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EFFECTS OF OMEGA 3 POLYUNSATURATED FATTY ACID AGAINST ACRYLAMIDE-INDUCED TOXICITY IN SUBMANDIBULAR SALIVARY GLANDS OF ALBINO RATS: A HISTOLOGICAL AND MOLECULAR STUDY

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KEYWORDS

Acrylamide, Omega 3, Oxidative stress, Salivary glands

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ABSTRACT

Introduction: Acrylamide (AA), a water-soluble compound with high chemical activity that can be found in widely consumed food products. **Aim:** To evaluate the protective effect of Omega 3 polyunsaturated fatty acid (ω3-PUFAs) against AA induced toxicity on the submandibular salivary glands (SMGs) of Albino rats. **Materials and methods:** Thirty male albino rats weighing 150 – 200 gm were equally and randomly divided into control group, which received normal saline vehicle daily via oral gavage for 30 days, AA group received 15 mg/kg body weight (bw) of AA dissolved in 0.2 ml saline solution daily via oral gavage for 30 days. ω3-PUFAs group received 15 mg/kg bw of AA combined with 0.4 g/kg of ω3-PUFAs 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 tumor necrosis factor (TNF-α), as well as analysis for heme-oxygenase-1 gene (HO-1) expression using real-time Polymerase chain reaction (RT-qPCR). **Results:** The SMG of AA group showed signs of toxicity and degeneration in the form of ill-defined outlines with different-sized cytoplasmic vacuolations, pyknotic and crescent-shaped nuclei that were statistically significant, with an increase in TNF-α immunoexpression and HO-1 gene expression. ω3-PUFAs administration mitigated the toxic effect following AA exposure and down-regulated the TNF-α and HO-1 gene expression. **Conclusion:** The study revealed a significant cytotoxic effect of AA on SMGs of albino rats, presumably by generation of oxidative stresses and mitochondrial dysfunction. ω3-PUFAs effectively alleviated these toxic effects, indicating its antioxidant potential.

INTRODUCTION

Acrylamide (AA) is an m industrial compound used in the synthesis of polyacrylamide [1,2]. Various levels of AA have been reported in many dietary products, particularly fried food that have reached a heating temperature exceeding 120 ◦C, as well as coffee and cigarette smoke[3-5].

Continuous exposure of individuals to AA in daily activities has increased public health attention to its possible toxic effects on different tissues[6,7]. Studies have proved that AA is a powerful chemical compound with the ability to induce toxicity on different organs including liver, kidney, brain, as well as carcinogenicity, reproductive and genotoxicity[3,6,8]. AA toxicity is strongly believed to produce oxidative stresses, inflammatory responses and apoptosis [9, 10]. The assessment of AA toxicity on different oral tissues including salivary

glands (SG), tongue and soft palate resulted in histological and ultrastructural changes proving its toxic effect on these tissues [11, 12].

Antioxidants are compounds that can interact with free radicals thereby mitigating their potential damage to biological molecules [13]. Their clinical applications are diverse, encompassing anti-inflammatory, anti-microbial, anti-cancer, as well as nephroprotective, hepatoprotective and neuroprotective effects [14]. Natural dietary products and supplements as Omega 3 polyunsaturated fatty acid (ω3-PUFAs) have antioxidant properties that has been proved effective against different toxic compounds including AA [15, 16].

Omega 3 polyunsaturated fatty acids (ω3-PUFAs) are natural supplements that have to be introduced through diet, fish and other seafood like algae are considered the main source for ω3-PUFAs as well as some nuts and seeds [17]. Several studies have reported the strong, anti-inflammatory, anti-apoptotic and antioxidant potentials of ω3-PUFAs against different food toxins [17, 18], which gives it the capacity to suppress oxidative stress, lipid peroxidation production and pro-inflammatory cytokines levels after exposure to different toxins [18]. This research aims to evaluate the possible ameliorative effect of ω3-PUFAs against AA-induced toxicity in the submandibular salivary gland (SMG) of Albino rats.

MATERIALS AND METHODS

Animals

This study was conducted following animal experimentation guidelines and granted ethical approval **(474/2022)** from the Faculty of Dentistry, Suez Canal University. Thirty male Albino rats weighing 150 – 200gm were housed in controlled and sterile environment and fed with standard pellets diet and tap water during the study. 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 [11].
- ***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 [11].

