Protective Effects of Nigella Sativa Against Acrylamide-Induced Toxicity in Submandibular Salivary Glands of Albino Rats: A Histological and Molecular Study

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Abstract

Acrylamide (AA), a chemical compound that is a major public health concern. This study aimed to evaluate the protective effect of nigella sativa (NS) oil against AA induced toxicity on the submandibular salivary glands (SMGs) of Albino rats. Thirty male albino rats weighing 150 – 200 gm were equally and randomly divided into the control group, which received normal saline vehicle daily via oral gavage for 30 days, AA group received 15 mg/kg body weight of AA dissolved in 0.2 ml saline solution daily via oral gavage for 30 days. NS group received 15 mg/kg bw of AA combined with 1 ml/kg bw of NS oil daily via oral gavage for 30 days. The rats were euthanized, and SMGs were dissected for histological evaluation, including hematoxylin and eosin staining (H&E) and immunohistochemistry for inducible nitric oxide synthase (iNOS), as well as analysis for heme-oxygenase-1 gene (HO-1) expression using real-time Polymerase chain reaction (RT-qPCR). The acinar and ductal cells of SMG of the AA group showed signs of degeneration and toxicity in the form of ill-defined outlines, pyknotic and crescent-shaped nuclei with different-

sized cytoplasmic vacuolations that were statistically significant, with an increase in iNOS immunoexpression and HO-1 gene expression ($p < 0.0001$). NS administration alleviated the toxic effect following AA exposure and down-regulated the iNOS and HO-1 gene expression. The study revealed a significant cytotoxic effect of AA on SMGs of albino rats ($p < 0.05$), presumably by the generation of oxidative stresses and mitochondrial dysfunction. NS effectively mitigated these toxic effects, suggesting its potential as a natural antioxidant.

Keywords: salivary glands, acrylamide, nigella sativa, oxidative stress

1. Introduction

Acrylamide, or acrylic amide (AA), is a solid, odorless, water-soluble compound in the form of white crystals with high chemical activity (1). AA is an industrial chemical compound widely used in chemical industries such as mining, manufacturing of paper, cosmetics, textiles, and wastewater treatment and is considered the foundation for the polymer polyacrylamide (2). Various levels of AA have been reported in many dietary products, particularly fried and baked food that have undergone high-temperature processing in daily life. AA is formed as a by-product of deep frying or cooking of any carbohydrate-rich foods at high temperatures (>120°C). It has been described as a cooking-associated carcinogen due to its spontaneous formation during the cooking process thus, it has become one of the major public health concerns (1).

Acrylamide (AA) is quickly absorbed through the gastrointestinal system and then distributed to other parts of the body via the bloodstream (2). In the liver, AA is detoxified by combining with glutathione (GSH) with the help of the enzyme glutathione S-transferase (GST). This process results in the formation of N-acetyl-S-cysteine, which is then broken down and eliminated through urine. However, this

detoxification process reduces GSH levels, leading to decreased antioxidant capacity and increased oxidative stress. Additionally, AA can be transformed by the cytochrome CYP2E1 enzyme to produce Glycidamide (GA), a compound linked to the harmful and cancer-causing properties of AA. The interaction between oxidative stress and GA production worsens the damaging effects of AA, including its mutagenic and carcinogenic properties (3).

Several studies indicated that AA exposure can produce neurotoxicity, hepatotoxicity, nephrotoxicity, and reproductive toxicity (1,4). Moreover, numerous studies have evaluated the toxic effect of AA on different oral tissues, including salivary glands, tongue, and soft palate (5,6).

Considering the possibility of extensive oxidative stress and genotoxicity caused by AA, investigating preventative measures is essential. One such measure could be using Nigella Sativa (NS), a herbaceous plant traditionally utilized for its medicinal properties. NS treats different conditions like asthma, headache, dizziness, hypertension, inflammation, cough, bronchitis, diabetes, eczema, fever and gastrointestinal disturbances (7). Notably, NS oil is recognized as a powerful antioxidant, anti-inflammatory, immunostimulatory, and anti-apoptotic agent. These properties position NS as a promising candidate for mitigating cellular damage caused by oxidative stress, particularly in the context of exposure to food toxins like AA (8). NS oil and its active constituents, thymoquinones, can decrease oxidative stress levels while upregulating GSH and other antioxidant enzymes such as catalase and superoxide dismutase (SOD) (7,8).

