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Antiatherogenic Effect of Tiger Nut Tubers *(Cyperus esculentus* L.) Supplemented Diet in Apolipoprotein E Knockout Mice

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THE PRESENT study was carried out to evaluate the physiological effect of tiger nut tubers on lipid metabolism and peroxidation stress in apolipoprotein E knockout (apoE$^{-/-}$) mice. Six male and five female mice, 6-7 wk old, was fed on AIN 93G control diet supplemented with 25 % of whole tiger nut tubers powder for 11 wk. Measurements were done for plasma, liver, aorta and brain lipid fractions. Peroxidation stress in mice serum and brain, *i.e.* vitamin E, nitric oxide and Thiobarbituric acid reactive substances (TBARS) were evaluated and aortic valve lesions area was also determined. The obtained results showed that, the type of diet and mice gender did not affect mice growth parameters. Serum and liver lipids were lowered in male mice fed on tiger nut diet than that of control diet. However, the effect of tiger nut diet on the peroxidation parameters was not prominent. The mice fed on chufa-diet resulted in a significant reduction in the aortic arch atherosclerotic lesions in both gender compared to that fed on the basal diet. Finally it can be concluded that tiger nut tubers have antiatherogenic effect and contain a gender dependent factor which regulates lipid metabolism in apoE$^{-/-}$ mice.

Keywords: Tiger nut, Chufa, ApoE- knockout mice, Atherosclerosis, Cholesterol, TBARS, Vitamin E, Aorta..

Introduction

The tiger nut tuber (chufa) is a perennial grass-like edible plant with a slightly sweet and nutty flavor belonging to the sedge family. This plant was originally native to the Mediterranean region but its cultivation has spread to many other warm countries. It is consumed widely in Nigeria, other parts of west and east Africa, parts of Europe particularly Spain as well as in the Arabian Peninsula (Abaejoh et al., 2006). It was an important food in ancient Egypt (Negbi, 1992). Its dry tubers have been found in Pharaohs tombs about 6000 years ago (Zohary, 1986). Nowadays, the tubers are consumed mainly as a refreshing beverage called locally "horchata de tiger nut" (tiger nut milk) in Spain (Pascual et al., 2000). It is also cultivated in Northern Nigeria, Niger, Mali, Senegal, Ghana, and Togo where they are consumed primarily uncooked as a side dish (Omode et al., 1995). The tubers may be consumed as nut or grated and used for ice cream, biscuits and beverage making. Tiger nut has been reported as a wholesome nutritive food. It has long been recognized for their health benefits, as they contain oil (~ 25%) and they are high in oleic acid (about 70% of total oil), fibers (~ 9%), proteins (~ 5%), minerals (~ 1.7%) and carbohydrates (~ 43%). It also contains several micronutrients such as vitamin E, phytosterols, isoflavones, etc. The chemical composition and nutritional value of tiger nut tubers is presented elsewhere (Mokady & Dolev, 1970, Oladele & Aina, 2007, Shaker et al., 2009, S´anchez-Zapata et al., 2012 and Zommara & Imaizumi, 2017). Tiger nut has been found to be good for preventing arteriosclerosis, since its consumption can help prevent heart problems and thrombosis and activate blood circulation (Chukwuma et al., 2010), mainly because its unsaturated fatty acid content is similar to that of olive oil (Linssen et al., 1988) and its arginine is a precursor of nitric oxide which helps the veins to expand (Mart´ınez-Valls, 2003).

Atherosclerosis is the most common causes of death in Western society's; it results from

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complex interactions among multiple genetic and environmental factors. It develops from low-density lipoprotein molecules (LDL), the major carrier of cholesterol in human blood. While LDL has an essential physiological role as a vehicle for the delivery of cholesterol to peripheral tissues, increased LDL cholesterol levels are associated with increased risk of cardiovascular disease**.** Atherosclerosis develops from LDL becoming oxidized by free radicals, particularly oxygen free radicals and nitrogen species produced by vascular cells (Glass & Witztum, 2001, Stocker & Keaney, 2004 and Kakadiya, 2009).

