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Nootkatone Mitigated Melamine-Evoked Hepatotoxicity by Featuring Oxidative Stress and Inflammation Interconnected Mechanisms: In Vivo and In Silico Approaches

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Abstract: Melamine (ML) is a common environmental contaminant, commonly used in food fraud, representing a serious health hazard and jeopardizing human and animal health. Recently, nootkatone (NK), a naturally occurring sesquiterpenoid, has garnered considerable attention due to its potential therapeutic advantages. We investigated the potential mechanisms underlying the protective effects of NK against ML-induced liver injury in rats. Five groups were utilized: control, ML, NK10, ML-NK5, and ML-NK10. ML induced substantial hepatotoxicity, including considerable alterations in biochemical parameters and histology. The oxidative distress triggered by ML increased the generation of malondialdehyde (MDA) and nitric oxide (NO) and decreased levels of reduced glutathione (GSH), catalase (CAT), and superoxide dismutase (SOD) activities. In addition, decreased expression of nuclear factor-erythroid 2-related factor 2 (Nrf2) and increased nuclear factor kappa beta (NF-κB) expression levels were observed in hepatocytes, which indicated the occurrence of inflammatory changes following ML exposure. These alterations were alleviated by NK supplementation in a dose-dependent manner. The data revealed that the favorable effects of NK were attributed, at least in part, to its antioxidant and anti-inflammatory properties. Moreover, our results were supported by molecular docking studies that revealed a good fit and interactions between NK

and antioxidant enzymes. Thus, the current study demonstrated that NK is a potential new food additive for the prevention or treatment of ML-induced toxicity.

Keywords: melamine; nootkatone; liver injury; oxidative stress; Nrf2; NF-κB; molecular docking

1. Introduction

Melamine (ML; 2,4,6-triamino-1,3,5-triazine) is a triazine heterocyclic chemical, frequently employed for the production of resins, plastics, enamel dyes, commercial filters, glues, dishware, and kitchenware [1–4]. Furthermore, this organic base contains 66% nitrogen, and has been added to food for human and animal consumption; the latter has been shown to fraudulently boost the apparent protein content for commercial gain [5]. Several studies have demonstrated contamination of various types of foods by ML, including fruits, baby formula, milk, yogurt, cheese, butter, eggs, processed meat, and bread [6,7], causing considerable concern, because it jeopardizes human and animal health [8]. Zhang and colleagues [9] found that ML was able to induce DNA damage in sperm with a significant increase in sperm abnormality rates in exposed mice. ML residues accumulate in different organs after intravenous administration, causing toxic effects in the brain, spleen, bladder, and kidney [10]. ML also damages the liver, the chief site for detoxification and elimination in the body [10]. Previous investigations have reported that ML induced significant alterations in hepatic histoarchitecture and elevated liver function markers, as well as the induction of oxidative stress, inflammation, and apoptotic changes [2,11,12].

Many compelling publications have supported the presence of oxidative stress following ML exposure, primarily due to the depletion of the intracellular antioxidant defense systems and the uncontrolled formation of reactive oxygen species (ROS) such as superoxide anion, $O_2^{\bullet-}$; hydrogen peroxide, H_2O_2; and hydroxyl radical, $OH^{\bullet}$; as well as reactive nitrogen species such as nitric oxide (NO) [11,13]. These events cause tissue damage, lipid peroxidation (LPO), protein cross-linking, and DNA oxidation [12,14,15]. Another proposed mechanism following long-term exposure to ML is the enhancement of pro-inflammatory and pro-fibrotic markers [16]. Currently, there is no specific treatment for ML toxicity and treatment depends on controlling the ML-induced damage, removal of renal stones, and dialysis, if needed [17]. Consequently, antioxidant supplementation might be an effective therapeutic strategy to repair ML-induced tissue damage and enhance tissue renewal.

Antioxidants of plant origin have garnered global attention recently and are frequently used due to their phytochemical content [18]. Among them, nootkatone (NK; $C_{15}H_{22}O$) is a sesquiterpenoid extracted from several plants including grapefruit and rhizomes of *Cyperus rotundas*, as well as essential oil derived from citrus [19]. Due to its pleasant aroma, this compound has been commercially employed in the chemical, food, and cosmetic industries [20]. NK has tremendous pharmacological properties, including antioxidant, anti-inflammatory, and antiapoptotic activities [19,21]. In addition, an increasing body of evidence supports the potential protective effects of NK against certain drugs and environmental toxicants such as isoproterenol [22], carbon tetrachloride [23,24], rotenone [25], D-galactosamine [26], lipopolysaccharide [27], and water pipe smoking [28].

