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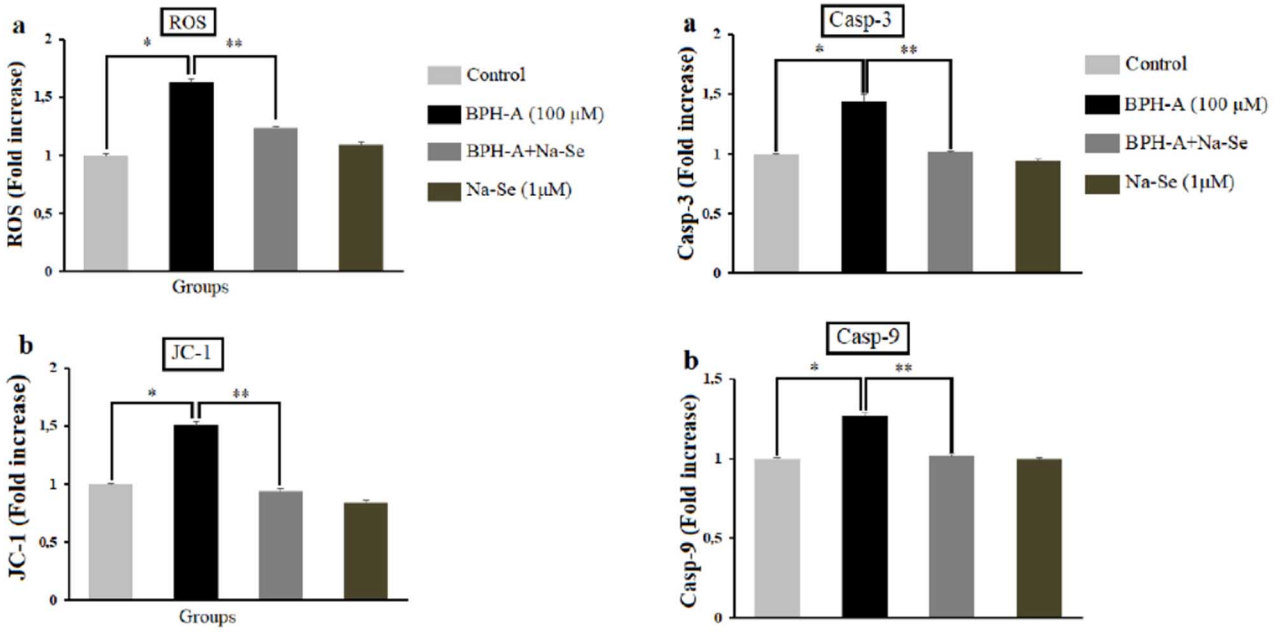

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B- Oxidative Stress (Antioxidant vitamins, antioxidant enzymes, metabolism of nitric oxide, oxidative stress, biophysics, biochemistry and physiology of free oxygen radicals)

C- Interaction Between Oxidative Stress and Ion Channels in Neuroscience
(Effects of the oxidative stress on the activation of the voltage sensitive cation channels, effect of ADP-Ribose and NAD^+ on activation of the cation channels which are sensitive to voltage, effect of the oxidative stress on activation of the TRP channels in neurodegenerative diseases such Parkinson's and Alzheimer's diseases)

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RESEARCH ARTICLE

J Cell Neurosci Oxid Stress 2020;12(3): 963-970.

Protective effects of tamoxifen and raloxifene on apoptosis and oxidative stress in the kidney and liver of ovariectomized rats

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List of Abbreviations :

17-β, *17β-estradiol;* ***GSH Px,*** *glutathione peroxidase;* ***GSH,*** *reduced glutathione;* ***LP,*** *lipid peroxidation;* ***MDA,*** *malondialdehyde;* ***PARP,*** *poly (ADP-ribose) polymerase;* ***RAL,*** *raloxifene;* ***ROS,*** *reactive oxygen species;* ***SERMs****, selective estrogen receptor modulators;* ***TAM,*** *tamoxifen.*

