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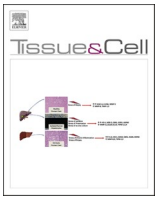

The effects of pentoxifylline and caffeic acid phenethyl ester on TNF-α and lung histopathology in D-galactosamine-induced pulmonary injury in rats

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ABSTRACT

In this study, we aimed to investigate the effects of pentoxifylline [PTX] and caffeic acid phenethyl ester [CAPE] in D-galactosamine [D-GAL]-induced pulmonary injury in rats. The rats were randomly divided into six groups: control, D-GAL, D-GAL+PTX, D-GAL+CAPE, PTX and CAPE. Each group included eight animals. Lung sections from the control, PTX and CAPE groups had a normal histological appearance. The D-GAL group showed histopathological changes in lung tissue, including haemorrhage, oedema, inter-alveolar septal thickening and widespread infiltration of inflammatory lymphocytes and macrophages. Administration of PTX and CAPE significantly reduced histopathological damage scores in the D-GAL+PTX and D-GAL+CAPE groups compared with the D-GAL group. PTX and CAPE treatment also significantly decreased malondialdehyde levels, increased levels of reduced GSH and increased catalase and superoxide dismutase activity in lung tissue samples. These results indicate that the destructive effects of D-GAL-induced inflammation in the rat lung are significantly reduced following administration of PTX and CAPE.

1. Introduction

A number of different animal models are used to study pulmonary injury and they are based on clinical issues such as sepsis, multiple transfusions, multiple traumas, aspiration of gastric contents and reperfusion of ischaemic tissues (Kurt et al., 2016; Lian et al., 2020). Research has shown that chemical agents and surgical methods used in experimental animal injury models can cause secondary organ failure (Bayrak et al., 2016). D-galactosamine [D-GAL] is a hepatotoxin that causes liver damage in vivo by depleting nucleotides with subsequent inhibition of protein and RNA synthesis (Decker and Keppler, 1974). Previously, Bayrak et al. reported that D-GAL-induced liver injury accompanied by lung injury also led to kidney injury in rats (Bayrak et al., 2016).

D-GAL induces the secretion of cytokines, mainly tumour necrosis factor-alpha [TNF-α], by activating macrophages (Kasravi et al., 1996). TNF-α is the primary mediator of the acute inflammatory response against infectious agents and is responsible for many systemic complications associated with serious infections. Released within a few minutes of local or systemic tissue injury, TNF-α damages the lung parenchyma by activating inflammatory mediators that regulate the migration and infiltration of neutrophils and macrophages into the pulmonary interstitial tissue (Iranzo Francisco et al., 2020).

Pentoxifylline [PTX] is a phosphodiesterase inhibitor with anti-inflammatory activity that targets TNF-α (Okumura et al., 2009). PTX also inhibits cell proliferation and extracellular matrix accumulation (Lin et al., 2008; Turhan et al., 2012). In addition, due to its reasonable cost and low toxicity PTX's antiviral, immunomodulatory and bronchodilatory effects suggest that it may have potential to be used alone, or as an adjuvant treatment in combination with other drugs, for the treatment of viral disease (Oliveira-Junior et al., 2010).

Caffeic acid phenethyl ester [CAPE] has been reported to have potent anti-inflammatory activity (Krol et al., 1996). In addition, CAPE has anti-oxidant, antiviral, antimicrobial, carcinostatic, immunostimulatory and immunomodulatory properties (Sud'ina et al., 1993).

The present study aimed to examine the role of PTX and CAPE in mitigating D-GAL-induced pulmonary injury in rats by evaluating histopathological changes and anti-oxidant, apoptotic and inflammatory parameters.

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2. Materials and methods

2.1. Animals

A total of 48 Wistar albino rats [3–4 months old] weighing 200–250 g were used. Rats were obtained from İnönü University Experimental Animals Research Center. The rats were randomly divided into 6 groups – control, D-GAL, D-GAL+PTX, D-GAL+CAPE, PTX and CAPE – with 8 animals in each group. The experiments were carried out in accordance with the guidelines for animal research from the National Institute of Health and were approved by the Committee on Animal Research at Inonu University, Malatya, Turkey. [Ethic number: 2010/71].

