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ORIGINAL RESEARCH

Nootkatone Counteracts Melamine-Mediated Nephrotoxicity via Modulation of Intermediate Filament Proteins, Oxidative, Inflammatory, and Apoptotic Events

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Background: Nootkatone (NK), a bioactive sesquiterpene ketone, is a major ingredient in grapefruit that has distinguished biological activities. Melamine (MM), a food adulterant, was reported to induce toxic effects including renal disorders. Hence, this protocol was devoted to evaluate the renoprotective impact of NK toward MM-evoked renal damage.

Methods: Rats were either exposed to MM (700 mg/kg) or a combination of MM and two doses of NK (5 and 10 mg/kg).

Results: The results showed that NK therapy notably decreased the kidney functional parameters, along with KIM-1 and NGAL expressions of MM group. Furthermore, a decrease in MDA and NO levels as well as an elevation in SOD, CAT, GSH, and SOD and NRF2 mRNA expression in the NK group demonstrated NK's ability to enhance the renal antioxidant defense of the MM group. Significant suppression in renal inflammatory markers was achieved by NK via lessening of IL-1β and TNF-α, besides downregulation of NF-κB and IL-1β expressions. NK also downregulated vimentin, nestin, and desmin in the MM group. Additionally, in response to the MM exposure, NK hindered renal apoptosis by decreasing caspase-3 expression and restoring renal histopathological features.

Conclusion: These outcomes suggest that NK can be considered as a prospective candidate to guard against MM exposure-mediated renal toxic effects.

Keywords: apoptosis, inflammation, intermediate proteins, melamine nephrotoxicity, nootkatone, oxidative stress

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Introduction

The industrial heterocyclic triazine compound; melamine (MM) has a high nitrogen content.[1] Due to its durability and inexpensive cost, a lot of bowls, cups, and other utensils that come into touch with food use MM.[2] Additionally, MM is a nonprotein nitrogenous element, therefore, it is used as a as controlled-release plant fertilizer and a pesticide.[3] Recent research has documented that MM monomer could be migrated from plastic dinnerware to foods.[4] Ghanati and et al[5] found that the MM content differed in various packaged milk, as it was 50.7 ppb in polyethylene bags, 57.7 ppb in plastic packaging and 790 ppb in packets.

MM intake is linked to deleterious influences, such as reproductive disruption,[6] renal damage,[7] bladder stones and cancers,[8] as well as behavioral and neural disturbances.[9,10] Recent investigations have reported that MM was detected in urine samples taken from children in US.[11] In addition, chronic exposure to MM is linked to the development of kidney injuries, such as renal stones and dysfunction in adults.[12] Lui et al[13] found that the urinary MM level was meaningfully and positively correlated with 8-OHdG and MDA in the urine samples of MM workers. Even though, the exposure of rats to MM at NOEL level (63 mg/kg/day) was reported to cause renal tubular injury via instigation of oxidative stress.[14] Yiu et al[7] reported that MM-exposed LLC-PK1 cells displayed significant increases in ROS levels, apoptotic and necrotic cells which resulted in acute kidney injury and promoted kidney stone formation. Former studies also verified that MM exposure induced an upsurge in liver and kidney function biomarkers, suppression of cellular antioxidant defense, and renal apoptosis.[15,16] Remarkable cytotoxic and genotoxic effects were observed in human embryonic kidney 293 cells upon being co-exposed to MM and cyanuric acid.[17]

Natural products such as plant extract are the most promising lead candidates, and as a result, these products play a crucial part in the future development of new drugs.[18,19] Nootkatone (NK), a naturally occurring sesquiterpene present in grapefruit, has drawn significant consideration because of its distinguished therapeutic activities.[20] NK administration mitigated liver fibrosis and kidney damage in mice exposed to carbon tetrachloride by suppressing oxidative, inflammatory, and apoptotic pathways.[21,22] It was reported that NRF2/HO-1 and NF-κB pathways are involved in the protective action of NK.[22] In addition, the study of Chen et al[23] verified that NK inhibited kidney damage, apoptosis, and fibrosis in mice with ureteral obstruction. Further, a former in vitro study showed that NK upregulated the gene expression level of HO-1 together with decreasing the level of NO, HMGB1 in lipopolysaccharide-treated Raw264.7 cells.[24] Notable mitigation of lung injury induced by diesel exhaust particles was noticed in NK-treated mice via decreasing lung inflammation, oxidative stress, and apoptosis mediated by repressing the NF-κB signaling.[25] NK also counteracted D-galactosamine-evoked hepatic damage by enhancing the Keap1/NRF2/HO-1 signaling and suppressing of hippocampal and cortical iNOS in mice.[26]

These empirical studies illustrated the potential applications of NK in the improvement of renal impairment in different animal models. Hence, this article was established to elucidate the potent pharmacological effect of NK toward MM-inflicted alterations in intermediate filament proteins, renal damage markers, antioxidants, inflammatory cytokines, and apoptosis in rats.

Materials and Methods

Animal Study

Male Sprague Dawley rats (8 weeks old, average weight 150 ± 5 g) were used for this experiment. These animals were provided by the Medical Experimental Research Center, Faculty of Medicine, Mansoura University, Egypt. A temperature range of 22–25°C and a light cycle of 12 h a day were maintained for all rats. During the experimental period, food and water were offered freely. Two weeks were allowed for the acclimatization of the rats before the beginning of the study.

Grouping and Treatment Strategy

Following 7 days of acclimatization, rats were randomly divided into 5 groups as follows; Group 1 (Control): orally received distilled water 1 mL/rat. Group 2 (MM): animals were dosed with MM (700 mg/kg).[27] Group 3 (NK-H): rats were provided with a therapeutic level of NK (10 mg/kg).[28] Group 4 (MM+NK-L): rats were given MM as the above-mentioned dose regimen and treated orally with a low dose of NK (5 mg/kg). Group 5 (MM+NK-H): animals were given

a combination of MM (700 mg/kg) and NK (10 mg/kg). Rats were orally received the abovementioned chemicals for consecutive 28 days and closely observed for any signs of ill health or mortalities throughout the experiment.

Sampling and Tissue Processing

On the 29th day, all animals were euthanized using pentobarbital sodium (60 mg/kg; i.p). Thereafter, serum samples were collected from blood and kidneys were dissected and allotted into three parts; one part was homogenized with 10% weight/volume PBS (pH 7.4). This homogenate was centrifuged, and the supernatant was collected and used for assaying biochemical biomarkers. For histopathological and immunohistochemical evaluation, another part was preserved in 10% formalin, while the last part was preserved at –80°C for total RNA isolation and assessment of mRNA expression.

Biochemical Analyses

Renal Function Assessment

A calorimetric kinetic method was used to determine creatinine and urea nitrogen levels in serum using spectrophotometric kits (Laboratory Biodiagnostics, Giza, Egypt).

