

## Original Article

# Ameliorating effects of CAPE on oxidative damage caused by pneumoperitoneum in rat lung tissue

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**Abstract:** We investigated the biochemical and histopathological effects of caffeic acid phenethyl ester (CAPE) against oxidative stress causing lung injury induced by pneumoperitoneum. Twenty-eight rats were selected at random and seven rats were assigned to each of the following groups. The control group (S) was subjected to a sham operation without pneumoperitoneum. The other groups were subjected to CO<sub>2</sub> pneumoperitoneum 15 mmHg for 60 min. The laparoscopy group (L) had no additional drugs administered, the laparoscopy + alcohol (LA) group had 1 ml of 70% ethyl alcohol administered 1 h before the desufflation period, and the laparoscopy + CAPE (LC) group had CAPE administered at 10 µmol/kg 1 h before the desufflation period. The total oxidative status levels of lung and plasma were significantly increased in the LA group as compared with the LC and S group. When the LC group was compared with the L group, there was a decrease in the level of total oxidant status and increase in the levels of total antioxidant status and paraoxonase in lung tissue. The level of total antioxidative status in the S group was increased compared with the L group in lung tissue and bronchoalveolar lavage fluid. TNF-α and IL-6 were found significantly elevated in the L group compared with the LC and S groups in bronchoalveolar lavage fluid. There was a similar increase in plasma levels of IL-6. These results were supported by histopathological examination. CAPE was found to considerably reduce oxidative stress and inflammation induced by pneumoperitoneum.

**Keywords:** Caffeic acid phenethyl ester, pneumoperitoneum, rat, lung, bronchoalveolar lavage

## Introduction

Laparoscopic surgery is a minimally invasive technique used in many therapeutic and diagnostic surgical procedures. It has been demonstrated to have high safety levels in a recent study [1]. The advantages of the laparoscopic technique include acceptable surgical trauma and restlessness duration, shorter recovery time, reduced postoperative scarring, diminished pain, and a shorter hospitalisation period [2, 3].

Despite the clear advantages of laparoscopic surgery in terms of patient outcome, increased intra-abdominal pressure (IAP) may give rise to significant organ ischaemia in the splanchnic organs and even in remote organs such as lung [4-6]. In other words, the insufflation of CO<sub>2</sub> into the abdominal cavity elevates the diaphragm and leads to an increase of intrathoracic pressure, thus decreasing respiratory system compliance, which is associated with pulmonary

barotrauma, pulmonary oedema, hypoxemia, and atelectasis [3, 7, 8].

Although these changes are well tolerated in patients without co-morbidity during laparoscopic surgery, there are greater risks of suppressed pulmonary function associated with an increased rate of peri-operative complications with especially overweight elderly patients and those with pre-existing lung diseases. Alternative approaches as well as antioxidant and anti-inflammatory drugs that reduce the effects of pneumoperitoneum may be useful [9].

CAPE is an active component of propolis, which is known to have anti-inflammatory, anticarcinogenic, vasorelaxant, antioxidant, and immunomodulatory properties [10, 11]. CAPE also inhibits lipid peroxidation and lipoxygenase activities with antioxidant properties [12-14]. Even though CAPE is commonly accepted to be

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an antioxidant [15], there has been no previous study investigating its effect on lung injury induced by CO<sub>2</sub> pneumoperitoneum.

To the best of our knowledge, this is the first study investigating the possible protective effect of CAPE on lung tissue. The aim of the current study is to evaluate the protective effects of CAPE on lung injury caused by pneumoperitoneum as measured by biochemical and histopathological parameters in bronchoalveolar lavage fluid (BALF), plasma, and lung tissue.

### Materials and methods

The study was approved by the Animal Care and Ethics Committee of Mustafa Kemal University, Faculty of Medicine. The experiment was performed on 28 adult male Wistar albino rats weighing from 270 to 320 g. Animals were reared separately with similar daylight conditions, temperature 22 ± 0.5°C, and 45-50% moisture; they were fed a pellet diet and tap water. Rats were assigned randomly into four groups of seven animals.

