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Article

An aqueous pomegranate peel extract (*Punica granatum*) protect against Elastase-induced pulmonary emphysema in Sprague Dawley rats model

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We investigated the effect of *Punica granatum* peel aqueous extract (PGE), on pulmonary inflammation and alveolar degradation induced by intratracheal administration of Elastase in Sprague Dawley rats. Lung inflammation was induced in rats by intratracheal instillation of Elastase. On day 1 and 2, animals received an intraperitoneal injection of PGE (200 mg/mL), three hours later, they were intratracheally instilled with 25U/kg pancreatic porcine Elastase. Animals were sacrificed 7 days later. Bronchoalveolar lavage (BAL) were collected and cellularity, histology and mRNA expression of Monocyte chemotactic protein 1(MCP-1), Tumor Necrosis Factor-Alpha (TNF-α), Interleukin 6 (IL-6), and Matrix Metalloproteinase-2 (MMP-2) were studied. In addition, activity of TNF- α, IL-6 and MCP-1 on BAL were also analyzed by ELISA Kit. Elastase administration increased: BAL cellularity, neutrophils recruitment and BAL MCP-1, IL-6 expressions. It also increased lung TNF-α, MCP-1, MMP-2 expressions, platelets recruitment, histological parameters at 7th day of elastase treatment. Intraperitoneal injection of 200 mg/kg of PGE reduced, significantly, BAL cellularity, and neutrophils recruitment. However, in animal treated with PGE, MCP-1, MMP-2 and IL-6 on day 7, were similar to the Sham group. Treatment with PGE (200 mg/kg) also significantly reduced lung TNF-α, and MCP-1 expression. This study reveals that PGE *Punica granatum* protects against elastase lung inflammation and alveolar degradation induced in rats.

Keywords: *Punica granatum*. Rat. Elastase. Lung inflammation. Lung oedema. emphysema

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a chronic inflammatory disease. It is the fourth cause of death world wide (Rabe *et al.*, 2007), the world health organization (WHO) announced that in 2030 this disease will be the third leading cause of mortality worldwide (WHO, 2014). COPD settles slowly through a chronic bronchitis and progresses to emphysema which is marked by the irreversible destruction of lung tissue. Alveolar degradation is caused by chronic inflammation induced by the increase of inflammatory cells such as neutrophil and macrophage releasing potentially destructive

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products essentially matrix metalloproteinases (MMPs) and Neutrophil Elastase (White*et al.*,1979; Houghton 2015). A directly proportional relationship has been established between the presence of COPD and several serum markers of systemic inflammation, leukocytes, Tumor Necrosis Factor-Alpha (TNF-α), Interleukin 6 (IL-6) (Gan *et al.*, 2004). Intratracheal administration of Elastase cause airspace enlargement in the lungs and a potential release of inflammatory mediators (Kuhn *et al.*, 1976; Fonseca *et al*, 2016).

In a previous work we showed that *Punica granatum* peel aqueous extractexhibited anti-inflammatory and antioxidant properties and protect mice against Lipopolysaccharide (LPS) lung induced inflammation (Bachoual *et al.*, 2011). *Punica granatum*, a member of the *Punicaceae* family, is a small tree originating from Asia. India, Iran and China are the most productive countries of these fruits. The pomegranate tree is widely cultivated in the Mediterranean countries such as Tunisia, Morocco, Spain, Italy and Greece (Wald, 2009). It produces orange-red flowers between May and April. The ripe of pomegranate fruit is composed of juicy seeds, surrounded by a leathery yellow peel. Several centuries ago, pomegranate was used in traditional medicine. At the beginning of the XIXth century, the fresh pomegranate peel was used as antidiarrheic and to treat the stomach aches (Jurenka 2008). In the West Indies, this peel was employed against irregular fevers. The peel of the trunk, twigs and roots are characterized by their deworming properties. It has recently been shown that the aqueous extract of the pomegranate peel attenuates the inflammation induced by Lipopolysaccharide in mice, and it is explained by the inhibition of the recruitment of the inflammatory cells as well as the inhibition of the activity of the myeloperoxidase (Bachoual *et al.*, 2011). Pomegranate extracts have been shown to have antioxidant activity (Aviram *et al*., 2000), and to protect against cancer (Mehta, Lansky, 2004). Pomegranate peel extract delays the proliferation of human breast and prostate cancer cell lines (Toi *et al.*, 2003).

