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THE EFFECT OF ESTROGEN RECEPTOR AGONISTS ON PANCREATICOBILIARY DUCT LIGATION INDUCED EXPERIMENTAL ACUTE PANCREATITIS

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The 17 β -estradiol plays a role in physiology of pancreas and may protect it from inflammation. To examine the possible anti-inflammatory effects of 17 β -estradiol in pancreaticobiliary duct ligated (PBDL) acute pancreatitis (AP) model, and the underlying mechanism that 17 β -estradiol acts on, *via* evaluating the direct and the receptor related effects by using 17 β -estradiol, ER- α and - β agonists. In the study both sexes of rats (n = 88) were used. Animals were divided into two groups as PBDL and PBDL + ovariectomized. ER- α agonist propyl-pyrazole-triol (PPT; 1 mg/kg/day), ER- β agonist diethylpropionitrile (DPN; 1 mg/kg/day) and 17 β -estradiol (10 mg/kg/day) were administered to the groups for 3 days following AP induction. On the 3rd day, lung and pancreas tissues and serum samples were taken for malondialdehyde (MDA), glutathione (GSH), myeloperoxidase (MPO), superoxide dismutase (SOD) and interleukin (IL) assays, and histological analyses. In both tissues of male and female AP groups MPO, MDA, SOD levels were increased (P < 0.05 – 0.01) and GSH levels were decreased (P < 0.05). Pancreas and lung MDA and SOD levels were improved with all treatments in female, except lung MDA levels of PPT-treated ones, while lung MDA and SOD levels were improved by PPT and 17 β -estradiol in females and *via* PPT in males (P < 0.05 – 0.001). The increased MPO levels were inhibited with PPT in male pancreas and female lung and with 17 β -estradiol in female pancreas (P < 0.05). The increased pro-inflammatory ILs were declined by treatments (P < 0.05 – 0.001). 17 β -estradiol and ER- α and - β agonists reduced oxidative pancreatic and pulmonary damage. Estrogen and agonists might have protective role in AP.

Key words: *acute pancreatitis, estrogen receptors, pancreaticobiliary duct ligation, oxidative stress, inflammation, 17 β -estradiol, proinflammatory cytokines*

INTRODUCTION

Acute pancreatitis (AP) is a widespread and highly lethal disease that is characterized by a reversible damage in the pancreas and the tissues adjacent to the pancreas in the form of oedema and necrosis, however sometimes by multi-organic complications such as shock, sepsis, metabolic disorders, and death as well (1). AP still constitutes a main problem for contemporary medicine. It results from the fact of diagnosis-related difficulties and from the lack of an effective therapy of the severe course of acute pancreatitis. In the cases of severe acute pancreatitis with multi-organic complications, micro-circulation disorders, primarily affecting lung, liver, digestive tract and circulatory system, and Systemic Inflammatory Response Syndrome (SIRS) and Multi-Organ Dysfunction Syndrome (MODS) may develop. Although the pathophysiology is not fully understood, there have been fundamental changes in the pathogenesis and treatment of acute pancreatitis in the last 10 years (2). While the old concept of disease was completely based

on the tissue damage *via* the activation of proteases, it was later stated that they had been only the precursor of the disease (3, 4), indeed, recent studies showed the importance of cytokines and agonists of estrogen in the inflammatory response of AP (5).

17 β -estradiol is an ovarian estrogen that has an important role in the physiology of the endocrine pancreas (6). It also protects human pancreatic islets from inflammation and apoptosis (7). Estrogens act on alpha (α) or beta (β) receptors in their variant activities in different tissues (8). In recent studies, it has been shown that alpha receptor of estrogen (ER- α) had effective roles in diabetes mellitus (DM), which is postulated as an inflammatory status, and also has been indicated that the deficiency of ER- α had stimulated the disease in males (9). Additionally, 17 β -estradiol and its agonists were reported to have different underlying genetic mechanisms (10). In the guidance of this previous data, estradiol may prevent anti-inflammatory process in the pancreas tissue *via* ER- α (11). Actually, both estrogen receptors (ERs) can mediate anti-inflammatory actions; in fact ER- β is a more desirable therapeutic target. ER- β -

selective agonists show their anti-inflammatory effects *via* repression on the transcription of pro-inflammatory genes. Although, the molecular mechanisms of their anti-inflammatory effects are not known yet, it was reported that ER- β was more potent than ER- α at repressing inflammatory parameters such as tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) (12, 13). As ER- β is more efficient in anti-inflammatory processes, it's probable effect in AP attracts attention.

Pancreatic enzymes may activate oxygen radicals. These reactive oxygen species (ROS) and their derivatives may be activated by direct or indirect routes in acute necrotizing pancreatitis resulting in the distribution of pro-enzymes following destruction of acinary cells. ROS have been considered as an important factor in the pathogenesis and progress of pancreatitis and pulmonary complications (14). In the pancreatic tissue, besides enzymatic activation, and the production of TNF- α by acinar cells, the secretion of interleukins (ILs) by macrophages and monocytes is the primary inflammatory process in the pathogenesis of local and systemic inflammation. The production of pro-inflammatory substances by these cells results in amplification of the inflammation to distant organs such as lungs and gastrointestinal tract and might result in multi-organ failure (15). The mechanism of direct inhibition of ROS in patients with acute pancreatitis is not clear, however there is limited information about the presence of estrogen receptors in lung tissue and their role in respiratory tract, and also there is an evidence that the estrogen receptor deficiency may lead to progress of Acute Respiratory Distress Syndrome (ARDS), which may be one of the important complications of AP (16, 17).

Based on the aforementioned evidence, the study was designed to examine primarily the possible anti-inflammatory effects of 17 β -estradiol in pancreaticobiliary duct ligated acute pancreatitis model in both genders and to show the potential distant organ damage such as lung injury. Secondly, we aimed to examine the underlying mechanism that 17 β -estradiol acts on, *via* evaluating the direct and the receptor related effects by using 17 β -estradiol, ER- α and - β agonists.

MATERIALS AND METHODS

Animals

Sprague-Dawley rats of both sexes (250 – 00 g) were obtained from Marmara University Experimental Animals Research and Implementation Centre (DEHAMER). Before the experiment, the animals were fed with standard rat chow and given water *ad libitum* and housed in standard cages in a climate-controlled room with an ambient temperature of (22 \pm 2°C) and a 12-h light/dark cycles at humidity (65 – 70%).

Our study has been carried out in accordance with the Declaration of Helsinki and with the guidelines for the care and use of laboratory animals that is accepted by properly appointed and approved by the Marmara University Animal Care and Use Committee (44.2012.mar).

Surgery and experimental design

Female rats (n = 48, 250 – 300 g) were divided into two main groups: sham operated (n = 8) and PDBL (n = 40) group. PDBL group was further divided into subgroups as vehicle-treated (n = 8) and OVX (n = 32) groups. Moreover, OVX group was separated to subgroups according to treatments as vehicle, PPT, DPN, and 17 β -estradiol groups.

Male rats (n = 40, 250 – 300 g) were grouped as sham operated and PDBL groups and PDBL group was further

divided into subgroups according to treatments. Each group consisted of 8 rats. Animals were anesthetized with ketamine and chlorpromazine (100 and 0.75 mg/kg; intraperitoneally, i.p.) and the abdomen was opened *via* midline laparotomy. The surgery was applied to the animals in the sham group with similar operative procedures, however pancreaticobiliary duct was not ligated. For the acute pancreatitis induction pancreaticobiliary duct ligation (PBDL) was performed in all groups except sham group. For PBDL, the common pancreaticobiliary duct was doubly ligated (PBDL; n = 8) with 5/0 silk (Ethicon, Edinburgh) adjacent to the duodenal wall. In a part of rats (n = 8), peritoneal cavity was exposed *via* a ventral abdominal incision and the ovaries in females were gently manipulated. Before the operation of PBDL some of female rats (n = 32) were subjected to bilateral ovariectomy and for the stabilization of hormone levels, animals were kept for 3 weeks in their cages. Following PBDL, estrogen receptor agonists, such as ER- α agonist propyl-pyrazole-triol (PPT; 1 mg/kg/day), ER- β agonist diarylpropionitrile (DPN; 1 mg/kg/day) and 17 β -estradiol (10 mg/kg/day) were dissolved in olive oil and administrated intraperitoneally for 3 days. After the postoperative 3th day, rats were decapitated and trunk blood was collected for the measurements of serum TNF- α , IL-1 β , IL-6 and IL-10 levels. The pancreas and lung tissues were immediately removed and tissue samples were stored at -80°C for the determination of MDA and GSH levels as well as MPO and SOD activities. For histological analysis, samples of the tissues were fixed in 10% buffered p-formaldehyde and were prepared for routine paraffin embedding.

