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Original Article

Flavocoxid Improves Bleomycin-Induced Respiratory Dysfunction and Pulmonary Fibrosis; *In vitro* Study

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ABSTRACT

Pulmonary fibrosis; a progressive and fatal lung disorder is a common interstitial lung disease affecting millions of individuals worldwide with a mean survival time of about 3 years. It has been reported to be the most serious side effect observed with bleomycin's (BLM) use as a chemotherapeutic agent. Flavocoxid is a potent anti-inflammatory and anti-oxidant agent. In the current study, flavocoxid has been investigated for its ability to ameliorate BLM-induced pulmonary fibrosis, respiratory and vascular dysfunction in rats. BLM (5 mg/kg) was instilled intra-tracheally and flavocoxid was administered (20 mg/kg) orally for 5 weeks; one week pre- and 4 weeks post BLM instillation. Flavocoxid administration significantly decreased lung contents of Nrf2, HO-1, TLR4, TNF-α, TGF-β1. Moreover, flavocoxid successfully restored vascular response to Kcl, PE and carbacol and tracheal response to carbacol. In conclusion; flavocoxid ameliorated BLM-induced pulmonary fibrosis and improved respiratory functions and can be proposed to be a potential effective therapeutic agent for management of pulmonary fibrosis.

Keywords: Bleomycin, flavocoxid, Nrf2, HO-1, TNF-α, TGF-β1

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INTRODUCTION

Pulmonary fibrosis is one of the most common types of interstitial lung diseases affecting over 5 million individuals worldwide with an average survival time of 3 years (1). It is a chronic, progressive respiratory disorder characterized by significant swelling and scarring of the alveoli and interstitial tissues of the lung, causing patients' lungs to stiffen and making breathing increasingly difficult (2).

To date, there is no medication that can significantly improve lung fibrosis (3). The only current effective approach is lung transplantation. Therefore, the search for novel drugs with significant efficacy and tolerability for the pulmonary fibrosis is inevitable (4). Viral infections, exposure to radiotherapy and chemotherapeutic drugs and environmental toxins are amongst causes implicated in development of lung fibrosis (4). Its pathogenesis remains incompletely understood, but increased line of evidences suggest that alveolar epithelial injury, inflammation, excessive ROS generation and abnormal lesion healing involving excessive extracellular matrix (ECM) deposition are main events involved in progression of pulmonary fibrosis (5).

Bleomycin (BLM) is anticancer antibiotic used for treatment of several types of malgenancies. The most severe side effect of BLM is pulmonary toxicity (6). BLM promotes pulmonary fibrosis through increasing oxidative stress and inflammation with concomitant cessation of the injury/repair process (7). Rats and mice models of BLM-induced pulmonary fibrosis have been repeatedly reported to be significantly important in studying pathogenic pathway of pulmonary fibrosis and in studying therapeutic efficacy of various therapeutic agents against pulmonary fibrosis.

BLM induces reactive oxygen species (ROS) that bind to DNA causing DNA damage and initiates inflammatory and fibro-proliferative responses. Moreover, BLM is reported to promote the depletion of endogenous antioxidant defenses, exacerbating oxidant mediated tissue injury (8).

Flavocoxid is a mixture of two flavonoids catechin and baicalin (9). It is an FDA-regulated medical food, for management of osteoarthritis in the United States. Flavocoxid modulates cycloxygenase (COX) enzymes via an anti-peroxidase activity and inhibits 5-lipoxygenase (5-LOX)-mediated leukotrines (LT) production. Flavocoxid has a wide range of strong antioxidant activities mediated mainly via down-regulation of inducible inflammatory gene expression and neutralization of ROS, preventing the conversion of arachidonic acid to oxidized lipids (10).

The present study was designed to evaluate the ability of flavocoxid to attenuate BLM-induced pulmonary fibrosis, respiratory and vascular responses in rats also to. *In vitro* vascular response of the pulmonary artery to Kcl, phenylephrine hydrochloride (PE) and carbacol and *in vitro* tracheal response of tracheal zigzag to carbachol were evaluated. The effect of flavocoxid administration on lung contents of nuclear factor, erythroid derived-2 like protein-2 (Nrf2), heme oxygenase-1 (HO-1) and Toll Like Receptor 4 (TLR4) and immunohistochemical analysis of lung tumor necrosis factor- α (TNF-α) and transforming growth factor-β1 (TGF-β1) were evaluated to draw a schematic conclusion about mechanisms involved in the observed improve in vascular and respiratory functions.

