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Protective effects of beta glucan in brain tissues of post-menopausal rats: a histochemical and ultra-structural study

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Abstract
Decline of estrogen during menopause has been associated with numerous significant changes that have been linked to many pathophysiological complications. In addition, ovarian hormone deficiency increases the production of reactive oxygen radicals which could result in oxidative stress and cell damage. While estrogen therapy is often considered to overcome the behavioral and physiological shortcomings, antioxidants are gaining popularity for their beneficial property. For this purpose, in the present study, utilizing the antioxidant properties of beta glucan has been examined in treatment of menopause induced oxidative stress in cerebral neurons. Four groups of female Wistar rats were used: control, ovariectomy, ovariectomy+beta glucan treated. We observed a significant increase in neural degeneration in ovariectomized rats as compared to controls. Moreover, increased oxidative stress in the brains of the ovariectomized rats has been detected by performing immunohistochemical analysis. A large number of immuno-positive cerebral neurons have been observed in ovariectomy group rat brains. Interestingly, providing beta glucan treatment to ovariectomized rats reduced the number of degenerated neurons. Our study is the first to examine light and electron microscopic examination and immunohistochemical and stereological analysis of estrogen depletion in rats and to test protective role of beta glucan in the experimental study.

Introduction
Natural menopause is defined as cessation of ovarian follicular activity and the menstrual cycle in the absence of a medical or surgical cause [1]. Changes in reproductive system result in diminishing of hormone production by the ovaries during menopause. Permanent decline in circulating 17β-estradiol (E2) and estrone (E1) levels associated with the onset of menopausal symptoms. Estrogens are potent regulators of neuron viability in females [2,3]. Estrogen receptors (ERs) are widely distributed in the brain, are present on both neurons and glia. Thus, along with its role in female physiology and reproduction, estrogen is a critical signaling molecule within the brain [4]. Previous studies have reported an importance of the ovarian hormones (especially estrogen) for optimal brain function [5]. It has been shown that prolonged period of E2 deprivation leads to neuronal cell death [6]. Result from different studies indicates a relationship between Alzheimer’s disease and low circulating levels of E2 in women [7]. Frequency of age-related neurodegenerative diseases occur more frequently after menopause [8,9]. Furthermore, in women who enter menopause prematurely, the risk for mortality from neurological disorders has increased [1,10].

Diminished estrogen levels at menopause have been associated with elevated oxidative stress during menopause [11]. As menopause has been reported to be linked with elevated oxidative stress, supplementation of antioxidants suggested to protect menopausal and post-menopausal deficits [12]. Recently, there has been renewed interest in the natural products to provide health and medical benefits. Beta glucan have been used for this purpose for the benefits of human health [13]. Beta glucan recognized by the C-type lectin receptor dectin-1. Down-stream signaling of dectin-1 activation promotes cellular responses such as phagocytosis, reactive oxygen radicals (ROS) production and inflammatory cytokine production [14,15]. One of the protective effects of the beta glucan mechanisms is related to antioxidant capacity of this molecule. Therefore, beta glucan has potential for the treatment of menopausal symptoms. However, there have not been yet enough studies for antioxidant ability of beta-glucans on brain tissues. The aim of this study was to investigate the antioxidant protective effects of beta glucan on brain tissues of ovariectomized (OVX) rats.

Materials and methods
This study was approved by Ataturk University Animal Ethical Committee and was carried out in accordance with the ‘‘Animal Welfare Act and the Guide for the Care and Use of Laboratory animals prepared by the Ataturk University, Animal Ethical Committee’’. The rats were housed in clean polypropylene cages having eight rats per cage and maintained under temperature-controlled room (23 ± 2°C) with a photoperiod of 12 h light and 12 h dark cycle. The rats were given standard pellets diet and water ad libitum throughout the experimental period.
A total of 32 adult female Wistar albino rats (250 ± 50 g) were randomly divided into four groups: control group, ovariectomized group, ovariectomized + estrogen-treated group, ovariectomized + beta glucan-treated group (n = 8 in each group). Three months before starting the experiment, three groups of animals (ovariectomized, ovariectomized + estrogen-treated and ovariectomized + beta glucan-treated groups) underwent bilateral ovariectomy.

