THE EFFECT OF HYPERCHOLESTEROLEMIA ON THE RAT PAROTID SALIVARY GLANDS (HISTOPATHOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY)

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ABSTRACT

Hypercholesterolemia is a common risk problem. It is a well-known aggravating factor in the pathogenesis of many diseases so that, the present study was conducted to induce hypercholesterolemia in rats by long-term high-cholesterol diet administration then investigate the histopathological and immunohistochemical changes in parotid salivary glands of these animals and discuss their potential significances in understanding the mechanism by which hypercholesterolemia had adversely affected the tissues. Thirty male albino rats (150 ±10 grams) were divided equally into control and experimental groups. The rats of the experimental group received hypercholesterolmic diet containing 1% cholesterol for 3 consecutive months, while the rats of control group were kept on normal diet. At the end of the experimental period, all rats were sacrificed. The parotid salivary glands were dissected out and prepared for histopathological and immunohistochemical examinations. Histopathological examination revealed severely atrophied serous acini and massive fibrosis formed of thick collagen fibers, thick walled lymphatics and numerous dilated blood vessels. The excretory ducts were degenerated with retained secretion. There were numerous intra and intercellular vacuoles. There were also numerous large vacuoles as well as severe haemorrhage in the connective tissue stroma. Some specimens revealed complete replacement of the salivary glands lobes by numerous large empty spaces. Immunohistochemical examination revealed intense positive immunoreactivity for Fas in the acinar and duct cells. Whereas, both of the acinar and duct cells showed negative p53 immunoreactivity. In conclusion, the major manifestations of hypercholesterolemia inducing salivary glands injury are apoptosis, fibrosis, fatty degeneration, lipid accumulation and microcirculatory disturbances.

INTRODUCTION

Hypercholesterolemia is a common abnormality. It is a well-known aggravating factor in the pathogenesis of many diseases. It is a strong, independent predictor for development of atherosclerosis (1). It often accompanies and aggravates early and advanced renal diseases (2&3). Experimental hypercholesterolemia induced renal dysfunction, inflammation, fibrosis and renal damage(4-7). It also produced glomerulosclerosis, activation of the renin-angiotensin system, glomerular hyperfiltration, increased renal lipid accumulation and structural changes.
in the kidney that may be the precursors of more severe glomerular injury (8&9).

Experimental and epidemiological studies demonstrated that elevated levels of cholesterol had constituted a major risk factor for coronary heart disease (10). Hypercholesterolemia was also reported to have toxic effects on the liver. It produced hepatic necrosis, macrophage infiltration and steatosis. There were Hepatic fibrosis, myofibroblast proliferation and Mast cell aggregation. So that, hypercholesterolemia was considered as a risk factor for hepatic fibrosis as well as atherosclerosis and coronary heart disease (11). Hypercholesterolemia was also involved in elevation of the blood pressure through excessive lipid oxidation (12). We have previously shown that administration of cholesterol-enriched diet for 8 weeks adversely affected the histological and ultra structure of the major salivary glands. It produced loss of the normal architecture of the secretory portions, degeneration and retained secretion of the ducts and severe fibrosis of the connective tissue stroma. There was abnormal nuclear form with pyknosis and numerous saturated fat globules were also detected (13).

 Approximately 50% of the middle-aged adult population has total cholesterol above the desirable levels (14). Experimental hypercholesterolemia was induced by feeding the model animals with a high cholesterol diet that in turn leads to elevation of the rates of aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase and total cholesterol (11). These alterations appear to be due to impaired catabolism rather than increased synthesis (15). However, the precise mechanisms by which fatty acids influence blood lipid levels are not fully understood (16). The type of dietary fat and the amount of cholesterol in the diet have been associated with several metabolic disorders. Hypercholesterolemic diet enriched with coconut oil and cholesterol had affected carbohydrate and lipid metabolism in a rat model. It produced a significant increase in serum total cholesterol, low-density lipoproteins, as well as increased liver cholesterol (17).

p53 (wild type) is a tumor suppressor gene found on chromosome 17. It is a short-lived protein with a half-life of about twenty minutes. It could be activated by several stressful conditions that produced DNA damage (18). It is localized and acts within the nucleus. It regulates the transition from G1 to S phase of the cell cycle. p53 was described as the guardian of the genome that accumulates in response to DNA damage and switches of replication in order to give the cells extra time for repair preventing genomic instability. So that, p53 induces apoptosis in cases where the DNA damage was too severe to be properly repaired (19). Mutations of the p53 tumor suppressor gene occurred in diverse tumors and it not only produced loss of the p53 suppressor function but also activate p53 as an oncogene (20). The mutated p53 is usually stable and has a prolonged half-life making it easier to be detected by the use of immunohistochemistry. The p53 immunexpression was detected in salivary glands tumors. p53 immunexpression was low in benign tumors, moderate in the malignant and high in the late stages of cancer that have an aggressive behaviour (21).