The aim of this work was to study the effect of PGE (peel aqueous extract) on pulmonary inflammation and alveolar destruction induced by intrathracheal instillation of porcine elastase in Sprague Dawley rats as a model of induced emphysema

MATERIAL AND METHODS

This study was approved by the ethical committee for animal experimentation of the Hospital Universitario de Gran Canaria, Dr.Negrín. The use of animals and the experimental protocols were performed in compliance with standard operating and quality procedures following guidelines by the European Commission (2010/63/EU). We followed the ARRIVE guidelines for reporting standards for preclinical animal studies (Rodríguez González *et al.*, 2014).

Animals and Housing

Sprague-Dawley male rats (wild type, bred at the animal facility, Research Unit, Hospital Universitario Dr.Negrín) 12 weeks old weighing 257 ± 3 g were randomized and assigned to four study groups (n=5 per group). Rats were kept together in threes in a clear 1500 cm^2 Euro standard type IV S mini isolator cage (Tecniplast, Buguggiate, Italy) with overall dimensions of 48 x 37.5 x 21 cm to allow social interactions and welfare.

Rats were housed under isolator conditionswith food (2014 Teklad Global 14% Protein Rodent Maintenance Diet®, Harlan, Spain), as well as tap water available *ad libitum* as bedding material (Lignocel ¾-S®; Harlan, Spain). For cage enrichment, rats were provided with paper tissue, and tubes. The light/dark cycle was 12/12 h: lights were turned off at 18:30 h and turned on at 06:30 h.

Ambient temperature was 21 ± 1 ºC and relative humidity was 55 ± 5% with an air change rate of 15 times/h. Routine microbiological monitoring, carried out in accordance with recommendations of the Federation of European Laboratory Animal Associations, revealed no evidence of infection or parasitosis with common rat's pathogens (Rodríguez González *et al.*, 2014).

Preparation of peel aqueous extract

Tunisian *P. granatum* peel (Hammouri variety) dried at 37 **ºC** was collected from Institut of Arides Regions (IRA, Medenine, Tunisia). The peel was blended and suspended in sterile 0.9% NaCl then centrifuged at 2000 rpm for 3 min. The supernatant (PGE) was used for all experiments.

Animal model

Studies were conducted on12-weeks-old male Sprague Dawley rats (257 ± 3 g) breeded in 4th generation in the Experimental Animal Service(Research Unit.

Hospital Universitario de Gran Canaria Doctor Negrín, from Charles River Laboratories ®)

Rats were randomly allocated to 4 groups (n=5): Sham (Sham), emphysema model rat (ELASTASE), healthy+PGE (PGE), and emphysema model+PGE treated (ELASTASE+PGE).

SHAM rats received a tracheal instillation of 80 µL of saline solution, ELASTASE rats received an intraperitoneal injection of 1mL/day of saline solution for 2 days before the instillation of 25U/kg of Porcine Pancreatic Elastase (cat: 324682-250U) in 80 µL of saline solution into exposed tracheal. PGE animals received an intraperitoneal injection of 200 mg/kg/day PGE in 1mL saline solution for 2 days and finally, ELASTASE+PGE rats received an intraperitoneal injection of 200 mg/kg/day PGE in 1mL saline solution for 2 days before Elastase instillation.

After Elastase instillation, in accordance with the humanitary end point, animals were assessed every 12 h (from 6 h post inoculation) for several vital signs: Ruffled fur, Ataxia, Weight loss, Tremor, Ocular discharge, Hypothermia, Lethargy, Cyanosis and Hunched posture (Martín-Barrasa*et al*., 2015).

Fifteen minutes before Elastase instillation, animals were anesthetized, with a cocktail of Ketamine (100 mg/kg) (Ketolar®; Pfizer, Madrid, Spain)and Medothomedine (0.25 mg/kg) (Domtor® Esteve, Barcelona, Spain).

For Histological studies, rats were sacrificed 7 days after Elastase instillation, and their lungs were removed and immediately stored at -80 ºC until use.

Bronchoalveolar lavage and lung sampling

Animals were euthanized by exsanguination via abdominal aorta, heart and lungs were removed. Left lungafter performing sternotomy, was exposed, ligated and remove from chest cavity. Next perform a Bronchoalveolar lavage (BAL) through a cannula (1.2 mm inner diameter) inserted into the trachea. Lavage fluid (5mL) was slowly injected into the right lung using a sterile syringe and then recovered by gentle suction to avoid tissue damage. The procedure was repeated four times (Rodríguez-González *et al*., 2014).

