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Effects of Selected Antioxidants on Atherosclerosis in Hyperlipidemic Wistar Rats

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Authors' contributions

This work was carried out in collaboration among all authors. Author KSA conceptualized and designed the study. Author SA carried out the study and the statistical analysis, wrote the first draft of the manuscript. Author TOO managed the literature searches and wrote the final document for publication. All authors read and approved the final manuscript.

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ABSTRACT

The interplay of hyperlipidemia and oxidative stress in atherosclerosis has been fairly established by previous studies. There remains however, paucity of data in this environment on the direct effects of antioxidants on atherosclerosis. This study therefore aimed at determining the protective effects of EDTA, vitamin C and Vitamin E on atherosclerosis in diet induced heperlipidemic wister rats.

Thirty Wister rats were investigated in this study. The rats were randomly divided into five groups (n=6). The control group was fed with growers mash and water only while group II-V were induced with hyperlipidemic diet for ten weeks. In addition to the hyperlipidemic diet; group III received 1 g/kg body weight of EDTA, group IV received 1 g/kg body weight of vitamin C, group V received 1 g/kg body weight of vitamin E, and group VI received EDTA, vitamin C and E. The group's treatments were orally for two weeks. C-reactive protein, Total cholesterol (TC), Triglyceride (TG), HDL-cholesterol, LDL-cholesterol, Total calcium and Total antioxidant status were analyzed using standard methods after the treatments.

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The study showed significant effect in the use of EDTA, Vitamin C and Vitamin E in the treatment of atherosclerosis in rats which could be due to their antioxidant and anti-hyperlipidemic properties. Therefore the combinations EDTA, vitamin C and vitamin E appear greatly protective against atherosclerosis.

Keywords: Antioxidants; atherosclerosis; hyperlipidemic Wistar Rats; hyperlipidemia; oxidative stress.

1. INTRODUCTION

In recent years, there seem to be a wide range of proposed treatments for atherosclerosis [1]; yet, this disease condition remains a major burden worldwide and a basic cause of death in most developed countries [2]. In Nigeria, Oladapo et al., 2013 [3] in a cross-sectional survey carried out on bodies referred for post-mortem, examined the circle of Willis of 44 consecutive patients ≥20 years of age for atherosclerosis using the AHA classification and reported an incidence of 45.5% of atherosclerosis in the intracranial cerebral vessels of the studied patients; an indication that atherosclerosis could now be a condition of concern in this region of the world as against what was earlier reported by other studies on ethnic or racial distribution of cerebral atherosclerosis, in which Chinese, Japanese, Hispanics, and African-Americans have higher rates of ICCA, and Caucasians have higher rates of extracranial carotid artery atherosclerosis (ECCA) [3,4].

Various prospective epidemiological trials have shown that the risk of developing the manifestations of coronary atherosclerosis is increased by smoking, hyperlipidemia, hypertension and diabetes [5]. Atherosclerosis develops from low-density lipoprotein molecules (LDL) becoming oxidized (ldl-ox) by free radicals, particularly oxygen free radicals (ROS). When oxidized LDL comes in contact with an artery wall, a series of reactions occur to repair the damage to the artery wall caused by oxidized LDL [6]. The body's immune system responds to the damage to the artery wall caused by oxidized LDL by sending specialized white blood cells (macrophages and T-lymphocytes) to absorb the oxidized-LDL forming specialized foam cells. Unfortunately, these white blood cells are not able to process the oxidized-LDL, and ultimately grow then rupture, depositing a greater amount of oxidized cholesterol into the artery wall. This triggers more white blood cells, continuing the cycle [6].

The interplay of hyperlipidemia and oxidative stress in atherosclerosis has been fairly established by previous studies [7]. Lipid peroxidation is among the deleterious effects of free radical-mediated attack on biological molecules. Among lipids, cholesterol and polyunsaturated fatty acids are very sensitive to free radical attack. As a consequence of lipid peroxidation a series of oxidation products are formed, including aldehydes, isoprostanes and oxysterols, which possess biological activity relevant for atherogenesis and which have been found in atherosclerotic lesions and plasma [8]. These products are responsible for derivation of apolipoprotein (apo)B100 and consequent recognition of LDL by the scavenger receptor. They induce platelet activation, vasoconstriction, and cytotoxicity. Furthermore, they promote apoptosis, and inhibit cholesterol de-loading from foam cells [8].

