

Original Article

Evaluation of the Olive Oil Effect on the Nitropropane-Induced Submandibular Gland Changes: An Immunohistochemical, histopathological and morphometric Study

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Abstract

Objectives: Nowadays there is great evidence of the possible health benefits of olive oil. 2-Nitropropane (2-NP) is a rat liver carcinogen. The present work is focused on the effect of olive oil on the nitropropane-induced submandibular gland changes and to determine the role of TNF- α .

Materials and Methods: BALB/c mice were divided into three groups (10 mice each): control mice, 2-Nitropropane inject group mice, and 2-Nitropropane inject group treated with Olive. Submandibular gland tissues in the previous groups were histologically studied. TNF- α is investigated by using immunohistochemical technique.

Results: Administration of 100 mg/kg 2-NP to BALB/c mice resulted in well-documented histopathological changes in the submandibular salivary glands manifested as degeneration, atrophy and preductal inflammatory cell infiltration. Olive oil administration reduced the 2-NP deleterious effect on the submandibular salivary glands. This was manifested less degenerative and atrophic changes. Immunohistochemical analysis for TNF- α was more intense in the second group and weak in the third group.

Conclusions: The findings conclude that 2-NP caused significant tissue damage. Olive oil may attenuate the alterations of 2-NP in BALB/c mice. The expression of TNF- α may be useful as an indicator of 2-NP effect.

Keywords: Olive oil, 2-Nitropropane, Submandibular Salivary Glands

1. Introduction

The submandibular gland is a major salivary gland located beneath the floor of the mouth. It is paired and contributes some 67% of unstimulated saliva. Various disorders affecting the major salivary glands are known. Sialadenitis is inflammation of a salivary gland caused by infections, irradiation, allergy or trauma (1). The nitropropane (2-NP) is a colourless, oily liquid with a mild odour used as a solvent, principally in

blends besides many industrial applications. It has also been used to separate closely related substances such as fatty acids, as intermediate in chemical syntheses, and as a fuel additive. 2-NP appears to be highly mobile in the natural environment. Since it is slightly water soluble, slightly adsorbed by sediment, slightly bio-accumulated, and it evaporates readily into the atmosphere, it will be distributed in both air and

water and not accumulated in any individual. General population exposure to 2-NP appears to be low and is derived from cigarette smoke, from residues in coatings, adhesives and print, and from vegetable oils fractionated with 2-NP (2). Previously, severe liver damage, as well as some kidney damage, has been observed in workers fatally poisoned from acute exposure to high concentrations of 2-nitropropane (3). Other results indicated that 2-NP inflicted DNA damage in the bone marrow cells and thus could be leukemogenic (4).

Studies were carried out in the rat in which micronucleus induction (bone marrow and liver) and unscheduled DNA syntheses (UDS) inductions (liver) were measured after treatment with nitropropane isomer. 2-NP induced UDS in the liver thus confirming the previous studies. In the bone marrow micronucleus test (BMMN test), occasional small increases in the incidence of micronuclei were found for both compounds, but results were interpreted as negative after considering the control background data and the lack of reproducibility. By contrast, the liver micronucleus test revealed a clastogenic effect of 2-NP in the liver. This indicated that 2-NP induces chromosome aberrations as well as DNA repair *in vivo*, but it seems to act organo-specifically. For 1-NP a slightly increased incidence of micronuclei was found in the liver, which was accompanied by a markedly increased mitotic index (5).

Olive oil is a liquid fat obtained from olives by pressing whole olives. It is commonly used in cooking, cosmetics, pharmaceuticals, and soaps, and as a fuel for traditional oil lamps. It was evidenced that increased dietary ω -3 polyunsaturated fatty acids and dietary extra virgin olive oil have beneficial synergistic effects on lipid metabolism and oxidative stress in patients with metabolic syndrome (6). Analysis of liver miRNAs showed a selective modulation of certain miRNAs by hybrid palm oil which has been proposed to be somehow equivalent to extra virgin olive oil (7). Use of extra-virgin olive oil (EVOO) combined with donepezil offered an