2.2. Experimental protocol

Rats in the D-GAL, D-GAL+PTX and D-GAL+CAPE groups received intraperitoneal [i.p.] D-GAL [BioChemica, Germany] 500 mg/kg body weight. Rats in the D-GAL+PTX and PTX groups received PTX [Trental ampule], 50 mg/kg/day, i.p, and those in the D-GAL+CAPE and CAPE groups received CAPE [Sigma Aldrich, Steinheim, Germany] 10 mmol/kg, i.p.

At the end of day 21, rats were sacrificed under ketamine–xylazine anaesthesia. After blood samples were collected from the tail vein, the lungs were excised and separated. The left lung from each animal was frozen for biochemical analysis, while the right lung was fixed in a 10% formalin solution for histological analysis.

2.3. Histopathological and immunohistochemical analysis

Paraffin-embedded blocks of lung tissue were sectioned at a thickness of 5 μm. Sections were stained with haematoxylin–eosin to observe the general histological structure, and immunohistochemical staining methods were used to visualise TNF-α [Thermo Fisher Scientific, Inc., Waltham, MA, USA] and caspase-3 [Thermo Fisher Scientific, Inc., Waltham, MA, USA] activity. Inter-alveolar septal thickness was measured in five randomly selected sections to evaluate the degree of interruption of the blood–air barrier (Zakaria et al., 2021). Samples were examined using a Leica DFC280 light microscope and a Leica Q Win Image Analysis system [Leica Micros Imaging Solutions Ltd., Cambridge, UK].

2.4. Biochemical analysis

Tissue homogenate from the lungs of each rat was used to analyse oxidative stress biomarkers. Protein levels in the tissue samples were measured using Bradford assays (Bradford, 1976). Tissue malondialdehyde [MDA] was evaluated colourimetrically as described by Buege and Aust (Buege and Aust, 1978) to assess lipid peroxidation in the form of thiobarbituric acid reactive substances. Measurement of reduced glutathione [GSH] was performed using a modification of Akerboom's method (Akerboom and Sies, 1981). Tissue superoxide dismutase [SOD] activity was evaluated using McCord and Fridovich's method (McCord and Fridovich, 1969), and catalase [CAT] activity was estimated using Gonzalez-Flecha's method (Gonzalez-Flecha and Demple, 1994).

2.5. Statistical analysis

A computer programme [SPSS 17.0] was used for statistical analysis. The results were compared using Kruskal–Wallis variance analysis. Where differences among the groups were detected, group means were compared using the Mann–Whitney U test. Values of $p < 0.05$ were considered significant. All results were expressed as mean ± standard error [SE].

3. Results

3.1. Light microscopy

Lung sections from rats in the control, PTX and CAPE groups showed intact lung architecture with thin inter-alveolar septa and normal alveolar, perivascular and peribronchiolar cells [Fig. 1A, B and C; respectively]. In the D-GAL group, induction of lung injury by D-GAL administration resulted in histopathological changes, including haemorrhage [Fig. 1D] and frequent cell infiltration by lymphocytes and macrophages, particularly in the inter-alveolar area [Fig. 1E, F; respectively].

Inter-alveolar septal thickness was measured to evaluate the degree of interruption of the blood–air barrier. When the D-GAL+CAPE and D-GAL+PTX groups were compared, no significant difference in inter-alveolar septal thickness was observed. However, there was a significant decrease in septal thickness when these groups were compared with the D-GAL group. Most alveoli in the PTX and CAPE-treated groups appeared intact with thin inter-alveolar septa, and these groups showed remarkable signs of improvement, with marked restoration of lung architecture. Moreover, administration of PTX and CAPE significantly reduced the histopathological damage score in the D-GAL+PTX and D-GAL+CAPE groups compared with the GAL group [$p < 0.0001$]. However, the D-GAL+PTX and D-GAL+CAPE groups showed slight haemorrhage [Fig. 1G] and mild inflammation [Fig. 1H], as well as partly focal areas of limited inter-alveolar septal thickening. The lung tissue damage scores of all groups are summarised in Table 1.

