Possible Protective Role of Capsaicin Against High Fat Diet Effects on Liver and Gall Bladder of Adult Male Mice

Original Article

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ABSTRACT

Introduction: Non-alcoholic fatty liver disease (NAFLD), cholecystitis and gallstones are important problems of public health which are associated with high fat diet intake and hypercholesterolemia. Capsaicin (CAP) is widely used in clinical practice. It can prevent obesity and lower blood lipids.

Aim of the Work: To clarify the possible protective role of Capsaicin against the effects of high fat diet (HFD) on the liver and gall bladder using histological and immunohistochemical examinations and real time polymerase chain reaction (PCR).

Material and Methods: Thirty-two healthy adult male mice were separated into four groups. Control group: Animals were fed normal chow diet for 8 weeks. CAP group: Animals were fed normal chow supplemented with 0.01% CAP. HFD group: Mice were fed HFD for 8 weeks. HFD + CAP group: Animals were fed HFD supplemented with 0.01% CAP for 8 weeks. At the end of the study period, animals were weighed, anesthetized, blood samples were collected and abdomens were opened. Liver and gall bladder were removed for histological preparation. H&E, Masson, Cyclooxygenase-2 (COX-2) immunohistochemically-stained sections and ORO-stained frozen liver section were examined.

Results: Ballooned and degenerated hepatocytes, thickened stratified hyperplastic gall bladder epithelium and inflammatory aggregations were observed in HFD group. Excess collagen fibers and strong positive COX-2 immunoreaction were also seen. Mean values of lipid profile levels, Peroxisome proliferator-activated receptor gamma (PPAR γ) and 3-hydroxy-3-methylglutaryl coenzyme-A (HMG CO-A) reductase gene expression group revealed an increase of very high significant difference from the control group. A decrease in these mean values was reported in HFD+CAP group which was of a very high significant difference from HFD and control groups. Normal hepatic and gall bladder architecture was markedly preserved in HFD+CAP and immunoreaction to COX2 was weak.

Conclusion: CAP is suggested to have a great protective effect against HFD-induced histological changes in the liver and gall bladder through downregulating PPAR γ and HMG CO-A reductase gene expression and by acting as anti-fibrotic and anti-inflammatory agent.

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Key Words: Capsaicin, gall bladder, high fat diet, liver, microscopic anatomy.

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INTRODUCTION

High fat diet (HFD) intake is one of the largest problems that face our communities. It leads to obesity and affection of body organs, including liver and gallbladder. HFD leads to accumulation of lipids in hepatocytes with resultant development of diseases. The prevalence of nonalcoholic fatty liver disease (NAFLD) has been recorded to reach epidemic level in parallel with the increasing prevalence of obesity[1]. It affects about 25% of the population[2]. NAFLD is associated with unhealthy dietary habits such as hypercaloric diets and excessive saturated fats[3].

Cholesterol infiltration in the liver may result in NAFLD, with evident hepatic steatosis (fatty liver disease)[4]. There is an evident correlation between steatosis and truncal obesity, insulin resistance, dyslipidemia, and impaired glucose tolerance. In fact, NAFLD is considered an early signal that indicates great future possibility of developing type 2 diabetes mellitus and also cardiac & vascular diseases[1].

According to Zaki *et al*.[5], lipids accumulation in hepatocytes is the main factor in liver damage. They observed sinusoidal dilatation and ballooning of hepatocytes around central vein and portal vein congestion in livers from rats fed HFD. HFD was found also to cause loss of normal hepatic tissue architecture with degeneration of hepatocytes[6]

High fat diet and hypercholesterolemia are considered major risk factors for cholecystitis and gall stones formation[7] It was reported that high fat diet induced histopathological affection of gall bladder in the form of increased wall thickness with inflammatory infiltration[8]. In addition, López-Reyes *et al*[9] observed hyperplasia of

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the gall bladder epithelium and type change from simple columnar to pseudostratified with expansion of connective tissue in the lamina propria[10].

Gallstone disease is a critical public health problem; it has a prevalence of about 10% in middle-aged individuals and 20% in old aged individuals. Most gallstones have cholesterol as its major constituent[11]. There is a strong association between gall stones and both atherosclerosis and metabolic syndrome[12].

Alteration of gene expression, especially those genes related to the liver has been reported in association with HFD. Jia *et al*.[13] reported overexpression of peroxisome proliferator-activated receptor (PPAR) γ gene in hepatocytes after HFD intake. PPAR γ is a ligand receptor that is responsible for stimulation of growth of fat cells[14].

In addition 3-hydroxy 3-methylglutaryl co-enzyme A (HMG CO-A) reductase, the enzyme that is involved in rate limiting step in the pathway of cholesterol synthesis and is responsible for cholesterol homeostasis, has been found by Ren *et al*.[15] to be over expressed due to feeding with high fat diet.

Capsaicin (8-methyl-N-vanillyl-6-nonenamide) is a compound with a spicy flavor which is present mainly in chili peppers. It has a molecular formula ($C_{18}H_{27}NO_3$)[16]. CAP has been widely used in clinical practice. In cardiovascular studies, capsaicin was found to prevent obesity, induce the apoptosis of cancer cells, lower blood pressure and reduce blood lipids[17]. In addition, CAP through stimulating transient receptor potential vanilloid type 1, can induce secretion of catecholamines from the adrenal medulla to increase thermogenesis and play a role in controlling body weight[18-19].

We studied in a previous work the effects of HFD on liver and gall bladder[20], for that we thought of a substance that can be naturally supplemented to ameliorate HFD harmful effects such as CAP. Therefore, we aimed in this study to clarify possible protective role of CAP against the structural changes that can occur in the liver and gall bladder of mice after administration of high fat diet.

MATERIALS AND METHODS

Chemicals

- Cholesterol ($C_{27}H_{46}O$) (3B-Hydroxy-5-cholestene, 5-Cholesten-3B-ol) in the form of white to off-white crystalline powder (02780, LOBA Chemie, India). Purchased from Saudi company for chemical and medical trading, Ismailia, Egypt.
- Cholic acid ($C_{24}H_{40}O_5$) in the form of white to light brown fine powder with purity 98% (C-02682 Oxford Lab Chem). Purchased from Saudi company for chemical and medical trading, Ismailia, Egypt.
- Animal fats: Buffalo fats were purchased from the local market (Ismailia, Egypt).
- Capsaicin ($C_{18}H_{27}NO_3$) (8-methyl-N-vanillyl-6-nonenamide) in form of capsaicin capsules each capsule containing 20mg of pure capsaicin manufactured by manufactured by "Medverita group company, Poland" it was purchased online. capsules were opened and 4mg CAP powder were added to each 4gm of rodent chow (average daily mouse meal) thus obtain a chow containing 0.01% CAP to be administered ad libitum through meal[21].

Animals and experimental design

The present study was carried on 32 healthy adult male mice, each weighing from 18 - 22 g. They were brought from the animal house unit, Faculty of Veterinary Medicine, Suez Canal University. All mice were housed in environmentally controlled rooms, in wire mesh cages under good hygienic conditions. The temperature was kept around 21-25°C. Animals were let to feed on a balanced diet and tap water for one week before beginning the experiment. The study was approved by "The Institutional Animal Care and Use Committee of Zagazig University"; reference number (Zu-IACUC/3/F/167/2019) and executed according to its instructions. Mice were equally divided into 4 groups (8 animals for each) as following:

- **Control group:** Mice were fed normal chow diet (El Gomhorya company, Ismailia, Egypt) consisting of 67% carbohydrates, 10% fat, and 23% protein for 8 weeks.
- **CAP group:** Mice were fed normal chow diet supplemented with 0.01% CAP which corresponds to dose of 1.33mg/kg body weight/day for 8 weeks[21].
- **HFD group:** Mice were fed HFD; 15% animal fat, 2% cholesterol and 0.5% cholic acid for 8 weeks[22].
- **HFD + CAP group:** Mice were fed HFD supplemented with 0.01% CAP for 8 weeks.

To ensure that mice took the daily requirement of the diet, each animal was separately caged[23].

