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THE PROTECTIVE ROLE OF NIGELLA SATIVA VERSUS LEPIDIUM SATIVUM ON THE SUBMANDIBULAR SALIVARY GLAND IN HYPERCHOLESTEROLEMIC ALBINO RAT (HISTOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY)

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ABSTRACT

Background: Direct relation between hypercholesterolemia and hyposalivation was suggested. Phytosterols of herbal origin have been used as prophylactic or curative agents against many disorders.

Objectives: Evaluate the protective role of Nigella sativa (Ns) versus Lepidium sativum (Ls) on the histological picture of submandibular salivary glands in hypercholesterolemic albino rat; and its immunomodulatory role of nuclear factor kappa B cell (NF-κB) and alpha smooth muscle actin (α-SMA) expression on the tissues.

Materials and method: This study was performed with forty male albino rats, divided into four equal groups as follow: Group 1 (n=10): served as negative control, Group 2 (n=10): were fed with hypercholesteremic supplement (HCS) for 8 weeks. Group 3 (n=10): were fed HCS with oral administration of (Ns) seeds for 8 weeks. Group 4 (n=10): were fed HCS with oral administration of (Ls) seeds suspension for 8 weeks.

Results: Histological results of submandibular salivary gland in group 2 revealed severe atrophic and degenerative changes in the secretory terminal portions & ducts. In group 3, the tissues appeared with nearly normal histological structures which reflect the beneficial effects of Ns. In group 4, a lesser degree of improvement was noticed with Ls. Quantitative analysis for α-SMA and NF-κB revealed highly statistically significant decrease in group 3 and 4 in comparison with group 2.

Conclusion: The immunohistochemical finding confirmed the histological results of the present study that proved the superior protective effects of Ns rather than Ls seeds against hypercholesterolemia on the submandibular salivary glands of albino rats.

KEYWORDS: Hypercholesterolemia, Nigella sativa, Lepidium sativum, α-SMA, NF-κB

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INTRODUCTION

Hypercholesterolemia (HC) may be caused due to familial or secondary causes as high lipid content, diabetes mellitus or liver dysfunction. It is characterized by elevated levels of plasma cholesterol. It plays a major risk factor for coronary heart disease, stroke, atherosclerosis, and ischemic heart disease[1,2].

Salivary glands are considered one of the most important determinants for oral health maintenance. Direct relation between hyperlipidemia and hyposalivation was suggested by several publications. Hypercholesterolemia as a lipid disorder may induce histopathological changes as fatty degeneration within the parenchymal cells and ductal enlargement result in salivary flow impairment[3,4].

Many years ago, phytosterols of herbal origin have been used as prophylactic or curative agents against many disorders due to their anti-inflammatory, lipid-lowering, anti-hypertensive, and antioxidant properties [5,6].

One of these promising medicinal plants is *Nigella sativa* (NS) which have many active constituents as thymoquinone, thymohydroquinone, thymol, and carvacrol[7]. It has multiple beneficial effects as antioxidant, anti-inflammatory, antidiabetic, antimicrobial, anticarcinogenic, immunomodulator and bronchodilator[8]. The choleretic effect of dietary NS can reduce serum lipids and peroxidation of lipids in hypercholesterolemic experimental animals[9]. Moreover, its hypolipidemic effect has been reported in some clinical trials[10,11].

Lepidium sativum (LS) represents another one of the medicinal plants that is native to Egypt. It has been widely used in traditional treatment from ancient time[12]. It has a broad pharmacological action as antioxidant, antidiabetic activity. Antibacterial, antifungal, anti-carcinogenic, hepatoprotective, anti-osteoporotic and anti-asthmatic activity[13,14]. It decreases the oxidative and metabolic disturbances in hypercholesterolemic rats[15].

Alpha-smooth muscle actin (α-SMA) is actin isoform that expressed mainly around the vascular muscle cells, fibrogenesis and during detection of myoepithelial cells (MECs). There is strong correlation between alpha-SMA expression and myofibroblasts activation[16,17]. In hyperlipidemic mice, there was significant increase in the expression levels of α-SMA[18].

Nuclear factor kappa B-cells (NF-κB) is considered as B-lymphocyte-specific nuclear proteins, essential for immunoglobulin kappa (κ) light chains transcription. It can control multiple physiological processes as proliferation, inflammation, cellular homeostasis, and apoptosis[19]. Increased levels of cholesterol enhancing the expression of NF-κβ that augmented the programmed cell death and inflammatory process[20]. Supplementation of exogenous plant antioxidants leads to scavenging for all free radicals, enhancing the antioxidant defense pathway and inhibiting the expression of NF-κβ[21].

Accordingly, the present work was designed to elucidate the protective role of *Nigella sativa* versus *Lepidium sativum* on the histologic picture of submandibular salivary gland in hypercholesterolemic albino rat; and its immunomodulatory role on NF-κB and α-SMA expression on the tissues.

