Comparison of tibolone and 17beta-estradiol administration on the expression of zonula occludens-1, occludin, glial fibrillary acidic protein and c-fos levels in the brain cortex and hippocampus of female rats

Ulviye Ceylan, Suleyman Engin Akhan, Ercan Bastu, Funda Gungor-Ugurlucan, Ahmet Cem Iyibozkurt, Samet Topuz

Department of Obstetrics and Gynecology, Istanbul University School of Medicine, Istanbul, Turkey

Correspondence to: Ercan Bastu, MD.
Istanbul University School of Medicine
Department of Obstetrics and Gynecology, Division of Infertility
Capa 34093, Istanbul, Turkey.
TEL: +90 532 413 4195; E-MAIL: ercan.bastu@istanbul.edu.tr

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Abstract

OBJECTIVES: To compare the effect exerted by oral tibolone or intramuscular 17β-estradiol administration on the expression of ZO-1, occludin, GFAP and c-fos levels in the brain cortex and hippocampus of ovariectomized rats.

RESULTS: Immunostaining for ZO-1 and occludin revealed similar staining patterns between controls and tibolone rats and between controls and E2 rats. When staining in tibolone and E2 rats were compared both for ZO-1 and occludin, staining patterns were again identical. Positive staining for the GFAP was detected in the controls, tibolone rats and E2 rats. Staining was more intense in the tibolone rats than controls and in the E2 rats than controls. In sections from the controls, tibolone rats and E2 rats, number of reactive cells for c-fos were 1.75±0.25, 3.75±0.36 and 4.50±0.50, respectively. There was a statistically significant difference between the three groups (p=0.0001). Comparison of tibolone and E2 rats revealed no statistically significant difference (p=0.246).

CONCLUSIONS: It is well known that natural hormones like E2 regulate brain development and function. Our results provide further information on the mechanism of action of tibolone in the brain cortex and hippocampus. These results will allow us to continue with further studies with different post-ovariectomy intervals, because tibolone can be proposed as an attractive alternative for hormone replacement therapy, acting as a neuroprotective agent for the prevention of neurodegenerative diseases in menopausal women.

ORIGINAL ARTICLE

INTRODUCTION

The population is aging and a tendency to better health care in the western world has led to greater longevity, especially among women (Miniño *et al.* 2004). Despite the increase in longevity, starting age of menopause has remained relatively stable, producing a situation in which many women are living a third of their lives in a postmenopausal, namely 17β-estradiol (E2)-deficient, state (Miniño *et al.* 2004). It is well established that cessation of E2 production is usually associated with physiologic symptoms, such as hot flushes, night sweats, genital dryness, and changes in psychological measures, such as cognition, anxiety, mood, resulting in decreased quality of life. Hysterectomy with bilateral salpingo-oophorectomy (BSO), also known as a surgical menopause, is associated with a decrease in sex hormone levels, leading to menopausal symptoms such as hot flashes, decreased libido, depression and vaginal dryness (Wild 2007; Gallicchio *et al.* 2006). BSO has also been associated with breast cancer risk in some premenopausal women (Meijer & van Lindert 1992; Schairer *et al.* 1997). Many premenopausal women undergoing BSO will require hormone replacement therapy (HT) unless there are contraindications. HT is usually continued until the average age of menopause (≈50 years) (North American Menopause Society 2010; Haney & Wild 2007). Unfortunately, there are no established guidelines describing the use of HT in women after BSO (Haney & Wild 2007). In addition, administration of exogenous E2 in postmenopausal women has been found to increase breast cancer risk (Colditz *et al.* 1995; Collaborative Group on Hormonal Factors in Breast Cancer 1997; Magnusson *et al.* 1999). Tibolone is a synthetic steroid that was approved in 90 countries for treatment of menopausal symptoms and in 55 countries for the prevention of osteoporosis by 2009 (Kenemans *et al.* 2009). Currently, many patients use tibolone to reduce menopausal symptoms.