environmental compartment. effective therapeutic approach by enhancing the noncholinergic mechanisms of donepezil and by providing additional mechanisms to attenuate attenuating amyloid- β (A β) -related pathology in Alzheimer's disease patients. They significantly reduced A β load and related pathological changes. Reduced A β load could be explained, at least in part, by enhancing A β clearance pathways including blood-brain barrier (BBB) clearance and enzymatic degradation, and shifting amyloid precursor protein processing toward the nonamyloidogenic pathway. Furthermore, EVOO combination with donepezil up-regulated synaptic proteins enhanced BBB tightness and reduced neuroinflammation associated with A β pathology (8). The olive oil phenolic compounds induced cell maturation *in vitro*, increasing alkaline phosphatase synthesis and reducing the expression of antigens involved in immune functions of the osteoblast which would improve bone density (9).

TNF- α expressed by leukocytes (10) is participating in the regulation of immune responses, inflammatory reactions, and hematopoiesis (11). Tumor necrosis factor receptor-1 (TNFR1) is also involved in apoptosis through extrinsic pathway initiation. The level of soluble TNFR1 is increased in primary Sjögren's syndrome patients. High concentrations of TNF α are detected in the tumor microenvironment, and infiltrating immune cells are thought to be a major source. TNF α is also a tumor-derived factor, expressed in estrogen receptor (ER+) tumor epithelial cells and regulated by 17- β -estradiol (E2) (12). The present work was focused on the effect of olive oil on the nitropropane-induced submandibular gland changes and to investigate the role of TNF α .

2. Material and methods

30 adult BALB/c male mice weighting 25–30 g/each were used. They were purchased from the Institute of Theodor Bilharz (Cairo, Egypt), maintained and monitored in a specific pathogen-free environment. All experimental animal protocols were performed according to regulations

set by the Institutional Animal Care and Use Committee and were approved by Al-Azhar University. All animal procedures were also performed according to the Declaration of Helsinki and the guidelines for the care and use of experimental animals established by the National Institutes of Health (NIH). They were allowed to acclimatize in cages (five animals per cage) inside a well-ventilated room for one week. They were maintained under standard laboratory conditions (temperature of 23°C, relative humidity of 60–70%, and a 12 h light/dark cycle) and were fed a diet of standard commercial pellets and water containing libitum. We made every effort to minimize animal stress. After 1 week of acclimatization, mice were randomly categorized into 3 groups (10 mice each): control mice, 2-Nitropropane inject group mice, and 2-Nitropropane inject group treated with Olive. Oral toxicity was induced in mice in the latter two groups (n = 20) by intraperitoneal (i.p.) injection of 2-Nitropropane (100 mg/kg body weight dissolved in canola oil) 2 times/week for 4 weeks; mice in the control group were injected with vehicle alone (canola oil, 5 mL/kg). At the same time of 2-NP injection, mice of the last group were orally drinking water supplemented with olive oil 50 (Mg/ML)/ 4 weeks.

2.1- Histological investigations

The animals were sacrificed at day 31 post- 2-NP injection. The submandibular salivary glands were removed and cut into small pieces in sterile saline. The pieces were fixed in 10% neutral buffered formalin. The pieces fixed in 10% formalin were micro-technically processed in order to obtain paraffinized tissue sections. The tissue sections were stained with hematoxyline and eosin (H&E) for morphological examination and viewed under light microscope (13).

2.2- Digital morphometric investigations

Digital morphometric studies were performed to evaluate the effect of 2-Nitropropane and olive oil efficiency in the 2-Nitropropane induced morphological alterations in submandibular salivary glands. Observations were made to count

the number of acini and ducts per microscopic field in both control and experimental groups.

