Title:

Cadmium-induced lung injury is associated with oxidative stress, apoptosis and altered SIRT1 and Nrf2/HO-1 signaling; protective role of the melatonin agonist agomelatine

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Abstract

Cadmium (Cd) is a hazardous heavy metal extensively employed in manufacturing polyvinyl chloride, batteries, and other industries. Acute lung injury has been directly connected to Cd exposure. Agomelatine (AGM), a melatonin analog, is a drug licensed for treating severe depression. This study evaluated the effect of AGM against Cd-induced lung injury in rats. AGM was administered in a dose of 25 mg/kg/day orally, while $CdCl_2$ was injected intraperitoneal in a dose of 1.2 mg/kg to induce lung injury. Pre-treatment with AGM remarkably ameliorated Cd-induced lung histological abrasions. AGM decreased reactive oxygen species (ROS) production, lipid peroxidation, suppressed NDAPH oxidase, and boosted the antioxidants. AGM increased Nrf2, GCLC, HO-1, and TNXRD1 mRNA, as well as HO-1 activity and downregulated Keap1. AGM downregulated Bax and caspase-3 and upregulated Bcl-2, SIRT1 and FOXO3 expression levels in the lung. In conclusion, AGM has a protective effect against Cd-induced lung injury via its antioxidant and anti-apoptotic effects mediated via regulating Nrf2/HO-1 and SIRT1/FOXO3 signaling.

Keywords: Agomelatine; Cadmium; Oxidative stress.

Introduction

Cadmium (Cd), a hazardous heavy metal, has been extensively employed in manufacturing polyvinyl chloride, batteries, and others (Ashizawa et al., 2012, Fulcher and Gibb, 2014). Smoking causes cadmium oxide to be deposited inside the lung or absorbed into the systemic circulation. As a result, a smoker's body has a Cd level about three times greater than non-smokers (Pappas, 2011). Although cigarette smoke is the main cause of Cd toxicity in humans, Cd pollution of water, air, and food also negatively influences human health (Rani et al., 2014). Acute lung injury has

been directly connected to Cd exposure (Li et al., 2018). Studies showed that Cd promotes oxidative stress (OS) and inflammation, which harms the lungs and induces the release of pro-inflammatory mediators by lung epithelial cells and the breakdown of extracellular matrix (Larson-Casey et al., 2020). OS mediated via excess reactive oxygen species (ROS) is linked to Cd-induced tissue damage and cell death. Chronic exposure to Cd leads to an imbalance of oxidants and antioxidants since it increases ROS production and lowers antioxidant levels (Sarkar et al., 2013, Rani et al., 2014). OS in the lung includes NADPH oxidases (NOXs)-mediated ROS generation (Lee and Yang, 2012). Excess ROS provokes inflammation and both orchestrate cell death via apoptosis (Shore and Nguyen, 2008). Therefore, attenuation of these pathologic processes can effectively protect the lung against Cd-induced injury.

Several transcription factors (TFs) can influence antioxidants and control OS. The nuclear factor erythroid 2–related factor 2 (Nrf2) is a TF that controls several antioxidant genes, including heme oxygenase 1 (HO-1) by binding to the antioxidant response elements (ARE) within the nucleus and hence confers protection against OS and cell death (Satta et al., 2017, Hassanein et al., 2020). In addition, sirtuin 1 (SIRT1) is the most extensively studied sirtuin that plays a crucial part in histone deacetylation-mediated transcriptional regulation (Sauve et al., 2006). SIRT1 has a significant redox regulator and essential for the antioxidant system (Singh et al., 2018). Also, it is critical for DNA damage repair, apoptosis prevention, and OS resistance (Haigis and Guarente, 2006, Sayed et al., 2020). The O type superfamily of forkhead transcription factors known as forkhead box O (FOXO) participates in many cell functions. FOXO3 is essential for cell survival (Fasano et al., 2019) and can protect cells against OS (Reed et al., 2004). Several studies reported that up-regulation of SIRT1/FOXO3 in lung tissue has a key role in mitigating oxidative lung injury in several models (Zhang et al., 2015, Mahlooji et al., 2022).

Agomelatine (AGM), a melatonin analog, is a drug licensed for treating severe depression in adults. It is a nonselective agonist for melatonin receptors 1 and 2 (MT1 and MT2) and has antioxidant effects as revealed by several experimental models of disease (Yigitturk et al., 2017, Eraslan et al., 2020, Cankara et al., 2021). Acute lung damage is reported to be improved by melatonin (Zhang et al., 2016). In this study, we examined the protective role of AGM against Cd-induced lung injury pointing to the involvement of OS and apoptosis, and the role of Nrf2/HO-1 and SIRT1/FOXO3 signaling.

Materials and methods

Animals and treatments

The present investigation included 32 male rats (*Rattus norvegicus*) weighing 190-210 g. These animals were housed in standard cages (4 rats/cage) under a standardized environment (22 ± 1°C and 50-60% humidity) with a 12 h light/dark cycle and had free access to water and food. The experiment was approved by the research ethics Committee at Al-Azhar University (Approval no.: AZ-AS/PHREC/26/2023).

The rats were randomly divided into four groups (n=8) based on the various treatments. The 1st group (Control) received 0.5% carboxymethyl cellulose (CMC) orally for 14 days and the 2nd group (AGM) received 25 mg/kg/day of AGM (Sigma, USA) suspended in 0.5% CMC orally for 14 days (Aguiar et al., 2013, Yucetas et al., 2019). Both the 1st and 2nd groups received a single intraperitoneal (i.p.) injection of physiological saline on day 7. The 3rd group (Cd) received 0.5% CMC orally for 14 days and a single i.p. injection of 1.2 mg/kg $CdCl_2$ (Sigma, USA) dissolved in physiological saline on day 7 (de Lima et al., 2020). The 4th group (AGM/Cd) received 25 mg/kg

AGM orally for 14 days and a single i.p. injection of 1.2 mg/kg $CdCl_2$ on day 7. The dose volumes for oral and i.p. administration were 5 ml/kg and 1 ml/kg body weight, respectively.

