Caffeic acid phenethyl ester ameliorates pulmonary inflammation and apoptosis reducing Nf-κβ activation in blunt pulmonary contusion model

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ABSTRACT

BACKGROUND: Pulmonary contusion (PC) is an important life-threatening clinical condition characterized by lung injury and inflammation. Caffeic acid phenethyl ester (CAPE) is a biological agent with potent antioxidant and anti-inflammatory effects. This study aimed to investigate the potential effects of CAPE on tissue damage, nuclear factor kappa-beta (NF-κB) activity, inducible nitric oxide synthase (iNOS) synthesis, and pulmonary apoptosis in an experimental PC model.

METHODS: Forty adult Wistar albino rats were used in this study and divided into four groups as follows: control, PC, PC + CAPE, and CAPE. CAPE was administered intraperitoneally for seven days following PC formation (10 µmol/kg, dissolved in dimethyl sulfoxide). Wet/dry weight ratio in lung tissue was determined. The pulmonary tissue was examined using hematoxylin–eosin and Masson’s trichrome histochemical staining and also by scanning electron microscopy. NF-κB and iNOS activities in the lungs were determined by the indirect immunohistochemical method. Pulmonary apoptosis was detected by the TUNEL method.

RESULTS: Increased leukocyte infiltration score, pulmonary edema, alveolar damage, and increased NF-κB and iNOS activities were determined in the PC group. CAPE administration inhibited NF-κB and iNOS activities and pulmonary apoptosis.

CONCLUSION: In this study, the findings showed that CAPE inhibited tissue damage by suppressing inflammatory mediators of NF-κB and iNOS activities. Also, CAPE was found to be protective in the lung tissue and could be used as a therapeutic agent.

Keywords: Apoptosis; inflammation; iNOS, NF-κB; pulmonary contusion.

INTRODUCTION

Pulmonary contusion (PC) is the most important intrathoracic injury of blunt chest injuries that arises from falls or traffic accidents in children and adults. PC is also the most important injury that triggers the local and systemic inflammatory response in multiple traumas. Cytokines and other pro-inflammatory mediators released from macrophages and leukocytes, which are active after blunt chest trauma, cause inflammation of lung tissue. Pulmonary edema, alveolar-capillary permeability, surfactant dysfunction, and ventilation-perfusion mismatch are known to be the consequences of PC. No specific treatment option is available for patients with blunt chest trauma, which is a concern in emergency medicine and trauma care and symptomatic treatment is generally administered.

Nuclear factor kappa-beta (NF-κB) is a transcription factor that has a central role in many biological processes, such as inflammation, apoptosis, and infections. Activation of NF-κB induces inflammation by triggering the transcription of pro-inflammatory genes. Recent studies reported that exposure to chest trauma caused NF-κB activation and increased the synthesis of pro-inflammatory cytokines, such as inducible nitric oxide synthase (iNOS) and interleukin-1 beta (IL-1β). Chest trauma resulting in macrophage activation and increased pro-inflammatory cytokine synthesis induced apoptosis in pulmonary alveolar epithelial cells.
Caffeic acid phenethyl ester (CAPE), one of the most important biological components of propolis produced by worker bees, has strong anti-inflammatory and antiapoptotic effects. [13,15] Koksel et al. [14] (2006) demonstrated that CAPE suppressed antioxidative stress in the lung tissue and reduced inflammation in the lipopolysaccharide (LPS)-induced lung injury model in rats. The present study aimed to reveal the potential effects of CAPE on inflammation and apoptotic processes using light and scanning electron microscopic methods in the rat chest trauma model.

MATERIALS AND METHODS

Experimental Design

This study was performed at Tekirdag Namık Kemal University Application and Research Centre for Experimental Animals after obtaining permission from the local ethics committee (Permission number: 17/10/2017-7). Forty adult male (8-week-old) Wistar albino rats were divided into four groups as follows: control (n=10), PC (n=10), PC + CAPE (n=10), and CAPE. Chest trauma was created with the model previously described by Raghavendran et al. [15] (2005). CAPE treatment was applied to the chest trauma model and chest trauma + CAPE groups for seven days dissolved in dimethyl sulfoxide (10 µmol/kg, intraperitoneally). During the experimental period, the animals were kept under standard laboratory conditions (22±2°C; 60% humidity; 12/12 dark–light mental period, the animals were kept under standard laboratory conditions). At the end of the seventh day, the rats were anesthetized (ketamine–xylazine; 90–10 mg/kg) and sacrificed by drawing blood from the heart-opening through the midline. The left lung lobe was evaluated for edema and the right lung lobe for light and electron microscopic analysis.

