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ORIGINAL PAPER

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Effect of high fat diet on structure of liver and gallbladder of adult male mice – an experimental study

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ABSTRACT

Introduction. High fat diet (HFD) intake induces obesity and adversely affects different body organs including liver and gallbladder.

Aim. It was to clarify the effects of HFD on the liver and gallbladder structure using light microscopic (LM) examination.

Material and methods. 16 healthy adult male mice were equally divided into 2 groups. Control group mice were fed normal diet. HFD group was fed using HFD. At the end of the 8-week experiment, mice were anesthetized. Liver and gallbladder were removed and prepared to histological processing. Sections were stained with hematoxylin and eosin (H&E) and immunostaining for cyclooxygenase-2 (COX-2) cellular localization. Oil Red O (ORO)-stained frozen liver sections were prepared.

Results. H&E-stained sections of HFD group revealed rounded swollen hepatic cells with pale cytoplasm suggesting cellular ballooning. Dilated congested sinusoids and portal vein, cellular degeneration and collection of inflammatory cells were observed between hepatic cells and in portal region. Gallbladder sections showed epithelial stratification and cellular vacuolation. Strong immunoexpression of COX-2 was observed in Kupffer and hepatic cells of the liver and gallbladder mucosal epithelial cells.

Conclusion. HFD is suggested to alter the normal histological features of liver and gallbladder represented by fatty liver and gallbladder epithelial hyperplasia and inflammatory reaction.

Keywords. COX-2, fatty liver, gallbladder epithelium, immunoexpression, Oil Red O staining

Introduction

Liver is the largest metabolizing organ in the body which regulates homeostasis of different body systems. Its main functions are synthesis, storage and metabolism of fats, carbohydrates and proteins, detoxification of hormones, drugs and toxins and excretion of bilirubin.[1] The gallbladder is a grey-blue pear-shaped organ found in a fossa at the right side of inferior surface of liver. Its main function is storage, concentration and then excretion of bile secreted by the liver; it has storage capacity of about 50 ml.[2] Despite the difference in the structure and functions of two organs, the liver and gallbladder are closely related in development where they firstly arise as a bud from the summit of duodenum. The bud then subdivides into cranial pars hepatica forming the liver and caudal pars cystica that dilates at its terminal end to form the gallbladder.[3]

High fat diet (HFD) intake is a large problem which

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faces modern societies where lifestyle has been changed and fast food and take-away foods become common. It induces obesity and adversely affects different body organs including liver and gallbladder.[4] Excess dietary fats could lead to fatty acids' deposition in hepatic cells with the possible development of nonalcoholic fatty liver disease (NAFLD).[5] It was recorded that about 25% of the world population are affected by NAFLD.[6]

Lipid deposition in tissues can be detected by use of ORO staining of frozen tissues' sections. ORO is a fat-soluble dye staining neutral lipids and cholesteryl esters but not membranes. In staining lipids, it depends on its hydrophobic character through moving away from the solvents to associate with lipids within tissues.[7]

Cyclooxygenase (COX) is an enzyme involved in production of prostaglandins (PGs) and other inflammatory mediators. It includes two subtypes; COX-1 and COX-2. COX-1 is responsible for physiological functions and cytoprotection of body organs. However, COX-2 is responsible for production PGs involved in inflammation. COX-2 is excessively expressed in pathological conditions of liver and gallbladder including hepatic inflammation, hepatitis, cirrhosis and cholecystitis while its expression is weak in physiological condition.[8] Therefore, COX-2 could be considered as an inflammatory marker.

