Title:

The melatonin receptor agonist agomelatine protects against acute pancreatitis induced by cadmium by attenuating inflammation and oxidative stress and modulating Nrf2/HO-1 pathway

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Abstract

Pancreatitis is a serious effect of the heavy metal cadmium (Cd) and inflammation and oxidative stress (OS) are implicated in Cd-induced pancreatic injury. This study evaluated the effect of the melatonin receptor agonist agomelatine (AGM) on Cd-induced acute pancreatitis (AP), pointing to its modulatory effect on inflammation, OS, and Nrf2/HO-1 pathway. Rats were supplemented with AGM orally for 14 days and a single injection of $CdCl_2$ on day 7. Cd increased serum amylase and lipase and caused pancreatic endocrine and exocrine tissue injury. Malondialdehyde (MDA), nitric oxide (NO) and myeloperoxidase (MPO) were elevated, nuclear factor (NF)-kB p65, inducible NO synthase (iNOS), interleukin (IL)-6, tumor necrosis factor (TNF)-α and CD40 were upregulated, and antioxidants were decreased in the pancreas of Cd-administered rats. AGM ameliorated serum amylase and lipase and pancreatic OS, NF-kB p65, CD40, pro-inflammatory mediators and caspase-3, prevented tissue injury and enhanced antioxidants. AGM downregulated Keap1 and enhanced Nrf2 and HO-1 in the pancreas of Cd-administered rats. *In silico* findings revealed the binding affinity of AGM with Keap1, HO-1, CD40L and caspase-3. In conclusion, AGM protected against AP induced by Cd by preventing inflammation, OS and apoptosis and modulating Nrf2/HO-1 pathway.

Keywords: Pancreatitis; Heavy metals; Oxidative stress; Inflammation.

1. Introduction

Cadmium (Cd) is a heavy metal environmental contaminant that can cause serious health effects. The main sources of human Cd exposure include smoking cigarettes, recharging nickel-Cd batteries, contaminated water and food, plastics, and some other industries such as quarrying, oil and mining [1-4]. Exposure to Cd has been linked to a range of health issues, including kidney and liver injuries, osteoporosis, bone fractures, neurological disease, cardiovascular disease, and cancer [5]. There may be detectable Cd concentrations in the

pancreas, indicating that Cd accumulates there after entering the body [6, 7]. Epidemiological investigations have shown that Cd exposure has a role in the development and progression of diabetes and pancreatic damage [8]. According to reports, exposure to Cd increased blood sugar, which changed the oxidative state and caused a decline in pancreatic β-cell function [9, 10]. While the chronic exposure to Cd leads to declined β-cell function, pancreatic damage and disrupted metabolism [11], acute pancreatitis (AP) could be a serious hazard. AP is a leading cause of hospitalization for gastrointestinal diseases, and patients generally have a death rate of about 5% [12]. AP can develop into the chronic form that is characterized by persistent abdominal pain, poor digestion, and a higher chance of developing pancreatic cancer [13]. If not resolved quickly, AP may lead to multi-organ failure and death by promoting a systemic inflammatory response [14].

Oxidative stress (OS) and inflammation have been linked to various disease processes, including diabetes, and represent major risk factors for several functional cell destruction [15]. When exposed repeatedly to high glucose levels and hazardous substances, pancreatic β-cells become vulnerable to oxidative injury, which results in malfunction and apoptosis [16, 17]. OS is linked to inflammation where excess reactive oxygen species (ROS) activates the transcription factor NF-kB and the release of various pro-inflammatory mediators. NF-κB activation has been linked to numerous experimental investigations as a crucial controller in the development of inflammation in AP. NF-κB is a key transcription factor that connects the initial acinar injury to systemic inflammation and perpetuates the inflammation [18]. OS is a key factor in cell injury and AP and severe AP is associated with redox imbalance and significant mortality [19]. OS can provoke cell injury by direct disruption of cell membrane, toxic lipid peroxides, altered signaling pathways, depletion of antioxidants and altered redox regulation of genes [19, 20]. Therefore, attenuation of OS could be effective against the development of AP. Mitigation of OS and enhancement of antioxidants could be achieved via

activation of the nuclear factor erythroid 2-related factor (Nrf2). Nrf2 is a redox-sensitive factor with antioxidant and cytoprotective properties that is found binding the cytosolic protein Kelch-like ECH-related protein 1 (Keap1) under normal cellular circumstances. Nrf2 is liberated upon oxidative or electrophilic stress and the free form binds to antioxidant response element in the nucleus and promotes the transcription of many genes, including heme oxygenase-1 (HO-1) [21, 22].

Agomelatine (AGM), a melatonin analog, is used to treat sleeping and depressive disorders by activating melatonin M1 and M2 receptors and inhibiting serotonin (5HT2C) receptors [23]. MT1/MT2 melatonergic agonists exert beneficial health effects mediated via both receptor-dependent and independent mechanisms and can scavenge ROS and prevent oxidative injury of the cells [24, 25]. AGM ameliorated inflammation as demonstrated in animal models of neuroinflammation, sepsis and nephrotoxicity [26-28]. It showed cardioprotective effects associated with attenuated OS and inflammation in animals challenged with isoproterenol [29] and lipopolysaccharide (LPS) [30]. Although AGM demonstrated significant beneficial effects against toxicity caused by many substances, its protective impact against Cd-induced AP has not yet been studied. Therefore, this study aimed to investigate the protective effect of AGM against Cd-induced OS and AP pointing to the role of Nrf2/HO-1 signaling.

2. Materials and Methods

2.1. Animals and treatments

This study included 32 male Wistar albino rats (*n*=8 per group), weighing 190-210 g, obtained from Assiut University, Egypt. These rats were kept in a standardized environment with a 12-h light/dark cycle and free access to food and water. The Research Ethics Committee of Al-Azhar University reviewed and approved the animal study protocol (ZA-AS/PH/39/C/28). The rats were allocated into four groups. The 1st group (Control) was given 0.5% carboxymethyl

cellulose (CMC; Sigma, USA) for 14 days orally. The 2nd group (AGM) was given 25 mg/kg/day AGM in 0.5% CMC orally for 14 days [31]. The 3rd group (Cd) received a single i.p. injection of 1.2 mg/kg $CdCl_2$ (Sigma, USA) on the 7th day [32]. The 4th group (AGM/Cd) was given AGM (25 mg/kg) for 14 days orally and an i.p. injection of 1.2. mg/kg $CdCl_2$ on day 7.

Blood was collected via cardiac puncture from rats anesthetized using ketamine (100 mg/kg i.p.) for serum preparation. The pancreas was excised immediately after sacrifice and divided into a portion fixed in 10% neutral-buffered formalin (NBF) for histological and immunohistochemical (IHC) analyses, one portion stored in RNALater for RNA isolation, and another portion that was homogenized (10% *w*/*v*) in Tris-HCl buffer, centrifuged and the supernatant was collected for biochemical investigations.

2.2. Biochemical assays

Serum lipase and amylase activities were determined using colorimetric kits from Biodiagnostic (Cairo, Egypt). ELISA kits (ELabscience, China) were employed to determine TNF-α and IL-6 in the pancreas. All assays were conducted following the provided instructions. Malondialdehyde (MDA) [33], reduced glutathione (GSH) [34] and nitric oxide (NO) [35] levels and activities of myeloperoxidase (MPO) [36], GSH peroxidase (GPx) [37], HO-1 [38], superoxide dismutase (SOD) [39] and glutathione-s-transferase (GST) [40] were determined in the supernatant of the pancreas homogenate.