The control group (S) was subjected to a 'sham' operation without pneumoperitoneum. The laparoscopy group (L) was subjected to 60 min of pneumoperitoneum with 15 mmHg IAP. The laparoscopy alcohol (LA) group was subjected to 60 min of pneumoperitoneum with 15 mmHg IAP and 1 ml of 70% ethyl alcohol administered as a single intraperitoneal injection 1 h before the desufflation period [13]. The laparoscopy + CAPE (LC) group was subjected to 60 min of pneumoperitoneum at 15 mmHg IAP, and CAPE at 10 µmol/kg was administered as a single intraperitoneal injection 1 h before the desufflation period [16, 17].

### Surgical technique

A mixture of ketamine hydrochloride and xylazine hydrochloride was used for the rats' anaesthesia (50 mg/kg and 3 mg/kg, respectively). When necessary, additional doses were administered during the procedure. The experiment was carried out by letting rats breathe spontaneously in a supine position on the operating table. An Abbocath catheter at 18 G was placed into the abdominal cavity as supraumbilical and fixed to the insufflator tube after the preparation of the surgical field [18]. Pneumoperitoneum was automatically established up to a 15-mmHg pressure for 60 min

via the insufflation of CO<sub>2</sub> by an abdominal CO<sub>2</sub> insufflator (Karl Storz GmbH, Tutlingen, Germany) and followed by immediate desufflation.

### Tested drugs

CAPE (Sigma, St. Louis, MO, USA) was administered intraperitoneally to rats in the LC group in doses of 10 µmol/kg; it was dissolved with 70% ethanol prior to use. One millilitre of 70% ethyl alcohol was administered intraperitoneally in the LA group.

### BALF

Tracheostomy was performed, and a 16-gauge cannula (Nova Cath IV cannula; Medipro, Istanbul, Turkey) was inserted into the trachea, using a technique described by Nakamura et al. soon after the desufflation [19]. The bronchoalveolar lavage was obtained twice with 3-ml saline to investigate biochemical parameters, such as paraoxonase (PON1) activity, total antioxidant status (TAS), total oxidative status (TOS) levels, and cytokine concentration. The lavage returned 5-7 ml, which was immediately centrifuged at 4000 rpm for 10 min at 4°C to eliminate mucus and cells, and the remaining supernatant fluid was frozen.

### Biochemical analysis

The levels of TAS, TOS, and PON1 were measured from the supernatants. The TAS and TOS of supernatant parts were assessed using a new colorimetric assay method developed by Erel [20, 21]. The TAS and TOS results were expressed as mmol Trolox equivalent/g protein and mmol H<sub>2</sub>O<sub>2</sub> equivalent/g protein, respectively, for lung tissue. The TAS and TOS results of BALF and serum samples in this assay were expressed as mmol Trolox equivalent/L and mmol H<sub>2</sub>O<sub>2</sub> equivalent/L, respectively [22].

Serum PON1 levels were measured spectrophotometrically by the modified Eckerson et al. method [23]. Paraoxonase activity was expressed as U/g protein for lung tissue and U/L for BALF and serum samples.

### Blood samples

After removal of the lungs, blood samples were taken intracardially and collected into tubes containing anticoagulant (2% sodium oxalate). The samples were centrifuged immediately at

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**Table 1.** Biochemical results of the lung tissue in the different study groups. Data are expressed as mean  $\pm$  SD

	Sham	Laparoscopy	Laparoscopy + alcohol	Laparoscopy + CAPE
TOS (mmol H <sub>2</sub> O <sub>2</sub> equivalent/g protein)	36.04 $\pm$ 18.84	61.53 $\pm$ 21.85 <sup>a</sup>	55.50 $\pm$ 7.66 <sup>c,d</sup>	30.94 $\pm$ 13.74
TAS (mmol Trolox equivalent/g protein)	2.08 $\pm$ 0.93 <sup>b</sup>	1.15 $\pm$ 0.38	1.45 $\pm$ 0.34	1.67 $\pm$ 0.73 <sup>a</sup>
PON1 (U/gr)	58.46 $\pm$ 10.53	35.20 $\pm$ 8.97	37.78 $\pm$ 11.57	55.59 $\pm$ 11.32 <sup>a,d</sup>

CAPE, caffeic phenethyl ester; TOS, total oxidant status; TAS, total antioxidant status; PON1, paraoxonase. Statistically significant differences ( $p < 0.05$ ) were noted as follows: (a) laparoscopy vs laparoscopy + CAPE group; (b) laparoscopy vs sham group; (c) laparoscopy + alcohol vs sham group; (d) laparoscopy + alcohol vs laparoscopy + CAPE group.

