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Impact of Experimental Hyperlipidemia on Histology of Major Salivary Glands

Deneysel Hiperlipideminin Büyük Tükürük Bezlerinin Histolojisi Üzerine Etkisi

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Objectives: To examine the histologic structure of major salivary glands in high-fat diet induced hyperlipidemic rats.

Materials and Methods: Sixteen Wistar Albino rats were grouped as control group (CG) and hyperlipidemic group (HG). CG was fed a standard chow and HG was fed a high-fat diet. At the end of three months, blood samples were obtained for lipid analyses. For histological analysis, major salivary glands were evaluted under light microscopy.

Results: In HG; the number of intracellular lipid droplets (ILD) in the major salivary glands significantly increased while the diameter of ILD significantly increased in the parotid and sublingual glands ($p< 0.01$). The cell types mainly affected were serous cells in the parotid and submandibular, serous demilunar cells and, to a lesser extent, mucous cells in the sublingual gland. Serum levels of all lipids were significantly correlated with the lipid numbers in the parotid and the sublingual glands. In the submandibular gland, only serum LDL and cholesterol levels were found to be significantly correlated with the lipid numbers ($p<0.05$).

Conclusion: High-fat diet caused intracellular lipid accumulation in the parotid and sublingual glands without any structural alteration. Submandibular gland exerted a partial resistance to intracellular lipid accumulation.

Key words: Experimental hyperlipidemia; major salivary glands; lipid accumulation and Oil red O.

Amaç: Yağ içeriği zengin diyet ile beslenerek hiperlipidemi oluşturulan sıçanlarda, büyük tükürük bezlerinin histolojik yapısını incelemektir.

Gereç ve Yöntemler: On altı adet Wistar Albino sıçanı kontrol grubu (KG) ve hiperlipidemik grup (HG) olmak üzere ikiye ayrıldı. CG standart yem, HG ise yağ içeriği zengin diyet ile beslendi. Üç ay sonunda, lipit analizi için kan örnekleri alındı. Histolojik değerlendirme için, büyük tükürük bezleri ışık mikroskobu ile incelendi.

Bulgular: Hiperlipidemik gruba ait büyük tükürük bezlerinde hücre içi lipit damlacıklarının sayısında anlamlı bir artış görülürken, sublingual ve parotis bezinin hücre içi lipit damlacıklarının çapında anlamlı derecede artış tespit edildi ($p<0.01$). Parotis ve submandibular bezlerde esas olarak seröz hücreler, sublingual bezde ise seröz yarımlar ve daha az derecede müköz hücreler hiperlipidemiden etkilendi. Tüm serum lipit düzeyleri ile sublingual ve parotis bezde biriken lipit damlacıkları sayısı arasında istatistiksel olarak anlamlı korelasyon saptandı. Submandibular bezde ise, sadece LDL ve kolesterol düzeyleri ile lipit damlacıkları sayısı arasında anlamlı bir korelasyon tespit edildi ($p<0.05$).

Sonuç: Yüksek yağ içeren diyet, parotis ve sublingual bezlerde yapısal bir değişikliğe neden olmaksızın, hücre içi lipit birikimine neden olmaktadır. Submandibular bez ise intraselüler lipit birikimine karşı kısmi bir direnç göstermektedir.

Anahtar sözcükler: Deneysel hiperlipidemi; büyük tükürük bezleri; lipid birikimi ve Oil red O.

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Salivary glands are one of the important determinants of the maintenance of oral health in human. There are many factors causing accumulation of lipid droplets in salivary glands such as actinomyocin-D,[1] diabetes mellitus (DM),[2-4] ethionine,[5] isoproterenol,[6] X-ray,[7] ingestion of liquid diet,[8] starvation,[9] prolonged hypoxia,[10] and ageing[11] and the like.

