The outcome of ketogenic diets on the liver and the protecting role of atorvastatin: A histological, immunohistochemical and ultrastructural study

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SUMMARY

The current study investigated structural and functional modifications of the liver following long-term ketogenic diet (KD) and every-other-day ketogenic diet (EODKD) usage. The probable role of atorvastatin (ATO) in the adjustment process was also investigated. It was carried out on 24 Sprague-Dawley rats, which were divided into four groups: control, KD, EODKD, and ATO. Various biochemical, histological, and immunohistochemical analysis were performed on the liver. The blood was tested for aspartate aminotransferase(AST),alanineaminotransferase (ALT), triglycerides, cholesterol, inflammatory markers, Bax, BCL2, NLRP3 inflammasome, and oxidative stress markers.

KDs induced damages to the liver mainly due to oxidative stress (increase TBARS, MDA/decrease SOD/GSH) and inflammation. In addition, the hepatic triglycerides and cholesterol levels are decreased. The KD group was worse off than the EODKD group. ATO administered concurrently with KD preserved liver architecture, reduced oxidative stress, normalized NLRP3, and further reduced intrahepatic triglycerides and cholesterol levels. Both KD and EODKD cause structural liver damage that is accompanied by an elevation of hepatic markers (AST and ALT) and a decrease in hepatic triglycerides, hepatic cholesterol, and serum cholesterol. KD has a more destructive effect. Oxidative stress, inflammation, and high fat concentrations are contributing factors to hepatic injury in these diets. ATO with KD is beneficial.

Keywords: Ketogenic diet – Every other day ketogenic diet – Liver – Atorvastatin – Rat

LIST OF ABBREVIATIONS:

BAX: Bcl-2-associated X protein.

BCL2: *B-cell lymphoma 2*.

CD11b: cluster of differentiation molecule 11b.

GSH: Glutathione.

HMG-CoA reductase: 3-hydroxy-3-methylglutaryl-Coenzyme A reductase.

MDA: Malondialdehyde.

NAFLD: non-alcoholic fatty liver disease.

NLRP3: The NACHT, LRR and PYD domains-containing protein 3.

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SDS- PAGE: sodium dodecyl sulfate- polyacrylamide gel electrophoresis.

SOD: superoxide dismutase.

TBARS: Thiobarbituric Acid Reactive Substances.

INTRODUCTION

The ketogenic diet (KD) has gained popularity among the public and scientific communities as a way to lose weight (Bolla et al., 2019). Additionally, they can alleviate several neurological illnesses, including Parkinson's disease, traumatic brain injury, Alzheimer's disease, autism, and Lou Gehrig's disease (Deng-Bryant et al., 2011; Hertz et al., 2015; Shaafi et al., 2016). KDs with very low carbohydrates can have effective weight-loss interventions that may reduce non-alcoholic fatty liver disease (NAFLD) and visceral adipose tissue, leading to decreased insulin resistance and end-organ damage (Marchesini et al., 2016). Additionally, very low-carbohydrate KDs are used as adjunctive therapy for brain cancer (Garbow et al., 2011). As a final note, the every-other-day KD (EDOKD) keeps seizures under control better (Hartman et al., 2013).

The KD is characterized by a high fat intake, moderate-to-low protein intake, and very low carbohydrate intake (<50 g) (Barrea et al., 2022). The fat-to-non-fat ratio in KD is 4:1 (Milder et al., 2010). After using KDs for a few days, the production of energy is dependent upon burning fat, with increased production of ketone bodies (Paoli et al., 2013). In addition, KDs promote the oxidation of dietary and adipose lipids (Barañano and Hartman, 2008).

However, these diets can pose several problems. In one study, mice fed with a KD over a period of 12 weeks accumulated hepatic lipids sequentially. The mice eventually developed endoplasmic reticulum stress, macrophage accumulation, cellular damage, and steatosis (Garbow et al., 2011). However, no research has been yet conducted on the specific structural and functional changes in the liver related to long-term KD.

Atorvastatin (ATO) was the most popular lipid-lowering drug in the early 2000s. It works by inhibiting the enzyme HMG-CoA reductase, a key component of cholesterol synthesis. This drug belongs to the statins family, which are applied as lipid-decreasing agents (Kogawa et al., 2019). Recently, some researchers reported promising results of ATO in the treatment of fatty liver disease (Gómez-Domínguez et al., 2006). However, studies are lacking on whether ATO prevents the damaging effects of KDs on the liver.

The purpose of the present research is to investigate the relationship between structural and functional liver changes following long-term KD and EODKD use. In addition, the causal mechanisms in hepatic affection were examined. The probable role of ATO in the adjustment process was also investigated.

MATERIAL AND METHODS

Twenty-four Sprague-Dawley rats weighing 150-200 g were used. They were housed in standard cages and acclimatized in the laboratory for two weeks before starting the study.

In keeping with the ethical standards set by the National Institutes of Health guide for the care and use of laboratory animals, this work was approved by the Ethics Committee at Cairo University (2545/2020).

Our rats were monitored for morphological and behavioural changes every day, and their health profiles were recorded. Four groups of six rats each were arranged:

- Control group: rats were fed standard chow (SC) (Holland et al., 2016).
- KD group: rats were fed KD (Holland et al., 2016).
- EODKD group: in the first 24 hours, the rats fasted. They then accessed KD every other day (second, fourth, sixth, etc.). Thus, they alternated fasting and feeding days (Hartman et al., 2013).
- ATO group: ATO was given concomitant with KD (Ji et al., 2011). ATO was purchased from the EIPICO Chemical Company in Cairo, Egypt, and given via gastric gavage in doses of 6 mg/kg/day (Schmechel et al., 2009).The essential nutrients of SC and KD were presented in Table 1 (Holland et al., 2016).

Table 1. Sources of macronutrients for each diet.

	KD	SC
Calories, g	5.2	3.1
CHO, % of calories	10.3	58
Protein, % of calories	20.2	24
Fat, % of calories	69.5	18
Fatty acid breakdown, % of fat • **Saturated fat** • **Monounsaturated** • **Polyunsaturated fat**	 70 13 17	 16 23 61
Cholesterol, % by weight	Not specified	Traces

Ketogenic diet (KD); Fat: MCT oil, flaxseed oil, and canola oil; Protein: casein as well as added L-cysteine; Carbohydrate: maltodextrin and cellulose; Standard chow (SC); Fat: soybean oil; Protein: soybean meal and corn gluten with added L-lysine and L-methionine; Carbohydrate: ground wheat and ground corn.

We measured the body weight (BW) of the rats at the beginning and at the end of the experiment. Blood was taken from the tail of the rats for biochemical analysis, followed by their sacrifice after 8 weeks.

The liver was dissected and weighted for calculating the relative body weight. Parts of the liver were processed for histological study, and other parts were processed for electron microscopy study and western blot analysis.

