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Effect of Walnut (*Tetracarpidium conophorum*)-oil on Cadmium-Induced Alterations in Lipid Metabolism in Male Albino Rats

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Abstract In order to investigate the effects of cadmium exposure on lipid metabolism, and the antidotal efficacy of walnut oil, 35 male rats were divided into 5 groups of 7 animals each. All groups were fed normal rat chow and distilled water or cadmium-poisoned water (200ppm of cadmium as cadmium chloride) for 4 weeks *ad libitum*. Groups 1 (control) and 2 received distilled water and cadmium-poisoned water respectively. Groups 3 and 4 received cadmium-poisoned water and 2.0g/kg and 4.0g/kg body weight of walnut oil respectively by oral intubation. Group 5 was given distilled water and 2.0g/kg body weight of walnut oil for 4 weeks. The cadmium exposure resulted in disruptions in lipid metabolism in the rats as evidenced in; reduced total and HDL cholesterol, high levels of plasma and HDL triglycerides, high levels of plasma, HDL, RBC phospholipids observed in the cadmium exposed animals compared to control animals. Walnut oil administered at both concentrations restored some of these lipid aberrations while causing an increase in total and LDL cholesterol. This is indicative that the oil could be associated with some cardiovascular risk despite its beneficial role in lowering cadmium levels in blood.

Keywords Walnut Oil, Cadmium, HDL, Cholesterol, Triglycerides, Phospholipids

1. Introduction

Heavy metal toxicity has attracted a lot of research interest in recent years[1-9], due to its far-reaching health implications. Consequently, research is on-going as to finding more effective means of managing heavy metal toxicity especially using antioxidant vitamins and natural food substances that will elicit minimum side-effects[1, 10-11]. African walnuts, *Tetracarpidium conophorum* (Müll. Arg.) Hutch & Dalziel Syn. *Plukenetia conophora* is one of such plants found to be rich in antioxidants and essential nutrients[12]. The African walnut belongs to the family Euphobiaceae. It is a climber found in the wet part of Southern Nigeria and West Africa. The fruits are greenish with four round seeds in each fruit. The seed testa is hard and the cotyledons are white in colour[13]. Several works have found different parts of this plant to have antioxidant [14], antimicrobial[15], chelating[16] and antidiabetic[17] properties. The fruits are edible; the plant is medicinal and used for various purposes, including masticatory, giddiness, thrush, antihelminthic, toothache, syphilis, dysentery and as an antidote to snake bite[18]. Consequently, we have attempted in this study to use walnut oil in ameliorating some effects of cadmium toxicity.

Cadmium (Cd) is the most toxic of the heavy metals with toxicity ten times that of other heavy metals. It is an important environmental pollutant present in soil, water, air and food. Anthropogenic sources add three to ten times more cadmium to the atmosphere than natural sources[19]. Major occupational exposure occurs from non-ferrous smelters during production and processing of Cd, its alloys and compounds and the exposure is increasingly common during recycling of electronic waste.

Cd is widely used in industrial processes as an anticorrosive agent, as a stabilizer in PVC products, as a colour pigment, a neutron-absorber in nuclear power plants, and in the fabrication of nickel-cadmium batteries[6]. Phosphate fertilizers also show a big cadmium load. Although some cadmium-containing products can be recycled, a large share of the general cadmium pollution is caused by dumping and incinerating cadmium-containing wastes[20- 21].

Cd has no known useful role in higher organisms,[22] although a role for it in lower life forms has been found. A cadmium-dependent carbonic anhydrase has been found in marine diatoms. Cd performs the same function as zinc in these anhydrases[23-24].

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Cadmium stimulates the formation of metallothioneins (a family of low molecular weight metal binding proteins unique in their high cysteine content) and reactive oxygen species, thus causing oxidative damage to erythrocytes and various tissues resulting in loss of membrane functions[25]. Long term exposure increases lipid peroxidation and causes inhibition of superoxide dismutase activity resulting in oxidative damage to liver, kidney and testes[26].

Cadmium toxicity causes hemorrhagic gastroenteritis, liver and kidney necrosis, cardiomyopathy, and metabolic acidosis can also occur. There is some proof that cadmium can also cause cancer[27]. In order to gain insight into the effect of cadmium on lipid metabolism and the possible ameliorative effects, if any, of walnut oil, this study was designed.