AA+ ω3-PUFAs group received 15 mg/kg bw of AA dissolved in 0.2 ml saline solution [11], and 0.4 g/kg (b.w) of ω3-PUFAs (Nordic naturals company, Watsonville, California, USA (LOT 224314)) daily via oral gavage for 30 days [19].

Histological and Immunohistochemical Procedures:

Following the experimental period, animals were sacrificed via extra dose anesthesia. The glands were excised and half the specimens were fixed overnight in buffered 10% formalin then embedded in paraffin sections of 5 microns (μ) thickness to be stained with Hematoxylin & Eosin (H&E) stain and immunohistochemical (IHC) detection of tumor necrosis factor (TNF-α). The other half was prepared for Polymerase chain reaction (PCR) examination. A light microscope was then used for slides examination (Leica DM 1000, Danaher Corporation, United States) [20].

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 executed 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

[21]. Then, extraction and purification of RNA was done via the RNeasy Mini kit (Qiagen, Hilden, Germany). QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) was then used for reverse transcription step, and the HO-1 gene expression level was amplified using QuantiTect primer assay and QuantiTect SYBR Green PCR Kit (Qiagen, Germany). 2-ΔΔCt method was used for calculation of the relative changes in gene expression between the two compared sequences [22].

Statistical analysis

SPSS software program (version 25.0) was used for data calculation and statistical analysis (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); where $p<0.05$ was considered statistically significant. Independent Student's T-test was performed for comparing the mean differences between the two materials at the same method at p-value <0.05.

RESULTS

Histological results

Histological results of the control group revealed normal histological features of the parenchymal element and connective tissue (CT) stroma. In the AA group, the serous acini showed different-sized cytoplasmic vacuolations, ill-defined outlines with pyknotic and crescent-shaped nuclei. The striated duct cells showed loss of normal cell lining, basal striations, cell height, and vacuolation in the ductal cells. The excretory duct showed loss of normal pseudostratification, cytoplasmic vacuolation, hyalinization of the surrounding CT stroma with areas of fiber dissociation. Conversely, AA+ω3-PUFAs group revealed that the gland has almost regained their normal histological appearance with minor degree of atrophic changes in some regions. The serous acini revealed an almost normal histological appearance and apparently less cytoplasmic vacuolations. Striated ducts maintained normal basal striations with apparently less cytoplasmic vacuolations. Excretory duct showed signs of degeneration with cytoplasmic vacuolations, hyalinization of the surrounding CT with areas of fiber dissociation **(Figure 1)**.

Immunohistochemical expression of TNF-α:

The control group showed a mild immunoreactivity for TNF-α among all glandular elements that slightly increased in the duct system compared to the acinar portions. The AA group revealed a marked increase in the cytoplasmic TNF-α immunoexpression throughout the whole gland's parenchyma. The acini showed moderate immunoreactivity, while the entire duct system revealed a strong immunostaining intensity. Meanwhile, an apparent decrease in TNF-α immunoreactivity was detected throughout the whole glandular parenchyma, where a mild to moderate cytoplasmic immunoreactivity was detected in the acini, while the duct system presented a moderate immunoreaction to TNF-α in ω3-PUFAs treated group **(Figure 2)**.

Image analysis revealed a statistically significant difference occurred between the whole studied groups (p <0.05). AA group recorded the highest mean area % of TNF-α immunoexpression, while the lowest was recorded in the Control group. A highly significant increase in TNF-α immunoexpression was recorded on comparing AA group to Control group (p <0.0001). Furthermore, a significant increase in TNF-α immunoexpression was recorded in AA+ω3-PUFAs group when compared to control group ($p = 0.0018$). Meanwhile, a highly significant decrease in TNF-α immunoexpression was found on comparing AA+ω3-PUFAs group to AA group (p <0.0001) **(Figure 3)**.

