The Role of p-Coumaric Acid on Reproductive and Remote Organ Damages Created by Adnexal Torsion/Detorsion: Biochemical and Immunohistochemical A Study

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ABSTRACT
Objective: We planned to search the effects of p-coumaric acid on ovary and lung injuries formed via bilateral adnexal torsion detorsion (T/D) in experimental rat model.
Methods: 24 female, Sprague-Dawley rats were sorted out as 3 groups. Design of the groups was performed as sham (group I) and T/D (group II), p-coumaric acid+T/D (group III) groups. Sham group; abdomen area was applied incision and repaired with no T/D model application. T/D group; 3 h of torsion phase completed and then 3 h of detorsion stage was established. P-coumaric acid+T/D group; p-coumaric acid was administered at the dose of 100 mg/kg for 15 days by oral gavage and then, T/D model was performed. Following detorsion phase, rats were sacrificed, lung and ovarian tissues were excised for biochemical and immunohistochemical evaluations.
Results: When it is compared to group I, oxidant parameters elevated significantly in group II (p<0.05) while the activity of antioxidant enzymes and TAS level decreased. On the other side, antioxidant enzyme activity raised and oxidant parameter levels diminished in group III compared to group II (p<0.05). Moreover, NF-kB, caspase 3 and LC3B protein expression levels increased in ovary and lung tissues of the group II. But NF-kB, caspase 3 and LC3B protein expression levels decreased in group III.
Conclusion: As a consequence, p-coumaric acid acted a protective performance against ovary and lung injuries arising from adnexal T/D model in rats.
Keywords: p-Coumaric Acid, Adnexal Torsion/Detorsion, Ovary, Remote organ, Rat.

1. INTRODUCTION

Adnexal/ovarian torsion (OT), which is more prevalent during reproductive age, is an emergency clinical condition rarely diagnosed preoperatively. In OT, the ovaries and fallopian tube portions rotate on the vascular pedicle. Ovarian/adnexal torsion causes to ischemia. In case of the OT, ischemia is defined as deterioration of organ blooding as a result of clogging due to clot or mechanical reasons of the blood vessels feeding ovaries. The regulation of this incident, re-oxygenation of the tissue and correction of blood flow is called as reperfusion. Pathologies which are caused by ischemia-reperfusion (I/R) are observed in many clinic cases such as burn, sepsis, shock, and torsion (1). There will be no permanent damage when it is noticed and operated in the early period, in ovarian tissue. However, in neglected or delayed cases, the necrosis in the ovaries occurs because the blood flow is cut off for a long time. In other words, irreversible damage is emerged in ovarian tissue. This case leads to infertility in woman. Some local and systemic consequences occur as a result of reperfusion of ovaries owing to detorsion of the torsional adnexa. Ovarian reperfusion results in activation, infiltration, and adhesion of leukocytes, because free radicals and proinflammatory substance levels increase (2). In addition to hypoperfusion in ischemia and I/R injury occurs inflammatory responses and many organ dysfunctions (3).
Numerous experimental studies have been conducted to alleviate ovarian tissue damage due to adnexal torsion/detorsion (T/D) (4, 5). Also, it is known that I/R directly leads to primary organ injury. Besides, I/R results in remote organ damage via starting an array of oxidative and inflammatory reactions in the secondary organs’ tissues (6, 7). To the best
of our knowledge, no studies were found on remote organ damage induced by adnexal T/D in the literature. Upon this reason, this search will make a significant contribution to the literature.

p-Coumaric acid is a phenolic compound commonly present in various plants (8, 9). The chemical structure of p-coumaric acid is given in Figure 1. The main dietary phenolic acid sources are beverages and fruits (10). Several researches have reported the association between the consumption of rich diets from phenolic acid derivatives and the protection of various diseases (11). The studies on food phenolics have enhanced due to their effects as antioxidants and their implication in the prevention of some diseases.

Here, we investigated the effects of p-coumaric acid on adnexal T/D-induced ovarian and lung tissue injury.

