Effects of melatonin and dexpanthenol on antioxidant parameters when combined with estrogen treatment in ovariectomized rats

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Abstract The purpose of the study was to assess whether it is possible to reduce the oxidative damage using antioxidant agents combined with hormone replacement therapy after menopause. In this prospective experimental study, 50 mature female Wistar albino rats weighing 270–310 g were used. Rats were divided into the following six groups: (1) *Ovx group* ($n=7$): the animals underwent bilateral ovariectomy. No drug was

administered following bilateral ovariectomy. (2) *Ovx+ E_2 group* ($n=7$): bilateral ovariectomy+17 β -estradiol (100 μ g/kg/day); (3) *Ovx+ E_2 +MT5 group* ($n=7$): bilateral ovariectomy+17 β -estradiol (100 μ g/kg/day)+melatonin (5 mg/kg/day); (4) *Ovx+ E_2 +MT20 group* ($n=7$): bilateral ovariectomy+17 β -estradiol (100 μ g/kg/day)+melatonin (20 mg/kg/day); (5) *Ovx+ E_2 +Dxp250 group* ($n=7$): bilateral ovariectomy+17 β -estradiol (100 μ g/kg/day)+dexpanthenol (250 mg/kg/day); (6) *Ovx+ E_2 +Dxp500 group* ($n=7$): bilateral ovariectomy+17 β -estradiol (100 μ g/kg/day)+dexpanthenol (500 mg/kg/day), and the activity of these antioxidative enzymes and oxidative stress products were measured. Enzymatic activity levels of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and glutathione reductase and levels of free radicals (malondialdehyde (MDA) and nitric oxide) were both analyzed. We observed an increase in the level of GSH activity, but no significant differences in levels of CAT, SOD, and GSH-Px enzymatic activity and in levels of free radical MDA following 17 β -estradiol or additional antioxidant treatment (melatonin or dexpanthenol). Despite the present study indicating that the addition of melatonin and dexpanthenol into the hormone replacement therapy regimen may contribute to the antioxidant effect of estrogen, the existence of limited data in this field indicates that further studies are warranted.

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Introduction

Estrogen loss following menopause is a contributing factor in cardiovascular disease (CVD) and is associated with an increased risk for health problems such as osteoporosis, hot flushes, depression, and neurodegenerative diseases. The most common agent in decreasing such problems is hormone replacement therapy (HRT) (Sowers 1998; Greendale et al. 1999). According to recent studies in the field, it is thought that decreased estrogen synthesis in postmenopausal women is responsible for an increase in oxidative stress (Bednarek-Tupikowska et al. 2004; Gura 1995; Trevisan et al. 2001; Ke et al. 2003). Here, we investigate the effect of melatonin and dexpanthenol, which are included into estrogen treatment in ovariectomized rats, on antioxidant parameters. Our results suggest that period-specific diseases such as CVD and osteoporosis can be avoided by minimizing the increase in oxidative stress which results as estrogen loss during menopause, with the use of antioxidants added to estrogen treatment.

Materials and methods

Materials

A Montage PCR centrifugal filter device (EMD Millipore, Bilerica, MA, USA) was used to obtain the double-distilled water used herein. Automatically adjustable pipettes of 5–50 μ l, 10–100 μ l, 20–200 μ l, and 200–1,000 μ l (Eppendorf, Enfield, CT, USA) were used in a Kotterman 3047 shaking water bath (Kotterman, Hanigsen, Germany) at 37 °C. Reduced glutathione (GSH) and total hemoglobin (Hb) concentrations were measured using a Shimadzu Model UV-160A UV–vis spectrophotometer (UV-160A, Shimadzu, Japan). For malondialdehyde (MDA) and nitric oxide (NO $\cdot$) analysis, a XL 800 microplate reader was used, and the results were automatically calculated with the device.

Experimental groups

Animals

The animal ethics and research committee of our center approved all protocols prior to the commencement of the study. The experiments were carried out on 50 mature female Wistar albino rats weighing 270–310 g

obtained from the experimental animals laboratory of this center. The animals were housed individually in cages in a light- and temperature-controlled (22 ± 2 °C) room on a 12:12-h light–dark cycle, where the relative humidity (65–70 %) was kept constant. The animals were fed a standard laboratory diet and water ad libitum for the duration of the study. The study was conducted at the Department of Pharmacology.

