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Investigation of protective effects of misoprostol against paclitaxel-induced ovarian damage in rats

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Abstract

Aim: The main objective of our study was to examine the protection of misoprostol (MP) on paclitaxel (PTX) side effects in rat ovaries.**Materials and Methods:** Twenty-one female Sprague-Dawley rats were provided to form 3 groups, each containing seven rats: In group 1 (control group), was given 1 mL of 0.9% NaCl intraperitoneally (i.p.) and 1 mL of 0.9% NaCl orally for 6 days. In treatment groups, each rat was injected 2 mg/kg PTX i.p. on days 0, 2, 4, and 6 of the study and 0.2 mg/kg/day MP was given by oral gavage for 6 days.**Results:** Levels of malondialdehyde (MDA), and glutathione (GSH), activities of superoxide dismutase (SOD), and catalase (CAT) of tissue samples were measured. Histopathological alterations in PTX administrated rats were seen such as, degenerative changes in granulosa cells, decrease in number of growing and tertiary follicles and increasing active caspase 3 (Casp-3) positive immunoreaction in theca, granulosa and stromal cells.**Conclusion:** As a result, it shows that the use of MP can improve PTX-induced ovarian damage by preventing oxidative damage.Copyright © 2022 The author(s) - Available online at www.annalsmedres.org. This is an Open Access article distributed under the terms of Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License.

Introduction

Paclitaxel (PTX), which is obtained from the bark and needles of the *Taxus brevifolia* tree, is known as a natural product [1]. This drug is a micro tubule inhibitor that promotes polymerization and inhibits the separation of microtubules. Besides being a new microtubule stabilizing agent, PTX is used as an effective drug against a wide variety of tumors such as ovarian [2]. In addition to showing high activity in epithelial ovarian cancer cases, it is also known to have the ability to penetrate many cell layers. Additionally, it is commonly used to ovarian cancer by promoting microtubule dysfunction [3]. PTX is a considerable anticancer agent applied in laboratory and clinical research. Although PTX, which we used in research, has effective protective activity against many tumors, it is known that it also exhibits serious toxic side effects. For example, Varbiro et al. (2001) reported in their study that PTX triggers mitochondrial permeability and formation of harmful radicals. Oxygen-induced stress is known to be an important toxicity to which PTX contributes [4]. Since the main target of oxidative stress is mitochondria, results such as protein losses and decreased calcium in-

take in mitochondria can be encountered [5]. Cells have developed many mechanisms to protect against damage caused by oxidative stress. As a result of ovarian toxicity caused by PTX, an increase in antioxidant capacity was observed in cellular membranes. It is known that antioxidant enzymes have serious functions in protecting against oxidative damage. In addition, studies have shown that antioxidant therapy reverses this increased oxidative damage [6].

PTX leads to the formation of abnormal microtubules in the cell cycle. In addition, PTX causes cell cycle arrest and apoptosis in mitosis [7]. Also, it is an extremely important drug in terms of side effects. Among the factors limiting the efficacy of PTX in clinical practice are its low water solubility, toxicity and lack of selectivity in antitumor activity. Several studies have shown that menstrual irregularities and amenorrhea may develop following anthracycline-based chemotherapy [8]. In young women diagnosed with breast cancer, lymphoma / leukemia, or non-malignant disease requiring the use of cytotoxic chemotherapy regimens (eg alkylating regimens) are at greater risk of developing ovarian failure and infertility following chemotherapy [9]. In animal studies, it has been observed that PTX causes cell death, damages healthy mature oocytes and affects reproductive potential [10]. The effects of taxans on

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ovarian histology and especially on the histological structure and numbers of ovarian follicles are controversial. Although it has been reported in some studies that PTX has a negative effect on the histological structure and number of especially primordial follicles [11], other studies have reported that this agent does not affect the primordial follicles but its affect the growing and tertiary follicles (9). It has also been shown that taxans increase apoptosis in stromal cells in the ovary [12].

Anticancer treatments such as PTX induce ovarian damage, which is one of the reasons to change or stop chemotherapy. Our main goal in this regard is to reduce the burst release of PTX, to minimize ovarian toxicity and to increase its anticancer activity. Accordingly, natural products can be used as a unique source of medicine to reduce or eliminate these side effects [13]. Misoprostol (MP) is a synthetic prostaglandin E1 analogue and was first used to treat stomach ulcers. In addition, MP has been used for different indications in the fields of obstetrics and gynecology [14]. In previous studies, MP treatment significantly protected many organs, including the ovaries, against oxidative damage by free radicals produced by various agents [15]. Recently, the potentials and low toxicity of MP have made it a good alternative in traditional therapeutic drugs. The use of PTX in the treatment causes excessive metabolic changes, suggesting that MP can be used as an alternative treatment [16].