Oxidative Stress-Related Biomarkers

Renal antioxidant and oxidative stress biomarkers were assessed by spectrophotometer (using ready-made kits obtained from Laboratory Biodiagnostics, Giza, Egypt). A lipid peroxidation marker (MDA) concentration was measured according to the technique described by Kei.[29] The enzymatic activities of catalase (CAT) and superoxide dismutase (SOD), were measured as declared by Aebi[30] and Misra and Fridovish,[31] respectively. Moreover, glutathione (GSH) was analyzed following the method of Beutler et al.[32] NO level was calorimetrically estimated following the protocol established by Green et al.[33]

Inflammatory Cytokines in Renal Tissue

The renal inflammation was assessed by ELISA kits for measuring tumor necrosis factor- α (TNF-α; Cat No: NBP1-92681), and interleukin-1β (IL-1β; Cat No: NBP1-92702) in renal tissue based on the manufactures' manual.

Gene Expression Level Evaluations

RNA Extraction and Reverse Transcription

As per the guidelines provided by the manufacturer, kidney tissues weighing close to 100 mg were homogenized in the Trizol reagent (Invitrogen, UK) using the Direct-zol RNA MiniPrep (catalogue no. R2050). A Nanodrop (UV-Vis spectrophotometer Q5000/USA) was used to determine quantity and purity, and gel electrophoresis was used to assess integrity. Following the manufacturer's procedure, RNA was subsequently reverse-transcribed into cDNA using the SensiFastTM cDNA synthesis kit from Bioline (catalogue no. Bio- 65053). The thermal program was set as follows: primer annealing at 25°C for 10 min, reverse transcription at 42°C for 15 min, and inactivation at 85°C for 5 min. Finally, the run was held at 4°C.

Quantitative Real-Time PCR

The expression levels of NF-κB, IL-1β, NRF2, SOD, KIM-1, NGAL, desmin, nestin, and vimentin mRNA in kidney tissue were assessed by a real-time PCR using SYBR Green PCR Master Mix (2x SensiFastTM SYBR, Bioline, catalogue No. Bio-98002). The primer sequences for targeted genes are listed in Table S1. The real-time PCR was achieved after 40 cycles of 94°C for 15s, annealing temperatures as listed in Table S1 for 30s, and extension temperature at 72°C for 20s, the process was run at 95°C for 4 min. The melting curve analysis, performed following the PCR amplification, was used to analyze the specificity of the PCR product. Using the $2^{-\Delta\Delta Ct}$ calculation, the expression level of each sample's target gene was compared to that of the Control group after normalization against β-actin.

Renal Histopathological Assessment

The formalin-preserved renal tissues were examined for histopathological changes through the standard histological procedures by dehydrating in ascending grades of ethyl alcohol, clearing with xylene, and embedding in molten paraplast. The resulting blocks were sectioned into 4–5-µm thickness sections and stained with hematoxylin and eosin.

Caspase-3 and Vimentin Protein Expressions

The paraffin-embedded renal sections were immune-stained with primary antibodies against caspase-3 and vimentin based on the manufacturer's information. Results were exported to Excel Sheet expressed as % positively stained cells in relation to total number of cells and % area of positively stained area in relation to total field area. Two slides from each rat were prepared, 5 random fields from each slide were analyzed. Imaging and scoring were done using Olympus® digital camera installed on Olympus® microscope Olympus BX51 microscope and Image-Pro plus 6.0 software, respectively.

Statistical Analyses

The obtained data are shown as means ± SE. All groups were compared using a one-way ANOVA, followed by the LSD as a post-hoc using IBM SPSS, version 26.0. When $P < 0.05$, the significant distinction was taken into account. For data analysis and visualization, GraphPad Prism software (version 8.4.3, GraphPad Software LLC, CA, USA) was used. Multivariate analyses (Clustering Heatmap, Debiased Sparse Partial Correlation (DSPC) network, Pattern Hunter, and Variable Importance Projection (VIP) scores) were done by RStudio software 2023.03.1+446 "Cherry Blossom" Release (6e31ffc3ef2a1f81d377eeccab71ddc11cfbd29e, 2023–05-09) for windows under R version 4.0.2.

Results

Effect of NK and/or MM on Kidney Functions and Damage-Related Factors

Figure 1 presents the modulating effect of NK and MM on renal dysfunction and injury. MM-dosed rats exhibited marked increases in urea and creatinine concentrations proportionated to the control rats. This elevation was significantly reversed in MM+NK-L and MM+NK-H treated groups relative to the MM group in a dose-dependent pattern. Notably, NK-treated

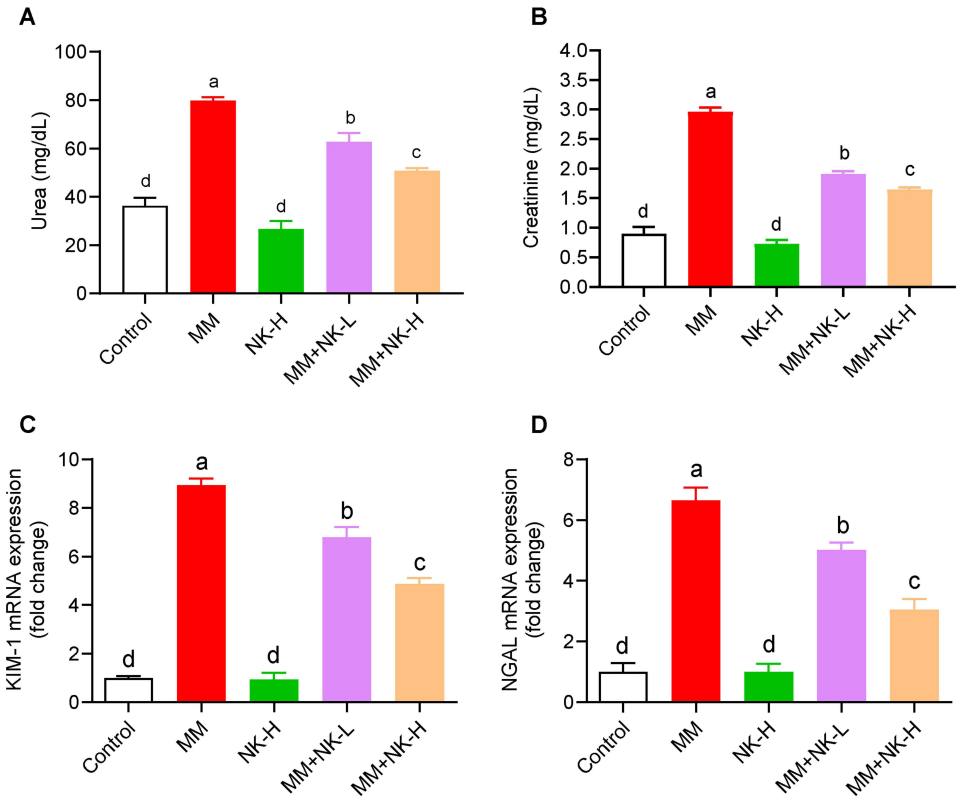

Figure 1 Effects of orally administered nootkatone (NK) on renal function and injury markers in melamine (MM)-administered rats, including serum levels of urea (**A**) and creatinine (**B**) and the mRNA expression levels of KIM-1 (**C**) and NGAL in renal tissue (**D**). Data are expressed as mean ± SE. The different letters denote the significant differences (P < 0.05) between groups.

groups with both tested doses showed no significant changes in kidney function indicators with respect to the controls. Further, the molecular Results revealed that MM evoked notable upregulation of the mRNA expressions of KIM-1 and NGAL in the examined renal samples. On the other side, the groups that received NK before MM had markedly lower gene expression levels of these renal-injury biomarkers with better effects of the higher dose of NK than the lower one (Figure 1).

