The Effect of Omega- 3 Fatty Acids on the Age Related Changes in Submandibular Salivary Glands of Albino Rats

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Abstract: Objective: The current study has been carried out to evaluate histologically, immunohistochemistry and statistically the effect of daily consumption of omega-3 on submandibular salivary gland of old aged rats. Material & methods: Eighteen male albino rats were used in this study. The rats were divided into three groups (6 rats/each): Group I: 4-6 months rats weighing 170-200 gm, represented the adult rats. Group II: 12-15 months rats weighing 250-300 gm, represented the old rats. Group III: : 12-15 months rats weighing 250-300 gm, represented the old rats, and receiving omega-3(60 mg/kg) once daily, for one month by intra- gastric intubation. All rats were sacrificed after 30 days. Soft tissue specimens were obtained from submandibular salivary gland at the floor of the mouth of the rats in all the studied groups. The sections were examined histologically with H&E, immunohistochemically using tumor necrosis factor alpha and the results were evaluated statistically. Results: Histopathological changes were observed in group II including; decrease in the overall size of the acini and granular convoluted tubules, an increase in the amount of fatty tissue, fibrous tissue and inflammatory infiltration. In group III, the acini and ducts appeared nearly normal and there was marked decrease in the inflammatory infiltration. Immunohistochemical examination revealed moderate expression of tumor necrosis factor alpha in group II, mild expression in group III and weak expression in group I. Conclusion: Omega-3 had significant effects in management of many age related changes of submandibular salivary gland.

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1.Introduction

Omega-3 fatty acids are fats commonly found in fish oils, algal oil, squid oils, and some plant oils such as flaxseed oil and hemp oil.Omega-3 polyunsaturated fatty acids (PUFA) are considered essential fatty acids, meaning that they cannot be synthesized by the human body but are vital for normal metabolism**(Wall *et al.,* 2010).**

PUFA have strong immunomodulatory activities, among the omega-3 PUFA, those from fish oil eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) which are more biologically potent than α-linolenic acid (ALA) **(Simopoulos,2002).**

Many studies indicated that omega-3 fatty acids have anti- inflammatory properties and therefore, might be useful in the management of inflammatory and autoimmune diseases. Their effects are mediated by modulation of eicosanoids and cytokines and by altering gene expression **(*Seed and Willoughby*, 1997*&Kehn and Fernandes,* 2001).**

The anti-inflammatory properties of omega 3 fatty acids, especially EPA, are due to competition with arachidonic acid (AA) as a substrate for cyclooxygenase and 5-lipoxygenase.The eicosanoids are considered a link between PUFA, inflammation and immunity **(Simopoulos, 2002).**

Aging is cumulative changes in an organism, organ, tissue or cell leading to decline in biological functions and inability to adapt to metabolic changes **(Kunlin, 2010).**

In humans and other animals, cellular aging has been attributed to the shortening of telomeres (strings of DNA located at the end of the chromosomes) with each cell cycle, when telomeres become too short, the cells die.Omega-3 has a direct effect on biological aging by slowing down the rate at which protective caps on the ends of chromosomes shorten **(Ramin, 2010).**

Cellular damage by oxygen free radicals is a primary driving force for aging. Omega-3 supplementation reduced oxidative stress, caused by excessive free radicals, by about 15 percent compared to effects seen in the placebo group **(Devanand and Paul, 2006).**

Although, there are many researches concerning the beneficial effects of omega-3, no interest had been directed toward the anti-aging effect of omega-3 on the oral tissues, hence the present study has been designed to evaluate histologically, immunohistochemistry and statistically the effect of daily consumption of omega-3 on submandibular salivary gland of old aged rats.

2. Materials and Methods

Eighteen clinically healthy male albino rats were used in this study. The rats were obtained from the animal house, Faculty of Medicine, Cairo University. The animals were housed in specially designed cages and maintained under good ventilation for 30 days. They were allowed to have free access to both water and standard rodent soft chow diet *ad-libitum*. The experiment was conducted according to the recommendations of the ethics committee in the Faculty of Oral and Dental Medicine, Cairo University.