Wet/Dry Weight Ratio

The fresh left superior lobe from each rat was weighed quickly after sacrificing for the evaluation of pulmonary edema. Wet/Dry (W/D) weight ratio was calculated by reweighing samples held for 24 h at 80°C according to a previous study.[16]

Histopathological Analysis

Lung tissue specimens were fixed in 10% neutral formalin. After fixation, paraffin blocks were prepared using routine histological methods. Five-micrometer-thick sections were stained with hematoxylin–eosin (H&E) and Masson’s trichrome stains. Histopathological changes, such as alveolar edema, vascular congestion, hemorrhage, and leukocyte infiltrations, were assessed in lung tissue. Extravascular leukocyte counts were determined and scored in H&E-stained sections (0, no extravascular leukocytes; 1, <10 leukocytes; 2, 10–45 leukocytes; 3, >45 leukocytes) to determine the severity of inflammation.[17] The distribution of connective tissue and the thickening of the alveolar wall were confirmed by Masson’s trichrome staining in lung tissue. All histological examinations were performed using Olympus CX41 (Olympus, Japan) light microscope and image analysis system (Kameram Gen 2.1 Image Analysis Software, Istanbul, Turkey).

Immunohistochemical Staining

iNOS and Nf-κB immunohistochemical markers were used for the indirect immunohistochemical method. Primer antibodies (iNOS: NB300-605; Nf-κB: NB100-56055) were obtained from Novus Biologicals (Littleton, CO, USA). Biotinylated secondary antibody and streptavidin peroxidase (Ultra Vision Detection System-HRP kit, Thermo Scientific/Lab Vision, Fremont, CA, USA) were used according to the manufacturer's instructions. Further, 3-amino-9-ethylcarbazole was used for iNOS staining, and 3,3’-diaminobenzidine chromogen was used for Nf-κB staining to create contrast. Contrasting stains were made with Mayer’s hematoxylin. iNOS and Nf-κB positive stained cell numbers were expressed as positive stained cells/mm² for each group in lung tissue.

Apoptosis

Apoptotic cells in the lung tissue were detected by the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) method (S7101 ApopTag Plus Peroxidase In Situ, Merck Millipore, Darmstadt, Germany). Apoptotic cells identified using intense brown nuclear staining were calculated as positive cells/mm² for each group.

Scanning Electron Microscopy Analysis

The specimens fixed in 2% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffered glutaraldehyde solution overnight at 4°C were used for ultrastructural analysis of lung tissue. After fixation, the tissues were rinsed with 0.1 M HEPES and dehydrated with ethanol. They were dried using hexamethyldisilazane. The specimens were examined using scanning electron microscopy (SEM) (Quanta Feg 250, FEI, USA) at Tekirdag Namık Kemal University Scientific and Technological Research and Application Center.

Statistical Analysis

Data were evaluated using the PASW Statistics 18.0.0 (SPSS Inc, IL, USA) statistical program. The numerical parameters of the groups were evaluated using a nonparametric test (Kruskal–Wallis), and the significance of the values obtained in the two-way comparison was measured using the Mann–Whitney U test. P-values <0.05 were considered statistically significant.

RESULTS

Effects of CAPE On Pulmonary Architecture

Histopathological changes in the lungs were assessed using H&E and Masson’s trichrome staining under a light microscope. Figure 1 shows the general tissue structure and infiltration score in H&E-stained lung sections. The control group exhibited a normal histological structure (Fig. 1a). Intense in-
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Inflammatory cell infiltration was seen in the PC group (Fig. 1b). Inflammatory cell infiltration in the PC + CAPE group was attenuated compared with that in the chest trauma group (Fig. 1c). The CAPE group showed normal histological architectures similar to that in the control group (Fig. 1d). As shown in Figure 1e, CAPE treatment caused a significant reduction in the leukocyte infiltration score.