This lung was preserved into formol to histologic study. The lavage fluid was immediately placed on ice, centrifuged at 3000 rpm for 10 min at 4 ºC. The supernatant was stored at minus 80 ºC until analysis. The cell pellet was resuspended in 150 µL of physiological saline and an aliquot was used to determine the total white cell count with a hemocytometer (Cell-Dyn Sapphire 4000®). For differential counts, the cell suspension was fixed in methanol, and stained with Diff Quick solution. One hundred cells were counted with an oil immersion lens (1000×).

Inflammation biomarker on Bronchoalveolar lavage

The concentration of MCP1, TNF-α, IL6 in BAL were determined by ELISA (Rat MCP-1(Monocyte Chemotactic Protein 1) ELISA Kit, RayBio Rat IL6 ELISA Kit, RayBio Rat TNF-α ELISA Kit) in accordance with manufactured indications.

Blood sampling

For haematological study, cytometry (Laboratorio de Hematología, Hospital Dr. Negrín, Investigación) (Rodríguez-González *et al*., 2014), 7 days after tracheal instillation rats were anesthetized, a midline thoracotomy/laparotomy was performed. For each group 2mL blood samples were obtained by jugular puncture and was put into EDTA (Becton Dickinson, Madrid, Spain).

RNA extraction and Real Time PCR (RT-qPCR)

RT-qPCR in left lung homogenates was performed to examine the mRNA expression proinflammatory and proteinases genes expression. Total RNA was extracted from left lung tissues using TRIzol (PureZOL RNA Isolation Reagent). For each sample, total RNA content was determined by measuring the absorbance by NanoDrop (Spectrophotomeer, INanoDrop ND-1000) at 260 nm and the purity was determined using A260/A280 ratio. The concentrations of extracted mRNA in lung samples were determined using RT-qPCR assay (iTaq TM Universal SYBR Green One-Step Kit, Bio-RAD), On one step a total of 1 µg mRNA sample from each group was reversely transcripted into cDNA and amplified for 35 cycles on a LightCycler1.5®thermocycler using the SYBR GREEN PCR mix.

Genes analyzed were Monocyte chemotactic protein 1(MCP-1), Tumor Necrosis Factor-Alpha (TNF-α), Interleukin 6 (IL-6), and Matrix Metalloproteinase-2 (MMP-2). The primer sequences are given in Table I. Gene expression was normalized usingglyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene.

TABLE I – Primers used for real-time PCR

Target genes	Primers	Cycles and conditions	Product size (bp)
GAPDH	F: 5′-AGG TCG GTG TGA ACG GAT TTG-3′ R: 5′-TGT AGA CCA TGT AGT TGA GGT CA-3′	2,25 mM $MgCl_2$ 95 ºC 15 seconds 58ºC Tªa 8 seconds extensions 40 cycles	37
MCP-1	F:5-CCAGAAACCAGCCAACTCTCA-3 R: 5-TGTGAACAACAGGCCCAGAA-3	3,0 mM $MgCl_2$ 95 ºC 15 seconds, 60 ºC Tªa 30 seconds extensions 35 cycles	90
TNF-α	F: 5′-CCC TCA CAC TCA GAT CAT CTT CT-3 R: 5′-GCT ACG ACG TGG GCT ACA G-3′		61
IL-6	F: 5-CGAGCCCACCAGGAACGAAAGTC-3 R:5-CTGGCTGGAAGTCTCTTGCGGAG-3		84
MMP-2	F: 5'-GAGTTGGCAGTGCAATACCT-3' R: 5'-CCAAAGAACTTCTGCATCTTCT-3'		104

Histologic study

7 days after tracheal instillation, either saline or Elastase according to animal groups, animals were sacrificed by supplemental anesthetic, followed by exsanguination by cutting the abdominal vessels. The lungs were removed from the chest, the trachea cannulated, and the right lung was fixed by intratracheal instillation of 3 mL 10% formalin. Random sections of the lung from each animal were obtained and slides were stained with hematoxylin and eosin and examined under light microscope. Each slide was examined by two expert investigators blinded to the experiment groups. In all lungs, the presence of oedemas around vessels was evaluated. In addition to this, lung injury was determined based on different signs, such as oedema, haemorrhage, leukocyte infiltration and alveolar septal thickening and pleuritis (Martín-Barrasa *et al.*, 2015).

Statistical analysis

The mean, standard deviation and quartiles have been calculated to describe the quantitative variables. The Shapiro-Wilk test was used to verify the normality of the data of the quantitative variables. Frequency and percentage have been calculated in the qualitative variables. The Kruskal Wallis test has been used to compare continuous variables in multiple categories. The statistical program used has been R Core Team (2017), R: A language and environment for statistical computing, R Foundation for Statistical Computing, Vienna, Austria, URL, http://www,R-project,org/.