MATERIALS AND METHODS

Experimental animals: Adult male Sprague Dawely rats (170-220 g) were purchased from Merck Research Center, Faculty of medicine, Mansoura University; they were kept under constant environmental and nutritional conditions throughout the experimental period. Research protocol complied with guidelines of "Research Ethics Committee", Faculty of Pharmacy, Mansoura University, Mansoura, Egypt.

Drugs and chemicals: Flavocoxid was purchased from Pimus pharmaceutical Inc. (Scottsdale, AZ, USA) and BLM was purchased from Nippon Kayaku Co. (LTD., Tokyo, Japan), PE and carbachol were purchased from Sigma Aldrich chemical Co. (St. Louis, MO, USA).

Experimental protocol:

Induction of pulmonary fibrosis: Pulmonary fibrosis was induced by intratracheal instillation of BLM (5 mg/kg) as sulfate salt dissolved in 0.1 ml of normal saline (11). Rats were anesthetized using intraperitoneal (IP) thiopental sodium (20 mg/kg). A midline incision was made in the neck, the trachea was exposed and BLM was instilled by slow injection. Rats were kept in vertical position and rotated several times to ensure uniform distribution of BLM within the lung tissues. The incision was surgically sutured and sodium fusidate 2% was applied topically to the wound to minimize any risk of infection.

Animals grouping: Rats were randomly allocated to three experimental groups (12 rats/group) as follows: Normal control; 0.1 ml of normal saline

was instilled to the trachea as previously described and rats received 0.2 ml of 0.5% carboxymethylcellulose (CMC) orally once daily for 5 weeks, BLM control: BLM was instilled intratracheally (5 mg/kg) and rats received 0.2 ml of 0.5 % CMC orally once daily for 5 weeks, flavocoxid treated group: rats received daily flavocoxid (20 mg/kg, orally) suspended in 0.5% CMC for one week prior to and for further 4 weeks post BLM instillation for an overall period of five weeks of flavocoxid administration.

Four weeks post BLM instillation; rats were deeply anesthetized with thiopental sodium (40 mg/kg). Then the tracheas and pulmonary arteries were separated for *in vitro* assay. Lungs were harvested, rinsed in ice-cold saline. The left lobes from all the lungs were isolated for preparation of lung homogenate and the right lobes were separated for histopathological examination and immunohistochemical (IHC) analysis.

Preparation of lung homogenate and assessment of lung nuclear factor, erythroid derived-2 like protein-2 (Nrf2), heme oxygenase-1 (HO-1) and Toll Like Receptor 4 (TLR4) contents: The isolated left pulmonary lobes were rinsed, weighed and homogenized in KCl (1.15%, pH 7.4) to yield 10% w/v tissue homogenate (Daba *et al*., 2004). The homogenate was centrifuged at 2000 rpm, 4ºC for 15 min, and the supernatant was separated and used immediately for assessment of .lung content of Nrf2, HO-1 and TLR4 using commercially available enzyme-linked immunosorbent assay (ELISA) kit (Uscn Life Science, INC. USA.), according to the supplied manufacturer's instructions.

***In vitro* assessment of vascular reactivity of pulmonary artery to potassium chloride (Kcl), phenylephrine (PE) (10^{-9}-10^{-6} M) and carbacol (10^{-8}-10^{-5} M):** The first branch of the main pulmonary artery was quickly separated and mounted in a cold oxygenated Krebs Hensilit solution (KHS). The vessels were dissected free of adherent fats and connective tissue, and cut into rings (2-4 mm). Pulmonary rings were mounted in a 10 ml organ bath at 37 °C and aerated with a mixture of 95% O_2 and 5% CO_2 and they were allowed to equilibrate under 0.8 g tension for 60 minutes. Isometric tension generated by the vascular pulmonary artery rings was measured using Riegestab K30 force transducer (Hugo Sachs electronic, D7806 march, Germany), and recorded with a Powerlab unit/400 linked to a PC running Chart v4.2 software (ADInstruments Pty Ltd., Australia). After equilibration, contractile response to 80 mmol/L of KCl was recorded. Contraction responses to semi logarithmic concentrations of PE (10^{-9}-10^{-6} M) were expressed as g tension. The maximum effect (E_{max}) and concentration inducing 50% of Emax (EC_{50}) were determined from the cumulative concentration-response curves. pD2 value (negative logarithm to base 10 of the EC_{50} values) was calculated. Relaxant responses to semi-logarithmic concentration of carbacol (10^{-8}-10^{-5} M) were expressed as the percentage decrease of the magnitude of the contraction induced by PE (1μM) before application of carbacol. The maximum effect (E_{max}) and inhibitory concentration 50% (IC_{50}) were determined from the cumulative concentration-response curves and pD2 value was calculated. Vascular relaxation in response to carbacol (10^{-8}–10^{-5} M) was recorded after pre-contraction of pulmonary artery rings with PE (1μM).