2. Methods

2.1. Experimental Animals

Thirty-five adult male albino rats (Wistar strain) weighing between 120-150g were used for the study. The animals were obtained from the animal house of University of Ibadan, Oyo State, Nigeria. The rats were divided into 5 groups of 7 animals each and kept in separate cages in the animal house of Bells University of Technology, Ota and acclimatized for 2 weeks under normal environmental conditions with 12 hour light/dark cycle. They were fed normal rat chow and water *ad libitum*. Rat weight was measured with a laboratory electronic scale, which is accurate to within 0.01grams.

2.2. Extraction of Walnut Oil

The walnuts were purchased from Oja Titun market in Ife, Osun State in Nigeria and were authenticated by Dr. P. I. Oni of the Biological Sciences Department of Bells University of Technology, Ota, Ogun State.

Walnuts were separated from their shells, air-dried and milled. Portions of the pulp (about 40g) were extracted at a time with n-hexane using a Soxhlet apparatus. After extraction the solvent was removed yielding the oil. Any remaining solvent in the oil was removed by gentle evaporation over a water bath at 60°C. The oil was stored in the fridge at about 4°C until used. When needed the oil was brought to room temperature before administration.

2.3. Experimental Protocol

The five groups of rats were treated according to the assay protocol summarized in Table 1. The walnut oil was administered by oral intubation.

2.4. Collection of Experimental Samples

At the end of four weeks, blood was collected from the animals into heparinized tubes by cardiac puncture under light ether anaesthesia after an overnight fast. Aliquots of blood samples were preserved for cadmium analysis and the remaining was centrifuged immediately at 4000 rpm for 10 minutes to separate plasma and erythrocytes. The plasma was then removed and stored in Eppendorf tubes for further analyses. The erythrocytes were washed with a wash buffer containing 20 mM Tris and 0.15 mM NaCl, pH 7.6. All samples were stored at -20°C until analysed.

Table 1. Summary of groups and treatment

Groups	Treatment
Group 1 (control)	Normal rat chow and distilled water for 4weeks
Group 2 (cadmium only group)	Normal rat chow and 200ppm of Cadmium in drinking water for 4weeks
Group 3 (cadmium and walnut oil group)	Normal rat chow, 200ppm of Cadmium in drinking water and 2.0g/kg body weight of walnut oil orally for 4weeks
Group 4 (cadmium and walnut oil group)	Normal rat chow, 200ppm of Cadmium in drinking water and 4.0g/kg body weight of walnut oil orally for 4weeks
Group 5 (walnut only group)	Normal rat chow, distilled water and 2.0g/kg body weight of walnut oil orally for 4weeks

2.5. Isolation of High Density Lipoprotein (HDL)

The HDL fraction was isolated by the method of Gidez *et al*.,[28] which involved precipitating very low density lipoproteins (VLDL) and low density lipoproteins (LDL) with heparin-manganese chloride (0.06 vol of heparin sodium and 1.0 vol of 1.06M $MnCl_2$) solution. Heparin - manganese chloride solution 0.025 ml was added to 0.25 ml of plasma in a test tube. The resultant mixture was vortexed and left to stand at room temperature for 10 minutes. The mixture was then centrifuged at 4000rpm for 10 minutes. The supernatant (HDL fraction) was carefully decanted into clean Eppendorf tubes and stored at -20 °C until analysed.

2.6. Extraction of Phospholipids from Plasma, Erythrocytes and HDL

Plasma lipids were extracted using chloroform-methanol mixture (2:1, v/v) as described by Folch et al.[29]. Briefly, to 0.1ml of plasma in an Eppendorf tube was added 0.9ml of the chloroform-methanol mixture (2:1 v/v). The mixture was then vortexed thoroughly and allowed to stand at room temperature for 30 minutes. It was centrifuged for 10 minutes and the chloroform layer was transferred into a separate tube using a syringe. This represented the lipid extract. Extraction of lipids from erythrocytes and HDL fraction also followed the same procedure as described for plasma but for erythrocytes, chloroform-isopropanol mixture (7:11, v/v) was used according to the method of Rose and Oklander[30].

2.7. Biochemical Analyses

2.7.1. Determination of Cholesterol in Plasma, HDL and Erythrocytes

Plasma and HDL cholesterol were determined spectrophotometrically according to the methods of Allain *et al*.[31] as outlined in the Cromatest diagnostic kits. The reagent was

made up of three enzymes: cholesterol esterase (CE), cholesterol oxidase (CO) and peroxidase (POD), and two substrates 4-aminoantipyrine (4 -AA) and phenol.

