EGYPTIAN DENTAL JOURNAL
www.eda-egypt.org
VOL. 70, 115:128, JANUARY, 2024
PRINT ISSN 0070-9484 • ONLINE ISSN 2090-2360
ORAL MEDICINE, X-RAY, ORAL BIOLOGY AND ORAL PATHOLOGY
Submit Date : 23-08-2023 • Accept Date : 27-10-2023 • Available online: 01-01-2024 • DOI : 10.21608/EDJ.2023.231256.2689

EFFECT OF LOW CALCIUM DIET ON THE SUBMANDIBULAR SALIVARY GLANDS IN RATS (HISTOLOGICAL, IMMUNOHISTOCHEMICAL AND ULTRASTRUCTURAL STUDY)

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ABSTRACT

Introduction: Calcium is a critical mineral. It is utilized by our body for blood pressure stability as well as building strong bones and teeth. It is completely utilized by the salivary glands for its secretory function induction. Low calcium intake leads to an increase in adipocytes formation and decrease in maturation, fusion, and exocytosis of the salivary granules therefore destruction in the gland function and structure.

Aim: Investigation of the role of low calcium diet on the histological structure of rat submandibular salivary glands.

Materials and Methods: Thirty adult male albino rats were haphazardly divided into 2 groups equally. Control group I, Low calcium diet group II. The animals in groups I ate normal diet while those in group II were fed low calcium diet for 8 weeks. All animals were sacrificed, at the end of the experiment and the calcium serum level was measured. submandibular salivary glands were dissected and assembled for histological, immunohistochemichal and ultra-structural analysis.

Results: Group II demonstrated dramatic loss of the acinar cell arrangement, vacuolization in cytoplasm, and shrunken nuclei. Significant fibrosis around the duct system was seen. A prominent increase in the α-SMA immunoreaction was seen at the peripheral part of acini and inter-lobular ducts.

Conclusion: This current research suggests that there is a prominent correlation between low calcium food consumption and histological and functional alterations in the submandibular salivary glands.

KEYWORDS: Low calcium diet, submandibular salivary glands, Rats.

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INTRODUCTION

The adult human body contains approximately 1kg of calcium (Ca), 99% of which is stored in bones and teeth as hydroxyapatite crystals. Although the Ca in bone appears to be in a static condition, it can be mobilized into the blood when necessary. The mobilization of Ca plays a significant physiological act in different processes comprising hormone secretion, muscular constriction, as well as blood coagulation [1].

Our body acquires its Ca needs by 2 ways. First, from the eaten foods such as dairy products, almonds and spinach or Ca contained supplements. The second way is by taking it from the body's Ca. If there is no enough Ca contained food intake, our body will fulfil its Ca needs from our bone. However, Ca consumed from our bone will be latterly replaced. Unfortunately, this couldn't always be accomplished by more Ca intake in diet [1].

Moreover, Ca has a crucial part in the salivary secretion from the glands, as it monitors the protein folding by the rough endoplasmic reticulum, as well as energy production by mitochondria that is highly recommended for cellular activity. In addition, it is preserved and released from the smooth endoplasmic reticulum upon the secretion of saliva [2].

Meanwhile, Ca may act as a popular intra-cellular mediator for all receptors [acetyl choline and β-adrenergic receptors] of the salivary glands. It regulates the protein kinase-A and-C activity, together with stimulating exocytosis. Also, it controls the open and closure of aquaporin, potassium and chloride channels [2].

Hypo-calcemia means that amount of Ca consumed by the body from the food is not enough. In this case, the body will try to compensate for the Ca deficiency by extracting Ca from bones to keep the blood calcium levels normally maintained to allow all the vital body functions such as heart, brain, muscles, and nerves to perform normally. This continued low Ca level will finally develop osteoporosis [3].

Meanwhile, hypo-calcemia quicken the danger of developing insulin resistance (IR) contributing to its powerless ability to enhance sensitivity of the insulin by activating the associated pathways, so Ca plays an essential role in decreasing the risk of developing type II diabetes by promoting the pancreatic B cell performance and increasing sensitivity of the insulin [4].

Previous investigation illustrated that Ca also plays an important part in bone tissue formation, and that a diet deficient in this element adversely affects the growth and development of the jaw bone. Therefore, it is natural to suspect that consuming a Ca-deficient diet will lead to dysfunction in the oral health [5].

However, extensive studies on the role of Ca on bones have been reported, relatively little is known about the Ca deficient diet on salivary gland function [6].

The proteins in acinar cells undergo three processes, synthesis, packaging, and storage and release. Each process is regulated by phosphorylation, a protein tyrosine kinase (PTK) and cyclic-adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) [7].

Therefore, cAMP stimulates transcription of genes encoding salivary proteins. cAMP also stimulates post-translational modification such as glycosylation. Finally, cAMP stimulates exocytosis [8].

Thus, an increase in the level of cAMP within a cell stimulates every step involved in protein secretion. In addition to PKA, acinar cells contain numerous other PK including PKC, that are involved in different aspects of protein synthesis and release [7,8].