Light microscopic study

Hematoxylin & Eosin (H&E) and Masson's trichrome stained: specimens were fixed in 10 % neutral buffered formalin and were processed to prepare 5 µm thick paraffin sections (Suvarna et al., 2019).

Analysis of the blood parameters

The plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured according to Monteiro et al. (2016).

An analysis of hepatic triglycerides, hepatic cholesterol and serum cholesterol was performed using the commercially available kit according to Schwartz and Wolins (2007).

Hepatic oxidative markers

Malondialdehyde (MDA) and Thiobarbituric Acid Reactive Substances (TBARS) were assessed conferring to the method of Oktay et al. (1995).

Glutathione (GSH) content and superoxide dismutase (SOD) activity were evaluated corresponding to Weydert and Cullen (2010) using the commercial kit (Biodiagnostic, Egypt).

Immunohistochemical study

Paraffin sections were dewaxed, rehydrated, and incubated in 3% hydrogen peroxide solution for 30 minutes to block endogenous peroxidase activity. In the next step, heat-mediated antigen retrieval was performed, and tissue antigen was retrieved using a microwave. The tissue sections were then cleaned and immersed for 5 minutes in phosphate buffered saline (PBS). Following PBS removal, serum was applied to the sections for 30 minutes at room temperature.

1. **Caspase-3:** the sections were incubated with primary anti-active caspase-3 antibody (Cat #: ab208161, Abcam, Cambridge, MA, USA), followed by secondary antibody labelled streptavidin biotin (LSAB) kit, Dako Carpentaria, CA, USA) (Bancroft and Gamble, 2008).
2. **Bax, Bcl-2:** The sections were incubated with the primary antibody Bcl-2, Bax (anti-human Bcl-2 protein, DakoCytomation, Denmark), followed by the secondary antibody (biotinylated link universal from the commercial kit LSAB: DakoCytomation, Denmark). The positive reaction for Bcl-2 and Bax showed a brown color of the cytoplasm (Panasiuk et al., 2006).

Image analysis and morphometric measurements

The content of collagen fibres and the expression of Bcl-2 and Bax were achieved using Leica LAS V3.8 image analyser computer system (Switzerland).

Western Blot

The liver was homogenized in Radioimmunoprecipitation assay (RIPA) buffer and centrifuged. Proteins were then loaded onto precast SDS-PAGE gels, then transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with rabbit anti-NLRP3 (1:500), ra-

bbit anti-β-actin (1:2000), and rabbit anti-CD11b antibody (1:2000). Then, the membranes were incubated with secondary antibodies. Immunoreactive proteins were imaged by an excellent chemiluminescent substrate (ECL) kit (GE Healthcare, Amersham, UK using iBright Imaging System (Yang et al., 2017; Qu et al., 2019).

Electron Microscopic examination

The sections were set according to Hayat (2000) and shot using a Joel, 100 CX II TEM.

Statistical analysis

We used SPSS 22 to conduct our analysis. We estimated the statistical significance of the data using ANOVA followed by a Bonferroni pairwise comparison.

RESULTS

Losses in the liver weight and body weight in the KD and EODKD groups

The mobility and health status of the groups were generally good. We detected no mortality in each group.

At the beginning of the study, the body weight (BW) was 180±15.6 g. At the end of the study, the BW increased in control and ATO groups to 250±15 and 220.1±13.1 respectively while it decreased in KD and EODKD groups to 154.4±10.6 and 164.8±12.9 respectively (Table 2, Bar chart 1).

A significant decrease of the relative liver weight was detected in KD and EODKD groups to 0.1±0.07 and 0.17±0.06 respectively as compared to the control group. The weights in ATO and control groups were comparable 0.3±0.01, 0.4±0.01 respectively (Table 3, Bar chart 2).

Table 2. Comparison of the body weight among the studied groups. The body weight at the beginning of the study was 180±15.6 g.

Body weight (g)	I-Control		II-KD		III-EODKD	IV-ATO
Mean ±SD	250±15		154.4±10.6		164.8±12.9	220.1±13.1
F-value	22.6					
Overall P-value	0.01					
Pairwise comparisons	II&I 0.01*	III&I 0.03*	IV&I 0.01*	III&II 0.5	IV&II 0.04*	IV&III 0.9

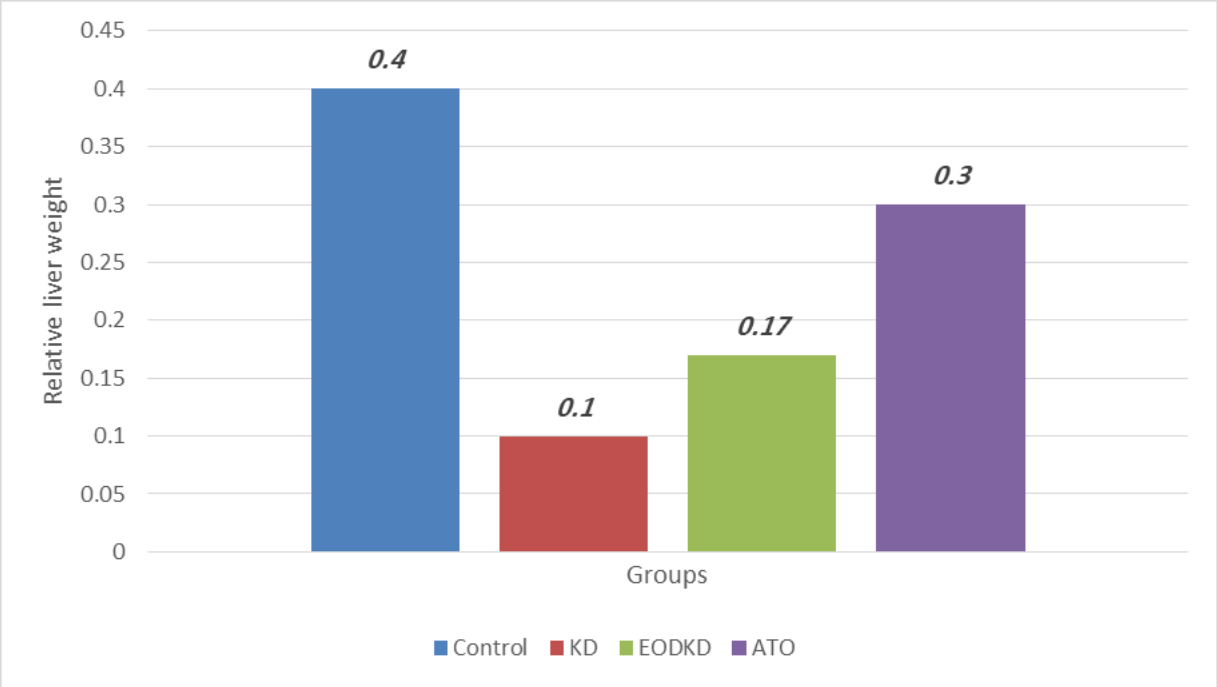
*= p-value significant

Bar chart 1. Comparison of the body weight among the studied groups.

