± 0. 09	0.05 ± 0.07	0.25
IgE (IU/ml)	333.3 ± 96.6	242.3 ± 96.8	0.25

*Significant at p =0.05 (2-tailed).

respectively. There was no significant difference between the two groups (p>0.05). There was a significant increase in chest circumference of babies born to helminth non-infested mothers compared with babies born to helminth infested mothers (p<0.05). Seventy-three percent (73%) of babies born to HP mothers were below 37 weeks of gestation compared with 68% of babies born by HN mothers. Also, babies of HP mothers have lower mean birth weight (2.3±0.3kg) compared with weight of babies born by HN mothers (3.04±0.4kg) (Table 3).

Table 5: Cytokine levels (±SD) in cord blood from babies of helminth infested compared with cord blood of babies from helminth non-infested mother.

	HP (n=17)	HN (n=21)	p - values
IL-6(pg/ml)	59.4 ± 3.4	49.9 ±3.9	0.42
IL-8(pg/ml)	23.7 ± 3.9	20.1 ± 5.9	*0.03
TNFα(ng/ml)	0.8 ±0.1	1.0 ± 0.2	0.68
IgE(IU/ml)	281 ± 224.9	263 ± 223.8	0.81

*Significant at p=0.05.

Table 6: Correlation of Cytokines and IgE levels of helminth infected mother- baby pairs.

Mothers' Samples / Babies' Samples	IL-6 (pg/ml)	IL-8 (pg/ml)	TNFα (ng/ml)	IgE (IU/ml)
IL-6 (pg/ml) p	0.580 0.015			
IL-8 (pg/ml) p		0.412 0.101		
TNFα (pg/ml) p			0.911 0.001	
IgE (IUml) p	`			0.084 0.758

*Correlation is significant at p<0.05.

Table 7: Correlation of Cytokine and IgE levels of helminth non-infested (HN) mother -baby pair.

Mothers' Samples / Babies' Samples	IL-6 (pg/ml)	IL-8 (pg/ml)	TNFα ng/ml)	IgE (IU/ml)
IL-6 (pg/ml) p	0.123 0.596			
IL-8 (pg/ml) p		0.378 0.091		
TNFα (pg/ml) p			0.402 0.109	
IgE (IUml) p				-0.004 0.987

*Significant at 0.05.

Inflammatory Cytokine and IgE

As shown in Table 4, HP pregnant women had slightly higher IL8 (24.3 ±3.5pg/ml), TNFα (0.09 ±0.0.09ng/ml) and IgE (333.3 ±196.6IU/ml) when compared with HN pregnant women. Significantly higher mean value of IL-8 (23.7 ± 3.9pg/ml) was observed in babies of HP when compared with babies born by HN mothers (20.1 ± 5.9, p=0.05) while the mean concentration of IgE and IL-6 were higher in babies born to HP mothers compared with babies born to HN mothers (Table 5). The serum levels of IL-6 and TNFα in baby born to HP mothers showed significant positive correlations with the serum levels of IL-6 and TNFα in their HP mothers. Such correlation was not observed in uninfested (HN) mother-baby pairs (Table 6 and 7 respectively).

DISCUSSION

Helminth infections during pregnancy cause significant morbidity in endemic areas through their effects on nutrition, growth and cognition (Crompton *et al.*, 2002; Bethony *et al.*, 2006; Cooper *et al.*, 2011) because intestinal parasites causes reduction in food intake, malabsorption, endogenous nutrient loss and anemia (Katona and Katona-Apte, 2008). Since mal-nutrition has been reported to cause immunodeficiency, it is hypothesized by this study that helminth infestation will modulate immune responses during pregnancy, thus affecting pregnancy outcomes.

This study reported 6.9% prevalence rate of *Ascaris lumbricoides* among pregnant women in their third trimester. This is contrary to the previous findings of Alli *et al*. (2011) and Omorhodion *et al*. (2012) who found prevalence rate among pregnant women to be 43.4% in Ibadan, and 23.7% in Warri. Obiezue *et al.* (2013) reported that helminth infection were found to be reduced during the third trimester while Aderoba *et al*. (2015) found 8.4% of pregnant women in the third trimester infested with Ascariasis in Benin City. *Ascaris lumbricoides* is transmitted faeco-orally and pregnant and pregnant women traditionally eat vegetables as source of micronutrients. This might explain the presence of Ascaris in the pregnant women.

The significant reduction in infestation rate between previous studies in Nigeria (Alli *et al*., 2011 and Omorhodion *et al*., 2012) compared with present study might be due to compulsory government policy on environmental sanitation. Obiezue *et al*, (2013) attributed reduction of helminth infestation in pregnant women observed during third trimester to the administration of anti-helminths drugs to pregnant women. Enforced regular sanitation exercise which complement monthly sanitation coupled with public enlightenment reduces unhygienic practices habit and indiscriminate waste disposal. Oranusi *et al*. (2013) suggested that consumption of contaminated fruits and vegetable causes parasitic infestation. Also, Ziegelbauer *et al.* (2012) reported that improved sanitation will reduce exposure to faecal contamination of soil-transmitted intestinal helminth infection.

Ascaris lumbricoides is a parasitic nematode that excretes a variety of molecules to evade immune attack of the host (Dzik, 2006), and also induces production of Th2-associated cytokines and IgE (Sykes *et al*, 2012). Based on this, raised levels of IgE in helminth infested pregnant women is expected as reported in this study. In this study, we reported that elevated IL-6 form part of the primary host response to helminth infestation and is frequently evident in altered cytokine profiles characteristic of unexplained adverse pregnancy outcomes that includes infertility, recurrent miscarriage, preeclampsia and preterm delivery (Prins *et al.*, 2012). Buonocore *et al* (1995) also associated increased IL-6 to fetal distress. In the present study, IL-6 was not significantly different in Ascaris infested mothers compared with Ascaris-free mothers. This might explain why mean of preterm birth and low birth weight were not statistically different in babies of helminth infested mother during pregnancy compared with babies of helminth free pregnant mothers. However, Sajjadian *et al.* (2011) reported maximum positive correlation between chest circumference and birth weight while Goto (2015) in his meta analysis of various studies reported that chest circumference is a better predictor of birth-weight less than 2500g. This study reported a significantly reduced chest circumference in babies born to helminth infested mothers.

During helminth infection, the Th2 response is suggested to have been moderated by parasite-expressed molecules (Fitzsimmons *et al.*, 2014). The significantly increased IL-8 observed in babies born to Ascaris infested mothers when compared with babies born to non-infested mothers signifies *in utero* IL-8 production induced by helminth antigen/molecules. Gebreegziabiher *et al (*2014) hypothesized that prenatal exposures to maternal helminth derived antigens elevate the level of total IgE in cord blood. Also, neonatal B cells have been shown to be intrinsically capable of IgE production (King *et al*, 1998; Soboslay *et al.*, 1999; Seydel *et al.*, 2012). Slight increase in the mean IgE levels in cord blood of babies born to helminth infested mothers compared to babies born to non-infested mothers in this study is supported by different studies conducted in helminth endemic areas in Africa (Seydel *et al.*, 2012) and could be attributed to the fact that the babies might have been sensitized *in utero* to produce Ascaris-specific B cells. Moreover, Terhell *et al.* (2002) also reported a positive correlation of IgE in sera of HP mothers and their babies as an indication of *in utero* sensitization to parasite antigens. The present study observed

significant positive correlations between IL-6 and TNF in cord sera of babies from Ascaris infested mothers and sera from their mothers during pregnancy. Such correlation was not observed in the cord sera of babies from helminth mothers compared with the sera of their mothers during. This is might be as a result of *in utero* sensitization by Ascaris molecules released into circulation of mothers during pregnancy.

The study concluded that the prevalence rate of helminth infestation among pregnant women in 3rd trimester is low and that IL-6, IL- 8, TNFα and and IgE were slightly raised in Ascaris infested pregnant mothers compared with un-infested pregnant women. Significantly, reduced chest circumference is the only negative neonatal anthropometric index observed in babies of Ascaris infected pregnant mothers.

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Spermatotoxicity and Testicular Pathology in Wistar Strain Rats fed Graded Levels of Pigeon Pea Diet

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Summary: Pigeon pea is an important grain legume in the tropics and subtropics and it is a valuable source of low-cost plant protein for humans and animals, but it remains an underutilized legume. Effects of feeding graded levels of raw pigeon pea seed inclusion diets on testicular function in Wistar rats was investigated. Thirty male Wistar rats weighing between 120 and 160 g were assigned into six groups (A-F) of 5 rats each. Group A rats served as the control and were fed with standard rat feed, Group B was fed with 10% pigeon pea inclusion diet, group C: 20% pigeon pea inclusion diet, Group D: 30% pigeon pea inclusion diet, Group E: 40% pigeon pea inclusion diet and Group F: 100% pigeon pea diet. Each rat received 30 g of feeds per day for 21 days with drinking water *ad libitum*. All analyses were carried out using standard methods. The motility scores were between 34.00±2.45 and 87.00±3.00 with the control group A having the significantly highest score ($P<0.05$) compared to the other groups. Group B rats had a significantly higher ($P<0.05$) values of (76.00±6.96) than groups C, D, E, and F while groups D, E and F were lower than normal range. This same trend was observed for the sperm viability and count across the groups. No lesion was observed in the testicular histology of rats in groups A, B, and D. The testis of rats in group C showed marked expansion of the interstitium by oedema, while the testis of group E rats showed immature germ cells in the seminiferous tubular lumen and the testis of group F rats revealed slightly reduced germinal depth. It was concluded in this study that feeding of pigeon pea seed diet to rats beyond 20% inclusion level is spermatotoxic having severe adverse effects on the sperm motility, viability and count and caused some testicular lesions. However, unprocessed pigeon pea must be incorporated with caution into animal feeds, especially the male animals used for breeding.

Keywords*:* Semen, Testes, Pigeon pea, Wistar rats, Motility, Histology

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INTRODUCTION

The increasing world population particularly in the developing countries like Nigeria calls for urgent improvement in livestock production. Deficiency of animal proteins is a major problem that needs to be addressed in Nigeria and other African countries (Okah and Ibeawuchi, 2011). The level of animal proteins in diets of most Nigerians is lower than the recommended level (Okah and Ibeawuchi, 2011).

Pigeon pea (*Cajunus Cajan* L.*) is* a diploid legume crop and is a member of the family *Phaseolea. (*Devi *et al.* 2016). It is a multipurpose, hardy grain legume crop grown in several developing countries in the semi-arid tropics and sub tropics (Zu *et al*., 2006). Pigeon pea plays a vital role in human and animal nutrition as a source of dietary protein in many countries (Abdelati *et al.* 2009). *Cajanus cajan* seeds are now well-thought-out to be a non–conventional source of feed in poultry and as a valuable source of protein in feeds *(*Devi *et al*. 2016). In Nigeria, it is grown extensively in Enugu, Anambra and Benue States.

It is also cultivated in Africa and the Americas, and it has been suggested as one of Africa's drought-tolerant crops referred to as 'orphan crop' because it falls into the group of least researched crops world-wide (Odeny, 2007). Most protein supplements are very expensive and their use in ruminant nutrition competes with monogastric animals and human nutrition. There is the need to address this problem of insufficiency of dietary supplements in animal nutrition especially the protein supplements (Okah and Ibeawuchi, 2011). This can be achieved by sourcing for alternative protein feedstuff that will attract minimal competition from monogastric animals and humans.

Pigeon pea has some medicinal and nutritional benefits like antibacterial (Siyabonga *et al*., 2016), anti-oxidative effects (Wu *et al*., 2009; Muangman *et al*., 2011), reportedly used in the treatment of diabetes, dysentery, hepatitis, malaria and diet-induced hypercholesterolaemia (Oke, 2014), an important

source of low-cost vegetable protein, minerals and vitamins for humans (Fasoyiro *et al.,* 2010; Okpala and Ekwe 2013) and a good supplementation of starchy foods (Mbaeyi-Nwaoha and Onweluzo, 2013). Despite the fact that pigeon pea is a valuable and economic crop for both human and animal nutrition, pigeon pea seeds have received little attention as compared to the leaves (Nix *et al*. 2015). Phytochemical constituents of pigeon pea leaves include alkaloids, flavonoids, saponins, tannins and terpenes but anthraquinones, phlobatannins and sterols were not detected (Oke, 2014).

The effects of processed and raw unprocessed pigeon pea seed meal have been investigated on the growth, meat and egg qualities of broilers and layers (Etuk *et al*. 2003; Ahmed, *et al*. 2008; Amaefule and Obioha, 2007; Saeed *et al*. 2007), on cockerels (Yisa *et al*. 2010), in sheep (Okah and Ibeawuchi, 2011), but there is dearth of information on its effect on the sperm motility, sperm viability and sperm count of rats. It is worthy of note, however, that most of the non-conventional and also conventional legumes used in animal nutrition, contain some antinutritional factors which limit their utilization in the raw (unprocessed) state by livestock (Ahamefule *et al.* 2008). There has been report that some farmers bypass the processing of legumes used in animal feeds, so as to reduce the processing costs of animal feeds (Bawa *et al*. 2003). This calls for a need to investigate the reproductive effects of using unprocessed pigeon peas on the male rats.

This study examines the effects of feeding graded levels of raw pigeon pea seed diets on the sperm characteristics and testes of Wistar strain rats.

MATERIALS AND METHODS

Chemicals and Reagents

Bouin solution was used for fixation of the testis for studies on sperm motility, viability and count of rats.

Feed Preparation

The contents of normal feed and the graded constitutions with pigeon pea are shown in Tables 1 and 2 below.

Table 1: Composition of Normal Feed

Ingredients	Percentage (%)
Carbohydrate (Maize)	40
Protein (GNC, PKC, SB)	32 (8,16,8)
Fiber Content (Wheat Bran, Rice Bran)	18 (16,2)
Bone Meal	6
Oyster Shell	3.8
Premix (Methionine and Lysine)	0.08
Salt	0.12

** GNC- Groundnut cake, PKC- Palm kernel cake, SB-SOYA BEAN* Source: Soetan *et al*. (2017a).

Table 2: Graded levels of Pigeon pea (*Cajanus cajan*) Inclusion Diets

GROUPS	NORMAL FEED (kg)	PIGEON PEA INCLUSION (kg)	TOTAL (Kg)
A (Control)	3	-	3
B (10%)	2.7	0.3	3
C (20%)	2.4	0.6	3
D (30%)	2.1	0.9	3
E (40%)	1.8	1.2	3
F (100%)	-	3	3

The pigeon pea seeds and the rat concentrate feed (3kg each) were ground into powder form using an electric miller. The feed was then reconstituted into different percentage inclusion of pigeon pea seed diets (10%, 20%, 30%, 40%, 100%) and normal concentrate feed as control.

Experimental Animals

Thirty male Wistar strain rats (weighing between 120g and 160g) were used for this study. The animals were kept in stainless-steel individual metabolic cages (Associated Crate Ltd., England) located at the Experimental Animal Unit of the Department of Animal science, University of Ibadan, Ibadan. They were allowed to acclimatize for a period of two weeks. The animals were weighed and the weights recorded before commencement of experiment.

Experimental Protocol

The rats were assigned into six groups (A-F) of 5 rats each. Group A rats served as the control and were fed with commercial rat feed, Group B was fed with 10% pigeon pea inclusion diet, group C: 20% pigeon pea inclusion diet, Group D: 30% pigeon pea inclusion diet, Group E: 40% pigeon pea inclusion diet and Group F: 100% pigeon pea diet. All the rats were given 30 g of feed per day with water *ad libitum* for the 21 days experimental period.

Weight Assessment

The weight of each rat was taken and recorded at the start of the experiment (initial weight) and on days, 7, 14 and 21 of the study using a laboratory weighing balance.

Semen Collection and Analysis

The rats were anaesthetized with diethyl-ether before sacrifice as described in earlier studies (Ola-Davies and Ajani, 2016; Oyeyemi and Ajani, 2015). The mid caudo-ventral abdominal incision was made with sterilized pair of scissors, permitting instant access to the testis once pushed upward from the scrotum with gloved hand. The testes were then separated from the epididymis. The right and left epididymides were trimmed off the body of the testes and semen sample was collected thereafter from the tail of the epididymis through an incision made with a scalpel blade.

Percentage Sperm Motility and Viability

Sperm percentage motility was carried out using 2 to 3 drops of 2.9% warm buffered sodium citrate kept at body temperature as described by Zemjanis (1977).

Also, a drop of semen sample was placed on warm glass slide and stained with eosin-nigrosin stain for viability study after which a thin smear was then made of mixture of semen and stain. The smear was air dried and observed under the microscope (Zemjanis, 1977).

Histopathology

The testes for each animal in each group were harvested, fixed in Bouin's fluid for 24 hours and routinely processed for histology. The prepared slides were then examined using an Olympus® CX21 light microscope.

Data analysis

The data generated was analyzed using one way analysis of variance (ANOVA). SPSS Version 15 for Windows (SPSS Inc, 2006) and Microsoft Excel Professional Plus (Microsoft Corporation, 2010) were used to carry out all procedures. P values less than 0.05 were considered as significant.

RESULTS

The results of the weights of rats are shown in Table 3. There were significant (P<0.05) decreases in the weights of rats in groups E and F compared with the weights of group A rats (control).

The results for the sperm motility, viability and sperm count are shown in Table 4. The motility scores were

Table 3: Mean weights of rats in different Pigeon pea (*Cajanus cajan*) Inclusion Diets

Groups	Day 1 (g) (Initial weight)	Day 7 (g)	Day 14 (g)	Day 21 (g)
A	121.6±1.1	126.2±3.6	129.4±4.0	135.6±1.1
B	137.8±8.9	135.4±9.9	131.6±9.8	126.0±9.6
C	150.6±8.4	145.4±9.2	139.6±7.2	132.0±7.8
D	144.4±16.3	139.6±15.2	134.2±15.1	125.6±16.7
E	142.2±17.5	136.6±20.1	128±19.3	112.2±16.5*
F	144.6±13.1	134.4±14.8	125.6±16.2	102.2±18.3*

* Significant difference at P<0.05

Table 4: Mean values for percentage motility, viability, and Sperm count of albino rats in different Pigeon pea (*Cajanus cajan*) Inclusion Diets

Parameters	Group A	Group B	Group C	Group D	Group E	Group F
Motility (%)	87.00±3.00	76.00±6.96*	67.00±3.74	34.00±7.48	34.00±2.45	34.00±2.45
Viability (%)	80.00±2.74	79.00±2.92*	73.00±3.74	70.00±2.74	69.00±4.58	66.00±1.87
Sperm count ($x10^6$sperm/ml)	294.60±7.87	207.40±6.19*	193.40±7.68	192.60±6.27	187.60±6.47	176.80±2.05

* Significant difference at P<0.05

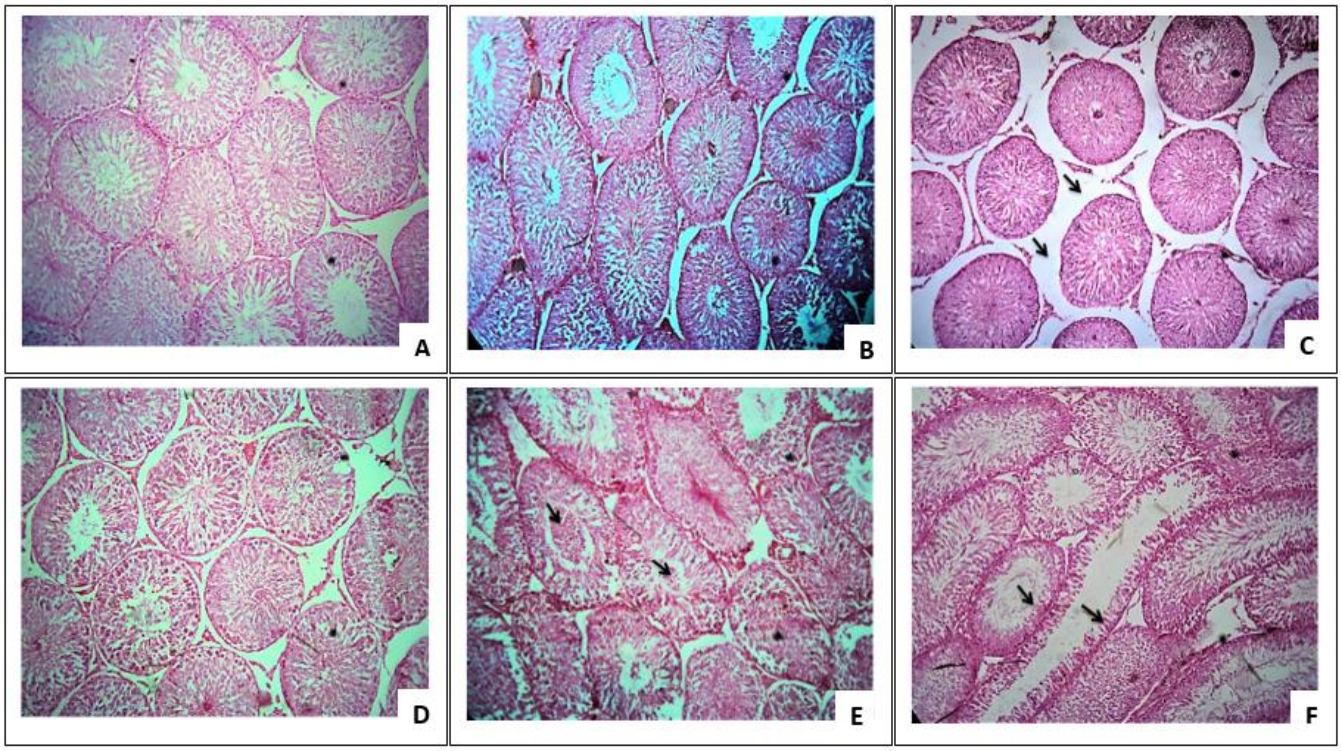

Figure 1. Transverse section of the testis of rats from groups A, B, C, D, E and F. Group C animals has marked expansion of the interstitial space (arrows) caused by oedema while group E and F showed immature germ cells in the seminiferous tubular lumen (arrows) and slightly reduced germinal depth (arrows). H&E, x150

between 34.00±2.45 and 87.00±3.00 with the control group A having the highest score (P<0.05). Group B rats fed with (10% pigeon pea inclusion diet) had a significantly higher (P<0.05) values (76.00±6.96) than groups C, D, E, and F of which mean percentage motility ranges between 34.00±2.45 and 67.00±3.74. Group E fed with 40% pigeon pea inclusion diet and group F fed with 100% pigeon pea diet had the lowest mean percentage motility of 34.00±2.45. This value was significantly lower than the mean values of groups A and B rats. The motility score was decreasing with increasing inclusion of pigeon pea. This same trend was observed for the sperm percentage viability and sperm count across the groups (Table 4).

