PROTECTIVE EFFECT OF ANTIOXIDANT ADJUVANT TREATMENT WITH HORMONE REPLACEMENT THERAPY AGAINST CARDIOVASCULAR DISEASES IN OVARIECTOMIZED RATS

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Objective. Since it is well known that estrogen deficiency or ovariectomy (OVX) results in a reduction of sexual steroids and increased prevalence of cardiovascular diseases (CVD), it was aimed to assess the benefits of hormone replacement therapy (HRT) alone or together with antioxidant vitamins (E and C) for the protection against CVD and oxidative stress.

Methods. The effect of ovariectomy and HRT alone or combined with antioxidants (Antiox) on lipid metabolism, insulin sensitivity, oxidative stress, antioxidant and markers of CVD was examined in four groups of female Wistar rats of 10 animals each: Group 1 (sham operated); Group 2 (OVX); Group 3 (OVX + HRT); Group 4 (OVX + HRT + Antiox). After four weeks of treatment total cholesterol, HDL-cholesterol (HDL-C), LDL-cholesterol (LDL-C), triglycerides (TG), Apo B lipoprotein, glucose and insulin as well as malondialdehyde (MDA; as index of lipid peroxidation), oxidized LDL (ox-LDL), homocysteine level, oxidized and reduced glutathione were determined in plasma.

Results. The lipid pattern of OVX rats showed a deviation from sham group in all lipid fractions and atherogenic indexes; LDL/HDL and TC/HDL. HRT group showed partial correction of these abnormalities and the antioxidant adjunct treatment significantly improved the lipid profile. OVX rats had significantly higher insulin and glucose levels compared to the sham group which was abolished by HRT and completely normalized by adjunct antioxidant treatment. HOMA was significantly decreased by HRT and showed normal value with adjunct antioxidant treatment. The oxidative stress parameters; MDA, ox-LDL and GSSG levels were increased, also homocysteine was greatly elevated in OVX rats. The HRT significantly corrected the levels of MDA, ox-LDL and GSSG while it had no effect on homocysteine and GSH. The adjunct antioxidant treatment potentiated the HRT and showed a significant correction of homocysteine and GSH levels.

Conclusion. Our data suggested that adjunct antioxidant treatment together with HRT may ameliorate the cardiovascular protection and improve metabolic syndrome as well as insulin resistance in OVX rats. Further studies are warranted to elucidate the beneficial role of antioxidant treatment on cardiovascular protection of menopause women.

Key words: Rats – Ovariectomy – HRT – Antioxidants – Cardiovascular diseases – Lipids – Insulin – Glucose

Ovariectomy in the animal model or lack of ovarian function in human menopause result in a reduction of sexual steroids (mainly estrogen) and in the occurrence of uncomfortable symptoms and serious diseases such as cardiovascular diseases (CVD), osteoporosis etc. (WILLIAMS 1997, 2004). The mechanism by which es-

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trogen deficiency (natural or postsurgical) could cause or accelerate the progression of CVD still remains to be clarified. It is known, however, that ovarian hormones deficiency increases the generation of reactive oxygen species (ROS) which induces oxidative stress and results in cells damage or death (Ha et al. 2006).

Many cardiovascular risk markers have been investigated in ovariectomized (OVX) rats and point to a significant changes in lipid parameters such as total cholesterol, low-density lipoprotein (LDL), high density lipoprotein (HDL) and atherogenic indexes (total cholesterol/HDL, LDL/HDL) (El-Swefy et al. 2002). The oxidative damage to LDL significantly increased LDL atherogenecity. Oxidized LDL (ox-LDL) may directly alter both the structure and function of endothelial cells and may induce the formation of foam cells of an atheromatous plaque (Jaarin et al. 2006).

Hyperhomocysteinemia has been identified as one of the risk factors for CVD. Hyperhomocysteine can damage blood vessels, cell structures, blood lipids and eventually lead to the development of atherosclerosis and other forms of heart diseases (Particia et al. 1997; Guo et al. 2003).

