IMPACT OF HYPERCHOLESTEROLEMIA ON THE PERIODONTAL LIGAMENT AND THE DENTOGINGIVAL JUNCTION OF THE ALBINO RAT (AN ULTRASTRUCTURAL AND IMMUNOHISTOCHEMICAL INVESTIGATION)

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ABSTRACT

The aim of the present work was to investigate the impact of short term and long term experimental hypercholesterolemia on the microscopic and submicroscopic structures of the dental periodontal ligament and the dentogingival junction of the first molar of adult male albino rats. The fibronectin immunohistochemistry of the aforementioned dental and oral structures, as a trial to find out an answer to the question: could the term dental angina be introduced in the literature as a new terminology of a dental disease, similar to the term abdominal angina which is newly introduced in the medical field?? Sixty healthy adult male albino rats were used and divided into 2 groups. Group I: consisted of 20 rats served as controls and received control diet. Group II: consisted of 40 rats served as the experimental hypercholesterolemic group and received hypercholesterolemic diet. The experimental period lasted for 4 months. Half the animals of each group were sacrificed by cervical dislocation after two months of the beginning of the experiment to study the short term effects of hypercholesterolemia on dental periodontal ligament and the dentogingival junction. The rest of the animals of each group were sacrificed by the same way after 4 months of the beginning of the experiment to study the long term effects of hypercholesterolemia on the tissues under investigation. The results of the present investigation showed that high dietary cholesterol can initiate marked degenerative changes in the periodontal ligament and dentogingival junction. Most of the vascular channels revealed thrombotic lesions in the form of total and subtotal occlusion of their lumina with marked increase in the thickness of their walls, a picture of typical dental angina.

KEY WORDS: Hypercholesterolemia, Dental pulp, degenerative changes, dental angina.

INTRODUCTION

Cholesterol is a type of fat which is very important for vital living. It is an important component of cell membrane providing them stability, since it makes the membrane’s fluidity stable over a bigger temperature interval. It is the major precursor for the synthesis of vitamin D, and various steroid hormones including cortisol, cortisone and aldosterone in the adrenal glands.
and of the sex hormones progesterone, estrogen and testosterone. Cholesterol also has an important role for the brain synapses as well as the immune system, including protecting against cancer (Anderson et al., 2003). The cholesterol in person’s blood originates from two major sources, dietary intake and internal production. Dietary cholesterol comes mainly from meat, poultry, fish, dairy products, egg yolk and organ meats such as liver which is especially high in cholesterol content. Internal synthesis of cholesterol occurs mainly in the liver, other sites of high synthesis rates include the intestines, adrenal glands and reproductive organs (Kwiterovich, 1998).

Cholesterol is minimally soluble in water, it cannot dissolve and travel in the water–based blood stream. Instead it is transported in the blood stream by lipoproteins which are water soluble. The largest lipoproteins which primarily transport fats and cholesterol from the intestinal mucosa to the liver are called chylomicrons. In the liver chylomicrons particles give up triglycerides and some cholesterol and are converted into low density lipoprotein (LDL) particles (bad cholesterol), which carry cholesterol to other body cells. In healthy individuals the LDL particles are large and few. Conversely large numbers of small LDL particles are very risky because they are associated with promoting atheromatous diseases within the arteries. High density lipoprotein (HDL) particles (good cholesterol) transport cholesterol back to the liver for excretion, but vary considerably in their effectiveness for doing this. Consciously having small amounts of large HDL particles is strongly associated with atheromatous disease progression within the arteries (Shepherd, 1995).

The average amount of blood cholesterol varies, rising gradually with age. Ochene et al., (2004) reported seasonal variation in cholesterol level in humans, more on average in winter.

Cholesterol is excreted from the liver in bile which aids in the digestion of fats and get reabsorbed from the intestines. When concentrated in the gall bladder it crystallizes and forms the major constituent of most gall stones (Russel, 2003).

Hypercholesterolemia is the term used to describe high levels of cholesterol in the blood. Most cases of hypercholesterolemia are caused by a combination of genetics and diet. The genes involved in hypercholesterolemia are usually multiple, however hereditary disorders in lipid metabolism may result from a defect in a single gene. The most common type of this hereditary disorder is the familial hypercholesterolemia (Brown, 1997).

Hypercholesterolemia can also occur as a result of other medical conditions such as diabetes mellitus, hypothyroidism, liver disease, alcoholism and certain types of kidney diseases. Various medications including estrogen, steroids and certain blood pressure drugs can cause hypercholesterolemia as well. Learning the facts about cholesterol is beneficial to health, to help understanding what cholesterol is and how it affects health.

**MATERIALS AND METHODS**

Sixty healthy adult male albino rats with an average body weight of 150± 10 grams were used in this investigation. The animals were obtained from laboratory animal colonies, Ophthalmology Institute, Giza, Egypt. They were divided into:-

**Group I:** consisted of 20 rats and served as controls. They received control diet composed of (g/ kgr): casein (120gr), salt mixture (50gr), vitamin mixture (10gr), soybean oil (80gr), choline (0.4gr), cellulose (10gr) and corn starch (729.6gr) (Torbino et al., 2003).