Measurement of myeloperoxidase (MPO) activity

MPO is an enzyme that is found predominantly in the azurophilic granules of polymorphonuclear (PMN) leukocytes. Tissue MPO activity is frequently utilized to estimate tissue PMN accumulation in inflamed tissues and correlates significantly with the number of PMN determined histochemically in tissues. MPO activity was measured in the tissue samples using a procedure similar to that documented by Hillegeass *et al.* All reagents for MPO assay were obtained from Sigma (St Louis, MO, USA). Lung and pancreatic tissues were homogenized in 10 volumes of ice-cold potassium phosphate buffer (50 mM K₂HPO₄, pH 6.0) containing hexadecyltrimethylammonium bromide (HETAB; 0.5% w/v). The homogenate was centrifuged at 12,000 g for 10 min at 4°C, and the supernatant was discarded. The pellet was then re-homogenized with an equivalent volume of 50 mM K₂HPO₄ containing 0.5% (w/v) HETAB and 10 mM ethylenediaminetetraacetic acid. MPO activity was assessed by measuring the H₂O₂-dependent oxidation of o-dianizidine·2HCl. One unit of enzyme activity was defined as the amount of MPO present that causes a change in absorbance of 1.0 unit/min at 460 nm and 37°C and was expressed in units per g tissue (18).

Measurement of malondialdehyde (MDA) and glutathione (GSH) levels

Oxygen free radicals cause peroxidation of lipids on membrane structure. Lipid peroxides cause serious membrane damage, organelle and cell damage by stimulating autocatalytic chain reaction. The most well-known end product of lipid peroxidation is MDA. In the present study, lipid peroxidation is evaluated by measuring MDA levels in pancreas and lung tissues. The samples were homogenized in 10 volumes of ice-cold 10% trichloroacetic acid and centrifuged at 3000 rpm for 15 min at 4°C. Supernatant was removed and recentrifuged at

15,000 rpm at 4°C for 8 min. Glutathione was determined by a spectrophotometric method which is a modification of Ellman procedure (19). Lipid peroxide levels were expressed in terms of MDA equivalents as nmol MDA/g tissue (20).

Measurement of superoxide dismutase (SOD)

SOD is the enzyme which catalyzes the dismutation of oxygen and hydrogen peroxide. SOD is an important antioxidant in the cell for the main reactive oxygen species of superoxide. Superoxide formed by the effect of riboflavin fluorescence light is converted to hydrogen peroxide under the influence of the SOD. H₂O₂ generates the color by reaction with o-dianisidine product. Determination of SOD activity depends on the formation of colored products. The lung samples were homogenized in 10 volumes and pancreas tissues were homogenized 1000 volumes and resulting colored products were measured at the 460 nm absorbance for lung and pancreatic tissues by spectrophotometrically. Superoxide dismutase levels were expressed as U/ml protein.min (21).

Measurement of serum cytokines

TNF- α , IL-1 β , IL-10, IL-6 levels were evaluated by ELISA (enzyme linked immunosorbent assay) method. All samples were assayed in triplicates using the commercial kits (BMS630, BMS629, BMS625) (Invitrogen, California, USA) and the values were expressed as pg/ml.

Histological analysis

For light microscopic investigations, pancreas and lung specimens were fixed in 10% formaldehyde and processed routinely for embedding in paraffin and experienced by a histologist who was unaware of the treatment conditions. The paraffin-embedded pancreas and lung tissues were cut at (5 μ m-thick) transversally and then were stained with Hematoxylin & Eosin (H&E) to examine the general morphology of lung and pancreas tissue microtomes, and were examined under an Olympus BH-2 photomicroscope (Tokyo, Japan). The histological scores were the combined scores of different criteria's graded from 0 to 3 (0: none, 1: mild, 2: moderate, 3: severe). The severity of pancreatitis was graded using the following criteria: (a) acinar cell degeneration, (b) interstitial edema, (c) leukocyte infiltration and (d) vasocongestion, yielding a maximum score of 12. Scoring

of the lung injury was done by grading the following criteria from 0 to 3: (a) vascular congestion, (b) interstitial edema, (c) alveolar structural disturbance, (d) leukocyte infiltration, and (e) general edema, giving a maximum score of 15 (22).

Statistical analysis

Statistical analysis was done using a Graph Pad Prism 5.0 (Graph Pad Software, San Diego, CA, USA). All data were expressed as means \pm S.E.M. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Values of two groups were compared by Student's test. Histological examination scores were evaluated by Mann-Whitney U test. Values of P < 0.05 were considered as significant.

RESULTS

The pro-inflammatory interleukins such as IL-1 β and IL-6 were increased *via* acute pancreatitis in both genders compared to sham operated groups (P < 0.05 – 0.001), additionally IL-10 levels were risen in males and TNF- α levels were increased in females with acute pancreatitis (P < 0.05 – 0.01) (Table 1 and 2). The risen IL-1 β levels were decreased back to control levels with DPN in males and with PPT in females (P < 0.05 – 0.01), moreover the increase in IL-6 levels were inhibited with both estrogen agonists in males and with DPN and 17 β -estradiol in females (P < 0.05 – 0.01), and the increased TNF- α levels in females were suppressed with PPT (P < 0.05). The increased IL-10 levels *via* acute pancreatitis were declined by 17 β -estradiol in males (P < 0.01). Although the increased IL-1 β levels with acute pancreatitis were suppressed by estrogen receptor agonists, DPN and PPT, *via* different receptor subtypes in different genders, DPN had a suppressive effect on IL-6 levels of both genders.

MDA levels indicating lipid peroxidation of pancreatic and lung tissues were increased in both genders with acute pancreatitis (P < 0.05 – 0.01) (Fig. 1A-1D). In females, all treatments improved the MDA levels of pancreatic tissue and DPN and 17 β -estradiol decreased the MDA levels of the lung tissue (P < 0.05 – 0.001) compared to both PBDL and PBDL + OVX groups, though in males PPT and 17 β -estradiol diminished the MDA levels of lung tissue (Fig. 1A, 1C and 1D).

As an indicator of tissue neutrophil infiltration, MPO activity of the acute pancreatitis group in both female and male

Table 1. Serum cytokine levels of female rats.

Groups	SHAM-OPERATED	PBDL n = 40				
	VEHICLE n = 8	VEHICLE n = 8	OVX n = 32			
			VEHICLE n = 8	DPN n = 8	PPT n = 8	17 β -estradiol n = 8
IL-1 β (pg/ml)	70.19 \pm 4.6	323.5 \pm 23.7 ⁺⁺⁺	329.1 \pm 10.1 ⁺⁺⁺	393.2 \pm 21.9 ⁺⁺⁺	141.7 \pm 44.2 ^{***}	193.5 \pm 43.4 ⁺
IL-6 (pg/ml)	182.1 \pm 21.85	885.6 \pm 29.38 ⁺	921.5 \pm 55.92 ⁺	111.7 \pm 66.01 [*]	878.9 \pm 331.7 ⁺	216.8 \pm 103.5 [*]
IL-10 (pg/ml)	888.9 \pm 74.05	851.3 \pm 73.5	872 \pm 42.5	710.3 \pm 56.77	846.2 \pm 41.75	641 \pm 62.23 ⁺
TNF- α (pg/ml)	31.14 \pm 0.16	83.55 \pm 5.25 ⁺	107.6 \pm 7.16 ⁺⁺	117.4 \pm 10.01 ⁺	31.11 \pm 0.18 ⁺	75.15 \pm 21.52

Values were compared with Student's t-test versus corresponding controls. *P < 0.05, **P < 0.01, ***P < 0.001; compared to vehicle-treated sham-operated group. *P < 0.05, ***P < 0.001; compared to vehicle-treated PBDL group. PBDL, pancreaticobiliary duct ligated; OVX, ovariectomy; DPN, diethylpropionitrile; PPT, propyl-pyrazole-triol. Each consist of 8 animals.