2.3- Immunohistochemical investigations

The fixed, paraffin sections were deparaffinized, rehydrated, and heated in a microwave oven in 0.01 M citrate buffer (pH 6.0; Química Contemporânea, Diadema, Brazil) for 30 min. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide for 10 min, followed by a wash with phosphate buffered saline. The sections were incubated overnight at 4°C with the Anti-TNF (rabbit polyclonal IgG, 100 µg/ml, 1:50 dilution, cat. no. sc-130220; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The primary antibody was then detected using avidin-biotin peroxidase detection solution (Dakocytomation labelled streptavidin biotin reagent; Dakocytomation, Glostrup, Denmark and System-horseradishperoxidase; Dako, Glostrup, Denmark) and the signal was visualized using diaminobenzidine (Dakocytomation) and Substrate Chromogen-System (Dako). Slides were counterstained with Harris's hematoxylin, dehydrated, cleared and mounted. The tissue sections were washed in tap water, dehydrated in ascending grades of alcohols, cleared in xylene. Slides then mounted with DPX, and a cover is slipped over. The samples were subsequently examined and the evaluation of cell staining was performed. Evaluation of IHC staining was done by Light microscope and Leica QWIN V3 image analyzer computer system (Switzerland), was used to assess area percentage of positive cells surface in IHC stained sections. Distribution of positive cells was measured in the form of area inside a standard measuring frame of area 59413.2 micrometer² per 10 fields using a magnification (x400) by digital light microscopy. The selected fields for evaluation had the most uniformly stained tissue areas.

2.4- Statistical analysis significance was evaluated by one way analysis of variance (ANOVA) test for comparison between the different groups.

3. Results

3.1- Histopathological results

Histologically, the submandibular glands of BALB/c male mice of the first group appeared with a well-known histologic features with no observable alterations. The animals in 2-Nitropropane treated group showed marked atrophy and degeneration of the acini. Glands in this group showed also proliferation of duct like structures and cytoplasmic vacuolations (Fig. 1A). The periductal inflammatory cell infiltration in the salivary glands was apparent in this group (Fig. 1B). In animals treated with olive oil there was apparent improvement from the 2-NP deleterious effect. This was manifested in the acini of the gland which appeared near normal (Fig.1C). However, this reversal of 2-NP deleterious effect did not acquire full normal case of gland.

3.2- Digital morphometric analysis

The morphological features of the submandibular salivary gland were calculated and analyzed by digital morphometric studies. Table (1) describes the number of serous acini per microscopic field, in which number of acini in the control group was (30.33 ± 7.37), and in the 2-NP treated group was (16.00 ± 4.57) which was a significant reduction in number of acini between

The microscopic analysis in the present work investigated that the acinar cells were atrophic and degenerated. In addition, these cells showed cytoplasmic vacuolations and periductal inflammatory cell foci in the 2-NP treated animals compared to the control group. These changes were supported by the morphometric studies and comparable to those demonstrated in other studies (2, 11). It was suggested that the liver damage induced by 2-NP is related to oxidative damage, lipid peroxidation (12). Previous results indicated that 2-NP inflicted DNA damage in the bone marrow cells (4) and induced chromosome aberrations as well as DNA repair (5).

In contrast, the findings of this study suggested that there was partial improvement from the 2-NP deleterious effect after administration of the olive oil. These results may be correlated with several studies which

the two groups. The number of acini in group III was increased when compared with GII (30.33 ± 2.31).

Table (2) describes the number of ducts per microscopic field, in which number of ducts in the control group was (6.33 ± 0.58), and in the 2-NP treated group was (10.67 ± 1.53) which was a significant reduction in number of acini between the two groups. The number of acini in group III was increased when compared with G II (7.33 ± 1.15).

3.3- Immunohistochemical evaluation of TNF- α staining.

Immunohistochemical staining for TNF- α was more intense in the 2-NP treated group. But, the analysis of the histological sections for TNF- α in the olive treated group showed absent or weak staining (Fig. 4).

Table (3) describes the mean of positive TNF- α immunostain area % in the three studied group. In the control group was (4.77 ± 1.19), and in the 2-NP treated group was (49.25 ± 12.84). In group III was decreased when compared with G II (7.84 ± 3.83) (Fig.5).

4- Discussion

evidenced possible health benefits of olive oil. It was evidenced that increased dietary extra virgin olive oil have beneficial synergistic effects on lipid metabolism and oxidative stress in patients with metabolic syndrome (6). On the other hand, analysis of miRNAs in the liver showed selective modulation by hybrid palm oil (7). Also, the olive oil phenolic compounds induced cell maturation in vitro, increasing alkaline phosphatase synthesis and reducing the expression of antigens involved in immune functions of the bone forming cells which would improve density of bone (9).