Ovariectomy procedure

Rats were anesthetized with an intraperitoneal injection of 20 mg/kg thiopental sodium. Under sterile conditions, the dorsal lumbar area was shaved. A longitudinal incision (0.5–1 cm) was made in the midline area of the lower abdomen and the ovaries were removed [16,17]. After ovariectomy procedure, 25 mg/kg metamizol sodium was administered as analgesic for 2 days.

Drug administration

17-β estradiol (Estrafem, Novo Nordisk, Denmark) was dissolved in vehicle (in 0.9% NaCl). About 8 weeks after ovariectomy, E2 or vehicle was administered 0.2 mg/kg by oral gavage once a day until the experiment finished.

Beta glucan (Mustafa Nevzat Company, Turkey) used as 1,3–1,6 β-d-glucan in the microparticulate form in this study. Prepared from Saccharomyces cerevisiae yeast beta glucan was suspended in saline and 8 weeks after ovariectomy was administered intraperitoneally 100 mg/kg until the experiment finished.

Tissue removing procedure

At the end of the study, all the rat groups were exterminated under ether anesthesia. The skulls of the animals were opened and the brain tissues were removed and placed in 10% neutral formaldehyde.

Light microscopic procedure

Hematoxylin and eosin staining

For light microscopy, the brain tissues were fixed in 10% buffered formalin. After the fixation, specimens were dehydrated in an ascending series of alcohol, cleared in xylene and embedded in molten paraffin. The paraffin blocks were cut 5 mm thick using a microtome (Leica RM2125RT) and sections were stained with hematoxylin and eosin (H&E) according to the conventional light microscope.

Histopathologic changes were examined in 30 randomly selected areas of eight sections for each group. Evaluations and scoring of the histopathologic changes for cerebral cortex were neuronal degeneration, hyperchromatic and pyknotic nuclei level [18].

Immunohistochemical (IHC) staining

Following steps were performed for 8-OHdG staining: the sections were deparaffinized and treated with protease K solution (20 µg/ml in PBS), washed in distilled water and immersed in 3% hydrogen peroxide. After several washes with PBS, the sections were immersed in an equilibration buffer. Sections were incubated with 8-hydroxy-2′-deoxyguanosine (8-OHdG) (1:50, Dako EnVision Kit JaICA, Fukuroi, Japan). After several washes, all sections were incubated in anti-digoxigenin-peroxidase. The reaction was revealed with 0.06% 3,3-diaminobenzidine tetrahydrochloride (Sigma Chemical, St. Louis, MO) in PBS and the sections were counterstained with Mayer’s hematoxylin.

Stereological evaluation procedure

For a quantitative estimation of the immuno-positive neurons, sections of control and experiment groups were processed in parallel. Estimation of neurons counts/μm area was performed using stereologic methods and visualized by light microscope with a camera attachment (Nikon Eclipse E600, Tokyo, Japan). For this purpose, immuno-positive cell counts of every cerebral tissue were calculated using the optic fractionator frame method via the stereo investigator system. Sections were obtained without any randomness in their orientation, and determination of immuno-positive cells were applied as described by Unal and Kalkan [19,20].

Electron microscopic evaluation procedure

For electron microscopic examinations, brain specimens from all groups were fixed by immersion in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), post-fixed in 2% osmium tetroxide, dehydrated in a graded ascending alcohol series and embedded in araldite. All specimens were cut into ultratomo sections, stained with uranyl acetate and lead citrate and examined using an electron microscope (100 SX Jeol, Tokyo, Japan).

Statistical analysis

For statistical analysis, we used the SPSS software (IBM, version 13.0, Chicago, IL). Results were presented as means ± SD. The data were analyzed by Duncan’s multiple-range test following one-way analysis of variance (ANOVA); significance was accepted at p < 0.05.