In general, the salivary glands provide fluid and protein secretion in reaction to specialized intracellular signals generated by neurotransmitters in acinar cells, which act as a primary site for both types of secretion. Stimulation of β-adrenergic receptors leads to cAMP generation, which activates

exocytosis and protein secretion. In contrast, Ca2+ signals are triggered in reaction to plasma membrane receptors stimulation in addition to the hydrolysis of phosphatidylinositol 4,5-bisphosphate and activation of phospholipase C, such as α1-adrenergic as well as muscarinic cholinergic receptors, and result in activating the secretion of the fluid. The elevation in cytosolic [Ca2+] ([Ca2+] i) is the triggering key for stimulating secretion of the fluid, that regulates ion channel activities which in turn creates the proper osmotic gradient needed for the fluid secretion to drive across the apical membrane [9,10]. Normally, the elevation in [Ca2+]i in acini of salivary glands is induced by inositol 1,4,5, trisphosphate (IP3)-mediated release of calcium ions from the endoplasmic reticulum and calcium ions stores by inositol trisphosphate receptor (IP3R), which is a well-known intra-cellular calcium ions release channel. The major sub-types of IP3Rs present in salivary acini: IP3R2 and -3, they are concentrated at the apex of salivary cells [11].

Recently, studies reported that rats having a low Ca food intake has a lower level of cAMP in their parotids rather than those having a normal food intake [6].

On the other side, hypocalcemia can increase lipid formation as well as decreasing lipd breakdown as Ca in food intake is essential for decreasing the synthesis of adipocyte fatty acid and inhibiting pre-adipocyte differentiation [12].

Salivary secretion (a fluid watery in nature and comprises mucus, enzymes and electrolytes) synthesis is considered the principle role of salivary glands. Furthermore, saliva has a protective function in regard to the gums and oral mucosa as well as their digestive function [13].

Impressively, the salivary glands subjected to low Ca intake are accompanied with some metabolic disorders as the development of IR resulting in type II diabetes emergence [13].

Common diabetes oral symptoms encompass not only destruction of teeth and tongue and oral mucosal lesions but also xerostomia, gingivitis and periodontitis [13].

Insulin resistance can be accompanied with atrophic and destructive alterations in salivary glands, demonstrated by the reduction in the weight and size of the glands reduction together with alteration in its histological architecture [13] .

The salivary glands are responsible for saliva secretion which plays a crucial role in maintaining the oral health such as protecting teeth from caries by dilution of sugars, providing a buffering capacity, a balance between demineralization and re-mineralization in addition to its antimicrobial effect. Thus, any alteration in the salivary gland structure will affect the salivary secretion that may lead to caries development and accumulation of bacterial plaque as well as development of periodontal diseases disrupting the oral health [13].

Therefore, the current experiment aimed at investigation the dangerous role of a low Ca diet on the histological architecture structure of rat submandibular salivary glands and the oral health.

MATERIAL AND METHODS

Experimental animals

The experiment was conducted in accordance with the research ethical guidelines of experimental animals at the Faculty of Dentistry, Alexandria University (IRB No. 00010556-IORG 0008839). Thirty male albino adult rats weighing from two-hundred to two-hundred and fifty grams and ages from three to seven months were included in this experiment. Rats stayed at neat cages which had a clear ventilation and persistent managed atmospheric conditions at Medical Research Institute, University of Alexandria [14]. Animals involved in an earlier experimental study or suffering from diseases or injuries were excluded.

Rats were haphazardly bisected by computer-assisted software into two groups equally: group I (control) and group II (LCD). Allocation was completed haphazardly by a random computer-generated sequenced numbers to allocate the status of treatment to minimize error probability, where all people involved in this experiment were unknowing of the assigned treatment [15].

- **Group I: (Control group)**: Rats were fed normal chow and normal Ca component (1.1-1.3%) for the 8 weeks [9, 10].
- **Group II: (low calcium diet group):** Animals were fed food with low Ca component (0.1-0.3%) for the 8 weeks [11].

METHODS

1) Clinical observations

Any abnormal sign or behavioral changes was recorded.

2) Body weight

Rats weights were measured weekly for 8 weeks to determine the changes throughout the experiment.

3) Biochemical evaluation

Rat's blood samples were collected before scarification under anesthetic conditions from their tail's veins. Centrifugation with three-thousands RPM for fifteen minutes was utilized to separate the serum and then frozen at minus eighty degrees centigrade. Serum calcium concentration was calculated by an auto analyzer [16].

4) Animal euthanization: [12]

Euthanization was done after 8 weeks from the beginning of the experiment with an overdose of diethyl ether. The right submandibular salivary glands of each rat were dissected out and assembled to light microscopic exploration and the left submandibular gland was assembled to transmission electron microscopic examination [17].

5) Histological examination:

For light microscopic examination, right submandibular salivary glands were fixed in ten percent neutrally-buffered formalin then undergo washing and dehydrating with ethanol in an ascending order. After that, they undergo clearing xylene, and embedding in blocks of paraffin wax. Finally, cutting the sections at a thickness of four micrometers and staining them with Masson's trichrome and Hematoxylin and Eosin stain before exploration by the light microscope [18].

6) Immunohistochemical examination:

For immunohistochemical analysis, 5μm sections were stained for alpha SMA using avidin-biotin peroxidase, the sections undergo de-paraffinization, re-hydration, incubation for twenty minutes with three percent hydrogen peroxide for endogenous peroxidase blocking, then rinsing with PBS and the slides were incubated with α SMA (BLR082G) at 4°overnight, sections were the rinsed with PBS, incubated with secondary antibody, the immune reaction was visualized with DAB chromogen, counter-stained with haematoxylin and visualized with light microscope [19].

