Journal of Biochemical and Molecular Toxicology

WILEY

RESEARCH ARTICLE OPEN ACCESS

New Potential Agent in Ovarian Ischemia Reperfusion Injury: Alpha Pinene

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Received: 11 April 2025 | **Revised:** 14 April 2025 | **Accepted:** 19 May 2025

Funding: This study was supported by the Ataturk University Scientific Research Projects Unit under project number TYL-2023-13255. This article has not been published or submitted for publication elsewhere.

Keywords: inflammation | ovarian ischemia-reperfusion injury | oxidative stress | α-Pinene

ABSTRACT

Ovarian torsion causes problems such as infertility in women due to ischemia-reperfusion (I/R) injury. α-Pinene (AP) is a monoterpene with known anti-inflammatory, antioxidant, and antiapoptotic impacts. In the present investigation, the protective impact of AP was examined in the ovarian I/R model. 28 Wistar-Albino female rats were used in the study. TNF-α, IL-1β, IL-10, MDA, IMA, SOD, and SIRT-1 levels were determined in ovarian tissue by ELISA method. Histopathological and immunohistochemical analyses were conducted to determine Bcl-2, Caspase-3, LC3B, and NFκB levels in ovarian tissues. TNF-α, IL-1β, IMA, and MDA levels were reduced in the treatment groups than the I/R group dose-dependent, while IL-10, SOD, and SIRT-1 levels increased substantially. Caspase-3 immunoreactivity declined in the treatment groups while Bcl-2 levels increased. LC3B and NFκB levels, which rise with I/R injury, were reduced considerably in the treatment groups. In addition, hemorrhage, edema, vascular congestion, and follicular degeneration due to I/R injury decreased in the treatment groups. The present investigation shows that AP has anti-inflammatory, antiapoptotic, and autophagy inhibitory effects against I/R damage in ovarian tissues and reduces oxidative stress. The results indicate that AP may be a potential protective agent in clinical use. Further research is needed before AP can be used in the clinic.

1 | Introduction

Ovarian torsion (OT) is a gynecological emergency that arises due to restrained or entire rotation of the ovary around its supporting ligaments. First, venous blood flow is disrupted, then arterial blood flow is affected, resulting in occlusion, edema, ischemic damage, and necrosis [1, 2]. The incidence of OT is between 2.5% and 7.4%. Although it can be seen in women of all ages, it most commonly affects women of reproductive age. Ovarian cysts, pelvic surgery, trauma, history of previous ovarian torsion, polycystic ovary syndrome, and pregnancy are among the main risk factors [3, 4]. If not treated immediately, OT can cause ischemia and related serious morbidity and mortality. Organ dysfunction is the outcome of reperfusion injury, which causes cellular damage. Particularly in teenagers and young adults, torsion-induced IR injury is a significant risk factor for ovarian function loss and, consequently, infertility [5]. Early diagnosis of OT followed by correct treatment is crucial to prevent possible necrosis, infertility, mortality, and morbidity. In this context, the first treatment is to reinstate blood flow by detorsion of the ovaries. Increased production of reactive oxygen species (ROS) and oxidative stress

(OS) may result from restoring blood flow to ischemic tissue. Reperfusion may be the primary cause of OS injury, which is a significant factor in I/R injury in ovarian tissue [6]. This increase in ROS production encourages the production of proinflammatory cytokines and causes further tissue damage. This situation refers to I/R damage [7]. The basic mechanism of I/R injury is not yet completely understood and involves many biochemical reactions and cellular signaling pathways [8]. Failure of ATPase-dependent ion channels to function causes an augmentation of both intracellular and mitochondrial calcium concentrations, resulting in cell swelling, membrane disruption, and cell death through necrotic, apoptotic, and autophagic mechanisms [9].

Delays in the diagnosis and treatment of OT can lead to ovarian loss and, thus, infertility. Additionally, severe I/R damage can increase mortality and morbidity. However, currently, there is no potential protective agent for OT other than surgical treatment. For this reason, many recent studies have investigated potential agents for therapy and prevention against ovarian I/R damage [10].

Coniferous trees contain AP, a bicyclic monoterpene molecule. According to experimental research, AP possesses antibacterial, anti-inflammatory, and antioxidant properties. In addition, research indicates that AP inhibits intracellular ROS production and significantly increases the expression of some antioxidant enzymes. AP exhibits various bioactivities and is used in multiple areas [11–13]. Examining AP's potential to prevent ovarian I/R injury was the goal of this study. Pro-inflammatory and anti-inflammatory cytokines, as well as apoptotic, OS, and autophagy indicators, were used to study a rat ovarian I/R model.

2 | Materials and Methods

2.1 | Reagents

As in earlier research, alpha-pinene (Sigma-Aldrich, USA, Cat no: 147524) was given intraperitoneally at 50 mg/kg and 100 mg/kg [14, 15].

2.2 | Animal Ethics

The Ataturk University Experimental Animals Ethics Committee approved the current study on June 20, 2023. 28 Wistar-Albino female rats weighing 200–250 g were used in the study, which was carried out at the Ataturk University Experimental Animals Research and Application Center. Rats were housed in a 12-h light-dark cycle during the experiment. The night before I/R experiments, food intake but not water intake of Wistar albino female rats was withheld, and tap water was used as drinking water.