Cholesterol and its esters are released from the lipoproteins through the action of a detergent. CE then hydrolyses the cholesteryl esters to cholesterol and fatty acids. Subsequent enzymatic oxidation of cholesterol by CO results in the production of H_2O_2. The H_2O_2 produced condenses with phenol in a reaction catalysed by a peroxidase. A quinoneimine dye is formed whose concentration is proportional to the concentration of cholesterol in the sample and read at 550nm against reagent blank. For cholesterol in erythrocytes, 0.1ml of the erythrocyte lipid (chloroform/methanol) extract was pipetted into different test tubes and evaporated to dryness at 60ºC. The dried extract was re-dissolved in 20 µl of a triton-X/chloroform mixture (1:1, v/v) and evaporated again as before. 1ml of the cholesterol reagent was then added to the dried extract, vortexed and taken through the procedure outlined in the kit.

2.7.2. Determination of Triglycerides in Plasma, HDL and Erythrocytes

Plasma and HDL triglycerides were determined spectrophotometrically by the method of Buccolo and David[32], as outlined in the Cromatest diagnostic kit. The method was based on the enzymatic hydrolysis of plasma triglycerides to glycerol and free fatty acids (FFA) by lipoprotein lipase (LPL). The glycerol was phosphorylated by adenosine triphosphate (ATP) in the presence of glycerolkinase (GK) to form glycerol-3-phosphate (G-3-P) and adenosine diphosphate (ADP). G-3-P was oxidized by glycerophosphate oxidase (GPO) to form dihydroxy acetone phosphate (DHAP) and hydrogen peroxide.

A red chromogen produced by the peroxidase (POD)-catalysed coupling of 4-aminoantipyrine (4-AA) and phenol with hydrogen peroxide (H_2O_2) was proportional to the concentration of triglyceride in the sample.

For erythrocyte triglycerides, 0.1ml of the erythrocyte lipid extract was used. 0.1 ml of the chloroform-isopropanol extract was pipetted into test tube and evaporated to dryness at 60ºC. The dried extract was re-dissolved in 200 µl of 95% ethanol. Then 1ml of the triglyceride reagent was added and vortexed. After incubation in the dark at room temperature for 30 minutes, absorbance was read at 500 nm against reagent blank.

2.7.3. Determination of Plasma Phospholipids

Plasma phospholipids were determined according to the method of Stewart[33]. The method is based on complex formation between ammonium ferrothiocyanate and phospholipids.

100 µl of plasma was extracted with 0.9ml of chloroform - methanol mixture (2:1,v/v). 0.1 of this chloroform lipid extract (3.7.3) was evaporated to dryness at 60ºC. The dried extract was then dissolved in 2ml chloroform. 2ml ammonium ferrothiocyanate was added and mixed well. The chloroform layer was removed using a syringe and the absorbance read at 488 nm against a blank. The blank was prepared by mixing 2ml chloroform with 2 ml ammonium ferrothiocyanate in a dry tube, and the chloroform layer removed and used as the blank.

2.7.4. Determination of Erythrocyte Phospholipids

0.1 ml of the erythrocyte lipid extract was taken in a test tube and evaporated to dryness at 60ºC. 2 ml of chloroform and 2ml of ammonium ferrothiocyanate were added to the extract and vortexed. The chloroform layer was removed using a syringe and the absorbance was read at 488 nm.

2.7.5. Determination of HDL Phospholipids

HDL phospholipids were determined in the HDL fraction using the same procedure for plasma.

2.7.6. Calculation of LDL and VLDL Cholesterol

LDL and VLDL cholesterol values were calculated using the Friedewald equation[34] which calculates these values using analysed values of Total cholesterol, HDL cholesterol and Triglycerides.

LDL-Cholesterol = Total Cholesterol – HDL- Cholesterol – Triglycerides/5

2.7.7. Determination of Cadmium in Blood

Blood cadmium was determined by atomic absorption spectrometry (Thermo Scientific Equipment S-series (model S4 AA system)). Concentrated nitric acid was used for the digestion of the blood samples which was then read with the AAS.

2.7.8. Statistical Protocol

Results are expressed as mean ± S.D. One way analysis (ANOVA) followed by Duncan's test was used to analyse the results with $p<0.05$ considered significant.

3. Results

Table 2. Cholesterol levels in different compartments of blood of the animals

GROUP	PLASMA (mg/dl)	HDL (mg/dl)	RBC (mg/dl)	LDL (mg/dl)	VLDL (mg/dl)
Group 1	82.81±19.10a	29.35±2.65b	59.34±21.48a	42.10±18.02a	11.36±0.69a
Group 2	76.72±15.99a	14.67±4.35a	82.02±11.15b	47.14±15.09a	14.92±0.84b
Group 3	107.81±11.47b	16.25±2.30a	68.57±12.90a	77.62±13.23b	13.94±0.55b
Group 4	105.20±1.35b	18.94±2.40a	78.75±7.62b	74.03±2.51b	12.23±1.22a
Group 5	84.19±6.85a	31.76±7.50b	54.09±4.12a	40.03±10.71a	12.40±1.01a

Values are mean ± Standard Deviation (SD). Values in a column having no letter (a-b) in common are significantly different from each other ($p<0.05$).