Table 2. Serum cytokine levels of male rats.

Groups	SHAM-OPERATED	PBDL n = 32			
	VEHICLE n = 8	VEHICLE n = 8	DPN n = 8	PPT n = 8	17β-estradiol n = 8
IL-1β (pg/ml)	79.86 ± 6.4	398.8 ± 63.8 ⁺⁺	68.83 ± 15.86 [*]	212.3 ± 76.74	264.8 ± 73.52
IL-6 (pg/ml)	1331 ± 118.1	1971 ± 15.89 ⁺⁺	1298 ± 204.6 ^{**}	1199 ± 77.15 ^{**}	1650 ± 128.1
IL-10 (pg/ml)	418 ± 92.22	856.3 ± 49.18 ⁺⁺	743.4 ± 122.2	945.1 ± 23.37 ⁺⁺⁺	377.5 ± 88.04 ^{**}
TNF-α (pg/ml)	42.92 ± 11.62	77.64 ± 18.25	40.99 ± 9.68	43.24 ± 11.94	215.9 ± 121.6

Values were compared with Student's t-test versus corresponding controls. ⁺⁺P < 0.01, ⁺⁺⁺P < 0.001; compared to vehicle-treated sham-operated group. ^{*}P < 0.05, ^{**}P < 0.01; compared to vehicle-treated PBDL group. PBDL, pancreaticobiliary duct ligated; OVX, ovariectomy; DPN, diethylpropionitrile; PPT, propyl-pyrazole-triol. Each consist of 8 animals.

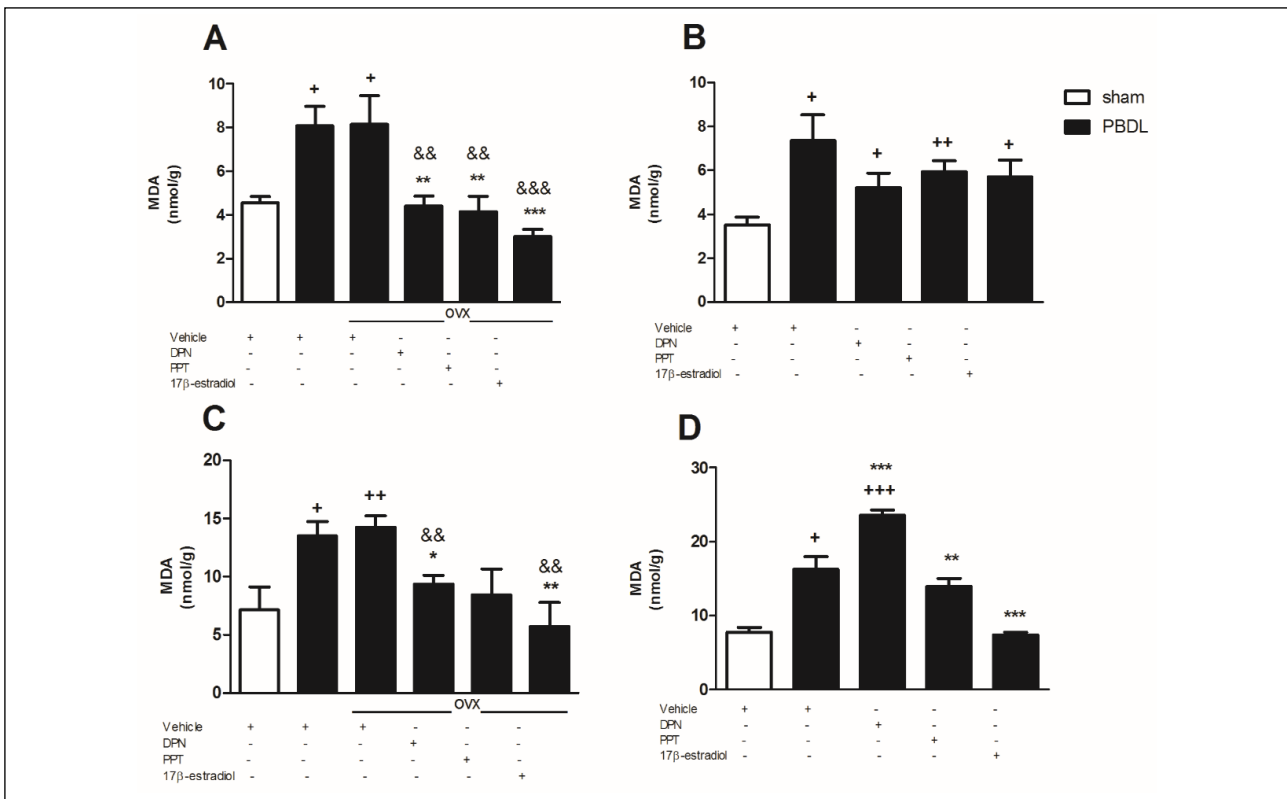


Fig. 1. Malondialdehyde (MDA) levels in pancreas tissues of female (A) and male (B) rats and lung tissues of female (C) and male (D) rats, which were either sham operated or pancreaticobiliary duct ligated (PBDL) with or without ovariectomy (OVX), and which were vehicle-, DPN-, PPT-, or 17β-estradiol-treated. ^{*}P < 0.05, ⁺⁺P < 0.01, ⁺⁺⁺P < 0.001, compared to sham group; ^{*}P < 0.05, ^{**}P < 0.01, ^{***}P < 0.001, compared to PBDL group; ^{&&}P < 0.01, ^{&&&}P < 0.001, compared to PBDL + OVX group. Each group consists of 8 animals.

pancreatic tissues, as well as in the lung tissues of both genders were increased significantly (P < 0.05 – 0.01), and decreased by 17β-estradiol treatment in pancreatic tissue of females, although it was ameliorated by PPT treatment in males (P < 0.05) (Fig. 2A-2D). On the other hand, in lung tissue, MPO activity was weakened by PPT treatment compared to both PBDL and PBDL + OVX groups in female rats (P < 0.05 – 0.001), and was increased with all treatments versus to PBDL group in males (P < 0.05) (Fig. 2C and 2D).

As expected, PBDL ligation resulted in a depletion of antioxidant GSH levels of both male and female pancreatic and lung tissues when compared to sham group (P < 0.05) (Fig. 3A-

3D). Although, pancreatic GSH levels were also depleted in all treated groups of females and DPN-treated males when compared with PBDL group (P < 0.05 – 0.001), lung GSH levels were increased by DPN and 17β-estradiol treatments when compared to both PBDL and PBDL + OVX groups in females, while they were improved by PPT application in males compared to PBDL group (P < 0.01) (Fig. 3A-3D).