Despite the known toxicity of AA on various tissues, few studies have explored its specific impact on submandibular salivary glands. This study aims to fill that gap and investigate the potential protective role of NS against AA-induced damage in the submandibular salivary gland of Albino rats. The null hypothesis is

that there is no significant protective effect of Nigella sativa (NS) oil against acrylamide (AA)-induced toxicity in the submandibular salivary glands of albino rats.

2. Materials and Methods:

2.1 Animals

This study was granted ethical approval (474/2022) from the Faculty of Dentistry, Suez Canal University. Sample size calculation was performed using G*Power version 3.1.9.2 (University Kiel, Germany). The effect size was 0.95 using α level of 0.05 and β level of 0.05, i.e., power = 95%; the estimated sample size (n) was a total of 30 rats (9). Thirty male Albino rats weighing 150 – 200gm were housed in a sterile, controlled environment (temperature 25 ± 2 C^{o} and 12-hour dark/light cycles) and fed with a standard laboratory diet and tap water during the study. The rats were kept in individual cages, 5 rats per cage. The size of the cage was 20 cm in width and 40 cm in length. Following an adaptation period of 1 week, the rats were equally and randomly divided into three groups (n=10) as follows:

Control group: received normal saline vehicle daily via oral gavage for 30 days.

AA group: received 15 mg/kg body weight (bw) of AA (Advent Chembio Private Limited Company, Navi Mumbai, India (CAS No. 79-06-1) dissolved in 0.2 ml saline solution daily via oral gavage for 30 days.

AA+NS group received 15 mg/kg bw of AA dissolved in 0.2 ml saline solution, and 1 ml/kg bw of NS oil (Imtenan Health shop company, Obour City, Cairo, Egypt) after AA administration, daily via oral gavage for 30 days (10).

2.2 Histological and Immunohistochemical Procedures

Following the experiment period, animals were sacrificed via an extra dose of anesthesia. All rats' major submandibular glands (SMG) were excised. Half the specimens were fixed overnight in buffered 10% formalin, then embedded in paraffin sections of 5 µ thickness and prepared for subsequent Hematoxylin & Eosin (H&E) stain and immunohistochemical (IHC) detection of inducible Nitric Oxide Synthase (iNOS). iNOS rabbit polyclonal antibody (Thermo Fisher scientific, Anatomical pathology, Tudor Road, Manor Park, Runcorn, Cheshire WA7 ITA, UK (7.0 ml) was used for reactive oxygen species (ROS) identification with brown cytoplasmic expression. In contrast, the other half was prepared for Polymerase chain reaction (PCR) examination. The slides were examined and photographed under a light microscope (Leica DM 1000, Danaher Corporation, United States) (11).

The assessment of the expression of iNOS involved determining the proportion of cells with positive immunostaining per 100 cells in 10 fields for each group. Image analysis was performed using Image J (1.46a, NIH, USA) software.

2.3 Quantitative real-time Polymerase chain reaction (RT-qPCR)

Analysis of Heme Oxygenase-1 (HO-1) gene expression using quantitative real-time Polymerase chain reaction (RT-qPCR) was performed using RT-qPCR to evaluate levels of ROS. Tissue Homogenization was performed using the Tissue Ruptor II (Qiagen, Hilden, Germany) in the presence of lysis buffer for 15‑90 seconds. Then, the mixture was centrifugated for 20 mins at 4000rpm. Finally, the cell supernatant was collected for RNA extraction (12). Then, RNA extraction and purification were performed using the RNeasy Mini kit (Qiagen, Hilden, Germany). After that, the reverse transcription step was performed by the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany), and the HO-1 gene expression level was amplified using QuantiTect primer assay and QuantiTect SYBR Green PCR Kit

(Qiagen, Germany). The relative changes in gene expression between the two compared sequences were calculated using the 2-ΔΔCt method (13).

