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Alterations in the Liver Histology and Markers of Metabolic Syndrome Associated with Inflammation and Liver Damage in L-arginine Exposed Female Wistar Albino Rats

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Abstract: Metabolic Syndrome (MES), a cluster of metabolic disorders, is pandemic and more prevalent in females. It was associated with inflammation, liver damage and reduced nitric oxide concentration. Since L-arginine (ARG) may enhance nitric oxide synthesis, this study investigated the effect of ARG on the liver histology and selected serum markers of MES related to inflammation and liver damage. Two groups (n = 8) of female Wistar albino rats were exposed to 60 mg kg^{-1} b. wt. of ARG and 3 mL kg^{-1} b.wt. of distilled water, respectively as treated and control groups. Per oral exposure to ARG for twenty eight days caused a non-significant increase ($p>0.05$) in the neutrophils count (22.50±10.35%, representing 38.46%) but a decrease ($p>0.05$) in the lymphocytes count (77.50±10.35%, representing 8.82%) and in the total bilirubin concentration (0.40±0.19 mg/100 mL, representing 52.38%) of the rats, suggesting non-treatment related influence on these parameters. However, the exposure elicited a significant decrease ($p<0.01$) in the serum alanine aminotransferase (ALT) activity (66.47±0.37 IU L^{-1}, representing 18.55%) and in the total White Blood Cell (WBC) count ($2.73±0.75×10^9$ L^{-1}, representing 43.24%), suggesting absence of inflammation and liver damage. ALT had a significant positive correlation with WBC ($r = 0.01$), while the liver histology revealed possible benefit in the ARG-fed rats, seeminlgly confirming benefit on these markers of inflammation and liver damage that could improve related MES features in the rats. Further studies using ARG rich nuts are required to harness insight gained from this study.

Key words: Alamine aminotransferase, liver damage, inflammation, L-arginine, metabolic syndrome

INTRODUCTION

Metabolic Syndrome (MES) is not a disease entity but a cluster of cardiovascular risk factors characterized by obesity, insulin resistance, atherogenic dyslipidemia and hypertension (Deedwania and Gupta, 2006; Gallagher *et al*., 2010). MES predisposes such an individual to further health challenges (Pelucchi *et al*., 2010; Siddiqui, 2011). Metabolic syndrome scourge is pandemic (Gotto *et al*., 2006; Grundy, 2008). The syndrome is prevalent in children (Pedrosa *et al*., 2011) and could aggravate the health burden of the rural populace (Mohan and Deepa, 2006). Globally, the syndrome afflicts about 20-30% of the adult population (Grundy, 2008). The figure corresponds with that (30.7%) reported by Ijeh *et al*. (2010) in adult Nigerian population. It is worrisome that the prevalence of MES is on the increase (Bakoma *et al*., 2011) and among the female gender, that is an independent risk factor for the development of MES (Ravikiran *et al*., 2010), the increase may be higher.

Nitric oxide, NO, regulates cardiovascular function (McGrowder and Brown, 2007) and its abnormal concentration could be pathological (Lokhande *et al* ., 2006). Earlier, Garlichs *et al*. (2000) associated reduced NO concentration with the pathophysiology of MES. A decrease in ARG availability affected the biological activity of NO (Subratty *et al*., 2007; Harisa, 2011) while possible benefit of ARG supplementation on MES features was suggested (Sepehri *et al*., 2006; Harisa, 2011). Indeed, ARG plays a key role in many metabolic processes (Van Waardenburg *et al*., 2007) and could play important role in insulin resistance and NO synthesis (Ezeanyika and Egbuonu, 2011) that could improve MES in animals.

In similar studies, L-arginine exposure improved the markers of MES related to renal function (Egbuonu and Ezeanyika, 2013) and glucose metabolism (Egbuonu and Ezeanyika, 2012), but aggravated those related to lipid metabolism (Egbuonu and Ezeanyika, 2013). Metabolic syndrome was associated with liver damage

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(Nakanishi *et al*., 2004; Liangpunsakul and Chalasani, 2005) and chronic inflammation (Gallagher *et al*., 2010). These therefore warranted this study.

Objectives set to achieve the aim include the study of the effect of ARG on the liver histology, serum ALT activity and total bilirbin (T-Bilirubin) concentraton as well as total White Blood Cell (WBC), neutrophils and lymphocytes counts, using female Wistar albino rats as model. Bilirubin is a product of heme metabolism and its concentration in serum reflects the functional capacity of the liver or the extent of liver necrosis (Dahiru and Obidoa, 2007) and response to oxidative stress from liver injury (Berk and Korenblat, 2009; Egbuonu, 2010). Inflammation is an immune response mediated by lymphocyte, a type of WBC (Ochei and Kolhatkar, 2008).