As an indicator of ROS scavenger system SOD activity was amplified in pancreatic tissue of the PBDL group of both genders and lung tissue of males compared to sham operated rats (P < 0.05 – 0.01) and was improved via PPT application in lung tissue males (P < 0.05) (Fig. 4A-4D). However, in the lung tissue

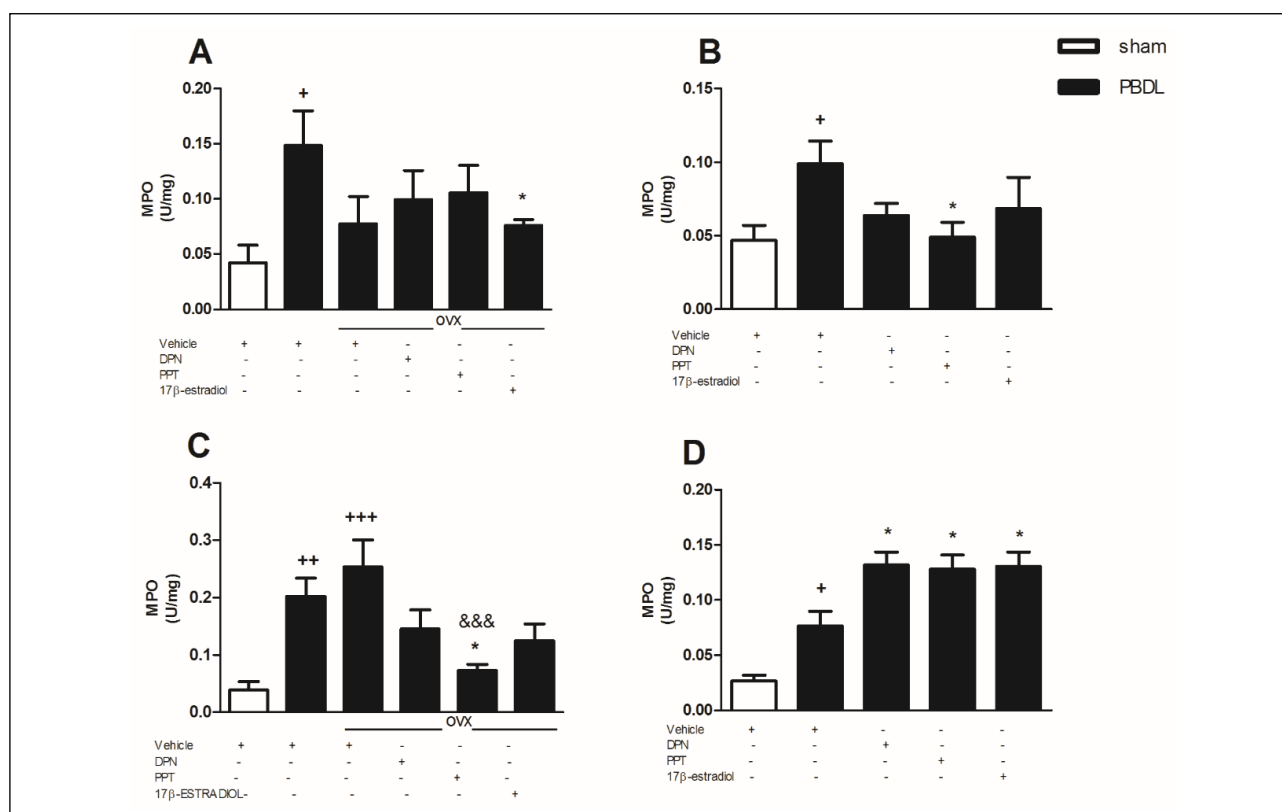


Fig. 2. Myeloperoxidase (MPO) activity in pancreas tissues of female (A) and male (B) rats and lung tissues of female (C) and male (D) rats, which were either sham operated or pancreaticobiliary duct ligated (PBDL) with or without ovariectomy (OVX), and which were vehicle-, DPN-, PPT-, or 17β-estradiol-treated. ⁺P < 0.05, ⁺⁺P < 0.01, ⁺⁺⁺P < 0.001, compared to sham group; ^{*}P < 0.05, compared to PBDL group; ^{&&&}P < 0.001, compared to PBDL + OVX group. Each group consists of 8 animals.

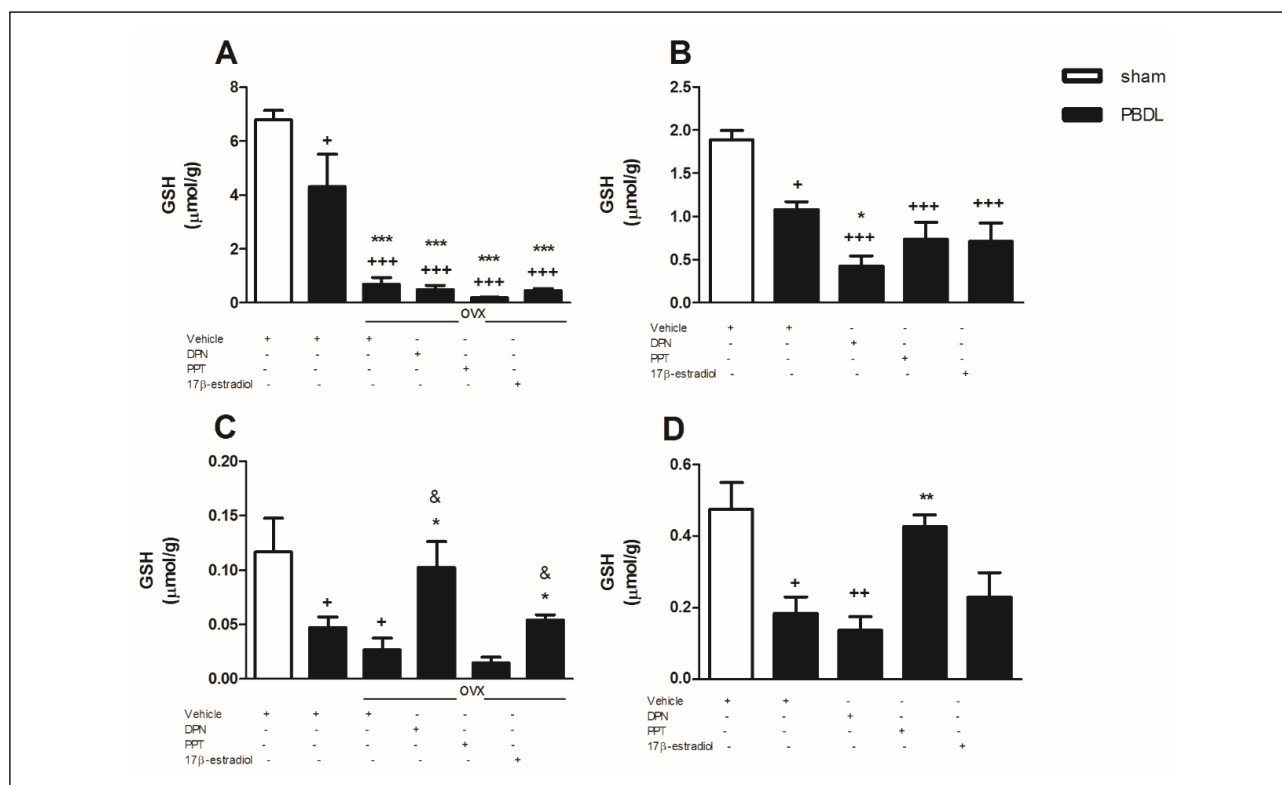


Fig. 3. Glutathione (GSH) levels in pancreas tissues of female (A) and male (B) rats and lung tissues of female (C) and male (D) rats, which were either sham operated or pancreaticobiliary duct ligated (PBDL) with or without ovariectomy (OVX), and which were vehicle-, DPN-, PPT-, or 17β-estradiol-treated. ⁺P < 0.05, ⁺⁺P < 0.01, ⁺⁺⁺P < 0.001, compared to sham group; ^{*}P < 0.05, ^{**}P < 0.01, ^{***}P < 0.001, compared to PBDL group; [&]P < 0.05, compared to PBDL + OVX group. Each group consists of 8 animals.