Hyperlipidemia may be familial or secondary to several conditions like high-lipid diet, DM, hypothyroidism and liver dysfunction.[12-14] Although the effect of lipid accumulation on the histology and function of salivary glands has been questioned in several clinical and experimental conditions, lipid deposition in salivary glands in these studies was mostly secondary to some metabolic disorders like DM, hypothyroidism or cirrhosis.[4,15-17]

Hyperlipidemia induced by metabolic conditions cause lipid accumulation mostly in parenchymal cells of major salivary glands with several morphologic alterations in granular ducts and acini.[3] However, the effect of isolated hyperlipidemia without other risk factors on salivary glands has not been studied yet. Therefore, in this study, we hypothesized that diet-induced hyperlipidemia without any metabolic disease may alter the pattern or the distribution of lipid accumulation in major salivary glands. Accordingly, we aimed to reveal the effect of diet-induced hyperlipidemia on the histological structure of major salivary glands. For this purpose, the distribution, the number and the size of accumulated lipid droplets in the salivary glands of control and hyperlipidemic rats were examined.

MATERIALS AND METHODS

Animals and Experimental Hyperlipidemia

The study was approved by the ethical committee of the Marmara University regarding the use of live animals in teaching and research. Sixteen Wistar Albino rats aged eight weeks were used in this study (approved date and number: 24.01.2003-71.2002mar). The animals were maintained under controlled conditions (temperature, 22-24 ºC, light - dark cycle, 12 h) with free access to water and food. They were divided into two groups, namely control group (CG; n=8) and hyperlipidemic group (HG; n=8). Each group had equal number of male and female rats. Rats were fed ad libitum on the following diets for three months: control group was fed a standard chow (standard commercial chow content: 20% crude protein, 2.85% crude oil, 5.95% cellulose, 8% crude ash, 0.47% calcium, 0.5% phosphorous, 1.03% lycine, 0.33% methionine, 0.65% methionine+cysteine, 0.14% sodium, 1.13% linoleic acid, vitamin A, 9000 IU/kg; vitamin D3, 2000 IU; vitamin E, 60 IU; ingredients used include 45% wheat, 20% soybean bagasse, 6% corn, 3% sunflower bagasse, 10% bran flour) and hyperlipidemic group was fed a standard chow supplemented with 1.63% cholesterol, 0.41% colic acid, 16.3% sunflower oil.[18,19] The texture and solidity of the hyperlipidemic diet was similar to the standard chow. Water consumption of each group was determined everyday throughout the experimental period.

At the end of the three months, animals were fasted overnight. Anesthesia was induced by intraperitoneal injection of 60% urethane solution (Sigma, St. Louis, MO, USA) (1.2 g/kg body weight) and then they were sacrificed. Intracardiac blood was obtained for determination of serum lipid levels. Major salivary glands were dissected out for morphologic investigation.

Morphologic Studies

For light microscopic analysis, the salivary glands were initially fixed into 10% neutral buffered formalin solution. Cryostat sections (10 μm thick) of formalin-fixed tissues were stained with Oil Red O (Acros organics, New Jersey, USA) to demonstrate the presence of lipids.[20] Stained salivary gland sections were examined by using x100 oil immersion objective (Olympus DPlan 100, Japan) under an Olympus light microscope (BH - 2, Japan). The intracellular lipid droplets in 10 different randomly selected microscopic fields without an artifact were counted. The diameter of two intracellular lipid droplets in each of microscopical fields was also measured by using spot advanced software program (Spot 4.1). Microscopic views of the

Table 1. Serum lipids and body weight in control and hyperlipidemic groups and their comparison

Parameters	Control group (n=8) (median (25-75 percentiles))	Hyperlipidemic group (n=8) (median (25-75 percentiles))	*p*
Triglyceride (mg/dL)	62.64 (58.6-67)	71.5 (69.8-76.7)	0.012
Cholesterol (mg/dL)	63.5 (58.2-76.5)	299.5 (279.7-316.2)	0.001
HDL (mg/dL)	28.1(24.6-30.8)	19.27 (19.1-20)	0.001
LDL (mg/dL)	14.5(3.2-16.7)	63.5 (60.2-73.7)	0.001
Total lipid (mg/dL)	228 (188.5-251.7)	874 (856.7-961-2)	0.001
Body weight (gr)	290 (239.7-398)	291.5 (229.7-358.7)	0.958

Mann Whitney U test was used to asses the differences between two group. LDL: low-density lipoprotein; HDL: high-density lipoprotein.

salivary glands were captured by insight - 2 Megasample Camera.