Gall stones are a common public health problem; about 10-15% of the world adult populations are affected. Such disease is closely associated with the nutritional lifestyle. Its prevalence is increasing proportionate with the worldwide increase in obesity prevalence. The NAFLD is considered a risk factor for occurrence of gallbladder stone disease.[9,10] The main component of gallstones is cholesterol. Therefore, hypercholesterolemia is a predisposing factor for the development of gallstones and cholecystitis.[11,12]

In addition, it was reported that functional changes in the form of decreased gallbladder response to neurotransmitters and diminished contractility have been reported in association with high fat diet.[13] Also, Li et al. recorded association between fatty liver change and gallbladder diseases.[14] They stated that fatty liver can be considered together with age a predictor for the risk of developing gallbladder disease.

Inflammation in association with fatty diet has been reported by Yu et al. in the liver and also by Van Erpecum et al. in the gallbladder.[15,16] They recorded granulocytes and lymphocytic infiltrations in lamina propria. However, Lavoie et al. observed mucosal inflammation in the gallbladder of mice fed fatty diet; and they stated that inflammation was preceded by muscular dysfunction.[17]

Aim

Despite the well-documented adverse effects of HFD, previous studies examining its effect on the histological structure of the liver and gallbladder are scarce. Therefore, in this study, we aimed to elucidate the potential adverse effects of HFD administration on the structure of these organs using the LM examination. This was performed by routine histological examination using H&E as well as detection of lipid accumulation by ORO within tissues and immunohistochemical analysis of inflammation using COX-2.

Material and methods

Animals and study design

Sixteen healthy adult male mice were used in this study. Their weights ranged from 18 to 22 gm. The mice were bought from animal house unit of the faculty of Veterinary Medicine Suez Canal University. They were kept under good aseptic & healthy conditions and standardized environment (e.g., temperature 23±2°C). The study was approved by The Institutional Animal Care and Use Committee Zagazig University (ZU-IACUC); reference number (Zu-IACUC/3/F/167/2019). Animals were divided into two groups; each contains eight mice; as the followings:

- G I (Control group): animals were fed normal chow diet for 8 weeks.
- G II (HFD group): animals were fed HFD; 15% total animal fat, 2% cholesterol (02780, LOBA Chemie, India) and 0.5% cholic acid (C-02682 Oxford Lab Chem) for eight weeks[18].

At the end of experiment, mice were anesthetized using thiopental. Their abdomens were opened to remove the liver and gallbladder that were immediately fixed in 10% neutral-buffered formalin solution for histological preparation. Parts of liver tissues were kept frozen (at -80°C) for the procedure of the ORO staining.

LM techniques

Liver and gallbladder were histologically processed and embedded in paraffin wax.[19] Then, 5 um thick sections were obtained and stained with H&E.[20]

Frozen liver tissue was embedded in Tissue-Tek as described by Mehlem et al.[7] Tissue sectioning was done and 12 µm thick sections were obtained to be stained with ORO and examined with LM.

Immunohistochemical staining for COX-2 (Inflammatory marker)

Liver and gallbladder tissues embedded in paraffin were cut into 5 µm thick sections, and then sections were processed for immunohistochemical staining by avidin biotin peroxidase method for COX-2 (DAKO, Germany) immunodetection.[21]

Morphometrical study

The area percentage (%) of ORO liver staining and COX-2 immunoexpression in liver and gallbladder were measured using Image J software.

Statistical study

The data were presented in the form of mean ± standard deviation (M±SD) statistical comparison of the mean values was done by independent sample student t test using Graph Prism 5.01 Software. The *P* value less than 0.05 was considered significant.

Results

H&E-stained sections

H&E-stained liver sections of the control groups showed normal hepatic lobule structure; polygonal acidophilic hepatic cells with single or two vesicular nuclei, arranged in cords around the central vein with hepatic sinusoids between the cords. (Fig. 1a). Portal vein that was a thin-walled vessel with wide lumen and bile ductile with its cuboidal epithelial lining were seen in the portal region (Fig. 1b). In the HFD group marked structural changes were observed; rounded swollen hepatic cells with pale cytoplasm were seen suggesting cellular ballooning. Dilated congested sinusoids, cellular degeneration and collection of inflammatory cells between the hepatic cells & in the portal region were observed. Also, marked dilatation and congestion of the Portal vein was seen (Fig. 1c, d).