2.3. Histopathology and IHC examination

The fixed pancreas samples were processed for standard paraffin embedding and sectioning into 4-µm thick sections. The sections were stained with hematoxylin and eosin (H&E) [41] and observed under a light microscope. For IHC analysis, sections were processed by

deparaffinization, clearing, and treatment with 50 mM citrate buffer. Following blocking in 1% BSA, the sections were treated with 0.3% hydrogen peroxide (H_2O_2), washed in PBS before incubation with primary antibodies against inducible NO synthase (iNOS), CD40, and cleaved caspase-3 (Biospes, China) overnight at 4°C. After washing and incubation with 2^{ry} antibodies, the color was developed using DAB in H_2O_2, counterstained with hematoxylin, and color intensity was measured using ImageJ.

2.4. qRT-PCR

To assess mRNA levels of Nrf2, Keap1, HO-1, and NF-κB p65, total RNA was extracted from the pancreas using Trizol (Invitrogen). The RNA was quantified, and cDNA was synthesized from samples with OD260/OD280 ≥ 1.8 using ThermoFisher Scientific Reverse Transcription Kit. SYBR Green Master Mix (ThermoFisher Scientific) and the primers in Table 1 were employed for amplification and the data were analyzed using the $2^{-\Delta\Delta Ct}$ method [42] and normalized to GAPDH.

2.5. Molecular docking (MD)

The binding affinity of AGM with Keap1 (PDB ID: 4L7B), HO-1 (PDB ID: 3HOK), CD40L (PDB ID: 1ALY) and caspase-3 (PDB ID: 3GJQ) was investigated as previously reported [43].

2.6. Statistical analysis

The results are displayed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) with Tukey's test were used to determine statistical significance. The statistical analysis was performed using GraphPad Prism 8.0, with statistical significance considered at $P < 0.05$.

3. Results

3.1. AGM attenuates Cd-induced pancreatic tissue injury

Histopathological examination (Fig. 1 and 2) and serum amylase and lipase activities (Fig. 3) were used to evaluate Cd-induced tissue injury and the effect of AGM. Control and AGM-treated rats exhibited normal appearance of pancreatic acini and islets of Langerhans (Fig. 1A-D). Cd caused alterations in both exocrine and endocrine parts, including lymphocytic infiltration, hemorrhage around the pancreatic fat, hyperemia, vacuolation and necrosis of tunica intima, marked enlargement and proliferation of the smooth muscle fibers nuclei, perivascular inflammatory cellular infiltrations, and dissociation and vacuolation of the cellular components of the islets of Langerhans (Fig. 1E-J). AGM ameliorated Cd-induced tissue damage and the rats exhibited slight vacuolation of the cellular components of the islets of Langerhans and lymphocytic infiltration of the pancreatic fat (Fig. 1K-N). AGM increased the number of cells in islets of Langerhans (Fig. 2A) and decreased inflammatory cells in pancreatic fat (Fig. 2B) of Cd-administered rats ($P<0.001$). Cd-administered rats showed elevated serum amylase (Fig. 3A) and lipase (Fig. 3B) as compared to control animals ($P<0.001$). AGM ameliorated serum amylase and lipase in Cd-administered rats.

3.2. AGM mitigates Cd-induced pancreatic OS

The administration of Cd elevated pancreatic MDA (Fig. 4A) and MPO (Fig. 4B) with a notable reduction of GSH (Fig. 4C), SOD, (Fig. 4D), GPx (Fig. 4E) and GST (Fig. 4F) in comparison to the control group ($P<0.001$). Treatment with AGM effectively decreased MDA and MPO and restored antioxidants.

3.3. AGM attenuates Cd-induced pancreatic inflammation

Cd increased pancreatic NF-kB p65, TNF-α and IL-6 when compared to the control rats as depicted in Fig. 5A-C. IHC revealed significant upregulation of iNOS (Fig. 6A-B) and NO levels (Fig. 6C) in the pancreas of Cd-administered rats. CD40 showed upregulation in the pancreas of Cd-administered rats as shown in Fig. 7A,B. AGM significantly ameliorated NF-kB p65, TNF-α, IL-6, iNOS, NO and CD40 in Cd-treated rats. *In silico* MD investigation

showed that AGM forms hydrophobic interactions with the residues Ala123, Ala124, His125, Thr147, Tyr170, Tyr172, His224 and leu259 of CD40L with a binding energy -6.0 kcal/mol (Fig. 7C & Table 2).

3.4. AGM prevents Cd-induced pancreatic apoptosis

Cd remarkably upregulated cleaved caspase 3 (Fig. 8A,B) and treatment with AGM resulted in significant downregulation. *In silico* investigation showed that AGM forms polar bonding with the residues Lys137 and Arg164 and hydrophobic interactions with Glu124, Gly125, Leu136, Tyr195, Tyr197 and Pro201 of caspase-3 with a binding energy -6.6 kcal/mol (Fig. 8C & Table 2).

3.5. AGM upregulates Nrf2/HO-1 signaling in Cd-administered rats

The administration of Cd significantly upregulated the mRNA of the Keap1 (Fig. 9A) and decreased Nrf2 (Fig. 9B) and HO-1 (Fig. 9C), as well as HO-1 enzymatic activity (Fig. 9D) relative to the control group ($P<0.001$). Treatment with AGM effectively counteracted these effects in Cd-administered rats. *In silico* findings revealed that AGM forms polar bonding with Ser555 and hydrophobic interactions with Tyr334, Ser363, Gly364, Arg415, Ser508, Ala556, Tyr572 and Ser602 of Keap1 (Fig. 9E) and hydrophobic interactions with HO-1 (Fig. 9F). AGM exhibited binding energy of -7.5 kcal/mol and -7.6 kcal/mol with Keap1 and HO-1, respectively (Table 2).

4. Discussion

Pancreatitis is a health hazard of Cd and the positive correlation between age and Cd levels in human islets suggests its negative impact on the pancreas [7]. This study investigated Cd-induced AP and the protective role of the melatonergic agonist AGM in rats. Cd caused pancreatic injury as revealed by the microscopic examination that revealed dissociation and vacuolation of the cellular components of the islets of Langerhans, lymphocytic infiltration,

hemorrhage around the pancreatic fat, hyperemia of blood vessel, necrosis of tunica intima, proliferation of the endothelial lining of blood vessels, and perivascular inflammatory cellular infiltrations. A previous study has suggested that chronic exposure to Cd causes vacuolar degeneration and disrupted β-cell function [44]. Additionally, necrosis and degeneration of β-cells were observed in mice exposed to Cd [45]. These findings were supported by the biochemical results of serum activities of pancreatic amylase and lipase. The pancreatic acinar cells produce lipase and amylase which are released into the blood upon destruction of these cells and hence used as markers for pancreatitis [46]. In AP, the permeability of acinar cells increases resulting in the release of lipase and amylase into the circulation. Lipase has a larger diagnostic window as compared to amylase because it is mainly produced by the pancreas and has a two-week persistent elevation [46]. The elevated circulating levels of lipase and amylase have been observed in rats that received Cd for 28 days [47]. AGM effectively prevented Cd-induced pancreatic injury and ameliorated serum amylase and lipase. In support of these effects, AGM ameliorated LPS-induced elevation of serum amylase and lipase, neutrophil infiltration in interstitial tissue and degenerative changes in exocrine and endocrine pancreatic tissues [48]. The current study supported the ability of AGM to protect the pancreas against injury induced by Cd.