Table 3. Comparison of the relative liver weight among the studied groups.

Relative liver weight	I-Control		II-KD		III-EODKD	IV-ATO
Mean ±SD	0.4±0.01		0.1±0.07		0.17±0.06	0.3±0.01
F-value	37.8					
Overall P-value	<0.001*					
Pairwise comparisons	II&I <0.01*	III&I 0.011*	IV&I 0.987	III&II 0.002*	IV&II <0.01*	IV&III 0.014*

*= p-value significant



Bar chart 2. Comparison of the relative liver weight among the studied groups.

Hepatic structural affection in the KD and EODKD groups

Normal hepatocyte architecture was observed around central veins in the control group (Fig. 1-A). KD hepatocytes exhibited pyknotic nuclei and congested central vein associated with mononuclear inflammatory cells in the KD group (Fig. 1-B). Hepatocytes with vacuolated cytoplasm associated with mononuclear inflammatory cells around dilated central vein in the EODKD group (Fig. 1-C). The ATO group showed normal hepatocyte patterns (Fig. 1-D).

Hepatocytes with vacuolated cytoplasm and dilated hepatic sinusoids in both KD and EODKD groups (Figs. 2-A, B)

A normal histological pattern was seen in the control group (Fig. 3-A). The portal veins were dilated and congested, hepatocytes with pyknotic nuclei and cytoplasmic vacuoles were seen in both KD and EODKD groups (Figs. 3- B, C). In the ATO group, the hepatocytes regain the normal histological pattern (Fig. 3-D).

Dilated, congested portal veins and hepatocytes with vacuolated cytoplasm were observed in both KD and EODKD groups (Figs. 4-A, B)

Collagen fibre content increased in the KD and EODKD groups

Collagen fiber content was minimal in the control group (Fig. 5-A). The fiber content increased significantly around the congested portal veins in the KD group (> two-fold) (Fig. 5-B, Table 4) and in the EODKD group (> one-fold) (Fig. 5-C, Table 4) in comparison to the control group. Compared to the EODKD group, the fiber content in the KD group was 30% higher (Table 4). The collagen fibres in the ATO group were minimal (Fig. 5-D).

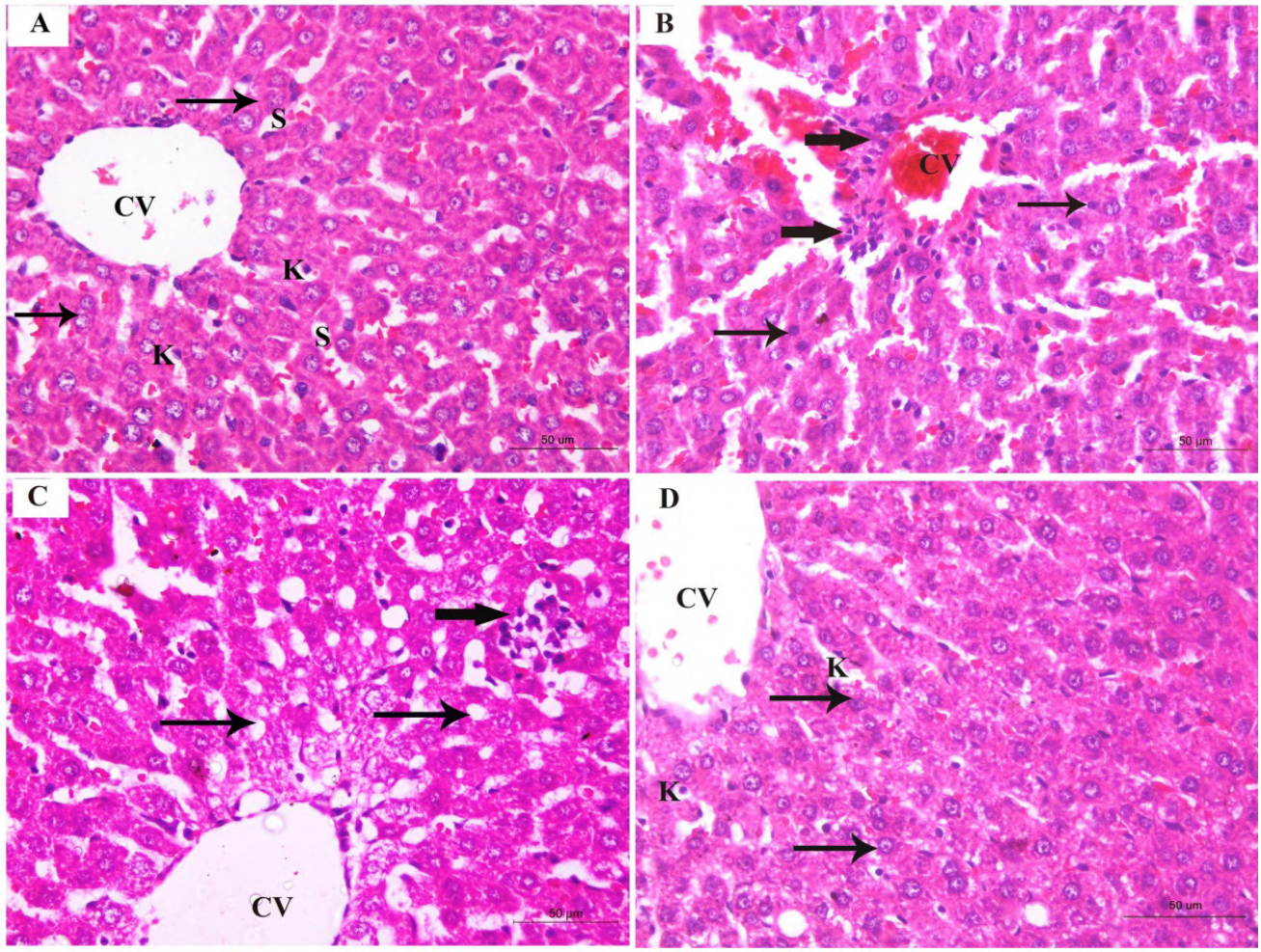


Fig. 1.- Sections around central veins. **A:** interconnected branching plates of hepatocytes (arrows) spreading from a central vein (CV) in the control group. Note blood sinusoids (S) and Von-Kupffer cells (K). **B:** Pyknotic hepatic cell nuclei (thin arrows), dilated congested central vein (CV), and mononuclear inflammatory cells (thick arrows) in the KD group. **C:** Hepatocytes with vacuolated cytoplasm (thin arrows) and inflammatory cellular aggregation (thick arrow) near a central vein (CV) in the EODKD group. **D:** Normal hepatocytes (arrows), Von-Kupffer cells (K), and slightly dilated central vein (CV) in the ATO group. H&E staining. Scale bars: A,B = 50 µm; C,D = 60 µm.