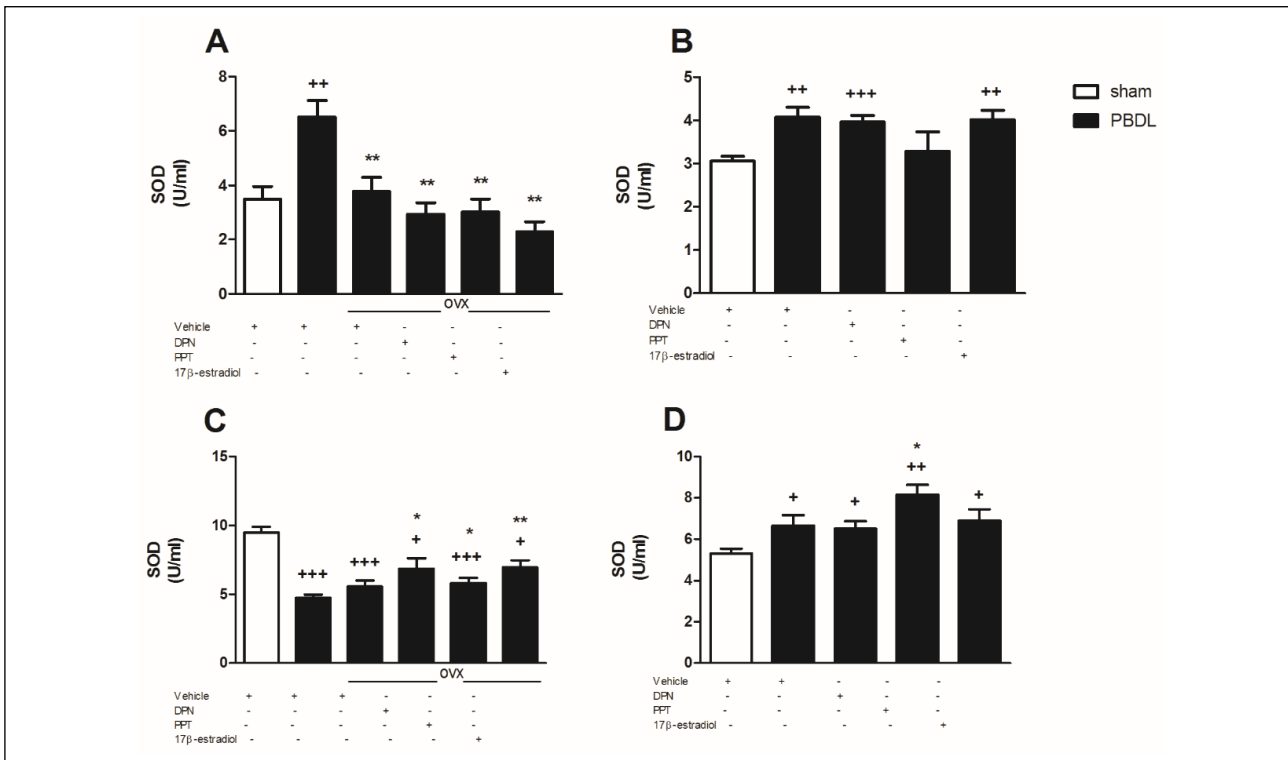


Fig. 4. Superoxide dismutase (SOD) activity in pancreas tissues of female (A) and male (B) rats and lung tissues of female (C) and male (D) rats, which were either sham operated or pancreaticobiliary duct ligated (PBDL) with or without ovariectomy (OVX), and which were vehicle-, DPN-, PPT-, or 17 β -estradiol-treated. +P < 0.05, ++P < 0.01, +++P < 0.001, compared to sham group; *P < 0.05, **P < 0.01, compared to PBDL group. Each group consists of 8 animals.

of females, the SOD activity was attenuated with PBDL and PBDL + OVX compared to sham group ($P < 0.001$), and increased *via* all treatments ($P < 0.05 - 0.01$) when compared to PBDL group (Fig. 4C).

According to histological analyses of female and male rats, normal acinar structures were observed in the pancreas tissue of sham-operated groups (Figs. 5a and 6a). However, PBDL (Figs. 5b and 6b) and PBDL + OVX (Fig. 5c) groups showed severe injury in the pancreatic acini, additionally vasocongestion, and inflammatory cell infiltration through the pancreatic tissue. DPN (Figs. 5d and 6c), PPT (Figs. 5e and 6d), or 17 β -estradiol (Figs. 5f and 6e) treatments significantly reduced the histopathological injury and damage scores (Fig. 7).

However, besides normal appearing acini, there were also injured acini and vasocongestion in the treatment groups (Fig. 6c, 6d and 6e). Inflammatory cell infiltration was still evident in the 17 β -estradiol group. When the lung tissue was analyzed as a distant organ, sham-operated groups showed normal alveolar structures in the lung tissues of both female (Fig. 5g) and male (Fig. 6f) animals. Although PBDL (Figs. 5h and 6g) and PBDL + OVX (Fig. 5i) groups showed severe lung injury resulting in high damage scores, DPN (Figs. 5j and 6h), PPT (Figs. 5k and 6i), or 17 β -estradiol (Figs. 5l and 6j) treatments significantly reduced the pulmonary injury scores (Fig. 7).

DISCUSSION

The present study was undertaken to evaluate the possible anti-inflammatory effect of estrogen in pancreaticobiliary duct ligated acute pancreatitis model and to show the potential distant organ damage such as lung injury. Secondly, our aim was to examine the underlying mechanism of estrogen acts on *via* evaluating the direct effects of 17 β -estradiol and *via* showing the receptor related effects

by using ER- α and ER- β agonists. The results demonstrate that pathogenesis of acute pancreatitis does not only involve the oxidative damage of the pancreas and pancreatic tissues, as assessed by increased MPO activity and elevated MDA levels and reduced GSH levels and raised SOD activity, additionally the lungs are also challenged by the oxidant injury. Furthermore, increased serum pro-inflammatory cytokines as a marker of systemic response were verified. Estrogen and/or its receptor agonists protected affected tissues by reducing oxidative tissue damage, which appears to involve an inhibitory effect on tissue neutrophil infiltration and lipid peroxidation in the pancreas and lung tissues, and by improving antioxidant system in the lung tissue, and by suppressing the systemic pro-inflammatory response, moreover by ameliorating the histopathological changes such as vasocongestion, and inflammatory cell infiltration in pancreatic acini and distant organ damage that occurred in the lung tissue.

It is well known that severe form of acute pancreatitis is characterized by the development of remote organ injury, which is reported to result in high mortality rates (23). In this injury, besides the direct damaging effects of free radicals on tissues, it is well established that they induce the attraction, adhesion and migration of leukocytes, such as polymorphonuclear cells (PMN), through the affected tissues (24). PMNs worsen the tissue injury by activating cytotoxic enzymes, including myeloperoxidase, elastase, proteases and lactoferrin (25). Therefore, in this study MPO activity was evaluated as an index of tissue neutrophil infiltration. Previously the increased MPO activity indicating the increased neutrophil infiltration through the pancreas and lung tissues in PBDL induced acute pancreatitis model was reported (26). Our results support these previous findings that included exacerbated MPO activity in pancreatic and lung tissues *via* acute pancreatitis induction. Furthermore, according to our results, pancreatic MPO levels were decreased