We thought that MP might be effective against ovarian damage caused by side effects in PTX treatment. There is no study showing the biochemical and histopathological changes in the ovaries as a result of oxidative stress and the preventive effect of MP. The main theme of our study is to reveal the effects of MP on ovarian damage caused by the side effects of PTX.

Materials and Methods

Chemicals

The PTX used in the experiment were supplied by Actavis (Little Island, Co. Cork, Ireland). The MP (purity > 98%) that we used in our study was purchased from Sigma. We aimed for the other chemicals in our study to be of the best quality and were purchased from Sigma Chemical Co.

Animals

In our study, 21 Sprague-Dawley type female rats weighing 200-250 g and 9-10 weeks old were obtained from Adiyaman University Research Center. Ethics Committee protocol rules were adhered to in our practices (Protocol # 2020/052). In the experiment, the room temperature of the animals was 21 ± 2 °C and the humidity was 60 ± 5 %. These created conditions were kept constant throughout the experiment and a light cycle was continued at 12:12 am.

Experimental Conditions

The 3 groups in our study were randomly generated and included seven rats: control; PTX; PTX + MP. Control group received 1 mL 0.9% NaCl intraperitoneally (i.p.) and 1 mL 0.9% NaCl oral administration for 6 days. On days 0, 2, 4 and 6, the second group was given 2 mg/kg

PTX i.p. [17]. Then, on days 0, 2, 4 and 6, the third group was given i.p. 2 mg/kg PTX. In addition, the third group was given 0.2 mg/kg MP (Cytotec, 0.2 mg) daily by oral gavage for 6 days (18).

At the end of the study, anesthesia was performed intramuscularly. Rats were sacrificed by dislocation of the spine after injection of ketamine hydrochloride and xylazine. The ovaries of all groups were quickly removed from the body and washed with cold saline solution containing 0.9% NaCl for histological and biochemical analysis. One of the ovaries was placed in liquid nitrogen until all treatments were finished and then kept at -86 °C until the analysis of malondialdehyde (MDA), glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) parameters. In order to perform histopathological analysis, the other ovary was immersed in 10% buffered neutral formalin solution and fixed at +4 °C for 24 hours. Tissues undergoing routine histological procedures as described in previous studies [19, 20] were stained with hematoxylin & eosin (H&E), Crossman's trichrome (CT) methods.

Biochemical Analyzes

Homogenization of the ovaries was carried out by means of a homogenizer (Turrax T 25, Wilmington, United States). It was then seen that the homogenates were obtained by centrifugation at 10,000 x g at approximately 4 °C and 15 minutes. The desired processes were achieved at 4 °C. The homogenates of the tissues were prepared as 0.5-1.0 g and biochemical analyzes were performed. For the measurement of MDA, GSH, SOD and CAT parameters, the determination of absorbances was carried out by means of a spectrophotometer (UNICO Instruments C., Dayton, USA). Analysis of protein concentrations was performed by the method developed by Lowry et al. (1951) [21].

Determination of the CAT parameter was performed by centrifugation of 10% tissue homogenates in 0.9% NaCl at 8,500 x g for approximately 4 °C and 15 minutes. Measurement of CAT was made by analysis of the hydrolysis of hydrogen peroxide (H₂O₂) at pH 7.0 by means of a phosphate buffer. CAT level was determined in absorbance at 240 nm and as nmol/mg protein [22].

Measurement of SOD parameter was demonstrated using the method of Marklund and Marklund (1974) by inhibiting autoxidation of pyrogallol. Enzyme activity at 440 nm was completed in 180 s (s). As a result, SOD measurement was determined as U/mg Hb [23].

Detection of MDA was made using the method developed by Draper and Hadley (1990). The reaction of MDA with TBA was done at pH 3, about 15 minutes and at 95 °C. In the measurement made at 532 nm, pink pigment was first obtained at maximum absorption. The measurement was then carried out by means of a spectrophotometer (UNICO Instruments C., Dayton, USA) [24].

