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Influence of menopause on biochemical markers of endothelial dysfunction—A case–control pilot study in North Indian population

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ABSTRACT

Objective: Menopause, an estrogen deficient state, is known to increase the cardiovascular risk. Lipid changes accompanying menopause account for only few cases of coronary artery disease (CAD). Endothelium-dependent nitric oxide-mediated vasodilatory mechanisms are also known to play a role in development of coronary artery disease, but studies in menopausal women are very few. This study was hence undertaken to see if nitric oxide (NO)–cyclic guanidine monophosphate (c-GMP) pathway is influenced by menopause.
Design: This study was a hospital-based case–control study involving 100 women in age group 40–55 years. Of these, 50 women were postmenopausal and 50 were premenopausal. Women with known risk factors for CAD were excluded. Fasting blood samples from these women were collected and analyzed for estradiol levels, lipid profile, apolipoprotein B, plasma nitric oxide, c-GMP and platelet nitric oxide using standard kits and reagents. Statistical analysis was done on SPSS and two-tailed p-value <0.05 was considered significant.
Result: Postmenopausal women had significantly lower estradiol, plasma NO, and c-GMP levels as compared to premenopausal women ($p < 0.05$). Cholesterol, low-density lipoprotein (LDL) cholesterol and apolipoprotein B (apo-B) levels were higher and HDL levels were lower in postmenopausal as compared to premenopausal women ($p < 0.05$). Plasma NO showed a significant positive correlation with estradiol, HDL levels and negative correlation with apo-B levels.
Conclusion: Menopause tends to downregulate NO–c-GMP pathway resulting in endothelial dysfunction. The mechanism may be directly through estrogen receptors or indirectly through potentiation of dyslipidemia.

1. Introduction

Menopause is an important physiological change in a woman's life, which is accompanied by functional and morphological changes in the vasculature apart from loss of reproductive function. Early in the menopausal transition when cycle irregularity is first seen, a decrease in estradiol and a fluctuating rise in serum follicle stimulating hormone (FSH) levels have been observed [1]. The average age at onset of menopause in northern India is 48 years which is about 3 years earlier than the west [2].

Premenopausal women have a lower incidence of coronary artery disease (CAD) than postmenopausal women and men of same age group. Although the mechanisms of this apparent relative protection against atherosclerosis remain ill defined, some of the past studies indicate that, estradiol, which is present in higher concentrations before menopause, may play a central role [3]. Angiographic studies have consistently found less coronary artery disease in postmenopausal women who receive estrogen replacement therapy [4].

The vascular endothelium helps to maintain homeostasis by generating vasodilator and vasoconstrictor substances [5]. It has been observed that in normal course, several circulating agonists and hydro-mechanic factors such as viscous drag result in shear forces in the bloodstream, which stimulate the release of nitric oxide (NO) from endothelial cells. This NO diffuses into the specific molecular targets (smooth muscle and platelets). In smooth muscles, it interacts and stimulates heme group of guanylate cyclase, leading to increased cyclic-guanidine mono phosphate (c-GMP) production, which leads to the activation of protein kinase G and the phosphorylation of proteins of the signaling cascade, which result in smooth muscle relaxation and hence vasodilatation [6–8]. A strong correlation between endogenous estrogen level and capacity for

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NO production has been shown in women. Several studies have reported that estradiol can increase endothelial and constitutive NO synthesis [9,10].

Decrease in estrogen levels associated with menopause adversely affects lipid and lipoprotein metabolism and the effects may be reversed by postmenopausal use of estrogen by women [11]. Studies have suggested that postmenopausal women tend to have higher plasma levels of cholesterol, triglycerides, LDL, VLDL and apolipoprotein B levels and lower levels of HDL and apolipoprotein A1 than their premenopausal counterparts, facilitating the atherosclerotic process. The atherogenic lipoproteins LDL, VLDL remnants, or IDL, and chylomicron remnants, each contain one molecule of apo-B as the structural protein. The plasma apo-B concentration thus, reflects the number of atherogenic lipoproteins [12].

