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STUDY THE INFLUENCE OF ANTIOXIDANT THERAPY ON AGE- RELATED CHANGES IN THE BUCCAL MUCOSA OF RATS (HISTOLOGICAL AND IMMUNOHISTOCHEMICAL INVESTIGATION)

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ABSTRACT

Aim The aim of the present work was to study the histological and immunohistochemical impact of antioxidant supplementation on age- related changes in the buccal mucosa of rats using routine H&E stain and immunohistochemical detection of any possible changes in NF-ϰB (nuclear factor kappa) in the surface epithelium, lamina propria as well as buccal salivary glands.

Materials and methods Fifty young adults albino male rats with average body weight 100± 10 grams and 2 months of age were used in this investigation. The animals were divided into 5 equal groups 10 aminals each. They were caged, 5 animals per cage and fed diet consisting of coarse corn barley and powdered milk. Diet and drinking water were supplied adlibitum throughout the whole experimental period which lasted for 9 months. **Group I:** animals served as control. 5 animals were sacrificed by cervical dislocation after one month to serve as young controls, while the rest remained till the end of the experiment to serve as old controls. **Group II:** animals received vitamin A. **Group III:** animals received vitamin C. **Group IV:** animals received Selenium. **Group V:** animals received combined vitamin A, C and Selenium in the same doses given to group II, III, IV respectively. Preparation of the diet mixed with antioxidants were repeated several times until the experiment was over. At the end of the experiment, The animals of different groups were scarified by cervical dislocation. Their buccal mucosa were dissected out, fixed in 10% neutral buffered formalin, washed, dehydrated in ascending grades of ethyl alcohol, cleared in zylene and embedded in paraffin. Six micron thick sections were cut and stained with Hematoxylin and eosin for histological examination. Masson's trichrome stains for collagen evaluation. Immunohistochemical localization of NF-ϰB (nuclear factor kappa) for detection of any possible changes.

Results The histological and immunohistochemical results revealed aging caused atrophic and degenerative changes in the oral mucosa associated with increased expression of nuclear factor kappa. Consuming antioxidant vitamins as vitamin A, vitamin C or selenium separately partially modulates the action of aging process and increase the regenerative capacity of the oral mucosa and oral salivary glands. Consuming combined antioxidant vitamin A, vitamin C and selenium had a powerful and beneficial synergizing antiaging effect on the aforementioned tissues.

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Conclusion The balance between oxidation and antioxidation (redox balance) is critical in maintaining a healthy biological system. Our histological and immunohistochemical investigation proved the important role of antioxidant for keeping the integrity and health of the mucous membrane and salivary glands of the buccal mucosa of aging rats under investigation.

Recommendation Improvement of dietary intake by consuming neutral foods rich in antioxidant vitamin A, C and selenium is of utmost importance to gain the antiaging benefits and to prevent age –related degenerative diseases.

KEY WORDS: aging, vitamin A, vitamin C, selenium, antioxidant, NF-ϰB, buccal mucosa.

INTRODUCTION

Aging is a normal physiological process that influences the structure and function of all tissues of the body, it is among the greatest known risk factors for most human diseases and death[1]. Ageing can refer to single cells within an organism which have ceased dividing and called cellular senescence. The causes of ageing are unknown; current theories are assigned to the damage concept (whereby the accumulation of damage such as DNA breaks, oxidized bases and/or mitochondrial malfunctions[2]) or to the programmed ageing concept (whereby internal processes such as DNA telomere shortening) may cause biological systems to fail and may cause ageing[3].

The free radical theory of aging (FRTA) states that organisms age because cells accumulate free radical damage over[4]. free radical damage is closely associated with oxidative damage. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reaction can produce free radicals (FR), which start chain of reactions that damage cells. Antioxidants terminate these chain of reactions by removing free radical intermediates, and inhibit other oxidation reactions by being oxidizing themselves. As a result, antioxidants are often reducing agents[5]. Free radicals are molecules with impaired electron causing them to be highly reactive and unstable so it react quickly with other molecules to steal electrons to gain stability. Antioxidant seek out free radicals and donate electrons to neutralize them. In other words antioxidants can counter the formation of free radicals and prevent free radical damage by donating electrons.

Oxidative stress has been described as a process derived from the inability of the body's endogenous antioxidant defenses to scavenge free radical species and has been related to many pathologies such as ageing, cardiovascular disease, neurodegenerative disorders, cancer, complex regional pain syndrome and many others[6]. When free radical-derived oxidative damage to nucleic acids, proteins and lipids of the cellular and extracellular matrix is observed, it produces damages of clinical importance and severity. The most crucial concerns is the mutation-induced carcinogenesis, the damage to cellular membrane lipoproteins and lipid-mediated oxidative damage leading to tegument ageing evolution.

The physiologic and pathologic changes in the human body depend on free radical and reactive oxygen species (ROS) interactions to maintain normal cellular activities. In aerobic organisms, the imbalance between ROS generation and antioxidant levels leads to increased oxidative stress and cellular degeneration.

NF-ϰB (nuclear factor kappa-light-chain-enhancer of activated B cells) is a dimeric transcription factor that is involved in the regulation of a large number of genes that control various aspects of the immune and inflammatory response. It is activated by a variety of stimuli such as stress, cytokines, various forms of radiation, heavy metals, oxidized LDL, bacterial or viral antigens, free radicals and oxidative stress such as exposure to H_2O_2[7]. Recent studies demonstrated the mechanism

by which NF-ϰB is regulated by oxidative stress. Incorrect regulation of NF-ϰB has been linked to cancer, inflammatory and autoimmune diseases, septic shock, viral infection, improper immune development and apopotosis[8] .