***In vitro* assessment of tracheal smooth muscles reactivity to carbacol (10^{-8}-10^{-5} M):** The exposed tracheas were excised and thoroughly cleared from extraneous connective tissue and the tracheal zigzag was prepared according to the method described by Emmerson and Mackay (12) and was incubated in an organ bath filled with 10 ml of KHS at 37 °C and bubbled with carbogen mixture. The tracheal tension was set and kept at 1g throughout a stabilization period of 60 min during which the KHS was refreshed every 15 min. Isometric tension generated by the tracheal smooth muscles was measured and recorded as previously described. The tracheal zigzag was initially contracted with carbacol (10^{-4} M) to check contractility and record the maximal contraction. The tracheal zigzag was then washed several times with fresh KHS until base line tension was restored. A cumulative concentration-response curve (10^{-8}-10^{-5} M) carbacol was constructed. Both E_{max} and effective concentration 50 (EC_{50}) were calculated from the individual concentration-response curves. Results are expressed as percentage of maximal contraction induced by 10^{-4} M carbacol.

Immunohistochemical analysis of Tumor necrosis factor- α (TNF-α) and transforming growth factor-β1 (TGF-β1) expression in lung specimen: Immuno-staining was performed using Avidin-Biotin Complex (ABC) method. The sections were immune-stained with primary antibody Rabbit polyclonal IgG to rat (TNF-α and TGF-β1). The intensity of staining was graded semi-quantitatively and each specimen was assigned a score on a scale from 0 to 3, designated as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong) (13).

Statistical analysis: Results are expressed as mean ± standard error of mean (SEM). Statistical analysis and graphing were carried out using Graphpad

software Prism V 5 (Graphpad Software Inc., San Diego, CA, USA). Significant differences between groups were determined using one-way analysis of variance (ANOVA) followed by Tukey-Kramers *post-hoc* test for parametric measures, Kruskal-Wallis test followed by Dunn *post hoc* test for non-parametric measures, linear regression for construction of standard curves and nonlinear regression for construction of sigmoidal curves. Statistical significance was accepted at $p<0.05$.

RESULTS

Effect of daily oral flavocoxid on lung Heme oxygenase-1 (HO-1) content: Lung HO-1 content significantly increased following intratracheal instillation of BLM in comparison to normal control. Flavocoxid significantly reduced lung HO-1 content compared to BLM control (figure 1).

Effect of daily oral flavocoxid on lung nuclear factor, erythroid derived-2 like protein-2 (Nrf2) content: Lung Nrf2 content significantly increased in BLM control group in comparison to normal control group. Daily flavocoxid significantly decreased lung Nrf2 content compared to BLM control (figure 1).

Effect of daily oral flavocoxid on lung Toll Like Receptor 4 (TLR4) content: Lung TLR4 content significantly increased after BLM instillation compared to normal control. Daily oral flavocoxid significantly decreased lung TLR4 content in comparison to BLM control (figure 2).

Effect of daily oral flavocoxid on vascular reactivity in isolated rat pulmonary artery rings: Intratracheal BLM instillation significantly decreased Kcl-induced contraction, PE-induced maximal contraction and carbacol induced maximal relaxation in isolated pulmonary artery rings compared to normal control (figure 3). On the other hand, flavocoxid significantly corrected BLM-induced damage, where pulmonary contraction response to Kcl and PE-induced contraction significantly increased, finally, carbacol induced relaxation significantly increased in comparison to BLM control (figure 3).

Effect of daily oral flavocoxid on carbacol-induced contraction in tracheal smooth muscle: BLM instillation induced a significant decrease in carbacol-induced maximal contraction in isolated tracheal zigzag compared to normal control. Daily oral flavocoxid significantly increased carbacol-induced maximal contraction compared to BLM control (Figure 4).