Wild mice are highly resistant to atherosclerosis. However, apolipoprotein (apo) E-knockout mice ($apoE^{-/-}$ mice) have recently been generated by gene targeting (Piedrahita et al., 1992, Breslow, 1996 and Jawieň & Korbut, 2004). These animals have elevated serum cholesterol levels (400-500 mg/dl) and spontaneously develop severe atherosclerosis on low-fat chow diets, which is apparent in mice of only 10 weeks of age (Plamp et al., 1992). They found that, $ApoE^{-/-}$ mice fed a high fat diet (21% fat and 0.15% cholesterol) have serum cholesterol levels of approximately 1,500-2,000 mg/dl and larger lesions at any given time. ApoE is an important legend for the uptake of lipoproteins by many receptors in the LDL receptor gene family, and deficiency of apoE leads to the accumulation of cholesterol ester enriched particles (Piedrahita et al., 1992). Therefore, $ApoE^{-/-}$ mice are considered one of the most relevant models for atherosclerosis since they are hypercholesterolemic and develop spontaneous arterial lesions (Nakashima et al., 1994).

Nitric oxide (NO) is produced in trace quantities by neurons, endothelial cells, platelets and neutrophils in response to homeostatic stimuli (Moncada, 1992 and Nathan, 1992). It was identified as the endothelium relaxing factor (EDRF) responsible for the relaxation of vascular smooth muscle (Ignaro et al., 1987 and Moncada & Higgs, 1993). Therefor, a disturbance in either production or availability of NO is thought to be responsible for the functional alterations that are associated with endothelial dysfunction and plays a key role in the development of atherosclerotic lesions. NO is a gaseous and unstable substance. The rapid metabolism and short half-life of NO ($t_{1/2}$ = 4 sec.) poses a considerable obstacle for its analytical assessment in the tissue. It consequently reacts readily with oxygen, yielding nitrite (NO_2•) or nitrate (NO_3•) called nitric oxides (NOx). The direct measurement of plasma nitrite/nitrate (NOx) value beings would enable us to evaluate the vascular endothelial function as NO release. Kawakatsu et al. (2002) demonstrated that plasma (NOx) was influenced by exogenous factors, aging, and difference of gender, and showed some correlations with hyperglycemic vascular impairments. In comparison with healthy volunteers, it was found that plasma from patient group with coronary artery disease showed a higher NOx value ($P \leq 0.001$).

In our previous studies, Tiger nut (chufa) oil and its water and methanol fractions exhibited in vitro anti-lipoperoxidation effect against peroxidation stress initiated by ferrous ions dependent peroxidation stress and in free radical mediated peroxidation by the water soluble initiator 2,2'-azobis (2-aminopropane) dihydrochloride (AAPH) and the lipid soluble free radicals initiator 2,2'-azobis (2,4- dimethylvaleronitrile) (AMVN) in phosphatidylcholine liposomes (Zommara & El-Shaer, 2001 and Zommara & Imaizumi, 2017). The aim of this study was therefore to investigate the influence of supplementing a diet with whole powder of tiger nut tubers on serum lipids and atherosclerosis parameters in $apoE^{-/-}$ mice. Since there seems to be a gender effect on the serum cholesterol concentration and aortic lesion development in this animal model (Breslow, 1996), both genders were used in the present study.

Materials and Methods

Animals and diets

Four to five-week-old, male and female, apoE-deficient mice initially engineered at the University of North Carolina (Chapel Hill, NC, USA) (Ni *et al*., 1998), were purchased from Jackson Laboratory, Bar Harbor, ME, USA. These mice were maintained at the Laboratory of Animal Experiments in Kyushu University Faculty of Medicine (Fukuoka, Japan). The mice were housed individually in a temperature-controlled room at 22-25°C with a 12-hr light: dark cycle and acclimatized on a commercially available nonpurified diet (NMF, Oriental Yeast, Tokyo, Japan) for two weeks. Then, the mice were divided into two groups, 6 male and 5 female mice each, and given free access to the AIN-93G based purified diets (Reeves et al., 1993 and AIN, 1997) for 10 weeks. The diet contained (weight percent) casein 20, olive oil 10, α-corn starch 13.2, sucrose 10, cellulose 5, vitamin mixture 1, mineral mixture 3.5, L-cysteine 0.3, choline bitartrate 0.25, tetra-butylhydroquinone (TBHQ) 0.0014 and corn

