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Effect of oleanolic acid for prevention of acute lung injury and apoptosis

Akut akciğer hasarı ve apoptozisi önlemede oleanolik asidin etkisi

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ABSTRACT

Background: This study aims to evaluate the efficiency of oleanolic acid on acute lung injury and acute respiratory distress syndrome.

Methods: The study included 70 female Wistar albino rats (weighing 180 to 200 g). We created seven groups, each consisting of 10 rats. Then, we generated acute lung injuries by intra-tracheal peroxynitrite injection in every group except for the control group. We investigated the effect of oleanolic acid. For this purpose, we measured the levels of malondialdehyde, interleukin 1 beta, interleukin 4, interleukin 10 and tumor necrosis factor alpha in the collected blood samples from the rats. In addition, we examined the lung tissue samples histopathologically and assessed the rate of apoptosis.

Results: Peroxynitrite injected groups at 24 and 48 h showed a statistically significant increase in interleukin 1 beta, tumor necrosis factor alpha, interleukin 4, interleukin 10 and malondialdehyde levels, which are accepted as mediators of the inflammatory process, compared to the control group. When peroxynitrite injected groups at 24 and 48 h were compared to the treatment groups of the same hour, a statistically significant decrease was detected. According to histopathological examination, peroxynitrite injected groups at 24 and 48 h showed a significant increase of tissue injury scores compared to the control group. However, the groups that were treated with oleanolic acid showed a significant decrease compared to the peroxynitrite groups ($p<0.001$ for tumor necrosis factor alpha and apoptosis results at 48 h).

Conclusion: In this study, we confirmed that oleanolic acid can be an effective agent for the prevention of acute lung injury generated via peroxynitrite.

Keywords: Acute lung injury, acute respiratory distress syndrome, oleanolic acid, peroxynitrite.

ÖZ

Amaç: Bu çalışmada oleanolik asidin akut akciğer hasarı ve akut solunum sıkıntısı sendromu üzerindeki etkinliği değerlendirildi.

Çalışma planı: Çalışmaya 70 dişi Wistar Albino sıçan (ağırlık, 180-200 g) dahil edildi. Her biri 10 sıçan içeren yedi grup oluşturuldu. Sonra, kontrol grubu hariç her grupta intratrakeal peroksinitrit enjeksiyonu ile akut akciğer hasarı oluşturuldu. Oleanolik asidin etkisi araştırıldı. Bu amaçla, sıçanlardan toplanan kan örneklerinde malondialdehit, interlökin 1 beta, interlökin 4, interlökin 10 ve tümör nekroz faktör alfa düzeyleri ölçüldü. Ayrıca, akciğer doku örnekleri histopatolojik olarak incelendi ve apoptozis oranı değerlendirildi.

Bulgular: Peroksinitrit enjekte edilen gruplarda 24. ve 48. saatte kontrol grubuna kıyasla enflamatuvar sürecin mediatörleri kabul edilen interlökin 1 beta, tümör nekroz faktör alfa, interlökin 4, interlökin 10 ve malondialdehit düzeyleri istatistiksel olarak anlamlı artış gösterdi. Peroksinitrit enjekte edilen gruplar 24. ve 48. saatte kendi saatlerindeki tedavi grupları ile karşılaştırıldığında, istatistiksel olarak anlamlı bir düşüş saptandı. Histopatolojik incelemeye göre, peroksinitrit enjekte edilen gruplar 24. ve 48. saatte kontrol grubuna kıyasla doku hasarı skorlarında anlamlı bir artış gösterdi. Diğer yandan, oleanolik asit ile tedavi edilen gruplar peroksinitrit gruplarına kıyasla anlamlı düşüş gösterdi (48. saat tümör nekroz faktör alfa ve apoptozis sonuçları için $p<0.001$).

Sonuç: Bu çalışmada, peroksinitrit ile oluşturulan akut akciğer hasarını önlemede oleanolik asidin etkili bir ajan olabileceği saptandı.

Anahtar sözcükler: Akut akciğer hasarı, akut solunum sıkıntısı sendromu, oleanolik asit, peroksinitrit.