Given the implication of OS in Cd toxicity, the protective effect of AGM against Cd-induced AP could be linked to its antioxidant and anti-inflammatory efficacies. Here, AP was associated with elevated pancreatic MDA and MPO and declined GSH and antioxidant enzymes in Cd-challenged rats, demonstrating OS. The increase in OS markers in AP has been well-acknowledged in animal and human studies. The evolution of AP is characterized by elevated LPO and decreased GSH and other antioxidants such as vitamins C and E along with concomitant increase in CRP in the circulation of patients [49, 50]. In addition, the circulating levels of superoxide radicals, myeloperoxidase and LPO were elevated, and SOD was declined

in patients with mild and severe [51, 52]. Superoxide and hydroxyl radicals and other oxidants are indirectly produced by Cd and not directly through its redox reactions due to its existence in the +2 oxidation [53, 54]. The exposure to Cd increased ROS generation in different cells and the generated ROS can oxidize lipids and damage proteins and DNA, resulting in cell death [53, 55]. This can explain the pancreatic injury, elevated MDA and declined antioxidants following Cd administration. The early stages of AP are characterized by a positive correlation between MDA and free iron, suggesting the role of iron release in provoking OS and cell damage in pancreatitis [56]. In this context, Cd promotes the release of free iron and the generation of ROS via Fenton-type mechanisms [57]. Moreover, Cd activated the neutrophil-derived enzyme MPO that functions to produce pro-oxidants and elicits protein crosslinking, nitration, oxidation and halogenation [58]. AGM effectively decreased MDA and MPO and restored pancreatic GSH and antioxidant enzymes in Cd-challenged rats. Previous reports have demonstrated the antioxidant efficacy of AGM in animal models of neurotoxicity and acute kidney injury [26, 59]. Through binding to melatonergic receptors, AGM can exert antioxidant effects similar to melatonin that can protect the cells against OS and oxidative injury by scavenging ROS and maintaining redox homeostasis [24, 25].

Besides the suppression of OS, AGM effectively attenuated inflammation in the pancreas of rats that received Cd as shown by the downregulated NF-κB p65, IL-6, TNF-α and iNOS. Inflammation is central in Cd toxicity and several *in vivo* and *in vitro* studies have demonstrated inflammatory responses. Treatment of the MIN6 cell with Cd upregulated IL-6, TNF-α and IL-1β and decreased glucose-stimulated insulin release [11]. The upregulation of pro-inflammatory cytokines and decreased IL-10 was also reported in mice treated with Cd for 6 weeks [11]. Cd-induced ROS can activate NF-κB that elicits an inflammatory response by regulating the production of pro-inflammatory mediators which in conjunction with OS orchestrate cell injury [60]. IL-6 and TNF-α initiate the inflammatory response linked to AP

[61] and work in concert with ROS to elicit apoptosis by deteriorating the mitochondrial function [62]. iNOS is another mediator regulated by NF-κB and its upregulation was supported by the elevated NO that can react with superoxide radicals to produce peroxynitrite, a powerful oxidant species capable of oxidizing DNA and further increasing ROS [63]. In turn, iNOS-generated NO can activate NF-κB, leading to further upregulation of inflammatory genes and aggravation of tissue damage [47]. AGM downregulated NF-κB, iNOS, and pro-inflammatory cytokines, showing its anti-inflammatory role in the protection against Cd-induced AP. These findings supported previous studies revealing the anti-inflammatory efficacy of AGM [26, 29, 64]. Given the role of OS in eliciting inflammation and the antioxidant efficacy of AGM, the suppression of Cd-induced AP could be explained in terms of the attenuated OS. The melatonergic agonist property of AGM reveals its ability to mitigate inflammation similar to melatonin [25]. Melatonin suppressed the activation of NF-κB in various cell types [65, 66] and AGM downregulated NF-kB in rat kidney [26].

The anti-inflammatory efficacy of AGM could also be related to the suppression of CD40–CD40L interaction. CD40L is a type II transmembrane protein belongs to the TNF superfamily and is expressed in immune and non-immune cells. Macrophages, neutrophils, platelets, B-cells, endothelial cells and activated T-cells express CD40L. The expression of CD40L in macrophages, and B-cells increases in response to cytokines [67]. CD40 is a transmembrane glycoprotein receptor involved in immune responses and inflammation. CD40L and its receptor CD40 participate in multiple inflammatory pathways implicated in different pathophysiological processes [68]. There is evidence that the CD40–CD40L interaction might be involved in ROS generation and OS. CD40L can provoke ROS generation and OS in endothelial cells, and the CD40–CD40L interaction orchestrates the development of inflammatory responses through the activation of several transcription factors and release of various cytokines and chemokines [69]. Besides the upregulation of TNF-α and IL-6, the

current study revealed upregulated CD40 in the pancreas of Cd-administered rats. Increased expression of CD40 in islet and ductal cells in the diabetic pancreas has been reported upon exposure to TNF-α and IL-1β [70]. Therefore, inhibition of CD40–CD40L could be an effective strategy to counteract AP. In the same context, inhibition of this interaction is a powerful plaque-stabilizing strategy as stated by Seijkens et al [71]. AGM effectively downregulated CD40 in the pancreas of Cd-administered rats, pinpointing the involvement of CD40L-CD40 inhibition in its anti-inflammatory activity. To further explore this result, we investigated the binding affinity of AGM toward CD40L using MD. AGM was shown to form hydrophobic interactions with several amino acid residues of CD40L. The obtained lowest binding energy (-6.0 kcal/mol) is lower than that of curcumin (-4.69 kcal/mol), cannabidiol (-4.22 kcal/mol) and other recently reported compounds [72], demonstrating the higher binding affinity and inhibitory effect of AGM.

The ability of AGM to mitigate inflammation and OS resulted in attenuated apoptosis in the pancreas of Cd-administered rats. ROS and cytokines orchestrate apoptotic cell death through the deterioration of mitochondrial membrane potential, resulting in the outflow of cytochrome c which activates caspase-3 [62]. Activated caspase-3 cleaves key structural and cell cycle proteins, and DNA followed by blebbing and condensing of cells and ultimately death of the cells [73]. Therefore, attenuation of OS and inflammatory response by AGM protected against apoptosis as shown by the downregulated caspase-3 cleavage in the current study. In addition, AGM exhibited binding affinity mediated via polar bonding and hydrophobic interactions with caspase-3, pointing to its ability to bind to and inhibit this executioner enzyme.

The protective role of AGM against Cd-induced OS and AP was associated with enhanced Nrf2/HO-1 signaling. AGM downregulated Keap1 and upregulated pancreatic Nrf2 and HO-1 mRNA as well as HO-1 activity in Cd-administered rats. Nrf2 is vital to the antioxidant and

anti-inflammatory responses that improve the prognosis of AP [74]. Activation of Nrf2 results in the expression of antioxidant genes including the powerful antioxidant HO-1 that catalyzes heme degradation into the radical scavenger bilirubin [75]. In addition, Nrf2 is an upstream regulator of pro-inflammatory mediators [76] and both Nrf2 and HO-1 have a direct inhibitory action on NF-κB [77]. In addition to the mRNA and HO-1 activity assays, *in silico* MD was employed to investigate the binding affinity of AGM toward Keap1 and HO-1. AGM exhibited binding affinity through the formation of polar bonding and hydrophobic interactions with Keap1 and bound to HO-1 with only hydrophobic interactions with low binding energies. The binding energy contributes to the stability of drug/target complexes and is notably influenced by hydrophobic interactions between lipophilic surface of the drug and active site hydrophobic regions. The agreement between the *in silico* and biochemical findings indicated that the elevated affinities between AGM, Keap1 and HO-1 may contribute to the activation of Nrf2/HO-1 signaling and the reported antioxidant and inflammatory activities.

