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Hyperhomocysteinaemia and cardiovascular risk in female ovariectomized rats: role of folic acid and hormone replacement therapy

Sahar E. El-Swefy, Sousou I. Ali, Mervat E. Asker and Hoda E. Mohamed

Abstract

Hyperhomocysteinaemia is an independent risk factor for arteriosclerosis, recurrent thromboembolic complications and osteoporosis. After menopause, a high level of total homocysteine seems to be secondary to the altered hormonal status. Hormone replacement therapy (HRT) limits the development of coronary artery disease through a variety of mechanisms. One such mechanism is through affecting homocysteine metabolism. Folate and vitamin B_{12} deficiencies are considered to be major risks for hyperhomocysteinaemia. This study, therefore, was undertaken to examine whether lowering homocysteine with HRT or folic acid in ovariectomized rats could attenuate cardiovascular complications. Sixty sexually mature female Wistar rats were ovariectomized. Three weeks later, they were treated with estradiol (15 μg kg^{-1}, every two weeks, i.m.) or folic acid (90 μg daily, orally), either alone or in a combined form for four weeks. In addition, groups of ovariectomized rats (positive control) and healthy rats (negative control) were given cottonseed oil. Blood samples were then collected for serum and plasma separation. Serum total homocysteine, folate, estradiol, plasma nitric oxide (NO), lipid profile, and susceptibility of non-high-density-lipoprotein cholesterol (non HDLC) content to oxidation were determined. In ovariectomized rats, hyperhomocysteinaemia was established and associated with significant increments of both atherogenic indexes (total cholesterol/HDLC, low-density-lipoprotein cholesterol (LDLC)/HDLC) and susceptibility of their non HDLC to oxidation. However, plasma NO, serum folate, and estradiol levels significantly decreased. HRT and folic acid significantly reduced total homocysteine and susceptibility of non HDLC to oxidation and increased plasma NO content. Moreover, a significant negative correlation was found between total homocysteine versus folate and estradiol ($r = -0.5$, $P < 0.01$; $r = -0.25$, $P < 0.05$, respectively). Meanwhile, a positive correlation with the susceptibility of lipoprotein to oxidation was observed ($r = 0.85$, $P < 0.001$). In conclusion, a low folate level is found to be associated with elevated total homocysteine. Folic acid supplementation, either individually or in a combined form with HRT, has a beneficial effect in low estrogen status subsequent to ovariectomy.

Department of Biochemistry, Faculty of Pharmacy, Zagazig University, Zagazig, Egypt

Sahar E. El-Swefy, Sousou I. Ali, Mervat E. Asker, Hoda E. Mohamed

Correspondence: S. E. El-Swefy, Department of Biochemistry, Faculty of Pharmacy, Zagazig University, Zagazig, Egypt.
E-mail: el-azzazy@egyptnetwork.com

Introduction

After natural or surgical menopause, women are more likely to develop coronary artery disease than premenopausal women of the same age (Barrett & Bush 1991). Clinical events support the concept that postmenopausal hormone replacement therapy (HRT) is cardioprotective (Mijatovic et al 1999). The putative mechanisms are related to its effects on lipid metabolism, coagulation and fibrinolysis, arterial

blood flow and glucose metabolism (Pines et al 1997). The list of candidate mechanisms is growing and includes homocysteine metabolism, which may be affected by endocrinological status (estrogen) (Mijatovic et al 1998).

Homocysteine is an intermediate sulfur-containing amino acid that is formed by demethylation of methionine (Selhub & Miller 1992). It may be retroconverted to methionine through vitamin B_{12} and folic acid pathways. It can also be degraded via the vitamin-B_6-dependent route (Verhoef et al 1996). Folate status appears to be the main determinant of the total homocysteine concentration (Brouwer et al 1998). Many authors have shown that total homocysteine levels can be lowered substantially by folic acid supplementation in both healthy subjects and patients with hyperhomocysteinaemia (Franken et al 1994; Rasmussen et al 1996). This disorder is thought to be an independent risk factor for premature atherosclerosis and thrombosis (Malinow et al 1999).

Hyperhomocysteinaemia associated with menopause has raised an interest in the possible effect of HRT on plasma homocysteine. From this point of view, we hypothesized strategies for lowering homocysteine concentration through folic acid administration (as an important cofactor in homocysteine metabolism) or HRT, as well as their combined administration in female bilaterally ovariectomized rats.