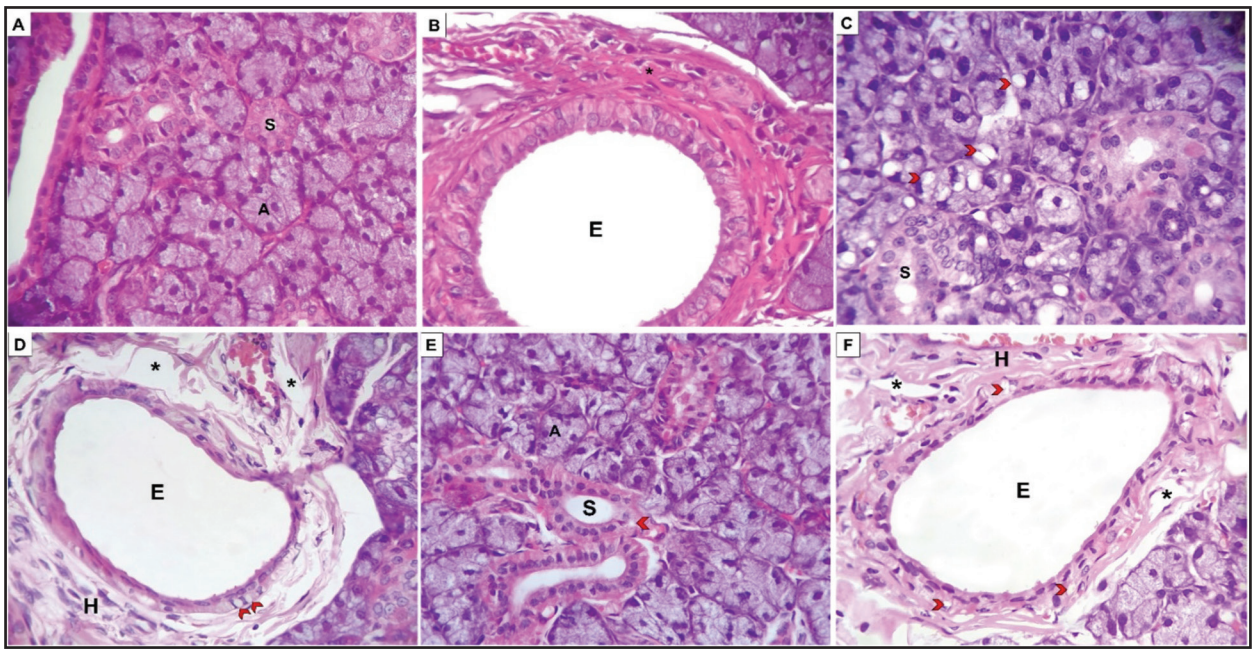

Fig. (1) Hematoxylin & Eosin (H&E) section of SMG of albino rats. (A, B) the control group showed normal gland architecture. (C, D) Acrylamide (AA) group showing serous acini with ill-defined outline, and different sized cytoplasmic vacuolations (arrow heads). Striated duct (S) with cytoplasmic vacuolations and loss of normal cell lining and basal striation as well as excretory duct (E) with loss of pseudostratification, cytoplasmic vacuolations (arrow heads), hyalinization (H) of the surrounding CT with areas of fibers dissociation (*). (E, F) ω3-PUFAs 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 excretory duct (E) showing signs of degeneration.