Abstract

In the postmenopausal period, women undergo physical and morphological changes that may result in insufficiency and deterioration in physiological functions. It is accepted that oxidative stress is involved in the etiology of postmenopausal changes. It is known that the decrease in ovarian hormones, especially 17β-estradiol (17-β) after menopause induces apoptosis and oxidative stress in many tissues. It is well known that 17-β has an antioxidant role in non-menopausal women. Recently, we observed that the treatments of 17-β, raloxifene (RAL), and tamoxifen (TAM) diminished apoptotic factors, oxidative stress, and mitochondrial membrane depolarization in the brain and dorsal root ganglia of ovariectomized rats. There is no enough information about the effects of triple therapy [17-β, and selective estrogen receptor modulators (TAM and RAL)] effects on liver and kidney tissues. We aimed to investigate the effects of 17-β, TAM, and RAL on apoptosis, cell viability (MTT), and oxidative stress in the kidney and liver of bilateral ovariectomized (OV) rats.

Forty female rats used in the experiment, and they were divided into five groups as control, OV, OV+17-β,

OV+TAM, and OV+RAL. 17-β, TAM, and RAL were subcutaneously given to three groups (OV+17-β, OV+TAM, and OV+RAL) for 14 days after ovariectomy.

While kidney and liver cells lipid peroxidation levels were high in the OV group, they were low in the OV+17-β, OV+TAM, and OV+RAL groups. The treatments of 17-β, TAM, and RAL in the groups of OV+17-β, OV+TAM, and OV+RAL increased the glutathione peroxidase (GSH Px) activity and glutathione (GSH) levels in the cells of kidney and liver. In addition, the MTT level of kidney and liver cells was low in the OV group and higher in the OV+17-β, OV+TAM, and OV+RAL groups. The treatments of OV+17-β, OV+TAM, and OV+RAL decreased the apoptosis and ROS levels in kidney and liver cells.

In conclusion, we observed that 17-β, TAM, and RAL administrations were beneficial on cell viability (MTT), apoptosis, and ROS levels in the kidney and liver cells of OV rats by modulating antioxidant systems.

Keywords: 17β-estradiol; Raloxifene; Tamoxifen; Apoptosis; ROS.

Introduction

Menopause is defined as a natural life process that occurs with the permanent cessation of menstruation in which women undergo physical and morphological changes (Thompson et al. 2019). It is well known that these changes are related to the decrease in the ovarian hormone, especially 17-β estradiol (17-β) (Nazıroğlu et al. 2004; Yazğan et al. 2016). 17-β has been shown to have a comprehensive organ protective role as well as anti-inflammatory and antioxidant effects (Brady, 2015; Yazğan et al. 2016; Yazğan and Nazıroğlu, 2017; Ltaif et al. 2020). It is known that the decrease in ovarian hormones after menopause induces apoptosis and oxidative stress in many body tissues (Dilek et al. 2010; Lamas et al. 2015). Mitochondria have important roles in cell metabolism, cell viability (MTT), apoptosis, and reactive oxygen species (ROS) homeostasis (Shukla et al. 2009; Ltaif et al. 2020). The treatment of 17-β has been shown to regulate the structure and function of mitochondria, particularly in tissues that have a high energy demand such as liver and kidney (Konyalioglu et al. 2007; Shukla et al. 2009). Estrogen deprivation following menopause causes damage to glomerular, tubular, or vascular kidney tissues due to an increase in the ROS generation.