RESULTS AND DISCUSSION

To our knowledge, this is the first report to provide evidence that the application of PGE modifies the lung

inflammatory development in the setting of emphysema. Our main findings are: (i) ip administration of PGE was associated with a more than threefold decrease of inflammatory markers compared to injured animals in an emphysema experimental model; and (ii) the action of PGE on inflammatory markers had a direct relationship on the clinical status of the animal, with a decrease in pulmonary oedema and inflammatory bronchoalveolar cellularity.

Vital sign evaluation

In the evaluation of vital signs, we saw a temperature around 35.33 ± 0.8, respiratory frequency on 48 rpm and about ruffled fur were found in less than 10% (Figure 1).

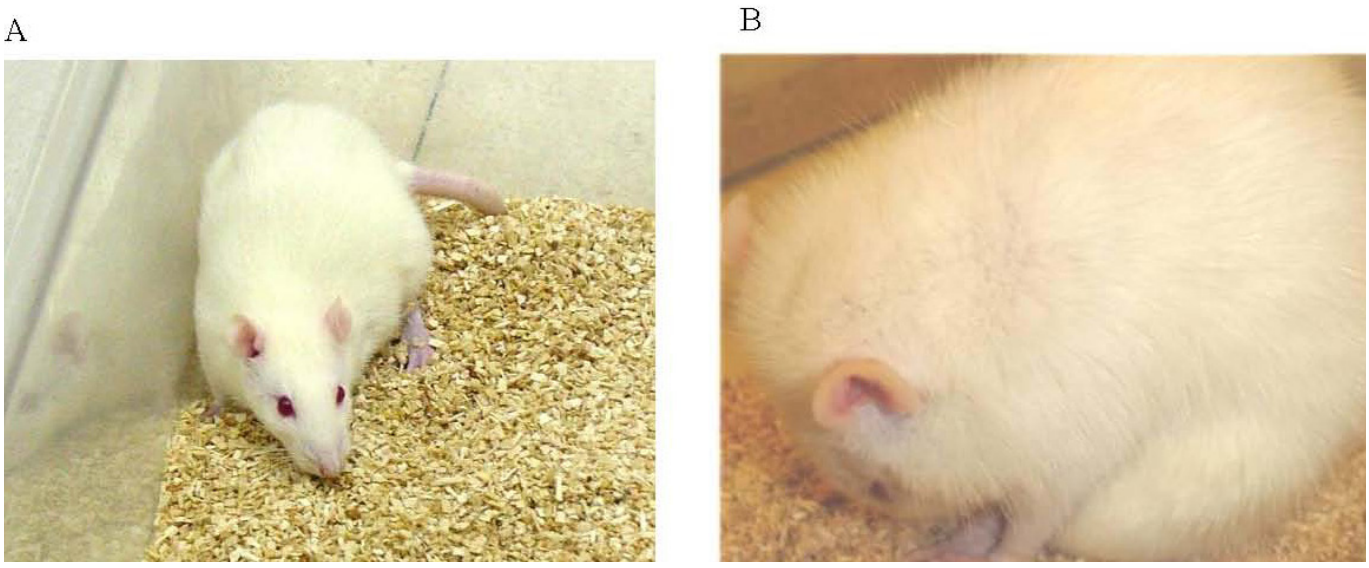

FIGURE 1 – (A) No ruffled fur (B) Ruffled fur.

Histologic study

At 7 days of PGE exposure, a decreased in the number of lung oedemas was observed in the treated rats, group. Relationship between size of oedema and experimental group are showed in the figures 2.

Many scientific approaches are available to induce emphysema in animal models. Including exposure laboratory animals to cigarette smoke, inflammatory stimuli like LPS or proteolytic enzymes (Ghorani *et al.*, 2017). Elastase is an elastolytic enzyme and it instillation in the lung induced tissue damage and developed emphysema, it induced progressive enlargement of alveolar spaces (Pera *et al.*, 2011). Mouse, rats and hamsters models are well characterized to develop Elastase-induced emphysema (Plantier *et al.*, 2007; Pera *et al.*, 2011). Four months after intra-tracheal instillation of Elastase, severe emphysema was observed in the respiratory mechanics system of the hamster (Plantier*et al.*, 2007; Ghorani*et al.*, 2017).