At the end of the study period, animals were weighed by digital balance, and then anesthetized using thiopental. Venous blood samples were obtained from mice by micro-capillary glass tubes from the retro-orbital venous plexuses[24]. Blood samples of about 2mL were collected in clean test tubes without anticoagulant then the blood was centrifuged at 3000 rpm for 10 minutes for serum separation. The serum was stored at -20°C[25] for performing lipid profile assessment; total cholesterol (TC)[26], triglycerides (TG)[27], high density lipoproteins (HDL)[28] and low-density lipoproteins (LDL)[29] were measured.

The abdomens of the anesthetized mice were opened and dissected carefully. The liver and gallbladder were removed, weighed by the digital balance and immersed in 10% neutral-buffered formalin solution to be fixed for

histological preparation. Parts of liver tissue were frozen at-80 °C for Oil Red O (ORO) staining procedure and real time PCR

Frozen liver tissue, for ORO staining procedure, was directly embedded in Tissue-Tek on the cryostat mold; Tissue-Tek was poured around the tissue with caution. Liver tissue was kept frozen during this process. Tissue was sectioned and 12 µm thick sections were obtained on a glass slide and stained with ORO[30].

Experimental methods

Light microscope techniques

Liver and gallbladder were fixed in 10% formalin for 24 hours to be processed and embedded in paraffin wax according to the procedure described by Hegazy and Hegazy[31] then 5µm thick sections were obtained and stained with Hematoxylin & Eosin (H&E) and Masson Trichrome stains[32].

Immunohistochemical staining for COX-2 (Inflammatory marker)

5µm thick sections were Immunohistochemically stained for localization of cyclooxygenase 2 (COX-2) (DAKO, Germany) using the avidin biotin peroxidase system[33].

Real time PCR (qPCR) detection of PPAR γ and HMG CO-A reductase

The mRNA expression levels, of PPAR γ and HMG CO-A reductase were detected by RT-qPCR. Briefly, total RNA extraction was done using RNeasy Mini Kit (Catalogue no.74104) and cDNA synthesis was performed by using Revert Aid Reverse Transcriptase (Thermo Fisher, EP0441). RT-qPCR was performed using the QuantiTect SYBR green PCR kit (Cat. No. 204141). The primer sequences utilized are shown in (Table 1). Thermal parameters and the amplification cycles were done according to the following: A first denaturation step was performed at 94°C for 15 minutes, then a following 40 cycles at 94°C for 15 seconds, and then a last 40 cycles at 60°C for 30 seconds and 72°C for 30 seconds. The cycle time values of PPAR γ and HMG CO-A reductase were normalized with ß-Actin. Calculation of the target genes relative expression levels was done confronting to ΔΔCt method stated by Yuan *et al*.[34].

Table 1: Primer sequence used in SYBR Green real time PCR

Gene	Primer sequence (5'-3')	Reference
Rat ß. actin	TCCTCCTGAGCGCAAGTACTCT GCTCAGTAACAGTCCGCCTAGAA	Banni *et al*.[35]
PPAR-γ	CATTTCTGCTCCACACTATGAA CGGGAAGGACTTTATGTATGCG	Ismail *et al*.[36]
HMG-COA reductase	CAGCACTGTCGTCATTCATTTCC ACATTCCACCAGAGCGTCAAGG	Morral *et al*.[37]

Morphometrical study

Image analysis and morphometrical assessment were performed by Image J (FIJI) software for measuring the epithelial thickness of the gall bladder in H&E-stained sections, area percentage (%) of collagen fibers in Masson trichrome-stained sections, and optical density of COX-2 immunoreaction and ORO-staining.

Statistical Analysis

Statistical evaluation was performed using Graph Pad Prism 5.01. Quantitative data were expressed in the form of mean ± SD. Differences between the mean values of the studied groups were evaluated by analysis of variance (ANOVA) & turkey post hoc tests. Significant difference was considered when *P value* <0.05.

RESULTS

Light microscopy and morphometrical study

The control and CAP groups showed similar structural appearance with non-significant difference between the two groups regarding the morphometrical parameters. So, we represented the histological results of one group only; the control group.

H&E-stained sections

Examination of H&E-stained liver sections of control group revealed normal architecture of the hepatic lobule with cords of tightly packed hepatocytes radiating from the central vein. Blood sinusoids were seen between hepatic cords. Hepatocytes appeared as polygonal cells with acidophilic cytoplasm and rounded vesicular nuclei. Many cells were binucleated (Figure 1a). A normal hepatic structure in the portal area was observed; portal vein with large lumen and thin wall, hepatic artery with small lumen and a thick wall of smooth muscle and bile ductules lined by cuboidal cells (Figure 1b).

In the HFD group there was a marked disturbance of the hepatic architecture; hepatocytes appeared ballooned; being rounded, swollen with rarified cytoplasm. Empty hepatocytes with ballooning degeneration and necrotic areas were seen (Figure 1c). Dilated congested portal vein and inflammatory aggregations were observed in the portal area (Figure 1d) and between ballooned and degenerated hepatocytes (Figure 2a). In HFD+CAP group, the liver revealed preservation of the normal hepatic lobule structure, but some ballooned hepatocytes were still noticed (Figure 2b). A preservation of the normal hepatic structure in portal area was observed with the portal vein appeared less dilated (Figure 2c).

Examination of H&E gall bladder-stained sections of the control group revealed mucosa formed of single layer of simple columnar epithelial cells with eosinophilic cytoplasm and oval nuclei. The epithelium rested on the loose connective tissue of lamina propria. The mucosa was thrown into folds containing core of lamina propria. Musculosa was formed of interlacing bundles of smooth

muscle fibers. Outer adventitia consisted of dense connective tissue (Figures 3a,b). In the HFD mucosal invagination into lamina propria, thickened hyperplastic stratified epithelium and inflammatory aggregation in lamina propria and musculosa were seen. Also, the musculosa appeared thickened (Figures 3c,d). In the HFD+CAP group there was normal mucosa of simple columnar epithelial cells, but areas with hyperplastic stratified epithelium were seen. (Figures 4a,b).

Statistical analysis of epithelial thickness means values of the gall bladder in the HFD group revealed an increase of significant difference (p <0.05) from the control group. A decrease in epithelial thickness mean values was recorded in HFD+CAP group which showed significant difference (p<0.05) from the HFD group and also from the control group (Figure 4c,Table 2).

ORO-stained liver sections

ORO-stained liver sections of the control group revealed weak red staining of the hepatocytes indicating low lipid content (Figure 5A). In the HFD group a strong red staining of the hepatocytes was observed indicating high lipid content (Figure 5b). While in the HFD + CAP group there was moderate red staining of the hepatocytes indicating moderate lipid content (Figure 5c).

Statistical analysis of optical density means values of ORO-stained liver sections in the HFD group revealed an increase of significant difference (p<0.05) from the control group. A decrease in the optical density was found in HFD+CAP group which showed significant difference (p<0.05) from the HFD group and also from the control group (Figure 5d, Table 2).

Masson trichrome-stained sections

Masson trichrome-stained liver sections of the control group showed little amount of green stained collagen fibers in the portal area (Figure 6a). In the HFD group excess amount of green stained collagen fibers was seen in the portal area (Figure 6b). While in HFD+ CAP group moderate amount of the green stained collagen fibers was observed in the portal area (Figure 6c).

Masson trichrome-stained gall bladder sections revealed collagen fibers only in the adventitia which is an insignificant finding so results were not shown

Statistical analysis of area % of collagen fibers in Masson trichrome-stained liver sections, revealed in the HFD group an increase of significant difference (p<0.05) from the control group. In HFD+CAP group, a decrease in the collagen fibers area % mean values was recorded which was of significant difference (p<0.05) from HFD group and also from the control group (Figure 6d, Table 3).

COX-2 immunohistochemically stained sections

COX-2 immunohistochemically stained liver sections of the control group showed negative immunoreaction in hepatocytes and Kupffer cells which appeared of variable shapes with cytoplasmic processes some Kupffer cells showed weak immunoreaction (Figure 7a). In HFD group a strong positive cytoplasmic immunoreaction to COX-2 in Kupffer cells and hepatocytes was seen (Figure 7b). In HFD+CAP group a moderate positive cytoplasmic immunoreaction reaction to COX-2 and negative immunoreaction in hepatocytes were observed (Figure 7c).

