EGYPTIAN DENTAL JOURNAL
www.eda-egypt.org
VOL. 71, 1305:1321, APRIL, 2025
PRINT ISSN 0070-9484 • ONLINE ISSN 2090-2360
ORAL MEDICINE, X-RAY, ORAL BIOLOGY AND ORAL PATHOLOGY
Submit Date : 09-12-2024 • Accept Date : 06-01-2025 • Available online: 20-04-2025 • DOI : 10.21608/edj.2025.343212.3302

THERAPEUTIC EFFECT OF ANTHOCYANINS ON HIGH-FAT DIET-INDUCED OXIDATIVE STRESS IN MALE RATS SUBMANDIBULAR SALIVARY GLANDS (HISTOLOGICAL, IMMUNOHISTOCHEMICHAL AND ULTRASTRUCTURAL STUDY)

Dina A. Nagui *, Mai M. Saleh** and Sara A. Hamza*

ABSTRACT

Introduction: Obesity is an epidemic health problem that has many adverse effects on submandibular glands. Anthocyanins are potent antioxidants; therefore, they might ameliorate the obesity-induced oxidative stress.

Aim: This study aimed at evaluating the therapeutic effect of anthocyanins on the high-fat diet induced oxidative stress on submandibular salivary glands in rats.

Materials and Methods: Thirty adult albino rats were distributed into three groups. Control group (I): received normal diet, High-fat diet group (II) and High-fat diet + Anthocyanin group (III): received high-fat diet for eight weeks. Then, group (III) received an intra-peritoneal injection of Anthocyanins (100 mg/kg) for 7 days. After euthanasia, blood samples were collected for serological analysis. The glands were dissected and prepared for histological immunohistochemical and ultra-structural examination.

Results: Group II showed significant increase in insulin resistance, blood glucose, cholesterol, triglycerides, LDL and insulin serum level and significant decrease in HDL and calcium serum level compared to group III. Histologically, group II showed alteration in acinar structure, cytoplasmic vacuoles, and lipid infiltration. Significant α-SMA and AnnexinV immunoreaction was seen at acinar borders and interlobular ducts compared to group III ($p < 0.001$*) which revealed moderate restoration of normal gland architecture, reduction in lipid infiltration and apoptotic immunofluorescence.

Conclusions: High-fat diet resulted in alterations in the glandular architecture with marked lipid infiltration. It showed the highest αSMA immunopositivity and Annexin V immunofluorescence denoting severe apoptosis. However, anthocyanins had a counteracting lipolytic effect, as it moderately restored the normal glandular architecture, expressed the lowest αSMA immunoreactivity, and apoptosis immunofluorescence.

KEYWORDS: Obesity, High-fat diet, Submandibular Salivary Glands, Anthocyanins.

* Assistant Professor of Oral Biology, Faculty of Dentistry, Alexandria University, Egypt.
** Lecturer of Oral Pathology, Faculty of Dentistry, Alexandria University, Egypt.

INTRODUCTION

Chronic obesity, which is regarded as one of the civilization illnesses, is characterized by large and unnatural adipose tissue aggregation. The World Health Organization (WHO) reported that between 1975 and 2016, the number of obese individuals nearly tripled. It is a worrying fact that overweight, and obesity are correlated to a wide range of illnesses, including cancer, insulin resistance (IR), diabetes type II, and hypertension [1, 2]. Indeed, a critical factor leading to the emergence of neuro-degenerative disorders, such as Alzheimer's disease, is having an excessive body weight [3].

For the objective of studying the causes and effects of human obesity, the highly pleasant and energetic diet-induced obesity paradigm is a valid and useful one. Research revealed the importance of hypercaloric diets in the development of obesity in animal models as it caused changes in the composition of the body, such as increased body weight (BW), fat deposits and altered glycaemic and lipid profiles which lead to oxidative stress [4, 5].

Reactive Oxygen Species (ROS) are chemically reactive molecules that, when out of equilibrium, cause oxidative changes of proteins, fats, carbohydrates, and nucleic acids. These modifications disrupts the equilibrium of the entire biological system and can lead to obesity-related problems [6].

Saliva performs a variety of necessary functions for maintaining general and dental health. It has several important roles, including lubricating and cleaning the teeth and oral mucosa, preserving a neutral pH through buffering action, preventing tooth demineralization, acting as an antimicrobial, promoting taste and bolus formation, starting the enzymatic digestion of starch, and being necessary for mastication, swallowing, and speech articulation [7].

The oral cavity homeostasis is regulated through salivary glands. As a result of their malfunction, the content and volume of saliva given to the oral cavity alter. The effect of ROS in the development and pathogenesis of salivary gland illness associated with diabetes, IR and obesity due to oxidative changes in proteins, lipids, and DNA, has been demonstrated. It is widely established that the capability of the salivary glands to produce antioxidants is impacted by IR, obesity, and diabetes mellitus [6, 8].

Scheduled physical activity, dietary therapy, and surgical treatment, are all effective treatments of obesity. The research showed that supplementing probiotics and, more broadly, phenolic substances like flavonoids and anthocyanins enhanced the health condition of obese patients [9].

Anthocyanins, which are naturally occurring flavonoids are extensively present in the cytoplasm of plant organs such as flowers, fruits, berries, leaves, and roots [10]. Studies have supported the biological advantages of dietary anthocyanins, which have been shown to have anticancer, anti-inflammatory, neuroprotective, anti-obesity, anti-diabetic, and cardiovascular disease preventive effects [11].

According to a literature review, anthocyanins' anti-obesity properties are due to their capacity to regulate food intake and energy metabolism, as well as their modulation of the inflammatory response, IR, glucose metabolism, and other related processes [12].

Several studies have been performed to assess the impact of high fat diet (HFD) on the mitochondrial respiratory system, the activity of prooxidant enzymes, ROS production, as well as antioxidant systems and oxidative modifications of proteins and lipids [6, 13], whereas no research have been conducted too assess the histological effect of HFD and the estimated antioxidant effect of anthocyanins on either light, ultrastructural or immunohistochemical microscopic levels.

As previously mentioned, nowadays there is a great concern about the use of complementary herbal medicine, and due to the antioxidant activity and

possible lipolytic effect of anthocyanins, the current study was performed to assess the therapeutic effect of anthocyanins on high fat-diet induced oxidative stress in rats' submandibular salivary glands.

MATERIAL AND METHODS

This research received the approval of the Scientific Research Ethics Committee at the Faculty of Dentistry, Alexandria University (IRB No. 00010556-IORG 0008839). This study conforms to the ARRIVE guidelines for animal pre-clinical studies and followed the institutional guidelines and the National Research Council's Guide for the Care and Use of Laboratory Animals [14, 15]

The sample size was calculated assuming 80% study power and 5% alpha error. The mean (±SD) area of submandibular serous acini was calculated to be 2765 μm (±493.6) between untreated rats, 3950 μm (±816.4) between high-fat diet rats and 2819 μm (±553.6) between high-fat diet rats and a comparable therapy (vitamin D) [16]. According to the variation between independent means using the highest SD=816.4, a sample of 9 rats per group was required, yielding an effect size of 0.67. this was increased to 10 rats. Total sample= Number per group × Number of groups = 10*3= 30 rats. Sample size was based on Rosner's method, calculated by G*Power 3.1.9.7 [17]. Any animal that was withdrawn from the study was replaced to maintain the sample size. This animal number has been estimated according to specific calculation done in the Department of Medical Statistics Medical Research Institute, Alexandria University.

Thirty healthy male adult Wistar albino rats (age ±12 weeks, weight 200-250 g) were included in this study. Rats were housed at Institute of Medical Research, Alexandria University and housed in cages with wire mesh bottoms. Animal groups were kept in separate cages, maintained in the same controlled laboratory conditions of temperature (22–25°C), good ventilation, 12:12 h day/night cycles, and with free access to standard diet and water. Rats showing any signs of illness or wounds, genetically modified or included in previous studies were excluded. After 2 weeks of acclimatization, rats were randomly distributed equally with a computer-assisted software into three groups (10 rats each).

- Group I (control group) (n = 10): Rats fed on normal rat chow with normal amount of fat (up to 20%) for 8 weeks [18, 19].
- Group II (high-fat diet group) (n = 10): animals fed on diet containing 59.8% fat, 20.1% protein, and 20.1% carbohydrates for 8 weeks [19].
- Group III (high-fat diet group + Anthocyanins group) (n = 10): animals fed on the same diet as group II for 8 weeks [19], then they were injected intraperitoneally with anthocyanins (Nature's Way, USA), 100mg/kg body weight, dissolved in distilled water once daily for 7 days [20].