Therefore, a literature review revealed that even though numerous studies have been performed regarding ML toxicity, research focused on ML-induced liver injury and its alleviation is minimal. Therefore, this study evaluated whether NK exhibited a modulatory effect on ML-triggered oxidative stress and inflammatory liver damage. Hepatic biochemical parameters, oxidative status, histological alterations, and expression of nuclear factor erythroid 2-related factor 2 (Nrf2) and nuclear factor kappa beta (NF-κB) were investigated.

2. Materials and Methods

2.1. Chemicals

Melamine and nootkatone were obtained from Sigma Chemical Company (St. Louis, MO, USA). The analytical kits used to assay the biochemical parameters and oxidative cascade markers were purchased from Bio-diagnostics Co., Giza, Egypt.

2.2. Animals and Ethical Endorsement

Male Wister albino rats weighing 150–170 g were used to complete this experimental protocol. Rats were procured from the Medical Experimental Research Center of Mansoura University (MERC) for experimental research, Faculty of Veterinary Medicine, Mansoura, Egypt. Before the trial, the rats were confined in comfortable, standard hygienic conditions for two weeks (temperature: ~25 °C, humidity: 50–60%). During the trial, all rats were fed a standard baseline diet and had unrestricted access to water.

2.3. Experimental Modeling

After being acclimated, animals were sorted into five equivalent groups (five rats/group). The control group received food and water *ad libitum* without any treatment. The ML group was the positive toxic group, in which rats received 700 mg/kg body weight ML [11]. In the NK group, rats were treated with NK10 (10 mg/kg body weight) [22]. In the ML-NK5 group, rats were given a low dose of both ML and NK, of 5 mg/kg, while in the ML-NK10 group, rats received ML and NK in high doses of 10 mg/kg (ML was given in the same regimen used with the ML group). All treatments were given orally, once a day for 28 successive days.

2.4. Sampling

At the end of the trial, the rats were euthanized by intraperitoneal injection of a xylazine and ketamine mixture (1:1 v/v; 0.15 mL/100 g body weight). Blood specimens were gathered from the heart of each animal. The serum was collected after centrifugation of the coagulated blood at 2000× g for 10 min and preserved at −20 °C until further analysis. Immediately following blood collection, the liver was rapidly extracted, washed in ice-cold physiological saline, and sliced into several pieces. One piece was placed in neutral buffered formalin (10%) for subsequent histopathological evaluation. The other fresh tissue samples were preserved at −80 °C for oxidative cascade marker investigation.

2.5. Estimation of Liver Biomarkers

Serum was used to estimate the liver biochemical markers (ALP, AST, and ALT) using kits from Bio-diagnostics Co. The analysis of all markers was performed according to the manufacturer's directions.

2.6. Liver Homogenate Preparation

Liver homogenates from each rat were prepared by centrifuging the tissue samples at 8000 rpm for 15 min at 4 °C in 400 mL of phosphate-buffered saline (PBS). The supernatants were collected in cold Eppendorf tubes for enzymatic analyses.

2.7. Estimation of the Hepatic Oxidative State

The non-enzymatic oxidative markers (MDA, GSH, and NO) and the enzymatic oxidative marker (CAT) were purchased from Bio-diagnostics Co, and measured according to the manufacturers' guidelines.

2.8. Histoarchitecture Analysis

Formalin-fixed liver samples were dehydrated in a graded alcohol series. Subsequently, xylene clearing was performed followed by routine paraffin embedding. The tissue was cut into 5 µm sections and stained with hematoxylin and eosin (H&E) for histological

evaluation. Representative images were captured using an integrated digital scanning camera system (DM300, Leica, Germany).