Determination of the GSH level was carried out by the method of Ellman (1959). A reaction was observed in the environment with the chemicals added to the sample, and then a yellow-green color was formed. The measurement of the GSH level was carried out by means of a spectrophotometer at 410 nm absorbance [25].

Immunohistochemistry

Sections were passed through xylol and alcohol series after being placed in an oven at 60°C for 1 hour to ensure stronger adhesion of the tissues on the slide. In the removal of endogenous peroxidase, tissue sections were kept in 3% H₂O₂ (prepared with methanol) for 10 minutes and then washed with distilled water for 5 minutes. In our study, the antigen retrieval protocol was performed in accordance with previous studies [26, 27]. Immunohistochemical staining was performed using the Avidin-Biotin-Peroxidase Complex (ABC) technique. After the antigen retrieval protocol, sections were washed with PBS and incubated using 10% normal goat serum at room temperature (10 minutes) to prevent binding of non-specific antibody. To determine active (cleaved) caspase 3 (Casp-3) expression, tissue sections were incubated with anti-caspase 3 polyclonal primary antibody (cat. AB3623, lot 2387464) in a humidified chamber at 4 °C for 16-20 hours. Prior to this procedure, Casp-3 was diluted 1:10. The sections were then incubated with biotinylated secondary antiserum for 1 hour followed by streptavidin horseradish peroxidase for 1 hour at 37 °C in a humid environment and washed with PBS solution for 10 minutes before each incubation. Sections were then immersed in 3-amino-9-ethylcarbazole (AEC) chromogen substrate (5 minutes), washed with distilled water, stained with hematoxylin (30 seconds) and covered with mounting medium. Histopathological examinations were performed with an Olympus BX-53 microscope and photographic images were taken with a microscope camera (DP 80 Olympus, Tokyo, Japan).

Immunohistochemically positive stained preparations were examined by two different researchers. Immunostaining intensity was graded as negative (-), positive (+), intensive positive (++), and variable (+/-, ++ / -).

Statistical Analysis

Statistical analyzes were performed with SPSS version 20.0. Data are means \pm SEM. Normality was evaluated by Shapiro-Wilk test. Inter-group and intra-group comparisons of parametric values in biochemical parameters were performed by post-way one-way ANOVA after LSD. In addition, non-parametric values were determined with the Kruskal Wallis test. Afterwards, the Kruskal-Wallis test was used to make a semi-qualified evaluation of the histopathological scores. Measurements of differences in parameters between groups were performed with the Kruskal-Wallis test. Comparison of the paired groups was made using the Mann-Whitney U test. Values for $p \leq 0.05$ were considered statistically significant.

Results

Data on enzymatic activities are presented in Table 1. It was determined that there were differences in MDA, GSH levels and enzyme activities between different treatment groups.

MDA Level

When the PTX group was compared to the control group in determining the MDA level, it was observed that MDA increased significantly in the PTX group ($p < 0.05$). A significant decrease of MDA level was observed in the PTX

Table 1. Ovarian tissue oxidative stress biomarkers

	Control	PTX	PTX + MP
Ovarian tissue oxidative stress biomarkers			
SOD (U/g)	3.68 \pm 0.28 ^b	2.85 \pm 0.17 ^{a,c}	3.87 \pm 0.32 ^b
CAT (K/g)	2.27 \pm 0.51 ^b	1.85 \pm 0.19 ^{a,c}	2.85 \pm 0.35 ^b
GSH (μ mol/g)	1.12 \pm 0.14 ^b	0.95 \pm 0.14 ^{a,c}	1.27 \pm 0.25 ^b
MDA (nmol/g tissue)	0.52 \pm 0.18 ^b	0.78 \pm 0.17 ^{a,c}	0.48 \pm 0.16 ^b

Data are means \pm SEM, n = 7. PTX, paclitaxel; MP, misoprostol; MDA, malondialdehyde; GSH, glutathione; SOD, superoxide dismutase; CAT, catalase. ^aSignificant difference from control, ^bsignificant difference from PTX, ^csignificant difference from PTX + MP.

+ MP-treated group compared to the PTX-treated group ($p < 0.05$). As a result of MP treatment, a significant decrease was observed in the MDA level, which was previously increased by PTX (Table 1).

GSH Level

The GSH level significantly decreased in the PTX treated group compared to the control group ($p < 0.05$). A significant increase of GSH level was observed in the PTX + MP-treated group compared to the PTX-treated group ($p < 0.05$). In our study, a significant increase in GSH level was observed as a result of MP treatment (Table 1).