Statistical analysis of optical density mean values of COX-2 in liver showed in HFD group an increase of significant difference (p<0.05) from control group. A decrease in optical density of COX-2 mean values was recorded in HFD+CAP group which was of significant difference (p<0.05) from HFD group and also from the control group (Figure 7d, Table 4).

Examination of COX-2 immunohistochemical gall bladder-stained sections of the control group showed negative immunoreaction in the mucosal epithelial cells (Figure 8a). In HFD group strong positive cytoplasmic immunoreaction to COX-2 in the mucosal epithelial cells was observed (Figure 8b). While in HFD+CAP treated group, moderate positive cytoplasmic immunoreaction to COX-2 in the mucosal epithelial cells was seen (Figure 8c).

Statistical analysis of optical density mean values of COX-2 in gall bladder showed in HFD group an increase of significant difference (p<0.05) from control group. In HFD+CAP group a decrease in optical density of COX-2 mean values was recorded which was of significant difference (p<0.05) from HFD group and also from the control group (Figure 8d, Table 4).

Body & liver weight assessment

There was none significant difference (p>0.05) in the initial body weight mean values among different groups. An increase in the body and liver weight was reported in HFD group which was of significant difference (p<0.05) from the control group. In HFD+CAP group a decrease in liver and body weight was recorded which was of significant difference (p<0.05) from the HFD group; the decrease in body and liver was still of significant difference (p<0.05) from control group (Table 5).

Biochemical assessment of lipid profile

Statistical analysis of lipid profile mean values revealed in the HFD group an increase in cholesterol, Triglycerides & LDL serum levels and a decrease in HDL serum level. These changes in the serum level mean values were of significant difference (p<0.05) from the control group. In HFD+CAP group, a decrease cholesterol, Triglycerides & LDL serum levels and an increase in HDL serum level were recorded. these improvement in the serum level mean values was of significant difference from HFD and also from control group (Table 6).

Real time PCR for PPAR γ and HMG CO-A Reductase detection

Statistical analysis of PPAR γ and HMG CO-A Reductase gene expression mean values revealed in the

HFD group an increase of significant difference (p<0.05) from the control group. In HFD+CAP group a decrease in gene expression mean values was recorded which showed a very highly significant difference (P<0.001) from the HFD group and also from control group (Table 7).

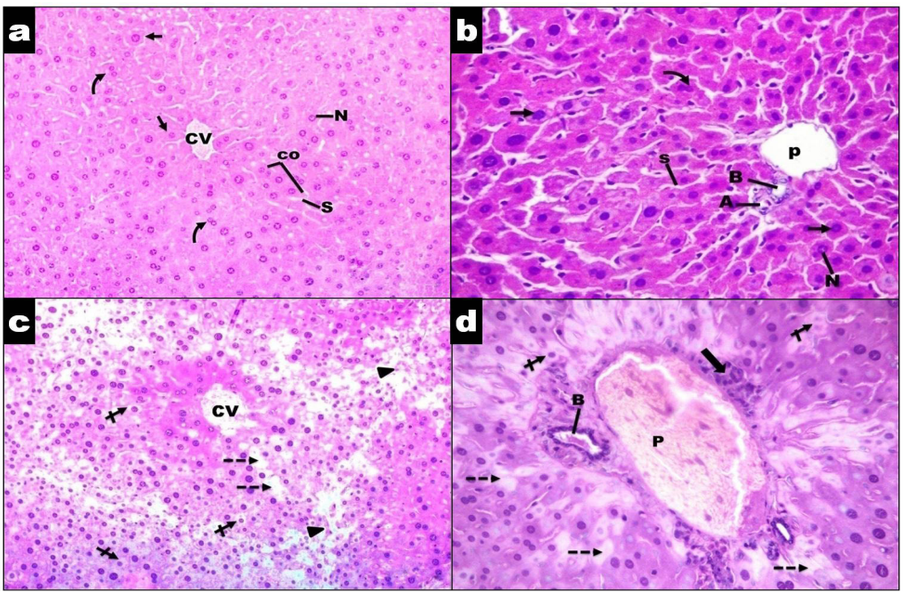

Fig. 1: H&E-stained liver sections a,c (x200), b &d (x400) of adult male mouse of a: Control group showing normal hepatic architecture with cords of tightly packed hepatocytes (co) radiating from the central vein (CV). Blood sinusoids (S) are seen between hepatic cords. Hepatocytes (arrow) appear as polygonal cells with acidophilic cytoplasm and rounded vesicular nuclei (N). Some cells are binucleated (curved arrow). b: Control group showing normal hepatic structure in the portal area; portal vein (P) with large lumen and thin wall, hepatic artery(A) with small lumen and a thick wall of smooth muscle and bile ductules (B) lined by single cuboidal cells. Note, Hepatocytes (arrow) appear as polygonal cells with acidophilic cytoplasm and rounded vesicular nuclei (N). Many cells are binucleated (curved arrow), hepatic sinuses (S) c: HFD group showing marked disturbance of the hepatic architecture; hepatocytes appear ballooned (crossed arrow); being rounded, swollen with rarified cytoplasm. Empty hepatocytes with ballooning degeneration (dotted arrow) and necrotic areas (arrow head) are seen. Note: central vein (CV). d: HFD group showing dilated congested portal vein (P) and inflammatory aggregations (thick arrow) in the portal area. Note: hepatocytes appear ballooned (crossed arrow), empty hepatocytes with ballooning degeneration (dotted arrow), bile ductules (B).

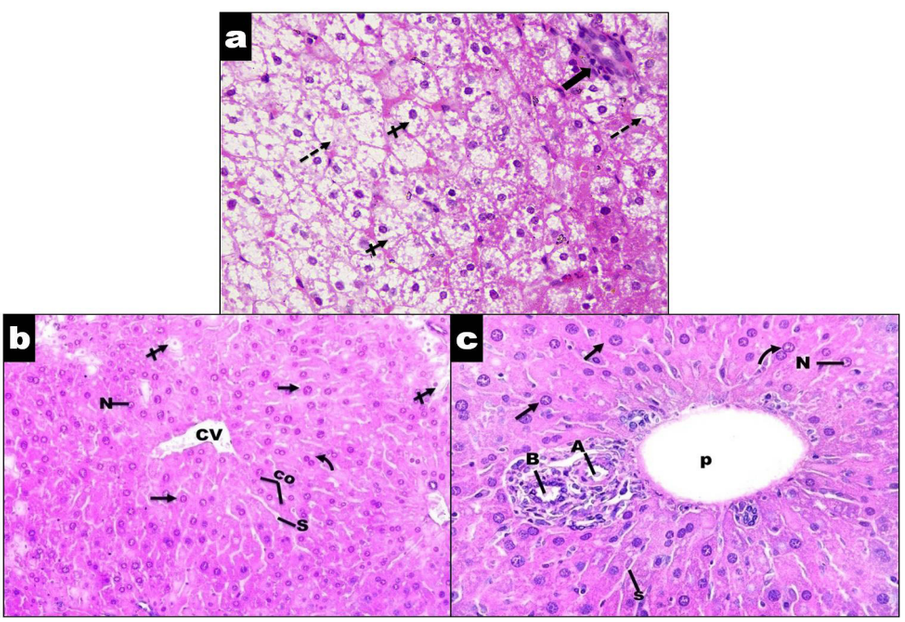

Fig. 2: H&E-stained liver sections a,c (x400), b (x200) of adult male mouse of a: HFD group showing inflammatory aggregations (thick arrow) between ballooned (crossed arrow) and degenerated (dotted arrow) hepatocytes b: HFD+CAP group showing a preservation of the normal hepatic lobule structure with cords (co) of tightly packed hepatocytes radiating from the central vein (CV). Blood sinusoids (S) are seen between hepatic cords. Hepatocytes (arrow) appear as polygonal cells with acidophilic cytoplasm and rounded vesicular nuclei (N). Many cells are binucleated (curved arrow). Some ballooned hepatocytes (crossed arrow) are seen. c: HFD+CAP showing preservation of the normal hepatic structure in portal area; portal vein appears less dilated (P). Hepatic artery (A) with small lumen and a thick wall of smooth muscle and bile ductile (B) lined by single cuboidal cells. Note: Blood sinusoids (S) and Hepatocytes (arrow) as polygonal cells with acidophilic cytoplasm and rounded vesicular nuclei (N). Many cells are binucleated (curved arrow).