**Group II:** consisted of 40 rats and served as the experimental hypercholesterolemic group. They received hypercholesterolemic diet which consisted of (g/ kgr): casein (120gr), salt mixture (50gr), vitamin mixture (10gr), soybean oil (250gr), choline (0.4gr), cellulose (130gr) and corn starch (429.6gr), bile salt mixture (2.5gr) necessary for intestinal absorption of cholesterol and cholesterol (10gr) (Torbino et al., 2003).

The animals were caged in a specially raised wire bottom cages, five animals per cage, in the animal
house of the faculty of Oral and Dental Medicine Cairo University. They were fed the corresponding diet and supplied drinking tap water ad libitum throughout the whole experimental period which lasted for 4 months. They were maintained under good ventilation.

Half the animals of each group were sacrificed by cervical dislocation after two months of the beginning of the experiment to study the short term effects of hypercholesterolemia on dental periodontal ligament and dentogingival junction of the first molar region. This time of sacrifice was chosen because rats develop hypercholesterolemia after one month of consuming high cholesterol diet, (Chiang et al., 1998).

The rest of the animals of each group were sacrificed by the same way after 4 months of the beginning of the experiment to study the long term effects of hypercholesterolemia on the tissues under investigation.

Blood samples were collected from the retro orbital plexus of veins at the inner canthus of the eye of 12-hour-fasted each animal of the different groups using glass capillary tubes to detect the total cholesterol level at the beginning and at the proposed times of the end of the experimental period. Data were recorded and treated statistically.

Immediately after sacrifice, the jaws of each animal were dissected, separated and then fixed in 3% phosphate buffered glutaraldehyde for 4 hours, washed in the buffer for 24 hours at 4°C, then the specimens were decalcified in 10% Ethylene diamine tetra acetic acid (EDTA) PH 7-7.4.

After complete decalcification, jaw specimens were washed in the phosphate buffer. Those of the right side were prepared for examination with the light microscope. The specimens were dehydrated, cleared, embedded in paraffin, sectioned and stained with:

1- Hematoxylin and eosin (H&E) for histological examination of the dental periodontal ligament and dentogingival junction of the first molars.

2- Immunoperoxidase staining technique for localization of fibronectin in the structures under investigation.

Primary Antibody
- Fibronectin Ab-11 (Clone FBN11).
- Molecular weight of antigen: 440 KDa (non-reduced) and 220 KDa (reduced).
- Ig Isotype: Ig G1.
- Clone Designation: FBN11
- Species Reactivity: Human, Mouse and Rat.
- Comments: Ab-11 is excellent for staining of formalin-fixed, paraffin-embedded tissue.

Universal Kit

A labeled streptavidin–biotin immunoenzymatic antigen detection system was applied. This technique involves the sequential incubation of the specimen with an unconjugated primary antibody specific to the target antigen. This kit was obtained from GOLDEN LAB Company.

Content of The Kit
1- Hydrogen Peroxide Block.
2- Ultra V Block.
3- Biotinylated Goat Anti-Polyvalent.
4- Streptavidin Peroxidase.
5- Diaminobenzidin (DAB) Chromogen.
6- DAB Substrate.

Interpretation

The immuno-stained sections were examined under the light microscope. The positive staining reaction appeared in the form of an orange to brown staining. Staining reactivity was interpreted (according to the intensity of staining) as follows:
-ve. Negative reaction.
+ve. Slightly positive reaction.
++ve. Moderately positive reaction.
+++ve. Strongly positive reaction.

Jaw specimens of the left side were used for transmission electron microscopic examination of the dentogingival junction, the periodontal ligament at cervical and middle regions of the roots of the first molars where small specimens about one cubic millimeter size were cut after complete decalcification using very sharp blade within the phosphate buffer from the corresponding sites. Specimens were then washed in freshly prepared phosphate buffer PH7, post fixed in 1% buffered osmium tetroxide, washed, dehydrated in ascending grades of ethanol and then embedded in epoxy resin. Ultra thin sections were cut and stained with uranyl acetate followed by lead citrate and examined with transmission electron microscope.

RESULTS

I- Biochemical results

There was a significant increase in the total blood cholesterol level of the hypercholesterolemic rats after two months and four months (P< 0.01) of the beginning of the experiment compared with their initial records. While there was no difference in the same parameter of the control rats after two and four months (P > 0.05) of the beginning of the experiment compared with their initial records (Table, I).

<table>
<thead>
<tr>
<th>Biochemical parameter mg/100ml blood</th>
<th>Group I (Control)</th>
<th>Group II (Hypercholesterolemic)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>2 months</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>50.8 ±2.5</td>
<td>53.5 ±3.1</td>
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II- Light Microscopic Results

A- The periodontal ligament

Control group

In the cervical region, the periodontal fibers arising from the cementum appeared to be directed toward the lamina propria of the gingiva forming the gingival group of fibers. The interdental (transeptal fibers) were horizontally directed and extended from the cementum of one tooth to the cementum of the adjacent one.