This link that exist between lipids and oxidative stress has made some researchers in recent years, to shift focus to studying the effect of antioxidants and other substances that may inhibit oxidation of lipids as a tool that may be useful in alleviating the burden of atherosclerosis. Previous studies by Adedapo et al (2017) [9] confirmed EDTA to be effective at reducing hyperlipidemia at a dose dependent rate thus supporting the hypothesis that EDTA chelation has a significant positive effect on atherosclerosis. Although few studies have attempted to determine the level of some antioxidants in individuals with cardiovascular diseases, there remains paucity of data in this environment on the direct effects of antioxidants on atherosclerosis.

This study aims at determining the protective effects of EDTA, vitamin C and Vitamin E on atherosclerosis in diet induced hyperlipidemic wistar rats.

2. METHODS

2.1 Subjects

Thirty Wistar rats obtained from the animal house of College of Medicine, University of Ibadan,

Ibadan, Oyo state, Nigeria were investigated in this study. The rats were divided into five groups (n=6) in cages and kept in a room maintained at 26-29ºC with a 12 hour light-dark cycle to acclimatize and were allowed libitum to food and water. The protocol conforms to the guidelines of the National institute of Health (NIH publication 85-23, 1985) for laboratory animal care and use.

2.2 Experimental Design

A total of thirty six Wistar rats were randomly divided into six (n=6):

- Group I: Control rats fed with growers mash and water only.
- Group II-V: Were induced with hyperlipidemic diet (palm kernel oil at 10 ml/kg body weight) for ten weeks. In addition to the hyperlipidemic diet;
- Group III received 1 g/kg body weight of EDTA.
- Group IV received 1 g/kg body weight of vitamin C.
- Group V received 1 g/kg body weight of vitamin E.
- Group VI: Received EDTE, vitamin C and vitamin E.

The group's treatments were orally for two weeks. At the end of the experiment (twelve weeks) the rats were made to fast for twelve hours and blood samples collected from the tails. The rats were anaesthetized with chloroform and the aorta checked for lesions by a histopathologist.

2.3 Biochemical Assays

Determination of C-reactive protein: C-reactive protein was determined using the enzyme linked immunosorbent assay (ELISA) technique.

Determination total cholesterol: This was determined using the method described by Allein et al. 1974 [10].

Determination of triglyceride: Triglyceride was determined by spectrophotometric method as described by Schettler, 1975 [11].

Determination of HDL-cholesterol: HDL cholesterol was determined using the CHOD-PAP method of cholesterol estimation after precipitation of other lipoprotein fractions using phosphotungistic acid.

Determination of LDL cholesterol: LDL-C was calculated using the Friedewald equation [12].

Determination of total calcium: Calcium was determined by spectrophotometric method, which is based on modified Ortho-cresolphtalein complex methodology [13].

Determination of total antioxidant status (TAS): TAS was determined using the method described by Koracevic et al., 2001[14].

Histopathological studies: After the blood collection, the animals were sacrificed; the aorta was removed as quickly as possible, fixed in 10% formalin and prepared for histological examination.

2.4 Statistical Analysis

All results were expressed as mean ± SE of the mean. The statistical package for the social sciences (SPSS) version 22.0 was used to analyze data. Groups were compared using Student's T-test with differences considered as significant when $p<0.05$.

3. RESULTS

A total of thirty six male Wistar rats were obtained for this study and divided into six groups (n=6). The atherogenic diet was well tolerated by the rats except one; thirty five rats therefore completed the study. The results obtained in this study are shown in Tables 1-5.

Table 1 shows the comparison of biochemical parameters analyzed between the control and hyperlipidemic diet fed Wistar rats (mean ± SD). Significant increase in the serum cholesterol, triglyceride and LDL were observed ($p< 0.05$) in the hyperlipidemic diet fed group when compared to control. Also, increase in the concentration of total antioxidant status and calcium were observed. There was however, no significant change in HDL and Hs-CRP between the case and control.

Table 2 shows the mean results of the comparison of the biochemical parameters observed in the pre and post treatment with 1g/kg of ethylene di-amine tetra acetic acid (EDTA). A significant decrease in the serum total cholesterol, triglyceride, LDL and calcium was

observed (p-value = 0.002, 0.042, 0.010 and 0.001 respectively), while a significant increase was observed in the serum HDL and TAS (P = 0.000 and 0.001 respectively).

In Table 3, a significant decrease in the mean serum total cholesterol, TG, LDL-C and Hs-CRP was observed (P = 0.009, 0.025, 0.008 and 0.000) pre and post treatment with vitamin C.