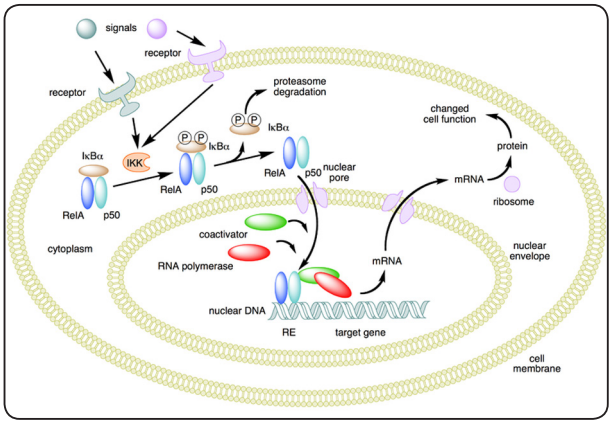

Fig. (1): Diagram showing Mechanism of NF-ϰB action. The NF-ϰB heterodimer between Rel and p50 proteins is used as an example. While in an inactivated state, NF-ϰB is located in the cytosol complexed with the inhibitory protein IϰBα. Through the intermediacy of integral membrane receptors, a variety of extracellular signals can activate the enzyme IϰB kinase (IKK). IKK, in turn, phosphorylates the IϰBα protein, which results in ubiquitination, dissociation of IϰBα from NF-ϰB, and eventual degradation of IϰBα by the proteosome. The activated NF-ϰB is then translocated into the nucleus where it binds to specific sequences of DNA called response elements (RE). The DNA/NF-ϰB complex then recruits other proteins such as coactivators and RNA polymerase, which transcribe downstream DNA into mRNA, which, in turn, is translated into protein, which results in a change of cell function [9].

Maintaining a good balance of oxidants and antioxidants is important for oral health as well as systemic health. Factors such as pollutants, alcohol, nicotine, hydrogen peroxide, and dental compounds and procedures can disturb the balance of oxidants in oral tissues, causing oxidative stress. Antioxidants can help to offset the imbalance. There are several thousand antioxidants, including enzymes, vitamins, minerals and other nutrients and compounds. Some antioxidants are produced within the body; others, such as vitamin A, vitamin C and selenium must be provided by external sources. A healthy, varied diet rich in fruits and vegetables, whole grains, and nuts is an excellent source of antioxidants.

Vitamin A is a group of unsaturated fat – soluble nutritional organic compounds that includes retinol, retinal, retinoic acid, and several provitamin A carotenoids such as all-trans-beta-carotene. Vitamin A has multiple functions: it is important for growth and development, for the maintenance of the immune system and good vision[10]. Vitamin A is needed by the retina of the eye in the form of retinal, which combines with protein opsin to form rhodopsin, the light-absorbing molecule necessary for both low-light (scotopic vision) and color vision[11]. Vitamin A also functions in a very different role as retinoic acid, which is an important hormone-like growth factor for epithelial and other cells[12]**.** Individuals who suffer from vitamin A deficiency are plagued by night blindness and longer vision-restoration times. Other changes include impaired immunity, increased risk of ear infections, urinary tract infections, Meningococcal disease, hyperkeratosis, keratosis pilaris and squamous metaplasia of the epithelium lining the upper respiratory passages and urinary bladder to a keratinized epithelium. With relations to dentistry, a deficiency in Vitamin A leads to enamel hypoplasia.

Ascorbic acid or "vitamin C" is a monosaccharide oxidation-reduction (redox) catalyst found in both animals and plants. As one of the enzymes needed to make ascorbic acid has been lost by mutation during primate evolution, humans must obtain it from the diet. Most other animals are able to produce this compound in their bodies in the liver from glucose and do not require it in their diets. Ascorbic acid is required for the conversion of the procollagen to collagen by oxidizing proline residues to hydroxyproline. In other cells, it is maintained in its reduced form by reaction with glutathione, which can be catalysed by protein disulfide isomerase and glutaredoxins. Ascorbic acid is essential for

collagen and neurotransmitter synsthesis. In addition to its effects as antixoxidants, antiatherogenic, anticarcinogenic and immunomodulator and prevents cold etc[13]. Deficiency in this vitamin cause the disease scurvy in humans. An adult need 10mg/ day of vitamin C to prevent scurvy[14].

Selenium (Se) is an essential trace element, and its low status in humans has been linked to increased risk of various diseases, such as Keshan disease, cancer[15], heart disease[16], rheumatoid arthritis[17]. In recent years, selenium research has attracted tremendous interest because of its important role in antioxidant selenoproteins for protection against oxidative stress initiated by excess reactive oxygen species (ROS) and reactive nitrogen species (RNS). Interest in Se research has led to the discovery of at least 30 selenoproteins; however, the biochemical functional roles of some of these selenoproteins are still unknown[18]**.** Other selenoproteins help regulate thyroid function and play a role in the immune system[19]**.**

Several forms of selenium enter the body as a part of amino acids within proteins. Selenium exist in many different chemical forms in biological materials either as organic compounds, such as selenomethionine and dimethylselenide, and inorganic selenites and selenates. In foods, Se is predominantly present as selenomethionine, which is an important source of dietary Se in humans, and also as a chemical form that is commonly used for Se supplements in clinical trials[20]**.** Plant foods are the main dietary sources of selenium in most countries throughout the world. Se also can be found in some meats, seafood and bread in addition to some nuts[21] .