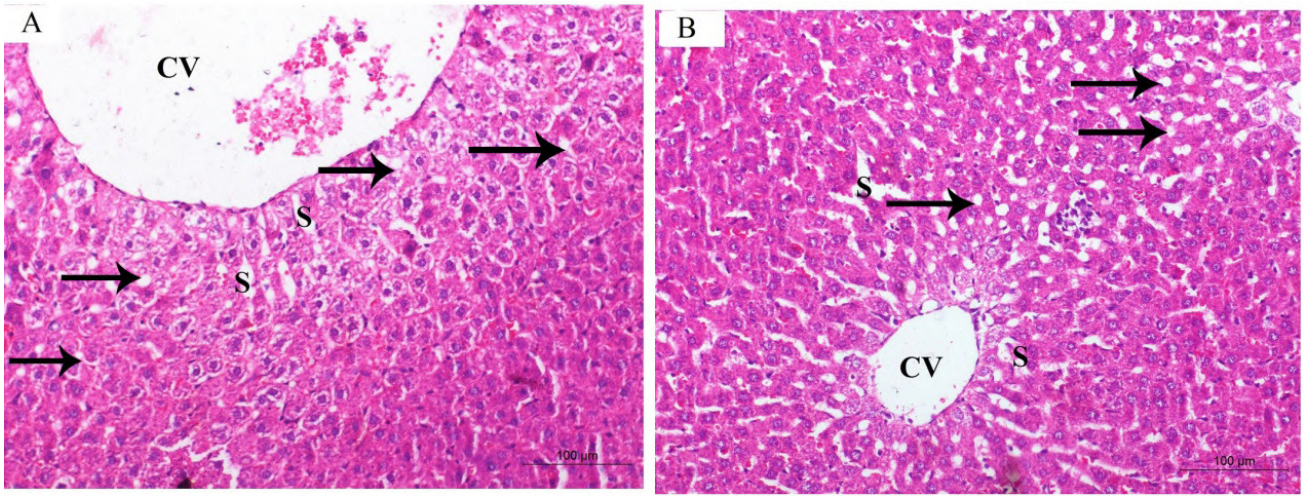


Fig. 2.- Sections around central veins. **A:** Dilated, congested central vein (CV), dilated hepatic sinusoids (S) and hepatocytes with vacuolated cytoplasm (arrows) in the KD group. **B:** hepatocytes with vacuolated cytoplasm (arrows) and dilated hepatic sinusoids (S) in the EODKD group. H&E staining. Scale bars = 100 µm.

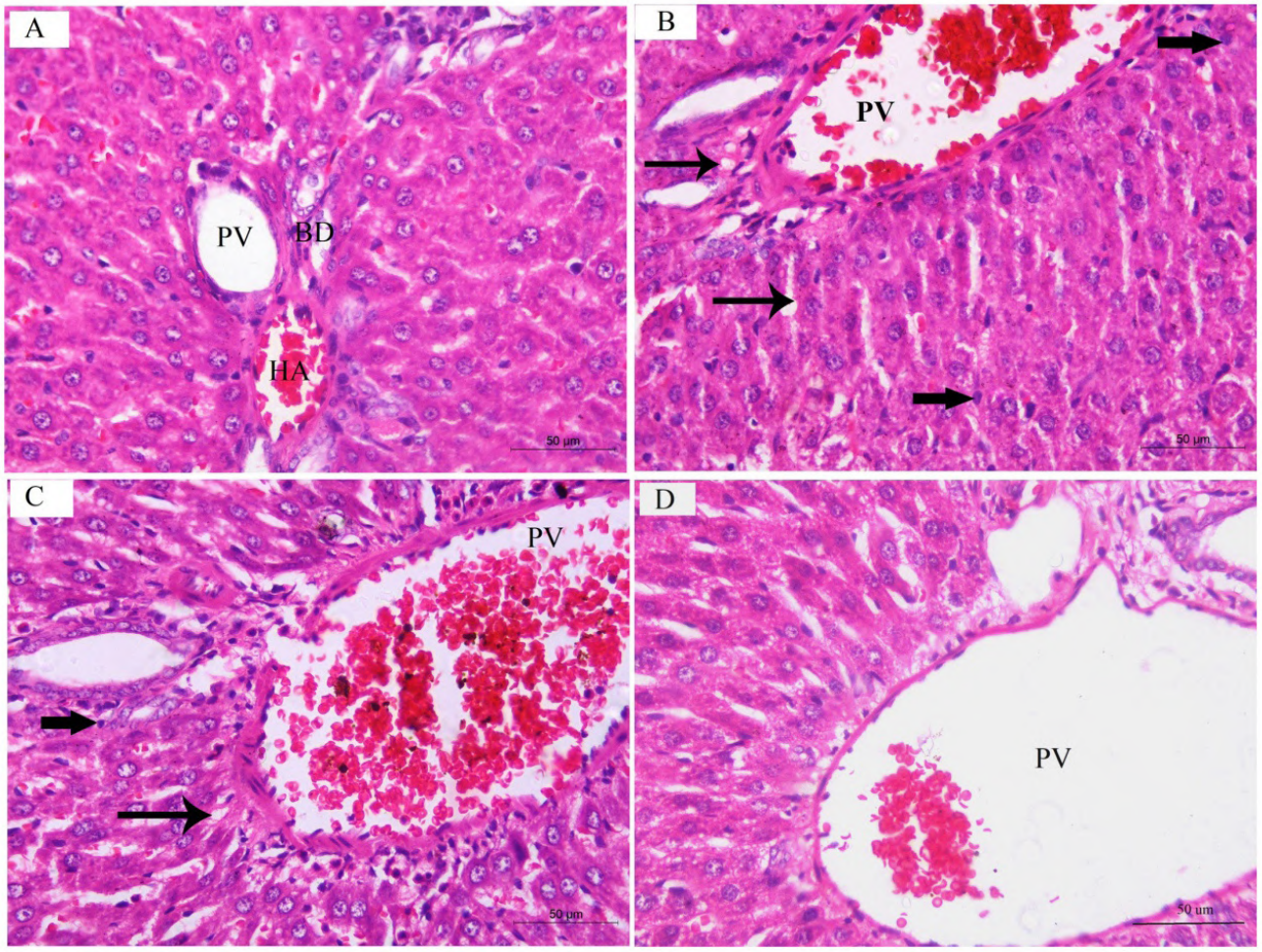


Fig. 3.- Sections around portal areas. Note hepatic artery (H), portal vein (PV), and bile ductule (BD). **A:** Normal architecture of the control. **B:** Pyknotic hepatic cell nuclei (thick arrows) and vacuolated cytoplasm (thin arrows) in the KD group. Note the portal vein is dilated and congested. **C:** Dilated and congested portal vein, hepatocytes with pyknotic nuclei (thick arrows) and vacuolated cytoplasm (thin arrow) in the EODKD group **D:** Normal hepatocytes and dilated portal vein in the ATO group. H&E staining. Scale bars A-D = 50 µm.