Materials and Methods

Animals

Sixty sexually mature female virgin Wistar rats, weighing 200±10 g, were obtained from Veterinary Medicine farm in Zagazig, Egypt. The rats were housed in stainless-steel cages at room temperature (25±2°C) with a 12-h light cycle. Moreover, food (laboratory folate depleted chow diet; El-Nasar Lab. Chem. Co., Egypt) and water were freely allowed. This protocol was approved by the Animal Care and Use Committee of the Biochemstry Department, Faculty of Pharmacy, Zagazig University.

Design of the experimental work

After two weeks of acclimatization, the rats underwent bilateral ovariectomy and prophylactic penicillin (40000 IU kg^{-1}, i.m.) was administered after surgery. Postoperatively (3 weeks), rats were randomly divided into 3 groups (n = 10) and received estradiol (E_2), folic acid (F) and estradiol plus folic acid (E_2+F), respectively. Depo-estradiol (estradiol valerate; Schering, Germany) was diluted with cottonseed oil and administered intramuscularly, in a dose of 15 μg kg^{-1}, twice during the 4 weeks of the experiment. A single intramuscular injection of depo-estradiol is sufficient to maintain a steady blood concentration of estradiol for at least two weeks (Kim et al 1997). Folic acid (Sigma, St Louis, MO) was given orally (90 μg daily) for 4 weeks. Groups of ovariectomized (OV) and healthy rats (C) received cottonseed oil and served as positive and negative controls. At the end of the study, blood samples were collected for plasma and serum separation and were stored at −20°C until biochemical analysis.

Methods of analysis

Plasma levels of total cholesterol (TC), high-density-lipoprotein cholesterol (HDLC), low-density-lipoprotein cholesterol (LDLC) and triacylglycerol (TG) were measured enzymatically using commercially available kits (Boehringer Mannheim, Germany). Atherogenic indexes were calculated from the ratio of TC/HDLC and LDLC/HDLC.

Lipoprotein oxidation susceptibility was estimated according to the principle of Dujovne et al (1994). Briefly, very-low-density-lipoprotein cholesterol (VLDLC) and LDLC were precipitated from 500 μL of plasma by 50 μL dextran sulfate/magnesium chloride. The pellet was dissolved in 4% saline solution. A volume of redissolved precipitate containing 100 μg non HDLC was mixed with 4% sodium chloride to give 500 μL of total solution. Copper solution (0.5 mM $CuCl_2$) was added and incubated at 37°C for 3 h in a shaking water bath. The solution was assayed for thiobarbituric acid-reactive substances as an index for oxidation. The nmoles of malonaldialdehyde (MDA) present in the samples were estimated from a standard curve prepared by 1,1,3,3-tetraethoxypropane.

Plasma NO was determined as nitrite concentration after the reduction of nitrate to nitrite using Griess reagent. The nitrite concentration was measured at 546 nm using sodium nitrite as standard (Moshage et al 1995).

Serum estradiol was determined according to the electrochemiluminescence immunoassay (ECLIA) using Elecsys Estradiol kits (cat. no. 776002; Roche, Germany). Serum total homocysteine was estimated using reverse-phase high-performance liquid chromatography with fluorescence detection after pre-column derivatisation with monobromobimane (Refsum et al 1989). Serum folate was measured as liquid-phase,

ligand-labelled, protein-binding chemiluminescent assay with in-situ immobilization and with an antiligand detection system using Immulte folic acid kits (DPC, CA).

Statistical analysis

Statistical evaluations of the data were analysed using SAS (Statistical Analysis Computer Program SPSS). The differences in mean values were determined by analysis of variance (one way) followed by Tukey's Student Rank test. The significance of relationships between variables was calculated by linear regression analysis; $P < 0.05$ was considered significant. Values are presented as mean$\pm$s.d.

Results

Lipid and lipoprotein oxidation

Cardiovascular risk markers were established in ovariectomized rats by a significant increment of lipid parameters such as TC, LDLC, atherogenic indexes (TC/HDLC, LDLC/HDLC) and a decrement of HDLC compared with normal rats. Estradiol and combined treatment with folic acid successively reduced the lipid risk parameters, whereas folic acid therapy alone failed to affect the above-mentioned parameters (Table 1). Lipoprotein oxidation susceptibility was significantly ($P < 0.0001$) higher (52%) in ovariectomized rats than in normal rats. Estradiol and folic acid administration induced 26% and 20% inhibition of copper-induced oxidation, respectively, compared with ovariectomized rats, whereas co-treatment resulted in 23% and 12% inhibition compared with ovariectomized and folic-acid-treated groups, respectively (Table 2).