Effect of daily oral flavocoxid on immunohistochemical analysis of lung TNF-α expression: Intratracheal BLM instillation significantly increased lung TNF-α expression (figure 5, B) compared to normal control group (figure 5, A). Daily oral flavocoxid significantly reduced lung TNF-α expression compared to BLM control (figure 5, C), (table 1).

Effect of daily oral flavocoxid on immunohistochemical analysis of lung TGF-β1 expression: Intratracheal BLM instillation caused a significant elevation in lung TGF-β1 expression (figure 6, B) compared to normal control (figure 6, A). Daily flavocoxid significantly reduced lung TGF-β1 expression compared to BLM control (figure 6, C), (table 1).

DISCUSSION

The present study was designed to investigate the pulmonary and vascular protective potential of flavocoxid against BLM-induced pulmonary fibrosis and vascular damage and to highlight the mechanisms responsible of the observed protective effect. In the current study, a well characterized rodent model of pulmonary fibrosis involving single intratracheal instillation of BLM was adapted. BLM instillation significantly impaired biochemical dynamics, lung histopathology and induced marked impairment in physiological and vascular pulmonary functions.

BLM has been reported to induce oxidative stress and inflammation with concomitant cessation of the injury/repair process, thus, promoting pulmonary fibrosis (14). BLM stimulates ROS generation (15), which target important bio-macromolecules such as DNA, protein, and lipid and induces lipid peroxidation. Lipid peroxidation contributes to biochemical, histopathological and physiological organs' dysfunctions (16).

Oxidative stress has been reported to play a crucial role in the pathological development of pulmonary fibrosis (17). Markers of oxidative stress have been identified in the lungs of pulmonary fibrosis patients and aberrant antioxidant activity exacerbated pulmonary fibrosis in animal models (15).

The redox sensing transcription factor Nrf2 plays a crucial role in regulation of cellular oxidant/anti-oxidant hemostasis. Under basal conditions, low levels of Nrf2 are expressed. ROS generation induces Nrf2 initiation and activates Nrf2-dependent genes. Dys-regulation of Nrf2 activity has been proposed to be a contributing factor in aggravating pulmonary fibrosis (18). In agreement, intratracheal BLM instillation in the current study stimulated lung Nrf2 expression and this

observation is in agreement with observations of Chitra et el., (15). On the other hand, flavocoxid administration decreased lung Nrf2 content. This effect might have arisen due to the antioxidant properties of flavocoxid which prevented BLM induced ROS from triggering Nrf2 activation.

Heme oxygenase (HO) is another contributor that gets up-regulated during oxidative stress and in coordination it has been reported to have key role in regulation of inflammation (19). Increased expression of HO-1 has been associated with a number of pulmonary disorders (20, 21). Up-regulated HO-1 activity exerts damaging effects due to excessive production of its products; bilirubin, CO, and iron (22). Levels of tissue HO have been reported to increase in response to oxidative injury. This increase is thought to protect against hyperoxic cellular injury *in vitro* (21) and *in vivo* (23).

In the current study, BLM instillation resulted in a significant increase in lung HO-1 content. On the other hand, flavocoxid administration caused significant decrease in lung HO-1 content further confirming the antioxidant effect of flavocoxid and its ability to prevent Nrf2 activation which represents the major transcriptional regulator of the HO-1 gene (13). This is in line with previous studies reporting cyto-protection afforded by HO-1 inhibition against BLM-induced pulmonary fibrosis due to combined diminished generation of toxic heme metabolites and increased tissue antioxidant capacity (24).

As previously mentioned, both oxidative stress and inflammation contribute to pathogenesis of BLM-induced pulmonary fibrosis. TLRs represent a conserved family of innate immune recognition receptors that play roles in both regulation of innate and adaptive immune responses (25) and in non-infectious inflammatory diseases (26). TLR4 is expressed in a wide range of cells in the lung (27). It has been reported to be essential for hemorrhage-induced lung TNF-α expression, neutrophil accumulation, and protein permeability (28). In association, stimulation of TLR4 receptors has been linked to fibroblast activation by augmenting TGF-β1 signaling (29). Moreover, the essential role of both of TNF-α and TGF-β1 in the pathogenesis of pulmonary fibrosis has been established (30, 31). Many researchers have reported direct correlation between TNF-α and enhanced TGF-β1 expression (32). In the current study, BLM instillation increased both lung content of TLR4, expression of TGF-β1 and TNF-α. Such association is in agreement with (33) who reported implication of TLR4 in BLM-induced pulmonary fibrosis. Flavocoxid administration significantly decreased lung TLR4, TGF-β1 and TNF-α expression, proposing down-regulation of TLR4 TGF-β1 and TNF-α to be additional possible pathway implicated in beneficial anti-inflammatory and anti-fibrotic effects of flavocoxid as seen with the results.

