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EVALUATION OF ANTIOXIDANT PROPERTIES OF SOYBEAN PHYTOESTROGEN-RICH EXTRACT IN 4-VINYLCYCLOHEXANE DIEPOXIDE-INDUCED MENOPAUSAL RATS

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ABSTRACT

The vasomotor symptoms of menopause, which include hot flashes, sweating, physical and psychological discomfort, and emotional changes, are real and experienced by a large portion of the menopausal and postmenopausal female population. In addition, it causes osteoporosis and slowed metabolism, both of which raise the chance of developing a number of different ailments. Given that hormone replacement therapy (HRT) has been linked to an increased cancer risk, this investigation was undertaken to identify viable alternatives. The study aimed to evaluate antioxidant properties of soybean phytoestrogen-rich extract in 4-vinylcyclohexane diepoxide-induced menopausal rats**.** Sixty five (65) female albino Wistar rats were employed in the investigation (n=20 for toxicity study, n=15 for preliminary and 30 for experimaental study) and each one of the experimaental animal was induced with 80mg/kg of 4-vinylcyclohexene diepoxide before being treated with either normal estradiol therapy (14ug/kg) or varying concentrations of the soybean phytoestrogen-rich extract (200 mg/kg, 400 mg/kg, and 600 mg/kg). The levels of total antioxidant, vitamin E, and oxidative stress marker (malonialdehyde (MDA) were all measured spectrophotometrically. Statistical software SPSS (IBM) version 23.0 was used to analyze the data. Treatment with soybean phytoestrogen-rich extract resulted in dose-dependent decreased malondialdehyde (MDA) levels compared to the positive control group. Compared to the positive control group, the measured antioxidant levels of the soybean phytoestrogen-rich extract treatment group increased significantly ($p<0.05$) in a dose-dependent manner. Data from this research clearly demonstrate increased anti-oxidants effects of a soybean phytoestrogen-rich extract therapy in menopause-induced female Wistar rats. Soybean phytoestrogen-rich extract therapy in a high-dose appears to be more effective compared to hormone replacement therapy as an alternate source of estrogen in treatment of oxidative stress in menopausal and post-menopausal women.

KEYWORDS: Antioxidant, Phytoestrogens, Menopause, Soybean extract, oxidative stress.

INTRODUCTION

Postmenopausal women are at an increased risk for developing chronic diseases, highlighting the need for preventative care. Consumption of phytoestrogens may be important strategies for minimizing or preventing the low-grade chronic inflammation and oxidative stress that are common in postmenopausal women.[1] Heart disease, oxidative stress, osteoporosis, irritability, sleeplessness, mood swings, anxiety, panic attacks, forgetfulness, and inability to focus are all linked to menopause.[2] Oxygen metabolism and other physiological processes driven by exogenous stimuli, such as phagocytosis, generate free radicals and reactive oxygen species (ROS).[3] Peroxidation of membrane lipids, oxidative damage to nucleic acids and carbohydrates, and oxidation of the sensitive groups in proteins are all possible outcomes of an overabundance of free radicals and reactive oxygen species (ROS).[4] For example, neutrophils and other inflammatory cells can be attracted to an area where reactive oxygen species (ROS) have been released, furthering the inflammatory response and increasing the risk of tissue damage. Human neutrophil reactive species can be directly removed by isoflavones.[5] Genistein has been shown to improve insulin sensitivity by increasing the production of antioxidant enzymes in the livers of streptozotocin-induced diabetic mice.[6] These enzymes

include catalase, superoxide dismutase, and glutathione peroxidase.

Although isoflavones are effective scavengers of peroxyl radicals and other reactive oxygen, nitrogen, and chlorine species, they are more vulnerable to oxidation by peroxynitrite and hypochlorous acid. In mice with type 1 diabetes caused by streptozotocin, genistein restores normal levels of superoxide anion generation and nitrotyrosine synthesis.[7] Blood platelet peroxynitrite-mediated protein changes and lipid peroxidation are both attenuated by isoflavone-rich Trifolium extracts.[8] Soy isoflavones and soy extracts also reduce serum nitrite, nitrate, and nitrotyrosine levels in rats that have been challenged with lipolysaccharide when consumed orally. The data presented here suggest that isoflavones scavenge the increased free radicals produced by activated macrophages during inflammation, thereby preventing NO reactions with free radicals and the subsequent production of peroxynitrite, which can directly oxidize LDL and result in irreversible damage to the cell membrane.