Testicular Histopathology

No observable lesion was observed in the testicular histology of rats in groups A, B, and D (Figures 1A, 1B and 1D respectively). The testis of rats in group C showed marked expansion of the interstitium by oedema (Figure 1C). The testis of group E rats showed immature germ cells in the seminiferous tubular lumen (Figure 1E) while testis of group F rats revealed slightly reduced germinal depth (Figure 1F).

Figure 1: Group A (Control) Testis: There are no observable lesions. H&E, x150

DISCUSSION

Sperm motility, viability and sperm count are essential parameters in the evaluation of fertility potentials in the male animals (Garner and Hafez, 1993).

In this study, it was observed that the mean percentage motility values obtained for rats in groups D, E and F decreased significantly (P<0.05) with increasing inclusion levels of pigeon pea. The values obtained were below the 60.00% minimum required value for potential fertility (Garner and Hafez, 1982). This implies that feeding pigeon pea to rats beyond 20% inclusion level had adverse effect on the sperm motility and can thereby reduce the spermatozoa fertilizing capacity and consequently precipitating infertility. The results of this study is similar to the report by Soetan *et al*. (2017b) on the adverse effects of 50% raw lima beans (Phaseolus lunatus) inclusion diets, on the testes of mice. The study observed a significant (P<0.05) rise in sperm abnormalities with a drop in sperm motility and counts.

The results from this present study implies that 10-20% inclusion level of unprocessed pigeon pea fed to rats had no adverse effect on sperm percentage motility, and since this same trend was observed for the mean percentage liveability and sperm count, it connotes that the inclusion level of pigeon pea beyond 20% in rats feeds will be spermatotoxic and this will have a grave consequence on fertility potentials of the rats.

Soetan *et al*. (2014) reported significant decreases in the absolute weights of testes in rats fed with Rongai brown and Rongai white varieties of lablab beans (Lablab purpureus), when compared with the control group. Lablab purpureus-fed rats also showed significant decreases in spermatozoa motility, epididymal spermatozoa number, viability, testicular spermatozoa number and daily spermatozoa production. The percentage of morphologically

abnormal spermatozoa was significantly increased in all the rats fed with the three varieties of Lablab purpureus (Rongai brown, Rongai white and Highworth black).

Findings from our present study also corroborates earlier studies in male Wistar rats using nicotine (Oyeyemi *et al*., 2014), gossypol (Hadley *et al*., 2013) Lagenaria Breviflora Roberts; (Saba *et al*., 2009), Aloe vera gel (Oyeyemi and Ajani, 2015) and sodium arsenite (Ola-Davies *et al*., 2017).

It is likely that there are some anti-nutritional and toxic factors contained in raw pigeon pea which at higher dose might give unfavourable outcome when not adequately processed before feeding to animals. The histopathology lesions observed in testis of rats in groups C, E and F could be attributed to the presence of some toxic factors in the raw pigeon pea.

In a similar study on the effects of graded levels of pigeon pea, male rats fed with 30% raw pigeon pea inclusion diet showed *m*oderate lymphocytic infiltration in the portal areas of the liver while 100% raw pigeon pea diet produced a marked lymphocytic infiltration of the portal areas in the liver of male rats (Soetan *et al*., 2017a).

In another study on the effects of feeding three different varieties of raw lablab beans to rats, Rongai brown variety of lablab beans produced disruptions in testicular basement membrane of seminiferous tubules and loss of spermatozoa of the rats while Rongai white and Highworth black varieties caused oedema and reduction of seminiferous tubular diameters on the testis of rats (Soetan *et al*., 2014).

Immature clumps in the lumen of very few seminiferous tubules, severe congestion of interstitial vessels and a severe germinal and sertoli cell necrosis and erosion of the seminiferous lumen were observed in the testes of mice fed 50% raw lima beans inclusion diets (Soetan *et al*., 2017b) while vacuolation of secondary spermatocytes and loss of spermiogenic epithelium were reported by Ola-Davies *et al*. (2017) in the testes of male rats administered with sodium-arsenite.

In the light of the low sperm qualities observed in groups D, E, and F rats, it can be adduced that the poor sperm quality occurred during storage and maturation in the epididymis. The exact mechanism for this cannot be determined at the moment and would need to be further investigated. The exact toxic factors in the raw, unprocessed pigeon pea which caused the testicular histopathology in rats and their mechanism of action would need further study.

It was concluded in this study that feeding rats with raw pigeon pea beyond 20% inclusion level is

spermatotoxic having severe adverse effects on sperm motility, viability and sperm count. Pigeon pea in raw, unprocessed form must be incorporated with caution into animal feeds, especially the male animals used for breeding.

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Undergraduate Students' Understanding of Physiology Subject, Opinions and Perception: The Case of Bayero University, Kano, Nigeria

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Summary: Human Physiology courses are compulsory in medical education but many students encounter challenges in studying them. This study investigates understandings, opinions and perceptions of students about physiology subject and the challenges they encounter in their study. Two hundred and eighty (280) students were interviewed using structured questionnaire. The data were analyzed using IBM SPSS statistics 22. The mean age of the students was 22.8 ± 4.1 years. Males constituted 174(62.1%), while females were 106 (37.9%). Majority of the respondents were in 300 level of study in the university 178(63.6%) as of the time of conducting this research. Most of the participants 257(91.8%) claimed to have good to excellent understanding of the physiology subjects. One hundred and nine (39.1%) alleged that academic staff had unfriendly attitude like rushing lectures, commencing lectures late and fixing tests and other continuous assessment close to examinations, and that lecturers have poor communication/ teaching skills. Less than one-third (28.7%) believed that the subjects have bulky course contents, and 56(20.1%) were of the opinion that the students' lack of seriousness or interest is among the reasons for failing physiology courses. On binary logistic regression, being of female sex, and in 300 level of study emerged as the independent predictors of the students' perception of physiology. Improving the pedagogy 102(36.4%), good attitude of staff towards students and teaching 73(26.1%), improving infrastructure and equipment 35(12.5%); and instituting guidance and counseling unit for both staff and student 31(11.1%) were the ways suggested by the students for improving performance in physiology subject. Most of the students had poor and negative perception about physiology subjects (83.2%). Guidance and counseling for students, through the level coordinators and the mentor-mentee program should be strengthened in all departments. Basic infrastructure and equipment, and teaching techniques should also be upgraded.

Keywords*:* Undergraduate students, Understanding of Physiology Subject, Opinions, Perceptions, Kano-Nigeria

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INTRODUCTION

Physiology is one of the prerequisite courses of medical and allied health disciplines. Courses in physiology subjects provide learners with the knowledge about the normal function of the human body and by so laying the foundation for identifying departures from health. Physiology is discussed repeatedly at all levels of study so that students not only acquire knowledge but recognize how it fits into their future medical practice. The human body is a complex system and physiology is thus acknowledged, from the views of both staff and students as a discipline that presents most students with a challenge (Somjen, 1999; Tufts & Higgins-Opitz, 2009; Tufts & Higgins-Opitz, 2014). According to Sturges & Marer (2014), Physiology is difficult because it makes connections among multiple disciplines, and has boundaries that are not rigid. It is a subject with a rapidly increasing knowledge and complexity (Somjen, 1999; Tufts & Higgins-Opitz, 2009; Sturges & Marer, 2014). However, the progression of students from the medical and allied health disciplines largely depends on their understanding of physiology courses, even though they may have different and diverse requirements.

The Faculty of Basic Medical Sciences, Bayero University, Kano offers physiology as compulsory course of study for undergraduate students in medical and allied health disciplines. The students were taught and examined based on their disciplines as follows: MBBS and Dentistry, BSc. Physiology, and the Allied programs consisting of B. Physiotherapy, B. Nursing, B. Medical Science and B. Radiography. The performance of these students was generally noticed to be poor across all disciplines during the semester examination conducted in October 2015. This study was planned to assess the perceptions of the different groups of students about their understanding of physiology, and their opinions about the factors associated with the poor performance with a view to proffer solution to the problem.

MATERIALS AND METHODS

Study population:

This comprised of three groups of students in their 200 and 300 levels of study from faculty of basic medical sciences; MBBS and Dentistry, BSc. Physiology, and the Allied programs.

Study design, Sample size and sampling:

A descriptive cross-sectional design was used to study a random sample of 280 students. The sample size was determined using the Fisher's formula for estimating minimum sample size for descriptive studies (Lwanga & Lemeshow, 1991); and an 85% proportion of students that believed that their absence from different academic activities can affect their performance, obtained from a previous study from Sudan (Kaddam *et al*, 2012). The sample size was proportionately divided among the three groups of students based on their sizes. Thus the sample size for MBBS/Dentistry was 112 students, the Allied program was 145, while physiology was 23 students.

Sampling frames for the three (3) groups of students was compiled and the required samples were systematically selected using sampling intervals obtained by dividing the number of students under each group by the required sample from that group. The sampling intervals were used to select respondents from the respective groups until the sample size for each group was met.

Instrument and method of data collection

A self-administered semi-structured questionnaire with mostly opened ended questions was used for data collection. The questionnaire had five (5) sections that elicited information on the respondents' bio-data, their opinions and perceptions of physiology as a course of study and as a subject; and the students 'understanding of physiology.

Pre-testing of the questionnaire was on thirty (30) students from a State University in Kano (Northwest University). Six trained research assistants administered the questionnaires. Data was collected in July/August 2015.

Data analysis

Data were analyzed using IBM SPSS Statistics for Windows, version 22. Armonk, NY: IBM Corp.IBM SPSS statistics 22. Quantitative variables were summarized using appropriate measures of location and variability, whereas categorical variables were presented as frequencies and percentages. Perception about physiology was scored and graded using a Likert scale, with 1 and 5 indicating lowest and highest level of agreement respectively with the items eliciting perception. Respondents that chose to "agree - 4" or "very agree - 5" were considered to have "good perception" about physiology while those that chose "very disagree - 1", "agree - 2" or "indifferent/ neutral - 3" were considered to have "wrong perception". Thus out of an aggregate points for all responses, the students that scored a minimum of 80 points (4 x 20 items) were considered to have good perception while those that score less than 80 points were considered to have wrong perception about physiology. The scoring and grading system was adapted from a previous study (Iliyasu *et al.*, 2010).

Pearson's chi-square and Fisher's exact probability tests were used to identify factors significantly associated with the students' perceptions about physiology at bi-variate level. $P \leq 0.05$ was considered significant. Covariates that were associated with the student' perceptions (adjusting for age and sex) with $p < 0.2$ were included in the multivariable analysis. Variables were included in model if they resulted in >10% change-in-estimate or a change in log likelihood with p-value<0.2. Variables that resulted in change in coefficient standard errors of already included variables by >20% were assumed to be collinear and excluded from the model.

Ethical considerations

Permission and ethical clearance for the study were obtained from BUK authority and the Institutional Review Board of Aminu Kano Teaching Hospital respectively. Informed consent was sought and obtained from all respondents before questionnaire administration.

RESULTS

Biological and academic characteristics of the students

The age of the students examined ranged from 17 to 40 years with a mean of 22.8 ± 4.1 years. About two-thirds were males 174 (62.1%), and in 300 level of study in the university 178 (63.6%). All the students assessed take courses in Physiology but only 23 (8.2%) of them are studying BSc. Physiology as shown in Table 1.

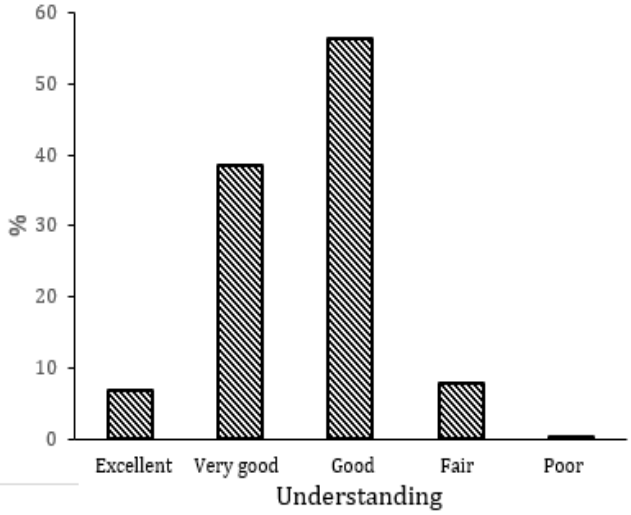
Table 1: Biological and academic characteristics of the students

Characteristic	Frequency (n = 280)	Percentage
Age (Years)		
17 - 24	208	74.3
25 - 32	61	21.8
33 – 40	11	3.9
Sex		
Male	174	62.1
Female	106	37.9
Programme of study		
BSc. Physiology	23	8.2
MB; BS	112	40.0
Harmonized programme	145	51.8
Level of study		
200 level	102	36.4
300 level	178	63.6

*Allied Sciences include; B. Physiotherapy, B. Radiography, B. Medical Laboratory Science, B. Nursing

Students' understanding of physiology courses

The students were asked questions to elicit their self-assessment about their understanding of physiology based on the content of the courses taught and the scores they obtained in the continuous assessment. Most of the students 257 (91.8%) claimed to have good to excellent understanding of the physiology courses as depicted in Figure 1.When the students were generally asked on the common challenges that affect students' performance in physiology, 80 (28.7%) mentioned that the subjects have bulky course contents, 62 (22.2%) said that the academic staff had unfriendly attitude like rushing lectures, commencing lectures late and fixing tests and other continuous assessment close to examinations, while 47 (16.9%) admitted poor communication/teaching skills of the lecturers; and 56 (20.1%) opined that the students' lack of seriousness or interest is among the challenges faced by students. In the same vein, when the students were asked on the ways to improve performance in physiology, the most common responses were improving pedagogy 102 (36.4%), improving attitude of staff towards students and teaching and improving infrastructure and equipment 35 (12.5%). Other responses are as shown in Table 2.



Opinions and perceptions of the students about physiology

Table 3 summarizes the parameters used to assess the perceptions of the students about physiology courses, its pedagogy and the academic staff teaching the

Table 2: Students' perceived challenges and ways of improving performance in Physiology

Responses	Freq (%) (n=280)
Challenges	
Bulky course content	80 (28.7)
Negative/ Unfriendly attitude of lecturers	62 (22.2)
Students' lack of seriousness/ interest	56 (20.1)
Poor communication/ teaching skills of the lecturers	47 (16.9)
Inadequate infrastructure and equipment	10 (3.6)
No guidance/ counseling	9 (3.2)
No revision session(s)	1 (0.4)
None	15 (5.4)
Ways of improving performance	
Improving pedagogy	102 (36.4)
Improving attitude of staff towards students and teaching	73 (26.1)
Improving infrastructure and equipment	35 (12.5)
Institute guidance and counseling for both staff and students	31 (11.1)
Capacity building for academic staff	10 (3.6)
Improve the number of experienced academic staff	4 (1.4)
No idea/ don't know	25 (8.9)

Table 3: Parameters used for assessing students' perceptions about physiology

Parameter	Frequency	Percentage
About course content, pedagogy and infrastructure		
Agreed that the course content of physiology subjects is adequate in scope	239	85.4
Agreed that notice for Continuous Assessments are usually given	230	82.1
Agreed that the course content of Physiology subjects is practical and easily understood by students	215	76.8
Agreed that contact hours are adequate in Physiology lectures	210	75.0
Agreed that examinations cover all areas taught	200	71.4
Agreed that Continuous Assessments are adequate in number	182	65.0
Agreed that lecture rooms/ halls are adequately spaced	173	61.8
Agreed that practical sessions in physiology are comprehensive	171	61.1
Agreed that Continuous Assessments cover all areas of teaching	170	60.7
Agreed that teaching aids and illustrations in Physiology is optimum	148	52.9
Agreed that lecture rooms/ hall are well lit	124	44.3
Agreed that temperature in lecture rooms is most times conducive for learning	98	35.0
Agreed that feedback to students on Continuous Assessments and examinations are usually given	89	31.8
About academic staff		
Agreed that the number of academic staff is adequate	236	84.3
Agreed that the academic staff in physiology adequately mastered their areas of teaching	193	68.9
Did not agree that lecturers give unnecessary notes without explanations	189	67.5
Did not agree that lecturers often come late for lectures	142	50.7
Agreed that the communication skill of the lecturers is satisfactory	130	46.4
Did not agree that lecturers commence lectures late into the semester	125	44.6
Perception grades		
Good/ correct	47	16.8
Poor/ wrong	233	83.2

Table 4: Factors associated with the students' perceptions about Physiology

Characteristic	*Bi – variate*			*Binary logistic regression*	
	Positive (n = 47) Freq (%)	***Total (N =280)***	***Statistical test (p value)***	***Z test (p value)***	***OR (95%C.I)***
Age > 24 years	15 (20.8)	72	$X^2 = 2.41$ (0.30)	0.88 (0.38)	
Female sex	21 (19.8)	106	Fisher's (0.19)	1.95 (0.05)*	1.99 (1.00; 3.96)
Other programmes	46 (17.4)	257	$X^2 = 6.79$ (0.03)*	1.14 (0.25)	
300 level of study	14 (7.9)	178	$X^2 = 27.8$ (0.001)*	4.51 (0.0001)*	0.21 (0.10; 0.41)
Good understanding of physiology	45 (17.5)	257	Fisher's (0.22)	0.07 (0.94)	

**Statistically significant*

course. Most of the students agreed that the content of physiology courses is adequate 239 (85.4%), practical and easily understood by students 215 (76.8%), and contact hours for lectures are adequate 210 (75.0%). Although 230 (82.1%) agreed that an advanced notice is usually given before continuous assessments, less than two-thirds 170 (60.7%) and 89 (31.8%) of the students agreed that the continuous assessments cover all areas of teaching, and that feedback is given to students on the continuous assessments and other examinations respectively. The responses on the other aspects of pedagogy and infrastructure are however not encouraging as shown in Table 3.

Except for the numbers of the academic staff in physiology that was adjudged adequate by 236 (84.3%) of the students, the responses on the other parameters used to assess the academic staff are not encouraging as summarized in Table 3. Overall, only 47 (16.8%) of the students were assessed to have good perception about physiology. The overall perception of the students about physiology was significantly associated with being at 300 level of study in the university ($X^2 = 27.8$, $p = 0.0001$) and being in other academic programmes in the college other than BSc. Physiology ($X^2 = 6.79$, $p = 0.03$). The perception was however not associated with being more than 24 years $X^2 = 2.41$, $p = 0.30$), being a female sex (Fisher's $p = 0.19$) or with the students' self- perception of having a good understanding of physiology (Fisher's $p = 0.22$). On binary logistic regression in a model consisting of all the five factors, being of female sex, and in 300 level of study emerged as the independent predictors of the students' perception of physiology as shown in Table 4.

DISCUSSION

All students admitted into the undergraduate courses in the college of health science of Bayero University fulfilled the pre-entry cut-off points of 180 minimum in the Joint Admission and Matriculation Board (JAMB) examination for the physiology and allied programs and 220 for the MBBS and BDS programs. The MBBS/BDS applicants who could not secure admission into the respective courses were assigned to study anatomy or physiology courses. Along the line, this category of students tends to lose interest in their new course and hence pay little attention to it. The teaching of physiology subject to all the programs in the faculty is based on the respective curricula, and students are taught and examined separately in three different groups: MBBS/BDS class, Physiology class and Allied Sciences class. The MBBS/BDS students are examined using essay and multiple choice question (MCQ) formats, and the questions are strictly based on their curriculum. On the other hand, the physiology students are examined using essay questions while the allied science students answer MCQ only.

Although most of the students interviewed in this study claimed to have good to excellent understanding of the physiology courses, wrong perception about the subjects prevails (83.2%) indicating having challenges with understanding. Previous studies from a South African medical school reported that students felt that they lacked the basic conceptual foundations essential for the learning and understanding of physiology, since the difficulties that the students identified were mainly terminological and conceptual in nature (Tufts & Higgins-Opitz, 2009). Our findings generally show that the students find physiology relevant to other clinical courses and useful in their future career but somehow face challenges that affect their performance in the course. A similar survey from a Sudanese medical school revealed that most of the students (90.7%) perceived lectures as the most valuable academic activity and 85% believed that their absence from different academic activities could affect their performance (Kaddam *et al*, 2012).