In vitro, flavocoxid has been reported to suppress gene expression and protein levels of inflammatory markers, such as TNF-α from immune inflammatory cells (34). *In vivo* studies have confirmed the strong anti-inflammatory activity of flavocoxid (35). Accumulating evidences suggest modulation of NF-κB and TNF-α production triggered primarily by antioxidant properties of flavocoxid to be implicated in its anti-inflammatory properties (9, 36). (Minutoli, Micali (37)) referred to the inhibitory effect of flavocoxid on TNF-α and TGF-β1 expression in cadmium-induced disruption of the blood-testis barrier model. These data are also consistent with the findings of (El-Kashef, El-Kenawi (38)) who reported the ability of flavocoxid to reduce kidney content of TNF-α in gentamycin-intoxicated rats.

In line with the aforementioned evidences of biochemical damage induced, BLM instillation induced vascular and tracheal functional impairments as seen with *in vitro* assessment of vascular reactivity of isolated pulmonary artery rings to Kcl, PE and carbacol. BLM instillation significantly decreased vascular reactivity of isolated pulmonary artery ring. Also, *in vitro* assessment of tracheal smooth muscle reactivity to carbacol revealed significant decrease in tracheal smooth muscle reactivity. On the other hand, flavocoxid through amelioration of BLM-induced inflammation and fibrosis was able to restore normal vascular tone and tracheal contractile response confirming respiratory functional improvement associated with the evident biochemical and histopathological flavocoxid induced improvement. In conclusion; flavocoxid ameliorates BLM-induced pulmonary fibrosis and vascular damage. Combined antioxidant, anti-inflammatory and anti-fibrotic effects of flavocoxid are believed to be implicated in the protective efficacy. Modulation of Nrf2 and HO-1 pathways is the main mechanism involved in the observed antioxidant effect. Down-regulation of TLR4 expression is the major pathway involved in the observed anti-inflammatory effect and finally, down-regulation of tissue expression of TNF-α and TGF-β1 is the major pathways implicated in the observed anti-fibrotic activity.

Conflict of interest: The authors declare that no conflict of interests existed in the organization, results, presentation and the finance of the article.

Table (1): Effect of daily oral Flavocoxid on lung TNF-α and TGF-β1 expression:

Treatment	No. of animals in each TNF-α expression score level				Average TNF-α expression scores	No. of animals in each TGF-β1 expression score level				Average TGF-β1 expression scores
	0	1	2	3		0	1	2	3	
Normal control	6	0	0	0	0 ± 0.0	6	0	0	0	0 ± 0.0
BLM control	0	0	3	3	2.5 ± 0.22*	0	0	2	4	2.3 ± 0.21*
BLM/Flavocoxid	2	4	0	0	0.67 ± 0.21$	3	3	0	0	0.50 ± 0.22$

Data are expressed as mean ± SEM, n=6; Data were statically analyzed using Kruskal-Walis test followed by Dunn multiple comparisons test ($p<0.05$); *$ significantly different from normal and BLM controls respectively.