Effect of NK and/or MM on Renal Oxidative/Antioxidant Status

MM-induced oxidative stress was identified by evaluating the activities along with the mRNA expression levels of the antioxidant enzymes in rats' renal tissue (Figure 2). It was noticed that MM administration significantly decreased the activities of SOD and CAT together with increased the mRNA expression of SOD in kidney homogenates compared with the control group. In contrast, NK at both tested doses prominently reduced the activities of these enzymes compared with MM group. Additionally, the MM-intoxicated group showed notable downregulation in the gene expression of NRF2 in renal samples in relation to the sham group. However, the NK therapy was able to counteract the MM-induced changes. It is noteworthy that the higher dose of NK was more effective than the lower one in terms of enhancing the tissue antioxidant status.

Regarding the non-enzymatic markers (Figure 3), remarkable decreases were depicted in the levels of GSH along with upsurges in MDA and NO in the rats' kidney in the MM group. NK-H group, in contrast, induced marked falls in both MDA and NO concentrations in comparison to the intoxicated group without any effect on the administered dose. GSH levels showed noticeable increases, dose-dependently, in NK-administered groups compared to the MM-intoxicated animals.

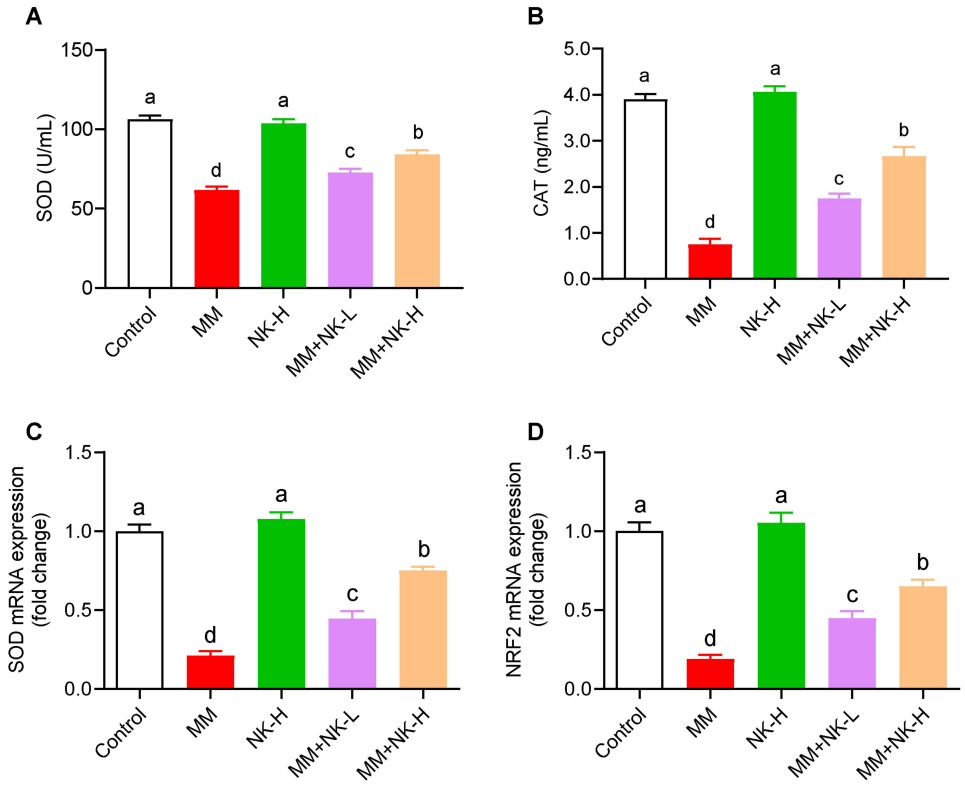

Figure 2 Effects of orally administered nootkatone (NK) on the antioxidant enzymatic activities of SOD (**A**) and CAT (**B**) as well as the mRNA expressions of SOD (**C**) and NRF2 (**D**) in the kidney of melamine (MM)-administered rats. Data are expressed as mean ± SE. The mRNA levels were quantified with β-actin as an internal control. The different letters denote the significant differences ($P < 0.05$) between groups.

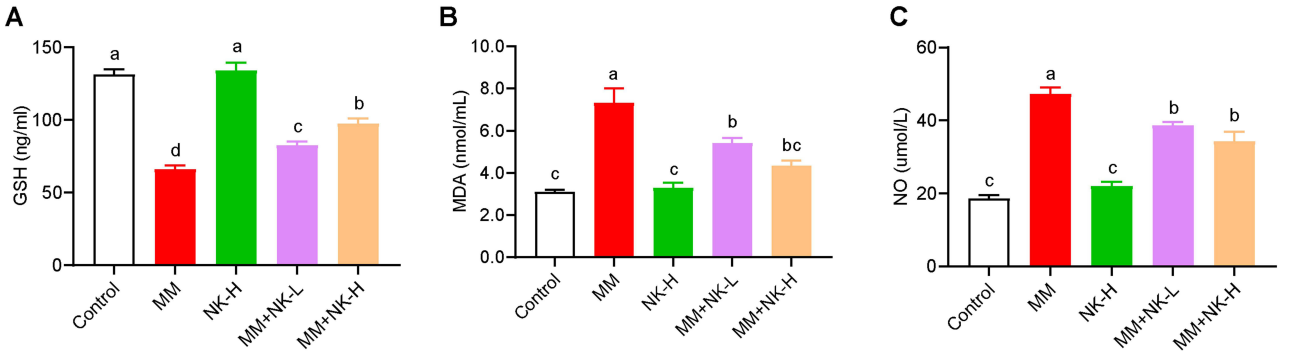

Figure 3 Effects of orally administered nootkatone (NK) on the levels of non-enzymatic oxidative stress markers including GSH (**A**), MDA (**B**), and NO (**C**) in the kidney of melamine (MM)-administered rats. Data are expressed as mean ± SE. The different letters denote the significant differences ($P < 0.05$) between groups.

Effects of NK and/or MM on Renal Inflammatory-Related Cytokines and Genes

In contrast to the control group, MM toxicity significantly caused marked upsurges in renal cytokines (IL-1β and TNF-α) along with up-regulation in the gene expression of IL-1β. However, NK-H elicited noticeable reductions in the aforementioned findings in a dose-dependent manner. To further understand the anti-inflammatory role of NK, the mRNA of NF-κB was measured in tested groups. Notably, MM exposure evoked increases in the NF-κB expression level compared to the controls. Alternatively, NK effectively, particularly at the higher dose suppressed its expression level in renal tissue (Figure 4).

