

**POMEGRANATE PEEL EXTRACT REDUCES
DIABETES-INDUCED COLLAGEN TURNOVER
AND BIOCHEMICAL IMBALANCE IN RAT LUNG**

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Received on July 21, 2022

Presented by I. Ivanov, Member of BAS, on September 27, 2022

Abstract

Diabetes causes lung function disorders. However, pulmonary collagen turnover under diabetes has not been well studied. The purpose of this study was to investigate collagen turnover in lung and to evaluate protective effect of pomegranate peel extract (PPE). Diabetes model in rat was induced by alloxan (250 mg/kg, i.p.). PPE treated rats received 100 mg/kg of PPE for 30 days. PPE was characterized by Gas chromatography–mass spectrometry (GC-MS). Hydroxyproline was determined by colourimetric assay kit, total collagen using ELISA kit and by histopathological assay. Collagenases (MMP-1,-8,-13) were analyzed by fluorometric method. Antioxidant enzyme activities and inflammatory marker C-reactive protein (CRP) were evaluated. Diabetes decreases SOD activity and increases CRP level by 33% ($p < 0.05$) and 125% ($p < 0.01$), respectively, when compared to the control group. PPE abrogated diabetes-induced SOD activity and CRP level disturbance. Collagenase activity was lower in diabetes and PPE group by 38% ($p < 0.05$) and 34% ($p < 0.05$), respectively, when compared to control. Diabetes increased significantly collagen and hydroxyproline by 32% ($p < 0.05$) and 192% ($p < 0.01$), respectively, and restored at similar level than controls in PPE group. Lung histopathological assay showed distended alveoli and peribronchial inflammatory cell infiltration

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The first and second authors contributed equally to this study.

DOI:10.7546/CRABS.2022.12.07

in diabetic group. Compared to diabetic, PPE limits inflammatory cell infiltration in lung tissue. Diabetes causes ECM imbalance and enhances inflammation in lung wall, which can be abrogated by PPE.

Key words: diabetes, Extracellular matrix (ECM) turnover, MMPs, collagen, inflammation, pomegranate peel extract

Introduction. Diabetes is a chronic metabolic disease characterized by a reduced insulin secretion by pancreatic cells and insulin resistance by target cells associated with several complications as a reduced lung function, nephropathy and retinopathy [19].

ECM is an essential component of pluricellular organisms, since it is the substratum secreted by cells to ‘condition’ their medium and the shape of the organ and organism. ECM is in permanent renewal, the result of the balance between the synthesis and their degradation by specialized enzymes such as matrix metalloproteinases (MMPs). The imbalance between ECM synthesis and degradation induces various pathologies such as osteoarthritis or pulmonary emphysema [5]. It has been described that ECM provides structural support and stability to the lung and that several chronic inflammatory diseases of the lungs develop with an altered composition of the ECM. Moreover, in response to injury cues, lung-resident fibroblasts are able to differentiate into muscle-like contractile proliferative myofibroblasts with a migratory phenotype. Myofibroblasts deposit collagen types III and I during fibrotic events, but also collagen type IV, various PGs, and glycoproteins such as laminins and fibronectin [3].

An altered composition of major ECM components was described in asthma involving accumulation of collagens I, III and IV and fibronectin [11]. ECM degradation was carried out especially by MMPs, which are a family of endopeptidases that degrade ECM molecules and basement membrane, and can function in many other proteolytic processes. MMPs are synthesized and secreted as inactive proenzyme forms and can be activated by other enzymes as plasmin [14].

Actually there are several evidences that pomegranate *Punica granatum* extract can prevent several diseases through the presence of abundant active biomolecules. Thus, the pomegranate bark is used to calm gastric disorders and to treat fungal-infected wounds [13]. Moreover, all parts of *Punica granatum* were described to prevent thyroid disorders and thickening of arteries due to their potent anti-inflammatory, antioxidant, and cardioprotective function, and to prevent degenerative diseases and neurotoxicity.