Ovariectomy procedure in experimental animals is an important model to investigate the effects of estradiol deficiency in menopause (Konyalioglu et al. 2007; Yazğan and Nazıroğlu, 2017). Removal of the ovaries increases oxidative stress with high ROS production, which leads to pathological changes in kidney and liver tissues (Konyalioglu et al. 2007; Ltaif et al. 2020). It was demonstrated that the postmenopausal women have higher serum concentrations of oxidative markers of lipid peroxidation and oxidized GSH (Doshi and Agarwal, 2013). ROS cause injury to cells and intracellular membranes resulting in lipid peroxidation and may lead to cellular destruction and subsequently, cell death in many tissues (Halliwell, 2006). Various antioxidant defense systems are available to clear ROS in body tissues including kidney and liver tissues. Glutathione peroxidase (GSH Px) is responsible for the reduction of hydro and organic peroxides in the presence of reduced glutathione (GSH). GSH is the most abundant thiol antioxidant in mammalian many cells and maintains thiol redox balance in cells (Schweizer et al. 2004; Nazıroğlu, 2009). Therefore, the antioxidant levels can be evaluated indirectly by measuring various antioxidants, including GSH Px and GSH.

Tamoxifen (TAM) and raloxifene (RAL) are non-steroidal selective estrogen receptor modulators (SERMs), and are used in the treatment of estrogen-dependent breast cancers and the preservation of bone tissue (Jordan, 2003; Shukla et al. 2009; Yazğan et al. 2016). SERMs act as estrogen antagonists in some tissues, such as the breast and uterus tissues, while acting as estrogen agonists in tissues such as bone and brain tissues (Jordan, 2003; Doshi and Agarwal, 2013; Yazğan and Nazıroğlu, 2017). Previous studies have shown that RAL can reduce lipid peroxidation and oxidative stress in the rat brain, and protects glucose-deficient astrocytes from oxidative injury (Konyalioglu et al. 2007; Yazğan and Nazıroğlu, 2017). The results of several studies indicated that the treatments of TAM and RAL acted neuroprotective effects on the central nervous system and also show pharmacological effects similar to that of 17-β in both postmenopausal women and OV rats (Yaffe et al. 2001; Moreira et al. 2005; Moreira et al. 2007; Yazğan et al. 2016; Yazğan and Nazıroğlu, 2017). Some compounds exhibiting antioxidant activity reduce the potential damage caused by oxidation by buffering endogenously

or exogenously produced free radicals. It has been reported that free radical production and apoptotic pathways are regulated by the treatments of 17-β, TAM, and RAL (Yang et al. 2004; Moreira et al. 2005; Konyalioglu et al. 2007; Moreira et al. 2007; Moreira et al. 2011; Yazğan and Nazıroğlu, 2017). Numerous studies have shown that estrogen is a powerful antioxidant (Kumar et al. 2011; Fidarov et al. 2015; Yazğan and Nazıroğlu, 2017; Gendy et al. 2019; Ltaif et al. 2020; El-; Xu et al. 2020). Recent studies documented that RAL has antioxidant properties in vitro (Arteaga et al. 2003; Mann et al. 2007). Lately, we observed 17- β, TAM, and RAL may reduce apoptotic factors (including caspase 3 and 9), mitochondrial membrane depolarization, and oxidative stress (Yazğan et al. 2016; Yazğan and Nazıroğlu, 2017). However, there are limited animal studies, showing the antioxidant properties of TAM and RAL.

The benefits effect of 17-β, TAM, and RAL on oxidative stress, MTT, apoptosis in the brain, neurons, and many other tissues are well known (Moreira et al. 2005; Konyalioglu et al. 2007; Moreira et al. 2007; Yazğan and Nazıroğlu, 2017).

However, their effects on lipid peroxidation, apoptosis, ROS, and antioxidants such as GSH and GSH Px in the kidney and liver cells have not been clarified yet, and there is no enough information about the effects of triple therapy [17-β, TAM, and RAL] effects on liver and kidney tissues. Therefore, we aimed to evaluate the effects of 17-β, TAM, and RAL on antioxidants, MTT, oxidative stress, and apoptosis status in kidney and liver tissue using a bilateral ovariectomy animal model.

Material and Method

Experimental Animals

Forty female Wistar albino rats (weighing 170 ± 10 g and aged 8–12 weeks) were used in the present study. All of the rats were housed under standard conditions of temperature (22 ± 2°C) and light (12 hours of daylight/12 hours of darkness). Animals were housed in individual plastic cages with bedding. The experimental protocol of the study was approved by the Ethical Committee of the Medical Faculty of Suleyman Demirel University (SDU).