The rats were divided into three groups (6 rats/each):

Group I:4-6 months rats, weighing 200-250 gm represented the adult rats.

Group II: 12-15 months rats, weighing 300-350 gm represented the old rats.

Group III: :12-15 months rats, weighing 300-350 gm represented the old rats, and receiving omega-3(60 mg/Kg) once daily **(Marjan *et al.*, 2012)**,for one month by intra- gastric intubation.

Omega-3 (EPA+DHA) used in this study was obtained from Sedico co.

All rats were sacrificed, by carbon dioxide inhalation, after 30 days.

Soft tissue specimens were obtained from submandibular salivary gland at the floor of the mouth of the rats in all the studied groups. These specimens were fixed in 10% neutral buffered formalin and processed for embedding in paraffin blocks.

Then sections of 4-5 microns were obtained from the paraffin blocks and subjected to the following investigations:

- Histological study:

Hematoxylin and eosin stain was used for interpretation of any histological changes.

-Immunohistochemical Study:

Particular interest was directed toward evaluation of the expression pattern of tumor necrosis factor alpha TNF in the submandibular gland of the rats in the studied groups.

Immunohistochemical procedures

The sections were deparaffinized and rehydrated routinely. For antigen retrieval, sections were treated by autoclave heating in citrate buffer for 10 minutes. The sections were treated with normal rabbit serum for 30 minutes,to block non-specific binding. Specimens were then incubated with the primary antibody TNF alpha (Dako Co.).Avidin biotin peroxidase complex was applied to the sections, and the slides were incubated for 30 minutes at room temperature. Then the slides were washed in phosphate buffer. Reaction products were visualized by diaminobenzidine DAB. Finally, tissue sections were dehydrated, cleared in xylene and mounted **(Falini and Taylor, 1983).**

Brown coloration of the cytoplasm was recognized as positive staining.

Statistical analysis

The mean area percent and the optical density of TNF- alpha immunoexpression were measured using an image analysis system (Leica DM LB2 with QWIN plus image analyzer computer system, Germany). The obtained experimental data were calculated, analyzed and computed to compare the changes in the area percent and the optical density of TNF alpha immunoexpression between the studied groups. Values were expressed as mean and standard deviation and were used for statistical analysis by ANOVA test.

Results were considered significant when probability (P) is ≤ 0.05 and highly significant when P is ≤ 0.01.

Histological results

Group I:

The submandibular glands of rats were mixed glands where the secretory end portions were mainly serous in addition to few numbers of mucous acini. Some mucous acini were capped with serous demilunes. The serous acini lined with pyramidal shaped cells with basally situated nuclei. The mucous acini consisted of low cuboidal cells with flattened nuclei. The duct system was composed of the intercalated, striated, excretory, and main excretory ducts. The intercalated duct lined by single layer of low cuboidal epithelial cells with centrally placed nuclei. The striated ducts were round and lined by single layer of columnar cells with centrally placed nuclei. The excretory duct was interlobular large duct, its epithelium consists of various types of columnar cells. The granular duct (granular convoluted tubule, GCT) is located between the intercalated and striated ducts in the rats submandibular gland. The duct wall is composed of a simple columnar epithelium. The principal cell type of the GCT was high-columnar secretory cell containing many secretory granules in its supra-nuclear cytoplasm. Connective tissue septa divided the gland into lobes and lobules **(Fig.1).**

Group II :

The acini and convoluted tubules of the submandibular salivary gland of this group showed shrinkage in the overall size. Few acini and granular ducts showed distortion. An inflammatory infiltration was obvious in the connective tissue septa. Dilated and congested blood vessels were observed **(Fig.2).** Also decrease in number of the acini was observed. The cytoplasm of the serous acini was faintly stained and the esinophilic granules were less. Both striated

and excretory duct cells appeared with no detectable changes from those of adult age.