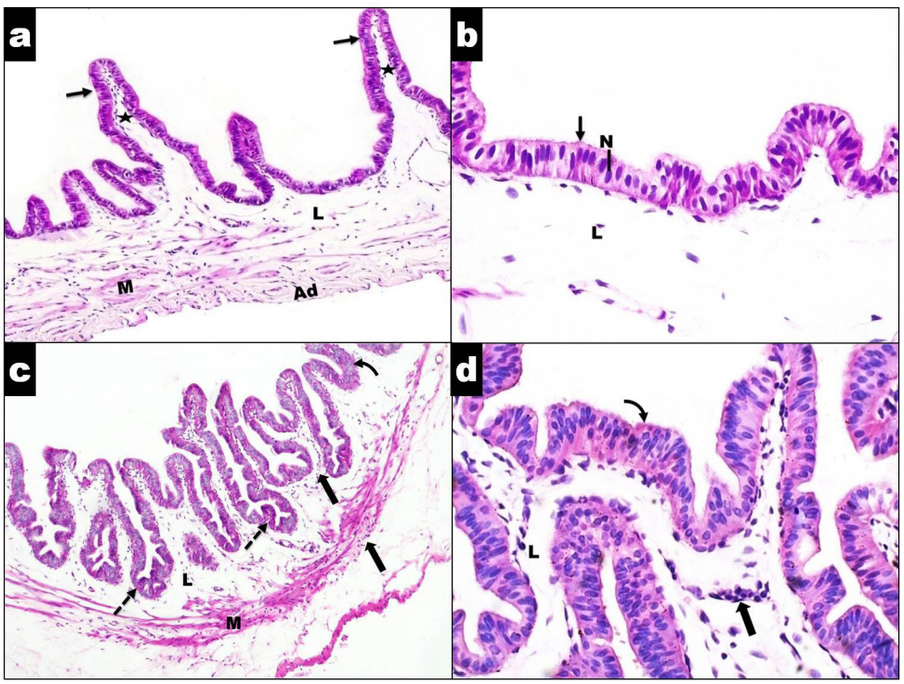

Fig. 3: H&E-stained gall bladder sections a&c (x100), b&d (x400) of adult male mouse of a: control group showing mucosa formed of single layer of simple columnar epithelial cells (arrow) with eosinophilic cytoplasm. The epithelium rests on loose connective tissue of lamina propria(L). The mucosa is thrown into folds (star) containing core of lamina propria. Musculosa (M) is formed of interlacing bundles of smooth muscle fibers. Outer adventitia (Ad) consists of dense connective tissue b: control group showing mucosa formed of single layer of simple columnar epithelial cells (arrow) with eosinophilic cytoplasm and oval nuclei (N). The epithelium rests on loose connective tissue of lamina propria (L).c: HFD group showing mucosal invagination into lamina propria (dotted arrow) and thickened hyperplastic stratified epithelium (curved arrow). Inflammatory Aggregations (thick arrow) are present in lamina propria (L) & Musculosa (M). The musculosa appears thickened d: HFD group showing thickened hyperplastic stratified epithelium (curved arrow). Inflammatory aggregations (thick arrow) are present in lamina propria (L).

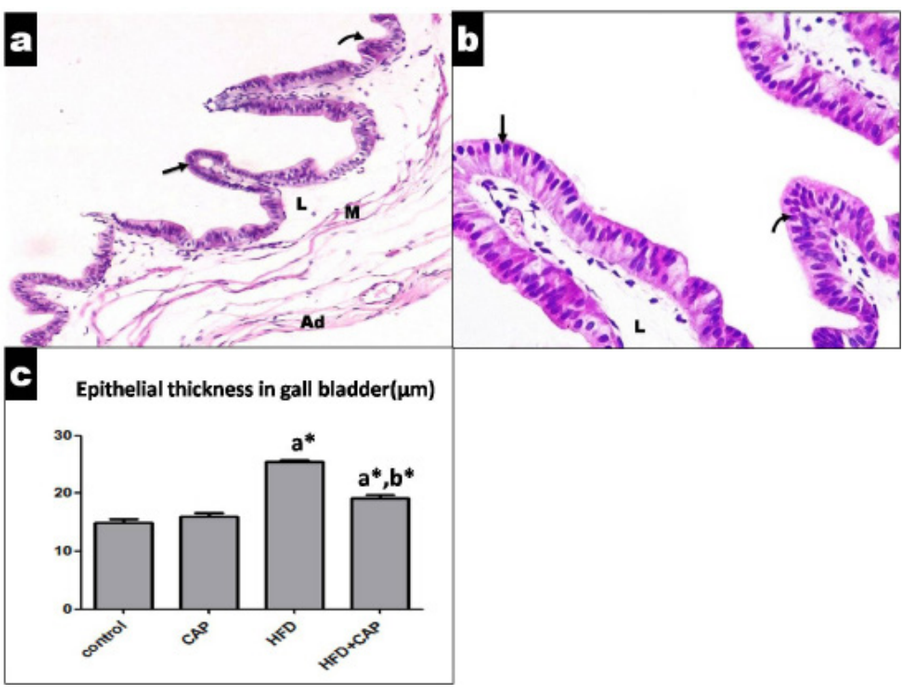

Fig. 4 a&b: H&E-stained gall bladder sections a (x100), b (x400) of adult male mouse of HFD+CAP group showing normal mucosa of simple columnar epithelial cells (arrow) but areas with hyper plastic stratified epithelium are seen (curved arrow). note lamina propria(L), Musculosa (M) adventitia (Ad). c: Bar chart showing Statistical comparison by ANOVA and Turkey post hoc test of the epithelial thickness in gall bladder among different groups. significant (*) when *P value* < 0.05. a: Comparison in relation to control group. b: Comparison in relation to treated group.

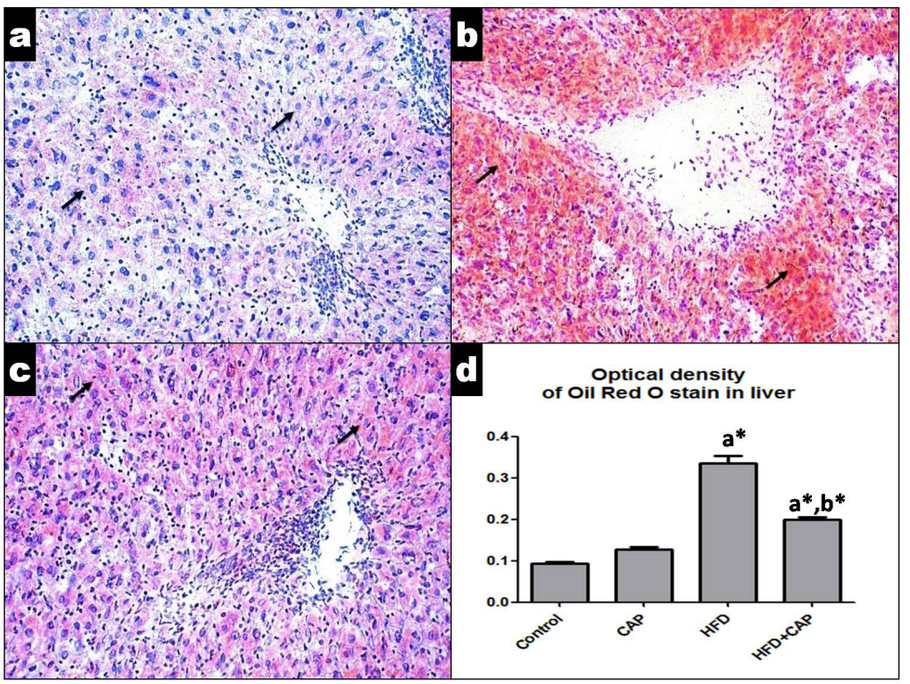

Fig. 5 a-c: ORO-stained liver sections (x200) of the adult male mouse of a: control group showing weak red staining of the hepatocytes (arrow) indicating low lipid content. b: the HFD group showing strong red staining of the hepatocytes (arrow) indicating high lipid content. c: HFD+CAP group showing moderate red staining of the hepatocytes (arrow) indicating moderate lipid content. d: Bar chart showing Statistical comparison by ANOVA and Turkey post hoc test of optical density of Oil Red O stain in liver among different groups. significant (*) when *P value* < 0.05. a: Comparison in relation to control group. b: Comparison in relation to treated group.