starch to 100. To the control diet was added 25 % of whole powder of tiger nut tubers (from local market at Tanta City, Egypt) at the expense of corn starch and designated as chufa-diet. Food and nonionized water were freely available throughout the experimental period. The average chemical composition of tiger nut tubers are as the fellows: 34% starch, 25% oil, 8% protein, 16% sucrose, 10% fiber and 7% others. The tubers were found to contain 12.4 and 8.5 µg/g of the isoflavons daidzein and genistein, respectively and about 18.6 mg of vitamin E /100 g oil (Zommara & Imaizumi, 2017). Accordingly, tiger nut-diet was adjusted for its content of oil, protein, corn starch and sucrose. The diets contained about 3606 kcal/kg diet. The body weight and food intake were recorded every other day. After food was removed for 6 hr, the rats were sacrificed by withdrawing blood from the right ventricle under anesthesia with an intraperitoneal injection of sodium pentobarbital (5 mg/ g body weight). This experiment was performed under the control of the guidelines for animal experiments of the Faculty of Agriculture and Graduate Course at Kyushu University and law No. 105 and notification No. 6 of the government of Japan.

Tissue preparation

Blood was collected for serum separation by centrifugation (GEAVI DNA, Sanyodennki Co., Tokyo, Japan) at 3000 rpm for 20 min. The aorta was perfused with 50 ml phosphate buffer saline, PBS (pH 7.4) via a cannula inserted in the right ventricle, allowing unrestricted efflux from an incision in the vena cava. Perfusion was continued with 50 ml of 10% neutral formalin buffer solution at pH 7.4 (Wako Pure Chemicals, Osaka, Japan), and the heart, aorta and liver were dissected. The bulk of the fat and tissue adhering to the adventitia was dissected from the aorta as much as possible. Liver and aorta were kept at -70°C until needed for further chemical analysis. The heart was fixed in a 20% neutral formalin buffer solution at pH 7.4 (Wako Pure Chemicals, Osaka, Japan) (Plamp et al., 1992).

Morphometric determination of atherosclerosis

To determine the cross-sectional lesion volume, hearts containing aortic roots were processed for quantitative atherosclerosis assay of the aortic root using a modification of the methods described by Paigen et al. (1987). Briefly, the heart was cut along a plane between the tip of the two atria and the top half was embedded in paraffin wax. Consecutive sections (10 mm thick) were prepared from the ascending aorta to the aortic sinus until the aortic tissue disappeared. The sections were mounted on glass slide and stained with elastic Van Gieson and hematoxylin (Ni et al., 1998). Five sections of each heart were selected for intimal area determination; the first and most distal sections to the heart were taken where the aortic valve cusp was barely discernible. From this section, moving to the base of the heart, every other 10 mm section was also photographed. The intimal area was measured by capturing the image using a video camera mounted on an Olympus LX70 light microscope and analyzed using Adobe Photoshop and Nih image/68K 1.57 software (National Institute of Health, Bethesda, MD, USA) on a Power Macintosh Computer. The mean intimal area was calculated for each animal, and subsequently for each group.

Analytical methods

Plasma lipids were enzymatically determined with commercially available kits (Cholesterol C Test, Phospholipids B Test and Triglyceride G Test, Wako pure chemicals, Osaka, Japan). Liver lipids were extracted by the method of Folch et al., (1957), and subjected to chemical determination for cholesterol (Sperry & Webb, 1950), triglycerides (Fletcher, 1968) and phospholipids (Bartlett, 1959). Tiger nut oil phytosterols were determined by GLC as previously described (Nagao, et al., 2001).

Statistical analysis

All data were expressed as mean ± SE and statistical differences were determined by Duncan's multiple range test (Duncan, 1955).

Results and Discussion

Data in Table 1 show the effect of tiger nut (chufa-diet) on growth parameters of male and female apoE$^{-/-}$ mice. Type of diet did not affect the growth parameters of both mice gender. There were no significant differences among all groups in food intake, body weight gain, food efficiency and liver relative weight. These findings may be explained by the equilibrium in energy content between the control and chufa-diet (about 3606 kcal/kg diet), and indicate no deleterious effect of tiger nut on mice metabolism when included in the diet.