Oil Red O Staining

Cryostat sections were collected in distilled water. Free-floating cryostat sections were transported into absolute propylene glycol for two minutes, immersed in Oil Red O solution for one hour and differentiated in 85% propylene glycol for one minute. They were rinsed in distilled water, counterstained in hematoxylin for 30 seconds and then rinsed in tap water to blue. Section were rinsed two times in distilled water and mounted on glass slides. After air-drying, stained sections were mounted with aqueous medium.

Biochemical Studies

Intracardiac blood samples were collected in test tubes, and serum was separated for biochemical analysis. Serum triglyceride, total cholesterol, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) levels were measured by using Human kits (Wiesbaden, Germany, cat. no: 10 028, 10 720P, 10 094, 10 084, respectively). Serum total lipid levels were measured using phosphovanilin method.[21]

Statistical Analysis

All statistical analyses were performed using SPSS for Windows 10.0 version program (Chicago, Ill, USA). According to Kolmogorov-Smirnov Z normality test, the distribution of the data was not normal, consequently a non-parametric test was used. Continuous variables were expressed as median (25-75 percentiles, %). Mann Whitney U test was employed for intergroup comparisons while Kendall's W test was preferred for multiple comparisons in each group. Kendall's tau-b test was computed to assess correlations between plasma lipid levels and the number of accumulated lipid droplets. A p value of less than 0.05 was considered significant.

RESULTS

The effect of hyperlipidemic diet on serum lipid profile was shown in Table 1. Hyperlipidemic diet increased serum triglyceride, cholester-

Table 2. Comparison of the number and diameter of the intracellular lipid droplets accumulated in control and hyperlipidemic groups

Glands	Parameter	Control group (n=8) (median (25-75 percentiles))	Hyperlipidemic group (n=8) (median (25-75 percentiles))	*p*
Parotid	Lipid number	27.6 (15.4-30.9)	73.1 (60.5-79.4)	0.001
	Lipid diameter (μm)	0.7 (0.6-0.8)	1.09 (1-1.1)	0.001
Sublingual	Lipid number	19 (17.6-29.2)	61.85 (56.4-67.6)	0.001
	Lipid diameter (μm)	0.8 (0.6-0.9)	1.09 (0.9-1.3)	0.009
Submandibular	Lipid number	14.2 (11.2-18.3)	18.25 (17-20)	0.035
	Lipid diameter (μm)	0.6 (0.5-0.7)	0.8 (0.6-0.8)	0.074

Mann Whitney U test was used to asses the differences between two groups.