It has been suggested that the antioxidant activity of isoflavones regulates thromboxane synthesis via the COX-1 pathway by neutralizing hydrogen peroxide in the cells.[9] Reduced platelet aggregation and a lower risk of thrombosis may result from isoflavones' capacity to inhibit platelet-ADP collagen receptors.[9][10]

Recently, it has been shown that in porcine mammary gland cells and human umbilical vein endothelial cells, soy isoflavone reduces reactive oxygen species (ROS) and malondialdehyde levels while simultaneously increasing the mRNA expressions and activities of superoxide dismutase and glutathione peroxidase.[11] Nuclear factor-erythroid 2 (Nrf2), a key transcription factor that leads to gene transcription of various antioxidant and phase II detoxifying enzymes, is associated with the modulation of enzymatic antioxidants by isoflavones.[12]

Fifty-four people with type 2 diabetes (aged 47 to 69) were studied to determine the impact of genistein consumption on oxidative stress, a metabolic parameter in postmenopausal women with diabetes. The participants were randomly assigned to receive either genistein (n = 28, 54 mg) or a placebo (n = 26) twice daily for 12 weeks.[13] Fasting blood glucose, glycated haemoglobin A1c, triglyceride, and serum malondialdehyde levels were all considerably lowered by genistein use, while overall antioxidant capacity was raised. Furthermore, genistein supplementation dramatically improved insulin sensitivity. Patients with NAFLD (n = 41) in another randomised, double-blind, controlled experiment were given 250 mg of genistein once day for eight weeks. Genistein significantly improved insulin resistance (homeostasis model assessment of insulin resistance (HOMA-IR), p = 0.041) and decreased blood insulin levels (p = 0.001) compared to the placebo group.[14] In addition, serum MDA, TNF-, and IL-6 levels were lowered due to genistein use. The waist-to-hip ratio (p = 0.021), body fat percentage (p = 0.015), and triglyceride levels (p = 0.018) were all considerably decreased with genistein compared to the placebo. But neither did indicators like fasting blood sugar or body mass index. Effects of genistein on glycemic control and insulin sensitivity were demonstrated in a meta-analysis of randomised controlled trials.[15] Seven randomised controlled studies with a total of 670 participants were included in the meta-analysis. Genistein significantly decreased fasting blood glucose levels compared to placebo (p = 0.005). In addition, genistein supplementation has been demonstrated to greatly enhance glycemic control and insulin sensitivity in women who have gone through menopause. Another trial found that postmenopausal women with metabolic syndrome who took genistein (n = 60) or a placebo (n = 60) for a year saw improvements in their blood sugar, fasting insulin, and homeostatic model assessment of insulin resistance (HOMA-IR).[16]

The aim of the study is to evaluate the antioxidant properties of soybean phytoestrogen-rich extract in 4-vinylcyclohexane diepoxide-induced menopausal rats.

MATERIALS AND METHODS

This is an experimental study design conducted at the Faculty of Pharmacy laboratory and animal house, University of Benin, Benin City. It involved 65 females matured (aged 6-8 weeks) Wistar rats (Rattus norvegicus) weighing between 120 and 240g. Toxicity (n=20), preliminary (n=15), and animal model (n=30) studies of chemically induced menopause were conducted on the animals. The animals were kept at the animal house at the University of Benin, Benin City for two weeks to stabilize before the experiments began. The Wistar rats were given a conventional rodent cube diet from Ewu feeds and flour mills limited Ewu, Edo state, Nigeria .and free access to water *ad libitum.* Before each experiment, all animals were fasted overnight. Because of their ability to mimic the symptoms of perimenopause and postmenopause in humans—such as estrous acyclicity and fluctuating, then undetectable, estrogen levels—Wistar rats were chosen for this study to enable the separation of the impact of hormone levels from that of ageing.

The approval for the study was sought from the Animal Studies Ethic Review Committee of the Faculty of Pharmacy, Department of Pharmacology and Toxicology, University of Benin, Benin City. The approval was given after experimental protocols (where animals were housed and cared for under natural lighting conditions). Because some of the Laboratory investigations were done at the Federal University of Technology, Akure, a second Ethical approval and clearance was obtained from the ethics and research committee of Federal University of Technology Akure, Ondo (protocol number FUTA/ETH/21/14).

