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Full Length Research Paper

Effects of ethanol extract of *Azadirachta indica* leaves on some immunological and haematological parameters of diabetic Wistar rats

Itemobong S. Ekaidem[1]*, Item J. Atangwho[2], Henry D. Akpan[3], Itoro F. Usoh[3], Oboso E. Etim[3] and Patrick E. Ebong[2]

[1]Department of Chemical Pathology, Faculty of Clinical Sciences, University of Uyo, Akwa Ibom State, Nigeria.
[2]Department of Biochemistry, Faculty of Basic Medical Sciences, University of Calabar, Cross Rivers State, Nigeria.
[3]Department of Biochemistry, Faculty of Basic Medical Sciences, University of Uyo, Akwa Ibom State, Nigeria.

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The effects of ethanol extract of *Azadirachta indica* (Family: Meliaceae) leaves on immunological and haematological parameters of alloxan-induced diabetic rats were investigated with a view to ascertaining its involvement in the immunological or inflammatory control of diabetic vascular complications. Total white blood cell, red blood cell, total lymphocyte and neutrophil counts were determined by microscopy. CD_4^+ cell counts by flow cytometry as well as packed cell volume (PCV), and haemoglobin determination by spectrophotometry were also performed on normal control (NC) and diabetic rats treated orally for 14 days with 400 mg/kg bodyweight of *A. Indica* leaf extract (DTAI) or 7.14 mg/kg bodyweight of chlorpropamide (DTCH) or distilled water (DC). The different groups comprised of six rats each. The result showed that total white blood cells, total lymphocytes and CD_4^+ cells were significantly ($p < 0.05$) increased in untreated diabetic (DC) rats compared to normal control (NC) rats, whereas the increase in neutrophil was not significant. These immunological parameters in diabetic rats treated with the extract (DTAI) and those treated with chlorpropamide (DTCH) were not different from those of the normal control group. Also PCV, haemoglobin concentration and red blood cell count were not significantly different among the test and control groups. The immune cell lowering effects of the *A. indica* leaf extract appeared to be of clinical significance in the control of atherosclerosis and other diabetic vascular complications in rats. These effects were comparable to those of chlorpropamide on the immune cells.

Key words: *Azadirachta indica*, immune cells, atherosclerosis, vascular complications.

INTRODUCTION

Diabetes mellitus is characterized by sustained hyperglycaemia and sometimes hyperlipidaemia. The chronic hyperglycaemia often results in non-enzymatic glycosylation of some protein molecules, setting in motion a chain of reactions that may end up in the distortion of proteins thus altering their structural conformations and physiological roles. These products often termed advanced glycosylation end products (AGEs) cause hardening of tissues and blood cells and thus lead to deteriorations similar to those observed in ageing. AGEs have been linked to stiffening of connective tissues, hardened arteries, loss of nerve functions and deterioration of kidney functions (Cerami, 1985). The degree of glycosylation of haemoglobin, the oxygen-carrying pigment of the red blood cells, has been shown to be a useful index of glycaemic control (Cohen, 1986). However, the effects of haemoglobin glycosylation on red blood cell structure and functions are not well known.

The morbidity associated with diabetes mellitus often results from a number of serious macrovascular and microvascular complications. Accelerated atherosclerosis among diabetics is the major pathologic cause of these

*Corresponding author. E-mail: seityjen@yahoo.com.

processes resulting in increased risk of myocardial infarction, stroke and lower extremity gangrene. Experimental and Clinical evidences suggest that these complications of diabetes are consequences of metabolic derangements such as hyperglycaemia and dyslipidaemia, particularly increase in LDL- cholesterol. The derangements are said to result from defects in insulin actions which are capable of causing cardiovascular diseases in diabetics and patients with insulin resistance (Haffner et al., 2000; Goldberg, 2001).