Many studies demonstrated that advance in age affect the dental and paradental structures so that our study aimed to investigate the histological and immunohistochemical impact of antioxidant supplementation on age- related changes in the buccal mucosa of rats using routine H&E stain and immunohistochemical detection of any possible changes in NF-ϰB (nuclear factor kappa) in the surface epithelium, lamina propria as well as buccal salivary glands.

MATERIALS AND METHODS

Fifty young adults albino male rats with average body weight 100± 10 grams and 2 months of age were used in this investigation. The animals were divided into 5 equal groups 10 aminals each. They were caged, 5 animals per cage and fed diet consisting of coarse corn barley and powdered milk. Diet and drinking water were supplied adlibitum throughout the whole experimental period which lasted for 9 months.

Group I: animals served as control. 5 animals were sacrificed by cervical dislocation after one month to serve as young controls, while the rest remained till the end of the experiment to serve as old controls.

Group II: animals received vitamin A, 0.7 mg/kg diet[22] for the whole experimental period. This means that 4.2 mg of vitamin A were mixed thoroughly with 6 kg diet using gloved hands in a large clean bowel.

Group III: animals received vitamin C, 60 mg/kg diet[23] for the whole experimental period. This means that 360 mg of vitamin C were mixed thoroughly with 6 kg diet using gloved hands in a large clean bowel.

Group IV: animals received Selenium, 150 mg/kg diet[22] for the whole experimental period. This means that 900 mg of selenium were mixed thoroughly with 6 kg diet using gloved hands in a large clean bowel.

Group V: animals received combined vitamin A, C and Selenium in the same doses given to group II, III, IV respectively as mentioned before. Preparation of the diet mixed with antioxidants were repeated several times until the experiment was over.

At the end of the experiment, The animals of different groups were scarified by cervical

dislocation. Their buccal mucosa were dissected out, fixed in 10% neutral buffered formalin, washed, dehydrated in ascending grades of ethyl alcohol, cleared in zylene and embedded in paraffin. Six micron thick sections were cut and stained with

1- Hematoxylin and eosin for histological examination.
2- Masson's trichrome stains for collagen evaluation.
3- Immunohistochemical localization of NF-κB (nuclear factor kappa) for detection of any possible changes.

Immunohistochemical staining procedure used for NF-κB **detection**[24]

- 4-μm-thick paraffin-embedded sections from buccal tissues were deparaffinized, rehydrated, immersed in target retrieval solution, and blocked for endogenous peroxidase activity.
- The sections were permeabilized in TNB-BB (100 mM Tris, pH 7.5, 150 mM NaCl, 0.5% blocking agent. 0.3% Triton-X, and 0.2% saponin) and incubated in primary monoclonal antibodies of NF-κB/p65, NF-κB/p50, and IκBα (Santa Cruz Biotechnology) at 1:200 dilution, respectively, overnight at 4°C.
- After washing three times in TBS, sections were incubated for 2 hours at room temperature with HRP-labeled polymer conjugated with secondary antibody.
- Immunoreactive complexes were detected using AEC^+ substrate chromagen consisting of 3-amino-9-ethylcarbazole. Slides were then counterstained in Mayer's hematoxylin, mounted in crystal mount media, and dried overnight on a level surface.
- Positive staining section appeared brown in color. The intensity of the immunohistochemical staining results was assessed as follows: negative, weakly positive, moderately positive and strong positive staining reactions.

RESULTS

I- Hematoxylin and eosin stain

Group I animals (control group)

a) Young controls:

The histological examination of the buccal mucosa of the young control rats showed normal histological features of the surface epithelium and lamina propria. The epithelium was formed of keratinized stratified squamous epithelium, characterized by folding toward the underlying lamina propria forming regular, broad, few and short epithelial ridges. The epithelium was formed of four layers, the basal cell layer formed of a single row of low columnar cells facing the connective tissue and resting on the basement membrane. The prickle cell layer was formed of several rows of irregularly polyhyderal cells with spherical central placed nuclei, intercellular spaces and intercellular bridges giving them the spinous or prickly appearances. The granular cell layer was formed of 2-3 rows of large flattened cells superficial to the prickle cells. The keratin cell layer was the most superficial and appeared as an eosinophilic amorphous layer.

The connective tissue of the lamina propria showed regular arrangement of collagen fibers, fibroblasts, progenitor cells and small sized blood vessels.

A submucosa layer connecting the lamina propria of the buccal mucosa to the buccinators muscles was seen. This submucosa was formed of densely packed collagen fibers, in between them there was loose connective tissue containing fat cells and buccal salivary glands which were mixed predominantly mucous. Parts of the buccal glands were seen between the muscle fibers (Fig. 2, A).

b) Old controls:

The buccal mucosa of old control rats presented severe atrophic and degenerative changes that involved the surface epithelium and lamina proporia as well as the buccal salivary glands. The

surface epithelium manifested a marked decrease in the thickness of keratinized stratified squamous epithelium with short and scanty rete ridges. The prickle cell layer showed edema and swelling of the cells. The surface epithelium presented abnormal cytoplasmic vacuolization of some epithelial cells. The lamina propria showed marked degeneration and dissociation of the collagen fibers with apparent reduction in the number of fibroblasts that usually presented abnormal cytoplasmic vacuolization with small and darkly stained nuclei. Dilatation of the blood vessel and with blood stagnation. The buccal salivary gland showed cystic transformation of some of the mucous acinar cells and others showed necrosis (fig.2,B).