Experimental Groups

The rats were randomly divided into five groups with eight rats per group as follows:

- **Control (CON) group:** A placebo (0.1 ml dimethyl sulfoxide [***DMSO***]+0.9 ml physiological saline [0.9 NaCl w/v]) was subcutaneously administrated to rats of the group for 14 days.
- **Ovariectomize (OV) group:** After inducing OV, DMSO (0.1 ml) was intraperitoneally (IP) supplemented for 14 days (Dilek et al. 2010).
- **Ovariectomize+17β-estradiol (OV+17-β) group:** Animals in the group received intraperitonal 17-β (80 µg/kg/day) for 14 consecutive days after OV treatment (Kramer and Bellinger, 2013).
- **Ovariectomize+Raloxifene (OV+RAL) group**: Animals in the group received intraperitonal RAL (1 mg/kg/day) for 14 consecutive days after OV treatment (Huang et al. 2007).
- **Ovariectomize+Tamoxifen (OV+TAM) group:** Animals in the group received intraperitonal TAM (1 mg/kg/day) for 14 consecutive days after OV treatment (Yazğan et al. 2016).

MDA levels determinations in kidney and liver cells

MDA levels in the hemolyzed kidney and liver cells homogenate were measured with the thiobarbituricacid reaction by the method of Placer et al. (1966). The values of MDA in the kidney and liver cells samples were expressed as µmol/g protein. The protein contents in the hemolyzed kidney and liver cells homogenate were measured by method of Lowry et al. (1951) with bovine serum albumin as the standard.

Reduced glutathione (GSH) and glutathione peroxidase (GSH Px) levels determinations in kidney and liver cells

The GSH contents of the kidney and liver cells were measured at 412 nm using the method of Sedlak and Lindsay (1968). GSH Px activities of kidney and liver cells were measured spectrophotometrically at 37 °C and 412 nm according to the Lawrence and Burk method (1976). GSH Px activity and GSH level in the kidney and liver cells samples were expressed as µmol/g protein.

Cell viability (MTT) levels determinations in kidney and liver cells

Viability assays were performed by measuring mitochondrial reductase activity with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma-Aldrich, Istanbul, Turkey) as described in previous studies (Yazğan and Nazıroğlu, 2017).

Absorbance in a microplate reader (Infinite pro200; Tecan Inc, Groedig, Austria) was read at 550 nm. The data are presented as percentage (%) increase over the pretreatment level.

Intracellular ROS production levels determinations in kidney and liver cells

Rhodamine 123 (Rh 123) is a cell-permeant, and green-fluorescent dye that is readily sequestered by active mitochondria without cytotoxic effects. Once getting in the cell, DHR 123 becomes fluorescent upon oxidation to (Rh 123, fluorescence being proportional to ROS generation. The kidney and liver cells were incubated with 20 μm DHR 123 at at 37 °C for 25 min (Espino et al. 2010). Then, the neurons were then washed in PBS. The fluorescence intensity of Rh123 was measured in an automatic microplate reader (Infinite pro200; Tecan Austria). Excitation was set at 488 nm and emission at 543 nm.

Apoptosis activities levels determinations in kidney and liver cells

The apoptosis assay was performed using a commercial kit according to the instructions provided by Biocolor Ltd. (Northern Ireland) and elsewhere (Yazğan and Nazıroğlu, 2017). When the membrane of apoptotic cell loses its asymmetry, the APOPercentage dye is actively transported into cells, staining apoptotic cells red, thus allowing detection of apoptosis by spectrophotometer. Substrate cleavage was measured with the microplate reader (Infinite pro200) with excitation wavelength of 360 nm and emission at 460 nm.

Statistical analysis

All results are expressed as means ± standard deviation (SD). Data were analyzed using the SPSS statistical program (version 17.0, software, SPSS. Chicago, IL, USA). The presence of statistical significance in the groups was evaluated with one-way ANOVA and Tukey HSD post hoc test. The level of significance was accepted as $p<0.05$ in all statistical comparisons.