**Table 2.** Biochemical results, TNF- $\alpha$  and IL-6 levels of plasma in the study groups. Data are expressed as mean  $\pm$  SD

	Sham	Laparoscopy	Laparoscopy + Alcohol	Laparoscopy + CAPE
TOS (mmol H <sub>2</sub> O <sub>2</sub> equivalent/L)	19.43 $\pm$ 7.20	30.19 $\pm$ 10.65	35.14 $\pm$ 8.08 <sup>c,d</sup>	22.00 $\pm$ 8.18
TAS (mmol Trolox equivalent/L)	2.09 $\pm$ 0.30	1.65 $\pm$ 0.24	1.70 $\pm$ 0.35	1.87 $\pm$ 0.51
PON1 (U/L)	58.46 $\pm$ 10.53	35.20 $\pm$ 8.97	37.78 $\pm$ 11.57	55.59 $\pm$ 11.32
TNF- $\alpha$ (pg/ml)	5.90 $\pm$ 1.03	6.95 $\pm$ 0.83	12.34 $\pm$ 2.20 <sup>c,d,e</sup>	5.73 $\pm$ 1.35
IL-6 (pg/ml)	8.30 $\pm$ 1.56	16.51 $\pm$ 8.62 <sup>a,b</sup>	15.75 $\pm$ 2.23 <sup>c,d</sup>	7.65 $\pm$ 4.02

CAPE, caffeic phenethyl ester; TOS, total oxidative status; TAS, total antioxidant status; PON1, paraoxonase; TNF- $\alpha$ , tumour necrosis factor; IL-6, interleukin. Statistically significant differences ( $p < 0.05$ ) were noted as follows: (a) laparoscopy vs laparoscopy + CAPE group; (b) laparoscopy vs sham group; (c) laparoscopy + alcohol vs sham group; (d) laparoscopy + alcohol vs laparoscopy + CAPE group; (e) laparoscopy + alcohol vs laparoscopy group.

**Table 3.** Biochemical results, TNF- $\alpha$ , and IL-6 levels of the BALF in the study groups. Data are expressed as mean  $\pm$  SD

	Sham	Laparoscopy	Laparoscopy + Alcohol	Laparoscopy + CAPE
TOS (mmol H <sub>2</sub> O <sub>2</sub> equivalent/L)	0.65 $\pm$ 0.16	2.96 $\pm$ 1.52	4.49 $\pm$ 2.58	1.28 $\pm$ 0.40
TAS (mmol Trolox equivalent/L)	1.32 $\pm$ 0.30 <sup>b</sup>	0.75 $\pm$ 0.16	0.80 $\pm$ 0.25	1.03 $\pm$ 0.13
PON1 (U/L)	20.83 $\pm$ 3.96	21.84 $\pm$ 17.56	20.50 $\pm$ 11.98	16.93 $\pm$ 12.35
TNF- $\alpha$ (pg/ml)	1.36 $\pm$ 0.20	2.30 $\pm$ 0.89 <sup>a,b</sup>	1.60 $\pm$ 0.16	1.40 $\pm$ 0.36
IL-6 (pg/ml)	5.58 $\pm$ 0.38	6.19 $\pm$ 0.36 <sup>a,b</sup>	5.89 $\pm$ 0.48	5.30 $\pm$ 0.17

CAPE, caffeic phenethyl ester; TOS, total oxidative status; TAS, total antioxidant status; PON1, paraoxonase; TNF- $\alpha$ , tumour necrosis factor; IL-6, interleukin. Statistically significant differences ( $p < 0.05$ ) were noted as follows: (a) laparoscopy vs laparoscopy + CAPE group; (b) laparoscopy vs sham group.