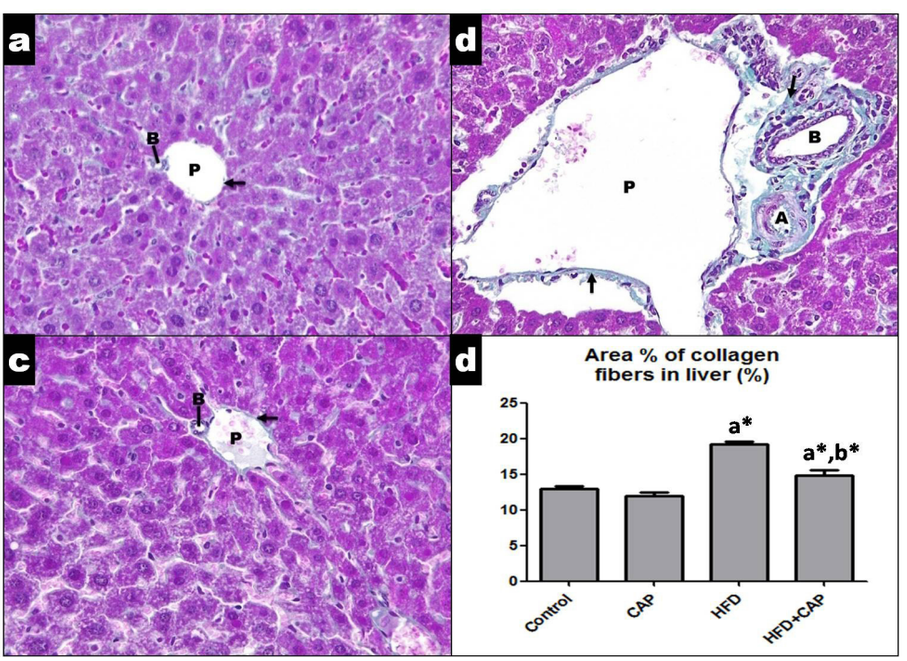

Fig. 6 a-c: Masson Trichrome (MT) -stained liver (x400) of the adult male mouse of a: control group showing little amount of green stained collagen fibers (arrow) in the portal area. Note: Portal vein (P) and bile duct (B). b: HFD group showing the excess amount of green stained collagen fibers (arrow) in the portal area. Note: Portal vein (P), hepatic artery (A) and bile duct (B). c: HFD+CAP group showing moderate amount of green stained collagen fibers (arrow) in the portal area. Note: Portal vein (P) and bile duct (B). d: Bar chart showing Statistical comparison by ANOVA and Turkey post hoc test of optical density of area% of collagen fibers in liver among different groups. significant (*) when *P value* < 0.05. a: Comparison in relation to control group. b: Comparison in relation to treated group.

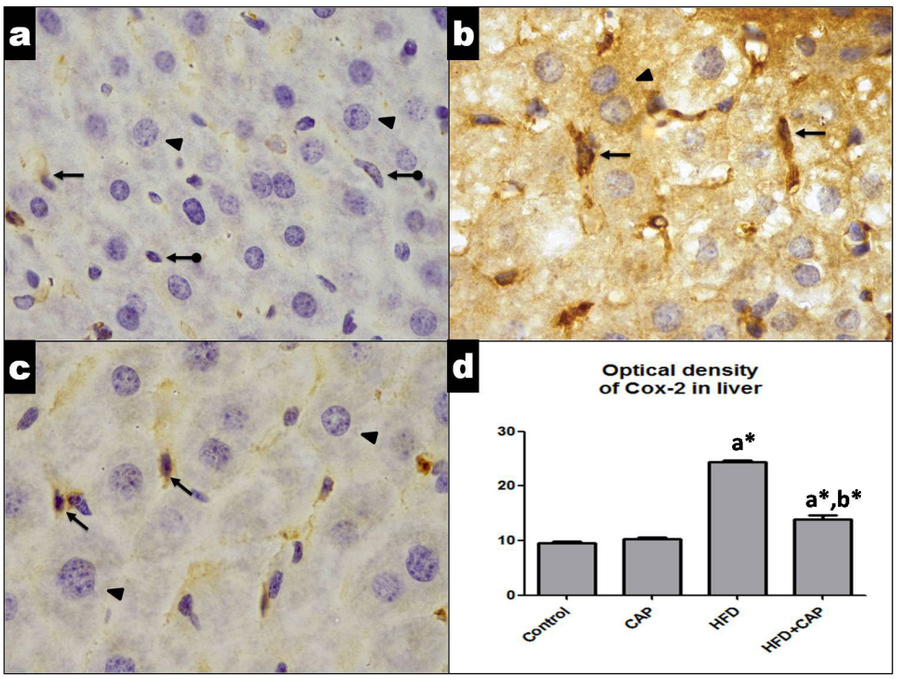

Fig. 7. a-c: Cyclooxygenase 2 (COX-2) immunohistochemically stained liver (x1000) of adult male mouse of a: control group showing negative immunoreaction in hepatocytes (arrow head) and Kupffer cells (round ended arrow) some Kupffer cells show weak immunoreaction (arrow). b: HFD group showing a strong positive cytoplasmic immunoreaction reaction to COX-2 in Kupffer cells (arrow) and hepatocytes (arrow head). c: HFD+ CAP group showing a moderate positive cytoplasmic immunoreaction to COX-2 in Kupffer cells (arrow) and negative immunoreaction in hepatocytes (arrow head). d: Bar chart showing Statistical comparison by ANOVA and Turkey post hoc test of optical density of optical density of COX2 immunoreaction in liver among different groups. significant (*) when *P value* < 0.05. a: Comparison in relation to control group. b: Comparison in relation to treated group.

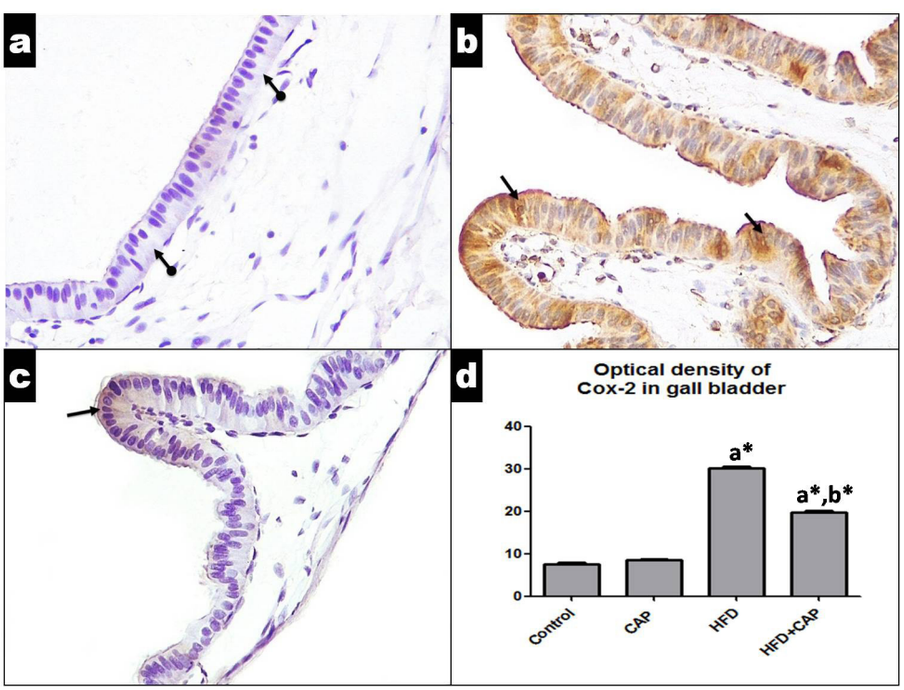

Fig. 8. a-c: Cyclooxygenase 2 (COX-2) immunohistochemically stained gallbladder sections (x400) of adult male mouse of a: control group showing negative immunoreaction (round ended arrow) reaction for COX-2 in the mucosal epithelial cells. b: HFD group showing strong positive cytoplasmic immunoreaction (arrow). for COX-2 in mucosal epithelial cells c: HFD+CAP group showing areas of moderate positive cytoplasmic immunoreaction (arrow) for COX-2 in mucosal epithelial cells. d: Bar chart showing Statistical comparison by ANOVA and Turkey post hoc test of optical density of optical density of COX2 immunoreaction in gall bladder among different groups. significant (*) when *P value* < 0.05. a: Comparison in relation to control group. b: Comparison in relation to treated group.