Results

MDA results in kidney and liver cells

The mean MDA (lipid peroxidation) values in liver and kidney cells of five groups are shown in **Figure 1A** and **Figure 2A**, respectively. The results showed that the MDA levels in the liver and kidney were significantly ($p < 0.05$) higher in the OV group than in the CON group. The 17-β, TAM, and RAL administrations caused decreases in MDA levels of the liver and kidney ($p < 0.05$) relative to the OV group.

GSH and GSH Px results in kidney and liver cells

The mean GSH levels and GSH Px activities in the liver and kidney cells of the five groups are shown in **Figures 1B, 1C, 2B, and 2C**. The GSH concertation and GSH Px activities in the liver and kidney cells of the OV group were significantly lower ($p < 0.05$) than in the CON group. The liver and kidney cells GSH and GSH Px concentrations were increased by the treatments of 17-β, TAM, and RAL treatments ($p < 0.05$).

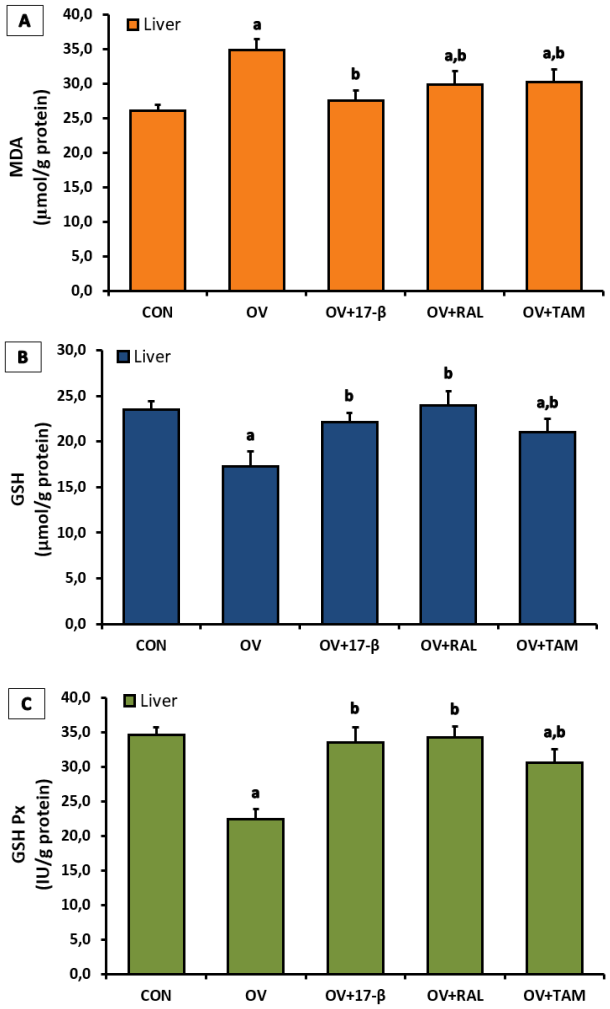

***Figure 1**. The effects of 17-β, RAL, and TAM on liver cells MDA, GSH, and GSH Px level in ovariectomized rats (n=8 and mean ± SD). ([a]p≤ 0.05 vs CON group. [b]p≤ 0.05 vs OV group).*