Fas is named also as Apo-1 or CD95. It is a member of the tumor necrosis factor (TNF) superfamily. A family of transmembrane receptors involved in cell death signaling. It is a cell surface glycoprotein (about 36 KD molecular weight) that involved in mediation of apoptosis (programmed cell death). Fas has three cystein rich extracellular domains and an intracellular death domain essential for signaling. Ligation of Fas by either agonistic antibody or by its natural ligand transmits a death signal to the target cells potentially triggering apoptosis (22).

Most of researches on hypercholesterolemia have revealed its effects on atherosclerosis, coronary heart disease and other tissues like liver or kidneys. We also demonstrated the toxic effects of cholesterol enriched diet on the histological and ultra structural changes of salivary glands in a previous study but the mechanism by which hypercholesterolemia had adversely affected the tissues was not detected. So that, the objective of this study was to induce hypercholesterolemia in rats by long-term high cholesterol diet intake, then investigate the histopathological and immunohistochemical
alterations in parotid salivary glands of these animals, and discuss their potential significances in understanding the mechanism by which hypercholesterolemia had adversely affected the tissues.

**MATERIALS AND METHODS**

Thirty healthy adult male albino rats weighing 150±10 grams were used in this study. The rats were obtained and housed in the animal house at the Faculty of Oral and Dental Medicine, Cairo University. The animals were divided into two main groups (15 rats each) as follows:

**Group I (Control group):**

The rats were kept on normal diet and water for 3 consecutive months.

**Group II (hypercholesterolemic group):**

The rats were kept on water and hypercholesterolemia inducing diet (hypercholesterolemic diet) containing 1% cholesterol for 3 consecutive months (Kunishima et al., 1999). Cholesterol crystals were dissolved in table butter and the hypercholesterolemic diet was prepared by mixing each 10 grams cholesterol dissolved in 100 grams table butter with 1 kilogram of the corresponding normal diet. Cholesterol crystals were purchased from Sigm chemical Co.

At the end of the third month, the rats were fasted for 16 hours. The rats of both experimental and control groups were anaesthetized with chloroform and blood was collected via the retro-orbital plexus with microhematocrit tubes. The blood was placed in clean centrifuge tube for serum separation for detection of cholesterol levels using commercial diagnostic kits (Bio-Merieux, France). Then, the rats were sacrificed by cervical dislocation. The parotid salivary glands were dissected free, cleaned rapidly of any adherent connective tissue and were fixed immediately in 10% calcium formol for 12 hours. Then, the specimens were washed by tap water, dehydrated in ascending grades of ethyl alcohol, cleared in xylol and embedded in paraffin wax. Sections of 6-7 M were obtained and mounted on clean glass slides and stained with:

- **Hematoxylin and Eosin stain:** for histopathological examination.
- **Masson’s trichrome stain:** for detection of newly formed collagen fibers (fibrosis).
- Additional sections were prepared for subsequent immunohistochemical staining:
  - **Fas Immunohistochemical staining:** for detection of apoptosis.
  - **p53 (mutant type) Immunohistochemical staining:** for detection of mutagenicity.

**RESULTS**

The cholesterol levels were measured in all blood samples of both control and experimental groups and statistical analysis of the data obtained was carried out using ANOVA test and comparing between means using LSD (Least Significant Difference) at P <0.05. Table (1) shows the effect of feeding a hypercholesterolemic diet on the cholesterol levels in blood. The table presents the mean of cholesterol level and the standard deviation of the control and the experimental groups. Results showed that the value of P < 0.05 which indicates that, there is a statistical significance difference in the cholesterol level between the control and the experimental animals. The data obtained revealed that, feeding animals with hypercholesterolemic diet containing 1% cholesterol for 3 consecutive months produced a significant elevation in total serum cholesterol concentrations (significant hypercholesterolemia)

**TABLE (1) shows the effect of hypercholesterolemic diet on the cholesterol levels in blood**

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>Experimental group</th>
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<tr>
<td>Mean</td>
<td>56.7</td>
<td>71.5</td>
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<tr>
<td>SD</td>
<td>±0.25</td>
<td>±1.67</td>
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<td>p</td>
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Histopathological Examination

**Group I (Control group):**

Light microscopic examination of the rat parotid glands of control group showed rounded medium sized serous acini packed in a connective tissue stroma. The acini were lined by pyramidal cells having rounded deeply stained nuclei and basophilic cytoplasm. Some intercalated ducts were scattered in between (Fig. 1).

