Journal of Advances in Medical and Biomedical Research | ISSN:2676-6264

Age Related Changes in Indices of Bone Metabolism and Oxidative Stress in Postmenopausal Women in Southern Nigeria

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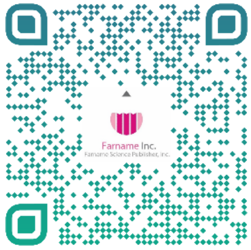
Article Info

10.30699/jambs.31.145.125

Received: 2022/01/19;
Accepted: 2022/11/21;
Published Online: 13 Mar 2023;

Use your device to scan and read the article online



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ABSTRACT

Background & Objective: Oxidative stress (OS) associated with estrogen deficiency in menopause has been implicated in various complications of menopause. Some indices of OS, bone metabolism and uric acid (UA) in postmenopausal women were assessed as possible predictors of gouty osteoarthritis.

Materials & Methods: This case-control study enrolled 40 postmenopausal women and 60 premenopausal women as participants. The levels of reduced glutathione (GSH), nitric oxide (NO), total plasma peroxides (TPP), total antioxidant capacity (TAC), malondialdehyde (MDA), inorganic phosphate (Pi), total calcium (tCa) and UA were estimated by colorimetry, estradiol (E_2) by ELISA and oxidative stress index (OSI) by calculation. Data were analyzed using t-test, correlation and regression at $P<0.05$.

Results: Postmenopausal women had higher UA, OSI and lipid peroxidation (higher MDA, TPP) with lower E_2, tCa and antioxidants (reduced GSH, NO, TAC) compared to premenopausal women ($P<0.05$). Aging correlated negatively with E_2 (r=-0.273, P=0.006), TAC (r=-0.484, P=<0.001), GSH (r=-0.306, P=0.002), NO (r=-0.337, P=0.001), tCa (r=-0.571, P=<0.001) and positively with TPP (r=0.445, P=<0.001), OSI (r=0.454, P=<0.001), MDA (r=0.505, P=<0.001) and UA (r=0.441, P=<0.001) in all women studied irrespective of menopause status. There were no associations between UA, tCa, Pi, E_2 and indices of oxidative stress (TPP, TAC, OSI, MDA, GSH, NO) with menopause (R^2=0.216, P=0.728).

Conclusion: Elevated UA, lipid peroxidation and oxidative stress and reduced tCa, E_2 and antioxidants observed in postmenopausal women may be associated with aging and not the menopausal status suggesting that their assessment may be utilized in predicting women at increased risk of gouty osteoarthritis.

Keywords: Menopause, Lipid peroxidation, Oxidative stress, Bone metabolism

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Introduction

Menopause accruing from reproductive aging is characterized by permanent cessation of ovarian follicular activity and progressive loss of estrogen and its beneficial effects on female reproductive health (1). Estrogen has been shown to perform a dual function as a sex hormone and antioxidant molecule that counterbalance oxidative stress (OS) (2). Estrogen exerts its antioxidant actions by direct inhibition of ROS generation and peroxidation process and upregulation of expression of antioxidant enzymes (3). The decline in estrogen associated with menopausal transition has been implicated in mechanisms of cellular senescence and age-related OS in postmenopausal women which is considered the common pathologic factor for the 'menopausal syndrome' that include vasomotor disturbances, cognitive impairment, urogenital dystrophy, redox imbalance, dyslipidemia and changes in body fat distribution (3,4). Previous studies have reported higher levels of lipid peroxidation markers and lower levels of low molecular weight enzymatic antioxidants in post-menopausal women when compared to premenopausal women (3).