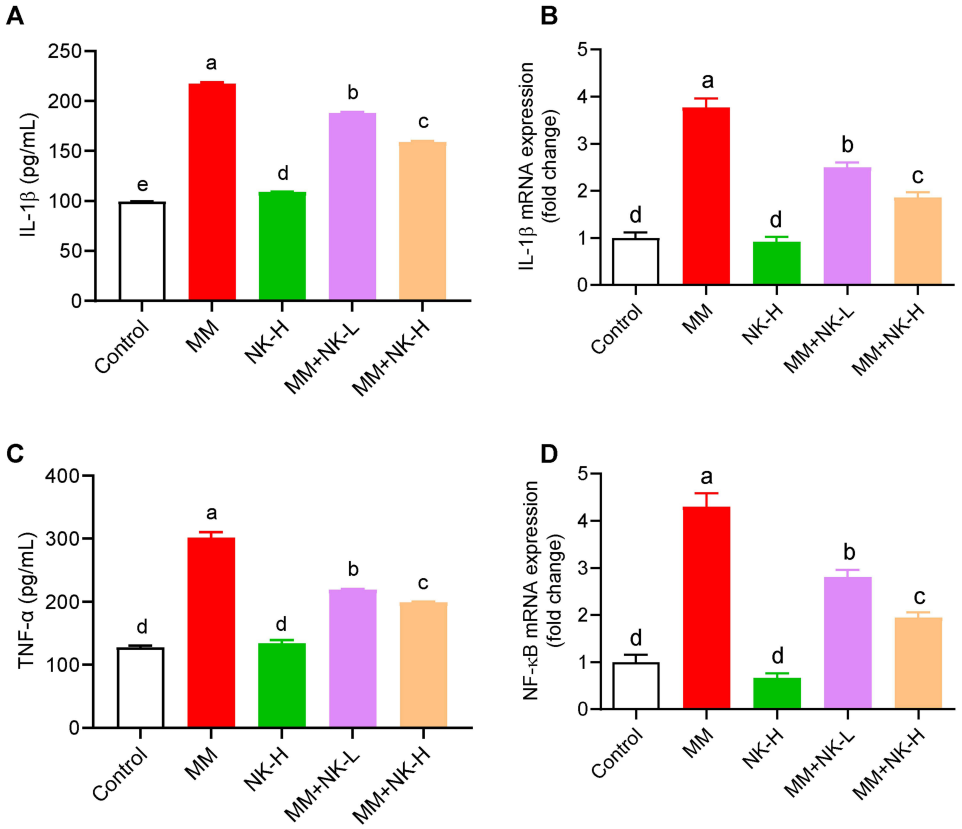

Figure 4 Effects of orally administered nootkatone (NK) on the renal levels of IL-1β (**A**) and mRNA expression of IL-1β (**B**) as well as the level of TNF-α (**C**) and the mRNA expression of NF-κB (**D**) in the kidney of melamine (MM)-administered rats. Data are expressed as mean ± SE. The mRNA levels were quantified with β-actin as an internal control. The different letters denote the significant differences ($P < 0.05$) between groups.

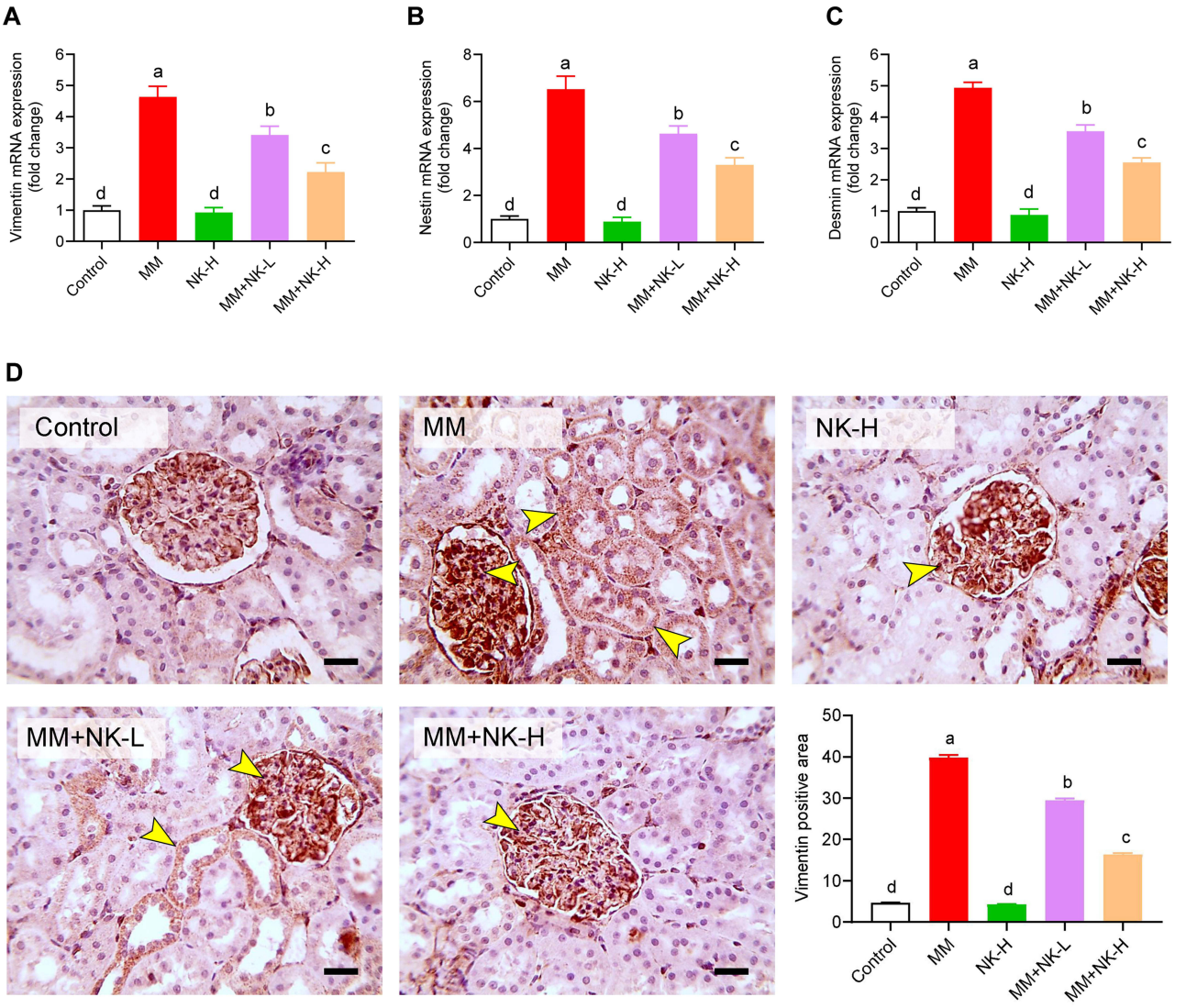

Figure 5 Effects of orally administered nootkatone (NK) on the expressions of vimentin (**A**), nestin (**B**), and desmin (**C**) mRNA and vimentin protein (**D**) in the kidney of melamine (MM)-administered rats. (**D**) Microscopic pictures of immunostained renal sections against vimentin show normal positive brown staining in glomeruli without tubular staining in the control and NK-H groups. Renal sections from the MM group exhibit increased positive brown glomerular staining, with prominent tubular positive brown staining. Renal sections from the MM+NK-L group presented decreased positive brown glomerular and tubular staining. Renal sections from the MM+NK-H group show normal positive brown staining in the glomeruli without tubular staining. The positive area is indicated by brown stain and counterstained with Mayer's hematoxylin (pointed by yellow arrows). Scale bars = 50 μm. Data are expressed as mean ± SE. The mRNA levels were quantified against β-actin as an internal control. The different letters denote the significant differences ($P < 0.05$) between groups.