The dose and route of administration of Cd in this study were selected based on a pilot study and previous investigations studied the toxicity of Cd (de Souza Predes et al., 2010, de Lima et al., 2020, Handan et al., 2020). We have conducted a pilot study and the use of 1.2 mg/kg did not cause mortality and resulted in blood Cd levels range between 1.36 and 1.75 µg/L which falls within the ranges reported in human populations exposed to Cd. For instance, Mortada *et al* have reported 0.8 − 4.5 µg/L blood Cd levels in an Egyptian population (Mortada et al., 2002).

The rats were anesthetized with ketamine (100 mg/kg i.p.) 24 h following the last treatment and after scarification, the lungs were removed and separated into several parts. For further histological and immunohistochemistry (IHC) examinations, one of these sections was collected on 10% neutral buffered formalin (NBF), others on RNAlater and kept at -80°C, and the remaining part was homogenized in Tris-HCl buffer (10% w/v), centrifuged, and the resultant supernatant was stored at -80°C.

Histopathology and immunohistochemistry (IHC)

The lung tissue was fixed in 10% NBF for 24 h, then cleaned, embedded in paraffin, sectioned (4-5 µm), and stained with H&E stain (Suvarna et al., 2013). Under the light microscope, the sections were inspected blindly. Other paraffin sections were rehydrated with ethanol, dewaxed with xylene, heated in citrate buffer, and then blocked with 1% BSA. The sections were incubated with anti-Bax, anti-Caspase-3, and anti-Bcl-2 primary antibodies (Biospes, China) overnight at 4 °C followed by washing and incubation with secondary antibody for 1 h. The color was developed using DAB in H_2O_2 and the sections were counterstained with hematoxylin. A digital camera and

an Olympus light microscope were used to capture images and intensity of the resulting color was evaluated using ImageJ.

Biochemical assays

ROS was determined by combining the samples with H_2DCF-DA (Sigma, USA) as previously reported (Hozayen et al., 2019). Malondialdehyde (MDA) (Mihara and Uchiyama, 1978), reduced glutathione (GSH) (Ellman, 1959) and activities of superoxide dismutase (SOD) (Marklund and Marklund, 1974), glutathione-s-transferase (GST) (Keen et al., 1976), HO-1 (Abraham et al., 1985), and GPx (Lawrence et al., 1976) were determined in the supernatant of the lung homogenates. ELISA kit (ELabscience, China) was utilized to determine NADPH oxidase following the provided instructions.

qRT-PCR

qRT-PCR was used to determine the changes in Nrf2, Keap1, glutamate-cysteine ligase catalytic subunit (GCLC), HO-1, thioredoxin reductase 1 (TNXRD1), NOX-2, SIRT1, and FOXO-3 mRNA levels. Using Trizol reagent (Invitrogen), total RNA was extracted from lung tissue and ThermoFisher Scientific reverse transcription kit was used to synthesize cDNA. SYBR Green Master Mix and primers listed in Table 1 were used to amplify cDNA and the data were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The results were normalized to GAPDH.

Statistical analysis

The data are presented as mean ± standard error of the mean (SEM). To compare the differences between the groups, one-way ANOVA followed by Tukey's test were performed using GraphPad Prism 8.0. A P value <0.05 was considered significant.

Results

AGM mitigates lung histopathological alterations induced by Cd

While control (Fig. 1A) and AGM-treated rats (Fig. 1B) showed normal histological structures, significant histopathological alterations were recorded in the lung of Cd-treated rats (Fig. 1C-E & Table 2). These included alveolar emphysema, interstitial hemorrhage, atelectasis, damage of the bronchial epithelium, hyperplasia of peribronchial lymphoid tissue, smooth muscle fibers fragmentation, and infiltration of the lamina propria with lymphocytes and interstitial connective tissue (Fig. 1C-E). Pre-treatment with AGM remarkably ameliorated Cd-induced lung histological abrasions with slight alveolar emphysema, foamy appearance, and hyperplasia of peribronchial lymphoid tissue could be seen (Fig. 1F & Table 2).

AGM attenuates Cd-induced pulmonary OS

Cd significantly increased ROS (Fig. 2A) production and dramatically increased NOX-2 mRNA (Fig. 2B) as well as NADPH-oxidase (Fig. 2C) in the lung compared to control group ($P<0.001$). The administration of Cd significantly increased lipid peroxidation marker MDA (Fig. 2D), and depleted enzymatic and nonenzymatic antioxidants, including GSH (Fig. 3A), SOD (Fig. 3B), GPx (Fig. 3C), and GST (Fig. 3D). AGM decreased ROS and MDA, downregulated NOX-2 mRNA and NADPH-oxidase enzymatic activity and restored GSH, SOD, GPx, and GST in Cd-administered rats.

AGM attenuates Cd-induced apoptosis in the lung

IHC we employed to study the impact of AGM on Cd-induced lung apoptosis (Fig. 4). The results revealed that Cd significantly downregulated Bcl-2 (Fig. 4A,B) along with increased Bax (Fig.

4A,C) and caspase-3 (Fig. 4A,D) levels compared to control group ($P<0.001$). AGM treated group showed significantly lower level of Bax and caspase-3 and higher levels of Bcl-2 protein.