Menopausal women are susceptible to disturbances in bone metabolism compared to women of reproductive age, because their redox balance is deranged not only by advancing age but also by declining levels of estrogens thereby predisposing them to the various sequel of menopause (4). Studies on indices of OS and bone mineral metabolism in relation to the menopausal status are rife (2, 3, 4) and often times contradictory among races and different ethnic groups, which have been attributed to individual peculiarities and variations in environmental, nutritional and genetic factors. Moreover, dearth of scientific information on the inter-relationship between indices of OS, bone mineral and purine

metabolism, female reproductive hormones and the menopausal status makes this present work a base line study in the area of study. Alterations in the homeostasis of markers of oxidative stress and bone metabolism may therefore herald the onset of metabolic bone disorder and other complications of menopause. This study evaluated some indices of OS and bone metabolism in relation to age-related decline in estradiol in pre and postmenopausal women and their possible use as effective indices for early detection of gouty osteoarthritis in a resource limited setting.

Materials and Methods

Study design

This case-control study was conducted in the Obstetrics and Gynaecology Clinic of the University of Calabar Teaching Hospital Calabar (UCTH), Nigeria. The study population comprised apparently healthy premenopausal and postmenopausal women residing in Calabar metropolis. Before commencement of the study, a written consent was obtained from all participants before enrollment, while approval of the research protocol was given by the health research ethics committee of Cross River State ministry of health (REC No. RP/REC/2018/540). The conduct of this study was in compliance with the ethical demands guiding medical research involving human subjects as declared in Helsinki in 1975 and more recent revisions.

Selection of Participants

The participants in this study were made up of 100 women comprising 40 postmenopausal women aged 50-75 years attending the Obstetrics and Gynaecology Clinic of UCTH for routine medical check-up and 60 premenopausal women aged 20-40 years who were apparently healthy volunteers among staff and students of UCTH. A structured questionnaire was used to obtain socio-demographic information, gynecological/ medical history; age at menarche and menopause, age at first pregnancy, parity, duration of breastfeeding, presence or absence of such symptoms as depression, joint pains, insomnia, hot flushes and irregular menstruation, use of hormone replacement therapy and supplements, smoking habit, use of alcohol, drug/substance abuse and diet. The body weight and height of the subjects were taken and used to determine the body mass index (BMI).

The sample size was obtained using the formula by Daniel and Cross; $n = Z^2P(1 - P)/d^2$ (5) (where Z = standard normal deviation at 95% confidence interval = 1.96; P = estimated prevalence of gouty osteoarthritis; d = precision limit 5% = 0.05) with a global prevalence of gouty osteoarthritis of 6.8% (6) giving a total of 100 participants.

Inclusion criteria:

Healthy premenopausal women (20-40 years) without any menstrual irregularities;

Healthy postmenopausal women (50-75) with history of natural menopause (>1year amenorrhea).

Exclusion criteria

Women who are pregnant or lactating, of perimenopausal status, undergoing dietary supplementation (with nutritional antioxidants, calcium and phosphorus), pharmacological and hormone therapies, with chronic illness or medication including bone disease, chronic alcoholics or smokers were excluded from the study.

Sample collection

Five milliliters of whole blood samples were aseptically collected from all the premenopausal women (during the follicular phase of the menstrual cycle) and postmenopausal women of the study population into plain anticoagulant free sample containers and sera collected for the estimation of inorganic phosphate (Pi), total calcium (tCa), uric acid, estradiol (E_2), malondialdehyde (MDA), nitric oxide (NO), reduced glutathione (GSH), total antioxidant capacity (TAC) and total plasma peroxides (TPP).

Laboratory methods

All analytical reagents used for analysis were procured from Sigma-Aldrich

Assay of total plasma peroxides

TPP was determined based on the ferrous-butylated hydroxytoluene-xylenol orange complex (FOX-2) test system. The TPP was estimated when plasma peroxides in sample reacts with ferrous-butylated hydroxytoluene-xylenol orange complex (FOX-2 reagent) to give a colour complex whose intensity at 560nm is proportional to TPP in the sample (7).