Clinical observations

Any abnormal sign or behavioural changes were recorded. Rats weight was measured every week for 9 weeks to determine the changes throughout the experiment. [20]

Biochemical analysis:

Intracardiac blood samples were collected under anaesthesia from the animals. The serum was separated by centrifugation (3000 rpm, 15 min) and frozen at -80 °C. Lipid profile including serum cholesterol, triglycerides, high-density lipid level (HDL) and low-density lipid levels (LDL) were quantified using routine laboratory procedures. [2] Fasting blood insulin level was measured using rat specific insulin ELISA kits (enzyme linked immunoassay). Serum glucose and calcium level were measured using an auto analyser.

Animal Euthanasia:

Euthanization was done after 9 weeks from the beginning of the experiment by intravenous injection of an overdose of sodium pentobarbital 100

mg/kg (Nembutal, Akorn, Illinois, USA). Rats were monitored with caution to confirm death. The submandibular salivary glands were dissected and processed for histological, immunohistochemical and transmission electron microscopic examination. [21]

Immunohistochemical Examination:[22]

α SMA (Smooth Muscle Actin) marker was used to detect the functional activity of myoepithelial cells.

The sections were mounted on positively charged glass slides, de-paraffinized, rehydrated, and treated with primary antibodies before being stained, as per the manufacturer's instructions. The amount of fibrogenesis in the various study and control groups was compared using the α SMA immunohistochemical marker. The Leica Qwin 500 software was utilized by the image analyser computer system to scrutinize the sections. A light microscope that is connected to a microcomputer for high-speed digital image processing is part of the apparatus. At a magnification of ×400, the amount of fibrosis was counted in ten fields within each group in the hot spot—the area with the highest level of fibrogenesis—and the average count was noted for every research group.

Antibody and Kit for Immunofluorescence analysis:[23]

Annexin V conjugated to fluorescein isothiocyanate (FITC) fluorochrome: This is an apoptosis detection kit (BD Pharmingen™, USA, Catalogue number 556547) Containing 3 bottles; 10x Annexin V Binding Buffer, FITC Annexin V and Propidium Iodide Staining Solution.

Alexa fluor®555 goat anti-mouse (Life Technologies, Catalogue number A21422). The kit was supplied by CERRMA to be used as secondary antibody for immunostaining applied for confocal laser scanning microscopy.

Utilizing Annexin V immunofluorescence, the amount of apoptosis in the study and control groups was compared. The universal immunostaining protocol was followed when carrying out the staining steps. Following deparaffinization, washes were carried out with a Coplins jar: Xylene for one hour and three minutes; 1:1 Xylene:100%, then in descending grades of Ethanol (100%, 95%, 70% and 50%) for three minutes each. The ethanol was rinsed off the slides by running tap water, then the samples were permeabilized by incubation in PBS containing 0.1% Triton X for 10 minutes. Following three separate five-minute washes in Tris buffer solution (TBS) (PBS + 0.1% Tween 20), the slides were blocked for one hour at room temperature using 1% BSA in TBS. After briefly draining the slides, tissue paper was used to wipe the various sections.

The 3rd step involves blocking and immunostaining, wherein 10 µg/ml of PCNA antibody was diluted in TBS with 1% BSA and incubated overnight at 4°C. Then, the solution was rinsed three times for five minutes in TBS with mild agitation. The slides were coated with a diluted (1:400) solution of fluorophore-conjugated secondary antibody (Alex Fluor 555) in TBS containing 1% BSA. (Goat anti-mouse secondary antibody Alex Fluor 555; excitation and emission wave lengths of 555 and 580 nm; Invitrogen, Cat # A-21422). Following a one-hour dark incubation at room temperature to prevent photobleaching, the slides were rinsed 3 times in 5 minutes using TBS. Lastly, cells were incubated for five minutes with 0.1 µg/ml Hoechst nuclear stain. Thermo Fisher Scientific's Hoechst 33342 fluorescent nuclear stain (Cat. # 62249; excitation and emission wavelengths of 361 and 497 nm) was utilized. It was rinsed three times in five minutes with TBS, mounted using a suitable mounting medium, and finally covered with a coverslip. Confocal laser scanning (CLS) was used for the examination; the Leica TSC SPE II/ DMI 8 units of Advanced Microscopy at Centre of Excellence for Research in Regenerative Medicine and Applications (CERRMA), Faculty of Medicine, Alexandria University.

Histological examination:

For light microscopic examination, submandibular salivary glands were embedded in paraffin wax blocks after being fixed in 10% formalin, neutrally buffered, cleaned, and dehydrated using increasing ethanol concentrations. Tissue sections were cut to a thickness of 4 μm, and stained with haematoxylin and eosin for routine examination under a light microscope. [2]

Oil red O staining for fat examination:

Oil red O staining was used for intracellular fat examination. The gland specimens were frozen and unfixed cryostat sections were cut at 10 μm. The sections were mounted on clean slides and fixed in 10% neutrally buffered formalin. Frozen sections were floated in water and attached to slides with albumin. Then, they were thoroughly dried (1-2 hours) to assure paper adherence, rinsed briefly with distilled water, a few dips in 60% isopropyl alcohol, application of Oil red O working solution for 20 minutes, a few dips in 60% isopropyl alcohol, rinsed with four changes of distilled water, counterstain with acidified Lillie-Mayer haematoxylin for 1 minute, rinsed in three changes of distilled water, immersed in blue haematoxylin in 0.3% sodium borate for 15 seconds, rinsed with four changes of distilled water and finally mounted with glycerine jelly. [24]

Transmission electron micrograph procedure:

Salivary glands were fixed in 2.5% glutaraldehyde, post fixed in 1% OsO4, then dehydrated in an ascending series of ethanol. After that, they were processed and embedded in Embed-812 resin. Semi-Thin sections (1μm) were cut and collected on a glass slide and stained with Toluidine blue 1%. Ultra-thin sections were stained with a saturated uranyl acetate. Grids were analysed and photographed in a Joel JEM-1200 EX II transmission electron microscope (Electron Microscope unit, Faculty of Science, Alexandria University) to detect cellular alterations on the ultrastructural level. [20]

Statistical analysis

Body weight as well as serological data were calculated and computer-analysed 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. One-way ANOVA test was utilized for comparing the 3 studied groups and followed by Post Hoc test (Tukey) for pairwise comparisons. Significance of the obtained results was judged at the 5% level.

RESULTS

Upon observation, all three groups showed a normal behaviour throughout the experimental period. The mean body weight among HFD, HFA and control groups throughout the experimental period was calculated (Table 1). The mean body weight of HFD group increased significantly 297.3 ± 9.5 compared to both the HFA and control groups 283.5 ± 8.5 and 251.6 ± 7.4 respectively ($p = 0.003^*$).

The following serum analysis tests were performed for the three groups at the end of the experimental. (Table 2). The calcium serum level values demonstrated that there was a statistically significant difference in the calcium level of HFD group = 6.9 ± 0.2 mg/dl, compared to control group = 8 ± 0.2 mg/dl, and HFA group = 7.7 ± 0.2 mg/dl ($p <0.001^*$). Lipid profile: HFD group exhibited the highest cholesterol= 91.5 ± 3.2, triglycerides= 91.0 ± 5.3 and LDL= 59.3 ± 6.1 levels and decreased HDL= 21.9 ± 2.6 level, followed by the HFA group cholesterol= 73 ± 7.1, triglycerides= 81.7 ± 8, LDL= 27.7 ± 7.9 and HDL= 24.9 ± 2.3, and control group= cholesterol 62.7 ± 7.9, triglycerides= 51.9 ± 7.8, LDL= 5.8 ± 1.1 and HDL= 28.2 ± 1.7.

HFD group had a significant increase in cholesterol, triglycerides and LDL levels and significant decrease HDL serum level when compared with control group ($P^1<0.001^*$). Indeed,

TABLE (1) Comparison between the three studied groups according to average Body Weight

Average Body weight (/gm)	Control (n =10)	HFD (n =10)	HFA (n =10)	F	p	Sig. bet. groups.
Min. – Max.	235.1 – 257.3	274.4 – 307.4	262.4 – 293.8	75.597*		$p_{1,2}$<0.001* p_3=0.003*
Mean ± SD.	251.6^c ± 7.4	297.3^a ± 9.5	283.5^b ± 8.5	<0.001*		

SD: Standard deviation, F: F for One way ANOVA test, Pairwise comparison bet. each 2 groups was done using Post Hoc Test (Tukey), p: p value for comparing between the three studied groups, p_1: p value for comparing between Control and HFD, p_2: p value for comparing between Control and HFD & Anthocyanin, p_3: p value for comparing between HFD and HFD & Anthocyanin. *: Statistically significant at p ≤ 0.05.