Table 2: Statistical comparison by ANOVA and Turkey post hoc test of the epithelial thickness in gall bladder and optical density of Oil Red O stain in liver among different groups

Parameter \ Group	Control Mean ± SD (N=8)	CAP Mean ± SD (N=8)	HFD Mean ± SD (N=8)	HFD+CAP Mean ± SD (N=8)	F-Test	ANOVA
Epithelial thickness in gall bladder (Um)	15.02±1.36	16.06 ±1.46	25.6 ±1.03^{a*}	19.17 ±1.6^{a*b*}	91.40	0.001*
Optical density of Oil Red O stain in liver	0.09±.009	0.12±.018^{ns}	.336±.048^{a*}	0.198±.017^{a*b*}	119.022	0.001*

significant (*) when *P value* < 0.05.
a: Comparison in relation to control group.
b: Comparison in relation to treated group.
N: Number of mice

Table 3: Statistical comparison by ANOVA and Turkey post hoc test for the morphometric area % of collagen fibers mean values in liver and gall bladder among different groups

Parameter \ Group	Control Mean ± SD (N=8)	CAP Mean ± SD (N=8)	HFD Mean ± SD (N=8)	HFD+CAP Mean ± SD (N=8)	F-Test	*P- value* of ANOVA
Area % of collagen fibers in liver (%)	12.9±1.35	12.01 ±1.31	19.25 ±0.8^{a*}	14.86 ±1.96^{a*b*}	41.60	0.001*
Area% of collagen fibers in gall bladder (%)	9.41± 1.31	10.51± 1.23	17.41± 0.68^{a*}	12.98± 0.79^{a*b*}	90.65	0.001*

significant (*) when *P value* < 0.05.
a: Comparison in relation to control group.
b: Comparison in relation to treated group.
N: Number of mice

Table 4: Statistical comparison by ANOVA and Turkey post hoc test for the optical density of Cox-2 immunoreaction in liver and gall bladder among different groups

Parameter \ Group	Control Mean ± SD (N=8)	CAP Mean ± SD (N=8)	HFD Mean ± SD (N=8)	HFD+CAP Mean ± SD (N=8)	F	*P- value* of ANOVA
Optical density of Cox-2 in liver	9.62± 0.7	10.36±0.81	24.34±0.88^{a*}	13.89±2.293^{a*b*}	202.4	0.001*
Optical density of Cox-2 in gall bladder	7.46± 1.09	8.44± 1.00	30.09± 1.18^{a*}	19.72± 1.04^{a*b*}	778.0	0.001*

significant (*) when *P value* < 0.05.
a: Comparison in relation to control group.
b: Comparison in relation to treated group.
N: Number of mice

Table 5: Statistical comparison by ANOVA and Turkey post hoc test for body weight and liver weight mean values among different groups

Parameter \ Group	Control Mean ± SD (N=8)	CAP Mean ± SD (N=8)	HFD Mean ± SD (N=8)	HFD+CAP Mean ± SD (N=8)	F-Test	*P- value* of ANOVA
initial body weight (g)	20.20 ± 1.36	20.51 ±1.43	20.58 ±1.5	20.31 ±1.25	0.1280	0.001*
body weight (g)	32±2.84	31.91±2.75	42.2±1.89^{a*}	36.17±2.45^{a*b*}	29.85	0.001*
Liver weight (g)	1.28± 0.12	1.27± 0.10	2.05± 0.19^{a*}	1.53± 0.12^{a*b*}	57.08	0.001*

significant (*) when *P value* < 0.05.
a: Comparison in relation to control group.
b: Comparison in relation to treated group.
N: Number of mice

Table 6: Statistical comparison by ANOVA and Turkey post hoc test lipid profile mean values among different groups

Parameter \ Group	Control Mean ± SD (N=8)	CAP Mean ± SD (N=8)	HFD Mean ± SD (N=8)	HFD+CAP Mean ± SD (N=8)	F-Test	*P- value* of ANOVA
Cholesterol level (Mmol /L)	120.5± 2.7	123.7±3.94	155.3±11.38a*	136.4±7.6a*b*	37.97	0.001*
Triglycerides level (Mmol /L)	115.0±16.67	113.6±17.14	167.6±15.44a*	139.3±5.83a*b*	24.40	0.001*
HDL level (Mmol /L)	63.53±1.8	63.25±1.97	57.55±2.52a*	60.49±1.3a*b*	16.45	0.001*
LDL level (Mmol /L)	29.64± 6.43	30.74± 6.31	67.03± 13.14a*	48.02± 12.28a*b*	24.48	0.001*

significant (*) when *P value* < 0.05.
a: Comparison in relation to control group.
b: Comparison in relation to treated group.
N: Number of mice

Table 7: Statistical comparison by ANOVA and Turkey post hoc test for PPAR γ and HMG CO-A reductase expression among different groups

Parameter \ Group	Control Mean ± SD (N=8)	CAP Mean ± SD (N=8)	HFD Mean ± SD (N=8)	HFD+CAP Mean ± SD (N=8)	F-Test	ANOVA
PPAR γ changes	0.26± 0.03	0.28±0.03	10.96±0.32a*	2.93±0.28a*b*	4501	0.001*
HMG CO-A reductase	0.11± 0.009	0.12± 0.01	8.08± 0.36a*	1.8± 0.24a*b*	2447	0.001*

significant (*) when *P value* < 0.05.
a: Comparison in relation to control group.
b: Comparison in relation to treated group.
N: Number of mice

DISCUSSION

The present work studied the possibility of using capsaicin as protective agent against HFD harmful effects on liver and gall bladder structure. For this purpose, we used male mice as an animal model, as mice have anatomical, functional and genetic resemblance to human in addition to its small size and easy maintenance being a rodent[38]. Mice were preferred over rats as the latter lack gall bladder in their bodies[39]. Moreover, male animals were used to avoid potential effect of estrus hormones of females on the results[40].

In this study, disrupted normal hepatic architecture with variable degrees of changes in hepatocytes were observed in HFD group. Some hepatocytes showed degrees of fatty degenerative changes and necrosis with inflammatory cells infiltrations while others showed cytoplasmic ratification and cellular ballooning. These results are compatible with that of Kim *et al*.[41] Cellular ballooning has been considered principle histological findings of progressive steatohepatitis in NAFLD[42]. This manifests with a progressive hepatic cellular injury which can advance to lytic necrosis[43]. Steatosis is the excess deposition of fats in the liver which involves more than five percent of hepatocytes[44].

We examined ORO-stained liver sections to assess hepatic lipid accumulation; we found in HFD group a strong red staining of the hepatocytes, indicating high lipid content. In addition, an increase in the optical density mean values of ORO staining was recorded in the HFD group which was of significant difference (p<0.05) from the control group. These findings were in accordance with Wu *et al*[45].

Necrotic areas were seen in liver sections of HFD group; high fat diet has been found to be associated with impaired mitochondrial respiration leading to ATP depletion which induces ischemic tissue injury and so release of oxidative markers that leads to cellular membrane rupture and necrosis[46,47].

We observed empty cells with ballooning degeneration in liver sections of HFD group. In fact, swollen ballooned hepatocytes were reported to be enlarged not only by accumulation of lipids but also by osmophilic droplets accumulation. This suggest a defect in cytoskeleton, and it was recorded to be deficient in cytoskeletal protein: Cytokeratin[18]. Furthermore, ballooned cells revealed degenerative changes[48].