Gallbladder sections stained with H&E showed in the control group mucosa consisted of one layer of simple columnar epithelium with cells having oval nuclei and lamina propria, underlying the mucosa, formed of loose connective tissue. The mucosal folds with central core of lamina propria were seen (Fig. 2a). In the HFD group epithelial stratification and cellular vacuolation were observed (Figs. 2b).

ORO-stained frozen liver sections

ORO-stained sections of the liver of control group revealed low lipid deposition specified by a weak cytoplasmic red staining of the hepatic cells (Fig. 2c). In the HFD group, there was excess lipid deposition specified by strong cytoplasmic red staining of the hepatic cells (Fig. 2d).

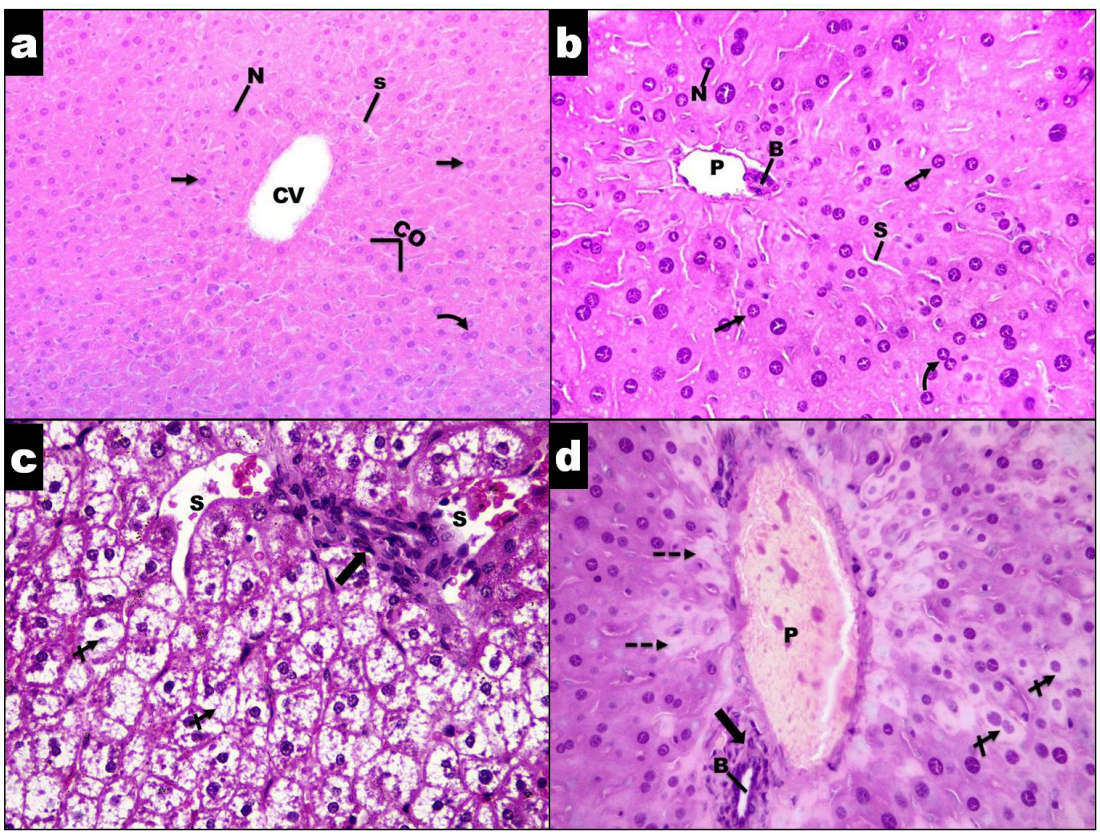

Fig. 1. Photographs of H&E-stained LM liver sections (x400) of adult male mouse: a – Control group showing normal hepatic lobule structure; polygonal acidophilic hepatic cells (arrow) with single (N) or two (curved arrow) vesicular nuclei, arranged in cords (co) around the central vein (CV) with the hepatic sinusoids (S) between the cords; b – Control group showing portal vein (P) that is thin-walled vessel with wide lumen and bile ductile (B) with its cuboidal epithelial lining in the portal region. Note: polygonal acidophilic hepatic cells (arrow) with single (N) or two (curved arrow) vesicular nuclei, hepatic sinusoids (S); c – HFD group showing marked structural changes; rounded swollen hepatic cells with pale cytoplasm suggesting cellular ballooning (crossed arrow); dilated congested sinusoids (S) and collection of inflammatory cells between hepatic cells (thick arrow) are seen; d – HFD group showing cellular degeneration (dotted arrow), aggregation of inflammatory cells (thick arrow) in the portal region and marked dilatation & congestion of the portal vein (P). Note: rounded swollen hepatic cells with pale cytoplasm (crossed arrow), bile ductile (B)