- The alveodental group of fibers were seen extending from cementum to bone and divided into: Fibers arising from the alveolar crest and obliquely directed to be attached to the cementum at a more occlusal level. These were the alveolar crest group of fibers.
- A group of fibers were horizontally directed at right angle to the tooth and bone surfaces forming the horizontal group of fibers.
- Oblique fibers were obliquely directed from bone to cementum with a more apical level of attachment in the cementum constituting the oblique group of fibers.
- Apical fibers were radiating from the cementum around the apex of the root to the bone of the fundus of the socket.
- The interradicular group were found only between the roots of multirooted teeth and running from the cementum to the bone of the crest of the interradicular septum.
The collagen fibers were grouped in bundles that presented small interstitial spaces in between them containing areolar connective tissue with blood vessels and nerves. The fibroblast cells had basophilic cytoplasm and nuclei that usually appeared elongated and having the direction of adjacent collagen fibers.

**Two months hypercholesterolemic group**

The periodontal ligament of rats that received cholesterol rich diet for two months revealed marked degenerative changes manifested as disorganized orientation of the collagen fibers in the cervical, mid root, and in apical areas. Marked fatty infiltration and degeneration of the fibroblasts. Widening of the blood vessels with blood cell stagnation. However stenosis of the vascular channels was commonly found forming thrombotic lesions. Frequent areas of loss of attachment of the PDL fibers to the bone and cementum surfaces were encountered together with surface bone resorption. Massive alveolar bone resorption resulting in marked rarefaction of bone trabeculae with a lot of reversal lines were seen. Moreover extreme widening of marrow cavities associated with massive fatty infiltration. Cementum showed increased thickness, with surface irregularity, extreme widening of the apical foramina was frequently encountered.

**Four months hypercholesterolemic group**

The periodontal ligament taken from rats that received cholesterol rich diet for four months revealed massive degeneration and loss of orientation in the collagen fibers in the cervical, mid root, and apical areas of all the examined PDL specimens in this group. Decreased density of the collagen fibers and frequent areas of loss of attachment of the PDL fibers to the bone and cementum were always found.

Cholesterol like clefts were demonstrated between the collagen fibers together with marked fibroblastic fatty infiltration and degeneration. Areas of fatty infiltration were found totally replacing the periodontal tissues. Massive alveolar bone resorption with osteoclastic activity and marked rarefaction of bone trabeculae, extreme widening of marrow cavities were seen associated with extensive fatty infiltration and chronic inflammatory cell infiltration. Cementum showed hypercementosis with surface irregularity (Fig. 1).

**B- The dento – gingival junction (Attachment epithelium and underlying connective tissue)**

**Control group**

The attachment epithelium of adult male control rats was usually thin and consisted of few layers of nonkeratinized stratified squamous epithelium. The epithelial cells were resting on smooth basement membrane with no evidence of epithelial ridges. The basal cells were cuboidal in shape and had their long axis almost perpendicular to the basement membrane and tooth surface. In the suprabasal layer, cells were polyhydral and gradually became flattened and almost parallel to the tooth surface with observable intercellular spaces. The subepithelial connective tissue (lamina propria) was formed of cells and intercellular substance formed of collagen fibers embedded in ground substance. The cells were mainly fibroblasts, progenitor cells and defensive cells. Sometimes, inflammatory cells could be detected in the lamina propria.
Two months hypercholesterolemic group

The dentogingival junction of rats that received high cholesterol diet for two months showed changes in the attachment epithelium and subjacent lamina propria. The cells of the attachment epithelium revealed signs of degeneration in focal areas manifested as hydropic degeneration and fatty infiltration. A tendency for apical migration with slight pocket formation was seen. Pathological folding of the basement membrane was also encountered. The subjacent lamina propria revealed areas of cellular and fibrous degeneration with fatty infiltration and increase in chronic inflammatory cell infiltration.

Four months hypercholesterolemic group

The dentogingival junction of rats treated with high cholesterol diet for four months revealed severe degenerative changes. The attachment epithelium was presented with extreme thinning and marked hydropic and fatty degeneration of its cells. Marked apical migration and deep pocket formation were frequently seen. The underlying lamina propria suffered massive degenerative changes in the cells and collagen fibers due to the extreme accumulation of fat and cholesterol in them. Collection of cholesterol like clefts between the fibers and in the walls of blood vessels were frequently seen (Fig.2).

III- Submicroscopic finding

A-The periodontal Ligament

Control group

The electron microscopic examination of the PDL of control rats revealed the normal ultrastructure of the collagen fibers that were cut longitudinally or transversely extending between the bone and the cementum. According to the direction of the section, the fibroblasts were either spindle, stellate, or rounded in shape. The fibroblasts were presented with large rounded or oval nuclei with peripheral chromatin condensation. The nuclear membrane was smooth and regular. The cytoplasm showed abundant R.E.R., variable sized mitochondria, Golgi bodies and occasional lysosomal bodies. Elements of the cytoskeleton (microtubules and microfilaments) in the fibroblasts were well defined.

Neutrophils, Mast cells, plasma cells and lymphocytes were sometimes encountered within the fibrous tissue of the PDL. The neutrophils presented the typical granular cytoplasm with electron dense granules of variable size and shape. Its nucleus showed multilobes. Mast cells were oval or rounded in shape with spherical centrally placed nucleus and granular cytoplasm with characteristic scroll-like structures within the granules. Plasma cells were ovoid with spherical eccentrically placed nucleus and their cytoplasm rich in R.E.R. and Golgi complexes. Interstitial spaces containing interstitial tissue, blood vessels, and nerves were detected.