The minimum sample size required was calculated using the formula (N) = $\frac{2\,(Z_{\alpha} + Z_{(1-\beta)})^2\, S^2}{(\mu_1-\mu_2)^2}$[17]

Where:

Z_{α} = Standard normal deviate corresponding to 5% level of significant= 1.96

$Z_{(1-\beta)}$ = Standard normal deviate corresponding to a power of 80%= 0.84

S = Standard deviation of SOD level in Wistar rat injected with VCD=3.16

$(\mu_1-\mu_2)^2$ = the mean differences in SOD level between the group =1.76

Calculation:

$$\frac{2\,(1.96+0.84)^2\,(3.16)^2}{(1.76)^2}$$

= 50.5 (approx)

This then gives a minimum of 51 rats for the study

The soybeans were purchased from the Oba market in Benin-City, and were authenticated by a plant taxonomist at the Department of Plant Biology and Biotechnology (PBB) laboratory, University of Benin, Benin City, and was given a voucher number (UBH-G628). In preparing the flour, the soybean seeds were carefully picked, separated from debris and rinsed in water. After washing, the grains were transferred to a large, clean bowl, and left to soak overnight. The soybean chaff was washed off, drained to eliminate as much water as possible, and dried in the sun until they were completely dried. The grains were heated in a frying pan over medium heat, and stir until they turned brown, but being careful not to allow them to burn. Once the beans have browned, the grains were removed immediately. The roasted soybeans were immediately ground into a fine powder in a Kitchen blender. A quick transfer from a hot frying pan into the blender ensures the seeds grind smoothly to powder. The powder was stored in an air tight container.

The method of Cvejic *et al.* (2009)[18] was used with modification. Here, a known quantity of the Soybean flour was loaded into a thimble and placed in the Soxhlet extractor chamber until it was defatted using hexane, in the Soxhlet extractor. After defatting, the powder was dried and then re-extracted with methanol, using the Soxhlet extractor to obtain the methanol extract (phytoestrogen - rich extract). The extract was concentrated using a rotary evaporator and then dried completely using a thermostatically controlled hot air oven.

Animals were divided into six (6) groups of five (5) animals in each group and induced intraperitoneally with 80mg/kg of VCD, obtained during the preliminary study.
Group 1: 80 mg/kg of 4-vinylcyclohexene diepoxide + 200mg/kg phytoestrogen - rich extract.
Group 2: 80 mg/kg of 4-vinylcyclohexene diepoxide + 400 mg/kg of phytoestrogen - rich extract.
Group 3:80 mg/kg of 4-vinylcyclohexene diepoxide + 600 mg/kg of phytoestrogen - rich extract.
Group 4: 80 mg/kg of 4-vinylcyclohexene diepoxide + 14µg/100g estrogen of body weight
Group 5: 80 mg/kg of 4-vinylcyclohexene diepoxide (positive control)
Group 6: Normal rats (negative control)

Sexually matured female Wistar rats were used in the study while those less than 6 weeks' old and male Wistar rats were excluded.

Blood sample was withdrawn directly from the abdominal aorta and the heart chamber with a needle mounted on a 10 mL syringe (Agary pharmaceutical LTD, Nigeria) into lithium heparin anticoagulant and plane sample bottles. Biochemical analysis were performed on the serum sample obtained after centrifugation of whole blood at 2500 rpm for 10 min. The serum they were kept frozen at -20^0 degrees Celsius until biochemical analysis was performed.

Determination of Total Antioxidant Status

Total Antioxidant Status was determined using enzymatic colorimetric method by Erel (2004).[19]

Principle

ABTS (1,2^1-Azino-di-3-ethylbenzthiazoline sulphonate) is incubated with a peroxide (metmyglobin) and to produce the radical cation BTS. This has a stable blue green colour which is measured at 600-660 nm. Antioxidants in the sample, suppress the formation of this colour, to a degree which is proportional to their concentration.

Assay procedures

There were three sets of test tubes: a blank set, a standard set, and a test set. Each of the blank, standard, and sample tubes received 800ul of TAS buffer. Both the sample and the calibrator were injected into their respective tubes at a volume of 50ul. In a spectrophotometer equipped with a 1 cm light cuvette, the initial absorbance of the test was read against a reagent blank at 660 nm. Next, 125ul of TAS chromogen was added to each of the aforementioned test tubes. It was well combined and incubated at 37^0 degrees Celsius for 5 minutes. At 660 nm, the absorbance was measured.

Results were calculated as follows

Calculation of Δabsorbances

A2-A1 = ΔAbsorbance sample/calibrator/blank

Results = $\frac{\Delta\text{Abs sample}}{\Delta\text{Abs calibrator}}$ x calibrator concentration

The serum levels of vitamin D, Vitamin E, MDA and total antioxidant status were measured using the ELISA method (Calbiotech Diagnostic Products Monobind Inc. Lake Forest, USA.) and colorimetric method respectively.

The antigen presents in the patient sample and calibrator bind to the coated antibody. The antigen –antibody complex reacted with enzyme conjugate which converted the substrate into colored solution. The intensity of the color change is proportional to the concentration of enzyme-antibodies present in the samples which was read at 450nm.

