Effect of Aminoguanidine on Methotrexate-Induced Lung Toxicity in Male Wistar Rats

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Abstract

The present work was designed to investigate the effect of aminoguanidine (AMG) on methotrexate (MTX)-induced lung toxicity in rats. Forty adult male rats weighing 200–220 gm divided into four groups, 10 each were used in the study. The control group received normal saline. AMG-treated group: rats received 50 mg/kg/day AMG. Whereas the MTX-treated group: rats received 0.5 mg/kg MTX twice weekly, and AMG+MTX-treated group: rats were co-administered AMG and MTX at the same doses as in the previous groups. All groups received their treatment via the IP route. Additionally, the experiment duration was 4 weeks. MTX administration revealed a significant decrease in SOD, CAT, GSH, Nrf2, and Bcl-2 accompanied by a significant increase in MDA, NO, IL-6, IL-1β, NF-κB, caspase-3, and Bax compared to the control group. Moreover, there were histological abnormalities in the lung tissue. However, the AMG+MTX-treated group produced a significant increase in SOD, CAT, GSH, Nrf2, and Bcl-2, and a significant decrease in MDA, NO, IL-6, IL-1β, NF-κB, caspase-3, and Bax compared to the MTX-treated group. Besides, the histological results strongly supported our biochemical findings. The present study demonstrated the protective effect of AMG against MTX-induced lung toxicity in rats through its antioxidant, anti-inflammatory, and anti-apoptotic actions.

Keywords: Methotrexate, Aminoguanidine, Lung toxicity, Oxidative stress, Apoptosis.

1. Introduction

Methotrexate (MTX) is an anti-proliferative drug that prevents folate metabolism by inhibiting dihydrofolate reductase. MTX is frequently used to treat autoimmune illnesses like psoriasis and rheumatoid arthritis (Hagner and Joerger, 2010; Valerio et al., 2021). It was initially used as an antineoplastic medication to treat various cancers, such as leukemia, breast, ovary, and brain cancers (Zaki et al., 2021). Because of its extreme toxicity, MTX’s therapeutic use is typically constrained (Zhu et al., 2014). The most significant life-threatening side effects include hematopoietic suppression, hepatotoxicity, and pulmonary toxicity. Long-term, low-dose MTX use increases the risk of pulmonary damage. Additionally, it could result in pulmonary toxicity when used intravenously and in high doses (Kalemei et al., 2018).
In order to pinpoint the mechanism of MTX-induced lung injury, a number of mechanisms have been investigated. One of these mechanisms is oxidative stress. MTX suppresses the antioxidant defense enzymes, lowers glutathione levels, and induces lipid peroxidation (Arpag et al., 2018). Reactive oxygen species (ROS), which cause parenchymal lung damage and interstitial alveolar fibrosis are produced more readily when antioxidant defenses are compromised (Mohamed et al., 2019). The MTX's inflammatory reaction is another potential mechanism of MTX-induced lung injury. The inflammatory response markers interleukin 1β (IL-1β) and tumor necrosis factor-α are both enhanced by MTX (Zaki et al., 2021). Likewise, MTX enhances apoptosis and reduces cell regeneration due to increased ROS production (Ohbayashi et al., 2010). Hence, compounds having combined anti-inflammatory and antioxidant effects would be beneficial in the prevention of MTX-induced lung toxicity.

Aminoguanidine (AMG), a substance that shares structural similarities with L-arginine (the nitric oxide substrate), is a specific inhibitor of inducible nitric oxide synthase (iNOS), which is required for the process of NO release. AMG substantially lowers inflammation and nitrosative stress by suppressing iNOS. AMG's antioxidant benefits are based on its ability to drastically lower xanthine oxidase activity and scavenge peroxyl and hydroxyl radicals. Data further indicate that AMG greatly suppresses the nuclear factor-κB (NF-κB) signaling pathway, which dramatically lowers the generation of inflammatory mediators. Research in vivo and in vitro revealed that AMG possesses considerable anti-inflammatory and antioxidant capabilities (Kostic et al., 2022).

The beneficial effects of AMG on the respiratory system have received little attention. Accordingly, we conducted this study to explore the potential protective role of AMG against MTX-induced lung toxicity in a rat model.