Group II, III, and IV animals (old rats receiving vitamin A, vitamin C and selenium separately)

The examined buccal mucosa of rats that received vitamin A, vitamin C and selenium separately showed slight to moderate improvement in their histological structures when compared to old controls. The surface epithelium showed less atrophic with minute degenerative changes of their cells. The lamina propria partially regained the density and arrangement of its collagen fibers with the presence of a lot of blood vessels. The buccal salivary glands showed partial improvement of the normal histological architecture of their acini and ducts (fig.3).

Group V animals (old rats receiving combined vitamin A, vitamin C, and selenium)

The light microscopic examination of group V animals that received combined vitamin A, vitamin C, and selenium, showed nearly the same histological feature of the buccal mucosa of the young control. There was marked increase in the thickness of the surface epithelium, in the numbers of its cells and in the length of the epithelial ridges. The rate of the mitotic activity are almost increased. The lamina propria showed marked increase in the cellularity and density of the collagen fibers which became highly organized with increased vascularity. The buccal salivary gland showed marked improvement in the histological structure of their acini and ducts (fig.4).

II- Massons' Trichrome Stain

Group I animals (Control group):

a) Young controls:

The histological examination of the buccal mucosa taken from young control rats, and stained

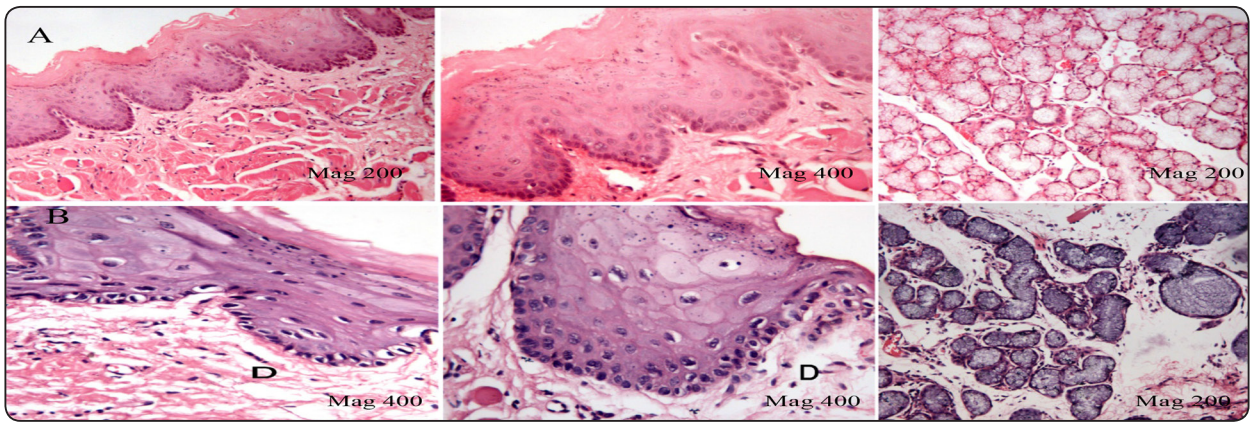

Fig. (2) A): Photomicrographs of buccal mucosa of a control rats showing surface epithelium of the keratinized stratified squamous type, regular, broad, few and short epithelium ridges toward the underlying lamina propria. The lamina propia and submucosa formed of densly packed collagen fibers and buccal salivery glands (H&E, ori. Mag.200, 400, 200). B): Photomicrographs of the buccal mucosa of the old control rats showing extreme attrophy and degeneration of the surface epithelium. The lamina propria showed degeneration of the collagen fibers (D), dilatation of the blood vessel, The buccal salivary gland showed cystic transformation of some of the mucous acinar cells and others showed necrosis (H&E, ori. Mag.400, 200).

with Massons' trichrome stain revealed a strong positive staining of the collagen fibers of the lamina propria as well as the connective tissue septa dividing the buccal salivary glands into lobes and lobules (fig.5, A).

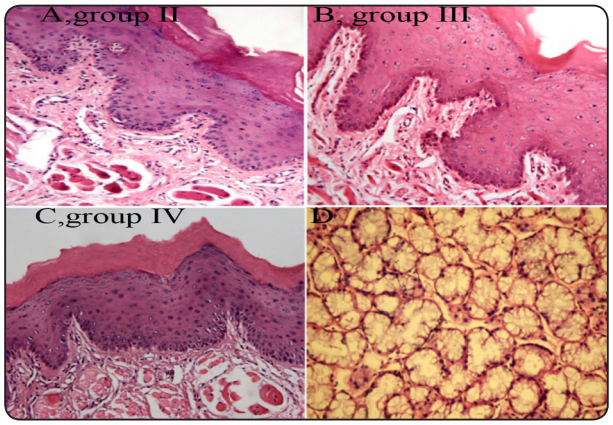

Fig. (3): Photomicrographs of the buccal mucosa of group II (A), III (B), IV (C) animals received vitamin A, C and selenium respectively showing slight strophic and degenerative changes in the surface epithelium with slight degeneration of the collagen fibers of the lamina propria (H&E, ori. Mag.200). (D) The buccal salivary glands showed partial improvement of the normal histological architecture of their acini and ducts (H&E, ori. Mag.400).