8) Transmission electron micrograph procedure:

Salivary glands undergo fixation in glutaraldehyde with 2.5% concentration, post-fixed in one percent osmiumtetraoxide, then followed by dehydration in an ascending series of ethanol. Next, they undergo processing in a routine manner and embedding in Epoxy resin. Then, cutting semi-thin sections with thickness1 μm with the microtome glass knife and gathering them on a glass slide. After that, staining with one percent Toluidine blue. Finally, staining the ultra-thin sections with a saturated uranyl acetate. Grids were analyzed and pictured by transmission electron microscope (Joel JEM-1200 EX II) at the unit of electron microscopy, in faculty of science Alexandria university) [20].

9) Statistical analysis

Body weight as well as serological data were calculated and were computer-analyzed with IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp). Normality was tested by the **Shapiro-Wilk test**. Quantitative results were demonstrated in the form of minimum and maximum range, standard deviation and mean. Normally distributed quantitative variables of groups I and II were compared by **Student t-test**. A five percent significance level was used to judge the acquired data.

RESULTS

Clinical observations

Any abnormal animal behavior was recorded daily throughout the experimental period, we observed that low calcium group rats in the revealed an irritable and nervous behavior expressed by striking their head into the wall of the cages during the final 3 weeks of experiment. On the other hand, control group, expressed no behavior changes appeared during the eight weeks of the experiment.

Body weight changes:

TABLE (1) Comparison between the two studied groups according to mean body weight (gm)

	Control (n = 15)	LCD (n = 15)	t	p
Mean body weight (gm)				
Min. – Max.	235.1 – 260.9	274.8 – 304.0	3.167*	0.004*
Mean ± SD.	253.0 ± 6.51	295.1 ± 7.74		

SD: Standard deviation t: Student t-test
p: p value for comparing between the two studied groups
****: Statistically significant at p ≤ 0.05***

Table (1) illustrates the mean body weight among the LCD and control groups throughout the experimental period. The mean body weight of low Ca group was increased significantly 295.1 ± 7.74 compared to controls 253.0 ± 6.51 (p = 0.004*).

Biochemical analysis:

Calcium serum level

Table (2) Shows comparison among the LCD and control groups throughout the experimental period, were their values were = 9.07 ± 0.44 and 7.04 ± 0.39 respectively. Regarding the calcium serum level in mg/dl. The present study demonstrated that the serum calcium level significantly decreased in the LCD group compared to controls (p <0.001*).

TABLE (2) Comparison between the two studied groups according to serum calcium level

	Control (n = 15)	LCD (n = 15)	t	p
Serum calcium level (mg/dl)				
Min. – Max.	8.5 – 9.8	6.5 – 7.9	13.448*	<0.001*
Mean ± SD.	9.07 ± 0.44	7.04 ± 0.39		

SD: Standard deviation t: Student t-test
p: p value for comparing between the two studied groups
****: Statistically significant at p ≤ 0.05***

Light microscope examination results

H & E stain:

Control (Group I)

The H & E stain revealed that the glands showed normal histological architecture containing serous acini, different types of ducts and connective tissue stroma. The serous acini appeared rounded with narrow lumen, consisting of pyramidal shaped cells having secretory granules at the apical cytoplasm. Their nucleus appeared huge, spherical, and located at the center or the basal parts of the cells. The duct system seen in between the acini comprised the intralobular ducts (intercalated (ID), secretory striated ducts (SD) as well as granular convoluted tubules (GCT) and the excretory duct (ED). The intercalated duct is small with narrow lumen lined by low cuboidal cells with nuclei situated in the center of the cells. Moreover, the SD appeared larger, rounded in shape with wide lumen and

surrounded by columnar cells arranged in one layer with basal striations, Normal appearance of the blood capillaries near to the secretory striated duct. The GCT revealed normal appearance with simple columnarar epithelial linning with acidophilic granules (Fig. 1a,b).

While the ED are present interlobular, with a larger size and wider lumen surrounded by pseudo-stratified columnar epithelial lining. (Fig. 1c).

Low calcium diet group (Group II)

In this group, the gland appeared with altered architectural structure with the ill-distinct contour of acinar cells. The serous acini showed severe structural disorganization as well as damage to their normal architecture and cell boundaries. Acinar cells had darkly stained nuclei some of it appeared pyknotic. The cytoplasm exhibited numerous intra-cellular vacuoles. Infiltration of some lipid droplets in between the acini together with congested blood capillaries and dilated irregular ducts (Fig. 1d).

The intralobular ducts and the excretory duct showed huge widening of the lumen surrounded with atrophic epithelial cells.in addition, there was lack of the SD striations and most of blood capillaries near the striated looks dilated engorged with red blood corpuscles. Moreover, the granular convoluted tubules appeared collapsed with degenerated epithelial lining (Fig. 1e). The ED were contoured by connective tissue fibrosis and showed dramatic intracellular vacuolation and degenerative changes of the lining epithelium as well as expansion in the luminal size (Fig. 1f).