2.3 | Experimental Ovarian Ischemia-Reperfusion Model

28 Wistar-Albino female rats were weighed (200–250 g) and separated into four groups ($n = 7$). Each rat's weight was recorded, and then ketamine (75 mg/kg) and xylazine (8 mg/kg) were injected intraperitoneally to induce anesthesia. In the sham group, their bilateral ovaries were removed and placed anatomically again, no clamps were applied to the rats. The incision site was closed with a stapler. Bilateral ovaries in the I/R group were clamped and ischemia was applied for 3 h. Then, the clamps were removed and reperfusion was provided for 3 h. Rats in the 50 mg/kg and 100 mg/kg AP treatment groups had their bilateral ovaries clamped, and they were subjected to ischemia for 3 h. After 3 h, the clamps were removed and intraperitoneal low and high doses (50 mg/kg and 100 mg/kg) of AP were applied and reperfused for 3 h [16, 17]. Immediately after reperfusion, anesthetized rats were euthanized by intracardiac blood collection, and then ovarian tissues were collected.

2.4 | Biochemical Analysis

100 mg of ovarian tissue was cut separately and placed in 1 mL of PBS (phosphate-buffered saline). The left ovaries were homogenized in a container filled with ice at 5000 rpm. They were then transferred to an Eppendorf and centrifuged for 20 min at 4°C at 1600 x g. The supernatants were stored in a deep freezer until the day of the experiment. To measure biochemical parameters, samples frozen at −80°C were thawed slowly without repeating these procedures just before analysis. All materials to be used on the day of the experiment were brought to room temperature (18°C–26°C). Ovarian tissue protein was analyzed using the Coomassie blue G-250 protein analysis procedure.

Malondialdehyde (MDA), superoxide dismutase (SOD), ischemia-modified albumin (IMA), tumor necrosis factor (TNF-α), interleukin 1- beta (IL-1β), sirtuin-1 (SIRT-1), and interleukin-10 (IL-10) levels were assessed by ELISA method using "Rat MDA ELISA Kit (Cat. No: 201-11-0157, Sunred, China), Rat SOD ELISA Kit (Cat. No: 201-11-0169, Sunred, China), Rat IMA ELISA Kit (Cat. No: 201-11-1672, Sunred, China), Rat TNF-α ELISA Kit (Cat. No: 201-11-0765, Sunred, China), Rat IL-1β ELISA Kit (Cat. No: 201-11-0120, Sunred, China), Rat SIRT-1 ELISA Kit (Cat. No: 201-11-1498, Sunred, China) and Rat IL-10 ELISA Kit (Cat. No: 201-11-0109, Sunred, China)" was measured as stated by the manufacturer's instructions.

2.5 | Histopathological Examination

After the experiment, the right ovaries were immediately placed in a 10% neutral formaldehyde solution. The solution was also renewed every 24 h. The tissues were kept in this solution for 72 h to ensure fixation. The tissues were then placed in paraffin blocks after being treated for an hour with xylene and a series of graded alcohols (70%, 80%, 96%, and 100%). Tissue damage was assessed using a light microscope after slices that were 5 μ thick were stained using the triple staining technique.

Microscopic examination was performed with a light microscope with a computer and camera attachment. In histopathological examination, vascular congestion, hemorrhage, edema, and follicular degeneration were evaluated with scores of 0