Dilatation and congestion of the portal vein recorded in the current work can be attributed to development of portal hypertension; enlarged ballooned hepatocytes compress the hepatic sinusoids leading to its narrowing and subsequent increase in the intrahepatic vascular resistance. Also, hepatic sinusoids lose their fenestrated epithelium (capillarization) as a response to ischemia and hypoxia present in fatty liver disease. This capillarization affect their function and cause portal hypertension[49].

In the present study, a restoration of normal hepatic lobule architecture was reported in HFD+ CAP group with some ballooned hepatocytes were still present. Moderate red staining of the hepatocytes was observed in ORO-stained sections indicating moderate lipid content. This was supported by statistical results which revealed a decrease in the ORO optical density mean values which was of a statistically significant difference from HFD and control groups. Our findings are in agreement with Shin *et al*.[50]

who observed suppression of lipid deposition in the liver after topical treatment with CAP. They stated that CAP could induce inhibition of lipogenesis and gluconeogenesis and elevation of the adiponectin hormone level which degrades lipids.

We observed a strong positive cytoplasmic immunoreaction to COX-2 in Kupffer cells and hepatocytes in COX-2 immunostained liver sections of the HFD group. Kupffer cells are liver macrophages that resides sinusoidal lumen close to the endothelium. Kupffer cells provide immunological function and also share in proteins and lipids metabolism and removal of dead cells[51].

Our immunohistochemical results in addition to the inflammatory infiltration seen in H&E-stained liver sections of HFD group suggest hepatic inflammation. HFD induces saturation of adipose tissue with lipid; it becomes unable to store more lipids and accordingly fat is deposited in other tissues such as the liver. This ectopic lipid deposition results in expression of proinflammatory mediators. In addition, the liver is excessively exposed to proinflammatory cytokines, lipoproteins and free fatty acids that reach the portal circulation from the gastrointestinal tract which has increased permeability to endotoxins due to a change in gut microbiota under the effect of HFD. This leads to activation of Kupffer cells and development of hepatic inflammation[52].

We observed moderate positive cytoplasmic immunoreaction to COX-2 in Kupffer cells in COX-2 immunohistochemically stained liver sections of the HFD+CAP group. A decrease in optical density of COX-2 mean values was recorded in HFD+CAP group which was of statistically significant difference (P<0.05) from the HFD group and also from the control group. This is in accordance with Pi *et al*.[16].

CAP is known to have anti-inflammatory properties[53]. It has the ability to inhibit macrophages infiltration in tissue and was considered beneficial to limit inflammation produced by HFD through controlling fat accumulation in the liver. It was found also to inhibit inflammatory mediators such as TNF- and IL-6[54].

In our work, Masson trichrome- stained liver sections of the HFD group showed excess collagen fibers in the portal area which were little in control group. These results go with that of statistical analysis which revealed an increase in area % mean values of collagen fibers in liver of the HFD group which was of a significant difference from the control group. This is in accordance with Chen *et al*.[55] who stated that HFD stimulate inflammation and fibrosis in hepatic tissue.

NAFLD is a state of chronic inflammation, it was found to stimulate hepatic fibrosis through transforming stellate cells, resident cells of the liver that store vitamin A, to myofibroblast. the last will produce matrix in the extracellular spaces inducing hepatic fibrosis. When fibrosis state is persistent and increasing, cirrhosis occurs[56,57].

In the present study, moderate amount of collagen fibers in the portal area was observed in HFD+CAP group. Moreover, a decrease in area % mean values of collagen fibers was recorded in HFD+CAP group which was of statistically significant difference from HFD group and also from the control group. CAP has been found to induce its antifibrotic effects through suppressing myofibroblasts differentiation[58].

In the present study, hyperplastic stratified epithelium and marked inflammatory aggregations were observed in the gall bladder of the HFD group. Statistical analysis of the gall bladder epithelial thickness mean values revealed in the HFD group an increase of a significant difference from the control group. These results go with that of López-Reyes *et al*.[9] who reported fast structural changes in the gall bladder epithelium after 2 days of cholesterol rich diet. The amounts Fat and cholesterol in diet lead to corresponding changes in the composition of bile a change which may make bile irritant to the bladder mucosa thus inducing such metaplasia[59].

Mucosal prolongation into the lamina propria was observed in the HFD group. This finding is most probably Rokitansky–Aschoff sinuses/crypts. It is an inward proliferation of the gall bladder epithelium that may pass even through bundles of the musculosa reaching the subserosa[60]. Similar observation was reported by Zuhair *et al*.[61] they stated the association of Rokitansky–Aschoff sinuses/crypts with the gall stones and especially cholesterol stones according to them cholesterol is the strongest stimulator for formation of Rokitansky–Aschoff sinuses. it is a benign condition being developed in case of bile outflow restriction, so intra-luminal pressure is increased with resultant inward bulging of the mucosa[62].

Muscular thickening was seen in gall bladder sections of HFD group. Similar results were recorded by Shahid *et al*.[63] Musculosa thickening, hypertrophy and hyperplasia were reported in association with cholesterol gall stone disease[64,65]. Lavoie *et al*.[66] stated that fatty diet caused gall bladder muscular dysfunction and hypertrophy that was insufficient to prevent or accommodate for the induced pathological changes.

In our work, the HFD +CAP group showed normal mucosa of simple columnar epithelial cells, but areas with hyperplastic stratified epithelium were seen. Statistically a decrease in gall bladder epithelial thickness mean values was recorded in HFD+CAP group which was of significant difference from the HFD group and also from the control group. This indicates improvement in gall bladder depending on using CAP.

Our study showed inflammatory aggregations in lamina propria and musculosa in H&E-stained gall bladder sections and strong COX-2 immunoreaction in mucosal epithelial cells of the HFD group which was weak in the control group. Statistical analysis of optical density mean values of COX-2 immunoreaction in gall bladder revealed an increase in the HFD group which was of a significant

difference from the control group. Pi *et al*.[16] reported that COX-2 was associated with gall bladder inflammation, lithogenesis and cholesterol synthesis enzyme activity.

In HFD+CAP group moderate positive cytoplasmic immunoreaction to COX-2 in the mucosal epithelial cells was seen. Statistically a decrease in optical density mean values of COX-2 immunoreaction was recorded in HFD+CAP group was of a significant difference from the HFD group and also from control group. CAP was reported to decrease expression of COX2 in peripheral blood mononuclear cells[67]. In fact, COX-2 antagonists have been found to improve gall bladder inflammation and motility with a good muscle contraction[68].

In the present study, statistical analysis of final mice body weight and liver weight mean values revealed an increase in the HFD group which was of a statistically significant difference ($p<0.05$) from the control group. This result is in accordance with Shin *et al*.[50]. Our work showed a decrease in liver and body weight in HFD+CAP group which was of significant difference from the HFD group and also from the control group. Our results indicated that there was not a marked increase in body and liver weights as in HFD mice. This is in agreement with Shen *et al*.[69].

Weight changes can be explained by the ability of capsaicin to elevate the basal metabolic rate[21]. CAP causes stimulation to TRPV1 receptors that stimulate catecholamines secretions from adrenal medulla to increase thermogenesis and play a role in controlling body weight[18], but on the other side Smeets & Westerterp-Plantenga[70] and Galgani *et al*.[71] reported that CAP had no effect on energy consumption or lipid oxidation.

In this study, an increase in cholesterol, triglycerides & LDL serum levels and a decrease in HDL serum levels were reported in the HFD group which were all of a statistically significant difference from the control group. Similar observations were reported by Pi *et al*.[16] Hyperlipidemia and hypercholesterolemia cause a decrease in the serum level of HDL and an increase in serum levels of LDL and VLDL lipoproteins. HDL returns excess cholesterol to the liver to be secreted in bile through a process called reverse cholesterol transport, while LDL and VLDL lipoproteins carry excess cholesterol. The increase LDL and VLDL lipoproteins, induced by Hyperlipidemia and hypercholesterolemia, is produced through decrease expression of LDL hepatic receptors with resultant decrease in LDL removal from the circulation and accordingly increased risk of exposure to NAFLD and atherosclerotic diseases[72,73].

