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New Insights on Nigella Sativa's Protective Effect against High Fat Diet Induced Alteration in Small Intestine and Liver of Rats: a Biochemical and Histological Study

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ABSTRACT

Background*: Excessive consumption of High Fat Diet (HFD) harmfully impacts body tissues and organs. Interestingly, there is a high concern towards the use of medicinal plants to ameliorate those harmful effects.* ***Objectives:*** *This study is aimed at investigating the effective possibility of Nigella Sativa (NS) seeds powder on liver and small intestine of the rats fed on HFD using biochemical, histological and morphometric techniques.* ***Material and Methods****: Eighteen adult male albino rats were randomly divided into three equal groups. Group I (control) was fed on standard rat pellets chow, Group II (HFD) was fed on standard diet mixed butter (20% fat of diet) and Group III (HFD + NS) was fed on HFD and concomitantly administrated Nigella sativa (300 mg/Kg daily orally) for 8 weeks. The biochemical study included lipid profile assessment and the histological study included paraffin sections of small intestine and liver stained by Hematoxylin and Eosin, Masson-trichrome for liver collagen and PAS for intestinal Goblet cells to evaluate the histological alteration. Quantitative statistical analysis of area percent of liver collagen content and goblet cells was done using Digital pro-image analysis.* ***Results****: HFD was associated with increased serum lipid profile. The histological analysis of hepatic sections revealed abundant fat deposition, inflammatory cell infiltrate, degeneration of hepatocytes with significant increase of collagen fibers as shown by image analysis. Inflammatory changes with significant reduction in the mean area percent of Goblet cells were observed in intestine of HFD group. NS intake significantly lowered serum level of total cholesterol, triglycerides and LDL, in concomitant with reversed HFD-induced histological alteration by decreasing hepatic collagen deposition and increasing intestinal goblet cells.* ***Conclusion****: Biochemical, histological and morphometric results provided further evidence that crude NS seeds powder can ameliorate high fat diet–induced alteration in liver and small intestine suggesting its beneficial use in preventive medicine.*

Key words: *High Fat Diet, Nigella Sativa, Liver, Small Intestine, Collagen, Goblet Cells.*

INTRODUCTION

It is known that increased consumption of diet with high fat content is associated with drastic clinical problems and it can be considered as a challenge for public health in developed and developing countries. It is highly correlated with obesity, which in turn can lead to insulin resistance and type II diabetes mellitus besides increased risk of cardiovascular diseases. In addition, it has been linked to some immune mediated diseases [1, 2]. Recent studies pointed to the link between consuming high fat diet and variable disorders affecting the digestive tract and its associated glands especially liver and pancreas. Liver is the largest vital gland maintaining homeostasis of the human body. Many studies illustrated the damaging impact of HFD on liver, such as *non-alcoholic* fatty liver disease (NAFLD), which can progress to increase oxidants production [3, 4]. The pathological changes of NAFLD start with steatosis and may lead to worse situations like steatohepatitis, cirrhosis, liver failure and hepatocellular carcinoma, and thus increased hepatic related mortality [4, 5]. Medical treatment for NAFLD is still inefficient. Lifestyle modifications and increased physical activity for weight loss and natural product intake are promoted as primary management [6].

Food digestion and absorption are crucial functions carried out by the small intestine. The functional integrity of the small intestine requires coordinated specialized features of its different components such as enterocytes, goblet cells and the layer of mucous, resident microflora and immune response [7]. However, excessive intake of HFD impacts the intestinal physiology and its microbiota by disturbing the intestinal epithelial barrier integrity and intestinal permeability and lowers the tight junction proteins, leading to leaked gut microbial products and stimulating the immune inflammatory process [8, 9].

Despite the considerable advance in current medicine, traditional herbal medicines are still widely used by public. Nigella sativa (Black Cumin) is an annual Ranunculaceae herbaceous plant grown in many parts of the world. It has been used as a flavor spice added to food in the Mediterranean region and the Middle East. Since ancient ages, people have considerrf it a miracle traditional herb for treating many diseases referred by the Arabic, Islamic and Indian popular medicine [10]. In Africa and Asia, it is a useful treatment for bronchial asthma [11]. Crude NS seed contains many active ingredients of which volatile oil and thymoquinone (TQ) are the most effective components as they have anti-inflammatory, antioxidant, anti-aging, antidiabetic and hypolipidemic effects besides other incredible benefits [12-16]. Based on the above-mentioned evidence, this study aimed to focus insight on the efficacy of using whole seed powder for amelioration of HFD impact on rat liver and small intestine using evidence of biochemical, histological and morphometric assessments.

MATERIAL AND METHODS:

The present experiment was conducted in King Fahd Medical Research Center at King Abdulaziz University, Jeddah, Saudi Arabia.