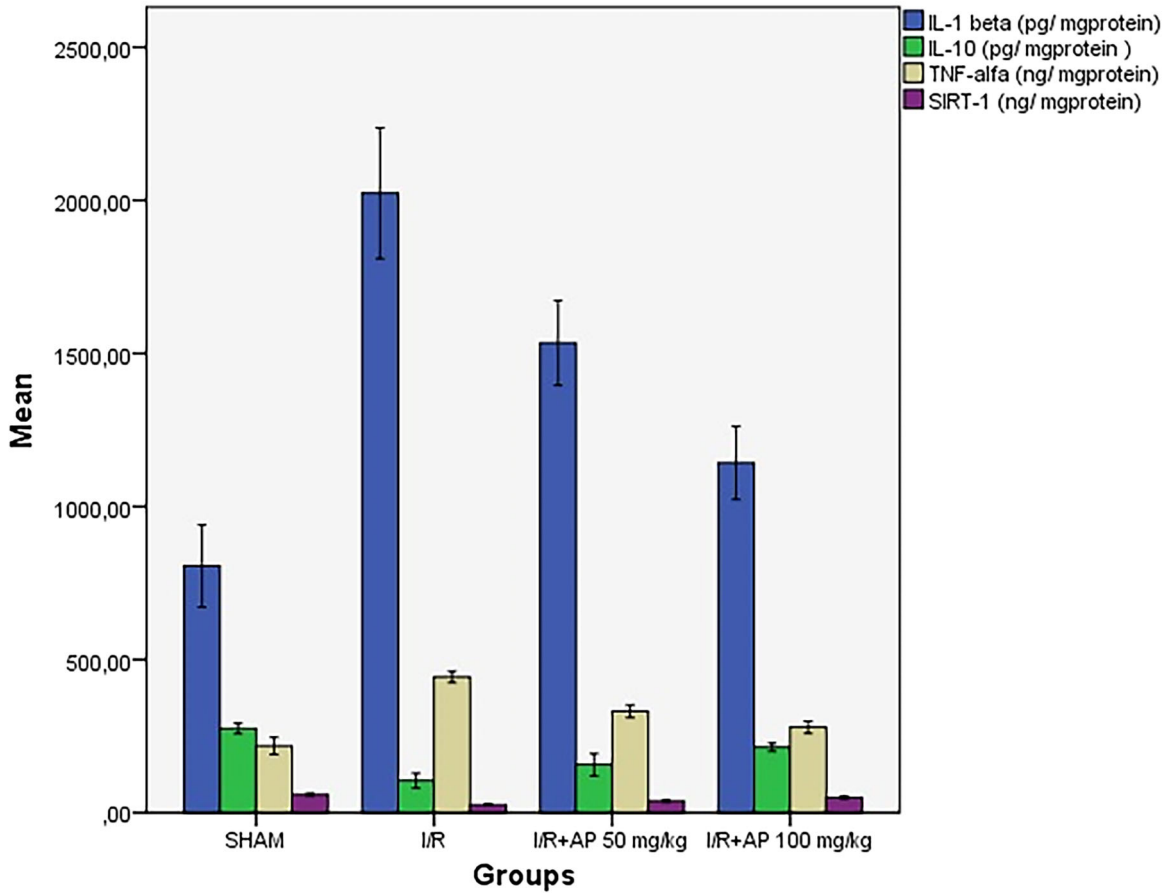

FIGURE 1 | IL-1B, TNF-a, IL-10 and SIRT-1 levels in ovarian tissues of ovarian ischemia-reperfusion rat model.

(normal), 1 (mild), 2 (moderate), and 3 (severe) [18, 19]. The results are presented in Figures 3 and 4.

2.6 | Immunohistochemical Analysis

Before starting the immunohistochemical staining process, sections obtained on positively charged slides were deparaffinized by waiting in two different xylene solutions for 15 min each. Deparaffinized sections were dehydrated by waiting in ethyl alcohol series (100%, 96%, 80%, and 70%), then rinsed in distilled water and dehydrated. Slides were boiled with citrate solution (pH: 6.0) to reveal antigens in the tissues. Then, they were kept in a 3% H_2O_2 solution (25°C) to inhibit endogenous peroxidase activation. Following this process, they received a PBS wash and a 5-min treatment with a protein-blocking solution. Next, anti-Bcl-2 (Santa Cruz, sc-7382, 1/50 dilution), anti-P Caspase 3 (Affinity, AF3311, 1/100 dilution), anti-P-LC3B (Affinity, AF4350, 1/100 dilution), and anti-P-NF-κB p65 (Affinity, AF2006, 1/100 dilution) Overnight, primary antibodies were stored at +4°C. After rinsing the slides with PBS between two solutions, they were treated for 30 min with secondary antibody and HRP. Following the manufacturer's instructions, HRP (Thermo Fischer, TP-125-HL) and secondary antibodies were used. DAB (Thermo Fisher, 3,3'-Diaminobenzidine) was used as a chromogen to stain PBS-washed slides. Following counterstaining, a light microscope was used to inspect them.

When the immunohistochemical staining process was completed, they were examined under a light microscope (Nikon Eclipse E600) at appropriate magnifications (100X for histopathological images, 200X for immunohistochemistry images), evaluated, and photographed. Staining intensity analysis was performed by selecting five different microscope fields on the sections. Staining intensity (0, 1, 2, 3) and percentage of positively stained cells (0 = 0.1% = 1%–33.2% = 34%–66.3% ≥ 66%) were scored numerically [20–22]. All data obtained are presented in Figures 5 and 6.

3 | Results

3.1 | Biochemical Results

3.1.1 | Effects on Inflammation

The I/R and AP-treated groups had considerably greater levels of TNF-α and IL-1β than the sham group ($p < 0.05$). Depending on the dose, IL-1β, and TNF-α levels were significantly lower in the low- and high-dose treatment groups relative to the I/R group ($p < 0.05$). The high-dose AP group had substantially lower levels of inflammation than the low-dose AP group ($p = 0.01$ for IL-1β, $p = 0.022$ for TNF-α) (Figure 1).

The I/R and AP treatment groups had considerably a greater decrease of IL-10 than the sham group ($p < 0.05$). IL-10 levels were substantially greater in the low- and high-dose treatment groups

compared to the I/R group ($p < 0.05$). The high-dose AP group had markedly greater levels of IL-10, which is linked to anti-inflammatory activity, than the low-dose AP group ($p = 0.017$) (Figure 1).

3.1.2 | Impact on Oxidant-Antioxidant System

The I/R and AP treatment groups had significantly greater MDA and IMA levels than the sham group ($p < 0.05$). When comparing the untreated group, the AP-applied groups' MDA and IMA levels were significantly reduced ($p < 0.05$). The high-dose AP group's MDA and IMA levels were substantially lower than those of the low-dose AP group ($p = 0.016$ and $p = 0.028$, respectively) (Figure 2).

Compared to the sham group, SOD levels were significantly lower in the I/R and AP-treated groups ($p < 0.05$). Relative to the I/R group, SOD levels were significantly higher in the AP-treated groups ($p < 0.05$). Relative to the low-dose AP group, the high-dose AP group's antioxidant levels increased significantly ($p = 0.021$) (Figure 2).