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Acute respiratory distress syndrome (ARDS) is regarded as a syndrome rather than a disease since it shows an acute presentation due to many different reasons, including the effect on lungs from airways and the circulatory system. It occurs in distinct diseases (pneumonia and extra pulmonary sepsis).[1-3] Acute respiratory distress syndrome results in a high rate of mortality. The aim of the acute lung injury (ALI)/ ARDS treatment is to prevent lung injury, to decrease lung edema, and to maintain tissue oxygenation.[4]

Apoptosis is the synchronized and selective type of cell death which balances the cell proliferation and cell death.[5] It is programmed to kill cells with degenerated functions as well as cells that are unnecessary in terms of connection.[6]

Cell death is related to tissue damage in most of the diseases and it results in reaction of two highly reactive free radicals: nitric oxide and superoxide.[7] Peroxynitrite can easily react with cell components such as lipids and proteins and oxidize and harm many molecules (deoxyribonucleic acid, lipids, proteins etc.) with its oxidative effect due to its high reactive anion nature.[7] These reactions lead to degenerated cell function and cell balance. Peroxynitrite damages physiological cell function by nitrification of many proteins and lead to cell apoptosis.[8] If peroxynitrite starts the cell damage and the repair mechanism cannot work properly, it triggers two different cell death types (apoptosis and necrosis) and causes tissue damage.[9] In addition to peroxynitrite being part of an inflammatory cascade, the application of peroxynitrite can also trigger an inflammatory response. In experimental studies, it is also shown that intra-tracheal application of peroxynitrite can lead to inflammation and degenerative changes in respiratory epithelia.[7]

Compounds that are found in nature are an important source for the drug industry.[10] Oleanolic acid is a triterpenoid which can be isolated in more than 120 plant leaves or roots and is naturally present in foodstuffs.[11-13]

The main precursor of the biosynthesis of triterpenoids and steroids is accepted as squalene. Triterpenoids have many of the same biological effects as steroids. Therefore, there is a growing interest in triterpenoids.[13]

Oleanolic acid has been shown to have anti-bacterial, anti-parasitic, anti-osteoporotic, antifertility, anti-hypertensive, anti-hyperlipidemic, anti-diabetic, anti-mutagenic, immunomodulatory, anti-inflammatory, gastro- and hepatoprotective, antiviral, anti-tumoral, anti-human immunodeficiency virus, and anti-oxidant effects.[13-18] In this study, we aimed to evaluate the efficiency of oleanolic acid on ALI and ARDS.

MATERIALS AND METHODS

This study was conducted at Mersin University Faculty of Medicine between April 2014 and May 2014. The study protocol was approved by the T.C. Mersin University Animal Experiments Local Ethics Committee. The study included 70 female Wistar albino rats (weighing 180 to 200 g). The rats were obtained from the Mersin University Experimental Research and Animal Experimentation Laboratory.

Seven groups were set up with 10 rats each. Seven groups were allocated as follows: the control group, peroxynitrite 24th h group (P24), peroxynitrite and bronchoalveolar lavage oleanolic acid 24th h group (POB24), peroxynitrite and intraperitoneal oleanolic acid 24th h group (POI24), peroxynitrite 48th h group (P48), peroxynitrite and bronchoalveolar lavage oleanolic acid 48th h group (POB48) and peroxynitrite and intraperitoneal 48th h oleanolic acid group (POI48), respectively. Prior to surgery and drug administration, the rats were anesthetized with 100 mg/kg of intraperitoneal ketamine.

Rats in the control group were not exposed to any treatment but they were also anesthetized with 100 mg/kg of intraperitoneal ketamine. Rats were sacrificed at 24 h in 24th hour groups and at 48 h in 48th h groups for biochemical examination by opening the abdominal cavity and taking blood samples by puncturing the inferior vena cava. The thorax cavity and neck were opened and the lower right lung lobe was excised for histopathological examination.

Rats in the other groups were injected with 100 mg/kg ketamine intraperitoneally and were anesthetized. Then, trachea was freed and 5 mM 0.25 mL of peroxynitrite was injected. Skin and subcutaneous tissues of the neck were sutured.

In the peroxynitrite groups, group P24 was injected again with 100 mg/kg of intraperitoneal ketamine and anesthetized 24 h after the first treatment and the P48 group was injected again after 48 h. Rats were sacrificed under anesthesia for biochemical examination by opening the abdominal cavity and taking blood samples by puncturing the inferior vena cava. The thorax cavity and necks of the rats were opened and the lower right lung lobe was excised for histopathological examination.