The association between diabetes and impaired lung function has been frequently observed. Various respiratory disorders have been described in patients with either type 1 or type 2 diabetes. However, effect of diabetes on lung collagen turnover and collagenases activities need further investigation. Thus, we aimed to explore pulmonary ECM turnover under diabetes and to investigate possible beneficial effect of PPE.

Materials and methods. Pomegranate peel extract (PPE) preparation. Pomegranate fruits (*Punica granatum*) were collected from farming in Zerkin (Gabes, Tunisia). Fruit peels were dried for 48 h at 35 °C, ground and stocked in obscurity until use. Fifteen grams of peels were extracted in 100 ml of ethanol (96%) under continuous agitation at room temperature for 24 h. Obtained extracts were filtered and evaporated using rotary evaporator.

Gas chromatography–mass spectrometry (GC-MS). The chemical identification of the volatile compounds from PPE was performed according to [16]. Briefly, the analysis was done on a gas chromatography system (7890A) coupled to a mass spectrometry system (5975C, MSD) (Agilent Technologies, Santa Clara, CA, USA), fitted with a fused silica capillary HP-5 column (5% phenylmethylpolysiloxane, 30 × 0.25 mm, film thickness 0.25 µm) (Agilent Technologies, J & W GC Columns). Chromatographic conditions were 50 °C hold for 1 min, up to 250 °C at the rate of 10 °C/min and then 1 min isothermally at 250 °C. Afterward, a second gradient was used 300 °C at 50 °C/min and finally 300 °C hold at 3 min. One microliter of each extract was injected in the split mode ratio of 1:10. Helium was used as carrier gas at 1 ml/min. The injector was operated at 200 °C. Mass spectrometer was adjusted for an emission current of 10 µA and electron multiplier voltage between 1400 and 1500 V. Trap temperature was 250 °C and that of the transfer line was 270 °C. Mass scanning was from 40 to 650 mAU. Compounds were identified by comparison of their mass spectra with those obtained in NIST 08.

Reducing power assay. Various concentrations of PPE (50, 100, 200, 300, 400 and 500 µg/ml) were mixed with 2.5 ml of 200 mmol/l sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min; 2.5 ml of 10% trichloroacetic acid (w/v) were added; the mixture was centrifuged at 3000 rpm for 10 min; 2.5 ml of the supernatant was mixed with 2.5 ml of deionised water and 0.5 ml of ferric chloride (0.1%). The absorbance was measured at 700 nm.

Animals. In our present study we used rats purchased from faculty of sciences of Gabes, Tunisia. Rats were kept in controlled conditions of temperature, humidity and light/dark cycle and were fed commercial diet and tap water ad libitum. Our protocols were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the University Animal Care and Use Committee of the University of Gabes, Tunisia.

Experimental design. Rats were divided into the following groups:

- CTRL group ($n = 8$): control rats received intraperitoneal injection of saline solution (NaCl 0.9%) for 30 days.
- ALX group ($n = 8$): diabetic rats induced by alloxan injection (250 mg/kg, i.p.) (Sigma Chemical Co. MO, USA) according to previous protocol [4].

- PPE group ($n = 8$): diabetic rats treated with PPE (100 mg/kg, i.p.) [18] for 30 days.

Blood glucose was recorded daily with a digital glucometer (ACCU-CHEK®[®], Roche, India).

At the end of the treatment, rats were euthanized with overdose of chloral hydrate (150 mg/kg, i.p.). The lung was then removed and stored at -80°C . Blood was collected and serum obtained by centrifugation (3000 rpm, 15 min, at 4°C) and stored at -80°C until use.

Protein extraction and quantification. Total protein was extracted from frozen lung pieces (100 mg) according to our previous protocol [4] and measured by colourimetric method using Bovine serum albumin as a standard.

Histological study of the lung. Histological investigations of the lung were performed in the Pathology, Anatomy and Cell Biology department of the University Medical Centre Habib Bourguiba, Medenine, Tunisia. Lungs from all used rats were fixed in 10% formalin (pH 7.4) and embedded in paraffin. Five micrometers thick slices were performed and stained with hematoxylin and eosin (H&E) (5 min) followed by light green staining (1 min). After that, slides were observed and representative photos were taken using light microscope (Leica) and interpreted by a pathologist.