5. Conclusion

The melatonin receptor agonist AGM protected against Cd-induced AP by attenuating OS and inflammation. AGM ameliorated circulating lipase and amylase, pancreatic LPO, NO, MPO, and pro-inflammatory cytokines, downregulated CD40 and upregulated Nrf2/HO-1 pathway and antioxidant enzymes. *In silico* investigation revealed the binding affinity of AGM toward Keap1, HO-1, caspase-3 and CD40L. Therefore, AGM is effective in conferring protection against AP caused by exposure to Cd, pending further investigation(s) to explore other mechanism(s) of action.

CRediT authorship contribution statement

Conceptualization; A.M.M. and E.H.M.H.: Data curation; A.M.M., R.S.A., E.H.M.H., M.S.A., A.B-A. and E.M.K.: Formal analysis; A.M.M. and E.H.M.H.: Funding acquisition; R.S.A.:

Investigation; A.M.M., R.S.A., E.H.M.H., M.S.A., A.B-A., M.K.A. and E.M.K.: Methodology; A.M.M., R.S.A., E.H.M.H., M.S.A., A.B-A., M.K.A. and E.M.K.: Project administration; A.M.M. and R.S.A.: Resources; A.M.M., R.S.A., E.H.M.H., M.S.A. and A.B-A.: Software; E.M.K.: Supervision; A.M.M.: Validation; A.M.M. and E.H.M.H.: Visualization; A.M.M.: Roles/Writing - original draft; A.M.M., R.S.A., E.H.M.H., M.S.A., A.B-A., and M.K.A.: and Writing - review & editing; A.M.M..

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Declaration of Competing Interest

All authors declare no conflict of interests in relation to the manuscript.

Availability of data and materials

The manuscript contains all data supporting the reported results.

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Tables:

Table 1. Primers used for qRT-PCR.

Gene	Sequence (5′-3′)
Keap1	F: TCAGCTAGAGGCGTACTGGA R: TTCGGTTACCATCCTGCGAG
Nrf2	F: TTGTAGATGACCATGAGTCGC R: TGTCCTGCTGTATGCTGCTT
HO-1	F: GTAAATGCAGTGTTGGCCCC R: ATGTGCCAGGCATCTCCTTC
NF-κB p65	F: TTCCCTGAAGTGGAGCTAGGA R: CATGTCGAGGAAGACACTGGA
GAPDH	F: TGCTGGTGCTGAGTATGTCG R: TTGAGAGCAATGCCAGCC

Table 2. Binding interactions of AGM with caspase-3, CD40L, HO-1 and Keap1.

	Binding affinity (kcal/mol)	**Polar bonds**	**Hydrophobic interactions**
Caspase-3	-6.6	Lys137 and Arg164	Glu124, Gly125, Leu136, Tyr195, Tyr197 and Pro201
CD40L	-6.0		Ala123, Ala124, His125, Thr147, Tyr170, Tyr172, His224 and leu259
HO-1	-7.6		Glu62, Glu63, Ile65, Glu66, Phe79, Pro80, Leu83, His84 and Tyr137
Keap1	-7.5	Ser555	Tyr334, Ser363, Gly364, Arg415, Ser508, Ala556, Tyr572 and Ser602

Figure legends:

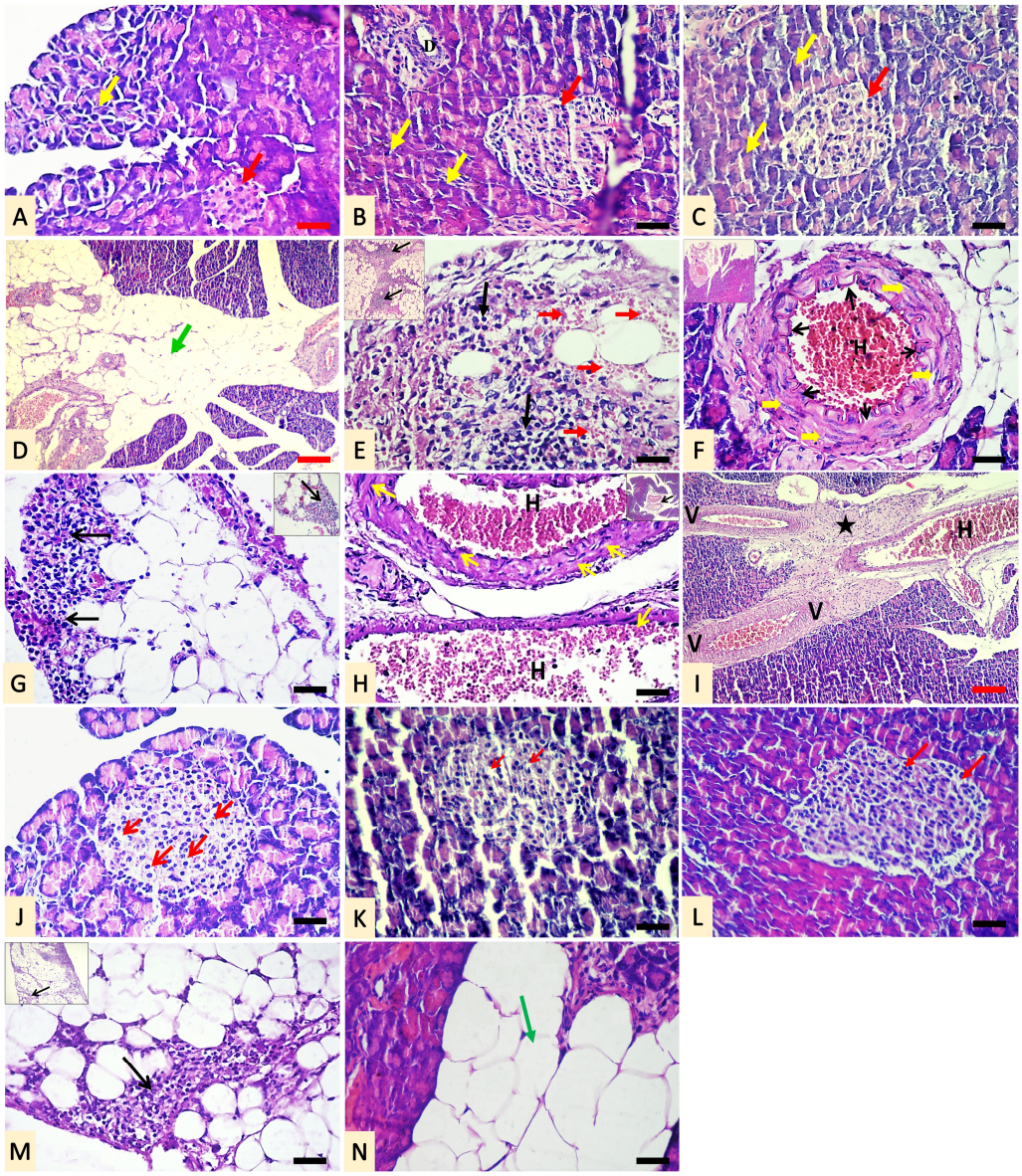

Figure 1. Photomicrographs of sections in the pancreas of (**A**&**B**) control and (**C**&**D**) AGM-treated rats showing normal appearance of pancreatic acini (yellow arrow) and islets of Langerhans (red arrow), pancreatic fat (green arrow) and pancreatic duct (D); (**E**-**J**) Cd-administered rats showing lymphocytic infiltration (black arrow), hemorrhage around the pancreatic fat (red arrow) [**E**], hyperemia of blood vessel (H), necrosis of tunica intima (black arrow), marked enlargement and proliferation of the smooth muscle fibers nuclei (yellow arrow) [**F**], lymphocytic infiltration around the pancreatic fat (black arrow) [**G**], hyperemia (H), proliferation of the endothelial lining of blood vessels (yellow arrow) [**H**], vacuolation of its tunica media (V), few perivascular inflammatory cell infiltrations (star) [**I**], and dissociation and vacuolation of the cellular components of the islets of Langerhans (arrow) [**J**]; (**K**-**N**) Cd-administered rats treated with AGM showing slight vacuolation of the cellular components of the islets of Langerhans (red arrow) [**K**], normal appearance of the islet of Langerhans with increased number of cells (red arrow) [**L**], few [**M –** black arrow] or no [**N** – green arrow] lymphocytic infiltration of the pancreatic fat. (H&E, black scale bar = 50 µm – red scale bar = 200 µm)

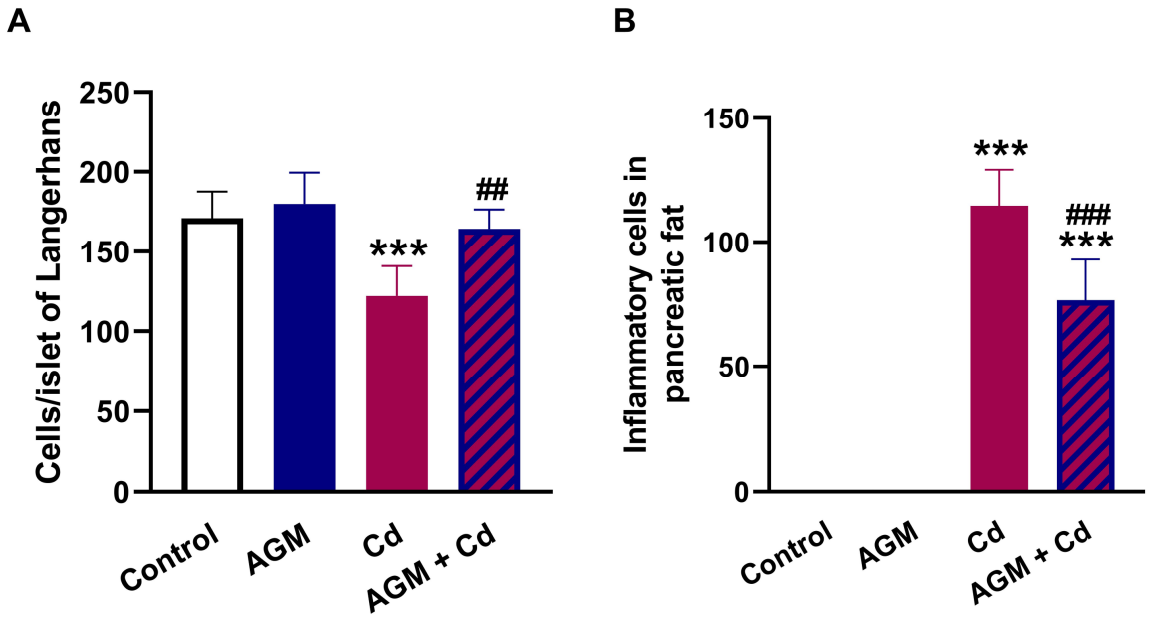

Figure 2. AGM increased cells in islets of Langerhans (**A**) and decreased inflammatory cells infiltration (**B**) in the pancreas of Cd-administered rats. Data are Mean ± SD, (*n* = 8). ***P<0.001 vs Control. $^{##}$P<0.01 and $^{###}$P<0.001 vs Cd.

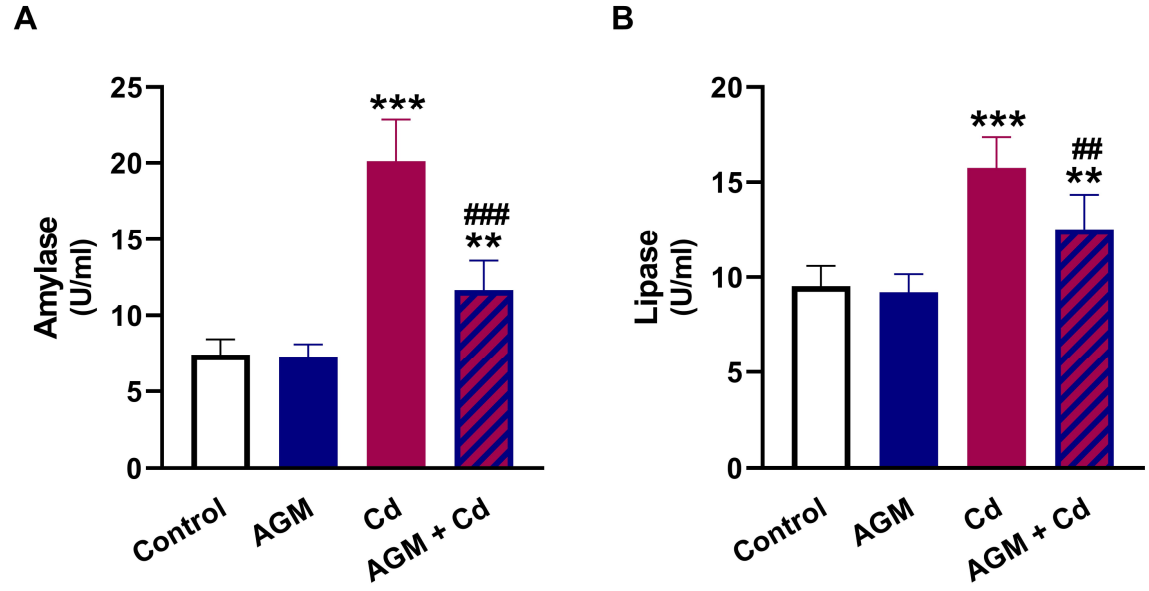

Figure 3. AGM ameliorated serum amylase (**A**) and lipase (**B**) in Cd-administered rats. Data are Mean ± SD, (*n* = 8). **P<0.01 vs ***P<0.001 vs Control. $^{##}$P<0.01 and $^{###}$P<0.001 vs Cd.