2.4 Statistical analysis

All data were calculated, tabulated, and statistically analyzed using the computer program SPSS software for Windows version 25.0 (Statistical Package for Social Science, Armonk, NY: IBM Corp) at significant levels 0.05 ($p<$ 0.05). One-way ANOVA (Analysis of variance) was used to compare data, and Tukey's post hoc test was performed to evaluate statistical significance among the groups. Data were expressed as mean±standard deviation and range (Max-Min); the value of $p < 0.05$ was considered statistically significant. Independent Student's T-test was performed to compare the mean differences between the two materials at the same method at *p*-value <0.05.

3. Results

3.1 Histological results

Histological results revealed regular histological features of the parenchymal element and connective tissue (CT) stroma in the control group. In the AA group, the serous acini had ill-defined outlines and pyknotic and crescent-shaped nuclei with different-sized cytoplasmic vacuolations. The striated duct cells showed signs of degeneration and loss of normal cell lining, basal striations, cell height with the presence of cytoplasmic vacuolation within the cells. Granular convoluted tubules (GCTs) showed cytoplasmic vacuolations and a marked decrease in granularity and eosinophilia. On the other hand, the NS group showed SMG regained their normal appearance. However, the minimum degree of atrophic changes among the acini and ducts were encountered in some regions. Serous acini lined by pyramidal-shaped cells with well-defined cell boundaries and apparently fewer cytoplasmic vacuolations were observed. The striated ducts showed an almost normal cell lining

maintaining their normal basal striations with few cytoplasmic vacuolations, while GCTs presented signs of degeneration (Figure 1).