Aging, both in animals and humans, is associated with significant structural and functional alterations in the blood-brain barrier (BBB) that are characterized by the molecular anatomy of the tight junctions (Mooradian 2003). The precise biochemical mechanics of these alterations is unknown. Level of expression of tight junction structural proteins such as occludin and zonula occludens-1 (ZO-1) may be altered with age. Importantly, studies on animal models of stress and mood disorders have also supported the concept of an astrocytic deficit in depression. Recently, it has been shown that early life stress results in a reduced density of glial fibrillary acidic protein (GFAP) astrocytes in various regions of brain of adult rats (Leventopoulos *et al.* 2007). On the other hand, c-fos activity and/or expression correlated with tumor grade, cell cycle-regulatory protein expression, estrogen receptor (ER) expression, and/or tamoxifen resistance and metastases in several studies (Milde-Langosch et at. 2000; Milde-Langosch et at. 2003; Bamberger *et al.* 1999; Johnston *et al.* 1999; Gee *et al.* 2000).

The aim of the present study was to compare the effect exerted by oral tibolone or intramuscular 17β-estradiol administration on the expression of zonula occludens-1, occludin, glial fibrillary acidic protein and c-fos levels in the brain cortex and hippocampus of ovariectomized rats.

MATERIAL AND METHODS

Animals

Adult female Sprague-Dawley rats (weighing 180–220 g) were included in the present study. All rats had 14 hours per day of illumination (lights on at 6 AM and off at 8 PM) and free access to standard rat chow and tap water. Following induction of anesthesia with an intraperitoneal injection of sodium pentothal (35 mg/kg), all animals were bilaterally ovariectomized. Animals were cared for in accordance with the recommendations of the *Guide for the care and use of laboratory animals* (U.S. National Research Council 2011).

Protocol

The ovariectomized animals (n=30) were housed for 28 days for acclimatization and were then divided into 3 groups of 10 rats each: (1) untreated rats (controls), (2) 10 rats that received 10-week oral treatment with tibolone (2.5 mg/kg/d) and (3) 10 rats that received 17β-estradiol 1.0 mg/kg/d intramuscularly for 10 weeks. The doses of tibolone and E2 were chosen according to those suggested in the literature as able to give exposures comparable to exposure in humans (Genazzani *et al.* 2000; Ederveen *et al.* 2001). We chose a treatment length of 10-week, because in previous studies, it has been reported that especially tibolone starts to influence cognitive processes after the first 2 months of treatment (De Aguiar *et al.* 2006; Wu *et al.* 2008).

Two rats in the control group were lost for unknown reasons, 2 rats in the tibolone group were lost during gavage and 2 rats in the E2 group were lost due to infection.

Twenty-four hours after the last treatment, all animals (including the control group) were killed by decapitation under deep pentobarbital anesthesia (35 mg/kg IP). For immunohistochemistry the rats were perfused through the left cardiac ventricle using 200 ml of fixative (4% paraformaldehyde in 0.1 M phosphate-buffered saline [PBS]) for 10 minutes. Afterwards, the brains were removed and kept the fixative at 4 degrees C for 24 hours. Paraffin-embedded brains were cut at 5 microns for immunohistochemistry evaluation of ZO-1, occludin and GFAP. Sections of 40 microns were also included for immunohistochemistry evaluation of c-fos.

The protocol was approved by the local animal ethics committee of the Institute of Experimental Medicine Research, Istanbul University. Special care was taken to minimize animal suffering and to reduce the number of animals used to the minimum necessary.

Immunohistochemistry evaluation

5-micron tissue sections for ZO-1 and occludin immunodetection were deparaffinated, rehydrated and incubated with 1 mg/ml protease (Sigma-Aldrich Co., St. Louis, MO, USA) for 10 minutes. 5-micron tissue sections for GFAP immunodetection were deparaffinated, rehydrated and boiled in 10 mM citrate buffer (pH 6.0) for 2 minutes under 1 atm pressure. Endogenous peroxidase activity was first blocked by pretreatment of tissue sections with 0.3% hydrogen peroxide for 30 minutes, followed by rinsing in PBS. After rinsing, a non-specific blocking reagent (Ultra-V-Block; Lab Vision Corp., Fremont, CA, USA) was used to prevent non-specific binding.

For ZO-1 immunohistochemistry, 5-micron sections were incubated with the polyclonal rabbit primary anti-ZO1 (1:50 dilution; Zymed Lab. Inc., San Francisco, CA, USA) diluted in PBS. Following primary immunoreaction, sections were rinsed in a wash buffer three times for 10 min before the incubation with the biotinylated goat anti-polyvalent secondary antibody (Lab Vision Corp., Fremont, CA, USA).

For occludin immunohistochemistry, 5-micron sections were incubated with the polyclonal rabbit primary anti-occlusin (1:50 dilution; Zymed Lab. Inc., San Francisco, CA, USA) diluted in PBS. Following primary immunoreaction, sections were washed in PBS three times for 10 min each before the incubation with the biotinylated goat anti-polyvalent secondary antibody (Lab Vision Corp., Fremont, CA, USA).