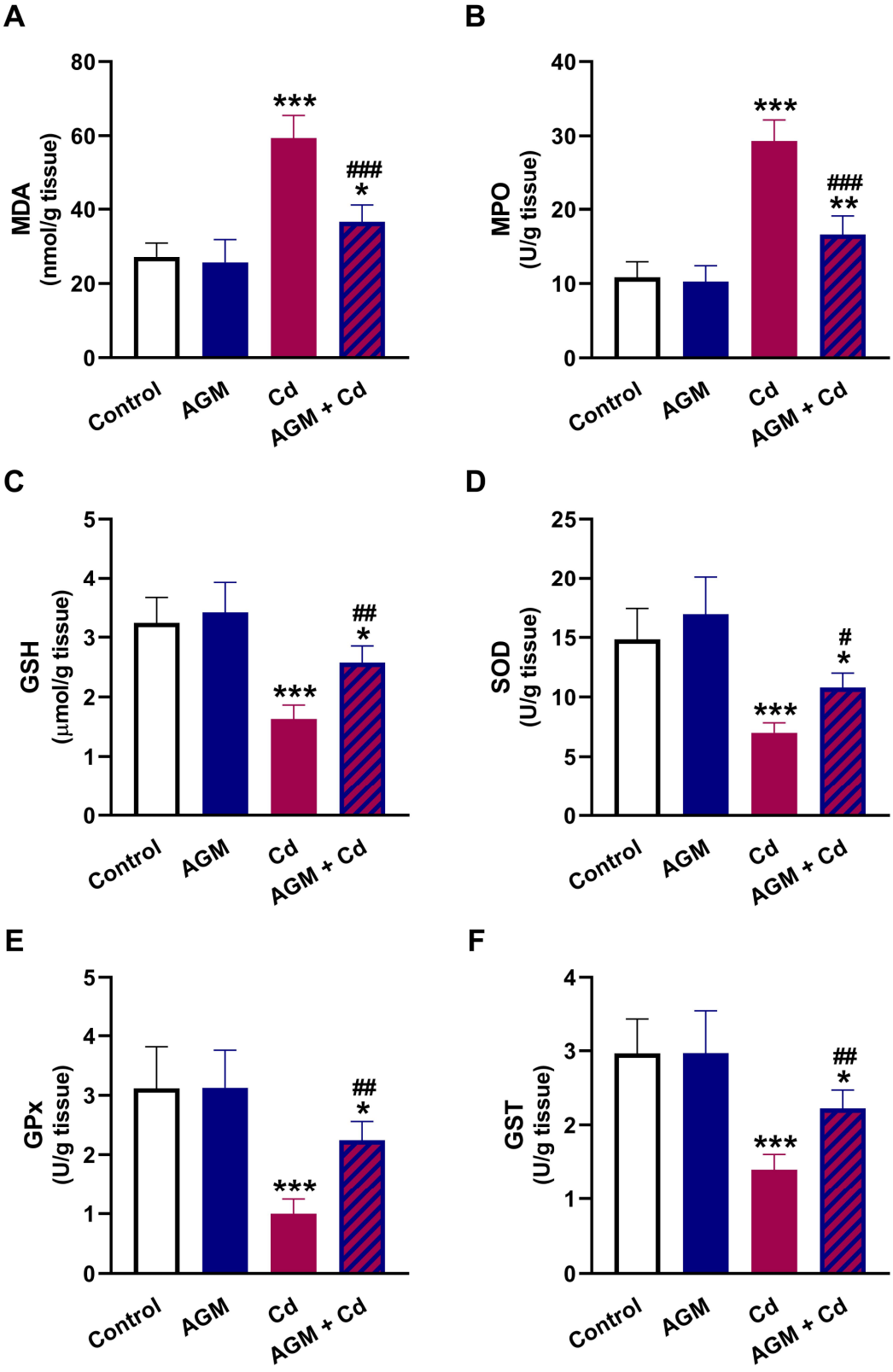

Figure 4. AGM attenuated Cd-induced pancreatic oxidative stress. AGM decreased (**A**) MDA, and (**B**) MPO, and increased (**C**) GSH, (**D**) SOD, (**E**) GPx and (**F**) GST in the pancreas of Cd-administered rats. Data are Mean ± SEM, (*n* = 8). *P<0.05 and ***P<0.001 vs Control. $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs Cd.

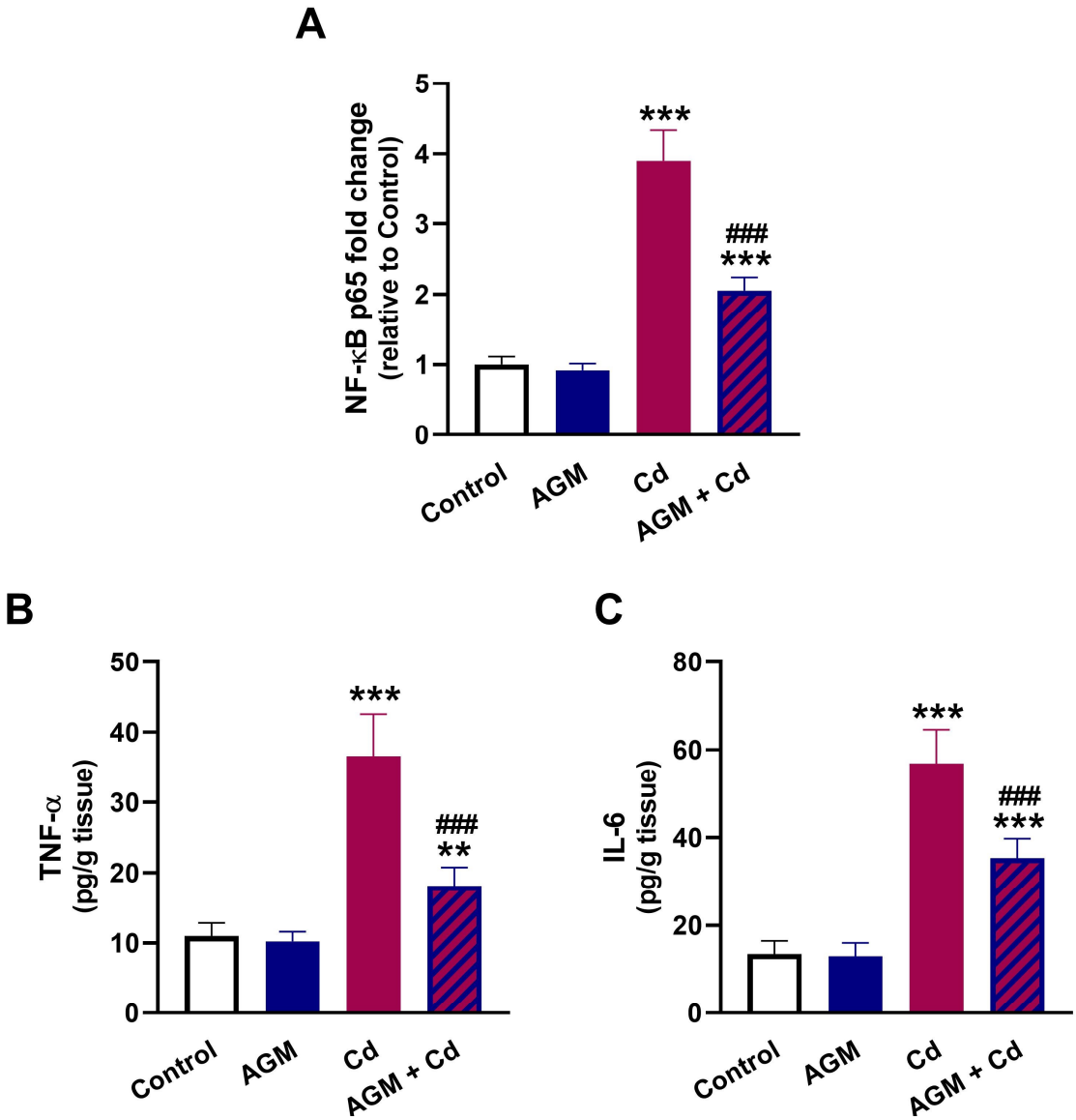

Figure 5. AGM downregulated (**A**) NF-kB p65, (**B**) TNF-α and (**C**) IL-6 in the pancreas of Cd-administered rats. Data are Mean ± SD, (*n* = 8). **P<0.01 and ***P<0.001 vs Control. ###P<0.001 vs Cd.