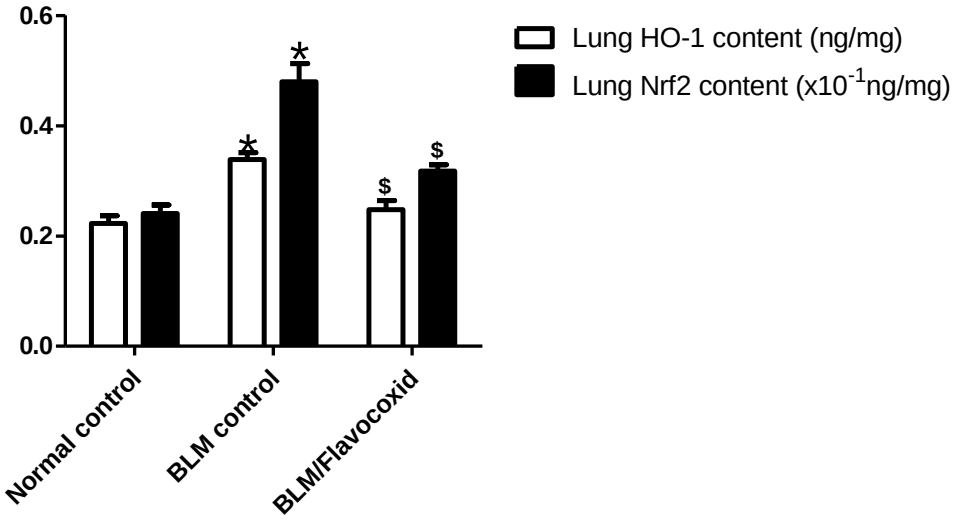


Figure (1): Effect of daily oral flavocoxid on lung nuclear factor, erythroid derived-2 like protein-2 (Nrf2) and Heme oxygenase-1 (HO-1) contents.

Data are expressed as mean ± SEM, n=12; Data were statically analyzed using One-Way ANOVA followed by Tukey-Kramer multiple comparisons test (p<0.05); *$significantly different from normal and BLM controls respectively.

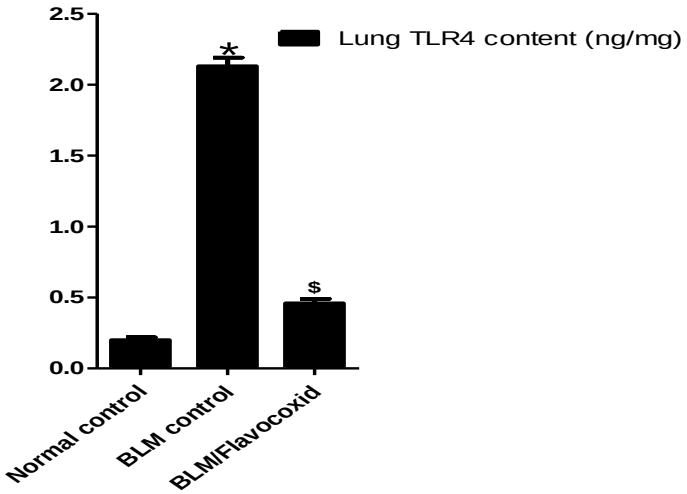
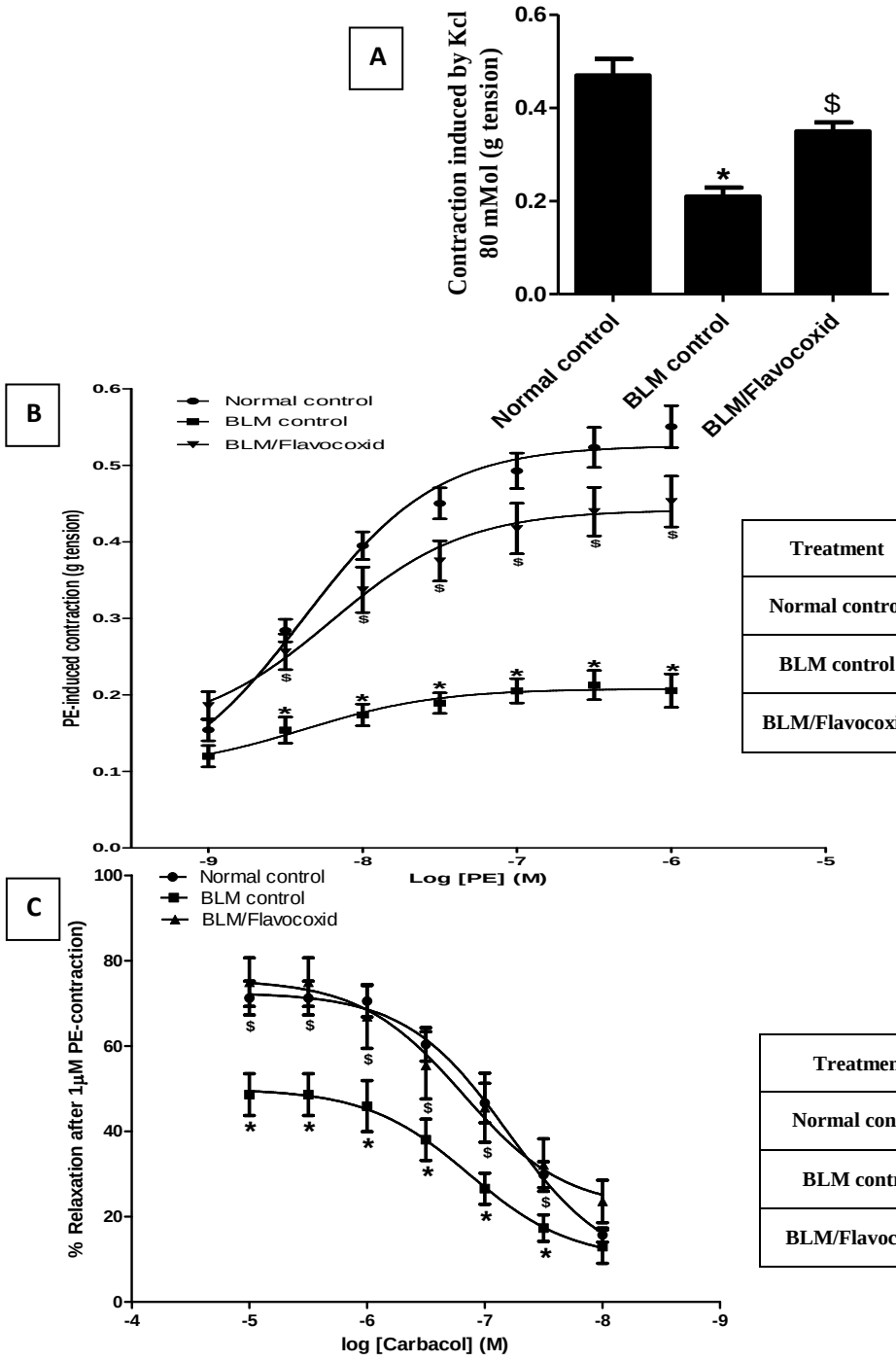