After peroxynitrite injection with oleanolic acid from bronchoalveolar lavage (POB24 and POB48), 5 mg/kg of intra-tracheal oleanolic acid was applied. Rats were sacrificed following the collection of blood and tissue samples at the 24 (POB24) and 48 (POB48) h after treatment.

As for the intraperitoneal oleanolic acid groups (POI24 and POI48), 10 mg/kg of intraperitoneal oleanolic acid was applied after the peroxynitrite injection. Rats were sacrificed following the collection of blood and tissue samples at the 24 (POI24) and 48 (POI48) h after treatment, respectively.

The level of interleukin (IL) 10 was measured with eBioscience Rat IL-10 Platinum enzyme-linked immunosorbent assay (ELISA) (eBioscience, San Diego, CA, USA, Catalog No: BMS629, Lot: 92553013), IL-4 level with eBioscience Rat IL-4 Platinum ELISA (eBioscience, San Diego, CA, USA, Catalog No: BMS628, Lot: 94252011), tumor necrosis factor alpha (TNF-α) level with eBioscience Rat TNF-α Platinum ELISA (eBioscience, San Diego, CA, USA, Catalog No: BMS622, Lot: 93556038) and IL-1 beta (β) level with eBioscience Rat IL-1β Platinum ELISA (eBioscience, San Diego, CA, USA, Catalog No: BMS630, Lot: 94640015) by the DSX™ Four-Plate Automated ELISA Processing System microELISA device. The amounts of biochemicals were measured according to the curve obtained from the absorbance and concentrations of standards.

The serum malondialdehyde (MDA) level was determined by using the thiobarbituric acid reactive substance (TBARS) assay kit (Catalog No: 10009055, Lot: 0449411; Cayman Chemical Company, MI, USA).

Pulmonary specimens of 70 cases (rats) were fixed in 1% formalin for one day, followed by standard dehydration and paraffin embedding procedures. Hematoxylin and eosin (H-E) stained sections were prepared by standard techniques. In addition, 4 μm

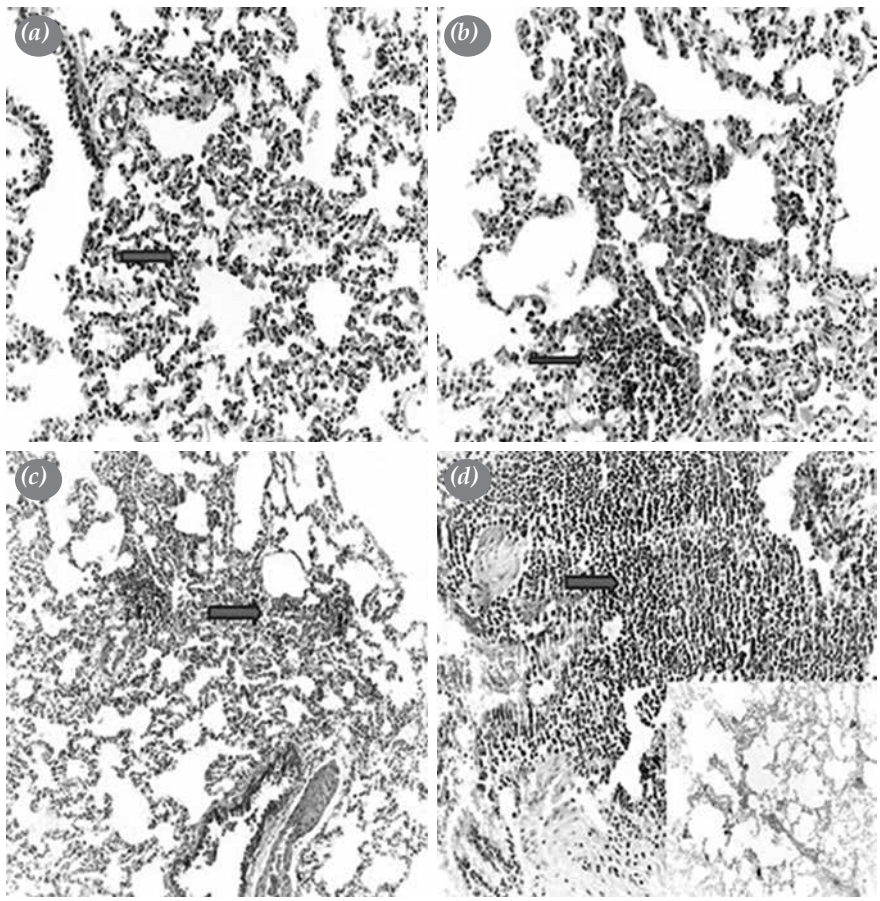

Figure 1. (a) Normal lung histology, score 1 (H-E ×20). **(b)** Perivascular minor neutrophil infiltration in lung, score 2 (H-E ×20). **(c)** Perivascular neutrophil infiltration and edema fluid in lung, score 3 (H-E ×20). **(d)** Intensive neutrophil infiltration, abscess intraalveolar exudates, fibrosis of small airways walls, and epithelial desquamation in lung, score 4 (H-E ×20).