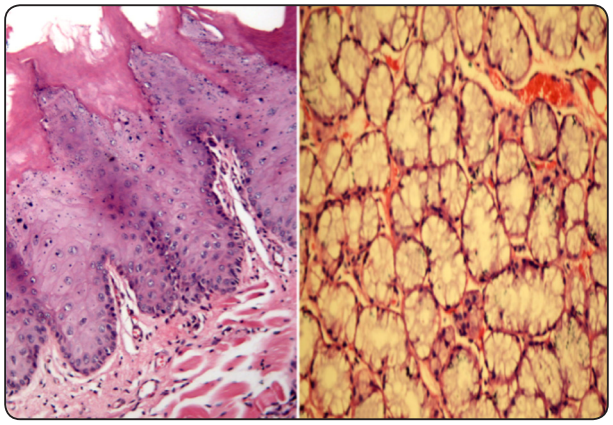

Fig. (4): Photomicrographs of the buccal mucosa of group V animals received combined vitamin A, C and selenium showing increased thickness of the surface epithelium with elongation of its epithelial ridges. The lamina propria showing increase in the cellularity and vascularity as well as normal density and organization of the collagen fibers. Improvement in the histological structure of the buccal salivary gland. (H&E, ori. Mag.400).

b) Old controls:

The buccal mucosa taken from old control rats showed the collagen fibers of the lamina proporia and the connective tissues septa of the buccal salivary glands dissociated, degenerated and stained weakly positive with Massons' trichrome stain (fig. 5, B).

1. Group II, III, IV animals (old rats receiving vitamin A, C and selenium respectively)

The histological examination of buccal mucosa obtained from old rats of group II, III and IV respectively showed slight improvement in the density and distribution of the collagen fibers of the lamina propria and connective tissues septa of the buccal salivary glands which became more integrated, organized and presented moderately positive stain with Masson, trichrome stain. (fig.5, C, D, E).

2. Group V animals (old rats receiving combined vitamin A, vitamin C, and selenium)

The examined buccal mucosa obtained from old rats received combined vitamin A, vitamin C, and selenium showed strongly positive reaction to Massons' trichrome stain and became more organized and integrated comparable to those of the young control animals (fig.5, F).

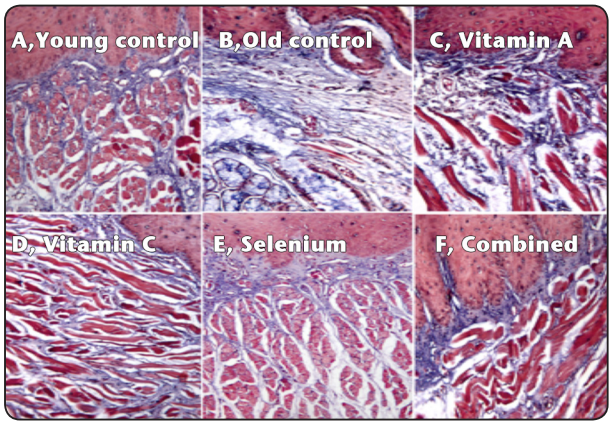

Fig. (5): Photomicrographs of the buccal mucosa of all groups of animals stained with Massons' trichrome stain showing improvement in the organization and staining reactivity of collagen fibers to Massons' trichrome stain compared with old control (Massons' trichrome, ori. Mag.200).

II- Immunohistochemical localization of NF-ϰB (nuclear factor kappa)

Group I animals (Control group):

a) Young controls:

Nuclear factor (NF)-ϰB expression in the surface epithelium and buccal salivary glands of buccal mucosa of young control rats using primary monoclonal antibodies of NF-ϰB/p65, NF-ϰB/p50, and IϰBα showed negative to weak positive staining reactivity (fig.6, A, G).

b) Old controls:

Nuclear factor (NF)- ϰB expression in the buccal mucosa of old control rats using subunits of NF-ϰB primary monoclonal antibodies showed strong positive staining reactivity of the different strata of the surface epithelium and buccal salivary glands (fig.6, B, H).

Group II, III,IV animals (old rats receiving vitamin A, C and selenium respectively)

Examination of sections taken from the buccal mucosa of old rats received vitamin A, C and selenium separately incubated with subunits of NF-ϰB primary monoclonal antibodies revealed weak to moderate staining reactivity of their basal and suprabasal cells. Cells of the duct and mucous acini of the buccal salivary gland showed weak positive reactivity (fig.6, C, D, E, I).

Group V animals (old rats receiving combined vitamin A, vitamin C, and selenium)

Nuclear factor (NF)- ϰB expression in the buccal mucosa of old rats received combined vitamin A, C and selenium using subunits of NF-ϰB primary monoclonal antibodies showed weak to moderate staining reactivity of surface epithelium and the buccal salivary glands (fig.6, F,I).