2. Materials and methods

2.1. Drugs and Chemicals

Aminoguanidine was provided by Merck (Germany). Methotrexate was purchased from Sigma Aldrich Co. (USA). Normal saline (0.9% NaCl) was taken from Bio-diagnostic Co. (Egypt).

Interleukin 6 (IL-6) measurement kit was purchased from Elabscience Biotechnology Inc. (USA).

Cusabio Technology LLC (USA) provided Kits for interleukin 1β (IL-1β), nuclear factor-κB (NF-κB), the nuclear factor erythroid 2–related factor 2 (Nrf2), B-cell lymphoma 2 (Bcl-2), and hydroxyproline. Bcl-2 Associated X-protein (Bax) was procured from Abcam Co. (USA). Caspase-3 was obtained from BioVision Inc. (USA).

Bio-diagnostic Company (Egypt) provided kits for the estimation of superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), malondialdehyde (MDA), and nitric oxide (NO). Analytical-grade chemicals were used.

2.2. Animals

The experiment involved 40 adult male Wistar rats that weighed 200-220g which were procured from the animal house at the Faculty of Medicine at Sohag University in Egypt. They were housed in typical cages with a temperature of (23±2°C), and normal light and dark cycles. Conventional laboratory feed and water were available for all rats. Rats were acclimatized for a week preceding the study. According to the authorization of the Institutional Animal Care and Use Committee of Sohag University, Faculty of Medicine, Egypt (Approval No. Sohag-5-5-2023-02), all experimental techniques were undertaken.

2.3. Experimental design

Animals were randomly allocated into four groups each consisting of 10 rats as follows:

Control group: rats were given normal saline intraperitoneally daily (IP) for 4 weeks.

AMG-treated group: rats were given AMG (50 mg/kg/day IP) (de Rezende et al., 2000) for 4 weeks.

MTX-treated group: rats were given MTX (0.5 mg/kg IP) twice weekly for 4 weeks (Mohamed et al., 2019).

AMG+MTX-treated group: rats of this group were given AMG (50 mg/kg/day IP) for 4 weeks and received MTX (0.5 mg/kg IP) twice weekly for 4 weeks.

2.4. Sample collection

Rats from all groups were given light ether anesthesia 24 hours following the last treatment. Then, to separate tissue samples, animals were sacrificed by cervical dislocation. The lung tissue
of every animal was precisely separated, cleaned in ice-cold saline (0.9% NaCl), and dried on filter papers. Then, by using a homogenizer, part of the lung was weighed and homogenized in phosphate-buffered saline (pH 7.4). In order to further examine the oxidative stress, inflammatory, apoptotic biomarkers, and hydroxyproline, the homogenate obtained was centrifuged at 4000 rpm for 15 min at 4°C. After that, the supernatant was gathered and held at -80. The other part was preserved in 10% formalin for further histological analysis.

2.5. Biochemical analysis

2.5.1. Estimation of oxidant/antioxidant biomarkers

Superoxide dismutase was analyzed consistent with the method of Nishikimi et al. (1972). The data were depicted as U/g tissue. CAT was measured using the method of Aebi (1984). The data were reported as U/g tissue. GSH was assayed according to Beutler et al. (1963) method. GSH level was stated as mg/g tissue. Lipid peroxidation was assayed by the measurement of MDA levels according to Ohkawa et al. (1979) approach. Values were presented as nmol/g tissue. NO levels were measured using Montgomery and Dymock (1961) technique. The level of NO was expressed in μmol/g tissue. A colorimetric approach was used to estimate each parameter.

2.5.2. Estimation of Nrf2

Nuclear factor erythroid 2–related factor 2 was measured using enzyme-linked immunosorbent assay (ELISA) consistent with the manufacturer's conventions. It was demonstrated as ng/g tissue.

2.5.3. Estimation of inflammatory biomarkers

Interleukin 6, IL-1β, and NF-κB were determined using ELISA according to the manufacturer's guidelines. IL-6 and NF-κB were expressed as pg/g tissue, while IL-1β was reported as ng/g tissue.

2.5.4. Estimation of apoptotic biomarkers

The levels of the pro-apoptotic caspase-3 and Bax and the anti-apoptotic Bcl-2 were assessed by ELISA using the manufacturer's recommended procedure. Caspase-3 and Bcl-2 were reported as ng/g tissue. Bax was stated as pg/g tissue.