MATERIAL AND METHODS

Material preparation

Hypercholesterolemia induction in albino rats by introducing high cholesterol supplement (HCS) 4% cholesterol (w/w) and 1% cholic acid (w/w), which purchased from El-Gomhoria Company, Egypt for 8-weeks[22].

Seed's preparation: *Nigella sativa* (Ns) and *Lepidium sativum* (Ls) seeds were purchased from Agricultural Research Center, Egypt. Seeds were dried and grinded to form powder. Adequate amount of distilled water was added to the seed's powder to form suspension[23].

Experimental setting

Sample size calculation

G*Power version 3.1.9.2 was used to calculate the sample size in this experiment[24]. The estimated sample size (n) was 40 samples, 10 samples in each group. The effective size was 1.25, using alpha level of 0.05, Beta level of 0.05 and the power = 95%.

Experimental design

This study was performed after approval of Research Ethics Committee (REC), Faculty of Dentistry, Suez Canal University (342/2021). Forty male albino rats (n=40) with average sizes of ~200-250 gm were included in this study. Rats were acclimated for 7 days before starting experiment. All animals were housed in air-conditioned rooms on a 12 h light/12 h dark cycle. They were randomly allocated into four main equal groups, 10 rats each, 5 rats per cage, classified as follow:

Group 1 (n=10): served as negative control that were fed a standard diet for 8 weeks.

Group 2 (n=10): served as positive control that were fed with hypercholestrolemic supplement for induction of hypercholestrolemia for 8 weeks.

Group 3 (n=10): were fed HCS with oral administration of (Ns) seeds by oropharyngeal tube in a dose of [1000mg/kg/BW][25] for 8 weeks.

Group 4 (n=10): were fed HCS with oral administration of (Ls) seeds suspension by oropharyngeal tube in a dose of [550 mg/kg/BW][23] for 8 weeks.

Histological procedures

At the end of 8th week of the experiment, all rats were euthanized by ether inhalation. The submandibular salivary glands were dissected out, fixed in 10% neutral buffered formalin, washed, paraffin-embedded and mounted. Five microns thick sections were cut and stained with hematoxylin and eosin to investigate the histological changes of submandibular salivary glands.

Immunohistochemical (IHC) procedures

Other five microns thick sections were cut, mounted on positively charged glass slides to detect the expression of α-SMA and NF-κB on the tissues. The immunostaining was performed using rabbit polyclonal anti-body α-SMA antibody (Cat No. GTX100034, Gene Tex) at dilution of 1:100 and rabbit monoclonal anti-body NF-κB (Cat No. A19653, Leader in Biomolecular Solutions for Life Science) at dilution 1:100. The steps of IHC were performed according to the instructions of manufacturer.

Digital image analysis

For immunohistochemical quantitative assessment, software image analyzer (image J / Fiji 1.46) was used. The slides were digitized under 400X objective magnification. This software was used to assess the optical density of the cytoplasmic expression and to count the number of immune positive cells of the nuclear expression. Then, the fraction of the positive cells for α-SMA and NF-κB was calculated.

Statistical Analysis

A normality test was performed to assess the normal distribution of the samples. The SPSS software for windows version 24.0 (Statistical Package for Social Science, Armonk, NY: IBM Corp) was used for statistical analysis at significant levels 0.05 (P- Value **<**0.05).

Descriptive statistics were represented in the form of Mean ± Standard deviation (SD). One-way ANOVA test was used to compare between the four groups for α-SMA and NF-κB under study. Duncan's post hoc test was used to evaluate the statistical significances among the different groups. Changes Percentage (RD%) was calculated as Follow formula:

*Reduction decrease (RD %) = (Disease-treatment)/Disease) *100*

RESULTS

Histological Results

Group 1: Examination of rats' submandibular salivary glands of the negative control group showed normal histological features **(Fig. 1 A, B)**. The connective tissue septa which surrounded the parenchymal elements of the gland divided it into lobes and lobules.

The parenchymal elements consisted of secretory end piece (serous acini) and a successive duct system. Serous acini formed of pyramidal cells with basally rounded nucleus surrounding central narrow lumen. The duct system of the gland consisted of different types known as the intercalated, striated, and excretory duct. Moreover, additional duct that belong to rodent's gland known as granular convoluted tubule (GCT's) that was located between the intercalated and striated ducts.

Group 2 (HCS): Examination of the submandibular salivary glands of rats which received HCS revealed degenerative changes involved both acini and duct system. Most of the acini showed multiple cytoplasmic vacuolization, hyperchromatic nuclei, and loss of their normal architecture. The duct system also was affected, the most obvious finding was related to the GCT's that appeared totally destructed in most of the exanimated tissues, while in other samples appeared shrunken with loss of its granules. The striated ducts appeared shrunken and vacuolated. The excretory ducts appeared atrophied, vacuolated with stagnation of their secretions. Some of them showed apoptotic signs in their epithelial lining in addition to hyalinization in the surrounding structures. The interlobular connective tissue septa showed widening, fibrosis with dilated engorged blood vessels with RBCs **(Fig. 1 C: F)**.