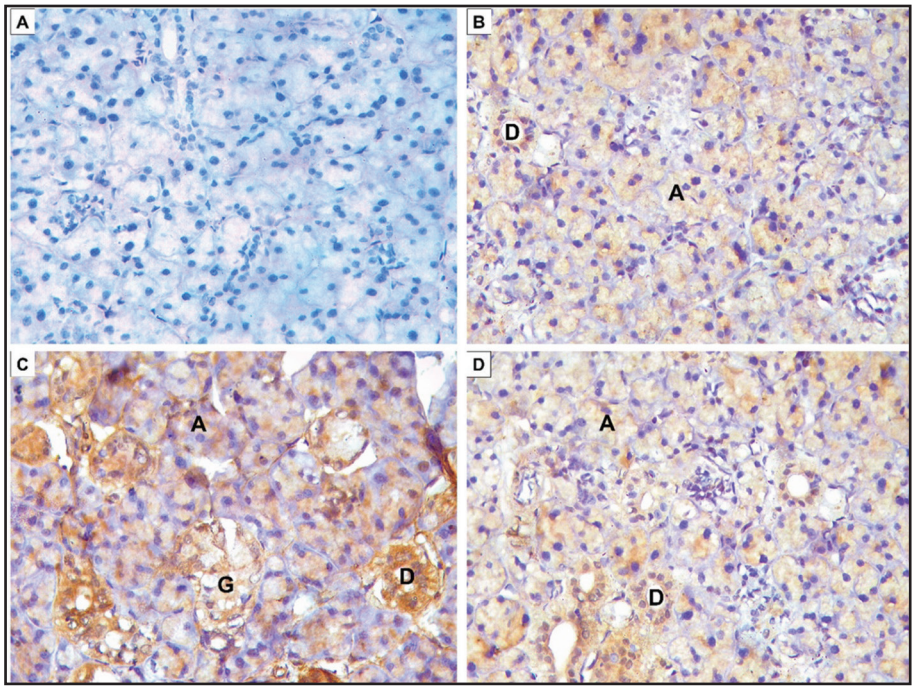

Fig. (2) An immunostained section of TNF-α antibody albino rats' SMG. (A) photomicrograph of the control group incubated with non-specific serum and color developed by DAP showing negative staining reaction of all the gland component. (B) control group showing mild immunoreactivity to TNF-α. (C) AA group showed moderate immunoreactivity to TNF-α in the acini (A), while the entire duct system revealed a strong immunostaining intensity (D). (D) ω3-PUFAs group showed mild to moderate reaction to TNF-α in the acini (A) and duct system (D) (original mag. X400).

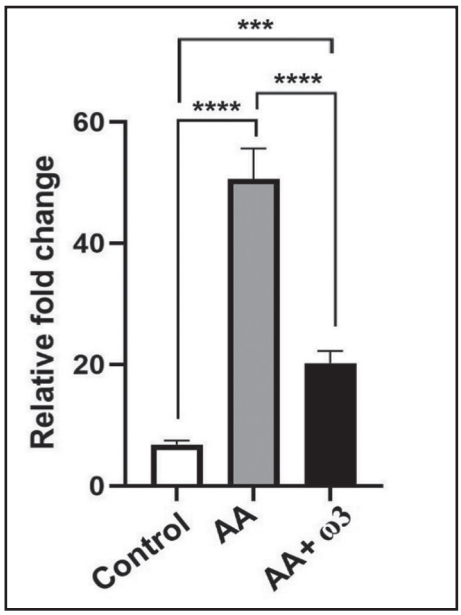

Fig. (3) Bar chart of means and standard deviations of TNF-α mean area percent expression within experimental groups. Significance levels are as follows *** p = 0.0018 and **** p < 0.0001.

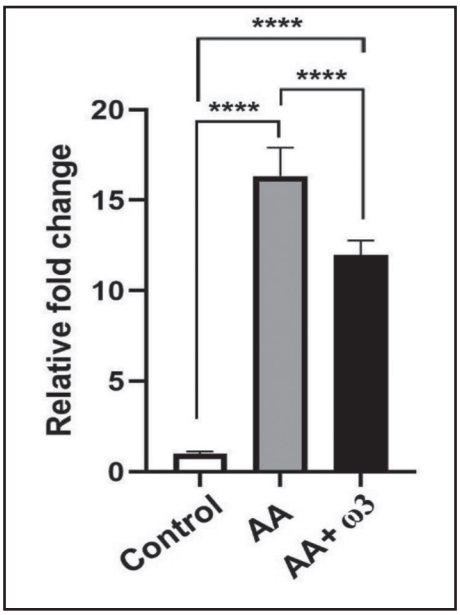

Fig. (4) Bar chart of means and standard deviations of HO-1expression within experimental groups. Significance levels are **** p < 0.0001.

RT-PCR

A statistically significant difference occurred between the whole studied groups ($p < 0.05$). The highest mean HO-1 gene expression was observed in the AA group, while the lowest was observed in the control group. Tukey's post hoc pairwise comparison revealed a highly significant increase in the mean HO-1 gene expression in the AA group and AA+ω3-PUFAs group compared to the control group ($p < 0.0001$) and a highly significant decrease in the mean HO-1 gene expression was recorded when comparing the AA+ ω3-PUFAs group to the AA group ($p < 0.0001$) **(Figure 4)**.

