The Effect of Hormone Replacement Therapy with or without Folic Acid on Homocysteine Level as Risk Factor for Cardiovascular Disease in Postmenopausal Female Rats

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ABSTRACT
This study was conducted to investigate the effect of menopause induced by ovariectomy of rats on serum level of some cardiovascular risk factors such as lipid profile and homocysteine (Hcy) as new risk factor for cardiovascular disease specially the coronary artery disease. Moreover, to determine whether supplementation of estrogen hormone as a replacement therapy or folic acid could be modulate the effects of ovariectomy on the previous parameters or not? Fifty adult female albino rats were divided into five equal groups: group one served as control, the rats in the second group were subjected to ovariectomy, the third group were exposed to ovariectomy and treated with estrogen, the fourth group were ovariectomized and supplemented with folic acid and the fifth group were consist of ovariectomized (ovx) rats treated with estrogen and supplemented with folic acid. Ovariectomized rats showed a significant increase in serum homocysteine, total cholesterol, LDL-c and triglycerides, with significant decrease in HDL-c levels than normal control, oral estrogen administration to ovariectomized rats, induced significant reduction of Hcy, total cholesterol, LDL-c levels than ovariectomized control group. Folic acid supplementation to ovariectomized rats revealed, significant decrease in serum Hcy, LDL-c with insignificant increase in HDL-c, while combined treatment of estrogen and folic acid resulted in significant decrease in Hcy and significant increase in triglycerides levels versus ovariectomized rats. We can conclude that supplementation of food with folic acid succeeded in alleviating the disturbances in homocysteine levels as well as lipid profile metabolism observed in ovariectomized rats.

Key words: Menopause, ovariectomy, cardiovascular risk factor, lipid profile, coronary artery disease

INTRODUCTION
Menopause is considered to be a natural change of life, which may be accompanied by various health problems which vary according to different groups (Taechakraichana et al., 2002). Ovarian weigh declines as menopause progress and plasma gonadotropin levels increase. As the negative feed back effect of estrogens and progesterone is reduced (Guyton and Hall, 2006).

Hormonal changes during the menopausal transition, resulting in estrogen deficiency plays a role in development of chronic disease such as coronary artery disease and cardiovascular disease which can contribute to impaired quality of life in postmenopausal women (Moorthy et al., 2004; Sormova and Donat, 2004).

Menopause occurs earlier in African women than European or American women. Also, symptoms of menopause may influence by social, economic and nutritional factors (Sidibe, 2005;
Nemati and Naghizadeh Baghi, 2008). The incidence of some associated diseases was reduced among HRT users compared to nonusers as cardiovascular problem (Naddaf and Semreen, 2005).

Coronary Heart Disease (CHD) occurs when menopause is produced by bilateral ovariectomy (Barnabei et al., 2002; Magyur and Fel, 2006). Also, estrogen deficiency in naturally or surgically postmenopausal women alters lipid metabolism (Berg et al., 2004).

A significant decline in the levels of total plasma cholesterol as well as low density lipoprotein cholesterol (LDL-c) following estrogen replacement therapy were observed (Battezzati et al., 2007).

In addition, the increase in plasma homocysteine (Hey) levels with natural menopause suggests a close relationship between Hcy metabolism and estrogen status and proposes one of the mechanisms through which menopause affects cardiovascular risk in women (Motivala et al., 2008). The level of total homocysteine seems to be secondary to the altered hormonal status (Kaleli et al., 2003).

With oophorectomy, the physiologic changes associated with menopause occur quickly with significantly reduced levels of estrogen and androgen (Bachmann, 2001). Also, hyperhomocysteinemia takes place after two weeks of ovariectomy (Kapral et al., 2002).

Hyperhomocysteinemia (HHcy) is multifactorial (Righetti, 2009); it may be due to congenital or acquired defects of metabolic pathways, vitamin deficiency such as folic acid, vitamin B6. Treatment of hyperhomocysteinemia is usually correctable with vitamin supplements containing folic acid. It appears to regulate the blood level of Hey (Lacey et al., 2002). Also, folic acid supplementation reducing an elevated Hey levels in a dose of more than 400-500 mg day^{-1} (Anderson et al., 2004; Shirodaria et al., 2007). So, folic acid may help to reverse the problems associated with hyperhomocysteinemia in postmenopausal women and may be useful in primary cardiovascular prevention in this high risk group (Sultan et al., 2007).