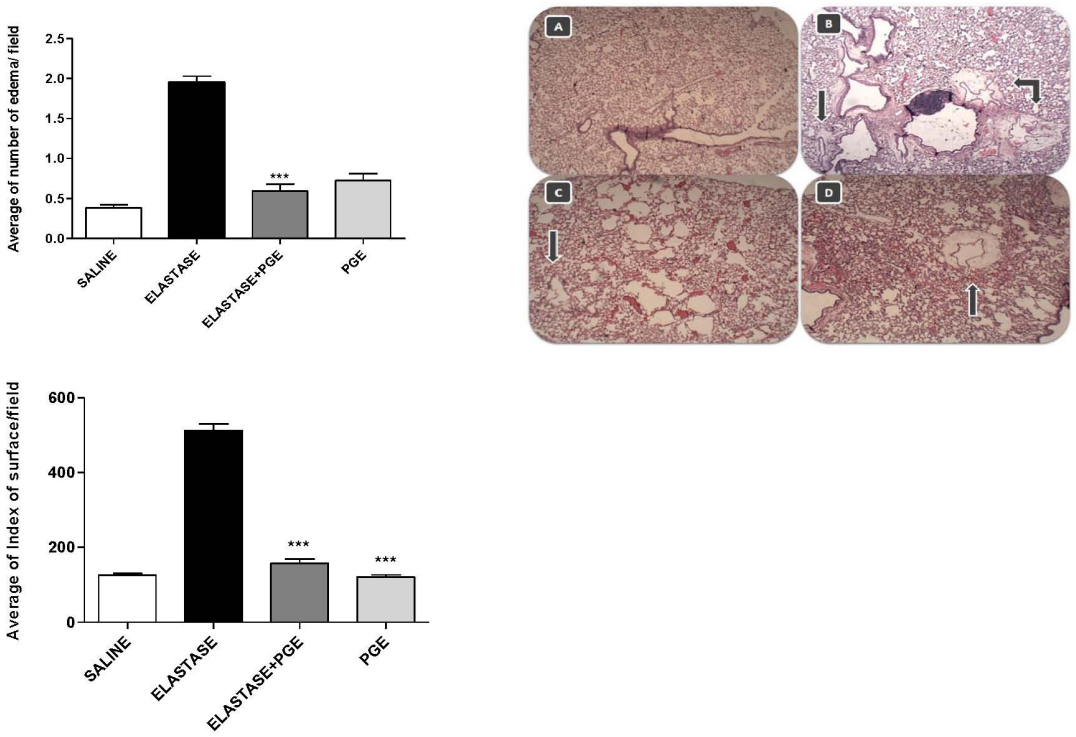

FIGURE 2 –The number and Index of surface of lung oedemas Significance pair to pair comparison are showed **: p for trend ≤ 0,05.

Effect of peel aqueous extract on Bronchoalveolar lavage fluid and on Blood parameters

Intratracheal administration of 25U/kg of Elastase induced a significant increase in the total number of cells in the BALF, as well as an increase in the number of neutrophils recruited. However, the intratracheal administration of 25U/kg of Elastase leads to a significant decrease of the number of platelets in the blood. Compared to animals treated by ELASTASE, animals pretreated with PEG at 200 mg/kg (IP route) showed a significant decrease of both BAL total cell count and the neutrophil recruitment. Furthermore, PEG at 200 mg/kg stimulates significantly the recruitment of platelets (Figure 3). These results show that pomegranate peel prevented the Elastase-induced influx of neutrophils in BAL and stimulates the recruitment of platelets to reward the loss of blood components in case of hemorrhage.

Using the same animal model, Roulet *et al.*(2012) showed that a dose of 25 U/kg of Elastase induce emphysema and histological inflammationin rats. Our results show that the composition of the inflammatory cell influx in BAL varied with time after Elastase instillation. While neutrophils were very rare in BAL of animals from the Saline group at all time points, they formed the main fraction of BAL cells at 7days after saline instillation. PGE prevented the Elastase-induced influx of neutrophils in BAL. Neutrophil numbers in BAL of rats from the PGE-Elastase group were reduced at D7 ($P < 0.014$), compared to animals from the Elastase group. In this study, emphysema was evaluated at 21 days after Elastase administration. However, in our case, emphysema was very low or impossible to observe, because animals were sacrificated at 7 days after Elastase administration.

In many animal models, intratracheally instillation of Elastase induced an increase of the number of neutrophils but also MMP-2, MMP-9, and tissue inhibitor of metalloproteinases (TIMP-1) in the BAL of the mouse (Plantier *et al.*, 2007). A combination of cigarette smoke exposure and Elastase instillation induced emphysema in mice by a parenchymal destruction in a shorter time and an increase in the numbers of cells (Rodrigues *et al.*, 2016). It was shown that intratracheal instillation of Elastase is responsible for emphysema, it increased the total cells, and the recruitment of neutrophils in BAL and it induced changes in pulmonary mechanics, airspace enlargement. It also induces MCP-1 and IL-6 expression in rats (Plantier *et al.*, 2007).