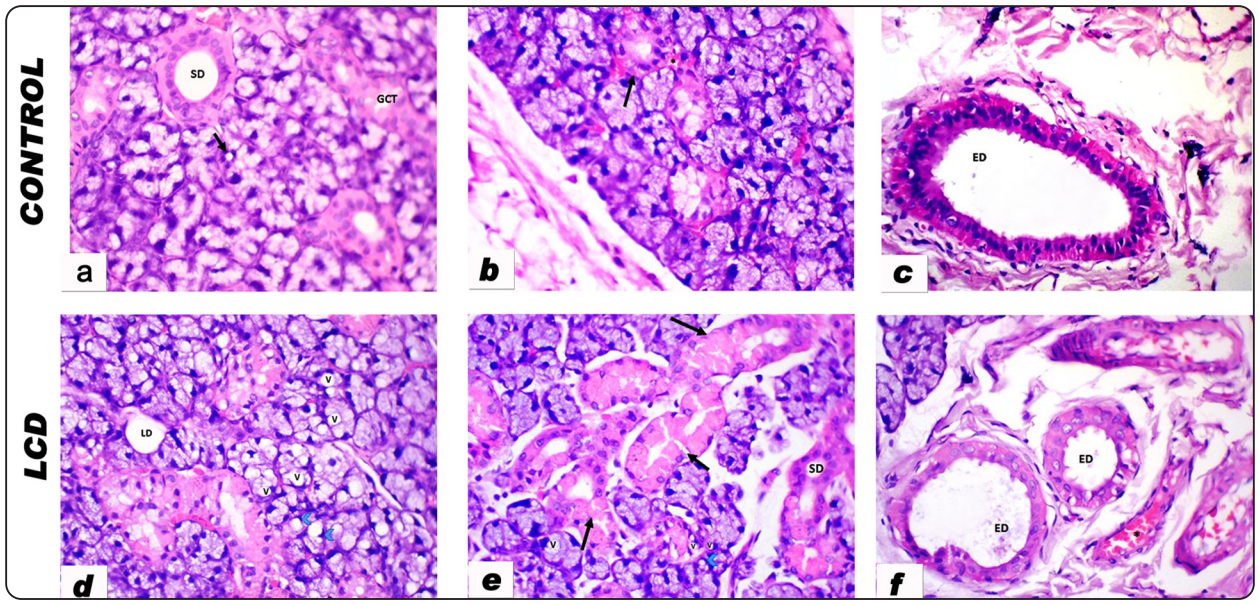

Fig. (1) Light micrograph (L.M) H & E stain: **Control group I (a-c)** showing normal histological architecture of the submandibular salivary gland consisting of rounded serous acini, the acinar cells are pyramidal in shape surrounding a narrow lumen with spherical nuclei at the basal part of the cell. Normal intercalated duct with narrow lumen lined by cuboidal cells (arrow) together with, secretory straited duct (SD) lined by columnar epithelial cells and normal granular convoluted tubules (GCT) lined with simple columnar epithelium with acidophilic granules. Normal rounded serous acini with well developed secretory striated duct lined by columnar epithelium with well-defined basal strations (arrow) at the base of the cell. Note the normal blood capillary (*) in close proximity to the striated duct. Typical excretory duct located in between the lobules, appeared large with wide lumen and surrounded by pseudostratified columnar epithelium and surrounded by normal connective tissue fibers and cells. **Low calcium diet group II (d-f)** showing abnormal presence of a lipid droplet (LD) in between the acini, together with atrophy in the acini with shrunken pyknotic nuclei (arrow heads) with numerous vacuolation (v) in the cytoplasm and loss of the normal architecture of the striated duct (SD) with loss of its striation. Collapse of the granular convoluted tubules (arrows) with loss of its granular appearance. Excretory duct (ED) with abnormal widening of the lumen associated with stagnated salivary secretion. Note degeneration of the epithelial lining and connective tissue fibrosis surrounding the duct as well as the dilated blood capillary (*) engorged with RBCs. (All images are stained with H&E stain; ×400).

Trichrome stain:

Control (Group I)

The trichrome stain showed sparse collagen fibers separating the lobules and surrounding the inter-lobular ducts as well as blood capillaries (Fig.2a, b).

Low calcium diet group (Group II)

Enormous amount of collagen fibers contouring the inter-lobular ducts and blood capillaries were illustrated by the trichrome stain (Fig. 2c, d).

α-SMA immunoreaction:

Control (Group I)

In addition, the cytoplasm of the myoepithelial cells at the periphery of the acinar cells demonstrated a negligible α-SMA immunoreaction in this group (Fig. 3a).

Low calcium diet group (Group II)

On the contrary, an outstanding α-SMA immunoreaction was seen at the acinar periphery and surrounding the interlobular ducts and blood capillaries in the LCD group (Fig. 3b, c).