The teaching of physiology in BUK has traditionally been hampered with a lot of negative drawbacks affecting the performances of both the lecturers and the students. Majority of teachers use the traditional face-to-face method largely due to limited resources, lack of adequate ICT knowledge and poor electricity supply. Despite these constraints, many institutions in Africa are increasing students' intake (Sofola, 2014). Mullan *et al.,* (2011) argue that, students enrollment in medical schools ranged from 20 to more than 600, whereas the number of lecturers ranged between 2 and 18, making it difficult for the lecturers to cope with the large volume of work. The situation is worst considering the population expansion in Nigeria and the need to increase internally generated revenue by

most of the Nigerian Universities, further widening the Lecturer – Student ratio and limiting the capacity of the academic staff to impart requisite knowledge on the students. Low lecturer-to-student ratio and large class sizes were identified as common causes of "non-ideal" lecture rooms and facilities as well as poor supervision during practical classes (Mullan *et al.*, 2011). This study observed that lecturer/ teaching factor was the most common factor leading to poor students' performance in physiology (Table 2). This is corroborated by the findings of Anyaehie *et al.*, (2011). Contrarily, previous studies from Europe reported that Lecturers' factor is the least cause of poor performance of students (Micheal, 2007; Carter & Brickhouse, 2004; Sozbilir, 2004). This discrepancy is perhaps due to better technological advancement in teaching that gives students less physical contact with staff but still provides better access to academic resources e.g the use of modern instructional gadgets such as videos, YouTube, and electronic blackboard. Furthermore, well developed structured pedagogy and robust staff welfare and remuneration in the developed countries are among important reasons that shape attitude of staff towards teaching and the students.

Bulky course content, students' factors including lack of seriousness/ interest in the course, and inadequate infrastructure and equipment have severally been cited as sources of poor students' performance in physiology subject (Abour, *et al*, 2004; Carter & Brickhouse, 2004; Sozbilir, 2004; Micheal, 2007). The findings of this study may not be generalizable to all the students studying physiology because participants were only sampled from 200 and 300 level students.

This study observed that most of the students (91.8%) claimed to have scored 50% and above in the continuous assessment for physiology subject, but only 16.8% think positively about the course. The lecturer/teaching factor was identified as the most common factor associated with poor performance of the students in physiology. In view of the finding of the study, guidance and counseling through the level coordinators and the mentor-mentee programme should be strengthened in all departments. Basic infrastructure and equipment, and teaching techniques should also be upgraded.

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Deteriorating Hemostatic Functions of Adult Female Wistar Rats Mediated by Activities of Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) – Piroxicam and Vitamin E

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Summary: The status of hemostatic parameters, are useful physiological markers of organ and tissue damage and dysfunction. This study investigated the effect of Piroxicam on some hemostatic parameters of albino Wistar rats. Twenty-four (24) female albino Wistar rats were used for this study, they were randomly divided into four (4) groups of six (6) rats each. Group A served as control, Group B and C were rats treated with 0.1 mg/kg and 0.2 mg/kg piroxicam while Group D served as 0.2 mg/kg piroxicam treated rats administered with Vitamin E. The experiment lasted for a period of 4 weeks, after which the rats were euthanized. Blood sample was collected for measurement of bleeding time, clotting time, fibrinogen level and platelets count. One-way ANOVA was used to compare, means and a $p<0.05$ was considered significant. **Result:** Data generated showed that Piroxicam significantly ($p<0.05$) decreased the clotting time, platelets count and fibrinogen level. Piroxicam also significantly ($p<0.05$) increased the bleeding time level of the rats. Co-administration of Vitamin E significantly ($p<0.05$) increased the bleeding time, it also significantly decreased the clotting time, fibrinogen level and platelets counts. **Conclusion:** This study therefore shows that Piroxicam impairs hemostasis while Vitamin E administration further enhances the activities of Piroxicam on hemostatic parameters.

Keywords*:* Hemostasis, NSAIDs, Piroxicam, Bleeding Time, Clotting Time, Fibrinogen.

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INTRODUCTION

Some synthetic agents including non-steroidal anti-inflammatory drugs have been found valuable in management of heart attacks and other complications of cardiovascular disorders (Handoll, Farrar, McBirnie et al., 2002). Non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen, indomethacin, aspirin, and naproxen are used as analgesics and anti-inflammatory agents and produce their therapeutic effects through the inhibition of prostaglandin synthesis (Klaassen, 2001). The drugs (NSAIDs) provide effective relief of pain and inflammation caused by a variety of clinical disorders, including arthritis, nonarthritic musculoskeletal conditions, dysmenorrhea, and headache (Taubert, 2008; Simmons, Botting, and Hla, 2004). Among the NSAIDS used in dentistry, piroxicam, a non-selective COX inhibitor, has been extensively studied (Barroso, Lima, Guzzo et al., 2006; Graziani, Corsi, Fornai et al., 2005). Piroxicam is a strong inhibitor of cyclooxygenase-2 (COX-2). It is known as a non-steroidal anti-inflammatory drug (NSAID) that has an analgesic effect. The effectiveness of piroxicam as an anti-inflammatory agent is likely due to the inhibition of PGE synthesis (Starek and Krzek, 2009).

Haematological parameters are a very important diagnostic tool and used as a routine clinical evaluation of health as well as an essential factor for surgeons and anaesthetist before initiating any surgical procedure (Saliu et al., 2012). The status of haemostatic parameters, are useful physiological markers of organ and tissue damage and dysfunction, but blood clotting, bleeding time, platelet level and prothrombin level has been used in the past to assess both the intrinsic and extrinsic pathways of coagulation cascade (Laffan and Bradshaw, 2000). Preoperative haemostasis evaluation has always been a significant factor in the minds of surgeons and anaesthetists before taking any case for surgery (Bharadwaj, 2001).

Evaluation of blood groups, bleeding and clotting time are the most important and initial haematological parameters investigated. Bleeding Time (BT) and Clotting Time (CT), are significant to study clinically. Bleeding time is mainly a test to assess the platelet adhesion and aggregating. It is found to be prolonged significantly in platelet defects, either congenital or

acquired (Issitt, 1985). While the time interval between the blood vessels puncture and fibrin threads formation is called clotting time (Franchini, Capra, Targher et al., 2007). These two parameters, although considered by many as obsolete provides enough information about platelet activation and function and may serve as a means of accessing clinical conditions such as disseminated intravascular coagulation (DIC), von Willebrand Disease (vWD) and thrombocytopenia. With increasing prescription of NSAIDs by physicians, and recent documentation of high haemostatic disorders prevalence, this study therefore aim to assess the effect of Piroxicam on some haemostasis parameters.

MATERIALS AND METHODS

Piroxicam was sold under the trade name Dolonex DT® and Vitamin E (α-tocopherol) tablets were purchased from the local chemist shop in Abraka, Delta State, Nigeria.

Ethical Consideration

Prior to the commencement of the study, ethical approval was sought and obtained (FBS/PHS/RBC/013) from the Research and Bio-ethical Committee of the Faculty of Basic Medical Sciences, College of Health Sciences, Delta State University, Abraka, Nigeria.

Study Design

The study is experimental in nature. A total of twenty-four (24), 12±2 weeks old female albino Wistar adult rats weighing between 180 ± 20 g from the breeding colony in the Animal House of the Department of Physiology, Delta State University, Abraka were used for the study. The animals were housed in wooden cages with wood shavings as bedding and allowed two (2) weeks for acclimatization. Subsequently, the rats were weighed and randomly divided into four groups (n = 6). Group A (Control) were fed with normal rat chow and water, Group B treated with 0.1mg/kg Piroxicam, Group C treated with 0.2 mg/kg Piroxicam, and Group D treated with 0.2 mg/kg Piroxicam + 150 mg/k Vit E. Freshly dissolved tablets of Piroxicam and Vitamin E were administered through oro-gastric cannula once daily for twenty-eight (28) days.

Sample Collection

At the end of 28 days of treatment, the bleeding and clotting time were assessed in rats restrained manually, after which the animals were euthanized via cervical dislocation. Two (2) mls of blood samples were collected via the common carotid into anti-coagulant free containers. collected from the retro-bulbar plexus of the medial canthus and amputating 5 mm of the tail tip with a scalpel blade.

Bleeding Time determination

Bleeding Time was determined using a modified Duke method (Ochei and Kolhatkar, 2000). A skin puncture was made quickly using disposable lancet and the stopwatch was started as soon as bleeding started. The puncture was dabbed with blotting paper every 15 seconds until there was absence of blood stains on the blotting paper. Bleeding time was recorded as the time when there was stoppage of blood flow from the puncture.

Clotting time estimation

Clotting time was determined using capillary glass tube method. A standard incision was made in the skin of the ear and the blood was taken into a capillary glass tube and the time of collection was noted. Pieces of capillary glass were broken from one end at every thirty seconds and the appearance of fibrin threads was used as the end point and the time was noted in seconds (Harris et al., 1956).

Platelet Count Determination

Platelet concentration was assessed under 40x magnification, scan to ensure even distribution. Platelets were counted in all twenty-five small squares within the large center square. Platelets appear greenish, not refractile. Count cells starting in the upper left of the large middle square. Continue counting to the right hand square, drop down to the next row; continue counting in this fashion until the total area in that middle squares) have been counted. Count all cells that touch any of the upper and left lines, do not count any cell that touches a lower or right line. Count both sides of the hemacytometer and take the average.

Fibrinogen analysis

Plasma fibrinogen concentration was determined as defined by the clot weight method of Ingram (1961). Blood was first collected into sample vials containing 3.2% sodium citrate in the ratio 1:9 with blood. Blood plasma was obtained by centrifuging blood in a stopped vial at 1000 g for 10 min. 0.2 ml of the test plasma was put into a test tube and incubated in a water bath for 3 min at 37°C. 0.2 ml of thrombin time-reagent was added to test plasma, mixed and the clot formed harvested with a wooden applicator stick. The resulting clot was transferred into a tube containing acetone to dry and harden for about 10 min; the acetone was decanted and the clot placed on a filter paper for the remaining acetone to evaporate. The clot was then recovered and weighed. The process of fibrinogen concentration determination was completed within 3 h of blood collection. Thus, fibrinogen concentration of citrated plasma in mg/dl equals clot weight (mg) divided by plasma volume (dl).

Statistical Analysis

Data were analyzed for mean, and standard deviation. Comparison for significance between the control and experimental group were analysed using One-Way Analysis of Variance (ANOVA) with Scheffe's Post

Hoc test. The level of significance for all experiment was $p< 0.05$.

RESULTS

Effect of Piroxicam on Bleeding Time

It was observed that bleeding time increased in a dose dependent manner following administration of graded doses of piroxicam. The mean bleeding time in control group was 7.50 ± 1.11 mins while those of group B and C were 8.33 ± 1.11 mins and 9.17 ± 1.00 mins respectively. The mean bleeding time of rats in Group D was 10.00 ± 1.16 mins. These changes in bleeding time due to the effect of Piroxicam were not significant in this experiment.

Effect of Piroxicam on Clotting Time

The mean clotting time in the control group was 127.00 ± 23.76 s while those of group B, C and D were 90.00 ± 13.40, 71.01 ± 6.32 and 60.00 ± 5.22 s respectively as shown in Fig 2. Piroxicam administration caused a dose dependent decrease in the clotting time of the albino Wistar rats with significance ($p<0.05$) recorded in a higher dose 0.2mg/Kg of Piroxicam. Further decrease in clotting time was

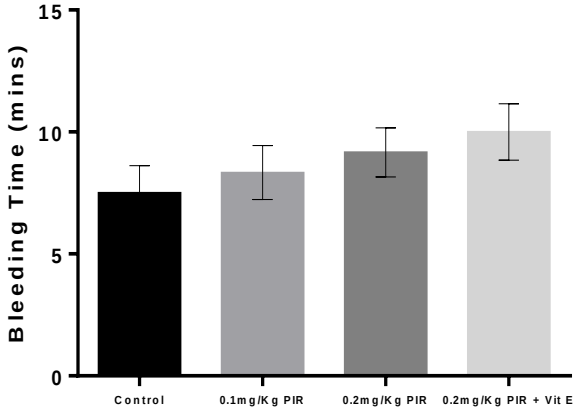

Fig 1 Effect of Piroxicam on Bleeding Time

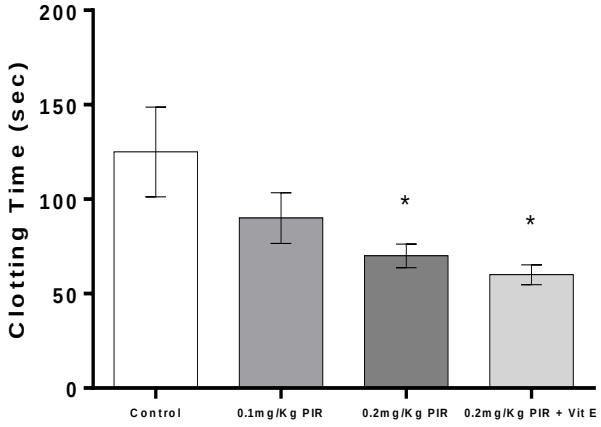

Fig 2 Effect of Piroxicam on Clotting time. *:significance ($p<0.05$) when compared to control;

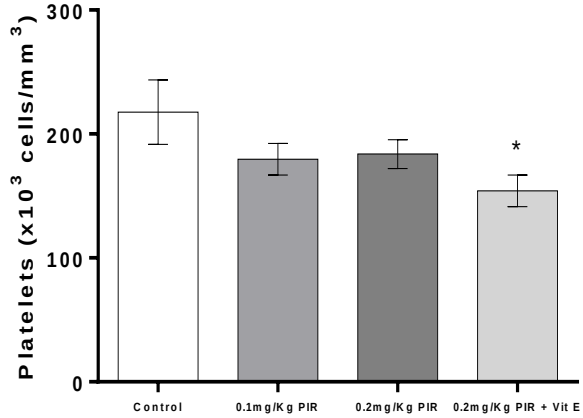

Fig 3 Effect of Piroxicam on Platelet count
*:significance ($p<0.05$) when compared to control;

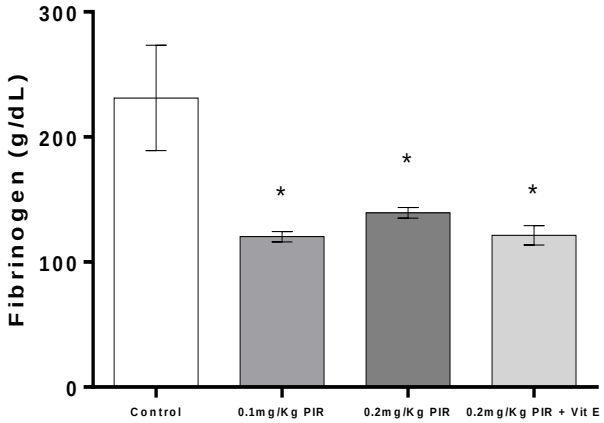

Fig 4 Effect of Piroxicam on Fibrinogen level. (n=6)
*: significance ($p<0.05$) when compared to control;

observed following administration of Vitamin E to the 0.2mg/Kg Piroxicam treated rats. This Vitamin E effect on the clotting time was also significant ($p<0.05$) when compared to control.

Effect of Piroxicam on Platelet count

The mean platelet concentration in the control group was 217.67 ± 25.90 $x10^3/mm^3$ while those of group B, C and D were 179.67 ± 12.82, 183.83 ± 11.69 and 154.16 ± 12.74 $x10^3/mm^3$ respectively as shown in Fig 3. Piroxicam decreased the platelets count, and the co-administration of Vitamin E enhanced the effect of piroxicam by showing significant ($p<0.05$) decrease in platelets count.

Effect of Piroxicam on Fibrinogen level.

The mean plasma fibrinogen in control group was 231.17 ± 42.40 g/dl while those of group B and C were 120.33 ± 4.15 and 139.33 ± 4.32 g/dl respectively. The mean fibrinogen of rats in Group D was 121.33 ± 7.67 g/dl. It was observed that piroxicam caused significant ($p<0.05$) decrease in fibrinogen level when compared to control. It was observed that co-administration of Vitamin E did little in changing the effect of 0.2 mg/kg of piroxicam on fibrinogen level as fibrinogen

concentration remained at the range of fibrinogen level of piroxicam treated rats, significance was also recorded when compared to control.

DISCUSSION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are one of the most commonly prescribed and self-administered drugs (Kaur, Singh, Bassi et al., 2015). However, up to 50% of patients taking these medications experience some variety of alteration in hemostasis (Wolfe 1999). This study showed the effect of piroxicam on the bleeding time, clotting time, platelets count and fibrinogen level. It was observed that piroxicam increased the bleeding time especially at higher doses. The increase in bleeding time may likely be as a result of decrease in platelets count reported in this study.

Cronberg et al. (2004) showed the effect of other NSAIDs on platelets function show similarities with piroxicam induced effect in this study. Ibuprofen inhibits platelet aggregation and thromboxane A2 synthesis. Ha et al (1999) showed that Ibuprofen inhibited platelet aggregation at 1.5, 3, and 6 hours after a single 800-mg oral dose. A single dose of ibuprofen between 300 mg and 900 mg blocked platelet aggregation 2 hours after administration; however, the effect was lost within twenty-four (24) hours (Mcintyre et al., 2008). Effect of Indomethacin, which is as potent inhibitor of platelet thromboxane production occurred within 2 hours of drug ingestion and persist for up to 8 hours. (Cronberg et al., 2004). Since most NSAIDs have similar properties, it is therefore understandable that decrease in the platelets count in this study could be as result of piroxicam inhibiting effect of platelets production and thus reducing its aggregating strength.

This was further confirmed by the increase in bleeding time following administration of graded doses of piroxicam. The normal BT by Duke's filter paper method is usually in the range of 1–5 min (Pal and Pal, 2010). These values were well exceeded due to the anti-thrombocytes activities of piroxicam. Data from this study was also in accordance with previous studies of other NSAIDs effect on bleeding time. Ibuprofen, Diflunisal (Green et al., 2001), Indomethacin (Taivainen, et al., 2009), and Ketorolac (Conrad, Fagan, Mackie et al., 2008) diclofenac (Taivainen, et al., 2009) all caused prolongation of bleeding time.

It might be expected that simultaneous inactivation of platelet and endothelial cyclooxygenase by Piroxicam would have deleterious effects on haemostasis by blocking thromboxane A2 and prostacyclin formation. The significant ($p<0.05$) decrease in fibrinogen following piroxicam administration, shows that piroxicam influence over haemostasis is not only platelet dependent. In support to the current finding, Danesh et al. (2005) showed other NSAIDs have a decreasing effect on fibrinogen. In their study, fibrinogen levels were lower in patients taking celecoxib and non-selective NSAIDs but not significantly different in those taking rofecoxib compared to controls.

It was observed that Vitamin E acted as a permissive agent for piroxicam as it enhanced the effect of piroxicam on the various haemostatic parameters of this study. Vitamin E further reduced the platelet count and fibrinogen level with significance ($p<0.05$). The possible explanation for the platelet incorporation of vitamin E both in vitro and in vivo leads to dose-dependent inhibition of platelet aggregation (Freedman et al., 1996). Another possible mechanism of Vitamin E inhibition on haemostatic function is the antagonistic interaction with Vitamin K, a known contributor to coagulation (Dowd and Zheng, 1995).

Piroxicam had similar effect to other NSAIDs on haemostatic parameters by decreasing the clotting time, platelets count and fibrinogen level whilst increasing bleeding time. With the widespread use of NSAIDs (Piroxicam) for a variety of clinical indications, physicians must be aware of their potential to cause bleeding complications. The chronic use of even very low dose Piroxicam can produce maximal inhibition of platelet function and primary haemostasis.

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Ameliorative Effects of *Raffia hookeri* Pulp Extract on Cisplatin-induced Brain Damage and Consequent Neurobehavioural Changes in Wistar Rats

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Summary: Cisplatin (CIS), a known anticancer drug, has side effects initiated by oxidative damage which hinders its use. *Raffia hookeri* pulp extract (RHPE), reported to possess antioxidant activity should mitigate cisplatin toxicity. The present study examined the potential of RHPE to reduce brain damage in rats exposed to cisplatin. Forty eight female rats (150 g – 220 g) were randomized into four groups (n = 12) viz: Group 1 served as control received distilled water daily, Group 2 received 100 mg/kg body weight of RHPE, Group 3 received CIS (7.5 mg/kg body weight, intraperitoneally) as single dose, Group 4 received 100 mg/kg body weight of CIS+RHPE. The RHPE was given orally via gavage for 14 days while the single dose of cisplatin was administered on the eighth day of experiment. Behavioral tests namely: transitions, rearings, groomings and forelimb grip strength were carried out on 15th day of the experiment after which rats were euthanized followed by histology and histomorphometry. Cisplatin significantly ($p<0.05$) reduced the percentage body weight changes, transitions, rearings, groomings and forelimb grip strength compared with the control group, whereas treatment with CIS+RHPE significantly ($p<0.05$) increased these parameters compared with Cisplatin treatment. Cisplatin also caused histological alterations of Purkinje neurons, pyramidal neurons of Cornu ammonis3, granule cells and cerebral cortex neurons. It significantly ($p<0.05$) reduced the diameter of Purkinje (9.1±0.59 µm) compared with control (14.41±0.31 µm) and pyramidal neurons (11.32±0.05 µm) compared with control (17.03±0.54 µm). Rats in the CIS+RHPE had their histology considerably improved compared with those of cisplatin. In conclusion, RHPE reversed the behavioural changes and demonstrated neuroprotection against CIS-induced behavioural changes and microanatomical alterations of cerebellar, hippocampal and cerebral neurons.

Keywords*:* Cisplatin, *Raffia hookerii*, neuroprotection, neurons, behavioural tests.