COX-2 immunohistochemically stained sections

COX-2 immunohistochemically stained liver sections of the control group of the liver showed a weak immunoexpression of COX-2 in hepatic cells marked by faint brown cytoplasmic staining in Kupffer cells. No immunoexpression of COX-2 was shown in hepatic cells (Fig. 3a). In HFD group a strong immunoexpression of COX-2 was observed as specified by markedly strong brown cytoplasmic staining in both Kupffer and hepatic cells was seen (Fig. 3b).

Examination of COX-2 immunohistochemical gallbladder-stained sections of the control groups showed weak immunoexpression of COX-2 noticed by a faint brown cytoplasmic staining in the mucosal epithelial cells (Fig. 3c). In HFD group strong immunoexpression of COX-2 specified by a strong brown cytoplasmic staining in the mucosal epithelial cells was observed (Fig. 3d).

Statistical analysis

Statistical analysis by independent student sample t test of the area % mean values of ORO staining in the liver tissue revealed a highly significant (P>0.001) increase in the HFD group as compared to control. Also, a very highly significant (P>0.001) increase in the area % mean values of COX-2 immunoexpression in the liver and gallbladder was recorded in the HFD group in comparison to the control group (Table 1; Fig. 4).

Table 1. Statistical analysis by independent sample t test of the area % of ORO staining in the liver and COX-2 immunoexpression in liver and gallbladder between the studied groups

Parameter	Control, mean ± SD (range)	HFD treated, mean ± SD (range)	t- test	P value
number of mice	8	8		
area % of ORO staining in the liver	14.13 ± 0.41 (12.58-15.74)	28.87 ± 0.44 (26.89–30.46)	24.47	0.0001
area % of COX-2 immunoexpression in liver	23.23 ± 1.61 (15.7-29.4)	47.01 ± 3.58 (33.49-60.09)	6.05	0.0001
area % of COX-2 immunoexpression in gallbladder	1.99 ± 0.2 (1.09-2.81)	14.63 ± 0.76 (11.33-17.96)	16.15	0.0001