AGM modulates Keap1/Nrf2/HO-1 signaling in the lung of Cd-administered rats

Cd markedly increased Keap1 mRNA (Fig. 5A), and decreased Nrf2 (Fig. 5B), GCLC (Fig. 5C), HO-1 (Fig. 5D), and TNXRD1 (Fig. 5F) mRNA levels as well as HO-1 activity (Fig. 5E) in the lung as compared with control group ($P<0.001$). AGM administration counterbalanced these effects by increasing Nrf2, GCLC, HO-1 and TNXRD1 mRNA, as well as HO-1 activity while it downregulated Keap1 mRNA significantly relative to that of Cd-intoxicated rats.

AGM upregulates SIRT1 and FOXO3 in the lung of Cd-administered rats

Cd significantly decreased SIRT1 (Fig. 6A) and FOXO3 (Fig. 6B) mRNA levels in the lung of rats. In contrast. AGM potently upregulated SIRT1 and FOXO3 mRNA in the lung of Cd-administered rats.

Discussion

Cadmium (Cd) is an extremely toxic heavy metal that has a detrimental effect on the body organs, including the lung, as it can produce acute and chronic lung injury (Bernard, 2008, Rafati Rahimzadeh et al., 2017). This study investigated the possible protective role of AGM against Cd-induced lung toxicity. AGM attenuated Cd-induced lung injury by regulating Nrf2/HO-1 and SIRT1/FOXO3 signaling and preventing OS and apoptosis.

Cd administration caused lung injury as shown by the tissue alterations such as damage to bronchial epithelium and alveoli, infiltration of inflammatory cells, hemorrhage, emphysema, and others. These findings showed the deleterious impact of Cd on the lung, effects that could be

explained by the surplus ROS generation which damage the cell macromolecules. The pathologies of Cd toxicity have been linked to increased ROS and OS (Rashid et al., 2013). Cd does not generate free radicals through redox reactions but generates H_2O_2 and NO and hydroxyl radicals indirectly (Ikediobi et al., 2004, Cuypers et al., 2010). It can increase ROS generation via Fenton-type mechanisms due to its ability to promote free iron release, leading to OS (Casalino et al., 1997). OS is an imbalance between oxidants and antioxidants, leading to cellular disruption mediated via oxidation of the cell macromolecules (Sies, 1997). Cellular antioxidants act as the body's first line of defense against oxidative injury (Saha et al., 2016). OS in the lung of rats received Cd was evidenced by elevated ROS and MDA, upregulated NOX2 and declined antioxidants. NOXs are membrane-bound enzymes that function to generate ROS and NOX2 generates superoxide radicals as its product (Bedard and Krause, 2007) which can activate several kinases, cytokines and TFs (Ma et al., 2017). The role of NOX-mediated ROS production in Cd toxicity and vascular damage was reported (Pinheiro Júnior et al., 2020) and its role in pulmonary fibrosis and airway and pulmonary diseases has been previously reviewed (Lee and Yang, 2012, Kato and Hecker, 2020). Owing to the role of OS in Cd toxicity, attenuation of excess ROS would be effective in protecting the lung against injury.

AGM attenuated lung injury, decreased ROS and MDA, downregulated NADPH oxidase and boosted antioxidants in the lung of Cd-administered rats. Thus, it is noteworthy assuming that the protective effect of AGM was mediated via attenuation of OS. In accordance, studies have shown that the role of AGM in protecting the lung against methotrexate toxicity has been associated with suppressed OS (Kamel et al., 2022). AGM prevented histopathological alterations induced by lipopolysaccharide (LPS) in the lung of rats by suppressing cytokines (Köse et al., 2021). The antioxidant efficacy of AGM is supported by studies involving disease models of nephro- and

198 neurotoxicity (Cankara et al., 2021, Mahmoud et al., 2021). Similar to melatonin, AGM can
199 protect the cells against injury by scavenging ROS and maintaining redox homeostasis through its
200 binding with melatonergic receptors (Paulis et al., 2012, Galano and Reiter, 2018). This protective
201 role was supported by the investigation of apoptosis markers that showed the ability of AGM to
202 prevent apoptotic cell death. Besides the surplus ROS, Cd upregulated pro-apoptotic proteins and
203 decreased Bcl-2 in the lung of rats demonstrating cell death via apoptosis. ROS activate Bax and
204 both can deteriorate mitochondrial membrane potential (MMP), resulting in the outflow of
205 cytochrome c into the cytosol to activate caspase-3 (Shi et al., 2003). Cleavage of DNA and
206 structural and cell cycle proteins by activated caspase-3 lead to cell death (Thomsen et al., 2013).
207 Hence, suppression of surplus ROS by AGM mediated its protective role against Cd-induced
208 apoptosis in the lung of rats.

209 To further explore the beneficial role of AGM against Cd-induced lung injury, we determined its
210 effect on Nrf2/HO-1 signaling, SIRT1 and FOXO3. Nrf2 is a TF widely expressed in several
211 organs and tissues, including the lung. The Keap1 repressor protein controls Nrf2 in the cytoplasm
212 through the ubiquitin-proteasome pathway degradation (Furukawa and Xiong, 2005). In response
213 to elevated ROS, Nrf2 dissociates from Keap1, relocates to the nucleus, and transactivates a
214 number of antioxidants, including HO-1 that exerts antioxidant and anti-inflammatory activities
215 (Satta et al., 2017). HO-1 catalyzes the degradation of heme into the radical scavenger bilirubin
216 (Siow et al., 1999), resulting in attenuation of OS and confers protection against cell death.
217 TXNRD1 plays a critical role in redox homeostasis by reducing and activating the oxidoreductase
218 thioredoxin. The latter contains a dithiol-disulfide active site and thereby can reduce the oxidized
219 cysteine residues on proteins subjected to oxidation by ROS. It binds ROS and therefore prevent
220 these oxidizing species from harming the cells via OS and can also inhibit apoptosis signal