SOD Activity

SOD activity decreased in the PTX-treated group compared to the control group ($p < 0.05$). Additionally, SOD activity increased in the PTX + MP-treated group compared to the PTX-treated group ($p < 0.05$), (Table 1).

CAT Activity

CAT activity significantly decreased in the PTX-treated groups compared to the control group ($p < 0.05$). Additionally, CAT activity increased in the PTX + MP-treated group compared to the PTX-treated group ($p < 0.05$), (Table 1).

Histopathology

Our histopathological findings are shown in Figure 1. The histological structures of primordial, primary, secondary and tertiary follicles and also stroma were found to be normal in the ovaries of the control group. Histological structure of the stroma was normal in the PTX group rats. In this group, although the histological structure and distribution of primordial follicles are normal, in multilaminar primary follicles, secondary follicles and Graafian follicles; degeneration, desquamation and pycnotic nucleus were observed in the granulosa cells. In addition, the ratio of follicles other than primordial follicles in the rat ovaries in this group seemed to have decreased compared to the control group. However, it was observed that the use of MP slightly reduced the pathological changes in granulosa cells in the PTX group.

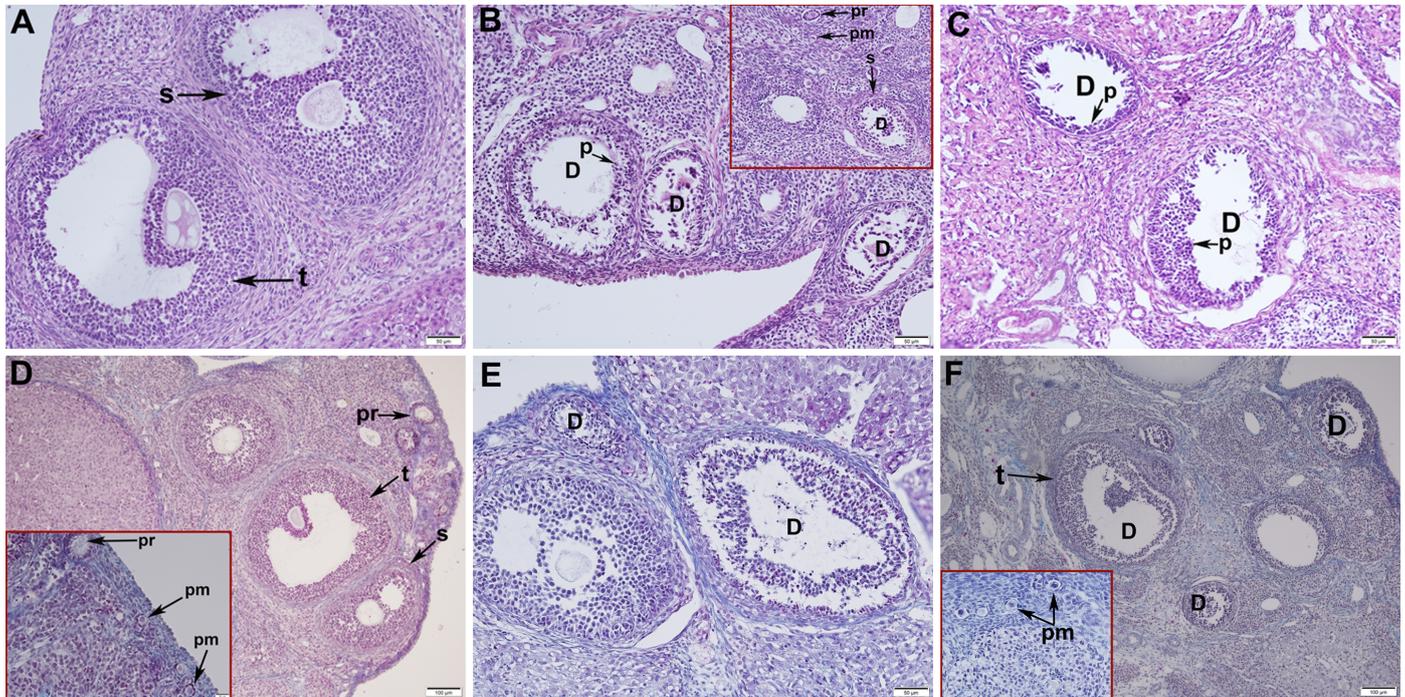


Figure 1. Light micrographs showing the control (A, D), PTX (B, E) and PTX + MP (C, F) groups' ovaries stained with hematoxylin-eosin (A, B, C) and Crossman's trichrome (D, E, F). Degeneration, desquamation and pycnotic nuclei were observed in the granulosa cells of multilayered primary follicles, secondary follicles, and tertiary follicles (in the PTX and PTX + MP groups). pm: primordial follicle, pr: primer follicle, s: sekunder follicle, t:tersiyer follicle, D: desquamation in granulosa cells, p: pycnotic nucleus.