TABLE (2) Comparison between the three studied groups according to Biochemical Blood Analysis

	Control (n =10)	HFD (n =10)	HFA (n =10)	F	p	Sig. bet. groups.
Insulin / IU						
Min. – Max.	4.1 – 6.1	11.3 – 14.5	7.7 – 10.1	167.763*	<0.001*	$p_{1,2,3}$ <0.001*
Mean ± SD.	5^c ± 0.7	12.9^a ± 1.2	8.8^b ± 1			
Glucose						
Min.- Max.	131 - 165	411 - 465	240 - 311	493.387*	<0.001*	$p_{1,2,3}$ <0.001*
Mean ± SD.	149.9^c ± 12.4	444.9^a ± 20.5	266.2^b ± 27.7			
Ca						
Min.- Max.	7.7 – 8.3	6.5 – 7.1	7.5 – 8	79.619*	<0.001*	$p_{1,3}$ <0.001* p_2=0.031*
Mean ± SD.	8^a ± 0.2	6.9^c ± 0.2	7.7^b ± 0.2			
Cholesterol						
Min.- Max.	50 – 74	87 – 96	64 – 84	52.313*	<0.001*	$p_{1,3}$ <0.001* p_2=0.003*,
Mean ± SD.	62.7^c ± 7.9	91.5^a ± 3.2	73^b ± 7.1			
Triglycerides						
Min.- Max.	40 – 63	78 – 98	63 – 90	81.454*	<0.001*	$p_{1,2}$ <0.001* p_3=0.019*
Mean ± SD.	51.9^c ± 7.8	91.0^a ± 5.3	81.7^b ± 8			
HDL						
Min.- Max.	26 – 30.5	18 – 25	22 – 28	19.423*	<0.001*	p_1<0.001* p_2=0.009* p_3=0.016*
Mean ± SD.	28.2^a ± 1.7	21.9^c ± 2.6	24.9^b ± 2.3			
LDL						
Min.- Max.	4.4 – 7.4	50.9 – 68.2	18.2 – 42	216.137*	<0.001*	$p_{1,2,3}$ <0.001*
Mean ± SD.	5.8^c ± 1.1	59.3^a ± 6.1	27.7^b ± 7.9			
HOMA-IR						
Min.- Max.	1.7 – 2	12.9 – 14.7	5.5 – 6	2530.355*	<0.001*	$p_{1,2,3}$ <0.001*
Mean ± SD.	1.8^c ± 0.1	14^a ± 0.6	5.7^b ± 0.2			

SD: Standard deviation, F: F for One way ANOVA test, Pairwise comparison bet. each 2 groups was done using Post Hoc Test (Tukey), p: p value for comparing between the three studied groups, p_1: p value for comparing between Control and HFD, p_2: p value for comparing between Control and HFD & Anthocyanin, p_3: p value for comparing between HFD and HFD & Anthocyanin. *: Statistically significant at p ≤ 0.05.

there was a statistically significant difference in cholesterol ($p=0.003^*$), triglycerides ($p<0.001^*$), LDL ($p<0.001^*$) and HDL ($p=0.009^*$) serum levels between HFA and control groups. Moreover, there was a statistically significant difference in cholesterol ($p<0.003^*$), triglycerides ($p^3=0.019^*$), LDL ($p<0.003^*$) and HDL ($p^3=0.016^*$) levels between HFD and HFA groups.

The insulin serum level values revealed that HFD group exhibited the highest insulin serum level=12.9±1.2 Ng/ml, followed by HFA group= 8.8±1 Ng/ml, and then control group = 5±0.7Ng/ml. The results showed that there was a significant increase in the insulin level in HFD and HFA groups in comparison with control group ($p^1<0.001^*$), ($p^2<0.001^*$) respectively. Moreover, there was a statistically significant difference in insulin level between HFA and HFD groups ($p^3<0.001^*$). The blood glucose level values were 149.9 ± 12.4 mg/dl, 444.9 ± 20.5 mg/dl, 266.2 ± 27.7 mg/dl in control, HFD and HFA groups, respectively. It was noted that the HFD group exhibited the highest blood glucose level followed by groups HFA and control groups. These results revealed that there was a significant difference in the blood glucose level in groups HFA and HFD in comparison with the control group ($p^1<0.001^*$), ($p^2<0.001^*$) respectively. Moreover, there was a statistically significant difference in the blood glucose level between HFA and HFD groups ($p^3<0.001^*$).

Insulin resistance (HOMA-IR) values for control group= 1.8 ± 0.1, HFD= 14 ± 0.6 and HFA= 5.7 ± 0.2. The results revealed that there was a significant difference in the IR in HFA and HFD groups in comparison with control group ($p1<0.001^*$), ($p2<0.001^*$) respectively. Moreover, there was a statistically significant difference in the IR between HFA and HFD groups ($p3<0.001^*$).

Light microscopic examination: In control group, the normal histological structure of the submandibular salivary gland was seen in H&E-stained sections. The serous acini were spherical in shape with basophilic pyramidal shaped cells having rounded basally situated nuclei. Normal striated ducts (SDs) lined by columnar cells with centrally located nuclei and basal striations associated with normal blood capillaries were noted (Fig. 1a & b). HFD group revealed disturbed glandular architecture with multiple degenerative changes, in the form of congested blood capillaries engorged with RBCs. Besides, acinar cells revealed many cytoplasmic vacuoles and some darkly stained pyknotic nuclei (Fig. 1c & d), together with the presence of a lipid droplet trapped in between the acini. Considering the glandular ducts; the intercalated duct showed slight dilatation of the lumen (Fig. 1c) while the striated ducts showed severe dilatation in the lumen with degenerated epithelial lining, and darkly stained nuclei (Fig. 1d). On the other hand, HFA group revealed a moderate improvement in the serous acini and restoration of the normal architecture of the ID and SDs as well as moderate improvement in blood capillary congestion. However, few cytoplasmic vacuoles can be still seen (Fig. 1e & f).

When specimens stained with Oil Red O stain were examined, the control group showed few intracellular lipid droplets in acinar cells (Fig. 2a). HFD group showed apparent increase in the intracellular lipid in acinar cells more than control group (Fig. 2b). HFA group revealed fewer intracellular lipid droplets in acinar cells compared with HFD group (Fig. 2c).

The evaluation of αSMA immunohistochemistry in each group revealed decreased αSMA expression in HFA group (mean: 3.60±0.93) as compared to HFD group (mean: 9.38±1.03). Also, HFD group showed increased immunopositivity, while HFA group revealed the lowest immunoreactivity compared with the control group (mean: 4.08±0.72) and HFD group (Fig. 3 a, b & c).

Utilizing immunofluorescence analysis, the apoptosis rates in the treated and control groups were evaluated. Initially, the cells 'outline in the treated and control groups was shown using the