Group 3 (HCS + Ns): Examination of rat's submandibular salivary glands which received HCS and Ns, showed great improvement in their histological picture in comparison with group 2. The serous acini restored their normal architecture, almost no intracytoplasmic vacuolization was recognized. The duct system revealed much better histological appearance, however some convoluted tubules appeared shrunken. Interlobar and interlobular connective tissues appeared within normal width, few dilated blood vessels noted around execratory ducts with little number of RBCs within them (**Fig. 2 A: C**).

Group 4 (HCS + Ls): Examination of rat's submandibular salivary glands which received HCS and Ls, showed some degree of improvement in their histological picture when compared with hypercholesteremic rats. The serous acini appeared histologically normal, while others still appeared with disorganized appearance. The GCT's still suffering from vacuolization and sometimes destruction. The striated ducts showed normal epithelial lining although some appeared shrunken. Abnormal lining in the excretory ducts was observed. The interlobular connective tissue septa showed widening with degree of fibrosis in addition to many dilated blood vessels engorged with large number of RBCs (**Fig. 2 D: F**).

Immunohistochemical (IHC) Results

The immunoreactivity of the rat's sub-mandibular salivary glands to α-SMA in group 1 showed positive immunoreactivity α-SMA poly-clonal antibody (brown cytoplasmic) at the periphery of myoepithelial cells (MECs) around the periphery of acini, intralobular duct and smooth muscle lining of blood vessel wall (**Fig. 3 A**). Quantitative analysis revealed highly statistically significant increase of α-SMA immunoreactivity at acini and ducts in group 2 (22.54 ± 1.8^{a}) in comparison with group 1 (6.44 ± 1.60^{d}) **(Fig. 3 B)**. For group 3 and 4, they showed statistically significant decrease (11.66 ± 1.4^{c}) and (15.98 ± 2.4^{b}) respectively in comparison to group 2 (22.54 ± 1.8^{a}) **(Fig. 3 C, D)**.

The immunoreactivity of the rat's sub-mandibular salivary glands to NF-κB in group 1 showed weak positive immunoreactivity NF-κB mono-clonal antibody (brown cytoplasmic and nuclear) within