Immunohistochemistry

Immunohistochemical observations were shown in Table 2 and Figure 2. Although active (cleaved) Casp-3 positive immunostaining was observed in theca and granular cells of tertiary follicles and some sekunder follicles in ovarian tissues of control group rats, primordial, primary and some secondary follicles were negative. In PTX and PTX + MP group rats, active Casp-3 positive immunoreaction was observed in theca and granulosa cells of other follicles except for primordial and unilayer primary follicles. The intensity and diffusivity of positive staining in these groups was higher than in the control group. Although cleaved Casp-3 positive immunostaining was observed in some of the stomal cells of all three groups, the ratio of positively stained cells in the PTX and PTX + MP groups and the staining intensity in these cells were excessive than in the control group. Positive immunostaining rate and intensity, observed in theca and granulosa cells of multilayered primary follicles and seconder follicles, and stromal cells of the ovary was slightly decreased in PTX + MP group compared to PTX group. These results, which showed that the rate of apoptosis in the PTX + MP group increased compared to the control group and slightly decreased compared to the PTX group, were consistent with the data obtained as a result of H&E and CT staining.

Discussion

The present study was undertaken to determine whether a chemotherapeutic agent such as PTX has adverse effects on ovarian damage, and whether an antioxidant drug such as MP can protect against this damage. Histological and

Table 2. Immunohistochemical localization of cleaved Casp-3 in ovarian tissue

Target stained immunohistochemically	Experimental groups		
	Control	PTX	PTX + MP
Primordial follicles	-	-	-
Unilayer primer follicles	-	-	-
Multilayer primer follicles			
-Theca cells	-	+/-	-
-Granulosa cells	-	++/-	+/-
Sekunder and tertiary follicles			
-Theca cells	+/-	++/-	++/-
-Granulosa cells Stroma	+/-	++/-	+/-

n = 7. PTX, paclitaxel; MP, misoprostol. a PTX increased ovarian damage vs. control. b MP reduced ovarian damage vs. PTX.

*Symbols are as follows: ++ = strong, + = moderate, - = negative, ++/-, +/- = variable, NE = not examined

biochemical changes on ovarian tissue in rats were evaluated (28). Supplying PTX + MP in combination allowed an assessment of the effectiveness of MP as a protective agent against ovarian damage induced by PTX.

Antineoplastic drugs are used in the treatment of many different tumors in scientific research. In addition, they are generally toxic to the gonads and may cause ovarian failure in patients (29). Therefore, the prevention of chemotherapy-induced gonadotoxicity caused by PTX has become a problem to be solved. In previous scientific stud-