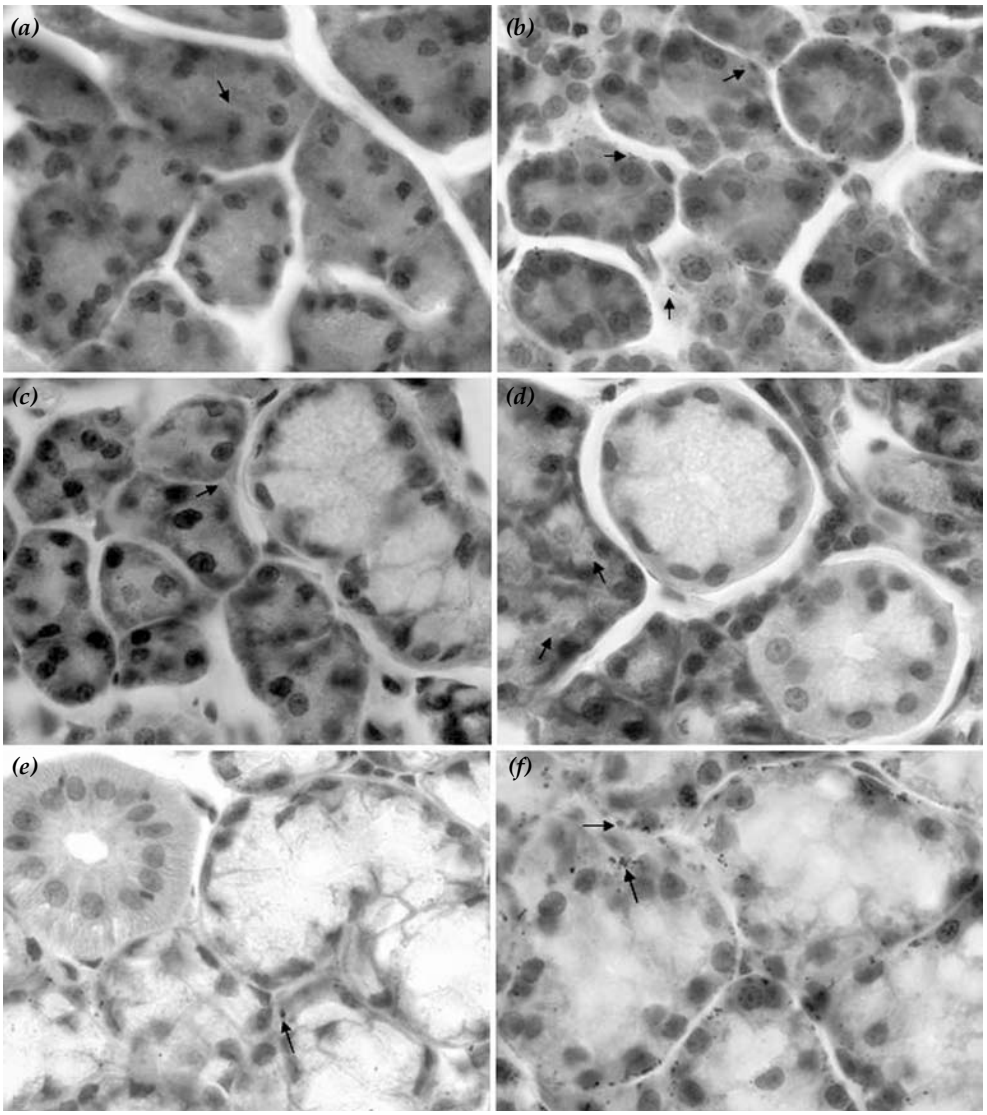

Fig. 1. ***(a)*** *Cryosections of control parotid gland stained with Oil Red O showing rarely intracellular lipid droplets (black arrow) in the acinar cells.* ***(b)*** *Hyperlipidemic parotid gland, Oil Red O staining showed increased intracellular lipid (black arrow) in the acinar cells,* ***(c)*** *Cryosections of control submandibular gland stained with Oil Red O showing rarely intracellular lipid droplets (black arrow) in acinar cells.* ***(d)*** *Hyperlipidemic submandibular gland, Oil Red O staining showed slightly increased intracellular lipid (black arrow) in acinar cells but more than controls'.* ***(e)*** *Cryosections of control sublingual gland stained with Oil Red O showing rarely intracellular lipid droplets (black arrow) in the serous demilunar.* ***(f)*** *Hyperlipidemic sublingual gland, Oil Red O showed increased intracellular lipid (black arrow) in the serous demilunar cells.*

ol, total lipid and LDL cholesterol levels and decreased HDL cholesterol levels significantly in the HG with respect to the CG (p=0.012; p=0.001; p=0.001; p=0.001; p=0.001 respectively). We did not determine the water consumption of the particular rat, instead water consumption of each group was determined. The mean value of water consumed by each rat was obtained by dividing the amount consumed by each group to the number of rats in the group (n=8). Although it was not statistically tested, in our study, hyperlipidemic rats consumed more water than the controls (30 ml/day versus 25 ml/day).