**Group II (hypercholesterolemic rats):**

Histopathological examination of the parotid glands of rats affected by hypercholesterolemia revealed severely atrophied serous acini with abnormal wide interlobular spaces (Fig. 2). There were numerous intracellular vacuoles within the serous acini. In some specimens, a large blood pool was detected within the lobes (Fig. 3). There were large vacuoles in-between the serous acini. There were also numerous large vacuoles as well as severe hemorrhage in the connective tissue stroma (Fig. 4). Some specimens revealed complete replacement of the salivary gland lobes by numerous large empty spaces. Some specimens revealed elongated and deformed serous acini with large empty spaces in between (Fig. 5). Other serous acini appeared degenerated with eroded margins and had many intracellular vacuoles. There were numerous large inter acinar vacuoles. Some extravasated red blood cells were detected in between the acini. The acinar cells had hyperchromatic nuclei with different shape and size (Fig. 6). The excretory ducts appeared dilated with retained secretion and degenerated epithelial lining. The ducts were surrounded by severe fibrosis and dilated blood vessels engorged with red blood cells (Fig. 7). The connective tissue stroma of the parotid glands of hypercholesterolemic rats showed extensive inter and intra lobular fibrosis. This fibrous connective tissue was characterized by presence of thick collagen bundles surrounding large dilated blood vessels engorged with red blood cells (Fig. 8).

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**Fig. (1):** A photomicrograph of rat parotid glands of control group showing the normal architecture of pure serous acini (a) and intercalated ducts in between (d) (H & E Orig.mag. X 200).

**Fig. (2):** A photomicrograph of parotid glands of hypercholesterolemic group showing severely atrophied serous acini with widening of intercellular spaces (a), degenerated excretory duct (d), severe fibrosis (f), and numerous dilated blood vessel engorged with red blood cells (bv) (H & E Orig.mag. X 200).

**Fig. (3):** A photomicrograph of parotid glands of hypercholesterolemic group showing atrophied deeply stained serous acini (a), some intracellular vacuoles (v) and large extravasated blood pool (b) (H & E Orig.mag. X 200).
The effect of hypercholesterolemia

Immunohistochemical Examination:

**Group I (Control group):**

Immunohistochemical examination of the rat parotid glands of control group revealed negative Fas immunoreactivity in the acinar cells and slight positive Fas immunoreactivity in the duct cells and blood vessels (Fig. 9). The acinar cells of control group also showed negative p53 immunoreactivity (Fig. 10).

**Group II (hypercholesterolemic rats):**

Immunohistochemical examination of the parotid glands of the rats affected by hypercholesterolemia revealed intense positive immunoreactivity for Fas in the acinar and duct cells (Fig. 11&12). On the other hand, both of the acinar and duct cells showed negative p53 immunoreactivity (Fig. 13).
Fig. (8): A photomicrograph of parotid glands of hypercholesterolemic group showing severe inter and intra lobular fibrosis with numerous newly formed collagen fibers and dilated blood vessels (Masson trichrome Orig.mag. X 200).

Fig. (9): A photomicrograph of rat parotid glands of control group showing negative Fas immunoreactivity in the acinar cells and slight positive Fas immunoreactivity in the duct cells and blood vessels (Fas Orig.mag. X 200).

Fig. (10): A photomicrograph of rat parotid glands of control group showing negative p53 immunoreactivity in the acinar cells (p53 Orig.mag. X 400).

Fig. (11): A photomicrograph of parotid glands of hypercholesterolemic group showing intense Fas positive immunoreactivity in both acinar and duct cells (Fas Orig.mag. X 200).

Fig. (12): A photomicrograph of parotid glands of hypercholesterolemic group showing intense Fas positive immunoreactivity in the acinar cells (Fas Orig.mag. X 400).