**Table 4.** Histopathological findings of lung. Values are expressed as median (minimum, maximum) for rats in each group

	Sham	Laparoscopy	Laparoscopy + Alcohol	Laparoscopy + CAPE
Intra-alveolar haemorrhage	1 (0-1)	1 (1-2)	1 (0-2)	1 (1-2)
Alveolar Oedema	1 (1-2)	2 (2-3) <sup>b</sup>	1 (1-2)	1 (0-2)
Congestion	1 (0-1)	2 (2-3) <sup>b</sup>	1 (1-1)	1 (0-1) <sup>a</sup>
Leukocyte infiltration	1 (0-2)	2 (2-3) <sup>b</sup>	1 (1-2)	1 (0-2) <sup>a</sup>

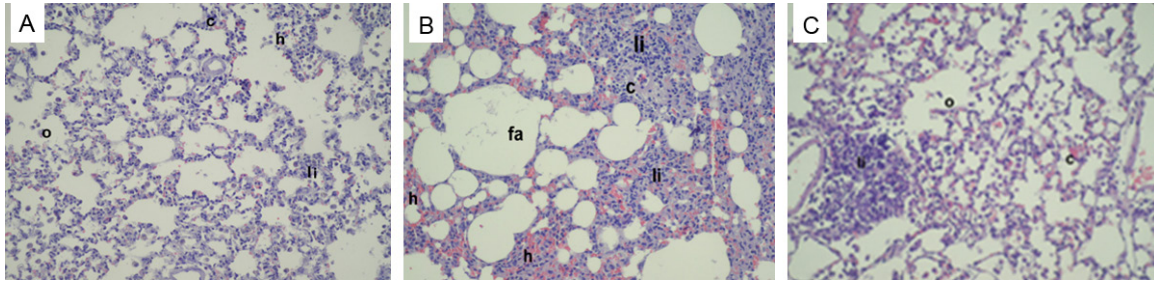
CAPE, caffeic phenethyl ester. Statistically significant differences ( $p < 0.008$ ) were noted as follows: (a) laparoscopy vs laparoscopy + CAPE group; (b) laparoscopy vs sham group.

4000 rpm at 4°C for 10 min, and both the serum and the supernatant fluid obtained were stored at -80°C until examination to estimate the cytokine concentration, TAS and TOS levels, and PON1 activity.

### Cytokine study

Interleukin-6 (IL-6) and tumor necrosis factor alfa (TNF- $\alpha$ ) levels in BALF and blood samples were measured by using the ELISA method

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**Figure 1.** Histopathological figures. A. The architectural integrity of alveoli was well preserved in the sham group. B. Destroyed alveolar architecture showing severe intra-alveolar fluid accumulation. C. Caffeic phenethyl ester (CAPE) group demonstrating preserved alveolar architecture compared to laparoscopy group. Intra-alveolar fluid accumulation (fa) and edema (o), massive leukocyte infiltration (li), hemorrhage (h) and congestion (c) (H&E x400).

(DIAsource ImmunoAssays SA, Nivelles, Belgium) in accordance with the method described by the manufacturer.

### *Histopathological examination*

The lungs of the rats were dissected after removal following median sternotomy. The left lungs were immediately stored at  $-80^{\circ}\text{C}$  for the biochemical study. Right lung tissue was fixed in 10% neutral buffered formaldehyde solution for histopathology. After dehydration procedures, the samples were embedded in paraffin, and whole surface of lungs were sectioned in 3- $\mu\text{m}$ -thick sections with a microtome. They were then deparaffinised, rehydrated, and stained with hematoxylin-eosin (H&E) to assess inflammatory changes with the number of leukocytes. The samples were examined under light microscopy by one blinded pathologist. Slides were examined using an Olympus BX50 light microscope at x400 magnification and photographed with an Olympus PM10SP camera.

All histopathological changes, including intra-alveolar haemorrhage, alveolar oedema, congestion, and leukocyte infiltration, were evaluated in all layers of each lung tissue. A scale from 0 to 3 was used for evaluating intra-alveolar haemorrhage, alveolar oedema, and congestion, where 0 = absence of pathology (< 5 % of maximum pathology), 1 = mild (< 10 % of maximum pathology), 2 = moderate (15-20 % of maximum pathology), and 3 = severe (20-25% of maximum pathology). Leukocyte inflammation was investigated to assess the severity of inflammation induced by pneumoperitoneum. The sections were examined at 400X magnification for 10 fields with the following scale:

0, no extravascular leukocytes; 1, < 10 leukocytes; 2, 10-45 leukocytes; 3, > 45 leukocytes. An average of the numbers was used for comparison [15, 24].