Table 2 shows the concentration of blood serum and liver lipids, aorta cholesterol and brain cholesterol and phospholipids of apoE-/- mice fed basal or chufa-diet. As previously mentioned (plamp et al., 1992), the data showed that feeding

apoE/- mice on the control diet resulted in elevated tissue cholesterol (824-1016 mg/ dl serum, 8.18-8.60 mg/g liver) and triglycerides (176-282 mg/ dl serum, (88.4-109.2 mg/ g liver) for male mice. These values are extremely high when compared with that obtained from normal male rats (*i.e.* Sprague-Dawley rats) fed on normal AIN 76™ diet (104-111 mg/dl plasma, 2.1-2.5 mg/ g liver and 122.7- 151.3 mg/dl plasma, 4.2-5.6 mg/ g liver for cholesterol and triglycerides, respectively) (Zommara et al., 1996).

TABLE 1. Effect of chufa diet on growth parameters of apolipoprotein E knockout mice

Groups		Food intake g/ day	Body weight gain (g)	Food efficiency (g gain/g food intake)	Liver %
Control					
	Male	4.03±0.28	10.67±3.93	0.43±0.19	4.29±0.82
	Female	3.86±0.32	9.60 ±4.51	0.47±0.20	3.95±0.77
Chufa-diet					
	Male	4.19±0.22	9.17±3.06	0.50±0.16	3.58±0.24
	Female	3.85±0.06	6.00±2.35	0.71±0.21	4.35±0.82

Data are the mean ± SE for 6 male and 5 female mice.
Liver %, g liver/100 g body weight.

TABLE 2. Concentration of serum and liver lipids and brain as well as aorta cholesterol of apolipoprotein E knockout mice fed on chufa diet

Parameter	Control		Chufa-diet	
	Male	Female	Male	Female
Serum (mg/dl)				
Cholesterol	920.0±96.0^{a}	684.0±119.0^{ab}	531.0±28.0^{b}	632.0 ±87.0^{b}
Triglycerides	229.0±53.0^{a}	107.0±37.0^{b}	91.6±2.4^{b}	115.0 ±24.0^{b}
Phospholipids	439.0±71.0^{a}	318.0±48.0^{ab}	238.0±29.0^{b}	308.0 ±50.0^{ab}
Liver (mg/g)				
Cholesterol	8.39±0.21^{a}	5.69±1.20^{ab}	4.68±1.24^{b}	5.14±0.85^{b}
Triglycerides	98.8±10.4^{a}	40.6±8.87^{a}	46.4±9.63^{b}	35.8 ±6.44^{b}
Phospholipids	25.9±0.88^{a}	19.8±4.47^{a}	19.6±3.42^{a}	20.6 ±0.43^{a}
Aorta (mg/mg)				
Cholesterol	3.60±0.40^{a}	2.51±0.50^{a}	2.89±0.47^{a}	2.43 ±0.43^{a}
Brain (mg/g)				
Cholesterol	17.0±0.40^{a}	16.8±0.52^{a}	16.8±0.33^{a}	15.7±0.48^{a}
Phospholipids	211.0±1.4^{a}	210.0±3.2^{ab}	209.0±1.6^{ab}	204.0±1.01^{b}

Data are the mean ± SE of 6 male and 5 female mice.
^{ab}Means within a raw with unlike superscript are significantly different at ($P< 0.05$).

On the other hand, female mice showed lower tissue lipid concentrations compared to the male one (cholesterol, 565-803 mg/ dl serum, 4.49-6.89 mg/ g liver; triglycerides, (70-144 mg/dl serum, 31.7-49.5 mg/ g liver); that indicates a gender effect. Hypercholesterolemia is a well-established risk factor for atherosclerosis and its pathologic complications. The diet supplemented with the tiger nut tubers resulted in a significant reduction in cholesterol and triglycerides by about 42% and 60% in blood serum and by about 44% and 53% in liver, respectively compared with the control diet. This effect was obvious in male mice than the female one although there were no significant differences between the two mice groups. Table 2 also show that chufa-diet had no significant differences in aorta and brain cholesterol compared with the control although the aorta from mice fed on chufa-diet tended to have less cholesterol concentration in their aorta.