3.1.3 | Effect on SIRT-1

Compared to the sham group, SIRT-1 levels were substantially lower in the treated and untreated groups ($p < 0.05$). The high-dose treated group had considerably greater SIRT-1 levels than the low-dose treated group ($p < 0.015$) (Figure 1).

3.1.4 | Histopathological Results

The ovarian tissues of the sham group exhibited typical follicular configurations with standard histological characteristics in the cortex and medulla. The ovarian tissues of the I/R group exhibited substantial arterial obstruction and bleeding, edema, and follicular degeneration. Rats in the low-dose therapy (50 mg/kg AP) group showed milder vascular congestion and bleeding in their ovarian tissues relative to the I/R group. Rats in the high-dose treatment (100 mg/kg AP) group showed less vascular congestion and hemorrhagic regions in their ovarian tissues. The low- and high-dose treatment groups did not, however, vary significantly (Figure 3) ($p > 0.05$). Figure 4 shows a graphic representation of each group's histopathological examination.

3.1.5 | Immunohistochemical Staining Results

Immunohistochemical staining of all groups was analyzed using light microscope images, as shown in Figure 5. The immunoreactivity of the Bcl-2 antibody was higher in other groups than in the I/R group. P-Caspase-3, P-LC3B and P-Nf-kB-p65 antibody immunoreactivities were substantially greater

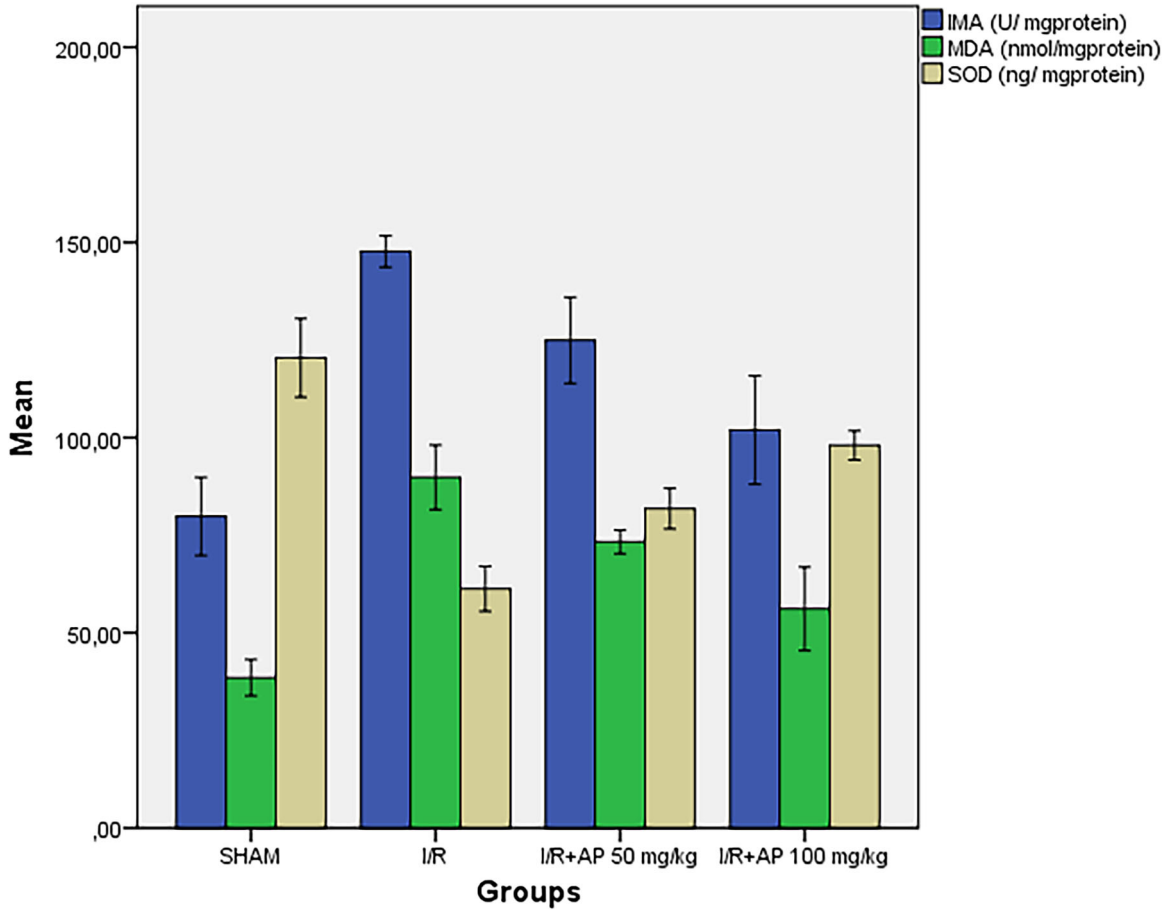

FIGURE 2 | IMA, MDA, and SOD levels in ovarian tissues of ovarian ischemia-reperfusion rat model.