Fig. (13): A photomicrograph of parotid glands of hypercholesterolemic group showing negative p53 immunoreactivity in both acinar and duct cells (p53 Orig. mag. X 400).
DISCUSSION

It has been well established that nutrition plays an important role in the etiology of hypercholesterolemia. Several animal and human studies have confirmed the hypercholesterolemic properties of saturated fatty acids and cholesterol, which include increasing total cholesterol and altering lipoprotein pattern (25,26). Cholesterol feeding has been often used to elevate serum or tissue cholesterol levels to assess hypercholesterolemia-related metabolic disturbances in different animal models (27,28). In this study, hypercholesterolemia was induced in rats by adding cholesterol crystals (1%) to the normal diet for 3 consecutive months. These results are in agreement with previous studies using different amounts and types of fats, in which the hypercholesterolemic effect was attributed to some saturated fatty acids (29-31), or feeding of cholesterol enriched diet (17,23). Furthermore, dietary fat saturation and cholesterol have been shown to affect lipoprotein response (32), apolipoprotein gene expression (33), in addition to changes in plasma cholesterol concentrations (34). These changes referred to impaired catabolism rather than increased synthesis (15).

In the present study, feeding of experimental animals with hypercholesterolemic diet had adversely affected the histological structure of the parotid salivary glands. Histopathological examination of the parotid glands of hypercholesterolemic animals revealed severely atrophied serous acini with abnormal wide interlobular spaces. There were numerous intra and intercellular vacuoles and some specimens revealed complete replacement of the salivary gland lobes by numerous large empty spaces that might be resulted from severe fatty degeneration and replacement of parenchymal cells by fat cells. On the same time, fatty degeneration of the connective tissue stroma and accumulation of fat cells resulted in pressure atrophy of the serous acini. These results were in agreement with other studies as hypercholesterolemia produced hepatic necrosis, fatty liver (steatosis) (11), and nonalcoholic fatty pancreatic disease (36).

The connective tissue stroma of the parotid glands of hypercholesteremic rats showed extensive inter and intralobular fibrosis. This fibrous connective tissue was characterized by presence of thick collagen bundles surrounding large dilated blood vessels engorged with red blood cells. Large blood pool was detected within the salivary glands lobes and there was also severe hemorrhage in the connective tissue stroma. These findings were in accordance with other studies as hypercholesterolemia was considered as a risk factor for hepatic fibrosis (11). Dyslipidemia was also reported to accompany and accelerate renal disease by promoting fibrosis mostly collagen IV expression. However, the mechanisms mediating this effect were unclear. It was hypothesized that hypercholesterolemia modulates several interlinked pathways that promote deposition and blunt degradation of extracellular matrix (37). It might be also as a response of fibroblast simulating factor that activated fibroblast to increase synthesis of collagen fibers and mucopolysaccharides. This increased fibrosis might be considered as a defensive and protective tissue response to limit the toxic effect of hypercholesterolemia (26). The dilatation and congestion of the blood vessels might be a part of inflammatory response to bring more blood to the areas of fibrosis or degeneration (28).
fat consumption was associated with gradual imbalance of homeostasis and alterations of capillaries in the pancreas (20). High fat diets were also reported to cause atherosclerosis and hypertension (7). The atherosclerotic vessels were fragile and easy to produce hemorrhage within the glands.

The excretory ducts appeared dilated with retained secretion. Their epithelial lining was degenerated. Dilatation of the ducts could be attributed to accumulation of the salivary secretion and failure of exocytosis due glandular injury and dysfunction as lipid accumulation promote gland dysfunction.

Immunohistochemical examination of the parotid glands of the rats affected by hypercholesterolemia revealed intense positive immunoreactivity for Fas in the acinar and duct cells indicating apoptotic cell death. These findings were in agreement with other studies as hypercholesterolemia increased postischemic myocardial apoptosis(38) and apoptosis of cerebellar neuronal cells(39). It was also found that, the major manifestation of hypercholesterolemia-induced renal injury is apoptosis(40). Apoptosis may be resulted from increasing the production of pro-apoptotic molecules, activating pro-apoptotic signaling pathways and inhibiting anti-apoptotic signaling(38). Meanwhile, other study revealed that EM-TUNEL positively labeled not only apoptotic but also some oncotic nuclei of macrophages indicating both apoptosis and oncosis in the aortic intima of hypercholesterolemic rabbits(41). Immunohistochemical examination also revealed that both of the acinar and duct cells showed negative p53 immunoreactivity indicating that hypercholesterolemia has no mutagenic effect and hypercholesterolemia produced DNA damage leading to apoptosis of the cells and not cell mutation. On the other hand, an increase in p53 expression was observed in tubuli, but not in glomeruli of the kidneys of hypercholesterolemic rats and p53 was reported to be involved in the pathogenesis of lipid-induced renal injury(40).

In conclusion, the major manifestations of hypercholesterolemia inducing salivary glands injury are apoptosis, fibrosis, fatty degeneration, lipid accumulation and microcirculatory disturbances.

**REFERENCES**

THE EFFECT OF HYPERCHOLESTEROLEMIA

(27)