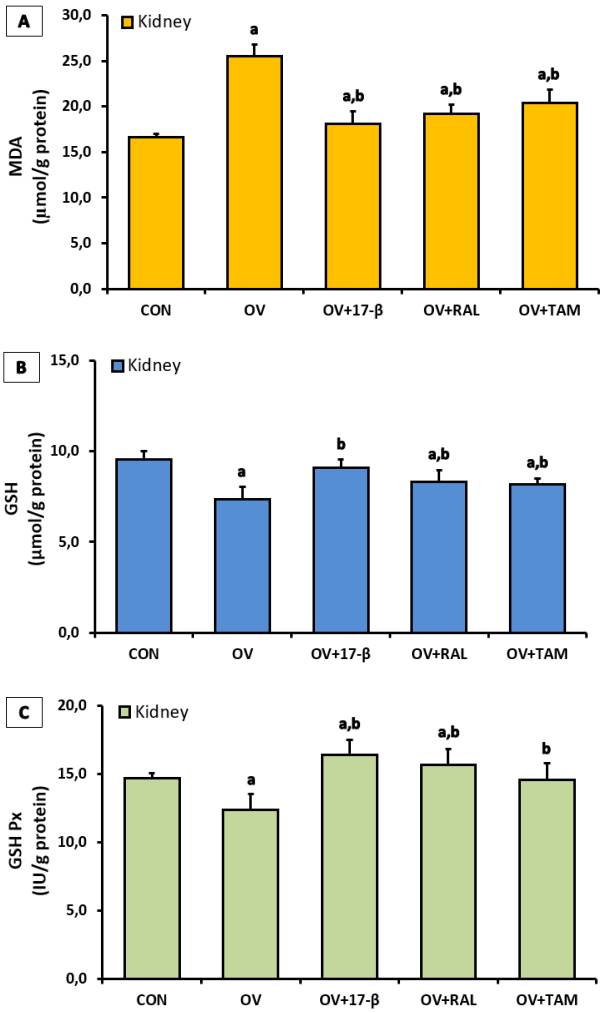

***Figure 2**. The effects of 17-β, RAL, and TAM on kidney cells MDA, GSH, and GSH Px level in ovariectomized rats (n=8 and mean ± SD). ([a]p≤ 0.05 vs CON group. [b]p≤ 0.05 vs OV group).*

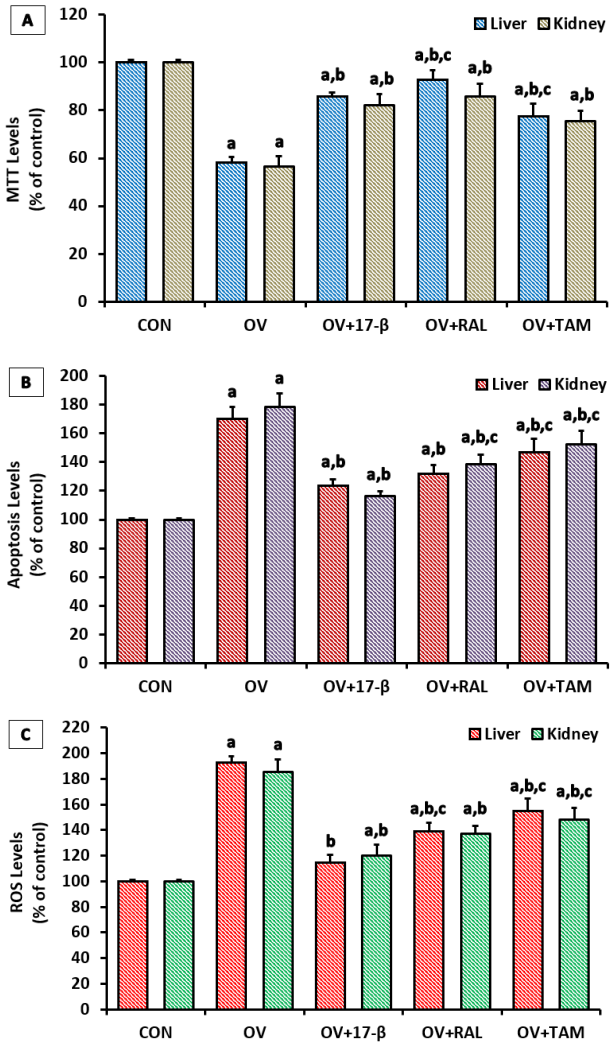

***Figure 3**. The effects of 17-β, RAL, and TAM on liver and kidney cells MTT, apoptosis,, and ROS level in ovariectomized rats (n=8 and mean ± SD). ([a]p≤ 0.05 vs CON group. [b]p≤ 0.05 vs OV group. [c]p≤ 0.05 vs OV +17- β group).*