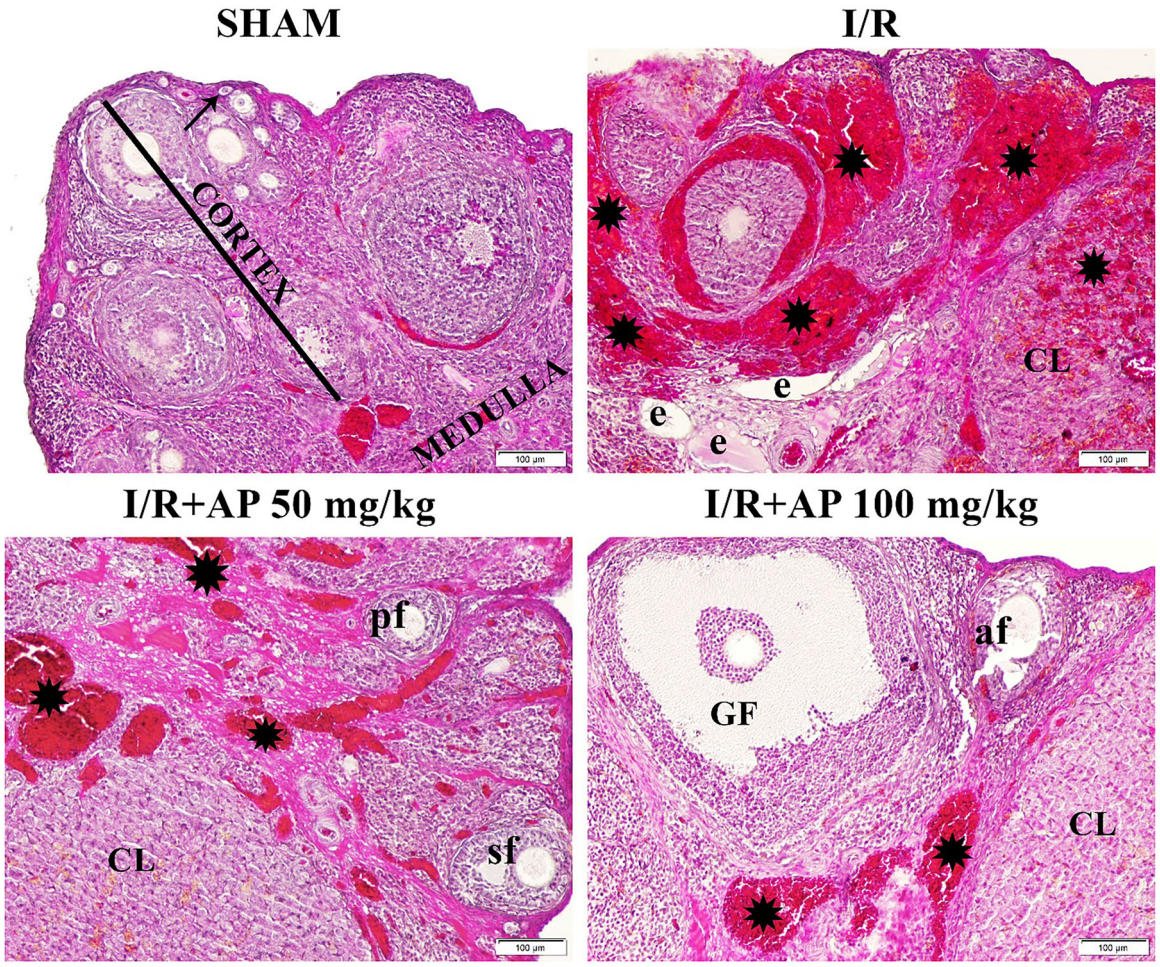

FIGURE 3 | Micrographs of ovarian tissues of all groups. Arrow; Primordial follicle, CL; Corpus Luteum, af; atrophic follicle, pf; primary follicle, sf; secondary follicle, GF; Graafian follicle, Star; Hemorrhage and vascular congestion, e; Edema. Staining: Mallory's Triple Stain Modified by Crossman. Magnification; 100X.

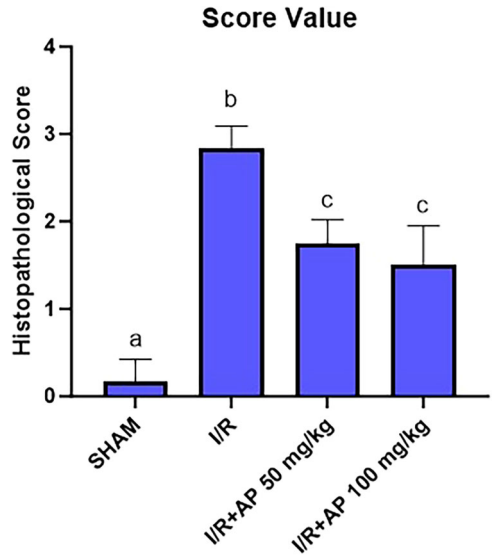

FIGURE 4 | Evaluation of ovarian tissue histologies of all groups. Values are given as mean ± SD and analyzed by one-way ANOVA followed by Tukey's test. Statistical differences between groups are demonstrated by the letters a, b, and c ($p < 0.05$).

in the I/R group than in the treatment and Sham groups. Figure 6 shows the immunohistochemical analysis of each group.