Histologically, the reversal effect of 2-NP did not acquire the full normal histology of the submandibular salivary gland. Regarding the morphometric results of the present study, it was demonstrated marked reduction in the number of serous acini.

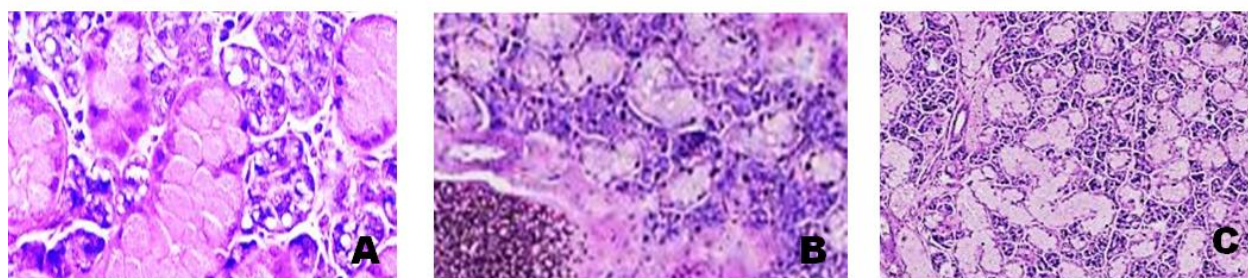


Fig. (1): Hematoxyline and eosin–stained sections of submandibular gland A) stained sections of 2-NP group has atrophic and degenerative acini and cytoplasmic vacuolations, B) stained sections of submandibular salivary gland tissue, showing focal inflammation in the NP group of BALB/c mice as indicated by immune cell infiltration and degenerated acinar cells, C) stained sections of submandibular gland of mice treated with olive reveals partially recovery as moderate acinar atrophy, acinar and ductal intracellular vacuolations and few cytoplasmic vacuolations

Table (1): Describes the number of serous acini in the control and experimental groups

No. of acini	Group I	Group II	Group III	Test value*	P-value	P1	P2	P3
Mean ± SD	30.33 ± 7.37	16.00 ± 4.57	30.33 ± 2.31	7.649	0.022	0.045	1.000	0.008
Range	22 – 36	16 – 27	29 – 33					

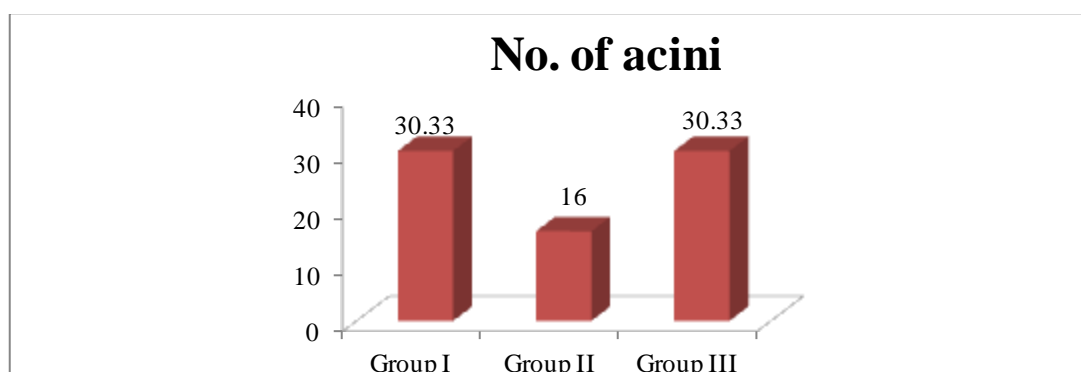


Fig (2): histogram showing the mean number of acini in the three studied groups.