Table 4 shows the mean results of the comparison of the biochemical parameters observed pre and post treatment in the group treated with vitamin E. A significant decrease in the concentration of total cholesterol, triglyceride, LDL-C and hs-CRP was observed (P = 0.037, 0.000, 0.018 and 0.002) while there was a significant increase in the mean concentration of calcium and TAS (P = 0.028 and 0.029).

Table 5 shows the mean results of the comparison of the biochemical parameters observed pre and post treatment in group treated with combination of Vitamin C, vitamin E and EDTA. A significant decrease in the serum total cholesterol, triglyceride, LDL-C and hs-CRP was observed (p = 0.028, 0.004, 0.018 and 0.027) while a significant increase was observed in the concentration of calcium and TAS (p = 0.016, and 0.009) while a significant increase was observed in the concentration of calcium and TAS (P = 0.016, 0.274.)

Table 1. Comparison of biochemical parameters analyzed between Control and hyperlipidemic induced rats

Parameter	Control N=6	Hyperlipidemic rats N=6	t-value	p-value
Total cholesterol (mg/dl)	96.21±15.09	182.34±2.05	-13.9	0.000*
Triglyceride (mg/dl)	70.33±27.27	140.70±1.73	-6.3	0.000*
HDL-Cholesterol (mg/dl)	41.22±0.87	39.97±16.68	0.29	0.771
LDL-Cholesterol (mg/dl)	42.17±11.65	112.86±1.61	-14.8	0.000*
Hs-CRP (mmol/l)	1.29±0.14	1.39±0.214	-0.81	0.306
Calcium (mg/dl)	4.36±1.28	4.68±0.63	-0.54	0.143
Total Antioxidant status (mmol/l)	1.26±0.16	1.36±0.03	-1.59	0.430

**Significant @ p < 0.05*

Table 2. Comparison of biochemical parameters analyzed between pre and post treatment of Wistar rats with 1 g/kg EDTA (Mean ± SD)

Parameter	Pre-treatment N=6	Post treatment N=6	t-value	p-value
Total cholesterol (mg/dl)	116.02±5.73	97.64±0.87	7.12	0.002*
Triglyceride (mg/dl)	97.64±13.18	69.24±4.87	2.94	0.042*
HDL-Cholesterol (mg/dl)	25.31±1.71	40.97±1.43	-22.07	0.000*
LDL-Cholesterol (mg/dl)	92.85±8.10	75.60±1.67	4.58	0.010
Hs-CRP (mmol/l)	2.19±0.53	1.52±0.28	2.04	0.111
Calcium (mg/dl)	5.16±0.09	4.28±0.35	8.41	0.001
Total Antioxidant status (mmol/l)	0.86±0.08	1.33±0.06	-8.78	0.001*

**Significant @ p< 0.05*

Table 3. Comparison of biochemical parameters analyzed between pre and post treatment of Wistar rats with Vitamin C (Mean ± SD)

Parameter	Pre-treatment N=6	Post treatment N=6	t-value	p-value
Total cholesterol (mg/dl)	117.03±11.33	101.07±10.48	4.12	0.009*
Triglyceride (mg/dl)	89.39±9.30	56.16±18.77	3.17	0.025*
HDL-Cholesterol (mg/dl)	32.66±6.52	40.48±6.81	-1.63	0.164
LDL-Cholesterol (mg/dl)	68.95±15.12	49.34±7.40	4.23	0.008*
Hs-CRP (mmol/l)	2.08±0.01	1.38±0.14	8.38	0.000*
Calcium (mg/dl)	7.01±1.08	8.27±1.67	-2.04	0.096
Total Antioxidant status (mmol/l)	0.99±0.20	1.13±0.08	-2.16	0.084

**Significant @ p< 0.05*

Table 4. Comparison of biochemical parameters analyzed between pre and post treatment of Wistar rats with Vitamin E (Mean ± SD)

Parameter	Pre-treatment N=6	Post treatment N=6	t-value	p-value
Total cholesterol (mg/dl)	136.23±26.74	101.04±9.30	2.82	0.037*
Triglyceride (mg/dl)	105.75±14.00	63.61±12.25	8.89	0.000*
HDL-Cholesterol (mg/dl)	29.51±10.86	35.01±0.38	-1.20	0.283
LDL-Cholesterol (mg/dl)	86.00±16.03	56.83±12.92	3.45	0.018*
Hs-CRP (mmol/l)	2.11±0.41	1.29±0.09	5.90	0.002*
Calcium (mg/dl)	8.15±0.51	9.12±0.48	-307	0.028
Total Antioxidant status (mmol/l)	0.81±0.38	1.27±0.05	-3.02	0.029*