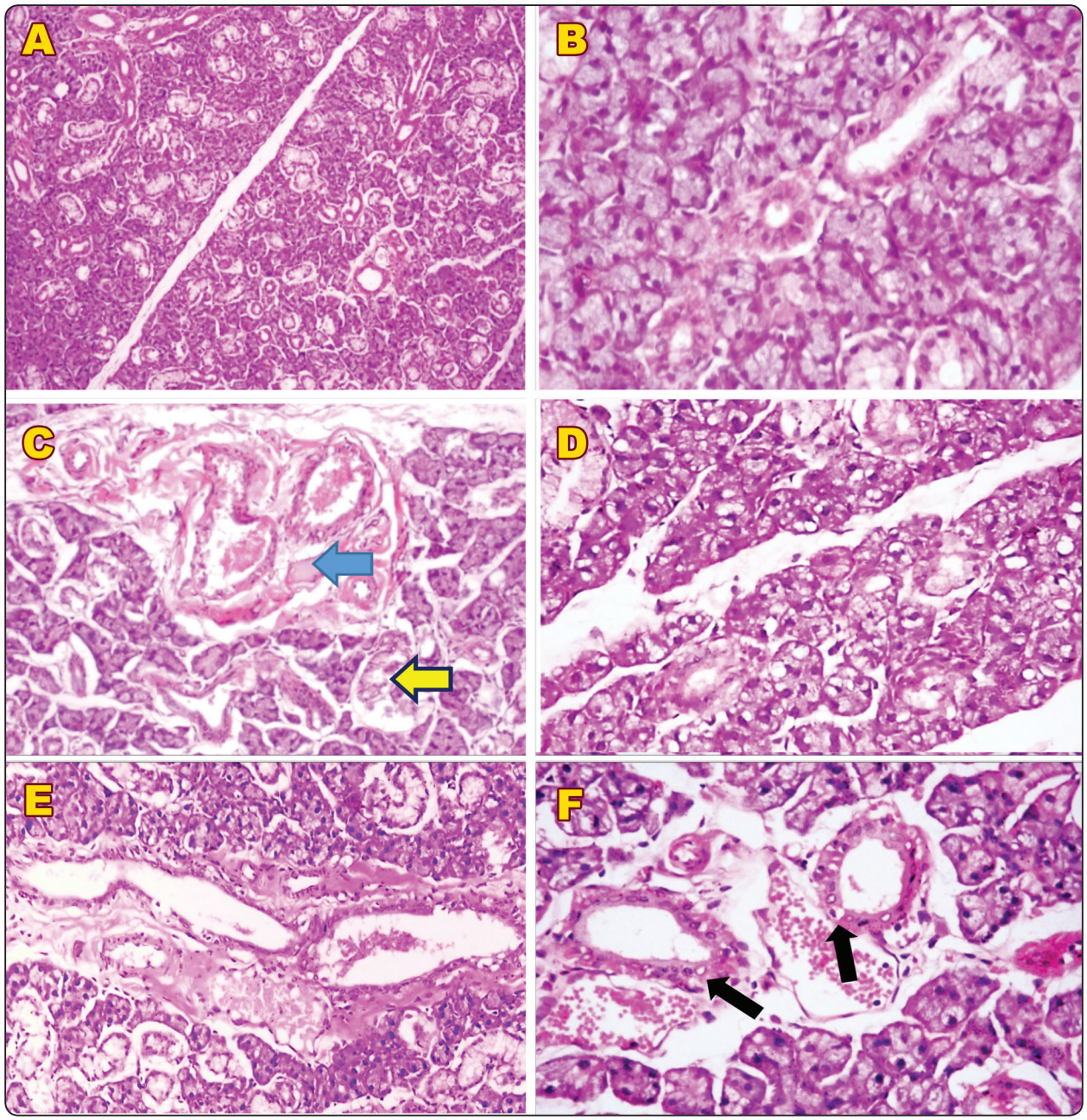

Fig. (1) Photomicrographs showing the histological pictures of rat's submandibular salivary glands. (A, B) Normal histological architecture of serous acini, intralobular and interlobar ducts in group 1 (x 100, 400). (C: F) Degenerative changes of hypercholesteremic rats in group 2. (C) Disorganized serous acini architecture with destruction of GCT's (yellow arrow), and hyalinization within the interlobular connective tissue septa (blue arrow). (D) Serous acini with cytoplasmic vacuolization. (E) Atrophic epithelial lining of excretory ducts surrounded by dilated engorged blood vessels. (F) Excretory ducts with apoptotic figures in their lining (black arrows). [C, E x 200] [D, F x 400].