17-β, RAL, and TAM treatment modulated ovariectomized induced cell viability (MTT), apoptosis, and ROS values in the kidney and liver cells

We investigated the protective effects of 17-β, RAL, and TAM on MTT, apoptosis, and ROS levels in the kidney and liver cells (**Figure 3**). The MTT levels (**Figure 3A**) in the kidney and liver were markedly ($p<0.05$) lower in the OV groups than in the CON group. The kidney and liver cells MTT levels were increased by 17-β, TAM, and RAL treatments. The apoptosis and ROS levels (**Figures 3B and 3C**) in the kidney and liver were markedly ($p<0.05$) higher in the OV groups than in the CON group. The kidney and liver cells apoptosis and ROS levels were decreased by the treatments of 17-β, TAM, and RAL treatments.

Discussion

17-β is a considerable hormone in the maintenance of the functions of the many tissues, including the liver and kidney tissues (Brady, 2015; El-Gendy et al. 2019; Ltaif et al. 2020; Xu et al. 2020). It is possible that the abrupt reduction in 17-β levels by OV can trigger complex functional and structural disturbances with consequent changes in this tissues (Kumar et al. 2011; Xu et al. 2020). Pathological changes in these tissues can be explained by the increase in oxidative stress parameters, and decrease in antioxidant levels as a result of the diminution in estradiol due to ovariectomy (Konyalioglu et al. 2007; Yazğan and Nazıroğlu, 2017; El-Gendy et al. 2019). It is also known that 17-β reduction activates apoptotic pathways (Mann et al. 2007; Yazğan and

Nazıroğlu, 2017; Xu et al. 2020). TAM and RAL are non-steroidal SERMs (Mann et al. 2007). It has been reported that apoptotic pathways and free radical production are regulated by TAM and RAL (Mann et al. 2007; Konyalioglu et al. 2007; Moreira et al. 2011; Schubert et al. 2016). Although the effects of 17-β, TAM, and RAL on lipid peroxidation, oxidative stress, and antioxidants have been investigated in various tissues, their effects in the kidney and liver are still not fully understood. For this purpose, we evaluated the effects of 17-β, TAM, and RAL on oxidative stress, antioxidants, and apoptosis status in kidney and liver tissue using a bilateral ovariectomy animal model.

We found that apoptosis, ROS, and lipid peroxidation (MDA) levels in the liver and kidney cells, were increased by OV induction. However, cell viability (MTT), GSH Px, and GSH activity were decreased by the induction of OV. For this reason, OV-induced estrogen deficiencies were characterized by increased oxidative stress and ROS along with decreased antioxidant levels. 17-β, RAL, and TAM applications decreased lipid peroxidation, apoptosis, and ROS levels in liver and kidney tissues; however, GSH, GSH Px activity, and cell viability levels were increased by the treatments. In this way, we have shown that 17-β, RAL, and TAM treatments modulated the balance of antioxidants in rats by down-regulating the levels of oxidative stress while up-regulating the GSH redox system.

Our results showing increased lipid peroxidation, apoptosis, and ROS levels, although the liver and kidney tissues GSH Px, GSH, and cell viability decreased in the OV rats were consistent with the findings in the literature (Arteaga et al. 2003; Konyalioglu et al. 2007; Nishi et al. 2013; Ltaif et al. 2020). El-Gendy et al. (2019) explained the effects of ovariectomy with an increase in the oxidative stress parameter (MDA), and a decrease in antioxidant levels. In a similar study, when rats in ovariectomized groups were compared with rats in non-ovariectomized groups, it was reported that MDA levels in brain, heart, and liver tissues increased (Konyalioglu et al. 2007).