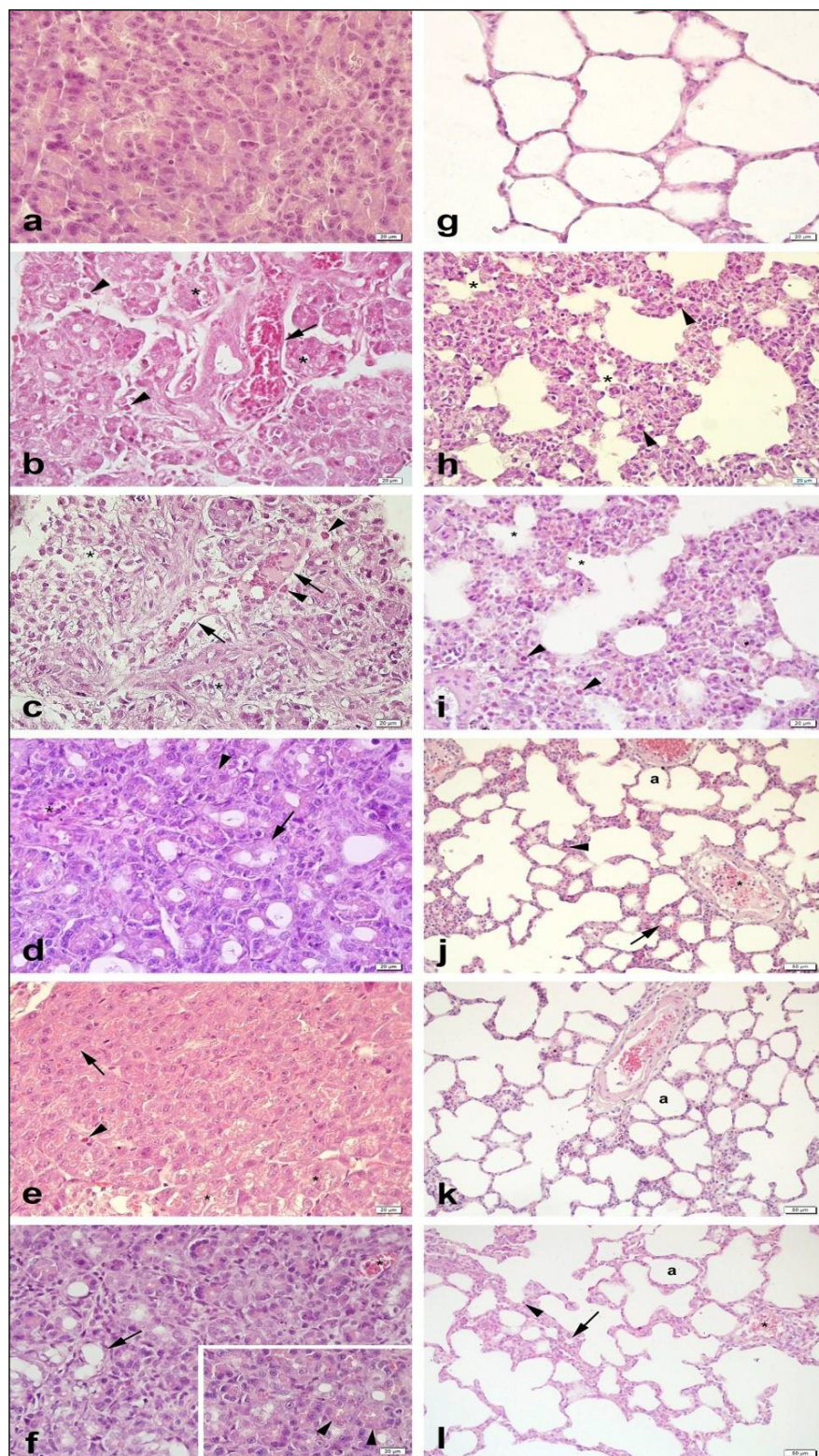


Fig. 5. Histological analyses of female pancreas (panels a-f) and lung (panels g-l) tissues. Normal appearing acinar cell structure in pancreas of sham-operated group (panel a). Severe acinar cell injury (asterisk), vasocongestion (arrow) and inflammatory cell infiltration (arrowhead) in PBDL (panel b) and PBDL + OVX (panel c) groups. Normal (arrowhead) and damaged (arrow) acini and vasocongestion (asterisk) in PBDL + OVX + DPN group (panel d). Normal appearing acini (arrow), injured acini (asterisk) and inflammatory cell (arrowhead) in PBDL + OVX + PPT group (panel e). Injured structures (arrow) and vasocongestion (asterisk) in PBDL + OVX + 17 β -estradiol group (panel f). Inset: Normal appearing acini (arrowhead). Normal appearing alveolar structures in the lung tissue in the sham-operated group (panel g). Severely affected alveoli (black asterisk), thickening in the interstitium (white asterisk) and inflammatory cell infiltration (arrowhead) in PBDL (panel h) and PBDL + OVX (panel i) groups. Normal (a) and injured (arrow) alveolar structures, vasocongestion (asterisk), and inflammatory cell infiltration (arrowhead) in PBDL + OVX + DPN group (panel j). Normal alveolar structures (a) and mild hemorrhage in the interstitium (asterisk) in PBDL + OVX + PPT group (panel k). Normal (a) and injured (arrow) alveoli, inflammatory cell infiltration (arrowhead), and vasocongestion (asterisk) in PBDL + OVX + 17 β -estradiol group (panel l). Hematoxylin and eosin staining. Each group consist of 8 animals.

by 17 β -estradiol treatment in females and by PPT treatment in males, and in lung tissue the activity was attenuated by PPT treatment in females. These results suggest that the protective effect of 17 β -estradiol and estrogen agonist on tissue neutrophil infiltration may be the direct effect of estrogen or by α -receptor depending on organ type and gender. Although, estrogen was reported to attenuate the lung injury in a rodent model of cerulein-induced acute pancreatitis, in our study the direct and receptor mediated effects of estrogen were investigated (27).

The elevated MDA levels of pancreas and lung tissues indicate increased lipid peroxidation levels which may act on ion permeability of cell membrane and may change activation of enzymes. Similar with our results, previous studies reported the increased MDA levels in taurocholate- and caerulein- induced acute pancreatitis models in rats (28, 29).

According to our results, the increased MDA levels were reduced by estrogen agonists and 17 β -estradiol treatments in female pancreas tissues, and this increase was continued in male