Eighteen adult male albino rats with average body weight 180-200 gm were acclimatized to lab conditions (12/12 hour dark/light cycle) and housed in controlled temperature of 22±2 C^o. They had free access to standard rat pellets and water. After one week, the rats were sorted randomly to three equal groups, 6 rats each.

Group 1 (control) was fed on standard rat pellets; while, group 2 (HFD) was fed on high fat diet includes standard rat pellets mixed with 20 % animal fat (19 gm animal butter +1 gm of soybean) per 100 grams of diet. After three weeks, serum lipid profile was assessed to ensure the occurrence of hyperlipidemia [17].

Group 3 (HFD+NS) was fed on high fat diet and concomitantly administrated NS seed powder, to provide 19.34 kj/g of diet, suspended in water at a dose of (300 mg/kg/BW) given daily by gastric tube [18, 19]. The duration of the whole study was 8 weeks after which blood was taken following deep ether anesthesia via retro-orbital venous plexus. Sera were subjected to lab analysis of triglycerides, cholesterol and LDL using the commercial kits as mentioned in [20].

For histological study, abdomen of animals was opened and livers and small intestines (jejunum regions) were excised and flushed with normal saline then fixed in 10% formalin for 48 hours, processed for paraffin embedding. Five microns thick sections were stained by Haematoxylin and Eosin stain (H&E) for general histological assessment. Masson′s trichrome stain was used to demonstrate collagen fibers contents in liver tissues. Periodic acid chief (PAS) stain was used to demonstrate mucopolysaccharides contents of intestinal goblet cells.

Morphometric analysis:

Leica Qwin 500 LTD image analysis was applied to determine the mean area percent of collagen fibers in Masson trichrome stained liver sections, and the mean area percent of PAS–stained Goblet cells in small intestinal sections. Ten low-power non-overlapping fields per slide from five slides of each animal were selected at random, assessed and expressed as a mean percentage of total area.

Statistical analysis

Statistical analysis was performed on SPSS package (version 16). Data were presented as the Mean ± SE (standard error). Differences between the study groups were detected by one-way analysis of variance (ANOVA) and post hoc comparisons and LSD test were also applied. P values <0.05 were considered statistically significant.

RESULTS:

The biochemical results:

The biochemical analysis revealed statistically significant increase in the levels of triglycerides, cholesterol and LDL-C in the high fat diet group as compared to the control group. N. sativa supplementation in Group III was

associated with significant decrease in triglycerides, cholesterol and LDL when compared with Group II (Table 1).

The histological results of the liver:

The control liver sections stained by H & E showed normal hepatic architecture (Figure 1) radiating as plates from the central vein. The plates were branching and anastomosing. The irregular boundary of hepatic lobule was ill-defined and bordered by portal tracts and sparse collagenous tissue and central vein location. Hepatocytes appeared as polyhedral cells with large round nuclei and prominent nucleoli. Occasional binucleated cells were present. Blood sinusoids could be demonstrated between hepatocyte cell plates. Masson trichrome stain demonstrated scanty fine collagen fibers adjacent to the central veins and around portal tract components (Figure 2).

Examination of H & E stained sections of group II (HFD) revealed disturbed normal hepatic appearance (Figure 3). Cytoplasmic vacuoles of variable sizes were evident within most hepatocytes. Mononuclear cellular infiltrate was seen between and at the site of degenerated parenchymal hepatocytes. The central vein region showed accumulation of fat droplets of various sizes within hepatocytes in perivascular region. Nuclear changes where many nuclei looked smaller and dark stained (Pyknosis) were observed. Hepatocytes looked shrunken with dark stained acidophilic cytoplasm (features of cell apoptosis) and necrotic foci with cell debris. Portal area (PA) showed congested portal veins (PV); while, bile duct (BD) showed karyolitic lightly stained nuclei and periductal inflammatory cell infiltrate. A marked increase in dark stained apoptotic cells was also evident.

In Masson trichrome stained sections, an apparent deposition of collagen fibers around central veins and within portal area triads is more prevalent compared to the control (Figure 2).

The liver sections of group III (HFD+NS) were stained with H & E demonstrated restored normal hepatic histological features (Figure 3). Most hepatocytes showing acidophilic granular cytoplasm with absence of vacuolar changes were seen in HFD group. The nuclei are vesiculars with normal appearance. Few cells were still having small cytoplasmic vacuoles. The central veins as well as blood sinusoids showed mild congestion. Obvious decrease of mononuclear cell infiltrate was observed in most sections.

Masson trichrome stained sections, of this group, revealed obvious decrease in collagen fibers contents either around the central vein or among portal area triads (Figure 2).