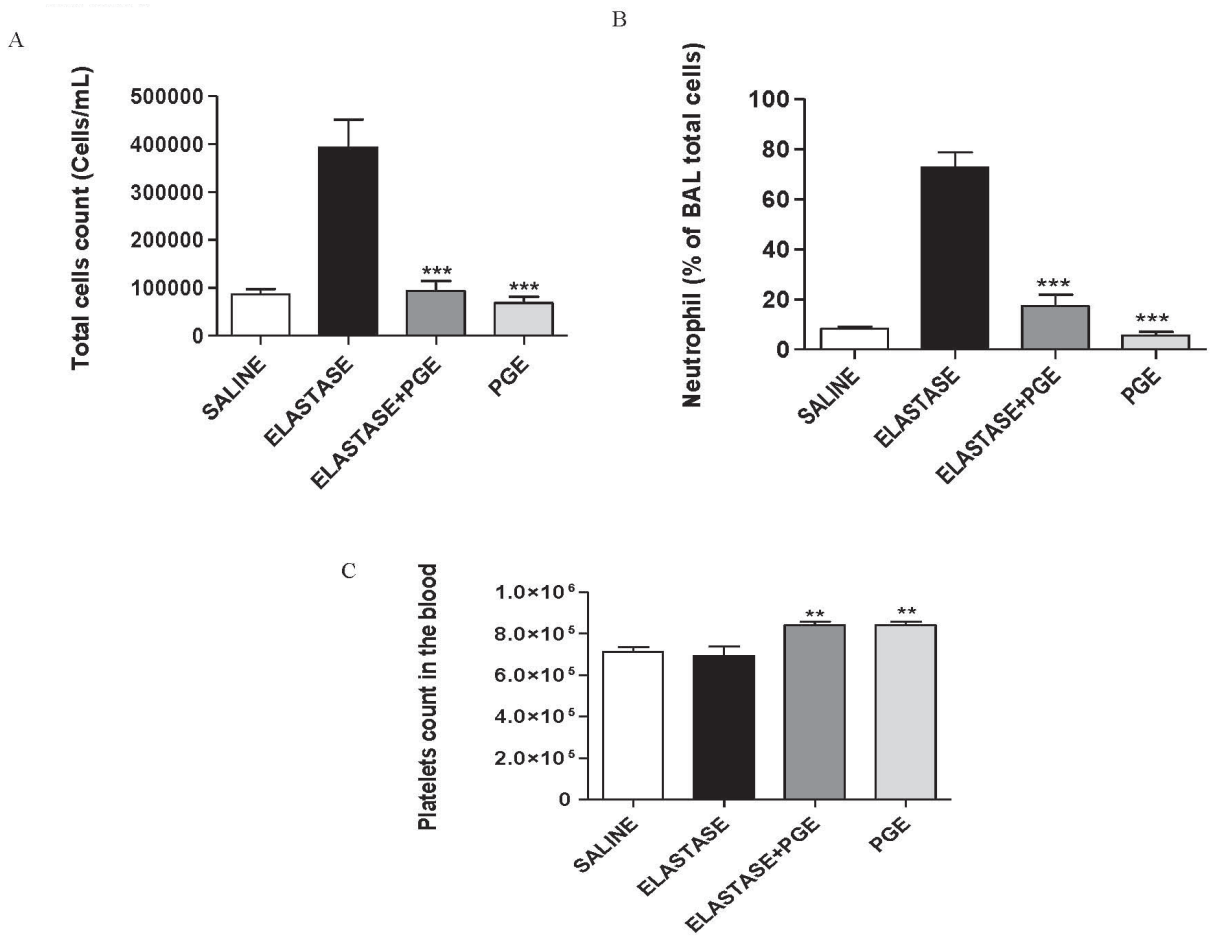

FIGURE 3 – Effect of PGE on cell content. Animals were treated with PGE (200 mg/kg), Elastase (25U/kg) or PGE (200 mg/kg) + Elastase (25 U/kg), and cells were counted 7 days after the treatment. (A), Total cell counts in BAL; (B) Neutrophil counts in BAL; (C) Platelets count (n=5, mean ± SEM *p<0.05).