MATERIALS AND METHODS

Chemicals: The chemicals used in this study were of analytical grade and were products of reputable companies based in Europe and America.

Concentration determination/justification: The test concentration, ARG (60 mg kg^{-1} b.wt.,) was calculated and adjusted based on the WHO reported daily ARG oral intake (Marshal, 1994) and the concentration used in earlier studies (Alexander *et al*., 2004; Egbuonu *et al*., 2010a-c).

Animals and treatment: Procurement of female Wistar albino rats (60-80 g) used in this study was from the animal house of the Faculty of Biological Sciences University of Nigeria, Nsukka. The animal study was according to International guidelines for the care and use of laboratory animals in Biomedical Research (CCAC, 1985; WMA/APS, 2002). The animal study was conducted between August and September, 2010.

The rats acclimatized for a week and were randomized into two groups (based on their body weight) with sample size of eight rats each. Group B rats were exposed to ARG (60 mg kg^{-1} b.wt.) whereas Group A rats were given distilled water (DW) (3 mL kg^{-1} b.wt.). Exposure route was by oral intubation, which was consecutive for 28 days.

The rats, housed in a well-ventilated stainless steel cages at room temperature (28±2°C) and tropical humid condition, were maintained under standard natural photoperiodic condition of twelve hours of light alternating with twelve hours of darkness. The rats were allowed unrestricted access to tap water and standard rat chow (Grand Cereals and Oil Mills Limited, Jos, Nigeria) for the experimental period.

Sample collection and preparation: The animals were fasted overnight before sacrifice after 28 days. Collection of the respective blood sample of the animals was by ophthalmic venous plexus or retro orbital sinus venipuncture. This involved inserting a sterile capillary tube into the medial canthus of the eye of the rat to puncture the retro-bulbar plexus resulting in out flow of blood into clean tubes: (i) non-anticoagulated tube for serum tests (total bilirubin (T-bilirubin) concentration and ALT activity) and (ii) anticoagulated tubes for whole blood tests (WBC counts).

Centrifugation of clotted blood at 3000 rpm for 10 min yielded the serum. The serum, after aspiration into separately stoppered polystyrene tubes, was stored in a deep freezer for subsequent use in determining the serum total bilirubin (T-bilirubin) concentration and ALT activity. Organ specimen (liver) excised from the sacrificed rats for histology were fixed in 10% formaldehyde buffered saline (formal saline) until used.

The choice of female rats in this study derived from recent reports of higher prevalence of MES in females (Titty *et al*., 2008; Mangat *et al*., 2010; Kilic *et al*., 2010) and the listing of female gender as an independent risk factor for the development of MES (Ravikiran *et al*., 2010).

Parameters determined

Total and differential white blood cell (WBC) count: The total White Blood Cell (WBC) count ($X10^9$/L) and differential WBC count (%) were determined as described by Ochei and Kolhatkar (2008). In particular, WBC was determined based on the principle that whole blood when diluted appropriately with a diluent haemolyses red cells, leaving all nucleated cells intact. Counting of the number of white cells in a known volume and known dilution was with a counting chamber.

The differential white blood cell count involved the identification and counting of various types of white blood cells (neutrophils and lymphocytes) and expressing the number of each type per 100 white blood cells (Ochei and Kolhatkar, 2008).

Serum alanine aminotransferase (ALT) activity: The serum alanine aminotransferase (ALT) activity was determined by the method of Reitman and Frankel (1957). The method is based on the coupling of pyruvate (pyruvic acid) formed from the alanine aminotransferase catalysed reaction with chromogen (2, 4-dinitrophenyl hydrazine) in alkaline medium to yield coloured hydrazone that was measured colorimetrically at 540 nm.

Serum total bilirubin (T-bilirubin) concentration: Total bilirubin in serum was estimated by a standard colorimetric method as described by Egbuonu (2010). This method involved Van de Bergh reaction based on the principle that bilirubin could form a stable complex with diazotized sulphanilic acid in dilute hydrochloric acid to give blue azobilirubin that was measured at 580 nm.