The histological results of the small intestine:

The sections of small intestine of the control stained by H & E revealed normal histological appearance. The wall consisted of three layers; mucosa with its characteristic villi and crypts, lamina propria and muscularis mucosa, submucosa and musculosa. Intestinal villi were covered by enterocytes and goblet cells. The villus core showed eosinophils and lymphocytes. In PAS –stained sections, Goblet cells were identified by their magmata red colored mucous content (Figure 5).

Examination of H & E-stained intestinal sections of group II (HFD) showed focal areas of sloughed mucosal surface in some sections. Others revealed distorted villi with denuded surface and shed out enterocytes into the lumen. Vascular congestion with blood extravasation was clearly noted within villus core. The cells with highly acidophilic cytoplasm and small dark stained nuclei (Pyknosis) were seen, indicating cell apoptosis. Some fused villi were also prominent. Intense mononuclear cellular infiltrate in lamina propria of villus core was evident. In the PAS-stained sections, a marked reduction was noticed in the goblet cells of intestinal villi (Figure 6).

The hematoxyline and eosin-stained sections of group III (HFD+NS) showed noticeable reversion of most histopathological changes in Group II. Apparently, normal villi with covering enterocytes and goblet cells were seen. Reduced inflammatory mononuclear cellular infiltrate within villus core was also evident. There was no signs of congestion or extravasation. In PAS –stained sections, Goblet cells could be recognized covering the villi together with enterocytes in a similar pattern to control sections (Figure 7).

The morphometric quantification analysis results:

In the small intestinal sections of control rat, the mean area percentage of collagen fibers was (6.13 ± 0.71). A significant statistical increase was observed in the mean area percentage of collagen fibers in high fat diet (Group II) which was (26.62±2.98). In Group III (received N. sativa), the mean area percentage of collagen fiber was (6.65±0.43), which was insignificant. Regarding the mean area percentage of goblet cells, they were significantly decreased in Group II (HFD) compared to the control (5.14±0.42 vs. 10.06±0.62); while, in the group III sections, the decrease was insignificant compared to the controls (9.89±0.54 vs. 10.06±0.62).

DISCUSSION:

Black seeds (*N. sativa*) powder was reported to have many biologically active compounds that were further identified to be highly antioxidants agent [21]. Moreover, the anti-inflammatory, anticancer, anti-diabetic, anti-hypertensive and hypolipidemic actions were previously reported [10, 12, 14-16, 22].

In the present study, the hypolipidemic effect of N. Sativa was investigated if it can ameliorate the injurious effects of HFD on rat liver structure as well alteration in structure and polysaccharide content of intestinal mucosa cells. For further investigation, assessment of serum lipid levels was also done. It was observed that HFD intake was associated with significant higher lipid profile parameters compared to the control values. In agreement with the present results, a significant increase was previously stated of total cholesterol and LDL-C in high diet fed mice over control [7, 23].

In this 8-week study, whole seed crude powder significantly declined the level of cholesterol, triglycerides, and LDL in Group III as compared to HFD Group. This beneficial effect was consistent with a previous study done on male and female rats but over longer duration (12-24 weeks), with favorable results in both sexes [24]. Similarly, lipid profile of cardiac patients given N. sativa regularly for six months was significantly lower than those treated with statin for the same period [25]. The decrease in the serum level of TC, LDL-C, TGs and HDL-C demonstrated in this study was also stated by others after thymoquinone intraperitoneal injection in rats [13].

The hypolipidemic effect of N. sativa is possibly attributed to its active ingredient Thymoquinone (TQ), which possesses powerful antioxidant effect. It was previously reported that the treatment with TQ decreased the serum level of lipid peroxidation marker, malondialdehyde (MDA) and increased the serum total antioxidant capacity (TAC) [26].

Recently, it has been stated that the protective and therapeutic properties of N. sativa for ameliorating oxidative stress caused by HFD were referred to its positive effect on the uncoupling Protein-1 (index protein of the brown adipose tissue) at the gene and protein levels [27]. This study revealed that consuming such HFD (20% fat) for 8 weeks induced intrahepatic fat deposition, relevant steatosis and inflammation in rats. Histological examination of liver sections of Group II (HFD) showed disturbed normal hepatic architecture. Hepatocytes with cytoplasmic vacuoles were seen ranging in size from small (microvesicular steatosis) up to large (macrovesicular steatosis). Hepatocytes with deeply stained pyknotic and lysed nuclei were also noted. Similar histopathological changes were previously described by others as non-alcoholic steatohepatitis induced by high fat diet [28]. This may be caused by accumulation of triglycerides, when the level of fatty acids is increased and it exceeds the oxidative capacity of hepatocytes. The increased intracellular fatty acids may lead to cytotoxicity [29].