3.2. TNF-α staining

Lung sections from control [Fig. 2A], CAPE [Fig. 2B] and PTX [Fig. 2C] groups showed mild TNF-α staining. In contrast, the number and signal density of TNF-α-positive cells were significantly greater in the D-GAL group [Fig. 2D]. CAPE [Fig. 2E] and PTX treatment reduced the reactivity and the number of TNF-α-positive cells relative to D-GAL treatment alone [Fig. 2F] [Table 1].

3.3. Caspase 3 staining

Lung sections from control [Fig. 3A], CAPE [Fig. 3B] and PTX [Fig. 3C] groups showed mild caspase 3 staining in lung sections. In comparison, the number and signal density of caspase-positive cells were significantly greater in the D-GAL group [Fig. 3D]. CAPE [Fig. 3E] and PTX treatment reduced the reactivity and the number of caspase-positive cells relative to D-GAL treatment alone [Fig. 3F]. [Table 1].

3.4. Biochemistry

When the D-GAL and control groups were compared, tissue MDA levels were significantly increased in the D-GAL group [$p < 0.05$], while GSH levels and CAT and SOD activity were significantly reduced [$p < 0.05$]. On the other hand, when the D-GAL+PTX and D-GAL+CAPE groups were examined, MDA levels were significantly reduced in these groups compared with the D-GAL group [$p < 0.05$]. These results suggest that while PTX and CAPE inhibited lipid peroxidation and the production of MDA, they stimulated the production of antioxidant enzymes. Mean tissue MDA and GSH levels, and SOD and CAT activity in all groups are summarised in Table 2.

4. Discussion

Experimental studies with animal models have described various chemical agents that protect against lung tissue damage. For example, dexmedetomidine has been reported to protect against lung damage induced by ischaemia–reperfusion injury (Zhou et al., 2018; Bringué, 2021). Mammadov et al. suggested that lutein helps prevent