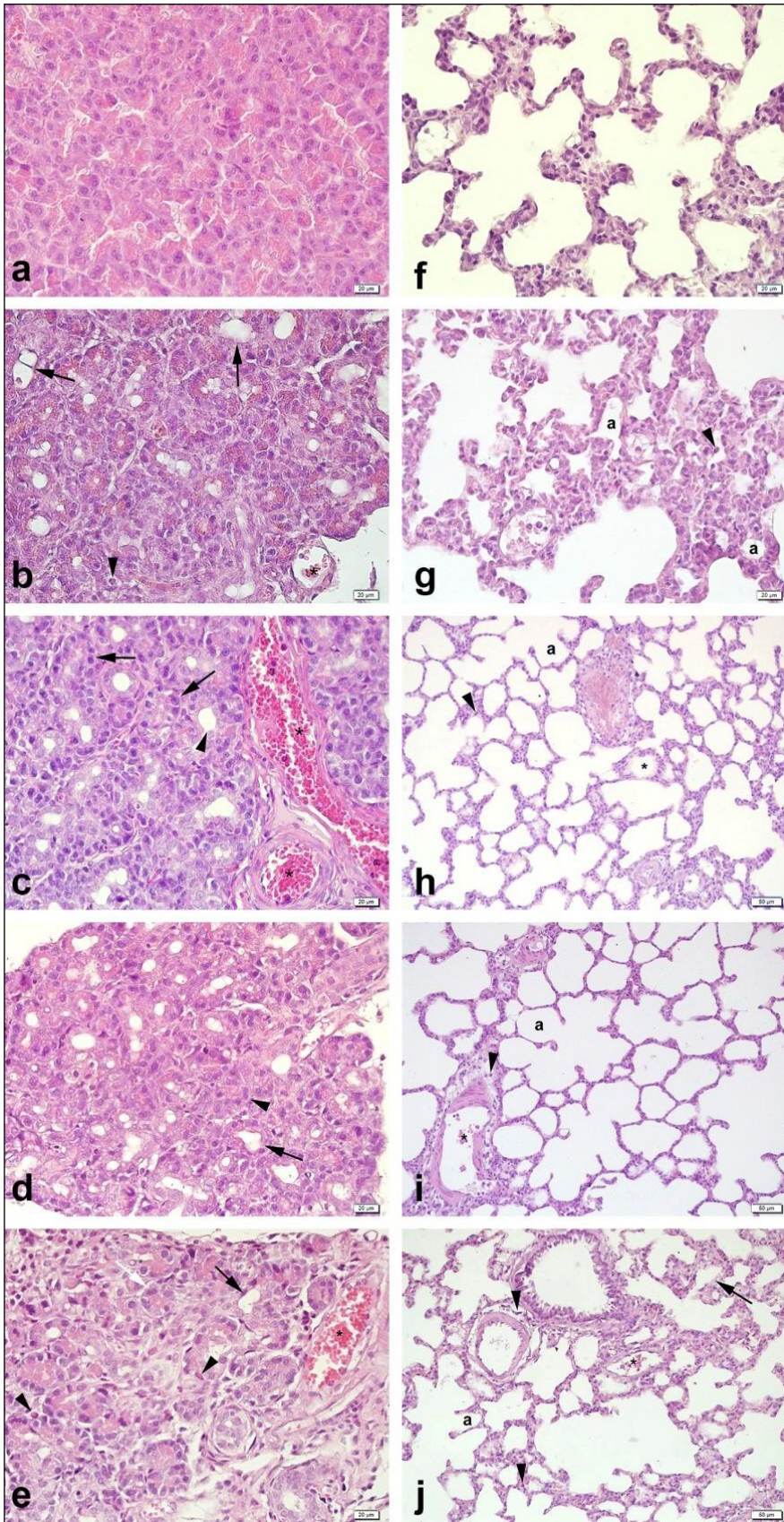


Fig. 6. Histological analyses of male pancreas (panels a-e) and lung (panels f-j) tissues. Normal appearing acinar cell structure in the pancreas of sham-operated group (panel a). Acinar cell injury (arrow), vasocongestion (asterisk), and inflammatory cell infiltration (arrowhead) in PBDL group (panel b). Normal (arrow) and injured (arrowhead) acini, and vasocongestion (asterisk) in PBDL + DPN group (panel c). Normal (arrowhead) and injured acini (arrow) in PBDL + PPT group (panel d). Normal (white arrow) and injured (black arrow) acini, vasocongestion (asterisk) and inflammatory cell infiltration (arrowhead) in PBDL + 17β -estradiol group (panel e). Inset: Normal alveoli in the lung tissue of sham-operated group (panel f). Severe damage in the alveoli (a) and inflammatory cell infiltration (arrowhead) of PBDL group (panel g). Normal (a) and injured (black asterisk) alveoli, thickening in the alveolar wall (white asterisk), and inflammatory cell infiltration (arrowhead) in PBDL + DPN group (panel h). Normal alveolar structures (a), vasocongestion (asterisk), and inflammatory cell infiltration (arrowhead) in PBDL + PPT group (panel i). Normal (a) and injured (arrow) alveoli, vasocongestion (asterisk), and inflammatory cell infiltration (arrowhead) in PBDL + 17β -estradiol group (panel j). Hematoxylin and eosin staining. Each group consists of 8 animals.

rats. On the other hand, in the lung tissue 17β -estradiol treatment and different estrogen agonists suppressed the elevated MDA levels according to gender. These results suggest that estrogen receptor agonists may have a protective role in suppression of lipid

peroxidation *via* variant receptor agonists in distinct organs and in different genders. Previously, in an *in vitro* study, the increased MDA levels were suppressed through 17β -estradiol treatment but not with PPT (30) and DPN applications in a methotrexate induced

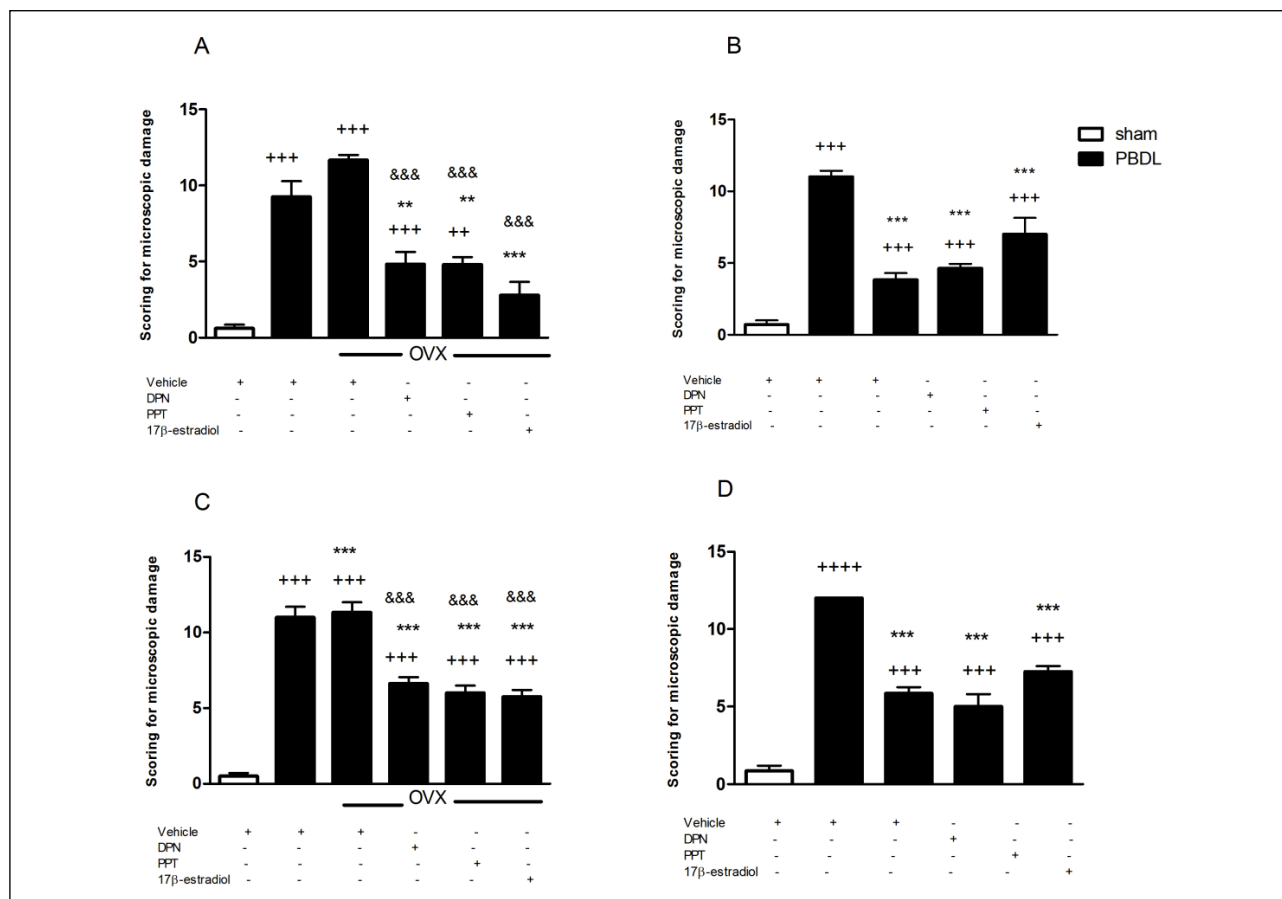


Fig. 7. Histological scores in pancreas tissues of female (A) and male (B) rats and lung tissues of female (C) and male (D) rats, which were either sham operated or pancreaticobiliary duct ligated (PBDL) with or without ovariectomy (OVX), and which were vehicle-, DPN-, PPT-, or 17 β -estradiol-treated. . ++P < 0.01, +++P < 0.001, compared to sham group; **P < 0.01, ***P < 0.001, compared to PBDL group; &&&P < 0.001, compared to PBDL + OVX group. Each group consists of 8 animals.

nephrotoxicity model. Our results supported these protective effects of 17 β -estradiol but also indicated the ameliorating effects of DPN and PPT treatments in pancreas tissue and inhibiting effects of DPN in female and PPT in male lung tissues.