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INTRODUCTION

Cisplatin (cis-DDP cis Diammine Dichloro Platinum (II)), a platinum-based drug is used as an antineoplastic agent with wide spectrum of use in various tumors including the lung, kidney, ovary, testis, bladder, head, neck, and brain (Mokhtari et al., 2012; Sanchez-Gonzalez *et al.* 2011). The antitumor activity of Cisplatin (CP) has been linked to its ability to form adducts with DNA, which could cause cross-linking of DNA strands (Dasari and Tchounwou, 2014; Hasheem et al., 2015). However, the use of CP is restricted due to its various side effects such as neurotoxicity, nephrotoxicity, ototoxicity, hepatotoxicity and testicular toxicity. These effects have been attributed majorly to the generation of reactive oxygen species (ROS) which could interfere with the antioxidant defense system (Tsimberidou et al., 2010).

Raffia palm leaf (*Raphia hookeri)*, a monocot belonging to the family Arecaceae is usually found in lowland swamps throughout Western and Central Africa, Asia and South America (Oduah and Ohimain, 2015). All parts of the plant are well utilised by locals for various things ranging from building materials as twine, rope; personalized items like baskets, placemats, hats, shoes to consumables like oil, wine and food (Akinola et al., 2010; Afolayan et al., 2014). Its fruit is large, cone-shaped with a single hard nut having an outer layer of overlapping reddish brown scales and in-between the outer layer of scales and the hard seed is a yellow, mealy, oil-bearing mesocarp or pulp (Mbaka *et al.,* 2012). The pulp extract of *Raphia hookeri* was shown to contain vitamins C and E, carotenes, niacin, alkaloid, saponins, flavonoids and phenols which explains its antioxidant activity (Edem et al., 1984; Akpan and Usoh, 2004; Dada et al., 2017). Flavonoids and tannins as phenolic compounds in plants are a major group of compounds that act as primary antioxidants by scavenging free radicals (Polterait, 1997).

The mammalian cerebral cortex is associated with cognitive and motor control. The hippocampus is involved in emotions, behaviour, memory coding and storage, while the cerebellum is the integrative center

for voluntary skeletal muscle control and equilibrium. The cerebellum also co-ordinates different muscle groups so that the muscle movements are fluent and precise (Fonnum and Lock, 2000; Afifi and Bergman, 2005; Ellis, 2006). The involvement of these important brain components in cisplatin toxicity may alter their microscopic anatomy and physiology as previously demonstrated (Owoeye and Onwuka, 2015; Owoeye et al., 2015). There is scanty information on the effect of the pulp extract on neural tissue. We hypothesized that the antioxidant activity of *Raphia hookeri* pulp extract (Edem et al., 1984; Akpan and Usoh, 2004; Dada et al., 2017) should be able to reduce oxidative damage that accompanies cisplatin-toxicity in brain tissue thus minimizing its effect.

The present study was carried out to assess the possible ameliorative effect of *Raphia hookeri* pulp extract (RHPE) in cisplatin-induced neurotoxicity and so answer the research question of whether RHPE may mitigate the neurotoxic effect of cisplatin in a Wistar rat model.

MATERIALS AND METHODS

Extraction Procedure

Ripe fruits of *Raphia hookeri* (RH) were obtained from the swamps of Oke Odan, Apete, Ibadan, Nigeria in December, 2016 and authenticated at Forestry Research Institute of Nigeria, Ibadan, Nigeria with FHI number 110540. The hard, tough and scaly exocarp of the fruits were removed and discarded and the soft, mealy mesocarps (pulp) scraped from the seeds. Extraction of the pulp was carried out applying a modified method of Afolayan et al. (2014). Briefly, 1 kg of the dried pulp was grounded into powdery form was soaked in 2.5 L of pure ethanol and stirred at 2 hours interval. After the first collection was filtered the pulp powder was soaked again in 1.5 L of ethanol and stirred at 2 hourly intervals for 72 hours to allow complete extraction. The ethanol solvent containing the extract was collected using muslin bag after which the effluent was further filtered using Whatman filter paper 1 and the filtrate then concentrated using rotary evaporator set at 40^{o}C after which it was stored in an air tight bottle and kept in a refrigerator at 4^{o}C till used. The final yield termed *Raphia hookeri* pulp extract (RHPE) was 130.8 g gave a percent yield of 13%.

Phytochemical screening

Phytochemical screening was performed using standard procedures (Sofowora, 1993). The pulp was screened for flavonoids, alkaloids, saponins, tannins, terpenoids, anthraquinones and cardiac glycosides.

Experimental animals

Forty-eight adult female Wistar rats weighing between 150 - 220 g were obtained from the Animal House of the College of Medicine, University of Ibadan, Nigeria. They were acclimatized at the Department of Anatomy, University of Ibadan, for two weeks before being assigned randomly to experimental and control groups using random numbers. They were housed in clean transparent plastic cages (39 x 29 x 27 cm) with wood shavings as bedding and were fed with rat chow and water *ad libitum*. Animals were humanely handled according to the acceptable guidelines on the ethical use of animals in research (Public Health Service, 1996). In chemotherapy, the use of drugs including cisplatin for cancer treatment might involve both male and female patients. It is needful therefore to mimic this true life exposure of females to cisplatin, hence we designed this present study using female rats as has been reported (Akman et al., 2015; Kumar et al., 2017).

Chemicals and drugs

Cisplatin manufactured by Korea United Pharm. Inc. (Naojang, Chungnam, Korea) and Ketamine hydrochloride was manufactured by Rotex Medica, Trittau, Germany were purchased from Kunle-Ara Pharmacy, Ibadan, Nigeria.

Research Design

The forty eight adult female rats were randomized into six groups of twelve animals each as follows:

Group 1 (n=12): CTRL, 0.3 mL distilled water daily, served as control.

Group 2 (n=12): RHPE, 100mg /kg body weight of *Raphia hookeri* pulp extract

Group 3 (n=12): CIS, Cisplatin at single dose 7.5 mg/kg body weight, i.p.

Group 4 (n=12): CIS+RHPE, 100mg /kg body weight *Raphia hookeri* pulp extract + Cisplatin 7.5 mg/kg body weight, i.p. as single dose.

The RHPE administration was oral via gavage for 14 days while the single dose of cisplatin at 7.5 mg/kg body weight, i.p. was administered on the eighth day of experiment, 1 hour after administration of RHPE extract. The dosage and route of administration of cisplatin were based on the method of Ko *et al.*, (2014), whereas those of RHPE was according to Mbaka *et al.* (2013).

Behavioural tests

Behavioural tests were performed on 6 rats in each of the groups of animals on day 15 after weighing each rat.

Open field test: Rats were placed in the center of the open field and allowed to explore the open field box for 5 minutes, after which, rats were returned to their cages and the floor of the box was cleaned with 70% ethyl alcohol and permitted to dry between tests to eliminate olfactory bias. This test assessed horizontal locomotion (number of squares crossed), and vertical locomotion (number of rearings) and grooming (number of times the rat cleaned its body)

(Mohammad et al., 2010). Each animal was given two trials at 30 minutes interval and the average taken.

Forelimb Grip Strength Test: It involves the forepaws of the rats being placed on a horizontally suspended metal wire of 2 mm in diameter and 1 m in length, placed one meter above a landing area filled with soft bedding. Given a maximum time of 2 minutes, the length of time each rat was able to stay suspended before falling off the wire was recorded. Each animal was given two trials at 30 minutes interval and the average taken. This test reflects forelimb muscular strength in the animals (Tamashiro *et al.*, 2000).

Sacrifice and Sample collection

After the behavioural tests, rats weighed and were thereafter euthanized using Ketamine 100 mg/kg intraperitoneally and euthanized by transcardiac perfusion with 10% neutral buffer formalin after an initial wash off with 200 mL of normal saline. The perfused rat was then laid prone on the dissecting board, cranium was opened and the whole brain carefully excised, rinsed in normal saline and weighed. The cerebellum was dissected out and the cerebral hemisphere divided into two sagittally and then fixed in 10% formalin for histological analysis.

Relative brain weight was calculated by the equation: weight of whole brain (g) / final body weight of rat (g) x 100.

Histology and Histomorphometry

The tissues were processed at the Histological Laboratory, Department of Anatomy University of Ibadan, Nigeria. Rats' brain specimens were processed through the stages of fixation, dehydration, clearing, infiltration, embedding and thereafter sectioned at 6 µm thickness with a Rotary Microtome (Leica RM2125 RTS, Germany). The ribbons were stained with haematoxylin and eosin according to the method of Bancroft and Gamble (2008) to demonstrate general histology of the brain and possible microscopic alterations. After 24 hours, the perfused brains separated for Golgi staining were immersed in potassium dichromate solution for 5 days (5 changes every 24 hours) and then silver nitrate for 3 days (3 changes every 24 hours). Thereafter tissues were infiltrated for 30 minutes in molten wax, embedded in paraffin wax and cooled overnight at 4^{o}C. The paraffin blocks were trimmed and sectioned at 60 µm, transferred into graded series of alcohol (80%, 90%, and two changes of 100%) for 2 minutes and cleared in xylene for 10 minutes. Tissues were thereafter mounted on glass slides using DPX as mountant. Thereafter, slides were viewed using Leica DM 500 digital light microscope (Germany) and images captured with Leica ICC50 E digital camera (Germany). Histomorphometric analyses were done using computerized image analyzers (Image J/Micro-Manager 1.4 and Digimizer Image Analysis Version 4.6.1). Using an objective lens (x 40) and an ocular lens (x 10), the viable and pyknotic neurons of the cerebral cortex, cornu ammonis3 (CA3) and frontal cerebral cortex of the brain were observed and counted. The pyknotic index (PI) according to the method described (Taveira et al., 2013), was calculated for in ten different areas of the slides of each of the interest area by 2 observers working independently. Photomicrograph calibrations were done using Image J/Micro-Manager 1.4 (Edelstein et al., 2014).

Statistical Analysis

Data was expressed as Means ± Standard Error of Mean (SEM). Significant differences between groups were calculated using Student's t-test and $p<0.05$ was considered statistically significant.

RESULTS

Phytochemical screening

The phytochemical screening of RHPE conducted showed the presence of flavonoids, terpenoids, saponins, alkaloids, steroids and tannins but tested negative for the presence of cardiac glycosides and anthraquinones.

Effects of RHPE on body weight and relative brain weight in rats treated with cisplatin

The immediate 48 hours following cisplatin administration, the rats had diarrhoea and weakness, but thereafter picked up gradually. There was significant percentage weight reduction in the CIS group compared with CTRL ($p<0.05$) whereas there was a significant increase in the RHPE compared with CIS group (Table 1). However, the relative brain weight alterations were not significant (Table 1).

Table 1: Effect of Cisplatin and RHPE on the body and brain weight changes in rats

Group	CTRL	RHPE	CIS	CIS+RHPE
Initial body Wt.	162.5±8.4	162±7.5	167±7.5	200.5±14.4
Final body Wt.	189±11.3	187±10.2	148±7.5	170±8.5
Body Wt. changes	26.5±1.5	25±1.3	19±1.1	30.5±1.7
% Wt. changes	16.3	15.4	11.4*	15.2$^{\#}$
Brain Wt.	1.69±0.02	1.63±0.02	1.63±0.02	1.71±0.03
R.B.W	0.89	0.87	1.10	1.01

Values are presented as Mean ± Standard error of mean for six rats per group. CTRL, Control, RHPE; *Raphia hookeri* pulp extract; CIS, cisplatin only, CIS+RHPE, cisplatin + *Raphia hookeri* pulp extract, Wt- Weight (g), R.B.W.-Relative brain weight. * P< 0.05 versus Control group, # P< 0.05 versus CIS group.

Effects of RHPE on behavioural and forelimb grip strength test in rats treated with cisplatin

Cisplatin significantly ($p<0.05$) reduced the transitions, rearings, grooming when compared with the control group as shown in Figure 1. Control values of number of (transitions 35±2.0; rearing 10.5±0.2 and grooming 10±0.5) were reduced by CIS (17±0.8, 5.5± 0.4 and 4.0±0.16 respectively). Similarly CIS significantly ($p<0.05$) reduced the duration of the forelimb grip strength to 2.2±0.3 seconds when compared with control of 4.9±0.58 seconds. However, pretreatment with RHPE significantly ($p<0.05$) ameliorated these changes as observed in the CIS+RHPE rats relative to CIS group findings for all the parameters with the exception of duration of the forelimb grip strength as depicted in the figure.

Effects of RHPE on the histology of cerebellar cortex, cornu ammonis3 (CA3), dentate gyrus (DG) and cerebral cortex in rats treated with cisplatin

The histology of the cerebellar cortex in the Control group (Figure 2) exhibited normal cytoarchitecture with three normal layers namely molecular, Purkinje and granular and the Purkinje cells (Pc) exhibiting basophilic nuclear and normal shapes. The Pc of the CIS group exhibited features of degenerative alterations ranging from deeply eosinophilic cell bodies, shrinkage of the neuronal bodies with loss of their regular outlines as in Figures 2C relative to those of the control. The representative photomicrographs of CA3, DG and cerebral cortex were similarly normal in all groups except the CIS groups which showed neuronal alterations when compared with the control as depicted in Figures 3, 4 and 5. It is observed that histologic features were returned to near control in CIS+RHPE groups with their neurons large, rounded or oval with open chromatin pattern and some with nucleoli. Plate 5 showed that the dendritic arborization of pyramidal neurons of frontal were reduced in CIS group.

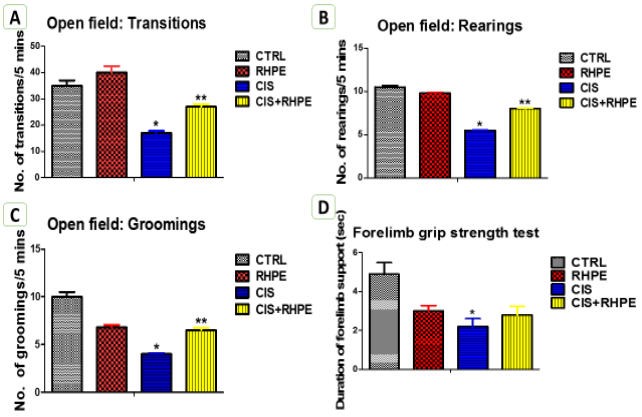

Figure 1: Histogram showing the effect of RHPE on behavioural parameters and forelimb grip test in rats treated with CIS. Values are presented as Mean ± S.E.M. of six rats. CTRL, Control, RHPE; *Raphia hookeri* pulp extract; CIS, cisplatin only; CIS+RHPE, cisplatin + *Raphia hookeri* pulp extract. *p< 0.05 versus CTRL group, **P< 0.05 versus CIS group.

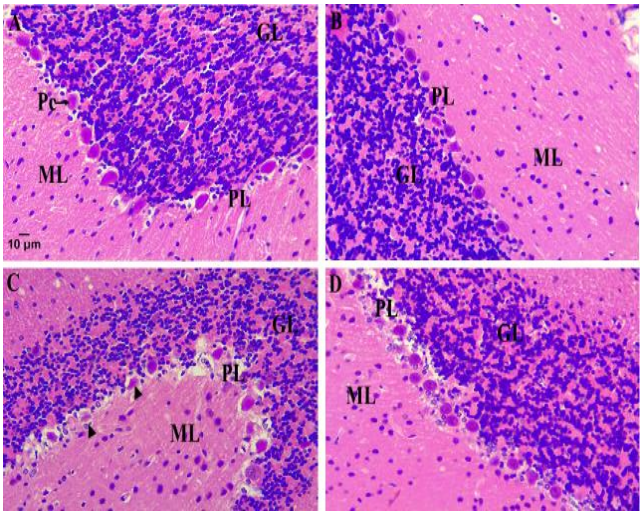

Figure 2: Representative stained sections of cerebellum of rats: (A) CTRL group (B) RHPE-treated (C) CIS-treated (D) CIS+RHPE treated. CTRL, Control; RHPE, *Raphia hookeri* pulp extract; CIS, cisplatin only; CIS+RHPE, cisplatin + *Raphia hookeri* pulp extract. ML, molecular layer; PCL, Purkinje cell layer; GL, granular layer. CIS-treated shows varying degree of degenerated Purkinje neurons (arrowheads). H&E. Scale bar = 10 µm for all figures.

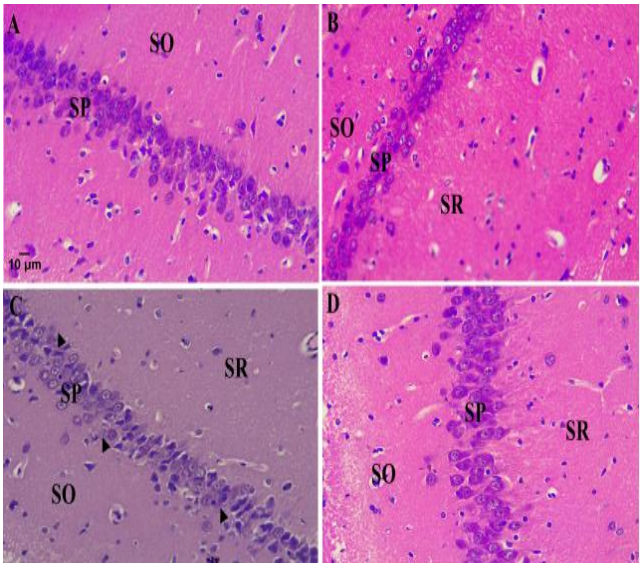

Figure 3: Representative stained sections of Cornu Ammonis 3 of rat hippocampus: (A) CTRL group (B) RHPE-treated (C) CIS-treated (D) CIS+RHPE treated. CTRL, Control; RHPE, *Raphia hookeri* pulp extract; CIS, cisplatin only; CIS+RHPE, cisplatin + *Raphia hookeri* pulp extract. SO, stratum oriens; SP, stratum pyramidalis; SR, stratum radiatum. Dark pyramidal neurons (arrowheads) are noted in CIS-treated brains. H&E. Scale bar = 10 µm for all figures.

Effects of RHPE on the histomophometry of Purkinje cells, Pyramidal neurons of frontal cerebral cortex and Pyknotic indices of frontal cerebral cortex and cornu ammonis3 in rats treated with cisplatin.

Figure 6 demonstrated that CIS significantly ($p<0.05$) reduced the diameters of Purkinje neurons of the cerebellum (9.10±0.59 µm) compared with the control (14.44±0.31 µm) and that of pyramidal neurons of frontal cortex (11.32±0.05 µm) when compared with the control (17.03±0.54 µm).

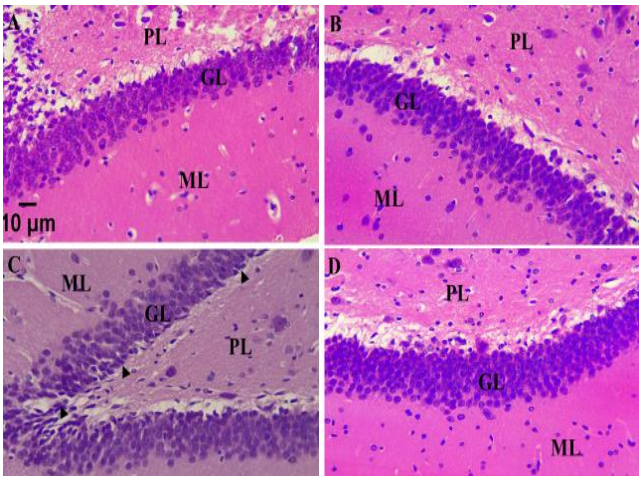

Figure 4: Representative stained sections of Dentate gyrus of rats: (A) CTRL group (B) RHPE-treated (C) CIS-treated (D) CIS+RHPE treated. CTRL, Control; RHPE, *Raphia hookeri* pulp extract; CIS, cisplatin only; CIS+RHPE, cisplatin + *Raphia hookeri* pulp extract. ML, molecular layer; PL, Polymorphic layer; GL, granular layer. Dark granule neurons (arrowheads) in CIS-treated brains are restricted to the innermost layer of cells. H&E. Scale bar = 10 µm for all figures.

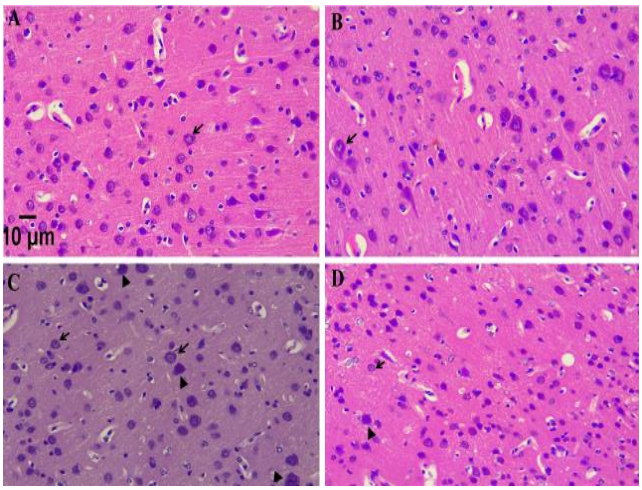

Figure 5: Representative stained sections of cerebral cortex of rats: (A) CTRL group (B) RHPE-treated (C) CIS-treated (D) CIS+RHPE treated. CTRL, Control; RHPE, *Raphia hookeri* pulp extract; CIS, cisplatin only; CIS+RHPE, cisplatin + *Raphia hookeri* pulp extract. Some of the CIS-treated cortical neurons exhibit pyknosis (arrowheads). H&E. Scale bar = 10 µm for all figures.