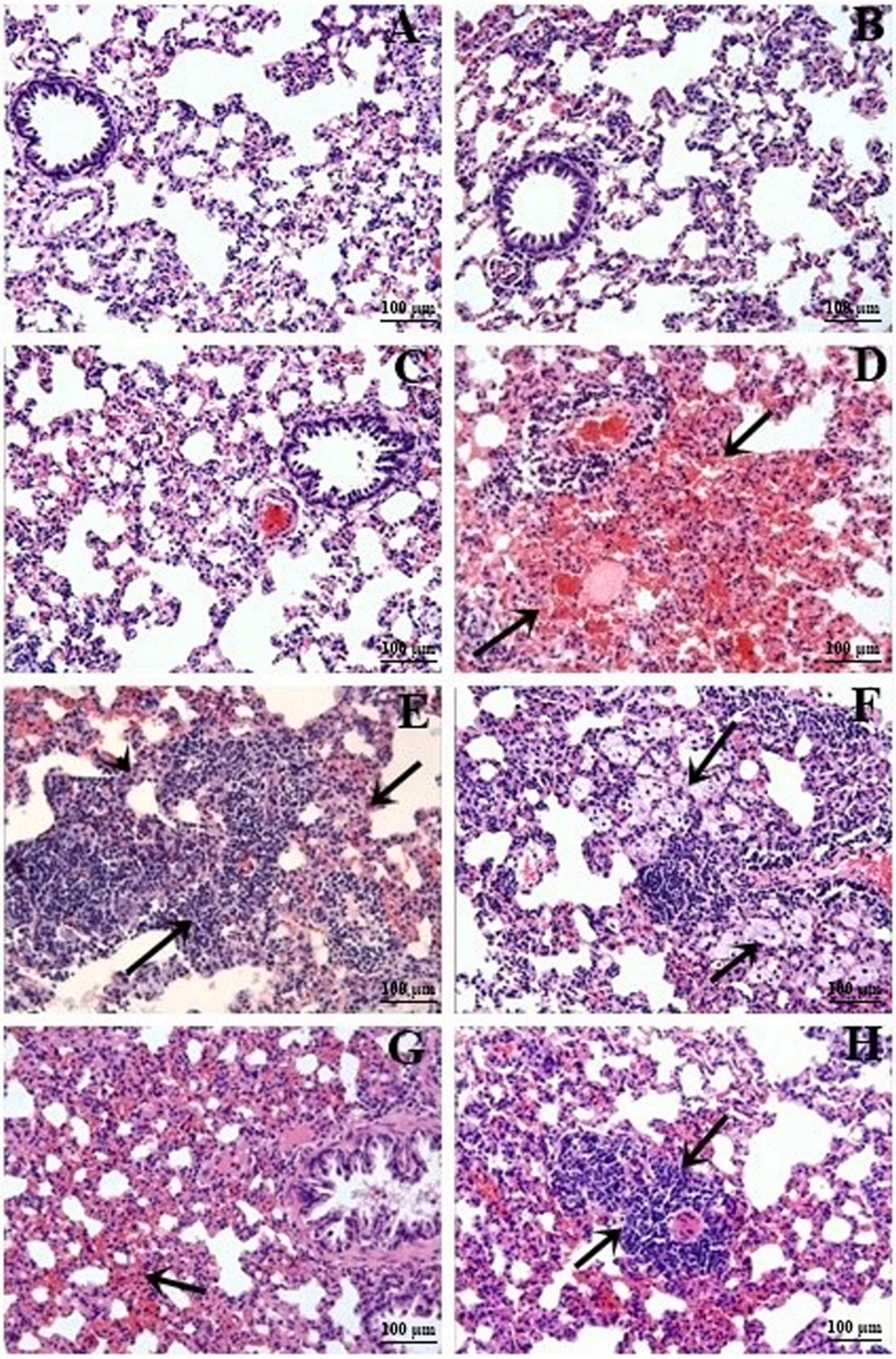

Fig. 1. Histopathological changes of lung tissues in different animal groups. Control, PTX and CAPE groups [A, B and C respectively] showing normal lung architecture. D-GAL group, [D] the appearance of hemorrhage within alveolar wall, [arrows] [E] notice thickening of inter-alveolar septa [arrow head] with massive lympocytes infiltration [arrows], [F] the view of alveolar, and septal macrophages accumulation [arrows]. D-GAL+PTX group, [G] the view of rare hemorhagia [arrows], D-GAL+CAPE group, [H] the appearance of mild inflammatuar cell infiltration [arrow]. H&EX200.

MTX-induced oxidative lung damage (Mammadov et al., 2019), and it has been reported that apocynin decreased bleomycin-induced lung damage (Kilic et al., 2015). In the present study, we found that D-GAL exposure led to oxidative damage in rat lungs by reducing the activity of antioxidant enzymes and increasing lipid peroxidation. This was accompanied by histopathological changes including inflammatory cell infiltration, haemorrhage and TNF-α and caspase-3 reactivity. However, the experimental data indicated that treatment with PTX or CAPE protected rats against D-GAL-induced lung damage.

D-GAL causes toxicity via a mechanism involving GSH depletion and inhibition of RNA synthesis (Gehrke et al., 2018; Yoo et al., 2008). Studies have also reported that D-GAL alone caused potent intracellular generation of reactive oxygen species (ROS) in cell culture, and that accumulation of ROS can induce oxidative stress in vitro and in vivo (Bak et al., 2018). MDA is a strong indicator of oxidative stress following lipid peroxidation in cellular structures. A significant increase in MDA has been reported in the tissue of animals following administration of D-GAL (Park et al., 2020). In our study, administration of D-GAL increased MDA levels and significantly decreased both GSH levels and the activity of the antioxidant enzymes SOD and CAT. Treatment with PTX and CAPE led to improvements in these parameters.