regulating kinase 1 (Cadenas et al., 2010). Nrf2 also increases the transcription of GCLC, a rate-limiting enzyme in the biosynthesis of GSH that protects against OS (Lu, 2013). Cd markedly upregulated gene expression of Keap1, and decreased Nrf2, GCLC, HO-1, and TNXRD1. Cd is known to downregulate Nrf2/HO-1 signaling and decrease the antioxidant levels in different organs (Alam et al., 2021, Bakr et al., 2022). Interestingly, AGM counteracted these effects and enhanced Nrf2, GCLC, HO-1, and TNXRD1, along with the downregulation of Keap1. Upregulation of Nrf2 signaling coincided with the suppressed ROS and OS and increased GSH and antioxidant enzymes. These findings pinpointed the role of Nrf2/HO-1 and Nrf2/TNXRD1 pathways in mediating the antioxidant activity of AGM.

Moreover, through the deacetylation of substrate proteins under OS, SIRT1 significantly safeguards the cell and promotes cell survival (Guarente, 2013, Sayed et al., 2020). SIRT1 targets FOXOs which have a role in many physiological and cellular processes, including cell proliferation, apoptosis, and response to ROS (Tzivion et al., 2011). FOXOs play a critical role in cell survival by transactivating antioxidant enzymes like SOD (Tzivion et al., 2011). SIRT1 inhibits the transcription of genes involved in apoptosis while increasing the expression of FOXO target genes involved in stress tolerance (Greer and Brunet, 2005). It deacetylates PPARγ coactivator 1 alpha that promotes the transcription of SOD and catalase (Olmos et al., 2013), and p53 resulting in inhibiting its OS-induced apoptotic cell death (Vaziri et al., 2001). Our results indicated that Cd downregulated SIRT1 and FOXO3, effects that were prevented by AGM. We have previously showed the ability of AGM to upregulate SIRT1 and prevent gentamicin nephrotoxicity in rats (Mahmoud et al., 2021). Thus, upregulation of SIRT1 contributed to AGM-mediated suppression of OS and apoptosis provoked by Cd. However, the lack of protein quantification of SIRT1 and FOXO3 could be considered a limitation of this study.

Conclusion

Our study provides new information on the protective effect of AGM against Cd-induced lung injury. AGM mitigated tissue injury, downregulated NADPH oxidase and ROS generation, decreased lipid peroxidation, and prevented apoptosis. These effects were linked to enhanced Nrf2, GCLC, HO-1, TNXRD1, and SIRT1. Taken together, AGM has a protective effect against Cd-induced lung injury via its antioxidant and anti-apoptotic effects. It is effective in conferring protection against Cd lung intoxication, pending further investigations to explore other mechanism(s) of action.

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Data availability

The authors confirm that the data supporting the findings of this study are available within the article.

Disclosure statement

The authors declare that no conflict of interest exists.

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Tables:

Table 1. Primers used for qRT-PCR.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
Keap1	TCAGCTAGAGGCGTACTGGA	TTCGGTTACCATCCTGCGAG
Nrf2	ATTGCTGTCCATCTCTGTCAG	GCTATTTTCCATTCCCGAGTTAC
HO-1	TGCTTGTTTCGCTCTATCTCC	CTTTCAGAAGGGTCAGGTGTC
GCLC	GTTGTTACTGAATGGCGGCG	CGGCGTTTCCTCATGTTGTC
TXNRD-1	TTGGAGTATGGCTGCTGTGG	TGCAGCCTTCTTCTACCTGC
NOX-2	CTGGGCTGTGAATGAGGGAC	TGTGAATGGCCGTGTGAAGT
SIRT1	CGGTCTGTCAGCATCATCTTCC	CGCCTTATCCTCTAGTTCCTGTG
FOXO3	GCCTCATCTCAAAGCTGGGT	AGTTCTGCTCCACGGGAAAG
GAPDH	TGCTGGTGCTGAGTATGTCG	TTGAGAGCAATGCCAGCC

426 Table 2. Scoring of the histopathological lesions.

	Control	AGM	Cd	AGM/Cd
Alveolar emphysema	-	-	+++	++
Interstitial hemorrhage	-	-	+++	-
Atelectasis	-	-	++	-
Damage of the bronchial epithelium	-	-	+++	-
Hyperplasia of peribronchial lymphoid tissue	-	-	+++	+
Infiltration of the lamina propria with lymphocytes and interstitial connective tissue	-	-	++	-
Smooth muscle fibers fragmentation	-	-	++	-