Two months hypercholesterolemic group

Electron microscopic examination of PDL of the rats of this group ensured the histopathological finding where there were marked degeneration of the collagen fibers associated with reduction in their density, destruction, fragmentation and loss of orientation, leaving a lot of debris. The fibroblasts showed definite signs of degeneration as they were markedly shrunken with pyknotic nuclei or nuclei undergoing karyorhexis or karyolsis, degenerated mitochondria and Golgi complex, vacuolated cytoplasm and marked fatty infiltration.

A lot of phagocytic and inflammatory cells were encountered, some of them showed nuclei with irregular
outlines and marked fatty infiltration and vacuolization of their cytoplasm. There was dilatation of blood vessels with blood cell stagnation. Endothelial cells showed marked cytoplasmic fatty infiltration and vacuolization. Thickening of the vascular walls with numbers of fatty streaks, fibrous plaques, lipid laden macrophages (foam like cells), platelets aggregation, blood monocytes and erythrocytes leading to stenosis of the vascular channels were observed forming thrombotic lesions.

Nerve fibers presented abnormal myelin configuration.

### Four months hypercholesterolemic group

Electron microscopic examination of PDL of the rats of this group ensured the histopathological finding where there marked augmentation of the degenerative effect of hypercholesterolemia on the structure of the PDL of this group where the collagen fibers presented massive destruction, fragmentation and loss of orientation, leaving a lot of debris. The fibroblasts showed a definite signs of degeneration as they were markedly shrunken with pyknotic nuclei or undergoing karyorhexis or karyolsis, degenerated organelles, vacuolated cytoplasm and marked fatty infiltration. Areas of tissue destruction, phagocytic and inflammatory cells infiltration were also encountered. The blood vessels showed stenosis of the vascular lumen forming thrombotic lesions (Fig. 3).

**The Dentogingival junction (attachment epithelium and underling lamina propria)**

**Control group**

The attachment epithelium of the control rats was composed of several layers of cells. The basal cells were cuboidal in shape with oval nuclei and were separated from the underlying connective tissue by a basal lamina and showed its two characteristic layers, lamina lucida and lamina densa. Hemidesmosomes were frequently observed in the areas of plasma membrane facing the basal lamina. The more superficial cells were polyhydral to flattened cells with centrally placed nuclei.

In general, cells of the attachment epithelium showed great amount of cytoplasm, well developed cell organelles especially R.E.R., Golgi sacules, and mitochondria.

Desmosomal junctions between adjacent epithelial cells were also observed. The intercellular spaces between attachment epithelial cells were large.

No epithelial ridges were found at the interface between the attachment epithelium and the underlying lamina propria. Polymorphnuclear leukocytes were rarely found in the intercellular space between the cells of attachment epithelium.

The underlying lamina propria was composed of normal connective tissue consisting of moderately dense collagen fibers, fibroblasts with the normal ultrastructure of the nucleus and cytoplasmic components together with a number of lymphocytes, plasma cells and macrophages.

**Two months hypercholesterolemic group**

The electron microscopic examination of the attachment epithelial cells in this group revealed less extensive degenerative changes manifested as marked cytoplasmic vacuolization, fatty infiltration, destruction of cell organelles, homogenization of the nuclear chromatin and/or pyknotis of the nucleus, marked reduction of desmosomal junctions and widening of the intercellular spaces with collection of intercellular inflammatory cells.
The underlying lamina propria showed marked fatty infiltration and degeneration. Fibroblasts showed marked fatty infiltration, cytoplasmic vacuolization, and destruction of cell organelles, pyknosis, karyorhexis or karyolysis of their nuclei. A lot of macrophages, plasma cell, lymphocytes were observed with massive ultrastructure degenerative changes, fatty infiltration and cytoplasmic vacuolization.

The blood vessels showed widening of their lumina and endothelial cells presented massive cytoplasmic fatty infiltration, vacuolization, destruction of cell organelles, pyknosis, karyorhexis or karyolysis of their nuclei.

**Four months hypercholesterolemic group:**

The electron microscopic examination of the attachment epithelial cells in this group revealed marked degenerative changes manifested as massive reduction of epithelium thickness, marked cytoplasmic vacuolization, fatty infiltration, destruction of cell organelles, homogenization of the nuclear chromatin or pyknosis of the nuclei, marked reduction of desmosomal junctions and widening of the intercellular spaces with collection of intercellular inflammatory cells.

The underlying lamina propria showed marked fatty infiltration and degeneration of its cells and fibers. Fibroblasts showed shrinkage, fatty infiltration and cytoplasmic vacuolization, with destruction of cell organelles, pyknosis, karyorhexis or karyolysis of their nuclei. Collections of macrophages, plasma cell, lymphocytes were observed with massive ultrastructure degenerative changes, fatty infiltration and cytoplasmic vacuolization.

The blood vessels showed marked thickening of the vascular wall with collection of cholesterol cleft (Fig. 4).

**IV- Immunohistochemical results**

**The periodontal ligaments (Table, II)**

**Negative control**

Examination of the negative control sections of the rat periodontal tissues incubated with the non specific serum, revealed a negative staining reaction to fibronectin of all elements forming the periodontal ligament.