Atherosclerotic lesions are asymmetric focal thickening of the innermost layer of the artery. Blood-born inflammatory and immune cells constitute an important part of atheroma, the remaining being vascular endothelial and smooth muscle cells (Hansson, 2005). In the centre of the atheroma, foam cells and extracellular lipid droplets form a core region, which is surrounded by a cap of smooth muscle cells and a collagen-rich matrix. The lesions are often infiltrated by T-cells, macrophages and mast cells which are activated to produce inflammatory cytokines (Jonasson et al., 1986; Kovanen et al., 1995, Hansson, 2005). These immune cells dominate early atherosclerotic lesion and their affector molecules accelerate progression of the lesion and inflammation to acute coronary syndrome. The predominant T-cells in the lesion are the CD_4^+ T-cells which recognise protein antigens presented to them as fragments bound to major histocompatibility complex (MHC) class II molecules. CD_4^+ cells reactive to the disease-related antigens such as oxidized LDL and heat shock protein 60 have been identified in human atherosclerotic lesions (Stemme et al., 1995; Hansson, 2001; Xu, 2002).

The balance between inflammatory and anti-inflammatory activities is important in controlling the progression of atherosclerosis. These processes are influenced to a greater extent by some metabolic factors. Metabolic breakdown in diabetes mellitus results in increased circulation and deposition of lipids in the artery which initiate new rounds of immune cell recruitment (Hansson, 2005). Increased production of inflammatory cytokines, particularly TNF and IL-6 by the adipose tissue has also been reported in patients with metabolic syndrome (Arner, 2003). The adipokines including leptin, adiponectin and resistin may influence inflammatory responses in affected patients. All these reactions can conceivably induce the activation and rupture of plaques, thrombosis and ischaemia. The management of diabetes mellitus with the aim of reducing its attendant complications can therefore be improved by the use of agents capable of controlling hyperglycaemia, hyperlipidaemia as well as inflammation.

Azadirachta indica, also known as neem, belongs to the family of meliaceae (Yanpallewar et al., 2003). It is one of the most useful medicinal plants (Kausik and Ranajet, 2002) . Blood glucose lowering activity of *A. indica* seed oil and leaf extracts have been reported in various models of diabetic animals (Dixit et al., 1986; Khosla et al., 2008; Halim, 2003; Gupta et al., 2004). Ethanol extracts of neem leaves have also been shown to demonstrate anti-lipid peroxidative, antihyperglycaemic and anti-hypercholesterolaemic activities as well as reduce serum triglyceride level in diabetic rat model (Ekaidem et al., 2007). The leaf extract of *A. indica* have been used traditionally in the control of diabetes mellitus in many countries including Nigeria, however, reports on studies of its effects on immune and other cells of the haematopoietic system is scanty. This study therefore examines the effect of ethanol extract of *A. indica* leaves on immune and red cell parameters of diabetic rats with a view to ascertaining its involvement in the immunological and inflammatory control of diabetic vascular complications.

MATERIALS AND METHODS

Plant materials and extract preparation

Fresh matured leaves of *Azadirachta indica* (Neem) were harvested from Endocrine Research Farm of the Department of Biochemistry, University of Calabar, Calabar. The leaves were washed with tap water, rinsed with distilled water and sundried to remove traces of water. The leaves were ground to form paste with an electric blender (Binatone, Japan) . One hundred and fifty grams of leaf paste was agitated in 500 ml of ethanol and stored overnight in a refrigerator at 4°C for complete extraction. The suspension was filtered and 50 ml aliquots of the filtrate were poured into separate flasks of known weight. The aliquots were dried at 50°C to constant weight using rotary evaporator. The dry extract was stored in a refrigerator at 4°C. Two grams of the dry extract was resuspended daily in 50 ml of distilled water for administration to the animals. 1.5 ml of the suspension, given to a 150 g rat was equivalent to 400 mg extract per kilogram body weight. The volumes of extract suspension were adjusted accordingly for various weights of the rats.