427

Figure legends

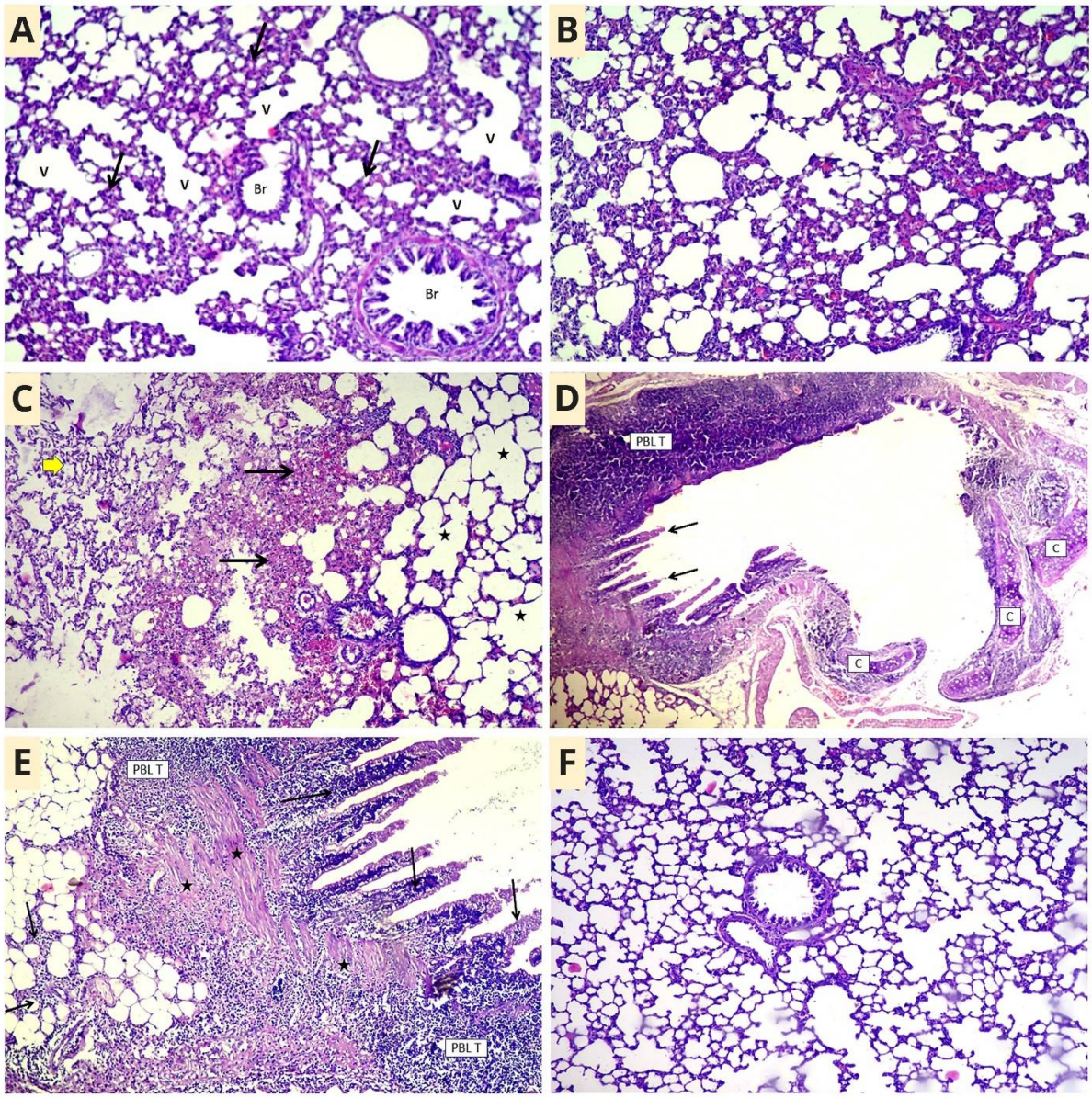


Fig. 1. AGM mitigated lung histopathological alterations induced by Cd. H&E-stained sections in lung of (A) Control and (B) AGM-supplemented rats showing the normal appearance of alveoli (v), interstitial tissue (arrow) and bronchioles (Br), (C-E) The lungs of Cd-intoxicated rats showed alveolar emphysema (star), interstitial hemorrhage (black arrow), slight atelectasis (thick yellow arrow) [C], damage of bronchial epithelium (arrow) and hyperplasia of peribronchial lymphoid

tissue (PBLT) [D], damage of bronchial epithelium and infiltration of the lamina propria with lymphocytes, interstitial connective tissue (arrow), hyperplasia of PBLT and smooth muscle fibers fragmentation (star) [E], and (F) Cd-administered rats treated with AGM showing noticeable improvement of the emphysema with an absence of interstitial hemorrhage. (Scale bar = 200 µm).