The presence of inflammatory mononuclear cell infiltrate, dilated congested central veins, blood sinusoids and portal veins as well as hepatocytes necrosis or apoptosis as sequels of inflammation seen in the sections of this group could be considered as diagnostic signs of steatohepatitis [30]. This progressive inflammatory damage induced by HFD is associated with increased proinflammatory cytokine IL-6 and TNF-α [31].

Morphometric analysis of Masson trichrome–stained sections revealed significant increase in the mean area percent of collagen fibers in liver sections of HFD group. They were seen around central veins as well as between components of portal tract. The current results are consistent with recent findings that reported similar histological results suggestive of steatosis and portal fibrosis in case of rats fed HFD and developed type 2 diabetes [32]. It has been found that peri-portal fibrosis is usually encountered in HFD-induced oxidative stress [33].

Microscopic examination of Group III hepatic sections, demonstrated the advantageous impact of the daily intake of N. sativa seed powder. Most of hepatocytes looked similar to those of the control as evidenced by having granular acidophilic cytoplasm and lightly stained active nuclei. Reduction of mononuclear cell infiltrate around slightly congested central veins and sinusoids was also observed. In addition, Masson trichrome stain for collagen fibers revealed decrease in their deposition around central veins and within portal tracts of this treated group compared to the liver of non-treated GII rats. Other authors, investigated the effect of N. sativa on experimentally induced liver fibrosis. Their results revealed that it effectively reduced fibrotic changes and attributed this effect to its antioxidant effect and free-radical scavenging activity [34]. A recent randomized double-blind clinical study revealed that patients with non-alcoholic fatty liver disease who received N. sativa oil versus placebo for 8 weeks, the lipid profile and liver enzymes decreased in NS group. It was suggested that the mechanism of decreasing liver fibrosis is via reducing the inflammation [35].

Progression of some liver diseases to fibrosis may be caused by the free radicals and lipid peroxides. Liver cirrhosis was initially considered as an irreversible process, but nowadays it is thought to be partly reversible [36]. Many studies stated that TQ acts as an antioxidant; thus, prevents the oxidative stress both in vivo and in vitro. This effect is achieved by inhibiting both lipid peroxidation and production of eicosanoids such as thromoxane B2 and leukotriene B4 [37].

In the current study, intense mononuclear cellular infiltrate noted in the lamina propria of distorted intestinal villi with vascular congestion and fusion of some villi, pointed to local inflammation in HFD group. The present findings go in hand with what was reported in a previous study conducted on C57BL /6 mice fed with a HFD, where histopathological analysis of ileum sections, revealed inflammatory cellular infiltrate [7]. Moreover, HFD supplementation in the rats resulted in an upregulation of both IL-10 and IL-1β, but not TNF-α in the intestinal tissues and their draining mesenteric lymph nodes [38]. In addition, the changes in the gene expression of the inflammatory and immune response mediators in the small intestine and distributed lipid metabolism are induced by HFD [39]. On the contrary, HFD intake in mice resulted in intestinal eosinophil depletion and not inflammation unlike liver or adipose tissue which may contribute to defective integrity of intestinal barrier [40]. This discrepancy may be explained by lack of proper standardization of HFD used or types of fats used whether animal or vegetable fat and its exact quantity.

The PAS-stained intestinal sections of Group II (HFD) demonstrated reduction of goblet cells; accordingly, the mucous production may be decreased. The changes in the proportion of immune cells and mucus secretion may lead to defective mucosal protective barrier [41]. Similar reduction in number of goblet cells was reported in HFD-induced obesity model in mice, associated with induced specific increase of stem cells in intestinal crypts in vivo, but with impaired intrinsic survival and growth in vitro [42, 43].

Endotoxins production by intestinal microflora and proliferation of Gram-negative pathogenic bacteria in case of increased fat diet intake consequently decreased PAS positive goblet cells either due to goblet cell exhaustion after prolonged infection or the immune system decreasing mucin production to reduce the possible nutrients for bacteria [44].

The examined intestinal sections of HFD treated group revealed distorted villi that have denuded surface with shedding of enterocytes within lumen. The daily intake of N. sativa reversed this effect, causing re-lining of villi as clearly shown in Group III intestine. Similar beneficial effect of N. sativa alcoholic extract was noted in domestic chicken experimentally infected with *Eimeria maxima* manifested as restoration of damaged intestinal tissues and relining of intestinal epithelium [45].

CONCLUSION:

The present study revealed that high fat diet has deleterious effects on the biochemical parameters for serum lipids and the histological structure of both small intestine and liver. Concomitant intake of Nigella sativa (NS) seeds along with high fat diet had potential favorable ameliorative effect on both histopathological and biochemical alterations. These results encourage further preclinical investigations on human cases in order to consider its use in preventive medicine.