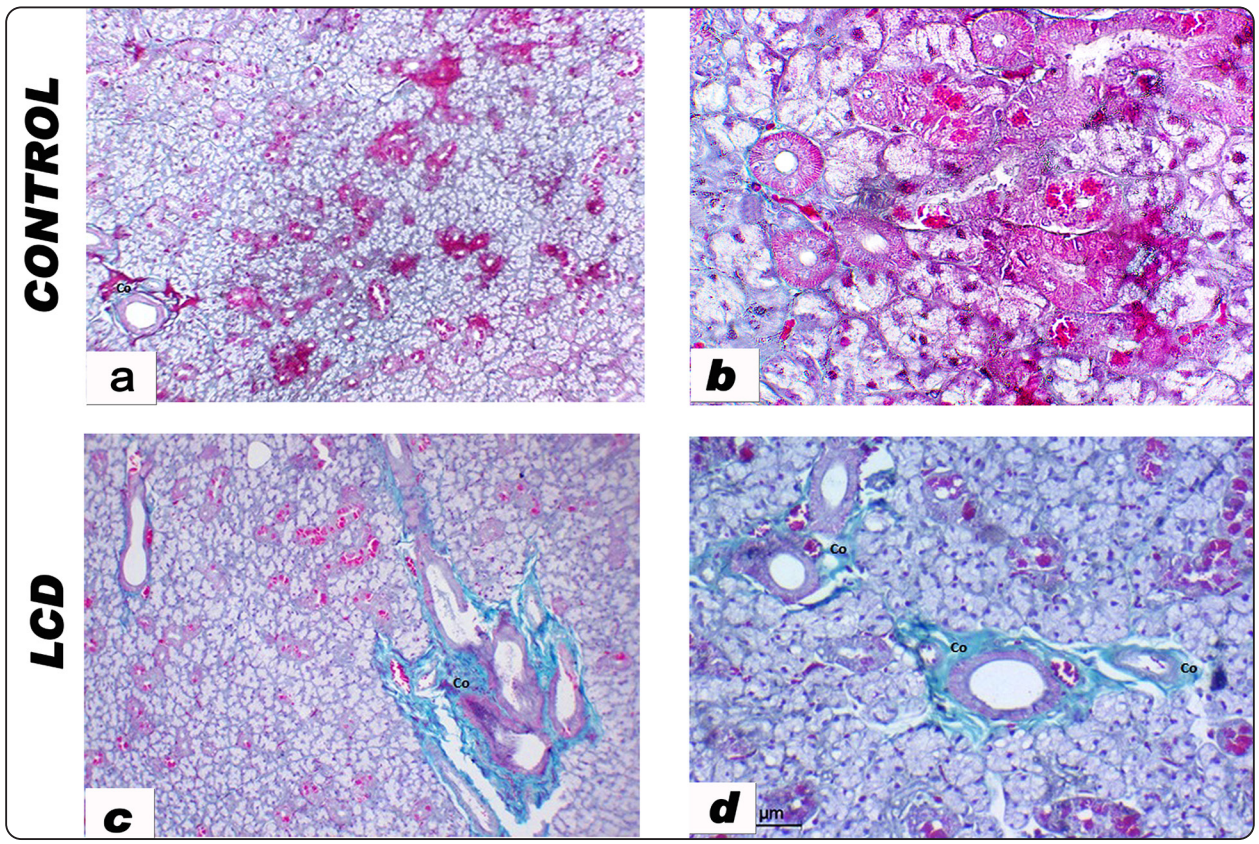

Fig. 2: Light micrograph (L.M) Trichrome stain: **Control group I (a,b)** showing normal sparse amount of collagen fibers (Co) between the lobules and surrounding the interlobular ducts and blood capillaries. **Low calcium diet group II (c,d)** revealing enormous amount of collagen fibers (Co) contouring interlobular ducts and blood capillaries. (All images are stained with Masson's trichrome stain; ×400).

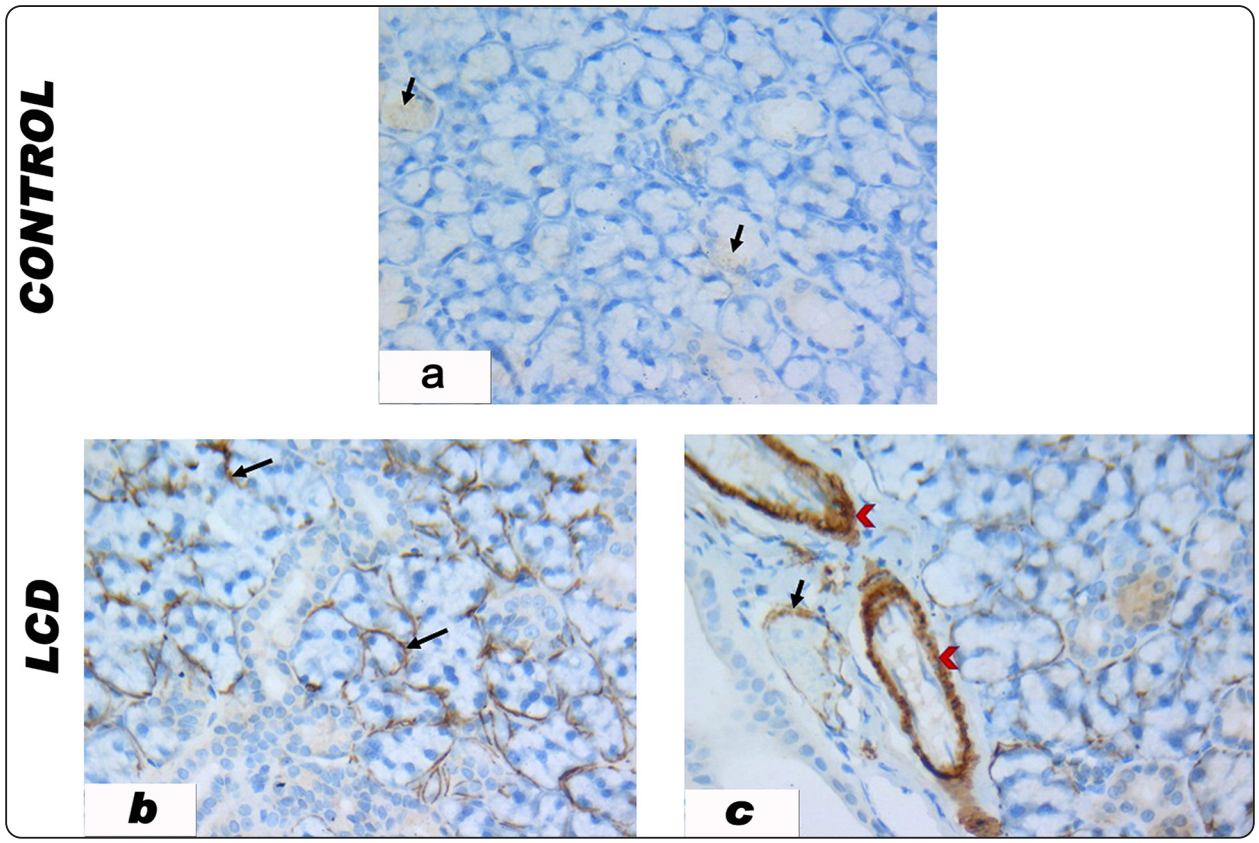

Fig. (3) Light micrograph (L.M) α-SMA immunoreaction: **Control group I (a)** demonstrated a negligible immunoreaction to α-SMA in the cytoplasm of the MECs at the periphery of the acini (arrows). **Low calcium diet group II (b,c)** illustrated an outstanding α-SMA immunoreaction at the acinar periphery (arrows) and interlobular ducts (arrow heads) as well as the blood capillaries (arrow). (All sections are at ×400 magnification).