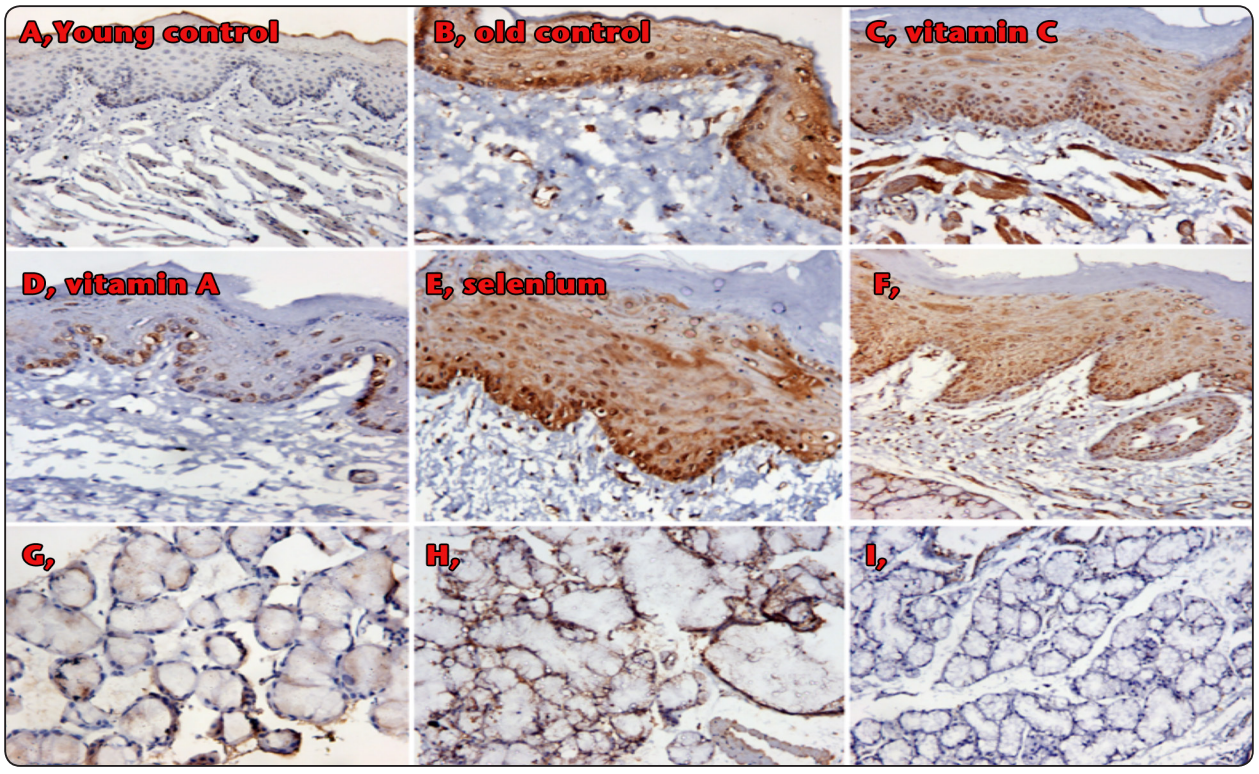

Fig. (6): Photomicrographs of the buccal mucosa of all groups of animals showing different staining reactivity to the primary monoclonal antibodies of NF-ϰB/p65, NF-ϰB/p50, and IϰBα subunits of NF-ϰB (orig. mag. 200, 400).

DISCUSSION

The oral cavity has correctly been described as a mirror that reflects the health of the individual. Age-induced changes can be manifested as alterations in the oral mucosa lining the mouth. The buccal mucosa and buccal salivary glands were chosen in our investigation to study the effects of aging and antioxidant therapy on these tissues.

Our histological result investigated that the buccal mucosa of old control rats presented severe atrophic and degenerative changes that involved the surface epithelium and lamina proporia as well as the buccal salivary glands. The surface epithelium manifested a marked decrease in the thickness of keratinized stratified squamous epithelium, edema and swelling of the prickle cells, some epithelial cells presented abnormal cytoplasmic vacuolization and short rete ridges. The underlying lamina propria showed marked degeneration and dissociation of the collagen fibers with apparent reduction in the number of fibroblasts. Masson' trichrome stain confirmed these marked degeneration and dissociation of the collagen fibers. In our opinion these degenerative changes most probably due to aging process. In addition to dilatation of the blood vessel and blood stagnation which results in stasis and decrease in the blood flow which most probably causes hypoxia and ischemia of the tissues that aggregates the degenerative effects of aging. This go with those of Reddy[25] who studied the age changes in the gingiva and showed decreased keratinization, reduced amount of stippling, atrophy of the connective tissue, decreased connective tissues cellularity, decrease in the mitotic activity, decrease in fibroblasts but increase in the number of mast cells, decrease in collagen fibers and mucopolysacharides, increased in elastic fibers, decrease in the vascularity with presence of arteriosclerotic changes and decreased oxygen consumption. Breustedt[26] stated that age– induced changes are brought about mainly by arteriosclerotic processes, the progressive obliteration of the capillaries and the reduction of cell metabolism. The author added that nerves and end organs in the oral mucosa may also be affected by age.

On the other hand Abu Aid and his colleges[27] studied the age and architecture of oral mucosa, they found no change in the oral epithelial thickness and found no change in the irregularity of epithelial connective tissue interface with advanced age. They found that epithelial cell shape changes with increasing ageing, the cells become larger and flatter as measured by convexity, concavity, roundness, circularity and sphericity. The authors added this might be attributed to changes in the level of maturation of the cells. This concur with our result that showed edema and swelling of the epithelial cells.

Our result revealed degeneration and cystic transformation of some of the mucous acinar cells similar to those of Nagler[28] who studied salivary glands and the aging process. He observed degenerative structural changes develop in the secretory tissues of most salivary glands including buccal salivary glands. The author positively correlate between the age- dependant decrements of secretory tissues and the reductions in salivary flow rate. Xerostomia, the subjective sensation of dry mouth caused by decreased saliva production, affects 29 to 57 percent of older persons. Saliva lubricates the oral cavity and maintain the health of the oral mucosa.