Effect of NK and/or MM on the Intermediate Protein Filament

As illustrated in Figure 5A, mRNA expression levels of desmin, nestin, and vimentin indicated remarkable upregulations in the MM-treated group compared to the controls. Meanwhile, the administration of NK with MM could meaningfully suppress the MM-induced upregulation of desmin, nestin, and vimentin mRNA expressions when compared with the MM group. But there were no discernible changes found between the groups receiving NK treatment and control.

In addition, the immune reaction of the renal sections against vimentin was investigated in different groups (Figure 5B). Normal positive brown staining in glomeruli without tubular staining was noticed in the control and NK-H groups, with a non-significant difference. However, renal sections from the MM group demonstrated considerably more glomerular staining in comparison to the control animals, with prominent tubular positive brown staining. Renal tissues from the MM+NK-L group showed significantly decreased positive brown glomerular and tubular staining

compared to the MM group. Renal sections from MM+NK-H group showed significantly decreased positive brown glomerular and tubular staining compared to both the MM and MM+NK-L groups.

Effect of NK and/or MM on Cellular Apoptosis

Tissue probed for Caspase-3 depicted mildly positive brown tubular staining in control rats and animals medicated with NK alone, with a non-significant difference. Meanwhile, renal sections from MM group exhibited significantly increased positive brown tubular staining with the presence of positive brown glomerular staining if compared to the control group. However, rats in (MM+NK-L) group showed significantly decreased brown tubular and glomerular staining in comparison to MM group. Moreover, renal sections from MM+NK-H group presented a significant decrease in positive tubular staining without glomerular staining compared to both the MM and MM+NK-L groups (Figure 6).

Effect of NK and/or MM on the Histopathological Features

The renal sections of normal control rats showed normal cortex and medulla with minimal interstitial tissue. Similarly, the kidneys of rats that received NK alone showed intact renal tissues without any inflammatory cells. Nevertheless, MM-challenged rats exhibited marked mononuclear cell infiltrations in the interstitial tissue, tubular degeneration, and necrosis as well as many degenerated glomeruli. Meanwhile, NK administration in low dose to MM-induced renal damage (MM+NK-L) group protects the renal architecture, as evidenced by decreased mononuclear cell infiltrations in the interstitial tissue, and few degenerated glomeruli. Moreover, Renal sections from (MM+NK-H) group showed no cellular infiltration in the interstitial tissue and normal glomeruli (Figure 7).

Multivariate Analyses

Clustering heatmap, DSPC network, pattern hunter, and VIP scores were performed to summarize as well as confirm the relationship between variables and different interventions, as depicted in Figure 8. As illustrated by the clustering heatmap (Figure 8A), there are significant discrepancies in concentration levels of whole measured variables resulting from NK and/or MM treatment across all datasets. In the study, rats exposed to MM were found to have greater damage than rats in the control group. However, the DSPC network (Figure 8B) uncovered the connectivity pattern among the studied features in response to NK and/or MM treatments. This visual

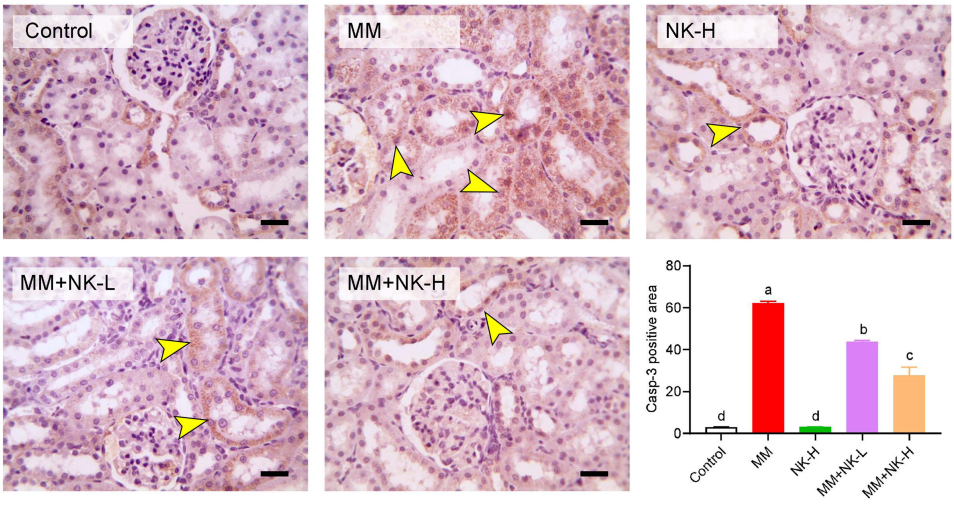

Figure 6 Effects of orally administered nootkatone (NK) on the expressions of Caspase-3 protein in the kidney tissue of melamine (MM)-administered rats. Microscopic pictures of immunostained renal sections against caspase-3 showed mild positive brown tubular staining in control and NK-H groups. Renal sections from MM group pointing increased positive brown tubular staining with presence of positive brown glomerular staining. Renal sections from the MM+NK-L group show decreased positive brown tubular and glomerular staining. Renal sections from MM+NK-H group presented mild positive brown tubular staining without glomerular staining. The positive area is indicated by brown stain and counterstained with Mayer's hematoxylin (pointed by yellow arrows). Scale bars = 50 μm. Data are expressed as mean ± SE. The different letters denote the significant differences ($P < 0.05$) between groups.