![Figure 1. The chemical structure of p-coumaric acid (4CA).](image)

2. MATERIALS AND METHODS

We established the experimental procedure at Atatürk University Experimental Animal Research and Application Center (ATADEM). The rats were provided by ATADEM. They were housed in standard cages with laboratory medium including appropriate humidity, light/dark cycle and temperature. Rats were fed with standard pellet feed and water. In order to avoid anesthesia complications, rats were famished before 12 hours of the experiment. This study was carried out via confirmation of Atatürk University Experimental Animals Local Ethics Committee (30.03.2018/94).

2.1. Groups and Torsion/Detorsion Model

24 female, Sprague-Dawley rats were weighted (220±10 g) and randomly divided into 3 groups. Group I; following the anesthesia, the rats were immobilized in the dorsal position. The abdominal region was prepared by shaving and cleaning. Povidone iodine was preferred for disinfection. Median laparotomy incision was made 1-2 cm, and it was repaired with silk 3/0 suture without any T/D model or medical process. Group II; all steps were established using anesthesia (100 mg/kg intraperitoneal i.p. Ketalar®, Pfizer, Istanbul, and 15 mg/kg i.p. xylazine hydrochloride, Rompun®, Bayer, Istanbul). When the detorsion ended, the rats were euthanized under overdose anesthesia. The ovarian and lung tissues were excised, washed, and preserved frozen for the biochemical analysis.

2.2. Biochemical Measurements

Malondialdehyde (MDA) levels were measured to find out the lipid peroxidation status (15). The results were demonstrated as µmol/g protein. The protocol defined by Sun et al. (16) was chosen to determine the superoxide dismutase (SOD) activity (U/mg protein). Myeloperoxidase (MPO) activity (U/g protein) was evaluated with a method defined by Bradley et al. (17). The total antioxidant status (TAS) was evaluated with a commercial kit (Rel Assay Diagnostics, Product Code: RL0017). Total oxidant status (TOS) was determined with an available kit (Rel Assay Diagnostics, Product Code: RL024). TAS and TOS levels were given as nmol/L. The ratio of TOS to TAS was accepted as the oxidative stress index (OSI).

2.3. Immunohistochemical Staining

Ovarian and lung tissues were taken into 10% buffered formalin solution. They were detected in neutral formaldehyde solution and washed with tap water. Tissues were blocked in the paraffin through the following-up of alcohol-xylol process. They were taken on the polylysine slide, incubated in 3% H₂O₂ to inactivate peroxidase, and washed in phosphate-buffered saline (PBS). Thereafter, incubation was executed with the antigen retrieval solution for 10 min at 500W. Nonspecific binding prevented by adding protein block solution. Cleaved Caspase-3 (Novus Biological, Cat. No. NB600-1235, Dilution: 1/100), microtubule-associated protein light chain 3 (LC3B) (Abcam, Cat. No. ab48394 Dilution: 1/200) and Nuclear Factor kappa-B (NF-kB) (Abcam, Cat. No: ab7971, Dilution: 1/150) were applied as the primer antibody. The exposure according to the mouse and rabbit specific procedure HRP/DAB detection IHC kit (Abcam: ab80436) was applied. 3,3’-diaminobenzidine chromogen was used and contrasted with hematoxylin. Positive cells were evaluated at 20x magnification in a light microscope.

2.4. Statistical Analysis

One-way ANOVA with the Tukey test was used for multiple comparisons. Descriptive statistics are given as the Mean±SD. In immunohistochemical examination, it was graded as intense immunopositivity (+++), moderate immunopositivity (++), mild immunopositivity (+), and negativity (-) in the ovary and lung tissues. Immunohistochemical findings were analyzed with Kruskal-Wallis test and Mann-Whitney U test was used for the binary group comparison. The statistically significant results were accepted as p<0.05.
3. RESULTS

3.1. Biochemical Results of Ovary and Lung Tissues

Biochemical analyses were performed on ovarian and lung tissues. MDA level was presented in Figure 2a. In group II, MDA value raised significantly compared to group I. Besides, MDA measurement declined in group III compared to group II (p<0.05). In group II, MPO activity was significantly higher than group I (Figure 2b). In group III, MPO activity diminished significantly compared to group II (p<0.05). Post hoc analysis of SOD revealed that it was low in group II compared to group. In addition, SOD activity elevated significantly in group III compared to group II (p<0.05). Post hoc analysis of SOD revealed that it was low in group II compared to group. In group II, OSI level raised in group II compared to group III (p<0.05, see Table 1).