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Table 1: Serum lipid profile of the studied groups (n=6)

	G I (Control)	G II (HFD)	G III (HFD + NS seeds)
Triglyceride (mg/dl)	71.9 ±1.4	109.2 ± 0.95*	61.7 ± 1 **
Cholesterol (mg/dl)	85.2 ± 1.6	211.8 ± 1.3*	102.4 ± 1.4**
LDL-C (mg/dl)	46.7 ± 1.4	153.5 ± 1.1*	64.6 ± 1.4**

* Significances versus G1 (Control).
** Significances versus GII (HFD).
Differences were considered significant as *P* values were < 0.05

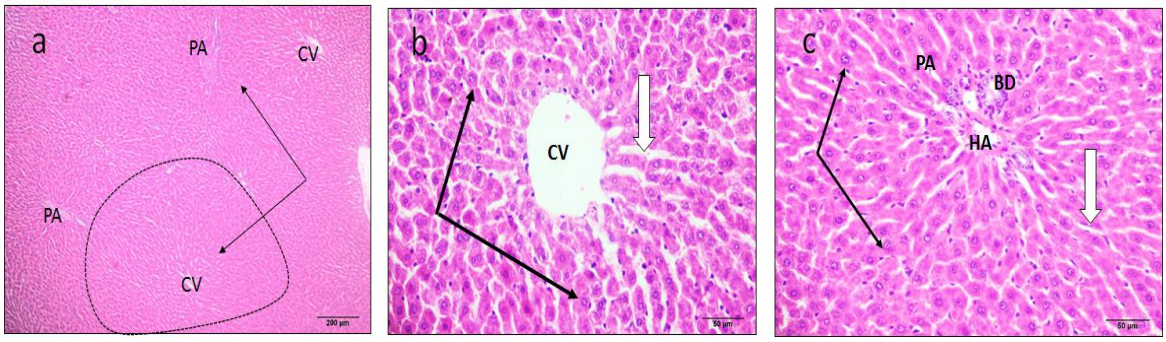

Figure 1: Liver sections of Group I (Control) stained by H&E:
a- Low power (x100) showing ill-defined liver lobules (dotted circle) identified by presence of central vein (CV) and peripherally located portal areas (PA).
b- High power (x400) showing central vein region (CV) with hepatocyte cell cords (black arrows) radiating from the central vein (CV) and separated by blood sinusoids (white arrows).
c- High power (x400) showing portal area (PA) with regular normal hepatocyte cell cords (black arrows), blood sinusoids (white arrow), bile duct (BD) and a branch of hepatic artery (HA).