The hypolipidemic effect of tiger nut may be explained by its content of, polyunsaturated fatty

acids (~ 9.9% of total fatty acids), isoflavones (daidzein and genistein (12.4 and 8.5 µg/g, respectively) (Zommara & Imaizumi, 2017) and phytosterols (168 mg/100 oil). Diets enriched with polyunsaturated fatty acids (PUFA) have been recommended for several medical and nutritional aims because of the lipid lowering effects of PUFA in the serum (An et al., 1995 and Schmid & Woollett, 2003). The cholesterol-lowering effect may also be linked to the presence of its isoflavones content (kirk et al., 1998, Potter, 1996 and Anderson et al., 1995).

Tiger nut content of phytosterols (mg/ 100g oil) are presented in Table 3. It contains a moderately high amount of phytosterols with a total content of about 168 mg/ 100 g oil. Plasma phytosterol levels in mammalian tissues are normally very low due to poor absorption from the intestine and faster excretion from the liver compared to cholesterol.

TABLE 3. Phytosterols content in tiger nut oil

Sterols	mg/ 100 g oil
Campesterol	6.8 ± 0.17
Campestanol	120.1 ± 2.12
Stigmasterol	17.5 ± 0.38
β-sitosterol	9.3 ±0.08
β-sitostanol	14.6 ± 0.34

Data are the mean ± SE of 3 replicates.

However, phytosterols may have a lot healthy benefits in animals/humans such as the reduction of cholesterol levels with decreaseding risk of coronary heart diseases (Bartnikowska, 2009 and ogbe et al., 2015). Michajlik & Bartnikowska, (1999) demonstrated that plant sterols inhibit the absorption of dietary cholesterol due to structural similarity, except that they always contain some substitutions at the C24 position on the sterol side chain. It takes the place of both dietary and endogenous cholesterol in micelles produced in intestinal lumen therefore, less cholesterol is delivered to enterocytes and is incorporated into chylomicrons and VLDLs synthesized in the intestine. However, in case of a high intake of phytosterols it competitively blocks the absorption of cholesterol, and at the same time phytosterols are not absorbed.

The progress of mice atherogenesis was evaluated by several parameters including serum and brain vitamin E content, serum nitrogen oxides (NOx) and brain TBARS (Table 4). The results showed no significant differences in vitamin E content in mice serum and brain although the serum from chufa-diet fed mice tended to have more vitamin E compared to that fed on the basal diet. Praticò et al., (1998) indicated that oxidative stress is increased in the apoE$^{-/-}$ mouse, is of a functional importance in the evolution of atherosclerosis and can be suppressed by oral administration of vitamin E. On the other hand, Imaizumi (2011), indicted that the rule of vitamin E (α- tochopherol) in the progress of atherogensis is inconclusive. As a useful predictor of the endothelial function, mice serum concentration of NO_2/NO_3 was significantly lower in female mice than the male fed on the basal diet. However, chufa-diet had no effect on this parameter. The brain of mice fed on chufa-diet resulted in low TBARS concentration compared to male mice fed on the basal diet.

TABLE 4. Some parameters related to peroxidation stress in serum and brain of apolipoprotein E knockout mice fed on chufa diet

Parameter	Control		Chufa-diet	
	Male	Female	Male	Female
Serum				
Vitamin E (mg/dl)	4.3±0.7^{ab}	2.7±0.5^{a}	4.7±0.1^{b}	3.4 ±0.7^{ab}
NO_2/NO_3(µmol)	22.5±2.4^{a}	16.7±0.8^{b}	18.2±1.7^{ab}	15.6 ±0.7^{b}
Brain				
Vitamin E(mg/g)	4.4±0.5^{a}	4.1±0.8^{a}	5.3±0.5^{a}	5.2±0.3^{a}
TBARS (mmol/g)	0.60±0.02^{a}	0.55±0.02^{ab}	0.53±0.02^{b}	0.48±0.03^{b}

Data are the mean ± SE of 6 male and 5 female mice.
^{ab}Means within a raw with unlike superscript are significantly different at ($P< 0.05$).