sections were taken for immunohistochemical staining method. ApopTag plus peroxidase *in situ* apoptosis detection kit (Millipore, Billerica, MA, USA) were used. The sections were evaluated at 5 and 10 high magnification fields (HPF) on light microscope (Olympus BX51;Olympus, Tokyo, Japan).

Specimens were assessed by modifying the study of Köksel et al.[19] in the form of lung architectural deformation, edema, inflammatory cell infiltration, tissue damage (as parenchymal damage parameters) and apoptotic cells.[20] The specimens were evaluated on light microscope by H-E sections.

The samples were divided into four scores according to tissue damage. Scoring was performed in the following way: score 1: normal histology, score 2: mild damage: perivascular minor neutrophil infiltration (<5 neutrophil infiltration at 10 HPF) for lung tissue, score 3: moderate damage: perivascular neutrophil infiltration (5-10 neutrophil infiltration at 10 HPF), perivascular and intra-alveolar edema fluid for lung, score 4: severe damage: intensive neutrophil infiltration (more than 10 neutrophil infiltrations in 10 HPF, (abscess formation), intraalveolar exudates, fibrotic thickening (fibrosis) of the small airways wall, and epithelial desquamation for lung and abscess formation (Figure 1).[19] Apoptotic cells were counted for mesenchymal cells at 5 HPF by light microscope with ApopTag plus peroxidase *in situ* apoptosisdetection kit (Millipore, Billerica, MA, USA).[20] The macrophages which showed positive staining by endothelial nitric oxide synthase were counted where the inflammation was most prominent in the lung (5 HPF). The alveolar cells stained by vascular endothelial growth factor receptor were also counted (5 HPF). Vascular endothelial cells stained by factor VIII were counted where the inflammation was most visible and vascularization was most frequently observed (5 HPF).

Statistical analysis

Normality based on continuous measurements was tested with Shapiro-Wilk test. For data with normal distribution, differences between groups were tested with variant analysis. Homogeneity of variations was tested with Levene's test. One-way analysis of variance was used for the results with homogeneous variation and Welch or Brown-Forsythe test was used for the results with non-homogeneous variation. Tukey honestly significant difference and Games-Howell tests were used for paired comparison. Average and standard deviation values were given for descriptive statistics. The chi-square test was used for categorized data analysis and the results were described using numbers (percentages). Data analysis was performed using the IBM SPSS version 21.0 software (IBM Corp., Armonk, NY, USA).

RESULTS

According to the TBARS results (Table 1), a statistically significant increase in the P24 and P48 groups compared to control group was confirmed (p=0.002). When P24 was compared to the POB24 and POI24 groups, a statistically significant decrease was observed compared to the treatment groups (p=0.010 and p=0.001, respectively). When P48 was compared to the POB48 and POI48 groups, a statistically

Table 1. Biochemical results

	TBARS (μM)	IL-4 (pg/mL)	IL-10 (pg/mL)	IL-1β (pg/mL)	TNF-α (pg/mL)
Groups	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
Control	5.889±0.876	0.374±0.367	24.289±6.190	86.163±31.855	50.976±9.885
P24	12.511±7.547	0.717±0.179	37.738±7.635	163.173±20.236	56.438±24.883
POB24	6.673±1.240	0.535±0.173	34.623±3.495	118.236±40.363	41.465±7.528
POI24	5.661±1.108	0.492±0.060	31.585±7.281	99.102±26.243	45.711±19.467
P48	10.353±4.491	0.736±0.168	42.941±14.430	141.449±46.718	81.602±45.420
POB48	6.503±1.061	0.548±0.107	41.514±15.919	90.240±24.860	45.964±12.885
POI48	6.442±1.291	0.583±0.147	31.382±4.508	88.701±28.891	43.478±8.177