Effect of peel aqueous extract on expression of inflammatory markers

IL-6 and MCP-1 expression in BAL was significantly increased in the Elastase group compared with saline group 7 days after Elastase instillation. In the group Elastase+ PGE this expression is significantly decreased to be like the saline group. This result shows that pomegranate peel significantly decreases the expression of IL-6 and MCP-1.Our results show that inflammation induced by Elastase doesn't modify the TNF-α level in BAL and we didn't detect any significant difference in the TNF-α level in the BAL between the 4 groups 7 days after each treatment (Figure 4).

RT-qPCR was performed to quantitatively measure the mRNA levels of IL-6, TNF-α, MCP-1, MMP-2, 7 days after treatment (after saline or Elastase instillation) mRNA levels of IL-6, TNF-α, MCP-1, and MMP-2 in the Elastase groups were significantly up-regulated compared with those in the control group. Intraperitoneal injection of PEG at 200 mg/kg reducedsignificantly this up-regulation 7 days after Elastase instillation (Figure 5).

In accordance with other authors, our results showed airspace enlargement, fragmentation of alveolar spaces, inflammation, and lung oedema in Elastase-treated rats (Ghorani*et al.*, 2017). These finding, showed that different inflammatory mediators such MCP-1, TNF- α, IL-8 but also matrix metalloproteinases are involved in emphysema pathogenesis. In agreement with these results, we have also demonstrated an increase of IL-6, TNF-α, MCP-1 and MMP2 in elastase treated rats. Elastase also induced an inflammatory response in the lung by increasing the expression of TNF-α, IL-1β, IL-6 and IL-8 and the infiltration of macrophages and neutrophils (Pera *et al.*, 2011).

FIGURE 4

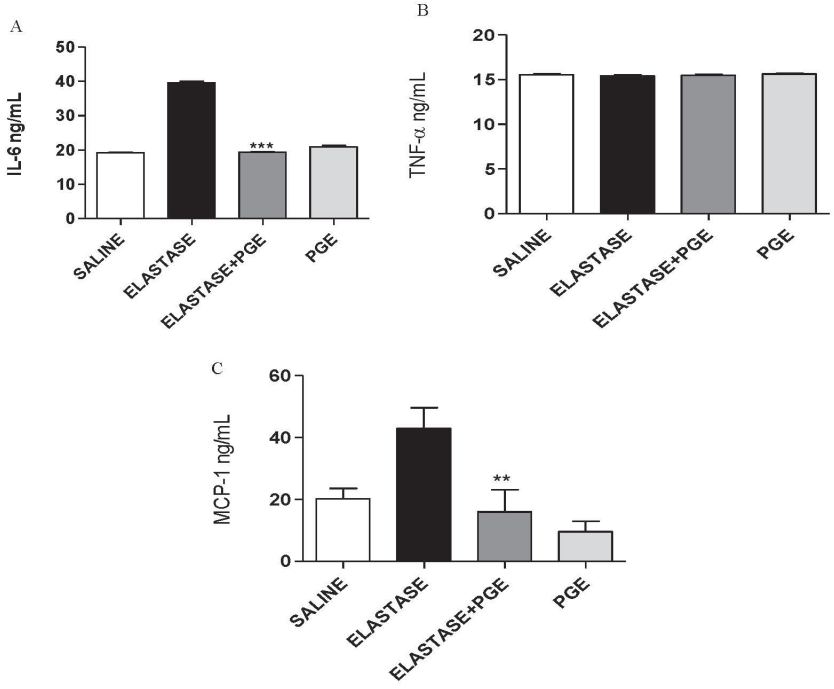

FIGURE 4 – Effect of PGE on BAL activity of TNF- α (A), IL-6 (B) and MCP-1 (C), 7 days after saline or Elastase instillation (D9). **: $p < 0.01$ *vs* Elastase. *: $p < 0.05$*vs* Elastase.