Plasma NO content

Ovariectomized female rats had a significantly ($P < 0.0001$) decreased level of plasma NO compared with the normal group (9.4$\pm$2.5 vs 17.7$\pm$5.2, respectively). Folic acid co-treatment with estradiol induced significant ($P < 0.03$) elevation in NO concentration in comparison with ovariectomized and estradiol-treated groups (Table 2).

Table 1 Plasma levels of lipid risk markers of cardiovascular disease in female ovariectomized rats after folic acid and HRT.

	Control (normal healthy rats)	Control (ovariectomized rats)	Estradiol treatment	Folic acid treatment	Estradiol plus folic acid treatment
TC (mg dL^{-1})	102$\pm$19	146$\pm$17#	114$\pm$17*	153$\pm$25	110$\pm$14*[b]
HDLC (mg dL^{-1})	37$\pm$9	30$\pm$7#	35$\pm$8	32$\pm$6	34$\pm$7
LDLC (mg dL^{-1})	54$\pm$9	107$\pm$17#	68$\pm$9*	110$\pm$16	64$\pm$6*[b]
TG (mg dL^{-1})	63$\pm$14	47$\pm$12#	54$\pm$17	48$\pm$11	57$\pm$17
TC:HDLC	2.8$\pm$0.29	5.00$\pm$0.49#	3.3$\pm$0.57*	4.70$\pm$0.36	3.1$\pm$0.29*[b]
LDLC:HDLC	1.51$\pm$0.28	3.69$\pm$0.49#	2.01$\pm$0.53*	3.45$\pm$0.38	1.89$\pm$0.32*[b]

Data are expressed as mean$\pm$s.d. $P < 0.05$ compared with #control group, *ovariectomized control, [a]estradiol-treated group, or [b]folic-acid-treated group. TC, total cholesterol; HDLC, high-density-lipoprotein cholesterol; LDLC, low-density-lipoprotein cholesterol; TG, triacylglycerol.

Table 2 Protective effect of estradiol and folic acid therapy in female ovariectomized rats.

	Control (normal healthy rats)	Control (ovariectomized rats)	Estradiol treatment	Folic acid treatment	Estradiol plus folic acid treatment
Oxidative lipoprotein susceptibility (nmol MDA (mg non HDLC)$^{-1}$)	93$\pm$12	141$\pm$25#	105$\pm$14*	113$\pm$12*	96$\pm$15*[b]
NO (μmol L^{-1})	17.7$\pm$5.2	9.4$\pm$2.5#	12.2$\pm$3*	13.7$\pm$2.1*	15.5$\pm$2.8*[a]

Data are expressed as mean$\pm$s.d. $P < 0.05$ compared with #control group, *ovariectomized control, [a]estradiol-treated group or [b]folic-acid-treated group. MDA, malondialdehyde.

Table 3 Serum estradiol, total homocysteine and folate concentration in ovariectomized female rats treated with estradiol, folic acid or their combination.

	Control (normal healthy rats)	Control (ovariectomized rats)	Estradiol treatment	Folic acid treatment	Estradiol plus folic acid treatment
Estradiol (pg mL^{-1})	96±16	36±12#	254±52*	41±9	252±34
Total homocysteine (μmol L^{-1})	2.5±0.66	6.1±1.5#	4.74±1.1*	3.26±0.74*	4.1±0.9*[b]
Folic acid (nmol L^{-1})	22±6	14.2±3.7#	16±5	70±21*	64±23*[a]

Data are expressed as mean±s.d. $P < 0.05$ compared with #control group, *ovariectomized control, [a]estradiol-treated group or [b]folic-acid-treated group.

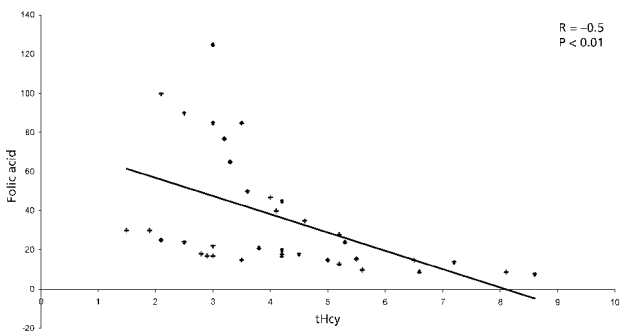

Figure 1 Correlation between serum total homocysteine (tHcy) and folate levels.