In our study, statistical analysis in HFD+CAP A decrease cholesterol, Triglycerides & LDL serum levels and a increase in HDL serum level was recorded. This improvement in lipid profile mean values revealed a statistically significant difference from both HFD and also control group. Effect of capsaicin in lowering serum levels of cholesterol could be explained by its ability to cause oxidation and decomposition of fat[74].

Statistical analysis of PPAR γ gene expression mean values in liver showed in the HFD group an increase of significant difference from the control group. This result is in accordance with Chen *et al*.[75] PPAR γ is a ligand receptor that is present in adipose tissue and liver cells especially expressed in hepatocytes. It is responsible for stimulation of fat cells growth. PPAR γ over expression induces increase in lipid storage and triglycerides level. Inflammatory mediators and cytokines that share in the pathology of NAFLD are also increased by PPAR γ overexpression. In addition, it was found that PPAR γ is incorporated in gall bladder inflammation and cholesterol synthesis. It is suggested that blocking this gene receptors helps in treatment of NAFLD[14,76].

A decrease in PPAR γ gene expression mean values was recorded in HFD+CAP group which showed a statistically significant difference from the HFD group and also from control group; this is in agreement with Mosqueda-Solís *et al*.[77] Inhibition of PPAR γ causes removal of excess lipid droplets, decrease lipogenesis and help in control of NAFLD[14].

On the other hand, statistical analysis of HMG CO-A reductase expression mean values, revealed in the HFD group an increase of significant difference from the control group. This is compatible with Ren *et al*.[15] Increased activity of HMG CO-A reductase was found to be associated with hypercholesterolemia[7]. HMG CO-A reductase is an enzyme that plays an important role in de novo synthesis of cholesterol by conversion of HMG CO-A to mevalonate. HMG CO-A reductase overexpression has positive feedback on cholesterol synthesis, cholesterol accumulation in hepatic cells in addition to blood and gall stones formation. HMG CO-A reductase down regulation is one of the most important steps in the control of cholesterol level and cholesterol production which help in NAFLD control[15,76,78].

Our work showed A decrease in PPAR γ gene expression mean values in HFD+CAP group which exhibited a statistically significant difference ($P<0.05$) from the HFD group and also from the control group. This is concomitant with Choi *et al*.[79] and Pi *et al*.[16] The down regulation of PPAR γ and HMG CO-A reductase gene expression induced by CAP explains the restoration of the hepatic architecture in the HFD+CAP group observed in H&E-stained sections, the less hepatic lipid content as detected by ORO-stained sections and the marked improvement of the lipid profile as proved by statistical comparison with the HFD group.

In the light of our results, it can be suggested that HFD induced histological changes on the liver and gall bladder in the form of fatty liver, epithelial hyperplasia of the gall bladder, collagen deposition and inflammatory aggregations. CAP has been found to have a great protective effect against these effects by down regulating PPAR γ and HMG CO-A reductase gene expression and acting as an anti-fibrotic and anti-inflammatory agent.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

الدورالوقائي المحتمل لمادة كابسيسين ضد تأثيرالغذاء عالي الدهن علي تركيب الكبد والحويصلة المرارية في ذكورالفئران البالغة

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المقدمة: تعد أمراض الكبد الدهني غير كحولي والتهاب الحويصلة المرارية وحصوات المرارة من أكثر الأمراض إرتباطا بتناول الأغذية عالية الدهن ، تلك التي تعد سببا او عاملا رئيسيا في العديد من المشكلات المتعلقة بالصحة العامه لما يترتب عليها من ارتفاع في مستوي الكوليسترول بالدم. وتعتبر مادة " كابسيسين " والتي يتم استخلاصها من الفلفل الأحمر والمستخدمة في الممارسات الاكلينيكية (السريرية) ، مادة فعالة تعمل علي تقليل مستوي الدهون بالدم ومن ثم تساعد في علاج السمنه .

الهدف : تهدف الدراسة الحالية الي الكشف عن الدورالوقائي لمادة كابسيسين ودورها في خفض مستوي الدهون بالدم الناتجه عن تناول أغذية عالية الدهن وما يترتب عليه من تأثيرات ضاره علي الكبد والحويصلة المرارية ، وذلك من خلال عمل مجموعه من الفحوصات النسيجية والهستوكيميائية المناعية ووضع ترجمة للجينات المؤثره.

المواد و الطـرق المستخدمة: تم تطبيق الدراسة الحالية علي اثنين وثلاثين من ذكورالفئران البالغة بعد تقسيمها إلى أربع مجموعات متساوية في العدد بحيث تتضمن كل مجموعه عدد ثمانية من الفئران البالغة ويمكن توضيح ذلك فيما يلي :المجموعة الضابطة : تلقت فئران هذه المجموعة النظام الغذائي الطبيعي طوال فترة التجربة ثمانية أسابيع.المجموعة المعالجة بالكابسيسين: تلقت فئران هذه المجموعة النظام الغذائي الطبيعي وماده الكابسيسين بنسبة ٠,٠١٪يوميا لمدة ثمانية أسابيع.المجموعة المعالجة بتناول أغذية عالية الدهون : تلقت فئران هذه المجموعة غذاء عالي الدهن لمدة ثمانية أسابيع.المجموعة المعالجة بتناول أغذية عالية الدهون وماده الكابسيسين : تلقت فئران هذه المجموعة غذاء عالي الدهن و ماده الكابسيبين بنسبة ٠,٠١٪عن طريق الفم يوميا لمدة ٨ أسابيع.وفي نهاية التجربة تم وزن الفئران ثم تخديرهم لسحب عينات الدم وشق البطن واستخراج الكبد والحويصلة المرارية واعدادهما للفحص بالمجهر الضوئي وصبغهم بالهيماتوكسيلين والأيوسينو "صبغة الماسون ثلاثية الألوان" بالاضافه الى الصبغة الهستوكيميائية المناعية مع صبغ الكبد بصبغة النفط الاحمر.

النتائج: أوضحت نتائج الدراسة: إنتفاخ الخلايا الكبدية وتراكم الدهون بها في شكل فجوات سيتوبلازمية ، كماوجد إلتهاب خلوي ونخر فى أنسجة الكبد وذلك في المجموعة المعالجة بتناول أغذية عالية الدهون، وتم ملاحظة زيادة في سمك النسيج الطلائى للحويصلة المرارية. كماأظهرت الدراسة أيضا زيادة فى الألياف الغروية بالكبد باستخدام صبغة الماسون الثلاثية.كما تم تسجيل تفاعلا إيجابيا قويا داخل سيتوبلازم الخلايا الكبدية وسيتوبلازم النسيج الطلائى للحويصلة المرارية بإستخدام الصبغة الهستوكيميائية المناعية وذلك في المجموعة المعالجة بتناول أغذية عالية الدهون. وقد تم تسجيل من خلال القياسات المورفومترية زيادة ذات دلالة إحصائية في مستوي الدهون بالدم ،ومستوي التعبير

الجيني لجين بابار جاماPPAR gamma وجين ٣-هيدروكسي-٣-ميثيل-جلوتاريل- كو- ا- المختزل HMG) CO-A reductase) في المجموعة المعالجة بتناول أغذية عالية الدهون، بينما اظهرت المجموعه المعالجة بالكابسيسين بالتزامن مع المجموعة المعالجة بتناول أغذية عالية الدهون انخفاضا دال احصائيا في تلك المتوسطات بالمقارنة مع المجموعة المعالجة بتناول أغذية عالية الدهون والمجموعات الضابطة ايضا وجود التركيب النسيجي الطبيعي للكبد والحويصلة المرارية مع وجود تفاعلا إيجابيا ضعيف للصبغة الهستوكيميائية المناعية لكوكس-٢ (COX-٢)بالمجموعة المعالجة بالكابسيسين بالتزامن مع تناول أغذية عالية الدهون.

الخلاصة: في ضوء ما توصلت اليه نتائج الدراسة الحالية نخلص إلي أن للكابسيسين تأثيرا وقائيا كبيرا ضد التغيرات النسيجية التي يسببها الغذاء عالي الدهن علي الكبد والحويصلة المرارية من خلال تنظيم التغييرات الجينية والعمل كمضاد للإلتهاب ومضاد للتليف