Catalase (CAT) and superoxide dismutase (SOD) activities assay. Catalase (CAT) activity was determined in sample of lung homogenate. Equivalent amount of 500 μg of protein was mixed with 500 μl phosphate buffer solution and absorbance recorded at 240 nm to set zero of spectrophotometer. 100 μl of H_2O_2 (200 mM) was added, mixed and absorbance was recorded each 15 s for 2 min. CAT activity was expressed in mmoles/second/ μg protein.

Superoxide Dismutase (SOD) activity was measured in lung homogenate according to previous protocol [4]. Results were expressed U/min/ μg protein. One unit of superoxide dismutase is defined as the amount of extract that inhibits the rate of adrenochrome formation by 50%.

Total collagen and hydroxyproline assay. Total collagen content and hydroxyproline in lung was determined by commercial kits K218-100 (BioVision) and K226-100 (BioVision), respectively.

Collagenase activity assay. Collagenase activity (MMP-1, -8, and -13) was measured as previously described [4] using assay kit (K792, Biovision). Results were expressed in U/ml.

C-reactive protein assay. CRP level was determined at the University Medical Centre Habib Bourguiba, Medenine, Tunisia, using an automatic analyzer BA400 (Biosystems S.A., Spain).

Statistical analysis. Comparison between means were performed by ANOVA followed by HSD Tukey test using Statistica tool (Statsoft, France); $p < 0.05$ is considered significant.

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Antioxidant enzyme activities and CRP level at the end of the study from control and treated rats. Data are mean $\pm$ SEM, $n = 8$, $n = 6$. * $p < 0.05$, ** $p < 0.01$ vs. CTRL, $\neq p < 0.05$ vs. ALX, $\neq p < 0.01$ vs. ALX. CTRL: control group, ALX: Alloxan-induced diabetic rats, PPE: Diabetic rats receiving injection of ethanolic pomegranate peel extract (100 mg/kg, i.p.) for 30 days

	CRP (ng/ml)	SOD (U/min/mg protein)	CAT (U/min/g protein)
CTRL	08.05 $\pm$ 1.402	10.49 $\pm$ 1.21	1.85 $\pm$ 0.22
ALX	18.37 $\pm$ 2.374**	7.60 $\pm$ 0.97*	2.32 $\pm$ 0.31
PPE	05.21 $\pm$ 1.051 $\neq$	10.63 $\pm$ 2.34	1.63 $\pm$ 0.33

Results. Antioxidant enzymes activities. Antioxidant enzyme activities of catalase (CAT) and superoxide dismutase (SOD) in lung from treated and control rats were illustrated in Table 1. A significant decrease ($p < 0.05$) of SOD activity was observed in diabetic rats and it was abrogated by PPE injection. However, no significant difference was observed in catalase activity between the three studied groups.

CRP level evolution. Analysis of CRP blood level showed a significant increase ($p < 0.01$) in diabetic rats (18.37 $\pm$ 2.374) compared to controls (8.05 $\pm$ 1.402). PPE injection to diabetic animals decreases significantly ($p < 0.01$) in CRP level to 5.21 $\pm$ 1.051 (Table 1).

Collagenase activity variation. Collagenases (MMP-1, MMP-8 and MMP-13) activity was carried out on lung homogenates containing 50 μ g/ μ l of protein. Collagenase activity was significantly reduced by 38% ($p < 0.05$) and 34% ($p < 0.05$) compared to the control group in ALX and PPF groups, respectively. However, our results did not show any significant difference in collagenase activities between ALX and PPE group (Fig. 1).

Total collagen and hydroxyproline content. Diabetes increases significantly total collagen and hydroxyproline by 32% ($p < 0.05$) and 192% ($p < 0.01$), respectively. Treatment with PPE lowered collagen and hydroxyproline by 43% ($p < 0.05$ and $p < 0.01$, respectively) as compared to the diabetic group (Fig. 2).