Some studies have shown that CAD initiation and progression may also result from the impaired synthesis or excessive oxidative degradation of NO in presence of dyslipidemia and/or hypertension [4,13]. Endothelial dysfunction occurring after menopause has been supported by studies in conduit arteries using high-resolution ultrasound, where evidence of a blunted flow-mediated dilatation in postmenopausal women and the beneficial role of estrogens was reported [14,15]. In the Indian population, menopause has recently been accepted as a health problem. At present, most of the Indian population is in the reproductive age group, which will be facing menopause sooner or later leading to a tremendous increase in the socio-economic burden of CAD associated with menopause. A total of 130 million Indian women are expected to live beyond the menopause into old age by 2015 [16]. So far, research pertaining to increased CAD risk in postmenopausal women has mainly focused on lipid profile and altered coagulation pathways. Though tremendous advances have been made, many questions pertaining to the NO–c-GMP-signaling pathway in menopause and the influence of decreased estradiol levels, lipid profile on this pathway remains unanswered. This was the first clinical study in India, undertaken to study if NO–c-GMP pathway is influenced in postmenopausal women and to find its correlation with age, estradiol levels and dyslipidemia.

2. Methods

One hundred women of age 40–55 years were selected from gynecology clinics/wards of a tertiary care hospital of a developing country. Of these, 50 postmenopausal women (either natural or surgical, who had their last period at least 1 year back and not later than 3 years) constituted the study group. The control group consisted of 50 premenopausal women without any gynecological complaints.

Women with gynecological cancers, cardiovascular diseases, and hypertension or on hormone replacement therapy/antihypertensive or hypolipidemic medications were excluded from the study. The Institutional Ethical Committee approved the study and all the women were included after a written and informed consent.

3. Sample collection and processing

Fasting blood samples were collected in plain vials and centrifuged at $1000 \times g$ for 5 min after clotting. Fresh serum was used for routine investigations and lipid profile. The rest of the serum was separated into two parts and stored at $-20\,^{\circ}C$ till estradiol and apo-B were batch analyzed.

Blood was also collected in a polyethylene container containing 3.8% trisodium citrate (1 unit for each 9 units of blood) and mixed thoroughly by inverting; care was taken to avoid any frothing in the sample. One millilitre sample of citrated sample was used for estimation of plasma NO and c-GMP. The samples were aliquoted and stored in polyethylene container at $-20\,^{\circ}C$ till batch analyzed.

4. Estimation of nitric oxide

Nitric oxide was estimated in plasma by modified Griess reaction [17]. Griess reaction involves the formation of a chromophore during the reaction of nitrite (NO_2^-) with sulfanilamide and heterocyclic amine of N-(1-napthyl) ethlenediamine (Griess reagent) under conditions of low pH.

In an oxygenated solution, NO decomposes to form nitrite (NO_2^-) and nitrate (NO_3^-) as shown below:

$$2^{\bullet}NO + O_2 \rightarrow 2^{\bullet}NO_2$$

$$2^{\bullet}NO + 2^{\bullet}NO_2 \rightarrow 2\,N_2O_3$$

$$2^{\bullet}N_2O_3 + 2H_2O \rightarrow 4NO_2^- + 4H$$

5. Determination of c-GMP

c-GMP was estimated by ELISA. The kit used a polyclonal antibody to c-GMP to bind, in a competitive manner, to the c-GMP in standard or sample or an alkaline phosphatase molecule which has c-GMP covalently attached to it [18].

6. Determination of estradiol

Serum estradiol was measured by competitive enzyme immuno assay [19], which is based on competitive interaction of estradiol and the hormone-enzyme conjugate for a limited number of immobilized anti estradiol antibodies (rabbit). Thus, the amount of bound hormone–enzyme conjugate in the well, unbound conjugate is removed by washing. When substrate solution is added, a blue color develops changing to yellow after stopping the reaction. The intensity of color is inversely proportional to the amount of estradiol in the specimen.

7. Determination of lipid profile and apolipoprotein B

Cholesterol and triglycerides were determined by enzymatic end point method and HDL was determined by precipitation method described by Burstein et al. followed by enzymatic end point method as for cholesterol [20]. LDL cholesterol was estimated using Friedewald's formula [21].

$$\text{LDL cholesterol (mg/dl)} = \text{total cholesterol} - [\text{triglycerides}/5 + \text{HDL}]$$

Serum apolipoprotein B was determined by immunoturbidimetric assay.

All the estimations were done on Beckman CX5 autoanalyser using standard kits, calibrators and controls from Randox (UK).

8. Statistical analysis

Statistical analysis was carried out using SPSS for windows 10.0 software (SPSS Inc., Chicago, IL, USA). Data are expressed in mean ± standard error of mean. The difference between groups was compared by independent sample *t*-test or Mann–Whitney test for continuous variables. Spearman's rank correlation was applied to test for association between continuous variables. Univariate logistic regression analysis was performed to assess the independent contribution of age, estradiol levels and lipid profile to nitric oxide and c-GMP levels in postmenopausal women. A two-tailed *p*-value <0.05 was considered statistically significant.