2.5.5. Estimation of hydroxyproline

Hydroxyproline concentration is used to quantify the total collagen content and has been used to explore the level and intensity of fibrosis. Hydroxyproline was stated as ng/g tissue.

2.6. Histopathological studies

Lung sections were taken from different groups and preserved for 24 hours in 10% formalin. After alcohol dehydration and paraffin embedding, tissues were cut to a thickness of 5 μm, stained with hematoxylin and eosin (H&E), and examined under the light microscope (Olympus, Tokyo, Japan) to check histological details.

2.7. Statistical analysis of data

Data were presented as mean ± SE. One-way analysis of variance (ANOVA) was performed to analyze the data using the statistical package for the social sciences (SPSS), version 25.0, SPSS Inc., Chicago, IL. Using Tukey's post hoc test, the group means were compared. P<0.05 was deemed significant.

3. Results

3.1. Effect of AMG on oxidant/antioxidant biomarkers

Methotrexate administration significantly (P<0.05) reduced lung SOD, CAT, and GSH levels and significantly (P<0.05) raised lung MDA and NO levels compared to the control group. Besides, AMG treatment (AMG+MTX-treated group) significantly (P<0.05) raised lung SOD, CAT, and GSH levels and significantly attenuated (P<0.05) oxidative damage in the lung (as indicated by decreasing MDA and NO levels) compared to the MTX-treated group (Table 1).

3.2. Effect of AMG on Nrf2

As shown in Fig. 1, MTX administration significantly (P<0.05) reduced lung Nrf2 level compared to the control group. AMG treatment (AMG+MTX-treated group) showed a significant (P<0.05) increase in lung Nrf2 level compared to the MTX-treated group.

3.3. Effect of AMG on inflammatory biomarkers

There was a significant (P<0.05) increase in the lung inflammatory biomarkers (IL-6, IL-1β, and NF-κB) in the MTX-treated group compared to the
Table 1. Effect of intraperitoneal AMG (50 mg/kg/day) on oxidant/antioxidant biomarkers in MTX-induced lung toxicity in rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>AMG</th>
<th>MTX</th>
<th>AMG+MTX</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD (U/g)</td>
<td>391.7±15.7</td>
<td>394.3±13.1</td>
<td>166.1±10.0*</td>
<td>354.2±20.3#</td>
</tr>
<tr>
<td>CAT (U/g)</td>
<td>505.4±18.1</td>
<td>506.0±20.7</td>
<td>230.3±18.5*</td>
<td>498.7±18.8#</td>
</tr>
<tr>
<td>GSH (mg/g)</td>
<td>105.8±4.5</td>
<td>104.9±4.3</td>
<td>41.8±2.9*</td>
<td>97.8±3.7#</td>
</tr>
<tr>
<td>MDA (nmol/g)</td>
<td>45.8±2.7</td>
<td>44.9±2.9</td>
<td>111.7±7.4*</td>
<td>64.8±4.0#</td>
</tr>
<tr>
<td>NO (μmol/g)</td>
<td>72.8±2.5</td>
<td>70.4±2.8</td>
<td>257.1±7.9*</td>
<td>93.0±4.1#</td>
</tr>
</tbody>
</table>

Mean±SE of 10 observations. AMG=Aminoguanidine, MTX=Methotrexate, SOD=Superoxide dismutase, CAT=Catalase, GSH=Reduced glutathione, MDA= Malondialdehyde, NO= Nitric oxide.

*Significant vs. control group (P< 0.05)  # Significant vs. MTX group (P< 0.05)

control group. Furthermore, the AMG+MTX-treated group showed a significant (P<0.05) decrease in the aforementioned inflammatory biomarkers in the lung compared to the MTX-treated group (Fig. 2).

3.4. Effect of AMG on apoptotic biomarkers

Methotrexate administration showed an apoptotic effect which is demonstrated by a significant (P<0.05) increase in the pro-apoptotic caspase-3 and Bax levels along with a significant decrease in the anti-apoptotic Bcl-2 level in the MTX-treated group compared to the control group. In contrast, the AMG+MTX-treated group showed a significant (P<0.05) decrease in the caspase-3 and Bax levels, and a significant elevation in the Bcl-2 level compared to the MTX-treated group (Fig. 3).