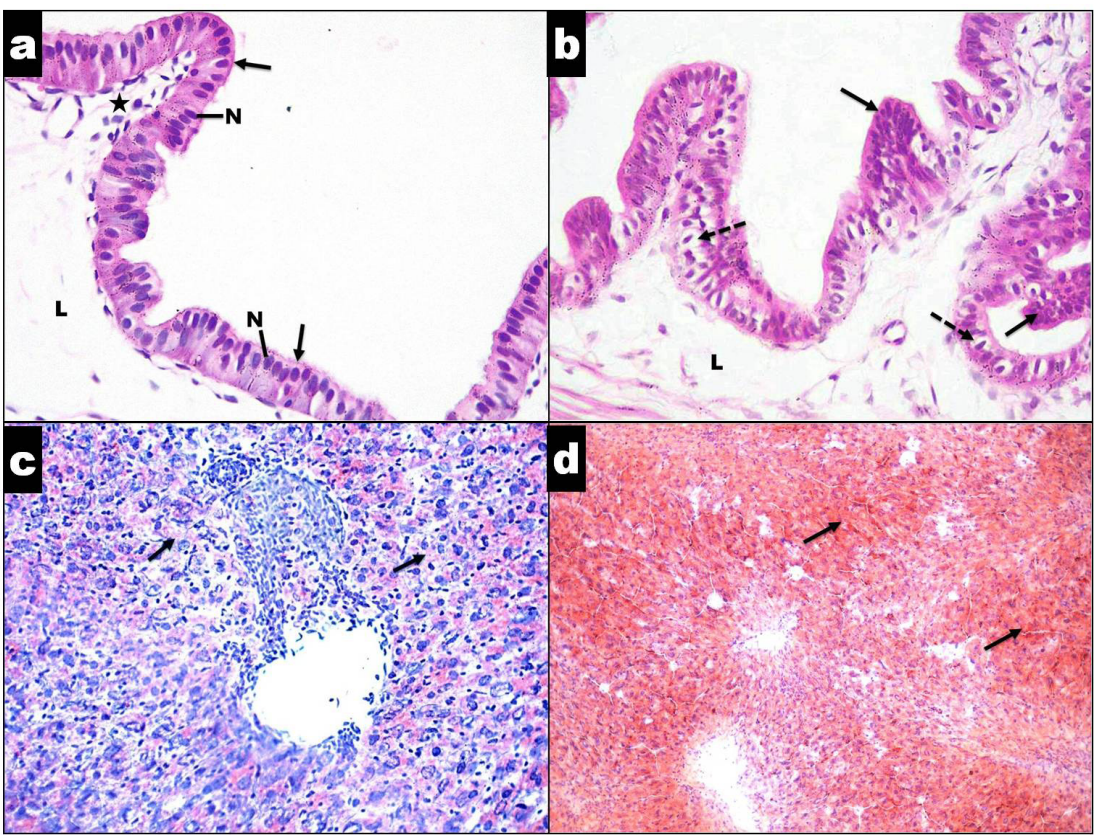

Fig. 2. Photographs of LM sections: a – H&E-stained gallbladder section (x400) of control group showing (showing) mucosa consisted of one layer of simple columnar epithelium with cells (arrow) having oval nuclei (N). Lamina propria (L), underlying the mucosa, formed of loose connective tissue. Mucosal folds (★) with central core of lamina propria are seen; b – H&E-stained gallbladder section (x400) of HFD group showing epithelial stratification (arrow) and cellular vacuolation (dotted arrow). Note: lamina propria (L); c – ORO-stained liver section (x200) of adult male mouse of control group showing low lipid deposition specified by weak cytoplasmic red staining of the hepatic cells (arrow); d – ORO-stained liver section (x200) of adult male mouse of HFD group showing excess lipid deposition specified by strong cytoplasmic red staining of the hepatic cells (arrow)