DISCUSSION

Acrylamide (AA) has become a significant public health concern following the identification of its spontaneous formation and accumulation during cooking [23]. AA toxicity has been thoroughly studied on human and experimental animals on different organs and systems [24]. Considering that the primary source of AA exposure in humans is thought to be dietary intake [25], oral administration was the route of choice in this study. The AA dose was chosen based on the findings of Al-Serwi et al. [11] who demonstrated that the long-term toxicity of this dosage caused histological and ultrastructural alterations 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 [26]. Dietary intake of ω-3PUFAs is necessary for maintaining the structural and biochemical integrity of all cells [17, 27]. The use of ω-3PUFAs as a

potential protective dietary supplement in this study was based on their wide range of physiological functions, including antioxidant, anti-inflammatory and anti-apoptotic effect [28].

In the present study, histological analysis of the SMG of AA group revealed a significant apoptotic and degenerative changes in the parenchymal elements of the gland, indicating a potential cytotoxic effect of AA on the parenchymal element of the gland. Similar results were noted in other studies following AA exposure on salivary glands. Both studies attributed these changes to reactive oxygen species (ROS) generation within the cells upon AA administration, leading to mitochondrial disfunction [11, 29]. once absorbed, AA has the ability to combine with glutathione (GSH), a naturally produced antioxidant, generating N-acetyl-S-cysteine. Although this metabolic pathway results in the detoxification of AA, it also consumes GSH body levels and reduces antioxidant balance, eventually leading to accumulation of ROS [25]. Moreover, AA hinders cell metabolic activity by suppressing the expression of complex I, III, and IV subunits, as well as anaerobic glycolysis and mitochondrial respiration. This mitochondrial dysfunction is linked to a decline in Bcl-2/Bax ratio and mitochondrial membrane potential, which in turn activates the mitochondrial-regulated apoptotic signaling pathway [30]. This mitochondrial dysfunction can also explain the loss of basal striations in the striated duct due to mitochondrial degeneration and loss of basal infoldings due to AA toxicity, which is consistent with the findings of **Al-Serwi *et al*.** [11] and **Mahmoud *et al*.** [29].

Acute inflammation results in the production of the inflammatory cytokines, one of which is tumor necrosis factor alpha (TNF-α), which is secreted primarily by macrophages and monocytes. Studies have linked ROS production to elevated levels of inflammatory cytokines, including TNF-α, elucidating the interplay between inflammatory process and oxidative stress, which could explain the elevated level of TNF-α in AA group [31, 32].

On the other hand, heme oxygenases are stress proteins and multifunctional enzymes that are responsible for acceleration of the breakdown of heme. Heme oxygenase-1 (HO-1) 1 is an inducible enzyme triggered by oxidative stress, responsible for heme degradation and preventing apoptosis due to inflammatory process, minimizing the detrimental effects of inflammation [33]. The increase in HO-1 gene expression in AA group aligns with Facchinetti [34] who noted that increased HO-1 expression could be attributed to excessive ROS production or inflammatory mediators.

The selected dose and duration of ω-3PUFAs have been proved effective in protection from oxidative stress damage in cases where oxidant/antioxidant defense mechanisms are disturbed [19]. In this research, ω3-PUFAs supplementation alleviated the detrimental effect of AA on rat SMG, as revealed via histological and immunohistochemical and molecular analysis. This aligns with **El-Baz D and Salem** [35] who reported that administration of ω3-PUFAs improved age-related changes in SMG. Moreover, **Hassanin and Shenouda** [36] noted an almost normal cell lining of the acini and ducts of the parotid gland with few cytoplasmic vacuolations following ω3-PUFAs administration against tetrazine toxicity. This cytoprotective effect could be accounted to the antioxidant, anti-inflammatory and antiapoptotic capacity of ω3-PUFAs [28]. This ameliorating effect was confirmed by the significant reduction in TNF-α immunoexpression in AA+ ω3-PUFAs group compared to AA group which come in line with **Elblehi *et al*.** [18] who reported that ω3-PUFAs effectively suppressed TNF-α levels in AA induced rats indicating its neuroprotective capacity.

Furthermore, a significant decrease in the mean HO-1 gene expression was also noted in the present study. This agrees with the findings of **Wiest *et al.***[37], who noted a down-regulation in HO-1 gene expression upon ω3-PUFAs administration against cigarette smoke-induced oxidative stress.

CONCLUSIONS

Acrylamide (AA) caused a marked degenerative change on the acini and ducts of SMG due to excessive production of oxidative stress. ω3-PUFAs treatment can reverse histological and immunohistochemical changes following AA exposure restoring the normal features of the SMG indicating its powerful cytoprotective effect.

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