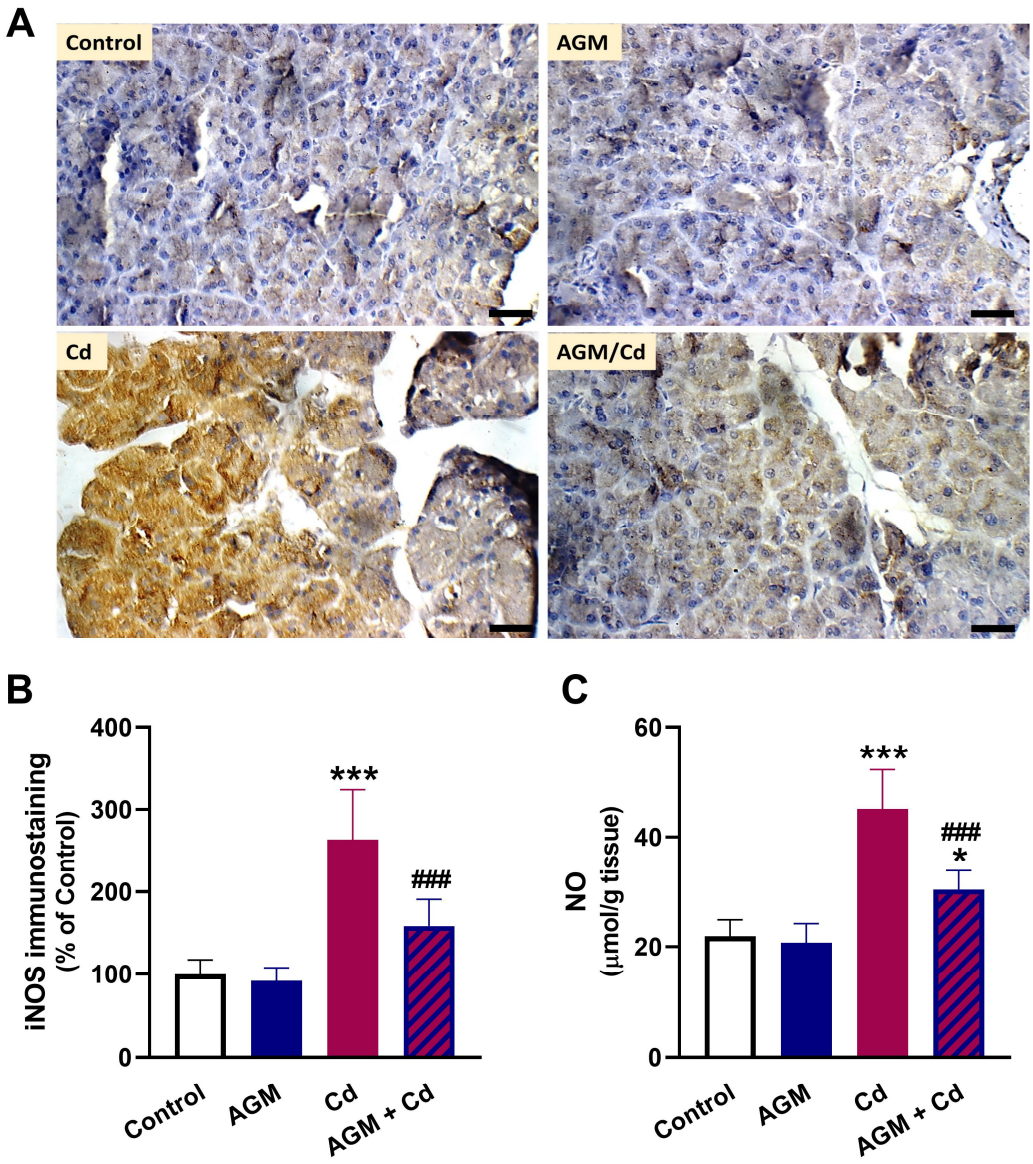

Figure 6. AGM ameliorated (**A**,**B**) iNOS and (**C**) NO in the pancreas of Cd-administered rats. Data are Mean ± SD, (*n* = 8). *P<0.05 and ***P<0.001 vs Control. $^{###}$P<0.001 vs Cd.

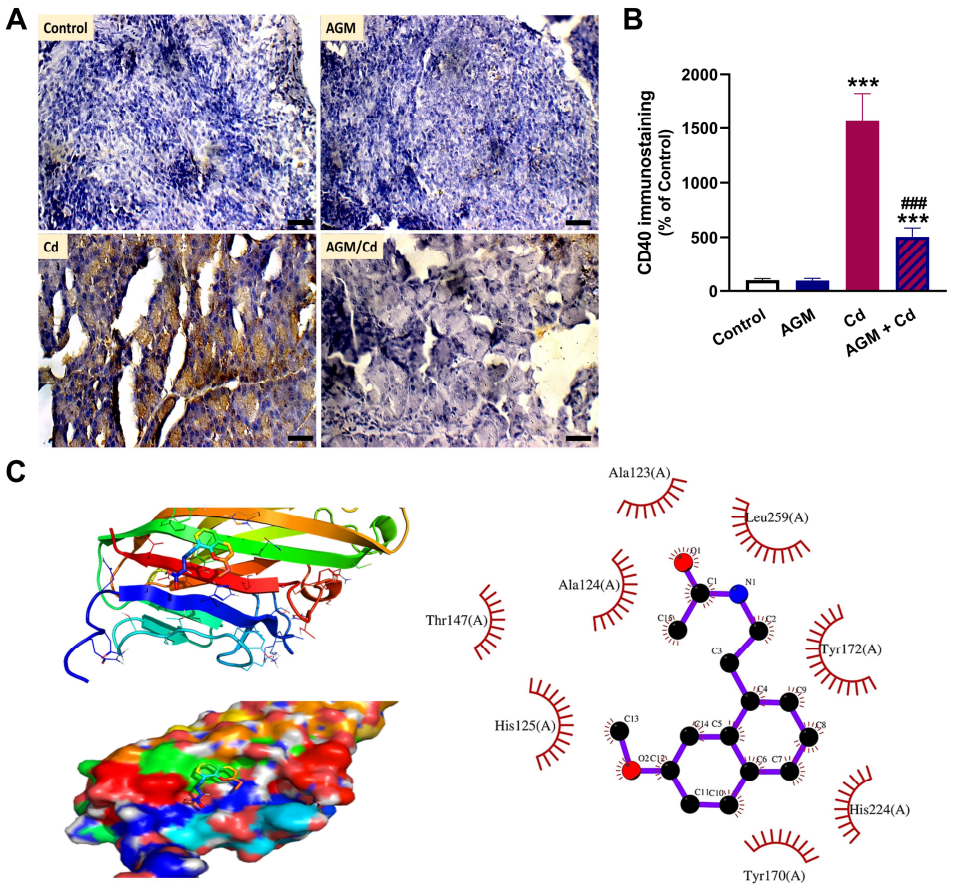

Figure 7. AGM downregulated (**A**,**B**) CD40 in the pancreas of Cd-administered rats. Data are Mean ± SD, (*n* = 8). ***P<0.001 vs Control and ###P<0.001 vs Cd. (**C**) Molecular docking showing the binding interactions of AGM with CD40L.