There was no significant difference in the macroscopic examination of the salivary glands

Table 3. Comparison of the number and diameter of the intracellular lipid droplets accumulated in the control and hyperlipidemic group

Group	Parameter	Parotid (n=8) median	Sublingual (n=8) median	Submandibular (n=8) median	Kendall' W test *p*
Control	Lipid number	27.6 (15.4-30.9)[a]	19 (17.6-29.2)[b]	14.2 (11.2-18.3)	0.016
	Lipid diameter (μm)	0.7 (0.6-0.8)[a]	0.8 (0.6-0.9)[b]	0.66 (0.5-0.7)	0.03
Hyperlipidemic	Lipid number	73.1 (60.5-79.4)[aa]	61.8 (56.4-67.6)[bb]	18.2 (17-20)	0.002
	Lipid diameter (μm)	1.09 (1-1.1)[aa]	1.09 (0.9-1.3)[b]	0.8 (0.6-0.8)	0.02

Kendall' W test was used to assess the differences within in each group. ([a] $p<0.05$, [aa] $p<0.01$ significant between parotid and submandibular gland, [bb] $p<0.01$, [b] $p<0.05$ significant between sublingual and submandibular gland)

between two groups. In the CG, lipid was mainly confined to stromal fat cells; however, small intracellular lipid droplets in acinar cells were occasionally seen. In the HG, no stromal fat cell was seen and accumulation of lipid was frequently seen in acinar cells. In comparison, the number of intracellular lipid droplets in parotid, sublingual and submandibular glands significantly increased in the HG with respect to the CG (p=0.001; p=0.001 and p=0.035 respectively) (Table 2). The distribution of intracellular lipid droplets varied with the type of the salivary gland. In the parotid gland, intracellular lipid droplets were present in acinar cells. No lipid was seen in duct cells (Fig. 1a, b). In the sublingual gland, a lipid-staining band in the peripheral region of each mixed acinus was observed due to the accumulation of intracellular lipid in serous demilunar cells (Fig. 1e, f). A significant lipid accumulation was observed in submandibular gland. Intracellular lipids were mainly observed in serous cells and to lesser degrees in mucous cells (Fig. 1c, d).

In the HG the diameter of intracellular lipid droplets in parotid and sublingual glands significantly increased (p=0.001 and p=0.009 respectively) when compared to those in the CG. However, the difference was not significant for the submandibular gland (p=0.074) (Table 2). In both groups, the number and the diameter of intracellular lipid droplets in submandibular gland were less than other glands (Table 3). Therefore, submandibular gland was found to be less severely affected in terms of intracellular lipid accumulation.

In the parotid and sublingual gland, there was a positively significant correlation between

Table 4. Correlation between serum lipids and the mean number of lipid droplets in salivary glands in both groups

Parameters	Kendall's tau-b *p*	Correlation coefficient (r)
Triglyceride - Number of the lipid in Parotid gland	0.0001	0.812
Triglyceride - Number of the lipid in Sublingual gland	0.002	0.577
Cholesterol - Number of the lipid in Parotid gland	0.022	0.427
Cholesterol - Number of the lipid in Sublingual gland	0.003	0.561
Cholesterol - Number of the lipid in Submandibular gland	0.024	0.420
Total Lipid - Number of the lipid in Parotid gland	0.030	0.403
Total Lipid - Number of the lipid in Sublingual gland	0.015	0.454
HDL - Number of the lipid in Parotid gland	0.0001	0.678
HDL - Number of the lipid in Sublingual gland	0.002	0.577
LDL - Number of the lipid in Parotid gland	0.0001	0.723
LDL - Number of the lipid in Sublingual gland	0.0001	0.689
LDL - Number of the lipid in Submandibular gland	0.006	0.515