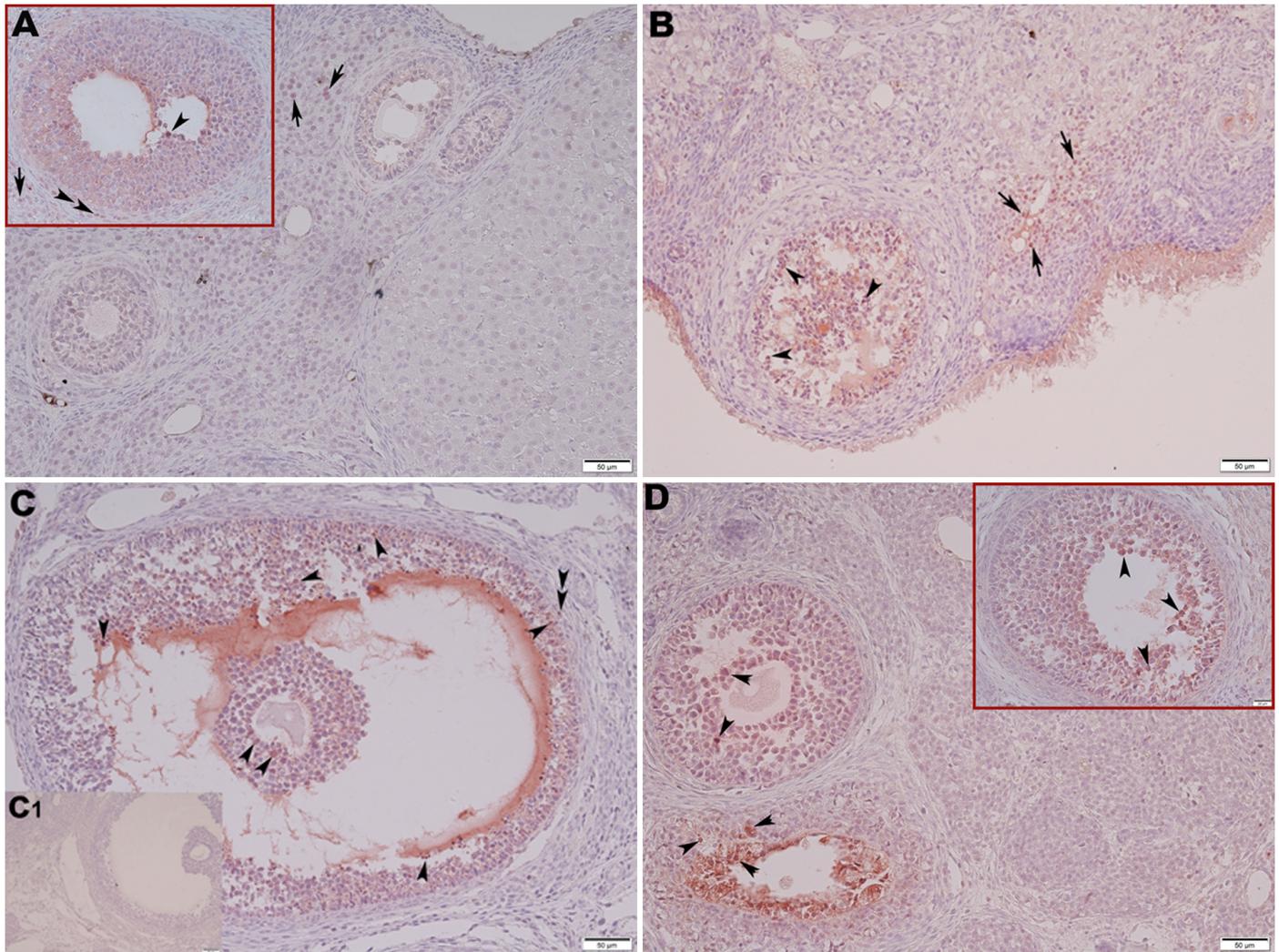


Figure 2. immunohistochemical localisation of active (cleaved) Casp-3 in control (A), PTX (B, C) and PTX + MP (D) groups' ovaries. C1: negative controls, Arrowhead: positive granulosa cells, double arrowhead: positive theca cells, arrow; positive stromal cells.

ies, it has been revealed that PTX leads to an increase in many functional disorders in the ovaries. At the same time, it has been reported that treatments have serious effects on patients' health, treatment processes and treatment costs [12]. In this study, it was shown that PTX caused a certain degree of toxicity in rat ovaries. The most serious side effect of PTX is that it leads to the production of oxygen radicals that are harmful to cells [4]. Additionally, PTX may damage the antioxidant defense system, which will lead to more effective oxidative damage in the ovaries [30]. In addition, it has been seen in studies on the ovaries that they can be more easily affected by PTX-induced oxidative stress as a result of lower antioxidant enzyme and GSH levels when compared to different organs [4]. In studies, antioxidants are mostly used to prevent the undesirable effects of PTX by preventing oxidative stress [31]. In our study treatment with MP reduced the negative biochemical effects that PTX produces on the ovaries. Additionally, we assume that these are modulated by the antioxidant and immunoregulatory effects of MP [16].

In our study, we determined MDA, one of the commonly used indicators of lipid peroxidation, in the evaluation of

oxidative stress and tissue damage caused by lipid peroxidation. As a result of experimental studies, it has been revealed that oxidative stress is effective in the development and evolution of ovarian damage. As a result of the therapy we applied in our study, it was determined that the efficiency of lipid peroxidation and oxidative stress decreased significantly in the group treated with MP. Therefore, MP can ameliorate PTX-induced ovarian damage through its protective and antioxidant mechanisms. This damage, possibly caused by oxidative stress, may be due to reactive intermediates of PTX. In the previous studies, harmful radicals inhibit the activity of the antioxidant enzymes in ovarian tissues with increased lipid peroxidation and oxidative stress [32]. Similar studies in the literature have shown that MP reduces MDA production [18].