D-GAL is a common agent used to sensitise rats and other animals to

Table 1
The results of semiquantitative histological assessment [mean ± SE].

Groups	Hemorrhagia	Lymphocyte accumulation	Macrophage accumulation	Septal thickness	TNFα density	Caspase-3-positive
Control	0.12±0.12	0.12±0.12	0.25±0.16	6.15±0.19	0.04±0.02	0.10±0.03
D-GAL	1.75±0.31[a]	1.50±0.26[a]	1.50±0.18[a]	12.99±0.36[a]	0.05±0.02	1.41±0.19[a]
D-GAL+PTX	0.75±0.25[b]	0.37±0.18[b]	0.50±0.18[b]	10.95±0.48[b]	0.04±0.02[b]	0.40±0.07[b]
D-GAL+CAPE	0.62±0.26[b,c]	0.62±0.26[b,c]	0.62±0.18[b,c]	10.70±0.39[b,c]	0.81±0.10[b,c]	0.42±0.08[b,c]
PTX	0.25±0.16	0.50±0.18	0.12±0.12	6.07±0.20	0.30±0.05	0.10±0.03
CAPE	0.12±0.12	0.25±0.16	0.12±0.12	6.28±0.17	0.25±0.05	0.14±0.04

[a] Significant increase [p<0.05], vs. Control group.
[b] Significant decrease [p<0.05], vs. D-GAL group.
[c] Not significant change [p<0.05], vs.D-GAL+PTX.