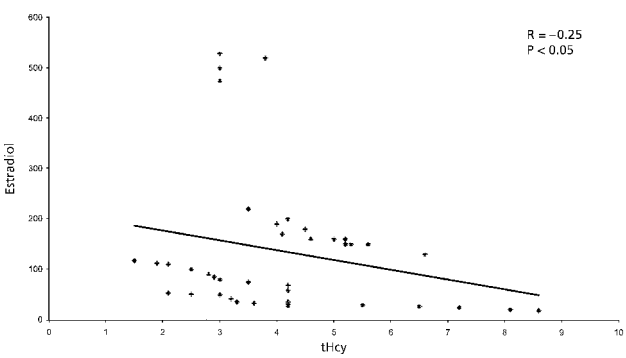

Figure 2 Correlation between serum total homocysteine (tHcy) and estradiol levels.

Serum estradiol, homocysteine and folate concentrations

Ovariectomized Wistar rats had significantly ($P < 0.05$) decreased levels of estradiol compared with healthy rats. Treatment of ovariectomized rats with estradiol, or its combination with folic acid, significantly ($P < 0.05$) increased serum estradiol concentration compared with the ovariectomized control group (Table 3). Hyperhomocysteinaemia was established in ovariectomized rats as shown by a significantly higher concentration of total homocysteine compared with the healthy control group. Treatment of ovariectomized rats with folic acid significantly reduced total homocysteine to a greater extent than estradiol compared with the positive control (3.26±0.74 and 4.74±1.1 vs 6.1±1.5, respectively). A second criteria of hyperhomocysteinaemia was a folate reduction in ovariectomized rats. Folic acid administration and combined treatment with estradiol significantly induced 5- and 4.5-fold higher folate concentration than that found in ovariectomized rats (Table 3). Using the combined results from all rats, there were significant negative correlations between total homocysteine and folate ($r = -0.5$, $P < 0.01$; Figure 1) and estradiol ($r = -0.25$, $P < 0.05$; Figure 2). On the other hand, total homocysteine was positively correlated with lipoprotein oxidation susceptibility ($r = 0.85$, $P < 0.001$; Figure 3).

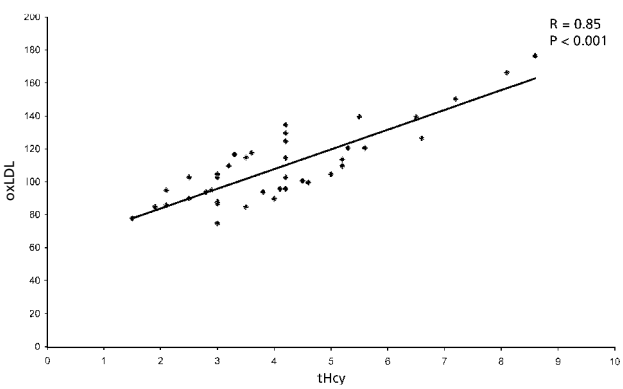

Figure 3 Correlation between serum total homocysteine (tHcy) level and lipoprotein oxidation (oxLDL).

Discussion

The results of this study clearly showed an elevated level of serum homocysteine in female ovariectomized rats compared with normal rats. The mechanism by which hyperhomocysteinaemia is involved in the pathogenesis of vascular disease has not been established with certainty, although experimental evidence has suggested several possibilities including endothelial cell toxicity (Stamler et al 1993), proliferation of smooth muscle cells and thrombus formation (Durand et al 1997). Recently, it has been suggested that the metabolic conversion of homocysteine to thiolactone and protein homocysteinylation is implicated in the protein damage and its toxicity specifically to the endothelium (Jakubowski 2000).

Genest et al (1992) showed that homocysteine potentiated the risk associated with hypertension, hypercholesterolaemia and smoking. A recent report suggested that homocysteine promoted binding of lipoprotein (a) to plasmin-modified fibrin (Foody et al 2000). This effect would potentially lead to enhanced atherogenesis and atherothrombosis. Thiols such as homocysteine are known to dissociate apolipoprotein (a) from the lipoprotein (a) binding site. This additional lysine binding site may increase the affinity of apolipoprotein (a) for plasmin-modified fibrin, thus impeding fibrinolysis (Harpel et al 1995).