4 | Discussion

Ovarian I/R is a pathological condition that occurs when blood flow to the ovaries is interrupted and the tissue is not adequately oxygenated. The ischemic tissue resulting from torsion causes a chemical reaction that, if uncontrolled, leads to cellular dysfunction and necrosis. The first intervention to be made to the ischemic ovarian tissue is to provide reperfusion of the affected tissue by detorsion of the torsioned ovary. However, sudden and intense oxygen delivery to the ischemic tissue can trigger a reaction process that can cause further damage to the tissue. This is called I/R damage and causes further tissue damage as it progresses [23]. Even if oxygen levels are brought back to normal by providing blood flow to the tissue due to reperfusion, this increases the amount of ROS [9]. Prolonged and excessive ROS production can cause OS, leading to mortality [24]. In addition, increased ROS production causes secondary damage by causing OS. It can lead to necrosis, apoptosis,

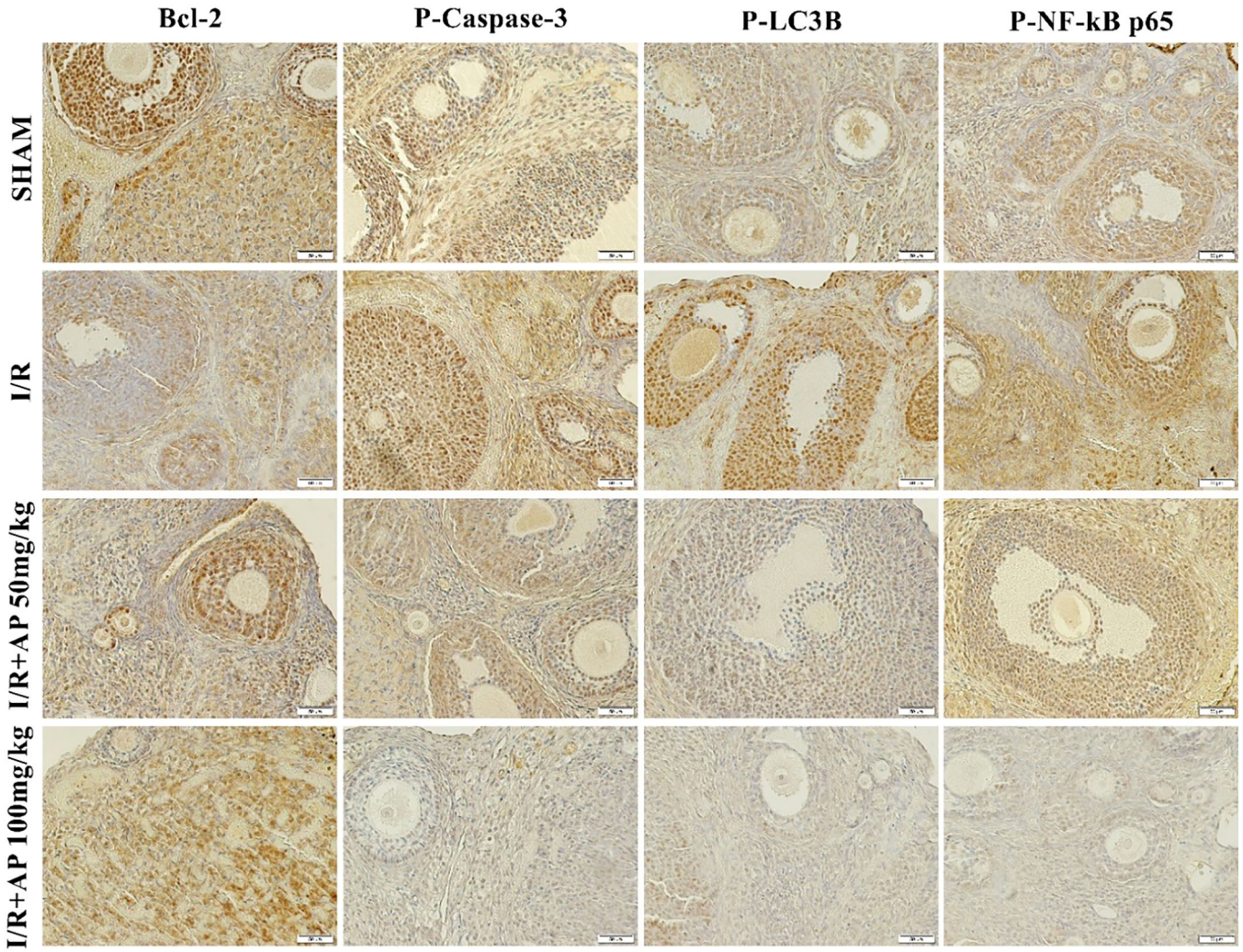

FIGURE 5 | Bcl-2, P-Caspase-3, P-LC3B, P-NF-kB p65 antibody immunoreactivities of all groups. Staining: Streptavidin Biotin Peroxidase Method. Magnification; 200X.

autophagy, or necroptosis [25]. In addition, ROS oxidizes cell membrane lipids, forming toxic compounds such as MDA, which disrupt the cell's membrane structure and function [26].