Our finding is in accordance with the free radical theory of aging, cells continuously produce free radicals and constant radical damage eventually kills the cell. When radicals kill or damage enough cells in an organism, the organism ages[29]**.** When we're young, our cells have a defense system known as superoxide dismutase (SOD) that reins in those free radicals, but as we get older, SOD doesn't work as well. That leaves the free radicals to have their way with our cells, and when the damage gets

to be too much, the cells die. Several previous studies investigated that free radicals are able to deform the cellular biological molecules, causing the damage to the cellular structure and ultimately obstructing cellular function. For example, high oxidative stress and free radical production damages the unsaturated fatty acids located in the lipid bilayer of cell membranes, causing a leaky cellular structure[30]. Furthermore, damage can be done with the oxidation of nucleotides, causing damage to DNA and resulting in the development of tumors[31]. Free radicals can also be responsible for denaturing protein structure, which results in the proteins becoming non-functional. Ultimately, free radicals lead to cellular damage, which is a common pathway for cancer, aging and a variety of diseases that become more common as we age, including dementia and heart disease.

Our results concur with the Mitochondrial Free Radical Theory of Aging (MFRTA) which proposes that mitochondrial free radicals, produced as by-products during normal metabolism, cause oxidative damage that driving force in the aging process. MFRTA proposes that these free radicals damage mitochondrial DNA (mtDNA) and in turn provoke mutations that alter mitochondrial function (e.g. ATP production). According to this, oxidative damage to mtDNA accumulates over time and shuts down mitochondria, causing cells to die and the organism to age[32]**.** Ziegler and co workers[33] studied the mitochondrial effectors of cellular senescence and stated that cellular senescence is a process that results from a variety of stresses, leading to a state of irreversible growth arrest. Senescent cells accumulate during aging and have been implicated in promoting a variety of age-related diseases. The authors explained that mitochondria are damaged over time leading to perturbation of mitochondrial homeostasis that promotes the establishment and maintenance of cellular senescence during aging through; excessive ROS production, impaired mitochondrial dynamics, electron transport chain defect, bioenergetics imbalance and increased AMP-activated protein kinase (AMPK) activity, decreased mitochondrial NAD+ and altered metabolism, and mitochondrial calcium accumulation. These mitochondrial signals trigger p53/p21 and/or p16/pRb pathways and ultimately lead to cellular senescence, which subsequently promotes age-related phenotypes, such as loss of tissue regeneration and function.

Low-density lipoproteins (LDL) that contain hundreds of phospholipids, cholesteryl esters and triglycerides are particularly targeted by the attack of free radicals and therefore subject to lipid peroxidation. The LDL oxidative modifications alter their biological properties increasing the atherogenic power. Oxidized LDL stimulate the endothelial cells to produce inflammatory markers leading to potential innate and adaptive immune responses; they are also involved in the formation of foam cells that inhibit macrophage motility and NO-induced vasodilatation[35]. This is in accordance with our finding.

The mechanism of action of antioxidants is probably due to their ability to quench free radicals and protect against oxidative damage to DNA. In addition to they inhibit the endogenous formation of N- nitroso compounds, some of which are potent carcinogens in animal experiments[34].

The histological results of the present work revealed that antioxidant supplementation using vitamin A, vitamin C, Selenium separately resulted in slight improvement in the histological structures of the surface epithelium, lamina propria as well as the buccal salivary glands. The surface epithelium showed less atrophic with minute degenerative changes of their cells. The lamina propria partially regained the density and arrangement of its collagen fibers with the presence of a lot of blood vessels. Masson· trichrome stain confirmed this improvement in the density and distribution of the collagen fibers of the lamina propria and connective tissues septa

of the buccal salivary glands which became more integrated and organized.

Our study confirmed that vitamin A is has an essential role cellular differentiation and in maintenance of epithelial integrity. Our result concur with[11] who stated the two main mechanisms involved in the prevention of visual disease are the effect of vitamin A on the immune system and on epithelial integrity. In addition to topical application of vitamin A in controlling oral leukoplakia showed a limited effect in reduction of the lesion and disappearance of dysplastic phenomenon[36].

Our demonstration is in agreement with Jacob[37] who reported that vitamin C is the major water-soluble antioxidant within the body. This property allow vitamin C for quenching of free radicals before they reach the cellular membrane in addition to it readily donates electrons to break the chain reaction of lipid peroxidation. Free radicals have been implicated in apoptosis and in DNA damage inducing alteration of the cell cycle. The antioxidant vitamin C is reported to inhibit damage induced by free radicals.

Kumar and et al[38] stated that ascorbic acid is a redox catalyst which can reduce, and thereby neutralize ROS such as hydrogen peroxide. In addition to its direct antioxidant effects, ascorbic acid is also a substrate for the redox enzyme ascorbate peroxidase, a function that is particularly important in stress resistance in plants. Previous study done by Levin[39] demonestrated that the physiological functions of ascorbic acid are largely dependent on the oxido-reduction properties of this vitamin. L-ascorbic acid is a co-factor for hydroxylases and monooxygenase enzymes involved in the synthesis of collagen, carnitine and neurotransmitters. In addition to ascorbic acid accelerates hydroxylation reactions by maintaining the active center of metal ions in a reduced state for optimal activity of enzymes hydroxylase and oxygenase.