Animals

Thirty albino Wistar rats weighing 150 - 200 g were obtained from the Animal House of the Department of Biochemistry, University of Calabar. The animals were kept in standards plastic cages and placed in a well ventilated room of temperature between 22 and 27°C. The animals were acclimatized for seven days. During this time and throughout the experimental period, they were fed with commercial rat feed (Pfizer Livestock Co. Ltd, Aba, Nigeria) and tap water *ad libitum*. Ethical approval for the study was obtained from the University of Calabar Medical College Ethical Committee.

Animal treatments

After acclimatization, the animals were assigned into four groups of six rats each as follows: Normal Control (NC), Diabetic Control (DC), Diabetic treated with leave extract of *A. indica* (DTAI) and Diabetic treated with Chlorpropamide (DTCH) groups. The animals for DC, DTAI and DTCH groups were made diabetic by a single intraperitoneal administration of 150 mg/kg bodyweight alloxan monophosphate (Sigma, St. Louis, USA) dissolved in distilled water (Battel et al., 1999). The animals were left for 7 days after which fasting glucose concentrations were determined in their tail-prick blood samples using One Touch Basic Glucometer (Life Scan, USA). Animals with consistent blood glucose greater than

200 mg/dl for 2 days were considered diabetic and randomly assigned to DC, DTAI or DTCH groups before administration of extract and chlorpropamide commenced. Group DTAI was treated daily with 400 mg/kg bodyweight of the extract by oral gavage while group DTCH was treated daily with 7.14 mg/kg bodyweight of chlorpropamide by oral gavage. Groups DC and NC which served as controls were given distilled water orally. Fasting blood glucose of the animals was monitored before and after 14 days treatment.

COLLECTION AND PREPARATION OF SAMPLES

At the end of the 14 day treatment, the animals were anaesthezised under chloroform vapour and sacrificed. Blood samples were obtained by cardiac puncture and poured into EDTA sample bottles. The samples were used for analysis within 12 h of collection.

Haematocrit determination

The packed cell volume (PCV) was estimated using the method of Alexander and Griffiths (1993) . Haematocrit capillary tubes were filled by capillary action to mark with whole blood and bottom end of the tubes were sealed with plasticine. The tubes were centrifuged for 5 min using haematocrit centrifuge. The percentage cell volume was read by sliding the tube along the haematocrit reader until the meniscus of the plasma intersects the 100% line.

Haemoglobin estimation

Cyamethaemoglobin (Drabkin) method (Alexander and Grifiths, 1993) of haemoglobin estimation was employed. Twenty microlitres of EDTA anticoagulated whole blood was added to 5 ml of Drabkin reagent mixed and incubated for 5 min at room temperature for the colour to develop. The absorbance was read against reagent blank at 540 nm using optima SP-300 Spectrophometer.

Total white blood cell count

The estimation of total white blood cells was done by visual method using New Improved Neubauer counting chamber. A 1 in 20 dilution of whole blood was made in Turk's fluid and the counting chamber with its cover glass already in position was filled with the diluted blood using a Pasteur pipette and ensuring that the chamber was filled in one action. The charged chamber was allowed to remain undisturbed for 2 min for the cells to settle. The cells were then counted microscopically using x40 objective lens. Four squares at the corners of the chamber were counted and the result was expressed in cells per litre of whole blood.

Red blood cell count

Red blood cells were counted by visual method using new improved Neubauer counting chamber. A 1 in 200 dilution of blood was made in formol citrate solution (Haymen's fluid) and the counting chamber with its coverglass in position was filled with the diluted blood using Pasteur pipette and ensuring that the chamber was filled in one action. The chamber was allowed to settle for 2 min for the cells to settle. Five squares, the four corners and the central squares were counted using x40 objective lens.

DIFFERENTIAL WHITE CELL COUNT

Differential white blood cell count was performed on Leishman's stained thin blood film and read microscopically using immersion oil objective and a differential manual counter. The different white cells were counted and expressed in cells/litre.