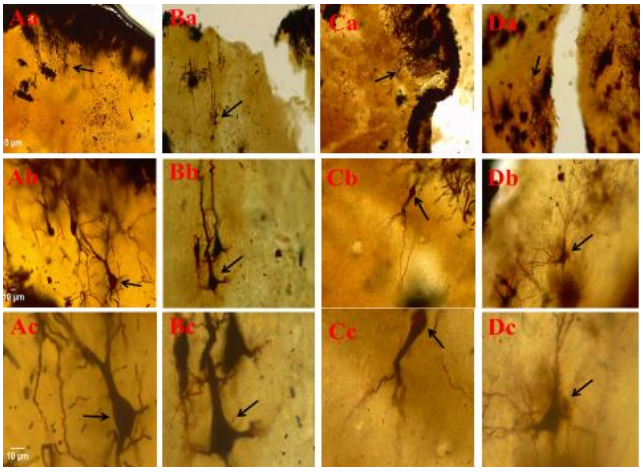

Figure 6: Representative Golgi stained sections of cerebral cortex of rats: (Aa-Ac) CTRL group; (Ba-Bc) RHPE-treated; (Ca-Cc) CIS-treated; (Da-Dc) CIS+RHPE treated. CTRL, Control; RHPE, *Raphia hookeri* pulp extract; CIS, cisplatin; CIS+RHPE, cisplatin + *Raphia hookeri* pulp extract. Upper panel, Aa-Da represents all the groups at ×100 magnification; middle panel, Ab-Db is at ×400 magnification while the lowermost panel, Ac-Dc is at ×1000 magnification. Dendritic arborization appears reduced in group C as shown in Cb and Cc. Scale bar = 10 µm for all figures.

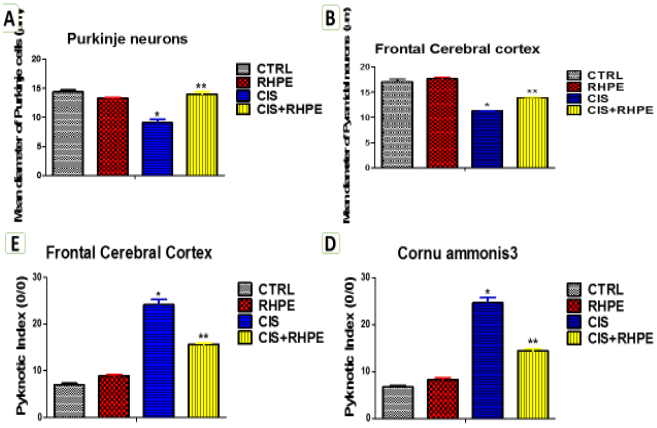

Figure 7: Histogram showing the effects of RHPE on the histomophometry of Purkinje neurons, Pyramidal neurons of frontal cerebral cortex and Pyknotic indices of frontal cerebral cortex and cornu ammonis3 in rats treated with cisplatin. Values are presented as Mean ± S.E.M. of six rats. CTRL, Control, RHPE; *Raphia hookeri* pulp extract; CIS, cisplatin only; CIS+RHPE, cisplatin + *Raphia hookeri* pulp extract. *p< 0.05 versus CTRL group, **P< 0.05 versus CIS group.

Similarly, CIS significantly ($p<0.05$) increased the pyknotic indices of both the pyramidal neurons of CA3 (24.62±1.12) compared with the control (6.7±0.33) and that of the frontal cortical neurons (24.14±1.09) relative to the control (6.93±0.38). However, all these parameters were significantly reversed to near control levels by pretreatment with RHPE when compared with CIS treatment.

DISCUSSION

The results showed that cisplatin (CIS) induced significant behavioural as well as micro-anatomical alterations in the cerebellum, cornu ammonis3 (CA3), dentate gyrus (DG), and frontal cerebral cortex (FCC) of adult albino rats.

The weight loss in the CIS group was possibly due to diarrhoea the rats had following CIS administration. This might be due to damage to water-absorptive mucosa epithelium of the rat colon from CIS injury. The water and possible salt loss might have caused the overall weight loss in agreement with previous studies (Ko et al., 2014; Ali et al., 2014; Adaramoye et al., 2016). The brain weight change in the groups were not significantly altered possibly because of the short duration of the diarrhoea and early recovery of the rats.

The reduction by CIS of both horizontal locomotion (number of lines crossed) and vertical locomotion (number of rearings) as well as forelimb muscle strength (duration of hanging on to the metal wire) by rats suggested possible reduction of the nervous and muscular activities of the rats which agreed with published reports (Ali et al., 2014; Owoeye and Onwuka, 2015). It was however, noted that co-treatment of CIS with RHPE reduced the effect of CIS alone thus ameliorating the reduction observed. The overall effect of these changes was to make the rats sluggish.

Damage to the Purkinje cells of the cerebellum as observed histologically agreed with previous reports of CIS on cerebellum (Bottone *et al.*, 2012; Owoeye and Onwuka, 2015; Owoeye et al., 2015). This damage posed a danger to the smooth and effective role the cerebellum performs as a motor stabilizing control system since it receives continual feedback information about intended movement and actual movements (Chaudhary et al., 2014). Degeneration of Purkinje cells, the principal efferent pathway of the cerebellum may lead to different forms of ataxia and an unstable gait (Kim *et al.,* 2009). Our findings of neural death in the cerebellum agrees with the report of induction of cell death *in vivo* and *in vitro* by CIS in both the cerebellum (Bottone *et al.,* 2012). The alteration of Purkinje neurons as shown in this study may be responsible for the associated behavioural and muscular strength changes observed.

The observations of CIS-induced alterations in rat CA3 and DG neurons showed distortion of the architecture of these hippocampal parts. Earlier reports indicated the inability of CIS to penetrate the blood-brain barrier (Gregg *et al.,* 1992). Further studies had shown that it could penetrate this barrier to induce neurotoxicity with histological alteration (Al Moundhri *et al.,* 2012; Gulec *et al.,* 2013; Owoeye and Onwuka, 2015; Owoeye *et al.,* 2015).

The effect of death of pyramidal neurons of CA3 and granule neurons of DG will be on the memory coding process in the brain as both will affect incoming perforant pathway projections from layer II of the entorhinal cortex. The death of granule neurons in the subgranular layer might affect the generation of new neurons as well as affect other cells of the dentate gyrus namely dentate pyramidal basket and mossy cells (Amaral *et al.,* 2007). The damage to mossy fibres from granule cells projecting to CA3 might also affect the quality of projections of Schaffer's collateral from CA3 to CA1 and its ultimate projection to subiculum and entorhinal cortex. We propose that the overall effect of these alterations, that is the elevated pyknotic index of CA3 pyramidal neurons and hippocampal components in general might lead to impairment of declarative memory formation, memory storage and behaviour of the rats (Ellis, 2006; Stepan *et al.,* 2015; Folarin *et al.,* 2017).

The observed death of FCC neurons, increased pyknotic index and reduction of dendritic arborization of the pyramidal neurons of the cortex in CIS-treated rats are in agreement with previous report of cisplatin injury in rat cortex (Karavelioglu *et al.,* 2015; Owoeye and Onwuka, 2015; Owoeye *et al.,* 2015). Since the final control of fragmented distal digital movements in mammals are controlled by the corticospinal tracts originating from the cortex, the death of cortical neurons induced by CIS might explain the reduced strength of the grip of the rats as observed in the forelimb grip test.

Cisplatin-induced neurotoxicity has been associated with histological damage (Al Moundhri *et al.,* 2012; Arrieta *et al.,* 2011) shown to be mediated via oxidative damage (Turan *et al.,* 2013). Brain tissue contains large amounts of long chain polyunsaturated fatty acids (PUFAs), low levels of antioxidants and high aerobic metabolism which make it very susceptible to oxidative stress (Ebokaiwe et al., 2013; Chaudahry et al., 2014). Our observation of reduction of the CIS-induced behavioural and micro-anatomic alterations in rats co-treated with RHPE suggested neuroprotection of Purkinje neurons, pyramidal neurons of CA3, granule neurons of DG of the hippocampal formation and neurons of the FCC. This supports the hypothesis of ameliorative potential of RHPE as shown in the CIS+RHPE group. *Raffia hookeri* pulp has been shown to possess antioxidant property (Edem et al., 1984; Akpan and Usoh, 2004; Dada et al., 2017) due to its flavonoid contents, but we did not study anti-oxidative property in this study.

Taken together, RHPE reversed the behavioural changes and demonstrated neuroprotection against cisplatin-induced and micro-anatomical alterations of cerebellar, hippocampal and cerebral cortex neurons possibly through its antioxidant property. Further studies are warranted to isolate and characterize the

active component in RHPE responsible for these observed effects.

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Dichlorvos Induced Oxidative and Neuronal Responses in Rats: Mitigative Efficacy of *Nigella sativa* (Black Cumin)

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Summary: Poisoning from Organophosphates (OPs), especially Dichlorvos (DDVP) has become endemic due to the increasing use in house hold and agricultural pests control, with most marked effects in the nervous system. However, it is evidenced that natural antioxidants are efficacious against OPs toxicity. Thus, this study investigated the possible antidotal efficacy of *Nigella sativa* oil (NSO) in Dichlovos (DDVP) induced oxidative and neuronal damages in Wistar rats. DDVP was administered at sub-chronic daily dosage of 8.8 mg/kg.bw for 7 days and a post-administration of NSO at 1 ml/kg.bw for the subsequent 7 days. The rats were euthanized on the 15thday, blood sample collected via cardiac puncture, centrifuged and the plasma used for biochemical analysis of total antioxidant capacity (TAC), reduced glutathione (GSH) and total reactive oxygen species (ROS), while the frontal, occipital and cerebellar cortices and the medulla were removed for histo-morphological examinations. The results showed significant ($P \leq 0.05$) decrease in plasma TAC and GSH, while a significant ($P \leq 0.05$) increase in ROS was recorded, and some vacuolation around the neurons especially in the frontal and cerebellar cortices following DDVP exposure. However, post treatment with NSO was observed to be efficacious in the recovery of the oxidative activities and the neuro-architectural integrities. Thus, it can be concluded that the antioxidant capacity of NSO could be efficacious against OPs induced oxidative damages, especially in dichlorvos accidents.
Keywords*:* Organophosphates, antioxidant capacity, antidote, *Nigella sativa* oil, neurotoxicity, poisoning.

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INTRODUCTION

Toxicity is an inevitable circumstance behind most human and animal diseases even more than the biological organisms, as toxic substances freely diffuse in air and water (Paliwal and Sharma, 2009). Many essential life supporting compounds that are necessary for human health and production are at the same time casualty to human wellbeing. An example of these compounds is the, irreversible acetyl cholinesterase inhibitors (ACHEIs) that are widely used in insect or pest control, meanwhile, their indiscriminate use and handling have resulted into high mortality in the developing world (Michael *et al*, 2008).

Dichlorvos (DDVP) is a common organophosphate (OP) used in diverged forms and applications in the tropical world (Uthman *et al*, 2013; Deka and Mahanta, 2015), mostly in the protection of domestic animals and livestock from parasite infestation, and in household or Agriculture insect and pests control, leaving residues in foods (Davies *et al*, 2016; Rashmikaa *et al*, 2016). Thus, the resulting accidental toxicity (Brown *et al*, 2015) is affecting the quality of life (Fariba *et al*, 2016) of the exposed individual and becoming a very important health concern (Farrukh *et al*, 2016).

Complicating the burden of OPs poisoning is the limitations of the available antidote (Yadav *et al*, 2012), thereby, requiring a search for alternative regimen. Phytomedicine is gaining high interest and almost becoming an alternative medicine, due to their perceived reduced side-effect, availability, and cost effectiveness. *Nigella sativa*, a phytonutrient antioxidant has been fairly reported to be efficacious in many diseases, and these are evidenced in its therapeutic efficacies as antioxidant (Ashraf *et al*, 2011), anti-inflammatory (Alemi *et al*, 2013), antineurotoxic (Beydilli *et al*, 2015), hepatoprotective (Ajao *et al.*, 2017a), anti-diabetic (Alli-oluwafuyi *et al.*, 2017), renal and hematoprotective (Ajao *et al.*, 2017b), efficacy in neurodegenerative diseases (Dariani *et al*, 2013) and memory enhancing effects (Imam *et al*, 2016a). Thus, the mitigative efficacy of NSO in DDVP induced oxidative stress and neuronal

toxicity was investigated due to its known antioxidant and/or anti-inflammatory properties.

MATERIALS AND METHODS

Chemicals and Drugs

Dichlorvos was purchased from the Sigma Chemicals (St. Louis, MO, USA), while the sunflower oil which was used to dissolve DDVP was purchased locally and of analytical grade. The *Nigella sativa* oil (100% pure natural oil) was obtained from Masra warda, Kingdom of Saudi Arabia.

Animal care and Ethics

This research work was approved by the University of Ilorin Ethical Review committee, following the recommendation of the College of Health Sciences Ethical Review Committee, University of Ilorin, Ilorin, Nigeria. The research was approved to be in compliance with the Institutional Animal Care and Use Committee (IACUC).

Twenty-four (24) adult male Wistar rats with an average weight of 200 ± 20 g were used in this study. The animals were housed (6 per cage) under standard laboratory conditions in the animal holding of the Faculty of Basic Medical Sciences, University of Ilorin, Nigeria. They were allowed free access to water and food *ad libitum* and euthanized at the end of the experiment with intraperitoneal injection of Ketamine (10 mg/kg. ip).

Treatments schedule

The rats were randomly distributed into four groups (n = 6) as follows:

Control: received sun flower oil (1 ml/kg by oral gavage), consecutively for 7 days

Experimental 1: received DDVP (8.8 mg/kg/day by oral gavage) (Sharma and Singh, 2012), consecutively for 7 days

Experimental 2: received DDVP (8.8 mg/kg/day by oral gavage) for 7 days (Day 1-7), then followed by NSO (1 ml/kg/day by oral gavage) consecutively for the next seven days (Day 8-14).

Experimental 3: received NSO (1 ml/kg/day by oral gavage) (Nahed and Bassant, 2011), for 7 days

Treatments of the control, experimental 1 and 3 were only commenced at the 8th day of the fourteen days experimental period.

Oxidative stress and Endogenous Antioxidant analysis

Twenty four hours after the completion of exposures, the animals were anaesthetized with Ketamine (10 mg/kg.ip), the thoracic cage was exposed, blood was collected from the heart via the right atria, then respective reagents were used to assay plasm levels of total antioxidant capacity (TAC), total reactive oxygen species (ROS), reduced glutathione (GSH) and C-Reactive protein (CRP) as markers of oxidative stress and inflammation.

Histopathology

After blood was collected for biochemical analysis, whole body transcardial perfusion fixation using 4% paraformaldehyde, the brains harvested after 30 mins and stored in 4% paraformaldehyde. 24 hours later, tissue blocks of the frontal cortices (from Bregma 2 mm to 4 mm), occipital cortices (from the occipital pole 2 mm to 4 mm), the cerebellar cortices (from Bregma –10 mm to –15 mm) and the medulla were separated. These later dehydrated in ascending grades of alcohol, cleared in xylene, embedded in paraffin block, and then sectioned in 5 µm thickness using a rotary microtome (MK 1110). The sections were stained with Cresyl fast violet (CFV) for general neural architecture and Nissl granulation following standard routine laboratory procedures (Bancroft & Gamble, 2008). Images of the general architectures were captured under 40X objective lens using the Zeiss Axiostar Plus Light microscope.

Statistical analysis

Data recorded in this study were reported as mean ± standard error of mean. The TAC, ROS, GSH and C-Reactive protein data were analyzed using one-way analysis of variance (ANOVA) and for post-hoc analyses, we used the Bonfferoni test. The software package Graph Pad Prism (version 6) was used to analyze and graphical presentation of the data.

RESULTS

Oxidative and inflammatory responses following DDVP and NSO exposures

DDVP significantly (P≤0.05) caused a reduction in plasma TAC and GSH levels in the DDVP only exposed rats, and increased total ROS levels with no significant effect on the levels of the C-reactive protein (Fig(s). 1, 2, 3 and 4).

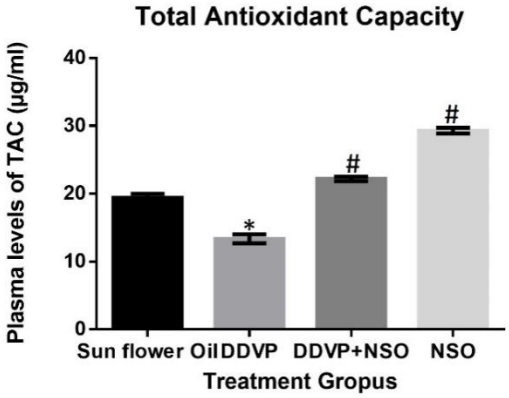

Fig 1: The effects of SFO, DDVP, DDVP+NSO, NSO on plasma TAC levels. ANOVA followed with Bonferroni. * indicates significant (P≤0.05) difference from DDVP+NSO and NSO while # indicates significant (P≤0.05) difference from DDVP only and SFO treated groups

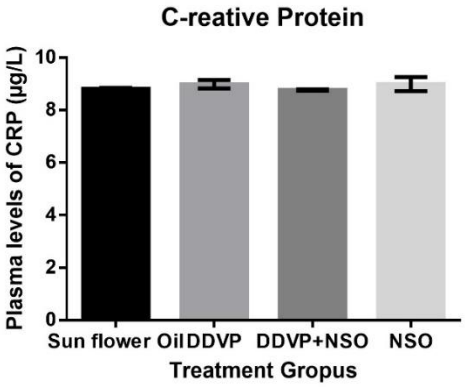

Fig 2: The effects of SFO, DDVP, DDVP+NSO, NSO on plasma C-reactive protein levels. There are no significant (P≤0.05) differences across the groups.

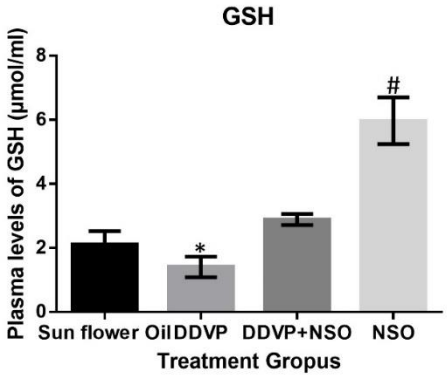

Fig 3: The effects of SFO, DDVP, DDVP+NSO, NSO on plasma GSH levels. * indicates significant (P≤0.05) difference from DDVP+NSO and NSO while # indicates significant (P≤0.05) difference from DDVP only and SFO treated groups

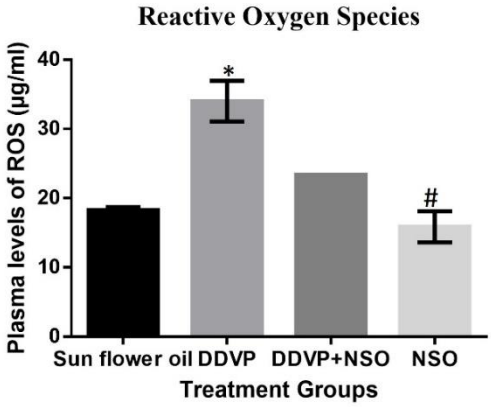

Figure 4: The effects of SFO, DDVP, DDVP+NSO, NSO on plasma ROS levels. * indicates significant (P≤0.05) difference from DDVP and NSO, while # indicate significant (P≤0.05) difference from the DDVP only and DDVP+NSO treated groups.

But NSO was observed to relieve these activities by effecting a significant (P≤0.05) increase in the levels of TAC and GSH, with a complementary reduction in ROS levels (Figs. 1, 2, 3 and 4) in the rats that received NSO only and those that received NSO after DDVP.

Neuronal responses to DDVP and NSO in various regions of the brain

Normal neuronal architectures were obvious in all the brain regions (frontal, occipital, cerebella and medulla) following SFO and NSO only treatments (Fig(s). 5-8). The brain regions of the DDVP treated animals, although not conspicuous appear to show some necrotic-like neurons with obvious vacuolations in the neuropils, especially in the cerebellar Purkinje cells and the frontal pyramidal neuron (Figures 5-8). However, the vacuolations were markedly reduced following a combined DDVP and NSO treatment (Fig(s) 5-8).