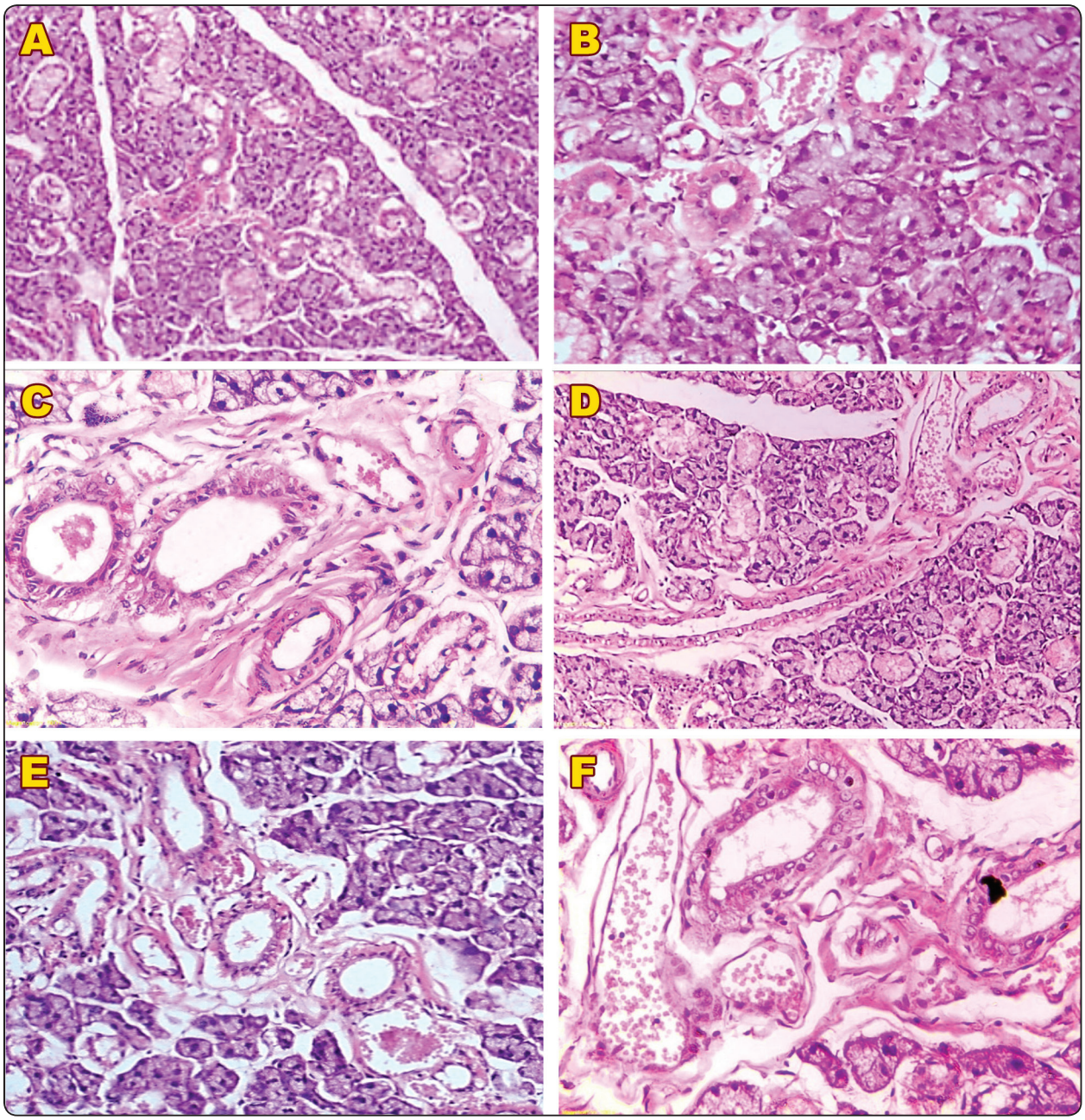

Fig. (2): Photomicrographs showing the histological pictures of rat's submandibular salivary glands. (A: C) In group 3, normal histological appearance of serous acini, interlobar connective tissue septa. Some striated ducts appeared shrunken. (D: F) In group 4, normal serous acini, destructed GCT's, dilated engorged blood vessels, abnormal appearance of excretory ducts and interlobar connective tissue septa widening were recognized. [A, D x 100] [B, E x 200] [C, F x 400]. Note: Dilatation of blood vessels and their engorgement of RBCs in both groups.

the acini and ducts (**Fig. 4 E**). Quantitative analysis revealed highly statistically significant increase of NF-κB immunoreactivity in acini and ducts in group 2 (83.38 ± 3.9^{a}) in comparison with group 1 (11.90 ± 2.5^{d}) **(Fig. 4 F)**. And for group 3 and 4, they showed statistically significant decrease (20.05 ± 2.5^{c}) and (43.05 ± 4.1^{b}) respectively in comparison to group 2 (83.38 ± 3.9^{a}) **(Fig. 4 G, H)**.

Reduction decrease percentage (RD%) was helpful in detecting which treatment more effective in protecting the rats from the harmful drawbacks of hypercholesterolemia. There was significant improvement in the rats receiving Ns (48.18% and 75.95%) rather than Ls (28.98% and 48.37%) through calculating *RD%* for α-SMA and NF-κB, respectively (**table 1**).