3.5. Effect of AMG on hydroxyproline

Lung hydroxyproline level showed a significant (P<0.05) increase in the MTX-treated group compared to the control group. However, the AMG+MTX-treated group resulted in a significant (P<0.05) decrease in the lung hydroxyproline level compared to the MTX-treated group (Fig. 4).
Figure 2. Effect of intraperitoneal AMG (50 mg/kg/day) on inflammatory biomarkers in MTX-induced lung toxicity in rats. Mean±SE of 10 observations. (A) IL-6= Interleukin 6, (B) IL-1β= Interleukin 1β, (C) NF-κB= Nuclear factor-κB. *Significant vs. control group (P< 0.05) # Significant vs. MTX group (P< 0.05)
Figure 3. Effect of intraperitoneal AMG (50 mg/kg/day) on apoptotic biomarkers in MTX-induced lung toxicity in rats. Mean±SE of 10 observations. (A) Caspase-3, (B) Bax= Bcl-2 Associated X-protein, (C) Bcl-2 = B-cell lymphoma 2.

*Significant vs. control group (P < 0.05)  
# Significant vs. MTX group (P < 0.05)
3.6. Histologic findings of lung tissue

Histological examination of the lung tissue of the control group showed normal histological architecture (Fig. 5A). Likewise, the AMG-treated group showed normal histological structure as those found in the control group (Fig. 5B).

The lung of the MTX-treated group showed remarkable thickening in the alveolar septum, which is made by numerous layers of cells. Congested blood vessels with the presence of extravasation of red blood cells. Moreover, inflammatory cell infiltrates were also observed (Fig. 6A). However, the AMG+MTX-treated group showed marked attenuation of the histopathological lesions observed in the MTX-treated group apart from some inflammatory cell infiltrates (Fig. 6B).

Figure 4. Effect of intraperitoneal AMG (50 mg/kg/day) on hydroxyproline in MTX -induced lung toxicity in rats. Mean±SE of 10 observations. *Significant vs. control group (P< 0.05)  ‡Significant vs. MTX group (P< 0.05)

Figure 5. (A) Photomicrograph of a section in the lung of the control group showing normal histological architecture (H&E x400). (B) Photomicrograph of a section in the lung of the AMG-treated group showing normal histological architecture as those found in the control group (H&E x400).
Figure 6. (A) Photomicrograph of a section in the lung of the MTX-treated group showing remarkable thickening in the alveolar septum (yellow arrow), congested blood vessels with the presence of extravasation of red blood cells (green arrow), inflammatory cell infiltrates (red arrow) (H&E x200). (B) Photomicrograph of a section in the lung of AMG+MTX-treated group showing normal histological architecture except some inflammatory cell infiltrates (red arrow) (H&E x200).

4. Discussion

The multiorgan damage brought on by cytotoxic medications like MTX is a big worry for patients taking them. The lung is one of the organs affected by MTX, and its dysfunction increases the risk of MTX-associated morbidity and mortality (Ohbayashi et al., 2010).

Three distinct pathways, including oxidative stress, inflammation, and apoptosis, were examined in order to better understand the mechanisms underlying MTX-induced lung toxicity and the protective effect of the AMG.

A number of factors contribute to lung ailment after MTX therapy. The primary factor is oxidative stress (Kurt et al., 2015). In the present study, MTX-induced oxidative lung tissue damage is expressed as a significant decrease in lung SOD, CAT, and GSH and a significant increase in MDA and the nitrosative stress marker NO levels in the MTX-treated group. Our results propose that the lung tissue of the MTX group had a disturbed oxidant-antioxidant equilibrium, favoring the oxidants. Identical outcomes were also mentioned by Kalemcı et al. (2015) who revealed decreased lung SOD and increased NO levels. Decreased lung SOD and CAT and increased MDA levels were also reported by Kaymak et al. (2022). In addition, Matouk et al. (2023) demonstrated that there is a decrease in lung SOD, and GSH and an increase in MDA activities in the MTX group. Oxidative damage and lipid peroxidation are both exacerbated by MTX, and the antioxidant system is disrupted by the extreme generation of ROS (Eki Nci-Akdemi et al., 2018).