**TABLE (II) Illustrates the intensity of staining reaction of the component of the periodontal ligament of different groups of fibronectin.**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Periodontal ligament</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Collagen fibers</td>
</tr>
<tr>
<td>Group I (control)</td>
<td>+++ve</td>
</tr>
<tr>
<td>Group II (two months group)</td>
<td>+ to ++ve</td>
</tr>
<tr>
<td>Group III (four months group)</td>
<td>- ve to +ve</td>
</tr>
</tbody>
</table>

- : negative, + : weakly positive, ++ : moderately positive, +++ : strongly positive
**A- Control group**

Localization of fibronectin in the periodontal ligament of the control group revealed strongly positive (+++ve) FN reaction in the fibroblasts, collagen fibers, ground substance and walls of the blood vessels.

**B- Two months hypercholesterolemic group**

Fibronectin localization in the periodontal ligament of two months hypercholesterolemic rat revealed weakly to moderately positive (+ve to ++ve ) FN reaction in the collagen fibers, fibroblasts, ground substances and walls of the blood vessels.

**The dentogingival junction (attachment epithelium and lamina propria ) (Table, III)**

**Negative control**

Examination of the negative control sections of the DGJ incubated with the non specific serum, revealed a negative staining reaction to fibronectin of both the attachment epithelium and the lamina propria

**TABLE (III) Illustrates the intensity of staining reaction of the component of the DGJ of different groups of fibronectin**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Surface epithelium</th>
<th>Subepithelial c.t.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>c.f.</td>
<td>g.s.</td>
</tr>
<tr>
<td>Group I (control)</td>
<td>-ve</td>
<td>+++ve</td>
</tr>
<tr>
<td>Group II (two months group)</td>
<td>-ve</td>
<td>+ve to +ve</td>
</tr>
<tr>
<td>Group III (four months group)</td>
<td>-ve</td>
<td>-ve</td>
</tr>
</tbody>
</table>

* c.f. : collagen fibers.     g.s. : ground substance.  fib. : fibroblasts.
* - : negative           + : weakly positive     ++ : moderately positive     +++ : strongly positive

**DISCUSSION**

In view of the results of the present investigation it is obvious that the hypercholesterolemia resulted in marked structural and ultrastructural degenerative changes in the dental periodontal ligament and dentogingival junction manifested as massive fatty infiltration and degeneration. The fibroblasts suffered a lot of degenerative changes including fatty infiltration, cytoplasmic vacuolization, swollen and degenerated mitochondria, shrinkage and nuclear pyknosis, karyolysis or karyorhexis. The collagen fibers presented massive degeneration and dissociation with collection of cholesterol like clefts between the fibers. Frequent areas of loss of attachment of the PDL fibers to the bone and cementum surfaces were observed.
Massive alveolar bone resorption resulting in marked rarefaction of bone trabeculae and extreme widening of the marrow cavities associated with massive fatty infiltration were an outstanding finding. The epithelium of the dentogingival junction was presented with extreme thinning, apical migration and marked fatty degeneration of its cells. The underlying lamina propria suffered massive degenerative changes in the cells and collagen fibers due to accumulation of fat and cholesterol in them. Collection of cholesterol clefts between the fibers and in the wall of the blood vessels were frequently seen. A lot of vascular changes demonstrated as thrombotic lesions in the form of marked thickening of their walls with partial or total occlusion of their lumina and stagnation of the blood were encountered. Inflammatory cell infiltration composed mainly of a lot of degenerating macrophages, neutrophils, lymphoctes and less frequently plasma and mast cells was found. The structural and ultrastructural changes were most severe in specimens of the four months hypercholesterolemic rats than those of the two months hypercholesterolemic rats.

It is well documented that dietary cholesterol plays an important role in the regulation of lipid metabolism in various organs (Mawatari et al., 2003 and Yoshida et al., 2004).

In the current study, high dietary cholesterol induced a significant increase in total serum cholesterol (hypercholesterolemia), a condition which has been proven to induce marked increase in the production of the cytokines interleukins and tissue necrosing factors-alpha (Han et al., 2002) and in macrophage infiltration (Hosoyamada et al., 2002), to affect T-cell-mediated immune functions (Han et al., 2003), and to increase the levels of systemic circulatory inflammatory molecules such as C-Reactive Protein (Satjjo et al., 2004; Warnberg et al., 2004). These impacts of hypercholesterolemia could indirectly result in an increase in circulation of inflammatory molecules that could cause and augment the pathological changes observed in the present investigation. Doxey et al., 1998 stated that such inflammatory molecules in the periodontium could contribute to periodontitis progression.

The results of the present study revealed infiltration of the tissue under investigation with macrophages and inflammatory cells that showed signs of degeneration and foam like cell appearance. However they may still be able to produce inflammatory cytokines such as interleukin -1β (IL-1 β) and tissue necrosing factor -α (TNF- α) which are known to cause degenerative changes in the epithelium and the connective tissue. Also IL -1β can stimulate the production of proteolytic enzymes including matrix metalloproteinase and plasminogen activation. Metalloproteinase are a family of structurally related endopeptidases capable of degrading various macromolecular components of the extracellular matrix (Berkedal, 1993).