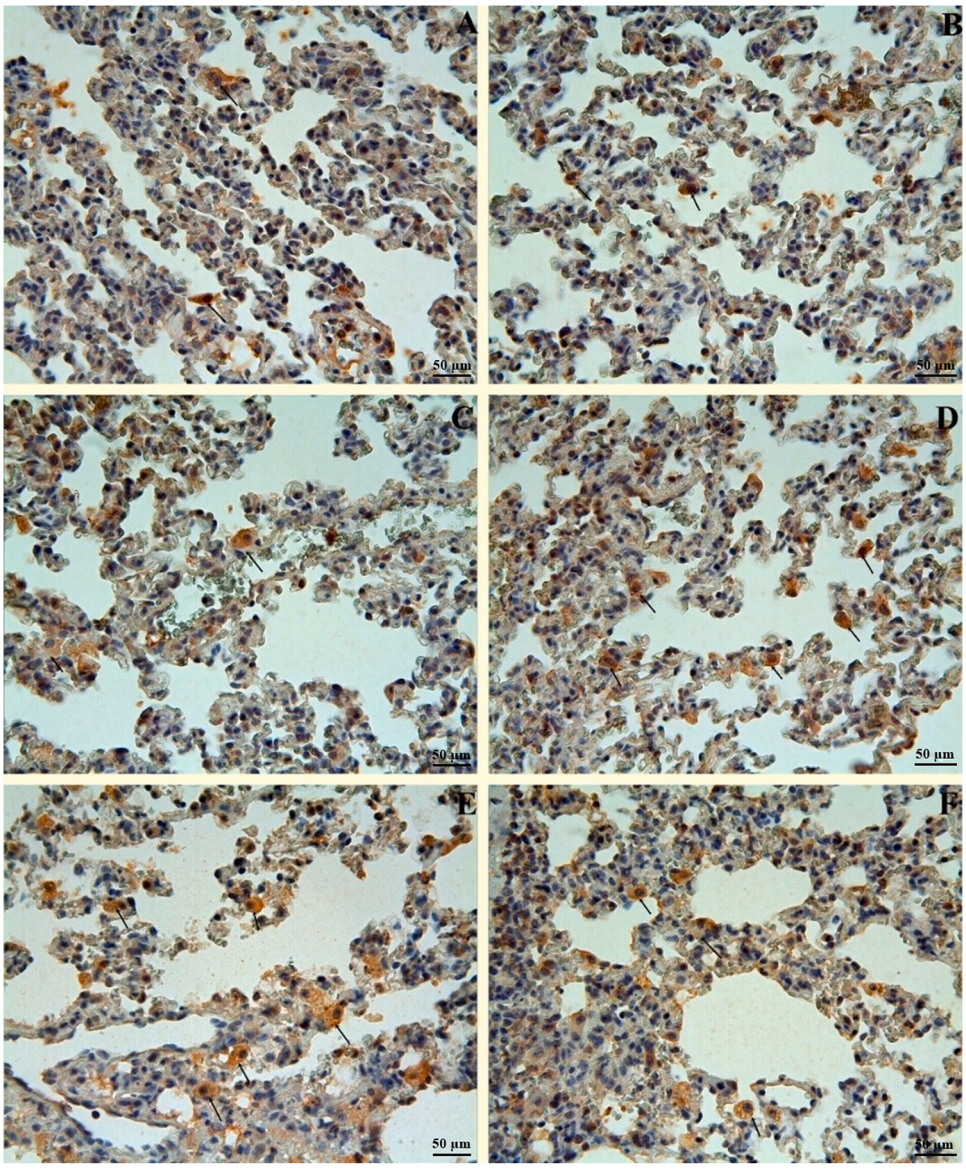

Fig. 2. Control, PTX and CAPE groups, [A, B and C; respectively] TNFα positive cell. D-GAL group, [D] the appearance of TNFα positive cell [arrows] D-GAL+PTX group, [E] the view of rare TNFα positive cell [arrows], D-GAL+CAPE Group, [F] the appearance of mild TNFα positive cell [arrow]. TNFα X400.

the lethal effects of TNF-α (Silverstein, 2004). TNF-α-related pathophysiological events are believed to play a key role in acute organ damage, including acute lung injury (Witkamp and Monshouwer, 2000). Some investigators have reported that administration of D-GAL to rats induced necrosis, and it has also been reported to induce apoptosis in the liver. This can be explained by the mediation of D-GAL toxicity through TNF-α, which is synthesized in alveolar macrophages and may be responsible for this damage. While there is considerable evidence on the intracellular effects of TNF-α, the mechanism of cytotoxicity is still not fully understood (Seckin et al., 2008; Wang et al., 2000; Chopra et al., 2009).

Caspase-3 is frequently used in studies to evaluate apoptosis because its activation is an irreversible stage in the induction of apoptosis (Nina and Mohamed, 2019). Previous studies demonstrated that D-GAL causes