Our study showed that vitamin C plays an important role in the maintenance of collagen which represents about one third of the total body protein. It constitutes the principal protein of skin, bones, teeth, cartilage, tendons, blood vessels, heart valves, inter vertebral discs, cornea and eye lens. Our result is in agreement with Boyera and his college[40] who stated that Ascorbic acid is essential to maintain the enzyme prolyl and lysyl hydroxylase in an active form. The hydroxylation of proline and lysine is carried out by the enzyme prolyl hydroxylase using ascorbic acid as co-factor. Ascorbic acid deficiency results in reduced hydroxylation of proline and lysine, thus affecting collagen synthesis.

Our investigation approved with that selenium is one of a group of antioxidants that may help limit the oxidation of LDL, cholesterol and thereby help to prevent coronary artery diseases. Our finding is in accordance with [41] who said that the selenoenzymes are found to have strong antioxidant activity and include six groups of the GPx – GPx1, GPx3, GPx4, GPx5 and GPx6. These GPx play a significant role in protecting cells against oxidative damage from reactive oxygen species (ROS) and reactive nitrogen species (RNS), which include superoxide, hydrogen peroxide, hydroxyl radicals, nitric oxide and peroxynitrite.

The microscopic examination of the buccal mucosa of rats that received combined vitamin A, vitamin C, and selenium, showed nearly the same histological feature of the buccal mucosa of the young control. There was marked increase in the thickness of the surface epithelium, in the numbers of its cells and in the length of the epithelial ridges. The rate of the mitotic activity are almost increased. The lamina propria showed marked increase in the cellularity and density of the collagen fibers which became highly organized with increased vascularity. The buccal salivary gland showed marked improvement in the histological structure of their acini and ducts.

It seems that when antioxidants were given together they potentiate their individual actions

and synergise each others. So a synergistic reaction between different types of antioxidants under investigation potentiate the action of each other in scavenging free radicals and preventing age related oxidative damage of different tissues of the body. Larry[42] confirmed this idea and reported that the four main exogenous antioxidant work together synergistically and form the mnemonic "ACES" which neutralizes the destructive free radicals that cause a cascading chain reaction that damage our bodies leading to many health problems.

The present immunohistochemical localization of NF-κB (nuclear factor kappa) in the buccal mucosa of old control rats using primary monoclonal antibodies of NF-κB/p65, NF-κB/p50, and IκBα showed strong positive staining reactivity of the different strata of the surface epithelium and buccal salivary glands. Our finding go with those of free radical theory of aging, and concur with Logan, et al[43] and Sonis[44] who stated that DNA damage and ROS trigger the activation of transcription factors such as nuclear factor-κB (NF-κB), AP-1, and p53. Among them, NF-κB seems to be a key transcription factor in the establishment of mucositis. NF-kB activation can upregulate the expression of pro-inflammatory cytokines including tumor necrosis factor (TNF) α, interleukin (IL)-1β, and IL-6. It seems likely that the increased levels of these cytokines induce inflammatory reactions in oral mucosa, promote the damage of the underlying connective tissues, reduce epithelial oxygenation, and ultimately result in epithelial basal cell death and injury. Since TNFα and IL-1β are efficient activators of NF-kB, the repeated NF-kB activation by them may amplify the mucosal damage in a vicious circle[45].

Examination of sections of old rats received separated or combined vitamin A, C and selenium incubated with subunits of NF-κB primary monoclonal antibodies revealed weak to moderate staining reactivity of their basal and suprabasal cells. Cells of the duct and mucous acini of the buccal salivary gland showed weak positive reactivity. Our result is in accordance with Ma and his college[8] who studied inhibition of Nuclear Factor κB by Phenolic Antioxidants. They analyzed of the NF-kappaB activation pathway and revealed that the antioxidants do not inhibit LPS-induced activation of the IκB kinase activity, degradation of IκBα, or translocation of activated NF-κB into the nucleus, but they do block the formation of NF-κB/DNA binding complexes. Structure-activity analyses suggest that inhibition of NF-kappaB function involves the redox cycling property of the antioxidants. These findings implicate a redox-sensitive factor important for the binding of NF-kappaB to its DNA recognition sequence as a target molecule in the inhibition of NF-κB function and inflammatory cytokine expression by phenolic antioxidants. Meyer, et al[46] stated that over expression of the antioxidant protein TRX1 was shown to diminish NF-κB activation by inhibiting IκB degradation.

Finally our histological and immunohistochemical investigations revealed aging caused atrophic and degenerative changes in the oral mucosa associated with increased expression of nuclear factor kappa. Consuming antioxidant vitamins as vitamin A, vitamin C or selenium separately partially modulates the action of aging process and increase the regenerative capacity of the oral mucosa and oral salivary glands. Consuming combined antioxidant vitamin A, vitamin C and selenium had a powerful and beneficial synergizing antiaging effect on the aforementioned tissues.

CONCLUSION

The balance between oxidation and antioxidation (redox balance) is critical in maintaining a healthy biological system. Our histological and immunohistochemical investigation proved the important role of antioxidant for keeping the integrity and health of the mucous membrane and salivary glands of the buccal mucosa of aging rats under investigation.

RECOMMENDATION

Improvement of dietary intake by consuming neutral foods rich in antioxidant vitamin A, C and selenium is of utmost importance to gain the antiaging benefits and to prevent age –related degenerative diseases.

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