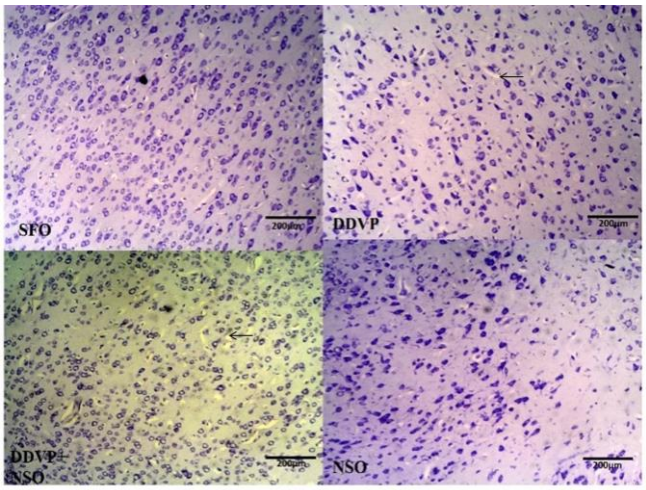

Figure 5: Representative photomicrographs of the frontal cortices of rats following administration of SFO, DDVP, DDVP+NSO, NSO. Arrow showing the vacuolation around a medium sized pyramidal neuron. (CFV 100X; Scale bar 200µm)

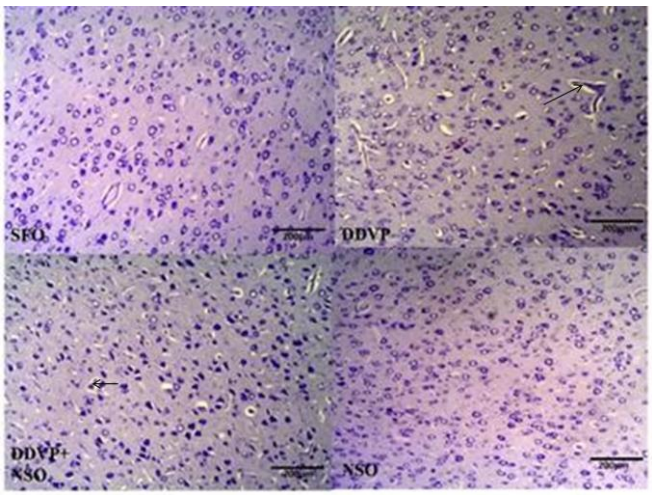

Figure 6: Representative photomicrographs of occipital cortices of rats following administration of SFO, DDVP, DDVP+NSO, NSO. Arrow showing the vacuolation around a small sized pyramidal neuron. (CFV 100X; Scale bar 200µm)

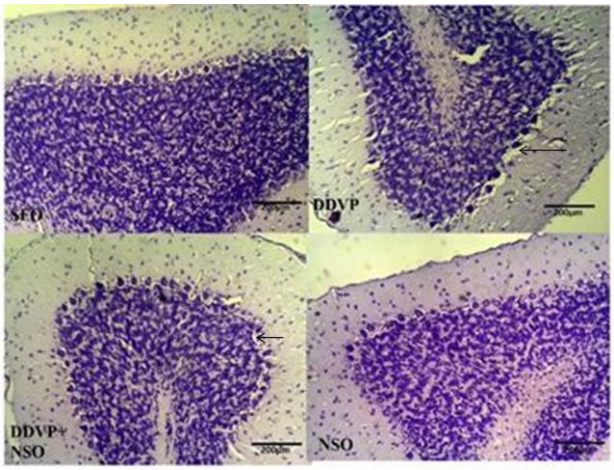

Figure 7: Representative photomicrographs of cerebella cortices of rats following administration of SFO, DDVP, DDVP+NSO, NSO. Arrow showing the vacuolation around purkinje cells in the purkinje cell layer. (CFV 100X; Scale bar 200µm).

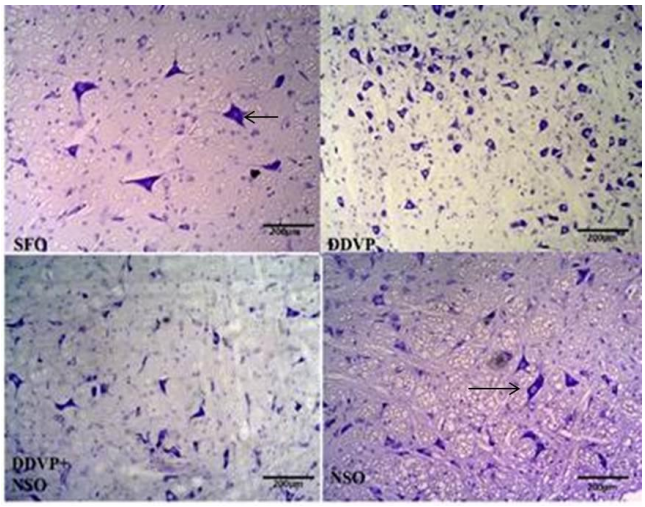

Figure 8: Representative photomicrographs of medulla oblongata of rats following administration of SFO, DDVP, DDVP+NSO, NSO. Arrow showing the large pyramidal cells in the rostral medulla. (CFV 100X; Scale bar 200µm)

DISCUSSION

The incidences of OPs poisoning in developing nations, have become endemic and threat to the quality of life in recent time, associated with high levels of depression, anxiety and stress (Fariba *et al*, 2016).

In this study, DDVP induced oxidative stress in the treated animals and markedly impaired anti-oxidant capacities, a report that is similar to what was reported in another OP (chlorpyrifos) which increased malondialdehyde (MDA), superoxide dismutase (SOD) and catalase (CAT) activities, complicated by a reduced cytosolic glutathione S-transferase (GST) levels (Asma *et al*, 2016), and the impaired antioxidant capacity, observed in the reduced TAC and GSH levels is in agreement with Owoeye *et al.* (2014). Ezeji and colleagues have also reported depletion in GSH level in response to OPs poisoning (Ezeji *et al*, 2012).

These deleterious activities or damaging effects of DDVP in the exposed rats, strengthens its previous report and of other OPs in impaired personality (Weidong *et al*, 2016), impaired neurocognitive behaviors, psych cognitive derangements (Alessandra *et al*, 2016; Farrukh *et al*, 2016).

NSO was able to cushion the oxidative damages caused by DDVP, this can be associated with its previously reported anti-oxidant capacities, and more so its reported therapeutic efficacies in OPs induced biochemical damages (Atef *et al*, 2010; Mohamadin *et al*, 2010; Nahed *et al*, 2011; Hashem, 2012; Halil *et al*, 2015). These activities against OPs induced oxidative stress or poisoning, can be validated by the facts that natural or phytonutrient antioxidants have been largely proven to be efficacious in OPs toxicity (Colovic *et al*, 2015; Beydilli *et al*, 2015; Lari *et al*, 2015; Elsaid *et al*, 2015; El-Demerdash and Nasr, 2014). These can also be strengthened by our previous reports using the same dosage of NSO as employed in this study on its neuroprotective efficacy against cannabis and scopolamine modelled amnesia, (Imam *et al*., 2016a; 2016b; Ajao et al., 2016)

Although, the activities of DDVP on oxidative stress and endogenous antioxidants in this work were damaging, the effects on the neuronal integrity of the various brain regions were not pronounced in this study, and thus, may be too exaggerating to report any damage. Such minimal effect may be due to the period of exposures and the dosage employed in the study (Alessandra *et al*, 2016), even though, such conclusion may contradict other reports with marked deleterious changes in the cyto-architectonic of different brain regions following OPs exposures (Du *et al*, 2014; Olatunde *et al*, 2014; Ojo *et al*, 2014; Omar *et al*, 2016), but partially supported with some marked distortion characteristics in the frontal and cerebella cortices.

Complementing the effects of NSO on re-installing oxidative activities and strengthening anti-oxidant capacities in the treated rats, was the improved neuronal integrities when given alone and its protective effects when co-administered with DDVP. These reports can be supported by the previous reports on its prophylactic, ameliorative and protective efficacies in the frontal cortical pyramidal neurons, dentate gyrus granule cells, hippocampal CA pyramidal neurons, cerebellar cortices, brain stem and spinal cord following degenerative exposures to scopolamine, toluidine, autoimmune encephalomyelitis, lead induced neuronal degeneration and axonal demyelination and spinal cord injury respectively (Kanter *et al*, 2008; Heba *et al*, 2015; Farimah *et al*, 2016; Imam *et al*, 2016; Norsharina *et al*, 2008; Khaled *et al*, 2014).

Following the results of this study, it can be concluded that NSO due to its antioxidant efficacy and effects on neuronal integrities, may be a potent

supplementary remedy in OP poisoning, especially the Dichlorvos.

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Effects of Clove and Fermented Ginger on Blood Glucose, Leptin, Insulin and Insulin Receptor Levels in High Fat Diet-Induced Type 2 Diabetic Rabbits

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Summary: The aimed of this research is to evaluate the effects of clove and fermented ginger supplements on blood glucose, serum insulin, insulin receptor and Leptin levels of high fat diet-induced type 2 diabetes mellitus in rabbits. Clove and ginger are spices with records of medicinal value over decades. Thirty males rabbits weighing, 1–1.5kg were used for the research. Type 2 diabetes was induced by feeding the animals with a high fat diet for a period of eight weeks. Blood glucose levels were determined after the induction period and rabbits having 140 mg/dL and above were selected for the study. The animals were grouped into six groups with five (n=5) rabbits in each group: Group 1 (Normoglycemic control group.) received normal feed and distilled water *ad libitum* for six weeks; Group 2 (Diabetic negative control group.) received normal feed and distilled water *ad libitum* for six weeks; Groups 3 (Diabetic positive control.) received cholestran 0.26g/kg and normal feed for a period of six weeks; Group 4 and 5 (diabetic rabbits) were fed on 12.5%, clove and 12.5% fermented ginger respectively for a period of six weeks; while Group 6 were co-fed on 12.5% clove and 12.5% fermented ginger for a period of six weeks. Fasting blood glucose levels were determined at weekly interval during the treatment period. At the end of the experiment, the rabbits were euthanized by cervical dislocation and blood samples were collected for the determination of insulin, insulin receptor and leptin levels. A significantly ($P<0.05$) decrease in blood glucose levels was recorded in the supplements treated groups compared to diabetic control group. Clove supplement been most effective and sustaining in antihyperglycemic activity, also appears with a significant decreasing effect on leptin levels compared to diabetic control group. A significant increase in insulin levels was also noted in the fermented ginger treated group along with higher levels of Leptin compared as compared to control group. In conclusion the result of the study show that clove and fermented ginger supplementation possesses anti-diabetic properties and may help in the control of hyperleptinaemia in type 2 diabetes.

Keywords*:* Clove, Ginger, Type 2 Diabetes, Leptin and Insulin.

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INTRODUCTION

Consumption of high Fat Diet (HFD) or western diet has been adopted in many population across the globe, which is associated with the largest incidence of metabolic syndrome in the world (Buettner *et al*., 2007). Chronic intake of HFD leads to obesity, defective insulin secretion or function which results to various metabolic aberrations and diabetes mellitus (Jimoh *et al*., 2015). The associated impairments include hyperglycaemia due to defective insulin-stimulated glucose uptake; up- regulated hepatic glucose production and dyslipidaemia (Baxter and Webb, 2009).

Excessive short term consumption of high fat diet has been associated with an increased incidence of enhanced oxidative stress, consequently, leading to high levels of circulating free fatty acids (FFA) and glucose which are potent inducers of reactive oxygen species (ROS) formation in cells (Supale *et al*., 2012). Lipotoxicity impairs cell function and viability due to chronic exposure to FFA, leading to the induction of β- cell endoplasmic reticulum stress, and glucose-induced β-cell dysfunction and apoptosis (Tang *et al*., 2013). The excessive consumption of high fat diet has been associated with an increased incidence of type2 diabetes mellitus (T2 DM) through insulin resistance associated with hyperleptinaemia (Keaney *et al*., 2003; Demarco *et al*., 2010; Otani, 2011).

Type 2 diabetes mellitus is now a common, growing, serious and costly, but potentially preventable disease appearing across populations globally (Adams, 2011). Continuous consumption of calories-rich meals, junk food and sedentary lifestyle has culminated into an epidemic of diabetes worldwide. The existing management of diabetes is indeed costly and associated with many side effects ranging from constipation, abdominal discomfort to hypoglycemia.

Hence, the need for an effective alternative therapy with fewer or minimum side effects.

Clove buds and ginger rhizome are important spices used across the world as natural remedy and in folklore for many disease management (Bode and Dong, 2011). Positive therapeutic effects of their individual extracts have been studied in type1 diabetic animal model with paucity in some results (Alnoory *et al*., 2013). This study aimed to determine the effects of Clove (*Syzygium aromaticum*) and Fermented Ginger (*Zingiber officinale*) Supplements on Blood Glucose, leptin, Insulin and Insulin Receptor Levels in High Fat Diet- Induced Type 2 Diabetes in Rabbits.

MATERIALS AND METHODS

Collection of Plant material.

Clove (*Syzygium aromaticum*) buds and Ginger (*Zingiber officinale*) rhizome were purchased at Tudun-Wada Market Zaria, Kaduna Nigeria. They were authenticated at the herbarium unit of the Department of Biological Sciences, Faculty of life Sciences, Ahmadu Bello University, Zaria, with voucher numbers 900127 and 2261 respectively.

Animals

A total of thirty (30) male rabbits (New Zealand) aged 5-8 weeks were used for the study, animals were housed in the Animal house of Department of Human Physiology, Faculty of Medicine Ahmadu Bello University Zaria, under standard laboratory conditions and had access to feed (Pellet Growers feed) and water *ad libitum.* Animal care and use was in accordance with the guide for the care and use of laboratory Animals, institute for Laboratory Animal Research, National Institute of Health (NIH Publication No.80-23; 1996).

Drugs /Reagents used

All chemicals used were of analytical gradesi. Cholesterol (powder) was purchased from KEM LIGHT Laboratory PVT. LTD, Mumbai India. (CAS: 57-88-5, m.w: 386.67, lot no.: 100814). Cholestran powder (made in Egypt PHARCO pharmaceuticals Alexandria was purchased from Amira Pharmacy, Tudun wada Zaria. Methylated spirit, cotton wool. Rabbit INS ELISA Kit (No.GA-E0015RB) LOT 20151012, REF E20151012001; v. Rabbit ISR ELISA Kit (No.GA-E0016RB) LOT 20151012, REF E20151012002, and LEP ELISA Kit (No.GA-E0017RB) LOT 20151012, REF E20151012003, GenAsia Biotech Co., Ltd., 7th floor,Wujiaochang Technology Building, No. 1675, Huangxing Road, Yangpu District, Shanghai, China. Digital glucometer (Accu-Chek Advantage, Roche Diagnostic, Germany). Glucose- test strips for assessment of plasma glucose levels, manufacture by accu-check Advantage II, Roche Diagnostics GmbH Germany.

Experimental Induction and Determination of Diabetes Mellitus

The rabbits were handled in accordance with the principles guiding the use and handling of experimental animals, ABU, Zaria. Ethical approval was obtained, with Approval No: ABUCAUC/2017/048. The animals were fasted from feeds for 12-14 hours prior to commencement of the experiment, but allowed water *ad libitum*. Type 2 diabetes mellitus was induced by feeding the animals with a high fat diet (2% cholesterol, 20% groundnut mill and 10% groundnut oil) as reported by Jimoh *et al*. (2015) for a period of eight weeks. Fasting blood glucose levels were determined by using the glucose oxidase method (Trinder, 1969) Rabbits having glucose levels greater than 140 mg/dl were considered Hyperglycemic. The results were reported as mg/dl (Rheney and Kirk, 2000).

Experimental Design

After the induction of type 2 diabetes mellitus, rabbits having fasting blood glucose levels of 140mg/dL (Jimoh *et al*., 2015) and above were selected for the study. The animals were randomly assigned into experimental and control group of five (5) rabbits each, as follows;

Group i: Normal rabbits fed on animal standard feed

Group ii: Diabetic rabbits fed on standard feed for six weeks

Group iii: Diabetic rabbits fed on standard feed and administered cholestran (0.26g/kg body weight) for six weeks.

Group iv: Diabetic rabbits fed on clove 12.5% supplement for six weeks.

Group v: Diabetic rabbits fed on 12.5% fermented ginger supplement for six weeks.

Group vi: Diabetic rabbits fed on clove 12.5% + fermented ginger 12.5% supplements for six weeks.

Blood Sample Collection and Serum Preparation

At the end of the six weeks treatment period, the rabbits were euthanized by cervical dislocation and blood samples were collected from the animals through cardiac puncture. About 5 mL of blood were collected into specimen bottles and allowed to clot and separated by centrifugation at 3,000 g for 10 minutes using Centrifuge Hitachi (Universal 32). The supernatant obtained were used for the determination of insulin, and leptin concentrations.

Biochemical Estimations

The sera were used for the determination of serum insulin and leptin levels, using rabbit ultra-sensitive enzyme-linked immunosorbent assay (ELISA) kits (GenAsia Biotech, Co., Ltd. Shanghai, China), with catalogue numbers, GA-E0015RB (insulin) and GA-E0017RB (leptin) according to the manufacturer's instructions. The principles were based on biotin

double antibody sandwich technology (Schmidt *et al*., 2012). Serum insulin receptor was estimated using rabbit ISR (insulin receptor) ELISA kit.

Statistical Analysis

Data obtained from the study were expressed as mean ± SEM. Statistical comparisons were performed by one way analysis of variance (ANOVA), followed by Tukey's post hoc test. Values of $P \leq 0.05$ were considered as significant (Duncan *et al*., 1977).

RESULTS

The results of blood glucose level (Table 1) revealed a significant ($P < 0.05$) decrease in blood glucose level at the second and fourth week of treatment in the diabetic model rabbits groups treated with clove 12.5%, fermented ginger 12.5%, and the combined clove 12.5% + fermented ginger 12.5% supplements, compared to diabetic model rabbits group fed on standard feed. While at the fifth week only, the group on clove 12.5% supplement showed a significant decrease in blood glucose level compared to that of diabetic model rabbits group fed on standard feed ($P < 0.05$).

Table 2) revealed a statistically significant ($P < 0.05$) increase in serum insulin level of all the HFD induced diabetic animal groups (groups 2-6), compared to the normal control group (group1). Moreover, a significant ($P < 0.05$) increase in group 5 (clove 12.5% supplement treated group), compared to serum level of insulin in diabetic rabbits fed on standard feed (group 2) was also recorded. Serum leptin level also showed a significant decrease in clove 12.5% supplement treated group (group 4) compared to the level seen in group 2 ($P < 0.05$). While a significant ($P < 0.05$) increase in serum insulin receptor was also seen in all HFD induced diabetic animals, compared to that of NC group.

Table 2: Effects of Clove, Fermented Ginger and co-administration of Clove and Fermented Ginger Supplements on Insulin, Leptin and Insulin-receptor in High Fat Diet Induced- type 2 Diabetes in Rabbits

Experimental Groups	Insulin (ng/ml)	Leptin (ng/ml)	Insulin Receptor (ng/ml)
Normal Control (NC)	9.00 ± 0.58	12.50 ± 1.44	15.00 ± 5.51
Diabetic Control (DC)	26.00 ± 2.51[a]	14.60 ± 1.96	29.40 ± 4.12 [a]
Diabetic + Cholestran (0.26g/kg)	25.60 ± 9.44[a]	12.60 ± 1.18	32.20 ± 4.05 [a]
Diabetic + Clove (12.5%)	31.20 ± 3.56 [a]	10.00 ± 1.60[b]	30.40 ± 3.64 [a]
Diabetic + Fermented ginger (12.5%)	37.20 ± 3.44[a,b]	14.20 ± 0.86	27.80 ± 2.58 [a]
Diabetic + Clove (12.5%) + Fermented ginger(12.5%)	26.40 ± 6.74 [a]	12.70 ± 2.63	28.60 ± 5.53 [a]

Superscripts: [a] = significant different compared to NC group; and [b] = significant difference when compared to DC group.

DISCUSSION

The observed decrease in blood glucose in the rabbit fed 12.5% clove and/or 12.5% fermented ginger could be explained by the effects of certain active constituents such as dehydrodieugenol and dehydrodieugenol B in clove, as well as Gingerol and shogaol present in ginger supplements which affect different level of insulin signaling cascade pathway or post insulin-receptor complex by increasing expression of glucose transporters and other molecular modulators of insulin activity resulting in increased glucose intake by muscle cells. (Arablou, *et al*., 2014; Hyun, *et al*., 2014).. Another mechanism of action of

Table 1: Effects of Clove 12.5%, Fermented Ginger 12.5% and co-administration of Clove12.5 and Fermented Ginger 12.5% Supplements on Blood Glucose Level in High Fat Diet Induced- type 2 Diabetes in Rabbits

Groups	BGL Week 0 (mg/dL)	BGL Week 1 (mg/dL)	BGL Week 2 (mg/dL)	BGL Week 3 (mg/dL)	BGL Week 4 (mg/dL)	BGL Week 5 (mg/dL)
Normal Control (NC)	109.80 ± 3.14	103.20 ± 4.62	101.80 ± 2.71	108.00 ± 1.92	95.20 ± 3.95	97.60 ± 2.04
Diabetic Control (DC)	140.00 ± 0.84[a]	119.00 ± 2.63[a]	123.40 ± 2.46[a]	140.80 ± 1.59[a]	125.40 ± 2.94[a]	114.00 ± 2.24[a]
Diabetic + Cholestran (0.26g/kg)	140.80 ± 1.53[a]	155.40 ± 2.87[a,b]	140.80 ± 0.86[a,b]	159.60 ± 2.84[a,b]	122.60 ± 0.98[a]	126.40 ± 1.17[a,b]
Diabetic + Clove (12.5%)	149.40 ± 2.04[a]	117.20 ± 1.59[a,]	128.00 ± 0.71[a,b]	162.60 ± 2.20[a,b]	104.60 ± 1.94[a,b]	105.20 ± 0.58[b]
Diabetic + Fermented ginger (12.5%)	149.20 ± 1.50[a]	132.80 ± 3.57[a,b]	134.00 ± 2.30[a,b]	141.80 ± 2.48[a]	112.20 ± 1.36[a,b]	132.00 ± 2.68[a,b]
Diabetic + Clove (12.5%) + Fermented ginger (12.5%)	149.40 ± 2.62[a]	143.20 ± 3.32[a,b]	136.60 ± 1.36[a,b]	168.00 ± 2.55[a,b]	112.20 ± 2.31[a,b]	127.80 ± 2.06[a,b]

Values are presented as mean ± SEM; n=5, P< 0.05 = significant. Values with superscripts: [a] = significant different in comparison to NC group, and [b] = significant different when compared to DC group.

ginger that may explain this action could be from inhibition of oxidative stress. Gingerol and shogaol are active ingredients present in ginger and may have contributed to the hypoglycemic effect observed in this study.). The findings of the present study on blood glucose level are similar to the work of Nafiseh*et al*. (2015), in which it was demonstrated that treatment with ginger significantly decreased blood glucose and other parameters tested in diabetic patients. Similarly, Mozaffari-Khosravi *et al*. (2014) demonstrated also that daily consumption of one-gram capsules of ginger powder for 8 weeks is useful for patients with type 2 diabetes as it reduced fasting blood glucose and glycated hemoglobin (HbA1c). This work is also in agreement with that of Khan *et al*. (2006), in which capsule of cloves were found to improve the function of insulin and to lower blood glucose and concluded that consumption of 1-3g of clove improve glucose level in diabetic patients. However, this work does not agree with the report of Ashade, *et al*. (2014), where ginger peel does not lower blood glucose levels in cat fish. The difference in the results may be as a result of method of preparation of ginger. The pronounced hypoglycaemic activity of the clove supplement may probably be also attributed to the activities of dehydrodieugenol and dehydrodieugenol B that had potent PPAR-γ ligand-binding activity, therefore causing increased insulin sensitivity via muffling the effect of lipotoxicity along insulin signaling pathway (Kuroda *et al*. 2012).