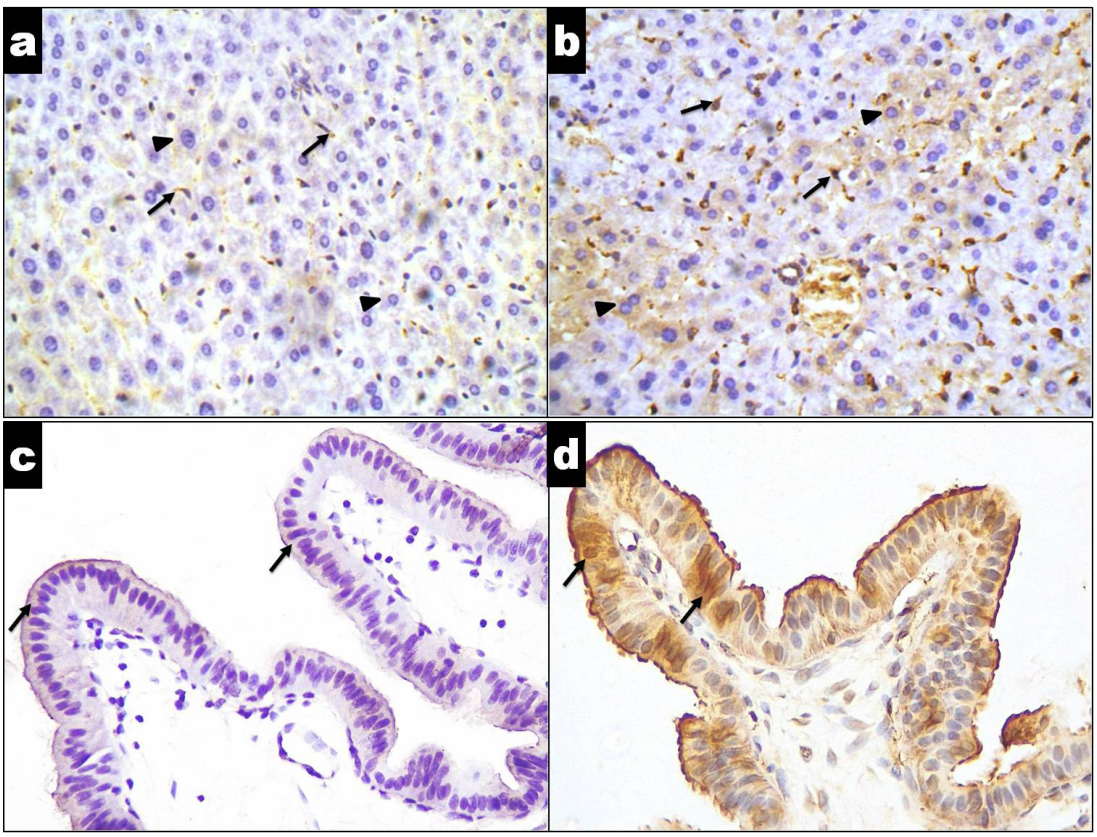

Fig. 3. Photographs of COX-2 immunohistochemically-stained LM sections: a – COX2 immunohistochemically-stained liver section (x400) of the control group showing a weak immunoexpression of COX-2 in hepatic cells specified by faint brown cytoplasmic staining in Kupffer cells (arrow). no immunoexpression of COX-2 is shown in hepatic cells (arrow head); b – COX2 immunohistochemically-stained liver section (x400) of the HFD group showing strong immunoexpression of COX-2 was observed specified by strong brown cytoplasmic staining in both Kupffer (arrow) and hepatic cells (arrow head); c – COX2 immunohistochemically-stained gallbladder section (x400) of the control group showing weak immunoexpression of COX-2 specified by faint brown cytoplasmic staining in the mucosal epithelial cells (arrow); d – COX2 immunohistochemically-stained gallbladder section (x400) of HFD group showing strong immunoexpression of COX-2 specified by markedly strong brown cytoplasmic staining in the mucosal epithelial cells (arrow)