5. Electron microscope examination results:

Control (Group I)

Electron microscopic examination of the serous acini of the submandibular gland illustrated, normal euchromatic nuclei at the basal part of the cells. The acinar cytoplasm showed a striking amount of electron lucent and electron dense secretory granules with different size together with multiple rough endoplasmic reticulum (rER) arrays. Moreover, well-defined mitochondria distributed between the organelles. The mitochondria appear rounded or oval in shape with different sizes and showed normal cristea. (Fig 4a).

The ID revealed a simple cuboidal epithelial lining with centrally located euchromatic nuclei and well-structured organelles contouring a small lumen (Fig. 4b).

The secretory striated ducts exhibited a typical normal appearance including columnar cell lining with centrally located euchromatic nuclei and normal basal infoldings adapting large number of mitochondria with well-developed cristae. A normal blood capillary is associated with the striated ducts. (Fig. 4.c).

Low calcium diet group (Group II)

The cells of the acini in this group illustrated a dark heterochromatic nucleus. Massive disorganization of the cytoplasm with swelling dilation of the rER, disintegrated mitochondria with degenerated cristae, some lipid droplets, multiple vacuoles (Fig 4d).

The intercalated ducts showed partial degeneration of the epithelial lining. The cells comprised mal-aligned and disintegrated organelles. The nuclei showed a heterochromatic electron dense and pyknotic appearance. Moreover, some electron dense secretory granules were seen in the cytoplasm (Fig. 4.e).

The secretory striated ducts showed dilatation of its lumen with severe alternations in the epithelial lining with shrunken nuclei, decrease in the basal infoldings associated with degenerated mitochondria, some dissolution and vacuolization in the cytoplasm. A dilated blood capillary fully occupied with red blood corpuscles is present in near to the SDs (Fig. 4f).