The data were statistically analysed using SPSS Software (IBM) version 23.0. The various results obtained from this study were expressed as Mean ± Standard deviation (SD). The differences between the groups were determined by one-way ANOVA. The Tukey-Kramer Multiple comparisons Test was used as the post hoc test for determination of significant difference between Means. A P-value (≤ 0.05) was considered to be statistically significant and P-value (>0.05) was considered not statistically significant.

RESULTS

Table 1: shows that soyabean phytoestrogen-rich extract (200mg/kg, 400mg/kg, 600mg/kg) and estradiol (14ug/100g) treatment improved antioxidant marker levels in female rats treated with 80mg/kg VCD. One-way analysis of variance followed by Tukey's post hoc test revealed that vitamin D ($F=19.55$, $p<0.001$), vitamin E ($F=5.41$, $p=0.002$), MDA ($F=16.86$, $P=0.001$) and total antioxidant ($F=9.58$, $p<0.001$) all differed significantly across groups. Vitamin D levels varied significantly ($p<0.05$) between groups, as shown by a post hoc multiple comparison test in Turkey, however the observed differences were consistent among groups 1, 2, and 4. Similar changes were seen between groups 2 and 3 and groups 1 and 4 and 5 in the vitamin E group. The total antioxidant level groups 1, 2, and 4 showed no significant variations from one another, but groups 3 and 4 did. It was observed that malondialdehyde (MDA) levels decrease significantly ($p<0.05$) between group 1 to group 3 and the control group (group 6) shown significant increase in level of malonialdehyde (MDA).

Table 1: Levels of Antioxidants and Oxidative Stress Markers In Female Wistar Rats Induced With 4-Vinylcyclohexane Diepoxide (Vcd).

Parameters	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	F	P-value
Vitamin E (ug/kg)	18.52±2.89^{a}	31.52±2.45^{b}	33.09±6.40^{b}	29.35±5.05^{a}	10.32±1.81^{a}	39.17±6.35^{c}	5.41	0.002
T.Antioxidan (mmol/l)	0.63±0.24^{a}	0.88±0.26^{a}	1.11±0.29^{b}	0.78±0.19^{a}	0.04±0.02^{a}	2.10±0.22^{c}	9.58	0.001
MDA (ng/ml)	638.62±68.06^{b}	576.08±44.32^{b}	296.68±31.63^{a}	273.04±58.11^{a}	843.50±95.69^{c}	235.34±37.64^{a}	16.86	0.001

Values are expressed in mean ± SD. The value with different superscript showed difference from each other ($p<0.05$) while value with same superscript are not statistically difference from each other ($p>0.05$).

KEY: Group 1 - 80mg/kg VCD+200mg/kg Soyabean, Group 2 - 80mg/kg VCD+400mg/kg Soyabean, Group 3 – 80mg/kg VCD+600mg/kg Soyabean, Group 4 – 14ug/100g Estradol, Group 5 - 80mg/kg VCD, Group 6 – Control. T-Total- Total antioxidant, MDA- Malonialdehyde

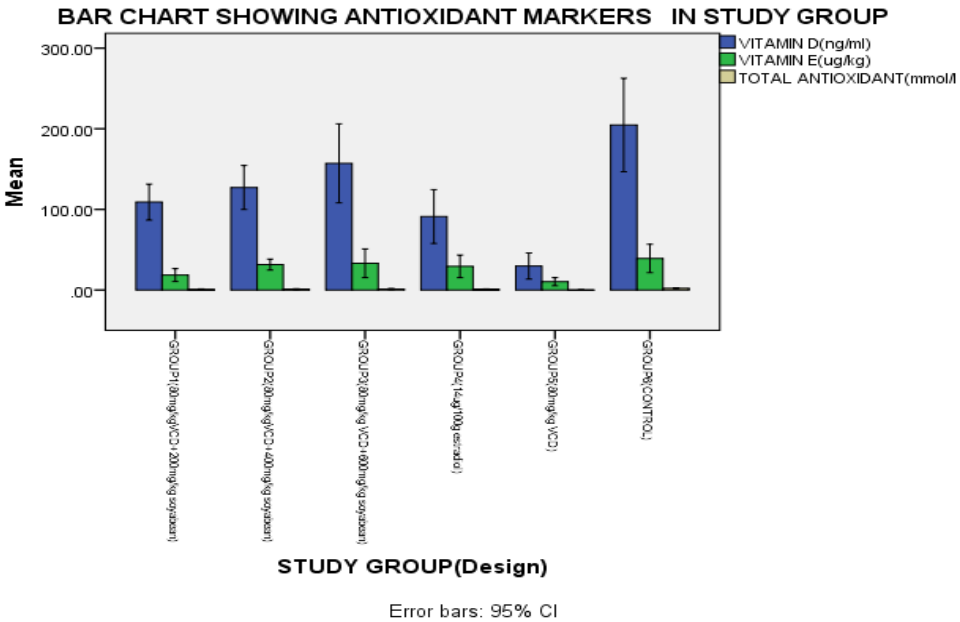

Figure 1: Bar Chart showing antioxidant markers in the study group.