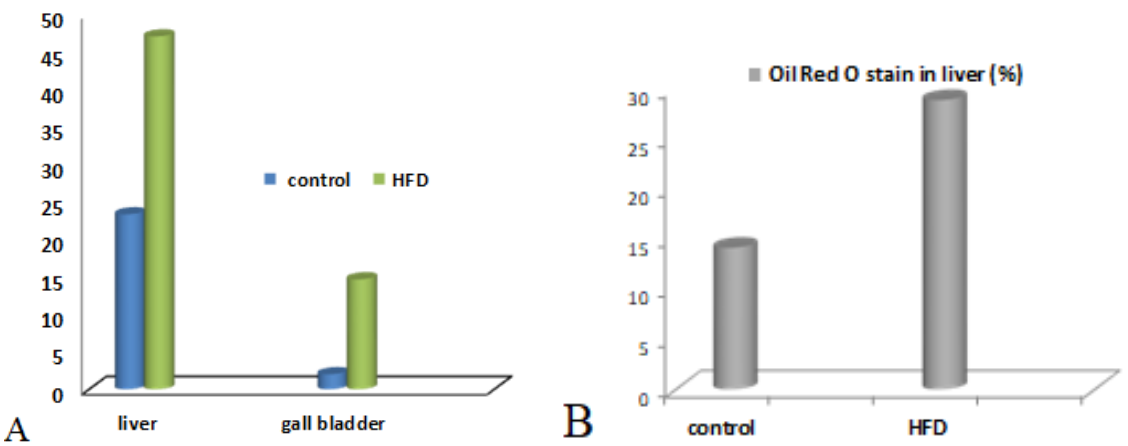

Fig. 4. Representative columns of the two groups: A – area percentages of COX-2 immunoexpression of liver and gallbladder; B – area percentages of ORO in liver

Discussion

HFD is a common bad food habit among many different societies. In this study, we aimed to investigate its effects on liver and gallbladder. We used male animals and not females for this purpose to avoid possible hormonal changes in female estrus cycles that might affect the results achieved.[22,23]

In this study, hematoxylin and eosin-stained liver sections of control group revealed normal hepatic lobule structure; polygonal acidophilic hepatic cells with vesicular nucleus, arranged in cords around the central vein with the hepatic sinusoids between the cords. Thin-walled portal vein and bile ductile with cuboidal epithelial lining were seen in the portal region. These results are consistent with the normal liver histology described by Ross and Pawlina.[24]

In the present study rounded swollen hepatic cells with pale cytoplasm were observed suggesting cellular ballooning. This picture is indicative of steatosis. Steatosis or fatty change of the liver is the accumulation of abnormal amounts of lipids in 5% or more of hepatic cells.[25]

In the present work, we assessed lipid deposition by examination of ORO-stained hepatic sections Low lipid deposition as specified by weak concise cytoplasmic red staining of the hepatic cells was seen in the control groups, while in the HFD group there was excess lipid deposition as specified by intense wide cytoplasmic red staining of the hepatic cells. In addition, statistically the area % mean values of ORO staining showed a very highly significant (P<0.001) increase in HFD group in comparison to the control group. These results are in accordance with findings reported by Meli et al. and Liu et al.[26,27]

In our work, dilated congested portal vein and hepatic sinusoids were observed in HFD group. This is in accordance with Altunkaynak et al. who attributed this finding to be a result of necrosis and inflammatory changes.[28] Arvanitidis et al. explained that hypoxia and ischemia induced by HFD intake could lead to vascular dilatation.[29] However, Elahi et al. stated that the cause of the vascular dilatation was hypertension occurring due to obesity induced by HFD.[30]

The current work revealed collection of inflammatory cells within portal area and between hepatic cells and strong immunoexpression of COX-2 was observed in HFD group which was weak in the control group. Statistically the area % mean values of COX-2 immunoreaction showed a very highly significant increase in HFD group in comparison to the control group. These results are in accordance with study of Yu et al.[15]

Our results suggest hepatic inflammation in HFD group that can be explained by Cyuela et al. who has stated that intracellular accumulation of fatty acids is associated with development of inflammatory response in addition to mitochondrial dysfunction that causes tissue ischemia.[31] Ischemic injury of the liver is associated with the inflammatory response that involves stimulation of Kupffer cells with subsequent production of proinflammatory cytokine like tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and collections of neutrophils and T lymphocytes.[32] TNF-α and IL-6 are considered main mediators responsible for hepatic inflammation, necrosis and fibrosis.[33] In addition, excess inflammatory PGs production by COX-2 enzyme stimulates hepatocytes for excess formation of triglycerides that accumulate in liver tissue.[8] Furthermore, Zhang et al. found that HFD intake during pregnancy might adversely affect the liver of male offspring through acceleration of fatty acid synthesis; and the damage could extend to second generation.[34] On the other hand, Romeijn et al. demonstrated the effective role of low-calorie diet (LCD) in reducing liver size and weight.[35] The authors added that LCD contains 800 to 1200 kcal/day for at least 5 days. The LCD is less effective than very-low-calorie diet (VLCD) of 450–800 kcal per day. However, LCD is preferred over VLCD to avoid unnecessary dietary restrictions and subsequent negative aspects such as LBM loss and other side effects.