Results

Light microscopic analysis

Sections of the cerebral cortex of the control group revealed normal histologic structure. However, histopathological examination of cerebral cortex tissues from OVX group revealed degeneration and cellular vacuolization in neurons. The number of hyperchromatic, pyknotic and condensed nuclei neurons were found significantly in OVX group sections (Figure 1). Most important finding of this group was the presence of the edema in neural cells. Changes were comparatively less severe in OVX + estrogen treated and OVX + beta glucan-treated groups as compared to OVX group (Figure 1). Microscopically, degenerative changes were mainly restricted in these groups. Examination of OVX + estrogen-treated and OVX + beta glucan-treated groups revealed that the number of neurons with chromatolysis of nuclear material and pyknotic neurons in cerebral cortex were reduced. Histopathologic changes of all groups are presented in Table 1.

Immunohistochemical analysis

The results of 8-OHdG positive cell numbers for all groups are presented in Table 2 and seen in Figure 2. We found the number of the 8-OHdG positive cells higher in the OVX group than the control group in neurons of the cerebral cortex. Difference between these groups was being statistically significant (p < 0.05). The number of the 8-OHdG positive cells were less in the OVX + estrogen and OVX + glucan-treated groups than OVX group. While the immuno-positive cells in OVX group were dominant, 8-OHdG positive cells have been reduced in OVX + estrogen and OVX + glucan-treated groups. Conversely, we did not found any statistically significant differences of 8-OHdG immuno-positive cell numbers in neurons of cerebral cortex between the OVX + estrogen-treated and OVX + beta glucan-treated groups.
Ultra-structural analysis

In electron microscopic examination, the neuron structures and cell membranes exhibit normal structure in the control group. However, in the OVX group, density of the myelin sheath were lost, somewhere compact lamella continuity was destroyed and some cytoplasmic vacuolizations were observed in neurons of cerebral cortex. In addition, edema was detected in micrographs of this group. The alterations in the myelin sheath were improved in OVX + estrogen-treated and OVX + beta glucan-treated groups. In addition, cytoplasmic vacuolizations, membrane discontinuity were reduced in neurons of OVX + estrogen-treated and OVX + beta glucan-treated groups and ultra-structural features of glia and axons were close to control group (Figure 3).

Discussion

The results of the present study demonstrate that the lack of estrogen hormones affects the cerebral cortical neuronal structures in the post-menopausal rats. The menopause is a mid life event involves dramatic declines in estrogen production and levels. Extensive studies indicates that estrogen is critical for maintaining normal neuronal viability and function. The results of numerous studies clearly have reported that E2 is a potent protective factor in the aging brain [21]. As estrogen hormones are implicated as a neuroprotective factor in a variety of neurodegenerative disorders, menopause is a risk factor for the occurrence of neurologic diseases [22,23].

In this study, our histopathologic examination revealed neuronal degeneration, pyknotic and hyperchromatic nuclei neurons and edema in neural cells especially in OVX rat sections.
Structural and degenerative changes in the cortical neurons could be associated with decreased estradiol levels [18,24]. The number of degenerated neurons, pyknotic and hyperchromatic nuclei were decreased in OVX + estrogen-treated and OVX + β-glucan-treated groups. The findings in these groups were close to the control group.

Ultra-structural findings for all groups were similar to light microscopic evaluations. Electron microscopic examination
revealed neural degeneration, degradation of the myelin sheath integrity and cytoplasmic vacuolizations in OVX rat micrographs. Mentioned findings were notably reduced in OVX + estrogen-treated and OVX + beta glucan-treated groups. Previous studies have been indicated that estrogens’ neuroprotection is mediated via a multimodal mechanism: genomic signaling, non-genomic signaling, antioxidative actions and regulation of mitochondrial bioenergetics [25]. In genomic signaling, E2, translocates to the nucleus in order to regulate the transcription of genes. E2 has been shown to regulate expression of anti-apoptotic and pro-apoptotic genes in cortical neurons [26]. Furthermore, as maximal mitochondrial respiratory rate in neurons and glia was increased by estrogen treatment, it has been considered that E2 reduces free radicals and oxidative damage [27]. Improvement of our findings in OVX + estrogen-treated and OVX + beta glucan-treated groups could be explained by estrogen’s and beta glucan’s antioxidative effects.