A significant increase in serum insulin level in high fat diet induced diabetic rabbits when compared to the normal rabbits was observed. Insulin activity may be low in these animals (diabetic control) due to the presence of excess FFAs in circulation that inhibit insulin signaling pathways. Consequent to this, higher plasma nutrients will be present in circulation that further leads to secretion of counter regulatory hormones such as glucagon and GLP1, which stimulates further secretion and release of insulin in to the circulation, resulting in hyperinsulinemia over time (Han *et al*., 2009). Treatment with fermented ginger also revealed a significant increase in insulin levels when compared to the level in diabetic control, fermented ginger may have exerted insulin mimetic effects thereby sparing serum insulin or may have contributed to increase insulin secretion and release. In the present study, the result of serum the insulin receptor level showed an increase similar to what was observe with serum insulin. The high fat diet could have induced insulin resistance as an underline mechanism leading to metabolic dysregulation in the animal model, resulting in the up-regulation of the insulin receptor, being a physiological response to sustained hyperglycaemia seen in pre-diabetic state or in diabetes with insulin resistance (Hyun *et al*., 2014). These findings could be explained by the proposed mechanism underlining the hypoglycaemic effect of ginger through increasing uptake of glucose into muscle cells without using insulin via regulating insulin inhibitory and stimulatory receptor proteins gene expressions (Arablou *et al*., 2014).

Serum Leptin level was significantly decreased in clove supplemented diet treated group compared with hyperglycaemic rabbits fed on normal feed in this study. Leptin, produced by adipocytes regulates energy expenditure, feeding behaviour and body weight. Abnormal higher levels of leptin are associated with hyperinsulinemia and insulin resistance (reference?). Leptin has a direct effect on glucose level independent of body weight and food intake (Kamohara *et al*., 1997). Nevertheless, leptin level is an indicator of better energy balance and control. It exerts an important role in regulation of glucose homeostasis; the mechanism of this effect may be at different level including glucose absorption, and as well secretion of insulin and down regulate counter regulatory hormones such as glucagon (Gabriela *et al*., 2015). So, the clove supplemented group may have an enhanced sensitivity effect on circulatory leptin which complements the down regulatory effect of the supplement on blood glucose level. Hence, the hypoglycaemic activity of the supplement may also be attributable to the physiological adaptive Leptin restoration effect on glucose homeostasis. Clove supplements could be acting centrally via Leptin effects on energy intake to down regulate blood glucose while the ginger supplements activity is likely at post receptor level via increased glucose transporters. In conclusion Clove, fermented ginger and their combined supplements treatments for six weeks improved glucose homeostasis and makers of energy balance in high fat diet induced type 2 diabetic rabbits.

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Moringa oleifera Ameliorates Histomorphological Changes Associated with Cuprizone Neurotoxicity in the Hippocampal *Cornu ammonis* (CA) 3 Region

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Summary: Cuprizone-induced neurotoxicity has severally been used to study demyelinating diseases like multiple sclerosis (MS), adversely affecting both the white and grey matters of the brain. Lesions have been observed in different regions of the brain including, corpus callosum, neocortex and the hippocampal formation. The current study explored the role of *Moringa oleifera* leaf extract in restoring the resultant histomorphological changes in cuprizone-induced hippocampal damage in Wistar rats. Twenty adult female Wistar rats with average weight of 163.74 ± 3.59 g were grouped into A: Control, administered with 1 ml of normal saline, B: received 0.4% cuprizone diet, C: received 1.875 mg/ml/day of *Moringa* extract, and D: received a combination of cuprizone and *Moringa* in similar doses. Administration was oral for 5 weeks. The weights of animals were assessed during treatment, and at the termination of experiment, the rats were euthanized and the brains were fixed in 4% paraformaldehyde. The tissue was processed for histological and histochemical examinations using the Haematoxylin and Eosin stain and cresyl fast violet stain to assess the general microarchitecture and neuronal cells respectively of hippocampal *cornu ammonis* (CA) 3 region. The body weight of cuprizone-treated rats was reduced and this was ameliorated significantly in animals that were co-administered with *Moringa*. Similarly, there were histological alterations in the CA3 region of the hippocampus with the presence of pyknotic pyramidal cells organized in clusters and CA3 cells with degenerative changes, but administration of *Moringa* led to a better organised and fairly intact histological appearance. Pharmaceutical development of *Moringa oleifera* into appropriate therapeutic formulations could offer some relief to patients of demyelinating conditions that have clinical features of neurological deficits.

Keywords*:* cuprizone, neurotoxicity, hippocampus, *Moringa*

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INTRODUCTION

Cuprizone intoxication has been used over time to study experimental demyelination and remyelination in the central nervous system (CNS) (Keegan *et al*., 2006). The copper chelator cuprizone causes a reproducible demyelination in corpus callosum within weeks of administration with spontaneous remyelination occurring after withdrawal (Matsushima *et al*., 2001).

The primary target of cuprizone toxicity is oligodendrocytes and various studies have demonstrated demyelination of hippocampal cortex in cuprizone model (Koutsoudaki *et al*., 2009; Kipp *et al*., 2011; Sun *et al*., 2016), with specific consequences on the behaviours and functions of the animal. The CA3 region of the hippocampus has more abundant internal connectivity compared with other regions of the hippocampus, and plays specific role in memory processes, susceptibility to seizures and neuro-degeneration (Cherubini and Miles, 2015). It is vulnerable to stress and seizure-induced damage (Belvindrah *et al*., 2014). Axonal fibres of CA3 pyramidal cells make excitatory contacts with neighboring excitatory and inhibitory neurons (Cherubini and Miles, 2015). Any injury therefore to this area of the hippocampus, though targeted at the myelin sheath, could affect the neuronal cells, especially pyramidal cells of the hippocampus, whose axons are myelinated by oligodendrocytes, with a resultant overall impairment in the function of the structure.

The cellular processes that occur during oligodendroglial damage involve other glial cells, such as microglia and astrocytes, which act as part of brain defense mechanism against the ongoing cellular injury occasioned by cuprizone cytotoxicity (Remington *et al*., 2007; Hibbits *et al*., 2012). Furthermore, evidence has emerged of damage to neuronal cells as well. A study by Hoffmann *et al*. (2008) demonstrated extensive neuronal degeneration in the hippocampus of cuprizone-treated mice. In addition, significant axonal loss has also been documented (Irvine and

Blakemore, 2008). According to Praet *et al*. (2014), most neurons are likely to survive acute cuprizone toxicity, but are however, more vulnerable to metabolic disturbance compared to oligodendrocytes (Praet *et al*., 2014).

The hippocampus is located in the medial temporal lobe. Its function is implicated in the processes of learning and memory,and is one of the structures of the CNS indispensable to long-term episodic memory (Bird and Burgess, 2008). Lesions in the hippocampus are associated with cognitive defects as seen in large proportions of multiple sclerosis patients (Rao *et al*., 1991). Despite the high functional impact, the knowledge about effective strategies for managing MS remains very low (Amato *et al*., 2006).

Traditionally, *Moringa oleifera* is used in many disease conditions throughout the world (mainly in Thai) (Rastogi *et al*., 2009). Many of its functions have been proven scientifically and these include antihypertensive, analgesic, anti-cancer, CNS depressant, antibiotics, anti-inflammatory, and antiepileptic properties (Faizi *et al*., 1994; Gupta and Mazumder, 1999). With a possibility of hippocampal damage following cuprizone intoxication, the current study explored the cytoprotective, anti-inflammatory, and anti-oxidative properties of *Moringa oleifera* leaf extract in restoring the resultant histomorphological changes in neuronal cells of the hippocampus.

MATERIALS AND METHODS

Twenty adult female rats were obtained and housed in the Animal Facility of the Faculty of Basic Medical Sciences, University of Ilorin. The animals were acclimatized for two weeks.

Experimental Design

The animals were fed on standard rat feeds (except those that received cuprizone diet) and water *ad libitum*. They were divided into four groups. Group A (control): were given 1 ml of normal saline daily; Group B (Cuprizone group): were given 0.4% cuprizone diet; Group C (*Moringa* group): received 1.875 mg/ml *Moringa* leaf extract; while Group D (cuprizone +*Moringa*): received simultaneously 0.4% cuprizone and 1.875 mg/ml *Moringa* (Omotoso *et al*., 2018).

Animal Treatment

Cuprizone (bis-cyclohexanone oxaldihydrazone) was procured from Sigma-Aldrich Inc, Germany. It was prepared as 0.4% cuprizone mixed with standard rodent chow. For all treatment, route of administration was oral. The weights of the rats were taken twice a week. *Moringa oleifera* leaves were obtained and authenticated at the Department of Plant Biology, University of Ilorin. The ethanolic extract of the plant was carried out using the silica gel open column method at the Department of Chemistry, University of Ilorin, Nigeria. Out of all the fractionations obtained during the fractionation process, the MoF_6 fraction had the highest yield quantitatively; this formed the basis of its use in this study.

Termination of experiment

At the termination of experiment at the end of 5 weeks of administration, the rats were euthanised. The head was removed from the rest of the body and the cranium was dissected to remove the brain, which was weighed and fixed in 4% paraformaldehyde solution. The tissues were dehydrated through grades of alcohol, cleared with xylene, infiltrated and embedded in paraffin and coronal sections of the cortex at 5 µ were obtained using rotary microtome. Tissue processing for histology and histochemistry was by the use of Haematoxylin and Eosin (for general architecture of the hippocampus) and cresyl fast violet stains (for demonstration of pyramidal neurons and Nissl bodies) respectively. The mounted sections were viewed with the aid of a light microscope.

Statistical analysis

The weights of the animals were analyzed using one-way analysis of variance (ANOVA). A Tukey post hoc test was used for group comparison when appropriate and a p value less than 0.05 was considered statistically significant.

RESULTS

Physical observation

The cuprizone group showed a decrease in body weight compared to control. A significant increase in body weight was observed in rats fed with *Moringa*. The rats in CPZ+MO group recorded a weight higher than CPZ group (Table 1).

Histological and histochemical observation

The histomorphological presentation of the *cornu ammonis* 3 (CA3) region of the hippocampus of rats in control group showed characteristically large pyramidal neurons with apical and basal dendrites projecting out of the large intensively stained soma. These pyramidal cells were laconically expressed and properly delineated in the CA3 region of *Moringa*-treated control animals (Figures 1 and 2). The pyramidal cells in the CA3 region of the hippocampus of rats treated with cuprizone showed pyknotic pyramidal cells organized in clusters and evidence suggestive of degeneration, including disintegration of neuronal cells, distorted cellular outline and irregular arrangement of cells (Figure 2). The cellular assortment of the CA3 region of the hippocampus alongside the polymorphic layer adjoining it on either side appeared distorted. Comparatively, the cellular assortment of pyramidal cells in the CA3 region of the rats that received a combined treatment of cuprizone and *Moringa oleifera* showed a better assortment and properly delineated cytoarchitectural manifestation

Table 1: Mean and standard error of initial weight, final weight and weight differences of experimental animals

Group	Final Weight (g)	Initial Weight (g)	Weight Difference (g)
Group A: Control	188.00 ±1.73	157.23± 1.65	30.77± 4.22
Group B: 0.4% CPZ	161.21± 2.41	168.93± 1.84	-7.72± 6.13*
Group C: 1.875 mg/ml *Moringa*	183.31 ±1.89	163.58 ±2.08	19.73± 6.44**
Group D: (CPZ+*Moringa*)	177.58 ±2.41	165.68± 1.97	11.9 ±7.95*

* and ** significant differences at p<0.05 relative to control and cuprizone groups respectively. CPZ=cuprizone.

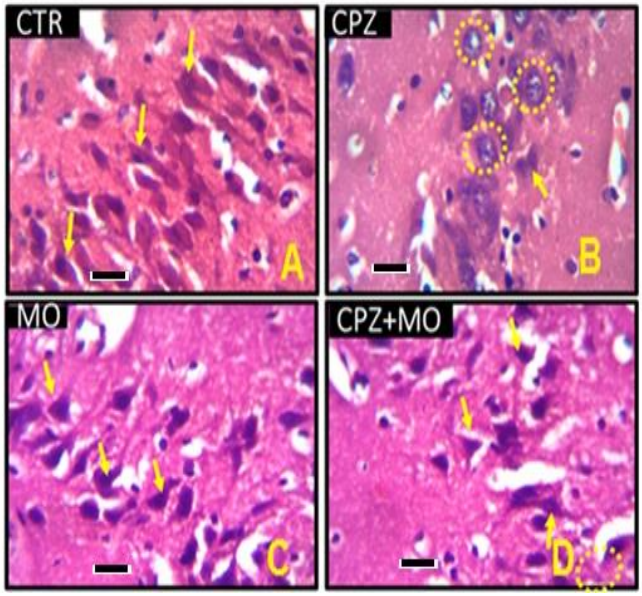

Figure 1: Representative photomicrograph of the *cornu ammonis* (CA3) region of the hippocampus of Wistar rats (H&E stain; Scale bar = 25µ).

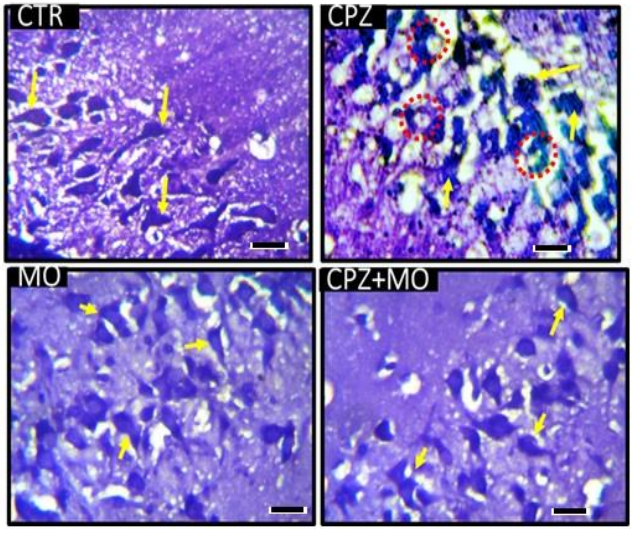

Figure 2: Representative photomicrograph of the *cornu ammonis* (CA3) region of the hippocampus of Wistar rats showing well demonstrated pyramidal cells in the Control, (CPZ) but marked architectural breakdown in the CPZ group (cresyl fast violet stain; Scale bar = 25µ)). CTR = control, CPZ = cuprizone, MO = *Moringa oleifera*, CPZ+MO = cuprizone plus *Moringa oleifera.*

when compared to that of the cuprizone-treated animals.

Histochemical demonstration of the *cornu ammonis* also showed well-preserved pyramidal neurons in the control group which were equally positive for Nissl bodies. The *cornu ammonis* of animals exposed to cuprizone diet had less positivity for Nissl staining, which was ameliorated following the use of *Moringa oleifera* (Figure 2).

DISCUSSION

Cuprizone administration is usually associated with weight loss (Franco-Pons *et al*., 2007; Hoffman *et al*. 2008; Benetti *et al*., 2010). However, according to the study by Benetti *et al*. (2010), mice fed on cuprizone diet lost weight up till the 5th week of exposure, but there was weight gain after this period. Similarly, in the current study, a reasonable level of weight loss was observed in the rats exposed to cuprizone (however, this study did not exceed 5 weeks). When co-administered with cuprizone, *Moringa* led to an appreciable weight gain, though not as high as the weight of rats treated with *Moringa* only.

Moringa oleifera has nutritional benefits and can support growth, as proteins are the most abundant nutrients in the leaf of *Moringa* (Brilhante *et al*., 2017). Although the weight increase in the current study was not up to that of the control, weight increase associated with *Moringa oleifera* leaf extract was able to ameliorate the weight loss associated with cuprizone diet.

In the current work, the micro-architecture of the *cornu ammonis* 3 of the hippocampus suffered some degree of distortion and disintegration in animals that received cuprizone diet, with the evidence of distorted cellular arrangement and degenerated cellular structures. Nissl bodies are rough endoplasmic reticulum present in the soma of neurons and are the site for neuronal protein synthesis (Fedorenko and Uzdensky, 2010). Cuprizone treatment in the current study led to depletion in Nissl bodies store, as revealed by the poor Nissl staining. However, co-administration of *Moringa* with cuprizone resulted in a more positive Nissl staining. This structural observation is corroborated by the work of Praet *et al*. (2014), where cuprizone treatment caused adenosine triphosphate (ATP) shortage and oxidative stress which led to the disruption of the proper functioning of the

endoplasmic reticulum, with a consequent impairment in protein synthesis.

This study further underscores the cytoprotective role of *Moringa oleifera*, as previously documented, in preserving the cellular architecture against toxic insults (Gupta and Mazumder, 1999; Rastogi *et al*., 2009).

Aside having a toxic effect on oligodendrocytes, our work revealed that cuprizone alters the morphology and integrity of pyramidal neurons of the hippocampus. However, co-administration with *Moringa* brought about a better cellular assortment and properly delineated cytoarchitecture of the hippocampal CA3 region. This intervention with *Moringa oleifera leaf extract* could help preserve the integrity of the hippocampus and the axonal internal connectivity vital for restoring and maintaining the neuronal functions of the hippocampus in individuals suffering from dymyelinating conditions of the central nervous system.

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Short Communication

Changes in Ocular Perfusion Pressure in Response to Short Term Isometric Exercise in Young Adults

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Summary: Ocular Perfusion Pressure (OPP) is the pressure difference between the Mean Arterial Pressure (MAP) and the Intra Ocular Pressure (IOP). Decreased OPP could be a major risk factor for glaucoma. The aim is to study the effect of Isometric exercise on OPP in apparently healthy young adults. Forty apparently healthy young adult volunteers comprising 20 males and 20 females in the age group of 18-21 years were selected among MBBS Phase I students of JSS Medical college, JSSU, Mysore. IOP and BP were recorded. Mean arterial pressure (MAP) and OPP were calculated. There was a statistically significant difference ($p<0.05$) in the mean OPP before and after performing Isometric exercise and between males (50.58±0.72 to 56.85±1.15 mm Hg) and females (49.35 ± 1.66 to 56.71 ± 1.61 mm Hg). Physical activity in the form of Isometric exercise improves ocular blood flow - OPP. Hence regular exercise of prescribed intensity may prove useful for glaucomatous patients which enhance their OPP.

Keywords*:* Ocular perfusion pressure, Isometric exercise, Glaucoma

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INTRODUCTION

Glaucoma is the leading cause of blindness in the world. It is a chronic optic neuropathy with characteristic changes in optic nerve head (ONH). The vascular hypothesis suggests that abnormal perfusion of the ONH causes ischemia and poor nutrition of retinal ganglion cells (Zheng et al., 2010). Glaucoma may continue to progress even after the reduction of Intra-Ocular Pressure (IOP) to targeted levels, which indicates that factors others than IOP, affect the pathogenesis.

A relatively new concept of Ocular Perfusion Pressure (OPP), defined as the difference between arterial pressure (BP) and IOP (Christina, 2009). It is an important determinant of ocular blood flow. A decrease in perfusion pressure may significantly decrease the ocular blood flow in absence of vascular autoregulation. Compromised ocular blood flow and deranged vascular auto regulation in the optic nerve head is emerging as an important causative factor contributing to glaucomatous optic neuropathy.

Isometric exercise is a form of physical exercise in which muscles are contracted and held in stationary position. Though isometric exercise increases muscle tension significantly, the length of the muscle remains the same (McArdle et al., 2006). In isometric exercise force is generated at constant muscle length without rhythmic episodes of relaxation. Isometric work intensity is usually described as percentage of maximal voluntary contraction (MVC), the peak isometric force that can be briefly generated for that specific contraction. Lower levels of physical activity are also associated with lower OPP (Jennifer et al., 2011).

Isometric exercise is known to cause an increase in the BP and decrease in IOP. These two components of OPP are strongly influenced by autonomic nervous system and the net result is an increase in the OPP. There are no studies yet on Indian population showing the effect of short term isometric exercise on OPP, particularly in healthy young adults. Hence this study aims to study the short-term effect of isometric exercise on OPP in young healthy adults.

MATERIALS AND METHODS

This comparative study was conducted on 40 healthy young adults of age 18-21 years (20 males and 20 females) who were selected randomly among MBBS students of JSS medical college. Ethical clearance was obtained from the institutional ethical committee, JSS Medical College, Mysuru. Subjects were screened using a questionnaire which included inclusion and exclusion criteria's and by physical examination for their age, history of hypertension, cardiac or pulmonary diseases, eye disorders, other factors affecting IOP, smoking and consumption of alcohols. Subjects were informed about the purpose of the study,

protocol was explained and informed consent was obtained.