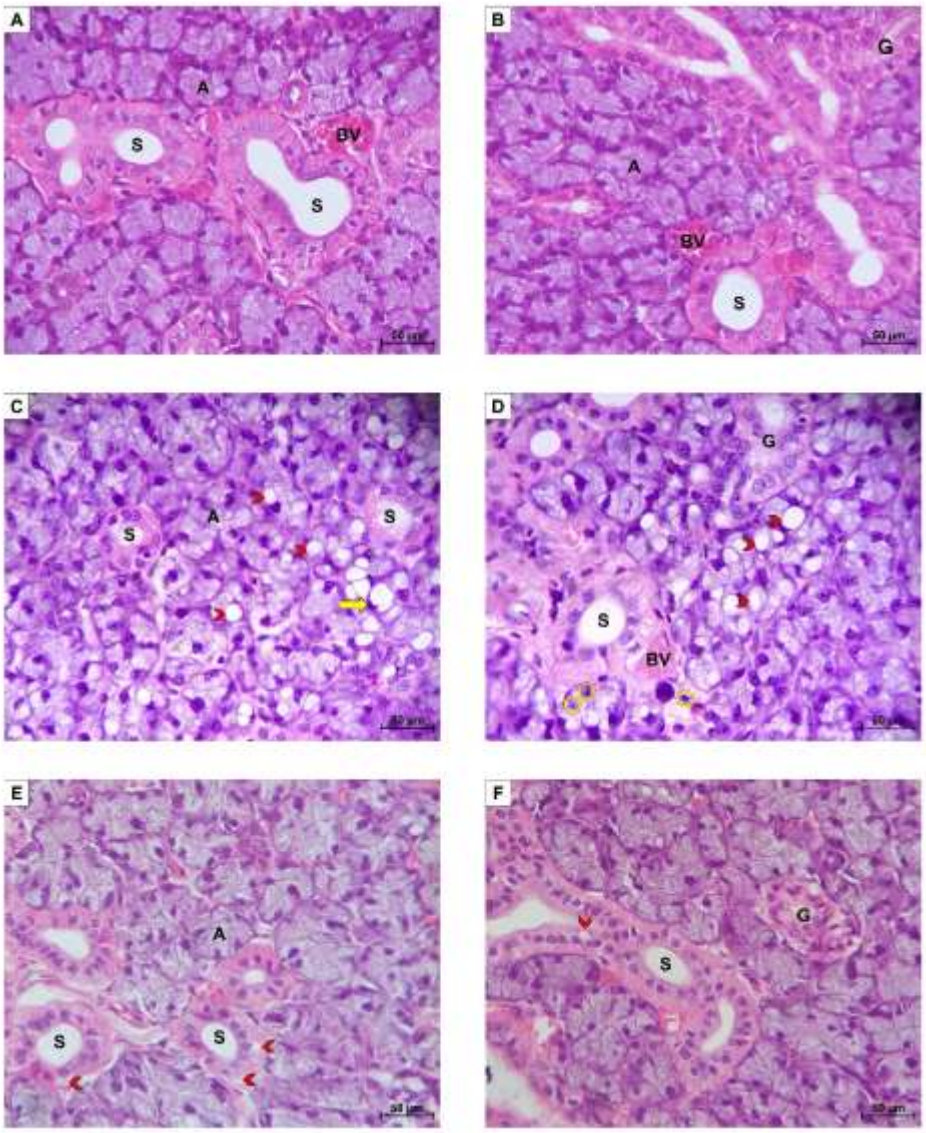

Figure 1. Hematoxylin & Eosin (H&E) section of SMG of albino rats. (A, B) the control group showed normal gland architecture. (C, D) Acrylamide (AA) group showing serous acini with ill-defined outline (A) and crescent shaped nuclei (arrow), pyknotic nuclei (yellow circle) and different sized cytoplasmic vacuolations (arrow heads). GCTs (G) with cytoplasmic vacuolations and marked decrease in their granularity and eosinophilia as well as striated ducts (S) with loss of normal cell lining, basal striations and different sized cytoplasmic vacuolations associated with congested blood vessels (BV) were also observed. (E, F) NS group showed serous

acini (A) with well-defined cell boundaries and apparently less cytoplasmic vacuolations, striated ducts (S) maintaining their normal basal striations with few cytoplasmic vacuolations (arrow heads) and GCTs (G) showing signs of degeneration (H&E original mag.x400). A, acini; S, striated duct; G, GCTs; BV, blood vessel.

3.2 Immunohistochemical expression of iNOS

The control group showed a very weak to mild immunoreactivity for iNOS among all glandular elements, which slightly increased in the duct system compared to the acinar portions. The AA group revealed a marked increase in the cytoplasmic iNOS immunoexpression throughout the SMG's parenchyma. The acini showed moderate immunoreactivity, while the entire duct system revealed a strong immunostaining intensity. As for NS treated group, a markedly reduced iNOS immunoreactivity was detected throughout the whole glandular parenchyma, where the acini reacted weakly to iNOS and the duct system presented a mild immunoreaction to iNOS (Figure 2).