2.9. Immunohistochemical Examination

Following the manufacturer's directions, immunohistochemical staining was used to evaluate the hepatic expression of NF-κB and Nrf2. Liver sections (4 mm) on microscope slides were incubated in 0.3% hydrogen peroxide for 20 min to prevent endogenous peroxidase activity. The sections were blocked for 30 min with 2% bovine serum albumin (BSA) for 10 min before incubation in a water bath at 100 °C. Then, the sections were incubated overnight at 4 °C in rat antibodies directed against a rat-targeted antigen. Subsequently, the sections were washed and then incubated with horseradish peroxidase (HRP) secondary antibody at 37 °C for 1 h. Then, the sections were exposed to a diaminobenzidine (DAB) working solution for 4 min and counterstained with Mayer's hematoxylin. Finally, the sections were evaluated and photographed. Images were taken using a Nikon integrated digital imaging system (Eclipse E200-LED, Nikon, Tokyo, Japan), at an original magnification of ×400.

2.10. Molecular Docking

ML was docked with rat superoxide dismutase (SOD1, SOD2, SOD3), catalase (CAT), glutathione peroxidase-1 (GPx-1), glutamate-cysteine ligase catalytic subunit (GCLC), glutathione reductase (GR), and glutathione synthase (GS). NK was docked against tumor necrosis factor receptor superfamily member 1A (TNFRSF1A), tumor necrosis factor receptor superfamily member 1B (TNFRSF1B), transforming growth factor beta-activated kinase 1 (TAK1), inhibitor of nuclear factor kappa-B kinase subunit beta (IKKB), interleukin-1 receptor type 1 (IL-1R1), interleukin-1 receptor type 1 (IL-1R2), caspase-3, and inducible nitric oxide synthase (iNOS). The three-dimensional structures of target proteins were retrieved from UniProt (https://www.uniprot.org/; accessed on 15 June 2023) and AlphaFold (https://alphafold.ebi.ac.uk/; accessed on 15 June 2023) protein structure databases. Proteins were prepared for docking using Molecular Operating Environment software (MOE 2022.02, Chemical Computing Group, Montreal, QC, Canada). In addition, the three-dimensional structures of ML and NK were retrieved from the PubChem (https://pubchem.ncbi.nlm.nih.gov/; accessed on 15 June 2023) database. Furthermore, the molecular docking, protein–ligand interactions, and visualization were carried out using MOE software (https://www.chemcomp.com/ accessed on 15 June 2023).

2.11. Statistical Data

One-way analysis of variance (ANOVA) was used to analyze the results, and differences between the groups were revealed using Duncan's post-hoc multiple tests. All values were judged to be statistically significant at $p \leq 0.05$ and expressed as means ± standard error of the mean (SE). GraphPad Prism version 8 was used for data analysis and the generation of column charts. Multivariate principal component analysis (PCA), the variable importance in projection (VIP) score, and clustering heatmaps were created using MetaboAnalyst software (version 0.5, developed by the XiaLab at McGill University, Montreal, QC, Canada).

3. Results

3.1. Effect of ML and/or NK Treatment on Liver Biochemical Parameters

As shown in Figure 1, ML treatment resulted in notable disruptions in liver functions, as indicated by a substantial increase in liver enzymes (AST and ALT) and ALP activity compared to control rats. On the other hand, preconditioning with a low dose of NK significantly attenuated the ML-induced injuries in liver tissue. Remarkably, administering a high dose of NK exerted noticeable amelioration of those parameters. These data suggested a dose-dependent change in ML-treated animals when co-administrated with different doses of NK.