Postmenopausal hormone therapy is the treatment of first choice to alleviate symptoms of estrogen deficiency and plasma concentration of Hey in postmenopausal taking HRT are lower than they are in those who do not take estrogen supplements (Gambacciani and Mannella, 2007). Estrogen replacement therapy is claimed to reduce CVD mortality by about 50% in postmenopausal women through improved in lipid and lipoprotein metabolism (Bachmann, 2001; Vrablik et al., 2009), also it can protect against some chronic diseases, such as CVD and colorectal cancer. From other side, hormone replacement therapy (HRT) have a risk of developing breast cancer (Kapral et al., 2002); increased risk of stroke (Anderson et al., 2004) and increased thrombotic risk (Duschek et al., 2004), but it still the best therapy.

Therefore, this study tries to shed light on the efficiency of estrogen as a replacement therapy and folic acid supplementation on serum level of homocysteine, lipid profile and coronary intima media thickness in ovariectomized rats.

MATERIALS AND METHODS

The experiment was carried out through Jul. 2009-Jul. 2010.

In this study 50 mature female albino rats weighting 200±50 g were used. The rats were housed under the prevailing atmospheric conditions allower the experimental period in the laboratory of physiology of the College of Science in AL-Qassim University.

Experimental procedure: Ovariectomy of rats as described by Oh et al. (2007). Two weeks after ovariectomy, rats were divided into five equal groups as follow:

Group I: Intact rats (without ovariectomy) served as control (I)
Group II: Ovariectomized rats served as control (II)

Group III: Ovariectomized rats received daily 2 mg of conjugated estrogen orally (Cagnacci et al., 2006)

Group IV: Ovariectomized rats received normal diet fortified with folic acid powder in a dose 7.5 mg day⁻¹ (Villa et al., 2005)

Group V: Ovariectomized rats received daily 2 mg of conjugated estrogen orally and maintained on the diet fortified with folic acid powder

Blood samples will be obtained from the orbital sinus all over night fasted rats under light ether anesthesia with capillary tubes.

Blood immediately centrifuged and serum will be collected and stored at -20°C until assayed for estimation of the following:

- Homocysteine level was performed using enzyme immunoassay kits (Candito et al., 1997)
- Total cholesterol level was performed according to the method of Allain et al. (1974)
- Low density lipoprotein cholesterol (LDL-c) was performed using the method of Friedwald et al. (1972)
- High density lipoprotein cholesterol (HDL-c) by using the method of Sawle et al. (2002)
- Triglyceride level according to the method of McGowan et al. (1983)

Also, histopathological examination of coronary arteries, were be done.

Statistical analysis: The data were analyzed using SPSS version 16. The analysis of covariance (one way ANOVA) was used to detect the differences in the mean between the control and different treated groups.

RESULTS

The results of the present study revealed a highly significant increase in serum homocysteine in ovariectomized rats.

In this study, oral estrogen administration to ovariectomized rats fed on normal diet showed a significant reduction in serum homocysteine level, while supplementation of folic acid to ovariectomized rats induced better effect on Hey level than did estrogen alone. Combined administration of estrogen and folic acid alleviating the increased level of Hey when compared with normal and ovariectomized groups (Table 1).

<table>
<thead>
<tr>
<th>Table 1: Effect of folic acid administration on serum homocysteine levels (μmol L⁻¹) in normal rats, ovariectomized and ovariectomized treated with estrogen</th>
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<tbody>
<tr>
<td>Groups</td>
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<tr>
<td></td>
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<tr>
<td>Control</td>
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<tr>
<td>Ovariectomized</td>
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<td>Ovariectomized treated with estrogen</td>
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<td>Ovariectomized treated with folic acid</td>
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<td>Ovariectomized treated with estrogen and folic acid</td>
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The mean difference is significant at the 0.05 level (*). (Ta): Significant as compared with normal control group. (Tb): Significant as compared with ovariectomized group.
Table 2: Effect of folic acid administration on serum lipid profile levels (mmol mL⁻¹) in normal rats, ovariectomized and ovariectomized treated with estrogen