Surgery

During the preoperative period, all animals were anesthetized by intraperitoneal (i.p.) injection of a mixture of ketamine (50 mg/kg) and xylazine (5 mg/kg). After checking withdrawal and blinking reflexes, animals were aseptically prepared for abdominal surgery. A lower abdominal cavity exploration was performed by midline incision, followed by bilateral ovariectomy. After a waiting period of 3 weeks to allow for the development of menopause, the animals were randomly assigned into seven experimental groups: the control group ($n=7$) received no surgical treatment and no drug treatment; The remaining six groups ($n=7$ for each group) underwent ovariectomy and drug treatment for 14 days.

Drugs

Powdered 17 β -estradiol (E8515-5 G, Sigma-Aldrich, Saint Louis, MO, USA) was dissolved in sesame oil and applied subcutaneously at 100 μ g/kg/day. Melatonin (M5250-1 G, Sigma-Aldrich) was dissolved in 96 % ethanol, diluted in saline (9.0 g/L NaCl), and i.p. injected at 5 or 20 mg/kg/day. Dexpanthenol (Bepanthen® 500 mg, 5 mL ampul; Bayer AG, Frankfurt, Germany) was also i.p. injected at 250 or 500 mg/kg/day. Following bilateral ovariectomy, intracardiac blood samples were taken under anesthesia (ketamine 50 mg/kg and xylasin 5 mg/kg), and the animals were killed by exsanguination during the operation.

Groups

The groups were assigned as follows:

1. *Ovx group* ($n=7$): The animals underwent bilateral ovariectomy. No drug was administered following bilateral ovariectomy
2. *Ovx+E₂ group* ($n=7$): Bilateral ovariectomy+ 17 β -estradiol (100 μ g/kg/day)

3. *Ovx+E₂+MT5 group* (n=7): Bilateral ovariectomy+17 β -estradiol (100 μ g/kg/day)+melatonin (5 mg/kg/day)
4. *Ovx+E₂+MT20 group* (n=7): Bilateral ovariectomy+17 β -estradiol (100 μ g/kg/day)+melatonin (20 mg/kg/day)
5. *Ovx+E₂+Dxp250 group* (n=7): Bilateral ovariectomy+17 β -estradiol (100 μ g/kg/day)+dexpantenol (250 mg/kg/day)
6. *Ovx+E₂+Dxp500 group* (n=7): Bilateral ovariectomy+17 β -estradiol (100 μ g/kg/day)+dexpantenol (500 mg/kg/day)

Blood sampling, analysis, and storage

Blood samples were collected from animals into two tubes; one tube treated with the anticoagulant EDTA (0.47 mol/L K₃-EDTA), and one sample tube not treated with EDTA (referred to as simple blood sample). In the EDTA-treated blood samples, Hb and GSH levels were measured using a UV-160A spectrophotometer (Shimadzu) and calculated according to the method of Tietze (85). The blood samples were centrifuged at 4,000 rpm for 10 min to separate the plasma. Erythrocytes were washed three times in cold saline (9.0 g/L NaCl) and hemolysed by adding the same weight of ice-cold demineralized ultrapure water to yield a 50 % hemolysate. Hemolysate samples were prepared for analysis of antioxidative enzyme capacity of catalase (CAT), CuZn-superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and glutathione reductase (GR). The activity of these antioxidative enzymes was measured using the methodologies of Aebi (1974) for assay of CAT activity, Sun et al. (1988) for assay of CuZn-SOD activity, Kakkar et al. (1998) with minor modification for assay of GSH-Px activity, and Racker (1955) for assay of GR activity. Hb levels in these samples were determined, and the values were given accordingly. On the other hand, simple blood samples were centrifuged at 4,000 rpm for 10 min in order to separate the serum. The serum levels of free radicals MDA and NO were determined using the methods of Ohkawa et al. (1979) and Navarro-Gonzales et al. (1998), respectively.

Statistics

Analysis of the data was performed using the SPSS for windows 11.5 package software. For statistical evaluation of the results, the data were taken as average $\pm$

standard error values. ANOVA was used in order to determine the differences between groups. Student–Newman–Keuls or Dunn comparison tests were used as post hoc tests in order to determine the sources of differences. The results were accepted as statistically significant for $p < 0.05$.

Results

Intracardiac blood samples were taken from the animals following 3 weeks of waiting period after the bilateral ovariectomy. Enzymatic activity levels of CAT, SOD, GSH-Px, and GR and levels of GSH and free radicals MDA and NO were both analyzed. We observed an increase in the level of GSH, but no significant differences in levels of CAT, SOD, and GSH-Px enzymatic activity and in levels of free radical MDA following 17 β -estradiol or additional antioxidant treatment (melatonin or dexpantenol).