### *Statistical analysis*

Statistical analyses of results were performed using SPSS software version 16. Variables were investigated using visual and analytical methods with Kolmogorov-Smirnov tests to determine whether or not they were normally distributed. One-way ANOVA was used for the statistical evaluation of results. Results were expressed as mean  $\pm$  standard deviation (SD). A *P* value less than 0.05 was accepted as statistically significant. When an overall significance was observed, pairwise post hoc tests were performed using Tukey's test with Bonferroni correction. Kruskal-Wallis tests were conducted to compare among groups for histopathological findings. The Mann-Whitney U-test was performed to test the significance of pairwise differences using Bonferroni correction to adjust for multiple comparisons. Because there were six comparisons in four groups, we regarded  $P < 0.008$  ( $0.05/6$ ) as statistically significant.

## **Results**

### *TOS levels*

When the L group was compared with the LC group, the L group showed significantly increased TOS levels ( $P < 0.05$ ) in lung tissue (**Table 1**). When the LA group was compared with the S and LC groups, the level of TOS of the LA group was significantly increased in lung tissue and plasma ( $P < 0.05$ ) (**Tables 1, 2**).



### TAS levels

Levels of TAS increased significantly in the LC and S groups compared with the L group in lung tissue ( $P < 0.05$ ) (**Table 1**). When the S group was compared with the L group, TAS levels were significantly increased in BALF ( $P < 0.05$ ) (**Table 3**).

### PON1 levels

PON1 levels in the LC group were significantly increased ( $P < 0.05$ ) when compared to the L and LA groups but not significantly increased when compared with the S group in lung tissue (**Table 1**). There were no significant differences between the L, LC, LA, and S groups in levels of TOS and PON1 in BALF (**Table 3**).

### TNF- $\alpha$ and IL-6 levels

When the L group was compared with the LC and S groups, levels of TNF- $\alpha$  and IL-6 of the L group were significantly increased in BALF ( $P < 0.05$ ) (**Table 3**). When the LA group was compared with the S, LC and L groups, levels of TNF- $\alpha$  of LA was significantly increased in plasma ( $P < 0.05$ ). Also, when the LA group was compared with the S and LC groups, levels of IL-6 in the LA group were significantly increased in plasma ( $P < 0.05$ ) When the L group was compared with the LC and S groups, levels of IL-6 of the L group were significantly increased in plasma ( $P < 0.05$ ) (**Table 2**).

### Histopathology results

Histopathological scores and severity of inflammation are shown in **Table 4**. Histopathological examination of the lung revealed several characteristics, including intra-alveolar haemorrhage, alveolar oedema, congestion, and leukocyte infiltration, as a consequence of increased pressure. During the histopathological examination of the lung specimens in the S group, the architectural integrity of alveoli was found to be well preserved, although slight congestion, alveolar oedema and intra-alveolar haemorrhage were observed. There were few leukocytes around the vascular walls in the S group (**Figure 1A**). In the L group the alveolar architecture was destroyed in large areas, intra-alveolar fluid accumulation and oedema were severe, and leukocyte infiltration and congestion were massive when compared with the S group;

these differences were statistically significant ( $P < 0.008$ ). Intra-alveolar haemorrhage was not statistically significant in the L group compared with the other groups ( $P > 0.008$ ) (**Figure 1B**). The architecture of the alveoli in the LC group was well preserved compared to the L group. Vascular congestion and leukocyte infiltration were less common than in the L group ( $P < 0.008$ ) (**Figure 1C**).

### Discussion

There is stability between oxidant and antioxidant defence system in the body [25, 26]. Some surgical procedures, such as laparoscopy, can disrupt the balance of this system. Previous studies have shown that even IAP levels as low as 10 mmHg may cause a reduction in the circulation of the intraperitoneal organs, resulting in biochemical signs of oxidative stress and histological signs of tissue injury [6, 27]. The antioxidant defence system of the human body is not always sufficiently effective to fully counteract the damaging effects of these oxidant species [28].