Assay of total antioxidant capacity

TAC was determined based on the reaction of hydrogen peroxide (H_2O_2) with ferric ion-ethylenediamine tetraacetic (Fe-EDTA) complex to form hydroxyl radicals (OH•). Briefly, on addition of H_2O_2 and Fe-EDTA to sample, the reactive oxygen species (ROS) (H_2O_2 and OH•) generated in the reaction mixture degrade benzoate leading to the release of thiobarbituric acid reactive substance (TBARS). The production of TBARS is suppressed by antioxidants present in the sample. This degree of inhibition of colour development is proportional to the concentration of the total antioxidant of the sample which was measured spectrophotometrically at 532nm (8)

Calculation of oxidative stress index (OSI)

The OSI was calculated as the ration of TPP/TAC and is commonly used to determine the degree of OS.

$OSI\% = [TPP/TAC] \times 100$ (7)

Estimation of Malondialdehyde

Malondialdehyde estimation was done based on the reaction of thiobarbituric acid (TBA) with malondialdehyde formed from the breakdown of polysaturated fatty acid to give a red coloured complex absorbing at 523nm. Malondialdehyde is used as an index of lipid peroxidation (9).

Assay of Reduced Glutathione

Estimation of GSH was carried out following the modified standard Ellman's method. The reduced glutathione in the sample reacts with Ellman's reagent to yield a coloured complex at 412nm (10).

Estimation of NO

Estimation of total nitrite or nitrous acid in the sample was done using the Griess test. The reagent α-naphthylamine react with NO containing compounds to give a pink azo dye whose intensity measured at 540nm is equivalent to NO concentration (11).

Estimation of Phosphorus

Phosphorus present in the sample combines with reagent ammonium molybdate in presence of strong acids to form phosphomolybdate. The absorbance of this complex is directly proportional to the phosphorus concentration in sample (12).

Estimation of Calcium

Total calcium was estimated using O-cresolphthalein complexon reagent. The reagent O-cresolphthalein complexone in an alkaline medium reacts with calcium in serum to yield a purple complex whose intensity is proportional to Ca content of the sample (13).

Assay of uric acid

Uric acid was estimated using enzyme colorimetric method. The enzyme uricase cleaves uric acid to yield allantoin and H_2O_2 which in the presence of peroxidase enzyme oxidizes 4-aminophenazone to give a colour complex whose intensity is proportional to the concentration of uric acid in the sample (14).

Estimation of Estradiol

Estimation of serum E_2 was done based on competitive binding enzyme immunoassay technique using commercial test kits procured from Alpco diagnostics, Salem USA. Unlabelled E_2 in target sample competes with an enzyme labeled estradiol (conjugate) for a limited number of antibody binding sites on the microtitre plate. The absorbance of complex formed after reaction with enzyme substrate at 450nm is inversely proportional to estradiol levels in the sample (15).

Data analysis

Data analysis was done using SPSS version 20.0, IBM USA. Determination of group mean differences was done using t-test analysis, associations between variables were determined by correlation analysis while associations between variables and menopausal status was determined using multiple regression analysis at $p<0.05$.

Results

The comparison of age, BMI, GSH, NO, TAC, TPP, MDA, OSI, UA, tCa, Pi, and E_2 in postmenopausal and premenopausal women was depicted in Table 1. Higher levels of TPP, OSI, MDA and UA with lower TAC, GSH, NO, tCa and E_2 were observed in postmenopausal women compared to premenopausal women studied ($P<0.05$). No significant differences were found in the BMI and Pi levels of the 2 groups ($P>0.05$).