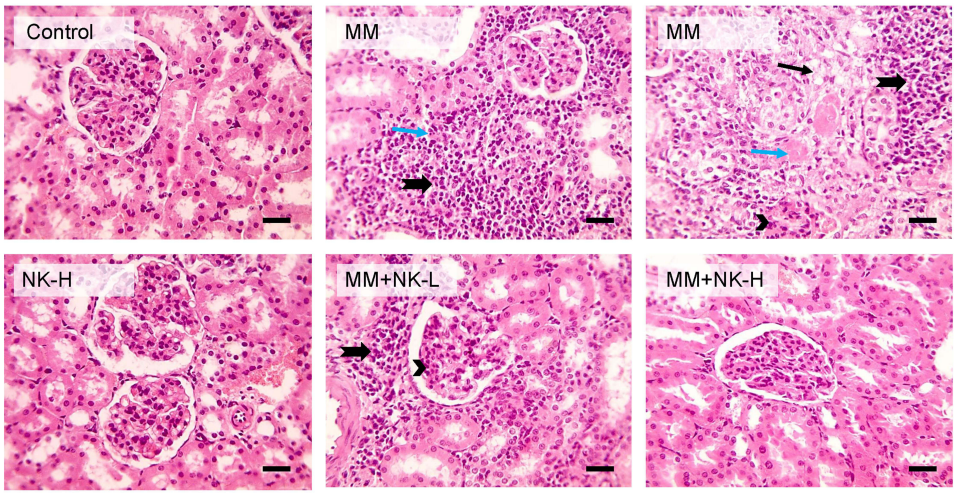

Figure 7 Effects of orally administered nootkatone (NK) on the histological picture of the renal tissue of melamine (MM)-administered rats. Microscopic pictures of H&E-stained renal sections show normal cortex and medulla with minimal interstitial tissue in control and NK-H groups. Renal sections from MM group showed marked mononuclear cells infiltration in interstitial tissue (thick arrows), tubular degeneration (black arrows) and necrosis (blue arrows), many degenerated glomeruli (arrowhead). Renal sections from MM+NK-L group depicted decreased mononuclear cells infiltration in interstitial tissue (thick arrows), few degenerated glomeruli (arrowhead). Renal sections from MM+NK-H group showed no cellular infiltration in interstitial tissue and normal glomeruli. Scale bars = 50 μm.

network confirmed the MM-evoked tissue injury, which indicated by positive correlations among the upregulated variables, including creatinine, urea, MDA, NO, IL-1β protein, TNF-α protein, and the mRNA expression of vimentin, NGAL, nestin, desmin, KIM-1, NF-κB, and IL-1β. These parameters were also inversely correlated to GSH, SOD activity, CAT activity, NRF2 mRNA, and SOD mRNA.

In another attempt to evaluate the correlation degree of the measured variables in response to MM-induced renal damage, a pattern hunter was performed, as seen in Figure 8C. The data revealed that all variables were linearly increased with the increase in the concentration level of creatinine (a documented kidney damage marker) except GSH, SOD activity, CAT activity, NRF2 mRNA, and SOD mRNA were linearly decreased along with the increase of creatinine, suggesting the sensitivity of those variables to the degree of renal injury in MM-exposed animals. In addition, as shown in Figure 8D, VIP scores revealed that urea, creatinine, NF-κB, IL-1β, and SOD were the strongest influences on our study, followed by others, which were sensitively altered in respect to MM intoxication.

Discussion

In the present experiment, a notable rise was observed in the kidney damage products that indicated a substantial renal injury caused by MM toxicity. In former studies, the first signs were regarded as the primary hallmarks of renal damage, which was later confirmed by significant renal histological injury.[16] Importantly, MM markedly elevated creatinine and urea levels in comparison to the controls which was in accordance with previous studies.[16,34] Additionally, these findings are consistent with earlier research that indicated a significant kidney impairment brought on by MM at doses of 12 mg/kg[35] and 120 mg/kg.[36] This also verified the histopathological damage in MM-treated rats, particularly the extensive coagulative necrosis of the renal tubules. In the current investigation, our data revealed an observable upsurge in the mRNA levels of nephropathy biomarkers (KIM-1 and NGAL) in MM-challenged rats. Both NGAL and KIM-1 are recognized as promising potential biomarkers for acute kidney injury.[37] After ischemia and nephrotoxic damage, drastic higher levels of extracellular KIM-1 were noticed in the apical membrane of proximal renal tubules.[38] In individuals with chronic nephropathies, NGAL has a prognostic value as it can not only predict acute but also chronic renal dysfunction.[39] Abd-Elhakim et al have reported that KIM-1 mRNA was dramatically upregulated in rats to MM (700 mg/kg) for 4 weeks.[16] Further, increased KIM-1 and NGAL concentrations were associated with detectable concentrations of MM and cyanuric acid in the urine samples taken from US children suggesting kidney injury.[11] In the kidneys of MM-treated rats for seven days, the expression of KIM-1/Havcr1, Timp1, and Spp1 was noticeably upregulated.[40] On the other side, NK therapy restored these biomarkers toward