CAPE, a widely studied antioxidant agent, is strongly affirmed to reduce the I/R injury in many animal models [10, 13, 15, 29]. In our study, we administered CAPE to prevent CO<sub>2</sub> pneumoperitoneum-induced oxidative stress and inflammatory reactions in lung tissue, and our results supported this hypothesis.

TAS is a significant indicator to ensure prevention of tissue damage caused by I/R-related oxidative stress. In the same way, TOS may display the level of all free oxygen radicals and provide a sensitive index of lipid peroxidation and I/R-related oxidative stress [22, 30]. We found that TOS levels were significantly increased in lung tissue of the L group; this may be a result of excessive production or decreased excretion of oxidative stress substances.

PON1 is an esterase, which has many enzymatic activities, and many studies on the role of PON1 have confirmed their antioxidant properties [31]. In the current study, decreased the TAS and PON1 levels in L group may have resulted from an increase in lipid peroxidation and the overconsumption of some endogenous antioxidants due to laparoscopy-induced I/R injury in the lung. Also, increased the TAS and PON1 levels in LC group support the hypothesis

that CAPE treatment clearly reduces inflammation and oxidative stress in lung tissue. Although the exact mechanism of CAPE is unclear, this effect may be attributed to its antioxidant properties [29, 32]. We suggest that the decrease of TOS levels in the CAPE group is probably due to its scavenging effect, and our results are consistent with previous studies [14, 29, 32, 33].

IL-1, IL-6, and TNF- $\alpha$  are proinflammatory cytokines and the major mediators in systemic and intraperitoneal immune response. In some animal studies, the use of CO<sub>2</sub> was found to be associated with impaired production of TNF- $\alpha$  by peritoneal macrophages during laparoscopic surgery and IL-6 and TNF- $\alpha$  levels were increased after pneumoperitoneum [34-36]. In our study, cytokine levels were in accordance with the biochemical parameters. These results are consistent with the results of previous studies [37, 38]. Anti-inflammatory properties of CAPE were possibly due to its specific inhibition of the transcription factor nuclear factor kappa B, which results in decreased cytokine production and inflammatory cell activation [10, 11, 13, 39]. In our study decreased levels of IL-6 and TNF- $\alpha$  in the CAPE-treated group may be related to this mechanism.

In general, CO<sub>2</sub> pneumoperitoneum-induced oxidative damage (I/R injury) gives rise to endothelial injury in lung tissue. This condition leads to alveolar oedema and intra-alveolar haemorrhage via the disruption of alveoli, resulting in the release of activated leukocytes and proteins into the bronchoalveolar system [9, 15]. In the current study, the histopathological examination of lung tissues was consistent with the biochemical data and cytokine levels. Although intra-alveolar haemorrhage is a strong indicator of tissue damage, we did not detect significant intra-alveolar haemorrhage in the L group. This situation may have resulted from the inadequate desufflation period of the study because the severity of intra-alveolar haemorrhage is directly associated with the level and duration of oxidative stress [9].

We observed that the degree of pulmonary tissue damage, including alveolar oedema, congestion, and leukocyte infiltration, was reduced in CAPE-treated rats as noted in the literature [13, 15, 40]. CAPE might have cytoprotective effects that decrease the degree of alveolar destruction.

Limitations of our investigation include the small number of rats and inadequate desufflation period. Our findings support the hypothesis that the use of CAPE may be effective for clinical purposes because of its antioxidant, anti-inflammatory, and immunomodulatory properties. For this reason, this experimental study should be supported by further large-scale human studies in order to minimise the morbidity and mortality of patients with pre-existing lung disease undergoing laparoscopy.

We conclude that prophylactic administration of CAPE may prevent lung injury induced by the CO<sub>2</sub> pneumoperitoneum model in rats. We showed these positive effects not only in terms of biochemical and histopathological examinations in lung tissue but also in BALF.

### Disclosure of conflict of interest

All of the authors declared the no conflict of interest.

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