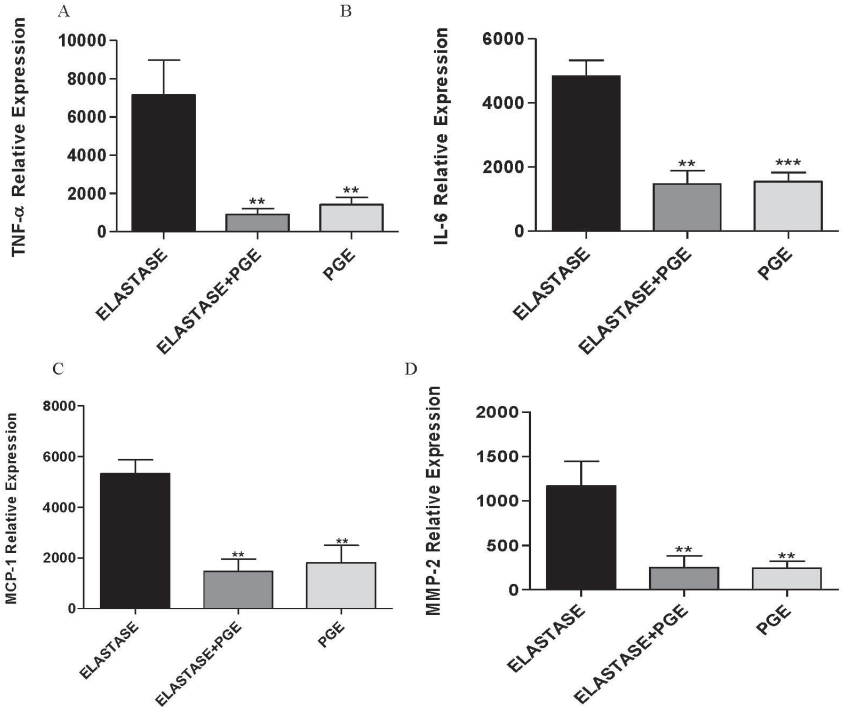

FIGURE 5 – Gene expression in 7 days after saline or Elastase instillation (D9) in animals receiving Saline solution, Elastase, and PGE alone and Elastase + PGE. Quantification of mRNA expression of TNF-a (A), IL-6 (B), MIP-1 (C) and MMP-2 genes at D9 by RT-qPCR. Data are expressed as the ratio to GAPDH as housekeeping gene. **: $p < 0.01$*vs* Elastase. n = 5.

The anti-inflammatory, anti-oxidant and anti-oncological characteristics of pomegranate, was already investigated by other studies. It was shown that pomegranate reduce overexpression of inflammatory mediators and overcome cellular degradation (Aviram *et al.*,2000; Mehta, Lansky, 2004; Bachoual *et al*., 2011; Adaramoye *et al*., 2017). Pomegranate fruit is characterized by high level of polyphenols involved in antioxidant activity and anti-inflammatory properties. Punicalagin (PUN), an ellagitannin isolated from pomegranate is characterized by its anti-inflammatory and anti-oxidant activities. Moreover, it induces an up-regulation of HO-1, a powerful endogenous antioxidant and anti-inflammatory enzyme in murine macrophages (Xu *et al.*, 2017). *Punica granatum* juice, at 5 and 8 mL/kg, abrogate the effect of trinitrobenzene sulfonic acid in provoked colitis in rats (Riaz *et al*., 2017).

In agreement with the latter work, in this study, we have demonstrated that Elastase increased lung TNF-α, MCP-1, MMP-2 expression, platelets recruitment, histological inflammation and induces severe spacing in the bronchi and arterioles characteristic of emphysema, indicating the risk of emphysema development 7 days after Elastase treatment. However, intraperitoneal injection of PEG at 200 mg/kg reduced both BAL total cell count and the neutrophil recruitment and it also decreased significantly BAL level of IL-6 and MCP-1. These finding suggest that, intraperitoneal injection of PEG at 200 mg/kg significantly reduced inflammatory cytokines and metalloproteinses involved in tissus destruction and reduces the spacing induced by the instillation of Elastase. Thereby, this also suggests that PGE protect and induce regeneration of tissus damedged by Elastase instillation.

Despite of the excellent statistical correlation between the genetic, histological and protein findings in our report, which provide a hopeful therapeutic pathway against emphysema, in future studies, we would like to purify the pomegranate extract and even try different states of fruit maturation, as well as vary the dose and time of treatment and see the effects derived from its use at a curative, preventive or harmful level.

CONCLUSIONS

The extract of pomegranate peel, at a dose of 200 mg/kg by intraperitoneal administration, reduces the lung oedema derived from the intratracheal administration of Elastase at a dose of 25 U/kg after seven days, in Sprague Dawley rat, it decrease the total cell count, as well as that of neutrophils, in BAL and it highly reduce the lung gene expression of IL-6, MCP-1, TNF-α and MMP-2. Thus, we can suggest that PGE *Punica granatum* protects against elastase lung inflammation and alveolar degradation induced in rats. However, further studies are needed to provide knowledge about the composition of the extract and about its pharmacodynamic, pharmacokinetic and toxicity properties, to fully address whether the attenuation or inhibition of mechanisms that triggered in PGE effect and these may offer a potential clinical therapeutic option in the setting of emphysema.

COMPETING INTERESTS

The author declares that they have no competing interests.

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