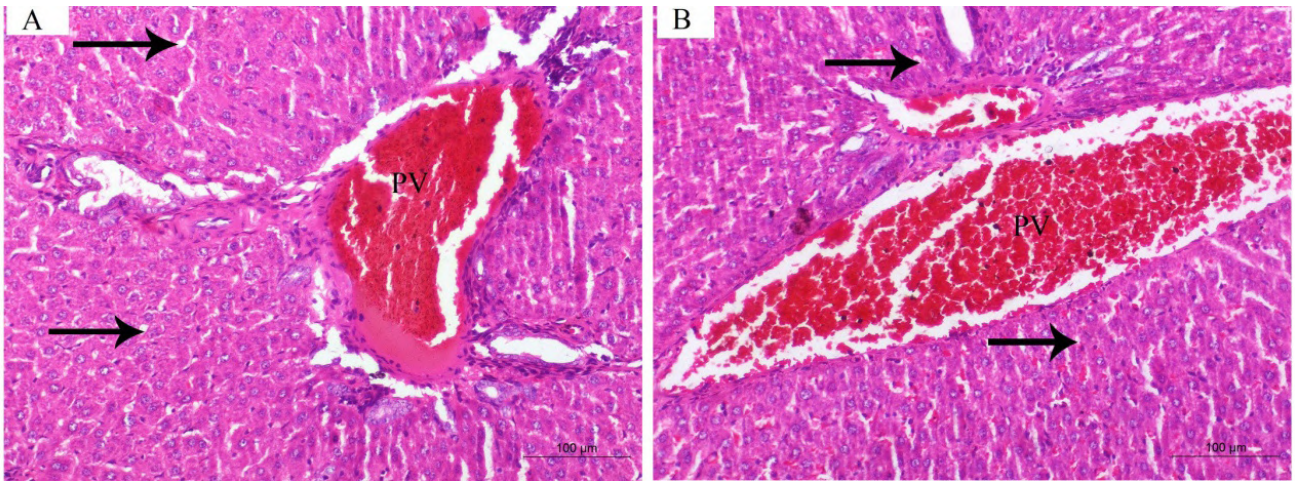


Fig. 4: A: Dilated, congested portal vein and vacuolated cytoplasm (arrows) in the KD group. **B:** Hepatocytes with vacuolated cytoplasm (arrows) in the EODKD group. Note the portal vein is dilated and congested. H&E staining. Scale bars = 100 µm.

Apoptosis was more prevalent in the KD and EODKD groups

Caspase-3 immunoreaction was minimal and comparable in control and ATO groups (Figs. 6-A, D, Table 4). Increased expression of apoptotic response was detected in the KD group (230%) (Fig. 6-B, Table 4) and EODKD (176%) group (Fig. 6-C, Table 4) when compared to the control group. The response in the KD group was 22 % higher than in the EODKD group (Table 4).

Table 4. Caspase3, Bcl2, Bax, Bcl2/Bax ratio and content of collagen fibers in the studied groups.

Group		Caspase 3	Bcl-2	Bax	Bcl-2/Bax ratio	Content of collagen fibers
Control	Mean ±SD	1.3±0.3	6.2±2.4	0.3±0.1	20.5±6.7	3.4±0.7
KD	Mean ±SD	4.3 ±0.4	2.2±0.3	3.5±0.2	0.43±0.01	11.1±0.9
	Versus control	0.001*	0.009*	0.001*	0.001*	0.001*
	Versus EODKD	0.563	0.952	0.019*	0.010*	0.005*
	Versus ATO	0.003*	0.001*	0.001*	0.001*	0.001*
EODKD	Mean ±SD	3.5±0.5	2.9±1.2	2.3±0.6	2.2±0.3	8.5±1.3
	Versus control	0.006*	0.001*	0.004*	0.003*	0.013*
	Versus KD	0.563	0.952	0.019*	0.010*	0.005*
	Versus ATO	0.001*	0.002*	0.005*	0.042*	0.014*
ATO	Mean ±SD	1.7 ±0.4	5.8±0.5	0.8±0.1	11.7±1.5	3.5±0.8
	Versus control	0.332	0.994	0.436	0.057	0.999
	Versus KD	0.003*	0.001*	0.001*	0.001*	0.001*
	Versus EODKD	0.001*	0.002*	0.005*	0.042*	0.014*