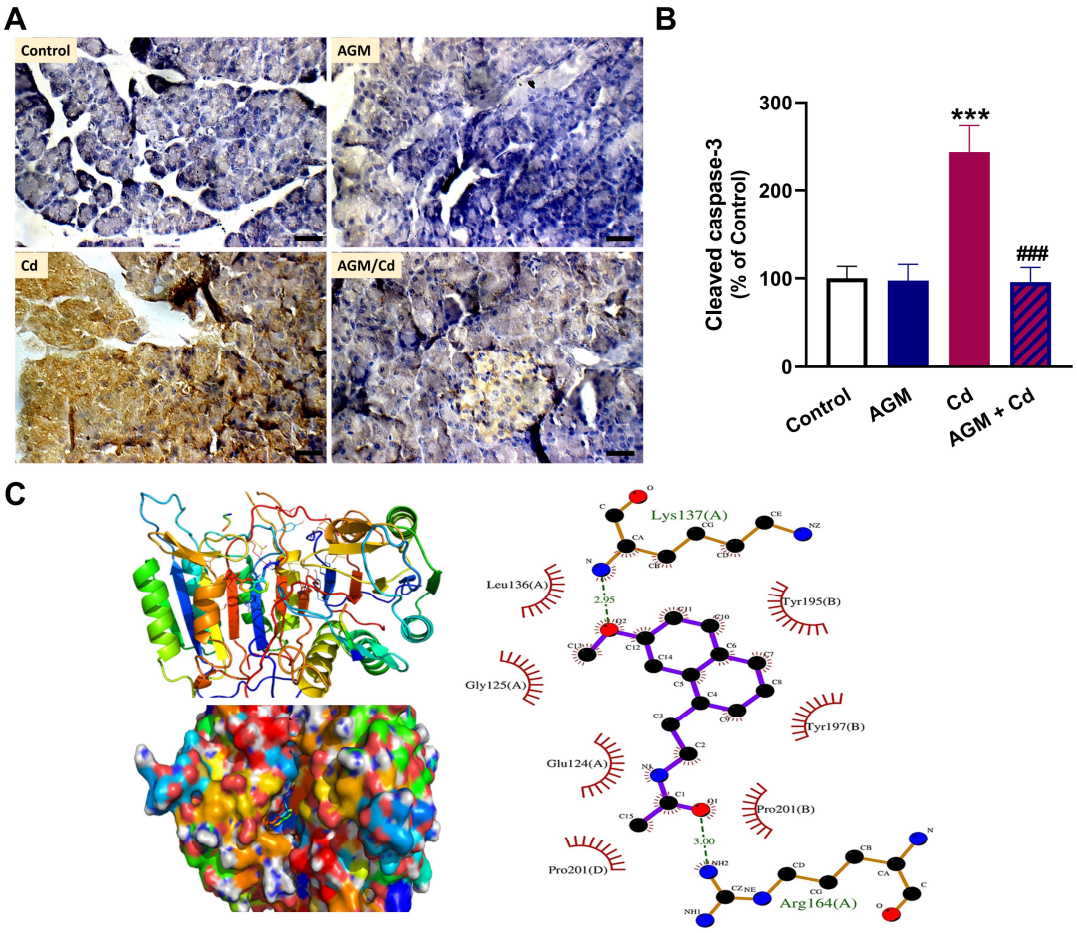

Figure 8. AGM decreased (**A**,**B**) cleaved caspase-3 in the pancreas of Cd-administered rats. Data are Mean ± SD, (*n* = 8). ***P<0.001 vs Control and $^{###}$P<0.001 vs Cd. (**C**) Molecular docking showing the binding interactions of AGM with caspase-3.

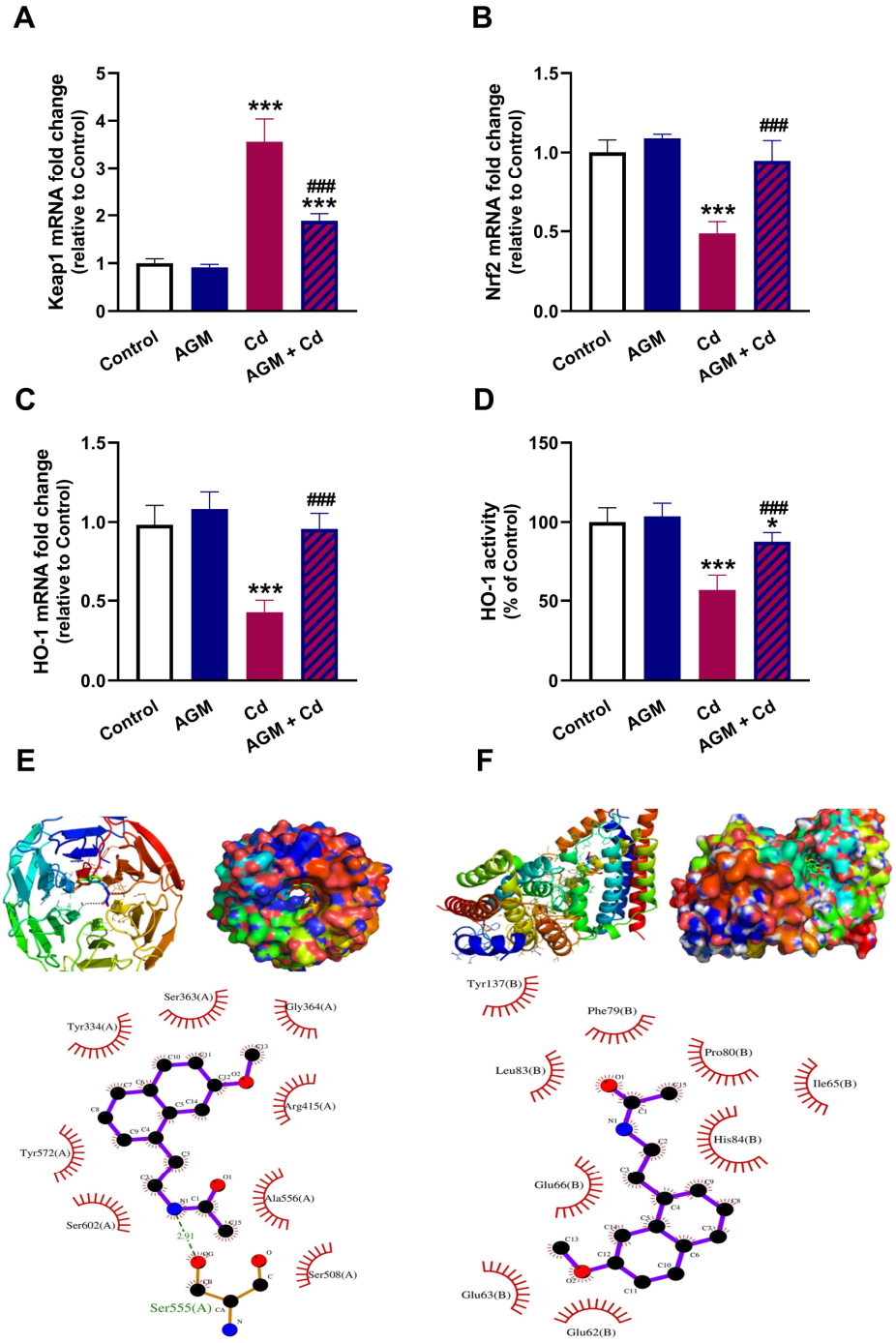

Figure 9. AGM decreased Keap1 (**A**) and increased Nrf2 (**B**) and HO-1 (**C**) mRNA and HO-1 activity (**D**) in the pancreas of Cd-administered rats. Data are Mean ± SD, (*n* = 8). *P<0.05 and ***P<0.001 vs Control. $^{###}$P<0.001 vs Cd. (**E-F**) Molecular docking showing the binding interactions of AGM with keap1 (E) and HO-1 (F).