No clear evidence exists that anti-inflammatory and antioxidant agents can inhibit I/R injury caused by laparoscopic detorsion in current OT treatment [27]. Considering this situation, future studies should seek to establish routine procedures for utilizing antioxidants in the clinic after laparoscopic treatment [28]. This study explored the protective effects of AP, which has anti-inflammatory, antiapoptotic, and antioxidant properties, against I/R damage for the first time. The results demonstrated that the low- and high-dose AP groups had fewer levels of the pro-inflammatory chemicals TNF-α and IL-1β than the I/R group. Compared to individuals not receiving treatment, IL-10 levels were substantially greater in the low- and high-dose AP groups. The MDA levels, a parameter of OS, were determined to be markedly lower in the groups receiving treatment. The SOD levels, which are antioxidant enzymes, were considerably higher in the groups receiving treatment, depending on the dose, compared to the untreated group. Antioxidant levels were significantly elevated in the high-dose AP group compared to the low-dose AP group. In an experimental study conducted on rats examining cisplatin (CP)-induced kidney damage, the protective effects of AP were evaluated MDA levels in the kidney tissue of rats induced with CP increased significantly; conversely, SOD levels were lower than the control group. The analyses revealed that 50 mg/kg AP treatment significantly lowered the MDA levels, and the antioxidant status was significantly improved than the CP-induced group. Additionally, it was found that the CP-induced group's TNF-α levels considerably increased than the control group, while the treatment group's TNF-α levels markedly dropped than the CP-induced group [29]. According to a rat study, the group treated with AP and the cerulein-induced acute pancreatitis model demonstrated considerably lower levels of IL-1β than the group given cerulein alone [30]. Albumin, the most abundant protein in plasma, is differentiated by OS, an increase of ROS, and acidosis. IMA levels rise within a few hours of ischemia and remain elevated for 6–12 h. In this current study, IMA levels were markedly greater in the treatment groups than in the I/R group. However, studies examining the relationship between IMA and AP are limited in the literature. However, the literature includes studies that evaluate the protective effects of AP on OS caused by testicular and cerebral I/R damage. For example, a study using the testicular I/R model in rats revealed

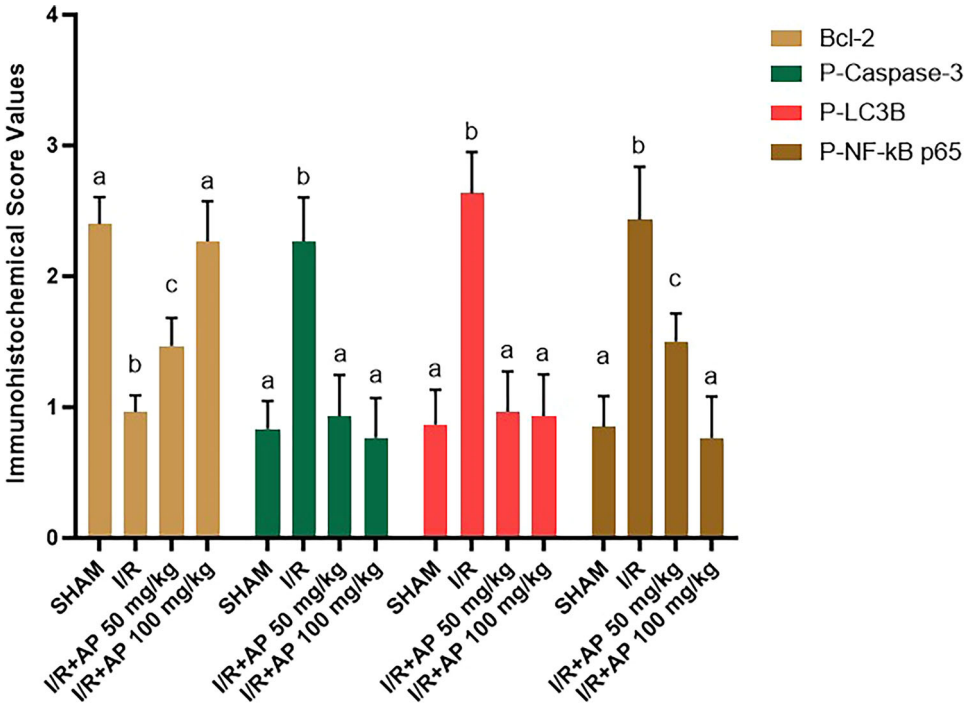

FIGURE 6 | Immunohistochemical analysis evaluation of all groups and all antibodies. Values are expressed as mean ± SD ($n = 6$) and analyzed by one-way ANOVA with subsequent Tukey's test. Statistical differences between groups are demonstrated by the letters a, b, and c ($p < 0.05$).

that AP had a protective effect on OS [31]. In addition, it was stated that AP reduced the level of OS in an experimental study in which cerebral ischemia was created in rats [15]. IMA is the end product of OS and an important biomarker [32]. In this current study, the significantly higher IMA levels in the treatment groups support the protective impacts of AP on OS. The IMA levels in the I/R group in the rat ovarian I/R model laboratory study were comparable to those in the current investigation [33]. However, more research is required to identify the specific properties of AP on IMA.