3.2. Immunohistochemical Results of Ovary and Lung Tissues

In the immunohistochemical staining for inflammatory reaction of NF-kB immunopositivity, it did not show immunopositivity in ovarian tissues of the group I (Figure 3a and Table 2). There was intense immunopositivity in the group II (Figure 3b). In group III, there was a significant decrease in the severity of immunopositivity (Figure 3c). But in inflammatory cells in the interstitial area and in lutein cells was found immunopositivity. In immunohistochemical staining for apoptotic cell death and autophagic cell
death; caspase-3 (Figure 4c) and LC3B protein (Figure 5c) immunopositivity was negative in group I, but the most intense caspase-3 and LC3B protein immunopositivity was found in group II (Figure 4b and Figure 5b). Whereas group III showed a decrease in caspase 3 and LC3B protein immunopositivity (Figures 4c and 5c). immunopositivity was also found in luteal cells and interstitial areas.

Immunohistochemical staining revealed no NF-κB immunopositivity in the group I in terms of inflammatory reaction (Fig. 6a and Table 2). The most intense immunopositivity was observed in the lung tissues of the group II (Fig. 6b). In the group III, the severity of immunopositivity decreased (Fig. 6c). Apoptotic cell death and autophagic cell death did not reveal caspase-3 and LC3B protein immunopositivity in the lung tissues of the group I (Fig. 7a and Fig. 8a). The most intense caspase-3 and LC3B protein immunopositivity was observed in the group II (Fig. 7b and Fig. 8b). In the lung tissues of the group III, there was a decrease in caspase-3 and LC3B protein immunopositivity (Fig. 7c and Fig. 8c).

Figure 3. a. Group I b. group II, intense NF-kB immunopositivity in interstitial region (star) and luteal cells (arrow) c. group III, mild NF-kB immunopositivity in luteal cells (arrow), moderate-intensity NF-kB immunopositivity in interstitial cells.

Figure 4. a. Group I b. group II, intense caspase-3 immunopositivity in luteal cells (arrow) c. in group III, mild caspase-3 immunopositivity in luteal cells (arrow).

Figure 5. a. Group I b. Group II, dense LC3B protein immunopositivity in luteal cells (arrow) c. Group III, mild LC3B protein (arrow) in luteal cells, moderate LC3B protein immunopositivity in interstitial cells (arrowhead).
Figure 6. a. Group I b. group II, dense NF-κB immunopositivity-arrow in inflammatory cells around the bronchiole (arrow) c. group III, decreased NF-κB immunopositivity-arrow in the inflammatory cell group around the bronchiole.

Figure 7. a. Group I b. group II, intense caspase 3 immunopositivity-arrow in inflammatory cells in the peribronchiolar region (arrow) c. group III, very light caspase 3 immunopositivity in inflammatory cell group around the bronchiole (arrow)

Figure 8. a. Group I b. group II, very dense LC3 protein immunopositivity in inflammatory cells in the peribronchiolar region (arrow) c. group III, mild LC3B protein immunopositivity-arrow in inflammatory cell group around the bronchioles (arrow).
Table 1. Total antioxidant status (TAS), total oxidant status (TOS) and oxidative stress index (OSI) values in lung and ovarian tissues were presented as a Mean±SD.

<table>
<thead>
<tr>
<th>Groups</th>
<th>TAS²</th>
<th>TOS²</th>
<th>OSI</th>
<th>Lung</th>
<th>Ovary</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1.094±0.215</td>
<td>8.931±1.538</td>
<td>0.845±0.226</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>II</td>
<td>0.795±0.172</td>
<td>12.476±0.753</td>
<td>1.633±0.355</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>III</td>
<td>1.031±0.140</td>
<td>8.259±1.062</td>
<td>0.815±0.149</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

Table 2. Nuclear Factor kappa-B (NF-kB), caspase-3 and LC3B protein immunopositivity as intense immunopositivity (+++), moderate immunopositivity (++), mild immunopositivity (+), and negativity (-) in lung and ovarian tissues.