GSH and GSH-Px enzymatic activities

The level of GSH was higher in the groups which were treated with antioxidants melatonin and dexpantenol than in the nontreated group (Ovx). The level of GSH was also significantly higher in the 17 β -estradiol treated group (Ovx+E₂) than in the Oxv group. When the group treated with 17 β -estradiol (Ovx+E₂) and the groups that received additional treatment to 17 β -estradiol were compared, a slight, albeit not statistically significant, increase in GSH level was observed in the groups that received additional treatment. The present data showed no significant difference between levels of GSH-Px activity ($p > 0.05$) in treated versus nontreated groups. Despite these results, it is possible the antioxidant system may be positively affected as a result of treatment because an increase in GSH-Px activity level was observed in the groups that underwent 17 β -estradiol and antioxidant (melatonin or dexpantenol) treatment compared with the Oxv group (Table 1).

GR enzymatic activity

We found a significant decrease in the level of GR activity in treated groups compared with the Oxv group. When the Oxv group was compared with treated groups, we observed a significant decrease in levels

Table 1 a-b Levels and statistical evaluation of GSH in all groups (in milligrams per gram hemoglobin)

GSH	Control	Ovx	Ovx + E ₂	Ovx + E ₂ + MT 5	Ovx + E ₂ + MT 20	Ovx + E ₂ + Dxp 250	Ovx + E ₂ + Dxp 500
mean	2,379	2,097	2,569	2,943	2,644	2,600	2,595
median	2,444	2,104	2,641	2,745	2,576	2,681	2,535
SD	0,264	0,245	0,320	0,701	0,349	0,269	0,219
Min	2,014	1,617	2,143	2,274	2,196	2,165	2,319
Max	2,728	2,436	2,899	4,316	3,352	2,911	2,929
Groups				<i>p</i>		<i>p</i>	
Ovx–Ovx+E ₂ + MT 5				0.008		<i>p</i> <0.05	
Ovx–Ovx+E ₂ +MT 20				0.011		<i>p</i> <0.05	
Ovx–Ovx+E ₂ + Dxp 250				0.009		<i>p</i> <0.05	
Ovx–Ovx+E ₂ + Dxp 500				0.005		<i>p</i> <0.05	
Ovx–Ovx+E ₂				0.003		<i>p</i> <0.05	

of GR activity in the treated groups, except for the *Ovx+E₂* and the *Ovx+E₂+MT5* treated groups. When the treated groups were compared with each other, the level of GR activity was shown to be lower in the groups that underwent dexpanthenol treatment than in the group treated with melatonin 5 mg (*Ovx+E₂+MT5*). Because GR participates in reducing the levels of oxidized glutathione, it is possible that the low levels of enzymatic activity of GR that we observed herein were due to the high levels of GSH (Fig. 1).

Levels of free radical MDA

There were no significant differences in levels of free radical MDA following 17 β -estradiol or additional antioxidant treatment (melatonin or dexpanthenol)

apart from a slight decrease in levels of MDA (that had been raised after ovariectomy) following dexpanthenol treatment, suggesting that dexpanthenol treatment may have preventive effects after oxidative damage.

CAT and SOD enzymatic activities

Our results did not demonstrate any significant difference in CAT and SOD enzymatic activity levels following 17 β -estradiol or additional antioxidant treatment (*p*>0.05). However, an increase in levels of SOD activity following dexpanthenol treatment demonstrates that dexpanthenol administration may have a positive effect in preventing oxidative damage.

Fig. 1 Levels of GR in all groups

Discussion

Many studies show that estrogen loss following menopause is associated with an increased risk of CVD as well as other health deficits, such as osteoporosis, hot flashes, depression, and neurodegenerative diseases. The most common agent in reducing such problems is HRT, a system of medical treatment that includes administration of 17 β -estradiol among other estrogens (Sowers 1998; Greendale et al. 1999). According to current studies, it is believed that decreased estrogen synthesis in postmenopausal women is responsible for an increase in oxidative stress (Bednarek-Tupikowska et al. 2004; Gura 1995; Trevisan et al. 2001; Ke et al. 2003). Being exempt from the protective effect of estrogen due to the estrogen loss during the menopausal period leads to serious metabolic diseases including oxidative stress (Kankofer et al. 2007).