TBARS: Thiobarbituric acid reactive substance; IL: Interleukin; TNF-α: Tumor necrosis factor alpha; P24: Peroxynitrite 24th h group; POB24: Peroxynitrite and bronchoalveolar lavage oleanolic acid 24th h group; POI24: Peroxynitrite and intraperitoneal oleanolic acid 24th h group; P48: Peroxynitrite 48th h group; POB48: Peroxynitrite and bronchoalveolar lavage oleanolic acid 48th h group; POI48: Peroxynitrite and intraperitoneal 48th h oleanolic acid group; After intra-tracheal peroxynitrite injection, increase in all of these molecular levels was statistically significant except for increase in TNF-α level in P24 group. But in P48 group, increase in all of these molecular levels was statistically significant. In treatment groups, particularly at 48 h, there was significant decrease in TBARS, IL-4, IL-1β and TNF-α levels.

significant decrease was observed compared to the treatment groups (p=0.007 and p=0.005, respectively). There was no significant difference between POB24 and POI24, or POB48 and POI48 (p=0.07 and p=0.121, respectively). These results show that oleanolic acid is effective in decreasing oxidative stress.

Compared to the control group, IL-4 levels (Table 1) showed a significant increase within the P24 and P48 groups (p<0.001). POB48 and POI48 showed a statistically significant decrease compared to P24 (p=0.007). Compared to P48, POB48 and POI48 showed a statistically significant decrease (p=0.003). There was no significant difference between POB24 and POI24; and POB48 and POI48 (p=0.067). These results show that oleanolic acid decreases the anti-inflammatory cytokine, which can cause continued inflammation.

The IL-10 level (Table 1) of P24 and P48 showed a statistically significant increase compared to the control group (p=0.003 and p=0.001, respectively). POB24 and POI24 showed a statistically non-significant decrease compared to P24 (p=0.137). POB48 did not show a statistically significant difference compared to P48 (p=0.117). When the P48 and POI48 groups were compared, a statistically significant decrease was observed (p=0.012). Although there was no significant difference between POB24 and POI24 (p=0.08), there was a statistically significant difference between the POB48 and POI48 groups (p=0.142). These results show that oleanolic acid decreases the anti-inflammatory cytokine, which is responsible for the continuation of inflammation, as indicated with the results of IL-4.

The IL-1β level (Table 1) of P24 and P48 showed a statistically significant increase compared to the control group (p<0.001 and p=0.008, respectively). POB24 and POI24 showed a statistically significant difference compared to P24 (p<0.001). POB48 and POI48 did not show any significant difference compared to P48 (p=0.019 and p=0.01, respectively). There was no statistically significant difference between POB24 and POI24; or between POB48 and POI48 (p=0.1 and p=0.072, respectively). Oleanolic acid was shown to prevent the trigger of inflammation.

The TNF-α level (Table 1) of P24 showed a statistically non-significant increase compared to the control group (p=0.139). However, P48 showed a statistically significant increase compared to the control group (p<0.001). POB24 and POI24 showed a statistically non-significant decrease compared to P24 (p=0.139). POB48 and POI48 showed a statistically significant decrease compared to P48 (p<0.001). There was no statistically significant difference between POB24 and POI24 or between POB48 and POI48 (p=0.05 and p=0.061, respectively). These results show that oleanolic acid decreases the release of pro-inflammatory cytokine during ALI.

According to the H-E staining results, the difference between the ratios of P24 and P48 compared to the control group was statistically significant when a normal histology (score 1) was considered (p=0.003) (Figure 2). Lung architecture was normal and 90% compatible with score 1 in the control group. There was a significant decrease between normal histology