McDevitt et al., (2000) stated that the pro-inflammatory cytokine IL-1 is a key regulator of the host responses to microbial infections and a major modulator of extracellular matrix catabolism and bone resorption. The authors added that variations in IL-1 gene cluster on chromosome 2 are associated with increased susceptibility to severe periodontitis. Ejeil et al., (2003) studying the possible correlation between cytokines and collagen degradation reported that IL-1 beta and IL-4 were particularly and intensively correlated with collagen loss. The authors added that these two cytokines could be used as markers of clinical severity during active periodontitis. Moreover Chang et al., (2003) published that pro-inflammatory cytokines may partially contribute to the destruction of pulpal and periodontal tissues through dysregulated proteolysis.

Ross., (1993) stated that atherosclerosis is a chronic inflammatory disease in which low-density lipoprotein (LDL) cholesterol plays a crucial role where oxidized LDL excessively taken up by macrophages via the scavenger receptor pathway (Haberland et al, 1982), leading to the formation of foam cells characteristic of the earliest atherosclerotic lesion.
Hypercholesterolemia can cause the formation and accumulation of plaque deposits in the arteries or atheroma. Plaque is composed of a necrotic central core containing lysed cells, cholesterol ester crystals and other fatty substances deposited from the blood stream, lipid laden foam cells, and surface plasma proteins including fibrin and fibrinogen. This central core is associated with a cellular infiltrate with hypertrophic smooth muscle cells, macrophages, and sparse of T lymphocytes. When it builds up in the arteries, it results in atherosclerosis. Atherosclerosis can lead to plaque ruptures and blockages of the arteries, which increase the risk of heart attack, stroke, circulation problems, and death.

When the innermost lining of the arteries (endothelium) is damaged, cholesterol particles deposit into the damaged wall and form plaques. More cholesterol and other substances incorporate into the plaque and the plaque grows, narrowing the artery. Atherosclerosis is associated with endothelial dysfunction, which may be caused by oxidative stress and subsequent lipid peroxidation (Cai and Harrison, 2000). Ren et al., (2001) reported that hypercholesterolemia can damage the vascular endothelium – dependant function severely but does not affect the vascular endothelium independant functions.

Plaque deposits can grow large enough to interfere with blood flow through the artery causing blockage. When the arteries supplying the heart with blood (coronary arteries) are blocked, chest pain (angina) may occur; when arteries in the legs are blocked, leg pain or cramping may occur; when arteries supplying the brain with blood are blocked, cerebral stroke may occur. The degeneration of the tissues under investigation which include the connective tissue of the pulp, PDL and lamina propria of denogingival junction together with the attachment epithelium in our opinion are due to blockage of the arteries supplying the tooth pulp and periodontium with blood. Thus dental angina is the result.

Ross., (1999) reported that a chronic inflammatory state occurs during atherosclerosis characterized by endothelial cell injury, increased endothelial cell adhesion molecule (CAM) expression, and leukocyte infiltration of the vessel wall. Lei and Buja., (1996) stated that cytokines such as tumor necrosis factor and interferon released from both the normal cellular components of the vessel wall and together with infiltrating leukocytes, allow this response to continue, culminating in plaque formation in the vessel wall. Furthermore, Kurose et al., (1998) stated that acute hypercholesterolemia appears to exacerbate the microvascular injury response to inflammatory stimuli such as ischemia-reperfusion resulting in decrease in microvessel density. Artete et al., (2002) stated that decrease in microvessel density could be related to failing vascular function and blood flow decrease.

The mechanism by which hypercholesterolemia initiates atherosclerotic lesion development is not completely understood, although cholesterol oxidation products such as those found in oxidized LDL cholesterol, have been implicated (Rong et al., 1999). Oxidized LDL promotes endothelial dysfunction and leukocyte chemotraction in both large vessels and the microcirculation (Liao et al., 1997). Increased oxidant stress, resulting from both increased oxygen free radical (OFR) production and decreased nitric oxide (NO) generation, appears to play an important role in the chronic inflammatory responses to hypercholesterolemia and atherosclerosis (Kojda, 1999). The proinflammatory effects of OFRs may result from the ability of these reactive species to (1) increase the expression of vascular adhesion molecules including selectins, vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule 1-1 (ICAM-1) on vascular endothelium (Elena, and Klaus, 2007) (2) enhance the production of leukocyte-activating substances (eg, platelet-activating factor, leukotriene B$_4$, and complement 5a); and (3) promote the rolling, firm adhesion and emigration of leukocytes in the vasculature (Carden and Granger, 2000). The recognition that OFRs may contribute to the vascular pathology associated with hypercholesterolemia and atherosclerosis has led to an interest in defining the sources of OFRs in these conditions. Potential sources of OFRs include mitochondrial oxidases, xanthine oxidase, lipoxigenase, and NAD(P)H-dependent oxidases (Ruef et al., 1999). A recent study by Guzik et al., (2000)
demonstrated an association between vascular superoxide production by NAD(P)H oxidase and the endothelial dysfunction that accompanies hypercholesterolemia in human blood vessels. The authors added that increased vascular NAD(P)H oxidase activity was associated with reduced NO-mediated vasorelaxation. Furthermore, reduced endothelial vasorelaxations and increased vascular NAD(P)H oxidase activity were both associated with increased clinical risk factors for atherosclerosis.