Many studies have suggested that ovarian hormones are able to modulate insulin sensitivity, but their exact role remain unclear. Thus the low level of ovarian hormones associated with menopause or ovariectomy is related to a decrease in insulin-mediated glucose uptake (Gonzalez et al. 2003). Insulin resistance is a common etiological factor for metabolic syndrome, comprising hypertension, dyslipidemia with or without hyperglycemia and abnormalities in homeostatic system. All of these increase the risk of CVD (Bonnefont-Rousselot 2002).

Several studies have been shown that estrogen replacement therapy (ERT) or hormone replacement therapy (HRT) could be able to modulate the progression of CVD by improving metabolic syndrome (Barbiker et al. 2002; Mendelsohn 2002). However, several lines of study indicated that ERT or HRT could not confer the cardiovascular protection (Gaspard et al. 2002; Gorodeski 2002).

To assess the benefits of HRT alone or together with antioxidant vitamins (E and C) for the protection against CVD and oxidative stress in ovariectomized rat, we systematically examined the effect of ovariectomy, HRT and antioxidants on lipid metabolism, insulin sensitivity, markers of oxidative stress, and antioxidant and markers of CVD in female Wistar rats.

Materials and Methods

The local Ethical Committee approved the animal experiment protocol. Forty female Wistar rats aged 4-6 months and weighing 200-220 g were used. They were fed a rat chow and water *ad libitum* and maintained on a constant 12 h light/dark cycle at 22 °C. The animals were randomly divided into four groups (10 rats each): Group 1 - (sham operated) underwent sham surgical procedure and was sc injected the vehicle (olive oil/ethanol 3:2 V/V); Group 2 – (ovariectomized - OVX), subjected to ovariectomy at the beginning of experiment through a medline incision under ether anesthesia and allowed to recover for 4 weeks, while being treated *sc* with the vehicle; Group 3 - (OVX + hormone replacement therapy - HRT); subjected to ovariectomy and daily receiving E_2 (30 μg/kg, sc) and progesterone (1 mg/kg, sc); Group 4 (OVX + HRT + antioxidant therapy - Antiox); subjected to ovariectomy and concurrently with HRT receiving daily ip injection of vitamin E (30 mg/kg) and C (20 mg/kg).

The four groups received the appropriate treatment at the same time every day for 4 weeks. The animals were killed by cervical dislocation on the next day following the last injection and 10 hours fasting blood samples were collected by cardiac puncture into heparinized tubes and the plasma was obtained by centrifugation at 1500 x g for 10 minutes.

The plasma was used for the determination of; total cholesterol (Katherman 1984), HDL-cholesterol (HDL-C) (Albers et al. 1978), LDL- cholesterol (LDL-C) (Friedwald et al. 1972), triglycerides (TG) (Buccolo and David 1973), Apo B lipoprotein (Steinmetz et al. 1995), glucose (Trinder 1969) and insulin by EIA kit (Mercodia Sweden). Also the level of malondialdehyde (MDA) as index of lipid peroxidation, was determined by thiobarbituric acid reaction (Draper and Hadley 1984), the level of oxidized LDL (ox-LDL) was determined by ELISA kit (Mercodia Sweden) (Shoji et al. 2002), and homocysteine level was determined by EIA Kit (Diazyme) (Frantzen et al. 1998). The levels of oxidized and reduced glutathione were determined according to Griffith (1980).

The insulin resistance index (IRI) was derived using the homeostasis model assessment (HOMA) as follows: IRI = fasting insulin (μU/ml) x fasting glucose (mmol/l)/ 22.5. HOMA has been validated as useful method of assessing insulin resistance (Kanauchi et al. 2003).