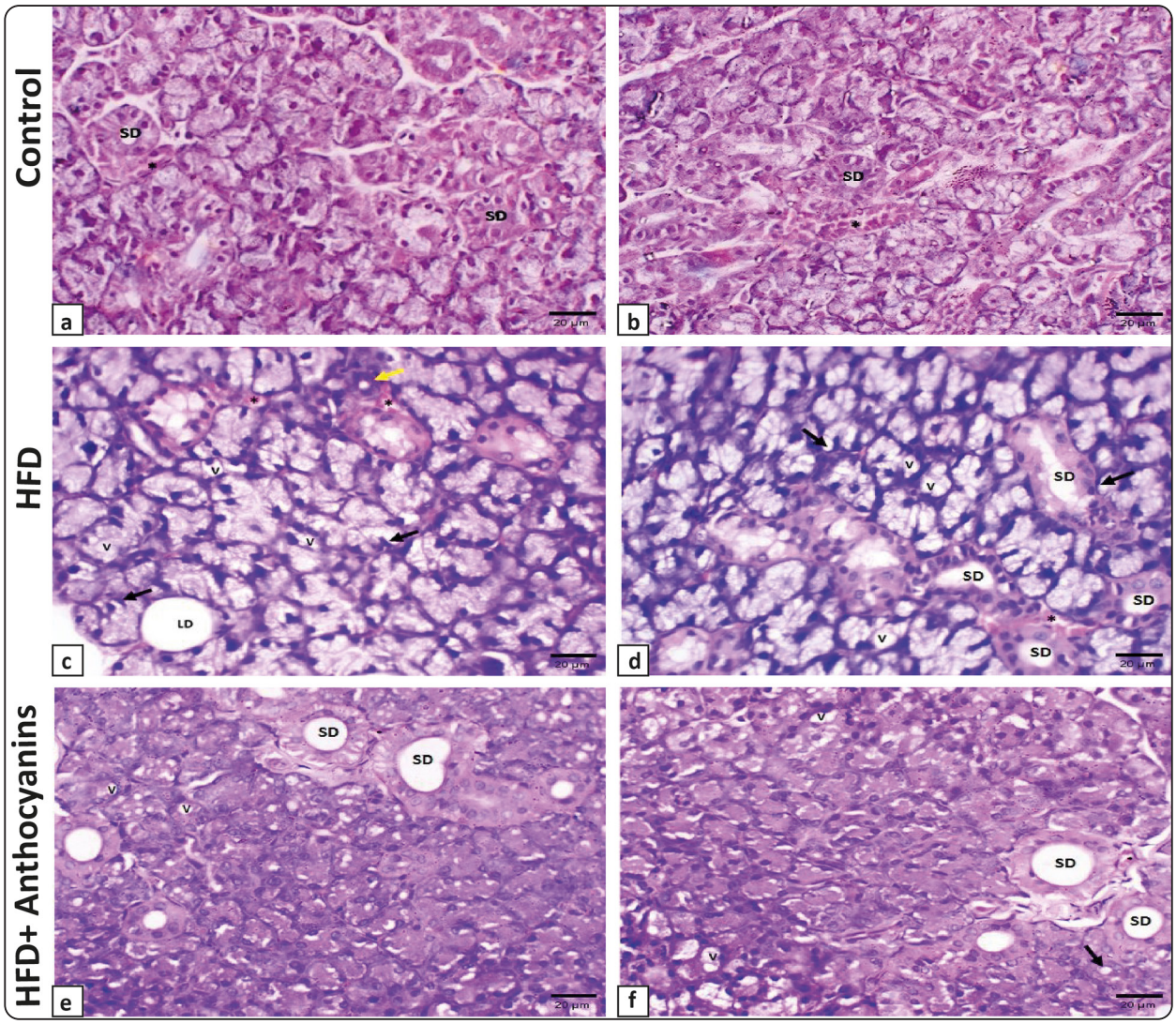

Fig. (1) Light micrograph (L.M) H & E stain: Control group I (a, b): showing normal histological architecture of the submandibular salivary gland consisting of rounded serous acini, the acinar cells are pyramidal in shape surrounding a narrow lumen with spherical nuclei at the basal part of the cell. Normal striated ducts (SD) lined by columnar cells with centrally located rounded nuclei and basal striations associated with normal blood capillaries (*). High-fat diet group II (c, d): revealing many cytoplasmic vacuoles (v) and some darkly stained pyknotic nuclei (black arrows) in the acini associated with congested blood capillaries engorged with RBCs (*). Slight dilatation in the intercalated duct (yellow arrow). The SD exhibited degeneration of the epithelial lining with darkly stained nuclei, sever dilatation of the lumen with dilated blood capillary engorged with RBCs (*) in close proximity to it. Note an abnormal lipid droplet (LD) trapped in between the acini. HFD +Anthocyanins group III (e, f): illustrating a moderate improvement in the serous acini and restoration of the normal architecture of the ID (arrow) and SD as well as moderate improvement in blood capillary congestion (*). Note few cytoplasmic v can be seen. (All sections are at ×400 magnification)

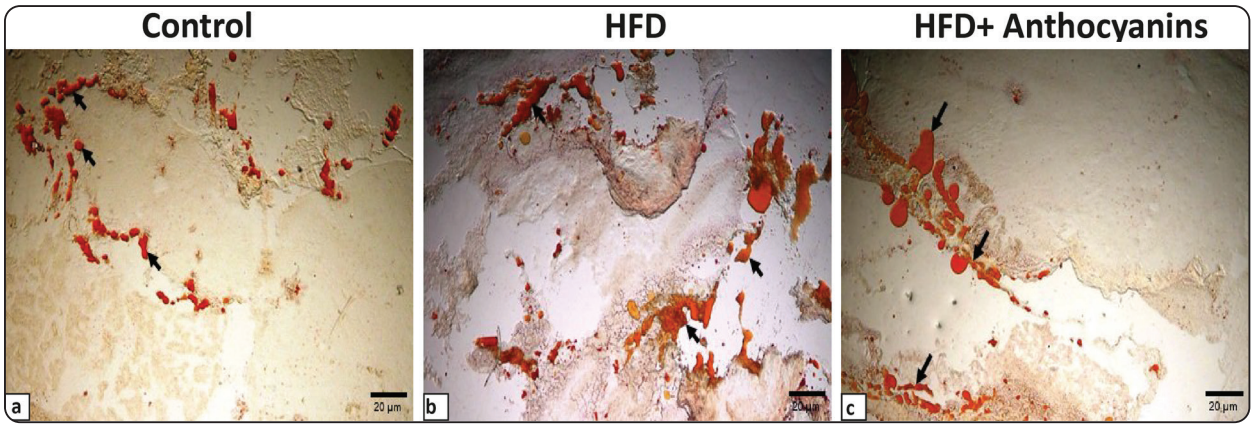

Fig. (2) Light micrograph (L.M) Oil Red O stain: Control group I (a): showing rarely intracellular lipid droplets (arrows) in acinar cells. High-fat diet group II (b): revealing an apparent increase in the intracellular lipid (arrows) in acinar cells more than controls. HFD +Anthocyanins group III (c): exhibiting few intracellular lipid droplets (arrows) in acinar cells compared with the HFD group. (All images are at ×400 magnification)

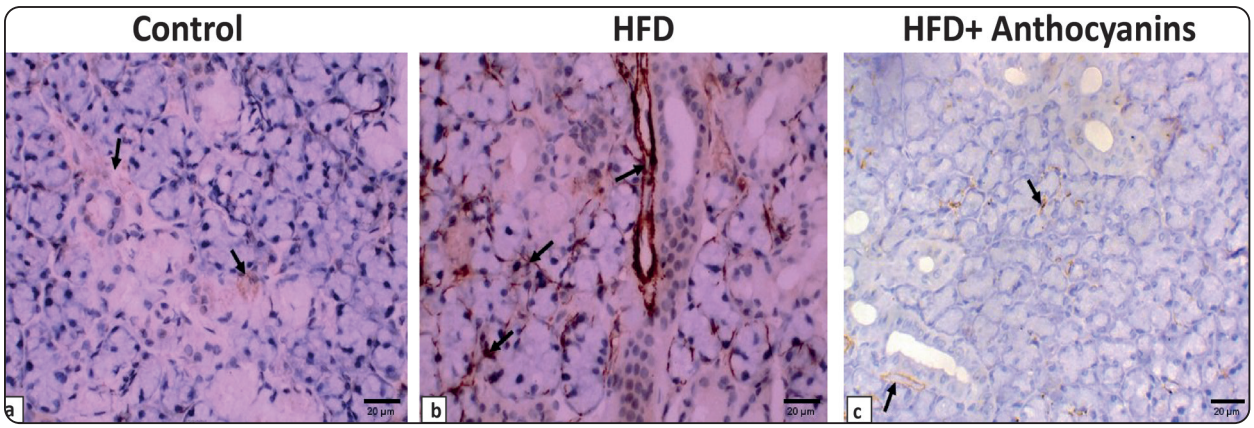

Fig. (3) Light micrograph (L.M) αSMA immune-expression: Immunohistochemical image demonstrated the immunopositivity among different study groups (a, b& c) (arrows indicating areas of immunopositivity). The HFA group (c) showed the lowest immunopositivity compared with control group and HFD group (αSMA X400)

DIC (Differential Interference Contrast) mode. Secondly, apoptosis was shown in the fluorescent images labelled for the detection of Annexin V in cells. The DIC mode of the control group, HFD group and HFA group revealed the ductal element and the acini of the salivary gland tissue (Fig. 4 a,b&c). Additionally, the fluorescent image of group I revealed the cells' DNA was blue in colour with very little green fluorescence, suggesting that the control group's apoptosis rate was low (Fig. 4d). In contrast group II showed that the cytoplasm of the acinar and ductal cells had higher production of the green fluorescence, indicating that these cells were extremely apoptotic (Fig. 4e). In the cytoplasm of the acinar and ductal cells, the fluorescent picture of group III showed reduced production of green fluorescence, suggesting a reduction in apoptosis (Fig. 4f).