*= p-value significant

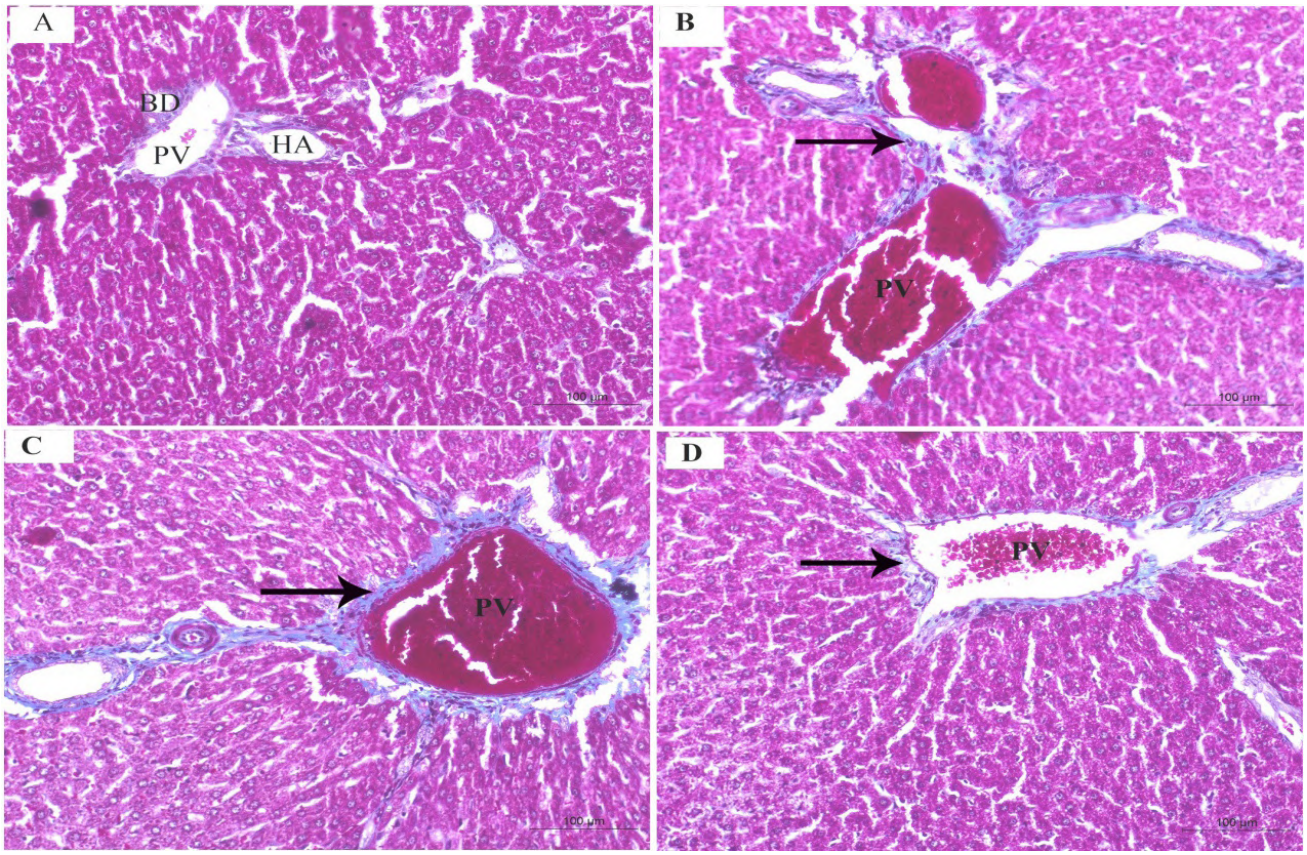


Fig. 5.- Collagen fibres (arrows) around the periportal area. Note the portal vein radical (PV), the hepatic artery (HA), and the bile ductule (BD). **A:** Negligible amount of collagen fibres in the control. **B:** The KD group exhibited more collagen deposition around congested portal vein (arrow). **C:** A relative increase in collagen deposition (arrow) around congested portal veins within the EODKD group. **D:** There are a few collagen fibres in the ATO group (arrow). Masson's trichrome. Scale bars A-D = 100 µm.

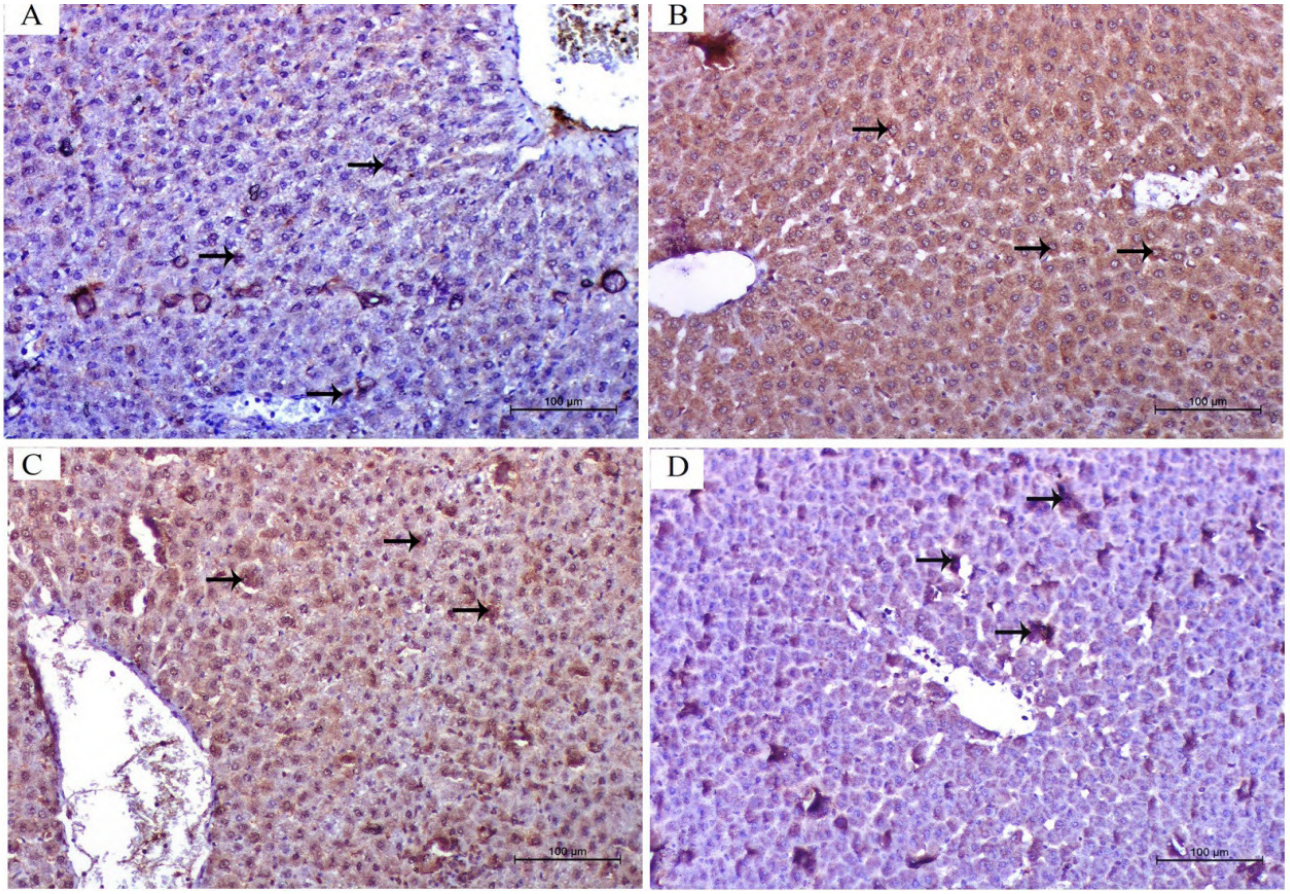


Fig. 6.- Immunoreaction of caspase 3 (arrows). **A:** A slight active expression of apoptotic reaction in the control group. **B:** increased expression of apoptotic reaction in the KD group. **C:** Apoptosis expression was less intense in the EODKD group. **D:** The ATO group showed a slight active reaction. Caspase 3 immunostaining. Scale bars A-D = 100 µm.

The BAX immunoreaction was minimal in the control group (Fig. 7-A, Table 4). A significant increase in the apoptotic reaction was observed in the KD group (> ten-fold) (Fig. 7-B, Table 4) and EODKD group (> six-fold) (Fig. 7-C, Table 4) as compared to the control group. The reaction in the KD group was 50% higher than that in the EODKD group (Table 4). ATO group shows minimal expression reaction (Fig. 7-D).

By comparison with the control group, the BCL2 immunoreaction was reduced in the KD and EODKD groups by 30% and 50%, respectively (Figs. 8-B, C, Table 4). However, the difference was not significant, since the last two groups were closely related (Table 4). The reaction in the ATO group was similar to the control group (Figs. 8-A, D, Table 4).

In comparison with the control group, the Bcl2/Bax ratio was 20-fold lower in the KD group and ten-fold lower in the EODKD group. The ratio in the KD group was four-fold lower than the EODKD group. Comparatively to the control group, the ratio dropped to 45% using ATO (Table 4).