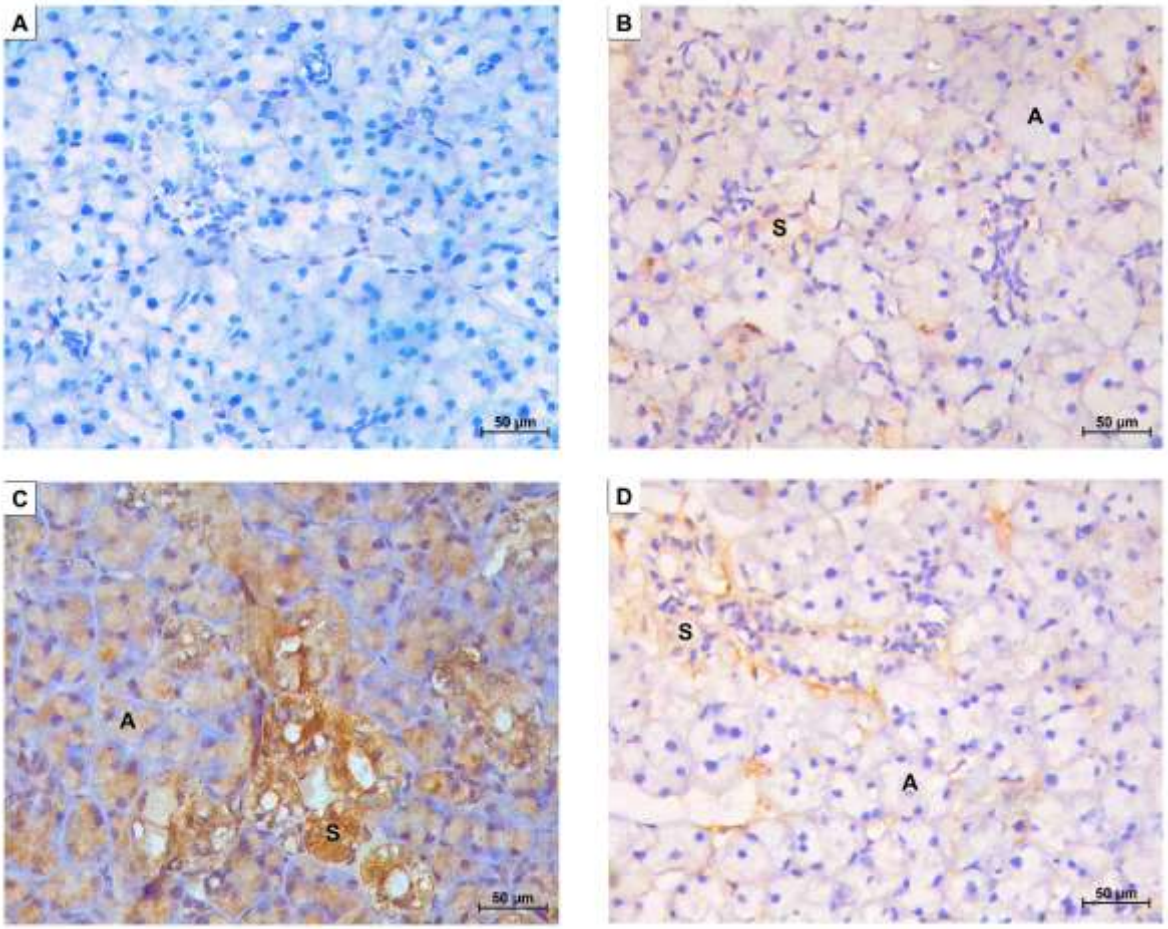

Figure 2. An immunostained section of iNOS antibody albino rats' SMG. (A) photomicrograph of the control group incubated with non-specific serum and color developed by DAP showing negative staining reaction of all the gland component. (B) control group showing weak to mild immunoreactivity to iNOS. (C) AA group showed moderate immunoreactivity to iNOS in the acini (A), while the entire duct system revealed a strong immunostaining intensity (S). (D) NS group showed weak reaction to iNOS in the acini (A) and a mild immunoreaction in the duct system (S) (original mag. X400).

Image analysis revealed that the AA group recorded the highest mean area % of iNOS immunoexpression. In contrast, the lowest was recorded in the control

group, and a statistically significant difference occurred between the whole studied groups ($p < 0.05$). A highly significant increase in the mean area % of iNOS immunoexpression ($p < 0.0001$) was recorded by comparing the AA group to the control group. Meanwhile, there was a non-significant increase in iNOS immunoexpression in the AA+NS group compared to the control group ($p = 0.0757$). Furthermore, a highly significant decrease in the mean area % of iNOS immunoexpression was recorded by comparing the AA+NS group to the AA group ($p < 0.0001$) (Figure 3).

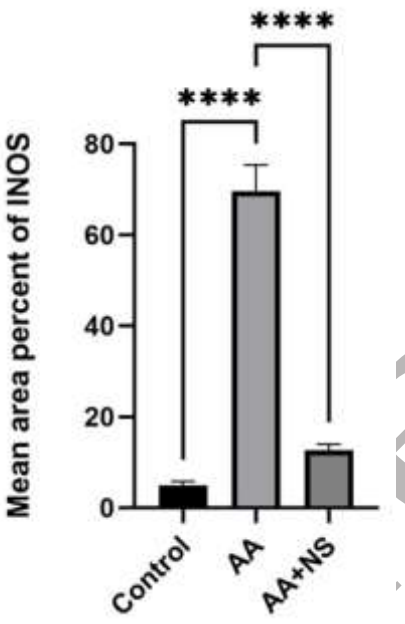

Figure 3. Bar chart of means and standard deviations of iNOS mean area percent expression within experimental groups. Significance levels are **** $p < 0.0001$