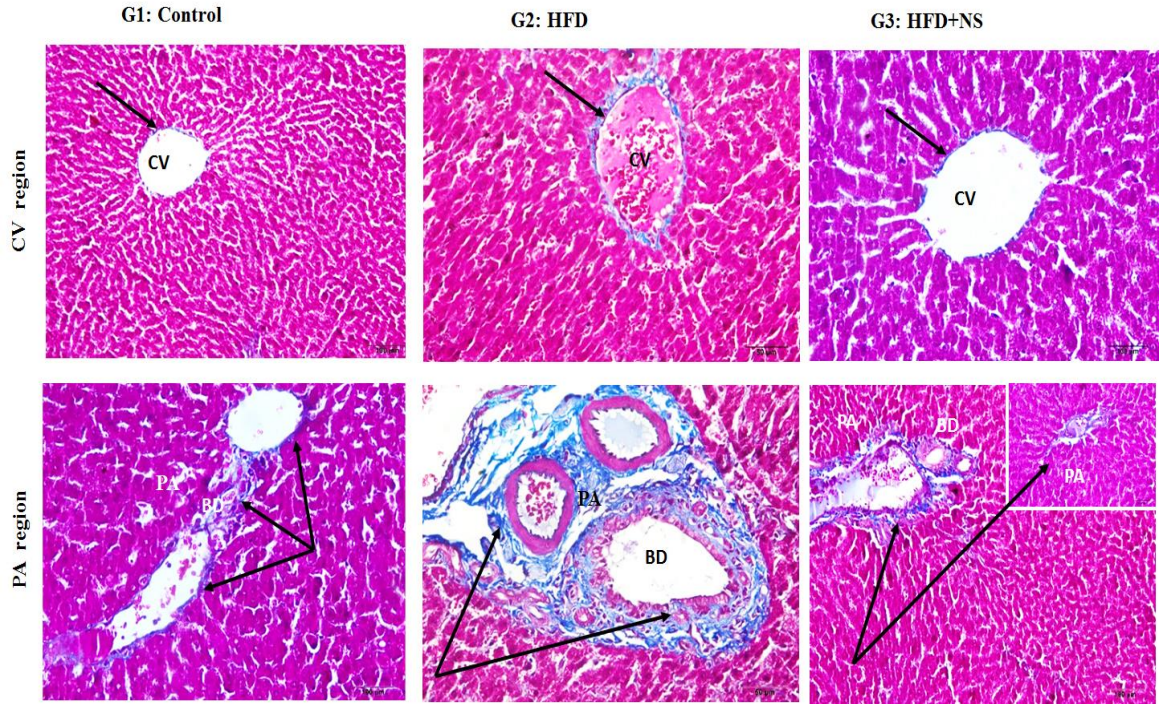

Figure 2: Liver sections of all Groups stained by Masson trichrome for collagen fibers showing:
G1: Control group: showing fine scanty collagen fibers (arrows) around central vein (CV) and portal area (PA).
G2: HFD group: showing an increase in collagen fibers (arrows) in both central vein (CV) and portal area (PA) and bile duct (BD).
G3: HFD+NS group: showing decrease in collagen fibers (arrows) in both regions.

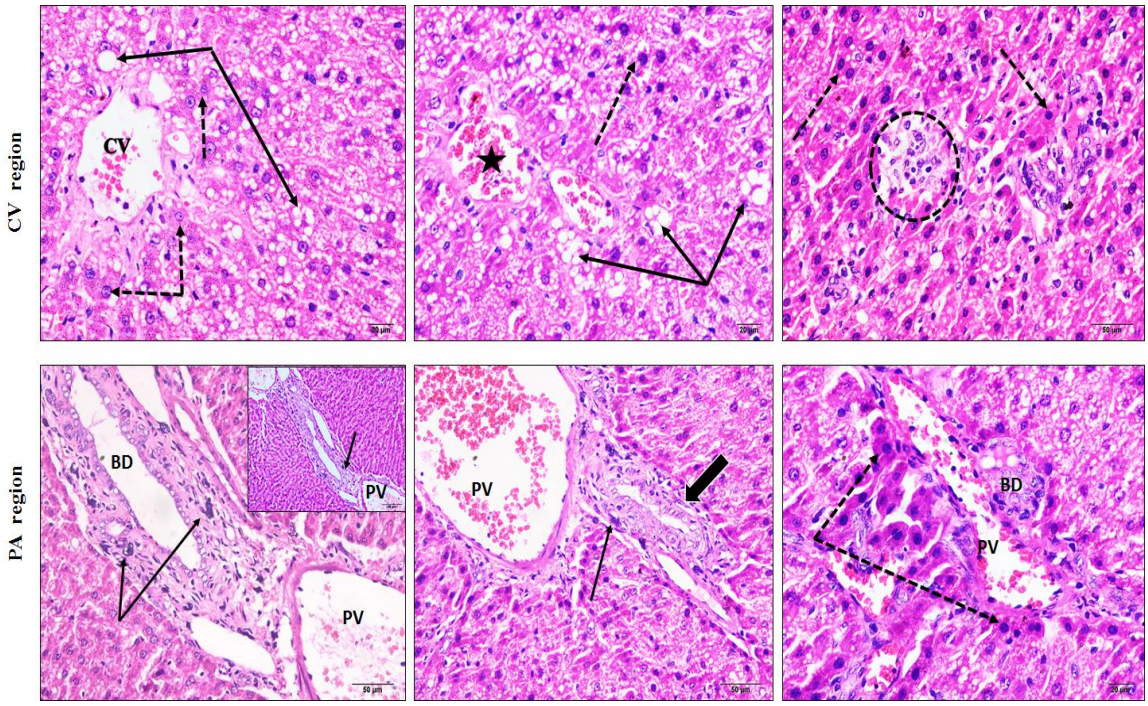

Figure 3: Liver sections of Group II (HFD) stained by H&E showing:

a. Sections of rat liver around and near central vein region (CV) showing:

Accumulation of fat droplets of various sizes (unstained) within hepatocytes in perivascular CV region (black arrows). Nuclear changes as many nuclei (dotted arrows) looked smaller and dark stained (Pyknosis), hepatocytes looked shrunken with dark stained acidophilic cytoplasm (features of cell apoptosis). Congested CV regions (star) or presences of necrotic cells with inflammatory cell aggregations (dotted circle).

b. Sections of rat liver at portal area region (PA) showing:

Dilated and congested portal vein (PV), dilated bile duct (BD) with karyolitic changes of their nuclei (black arrows). Periductal fibrosis (stars) and mast cell infiltrates (thick black arrows). Marked increase in periportal hepatocyte apoptosis (dotted arrows) with dark stained cytoplasm and small dark nuclei.