Intracytoplasmic vacuolization was observed in some cells of the acini, intercalated and granular convoluted tubules. The esinophilic granules of granular convoluted tubules were less.In some specimens, the epithelial lining of the excretory ducts showed metaplasia (epithelium changed to flattened cells) and their lumen became wider. Many ducts showed stagnation of the secretion in their lumens. There was an increase of the fatty and fibrous tissues **(Fig.3).**

Group III :

Specimens of this group revealed nearly normal size of the acini and granular convoluted tubules. The inflammatory infiltrate significantly decreased. The acinar cells showed darkly stained cytoplasm **(Fig.4).**

Immunohistochemical result:

Group I:

The TNF alpha expression in this group was weak in the ductal cells. The acinar cells showed negative reaction **(Fig.5).**

Group II :

Specimens of this group showed moderate immunoreactivity of TNF alpha in the ductal cells and negative one in the acinar cells **(Fig.6).**

Group III:

Marked decrease in the immunoexpression of TNF alpha was observed in sections of this group compared to those of group II. The ductal cells showed mild expression and the acinar cells revealed negative immunoexpression **(Fig.7).**

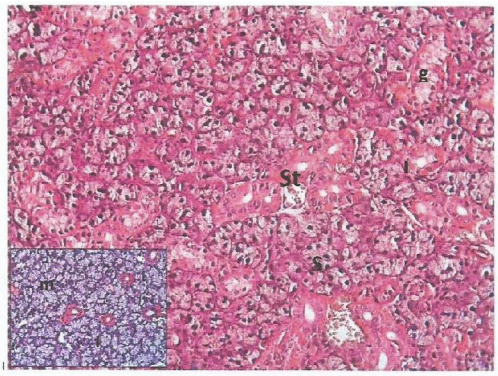

Fig.1 Photomicrograph of group I, showing normal architecture of submandibular gland: serous acini (S), intercalated ducts (I), striated ducts (st) and granular convoluted tubules (g).Inset: showing normal mucous acini (m),and intercalated duct (I). H&E× 200.

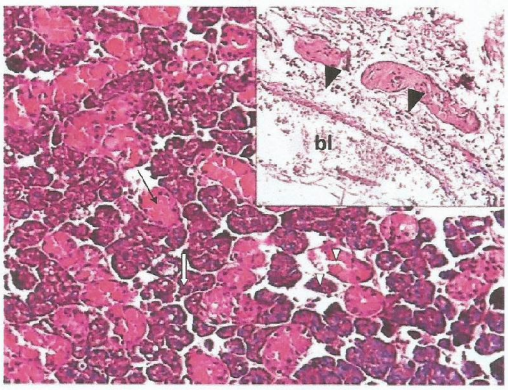

Fig.2 Photomicrograph of group II, showing shrinkage in the overall size of the acini (white arrow) and convoluted tubules (black arrow). Note the distortion of some ducts and acini (white arrow heads). Inset: showing an inflammatory infiltration in the connective tissue (black arrow heads) and dilated and congested blood vessel (bl). H&E× 200.

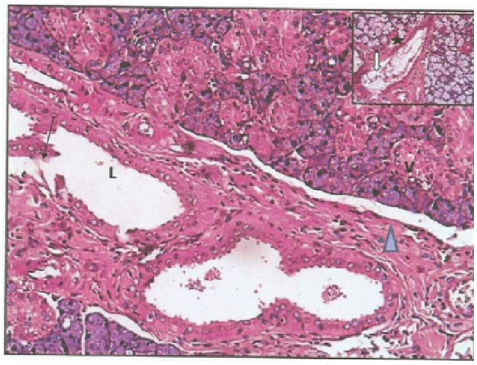

Fig.3 Photomicrograph of group II, showing distortion of the lining of the excretory ducts (arrow), widening of their lumen (L) and an increase in the fibrous tissue (arrow head). Intracytoplasmic vacuolization was observed in some cells of the ducts (v).Inset: showing metaplasia of the epithelial lining of the excretory duct(white arrow), and stagnation of secretion (asterisk). H&E× 200.