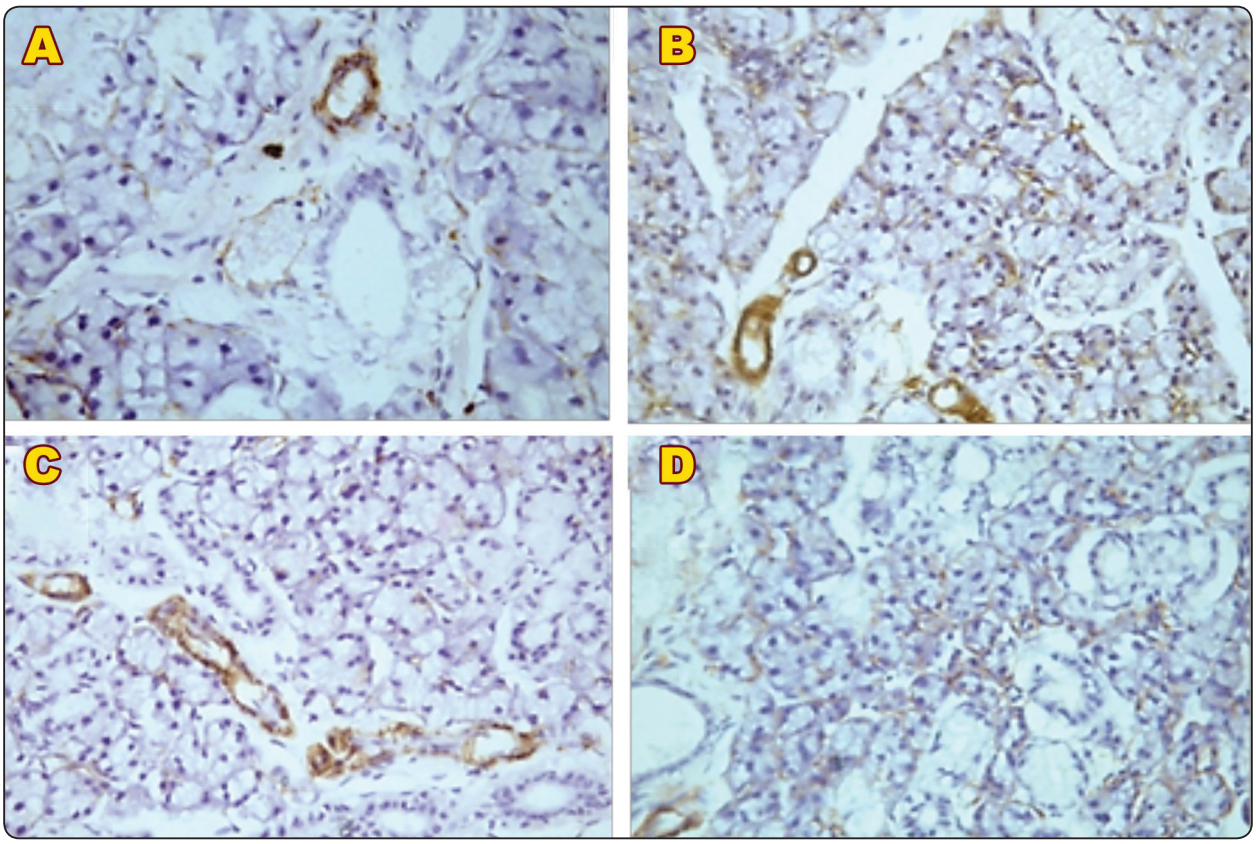

Fig. (3): Photomicrographs of the immunohistochemical stained sections from (A to D) showing the immunoreactivity reaction to α-SMA antibodies for all group X 400; A) control group showing weakly staining reactivity limited to periphery of the acini (MECs) and blood vessels, B) Hypercholesteremic group showing intense staining reaction at acini periphery, duct, and blood vessels. C, D) Group 3 and 4 showed weakly to moderately staining reactivity at periphery of the acini and blood vessels. [A x 400] [C: D x 200]