Kendall's tau-b test was used to assess the correlations. LDL: low-density lipoprotein; HDL: high-density lipoprotein.

the lipid number and serum lipid levels (for triglyceride levels r=0.812, p=0.001; r=0.577 p=0.002 respectively; for cholesterol levels r=0.427, p=0.022; r=0.561, p=0.003 respectively; for total lipid levels r=0.403 p=0.03; r=0.454, p=0.015 respectively and for LDL cholesterol levels r=0.723, p=0.0001, r=0.689 p=0.0001 respectively). A negative correlation was found between lipid numbers in the parotid and sublingual gland and serum HDL cholesterol levels (r=0.678, p=0.0001; r=0.577, p=0.002 respectively). In the submandibular gland, although there was a positive significant correlation between lipid numbers and serum levels of cholesterol and LDL cholesterol (r=0.420, p=0.024; r=0.515, p=0.006 respectively), we did not find any correlation between lipid numbers and serum HDL, total lipid and triglyceride levels (Table 4).

DISCUSSION

Hyperlipidemia is characterized by elevated serum levels of triglycerides, cholesterol or both.[22,23] It causes lipid accumulation in various tissues such as liver, kidney, muscle, arterial vessel wall and pancreas.[24-27] In this experimental study, we examined the effect of diet-induced hyperlipidemia on the morphologic structure of salivary glands. Our results show that hyperlipidemia (including hypercholesterolemia and hypertriglyceridemia) caused moderate to severe degrees of intracellular lipid accumulation in major salivary glands. Furthermore, submandibular gland exerted relative resistance to intracellular lipid accumulation with respect to parotid and sublingual glands.

Marked accumulation of intracellular lipid droplets in major salivary glands is caused by various experimental and clinical challenges. Feeding is one of the major factors for lipid deposition in salivary glands. As lipid intake is greater than usage during the first several days, excess lipid is temporarily preserved in the secretory cell cytoplasm of salivary glands as droplets.[28] Similarly, in our study, excess lipid as a result of feeding high fat diet is thought to be taken up by the secretory cells. Out of which, DM is a major factor for secondary hyperlipidemia and causes intracellular lipid accumulation (mainly triglyceride) in salivary glands.[29] Different from our results, Anderson and Garrett[29] reported intracellular lipid in the intercalated ducts of parotid and submandibular glands of diabetic rats. In our study, the lipid deposition was purely intracellular, however we did not perform cholesterol staining to differentiate the type of intracellular lipid. Moreover, the volume density of the acini increased whereas that of the granular ducts decreased, and some cellular degeneration was observed which might be due to the cytotoxic effects of DM.[3,30] However, since DM was not induced in our study, we observed extensive intracellular lipid accumulation without any morphological change.

There have been several proposed mechanisms for abnormal lipid accumulation in salivary glands including impaired lipid metabolism in the secretory cells, intake of excessive lipid, decreased use of lipids for the packaging and secretion of secretory granules and alteration in fatty acid composition of plasma membrane.[2,8,29,31] A number of studies showed that dietary lipids could modify the composition of the structural lipids of cell membranes and the fluidity of lipid bilayer. It is established that these changes can alter some of the membrane functions, such as transport, receptor characteristics and activities of membrane-associated enzymes.[32-37] Adenylate cyclase (AC) system is important in cellular signaling and it is sensitive to the change in the composition of dietary lipids.[34,36,37] Alam et al.[34] showed that feeding a diet rich in cholesterol could decrease AC activity and membrane fluidity. In our study, although membrane fluidity and AC activity were not examined, increased cytoplasmic lipid accumulation may be due to intake of excessive lipid and/or the alteration of the composition of structural membrane lipids.