For GFAP immunohistochemistry, 5-micron sections were incubated with the monoclonal mouse primary anti-GFAP (1:100 dilution; Lab Vision Corp., Fremont, CA, USA) diluted in PBS. Following primary immunoreaction, sections were washed in PBS three times for 10 min each before the incubation with the biotinylated goat anti-mouse secondary antibody (Lab Vision Corp., Fremont, CA, USA).

40-micron tissue sections for c-fos were prepared using a cryostat microtome (Leica CM-1900, Nussloch, Germany) and stored for 48 hrs in 0.01 M PBS. For c-fos immunohistochemistry, 40-micron sections were incubated with polyclonal anti-c-Fos primary antibody (1:20 000 dilution; Calbiochem, CA, USA) diluted in PBS. Following primary immunoreaction, sections were rinsed in a wash buffer three times for 10 min and incubated with a biotinylated goat anti-rabbit secondary antibody (1:200 dilution; Vector Laboratories, Burlingame, CA, USA) diluted in PBS.

In all sections, the immune activity was visualized by aminoethylcarbazole chromegen (Zymed Lab. Inc., San Francisco, CA, USA) and biotin-streptavidin (Lab Vision Corp., Fremont, CA, USA) solutions. Sections were finally counterstained with Mayer's hematoxylin. Afterwards, glass sealed sections were viewed with an Olympus BX60 fluorescence microscope and photomicrographs were taken using a digital camera. Immunoreactive cells were counted by a researcher who was experimentally blind to the rat strain.

Statistical analysis

Data were stored and analyzed using the SPSS statistical software (version 16.0, SPSS, Chicago, IL). All the results are reported as mean±SD. Analysis of variance (ANOVA) followed by the Student's t-test for paired variables was used to evaluate the statistical significance of the differences. A $p<0.05$ was considered the limit for statistical significance.

RESULTS

Successful immunostaining for, ZO-1, occludin, GFAP and c-fos was carried out in all biopsies from controls, rats that received tibolone and rats that received E2.

Figure 1a, b and c shows immunostaining for ZO-1 in the cortical and subcortical regions from the controls, tibolone rats and E2 rats, respectively. Similar staining patterns were observed between controls and tibolone rats and between controls and E2 rats. When staining in tibolone and E2 rats were compared, staining patterns were again identical.

Figure 2a, b and c shows immunostaining for occludin in the cortical and subcortical regions from the controls, tibolone rats and E2 rats, respectively. Similar staining patterns were observed between controls and tibolone rats and between controls and E2 rats. When staining in tibolone and E2 rats were compared, staining patterns were identical.

Figure 3a, b and c shows immunostaining for GFAP in the three histological divisions of the hippocampus (CA1, CA2 and CA3) from the controls, tibolone rats and E2 rats, respectively. Positive staining for the GFAP was detected in all three groups. Staining was more intense in the tibolone rats than controls. Staining was also more intense in the E2 rats than controls. When staining in tibolone and E2 rats were compared, there was no significant difference.

For c-fos evaluation, 8 sections that represent general immunostatining were chosen in CA1, CA2 and CA3 from the controls, tibolone received rats and E2 received rats each. Reactive cells were counted. In sections from the controls, tibolone rats and E2 rats, number of reactive cells were 1.75±0.25, 3.75±0.36 and 4.50±0.50, respectively. There was a statistically significant difference between the three groups as determined by one-way ANOVA ($p=0.0001$). Comparison of tibolone and E2 rats revealed no statistically significant difference as determined by Student-t test ($p=0.246$).

DISCUSSION

Tibolone is a compound that shows tissue selective estrogenic responses in postmenopausal women. It also lowers sex hormone-binding globulin, thus in- creasing estradiol and testosterone levels (Moore 2001; Davis