TEM examination of the control group showed open-faced nuclei with prominent nucleoli and regularly delineated nuclear membrane. The rough Endoplasmic Reticulum (rER) appeared as parallel arrays of regular cisternae arranged at the basal and lateral parts of the cell. Normal mitochondria were dispersed within the cytoplasm. The membrane bound secretory granules appeared with variable

sizes and electron densities and regular intercellular spaces were seen in between the acinar cells (Fig.5a). Examination of the striated duct cells revealed euchromatic nuclei, prominent basal infoldings with numerous uniform mitochondria radially arranged between the basal striations (Fig. 5b).

Examination of HFD group by TEM demonstrated pyknotic nuclei with irregularly demarcated nuclear membrane, rER was slightly dilated and fragmented while the mitochondria were shrunken with internally degenerated cristae. Large fat droplets were obviously seen in the form of isolated islands of different sizes. Abnormal widening of the intercellular spaces was also noticed (Fig. 5c & d). The striated duct cells revealed pyknotic shrunken nuclei, disrupted blurred basal infoldings with disorganized, pleomorphic mitochondria (Fig. 5e).

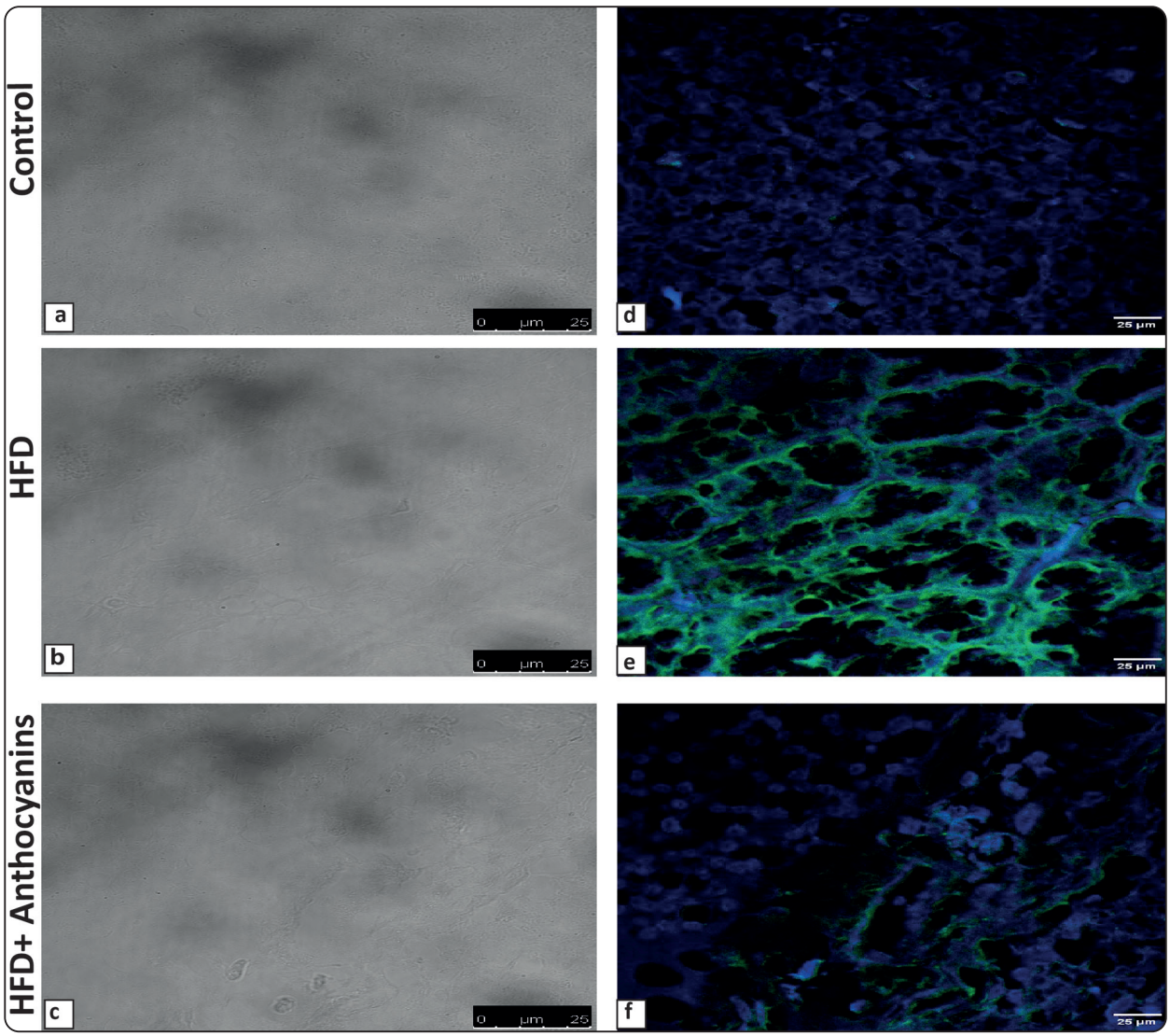

Fig. (4) Confocal Laser Scanning Photomicrograph of normal control group, HFD group and HFA group showing the ductal element and the acini of the salivary gland tissue by The Phase Contrast Mode (DIC) (a, b, c). The fluorescent image of the control group(d): showing the DNA of the cells by blue fluorescence with minimal green fluorescence indicating low apoptosis. The fluorescent image of HFD group (e): showing increased expression of the green fluorescence in the cytoplasm of the acinar and ductal cells indicating highly apoptotic cells. The fluorescent image of HFA group (f): showing decreased expression of the green fluorescence in the cytoplasm of the acinar and ductal cells indicating decreased apoptosis

However, examination of HFA group by TEM revealed euchromatic nuclei with prominent nucleoli, rER cisternae were more regularly arranged, however, they appear fragmented in some regions. Some secretory granules were variable in size and electron density while others showed coalescence in large masses (Fig. 5f & g). Examination of the striated duct cells showed open-faced nuclei with regularly demarcated nuclear membrane. The basal infoldings were evident with numerous normal mitochondria arranged parallel to each other in a palisaded appearance (Fig. 5h).