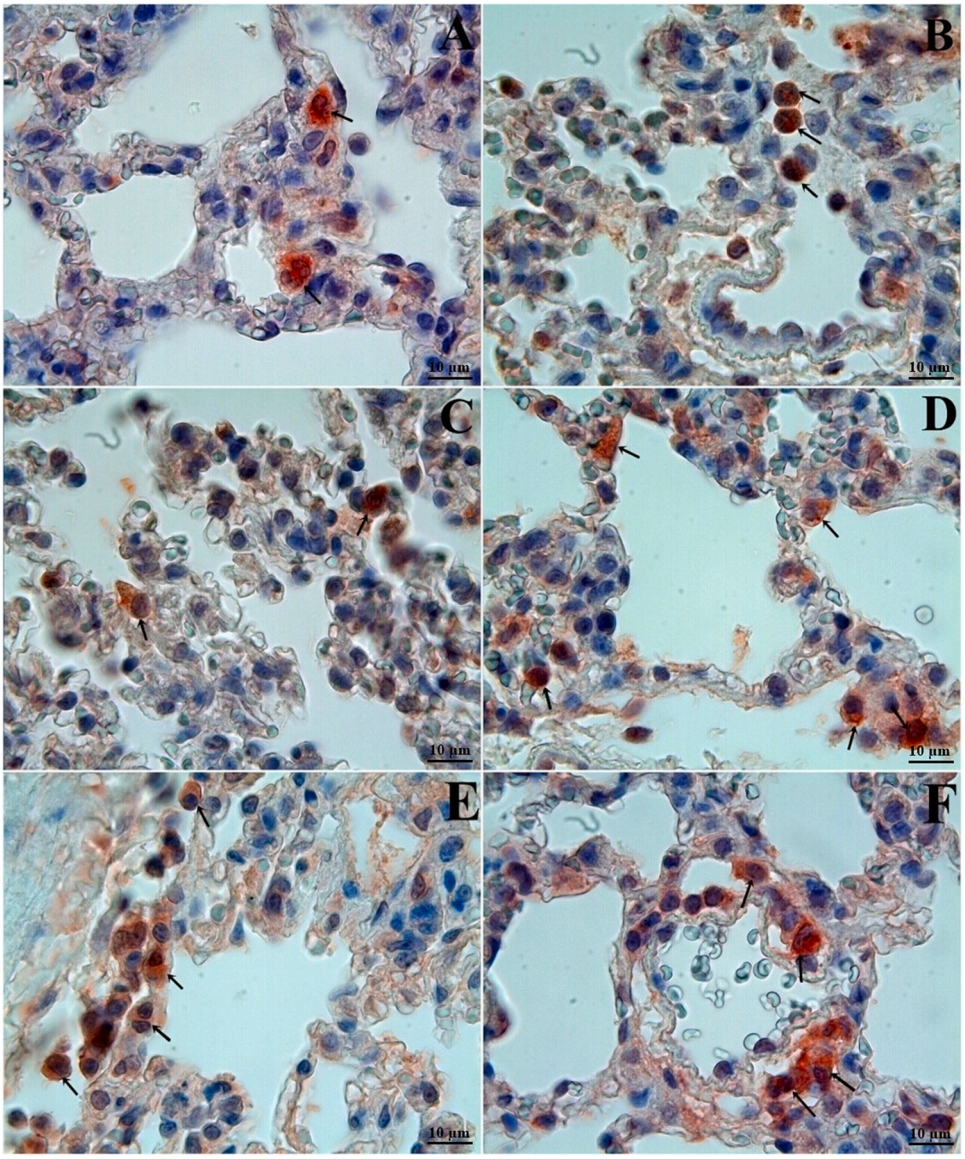

Fig. 3. Control, PTX and CAPE groups, [A, B and C; respectively] caspase-3 positive cell. D-GAL group, [D] the appearance of caspase-3 positive cell [arrows] D-GAL+PTX group, [E], the appearance of mild TNFα positive cell [arrow] D-GAL+CAPE Group, [F] the view of rare caspase-3 positive cell. Caspase-3 × 1000.

Table 2
The levels of biochemical parameters of all groups [mean ± SE].

Groups	MDA	GSH	SOD	CAT
Control	18.74±2.76	1.39±0.18	8.82±1.40	9.07±0.49
D-GAL	34.90±2.33[a]	0.69±0.13[b]	7.53±0.24[b]	7.76±0.18[b]
D-GAL +PTX	24.53±2.41[c]	1.11±0.12[d]	9.07±0.39[d]	8.85±0.30[d]
D-GAL +CAPE	21.43±2.37[c,e]	1.25±0.16[d,e]	9.92±0.57[d,e]	8.68±0.36[d,e]
PTX	19.72±3.51	1.02±0.03	9.08±0.40	8.64±0.17
CAPE	18.23±1.42	0.90±0.01	8.82±0.50	7.73±0.23

[a] Significant increase [p=0.002], vs. Control group.
[b] Significant decrease [p=0.032], vs. Control group.
[c] Significant decrease [p=0.002], vs. D-GAL group.
[d] Significant increase [p=0.032], vs. D-GAL group.
[e] Not significant chance [p<0.05], vs. D-GAL+PTX.

upregulation of caspase-3 (Zi-fengZhang et al., 2010; Yang et al., 2019). Similarly, Tapalaga et al (Tapalaga et al., 2002). reported that D-GAL treatment increased caspase-3 activity. The present study revealed that D-GAL treatment significantly increased the expression of caspase-3, while PTX and CAPE treatment significantly decreased its expression. Thus, the findings of our study suggest that PTX and CAPE may protect against D-GAL-induced lung injury by regulating the expression of caspase-3.