Table 1. **Comparison of age, BMI, TPP, TAC, OSI, MDA, GSH, NO, UA,tCa, Pi, and E_2 in postmenopausal and premenopausal women.**

Parameters	Postmenopausal women n=40	Premenopausal women n=60	P-value
Age (years)	62.98±13.31	31.28±10.35	<0.001*
BMI (kg/m2)	26.20±5.18	25.47±5.31	0.497
TPP (mmol/dl)	61.57±10.55	53.72±11.34	0.001*
TAC (mmol/l)	47.89±29.23	69.58±24.86	<0.001*
OSI (%)	27.21±25.79	10.85±10.55	<0.001*
MDA (mmol/l)	86.30±52.80	33.20±17.30	<0.001*
GSH (mmol/l)	47.04±5.70	49.94±4.72	0.009*
NO (mmol/l)	22.80±12.99	32.62±14.07	0.001*
UA (mmol/l)	0.34±0.08	0.26±0.07	<0.001*
tCa (mmol/l)	2.19±0.09	2.35± 0.06	<0.001*
Pi (mmol/l)	1.55±0.39	1.42±0.43	0.709
E_2 (pg/ml)	14.34±3.75	26.22±17.57	0.003*

Data presented as mean±SD, *= significant at P< 0.05 using t-test analysis, BMI= body mass index, TAC=total antioxidant capacity, TPP=total plasma peroxides, OSI= oxidative stress index, GSH=reduced glutathione, MDA=malondialdehyde, NO=nitric oxide, UA=uric acid, Pi=inorganic phosphate, E_2=estradiol, tCa=total calcium.

Table 2 shows the correlation of age with biochemical indices in premenopausal and postmenopausal women studied. Significant negative correlations were observed between age and TAC (r=-0.484, P=<0.001), GSH (r=-0.306, P=0.002), NO (r=-0.337, P=0.001), tCa (r=-0.571, P=<0.001) and E_2 (r=-0.273, P=0.006); and positive correlations between age and MDA (r=0.505, P=<0.001), TPP (r=0.445, P<0.001), OSI (r=0.454, P=<0.001) and uric acid (r=0.441, P=<0.001) in premenopausal and postmenopausal women studied.

Table 2. **Correlation of age with biochemical indices in Premenopausal and postmenopausal women studied.**

Variables		R	P-value
Age	tCa	-0.571	<0.001*
	TAC	-0.484	<0.001*
	GSH	-0.306	0.002*
	NO	-0.337	<0.001*
	E_2	-0.273	<0.001*
	MDA	0.505	<0.001*
	TPP	0.445	<0.001*
	OSI	0.454	<0.001*
	UA	0.441	<0.001*

TAC=total antioxidant capacity, TPP=total plasma peroxides, OSI= oxidative stress index, GSH=reduced glutathione, MDA= malondialdehyde, NO=nitric oxide, UA=uric acid, E_2=estradiol, tCa=total calcium.

The relationship between UA, tCa, Pi, E2 and indices of oxidative stress with menopause was shown in Table 3. The relationship between UA, tCa, Pi, E2 and indices of oxidative stress with menopause indicates that the model was not statistically significant (Constant = 140.992, R^2 =0.216, p=0.728) with only 21.6% probability of predicting menopause. There were no significant associations between UA (β=9.509, P= 1.000), tCa (β=-136.106, P= 0.998), Pi (β=10.599, P= 0.999), E2 (β=-0.221, P= 0.998), TPP (β=0.984, P= 0.997), TAC (β=-0.393, P= 0.996), OSI (β=-0.696, P= 0.995), MDA (β=2.938, P= 1.000), GSH (β=-1.162, P= 0.999), NO (β=0.130, P= 0.999) with menopause.

Table 3. **Relationship between UA, tCa, Pi, E_2 and indices of Oxidative Stress with menopause**

Predictors C=140.992, R^2=0.216, P=0.728	B	P- value
UA	9.509	1.000
tCa	-136.106	0.998
Pi	10.599	0.999
E_2	-0.221	0.998
TPP	0.984	0.997
TAC	-0.393	0.996
OSI	-0.696	0.995
MDA	2.938	1.000
GSH	-1.162	0.999
NO	0.130	0.999