Accumulation of lipids shows differences between humans and rats. In humans, fatty infiltration is observed in glandular stroma, however in rats, accumulation of lipid droplets is observed in acinar cells.[38,39] Alcoholic cirrhosis has been reported to cause elevated amount of lipid in submandibular and sub-

lingual glands.[17] This might be the result of disturbance of fat metabolism consequent to the disturbed liver function in hepatic cirrhosis. Furthermore, Rothbell and Duggan[15] suggested that parotid enlargement have ''much the same significance as a fatty liver, pointing to disturbed nutrition or metabolism but not defining the disturbance''.

Nagato and Masuno[28] reported that there was no difference in lipid accumulation between serous and mucous cells in neonatal rat salivary glands, indicating that, both types of cells take in and consume the same amount of lipid in the process of synthesis and secretion. In our study, serous cells revealed a predilection for lipid accumulation in both the control and hyperlipidemic group. The reason for that remains unclear, but it may be due to the type of their secretory products or to different effects of lipid metabolism on cell types. However, without electron microscopic evidence, it is difficult to explain these differences. An alternative explanation would be that intracellular lipid bodies are smaller in mucous cells. Furthermore, secretory granules of mucous cells are numerous and tend to undergo fusion. This could affect the dispersal of small lipid bodies since their light microscopic recognition becomes difficult.

A close relationship has been shown between elevated levels of plasma triglycerides and parotid gland swelling.[38] Moreover, decreasing the level of triglycerides by diet and drug therapy resolved parotid gland swelling in cases with high levels of cholesterol and triglyceride.[40] However, it is difficult to ascribe an etiologic relationship between parotid gland enlargement and hypertriglycemia in the presence of other risk factors like mild alcoholism, obesity, or DM.[39] Clinically Sjögren's syndrome is frequently associated with metabolic alterations such as dyslipidemia, DM and hyperuricemia. An autoimmune reaction against salivary glands also leads to the dysfunction of parotid gland.[38,42] To our knowledge, this is the first study which investigated the relationship between serum lipid levels and intracellular lipid accumulation in salivary glands in diet-induced hyperlipidemia without any metabolic disease. We found a significant correlation between serum all lipids and the lipid numbers of parotid and sublingual glands. However, in the submandibular gland, only serum LDL and cholesterol levels were found to be significantly correlated with the lipid numbers. In accordance with the results from the literature,[29,38] submandibular gland reveals a partial resistance to the hyperlipidemic process compared to the other glands. Consequently, in our study, the number of the lipid in the submandibular gland was not correlated with serum TG levels. Although submandibular gland has more serous cells which have been shown to have a great tendency for lipid accumulation, it is difficult to explain the reason for the resistance against hyperlipidemia in this study.

Impairment of salivary flow is suggested to be associated with elevated levels of plasma cholesterol.[38] Anttila et al.[43] reported reduced salivary flow rates in systemic diseases such as Sjögren's syndrome, rheumatoid diseases and hyperlipidemia. In this study, we did not measure the salivary flow rate. However, increased consumption of water could be due to decreased salivary flow in the HG. On the other hand, the unpalatability of the cholic acid-containing food and the lack of desire to consume it might have forced these animals to drink more water to wash it down when hunger makes them eat the diet with respect to the other group.

This preliminary study has some obvious limitations. Our prospective aims are (*i*) to perform an electron microscopic study, which would provide some insights in to the mechanisms of lipid accumulation secondary to diet-induced hyperlipidemia, (*ii*) to measure salivary flow rates that would highlight the physiologic impact of hyperlipidemia and increase the statistical power of the study, (*iii*) to use a specific staining method for cholesterol which would be beneficial to document the main intracellular lipid type.

Overall, our results showed that diet induced hyperlipidemia causes lipid accumulation in serous and to a lesser extent, in mucous cells

of major salivary glands, without causing any major degenerative alteration. Further studies are warranted to document the effect of lipid deposition on glandular function which could affect the oral milieu.

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