The antioxidant enzyme system inherent in the cellular defense system is the most important defense mechanism against ROS. GSH and GSH Px act as antioxidants, and have a preventive effect against the extensive production of ROS by OV induction (Konyalioglu et al. 2007; El-Gendy et al. 2019). In the current study, GSH and GSH Px activity values were increased in the liver and kidney of 17-β, RAL, and TAM treated rats by inhibiting oxidative stress. Similarly, Moreira et al. (2005) reported that oxidative stress levels in the brains of TAM treated rats were reduced by supporting antioxidant thiol groups and GSH levels as well as the inhibition of mitochondrial permeability transition pores. Recently, we reported lipid peroxidation values in the erythrocytes and brain tissues were decreased by 17-β, RAL, and TAM administrations; however, GSH and GSH Px values in the brain tissues were increased by the treatments. Thus, we have shown that 17-β, RAL, and TAM treatments modulated the balance of oxidants and antioxidants in rats by downregulating the levels of oxidative stress while upregulating the GSH redox system (Yazğan et al. 2016; Yazğan and Nazıroğlu, 2017).

Numerous studies have shown that 17-β is a powerful antioxidant (Kumar et al. 2011; Fidarov et al. 2015; El-Gendy et al. 2019). In the present study, a significant decrease was observed in the oxidative parameters of the 17-β-treated groups. These results were in agreement with the observations made by Kumar et al. (2011) and Azarkish et al. (2013). Recent studies have documented that RAL has antioxidant properties in vitro and in animal studies (Arteaga et al. 2003; Konyalioglu et al. 2007; Nishi et al. 2013; Yazğan and Nazıroğlu, 2017). Our results were in agreement with previous reports showing that RAL significantly reduced MDA levels and significantly increased GSH levels in liver and kidney tissues. However, while there are limited animal studies showing TAM to be an antioxidant (Moreira et al. 2005; Zhang et al. 2007; Yazğan et al. 2016; Yazğan and Nazıroğlu, 2017), there also are reports demonstrating it to be oxidant and cytotoxic (Parves et al. 2006; Nazarewicz et al. 2007; Gong et al. 2018). In this study, we found that TAM has a therapeutic effect on OV-induced oxidative stress, similar to our previous studies.

The current study provides a mechanistic underpinning for the antioxidant actions of 17-β, RAL, and TAM, and by demonstrating that it reduces lipid peroxidation production in the liver and kidney, reduces cell death due to excessive ROS production after OV induction. Mann et al. (2007) and Schubert et al. (2016) were reported the SERMs treatment led to a significant reduction in apoptosis (Zhang et al. 2007). These results

are in agreement with our study. We also proved in our previous study that while levels in the brain, hippocampus, and peripheral pain sensory neurons PARP were increased, pro-apoptotic pro-caspase 3 and 9 activities were decreased by the induction of OV (Yazğan et al. 2016; Yazğan and Nazıroğlu, 2017).

In conclusion, the current results support the idea that the protective roles of 17-β, RAL and TAM are primarily due to their antioxidant-like actions. Lipid peroxidation, apoptosis and ROS levels in liver and kidney were decreased by 17-β, RAL and TAM administrations, however, GSH, GSH Px activity and cell viability levels were increased by the treatments. Therefore, 17-β, RAL and TAM can reduce lipid peroxidation, apoptosis and ROS levels in the liver and kidney of rats with OV by virtue of their inherent antioxidant properties. The antioxidant effects of 17-β, RAL and TAM on the liver and kidney might be induced by increases in the GSH redox system. The results of this study suggested that TAM and RAL treatment and estradiol replacement therapy improved antioxidant enzyme activity and apoptosis in the kidney and liver tissues of OV rats. Therefore, RAL and TAM treatments may have a beneficial effect in chemotherapeutic adjuvant effects as well as their preventing oxidant complications.

Financial disclosure: There is no financial disclosure for the current study.

Authorship contributions: BY and YY formulated the hypothesis and was responsible for writing the report. BY and YY was responsible for the animal experiments and analyses.

Conflict of interest: The authors declare that they have no conflicts of interest.

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