Organ histology: Organ specimen (liver) promptly excised from the sacrificed rats for histological examination were fixed in 10% formaldehyde buffered saline (formal saline) until used as reported in Egbuonu *et al.* (2010c). In brief, after dehydration (in graded levels (70-100%) of alcohol), clearing (in xylene impregnated with paraffin wax) and sectioning (at 5 microns thickness using rotary microtome) the sections were floated on a water bath maintained at a temperature of 2-3°C below melting point of the paraffin wax. Thereafter, drying of the sections was performed on a hot plate maintained at a temperature of 2-3°C above the melting point of the paraffin followed by staining and mounting of the sections using haematoxylin and eosin.

Statistical analysis: Analysis of data to determine the significant differences in means was by Student's t-test, using the Statistical Package for the Social Sciences (SPSS) for Windows version 16.0 (SPSS Inc., Chicago, IL., USA). Results were expressed as mean and standard deviation (Mean±SD) of eight rats per group at significance levels of $p<0.05$, $p<0.01$. Furthermore, correlation of the results for possible association among the studied parameters was by Pearson's bivarate methods ($r = 0.05$, $r = 0.01$).

RESULTS

Serum alanine aminotransferase (ALT) activity: As shown in Fig. 1, the serum ALT activity decreased in the ARG-treated group (66.47±0.37 IU L^{-1}) when compared with the control group (81.61±2.22 IU L^{-1}). The observed decrease (representing a decrease of 18.55%) was significant ($p<0.01$).

Serum total bilirubin (T-bilirubin) concentration: The serum total bilirubin concentration results, as depicted in Fig. 2, decreased ($p>0.05$) in ARG-treated rats (0.40±0.19 mg/100 mL) relative to the control (0.84±0.34 mg/100 mL). The observation represents a decrease of 52.38% in ARG-fed rats relative to the control.

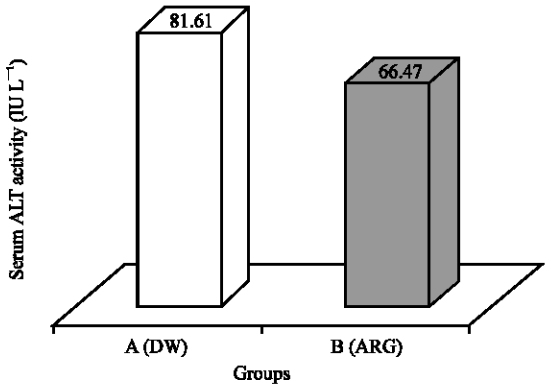

Fig. 1: Influence of DW and ARG on serum ALT activity of rats

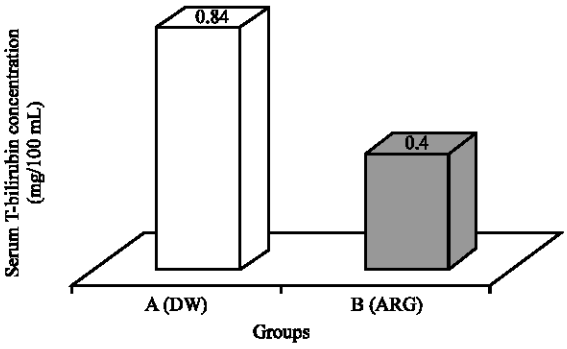

Fig. 2: Influence of DW and ARG on serum T-Bilirubin concentration of rats

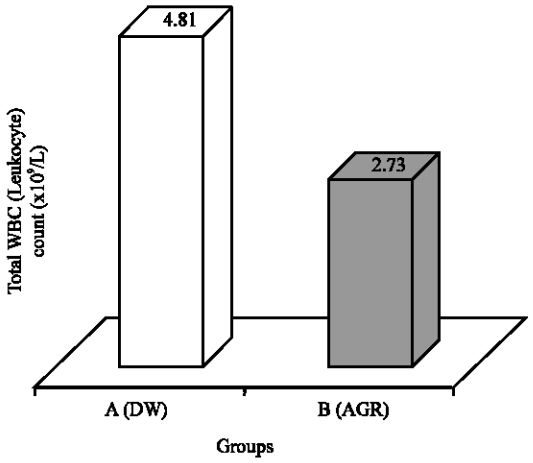

Fig. 3: Effect of DW and ARG on total WBC count of rats

Total white blood cell (WBC) count: The results of this study as presented in Fig. 3 show that the total WBC count of the ARG-treated rats ($2.73\pm0.75\times10^9$/L) significantly ($p<0.01$) decreased in comparison to the control rats ($4.81\pm0.29\times10^9$/L). This represented a decrease of 43.24% relative to the control.