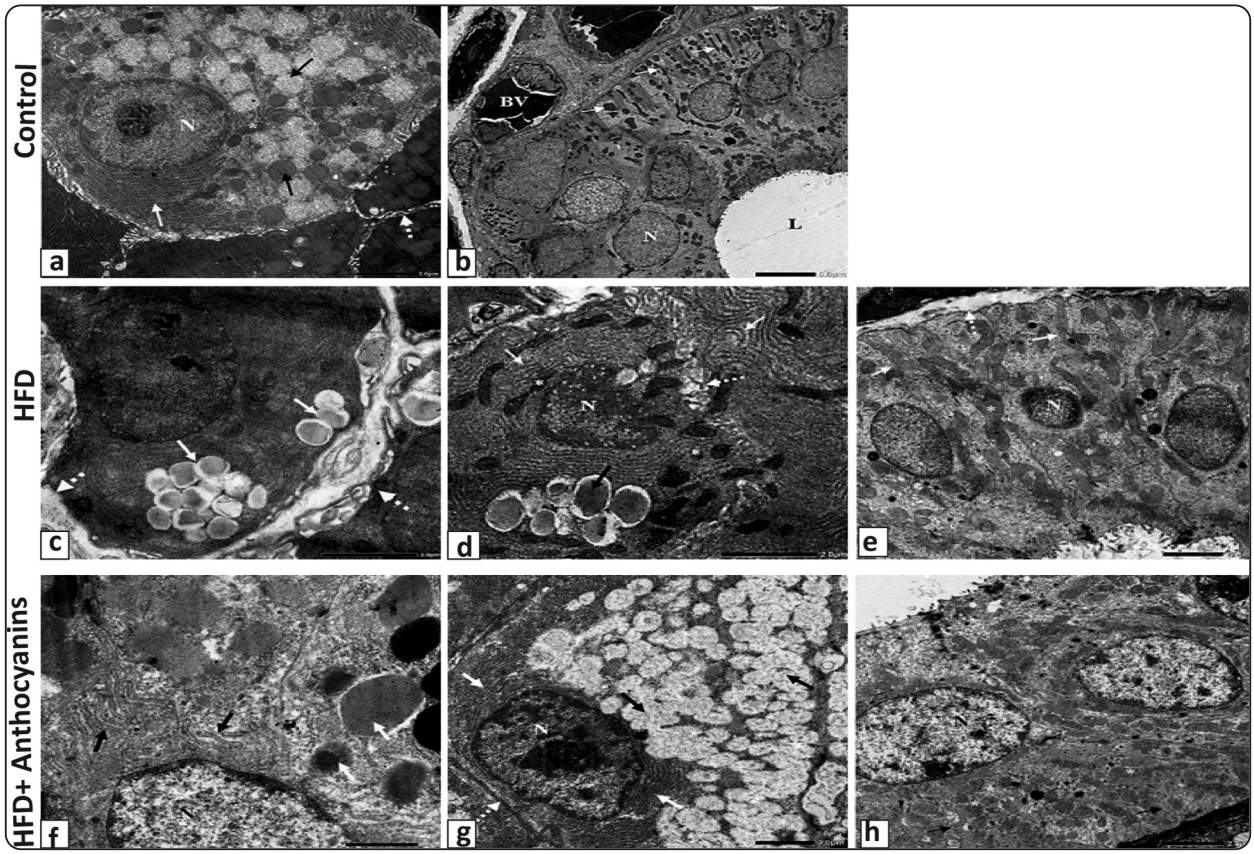

Fig. (5) A Transmission electron micrograph of rat's submandibular salivary gland. Control Group I a. revealing secretory cells with open-faced nucleus (N), normal mitochondria (asterisk) uniform parallel arrays of rER (white arrow), secretory granules of different sizes and electron densities (black arrows). Note the narrow intercellular spaces (dotted arrow) (mag.x2500). b. showing part of the secretory duct lined by columnar cells around a wide lumen (L), open-faced nuclei (N), multiple radially arranged mitochondria (white arrows). Note the prominent blood capillaries adjacent to the ductal basement membrane (BV) (mag. x 1000). High-fat diet group II c. showing shrunken acinar cell, abnormally widened intercellular spaces (dotted arrows), accumulation of fat droplets (white arrows) (mag.x4000). d. revealing pyknotic nucleus with irregular nuclear membrane (N), degenerated mitochondria (asterisk), dilated fragmented rER (white arrows), widened intercellular spaces (dotted arrow). Note the fat droplets at the basal part of the cell (black arrow) (mag.x4000). e. showing part of the secretory duct with pyknotic nucleus (N), pleomorphic mitochondria (asterisk), degenerated basal infoldings (white arrows). Note the wide space around the basement membrane of duct cells (dotted arrow) (mag.x2500). HFD +Anthocyanins group III f. illustrating the acinar cell with euchromatic nucleus (N), fragmented rER (black arrows), secretory granules of different sizes and electron densities (white arrows). (mag. x 2500). g. showing the secretory cell nucleus with prominent nucleolus (N), regular cisternae of rER (white arrows), coalescent secretory granules (black arrows). Note the narrow intercellular spaces (dotted arrow) (mag. x 2500). h. revealing a secretory striated duct with open-faced nuclei (N), numerous regularly arranged mitochondria (asterisk), uniform basal infoldings (black arrows) (mag. x 2500)

DISCUSSION

Hyperlipidemic diets are closely correlated with the onset of obesity. HFD increases adipocyte growth, hypertrophy, accumulation and activates the macrophages and adipocytes inflammatory signalling, thus it can result in inflammation. Pro-inflammatory cytokines such TNFα, IL-1β, and IL-6 are secreted in greater amounts, which leads to further adipocyte hyperplasia [4, 25].

Since obesity is a long-term, low-grade inflammatory disease it elicits oxidative stress. Over-production of ROS alters gene expression, signal transduction, cell development, and apoptosis, as well as destroys essential cellular constituents, thus interferes with cellular metabolism. Indeed, one of the pathogenic elements contributing to the development of IR is obesity, which may change oxygen metabolism and cause considerable oxidative stress [26, 27].

Upon clinical observation, the current study revealed that the body weights of HFD group were significantly greater than the control group. This is in agreement with Hariri N. et al who stated that rats receiving HFD showed a significant increase in their body weights. The failure to regulate fat oxidation with the excess fat in the diet, elevated lipoprotein lipase activity, and larger meal sizes are all contributing factors to the increase in adiposity[25]. Furthermore, they found that fat cells bind to the hypothalamic microglia's Toll-like receptors (TLR2 and TLR4), which triggers the release of pro-inflammatory cytokines, that in turn destroys the neurons in charge of controlling appetite and thermogenesis.

On the other hand, the present study revealed a decrease in the mean body weight of HFA group, which is in agreement with Azzini et al who reported that the weight loss and the reduction of adipose tissue are two mechanisms by which anthocyanins may help to mitigate obesity [28].

Several studies highlighted the correlation between calcium level and obesity; that's' why serum calcium level was measured in this study. Our findings revealed that HFD group showed a significant difference in the serum calcium level compared to control and HFA groups. It was demonstrated that calcium has anti-obesity properties through modulation of fat metabolism by decreasing lipogenesis and increasing lipolysis, promotion of adipocyte (precursor) proliferation and/or apoptosis and enhancing brown adipocyte tissue (BAT) thermogenesis which improves energy expenditure and helps in the loss of body fat [29, 30].

In this study, lipid profile parameters demonstrated mild to moderate intracellular lipid accumulation in submandibular salivary glands. This finding could be explained by the fact that HFD is characterized by increased levels of serum triglycerides, cholesterol and LDL [31, 32]. It causes aggregation of lipid in various tissues such as pancreas, kidney, liver, muscle and arterial walls [33, 34].

Indeed, biochemical analysis in the present study also found that rats fed with HFD showed a marked increase in blood insulin level. Animal studies mentioned that rats receiving HFD for a period of 8-weeks developed IR, obesity and impairment of insulin receptor function in several sites, such as salivary glands [4]. Zalewska et al. demonstrated that the stimulation of stress signalling mitogen-activated protein kinases (MAPKs) by oxidative stress induced by HFD can result in the blockage of insulin signalling, which causes IR [35]. Indeed, Kołodziej et al. stated that oxidative stress can induce inflammatory response by upregulating the expression and activity of pro-inflammatory cytokines such as TNFα and IL-6. These cytokines lead to phosphorylation of insulin receptor substrate-1 (IRS-1) at inhibitory sites and induce a considerable reduction in insulin sensitivity [4].

On the other hand, HFA group revealed improvement in IR compared to HFD group. According to rodent research, AMP-activated

protein kinase (AMPK) is activated by anthocyanins, which stimulates pancreatic β cells' production of insulin and glucose absorption. This accounts for the improvement in insulin sensitivity [36]. This is in agreement with the findings of Jennings A. et al. who found that diet rich in anthocyanins and flavones can reduce IR [37].

Besides the blood serum analysis included in the current study, light microscopic results of HFD group showed intra-cytoplasmic vacuolations which may be related to fatty degeneration in the glandular acini. It was reported that glandular secretory cells temporarily store excess lipid as droplets in their cytoplasm, which modifies the lipid fractions of the salivary glands and results in hypofunction [38].

The same findings were recorded by some investigators who found a huge aggregation of lipid droplets in the cells of the salivary glands in diabetic rats [12]. Besides, it was stated that the excess fatty acid induced by HFD disrupts the SMGs antioxidative systems, leading to DNA, protein and lipid oxidation, and increases the ROS production that results in oxidative stress with subsequent cellular damage [35]. Moreover, The total antioxidative status of sublingual and submandibular saliva have been demonstrated to be much lower than that of parotid saliva due to their lower amounts of enzymatic and nonenzymatic antioxidants [39].

In this study, HFD provoked inflammatory signs that were in the form of congested dilated blood vessels with inflammatory cellular infiltrations. It might be explained as an inflammatory response to deliver more blood to the areas of degeneration. Diffusion of oxygen and nutrients to parenchymal cells might be restricted by these inflammatory changes decreasing parenchymal cells survival and functions [40].

On the contrary, HFA group revealed a moderate restoration of the normal architecture in the serous acini and ductal elements as well as moderate improvement in blood vessel congestion. As antioxidants, anthocyanins significantly lower leptin levels in obese rat models and reduce obesity-related proinflammatory cytokines including CRP, IL-6, and TNF-α. Therefore, they can stop inflammation brought on by fat. In fact, research conducted in vitro on fruit extracts rich in anthocyanins demonstrated a considerable reduction of IL-6 gene expression in adipose stem cells generated by lipopolysaccharide [41].

The light microscopic findings of the current study were further supported by TEM results which illustrated that HFD group exhibited accumulation of lipid droplets in the cytoplasm of secretory cells. These findings were in agreement with Yan et al. [42] who mentioned that rats eating HFD for long time showed an increase in lipid aggregation in different organs such as pancreases, kidneys and liver. Eating habits is considered one of the main factors that affect accumulation of lipids in salivary glands. Ingestion of unlimited amount of lipids, results in temporary storage of unused lipids in the cytoplasm of secretory cells [12].