Inclusion criteria

The study subjects were in the age group 18-21 years of either sex, non-obese: BMI 18 – 22.9 kg/ m^2 and were normotensives.

Exclusion criteria

Those with pre-existing refractive errors, glaucoma, migraine and any systemic illness, with any drug history affecting IOP, smokers and alcoholics were excluded from the study.

Procedure

Study was carried out in the research laboratory in Department of Physiology, JSSMC, Mysuru, by a single examiner between 3pm to 5pm to minimize the bias of examiners and diurnal variations of IOP. Subjects were instructed about the study before the experiment was done and asked to relax for 15 minutes in supine position. Resting Heart rate was recorded using pulse-oximeter. Resting BP was measured using Mercury Sphygmomanometer and IOP using Schiotz tonometer in supine position. Mean arterial pressure and OPP was calculated using the formula, MAP = DBP+1/3 Pulse Pressure, OPP = 2/3(MAP-IOP) (Hayreh, 2001)

Then subjects were asked to do isometric exercise to their maximum strength with Digital back-leg lift dynamometer to determine Maximum Voluntary Contractions (MVC). 40 % MVC was calculated. Subjects did the isometric exercise at 40 % MVC until the point of fatigue. Both IOP and BP were recorded as the average of 3 sequential measurements in mm Hg in supine posture immediately, at five minutes, ten minutes and fifteen minutes after the exercise. Systolic Ocular Perfusion Pressure (SOPP) was calculated as (SBP – IOP) and Diastolic Ocular Perfusion Pressure (DOPP) was calculated as (DBP – IOP).

Statistical Analysis

Mean and standard deviation were worked out to assess the estimate of various parameters under study. Paired t-test was applied to test the significance of changes in parameters studied. Microsoft Excel and SPSS version 19 software were used for data entry and statistical analyses respectively.

RESULTS

The study included 40 healthy young adults in the age group of 18 to 21 years (male n=20 & female n=20). There was no significant statistical difference between the two groups in terms of age, Body mass index and Waist circumference.

At Rest:

Resting mean values of systolic BP, MAP, Systolic and Mean OPP among males was significantly higher ($p<0.05$) as compared to the female group as shown in Table 1. Baseline IOP, diastolic BP and OPP were also higher in the male group but is statistically non-significant.

After Isometric exercise:

Immediately after exercise, OPP was raised in both male and female groups significantly compared to the resting level. There was a statistically significant difference($p<0.05$) in Mean OPP before and after performing Isometric exercise and between males (50.58±0.72 to 56.85±1.15) and females (49.35 ± 1.66 to 56.71 ± 1.61) as shown in fig 1. Mean increase in male group was 6.27 mm Hg where as in females it was 7.44 mm Hg. Thus, the increase in OPP immediately after exercise was higher in females and was statistically significant. The difference between the two groups was statistically significant and the mean difference was higher in female group compared to that of males for OPP at 5^{th}, 10^{th} and 15^{th} mins after exercise. At the end of 15 mins the OPP almost reached the baseline in both the groups. SOPP and DOPP were also increased immediately after exercise and the mean increase was comparatively higher in females.

Table 1: Resting mean values of SBP, MAP, SOPP and MOPP in both groups

REST	MALE	FEMALE	P value
SBP (mm Hg)	119.8	116.4	0.002*
DBP (mm Hg)	78.4	77	0.144
MAP (mm Hg)	92.19	90.13	0.016*
IOP (mm Hg)	16.33	16.14	0.567
SOPP (mm Hg)	103.47	101.9	0.000*
DOPP (mm Hg)	62.07	61.47	0.123
MOPP (mm Hg)	50.57	49.34	0.005*

* $p < 0.05$. SBP-systolic blood pressure, DBP - diastolic blood pressure, MAP-mean arterial pressure, IOP-intra-ocular pressure, SOPP- systolic OPP, DOPP-diastolic OPP, MOPP- mean OPP

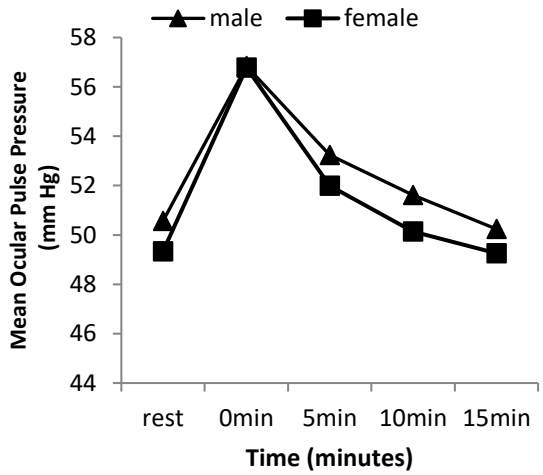

Figure 1: Ocular Pulse Pressure changes before and after Isometric exercise

DISCUSSION

The aim of the conducted work was to study the effect of short term isometric exercise on ocular perfusion pressure in healthy young adults of either gender. This study involved young adults of 18 to 21 years with normal BMI in two groups. IOP, MAP and OPP responses to autonomic function tests like Isometric Exercise was carried out. There was a statistically significant increase in OPP, MAP following Exercise.

In recent years, new knowledge about physical activity like exercise and the associated positive implications on health are gaining clinical significance. Almost all physiological process will be benefited by the regular physical activity. Likewise, exercise is also said to be beneficial for eyes (Hilton, 2003).

In our study we found out that IOP has declined & BP is increased, thus OPP has been increased. This effect could be due the fact that Isometric Leg press exercise can stimulate ocular sympathetic nervous system to increase the facility of outflow of aqueous humor and thus decreases IOP (Sears and Mead,1983) [11]. Stimulation of sympathetic nervous system during and post exercise is well documented and causes the release of large quantities of epinephrine and nor epinephrine from adrenal medulla. This epinephrine released as a result of exercise stimulates synthesis of cAMP. Activation of cAMP decreases IOP by decreasing aqueous humour production (Hilton, 2003).

Many studies have reported that epinephrine reduces IOP by lowering outflow resistance and by lowering the rate of aqueous formation (Richard and Drance, 1967). Also, after leg press exercise there is rise in blood lactate levels. Increased Lactate levels causes outflux of water from eye which is responsible for fall in IOP (Khurana, 2007). Rapid decrease in IOP during the first few minutes of exercise been postulated to be due to osmotic effect of increasing lactate leading to dehydration and decreasing pH, resulting in hypo-secretion of aqueous (Kielar et al., 1975).

Increase in Blood pressure recording has been suggested to be mediated primarily by the central command which is related to number of motor units activated and to reflex effects from active muscle mechanoreceptors (McArdle et al., 2006). The same may also occurs through withdrawal of vagal dominance. Sympathetic stimulation appears to be a secondary mechanism for increasing the HR, BP, as it becomes operative only after first mechanism of vagal withdrawal has been utilized (Martin et al., 1974). The increased systemic pressure post isometric exercise decreases IOP, elevates BP- which in turn produces an elevated OPP (Beck et al., 2003).

In conclusion, the present results indicate that ocular perfusion pressure increases immediately after isometric exercise which is believed to be beneficial for ocular health. Regular exercise also helps to control blood pressure which can help protect capillaries which would otherwise be predisposed to damage by raised blood pressure. Hence exercise offer a safe and simple method for reducing the risk of developing glaucoma and may proves to be useful in normotensive glaucomatous patients. Further studies should be carried out using glaucoma patients to investigate the probable beneficial effect of exercise.

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Short Communication

Erythrocyte Sedimentation Rates and Leukogram Changes in Canine Model of Osteoarthritis

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Summary: Inflammatory markers such as erythrocyte sedimentation rates (ESR) have been evaluated in humans with osteoarthritis (OA). However, there has been no record of evaluation of ESR during OA in dogs. Changes in erythrocyte sedimentation rates (ESR) and leukogram functions were evaluated following experimental knee osteoarthritis (OA).Ten dogs of both sexes with (mean weight = 12.4 ± 1.8kg) were used. Experimental OA was induced in the right knee, using the groove model and confirmed radiographically using evidence of joint space narrowing and presence of osteophytes. Gait was assessed subjectively and scores (GAS) were assigned. Blood was obtained fortnightly for the determination of ESR, total white blood cell (tWBC), neutrophil and lymphocyte counts, while knee radiographs were obtained fortnightly for twelve weeks. Radiographic scores (RAS), GAS, ESR and leukocyte parameters between the different time points were compared with ANOVA. Correlation between parameters was evaluated using Pearson's correlation. A "P" value less than 0.05 was considered significant. Both ESR and neutrophil/lymphocyte (N/L) ratio increased from week 0 to week 12 of OA. However, tWBC, neutrophil and lymphocyte counts did not differ significantly. Both GAS and RAS increased up to week 4 and 6 of OA respectively. Erythrocyte sedimentation rates was significantly (p= 0.033) and positively correlated (r=0.793) with N/L ratio, but negatively and slightly correlated (r= -0.843) with GAS. There was no significant correlation between ESR and RAS. It was concluded that both ESR and N/L ratio might be useful in monitoring progression of OA in dogs.

Keywords*:* Canine, Osteoarthritis, ESR, Neutrophils.

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INTRODUCTION

Osteoarthritis (OA) is a degenerative joint disease characterized by damage to the articular cartilage and changes in the subchondral bone (Lepine and Hayek 2001).It is frequently accompanied by inflammation of the synovium (Marijnissen *et al.* 2002). Osteoarthritis is more frequent in dogs than in cats. The first change in OA is probably biomechanical stress that feeds back onto the cartilage surfaces and later subchondral bone (Marijnissen *et al.* 2002). This leads to biochemical changes in the joint tissues. Once an injury occurs in the joint; there is an attempt to repair the injured part. This repair may be an inflammatory response with mononuclear cellular infiltrate or a fibroblastic response with the formation of fibrocartilage (Solomon 1997). Other definitive changes such as subchondral bone sclerosis, osteophytic proliferation and cartilage loss occur later in the course of the disease (Frost- Christensen *et al.* 2008).

Clinical parameters of OA such as pain, stiffness and functional ability can be measured relatively simple. However, evaluation of structural changes demands much greater effort. Imaging technique and biomarker analyses are done to obtain information on structural changes in OA joints (Frost-Christensen et al. 2008). The ideal molecular marker should not only relate to the nature of the disease, but also the stage of degradation either directly or in proportion to the degeneration (Rorvik and Grondahl 1995). Inflammatory markers such as ESR have been evaluated in humans with osteoarthritis and have been shown to be mildly elevated in osteoarthritic patients (Dieppe and Lim 1998), however it has not been evaluated in dogs. Most inflammatory OA markers evaluated in dogs such as C- reactive proteins and interleukins are relatively expensive. Till now, there appears to be no simple biochemical test that can be used for the confirmation and /or monitoring the progression and severity of osteoarthritis in dogs. The aim of this study therefore was to evaluate the changes in ESR and leukogram during experimental knee osteoarthritis in dogs, and to determine if these parameters can be used as a biomarker for monitoring the progression of osteoarthritis.

MATERIALS AND METHODS

Ten adult local dogs of both sexes with mean weight age ranging from 1-3 years and mean weight of 12.4 ± 1.8 years were used. They were adjudged to be free of any musculoskeletal disease based on the visual assessment of gait and radiographic evaluation of the joints. The dogs were housed individually in concrete -floored kennels and were fed once daily on cooked rice supplemented with sufficient amount of fish and palm oil, while water was provided *ad-libitum*. Ethical approval for this study was obtained from the Research Ethics Committee, College of Veterinary Medicine, Federal University of Agriculture, Abeokuta, Ogun State.

Dogs were premedicated with intramuscular injections of 0.04mg/kg Atropine (Amopin®, Yanzhou Pharmaceuticals, China) and 1mg/kg Xylazine (XYL-M2®, V.M.D, Germany). Fifteen minutes later, anaesthesia was induced with intravenous injection of 0.5mg/kg Diazepam (Calmpose® Ranbaxy, India) and 15mg/kg Ketamine (Ketamin hydrochloride USP®, Rotex Medica, Germany). Following induction of anaesthesia, the right knee was prepared aseptically. Experimental OA was induced as described by Frost-Christensen et al. 2006.Thereafter, the incision was then closed in three layers. The dogs were allowed to recuperate for two weeks. During this period, the dogs were treated with Penicillin-Streptomycin (PenStrep®, Kepro, Holland) and pain was controlled using a combination of Dipyrone and Sodium salicylate (Febralgina®, Agrovet, Peru) administered for three days.

The dogs were assessed radiographically, two weeks after arthrotomy of the right knee for confirmation of OA and then fortnightly, for twelve weeks. Similarly the gaits of the dog were assessed subjectively every two weeks up to twelve weeks of OA. In addition, 5 ml of blood was obtained from the cephalic vein before knee arthrotomy, immediately after radiographic confirmation of OA and fortnightly up to twelve weeks, for the determination of ESR, tWBC, and leukocyte differentials. The ESR was determined using the Wintrobe technique (Briend- Marchal et al. 2003), while the tWBC and the leukocyte differentials were determined with automated blood analyzer. Neutrophil lymphocyte ratio was taken as the ratio of the absolute counts of neutrophils and lymphocytes.

Statistical Analysis

Data were expressed as mean ± standard deviation. Gait assessment scores (GAS) and radiographic scores (RAS) were compared at six and twelve weeks using Wilcoxon sign rank test, while ESR and leukocyte parameters were compared between different time points with ANOVA. Correlation between parameters was evaluated using Pearson's correlation. A "P" value less than 0.05 was considered significant.

RESULTS

Radiographic signs of OA were first observed four weeks following knee arthrotomy and were characterized by joint space narrowing and presence of osteophytes. The radiographic scores of the dog increased progressively up to week 6 of experimental OA (Fig. 1a). However, there was no significant difference in the radiographic scores of the dog between week 6 and week 12 of OA. The gait assessment scores also increased progressively up to week 4 of experimental knee OA (Fig. 1b). Similarly, the gait assessment did not differ significantly between week 6 and week 12 of OA.

The mean ESR of the dogs increased from week 4 up to week 12 of experimental knee OA (Fig. 2a). The WBC decreased following knee arthrotomy in the dogs and up to ten weeks of experimental knee OA (Fig. 2b). However, the absolute neutrophil counts (ANC) increased gradually up to twelve week following experimental knee OA. The lymphocyte counts increased gradually up to four weeks after experimental knee OA, and then decreased up to ten weeks after experimental OA. The N/L ratio of the

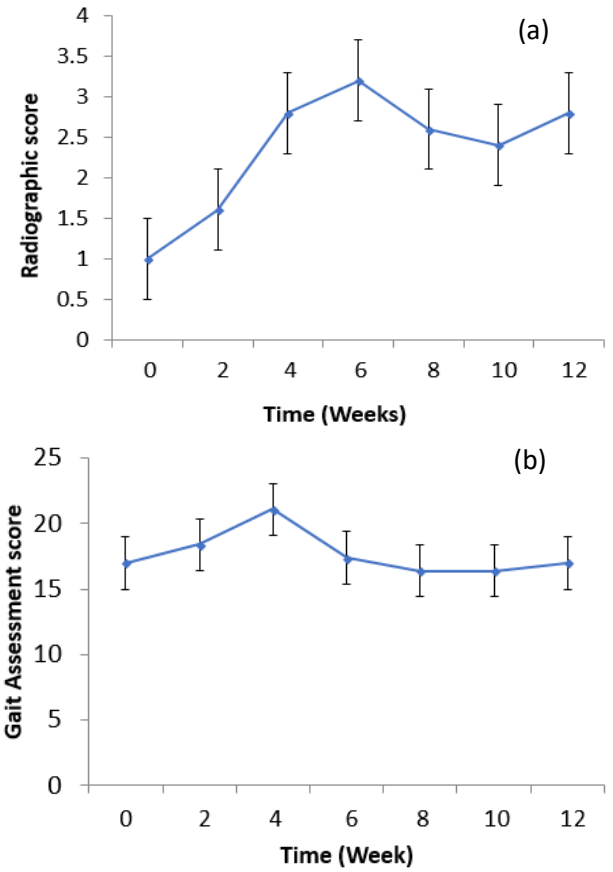

Fig. 1: (a) Radiographic scores and (b) Gait assessment scores of dogs immediately after confirmation of osteoarthritis (Week 0) and at two weeks interval up to a period of twelve weeks.

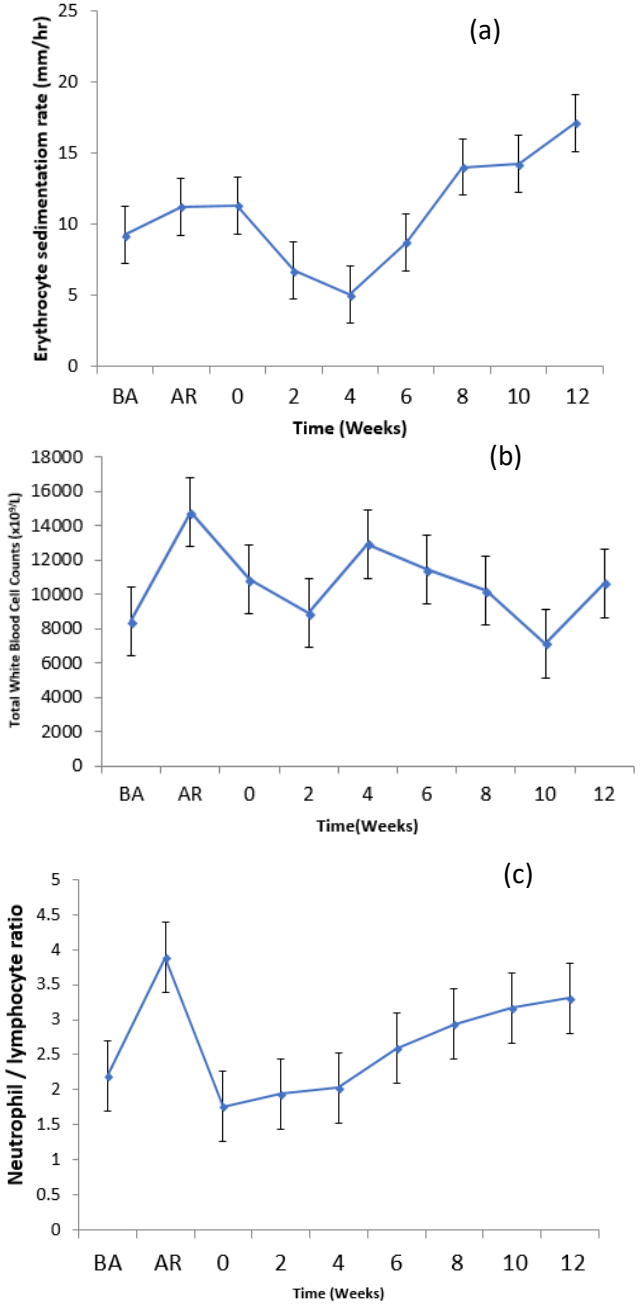

Fig. 2: ESR (a), WBC (b) and N/L ratio (c) of dogs before arthrotomy (BA), immediately after arthrotomy (AR), immediately after confirmation of osteoarthritis (Week 0) and at two weeks interval up to a period of twelve weeks.

dogs also increased from week 0 of experimental OA up to week 12 of osteoarthritis (Fig. 2c). The ESR was positively correlated to the neutrophil-lymphocyte ratio and negatively correlated to the gait assessment. The radiographic scores did not correlate with any of the measured parameters. Gait assessment scores and radiographic score were positively correlated but not significantly.

DISCUSSION

The results from this study showed that both the erythrocyte sedimentation rates (ESR) and neutrophil-lymphocyte (N/L) ratio increased progressively from the onset of osteoarthritis up to twelve weeks of osteoarthritis in an experimental model of knee osteoarthritis in dogs. In addition, there was a positive correlation between ESR and N/L ratio following experimental knee osteoarthritis in dogs. However, these parameters were negatively correlated to both gait assessment score (GAS) and radiographic scores (RAS) following OA in the dogs.

Involvement of phagocytic leukocytes during inflammatory response is an important aspect of the natural immune response (Hughes et al. 2010). Changes in leukocytes and endothelial markers are indicative of increased inflammatory reactions. It has been reported that the groove model of OA is characterized by minor inflammation and that the associated synovial inflammation tended to decrease from week 20 up to week 40 of OA (Marijnissen et al. 2002; Frost-Christensen et al. 2008). The progressive increase in the L/N ratio in this study up to week 12 of OA might be an indication of the progressive synovial inflammation secondary to the experimental OA.

ESR is probably the most widely used laboratory marker of the activity of joint diseases. A rise in ESR is one of the main hallmarks of inflammatory and non – inflammatory arthropathies (Punzi et al. 2005). In this study, ESR significantly increased following arthrotomy and then gradually declined up to week 0 of OA before it then progressively and steadily increased up to week 12 of OA. This changes were however, not significant. This finding further confirms that following the surgically induced inflammation which subsided thereafter, there was a mild component of synovial inflammation which accompanies the groove model of OA as earlier described (Frost-Christensen et al. 2008).

Although both ESR and N/L ratio progressively increased during the twelve week of OA, the lack of significant correlation between this parameters and radiographic and gait assessment scores makes it difficult to conclude that they are reliable indicators of OA in dogs. The lack of significant correlation further supports the previous findings that there is no association between the severity of OA and radiographic findings. This further confirms that OA is more than just damage to the cartilage and that complex mechanisms are involved in the response of cartilage to trauma during OA. In conclusion, although both ESR and N/L ratio may be useful to monitor the progression of knee OA in dogs, the lack of significant increase in their values would suggest further evaluation of their role as markers of inflammation in patients with OA.

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