Table (2): describes the number of serous acini in the control and experimental groups

No. of ducts	Group I	Group II	Group III	Test value*	P-value	P1	P2	P3
Mean ± SD	6.33 ± 0.58	10.67 ± 1.53	7.33 ± 1.15	11.583	0.009	0.004	0.330	0.012
Range	6 – 7	9 – 12	6 – 8					

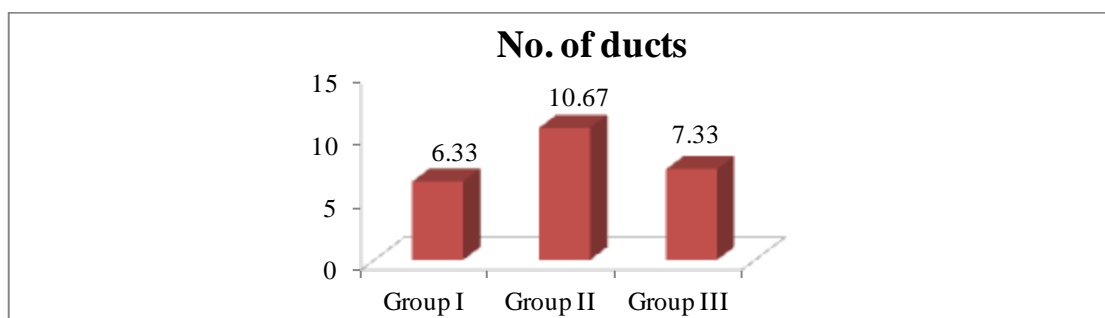


Fig (3): histogram showing the mean number of ducts in the three studied groups.

Table (3): describes the mean of positive TNF- α immunostain area %in the three studied group.

Area %	Group I	Group II	Group III	Test value*	P-value	P1	P2	P3
Mean ± SD	4.78± 1.19	49.25 ± 12.84	7.84± 3.83	13.583	0.05	0.004	0.330	0.002
Range	3-7	26 – 70	4 – 15					

*: One Way ANOVA Test, P1: Group I VS Group II, P2: Group I VS Group III, P3: Group II VS Group III

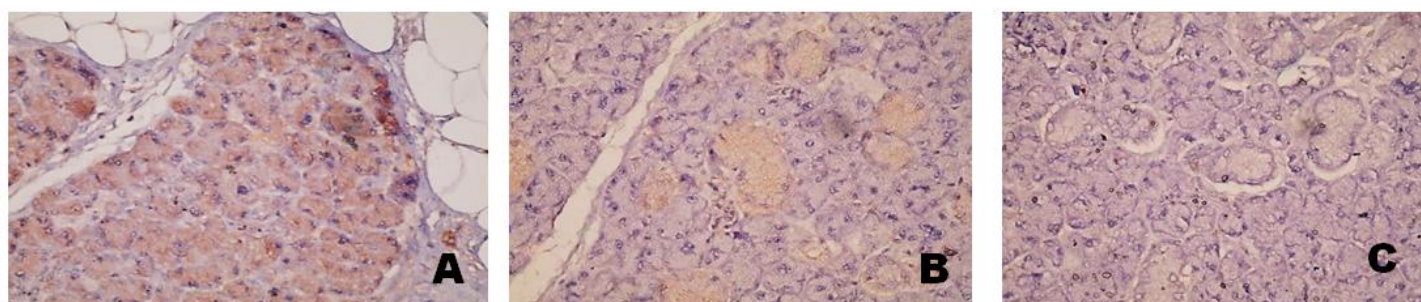


Fig. (4): immunohistochemical stained sections of submandibular gland showing the re-localization assessment of TNF- α A,B) 2-NP group has moderate or strong staining C) mice treated with olive reveals weak or absent staining.