TAC=total antioxidant capacity, TPP=total plasma peroxides, OSI=oxidative stress index, GSH=reduced glutathione, MDA= malondialdehyde, NO=nitric oxide, UA=uric acid, E_2=estradiol, tCa=total calcium, Pi=inorganic phosphate, C= constant

Discussion

In this study, postmenopausal women had lower E_2 and reduced TAC compared to premenopausal women studied. Lower levels of estradiol in postmenopausal women compared to premenopausal women have also been described by previous studies (16, 17). Lower E_2 in postmenopausal women have been attributed to the physiological decline in E_2 production from the ovaries after menopause hence symptoms and complications of E_2 deficiency are more pronounced in postmenopausal women (17). Seven to ten-fold decrease in estradiol levels has been observed between pre- and postmenopausal phase (17, 18). Decline in E_2 levels in menopause has been associated with alteration in the redox status that can expose to OS since estrogens acts as both sex hormones and antioxidant molecules that counterbalance oxidative damage (8). Their antioxidant activity is exerted directly by virtue of its chemical composition and indirectly by upregulation of the expression of intracellular anti-oxidative defense mechanisms and longevity-related genes (19). The phenolic A- ring of 17β-estradiol molecule is responsible for eliciting free radical scavenging antioxidant activity similar to that of simple phenolic antioxidants such as vitamin E (20). The natural state of estrogen deficit associated with menopause may therefore result in increased generation of ROS and OS and consequently reduction in antioxidants which are consumed in the process of maintaining redox balance suggesting that reduced antioxidant status in postmenopausal women may be linked to estrogen deficit (2). Decline in E_2 levels in menopause may therefore account for lower TAC levels seen in postmenopausal women studied. Estrogen replacement therapy has been shown to restore antioxidant status and prevent OS related menopausal complications (21).

The NO and GSH levels of postmenopausal women were lower than those of their premenopausal counterparts. Consistent with our findings, lower NO concentrations have been reported in postmenopausal women who are not on estrogen replacement therapy. Estrogen has been shown to induce nitric oxide synthase and increase NO release (22). Nitric oxide bioactivity has been shown to be reduced in postmenopausal women but returns to premenopausal levels with estrogen replacement (23). Therefore, decreased E_2 levels in postmenopausal women may be responsible for the observed lower levels of NO (24). Glutathione has been described as the major intracellular antioxidant involved in cellular detoxification and maintenance of cellular redox balance (21). Lower GSH levels seen in postmenopausal women compared to premenopausal may result from increased consumption in free radical scavenging activities in order to inhibit membrane lipid peroxidation and OS associated with E_2 decline in menopause. In vitro, 17-β estradiol has been shown to increase GSH levels and thioredoxin reductases activity (25). Lower glutathione peroxidase activity has also been reported in postmenopausal women (26).

Increased lipid peroxidation (higher MDA and TPP) and OS (higher OSI) were observed in post menopausal women compared to premenopausal women studied. Decreased E_2 levels associated with menopause may account for increased lipid peroxidation and OS observed in postmenopausal women. E_2 has been shown to exert both pro-oxidant and antioxidant effects depending on the concentration in-vivo (27). Higher estrogen levels have been associated with beneficial antioxidant effects and lower levels with enhanced ROS generation (1). Thus, redox imbalance characterized by increased lipid peroxidation and OS observed in postmenopausal women may be a consequence of either enhanced ROS production or impaired antioxidant function as a result of estrogen deficiency associated with menopause (28). Significantly higher lipoperoxide level has been demonstrated in postmenopausal than premenopausal women by a previous study (26).