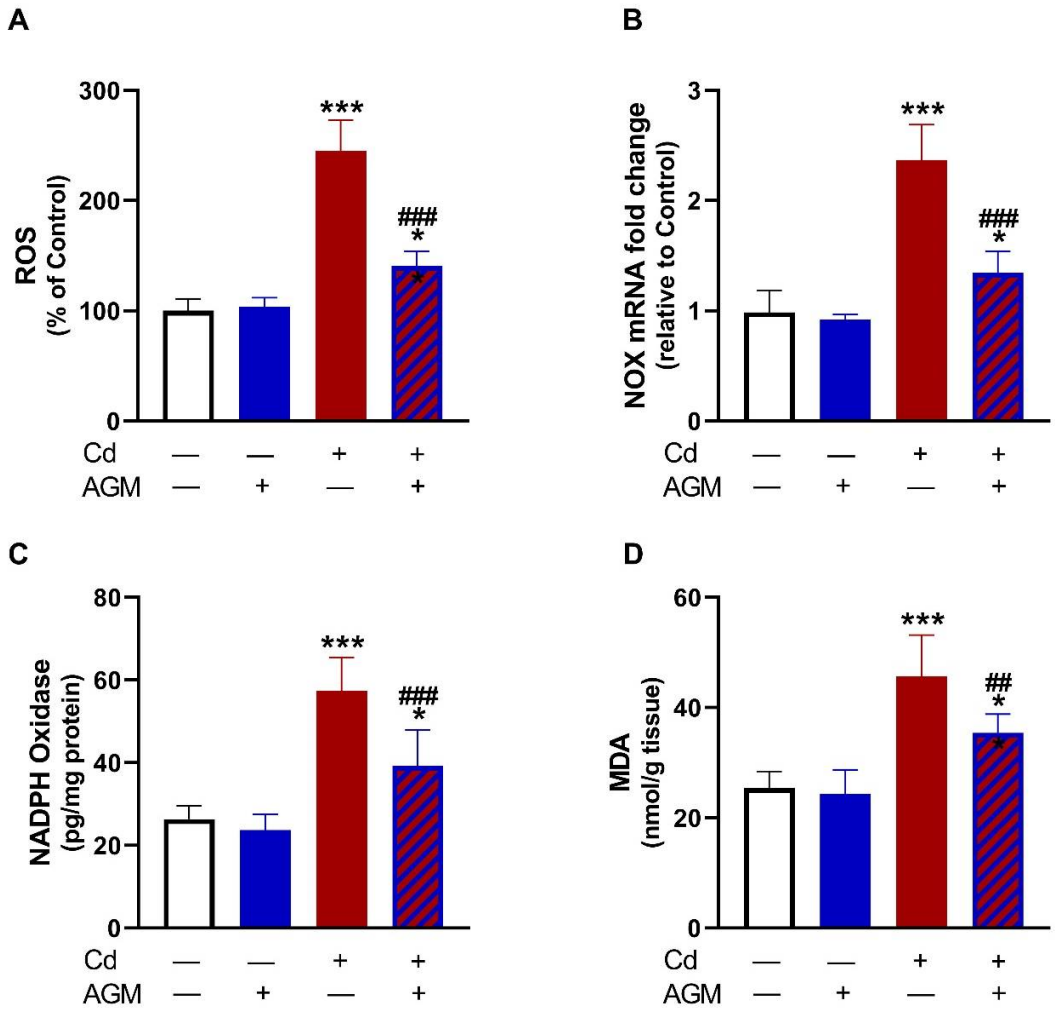
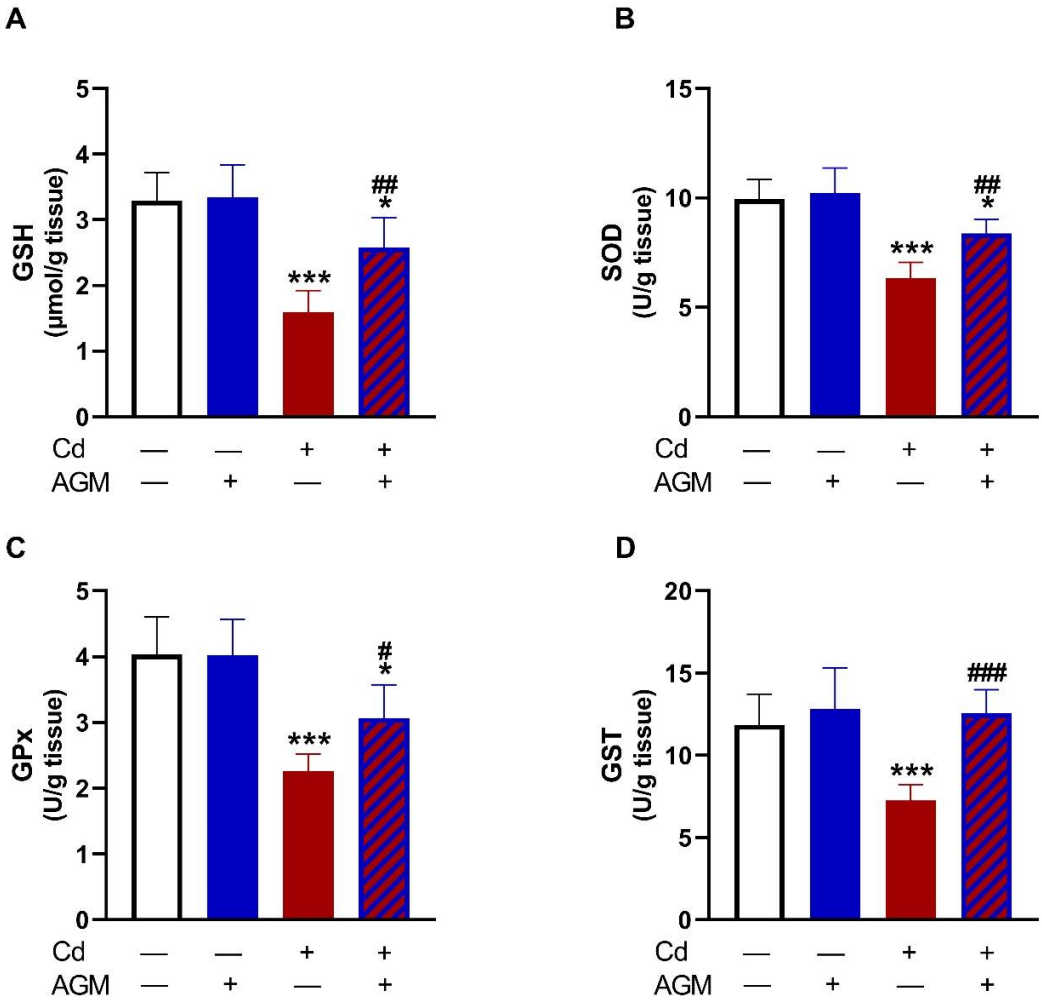


Fig. 2. AGM decreased ROS (A), NOX-2 mRNA (B), NADPH-oxidase (C) and MDA (D) in the lung of Cd-administered rats. Data are Mean ± SEM, (n = 8). *P<0.05, **P<0.01 and ***P<0.001 vs Control. $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs Cd.