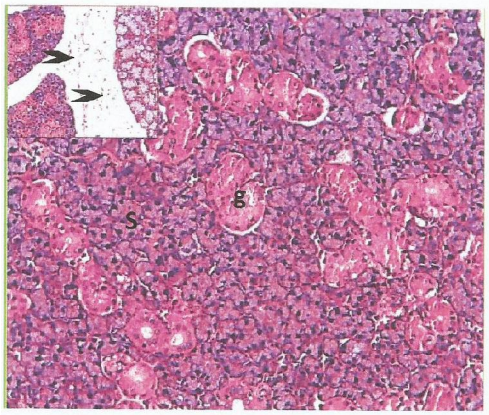

Fig.4 Photomicrograph of group III, showing nearly normal size and darkly stained cytoplasm of the acini (s) and granular convoluted tubules (g). Inset: showing the inflammatory infiltrate was significantly decreased (arrow heads). H&E × 200

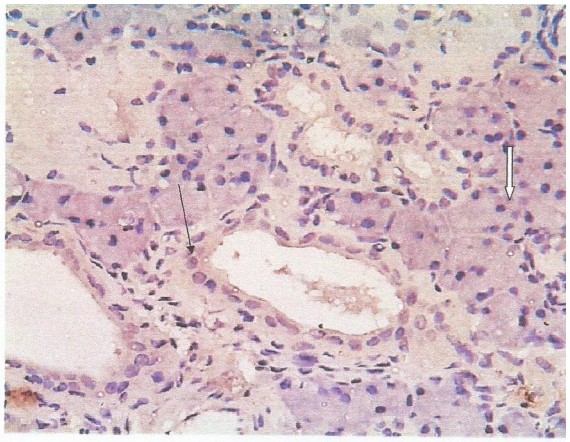

Fig.5 Photomicrograph of group I, showing weak cytoplasmic reaction to TNF alpha mainly in the ducts (black arrow) while negative reaction in the acini (white arrow). TNF alpha × 200.

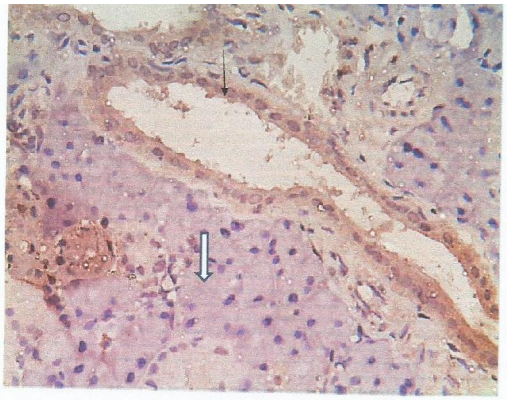

Fig.6 Photomicrograph of group II, showing moderate immunoreactivity of TNF alpha in the ductal cells (black arrow), and negative one in the acinar cells (white arrow). TNF alpha × 400

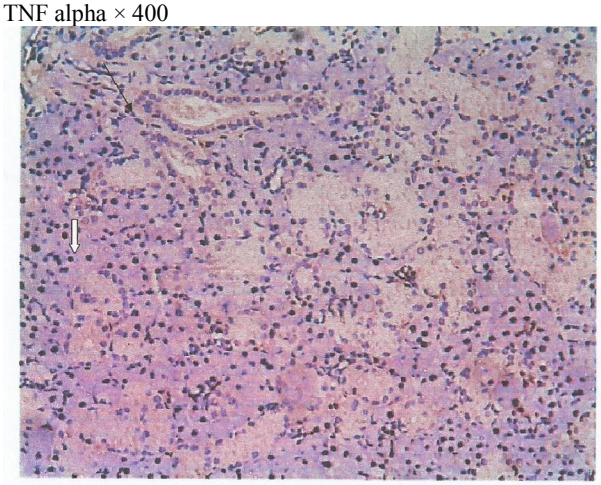

Fig.7 Photomicrograph of group III, showing mild reaction of TNF alpha in the ductal cells (black arrow) and the acinar cells revealed negative immunoexpression (white arrow). TNF alpha × 200