In this work, H&E gallbladder-stained sections of the control group showed mucosa consisted of one layer of simple columnar epithelium with cells having oval nuclei and lamina propria, underlying the mucosa, formed of loose connective tissue. Mucosal folds with central core of lamina propria were seen. These findings are in accordance with that reported by Lindberg and Lamps.[36]

In the present study, stratified epithelium and cellular vacuolations were observed in the gallbladder of HFD group. It was stated that excess cholesterol in bile irritates gallbladder epithelium.[37] This can explain epithelial metaplasia observed. Also, Zaki and Al-Refeidi reported cellular vacuolation and distorted gallbladder epithelium in association with gall stone disease.[38] Van Erpecum et al. stated that histological changes and affection of gallbladder cause a decrease in its ability to concentrate bile and this malfunction may reach up to gall stones formations.[16] HFD might cause gallbladder hypomotility and changes in composition of bile.[39] These could be additional factors for risk of gallbladder stones. Moreover, stress and anxiety during the COVID-19 pandemic that mostly associated with high HFD consumption could increase the incidence of acute calculous cholecystitis.[40]

Our study showed a strong COX-2 immunoexpression in gallbladder mucosal epithelial cells of HFD group which was weak in control group. Statistically the optical density mean values of COX-2 immunoreactions in gallbladder showed a very highly significant (P<0.001) increase in HFD group compared to control group. Our

result of inflammatory reaction is in general accordance with Van Erpecum et al. who reported granulocytes and lymphocytic infiltration in lamina propria.[16] According to Patel et al., HFD intake with elevated cholesterol level can induce gall stone formation and increase level of cholecystitis occurrence.[41] It was stated that inflammation and over production of PGE2 induced by COX-2 production has a cytoprotective role against cholesterol effects, but still, it may not be working as high levels of cholesterol decrease receptor binding capacity with resultant COX-2 overproduction.[42]

Conclusions

HFD could cause detrimental changes in the structure of liver and gallbladder in the form of fatty liver, gallbladder epithelial hyperplasia and inflammatory reaction. Therefore, it might be essential to lower fats in the usual diet. Furthermore, future studies are recommended to explore which drug or protocol can be administered in order to improve the detected changes.

Declarations

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Author contributions

Conceptualization, A.A.H, N.M.Q. and E.M.E.; Methodology, N.M.Q., N.M.A, and E.M.E.; Software, N.M.A.; Validation, A.A.H, N.M.Q. and E.M.E.; Formal Analysis, N.M.A, and E.M.E.; Investigation, All authors; Resources, N.M.A.; Data Curation, N.M.A. and E.M.E.; Writing – Original Draft Preparation, N.M.A. and E.M.E.; Writing – Review & Editing, A.A.H.; Visualization, N.M.Q. and E.M.E.; Supervision, A.A.H., N.M.Q. and E.M.E.; Project Administration, A.A.H., N.M.Q., N.M.A. and E.M.E.

Conflicts of interest

The authors declare no conflict of interest.

Data availability

Data supporting the results of this study shall, upon appropriate request, be available from the corresponding author.

Ethics approval

The study was approved by The Institutional Animal Care and Use Committee Zagazig University (ZU-IACUC); reference number (Zu-IACUC/3/F/167/2019).

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