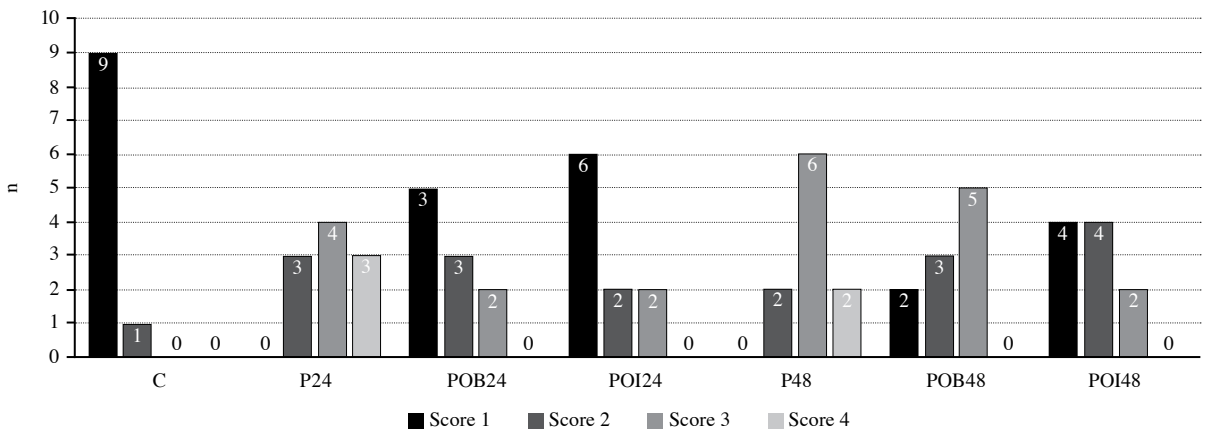

Figure 2. Parenchymal damage scores (H-E).

C: Control group; P24: Peroxynitrite 24th h group; POB24: Peroxynitrite and bronchoalveolar lavage oleanolic acid 24th h group; POI24: Peroxynitrite and intraperitoneal oleanolic acid 24th h group; P48: Peroxynitrite 48th h group; POB48: Peroxynitrite and bronchoalveolar lavage oleanolic acid 48th h group; POI48: Peroxynitrite and intraperitoneal 48th h oleanolic acid group; Score 1: Normal histology; Score 2: Minor neutrophilic infiltration; Score 3: Moderate neutrophilic infiltration, perivascular edema, mild fibrosis; Score 4: Intensive neutrophilic infiltration, abscess formation, marked fibrosis. The most tissue damage is seen in P48 groups. In treatment groups at 48 h (POI48 and POB48), there is a significant decrease in pathology scores.

values in P24 and P48 groups compared to the control group. The difference between the ratios of the control group and POI24 was statistically significant when a normal histology was considered (p=0.011).

Considering a normal histology, the difference between the ratios of the control group and POB24; and the control group and POI48 were statistically significant (p=0.018 and p=0.033, respectively).

The difference between the ratios of the control group and P24; and the control group and P48 were statistically significant when moderate damage (score 3) was considered (p=0.0121). These results show that treatment leads to decreased tissue damage.

In the lung tissue, the difference between the ratios of POB24 and POI24 compared to P24 were statistically significant when score 4 was considered (p=0.03).

In the lung tissue, the difference between the ratios of POB48 and POI48 compared to P48 were statistically significant when score 3 was considered (p=0.041).

Apoptosis results showed a statistically significant increase in apoptotic cells when P24 and P48 were compared to the control group (p=0.031 and p<0.001, respectively) (Figure 3). There was no statistically significant difference between the P24 and POB24 groups (p=0.149). POI24 showed a statistically significant decrease compared to P24 (p=0.014). There was a significant decrease when POB48 and POI48 were compared to P48 (p=0.001 and p<0.001). These results show that oleanolic acid might decrease the apoptosis in lung parenchyma, particularly at the 48 h.

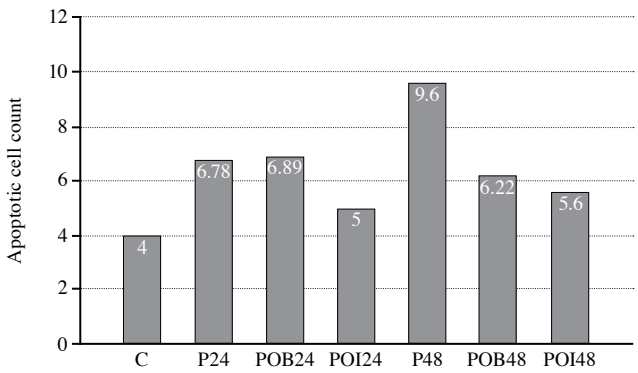

Figure 3. Lung tissue ApopTag staining results.

C: Control group; P24: Peroxynitrite 24th h group; POB24: Peroxynitrite and bronchoalveolar lavage oleanolic acid 24th h group; POI24: Peroxynitrite and intraperitoneal oleanolic acid 24th h group; P48: Peroxynitrite 48th h group; POB48: Peroxynitrite and bronchoalveolar lavage oleanolic acid 48th h group; POI48: Peroxynitrite and intraperitoneal 48th h oleanolic acid group; Lung tissue mean apoptotic cell count in high power fields. The most apoptotic cell count is in P48 group and there is a significant decrease in treatment groups (POI48 and POB48).