Hypercholesterolemia may increase the risk for cardiovascular disease not only by rendering tissues more likely to experience an ischemic episode (due to vessel obstruction with plaques) but also by exacerbating the inflammatory and tissue injury responses to a given ischemic insult.

Our study revealed severe degenerative changes and extreme thinning of the attachment epithelium with apical migration and pocket formation and degeneration of the underlying collagen fibers and fibroblasts. Ekuni et al., (2005) stated that lipopolysaccharides and proteases can induce periodontal ligament fibroblast apoptosis and collagen destruction. Imatani and Coworkers., (2001) reported that lipopolysaccharides are pathological mediators in periodontal disease and if coupled with inflammatory cytokines the condition will be greatly augmented.

The increase in apoptotic periodontal ligament fibroblasts induces the detachment of connective tissue from tooth surfaces (Sakai et al., 1999). The junctional epithelium would migrate apically to these areas (Ekuni et al., 2005).

The results of the present study revealed areas of fatty infiltration of the periodontal ligament that led to massive degeneration and destruction of the collagen fibers. Kim et al., (1991) reported that feeding a high-cholesterol diet has been demonstrated to increase lipid deposition within various organs. Also Assy et al., (2000) stated that hypercholesterolemia causes fat deposition in the liver and depletion of hepatocytes and liver cirrhosis. Octtinger et al., (2001) stated that in liver cirrhosis patient exhibited greater gingival pocketing and attachment loss due to altered immune response and increased serum cytokines compared to controls. Nicholas et al., (2001) reported that lipid penetration into fibrous tissues in combination with chronic inflammatory response may substantially potentiate prostaglandin secretion from the fibroblasts, promoting tissue destruction. Noguchi et al., (2001) stated that prostan-1ndins especially prostaglandin E1 and E2 act as long term mediators of inflammation and collagen production and release from stimulated macrophages resulting in collagen breakdown in this tissue (El Attar et al., 1984).

Our results revealed that hypercholesterolemia resulted in massive alveolar bone resorption causing marked rarefaction of bone trabeculae. The increase in hypercholesterolemic alveolar bone resorption is either dependent upon osteoclastic bone resorption and/or inhibition of osteoblastic differentiation. Osteoclast differentiation and activation can be induced by receptor activator of NF-κB (RANK) ligand (RANKL) and inflammatory cytokines such as interleukin (IL)-1β and tumour necrosis factor (TNF)-α. However, Ekuni et al., (1994) published that hypercholesterolemia-activated osteoclasts contribute to alveolar bone loss by directly increasing production of RANKL in osteoblasts and decreasing osteoprotegerin (OPG)- expression, and not via IL-1β and TNF-α. Goodson, (1973) described that prostaglandin act as potent mediators of bone resorption in adult rats. Kobayashi et al., (2005) reported that prostaglandin E2 has been shown to activate existing osteoclasts and to initiate (RANKL)-induced osteoclastic proliferation and differentiation of the precursor cells. Heasman and Seymour, (1990) said that the osteoclastic activating factor which is a potent mediator of bone resorption is released from activated lymphocytes only in the presence of prostaglandin of the E. series.

Electron microscopic examination of the degenerated fibroblasts, odontoblasts and inflammatory cells of the pulp, periodontal ligament and lamina propria of the
dentogingival junction revealed massive degenerative changes including fatty infiltration, cytoplasmic vacuolization, swollen and degenerated mitochondria, shrinkage and nuclear pyknosis, karyolysis or karyorhexis. In our opinion, these may be due to apoptosis (programmed cell death) caused by ischemic episode (due to vessel obstruction with plaques) and also by exacerbating the inflammatory and tissue injury responses to a given ischemic insult. Morphologic alteration of the nuclear pattern and chromatin distribution in our opinion are due to irreversible cellular damage leading to subsequent cell death.

The most extensively affected organelles in the cells of the tissue under investigation were the mitochondria which appeared pleomorphic, vacuolated, swollen with total or subtotal loss of cristae and their possible rupture. The major function of the mitochondria is the production of ATP by means of the Krebs cycle, and oxidation. Phosphorylation of the ATP which is formed inside the mitochondria is used in various energy activities, thus any reduction in the production of ATP will affect the activity of the cell. In addition, the mitochondrial matrix contain many ribosomes that can carry out protein synthesis and the fine circular distribution of DNA. Masayesva et al.,(2006) stated that mitochondria are the major source of endogenous reactive oxygen species (ROS) and also play a critical role in apoptosis, or programmed cell death, by the release of the enzyme cytochrome c and other soluble factors into the cytoplasm, thereby providing a critical role in the maintenance of cell proliferation. This study is in constant with Knight-Lozano et al., (2002) who found that hypercholesterolemia, a major lipid disorder contributing to increase atherosclerosis risk with increase oxidative stress. Because mitochondria are susceptible to damage mediated by oxidative stress and associated with increase mitochondrial damage, cardiovascular disease risk factors cause mitochondrial damage and dysfunction. Puddu et al., (2005) stated that oxidant stress and the ensuing endothelial dysfunction play a key role in the pathogenesis of atherosclerosis and cardiovascular diseases. The mitochondrial respiratory chain is the major source of reactive oxygen species as byproducts of normal cell respiration. Mitochondria may also be important targets for reactive oxygen species, which may damage mitochondrial lipids, enzymes and DNA with following mitochondrial dysfunction. Free cholesterol, oxidized low-density lipoprotein and glycated high-density lipoprotein are further possible causes of mitochondrial dysfunction and/or apoptosis.