These findings were online with ultrastructural results of the present study. The present work revealed pyknotic heterochromatic nuclei with irregularly demarcated nuclear membranes, in the HFD group. Different studies demonstrated that the increased amount of heterochromatin denotes low metabolic activity of the cell. These results were in accordance with other experiments, mentioning that apoptosis is the main manifestation of renal injury induced by hyper-cholesterolemia [43].

Also, the internal degeneration of mitochondrial cristae demonstrated in this study could be attributed to changes in the structure of the inner membrane with subsequent disruption in its pump mechanism resulting in a decrease in Ca^{+} retention. This leads to alteration in cell respiration, de-granulation of the rER leading to disorders in the cell-specific proteins synthesis [44].

However, examination of HFA group by TEM revealed evident improvement in the acinar and ductal structure except for few rER fragmentation in some regions. These findings could be due to the

potent antioxidant properties of anthocyanins that alleviate the histopathological deterioration and localized oxidative stress in the salivary glands [45].

In our study, early in the smooth muscle cells' differentiation process, αSMA immunohistochemistry has been identified as a particular marker expressed by these cells. Quantitative analysis revealed that HFD rats had a highly significant increase in αSMA immunoreactivity, which was consistent with findings from other earlier investigations [20, 46].

The prevention of vascular smooth muscle cell growth, scavenging of free radicals and antioxidant effect, and the anti-aggregatory platelet property may contribute to the health benefits of fruits and vegetables [47]. HFA group showed the lowest immunoreaction to αSMA compared to the other groups which go with El-Messiry et al [48]. Numerous research endeavours examine the impact of distinct substances on cellular parameters and apoptosis to ascertain their methods of action inside tissues. Nonetheless, limited research has examined the impact of combining phytochemicals with certain diets in this regard [49, 50].

Any alteration in the apoptosis process is harmful and causes tissue damage, that can be brought on by nutritional deficiencies. According to Chiang et al. [51], mice on a high-protein diet changed how apoptosis and fibrosis were expressed. Fatty acid oxidation and oxidative phosphorylation, which produce adenosine triphosphate (ATP), are boosted by excessive fat ingestion. Energy deficits and/or the generation of ROS, which cause cell damage, are caused by the pathway's dysregulation [48, 52]. This was found in our study that the HFD group showed increase in the apoptotic cells.

The current study concluded that high-fat diet has a significant effect on the disruption of structure and activity of the submandibular salivary glands which affects the oral health. Moreover, Anthocyanins have an effective therapeutic effect on antagonizing these hazardous effects of the high-fat diet.

Statements and Declarations

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Competing Interests:

Authors confirm that they have no competing financial interests or personal relationships to declare that are related to this research article.

Ethics Approval

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

Data Availability

All data presented in this article are available from the corresponding author upon request.

Credit Authorship Contribution Statement

D. A. Nagui: Investigation, Conceptualization, Data curation, Writing – original draft, Visualization, Writing – review & editing, Final approval. M. M.Saleh: Conceptualization, Methodology, Data curation, Validation, Writing – review & editing, Final approval. S. A. Hamza: Conceptualization, Data curation, Methodology, Writing – review & editing, Final approval.

Declaration Of Competing Interests

Authors confirm that they have no competing financial interests or personal relationships to declare that are related to this research article.

ACKNOWLEDGEMENTS

The authors would like to thank all the laboratory technicians at the faculty of Dentistry, and Centre of Excellence for Research in Regenerative Medicine and Applications (CERRMA), Faculty of Medicine,

Alexandria University for their assistance in the histological and immunohistochemical tissue preparation. Indeed, the authors would like to thank the members of the Medical Statistics department, Medical Research Institute, Alexandria University for assistance with the sample size calculation and statistical analysis of this study.

REFERENCES

1. Swinburn B, Hovmand P, Waterlander W, Allender S. The global syndemic of obesity, undernutrition, and climate change. Clinical Obesity in Adults and Children. 2022:409-27. DOI:10.1016/S0140-6736(18)32822-8.
2. Bosello O, Donataccio MP, Cuzzolaro M. Obesity or obesities? Controversies on the association between body mass index and premature mortality. Eating and Weight Disorders-Studies on Anorexia, Bulimia and Obesity. 2016;21:165-74. DOI: 10.1007/s40519-016-0278-4
3. Flores-Cordero JA, Pérez-Pérez A, Jiménez-Cortegana C, Alba G, Flores-Barragán A, Sánchez-Margalet V. Obesity as a risk factor for dementia and Alzheimer's disease: the role of leptin. International journal of molecular sciences. 2022;23(9):5202. DOI: 10.3390/ijms23095202
4. Kołodziej U, Maciejczyk M, Miąsko A, Matczuk J, Knaś M, Żukowski P, et al. Oxidative modification in the salivary glands of high fat-diet induced insulin resistant rats. Frontiers in Physiology. 2017;8:20. DOI: 10.3389/fphys.2017.00020
5. Roa I, Del Sol M. Obesity, salivary glands and oral pathology. Colombia Médica. 2018;49(4):280-7. DOI: 10.25100/cm.v49i3.3919
6. Zalewska A, Kossakowska A, Taranta-Janusz K, Zięba S, Fejfer K, Salamonowicz M, et al. Dysfunction of salivary glands, disturbances in salivary antioxidants and increased oxidative damage in saliva of overweight and obese adolescents. Journal of Clinical Medicine. 2020;9(2):548. DOI:10.3390/jcm9020548
7. Bechir F, Pacurar M, Tohati A, Bataga SM. Comparative study of salivary pH, buffer capacity, and flow in patients with and without gastroesophageal reflux disease. International Journal of Environmental Research and Public Health. 2021;19(1):201. DOI: 10.3390/ijerph19010201.
8. Hatipoğlu Ö, Maraş E, Hatipoğlu FP, Saygin A. Salivary flow rate, pH, and buffer capacity in the individuals with obesity and overweight; A meta-analysis. Nigerian journal of clinical practice. 2022;25(7):1126-42. DOI: 10.4103/njcp.njcp_1760_21.
9. Sivamaruthi BS, Kesika P, Chaiyasut C. The influence of supplementation of anthocyanins on obesity-associated comorbidities: A concise review. Foods. 2020;9(6):687. DOI: 10.3390/foods9060687.
10. Mattioli R, Francioso A, Mosca L, Silva P. Anthocyanins: A comprehensive review of their chemical properties and health effects on cardiovascular and neurodegenerative diseases. Molecules. 2020;25(17):3809. DOI: 10.3390/molecules25173809.
11. Liu J, Zhou H, Song L, Yang Z, Qiu M, Wang J, et al. Anthocyanins: Promising natural products with diverse pharmacological activities. Molecules. 2021;26(13):3807. DOI: 10.3390/molecules26133807.
12. Al-Serwi RH, El-Sherbiny M, Eladl MA, Aloyouny A, Rahman I. Protective effect of nano vitamin D against fatty degeneration in submandibular and sublingual salivary glands: A histological and ultrastructural study. Heliyon. 2021;7(4). DOI: 10.1016/j.heliyon.2021.e06932
13. Zalewska A, Ziembicka D, Żendzian-Piotrowska M, Maciejczyk M. The impact of high-fat diet on mitochondrial function, free radical production, and nitrosative stress in the salivary glands of wistar rats. Oxidative medicine and cellular longevity. 2019;2019. DOI: 10.1155/2019/2606120
14. Care IoLARCo, Animals UoL. Guide for the care and use of laboratory animals: US Department of Health and Human Services, Public Health Service, National …; 1986.
15. Percie du Sert N, Hurst V, Ahluwalia A, Alam S, Avey MT, Baker M, et al. The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. Journal of Cerebral Blood Flow & Metabolism. 2020;40(9):1769-77. DOI:10.1177/0271678X20943823
16. Rosner B. Fundamentals of biostatistics: Cengage learning; 2015.
17. Suresh K. An overview of randomization techniques: an unbiased assessment of outcome in clinical research. Journal of human reproductive sciences. 2011;4(1):8. DOI: 10.4103/0974-1208.82352
18. Lobstein T, Brinsden H, Neveux M. World obesity atlas 2022. 2022.
19. Rehman SU, Shah SA, Ali T, Chung JI, Kim MO. Anthocyanins reversed D-galactose-induced oxidative stress

and neuroinflammation mediated cognitive impairment in adult rats. Molecular neurobiology. 2017;54:255-71. DOI: 10.1007/s12035-015-9604-5