DISCUSSION

Acute respiratory distress syndrome is an acute respiratory failure syndrome which can affect both lungs, characterized by non-cardiogenic diffused infiltration, and non-responsiveness to oxygen treatment.[21]

There are many studies for the treatment of acute respiratory failure due to its high morbidity, mortality and the financial distress that it causes.[22] Although various drugs and methods were tested up to this point, the only trial that has been shown to contribute to the survival of patient is the lung-protective, lower-tidal volume mechanical ventilation.[23]

Oleanolic acid can be naturally present in foodstufs.[11-13] Most of the therapeutic effects of oleanolic acid were confirmed by recent scientific studies.[13] The effect of oleanolic acid on ALI which is induced by lipopolysaccharides was examined in terms of lung mechanics and histopathology.[15]

The aim of this study was to assess the effect of oleanolic acid using the ALI model obtained via intra-tracheal peroxynitrite application. Overall, an increase in serum MDA, IL-1β, IL-4, IL-10, and TNF-α levels was detected at the 24 and 48 h via the intra-tracheal peroxynitrite application. The increase in all of these molecular levels was statistically significant except for the increase in the TNF-α level in the P24 group.

A wide-spread cause of an excessive normal-inflammatory response is lipid peroxidation. Biochemical indication of oxidation damage is found in tissue and blood MDA levels.[24] Malondialdehyde levels were found to increase in various studies concerning ALI.[25,26] In our study, MDA levels in peroxynitrite-induced ALI increased in all groups. This is consistent with the literature. There was a statistically significant decrease in MDA levels with the olenaolic acid treatment. However, there was no statistically significant difference between bronchoalveolar lavage or intraperitoneal applications of oleanolic acid. These results suggest that oleanolic acid might decrease oxidative damage, as expected from previous studies.

It was shown that IL-1β and TNF-α are prime mediators in the activation of cytokines. They cause activation of leukocyte chemotaxis with adhesion molecules in ALI/ARDS.[27] In our study, there was an increase in IL-1β levels in groups of ALI consistent with the literature. Even though there was an increase

in TNF-α level at the 24 h, it was not statistically significant. However, the increase at the 48 h was statistically significant. Thanks to oleanolic acid treatment, there was a statistically significant decrease in IL-1β and TNF-α levels. These results suggest that oleanolic acid has an anti-inflammatory effect and it can be useful for ALI/ARDS treatment.

When the lung injury scores were considered, the P24 and P48 groups showed a statistically significant decrease compared to the control group in terms of moderate and severe damage scores. With oleanolic treatment, there was a significant decrease in damage scores. These results suggest that oleanolic acid might protect normal histology in ALI.

It has already been indicated experimentally and clinically that apoptosis takes place in the first 48 h in ALI/ARDS. Systemic inflammatory response and increased neutrophil accumulation, together with toxic metabolites released from neutrophils, form the first step of apoptosis.[28] Consistent with the literature, in our study, it was observed that the apoptotic cell count was statistically significant for the lung tissue of rats with ALI. However, there was a significant decrease in apoptotic cell count of rats treated with oleanolic acid. These results suggest that oleanolic acid might be effective during the treatment of ALI/ARDS.

Acute lung injury and ARDS have serious morbidity and mortality rates, but effective treatment has not yet been found. New drug treatments tested for these syndromes have not been effective, although there are various studies in the literature. Oleanolic acid, known to have inflammatory effects, has been observed to be effective for the treatment of these cases. However, further clinical studies are required to apply this as a standard treatment.

Since the study was planned on biochemical and pathological parameters, no blood gas analysis or radiological imaging was performed. This can be considered as a limitation for our study.

In conclusion, this study confirms that oleanolic acid treatment decreases macrophage infiltration in lung tissue, regulates the inflammatory process by decreasing the release of proinflammatory and anti-inflammatory cytokines, and decreases lung injury and oxidative stress. As a result, oleanolic acid, as an anti-inflammatory and antioxidant agent, might be useful for the treatment of acute lung injury and acute respiratory distress syndrome, which have no effective treatment up to this date.

Declaration of conflicting interests

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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