444

445 Fig. 3. AGM increased GSH (A), SOD (B), GPx (C) and GST (D) in the lung of Cd-administered

446 rats. Data are Mean ± SEM, (n = 8). *P<0.05 and ***P<0.001 vs Control. $^{\#}$P<0.05, $^{\#\#}$P<0.01 and

447 $^{\#\#\#}$P<0.001 vs Cd.

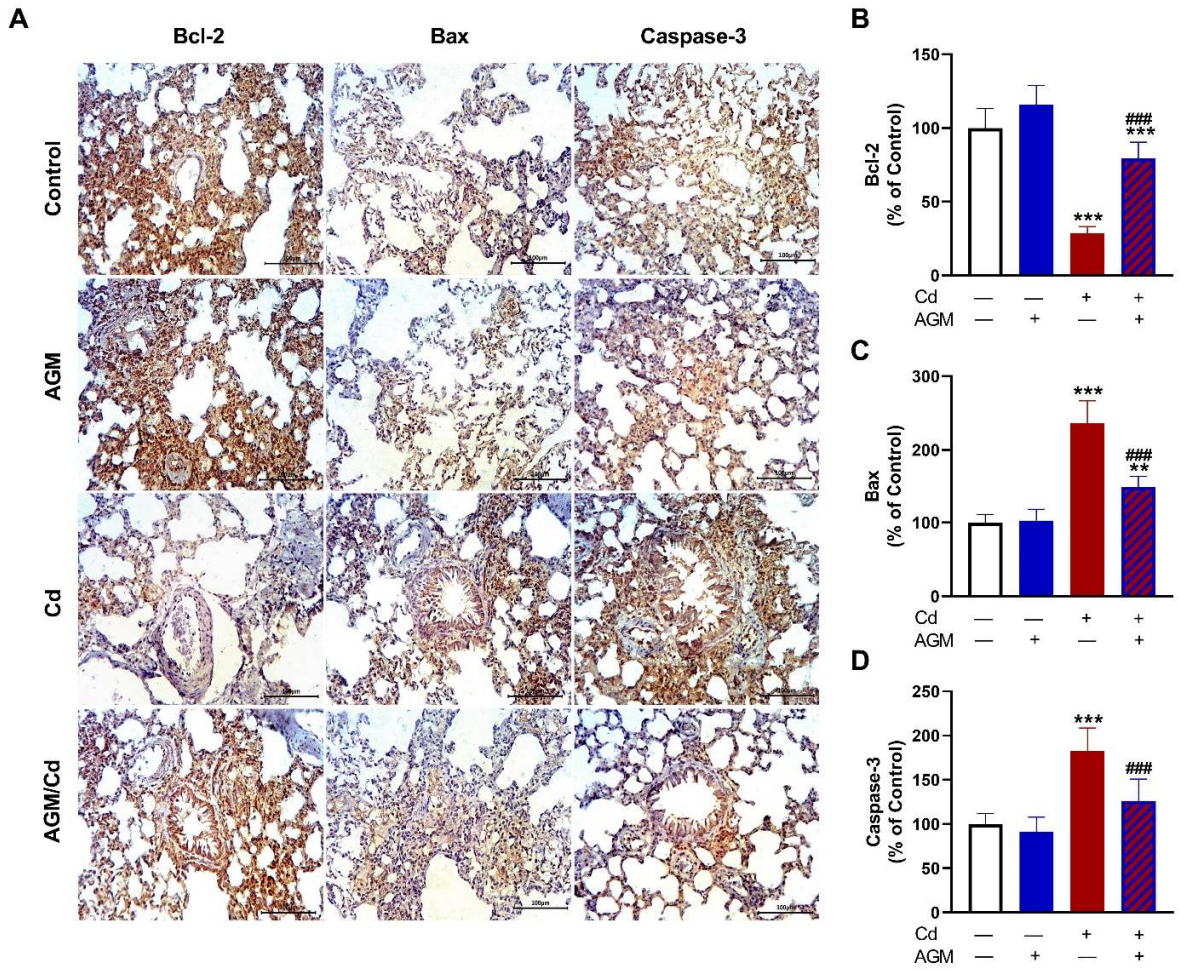
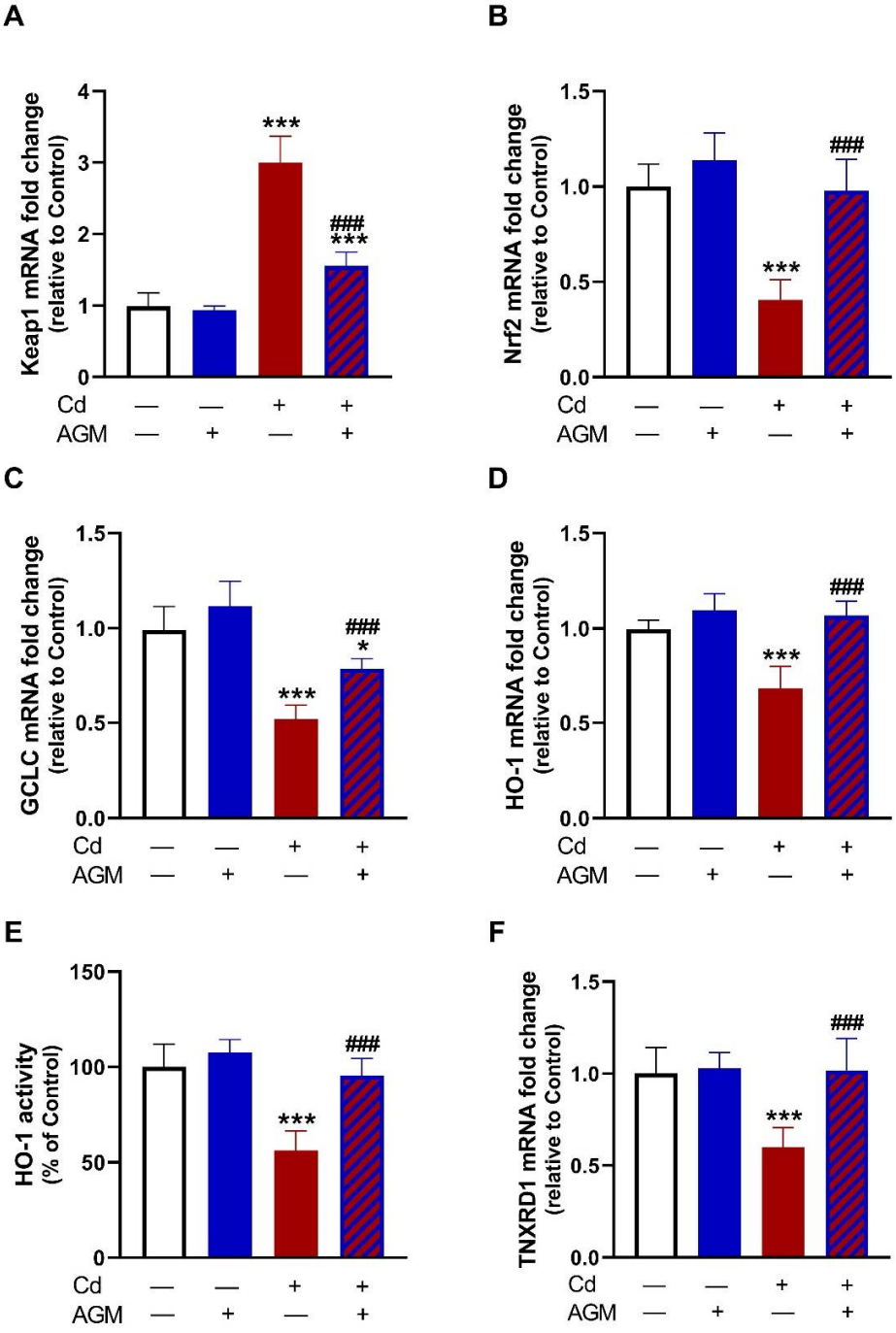