<table>
<thead>
<tr>
<th>Ovarian tissue</th>
<th>NF-B</th>
<th>Caspase 3</th>
<th>LC3B protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>-</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Group II</td>
<td>+++</td>
<td>++</td>
<td>+</td>
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<tr>
<td>Group III</td>
<td>+++</td>
<td>++</td>
<td>+</td>
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<table>
<thead>
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<th>Lung tissue</th>
<th>NF-B</th>
<th>Caspase 3</th>
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<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Group III</td>
<td>+++</td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>

4. DISCUSSION

The basic functions of the ovaries are the production of hormones and the development of the ovum. These functions can be interrupted by many factors. Torsion may occur and vascular insufficiency may cause necrosis. For this reason, OT must be recognized and treated quickly to avoid potential necrosis causing to infertility. Adnexal torsion is a gynecological condition. It decreases ovarian blood flow leading to tissue injury. The main goal in the treatment of ischemia is to renew blood circulation and ameliorate the tissue oxygenation (3, 18-20). Besides, ischemic organ reperfusion may cause much more critical injury than ischemia. This case is accepted as reperfusion damage (21). Neutrophil infiltration, reactive oxygen species (ROS) generation, release of cytokines, and inflammation are enounced to have important act in the etiology of this injury (22). After reperfusion, free radicals and ROS generate in severe quantity. Free radicals can cause damage by interacting with the entire biomolecules due to their extreme activities. Lipids, carbohydrates, proteins, amino acids, DNA, and complex molecules are damaged by free radicals. This is called as peroxidation of biomolecules (23-25). The main antioxidant defense in cell against radical damage is maintained by antioxidant enzymes including SOD and catalase (CAT) (25, 26). MDA is an important lipid peroxidation product and a toxic substance indicating oxidative stress (23, 24). SOD enzyme catalyzes the ROS neutralization (23, 24). MPO acts as a catalysor in hydroxyl radical formation (27). Until today, it has been made numerous experimental adnexal-T/D models, as well as remote organ damage studies induced by I/R in different organs (4, 12, 28-30). In a previous study, MPO activity and MDA levels increased in I/R groups (31). SOD activity decreased due to I/R in another adnexit T/D model [28, 32]. MDA levels significantly increased in an I/R experimental study (33). MDA level raised and antioxidant enzyme level diminished in another I/R research (7). In current study, MDA level and MPO activity elevated, and SOD activity diminished in both lungs and ovaries.

NF-kb plays role in different physiologic processes such as neurodegeneration, cell growth, tumorigenesis, immunity, inflammation, and apoptosis (34). During an inflammatory response, NF-kB plays as role in gene expression (35). Oxidative stress induces NF-kB transcription factor activation (36). Further, there is a powerful relationship between autophagic response and NF-kB (37). Hunger, hypoxia and various stress conditions stimulate cellular autophagic activity. ROS have been shown to be effective in autophagic vesicle and regulation of autophagia on cell death or survival (11, 26). The autophagy regulation of ROS takes place via Atg4, which is important in the formation of autophagosomes. The Atg4 gene is involved in the ripening of LC3B proteins and their degradation from fat molecules (38-40). Caspase 3 is vital for apoptosis and overexpressed during apoptosis events. Apoptosis was shown by increased caspase-3 levels in experimental animals (41). In this study caspase 3, NF-kB, and LC3B immunopositivity was intense in group II. Autophagy and apoptotic markers have been shown to be triggered by oxidative stress induced by ROS production. Whereas NF-kB, caspase 3 and LC3B protein showed less immunopositivity in ovarian and lung tissues owing to p-coumaric acid treatment.

In addition to this knowledge, p-coumaric acid was effective against lung and ovarian damage in adnexal T/D. Based on current biochemical data, p-coumaric acid has provided effective protection against ovarian injury induced by adnexal T/D.

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Data availability statement

Research data not shared

Declaration of conflict of interest

None

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