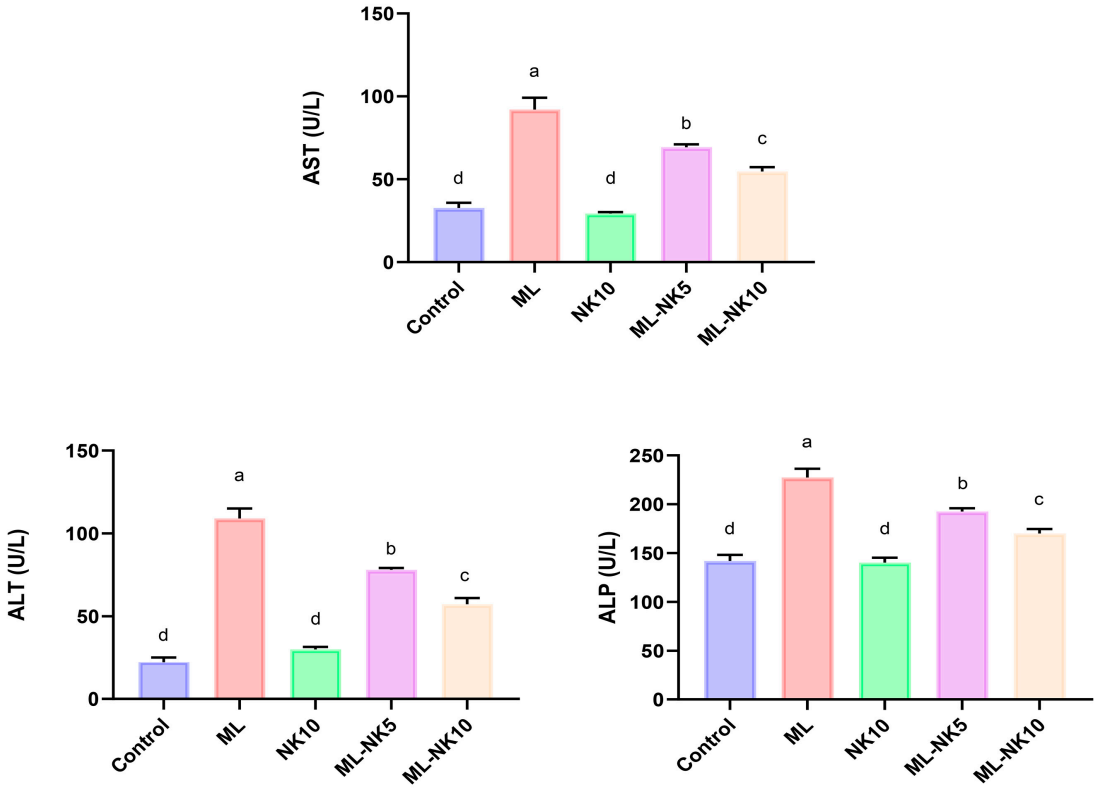

Figure 1. Bar plots of liver parameter levels with ML and/or NK exposure (n = 5). ALT, alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase. Control, nootkatone (NK10), melamine (ML), ML-NK5 combination, and ML-NK10 combination. Significant differences existed across groups with different letters ($p \leq 0.05$).

3.2. Effect of ML and/or NK Treatment on Oxidative Cascade and Lipid Peroxidation

The lipid peroxidation (MDA) index and oxidative status (NO, CAT, SOD, and GSH) following ML and/or NK administration are presented in Figure 2. ML treatment prompted considerable oxidative stress, as indicated by a drastic reduction in CAT and SOD activity as well as the level of GSH, along with increased MDA and NO levels, compared to controls. However, rats co-administrated ML and NK at high and low doses exhibited significant elevations in CAT and SOD activities and GSH levels, as well as a reduction in MDA and NO levels. An improvement in the oxidative state appeared in the ML-NK10 group compared to the ML-NK5 group in the CAT, SOD, and GSH expression levels. The MDA and NO levels exhibited no significant differences between the ML-NK5 and ML-NK10 groups (Figure 2).

3.3. Liver Histopathology following ML and/or NK Treatment

The hepatic histoarchitecture of the control and NK groups revealed normal hepatic cords radially arranged around central veins and normal portal areas and sinusoids (Figure 3A,C). Hepatic sections from the ML group showed marked portal fibrosis with disruptions in the radial arrangement of the hepatocyte cords. Marked infiltration of inflammatory cells was observed in the hepatic lobules. Congested portal blood vessels, sinusoidal enlargement, and also dilation and proliferation of biliary epithelium were observed (Figure 3B). Sections from the ML-NK5 group demonstrated limited portal fibrosis with sparse inflammatory infiltration. In addition, there was decreased dilation and proliferation of the biliary tracts (Figure 3D). Co-treatment of rats with ML and NK10 resulted in strikingly nearly normal hepatic lobules with minimal inflammatory cell infiltration in the portal areas (Figure 3E).

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Institutional Review Board Statement: The animal study protocol was approved by the Ethics Committee of Mansoura University's Institutional Animal Care and Use Committee (Approval code MU-ACUC (V.M.R.23.07.115).

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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