As expected, pancreaticobiliary duct ligation resulted in a depletion of antioxidant GSH levels in pancreatic and lung tissues of both genders. There was an additional decrease in GSH levels of ovariectomized PBL group of female pancreatic tissue when compared to PBDL group. This result underlies the supportive effect of endogen estrogens on anti-oxidative system. Although the decrease in GSH levels of pancreas tissue was not improved by all treatments, the GSH levels were enhanced *via* PPT application in lung tissue of males, and *via* DPN and 17 β -estradiol treatments in females. This might be resulted because of the severity of inflammation occurs in acute pancreatitis. As a severe inflammation was developed in the pancreas tissue, the endogenous antioxidant GSH was consumed and was not improved by treatments. Similar with our results, the depleted GSH content of pancreas and lung tissues with acute pancreatitis was shown previously (31). On the other hand, in our study lung tissue was taken as a distant organ, the severity of inflammation in lung tissue might be lighter than pancreas itself which was also supported by the histological analysis. Probably by this reason, the treatments did not inhibit the decrease in GSH content in pancreas however they were effective in inhibiting the depletion of GSH content of the lung tissue. Although to our knowledge, there is no data

about the effects of estrogen on GSH content in acute pancreatitis, previously, the anti-oxidative GSH improving effects of estrogen in a pulmonary arterial hypertension model was reported (32).

The intracellular concentration of ROS scheduled by the production and/or removal of oxygen radicals by the antioxidant system. Cells contain a large number of antioxidant enzymes, such as SOD or catalase, to prevent or repair the damage caused by ROS. The physiological function of SOD is to protect the cells from the possible harmful effect of superoxide radical by converting it to hydrogen peroxide molecule. The activity of SOD increases through elevated production of superoxide radicals (33). Likewise, in this study, the amplified SOD activity was observed *via* pancreatitis induction in pancreatic tissue of both genders. Moreover, depressed levels of SOD activity were shown in females *via* estrogen agonists and 17 β -estradiol treatments. Our results imply that estrogen agonists may decrease the inflammatory status and superoxide levels therefore may also decrease the SOD activity. On the other hand, the SOD activities of female lung tissues were decreased with acute pancreatitis and were improved with all treatments. According to our results the response of SOD activity was different depending on the tissue type. Previously, similar with our results, severe necrotizing pancreatitis suppressed SOD activity in the lung tissue of rats (14).

Additionally, depleted SOD activity levels of variant organs other than lung and pancreas were also reported in an inflammation process (34). These previous studies support our

results. Moreover, our results suggest that SOD activity levels were improved *via* estrogen treatments. In a prior study, serum E2 levels were shown to be positively correlated with SOD activity in an oxidative stress model of myocardium (35). Furthermore, estrogens have been implicated in an antioxidant response element-mediated gene transcription that is related to the upregulation of SOD and GPX activity in hepatic cells (36). In accordance with our results, these aforementioned studies point out the improving effect of estrogen on SOD activity.

During acute pancreatitis, some inflammatory cells and pancreatic tissues release inflammatory mediators and cytokines, which influence the whole process of inflammation. The most important cytokines about this process are TNF- α , interleukins and transforming growth factor (37). Lipsett and Hirota *et al.* independently reported that the levels of inflammatory cytokines rise during acute pancreatitis and that the degree of the rise is closely linked to the severity of the disease (37, 38). Many other studies have reported that self-tissue injured with over-activated neutrophil leucocytes and increased cytokine levels are important causal factors of systemic complications that according to our results one of these complications may be lung injury (39, 40). Following inflammatory stimulation, the neutrophilic granulocyte may generate and release inflammatory cytokines such as TNF- α that participates in the pathophysiology of pancreatitis (37). Additionally, the injection of TNF- α antibody into rats was reported to improve the state and survival of rats with necrotizing pancreatitis (41) also TNF- α was indicated to directly injure pancreatic duct cells and to cause pancreatic acinus ischemia, hemorrhage, necrosis, inflammation and edema (42). Following the entrance of excessive TNF- α in circulation, neutrophilic granulocytes are activated and cytokines, such as IL-1 β and IL-6, are released that cause a cytokine cascade reaction and trigger local and systemic injury (43). IL-1 is a pro-inflammatory cytokine generated by the pancreas that has an important role in the initial stage of severe acute pancreatitis (44). Moreover, IL-1 β and TNF- α have many of the same biological activities, including the production of protein in the acute reaction period, effecting the release of PGI₂ and platelet activating factor, which causes the enlargement of the inflammation area and promotes the levels of inflammatory mediators, destructive enzymes and ROS secretion (45). IL-1 β can interact with TNF- α to induce or aggravate organ injury (45) and can stimulate the production of other inflammatory mediators, such as IL-8, IL-6 and other inflammatory cytokines (2). The level of IL-6 in the serum was shown to reflect the state of necrotizing acute pancreatitis (46) and was reported to be correlated with markers of the severity of acute pancreatitis (47). When the levels of IL-6 is over 40 μ l, it is considered to be severe acute pancreatitis (48). While in our study, the IL-6 levels of the pancreatitis groups were above this level. Eventually, in our study the increased levels of pro-inflammatory cytokines, such as TNF- α , IL-1 β , and IL-6, were demonstrated *via* acute pancreatitis induction, and different estrogen agonists had inhibiting effects on serum cytokine levels, that significant reductions were observed in TNF- α and IL-1 β levels through PPT application and in IL-6 levels *via* DPN, PPT, and 17 β -estradiol applications. Additionally, IL-1 β levels were suppressed by DPN treatment. A previous study has reported 17 β -estradiol-mediated protection of lung injury following acute pancreatitis by attenuation of pro-inflammatory mediators such as IL-6 and TNF- α (27). Moreover, Pishva *et al.* have suggested that estrogen hormone with its anti-inflammatory activity reduced TNF- α gene expression level and might be an efficient molecule in spinal cord injury (49). Besides, Cuzzocrea *et al.* have indicated an important protective role of estrogens against carrageenan-induced acute lung inflammation by decreasing the expression of TNF- α and IL-1 β levels in pleural exudates (50). Additionally, pro-inflammatory cytokines have a main role in activation of coagulation and also inhibition of this

coagulation protects from acute pancreatitis (51). According to this data, as an anticoagulant factor, estradiol may have a protective role in acute pancreatitis (52). Although, the effects of estrogens on inflammatory mediators and coagulation factors are not known in acute pancreatitis, these previous studies with variant inflammatory models support our results.

Additional to these pro-inflammatory interleukins, anti-inflammatory cytokine IL-10 levels were risen in males with acute pancreatitis and were depressed back to the control levels by 17 β -estradiol treatment. Corresponding with our results, IL-10 is believed to have a protective role in acute pancreatitis (53). Administration of IL-10 in an experimental acute pancreatitis model was reported to reduce the local inflammatory response and subsequent mortality (54). Similar with our results, estrogen had suppressing effect on increased levels of IL-10 in an ischemia-reperfusion injury model on rats (55). Eventually, these pro- and anti-inflammatory responses were related to not only the estrogen itself, but also the receptor subtypes that differs according to gender and cytokine type. Moreover, by ameliorating the histopathological changes such as vasocongestion, and inflammatory cell infiltration in pancreatic acini and distant organ damage that occurred in the lung tissue.

Similar with a previous study of our group, acute pancreatitis resulted with a histological apparent damage both in pancreas and lung tissues of male and female rats (26). PBDL induced severe injury in the pancreatic acini and alveoli, moreover vasocongestion, and inflammatory cell infiltration in pancreatic and lung tissues. These histopathological changes were ameliorated with all treatments. Although there is limited data on this topic, parallel to our results, estradiol treatment was shown to alleviate acinar cell apoptosis in a chronic pancreatitis model in rats (56). Overall, these results suggest that estrogen might have ameliorating effects on the cell injury in both pancreas and lung tissues.

In conclusion, 17 β -estradiol and ER- α and - β agonists reduced oxidative pancreatic damage in an acute pancreatitis model that was induced by PBDL, while ameliorating the severity of lung injury as a distant organ. These anti-inflammatory and anti-oxidant effects suggest that estrogen and its agonists might have protective role in obstructive pancreatitis. Considering the decrease in oxidative stress and the intensity of inflammation, 17 β -estradiol and the receptor subtypes have preserving role depending on the gender and the investigated parameter not only in pancreas but also in lung tissue as a distant organ.

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