The W/D weight ratios determined as pulmonary edema size are shown in Figure 1f. The PC group showed increased W/D ratio compared with the control group (p<0.001). The CAPE-administered group exhibited significantly decreased W/D ratio compared with the PC group (p<0.001). No significant difference in W/D ratio was observed between the control and CAPE groups.

Figure 1. Effects of CAPE on pulmonary tissue. (a) Control, normal histological architecture. (b) PC, peribronchial and alveolar inflammation. (c) PC + CAPE, decreased inflammation in lung tissue. (d) CAPE, no histopathological change. (e) Lung leukocyte inflammation scores. (f) Effects of CAPE on W/D weight ratios (arrowhead; leukocyte infiltration, scale bar; 100 µm, H&E, *p<0.001 compared with the control group; **p<0.001 compared with the PC group).

Figure 2. Masson’s trichrome–stained pulmonary tissue. (a) Control. (b) PC, increased edema and alveolar infiltration. (c) PC + CAPE, decreased infiltration. (d) CAPE group similar to the control group (Masson’s trichrome, scale bar; 100 µm).
The findings of Masson's trichrome staining revealed that the chest trauma caused thickened alveolar wall and edema or increased connective tissue in areas of inflammation on comparing the control and PC groups (Fig. 2a and 2b). In addition, Masson's trichrome staining showed evidence of decreased inflammation in the CAPE-treated group (Fig. 2c).

SEM findings of lung tissue examined at different magnifications showed that the control and PC groups exhibited increased infiltration and alveolar degeneration (Fig. 3c and 3d). In contrast, the CAPE-treated group displayed decreased parenchymal infiltration and improved alveolar structure (Fig. 3e and 3f).

Immunohistochemical examination of nucleus-located NF-κB expression in lung tissue revealed a significant decrease in the PC group compared to the control and CAPE-treated groups (Fig. 4d). The distribution of NF-κB-positive nuclei indicated a marked reduction in the PC + CAPE group (Fig. 4c) compared to the other groups. The graph (Fig. 4e) showed that the number of NF-κB-positive cells per mm² was significantly lower in the PC + CAPE group compared to the control and PC groups.

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tions are presented in Figure 3. SEM analysis results were consistent with the findings of H&E and Masson's trichrome staining. Increased edema, infiltration, and alveolar disruption were found in the chest trauma group (Fig. 3c-d). Decreased pathological degeneration was detected in the PC + CAPE group, and the observations were consistent with the findings of the light microscopic examination (Fig. 3e-f).

Effects of CAPE on iNOS and Nf-κβ Immunoreactivity

The immunohistochemical expression of iNOS is shown in Figure 4. The control group showed rare iNOS immunoreactivity in some alveolar epithelial cells (Fig. 4a). iNOS expression increased, especially in the areas of inflammation, in the PC group (Fig. 4b). Decreased expression was detected in the PC + CAPE group compared with the PC group (Fig. 4c). The CAPE group was similar to the iNOS expression control group (Fig. 4d). iNOS-positive staining cell counts of the groups are presented in Figure 4e.

The Nf-κβ immunohistochemical expression was observed as nuclear brown staining. A statistically significant decrease in the number of positively stained cells was noted in the group treated with CAPE despite the increase in the Nf-κβ immunohistochemical expression in the PC group (p<0.001, Fig. 5b and c). The control and CAPE groups were similar in

![Figure 5. Effects of iNOS activity of CAPE.](image)

(a) Control. (b) PC. (c) PC + CAPE. (d) CAPE. (e) Distribution of iNOS-positive cell numbers in groups (arrows, iNOS-positive cells; counterstain, Mayer's hematoxylin; scale bar, 100 µm; *p<0.001 compared with the control group; **p<0.001 compared with the PC group).

![Figure 6. Apoptotic cells in lung tissue.](image)

(a) Control, a few apoptotic cells. (b) PC, increased number of apoptotic cells in contused lung tissue. (c) Decreased number of apoptotic cells. (d) Rare apoptotic cells in lung tissue. (e) Distribution of apoptotic cells in groups (scale bar, 100 µm; arrows, TUNEL-positive stained apoptotic cells; *p<0.001 compared with the control group; **p<0.001 compared with the PC group).
Effects of CAPE On Pulmonary Apoptosis

The number of apoptotic cells in the lung tissue was determined by the TUNEL method, which marked the broken DNA ends. The findings are presented in Figure 6. An increased TUNEL-positive cell number was detected in the PC group compared with the control group (p<0.001, Fig. 6b and c). The apoptotic cell number showed a significant decline in the CAPE-treated group compared with the PC group (Fig. 6e). These results clearly demonstrated the potential antia apoptotic effects of CAPE in the lung tissue after chest trauma.