Table 1
Effect of OVX, HRT and antioxidant treatment on lipid profile, apo B and atherogenic indexes

	Sham	OVX	OVX+HRT	OVX+HRT+ Antioxidants
TG (mmol/L)	1.17±0.07	0.92±0.29 [a]	1.17±0.08 [b]	1.09±0.09
T-C (mmol/L)	3.55±0.28	4.56±0.19 [a]	4.21±0.19 [a,b]	3.77±0.31 [b]
LDL-C (mmol/L)	2.06±0.12	2.64±0.11 [a]	2.55±0.07 [a]	2.16±0.06 [b, c]
HDL-C (mmol/L)	1.17±0.08	1.22±0.04	1.33±0.07 [a]	1.39±0.05 [b]
LDL/HDL	1.7±0.14	2.2±0.13 [a]	1.9±0.08 [b]	1.6±0.18 [b,c]
T-C/HDL	3.1±0.37	3.7±0.18 [a]	3.2±0.17 [b]	2.7±0.22 [b,c]
Apo B (g/L)	0.93±0.04	1.06±0.07 [a]	0.99±0.04 [a,b]	0.95±0.02 [b]

Data presented as the mean ± SD
[a] Significantly different from sham group ($p<0.05$)
[b] Significantly different from OVX group ($p<0.05$)
[c] Significantly different from OVX+HRT group ($p<0.05$)

Statistical evaluation: All data are presented as mean ± SD. One-way analysis of variance (ANOVA) followed by Bonferroni test was employed to compare the mean values of the groups. Differences were considered significant at a level of probability <0.05. All statistical analyses were performed using SPSS statistical software version 13.

Results

Data on the plasma lipid profile of sham and ovariectomized rat were shown in Table 1. The lipid pattern of OVX rats showed deviation from sham group in all lipid fractions assessed. The TG showed definite decrease by about 21 %**,** while TC, LDL-C and Apo B level were significantly increased by about 28.5, 28.2 and 14 % respectively. The level of HDL-C showed no significant change. The atherogenic indexes; LDL/HDL and TC/HDL in OVX group showed a significant increase by about 22.2 and 16.2 % compared to sham group (Table 1).

HRT group showed a partial correction of these abnormalities of lipid pattern as TG was normalized and TC, LDL-C and Apo-B were significantly decreased compared to OVX group. Also the atherogenic indexes were normalized (Table 1). The antioxidant adjunct treatment significantly improved the lipid profile as TC, LDL-C and Apo B were significantly decreased compared to HRT group. (Table 1).

The results of insulin, glucose and HOMA are summarized in Table 2. OVX rats had significantly higher insulin level compared to sham group which was abolished by HRT and completely normalized by adjunct antioxidant vitamins treatment (Table 2). Ovariectomy also affected fasting glucose level. Similarly the ovariectomy induced an increased level of glucose which was attenuated by HRT, also the antioxidant treatment produced further improvement. Also the insulin resistance index derived using HOMA showed a state of insulin resistance as HOMA in OVX group is 6.0 which is higher than sham group. HOMA was significantly

Table 2
Effect of OVX, HRT and antioxidant treatment on the level of fasting insulin and glucose and insulin resistance index

	Sham	OVX	OVX+HRT	OVX+HRT+ Antioxidants
Insulin (μU/ml)	17.6±1.65	25.7±1.89 [a]	20.2±1.93 [a,b]	17.8±2.70 [b,c]
Glucose (mmol/L)	4.9±0.13	5.3±0.16 [a]	4.6±0.21 [a,b]	4.5±0.17 [a,b]
IRI (HOMA)	3.8±0.40	6.0±0.43 [a]	4.1±0.27 [b]	3.6±0.58 [b,c]

Data presented as the mean ± SD
[a] Significantly different from sham group ($p<0.05$)
[b] Significantly different from OVX group ($p<0.05$)
[c] Significantly different from OVX+HRT group ($p<0.05$)

Table 3
Effect of OVX, HRT and antioxidant treatment on the level of oxidative stress parameters and homocysteine.