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total cholesterol</th>
<th></th>
<th>LDLc</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>(Ta) Mean±SE</td>
<td>(Tb) significant test</td>
<td>(Ta) Mean±SE</td>
<td>(Tb) significant test</td>
</tr>
<tr>
<td>Control</td>
<td>1.41±0.099</td>
<td>-</td>
<td>0.008*</td>
<td>0.07±0.020</td>
</tr>
<tr>
<td>Ovariectomized</td>
<td>1.70±0.0844</td>
<td>0.008*</td>
<td>-</td>
<td>0.29±0.143</td>
</tr>
<tr>
<td>Ovariectomized treated with estrogen</td>
<td>1.30±0.058</td>
<td>0.375</td>
<td>0.001*</td>
<td>0.06±0.016</td>
</tr>
<tr>
<td>Ovariectomized treated with folic acid</td>
<td>1.58±0.085</td>
<td>0.169</td>
<td>0.151</td>
<td>0.12±0.031</td>
</tr>
<tr>
<td>Ovariectomized treated with estrogen and folic acid</td>
<td>1.56±0.115</td>
<td>0.258</td>
<td>0.137</td>
<td>0.14±0.034</td>
</tr>
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<table>
<thead>
<tr>
<th>HDLc</th>
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</thead>
<tbody>
<tr>
<td>(Ta) Mean±SE</td>
<td>(Tb) significant test</td>
<td>(Ta) Mean±SE</td>
</tr>
<tr>
<td>Control</td>
<td>1.32±0.061</td>
<td>-</td>
</tr>
<tr>
<td>Ovariectomized</td>
<td>1.03±0.068</td>
<td>0.010*</td>
</tr>
<tr>
<td>Ovariectomized treated with estrogen</td>
<td>0.90±0.087</td>
<td>0.000*</td>
</tr>
<tr>
<td>Ovariectomized treated with folic acid</td>
<td>1.18±0.066</td>
<td>0.186</td>
</tr>
<tr>
<td>Ovariectomized treated with estrogen and folic acid</td>
<td>1.04±0.106</td>
<td>0.019*</td>
</tr>
</tbody>
</table>

The mean difference is significant at the 0.05 level (*); (Ta): Significant as compared with normal control group. (Tb): Significant as compared with Ovariectomized group.

Ovariectomized rats fed on normal diet showed a significant increase in the serum levels of cholesterol, LDL-c and triglycerides, with significant decrease in HDLc level in comparison to normal control group (Table 2).

Oral estrogen treatment of ovariectomized rats resulted in significant reduction of total cholesterol, LDL-c and insignificant decrease in triglyceride with insignificant elevation of HDL-c.

Folic acid supplementation to ovariectomized rats revealed insignificant decrease in total cholesterol, triglyceride with significant decrease in LDL-c and insignificant increase in HDL-c, while estrogen treatment with folic acid supplementation induced insignificant changes in serum cholesterol, LDL-c and HDL-c and significant increase triglyceride level versus ovariectomized group (Table 2).

**Histological results:** In normal control group, histological examination of the coronary artery revealed normal architecture the arterial wall (Fig. 1a).

On the other hand, ovariectomized rats showed irregular and interrupted tunica intimae with areas of fibrosis in the tunica media. The tunica adventitia becomes thick, irregular with deposition of fat and cellular infiltration with congested wall of the artery (Fig. 1b).

Estrogen treatment of ovariectomized rats revealed no difference versus ovariectomized group (Fig. 1c).

Folic acid supplementation alone or combined with estrogen produced less degenerative changes and less infiltration but not reach to the normal morphology (Fig. 1d-ε).
Fig. 1: (a) (H. and E.x.250): A photomicrograph of a transverse section of the coronary artery in control rats showing normal architecture arterial wall. (b) (H. and E.x.250): A photomicrograph of a transverse section of the coronary artery in ovariectomized female rats showing irregular and interrupted tunica intima. (Notice that areas of fibrosis in the tunica media). The tunica adventitia is irregular in its thickness with deposition of fat and cellular infiltration. There are haemorrhage and congestion in the wall of artery. (c) (H. and E.x.400): A photomicrograph of a transverse section of the coronary artery in ovariectomized rats treated with conjugated estrogen showing marked thickness of the wall of the artery. Notice the atrophy of the endothelium of squamous cells followed by irregular wavy lamina of elastic connective tissue. Both tunica media and the tunica adventitia are showed marked fibrosis, fat deposition and cellular infiltration. (d) (H. and E.x.400): A photomicrograph of a transverse section of the coronary artery in ovariectomized rats supplemented with folic acid showing dilatation of the artery with marked congestion and fat deposition in its lumen. There is marked fibrosis in both tunica media and the tunica adventitia and (e) (H. and E.x.400): A photomicrograph of a transverse section of the coronary artery in ovariectomized rats treated with conjugated estrogen and supplemented with folic acid showing an improvement in the arterial wall with less congestion.
DISCUSSION

In this study we induced hyperhomocysteinemia in female rats by bilateral ovariectomy, to stimulate hyperhomocysteinemia induced by menopause.