Histology. Light green hematoxylin-eosin-stained lung sections were examined under a light microscope and interpreted by a pathologist. Lung sections showed that in control rats alveoli have a normal appearance (not distended) with uniform interalveolar septum (Fig. 3A). In diabetic rats, lung sections show rather distended alveoli and significant peribronchial inflammatory cell infiltration (Fig. 3B, C) as well as the abundance of alveolar macrophages (Fig. 3D). Moreover, there are several signs of pulmonary edema. PPE treatment improves inflammatory cell infiltration and lung structure compared to diabetic group (Fig. 3E). The sections studied from the different treated and control rats did not show a significant difference in the distribution of collagen in the lungs.

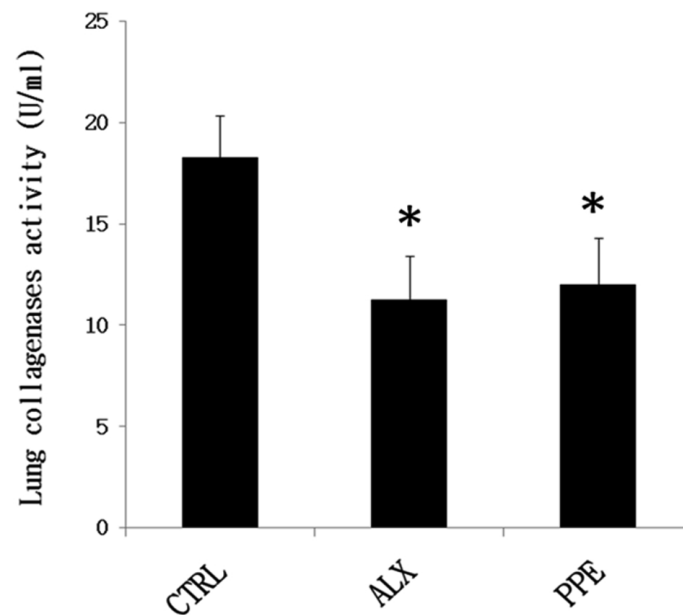


Fig. 1. Collagenases activity (U/ml) in lung homogenates from control and treated rats. Data are mean $\pm$ SEM, $n = 8$, $n = 8$. * $p < 0.05$. CTRL: Control group, ALX: Alloxan-induced diabetic rats, PPE: Diabetic male rats receiving injection of ethanolic pomegranate peel extract (100 mg/kg, i.p.) for 30 days