	Sham	OVX	OVX+HRT	OVX+HRT+ Antioxidants
MDA (μmol/L)	4.91±0.35	5.94±0.24 [a]	5.31±0.18 [a,b]	5.08±0.22 [b]
Ox-LDL (U/L)	41.0±1.43	56.3±1.53 [a]	52.1±1.37 [a,b]	43.7±0.64 [a,b,c]
Homocysteine (μM)	8.2±0.30	11.1±0.23 [a]	10.9±0.25 [a]	9.9±0.37 [a,b,c]
GSH (μmol/L)	2.71±0.11	1.82±0.09 [a]	1.84±0.08 [a]	2.29±0.11 [a,b,c]
GSSG (μmol/L)	0.12±0.01	0.18±0.02 [a]	0.15±0.01 [a,b]	0.14±0.09 [a,b]
GSH/GSSG	23.5±1.9	10.1±0.9 [a]	12.2±0.7 [a,b]	16.2±1.1 [a,b,c]

Data presented as the mean ± SD
[a] Significantly different from sham group ($p<0.05$)
[b] Significantly different from OVX group ($p<0.05$)
[c] Significantly different from OVX+HRT group ($p<0.05$)

decreased by HRT and showed normal value with adjunct antioxidant treatment (Table 2).

Table (3) shows the results of lipid peroxidation, ox-LDL homocysteine and glutathione. The oxidative stress parameters; MDA, ox-LDL and GSSG levels were increased by about 21.2, 37.3 and 50 % respectively, as a result of ovariectomy compared with sham rats. Also homocysteine was greatly elevated by about 35.4 % in OVX group. The ovariaectomy also results in a redox imbalance in the glutathione system as GSH decreased by about 32.8% and GSH become about 10 times GSSG concentration instead of 23.5 times in sham group (Table 3). The HRT significantly corrects the levels of MDA, ox-LDL and GSSG while it has no effect on homocysteine and GSH. The adjunct antioxidant treatment potentiates the HRT and show a significant correction of homocysteine and GSH levels (Table 3).

Discussion

This study demonstrates the protective effect of adjunct antioxidant treatment beside HRT against CVD and insulin resistance in ovariectomized rats.

Insulin resistance is a common etiological factor for the individual components of metabolic syndrome. The metabolic syndrome is a high risk factor for CVD and type 2 diabetes mellitus (Bertoni et al., 2007). Several clinical data and experimental studies suggest that insulin and sex hormones interact (Li et al., 2003; Os et al., 2005; Clegg et al., 2006), the low levels of female sex steroids associated with the menopause (natural or surgical) are related to a decrease in whole body insulin-mediated glucose uptake. The cellular mechanism behind this insulin resistance and the role of low female sex steroids are not fully understood.

Many studies have indicated that ERT or HRT ameliorates symptoms in postmenopausal women and OVX rats (Liu et al., 2004) as shown by preventing high plasma lipid and insulin resistance. Our study confirmed the previous observations that ovariectomy or estrogen deficiency significantly increased total and LDL-cholesterol, Apo B and insulin resistance. These effects were attenuated and reversed by HRT.

The ovariectomy was shown to result in the formation of a spectrum of apo B and apo E containing particles in plasma that are not normally present in female rats (Lenten et al., 1983). It was shown that the increase of apo B is due to the increase of total mass of LDL and also to increased LDL turnover rate in OVX rats which indicated that both LDL synthetic and catabolic rates were increased. So it may be postulated that the increased level of apo B fraction of LDL in OVX rats could be due to the enhanced synthesis. Also ovariectomy may stimulate the production of very low density lipoprotein (VLDL) destinated to become long lived LDL. Alternatively the increased LDL may be attributed either to a direct effect of low estrogen levels on the interconversion of VLDL to LDL or to a direct secretion of LDL by the liver (Lenten et al., 1983). The lowering effect of estrogen or LDL-C level is mediated by increasing the number of LDL-receptors on hepatic cells (Kishida and Kiyoshi, 2000).

Triglycerides level was significantly reduced in OVX rats which was in accordance with previous studies (Liu et al., 2004). The triglycerides level probably is not of clinical importance except in cases with basal hypertriglyceridemia.