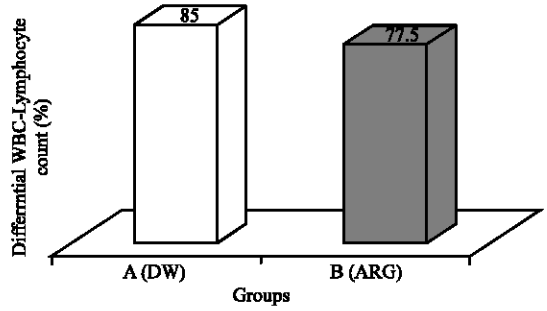

Fig. 4: Influence of DW and ARG on differential WBC-lymphocyte count of rats

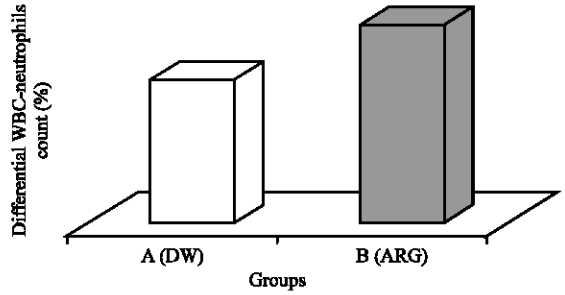

Fig. 5: Effect of DW and ARG on differential WBC-neutrophils count of rats

Differential WBC-Lymphocyte count: The results of this study as presented in Fig. 4 show that the differential WBC-lymphocyte count of ARG-fed rats (77.50±10.35%) decreased contrary to the control (85.00±5.34 %). The decrease (8.82%) however was statistically non-significant ($p>0.05$).

Differential WBC-Neutrophils count: The results of the present study as presented in Fig. 5 reveal that the differential WBC-neutrophils count of rats fed ARG (22.50±10.35%) increased ($p>0.05$) contrary to the control (16.25±7.44%). This increase, representing 38.46% relative to the control, was not statistidally significant.

Histomorphology of the liver: Liver histomorphology of rats in the control (Group A) showed typical lobular histology but with mild degenerative changes (Fig. 6). Sections collected from rats treated with ARG (Group B) showed normal histologic features devoid of degenerative changes (Fig. 7).

Correlation of results: The results of Pearson's correlation analysis revealed that ALT had a significant positive correlation with T-Bilirubin ($r = 0.05$) and T-WBC ($r = 0.01$), an insiginificant negative correlation with

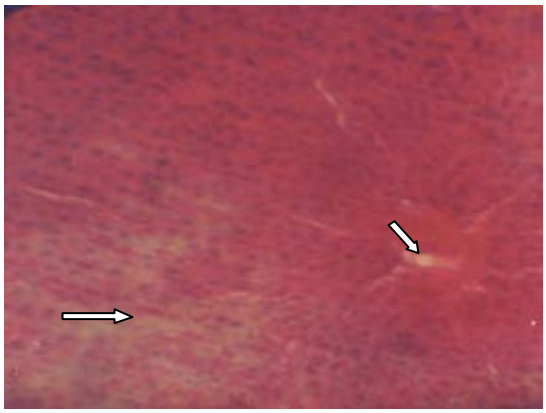

Fig. 6: Liver section of untreated, control (Group A) rats showing mild degenerative changes (arrow heads). H and E stains, ×400

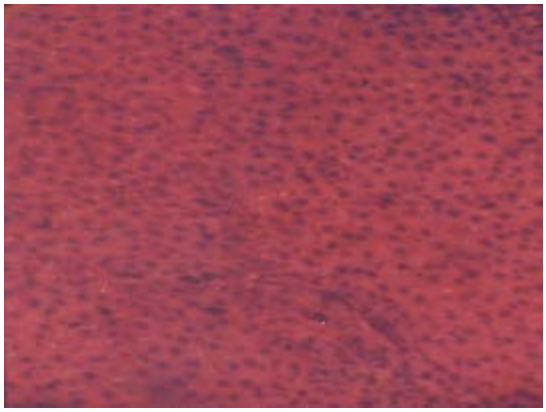

Fig. 7: Liver section of rats exposed to high ARG (Group B) showing normal histology devoid of degenerative changes. H and E stains ×400

neutrophil count but a positive correlation with lymphocyte count. On the other hand, neutrophils had a significant negative correlation ($r = 0.01$) with lymphocytes (Table 1).