In addition, we examined the neuroprotective effects of beta glucan in OVX-induced oxidative stress by performing immunohistochemical analysis. 8-OHdG is a marker that allows detection of oxidative DNA damage. No positive staining was observed in the control group. However, strong 8-OHdG-immunoreactivity was detected in the OVX group neurons of cerebral cortex. The number of 8-OHdG immuno-positive neurons were significantly reduced in OVX + estrogen-treated and OVX + beta glucan-treated groups. Moreover, we used stereological methods for expression of 8-OHdG immuno-positive neurons. Due to this method, we obtain information about a three-dimensional material from measurements conducted with two-dimensional planar sections. According to literature review, none of the previous studies has not performed stereological method. This is the first study, in which the number of the 8-OHdG immuno-positive neurons were determined in cerebral cortical tissues by applying stereological method. The numerical density of neurons for all group sections was evaluated via optic fractionator frame method (according to the antibodies’ positivity). Our results showed that the number of immuno-positive neurons were higher in OVX rat sections compared to control and treatment groups. In addition, the number of immuno-positive neurons were decreased in OVX + estrogen-treated and OVX + beta glucan-treated groups. Ovariectomy triggers an increased oxidant capacity in the brain tissues [28]. Oxidative stress, which occurs due to an imbalance between production and elimination of ROS causes oxidative damage in neurons. Estrogen acts as a free radical scavenger and degrades ROS produced during membrane oxidation processes. Ovarian hormone replacement reduces oxidative stress and supports bioenergetics via mitochondrial alterations in the central nervous system [27]. Decreasing number of 8-OHdG-positive neurons in OVX + estrogen-treated group could be explained by neuroprotective effects of estrogen. In addition, the results of OVX + beta glucan-treated group were similar to the results of OVX + estrogen-treated group. Previous experimental researches have reported the protective effects of beta glucans due to their antioxidative capacity [29]. The antioxidative properties of beta glucan were extensively investigated and postulated that its protective activity may partially be attributed to its ability to exert free radical scavenging properties [30,31]. Beta glucan exerts the protective effect by the inhibition of early activation of cytokines which are important in oxidative stress. Administration of beta glucan inhibited the tumor necrosis factor-α (TNF-α) response, suggesting a mechanism in blunting the host’s response through the inhibition of cytokine activity [32]. Inhibition of the cytokines results in inhibition of cellular infiltration, scavenging of the ROS produced by cytokines activation during oxidative stress. Oxidative stress causes damage of proteins by an addition of products of glycoxidation and/or lipid peroxidation and by a direct oxidation of protein side chains. Lipid peroxidation mediated by free oxygen radicals is believed to be an important cause of destruction and damage to cell membranes, leading to consequent disruption of membrane integrity, and also enhanced rates of protein degradation with eventually cell lysis [33]. Numerous studies [32,34,35] confirm that administration of beta glucan were effective against oxidative tissue damage and significantly reversed the elevations in MDA levels, reliable marker for the ROS-induced lipid peroxidation, and reduced GSH levels, defense components against oxidative injury, due to both their immunomodulatory and antioxidative properties. All of these studies proposed antioxidant and radical scavenging properties in addition to immunomodulatory effect for beta glucan. As the central nervous system largely consists of lipids, free-radical-induced lipid peroxidation is considered important in the second autodestruction. Thus, the authors of numerous studies have evaluated the neuroprotective efficacy of pharmacological agents with antioxidant activity. In our study, the beta glucan treatment was found to be effective against oxidative injury and significantly decreased the number of the degenerated neurons, and 8-OHdG-positive neurons, indicating decreased degree of oxidative stress. These findings suggest the protective effects of beta glucan on cerebral cortical tissues during oxidative stress. Our results were in agreement with the previous studies [36].

As a conclusion, our results support that beta glucan may be considered to reduce oxidative stress in post-menopausal brain tissues. Further studies are needed to highlight receptors and transduction pathways involved in these protective effects.

Declaration of interest
There is no conflict of interest for all authors. Appropriate Institutional Review Board approval was obtained and procedures were used concerning animal subjects.

References

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