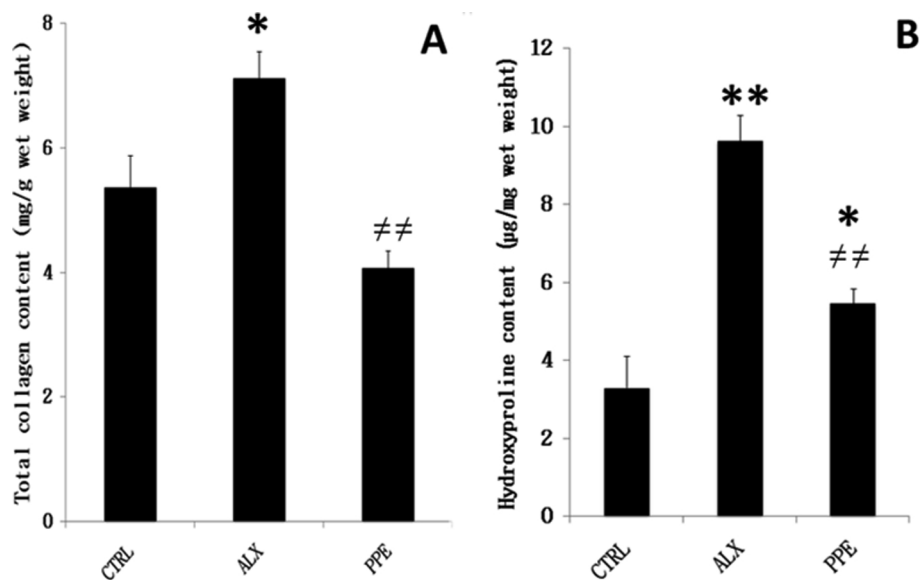


Fig. 2. Total collagen (A) and hydroxyproline (B) content in lung homogenates from control and treated rats. Data are mean $\pm$ SEM, $n = 8$, $n = 8$. * $p < 0.05$, ** $p < 0.01$ vs. CTRL, $\neq \neq p < 0.05$ vs. ALX, $\neq \neq p < 0.01$ vs. ALX. CTRL: Control group, ALX: Alloxan-induced diabetic male rats, PPE: Diabetic male rats receiving injection of ethanolic pomegranate peel extract (100 mg/kg, i.p.) for 30 days