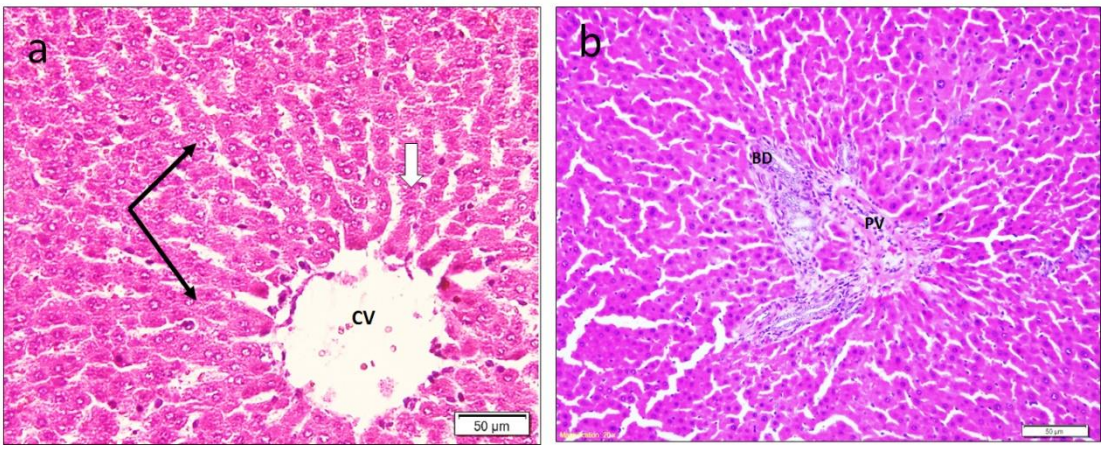

Figure 4: Liver sections of Group III (HFD+NS) stained by H&E showing:

a- Central vein region (CV) showing nearly normal hepatocytes (black arrows) with absence of lipid droplets and nuclear changes observed in HFD group.

b- Portal area with normal portal triad contents (BD: bile ducts, PV: portal vein)

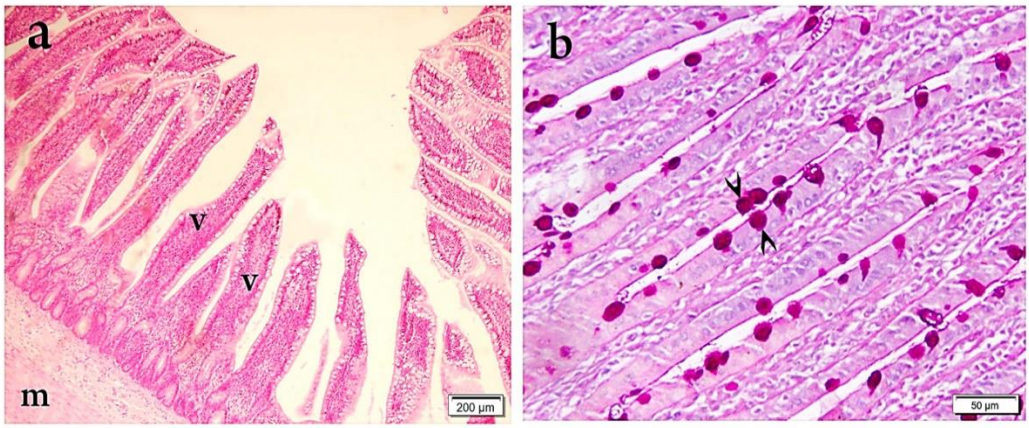

Figure 5: Small intestinal sections of Group I (Control) showing:
a- Mucosa with its characteristic villi (v) and crypts, submucosa and musculosa (m).
b- Goblet cells with red color mucous (arrowheads).
(a-H&E x40, b-PAS x200)