On the other hand, after AMG treatment there was a significant increase in lung SOD, CAT, and GSH and a significant decrease in MDA and NO levels which signifies an improvement in the antioxidant defense mechanism. This agreed with Eroglu et al. (2008) who showed significantly higher levels of lung SOD, and CAT and significantly lower levels of MDA, NO. Furthermore, Saadat et al. (2019) revealed that serum and bronchoalveolar lavage fluid SOD, CAT, and total thiol content were increased, and MDA and total nitrite were decreased after AMG treatment. Additionally, elevated hepatic GSH and reduced NO levels were shown by Kostic et al. (2022). AMG can function as an antioxidant and reduce the severity of lipid peroxidation and protein changes caused by free radicals by binding aldehydes and other ROS. AMG may also have numerous other benefits, including the inhibition of inflammation and the lowering of iNOS expression (Eroglu et al., 2008; Chen et al., 2017; Beheshti et al., 2020).

Nuclear factor erythroid 2-related factor 2 (Nrf2), a transcriptional factor that is essential for cell survival, improves cellular resistance to oxidative damage. It also controls the expression of the genes for antioxidant enzymes, like SOD, and CAT. In the present study, our results demonstrated a
significant decline in lung Nrf2 levels in the MTX-treated group. This is in harmony with the results of Dar et al. (2021), and Matouk et al. (2023). In order to trigger the activation of antioxidant genes, Nrf2 binds to antioxidant response elements (ARE) after being activated and translocated to the nucleus (Ma, 2013). Evidence suggests that MTX inhibits Nrf2's translocation to the nucleus, which inactivates it (Kawami et al., 2022). In contrast, after AMG treatment there was a significant increase in lung Nrf2 levels. This is agreed with Afifi et al. (2021) who demonstrated increased hepatic and cerebellar Nrf2 expression in AMG-treated rats suggesting that AMG has an antioxidant action.

It has been proven that there is a crosstalk between the NF-κB and Nrf2. The lack of Nrf2 boosts the NF-κB expression thus increasing the inflammatory mediators generation. Additionally, NF-κB can influence the downstream target genes expression by controlling the transcription and activity of Nrf2 (Gao et al., 2022).

An important mechanism that has been previously found to be implicated in the MTX-induced inflammatory reaction by boosting the expression of the interleukins is the inflammatory pathway dependent on NF-κB transcription (Dar et al., 2021). In the present study, MTX mediated the inflammatory response in the lung through a significant increase in the levels of lung IL-6, IL-1β, and NF-κB. This corroborates earlier reports (Zaki et al., 2021; Kaymak et al., 2022; Matouk et al., 2023). NO is recognized as an inflammatory response mediator and regulator. Inflammatory stimuli cause the production of high levels of iNOS-derived NO, which then mediates the pro-inflammatory and damaging consequences. These consequences are mediated indirectly by a very rapid interaction of NO with ROS, which is synthesized by activated inflammatory cells resulting in the creation of the cytotoxic reactive nitrogen species peroxynitrite. NF-κB, a principal regulator of several genes implicated in inflammatory reactions, can also be activated by NO, which results in the marked production of cytokines that are pro-inflammatory such as IL-1, IL-8, IL-6, and tumor necrosis factor-α (TNF-α) (Al-Taher et al., 2020; Matouq et al., 2023).

Conversely, our study showed that after AMG treatment there was a significant reduction in lung IL-6, IL-1β, and NF-κB levels. These findings agreed with the previous studies (Li et al., 2008; Ma et al., 2020; Aleksandrov et al., 2022) which demonstrated a significant lowering of IL-6, IL-1β, and NF-κB levels concluding that the AMG treatment diminished the inflammatory reaction by lowering the pro-inflammatory cytokines in lung tissue. The AMG, therefore, has anti-inflammatory efficacy against the MTX-induced inflammatory response, according to our data. AMG has been reported to decrease DNA-binding NF-κB, which ultimately prevents the production of inflammatory cytokines, and prevents the dysregulation of proinflammatory cytokines expression involved in chronic inflammatory processes (Diaz et al., 2014).