3.3 RT-PCR

The highest mean HO-1 gene expression was recorded in the AA group. In contrast, the lowest was recorded in the control group, and a statistically significant difference occurred between the whole studied groups ($p < 0.05$). Tukey's post hoc pairwise comparison revealed a highly significant increase in the mean HO-1 gene expression in the AA group and AA+NS group compared to the control group ($p <$

0.0001). On the other hand, a significant decrease in the mean HO-1 gene expression was recorded when comparing the AA+NS group to the AA group ($p < 0.001$) (Figure 4).

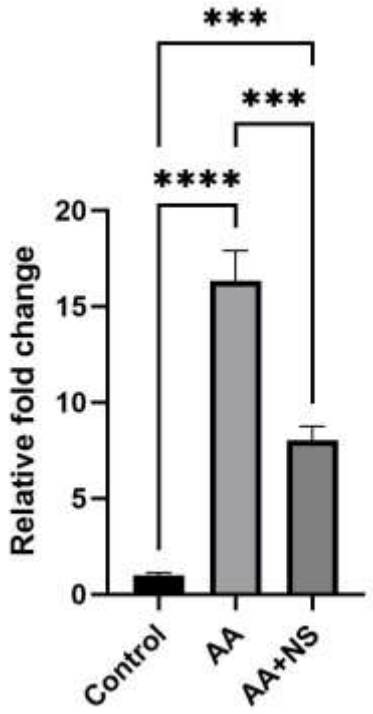

Figure 4. Bar chart of means and standard deviations of HO-1expression within experimental groups. Significance levels are as follows: *** $p < 0.001$, and **** $p < 0.0001$

4. Discussion

Acrylamide (AA) is an unsaturated amide with a high chemical activity that has been widely used in chemical industries and various consumer products. Owing to its various exposure routes, small molecular size, and high-water solubility that facilitate its rapid absorption and distribution throughout the body, AA toxicity has been thoroughly studied on human and experimental animals on different organs and systems. Given that dietary intake is considered the key source of AA exposure in humans (3), it only seemed logical that oral administration should be the route of choice in this study. AA dose was chosen based on the findings where it induced

chronic AA toxicity in the parotid gland of albino rats. Furthermore, the selected dose is safely below the lethal dose (LD50) for AA in rats, which is 150 mg/kg.bw (14).

In the present study, histological examination of the SMG of the AA group showed marked signs of degeneration in the parenchymal elements of the gland, suggesting that AA has a potential cytotoxic effect on the acinar and ductal cells. Similar signs of degeneration in the acini and ducts of SMG were observed in another study following AA exposure (5). Moreover, the same dose and duration of AA used in our study resulted in degenerative changes in the parotid gland architecture (15). Both studies attributed these degenerative processes to AA generating excessive oxidative stresses that lead to mitochondrial dysfunction.

Excessive oxidative stress production, in turn, impairs mitochondrial function, which is crucial for energy production and cell survival, resulting in mitochondrial membrane damage and a decline in the Bcl-2/Bax ratio, initiating the intrinsic apoptotic pathway causing cell death (16). Additionally, mitochondrial dysfunction impairs cellular metabolic processes such as glycolysis and respiration, exacerbating oxidative damage. The observed histological changes in this study in the acini as well as the loss of basal striations in the striated ducts, can be directly linked to AA-induced oxidative stress and mitochondrial dysfunction. Accumulation of oxidative stresses leads to mitochondrial degeneration and loss of basal infoldings due to AA toxicity (5). This unified mechanism of damage aligns with previous studies, further highlighting the role of ROS in AA toxicity (16,17). Moreover, the current results are consistent with the findings of Liu, Song (17), who concluded that AA hinders cell metabolic activity by suppressing the expression of complex I, III, and IV subunits and anaerobic glycolysis and mitochondrial respiration.