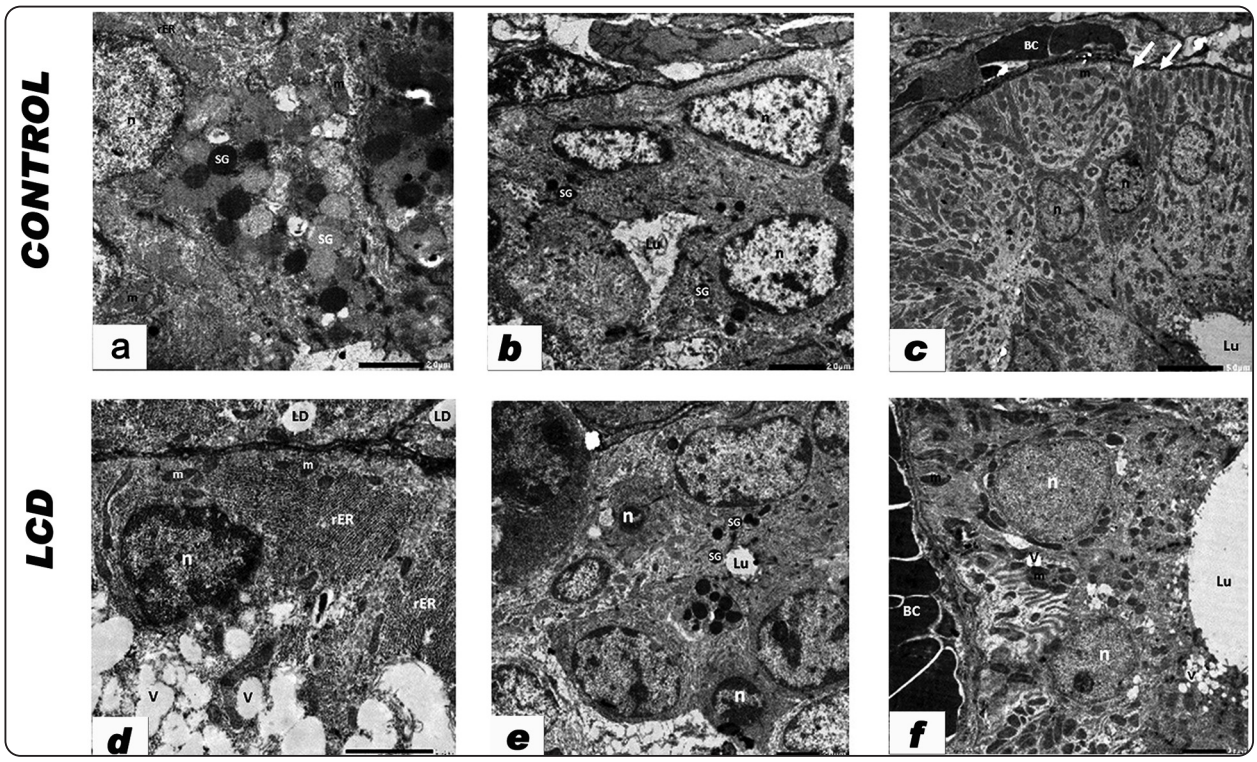

Fig. (4): A Transmission electron micrograph of rat's submandibular salivary gland. **Control group I a,** revealing a part of a normal serous acinus with normal euchromatic nuclei (n) at the base of the cells. The cytoplasm of acinar cells contained numerous electron-dense and electron lucent secretory granules (SG) of variable sizes and parallel arrays of rough endoplasmic reticulum (rER). Moreover, well-defined mitochondria distributed between the organelles. The mitochondria (m) appear rounded or oval in shape with different sizes and showed normal cristea (x 3000). **b,** the intercalated duct lined by cuboidal cells with centrally located euchromatic nuclei (n), well-structured organelles, scarce electron dense secretory granules (SG) at the apical part of the cell surrounding a narrow lumen (Lu) (x 2500). **c**, the secretory striated ducts exhibited a typical normal appearance including columnar cell lining with centrally located euchromatic nuclei (n) and normal basal infoldings (arrows) adapting large number of mitochondria (m) with well-developed cristae surrounding a normal lumen (Lu). A normal blood capillary(BC) is associated with the striated ducts (x 1200). **Low calcium diet group II d,** showing that acinar cells had dark electron dense heterochromatic nuclei (n). The cytoplasm contained extensive disorganization and dilation of the rough endoplasmic reticulum (rER), degenerated mitochondria (m) with destructed cristae, some lipid droplets (LD) and multiple vacuoles (v) (X 4000). **e,** the intercalated ducts showed partial degeneration of the epithelial lining. The epithelial lining comprised disorganized and degenerated organelles, electron dense heterochromatic and pyknotic nuclei (n) and some electron dense secretory granules (SG) in the cytoplasm surrounding a narrow lumen (Lu) (X 2000). **f**, the secretory striated ducts showed dilatation of its lumen (Lu) with severe alternations in the epithelial lining with shrunken nuclei (n), decrease in the basal infoldings associated with degenerated mitochondria (m), some dissolution and vacuolization (v) in the cytoplasm. A dilated blood capillary (BC) engorged with RBCs is present in close proximity to the striated ducts (X 2000).

DISCUSSION

Ca and P are vital minerals required for maintenance of bone tissue. Ca deficiency can lead to the development of osteoporosis and other metabolic bone diseases [21]. Ca deficiency is also the cause of numerous bone diseases such as rickets and osteomalacia. Studies on Ca deficiency have clarified the pathology of various diseases [22,23]. In research on salivary glands, several previous reports [23, 24] have shown the effects of a diet deficient in vitamin A or D on salivary gland function. Moreover, Glijer, B et al [25] showed that rats deprived of vitamin D have reduced salivary gland secretion. However, it was not clear whether this reduction in salivary gland function was due to secondary hypocalcaemia. Therefore, the current experiment study was conducted to investigate the role of low Ca diet on submandibular salivary gland structure.

Hypo-calcemia or in other words low Ca level in blood. Therefore, the body will try to compensate for this loss and maintain blood Ca level by extracting Ca from other sources such as teeth and bone leading to tooth decay, osteopenia and osteoporotic changes in bone. Furthermore, it leads to the development IR leading to the risk of developing type II diabetes[26]. Moreover, Ca deficiency provokes lipid formation and declines the breakdown of fats resulting in obesity [27].

Meanwhile, low calcium intake may have hazardous effect on general health as fatigue, muscle cramps, numbness in the hands as well as convulsions, irritability and hair loss. Also, it may minimize the salivary secretions resulting alterations in the oral health[28]. Interestingly, saliva has a protective role guarding the gingiva and the oral tissues, maintaining good oral health that can be disrupted by Ca deficiency [29].

The rats were chosen in the present work because of their close similarity to humans anatomically, physiologically, and genetically. Moreover, their availability, low cost, and having relatively longer life span than other animals [30]. This was in agreement with the food and drug administration (FDA) guidelines that recommended the use of rats in experimental studies and pre-clinical investigation of new materials that has a preventive or therapeutic role in Ca deficiency developed obesity [31].

Male rats were chosen to deny the role of hormones that may alter our findings [31]. Other studies suggested that 8 weeks duration was capable of inducing LCD obesity in rats and to undergo oxidative stresses imbalance owing to altering the structure of salivary glands [26].

The present experiment illustrated that mean body weight of the rats in the LCD group increased significantly in comparison to the controls. This was online with Schrager S et al [32], who concluded that LCD consumption leads to elevation in PTH and 1,25-hydroxy vitamin D levels, which in turn induces an increase in intracellular Ca levels in fat cells owing to promoting lipid formation and prohibiting lipid breakdown [32] .

The present work, observed that rats in LCD group showed a decrease in the physical activity in the final 3 weeks of the experiment this can be explained by the lack of Ca that leads to loss of the normal muscular tone leading to fatigue, muscle cramps and weakness [33].

Moreover, rats in the LCD group expressed an abnormal irritability and nervous behavior during the final 3 weeks of the study. This may be attributed to the Ca deficiency where the Ca ions play a vital role in controlling the release of the neurotransmitters and intra-cellular signal transduction in the nervous system [34].

The serum Ca level was showed a statistically significant decrease in the LCD rats in comparison with the controls. Normally enough calcium is obtained from healthy food intake and consumed by the upper intestinal tract to the circulatory system. Therefore any alteration in the Ca amount in food affects its level in blood [35] .