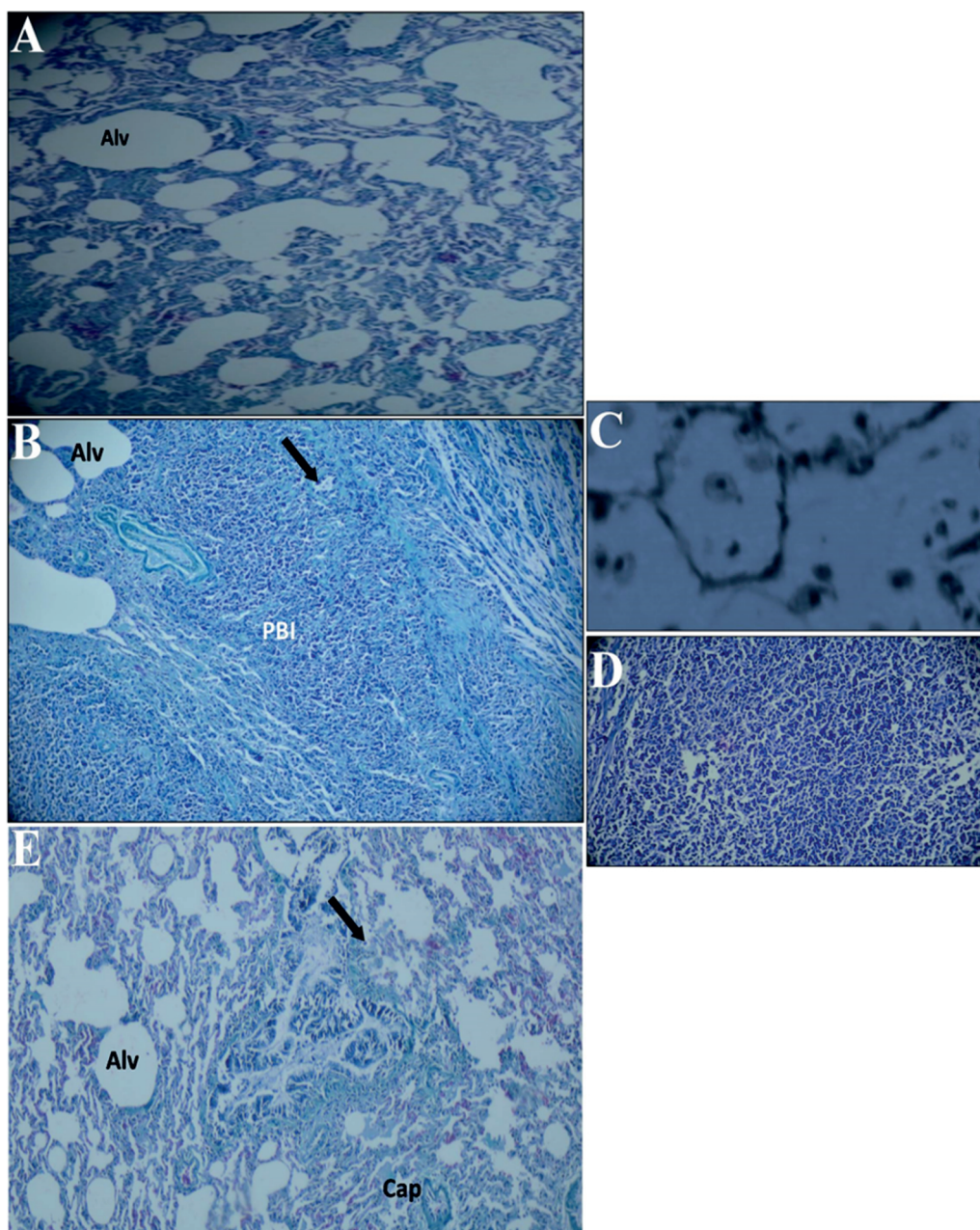


Fig. 3. Photomicrograph of cross section of lung stained with Light green hematoxylin-eosin of control rats. (A) Normal alveolar appearance (Alv). B. Lung section of alloxan-induced diabetic rat showing abundance of peribronchial inflammatory cell infiltration (PBI) and the presence of alveolar macrophages (arrow). C. Higher magnification of alveolar macrophage. D. Higher magnification of PBI. E. Lung section of PPE treated diabetic rat showing peribronchial inflammatory cell infiltration. Alv: alveolus, PBI: peribronchial inflammation, Cap: blood capillary, Lym: lymphatic vessel. Arrow: alveolar macrophage

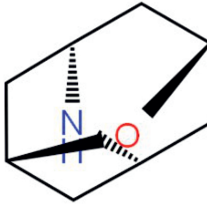
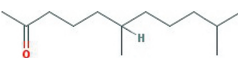
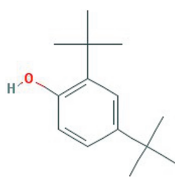
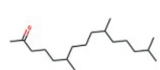
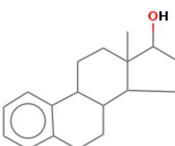
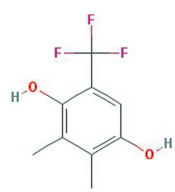
Gas chromatography–mass spectrometry (GC-MS) analysis of PPE. The ethanolic extract of the pomegranate peel was analyzed by GC-MS. The formulas and retention times of the six identified volatile compounds are presented in Table 2.

Reducing power assay. We conducted a reducing power assay which is a fast technique allowing measuring the power of pomegranate peel to reduce ferric iron Fe^{3+} to ferrous iron Fe^{2+} . From our results, we discern that the iron reduction capacity is proportional to the increase in the concentration of the pomegranate peel extract and that the EC_{50} concentration is equal to $330 \mu\text{g}/\text{ml}$.

Discussion. Diabetes is the most common metabolic disease worldwide and it is the primary cause of death in developed countries. It is well known that diabetes causes several pathophysiological alterations in lung tissue by decreas-

T a b l e 2

Identified compounds in PPE by Gas chromatography–mass spectrometry

Compound	rt (min)	Formula
2-Oxa-6-azatricyclo[3.3.1.1(3,7)]decane*	6.64	
Hexahydropseudoionone	7.89	
Phenol, 2,4-di-tert-butyl-	8.63	
Hexahydrofarnesyl acetone	10.50	
Estra-1,3,5(10)-trien-17β-ol	11.08	
Phen-1,4-diol, 2,3-dimethyl-5-trifluoromethyl-	16.08	

ing microvascular reserve of the lungs and leading to development of hypoxia. However, extracellular matrix alteration in the lung under diabetes remains not well investigated. In this context, this is the first study to investigate collagen and MMPs activity in the lung under experimental model of diabetes and to test potential protective effect of PPE injection. Our main results showed that: i) diabetes increases total collagen and hydroxyproline level in the lung and decreases collagenases activity; ii) diabetes is associated with an increased tissue inflammatory reaction and systemic increase of CRP level; and iii) PPE treatment abrogates diabetes-induced collagen; hydroxyproline and CRP improve antioxidant enzyme activity in the lung.

In this study, we used alloxan to induce diabetes, which is an analogue of glucose. Alloxan causes rapid destruction of pancreatic β cells by reactive oxygen species production and increases intracellular calcium concentration [9]. Diabetes was induced with a single intraperitoneal injection of 250 mg/kg in fasting rats as previously described [4]. Fasting glucose level higher than 200 mg/dl was considered diabetic as previously reported [2].