Similar to previous reports (Kasravi et al., 1996; Malaviya et al., 2012), our study showed significant histopathological changes in D-GAL-induced pulmonary injury, including inflammatory cell accumulation in the lung, oedema, haemorrhage and increased cytoplasmic expression of TNF-α.

PTX is a nonselective phosphodiesterase inhibitor with important immunoregulatory actions. Its anti-inflammatory, anti-oxidative, bronchodilatory and respiratory supportive properties have recently been investigated in clinical and experimental studies, particularly as adjuvant therapy in combination with other drugs (Seirafianpour et al., 2020). PTX supports tissues by accelerating blood flow and increasing tissue oxygenation (Emrecan et al., 2006; Zhang et al., 2022). PTX has been investigated in a number of different studies of inhibition of TNF-α gene transcription in vitro and in vivo. The process by which PTX terminates the transcription of TNF-α for in vivo experiments (Doherty et al., 1991) prevents its synthesis. *In vivo*, PTX attenuates the increase of TNF-α in response to intestinal mucositis (Melo et al., 2007), gastric

ulcer (Baraka et al., 2010) and allergic encephalomyelitis (El-Bassossy et al., 2009). In addition, PTX inhibits TNF-α by reducing its release from human alveolar macrophages. As a result, the production of chemotactic mediators including interleukin 1 [IL-1] and IL-6 (Marques et al., 1999) is also reduced. PTX has been shown to reduce histologic signs of pulmonary inflammation and injury after treatment. Similar suppression of inflammation and tissue damage have been reported following PTX administration in models of lung damage (Almario et al., 2012). PTX regulates inflammation through inhibition of leukocyte–platelet interactions, reduction of activation of pro-inflammatory cytokines and ROS (Lin et al., 2022).

CAPE, another drug used in our study, is known to have antioxidant properties as well as antitoxic, anti-inflammatory, antiviral, immunomodulatory, neuroprotective, and cytostatic effects (Dobrowolski et al., 1991; Castaldo and Capasso, 2002; Wan et al., 2022). It has been demonstrated that CAPE inhibits oxygen radicals and inflammation by potently and specifically inhibiting the activity of the nuclear transcription factor NF-kβ (Liao et al., 2003). NF-kβ is a redox-sensitive transcription factor that is activated in response to oxidative stress and induces cytokine expression during the inflammatory response (Karaboğa, 2019). It has also been shown that NF-kβ levels increase following D-GAL administration, (Osawa et al., 2018) and that CAPE administration specifically inhibits NF-kβ (Wang LC et al., 2010; Akgun et al., 2018). Lee et al (Lee et al., 2008). reported that CAPE had an anti-inflammatory effect on carbon tetrachloride-induced liver damage, and Sirmali et al. reported that CAPE inhibited tissue damage via antioxidative action in a rat pulmonary contusion model (Sırmalı et al., 2013). Our microscopy analyses showed that PTX and CAPE ameliorated D-GAL-induced haemorrhage, oedema and widespread inflammatory cell infiltration in the rat lung.

The present study shows that i.p. administration of D-GAL induced a well-organised lung inflammatory response, starting with a time-dependent invasion of the alveolar space by lymphocytes and macrophages, and ending in haemorrhagic lung damage. The results of our study show that the harmful effects of D-GAL-induced lung inflammation are significantly reduced by the administration of PTX and CAPE. In addition, PTX and CAPE achieved this effect by reducing the oxidative stress protein MDA and significantly increasing the activity of antioxidant enzymes SOD, GSH and CAT.

In conclusion, our results suggest that PTX and CAPE exert anti-inflammatory, anti-apoptotic and anti-oxidative effects, which may contribute to ameliorating D-GAL-induced lung damage in rats. PTX and CAPE may therefore have the potential to be protective agents in the lung.

Conflict of interest

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

Data Availability

The data that has been used is confidential.

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