Statistical results

Mean area percent of tumor necrosis factor alpha immunoexpression

The lowest mean area percent of tumor necrosis factor alpha immunoexpression was observed in group I, while the greatest value was recorded in group II. Analysis of variance (ANOVA) test revealed that the difference between the studied groups was extremely statistically significant ($p \leq 0.0001$) **(Table 1, Fig. 8).**

Table (1) Mean area percent of tumor necrosis factor alpha immunoexpression

	Group I	Group II	Group III
Mean	0.5	25.626	4.65
Std. Dev	±2.87	±4.671	±0.334
Max	6.89	29.561	4.993
Min	0.0754	18.41	3.022

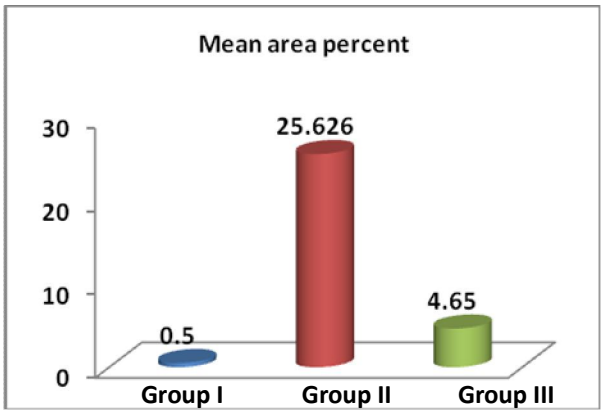

Fig. (8) Mean area percent of tumor necrosis factor alpha immunoexpression

Mean optical density of tumor necrosis factor alpha immunoexpression

A lowest mean optical density of tumor necrosis factor alpha immunoexpression was observed in group I, while the greatest value was recorded in group II. Analysis of variance (ANOVA) test revealed that the difference between groups was extremely statistically significant ($p \leq 0.0001$) **(Table 2, Fig. 9).**

Table (2) Mean optical density of tumor necrosis factor alpha immunoexpression

	GroupI	Group II	Group III
Mean	14.5	71.65	45.32
SD	±0.52	±0.52	±6.67
Min	9.5	67.12	29.33
Max	23.65	79.54	45.56

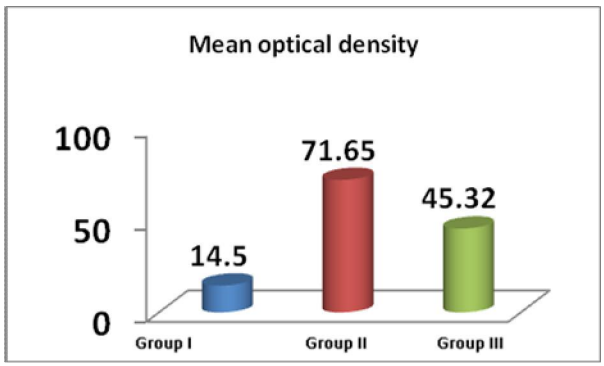

Fig. (9) Mean optical density of tumor necrosis factor alpha immunoexpression

4. Discussion

Since 1980 several studies were carried out about omega-3 fatty acids and their important role in normal growth, development, prevention and treatment of many inflammatory and autoimmune disorders **(Simopoulos, 2002)**.

In the current study the immunoexpression of TNF alpha was evaluated in the submandibular salivary gland of the rat. Cytokines play important roles as pro-inflammatory mediators,tumor necrosis factor TNF is one of the most important cytokines produced by monocytes and macrophages. Production of appropriate amounts of TNF is beneficial in response to infection, however inappropriate amounts or overproduction can cause some of the pathological responses that occur in inflammatory conditions. The link between inflammation and aging is the presence of pro-inflammatory mediators **(Devanand and Paul, 2006).**

In the present study, the acini and convoluted tubules of submandibular gland of group II showed shrinkage in the overall size. **Dayane *et al.* (2000)** supported this result as they studied the age related changes of salivary glands, and they reported that the mean volume of the acini decreased**.**