Diabetes is characterized by hyperglycemia, and leads to development of microvascular and macrovascular complications. Microvascular complications are generally nephropathy and retinopathy and macrovascular complications include coronary artery disease, cerebrovascular disease and peripheral arterial disease [6]. Previous investigations of wound healing regulation confirm that diabetes is associated with an aberrant redox signalling and increased oxidative stress which contribute to the development of diabetic complications [17]. Moreover, reducing lung function was reported in diabetes through a disrupted ECM balance [15]. In our study, we established that diabetes enhances significantly total collagen level and hydroxyproline in the lung, which can explain the observed lung dysfunction. In this context, previous investigations reported several histological changes in the lung parenchyma, such as nodular fibrosis by comparison of autopsies of 61 diabetic and 50 non-diabetic patients and that diabetes appears to accelerate changes in collagen metabolism [15].

Enhanced collagen and hydroxyproline content in alloxan-induced diabetes corroborates finding of previous studies of wound healing that suggest an abnormal morphology of fibroblasts associated with a decreased adhesion, diminished response to growth factors and cytokines, and decreased production of collagens and fibronectin [1]. Moreover, overexpression of collagen in diabetes can be related to reactive oxygen species which promote fibroblast proliferation and migration, and mediate TGF- β 1 signalling, which results in migration, collagen and fibronectin production, and basic fibroblast growth factor (bFGF) expression [8]. Enhanced collagen and hydroxyproline in diabetes rats was in concordance with observed decrease of collagenase activity in the lung homogenate. Decreased collagenase activity observed in lung from diabetic rats and enhanced collagen and hydroxyproline level confirms a direct involvement of diabetes in the remodelling

of ECM. Decreased MMPs activity can be also associated with an imbalance between activators and tissue inhibitors of MMPs.

Pathohistological study reveals an abundance of peribronchial inflammatory cell infiltration and presence of macrophage in the alveoli in diabetic rats. In the same context CRP level was significantly higher in alloxan-diabetic rats. Similar observations in humans was reported which stated a diminished lung function several years before diabetes diagnosis linked to an abnormal regulation of inflammatory mechanisms and an exaggerated inflammatory responses in the lung [10]. Our results show that diabetes was associated with a reduced antioxidant enzyme SOD activity in concordance with the investigations of [7] which demonstrated that experimental model of diabetes in rabbit and rat was associated with a reduction in antioxidant enzyme activity and increased lipoperoxidation which is revealed by a decrease in SOD activity in the lung.

In this study, we report that PPE injection improves hydroxyproline and collagen in diabetic rats as well as a reduced inflammatory response in the lung tissue and a reduced CRP level. These results can be explained by the richness of PPE of bioactive compounds, which improve antioxidant enzymes as confirmed by GC-MS and HPLC in our previous study [4]. Another study also reported richness of pomegranate peel of flavonoids, phenolic acids and their derivatives, including quercetin, rutin, gallic acid, elagic acid and punicalagin as ellagitannin [12]. On the other hand, a previous study reported that pomegranate decreases TGF- β 1 and type I collagen expression in the right ventricle in rats with pulmonary arterial hypertension. Moreover, pomegranate peel extract reduces fibrosis and improves liver function through a decrease of TGF- β and oxidative stress markers via p38MAPK/Nrf-2 pathway [20].

Limitations of the study and conclusion. In our study we established that diabetes disturbed collagen turnover and enhanced inflammatory infiltration in the lung tissue and that pomegranate peel extract restored diabetes induced collagen hydroxyproline and CRP disturbance in the lung as well as improved antioxidant enzyme activities. We demonstrate that pomegranate peel extract might play a protective role in disorders associated with diabetes through action of various bioactive molecules and higher antioxidant capacity.

This study demonstrated interesting preliminary results in favour of the protective effect of pomegranate peel against diabetes complication in lung. However, a larger-scale study involving a higher number of rats and more specific parameters related to lung function is necessary in order to fully explore the beneficial effects of pomegranate peel.

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