Fig. 4. AGM attenuated Cd-induced apoptosis in the lung of rats. (A) Immunostaining of Bcl-2, Bax and caspase-3 in the lung of control and treated rats. (B-D) Image analysis showing decreased Bcl-2 (B) and increased Bax (C) and caspase-3 (D) in the lung of Cd-administered rats and the protective role of AGM. Data are Mean ± SEM, (n = 8). **P<0.01 and ***P<0.001 vs Control. $^{###}$P<0.001 vs Cd.



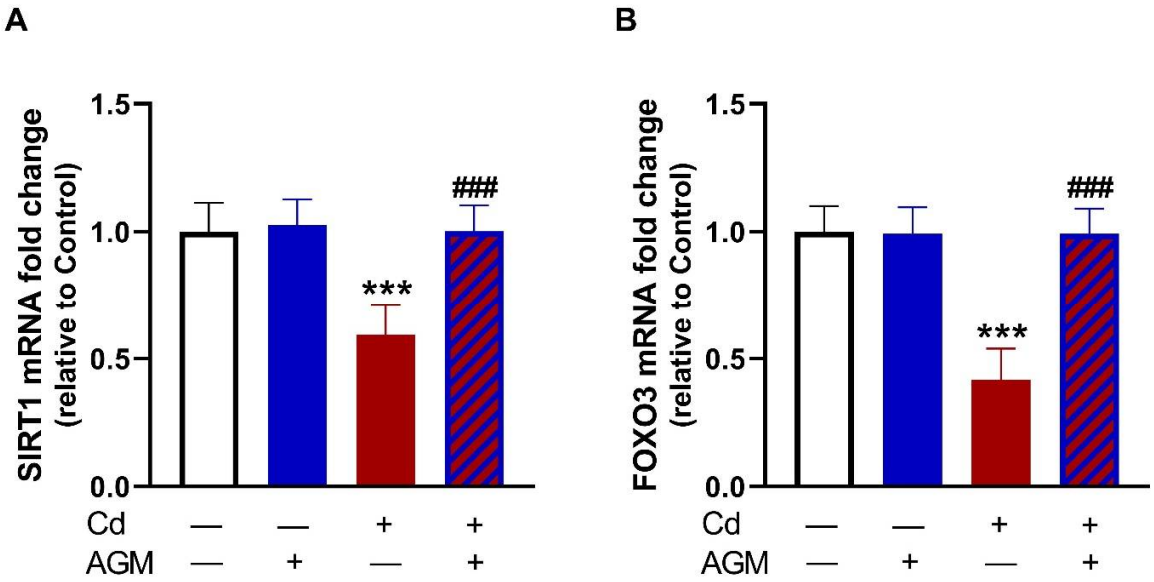
454

455 Fig. 5. AGM upregulated Nrf2 signaling in the lung of Cd-administered rats. AGM decreased

456 Keap1 mRNA (A), and increased Nrf2 (B), GCLC (C), HO-1 (D) and TNXRD1 (F) mRNA and

457 HO-1 activity (E) in Cd-administered rats. Data are Mean ± SEM, (n = 8). *P<0.05 and ***P<0.001

458 vs Control. $^{###}$P<0.001 vs Cd.



459

460 Fig. 6. AGM upregulated SIRT1 (A) and FOXO3 (F) in the lung of Cd-administered rats. Data are

461 Mean ± SEM, (n = 8). ***P<0.001 vs Control and ###P<0.001 vs Cd.

462