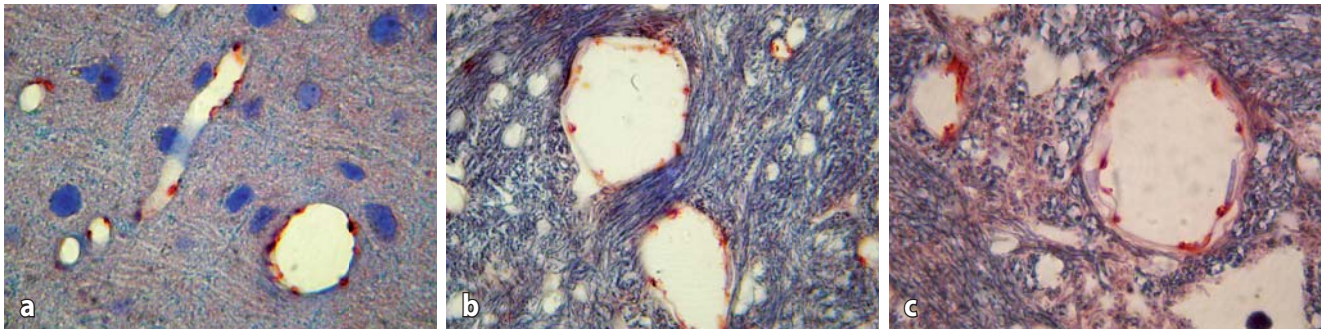

Fig. 1. a: Immunohistochemical analysis of ZO-1 in controls. **b:** Immunohistochemical analysis of ZO-1 in tibolone rats. **c:** Immunohistochemical analysis of ZO-1 in E2 rats.

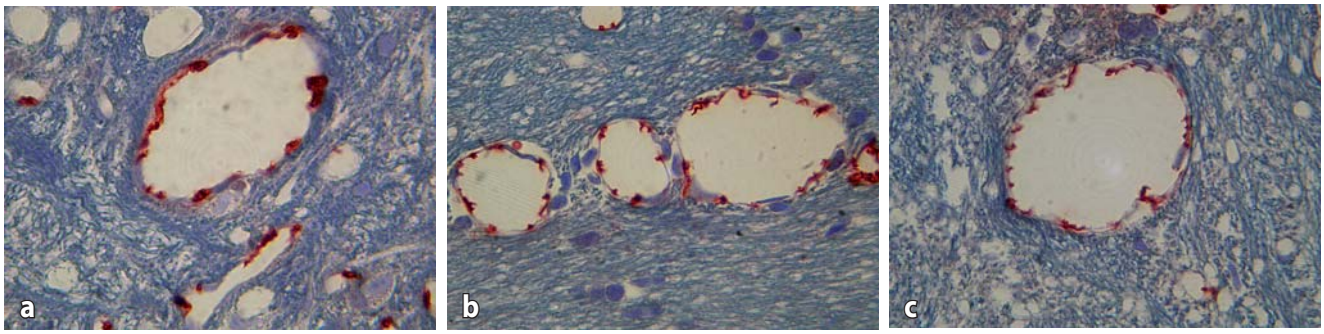

Fig. 2. a: Immunohistochemical analysis of occludin in controls. **b:** Immunohistochemical analysis of occludin in tibolone rats. **c:** Immunohistochemical analysis of occludin in E2 rats.

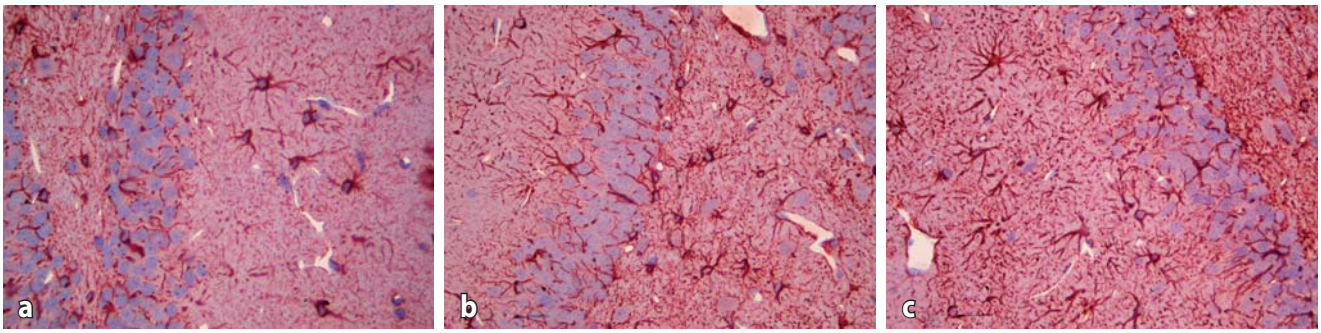

Fig. 3. a: Immunohistochemical analysis of GFAP in controls. **b:** Immunohistochemical analysis of GFAP in tibolone rats. **c:** Immunohistochemical analysis of GFAP in E2 rats.