Oxidative stress plays an essential role in the pathophysiology of some diseases, such as carcinoma, atherosclerosis, osteoporosis, and aging. Lipid peroxidation is known as a free radical chain reaction mechanism whereby polyunsaturated fatty acids interact with reactive oxygen radicals. Furthermore, proteins and lipids, in particular low density lipoprotein (LDL), are also subject to oxidative stress. Importantly, LDL oxidation plays a key role in the development of atherosclerosis (Şendağ et al. 2005; Muthusami et al. 2005). The studies of Krstevska et al. (2001) confirmed that serum total antioxidant levels significantly decrease in menopausal women who have coronary heart disease; therefore, antioxidants may have a protective effect against atherosclerosis. Because the increase of life span brings together longer postmenopausal periods, women are more frequently subject to period-specific diseases. Accordingly, the importance of treatment of these period-specific diseases is gradually increasing.

Melatonin is widely used as a protective therapy against the various agents that cause tissue damage by increasing free radical formation. It is a hormone that acts as an agent in catching free radicals both in physiological and pharmacological settings and has antioxidant characteristics. It not only significantly decreases oxidative stress, but also stimulates the antioxidant system (Tirentini et al. 1987; Reiter et al. 2001; Okatani et al. 2002; Reiter et al. 2003). In their study on rats, Baydaş et al. (2001) report that inhibition of melatonin synthesis increases levels of MDA.

The authors also report that a 2-week melatonin treatment decreases lipid peroxidation and increases the activity of the antioxidant enzyme GSH-Px. Based on the above, these authors conclude that melatonin is not only a free radical scavenger but also stimulates the activity of GSH-Px.

Dexpanthenol on the other hand is the biologically active alcohol of pantothenic acid; it acts as an antioxidant compound by means of increasing the content of GSH and the activity of glutathione peroxidase, both of which are the most important defense system against lipid peroxidation and oxidative stress (Slyshenkov et al. 1995; Wojtczak and Slyshenkov 2003). Etensel et al. (2007) investigated the effect of two different doses of dexpanthenol (250 or 500 mg/kg) on tissue damage and lipid peroxidation in the rat experimental testis torsion model and found a significant decrease in serum MDA levels in the 500 mg/kg dexpanthenol group. However, no significant differences were observed in serum MDA levels in the 250 mg/kg dexpanthenol group. The authors concluded that the effect of dexpanthenol on lipid peroxidation and tissue damage depends on the dose administered, and that the effect is significant at the 500-mg/kg dose.

The various studies which have investigated the effect of HRT and additional antioxidant treatment on the oxidative system have concluded that HRT and additional antioxidant treatment positively affect the oxidative system, and these treatments may have positive effects on CVD and on diseases related to oxidative stress (Shehata and Kamel 2008; Öztekin et al. 2007; Unfer et al. 2006; Naziroğlu et al. 2004). Here, we hypothesized that the increase in oxidative stress which occurs as a result of estrogen loss due to menopause may be decreased following supplementation of antioxidants administered in addition to estrogen treatment, and that consequently, diseases such as CVD and osteoporosis can be prevented. In the present study, the effect of melatonin and dexpanthenol on antioxidant parameters is analyzed following addition onto estrogen treatment in ovariectomized rats.

Our results demonstrate high levels of GSH in the groups that are administered with melatonin and dexpanthenol. Furthermore, following dexpanthenol treatment, we observe a slight increase in the level of SOD activity and a slight decrease in the level of free radical MDA, the latter which was raised during the post-ovariectomy period, although these differences

are not statistically significant. The fact that we also observe a slight, albeit not statistically significant, increase in levels of GSH-Px activity in Ovx+E₂+MT5 and Ovx+E₂+Dxp250 groups compared with Ovx+E₂ group suggests that both melatonin and dexpanthenol may have a positive effect on increasing the antioxidant efficiency of HRT.

We observe a significant decrease in the levels of GR activity in the treated groups compared to the control group (no treatment; Ovx group): when comparing the Ovx group with the treated groups, we find a significant decrease in the levels of GR activity in the treated groups except for the groups treated with estrogen (Ovx+E₂) and melatonin 5 mg (Ovx+E₂+MT5). When treated groups are compared to each other, we find the level of GR activity to be lower in the groups which are administered with dexpanthenol than the groups which are given melatonin 5 mg. Because GR plays a role in reducing oxidized glutathione, the low levels of GR that are observed herein may be due to high levels of GSH substrate. A decrease in levels of the free radical NO following melatonin 20 mg (Ovx+E₂+MT20) and dexpanthenol 250 mg (Ovx+E₂+Dxp250) treatments suggest that these treatments may have a positive atheroprotective effect.

In conclusion, despite the present study indicating that the addition of melatonin and dexpanthenol into the HRT regimen may contribute to the antioxidant effect of estrogen, the existence of limited data in this field indicates that further studies are warranted.

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