The present H & E stain examination of LCD group illustrated acinar degenerative changes including eminent intracellular vacuolization in the acinar cytoplasm together with thinning of the ED epithelial lining, the presence of adipocytes leads to the development of a chronic inflammatory stage which in turn induces the neutrophils and monocytes to produce oxygen free radicals using NAPDH oxidase enzyme activity that is active in phagocytosis process. These free radicals are responsible for the degenerative changes seen in the submandibular salivary gland [36].

The Masson's Trichrome stain examination illustrated an enormous amount of collagen fibers contouring the inter-lobular ducts and blood capillaries, this could be contributed to chronic inflammatory state that leads to continuous accumulation of the collagen fibers leading to fibrosis together with dissolution and degeneration of the tissues. However, the connective tissue fibrosis and the inflammatory state are considered part of the defense mechanism of the tissues as well as the repair and regenerative response [37].

Furthermore, the current light microscopic examination demonstrated a huge widening of the lumen of the inter-lobular and intra-lobular ducts, and congestion of associated blood capillaries. These findings may lead to alteration in the salivary glands function. These results were online with Garant PR [38] who illustrated that ductal dilatation might be explained by the saliva accumulation and failing of exocytosis. Meanwhile the presence of congested blood capillaries may be contributed to the passive hyperemia hindering the blood from getting out of the organs normally, so it accumulates in the blood capillaries [38] .

In the current work, α-SMA immunohistochemical marker was used as it is specialized marker exhibited by the cells of the smooth muscle in the early differentiating stage so it was used to demonstrate the myoepithelial cells at the periphery of the acini. It was observed that an outstanding α-SMA immunoreaction was seen at the acinar periphery and surrounding the interlobular ducts and blood capillaries in the LCD group. This results were in agreement with Cotroneo E etal [39] who found that staining with actin demonstrated the acinar shrinkage and atrophy and the myoepithelial cells showed changes in its morphology and its proliferation during acinar atrophy and regeneration with a following increase in their count and size [39]. During stressful conditions, the gland needs the increase the secretion potential of the remaining cells to restore the gland function [40].

The electron microscopic examination of the current study illustrated that the cells of the acini in this group had a dark heterochromatic nucleus in the LCD rats. This increased heterochromatin amount indicating a decrease in the metabolic activity of the cell. On the contrary, the increased proportion of euchromatin with a prominent nucleolus indicates the activity in synthesizing RNA and indicates an increase in the cell metabolic activity [41].

Moreover, the present work demonstrated massive disorganization of the cytoplasm with swelling and dilation of the rER in LCD rats. This finding were explained by Takahashi S etal who discussed that the dilataion and swelling of the rER is accompanied with cell apoptosis. rER degranulation might indicate an alteration in the cell-specific proteins formation [42].

In the present experiment, disintegrated mitochondria with different degrees and with degenerated cristae, was observed in LCD group. Degeneration and loss of the cristae in the mitochondria might be due to alterations in the structure of the internal membrane followed by disruption in pump channel leading to decreasing Ca storage. Any mitochondrial degeneration will alter the respiratory process of the cell and ATP production [43].

Moreover, ultrastructural findings showed, some lipid droplets inside the acinar cells. This could be explained by the type of food intake which is the

most responsible cause for lipid accumulation. LCD is capable to enhance lipogenesis and the extra lipids are stored for a short time in the cytoplasm of the acinar cells in the droplet form [44].

Likewise, in the current work, the extra lipid formation was because of the LCD and was believed to be picked up by the acinar cells. These observations were in agreement with different researches as using LCD for a prolonged time leads to lipid aggregation in various body organs such as pancreas, kidney and liver [45,46]. Lipid aggregation varies among human beings and rat models. In rat model, lipid droplets are aggregated and stored in the acinar cells. While in human beings they are seen in the connective tissue [47].

In addition, numerous intra-acinar vacuoles were observed in LCD rats. The presence of this vacuolization in the cytoplasm is a sign of degeneration that is occurring the acinar cells and it is mainly a fat degradation [44].

Also, the intercalated and secretory striated ducts exhibited a dilated lumen. The SD showed severe alternations in the epithelial lining with shrunken nuclei and a decrease in the basal infoldings associated with degenerated mitochondria, some dissolution and vacuolization in the cytoplasm. Ductal dilatation might be accredited to saliva accumulation and failing of exocytosis caused by gland malfunction., as lipid aggregation enhances gland malfunction [48].

CONCLUSION

The current study concluded that low calcium diet has a powerful role in altering the histological structure and function of the submandibular salivary glands which affects the oral health and calcium level is important for maintaining oral health stability.

Competing interests

There is no conflict of interests.

Funding

No funding is reported in this research.

Ethics approval

The ethical guidelines for experimental animals was followed in this research by the Faculty of Dentistry, University of Alexandria (IRB No. 00010556-IORG 0008839).

Informed consent

Not applied.

Availability of data and materials

The study data are ready with the corresponding author when requested.

Authors' contributions

SH and DA equally contributed to the methodology, conceptualization, investigation, validation, data curation, resources, writing-original draft preparation, visualization, and writing-reviewing and editing of the paper.

Recommendations

From the findings of the current experiment, the following is recommended:

1. Further researches are needed to explore the efficacy of calcium deficient diet on other body organs especially dental and paradental tissue
2. Further investigations should be performed to investigate the role of LCD with different durations on the salivary glands structure and oral health
3. Manipulate different percentages of calcium in diet on other dental and paradental tissues.

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