DISCUSSION

PC is an important clinical problem accompanied by multiple traumas or chest trauma. PC affects the patients’ prognosis in emergency medicine.[18] One of the most important pathophysiological factors in PC that arises from blunt chest trauma is the progressive inflammatory response.[11,19] An excessive inflammatory response leads to increased alveolar–capillary permeability, leukocyte infiltration, edema, and respiratory distress.[20,21] Moreover, PC is associated with the increased pro-inflammatory cytokine release and alveolar cell apoptosis.[3,22] Thus, effective treatment is the most important issue in managing patients with PC and preventing complications. This study used different methods in the experimental PC model to demonstrate that CAPE supported tissue healing by suppressing inflammation in the lung tissue.

Histopathological changes, such as infiltration, edema, and alveolar deterioration, in the pulmonary tissues of experimental PC models, have been revealed in many studies.[22,24] Sirmali et al.[25] (2013) showed that CAPE inhibited tissue damage by its antioxidative action in the rat PC model. Consistent with the findings of the aforementioned study, CAPE improved lung tissue damage in the present study. In addition, histopathological changes in the lung tissue were also demonstrated in this study using the Masson’s trichrome stain. Moreover, SEM analysis results supported the findings. This novel study showed that CAPE inhibited apoptosis by reducing the pulmonary levels of pro-inflammatory mediators iNOS and NF-κB in the PC model.

The NF-κB/Rel transcriptional family has a vital role in the inflammatory process because of the ability of pro-inflammatory genes to induce transcription.[26] Nuclear translocation of cytoplasmic complexes of NF-κB leads to the induction of pro-inflammatory cytokines, chemokines, adhesion molecules, and iNOS.[10] NF-κB [κβ] has been reported to be overexpressed in many inflammatory diseases, such as inflammatory bowel diseases, asthma, multiple sclerosis, and rheumatoid arthritis.[27,28] Experimental studies demonstrated the role of NF-κB activation in pulmonary damage that arises from chest trauma.

Conclusions

In addition to the data obtained from a previous study reporting that CAPE inhibited oxidative stress and inhibited tissue damage,[23] this study used light and electron microscopic methods to demonstrate that CAPE reduced tissue damage by suppressing NF-κB and iNOS activity in the lung tissue. Therefore, CAPE can be used as a powerful anti-inflammatory agent to prevent tissue damage resulting from chest trauma and to suppress inflammation.

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DENENEYÇİLİK BAŞ,DÖŞEMESİ

Kafeik asit fenetil ester kötüm pulmoner kontüzyon modellinde pulmoner enfalmasıyon ve apoptozisi NF-κB aktivasyonunun azaltarak iyileştirdi

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AMAC: Pulmoner kontüzyon (PC), akciğer hasan ve enfalmasyon ile karakterize, hayattı tehdit eden önemli bir klinik durumdur. Kafeik asit fenetil ester (CAPE), güçlü antioksidan ve anti-enflammatuar etkileri olan biyolojik bir ajandır. Bu çalışmada, CAPE’nin deneysel PC modelinde doku hasarı, nükleer faktör kappa-beta (NF-κB) aktivitesi, indüklemebilir nükleer sentez (iNOS) sentezi ve pulmoner apoptozis üzerindeki etkileri araştırıldı.


TARTIŞMA: Çalışmanın sonuçları, CAPE’nin NF-κB ve iNOS enfalmasyonu ve pulmoner kontüzyonunun azaltılmasında etkili olduğunu göstermektedir. Ayrıca, CAPE’nin akciğer dokusunda korojüencyon olduğunu ve terapotik bir ajan olarak kullanılabilirğini göstermektedir.

Anahtar sözcükler: Apoptozis; enfalmasyon; indüklemebilir nükleer sentez; nükleer faktör kappa-beta; pulmoner kontüzyon.