Sirtuins activate proteins involved in the pathophysiology of various diseases through the process of deacetylation [34]. SIRT-1 significantly affects cell viability, apoptosis, and inflammatory processes [35]. The NAD-dependent histone deacetylase SIRT-1 inhibits the transcriptional activity of apoptosis mediators like the tumor suppressor p53 by deacetylating them. It stops Bax, a member of the proapoptotic Bcl-2 family, from being transported to the mitochondria by deacetylating the DNA repair protein Ku70 [34]. SIRT-1 is recognized for its antioxidant and anti-inflammatory features [36]. In addition, SIRT-1 has inflammation-reducing effects through autophagy, expression of antioxidant enzymes, and suppression of NFκB [37]. There are narrow studies directly investigating the relationship between SIRT-1 and AP. In the present study, NFκB levels were lower and SIRT-1 levels were greater in the treatment groups than in the I/R group. Additionally, the treatment groups' levels of autophagy and apoptosis biomarkers were considerably reduced than those of the I/R group. From this perspective, The outcomes of this study reveal that SIRT-1 is effective in reducing inflammation and suppressing apoptosis and autophagy by suppressing NFκB. Furthermore, research on AP's anti-inflammatory and antioxidant qualities indicates that indirect links to SIRT-1's function in cellular stress responses may be possible [38]. However, more research is required to identify the specific effects of AP on SIRT-1.

According to the histopathological analysis results, typical follicular structures and normal histological medulla were observed in the cortex of rat ovarian tissues in the sham group. Rat ovarian tissues in the I/R group demonstrated substantial blood vessel congestion and bleeding, edema, and follicular degeneration. In comparison to the I/R group, the treatment groups' ovarian tissues showed less vascular congestion and bleeding. Previous studies have also found that ovarian I/R injury potentially leads to follicular cell degeneration, vascular congestion, hemorrhage, inflammation, and ovarian tissue loss [39, 40]. Accordingly, in the experimental study in which brain I/R injury was induced, the finding showed that AP improved brain dysfunction and significantly reduced infarct volume [41]. Substantial cellular abnormalities in the seminiferous tubules and widespread necrosis in the intertubular region were seen in the low-dose AP group in a study using an experimental model of CP-induced reproductive damage in rats, and decreases in these were found. On the other hand, the high-dose AP group was found to have fully recovered from severe cellular abnormalities in the seminiferous tubules, widespread necrosis in the intertubular area, blood vessel congestion, and edema [42].

The results of the immunohistochemistry research demonstrated that the sham and low- and high-dose therapy groups had greater Bcl-2 antibody immunoreactivity than the I/R group. P-caspase-3, P-LC3B and P-NF-κB-p65 antibody immunoreactivities were substantially greater in the I/R group than in the sham and treatment groups. Apoptosis is controlled by cytochrome c, Bcl-2, and caspases. An essential enzyme called caspase-3 triggers other caspases to start the apoptotic process [29]. In one study, a brain I/R model was created in rats,

and AP's protective properties were investigated. According to the current study, the treatment group's levels of the apoptosis biomarker caspase-3 and the inflammatory biomarker NFκB were determined to be lower than those of the I/R group [43]. In another investigation with rats, the neuroprotective impact of AP was investigated in the beta-amyloid $(A\beta)_{1\text{-}42}$ induced Alzheimer's model experiment. It was found that NFκB levels rose substantially in the group given only Aβ1-42, while NFκB levels reduced markedly in the treatment group given 50 mg/kg AP [44]. In an experimental study on liver toxicity induced by carbon tetrachloride in rats, Bcl-2 levels were evaluated. According to the study's results, the AP-treated group's Bcl-2 levels rose substantially than in the control group [45]. Autophagy is a destruction process in which the cell breaks down its components in situations such as energy deprivation and cellular stress [46]. Several studies have examined the impact of AP on the autophagy mechanism. However, studies directly demonstrating the relationship between AP and LC3B are narrow in the current literature. In a temporal lobe epilepsy model study in rats, AP was shown to prevent autophagy in the hippocampus [47]. An experimental study in mice showed that LC3B expression increased in brain tissues following brain I/R injury, as in this current study [48]. In this current study, LC3B levels were low in the treatment group. This suggests that AP is an autophagy inhibitor. However, more research is required to reveal more clearly the connection between AP and LC3B.

5 | Conclusion

Consequently, AP demonstrated protective efficacy by lowering vascular congestion, edema, follicular degeneration, and hemorrhagic regions in the treatment groups compared to the I/R group's ovarian tissues as seen by light microscopy. AP showed anti-inflammatory activity in ovarian I/R injury by decreasing TNF-α, IL-1B, and NFkB levels and increasing IL-10 levels. The analysis indicated that it had a reducing effect on OS by reducing MDA levels and rising SOD levels. It was also found to have an antiapoptotic effect by decreasing caspase-3 and rising Bcl-2 levels. Furthermore, AP acted as an autophagy inhibitor by decreasing LC3B levels. AP reduced the increased levels of IMA after I/R injury, increased the SIRT-1 enzymes that function in cell proliferation and apoptosis, and reduced proinflammatory cytokines, thereby reducing I/R injury in the ovary. In conclusion, it was observed that AP, which acts as an antioxidant, antiapoptotic, anti-inflammatory, and autophagy inhibitor in ovarian I/R injury, has a protective effect. The analysis suggests that AP may be recognized as a possible protective agent for clinical use. However, further studies are needed to determine whether AP can be used in the clinic.

Consent

All authors have approved the publication of the article. Ataturk University Animal Experiments Local Ethics Committee approved animal experiments and procedures with decision no. 96 in its session numbered 2023/06.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Due to confidentiality and ethical restrictions, the data obtained in this study are not publicly available but are available from the corresponding author upon request.

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