DISCUSSION

The study aims to evaluate the antioxidant properties of soybean phytoestrogen-rich extract in 4-vinylcyclohexane diepoxide-induced menopausal rats. Malondialdehyde (MDA), a marker of oxidative stress, was significantly reduced in a dose-dependent manner, with no observable difference between 200mg/kg and 400mg/kg of phytestrogen treatment, but a significant difference between 600mg/kg of phytestrogen isoflavone and 14ug/kg of estradiol therapy. Isoflavone, a phytestrogen, has been shown to improve antioxidant status by decreasing malondialdehyde (MDA), which was find in this study. This study's findings are consistent with those of Yamagata (2019),[20] Bakhtiari (2019),[21] Yan (2017),[22] Pereira (2018),[23] Khan (2012),[24] and Hsu (2007).[25] To counteract the effects of hydrogen peroxide on cells, soy isoflavone has been shown to increase the expression of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase in both porcine mammary gland cells and human umbilical vein endothelial cells.[11][26] According to these findings and a previous report,[27] isoflavones modulate enzymatic antioxidants (in addition to ROS scavengers), and nuclear factor-erythroid 2 (Nrf2), a key transcription factor that leads to gene transcription of various antioxidant and phase II detoxifying enzymes, is associated with this modulation.[28][12] The reactive species malondialdehyde may be lowered in postmenopausal women who consume 100 mg of isoflavones daily, according to certain research.[29] Also, postmenopausal individuals whose diets included 138 milligrammes of isoflavones and 81 milligrammes of dadzein saw an increase in the antioxidant enzyme superoxide dismutase activity. These compounds (isoflavone) have also been proven to enhance total antioxidant capacity in animal models. In this study, it is worthwhile to know that phytoestrogen rich extract of soyabean at dose –dependent manner decrease MDA, increases the total antioxidants and vitamin E.

Total antioxidant and vitamin E levels were all found to be considerably ($p<0.05$) higher than in the positive control group. Soyabean milk may enhance antioxidant activity scavenging free radicals, as evidenced by the increases in vitamin E, and total antioxidant found. Human neutrophil reactive species can be directly scavenged by isoflavones.[5][30] Although isoflavones are effective free radical scavengers, they are more resistant to oxidation mediated by peroxynitrite and hypochlorous acid than they are to peroxyl radicals.[31][32] To prevent nitration of tyrosine, isoflavones have been shown to scavenge peroxynitrite, a powerful oxidant formed in vivo from the reaction of nitric oxide with superoxide, while genistein and daidzein inhibit peroxynitrite-mediated low-density lipoprotein (LDL) oxidation in a dose-dependent manner. In mice with type 1 diabetes caused by streptozotocin, genistein restores normal levels of superoxide anion generation and nitrotyrosine synthesis.[7]

CONCLUSION

This study shows that soyabean phytoestrogen-rich extract and estradiol treatment improved antioxidant marker levels in female rats treated. This demonstrated that pituitary and gonadal hormone levels can be altered due to 4-vinylcyclohexane diepoxide (4-VCD). In animal models, administration of phytoestrogen -rich extract at varying concentrations exhibited fantastic antioxidative activities than estrogen therapy commonly used as hormone replacement therapy(HRT). This study has the potential to reawaken interest in natural diets and to refresh some of the public's understanding of the role of isoflavones in the oxidative stress process.

CONFLICT OF INTERESTS

There are no stated conflicts of interest by the authors.

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AUTHORS CONTRIBUTIONS

Olaniyan, Edusola Juliana designed and initiated the study, Emokpae, Mathias Abiodun reviewed the manuscript for important intellectual content, Oyakhire, Fidelis Ohiremen assisted in data analysis, Ahmed Liasu Adeagbo assisted in analysis and interpretation of data, Esezobor, Iria Kelly assisted in collection of blood sample from animals, and Olaniyan Stephen Olawale and Oyakhire, Fidelis Ohiremen assisted in draft of the manuscript.

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