20. Selim SA. The effect of high-fat diet-induced obesity on the parotid gland of adult male albino rats: histological and immunohistochemical study. Egyptian Journal of Histology. 2013;36(4):772-80. DOI: 10.1097/01.EHX.0000437624.80956.78
21. Zatroch KK, Knight CG, Reimer JN, Pang DS. Refinement of intraperitoneal injection of sodium pentobarbital for euthanasia in laboratory rats (Rattus norvegicus). BMC veterinary research. 2016;13:1-7. DOI: 10.1186/s12917-017-0982-y
22. Goldstein MA. The MassGeneral Hospital for children adolescent medicine handbook: Springer; 2011.
23. Kim S-W, Roh J, Park C-S. Immunohistochemistry for pathologists: protocols, pitfalls, and tips. Journal of pathology and translational medicine. 2016;50(6):411-8. DOI: 10.4132/jptm.2016.08.08
24. Lillie RD. Histopathologic technic and practical histochemistry. (No Title). 1965.
25. Hariri N, Thibault L. High-fat diet-induced obesity in animal models. Nutrition research reviews. 2010;23(2):270-99. DOI: 10.1017/S0954422410000168
26. Shao A, Campbell WW, Chen CO, Mittendorfer B, Rivas DA, Griffiths JC. The emerging global phenomenon of sarcopenic obesity: Role of functional foods; a conference report. Journal of Functional Foods. 2017;33:244-50. DOI: 10.1016/j.jff.2017.03.048
27. Naomi R, Teoh SH, Embong H, Balan SS, Othman F, Bahari H, et al. The Role of Oxidative Stress and Inflammation in Obesity and Its Impact on Cognitive Impairments—A Narrative Review. Antioxidants. 2023;12(5):1071. DOI: 10.3390/antiox12051071
28. Azzini E, Giacometti J, Russo GL. Antiobesity effects of anthocyanins in preclinical and clinical studies. Oxidative medicine and cellular longevity. 2017;2017. DOI: 10.1155/2017/2740364
29. Zhang F, Ye J, Zhu X, Wang L, Gao P, Shu G, et al. Antiobesity effects of dietary calcium: the evidence and possible mechanisms. International journal of molecular sciences. 2019;20(12):3072. DOI: 10.3390/ijms20123072.
30. Abdallah d, Hamza sa. Effect of low calcium diet on the submandibular salivary glands in rats (histological, immunohistochemical and ultrastructural study). Egyptian Dental Journal. 2024;70(1):115-28. DOI:10.21608/edj.2023.231256.2689
31. Naito H. Hyperlipidemia, laboratory approaches. Methods in clinical chemistry Washington DC: Mosby Company. 1987:1140-5.
32. Nguyen NT, Varela E, Sabio A, Tran C-L, Stamos M, Wilson SE. Resolution of hyperlipidemia after laparoscopic Roux-en-Y gastric bypass. Journal of the American College of Surgeons. 2006;203(1):24-9. DOI: 10.1016/j.jamcollsurg.2006.03.019
33. Davis HR, Vesselinovitch D, Wissler RW. Reticuloendothelial system response to hyperlipidemia in rhesus and cynomolgus monkeys. Journal of leukocyte biology. 1984;36(1):63-80. DOI: 10.1002/jlb.36.1.63
34. Wolf AM, Busch B, Kuhlmann HW, Beisiegel U. Histological changes in the liver of morbidly obese patients: correlation with metabolic parameters. Obesity surgery. 2005;15(2):228-37. DOI: 10.1381/0960892053268408
35. Zalewska A, Knaś M, Gińdzieńska-Sieśkiewicz E, Waszkiewicz N, Klimiuk A, Litwin K, et al. Salivary antioxidants in patients with systemic sclerosis. Journal of Oral Pathology & Medicine. 2014;43(1):61-8. DOI: 10.1111/jop.12084
36. Różańska D, Regulska-Ilow B. The significance of anthocyanins in the prevention and treatment of type 2 diabetes. Adv Clin Exp Med. 2018;27(1):135-42. DOI: 10.17219/acem/64983
37. Jennings A, Welch AA, Spector T, Macgregor A, Cassidy A. Intakes of anthocyanins and flavones are associated with biomarkers of insulin resistance and inflammation in women. The Journal of nutrition. 2014;144(2):202-8. DOI: 10.3945/jn.113.184358
38. Matczuk J, Zalewska A, Łukaszuk B, Knaś M, Maciejczyk M, Garbowska M, et al. Insulin resistance and obesity affect lipid profile in the salivary glands. Journal of Diabetes Research. 2016;2016. DOI: 10.1155/2016/8163474
39. Onopiuk BM, Dąbrowska ZN, Rogalska J, Brzóska MM, Dąbrowski A, Bijowski K, et al. The beneficial impact of the black chokeberry extract against the oxidative stress in the sublingual salivary gland of rats intoxicated with cadmium. Oxidative Medicine and Cellular Longevity. 2021;2021. DOI: 10.1155/2021/6622245

40. Ertunc ME, Hotamisligil GS. Lipid signaling and lipotoxicity in metaflammation: indications for metabolic disease pathogenesis and treatment. Journal of lipid research. 2016;57(12):2099-114. DOI: 10.1194/jlr.R066514

41. Ngamsamer C, Sirivarasai J, Sutjarit N. The benefits of anthocyanins against obesity-induced inflammation. Biomolecules. 2022;12(6):852. DOI: 10.3390/biom12060852

42. Yan M-X, Li Y-Q, Meng M, Ren H-B, Kou Y. Long-term high-fat diet induces pancreatic injuries via pancreatic microcirculatory disturbances and oxidative stress in rats with hyperlipidemia. Biochemical and biophysical research communications. 2006;347(1):192-9. DOI: 10.1016/j.bbrc.2006.06.063

43. Ying Y, Xingkui T, Xiaocheng L, Jufang S, Yongman L. The p53-mediated apoptosis in hypercholesterolemia-induced renal injury of rats. Journal of Huazhong University of Science and Technology [Medical Sciences]. 2005;25:408-11. DOI: 10.1007/BF02828209

44. Matuz-Mares D, González-Andrade M, Araiza-Villanueva MG, Vilchis-Landeros MM, Vázquez-Meza H. Mitochondrial calcium: effects of its imbalance in disease. Antioxidants. 2022;11(5):801. DOI: 10.3390/antiox11050801

45. Özgür A, Terzi S, Özdemir D, Tümkaya L, Rakıcı S, Mercantepe T, et al. Protective effect of whortleberry extract on salivary gland damage induced by neck irradiation in rats. Ear, Nose & Throat Journal. 2019;98(6):E64-E9. DOI: 10.1177/0145561319846868

46. Hamada AS, Hendawy M, El Hawary AAEM. Immunohistochemical Expression of Aquaporin 8 in the Sublingual Salivary gland of High Fat Diet/Fructose Rat Model. Zagazig University Medical Journal. 2024;30(1):199-210. DOI: 10.21608/ZUMJ.2023.182720.2705

47. Shahidi F, Ambigaipalan P. Phenolics and polyphenolics in foods, beverages and spices: Antioxidant activity and health effects–A review. Journal of functional foods. 2015;18:820-97. DOI: 10.1016/j.jff.2015.06.018

48. El-Messiry H, El Shaer DF, Fathy IA. A Comparative Histological and Immunohistochemical Study on the Effect of Moringa Oil and Sesame Oil on the Submandibular Glands of Cisplatin Treated Albino Rats. Egyptian Dental Journal. 2023;69(2):1161-80. DOI: 10.21608/edj.2023.195400.2458

49. Dudley KJ, Sloboda DM, Connor KL, Beltrand J, Vickers MH. Offspring of mothers fed a high fat diet display hepatic cell cycle inhibition and associated changes in gene expression and DNA methylation. PloS one. 2011;6(7):e21662. DOI: 10.1371/journal.pone.0021662

50. Fausto N, Campbell JS, Riehle KJ. Liver regeneration. Journal of hepatology. 2012;57(3):692-4. DOI: 10.1016/j.jhep.2012.04.016

51. Chiang W-D, Shibu MA, Lee K-I, Wu J-P, Tsai F-J, Pan L-F, et al. Lipolysis-stimulating peptide-VHVV ameliorates high fat diet induced hepatocyte apoptosis and fibrosis. journal of functional foods. 2014;11:482-92. DOI: 10.3402/fnr.v60.31417

52. Valenzuela M, Bastias L, Montenegro I, Werner E, Madrid A, Godoy P, et al. Autumn royal and ribier grape juice extracts reduced viability and metastatic potential of colon cancer cells. Evidence-Based Complementary and Alternative Medicine. 2018;2018. DOI: 10.1155/2018/2517080