**Significant @ p < 0.05*

Table 5. Comparison of biochemical parameters analyzed between pre and post treatment of Wistar rats with EDTA, Vitamin C and Vitamin E (Mean ± SD)

Parameter	Pre-treatment N=6	Post treatment N=6	t-value	P-value
Total cholesterol (mg/dl)	145.19±29.10	99.85±7.30	3.06	0.028*
Triglyceride (mg/dl)	106.36±14.86	59.42±9.80	4.90	0.004*
HDL-Cholesterol (mg/dl)	37.05±9.82	47.14±21.45	-1.42	0.214
LDL-Cholesterol (mg/dl)	87.86±20.6	49.14±8.36	3.47	0.018*
Hs-CRP (mmol/l)	2.20±0.28	1.64±0.26	3.09	0.027*
Calcium (mg/dl)	8.18±1.17	9.53±0.77	-3.57	0.066
Total Antioxidant status (mmol/l)	1.12±0.12	1.28±0.03	-4.12	0.009*

**Significant @ p < 0.05*

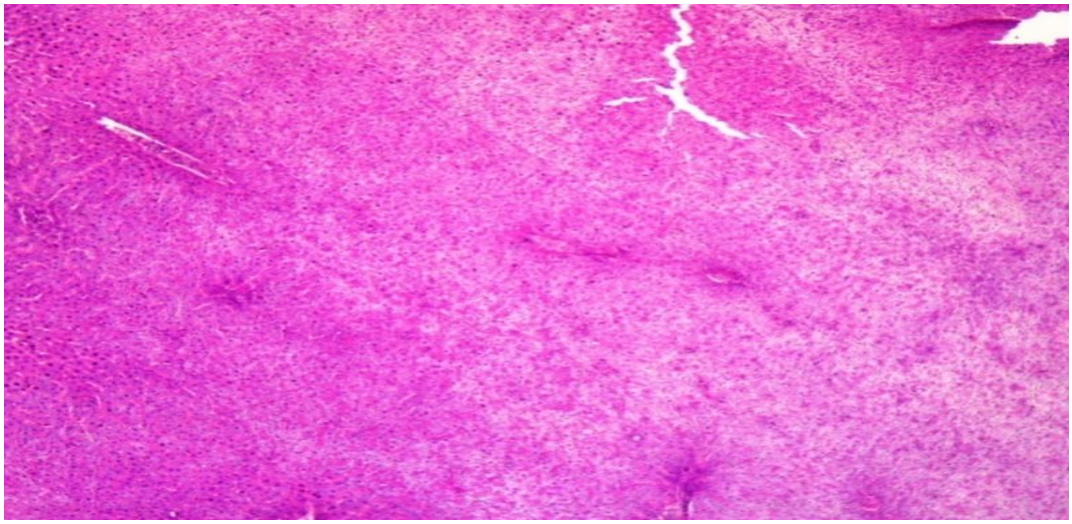

Fig. 1. Photomicrograph showing no development of plaque (H&E ×100) in the aorta of albino rat (Control group)

4. DISCUSSION

Atherosclerosis is characterized by the accumulation of lipids and fibrous elements in the large vessels, elevated serum total cholesterol, low density lipoprotein (LDL) and decreased high density lipoprotein (HDL). Blood cholesterol increases with free radical stress and oxidized LDL is reported to be directly involved in the initiation of atherogenesis [15].

This study showed alterations in the lipid profile of rats fed with atherogenic diet for ten weeks and is manifested by the increase in the serum

levels of total cholesterol, triglyceride and LDL-C concentration and decreased in HDL concentration in comparison with the control. Thus observation in agreement with earlier reports [16].

The hypercholesterolemia observed in the positive control (untreated rats) is due to the activities of the enzymes, cholesterol ester synthetase, the enzymes involved in blood cholesterol homeostasis through their esterifying activity [15]. Increased esterification can lead to accumulation of the ester form of cholesterol which is transported by LDL and increased deposition can lead to plaque formation, vascular calcification and aortic thickness. Plaque was observed in the positive control group of this study. This finding is in agreement with previous studies [17,18].