Table 3. Triglyceride levels in different compartments of blood

GROUP	PLASMA (mg/dl)	HDL (mg/dl)	RBC
Group 1	56.80±3.46a	19.80±1.88a	63.89±3.58a
Group 2	74.58±4.23b	27.06±2.43b	67.43±13.95a
Group 3	69.71±2.72b	20.53±1.91a	65.21±3.40a
Group 4	61.40±6.11a	21.48±2.61ab	66.20±5.21a
Group 5	62.00±5.04a	17.79±1.79a	58.66±11.60a

Table 4. Phospholipid levels in different compartments of blood

GROUP	PLASMA (mg/dl)	HDL (mg/dl)	RBC (mg/dl)
Group 1	122.50±12.10a	53.95±5.54a	70.08±5.53a
Group 2	159.50±11.58b	74.36±2.77b	109.50±1.51b
Group 3	145.31±6.29c	70.96±4.67b	94.38±7.76c
Group 4	148.72±6.52c	71.44±3.69b	97.20±11.68c
Group 5	114.22±12.98a	56.38±3.99a	66.68±4.89a

Values are mean ± SD. Values in a column having no letter (a-b) in common are significantly different from each other ($p<0.05$)

The cholesterol levels of the different compartments of blood studied - plasma, high density lipoprotein (HDL), red blood cell (RBC), low density lipoprotein (LDL), very low density lipoprotein (VLDL), are depicted in Table 2. Total cholesterol levels in plasma of the animals in groups 2 and 5 did not show any significant difference ($p<0.05$) compared with control, whereas those in groups 3 and 4 increased significantly ($p<0.05$). The cholesterol levels in HDL of the animals in groups 2, 3 and 4 were significantly decreased ($p<0.05$) compared with control while group 5 showed no significant difference ($p<0.05$) with control. The cholesterol levels in RBC of the animals in group 3 and 5 did not show any significant difference ($p<0.05$) compared with the control group, while groups 2 and 4 increased significantly ($p<0.05$). The cholesterol levels in LDL of the animals in group 2 and 5 did not show any significant difference ($p<0.05$) as compared with the control group, while the cholesterol levels in LDL of the animals in group 3 and 4 increased significantly ($p<0.05$). The cholesterol levels in VLDL of the animals in group 4 and 5 did not show any significant difference ($p<0.05$) as compared with the control group, while the cholesterol levels in VLDL of the animals in group 2 and 3 increased significantly ($p<0.05$).

Table 3 shows triglyceride levels in plasma, HDL, RBC of the animals. The triglyceride levels in plasma of the animals in group 4 and 5 did not show any significant difference ($p<0.05$) as compared with the control group, while groups 2 and 3 increased significantly ($p<0.05$). It appears that the walnut oil restored the increase of the triglycerides in the cadmium administered group towards control values but this was not statistically significant ($p<0.05$) in group 3. Walnut oil at 4.0g/Kg body wt appeared to be more effective in this instance as evidenced in the values in group 4. The triglyceride levels in HDL of the animals in group 3 and 5 did not show any significant difference ($p<0.05$) as compared with the control group, while the triglyceride levels in HDL of the animals in group 2 and 4 increased significantly ($p<0.05$). The walnut oil at 2.0g/Kg body wt restored the observed increase of the triglyceride level of HDL of the animals in group 3 towards control values. There was no significant difference ($p<0.05$) between the triglyceride levels in the RBC of the groups.

Table 4 shows phospholipid levels in plasma, HDL, RBC of the animals. There was cadmium - induced phospholipid osis observed in group 2. This phenomenon was sustained in groups 3 and 4. Walnut oil appeared ineffective in reversing this trend. The phospholipid levels in HDL of the animals in group 3 and 5 did not show any significant difference ($p<0.05$) as compared with the control group, while the phospholipid levels in HDL of the animals in group 2, 3 and 4 increased significantly ($p<0.05$) showing that, the walnut oil was ineffective in restoring the cadmium-induced increase in phospholipids. The phospholipid levels in RBC of the animals in group 5 were similar to those in the control group. While the phospholipid levels in RBC of the animals in groups 3 and 4 decreased towards control, the decrease was not significant ($p<0.05$). It appears that the walnut oil was effective to some extent.