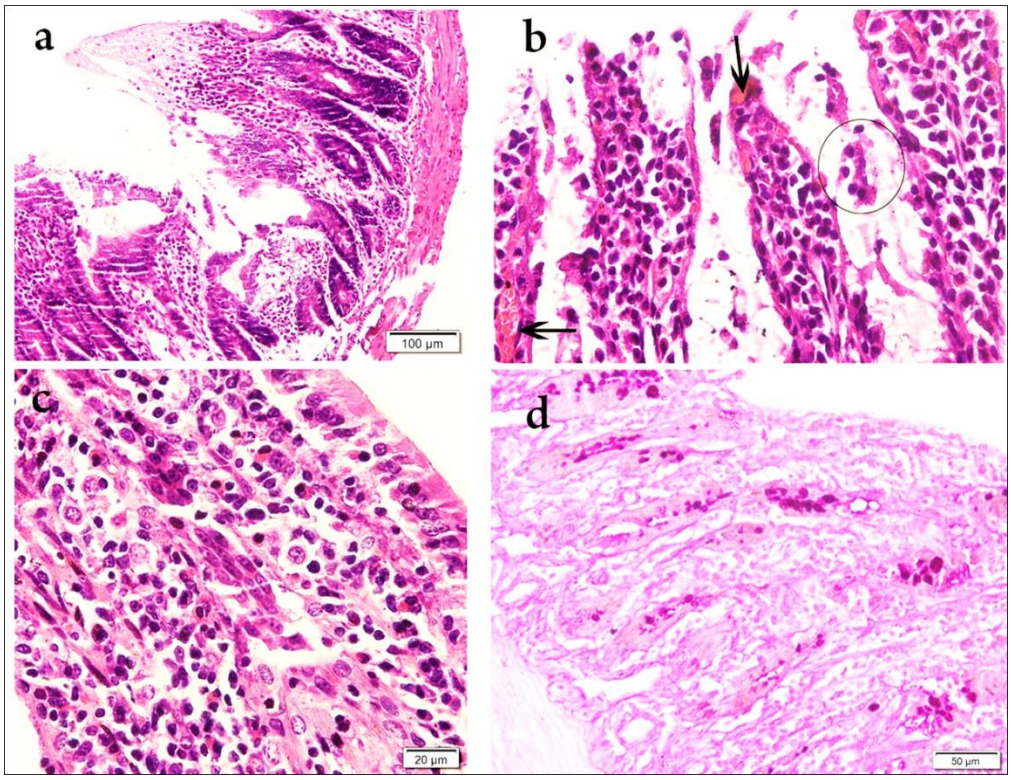

Figure 6: Small intestinal sections of Group II (HFD) showing:
a- Focal area of sloughed mucosal surface.
b- Distorted villi with denuded surface and shedded enterocytes into lumen (circled). Congested blood vessels within villus core are seen (arrows).
c- Fused villi with intense mononuclear cell infiltrate.
d- Reduced Goblet cells are noted.
(a-H&E x100, b-H&E x400, c-H&E x400, d-PAS x200)

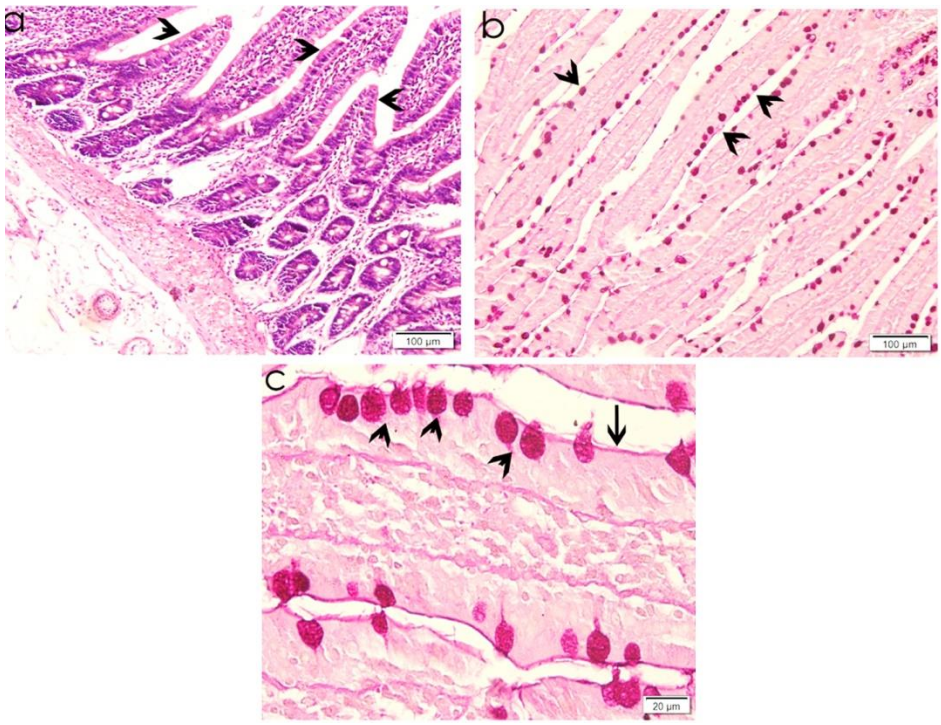

Figure 7: Small intestinal sections of Group III (HFD+NS) showing:

a- Apparently normal villi with covering enterocytes (arrowheads).

b- and c- Goblet cells were recognized in PAS-stained sections (arrowheads) with clear brush border membrane (arrow).

(a-H&E x100, b-PAS x100, c-PAS x400)