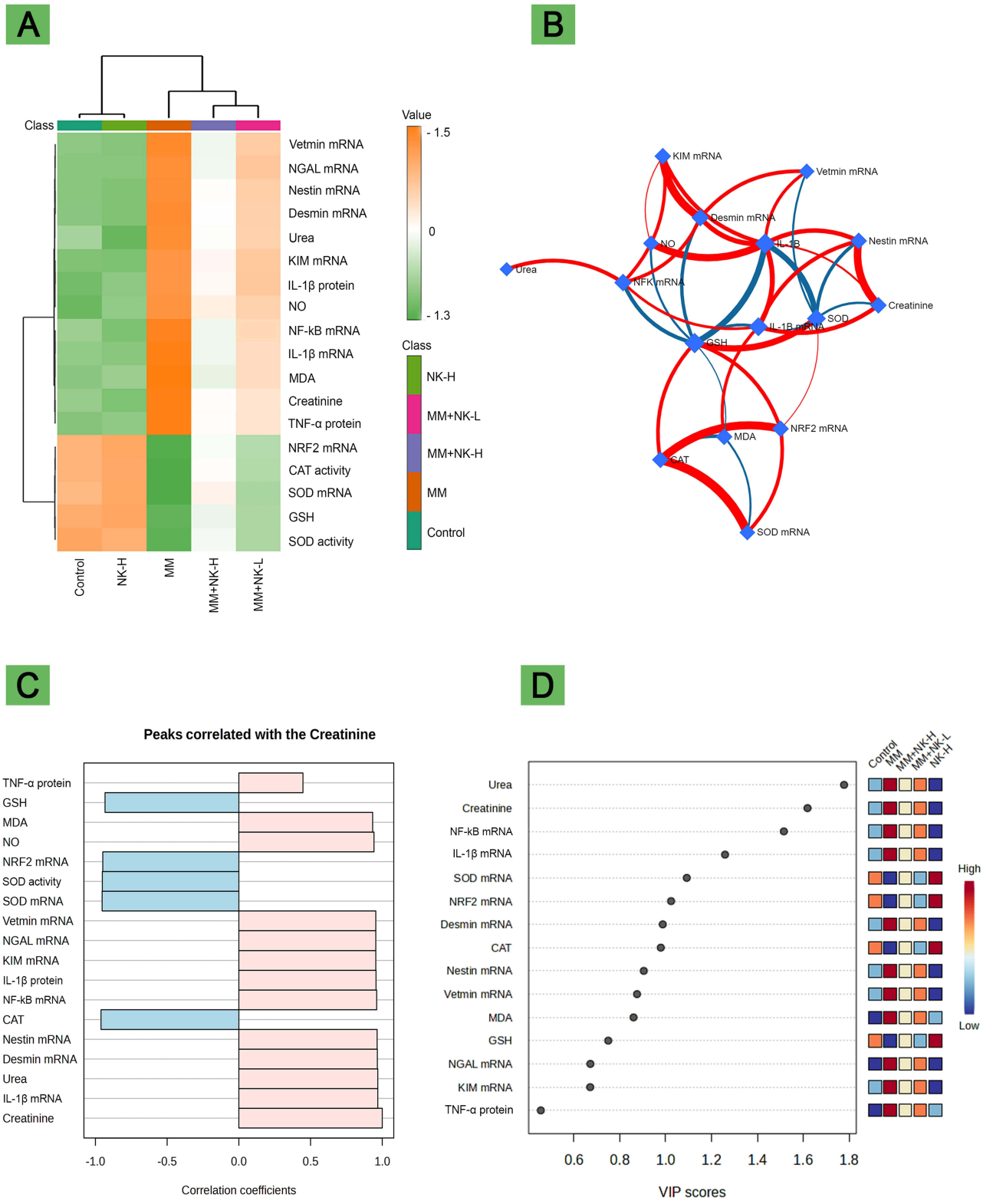

Figure 8 Multivariate analyses after NK and/or MM treatments. (**A**) Clustering heatmap. (**B**) Debiased Sparse Partial Correlation (DSPC) network. (**C**) Pattern hunter. (**D**) Variable Importance Projection (VIP) scores.

the normal levels indicating the improved kidney function and repaired tubular architecture. Dai et al[22] reported marked reductions in the urea and creatinine levels caused by CCl_4 exposure in mice. Similar findings were also documented in doxorubicin-induced nephrotoxicity in rats.[41]

MM-induced kidney injury is caused by unknown mechanisms, while two hypotheses have been put forth. Firstly, MM might act as a nidus to start the creation of stones. This theory discovered that MM significantly increased the renal precipitation of calcium oxalate.[42] According to the second mechanism, exposure to MM may cause tissue oxidative stress in the renal parenchyma, which subsequently results in renal damage, primarily the injury to renal tubules, which promotes the production of stones. This mechanism was confirmed by the investigation of Hsieh et al[43] who found that MM caused tubular damage in human renal proximal tubular HK-2 cells, via increasing oxidative stress, which supports the latter process. Moreover, MM has been shown to enhance ROS production, cause apoptosis, and cause kidney damage in different cell lines.[44] In the same line, inflammation and renal stones caused by MM have been studied by Tian et al,[45] who found that MM reduced the renal blood flow and disrupted the renal and vascular functions along with an upregulation in inflammatory biomarkers in renal tissue. Likewise, MM was reported to activate Ca^{2+}-sensing receptors, causing a sustained Ca^{2+} entry in the renal epithelial cell line, LLC-PK1 which resulted in a rise in calcium ion and greater ROS production.[7] In the current study, MM exposure induced notable decreases in the GSH concentration and in the SOD and CAT activities alongside increases in the MDA and NO levels in the renal tissue. It was also established that oral administration of MM induced notable oxidative stress in various organs.[28,46] Additionally, mRNA level of antioxidant (SOD and NRF2) was markedly downregulated in respect to control animals. As was already noted, NRF2 is a crucial transcription factor that protects cells from damage brought on by oxidative and inflammatory stresses. Cytoplasmic NRF2 translocate to the nucleus in order to produce the enzymes involved in the antioxidant defense and detoxification mechanism.[47–49] Combined exposure of MM and oxalate enhanced the levels of ROS, MDA, oxidative DNA damage and NRF2 expression level in the HK-2 cells and kidney tissue of rats.[14] Similarly, MM evoked noticeable downregulation of mRNA expression levels of NRF2 and HO-1 in the brain tissue of the exposed rats.[10] Remarkably, several reports claim that antioxidant therapy can reverse the renal damage caused by MM in animal models. Li and collaborators[50] confirmed that catechin administration to male rats resulted in the inhibition of ROS formation and modification of apoptosis in MM-associated urolithiasis. According to Meeran et al,[28] using a rat model with damaged myocardium, NK restored GSH and NO levels, hence producing an antioxidant effect. Likewise, NK induced the heme oxygenase-1 transcription in septic mice and LPS-activated-RAW264.7 cells which reflected its antioxidant and anti-inflammatory properties.[24] NK elicited noteworthy inhibition for ROS generation and upregulated the expression of NQO1 and HO-1 in LPS-treated BV2 cells as well as in exposed mice which have a crucial antioxidant role.[51]

In addition, our data revealed a significant rise in the renal cytokines as well as mRNA levels of inflammatory (NF-κB and IL-1β) markers in the MM-treated rats. In the same respect, oral MM exposure resulted in significant overexpression of bone morphogenic protein 4 and cyclooxygenase-2 in the kidneys of exposed rats.[45] In the kidney, macrophages, tubular epithelial cells, and mesangial cells all produce IL-1β, which is a crucial pro-inflammatory cytokine. NF-κB, a transcription factor family member, is a central controller of a number of biological processes, such as inflammation and innate and acquired immunity.[52–54] Upon being activated, NF-κB moves to the nucleus and phosphorylates its p65 subunit, this triggers elevation of pro-inflammatory cytokines eg, IL-1β.[55,56] Wang et al[57] reported that MM and cyanuric acid co-treatment increased the phosphorylation level of NF-κB as well as the mRNA expression of NLRP3 inflammasome in the kidney in rats. Increased NF-κB expression levels in hepatocytes of MM-exposed rats was observed which indicated that inflammatory reaction mediated by MM exposure.[58] In addition, MM induced oxidative and inflammatory stresses in macrophages and human embryonic kidney cells by activating the NF-κB/COX-2 and NOX/ROS pathways, besides increasing prostaglandin E2 production.[44] Therefore, a marked alteration in the inflammatory, and antioxidant markers in MM-intoxicated group might be attributed to the production of calcium oxalate crystals.[59] Besides, activation of NF-κB could also accelerate the production of cytokines, chemokines, and adhesion molecules, which favor the infiltration of inflammatory cells, and cell apoptosis.[60] On the contrary, NK also acts by hampering the NF-κB activating pathway and the activation of NRF2/HO-1 signaling.[22]

Moreover, our data revealed significantly upregulated mRNA levels of intermediate filament proteins (desmin, nestin, and vimentin) in the renal tissues of MM-treated rats. The cytoskeletal proteins known as intermediate filaments are those that are most distinctive to podocytes. The upregulation of desmin, type III IF protein, can be considered a sensitive

marker of podocyte injury, and it occurs after the epithelial-mesenchymal transition of damaged cells via activation of the TGF-beta pathway.[61] A type VI IF protein known as nestin is closely related to vimentin.[62] In adults, nestin expression is downregulated during kidney development, but when tissue is injured, nestin expression is increased throughout the healing process.[63] Upregulated levels of the expression of desmin, nestin, and vimentin were observed in the kidneys of tilmicosin-administered rats, and these results were reversed by Rhodiola rosea treatment[64] and Moringa oleifera ethanolic extract.[65] In another study, stabilized-chitosan selenium nanoparticles downregulated the expression levels of TGF-β1, nestin, desmin, and vimentin in a rat model of diabetic-nephropathy.[66]