CD_4^+ count

The CD_4^+ lymphocyte was estimated by flow cytometry (Center for Disease Control and Prevention, 1997) using the cyflow automated cell counter (Partec, Germany). Ten microlitres of CD_4^+ PE antibody (Partec, Germany) was mixed with 50ml of EDTA anticoagulated whole blood in a test tube. The mixture was incubated in the dark chamber for 15 min at room temperature of 22 - 28°C. During incubation, the content of the tube was mixed every five min. Eight hundred microlitres of buffer solution was added, mixed and plugged into the counter. After counting the CD_4^+ cells, monocytes and noise were separated gated and the result was recorded.

Statistical analysis

Data are presented as mean ± SD. The differences between and within groups were tested using ANOVA Posthoc analysis. A probability of 0.05 was chosen as a level of significance.

RESULT

The effects of treatment of diabetic rats with ethanolic extract of *A. indica* leaves and chlorpropamide on blood glucose concentration are presented in Table 1, while Table 2 shows the effects of the extract and chlorpropamide on immune and red cell parameters of diabetic and non-diabetic rats. The results show significant ($p < 0.05$) decrease in blood glucose following treatment of diabetic rats with *A. indica* leaf extract and chlorpropamide compared to untreated diabetic rats (DC). The level of blood glucose in rats treated with *A. indica* extract (DTAI) was not different ($p > 0.05$) from those treated with chlorpropamide (DTCH).

Total white blood cell count, total lymphocyte and CD_4^+ cell counts were significantly higher ($p > 0.05$) in untreated diabetic rats (DC) compared to normal control (NC) rats, groups treated with *A. indica* leaf extract (DTAI) and chlorpropamide (DTCH). However, circulating neutrophils and red blood cell parameters were not significantly ($p > 0.05$) different among all the groups.

DISCUSSION

The untreated diabetic rats showed significant increase in total white blood cell, lymphocyte and CD_4^+ counts compared to the extract and chlorpropamide treated diabetic groups and the control. The increased immune cell counts may be the manifestations of the low grade inflammatory reactions associated with the complications of diabetes mellitus.

Atherosclerosis is regarded as the major pathologic cause of macrovascular complications of the disease. Immune cells have been shown to dominate early atherosclerotic lesions and their effector molecules

Table 1. Blood glucose levels of rats before and after treatment.

Blood glucose (mg/dl)	Before treatment	After treatment
DTAI	250.4 ± 11.2*	113.2 ± 15.8[ab]
DTCH	236.5 ± 15.4*	100.8 ± 16.8[ab]
DC	243.8 ± 13.2*	238.3 ± 14.2*
NC	98.4 ± 10.5[b]	96.2 ± 11.3[b]

Mean ± SD: *significantly different from normal control at P < 0.05. [a]Significantly lower (p < 0.01) than its basal level (before treatment data). [b]Significantly lower (p < 0.01) than diabetic control after treatment.

Table 2. Effect of treatment on immunological and haematological parameters of diabetic and non- diabetic rats.

	WBC × 10^9/L	Neutrophil × 10^9/L	Lymphocyte × 10^9/L	CD_4^+ × 10^9/L	RBC × 10^{12}/L	Hb (g/l)	PCV (%)
DTCH	11.26 ± 3.84[b]	2.71 ± 0.61	8.33 ± 0.81[b]	1.32 ± 0.15[b]	5.98 ± 0.32	135.4 ± 25.8	41.82 ± 6.5
DTAI	11.92 ± 3.86[b]	3.05 ± 0.53	8.63 ± 0.48[b]	1.02 ± 0.12[b]	6.21 ± 0.17	136.7 ± 28.7	42.03 ± 7.2
DC	16.86 ± 4.45*	3.28 ± 0.48	13.78 ± 0.81*	1.78 ± 0.27*	6.68 ± 0.20	143.6 ± 18.9	44.85 ± 8.4
NC	12.08 ± 3.01[b]	2.97 ± 0.38	8.87 ± 0.41[b]	1.21 ± 0.17[b]	6.64 ± 0.28	131.0 ± 23.4	40.30 ± 5.3