Liver ultrastructural degeneration in the KD and EODKD groups

Control and ATO groups displayed classic hepatic architectures ((Figs. 9A, F). Hepatocytes from the KD and EODKD groups displayed degenerative nuclear changes, as well as mitochondria with lost cristae and ruptured outer membranes. Rarefied cytoplasm with prominent lysosomes was also observed (Figs. 9 B-E).

Enhanced hepatic enzyme markers and reduced hepatic triglycerides and cholesterol in the KD and EODKD groups

The levels of hepatic triglycerides were significantly lower in the KD and EODKD groups compared with the control group. In the KD group, they were 14% lower than those in the EODKD group. With ATO, they were 45% lower than those in the control group (Table 5).

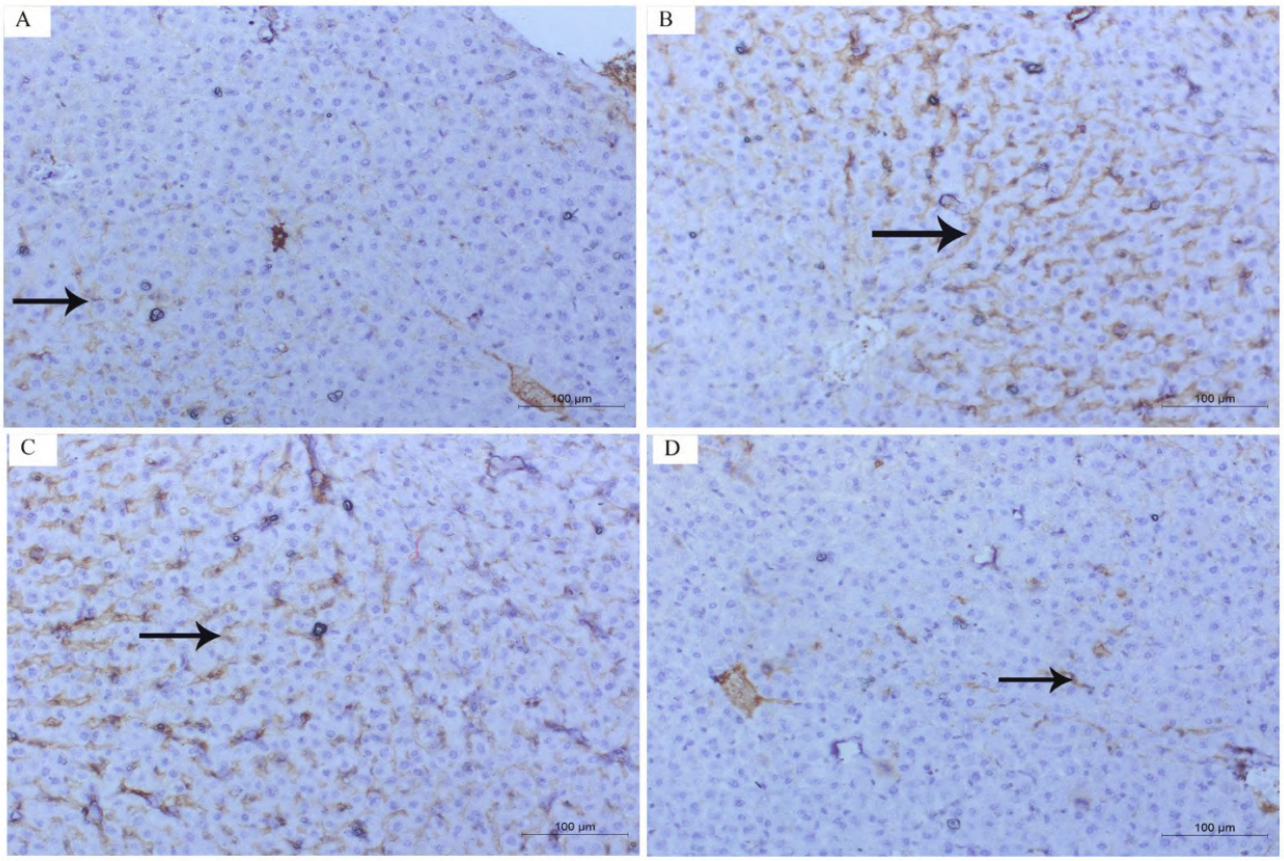


Fig. 7.- Immunoreaction of BAX (arrows). **A:** slight active expression of apoptotic reaction in the control group. **B:** A greater expression of apoptosis in the KD group. **C:** EODKD cells expressed fewer apoptotic reactions. **D:** ATO group shows minimal expression reaction. BAX immunostaining. Scale bars A-D = 100 µm.