Antioxidant enzymes are thought to have a functional role in preventing oxidative damage in cells. In many studies, it has been shown that the increased enzyme activities as a result of antioxidant treatment are reversed [33]. As a result of experimental studies, it has been revealed that SOD and CAT activities and GSH levels should be increased in order to prevent damage to the ovaries. Enzymes with

this protective function in the cell are known as the best method of protection from damage [34]. However, in cases of significant oxidative stress of cells, antioxidant mechanisms may be damaged. In this case, there may be a decrease in the activity levels and gene expression of antioxidants. In previous similar studies, it was determined that PTX application resulted in a decrease in SOD, CAT and GSH levels in tissues [31]. These decreased antioxidant enzyme activities in ovarian tissues are estimated to be the result of damage caused by PTX. In our study, the presence of decreased GSH level in the PTX group is accepted as an indicator of increased oxidative damage as a result of PTX treatment. In addition, as a result of MP treatment, an increase was observed in SOD, CAT and GSH levels in the MP + PTX group. For this reason, we found that the MP treatment we applied prevented the damage caused by PTX-induced oxidative reactions in the ovaries [35].

Gücer et al. (2001) and Yucebilgin et al. (2004) reported that PTX significantly reduced the number of primordial follicles [36, 37]. Ozcelik et al. (2009) stated that PTX significantly reduced the number of primordial follicles, decreased the number of primary and secondary follicles although not statistically significant, and almost never affected the number of tertiary follicles [11]. However, Tarumi et al (2009) stated that exposure to PTX increases apoptosis in antral follicles, decreases the number of antral follicles, and does not affect the number of primordial and pre-antral follicles [38]. Bildik et al. (2015) declared that the use of PTX does not affect the number of primordial follicles, but reduces the number of antral and preantral follicles [9]. In addition, they reported that PTX has toxic effects on granulosa cells but not stromal cells. Lopez et al. (2014) reported that docetaxel, a taxane, does not affect the number of primordial follicles, but causes a decrease in the number of growing follicles, especially primary follicles [12]. In addition, they reported that PTX exerts its negative effects on follicles mainly by damaging granulosa cells. They also reported that they detected pycnosis in granulosa cells and cleaved Casp-3 positive immunohistochemical staining was observed in both granulosa cells and stromal cells, and these findings were indicative of the increase in apoptosis, that is, damage to granulosa cells. As can be seen, it has been reported in previous studies examining the effects of PTX on the ovary that PTX affects especially primordial follicles, whereas in more comprehensive recent studies including molecular analyzes, PTX has been reported to affect tertiary and growing follicles. The results obtained in the present study regarding the effect of PTX on the ovary are in line with the results of many recent studies [9, 38, 12].

MP is a drug mainly used to prevent stomach ulcers and initiate birth. This drug has also been reported to have antioxidant, cytoprotective and antiapoptotic effects [38]. Bilgic et al. (2019, 2020) declared that MP reduces doxorubicin-induced liver and heart damage due to its antioxidant and antiapoptotic effects [39,36]. In the present study, it was determined that MP administration slightly reduced the pathological changes formed in PTX-induced granulosa cells and the intensity of cleaved Casp-3 positive immunostaining. In the evaluations, it was observed that

the positive changes observed in biochemical parameters in the PTX + MP group were not yet fully reflected in the histological structure at light microscopic level. Considering the results obtained, it is predicted that increasing the applied MP dose may increase the ameliorative effects on histological structure.

In recent experimental studies, MP has received great attention as a harmful radical scavenger in oxidative stress. Therefore, it is used for therapeutic purposes in the case of oxidative stress in many tissues and organs. In addition to antioxidants, MP also has many other properties such as antiapoptotic and cytoprotective effects [40].

Conclusion

In this study, we evaluated the protective effect of MP against damage to ovarian tissue caused by the side effects of PTX. In this experimental animal study, we found that MP decreased MDA level and increased protective antioxidant enzyme activities in rat ovaries due to PTX-induced damage. For all these reasons, MP can be used as a potential drug for the reduction and prevention of PTX-induced complications in experimental and clinical applications.

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