Also distortion was observed in some acini and granular ducts of group II, and this finding could be attributed to the increased levels of pro-inflammatory cytokines (interleukin 1) as IL-1and TNF alpha which may cause degradation of the basement membrane and disruption of acinar and ductal cell architecture by age, through matrix metalloproteinase (MMP-2) modulation. **(Azuma *et al.*, 1997).**

Histological examination of sections of group II revealed marked increase in the fibrous as well as the fatty tissues of the submandibular salivary gland, these results were in agreement with the observations of **Vered *et al.* (2000**) who demonstrated that the volume of stromal components, blood vessels, lymphatics, fat cells and inflammatory infiltrates of labial salivary glands, increased with age.

Moreover, the current study showed marked chronic inflammatory infiltration in the connective tissue septa. These results could be related to an increase in the production of oxygen free radicals and metabolites by age, with ultimate results of inducing chronic inflammatory conditions **(Larsson *et al.*, 2001).**

In group III, the present study demonstrated improvement of some age related changes in submandibular salivary gland, the acini and granular convoluted tubules appeared nearly normal and the inflammatory infiltrate markedly decreased. These results were supported by the findings of **Katsumata, 1998,** who reported that omega-3 fatty acids improved the blood flow and consequently metabolism in the cells. Also, omega-3 fatty acids stabilized the cell and protect it from damage by inhibiting the release of arachidonic acid from the cell membrane (**Xiao and Li, 1999).**In addition **Simopoulos, (2008)** reported that omega-3 fatty acids rapidly incorporated into the membrane phospholipids of human cells, suggesting that they have an effect on several aspects of cell function and the author also added that omega-3 PUFA have anti-inflammatory properties and, therefore, might be useful in the management of many inflammatory disorders .

The immunohistochemical results of the present study revealed that group I had the weakest immunoreactiviy for TNF alpha, while the greatest one was recorded in group II. These results were supported by the statistical results of the current study, where group I showed the lowest mean area percentage and mean optical density of TNF alpha expression and the highest scores were in group II.

These results were supported by **Bruunsgaard *et al.* (2001)** who demonstrated increased levels of pro-inflammatory cytokines such as IL-1, (interleukin 6) IL-6 and TNF alpha in aged individuals as compared to young individuals. Also, it was reported that Coronary heart disease, aging and cancer are characterized by an increased levels of IL-1 and TNF pro-inflammatory cytokines **(Simopoulos, 2002).** These results could be related to that in aging there is marked production of oxygen free radicals that leads to increased activation of redox-regulated,transcription factors such as NF-κB, that regulate the expression of pro-inflammatory molecules such as of IL-1,IL-6 and TNF **(Sarkar and Fisher, 2006).**

Additionally,in group III,receiving omega-3, decreased immunoreaction of TNF alpha was reported, as compared to that of group II. This result

was in agreement with those of **Simopoulos,(2008)** who reported that omega-3 supplementation suppressed the metabolism and production of pro-inflammatory cytokines such as IL-1 and TNF.

The present study demonstrated only immunoreactivity for TNF alpha in ductal cells and negative reaction in acinar cells. These results were in agreement with **Koski *et al.*(2001)** who demonstrated the expression of TNF alpha in vascular endothelial cells, ductal epithelial cells and fibroblasts of labial salivary glands in Sjogrens syndrome. They added that acinar end piece cells did not express TNF-alpha. The different behavior pattern between the acinar and ductal cells probably derived from the impact that the chronic inflammatory infiltrate might have on the ductal epithelium adjacent to it. The presence of chronic inflammation is associated with the generation of reactive oxygen species within the adjacent cells resulting in marked increase in the pro-inflammatory molecules and DNA damage **(Vered *et al.*, 2004).**

However, **Fox *et al.* (1994)** and **Tanda *et al.* (1998)** reported that inflammatory cytokines were synthesized and released by salivary gland acinar cells**,** lymphocytes and macrophages.

Conclusion

The present study revealed that omega-3supplements could represent unique nutritional intervention that has great potentional to decrease the deterioration and concomitant functional changes associated with aging.

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