Another major cause of lung damage in the MTX-treated group is apoptosis. Our results showed an apoptotic response mediated by elevating the pulmonary pro-apoptotic caspase-3, and Bax and lowering the anti-apoptotic Bcl-2 in the MTX-treated group. This concurred with Saygin et al. (2016) who showed a significant rise in lung caspase-3 expression in the MTX group. Likewise, Al-Taher et al. (2020) revealed high cardiac cytoplasmic and nuclear expression of caspase-3 in many cells. Elevated renal and hepatic Bax and lowered Bcl-2 in the MTX group were also mentioned by Dar et al. (2021). Apoptosis could be initiated either by extrinsic or intrinsic pathways (Kiraz et al., 2016). Cellular exposure to apoptosis can be controlled by the interaction between the pro-apoptotic Bax and the anti-apoptotic Bcl-2 (Abdel Moneim, 2016). MTX-induced apoptosis results from the initiation of mitochondrial stress which affects the depolarization of the mitochondrial membrane and causes caspase-3 activation, a key enzyme in apoptosis, and Bax/Bcl-2 release which activates the mitochondrial apoptotic pathway (Dar et al., 2021). Lipid peroxidation (increased MDA) can also trigger apoptosis, which harms cells and organs. Increased apoptosis causes excessive cytokine production and elevated ROS, which ultimately destroys lung tissue (Kurt et al., 2015).

On the contrary, our study revealed that AMG treatment causes a significant increase in lung Bcl2 and a significant decrease in lung Bax and caspase-3 levels. These findings are similar to Firouzjaie et al. (2014) who exhibited an increase in Bcl-2, and a decrease in Bax mRNA in the hippocampus of the streptozotocin-induced diabetic group after treatment with AMG. As well, marked amelioration with the decrease of hepatic and cerebellar caspase-3 expression in the AMG-treated
group was recorded by Afifi et al. (2021). It has been established that antioxidants, such as AMG, can prevent apoptotic cell death (Sun et al., 2010). AMG functions as an antioxidant in vivo by halting the production of ROS and lipid peroxidation in cells and tissues, hence preventing oxidant-induced apoptosis (Giardino et al., 1998).

Focusing on MTX-induced lung damage specifically, pulmonary fibrosis is the prominent manifestation (Fragoulis et al., 2019). In the present study, our results showed a significant increase in collagen content as represented by the elevation in lung hydroxyproline in the MTX-treated group. Our data are compatible with Ali et al. (2022) who demonstrated a significant increase in lung hydroxyproline in the MTX group. According to earlier research, oxidative stress and inflammatory damage are key factors in the development of fibrosis (Wynn, 2011).

Our study revealed that AMG treatment causes a significant decrease in lung hydroxyproline. These results are in accordance with the findings of Chen et al. (2009), Lanzetti et al. (2012) and Baraka and Guemei (2015). AMG inhibits the polymerization of collagen molecules by lowering advanced glycosylation end products in diabetes mellitus-related clinical situations and experimental models, as well as by lowering lysyl-oxidase, an oxidase implicated in the initial stages of collagen polymerization (de Rezende et al., 2000).

Inducible nitric oxide synthase inhibitors such as AMG have been shown to possess imperative cellular protective effects on lowering lipid peroxidation and total nitrite level. In addition, an earlier study reported their anti-inflammatory, antioxidant, and antiapoptotic capabilities (Anaegoudari et al., 2016).

In the present study, the MTX-treated group showed remarkable thickening in the alveolar septum, and congested blood vessels with the presence of extravasation of red blood cells, and inflammatory cell infiltrates. This is in harmony with Zaki et al. (2021) and Matouk et al. (2023). In contrast, AMG treatment revealed marked attenuation of MTX histopathological alterations apart from some inflammatory cell infiltrates which is compatible with the findings of Lanzetti et al. (2012) and Saadat et al. (2019).

**Conclusion**

According to the findings of the current study, AMG has antioxidant, anti-inflammatory, and anti-apoptotic actions that protect rats’ lungs from MTX-induced lung damage. SOD, CAT, GSH, and Nrf2 are all raised in AMG's antioxidant defenses against MTX-induced lung damage, while MDA and NO are decreased. The pro-inflammatory mediators (IL-6, IL-1β, and NF-B) are reduced as part of AMG's anti-inflammatory actions. Additionally, AMG anti-apoptotic actions boost Bcl-2 and decrease caspase-3, and Bax. Along with a reduction in lung hydroxyproline and normalization of the histopathological abnormalities.

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**Conflicts of Interest**

The authors state they do not have any conflicting interests.

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