Mean ± SD: *significantly different from normal control at P < 0.05.

activate inflammatory cells and accelerate the progression of the lesion to acute coronary syndrome (Hanson, 2005). T lymphocyte, predominantly CD_4^+ cells were shown to infiltrate atherosclerotic lesions and react with antigenic oxidized LDL leading to proliferation and circulation of more lymphocytes and neutrophils in human diabetes and animal models. Reduction of circulating neutrophils, total lymphocyte and CD_4^+ cells by the extract of *A. indica* leaves as well as chlorpropamide have been observe in this study. Although the mechanism of this effect is not well known, anti- inflammatory activities have been reported for neem leaf extract (Kaur et al., 2004; Udeinya et al., 2004). The extract effects were attributed to inhibition of immune cells migration and phagocytosis, particularly for macrophages and neutrophils in respect to inflammatory stimuli. The extract also inhibited the induction of inducible nitric oxide synthase, prostaglandin E_2 (PG E_2) and interleukin 1 (IL - I) productions (Kaur et al., 2004), thus controlling the increased vascular permeability associated with inflammatory reactions. Adhesion of malaria-infected erythrocyte and cancer cells to endothelial cells which are mediated by vascular cell adhesion molecule (VCAMI) were also inhibited by neem leaf extract (Udeinya et al., 2004).

In diabetic patients with hypercholesterolaemia, excess LDL lead to the release of inflammatory lipids that induce endothelical cells to express leukocyte adhesion molecules (VCAM-1) which cause blood cells rolling along the vascular surface to adhere at the site of activation (Cybulsky and Gimbrone, 1991; Eriksson et al., 2001). Once the blood cells have attached, chemokines produced in the underlying intima stimulate them to migrate through the interendothelial junctions and into the subendothelial space to enhance vascular damage. Genetic abrogation or pharmacologic blockade of certain chemokines and adhesion molecules for mononuclear cells has been shown to inhibit atherosclerosis in mice (Boring et al., 1998; Lesnik et al., 2003; Luthers et al., 2004).

The inhibition of cell migration, phagocytosis, cell adhesion and production of some chemokines by extract of *Azadirachta indica* leaves may be responsible for the reduction in circulating neutrophil, total lymphocyte and CD_4^+ cells in the diabetic rats. These properties of the extract may be of clinical significance in the prevention of atherosclerosis and coronary artery complications in diabetes mellitus. However, the primary pathogenic factors of vascular complications in diabetes are prolonged hyperglycaemia and hypercholesterolaemia.

Chronic hyperglycaemia results in the formation of advanced glycosylation end products (AGES) with some proteins. AGEs have a number of chemical and biological properties that are detrimental to extracellular matrix and target cells. Circulating plasma protein which modified by addition of AGE residues, bind to AGE receptors on endothelial cells, mesangial cells and macrophages. The AGE-receptor ligation results in generation of reactive oxygen species, activation and nuclear translation of the pleotropic transcription factor NK-KB, generating a variety of cytokines, growth factors and other proinflammatory molecules. Thus enhancing the initiation and progression of atherosclerosis (Brownlee, 2001).

Hypercholesterolaemia especially in the presence of increased free radical generation is atherogenic and may be associated with increased circulating immune cells. Therefore the reduction of circulating neutrophil, total lymphocytes and CD_4^+ cells of diabetic rats by A*. indica* leaf extract may also be associated with its plasma

glucose, cholesterol and free radical lowering effects as earlier reported (Ekaidem et al., 2007). This opinion may be further supported by the fact that diabetic rats treated with chlorpropamide, an oral hypoglycaemic drug for diabetes management, also showed reduced levels of circulating neutrophil, total lymphocyte and CD_4^+ cells. Ethanol extract of *Azadirachta indica* leaves have been shown to reverse the diabetes-associated increase in circulating immune cells and hence may play a significant role in the control of atherosclerosis and management of diabetic vascular complications.

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