Figure (2): Effect of daily oral flavocoxid on lung Toll like receptor 4 (TLR4) content.

Data are expressed as mean ± SEM, n=12.

Data were statically analyzed using One-Way ANOVA followed by Tukey-Kramer multiple comparisons test (p<0.05).

* $ significantly different from normal and BLM controls respectively.



Treatment	E_{max}	pD_2
Normal control	0.526 ± 0.013	8.38 ± 0.133
BLM control	0.208± 0.01*	8.46± 0.744
BLM/Flavocoxid	0.443± 0.017$	8.22± 0.237

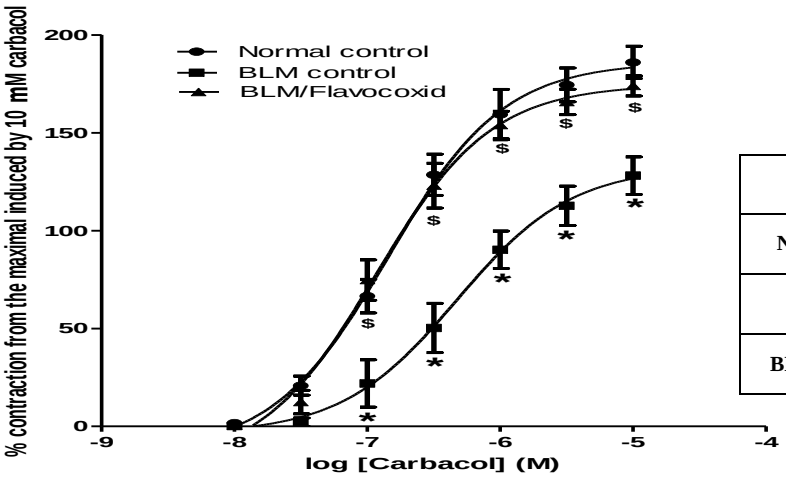
Treatment	Emax	pD_2
Normal control	72.52 ± 2.18	7.19 ± 0.131
BLM control	49.96 ± 3.09 *	6.86 ± 0.234
BLM/Flavocoxid	75.58 ± 4.66$	6.82± 0.254

Figure (3): Effect of daily oral flavocoxid on vascular reactivity of isolated rat pulmonary artery rings.

A) Effect of flavocoxid on Kcl-induced contraction in isolated rat pulmonary artery rings following intratracheal instillation of BLM. B) Effect of flavocoxid on PE-induced contraction in isolated rat pulmonary artery rings following intratracheal instillation of BLM. C) Effect of flavocoxid on carbacol-induced relaxation in isolated rat pulmonary artery rings following intratracheal instillation of BLM.

Data are expressed as mean ± SEM, n=6.
Data were statically analyzed using One-Way ANOVA followed by Tukey-Kramer multiple comparisons test ($p<0.05$).
* $ significantly different from normal and BLM controls respectively.