It was demonstrated that progesterone did not have a beneficial effect when combined with estrogen on lipid metabolism. The opposing effects of estrogen and progestines are seen on their divergent effects on lipoprotein transport. Estrogen enhances the flow of cholesterol from the diet subsequently to cells and from cells via HDL to liver. On the other hand, progestines appear to slow the stimulatory effect of estrogen on lipoprotein transport (Mikkola and Clarkson, 2002). Estrogen affects the plasma lipid profile, lipoprotein, and total cholesterol and has anti-insulin resistance effect. Also additional mechanisms whereby estrogen exerts its cardiovascular action may exist. Since it is believed that estrogen can act as an antioxidant due to similarity in structure to vitamin E, a decrease in endogenous levels can increase free radical production, thereby potentially causing adverse effects in postmenopausal women and ovariectomized rat (Persky et al., 2000). In the present study ovariectomized rats show a significant increase of plasma levels of MDA (lipid peroxidation end product) and GSSG and low level of GSH which indicate a state of oxidative stress. Also the disturbed distribution of plasma glutathione results in a deviation in plasma redox state as indicated by the change in GSH to GSSG ratio to be 10 times in OVX rats instead of 23.5 times in sham rats. This indicated that plasma redox state was shifted toward an oxidizing environment which generate a state of oxidation all over the body with consequent deleterious effect on proteins, lipids and nucleic acids (Schafer et al., 2001). HRT shows a significant correction of the plasma lipid peroxidation but with no significant effect on GSH level.

Many of the risk factors and some of the consequences of atherosclerosis have been associated with evidence of oxidant stress *in vivo*, one of these being oxidized LDL formation (Jaarin et al., 2006). ox-LDL interaction with endothelium is the initial injury leading to the formation of fatty streaks and ultimately atherogenesis (Jaarin et al., 2006). The ovariectomy of rats results in a great increase in ox-LDL level with subsequent predisposition for atherosclerosis. HRT cause attenuation of this increase and significantly decreases ox-LDL level through the antioxidant properties of estrogen and its LDL lowering effect. But the level of ox-LDL was still higher than that in a sham group. Another important independent risk factor for CVD is the hyperhomocysteinemia observed in OVX rats which is not corrected by HRT. In accordance with our results Martins *et al*., 2005 showed no relationship between female sex steroid hormones and homocysteine level.

These results and several lines of study indicated that ERT or HRT could not confer the cardiovascular protection (Liu et al., 2004). In the present study we use two antioxidant vitamins (E and C) as adjuvant treatment with HRT in trail to improve the status of CVD and oxidative stress parameters of OVX rats. This antioxidant combination results in a great improvements in CVD risk factors, ox-LDL and homocysteine levels, also significantly correct GSH concentration and shift plasma redox toward more reducing environment than OVX rats and HRT rats as indicated by increased GSH/GSSG ratio. Unexpectedly the adjunct antioxidant treatment greatly improved the levels of LDL-cholesterol, insulin, glucose and insulin resistance significantly. These effects probably reflected a tendency toward better overall improvement in animal general health, better tissue metabolic status and alleviation of oxidative stress. Various studies have suggested that vitamin E may improve the metabolism of glucose by muscle cells and the circulation to ß-cells and other tissues (Paolisso et al., 1994). Also Lucas *et al.,* 2006 demonstrated that vitamin E supplementation moderately improves lipid parameters in ovarian hormone-deficient rats. In our study the use of two vitamins, one lipophilic (vitamin E) and the other hydrophilic (vitamin C), ensure complete protection against oxidative stress in lipids, lipoprotein, cytosols and plasma and also ensure the recycling of active vitamin E by vitamin C.

In conclusion, our data suggested that the adjunct antioxidant treatment together with HRT may ameliorate the cardiovascular protection and improve metabolic syndrome and insulin resistance in ovariectomized rats. Further studies are warranted to elucidate the beneficial role of antioxidant treatment on cardiovascular protection of menopause women.

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