There is increasing evidence that total homocysteine has induced vascular dysfunction through an oxidative mechanism (Loscalzo 1996). Autooxidation of total homocysteine generates several potent reactive oxygen species that may impair endothelial function (Tawakol et al 1997). Such mechanism is in good accordance with our finding in which hyperhomocysteinaemia is established in ovariectomized rats and is associated with an increased susceptibility of their lipoprotein content to oxidation and decreased levels of NO and estradiol. Hence, postmenopausal women are particularly at risk from the loss of the protective effects of endogenous estrogen on the cardiovascular system. The goal of this study was to investigate possible strategies for lowering homocysteine concentration either through folic acid or HRT, or their combined administration, in ovariectomized rats.

Our results have shown that estradiol replacement therapy has resulted in a decreased level of total homocysteine, atherogenic indexes, susceptibility of non HDLC to oxidation, and increased NO production. Therefore, the benefit of this regimen appears to be multifactorial within a particular improvement of the lipid profile.

Emerging evidence has suggested that estrogen may act as an antioxidant (Clemente et al 1999). The phenolic structure of estrogen has protected LDL from both cellular and copper mediated oxidation in vitro (Maziere et al 1991). In addition, HRT can preserve the α-tocopherol and β-carotene contents of LDL (Clemente et al 1996). Other possibilities include preservation of endothelial function by estrogen, in part, through limiting oxidized LDL and its deleterious effect on agonist-mediated NO release and NO degradation (Keaney et al 1994).

There is also accumulating evidence to support the suggestion that elevated total homocysteine levels can be determined genetically. However, these levels may be affected by non-genetic factors such as nutritional status, impaired kidney function and reproductive hormones (Finkelstein 1972). Our data have shown there to be decreased levels of total homocysteine in estradiol-treated rats. This effect may be attributed to an increased kidney methionine synthase activity, or alternatively, may be associated with transamination of methionine (Blom et al 1988).

Our findings demonstrate that supplementary daily doses of folic acid 90 μg induced a significant reduction of total homocysteine levels and an elevation of folate concentration in ovariectomized rats. Thus, they confirm previous observations made in chronic renal insufficiency (Thambyrajah et al 2000). Moreover, there is an inverse correlation between serum total homocysteine and folate concentration. The most probable mechanism by which folic acid therapy could reduce total homocysteine is through remethylation of homocysteine to methionine (Tucker et al 1996). Folic-acid-induced reduction in serum homocysteine to near normal levels could decrease the cardiovascular risk and underscore the beneficial effect of folic acid supplementation in cardiovascular prevention strategies.

Recent reports have documented that folic acid may completely prevent the increase in oxygen radical stress through its effect on redox state and endothelial function (Verhaar et al 1999). This study shows that folic acid co-treatment with estradiol has a more prominent and significant effect on improving endothelial dysfunction than does estradiol treatment alone. This effect can be explained in several ways: firstly, folic acid has been suggested to increase endogenous regeneration of tetrahydrobiopterin, an essential cofactor for NO synthase, with subsequent decrease in NO-synthase-dependent O_2 formation as well as increased NO production (Verhaar et al 1998). Alternatively, as folate is reduced by the gastrointestinal tract into 5-methyl tetrahydrofolate, endothelial uptake of the methylated folate can reduce

the oxidative defence mechanism into an active oxidant state leading to an improvement of the endothelial redox state (Wilmink et al 2000). In general, folic acid possess a direct scavenging effect in-vitro (Stroes et al 2000). Finally, the well-known homocysteine-lowering effect of folic acid could contribute to the improvement of endothelial dysfunction (Stroes et al 2000).

In this study, the relation between hyperhomocysteinaemia and increased cardiovascular risk is confirmed by direct correlation between total homocysteine levels and lipoprotein susceptibility to oxidation in ovariectomized rats. HRT and folic acid may be vasculoprotective, as shown from the inverse correlation between total homocysteine and estradiol or folate. In conclusion, our finding warrants further exploration of the potency of oral folic acid therapy as a safe and inexpensive tool that can enhance the therapeutic efficiency of estradiol.

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