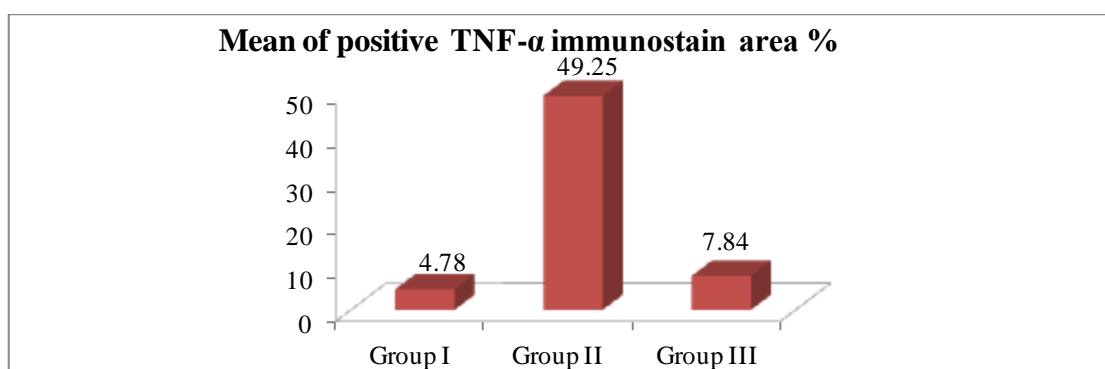


Fig (5): histogram showing the mean of positive TNF- α immunostain area %in the three studied group

In contrast, the ducts described increase in their number. This may correlate with others who showed a significant reduction in the number of parotid acinar cells at one month in olive oil/irradiated and irradiated rats. In rats evaluated over time a significant reduction was shown at one month in olive oil/irradiated and irradiated animals. The vitamin E/irradiated group presented more acinar cells than the irradiated, but no statistically significant difference was observed ($p>0.05$). It was concluded that vitamin E seems to have failed as a radioprotective agent on acinar cells in rat parotid salivary glands (14).

From the immunohistochemical point of view, staining of the TNF- α was moderate to strong in the 2-NP treated group. In contrast, the intensity was weak or absent in the olive treated animals. Previously, it was known that the salivary gland cells release several cytokines that may play important roles in the inflammatory process. This may explain the beneficial effect of olive oil. In respect to the role of cytokines, the present investigation is in agreement with several studies (15-20). It was found that stimulation with IL-1 β and TNF- α increased submucosal gland secretion in a concentration-dependent manner. The cytokine effect was dependent on cAMP, nitric oxide. Most importantly, IL-1 β - and TNF- α -stimulated secretion was blocked by the cystic fibrosis trans-membrane conductance regulator (CFTR) blocker. Furthermore, the data suggested that during bacterial infections and resulting release of proinflammatory cytokines, the glands were stimulated to secrete fluid, and this response is mediated by cAMP-activated CFTR, a process that would fail in patients with CF (21).

Additionally, higher expression of IL-6 was found in salivary gland cancer (SGC) (70.7%) than in normal tissue (20%). Positive expression of CMV antigens in a high percentage of SGC cells suggested that it might play role in

carcinogenesis by increasing IL-6 production and leading to inhibition of apoptosis and tumor development (22). It was demonstrated that IL6 pretreatment prevented both senescence and salivary gland hypofunction via a mechanism involving enhanced DNA damage repair. The IL6 pretreatment represented a promising therapeutic strategy to preserve salivary gland function in head and neck cancer patients undergoing radiotherapy (18). Similarly, protein expression levels of IL-17 and IL-6 were detected parotid and submandibular salivary glands by ELISA. Compared with the normal group, mRNA transcriptional levels and protein expression levels of IL-17 and IL-6 were significantly up-regulated after administration of deionized water (23).

Our results revealed significant difference in tissue expression of TNF- α between different groups. Expression of TNF- α significantly increased in 2-NP treated group in relation to normal group and significantly decreased in the olive treated in relation to 2-NP treated group. These results are in agreement with recent study which demonstrated significant increase of TNF- α expression associated with degeneration of submandibular salivary gland and significant decrease of TNF- α expression associated with regeneration of its acinar cells (24). In general, several studies have demonstrated an association between inflammation and tumorigenesis, suggesting that TNF- α is critical in the regulation of invasion, angiogenesis, and tumor metastasis (24) observed the increased production of TNF- α in peripheral blood mononuclear cells in patients with malignant ovarian cancer, compared with nonmalignant cases). (25). On the other hand, TNF- α is not always detectable in cancer patients, and concentrations may vary between individuals and throughout the course of the disease (26, 27). In conclusion, 2-NP caused significant tissue damage and olive oil may attenuate the alterations of 2-NP and TNF- α may be useful tissue marker in determining these alterations.

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