2002; Albertazzi *et al.* 1998). RCTs indicate that tibolone has positive effects on mood compared with placebo, relieving adverse mood disorders similar to standard HT (Davis 2002) and also improves sexual function more than standard HT (Davis 2002; Palacios *et al.* 1995; Nathorst-Boos *et al.* 1997). Tibolone seems to improve semantic memory without significantly modifying recognition memory (Fluck *et al.* 2002; Albertazzi *et al.* 2000). The model of ovariectomized rats has been used to study the effect of tibolone on hot flashes, showing that this compound is able to reduce the change in rat-tail temperature caused by ovariectomy (Berendsen *et al.* 2010).

It is well known that E2 alters the dendritic arbor of several areas in brain involved in cognition-related processes (Gibbs 2010), including prefrontal cortex and hippocampus (McEwen 2002; Wallace *et al.* 2006; Tang *et al.* 2004; MacLusky *et al.* 2005). In this sense, E2 replacement increases the density of dendritic spines in hippocampal CA1 pyramidal cells in rats and monkeys (Gould *et al.* 1990; Woolley & McEwen 1993; Leranth *et al.* 2002). This change seems to be related with long-term memory (Durand *et al.* 1996; Warren *et al.* 1995). Similar findings have been found in pyramidal neurons from the prefrontal cortex (Hao *et al.* 2007). Ovariectomized rat model to study menopause has been used to study some behavioral symptoms associated with human post-menopause such as anxiety (Picazo *et al.* 2006; Rodríguez-Landa *et al.* 2009), depression (Estrada-Camarena *et al.* 2011; Bekku & Yoshimura 2005), learning and memory (Rodríguez-Landa *et al.* 2009; Daniel *et al.* 2006; Espinosa-Raya *et al.* 2011).

HT that is initiated immediately after menopause, prevents the decline of cognition (Gibbs 2010). To the

best of our knowledge, there are limited number of studies about the influence of E2 or tibolone, on specifically ZO-1, occludin, GFAP and c-fos, while no previous study comparing the influence of E2 and tibolone on the aforementioned areas. In our study, we demonstrated that tibolone or E2 administration increased GFAP and c-fos expressions in the brain cortex and hippocampus of female rats; however, causing no significant effect on ZO-1 and occludin expressions in the same regions. Comparison of tibolone and E2 expressions revealed identical results in all evaluated sections. In line with our findings, it was previously demonstrated that GFAP expression in the interpeduncular nucleus (IPN) was responsive to testosterone in male rats (Hajos *et al.* 1999) and in females, the intensity of GFAP-immunoreactivity followed the periodic hormonal changes of the estrous cycle (Hajos *et al.* 2000). In another study on rats, Zsarnovszky *et al.* (2002) revealed that E2, in the absence of other ovarian hormones, can influence GFAP expression within individual subnuclei of the IPN. The authors argued that in the IPN, E2 may directly modulate GFAP expression through estrogen receptor β-mediated mechanisms. These observations suggest that the anatomical structures in regions of significant changes in GFAP expression are responsive to E2. A recent study of Camacho-Arroyo *et al.* (2011) on female rats, also demonstrated that chronic administration of ovarian hormones immediately after menopause modifies the content of GFAP in hippocampus and prefrontal cortex of the rat. Study of Pinto-Almazán *et al.* (2012) was maybe the first to evaluate E2 and tibolone (and also P4) expressions of female rats. Their findings indicated that chronic administration of E2, tibolone and P4 regulates the phosphorylation of tau in the hippocampus and cerebellum of adult ovariectomized rats. A study by Maggiolini *et al.* (2004) demonstrated that E2 and the two major phytoestrogens genistein and quercetin are able to induce rapid c-fos up-regulation in breast cancer cells. The up-regulation of c-fos by extracellular stimuli could represent an early molecular sensor associated with relevant biological responses, including those involved in cell proliferation.

CONCLUSION

It is well known that natural hormones like E2 regulate brain development and function, acting on neurons, synapses and glial cells. Our results provide additional information on the mechanism of action of tibolone in the brain cortex and hippocampus. These results will allow us to continue with further studies with different post-ovariectomy intervals, both at the level of specific neurobiological markers as well as the behavioral level, because tibolone can be proposed as an attractive alternative for HT, acting as a neuroprotective agent for the prevention of neurodegenerative diseases in menopausal women.

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