DISCUSSION

The liver is a target organ for insulin resistance and the development of metabolic syndrome (Grundy, 2007) and elevation in liver enzymes was associated with MES in animals (Kazumi *et al*., 2006; Rejeb *et al*., 2010). In similar studies, L-arginine exposure improved the markers of MES related to renal function (Egbuonu and Ezeanyika, 2013) and glucose metabolism (Egbuonu and Ezeanyika, 2012), but aggravated those related to lipid metabolism (Egbuonu and Ezeanyika, 2012). Metabolic syndrome was

Table 1: Correlations output of Serum ALT, T-Bilirubin, Total WBC, Neutrophils and Lymphocytes

		SERUM ALT	SERUM T-Bilirubin	Total WBC	Neutrophils	Lymphocytes
SERUM ALT	Pearson Correlation	1	0.581*	0.878**	-0.409	0.469
	Sig. (2-tailed)		0.018	0.000	0.116	0.067
	N	16	16	16	16	16
SERUM T-Bilirubin	Pearson Correlation	0.581*	1	0.626**	-0.302	0.355
	Sig. (2-tailed)	0.018		0.010	0.256	0.177
	N	16	16	16	16	16
Total WBC	Pearson Correlation	0.878**	0.626**	1	-0.268	0.340
	Sig. (2-tailed)	0.000	0.010		0.315	0.198
	N	16	16	16	16	16
Neutrophils	Pearson Correlation	-0.409	-0.302	-0.268	1	-0.963**
	Sig. (2-tailed)	0.116	0.256	0.315		0.000
	N	16	16	16	16	16
Lymphocytes	Pearson Correlation	0.469	0.355	0.340	-0.963**	1
	Sig. (2-tailed)	0.067	0.177	0.198	0.000	
	N	16	16	16	16	16

*Correlation is significant at the 0.05 level (2-tailed). **Correlation is significant at the 0.01 level (2-tailed)

associated with liver damage (Nakamishi *et al*., 2004; Liangpunsakul and Chalasani, 2005) and chronic inflammation (Gallagher *et al*., 2010). These therefore warranted this study.

Comparable to previous report (Sokolovic *et al*., 2009), serum T-Bilirubin concentration decreased in the ARG-fed rats. However, the effect of ARG on T-bilirubin concentration, lymphocyte neutrophils counts of the rats were not significant ($p>0.05$), suggesting that these observations in the rats were either non treatment related (Egbuonu, 2012) or influenced by an underlying factor beyond the control of the present study.

Exposing ARG to the rats caused a significant ($p<0.01$) reduction in their serum ALT activity, indicating absence of hepatitis or inflammation of the liver (Ochei and Kolhatkar, 2008) and perhaps, improvement on the related MES parameters in the rats. Possible benefit on the liver following agent-induced reduction in ALT activity was reported (Egbuonu *et al*., 2012). Elevated ALT activity, a specific bio-marker of hepatic function, significantly correlated with liver damage (Fraulob *et al*., 2010) and parameters of MES in patients with MES (Saely *et al*., 2008; Mojiminiyi *et al*., 2010; Koller *et al*., 2010; Gunji *et al*., 2010).

The result of the present study revealed that intake of ARG by the rats decreased their WBC count, precluding chronic inflammation and, possibly, associated MES features (Shim *et al*., 2006; Lee *et al*., 2010) in the rats. Increased WBC count was strongly associated with chronic inflammation and MES components (Park *et al*., 2009; Wu *et al*., 2010; Lee *et al*., 2010). Absence of inflammation, indicated by decreased WBC count may result from enhanced utilization of lymphocytes due, perhaps, to the decreased lymphocyte count observed in this study.

These were supported by the observed improvement in the liver histology of the ARG-fed rats. Histomorphologic alterations in organs were the most consistent treatment-related changes (Egbuonu *et al*. (2010c). Agent-induced physiological and biochemical disturbances (Adeniran *et al*., 2006), as well as alterations in liver and kidney histology (Farrag and Shalby, 2007; Egbuonu *et al*. (2010c) have been reported. The significant positive correlation between ALT and WBC may suggest apparent synergy in the ARG-induced effects in the rats. In a similar study (Egbuonu and Ezeanyika, 2013) L-arginine induced improvement of renal function markers was suggested.

In conclusion, the study suggests that ARG improved these markers of inflammation and liver damage which could improve related MES features in the female rats. With the relative abundance of ARG in nuts (groundnuts, cashew nuts e.t.c), further studies using such ARG rich nuts are therefore required to harness insight gained from this study.

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