Different-sized cytoplasmic vacuolations observed histologically in the acinar and ductal cells in the experimental groups were consistent with other studies

that investigated AA toxicity on submandibular and parotid salivary glands (5,15). According to Hamza, Aly (18) in cases of high oxidative stress and lipid peroxidation (LPO), vacuolization reflects cellular swelling, where the failure of the energy-dependent Na^+- K^+ ion pumps in the plasma membranes occurs. Consequently, this leads to intracellular accumulation of Na+ and gradual osmolarity shifts that allow water entry into the cells.

Nitric oxide synthases (NOSs) are a class of enzymes that convert L-arginine to L-citrulline, resulting in the formation of nitric oxide (NO), a free radical and essential cellular signaling molecule. Inducible nitric oxide synthase (iNOS), an isoform of NOS, is produced only when a cell is activated or triggered, usually by proinflammatory cytokines and/or bacterial lipopolysaccharides (19). Moreover, several studies demonstrated an elevation in iNOS expression in cases of oxidative stress, leading to NO generation (19). This may explain the elevation in iNOS immunoexpression noted in the AA group, which supports the hypothesis that the cytotoxic damage may indicate an inflammatory process and oxidative stress.

On the other hand, heme oxygenase-1 (HO-1) is an inducible enzyme triggered by oxidative stress, catalyzing heme degradation and preventing apoptosis in response to proinflammatory agonists, thereby minimizing the detrimental effects of inflammation. The up-surge of HO-1 gene expression in the AA group also aligns with Facchinetti (20), who reported elevated ROS or inflammatory mediators could account for increased HO- 1 expression. Moreover, HO-1 expression is usually minimal or nonexistent under homeostatic conditions but is dramatically upregulated in response to pro-oxidant stimuli, protecting against oxidative damage (21).

This research chose Nigella sativa (NS) oil as a protective dietary component against AA toxicity, owing to the reported antioxidant, anti-apoptotic, anti-inflammatory, antiviral, and immunomodulatory activities. The selected dose has

been reportedly effective in ameliorating oxidative stress damage in different brain regions of rats, including the cerebellum, cortex, and hippocampus (10). In the present study, NS oil intake demonstrated a cytoprotective effect against the harmful impact of AA on the SMG of rats, as evidenced by histological and immunohistochemical analysis and molecular analysis. This agrees with several studies investigating NS oil's effect on oxidative stress-induced toxicity in different tissues (22). This cytoprotective effect of NS oil can be attributed to its anti-apoptotic, antioxidative, and anti-inflammatory properties, which was evident by a significant down-regulation of iNOS immunoexpression and HO-1 gene expression. It was reported that NS supplementation significantly increased antioxidant enzyme levels (GSH and SOD), reduced LPO levels, and down-regulated pro-inflammatory mediators following AA-induced oxidative stress (23). Studies have also reported the ability of NS to down-regulate iNOS immunoexpression, inhibiting NO production and highlighting its powerful antioxidant ability (24,25). Moreover, studies have confirmed the capacity of NS oil to mitigate oxidative stresses and inflammation through a dose-dependent inhibition in HO-1 gene expression after NS oil administration (26,27).

AA administration has a cytotoxic effect on the parenchymal element of SMG due to excessive production of oxidative stress. NS oil treatment exerted an apparent therapeutic effect against AA-induced toxicity on SMG making. It is a promising candidate to combat oxidative stress, inflammation, and apoptosis.

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Authors' contribution

Study concept and design: S.A.T, E.F.M.

Analysis and interpretation of data: R.M.T, M.S.

Investigation: S.A.T, M.S.

Drafting of the manuscript: S.A.T

Critical revision of the manuscript for important intellectual content: E.F.M, R.M.T, M.S.

Study supervision, E.F.M, R.M.T.

Ethics

The research was granted ethical approval (474/2022) under the guidelines of animal experimentation and reviewed by the Faculty of Dentistry's research ethics committee (REC), Suez Canal University, Egypt.

Conflict of interest

The authors declare that they have no conflict of interest.

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Data Availability

The data used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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