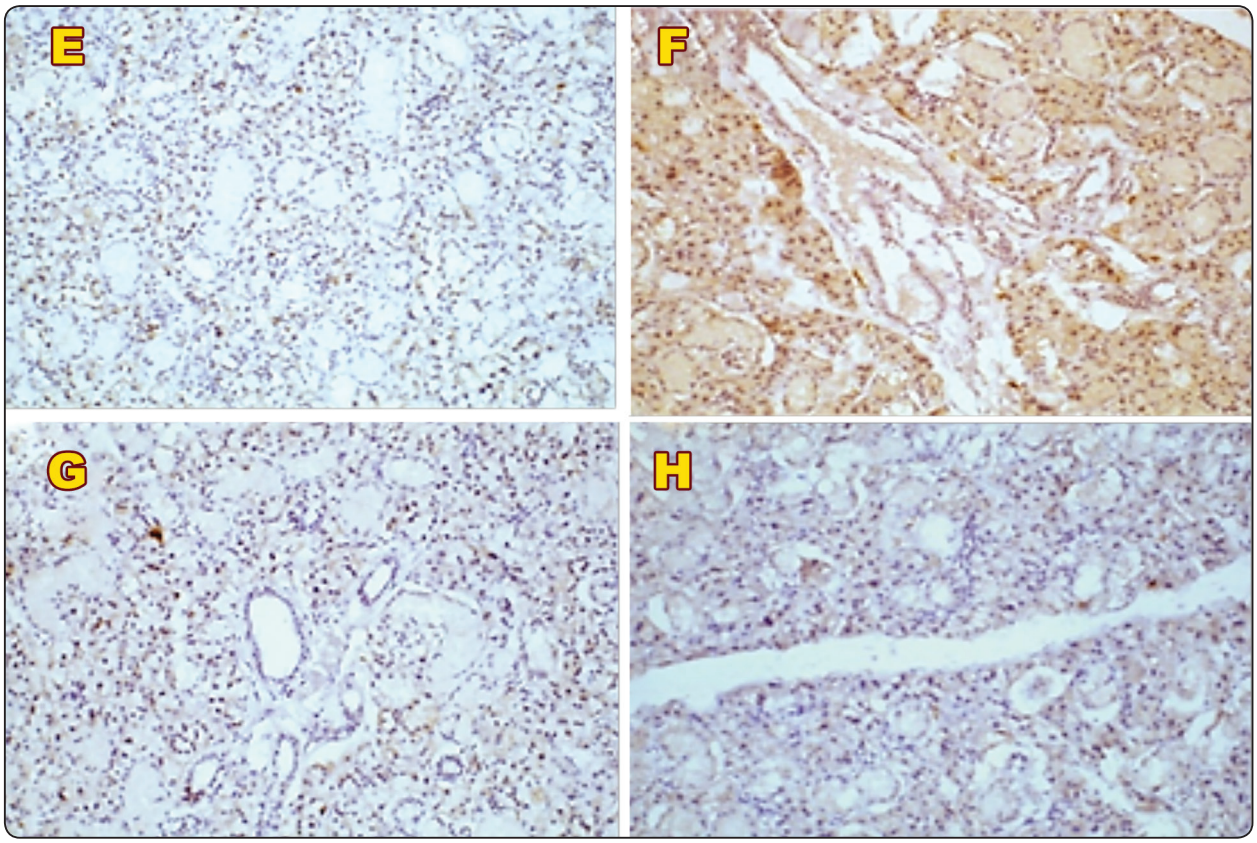

Fig. (4): Photomicrographs of the immunohistochemical stained sections from (E to H) showing the immunoreactivity reaction to NF-kB for all group X 200; E) Control group showing weak positive NF-ϰB immunoreactivity in the acini and ducts, F) Hypercholesteremic group showing increase of NF-ϰB immunoreactivity in both acini and ducts. G, H) Group 3 and 4 showing weak to moderate NF-ϰB immunoreactivity in both acini and ducts, respectively. [E: H x 100]

TABLE (1): Statistically immunohistochemical expression of NF-ϰB and α-SMA in different groups

Groups	NF-ϰB	α-SMA	RD % NF-ϰB	RD % α-SMA
Group 1	11.90±2.5[d]	6.44±1.6[0]d		
Group 2	83.38±3.9[a]	22.54±1.[8]a		
Group 3	20.05±2.5[c]	11.66±1.4[c]	75.95% (RD%)	48.18%
Group 4	43.05±4.1[b]	15.98±2.4[b]	48.37% (RD%)	28.98%
F_{cal}-Test	264.96	76.67		
P values<0.05	<0.0001**	<0.0001**		

Reduction decrease (RD%) = (Disease-treatment)/Disease) *100

***** a,b; means significant differences between groups using ANOVA test at P-value<0.05***

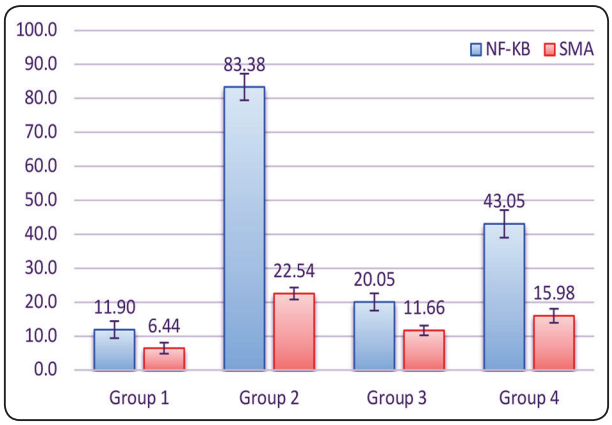

Fig. (5): Showing statistically immunohistochemical expression of NF-ϰB and α-SMA in different groups.

DISCUSSION

Salivary glands are responsible for production of saliva which plays an obvious role in preserving proper health of oral and gastrointestinal tract. However, impairment of salivary gland function is mediated by aging, inflammatory conditions, drugs, and multiple diseases[26]. The main disadvantages of medication which are utilized in treating hypercholesterolemia are their side effects and high price. So, attentions were directed to the medicinal plants which characterized by their easy availability and relatively minimal adverse effects[27, 28].

The main cause of the present structural deterioration of the submandibular salivary glands in hypercholesterolemic group might be due to the possible adverse reaction of hypercholesterolemia leading to disturbance in the protein secreting machine of the cells and reduction in the secretory cellular activity. In addition, the current investigation showed appearance of apoptotic bodies. This finding was going hand in hand with the data recorded by other studies which demonstrated that increasing the number of apoptotic bodies in hyperlipidemic submandibular salivary glands could be explained by different mechanisms of apoptosis. The inflammatory reaction plays a crucial role in stimulating apoptotic process and collagen synthesis[29,30].

Histological results of hypercholesterolemic group revealed severe atrophic and degenerative changes in the secretory terminal portions and ducts. Some cells showed intracytoplasmic vacuoles which were explained by *Selim*[31] as a kind of fatty degeneration when investigated the effect of high fat diet on parotid glands ultra-structurally. Other acini lost their normal architectures and appeared eroded. In the present study, atrophy of the lining of excretory duct, dilatation and stagnation of their secretion were evident. These findings might result from dysfunction of the gland and failure of exocytosis. Similar findings were obtained by *Izumi et al*.,[4] who suggested that there was strong correlation between impairment of salivary flow and elevated levels of plasma cholesterol. Furthermore, there was significant reduction in the salivary flow of the patients suffering from systemic disorders as rheumatoid diseases, hyperlipidemia, and Sjögren's syndrome.