Cytoplasmic vacuolization was a constant finding in the cells of the tissues under investigation, denoting cellular degeneration and functional impairment.

The reported massive degeneration of the collagen fibers of the pulp and periodontal ligament and the lamina propria of the dentogingival junction in our opinion is primarily and mainly due to the extreme degenerative changes that occurred in the fibroblasts as result of ischemia causing failure or defective collagen synthesis. This may be coupled with excessive collagenase production by neutrophils and macrophages (Sasaki et al., 1990).

Fibronectin is a member of fibronexal family associated with fibroblasts and collagen fibers and composed of extracellular filaments, intracellular microfilaments, transmembrane proteins responsible for the high density of the plasma membrane of the fibroblasts, and the fibronectin which is a sticky glycoprotein that sticks collagen and a number of extracellular components (TenCate 1998).

The results of the present investigation revealed that fibronectin was found all over the pulp tissue, periodontal ligament and lamina propria of the dentogingival junction of the control group where the collagen fibers are one of the main constituents of these structures, this is in agreement with Cho et al., (1986) who stated that fibronectin coats collagen fibrils, specially type I and III collagen. The authors added that fibronectin may sometimes fill spaces between the cell membrane and collagen fibrils. Moreover, our results confirm those of Kapila et al., (1998) who said that fibronectin has been localized in the periodontal extracelular matrix and is capable of participating in healing and regeneration of these tissues. In addition, formation of new fibroblasts and collagen is most active in the middle of the P.L.
suggesting that there is an overlap in the location of fibroblast turnover and in the presence of the collagen and thus FN. Therefore fibronectin and its filaments are involved in the cell-extracellular matrix interactions that underlie the maintenance, regeneration and healing of periodontal tissues.

The results of the present investigation revealed marked decrease in fibronectin localization in the pulp tissue, periodontal ligament and lamina propria of the dentogingival junction of two months and four months hypercholesterolemic tissue specimens. The decrease was most severe in specimens of the four months hypercholesterolemic rats than those of the two months hypercholesterolemic rats. Johansson et al., (1979) reported that normal macrophages synthesize and secrete fibronectin. Coito et al, (1995) stated that both fibronectin and laminin are primarily produced by healthy macrophages. The present study ensured that the fibroblasts and the macrophages suffered fatty degeneration and vacuolization forming foam like cells and consequently resulted in decreased expression of fibronectin. Song et al, (2001) suggested that the degenerating macrophages could be considered as a potential effector mechanism for the noxious effects of hypercholesterolemia. Because transforming growth factor (TGF)-beta 1 is secreted by activated macrophages and also stimulates fibronectin production.

Localization of fibronectin in the blood vessels of the samples the present investigation revealed weakly to moderately positive reaction in the two months HC. rats and negative FN. reaction of four months HC rats. Jun et al., (1992) stated that fibronectin appeared early and disappeared later in the intima during the process of fatty streak initiation and maturation. The authors suggested that in hypercholesterolemia without mechanical endothelial injury, fibronectin may play an important role in an early process of atherogenesis.

Shih et al., (1999) suggested that involvement of FN. in the development of atherosclerosis may be through monocyte rolling and adhesion in atherosclerotic lesions. Izabella et al., (2000) stated that blood monocytes are systemically activated by high serum cholesterol levels so that following increasing macrophage number and maturation to macrophages they elaborate soluble factors that can stimulate mesangial cell fibronectin production, cell proliferation, and growth factor secretion. In atherosclerotic lesions, both cytokines (Kishikawa et al., 1993) and chemokines (Terkeltaub et al., 1998) have been detected in addition to oxidized lipoproteins. The profile of adhesion molecule expression is likely to result from a combination of these factors. Swirski et al., (2007) stated that hypercholesterolemia-associated monocytosis (HAM) developed from increased survival, continued cell proliferation, and impaired monocytes that secrete serum C-reactive protein and other inflammatory mediators that stimulate smooth muscle cell migration and proliferation and participate in plaque development and rupture as well as thrombosis.

CONCLUSION

- High dietary cholesterol can initiate degenerative changes in the periodontal ligament and DGJ. and augment the inflammatory responses induced by bacterial pathogens. Analysis of these data supports high dietary cholesterol being a risk factor for pulp and periodontal disease progression.
- Most of the vascular channels revealed thrombotic lesions in the form of total and subtotal occlusion of their lumina with marked increase in the thickness of their walls, a picture of typical dental angina.
- Thrombosis leads to ischemia with its pathologic sequelae as a result of the evoked hypoxia. The later ultimately lead to infarction of the relevant organs and tissues. The dental and parodontal tissues are not exceptions.
- In conclusion it should remain an everlasting warning that hypercholesterolemia is a major health problem that leads not only to termination of lives if it massively affects the blood vessels of the vital organs like the heart, brain, kidneys as well as the abdomen and limbs, but also it can render lives so hard if it affects the vessels of tissues that endangers the life of the masticatory apparatus of the affected persons.
REFERENCES