Emax The maximum effect. pD2 negative logarithm to base 10 of the EC50 values



Treatment	E_{max}	pD_2
Normal control	186.2 ± 5.95	6.84 ± 0.09
BLM control	132.8± 8.48*	6.33± 0.13
BLM/Flavocoxid	174.7 ± 5.09$	6.94± 0.083

Figure (4): Effect of daily oral flavocoxid on carbacol-induced contraction in tracheal smooth muscle.

Data are expressed as mean ± SEM, n=6; Data were statically analyzed using One-Way ANOVA followed by Tukey-Kramer multiple comparisons test ($p<0.05$).
* $ significantly different from normal and BLM controls respectively.
Emax The maximum effect. pD2 negative logarithm to base 10 of the EC50 values

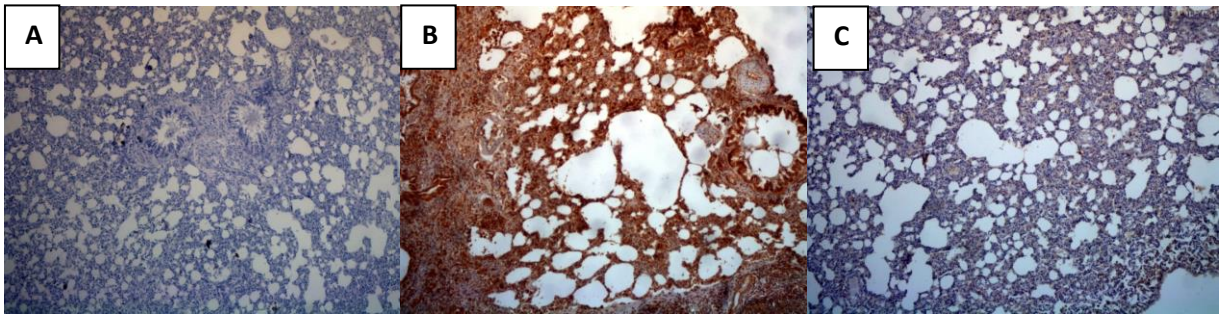


Figure (5): Effect of daily oral flavocoxid on lung TNF-α expression (IHC, 100X). (A) Normal control, revealing negative brown staining reaction of TNF-α in fibroblasts and pneumocytes. (B) BLM control, revealing sever brown staining denoting diffuse strong positive cytoplasmic reaction of TNF-α in fibroblasts and pneumocytes. (C) Flavocoxid, revealing negative brown staining reaction of TNF-α in fibroblasts and pneumocytes.

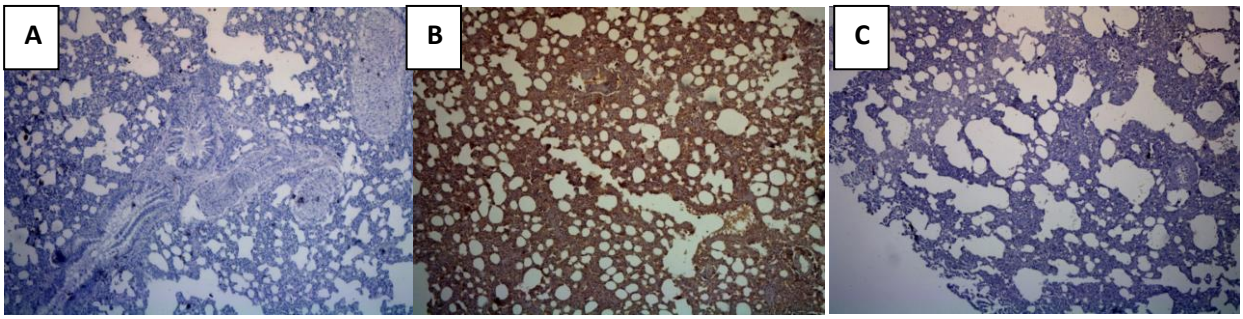


Figure (6): Effect of daily oral flavocoxid on lung TGF-β1 expression (IHC, 100X). (A) Normal control, revealing negative brown staining reaction of TNF-α in fibroblasts and pneumocytes. (B) BLM control, revealing sever brown staining denoting diffuse strong positive cytoplasmic reaction of TNF-α in fibroblasts and pneumocytes. (C) Flavocoxid, revealing negative brown staining reaction of TNF-α in fibroblasts and pneumocytes.

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