Table 5. Blood cadmium levels in the animals

GROUP	BLOOD Cd(μg/ml)
Group 1	13.57±0.43a
Group 2	15.26±0.43a
Group 3	12.36±2.15a
Group 4	12.80±0.66a
Group 5	13.64±1.85a

Values are mean ± SD. Values in a column having no letter in common are significantly different from each other ($p<0.05$)

Table 5 shows the blood levels of cadmium in the animals. There was a non-statistically significant ($p<0.05$) accumulation of cadmium in the cadmium - exposed animals as compared with the control group. Walnut oil administered at 2.0g/Kg and 4.0g/Kg body appeared to reverse this observed increase in cadmium.

4. Discussion

The use of traditional medicine and medicinal plants in Africa and Nigeria specifically as a normal approach to the maintenance of health is an age-long approach and is gaining more awareness due to its efficacy and recent advances in research in this area[14-15, 17, 35]. This present study was carried out to investigate the effect of walnut oil, a well-known fruit with antioxidant properties, on cadmium-induced alterations in lipid metabolism.

In comparison to controls, rats poisoned with cadmium in this study displayed lower HDL-cholesterol concentrations in plasma. This was also associated with increase in triglycerides or hypertriglyceridemia and increase in phospholipids.

Evidence for further cadmium-induced disruptions in lipid metabolism is shown in the increase in cholesterol and phospholipids in the red blood cells without a concomitant increase in triglycerides; high levels of plasma and HDL

triglycerides; high levels of plasma, HDL, RBC phospholipids observed in the cadmium-exposed animals compared to control animals.

HDL enables lipids like cholesterol and triglycerides to be transported within the water-based bloodstream. In healthy individuals, about thirty percent of blood cholesterol is carried by HDL. Blood tests typically report HDL-C level, i.e. the amount of cholesterol contained in HDL particles. It is often contrasted with low density or LDL cholesterol or LDL-C. HDL particles are able to remove cholesterol from within artery atheroma and transport it back to the liver for excretion or re-utilization, which is the main reason why the cholesterol carried within HDL particles (HDL-C) is sometimes called "good cholesterol" (despite the fact that it is exactly the same as the cholesterol in LDL particles). Those with higher levels of HDL-C seem to have fewer problems with cardiovascular diseases, while those with low HDL-C cholesterol levels (less than 40 mg/dL or about 1 mmol/L) have increased rates for heart disease,[36- 37].

Because LDL particles appear harmless until they are within the blood vessel walls and oxidized by free radicals,[38], it is postulated that ingesting antioxidants and minimizing free radical exposure may reduce LDL's contribution to atherosclerosis, though results are not conclusive[39]. Walnuts have been found to be rich in antioxidants[14].

In this study, the walnut oil proved to be quite effective in lowering the increased RBC cholesterol and the increased triglycerides in the plasma and HDL fraction. This was also the trend concerning the observed phospholipidosis in the plasma and RBC. Administration of the lower concentration of the oil reversed this increased levels of phospholipids. Increased cadmium in the blood was significantly reduced upon administration of the oil which might be a pointer to the fact that the oil could possess some chelating properties. This is in consonance with the work of Olabinri et al.[16], who observed a dose-dependent increase in the chelating properties of the aqueous fraction of walnut in vitro.

On the other hand, administration of the walnut oil appeared to lead to increase in LDL levels in the cadmium exposed animals in this study which implies an exacerbating effect on cadmium toxicity. The oil seemed to raise LDL cholesterol above the levels found in the cadmium-exposed animals upon its administration. This might be a pointer to the fact that the oil might not be so effective in the amelioration of cadmium toxicity despite its other useful effects. This is contrary to the host of studies that have shown that increasing the dietary intake of monounsaturated-dense walnuts has favourable effects on cholesterol levels and other cardiovascular risk factors. In one such study, involving an 8-week crossover feeding trial in subjects with moderate hypercholesterolemia, it was found that substituting walnuts for 32% of the energy from monounsaturated fatty acids in a cholesterol-lowering Mediterranean diet improves vascular endothelial function[40]. A lot of these studies however dealt with the whole nut in a diet-based study and not the oil alone like was used in this study and there was no heavy metal introduced[41-45].

5. Conclusions

Further research into the use of walnut oil in diets is needed in order to authenticate these results although we postulate that incorporating the whole fruit in a diet-based study might be more effective in appropriating the antioxidant properties of the fruit more than extracting the oil and ingestion. This could account for the disparity in results of this study and other studies that have shown the fruit to lower LDL levels.

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