Our study showed widening and fibrosis of interlobular connective tissue septa with dilated engorged blood vessels. Zidan and Alazouny[32] interpreted the presence of fibrosis might be considered as a method of defense mechanism due to the harmful effect of hyper-cholesterol. Dilated blood vessels which engorged with RBCs were observed to allow diffusion of high amount of blood to the degeneration areas. Rahmanzadeh et al.,[33] reported that the inflamed blood vessels adversely affected the parenchymal cells function due to the defect in transportation of oxygen and nutrients to different types of cells.

The histological results of the rat's submandibular salivary gland-treated with Ns, revealed nearly normal histological structures which reflected the beneficial effects of Ns. These results were in coincidence with Al-Naqeep et al.,[34] who observed that hyperlipidemia was improved following the

administration of Ns seeds and oil which has been proven to protect against development of atherosclerosis. Online with our study, Sahebkar et al., [35] carried out a meta-analysis of clinical trials suggested the modulatory effects of Ns and its ability to reduce the triglycerides and total cholesterol.

Thymoquinone represents one of the major components of Ns which has been shown to promote the function of enzyme in lipid metabolism. It is also responsible for cellular protection against lipid peroxidation and augment the superoxide dismutase (SOD) activity[36]. Other constituents of Ns as flavonoids, phenolic component and vitamins as ascorbic acid may also participate as antioxidants which ameliorate the non-enzymatic system through direct scavenging of carbon-centered radicals and hydroxyl radicals[36,37]. Other studies supported our results through improving lipid profile and anti-atherosclerotic properties of Ns, reducing intestinal cholesterol absorption, raising biliary and fecal excretion of cholesterol[38,39].

Concerning the Ls group, it demonstrated some degree of improvement in the histological picture of the submandibular salivary glands. Chauhan *et al*.,[40] stated that, the hypocholesterolemic effect of Ls might be attributed to inhibition of cholesterol biosynthesis, lipids absorption and enhancing lipids excretion. This is mediated through the inhibition of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase), the rate-limiting enzyme that initiates the first step in biosynthesis of cholesterol. Our findings agreed with those of Al-Bazii [41] who demonstrated that Ls might stimulate the cellular differentiation and proliferation of mammary glands due to its flavonoids content.

On the other hand, the constituents of Ls, tannin and flavonoids possess antioxidant activity as well as glutamate, glycine and cysteine which are intermediate for production of endogenous antioxidant glutathione These compounds had the ability to scavenge free radicals by single electron transfer [42]. El-Zawahry *et al*.,[15] confirmed that Ls ameliorated the oxidative disorders induced by high fat diet with significant improvement in the levels of apelin, triglycerides and very low-density lipoprotein (VLDL).

In the present study, the differentiation of MECs were evaluated immunohistochemically by detection of α-SMA expression. Quantitative assessment of the α-SMA of the submandibular glands of hypercholesteremic rats reflected highly significant increase in the MECs around periphery of acini, intralobular duct and smooth muscle lining of blood vessel wall. These results were confirmed by some studies which reported that proliferative and morphological changes of MECs resulted in increased of their sizes and numbers even when the acini were subjected to atrophy[29,43]. Quantitative analysis of NF-κB in the submandibular salivary glands of hypercholesterolemic rats revealed high statistically significant increase in its cytoplasmic and nuclear expression in the acini and ducts. NF-κB is present in the cytoplasm as an inactive non-DNA-binding form. NF-κB plays a pivotal role in different biological processes as, oxidative stress and inflammation. Any increase in the expression / nuclear translocation of NF-κB could result in histological changes of the salivary glands through inducing the inhibitors of kappa B cell (IκB) degradation and phosphorylation through effect on markers of inflammation as IFNα, TNFα and IL-6[44].

Significant decrease in the expression of α-SMA and NF-κB was detected in the rat's submandibular salivary glands of group 3 and 4 treated with Ns and Ls respectively in comparison to hypercholesteremic rats in group 2. Polyphenols could play a prominent effect in the prevention of chronic inflammatory diseases. They inactivated NF-κB cascades through regulation the immune cell, proinflammatory cytokines or their binding to DNA. They had the ability to decrease the expression of pro-inflammatory genes, toll-like receptor (TLR)

and glutathione which affect mainly on their anti-inflammation and antioxidant properties. They inhibited mammalian target of rapamycin complex 1 (mTORC1) which is necessary for protein formation[45]. From the point of view of Al-Refai *et al*.,[46] in stressful conditions, the submandibular salivary glands tried to compensate the defect in the secretory function by increasing the numbers of MECs to squeeze the accumulated secretion. Then, the antioxidants compounds had the ability to improve the harmful effects of free radicals and decreasing the expression of α-SMA. Polyphenols

CONCLUSION

The immunohistochemical findings confirmed the histological results of the present study that proved the superior protective role of the Ns rather than Ls seeds on the submandibular salivary glands of hypercholesterolemic rats.

RECOMMENDATIONS

More studies will be required to investigate different blood parameters and lipid profile to be sure about the effectiveness of the drugs and avoid any health problems.

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