As a direct evaluation of the probable antiatherogenic effect of chufa-diet, the aortic arch atherosclerotic lesions (mm^2) in male and female mice fed the basal or chufa-diet was measured (Fig. 1). Although, no significant differences were found between male and female mice from the two diets group, the female mice tended to have less atherosclerotic lesions compared to the male one. However, the mice fed on chufa-diet resulted in a significant reduction in the aortic arch atherosclerotic lesions in both gender compared with that fed on the basal diet.

Tiger nut oil has a high oleic acid content (~ 70%) and low content of polyunsaturated fatty acid (linoleic acid and linolenic acid) (~ 9.9%) (Zommara & Imaizumi, 2017). Belewu & Abodunrin, (2006) demonstrated that tiger nut oil reduces blood low density lipoprotein-cholesterol (LDL-C) and increases high density lipoprotein-cholesterol (HDL-C), reduces blood triglycerides level and the risk of forming bloody clots, thereby preventing arteriosclerosis. Besides its hypocholesterolemic effects, isoflavones have been reported to modify certain metabolic processes associated with atherosclerotic lesion development, such as inhibiting endothelial cell proliferation and macrophage expression of cytokines (Raines and Ross, 1995).

In conclusion, the obtained results confirmed that tiger nut has a gender dependent hypolipidemic effect and reduces atherosclerotic lesion development in apoE-deficient ($apoE^{-/}$) mice. This reduction may be explained by its hypolipidemic effect and reduced peroxidation stress in mice tissue. More investigation is needed to explore the mechanism of its hypolipidemic action, as well as its active fraction(s).

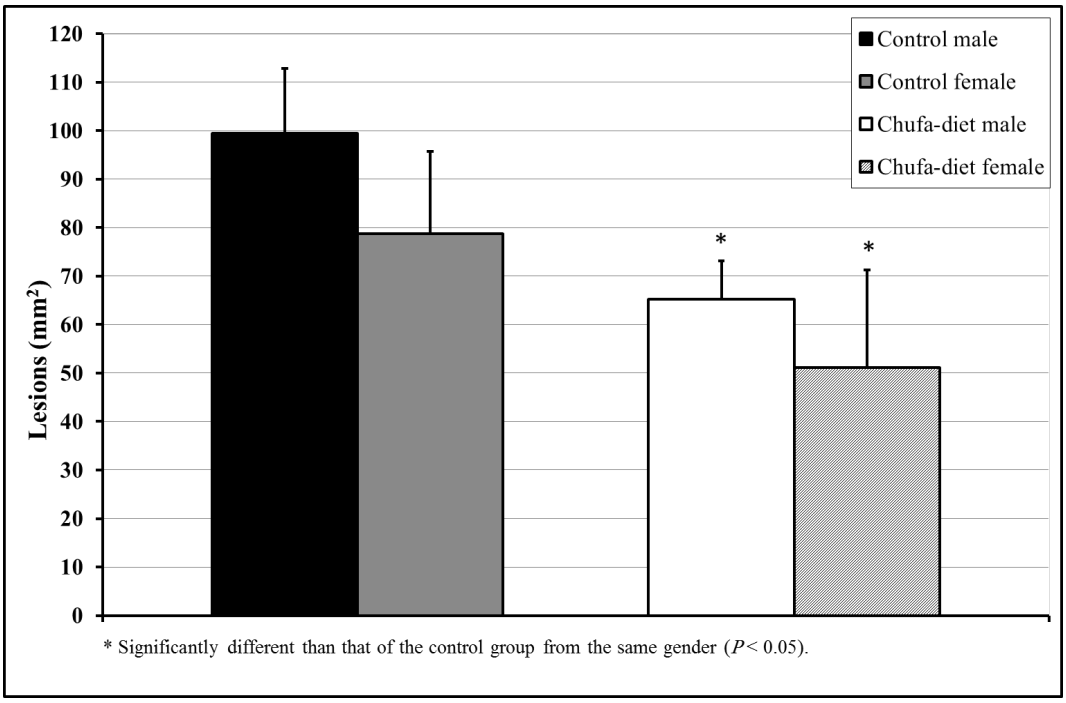

Fig. 1. Atherosclerotic lesions (mm^2) in the aortic arch of apolipoprotein E knockout mice fed on chufa diet

Data are mean ± SD for 6 male and 5 female mice.

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