Postmenopausal women had higher uric acid and lower total calcium compared to premenopausal women studied. Higher uric acid in postmenopausal women may be the consequence of estrogen deficiency associated with menopause. A similar observation on uric acid levels in postmenopausal women has been made. Higher uric acid levels have also been independently associated with menopause and lower uric acid levels with postmenopausal hormone therapy. Uric acid lowering effect of estradiol has been shown to be through mechanisms involving renal clearance, secretion and reabsorption (29). Elevated uric acid levels have been associated with increased risk of gout in aging women (30). These observations suggest that the risk of gout may be increased by menopause, while postmenopausal hormone therapy may reduce gout risk among women (31). However, uric acid has been reported to be protective for bones in menopause since uric acid have been hypothesized to exhibit some antioxidant properties (30). Lower calcium levels in postmenopausal women have been linked to E_2 decline in menopause. Alterations in the homeostasis of Ca, Mg and phosphate has been linked to estrogen deficiency in menopause by mechanisms directly or indirectly modulating the regulation of these minerals at the level of the intestine, bone and the kidney (32). Calcium loss as a result of estrogen deficiency may be due to decreased intestinal absorption and renal reabsorption. Decrease in bone osteoblastic activity and increase in the osteoclastic activity leading to bone demineralization and eventually osteoporosis has been associated with E_2 decline in menopause (33). Consistent with our findings, the mean value of serum calcium was reported to be significantly decreased in postmenopausal women when compared to premenopausal women (16). However, the findings of lower tCa levels in postmenopausal women in this present study contradicts higher levels observed in our previous study (34). The disparity in the two results may be attributed to non-exclusion of women on

estrogen replacement therapy from the earlier study as confounders. The phosphate levels of both pre and postmenopausal women were not statistically different.

Aging correlated positively with TPP, MDA, OSI and UA and negatively with E_2, tCa, TAC, GSH and NO in all women studied irrespective of the menopause status. Ageing has been associated with decreased calcium levels as a result of decline in estradiol and progesterone production in elderly women. Decrease in calcium absorption with increasing age after menopause has been reported **(32)**. Our findings of negative associations between ageing and calcium and E_2 levels in all women studied corroborate these observations. Declining E_2 levels may represent the onset of the aging process in women **(24)**. Progressive loss of tissue and organ function due to accumulation of oxidatively damaged macromolecules (proteins, lipids, DNA) and consequently of cells and tissues as a result of aerobic metabolism to which individuals are exposed to throughout their life time characterize the ageing process. Increasing age is therefore associated with increased ROS generation, lipid peroxidation, OS and consequently antioxidant depression **(26)**. Age related increase in lipoperoxide and decrease in TAC and glutathione peroxidase activity has been previously described. These findings may account for the positive associations between increasing age with lipid peroxidation and oxidative stress (TPP, MDA, OSI) and negative associations with TAC, GSH and NO. OS has been implicated in several age-related conditions including gouty osteoarthritis and osteoporosis **(35)**.

Our study is limited by small sample size and single spot sampling method, larger sample size is needed in future studies. This study employs a case-control design and may prove association but cannot establish causation. The strength of the study lies on its ability to demonstrate that increased lipid peroxidation, oxidative stress and depressed antioxidants in conjunction with changes in the homeostasis of estradiol, calcium and uric acid metabolism is associated with ageing and not the menopausal status. To the best of our knowledge, this study is the baseline study to demonstrate such associations between the targeted biochemical indices with ageing and risk of gouty osteoarthritis among women in the area of study.

Conclusion

The findings of this study suggest that enhanced lipid peroxidation and oxidative stress, increased uric acid and decreased calcium, estradiol and antioxidants may be related to ageing and not the menopausal status in postmenopausal women studied. Assessment of these biochemical indices may be effective in predicting women at increased risk of developing gouty osteoarthritis.

Acknowledgments

None.

Conflict of Interest

Authors declare no conflict of interest with regards to publication of this work.

Funding

There was no funding for this research work.

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How to Cite This Article:

Nsonwu-Anyanwu A C, Eworo R E, Nsonwu M C, Akpan U O, Kalu F A, Nwevo E O. Age related changes in indices of bone metabolism and oxidative stress in Postmenopausal women in Southern Nigeria. J Adv Med Biomed Res. 2023; 31(145): 125-32.

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