The results of the present study revealed a highly significant increase in serum homocysteine in ovariectomized rats are in agreement with Castelao and Gago-Dominguez (2008), Camafferi and Manni (2007) whom reported a higher plasma homocysteine concentration in postmenopausal women rather than premenopausal women. Also, Kaleli et al. (2003) contributed the higher level of total homocysteine in ovariectomized rats to the alteration of the hormonal status due to estrogen hormone deficiency.

The mechanism by which hyperhomocysteinemia is in valued in the pathogenesis of vascular disease may be due to endothelial cell toxicity, proliferation of smooth muscle cells and thrombus formation (Bednarek-Tupikowska and Tupikowski, 2004). A recent suggestion by Perlak-Kajan et al. (2007) that the metabolic conversion of homocysteine to thiolactone and protein homocysteinylations is implicated in the protein damage and its toxicity especially to the endothelium and endoplasmic reticulum stress. The cytotoxic effect of homocysteine to the vascular endothelium, the enhancement of platelet activity, promotion of platelet aggregation increase the genesis of fibrin and proliferation of smooth muscle cells, in addition to the inhibition of antioxidative processes (Rossi et al., 2006; Almeida et al., 2007). Also, reported that homocysteine promoted binding of lipoprotein to plasmin modified fibrin and this effect would potentially lead to enhanced atherogenesis and atherothrombosis (Perlak-Kajan et al., 2007).

In agreement with our results, evidence of Papatheodorou and Weiss (2007) that HHcy is associated with alterations in vascular morphology, loss of endothelial function, with induction of a procoagulant environment, most known forms of damage are due to Hcy mediated oxidative stresses and inherent cytotoxicity (Glushchenko and Jacobsen, 2007; Hachul de Campos et al., 2006).

The effect of gonadal steroids on homocysteine is not yet full understood. Most studies reported on the effect of combined estrogen progesterone replacement therapy leaving the question of wither the estrogen or progestogen component of this therapy was primarily responsible for the observed reduction of homocysteine (Ventura et al., 2001).

In the present thesis administration of oral conjugated estrogen to ovariectomized rats resulting in a significant decrease in serum homocysteine level when compared to ovariectomized rats. This reduction is supported by the findings of Duschek et al. (2004) and Hsu et al. (2005) who found that oral estrogen treatment as a replacement therapy in postmenopausal women reduced their high levels of plasma homocysteine which may contribute to the beneficial effect of estrogen replacement therapy on cardiovascular risk (Gol et al., 2006). In contrast with Marchesoni et al. (2003) who mentioned that in surgical hysterectomy and bilateral oophorectomy postmenopausal women received transdermal estrogen treatment does not modify homocysteine levels.

The mechanism underlying the observed HRT induced decrease in homocysteine may be related to an increased kidney methionine synthetase, the regulating enzyme responsible for the remethylation of homocysteine to methionine (Bonassi Machado et al., 2007). Also, hormone induced change in transamination of methionine could be a potential mechanism by which HRT can lower homocysteine concentration (Farag et al., 2003).

Supplementation of folic acid (powder) on diet of ovariectomized rats, in the present study resulting in a highly significant reduction in serum total homocysteine levels.
This is suggested also by Villa et al. (2005) and Ziaikka et al. (2001) who mentioned that folic acid is required for remethylation of homocysteine to methionine which account for significant intracellular homocysteine consumption.

Chen et al. (2005) had documented that folic acid may prevent the increase in oxygen radical stress through its effect on redox state and endothelial function.

Folic acid supplementation may determine a greater availability of methyl sulphate groups as cofactor, substrates leading to a new synthesis of methionine and also activate other enzymatic pathways in the liver and kidneys (Wolters et al., 2004). Also, homocysteine and cysteine-Glycine in plasma inter activate redox and disulfide reactions becoming part of dynamic system referred to as redox thiol status, which is linked to the antioxidants defense system (Petrama et al., 2009).

Oral estrogen administration to ovariectomized rats supplemented with folate powder resulted in a highly significant reduction in total homocysteine level.

These results are in accordance with Kalali et al. (2003), Toprak et al. (2005) who evaluate the effect of postmenopausal oral HRT with or without folic acid supplementation on plasma homocysteine levels, they found that folic acid and estradiol has a more potent lowering effect on plasma homocysteine level. Also, they suggested that co-treatment has a more prominent and significant effect on improving endothelial dysfunction than did estradiol treatment.

This effect was explained by Wilmink et al. (2000) that folic acid has been suggested to increase endogenous regeneration of tetrahydrobiopterin, an essential cofactor for nitric oxide synthase, with decrease in nitric oxide-synthase-dependent $O_3$ formation as well as increase nitric oxide production.