Regarding the effect of MM on renal apoptosis, marked caspase-3 triggering was noticed by IHC analysis. Abu-Zeid and collaborators have reported that MM induced apoptosis through variations in the expression of Bax and caspase-3 genes.[67] High percent of apoptotic cells were found in the renal tubules of the co-exposed rat to MM and oxalate and this apoptotic damage resulted in tubular atrophy in some regions.[14] In the same way, the percentage of apoptotic neurons as well as the cellular level of activated caspase-3 were increased in MM and cyanuric acid combined exposure in cultured hippocampal neurons.[68] In contrast, NK could protect against cell apoptotic death in the kidney which is in agreement with other authors.[21,69] Chen et al,[23] elucidated that NK alleviated the expression of renal apoptotic and fibrotic markers in mice with unilateral ureteral obstruction. Overall, NK therapy mitigated the MM-induced alterations in the renal markers of inflammation, nephropathy (KIM-1 and NGAL), oxidative stress as well as disruption of the intermediate filament proteins.

Interestingly, the multivariate analyses including the clustering heatmap and DSPC network affirming the above-mentioned findings by confirming the strong correlation between measured variables and different interventions which supports the severe occurrence of renal damage evoked by MM exposure. However, the pattern hunter and the VIP scores

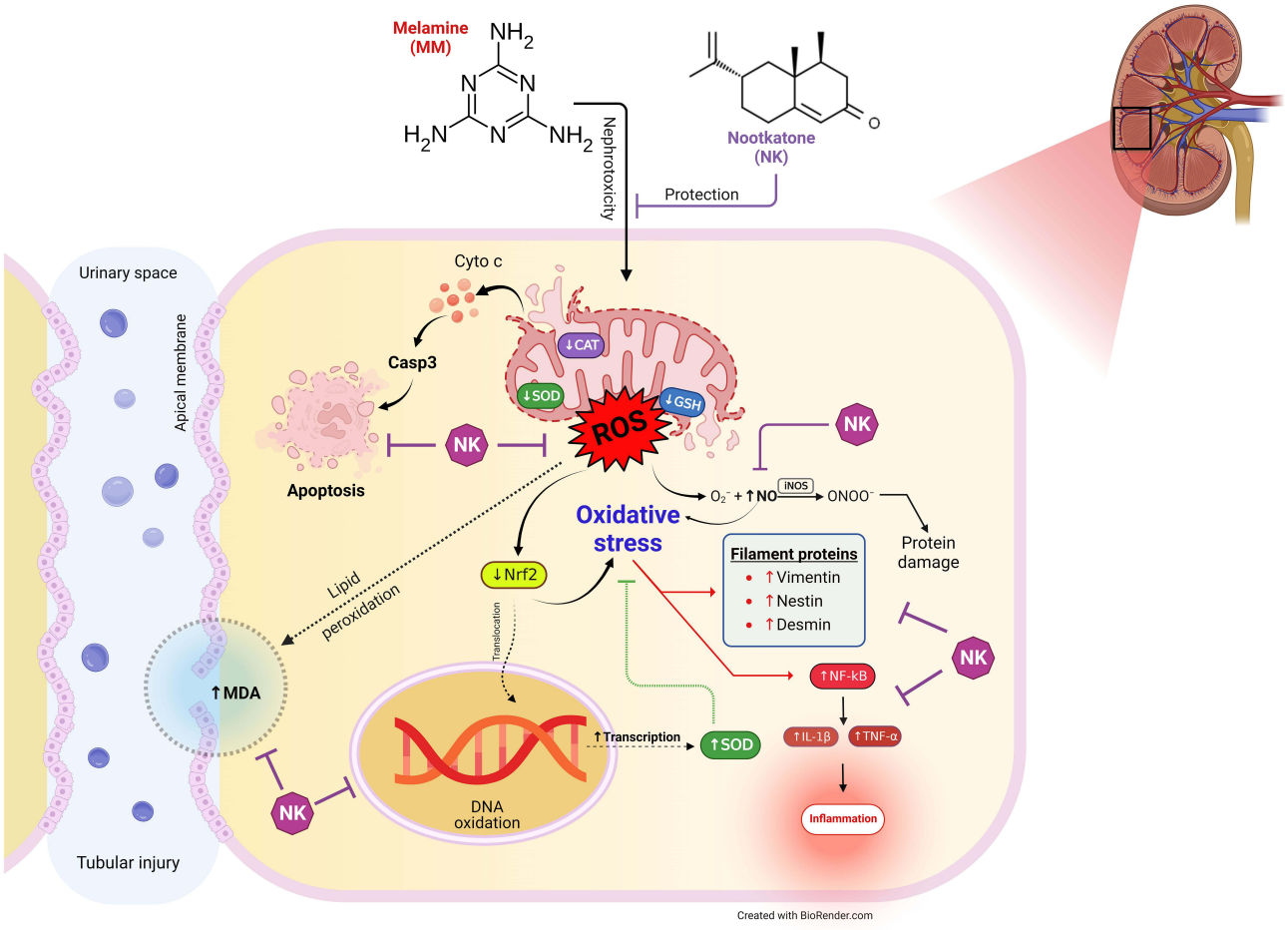

Figure 9 Proposed mechanisms located behind the renoprotective activity of NK against MM-induced renal injury.

revealed that the measured parameters were successfully selected where they exhibited a good sensitivity to the alterations in the kidney in response to MM exposure. Therefore, this study strongly supposed employing those features as potential biomarkers for evaluating MM-induced kidney injury. The summary of the proposed mechanisms associated with the renoprotective potential of NK toward MM-induced renal injury is illustrated in Figure 9.

Conclusions

The study's findings highlighted the potential molecular pathways for the harmful effects of MM exposure on renal function, and more research into the underlying mechanisms is strongly advised. Current research also reveals potential roles for NK in reducing MM-induced kidney damage by altering genes involved in oxidative, inflammatory, and apoptotic scenarios. Our findings offer a potential therapeutic option for treating kidney illness linked to MM with natural remedies that have minor or negligible side effects. For people who are at a high risk of MM exposure, such as MM manufacturing facilities, NK supplementation can be considered a continuous prophylactic precaution. In the future studies, further investigations are required to unveiling the in vitro effect of NK against toxic effects of MM. Additionally, the antagonistic efficacy of NK against MM-induced damage in cellular organelles are needed for better interpretation of the current results.

Institutional Review Board Statement

All experimental procedures were carried out according to the NIH guidelines, and the study protocol was approved in advance by the Medical Research Ethics Committee for animal research studies, Mansoura University, Mansoura, Egypt (Code No. R/MU-ACUC (VM.R.23.03.58)). All conceivable attempts were made to minimize animal suffering.

Data Sharing Statement

All data will be available upon request from the corresponding authors.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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