Conversely, other hypercholesterolemic groups treated with vitamins C, E and EDTA showed significant decrease in lipid profile in comparison to the pre treatment results ($p < 0.05$). EDTA improves lipid profile by its ability to chelate ectopic calcium from the atherosclerotic plaque and inhibit cell mediated LDL oxidation [19]. EDTA greatly reduces the excessive production of free radicals by binding to them and making them chemically inactive [20].

Vitamin C helps in the metabolism of cholesterol by promoting its metabolism through bile acid formation which is the most important pathway of cholesterol metabolism and reducing plasma cholesterol. Similarly, Vitamin E decreases the susceptibility of LDL to beta oxidation thereby decreasing cholesterol concentration.

Furthermore, decrease in triglyceride concentration observed in this study is in agreement with what has been reported by previous studies [21]. The decrease could be as a result of the effect of the antioxidant vitamins decreasing glucocorticoid secretion, inhibiting stimulation of lipoprotein and tissue lipases thereby decreasing lipid mobilization from tissue [15]. The decrease could also be due to inhibition of fatty acid synthesis and some metabolic enzymes such as fatty acid synthetase and glucose-6-phosphate dehydrogenase.

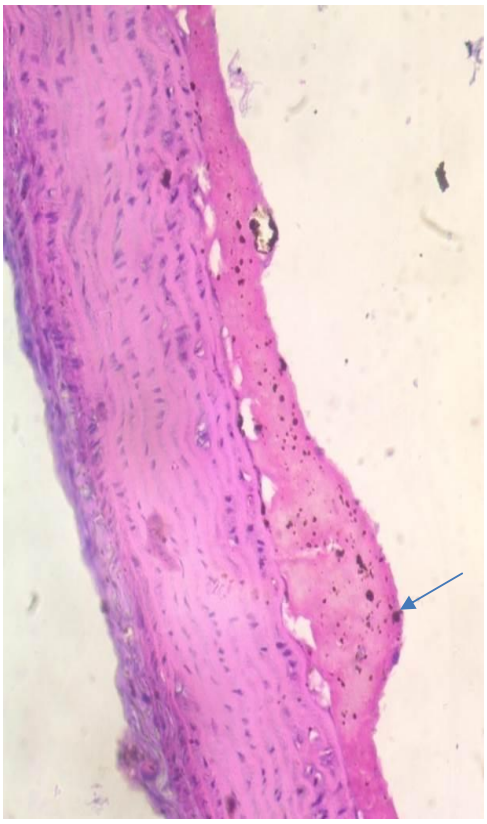

Fig. 2. Photomicrograph showing moderate development of plaques (blue arrow) H&E ×100 in the aorta of hyperlipidemic rat

An increase in the post treatment serum concentration of HDL was observed in all groups. HDL is reported to possess anti-atherogenic properties [22], it is involved in the transport of cholesterol from the peripheral tissues to the liver thereby reducing the amount stored in the tissues. This mechanism reduces the possibility of developing atherosclerosis. Reduction in the blood cholesterol has been reported to reduce vascular resistance by improving endothelial function [23].

The serum total antioxidant activity after treatment increased in all of the treatment groups which could be attributed to the addition of the vitamin C, vitamin E and EDTA antioxidant activity to the depleted antioxidant store in diet induced atherosclerotic rats. Free radicals have been implicated in atherosclerosis causing oxidation of lipid and inflammatory responses. These antioxidants inhibit susceptibility of lipids to peroxidation.

This study observed a decrease in the serum concentration of high sensitivity CRP in the post treatment groups of 3-6. This reduction may be due to the free radical activity salvaging by antioxidants and possible clearance by the immune system.

The effect of antioxidant activity on lipid profile was greatest in the group treated with combination of EDTA, vitamin C and vitamin E, this could be due to the synergistic effect of the individual antioxidant properties, vitamin C is an efficient reducing antioxidant and is a very important antioxidant, because it is stable and prevents the peroxidation of vitamin E by decreasing the activity of α tocopheroxyl radical to α-tocopherols thereby acting as a co-antioxidants and further contributing to increased total antioxidant status [24]. Vitamin E increase the level of superoxide dismutase and glutathione, enzymes that scavenge free radicals and prevent oxidative damage [25].

5. CONCLUSION

This study showed a significant lipid lowering effect in the use of EDTA, Vitamin C and Vitamin E in the treatment of atherosclerosis in rats which is due to their antioxidant and anti-hyperlipidemic properties. Therefore the combinations EDTA, vitamin C and vitamin E appear greatly protective against atherosclerosis.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Animal ethic Committee approval has been collected and preserved by the author.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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