From other side, Ventura et al. (2001) reported that Hey levels significantly decline in postmenopausal women treated with HRT and this effect due to an improvement in the transulfuration pathway of Hey metabolism. They also mentioned that this effect is due to the HRT and was not dependent on vitamin status.

In this study, ovariectomized rats fed on normal diet showed significant increase in fasting level of serum cholesterol, LDL-c and serum triglyceride level. On the other hand, the level of HDL-c showed a significant decrease.

These results are in agreement with Ziaikka et al. (2001) whom mentioned that the increased levels of LDL-c are due to an initial increased its production, followed by an enhanced catabolism of LDL-c establish a steady state at higher concentration. Also, the increased level of total cholesterol and triglyceride are in agreement with Picard et al. (2000) who attributed the increase in triglyceride level to the increase in endogenous production because they found that both plasma and liver TG levels are increased.

Ovariectomy induced reduction in HDL-c and this may be due to the increase of lipoprotein lipase activity (Suda et al., 1998). Also, Bednarek-Tupikowska et al. (2005) and Buckler (2003) mentioned that estrogen deficiency induces changes in lipid profile.

Further findings reported that ovariectomy may worsen arterial pathophysiology by disrupting the arterial endothelial layer with increase permeability and increase atheroma formation (Lucas et al., 2006).

Findings of the results revealed that, oral estrogen treatment of ovariectomized rats resulted in a significant reduction of total cholesterol, LDL-c and insignificant reduction in serum triglycerides level and HDL-c are in agreement with Ozer and Bayram (2006). The significant reduction of lipid profile may attributed to the estrogen effect on the up regulation of LDL apolipoprotein B100 receptors in the liver and other sites in addition to the partial depletion of LDL
particles from their cholesterol content. So estrogen prevented ovariectomy induced hypercholesterolemia (Man et al., 2001). Also, LDL-c reduction induced by estrogen may be due to its effect on the synthesis and clearance of LDL precursors such as VLDL (Greaves et al., 2000). As regard to insignificant reduction in TG may be due to the route of administration of estrogen that affecting the TG levels (Godsland, 2001), since the transdermal estrogen therapy has a lowering effect on TG and is the route of choice in women with high plasma TG levels (Nanda et al., 2003).

The insignificant change of HDL-c after estrogen treatment is consistent with Castelae and Gago-Dominguez (2008) who reported a significant decrease in HDL-c in ovariectomized rats after oral estrogen treatment, however in contrast to this result Murano et al. (2003) and Milewicz et al. (2007) recorded a significant increase in HDL-c following oral estrogen therapy in oophorectomized women.

The data of the present study showed insignificant decrease in total cholesterol, triglyceride and significant decrease in LDL-c with insignificant increase in HDL-c after folic acid supplementation.

The results are in agreement with Ziakka et al. (2001) and Semmler et al. (2010) who found no significant change in total cholesterol in postmenopausal women after folic acid treatment, while Choy et al. (2004) reported that folic acid has a cholesterol lowering effect by reducing the plasma Hcy level. However, they disagree the insignificant decreases in HDL-c in the present results and reported a 6% increase in HDL-c concentration after one month supplementation with folic acid and contribute the increased HDL-c level to the lowering effect of folic acid on hyperhomocysteinemia and improvement of hepatic metabolism. In agreement with the insignificant decrease in TG (Semmler et al., 2010; Olthof et al., 2005) reported that folic acid supplementation did not seem to effect blood lipids since folic acid reducing the hyperhomocysteine induced generation of O$_2$ radicals, has been to decrease the extent of LDL-c oxidation and to increase HDL-c in subjects at high risk for CVD (Toprak et al., 2005).

The significant increase in triglyceride after co-treatment with estrogen is in agreement with Toprak et al. (2005) whom reported the same result in postmenopausal women. The increased level of TG is due to the effect of estrogen therapy (Floter et al., 2004), which may abolish the decline of TG achieved with folic acid supplementation.

The insignificant increase in HDL-c with combined estrogen treatment and folic acid are in agreement with Milewicz et al. (2007) and may be due to the synergistic effect of both estrogen and folate.

The present study found that supplementation of food containing folic acid powder produced the same effect of estrogen succeeded in alleviating the disturbances in lipid and lipoprotein metabolism as well as homocysteine levels as observed in ovariectomized rats. The histopathological examination supports this effect by reduced destruction, congestion and infiltration of the coronary artery.

So, the study recommend a diet rich in folic acid to premenopausal and postmenopausal women as a primary protective measure against CVD and avoid the risks of estrogen treatment during these period.

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REFERENCES


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