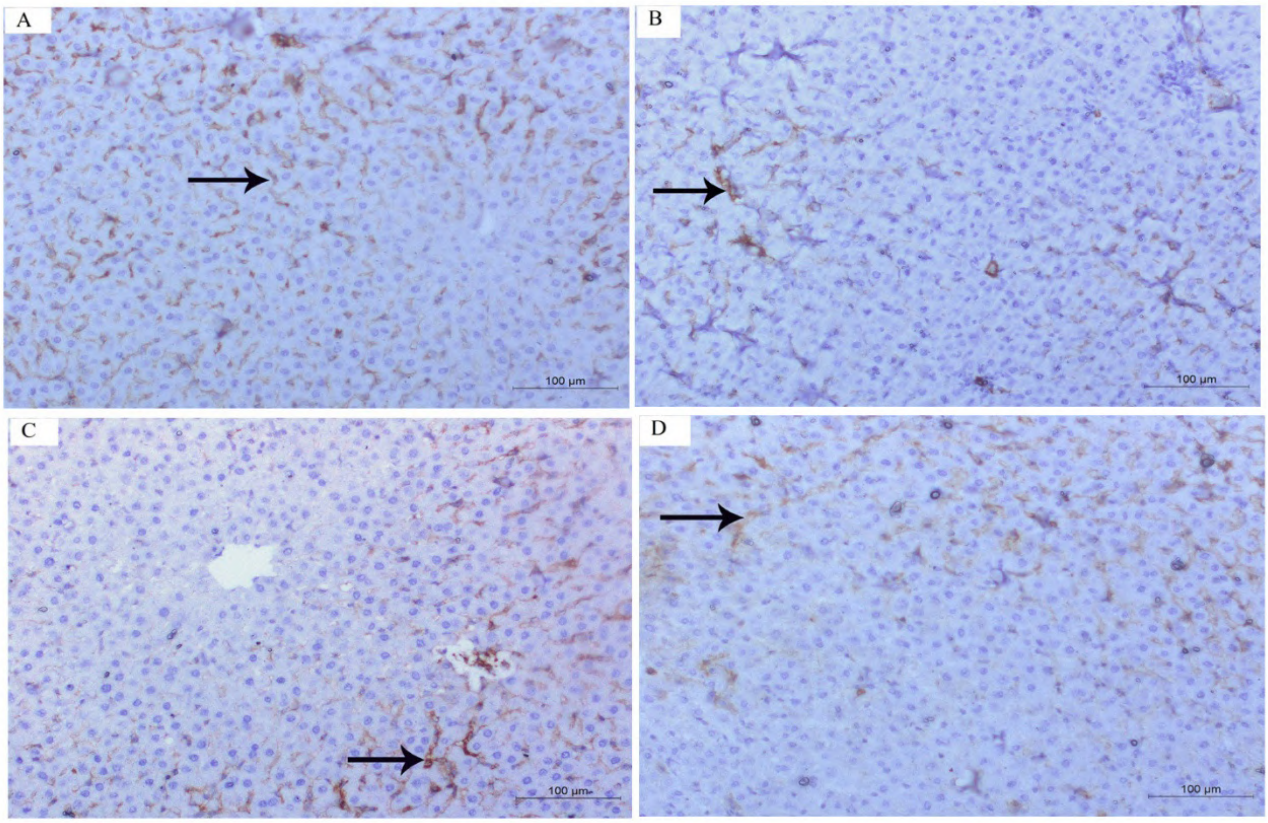


Fig. 8.- Immunoreaction of BCL2 (arrows). **A**: Normal reaction of the control group. **B**: The KD group showed a marked decrease in reaction. **C**: EODKD showed a less pronounced decrease in reaction. **D**: ATO group reactions were normal. BCL2 immunostaining. Scale bars A-D = 100 µm.

In the KD and EODKD groups, the levels of hepatic cholesterol were 54% and 32% lower than the control group, respectively. In the KD group the levels were 20% lower than the EODKD group. With ATO, the levels were 67% lower than the control group (Table 5).

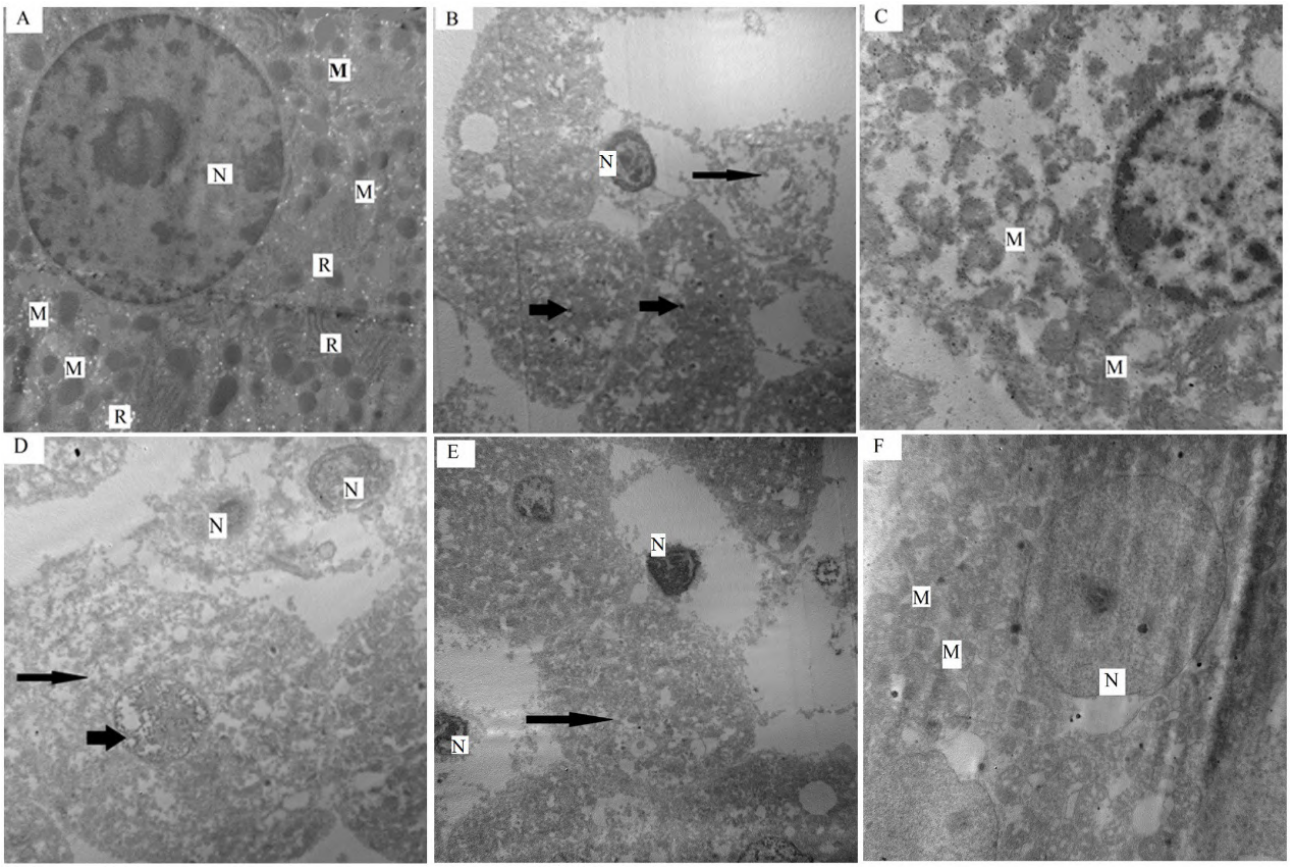


Fig. 9.- A: Classic hepatic architecture in a control rat. Note a hepatic nucleus (N) with a prominent nucleolus, mitochondria (M) of moderate electron density, and rER (R) with the normal organization (x 8000). **B:** rarefied cytoplasm (thin arrow), pyknotic hepatic nucleus (N), and prominent lysosomes (thick arrows) in the KD group (x 3000). **C:** mitochondria (M) with lost cristae and ruptured outer membranes in the KD group (x 8000). **D, E:** hepatocytes with degenerative nuclear changes (thick arrow), pyknotic nuclei (N), and rarefied cytoplasm (thin arrow) in EODKD (x 4000, 3000). **F**: normal hepatic architecture in the ATO group. Note a hepatic nucleus (N) with regular nuclear membrane, mitochondria (M) of moderate electron density (x 6000).

Table 5. Biochemical parameters of the studied groups.

Group		Hepatic triglyceride (mg/g liver)	Hepatic cholesterol (mg/g liver)	Serum cholesterol (mg/dl)	ALT (U/L)	AST (U/L)
Control	Mean ±SD	110±20	37.0±2.8	122±10.5	14.5±3.5	15.0±1.4
KD	Mean ±SD	70.0±18.4	17.0±8.2	90.7±15.6	50.3±5.8	55.3±6.4
	Versus control	0.008*	0.001*	0.003*	0.001*	0.001*
	Versus EODKD	0.03*	0.010*	0.001*	0.011*	0.003*
	Versus ATO	0.002*	0.001*	0.005*	0.001*	0.001*
EODKD	Mean ±SD	80.5±19.6	25.3±9.8	100.0±17.3	34.0±5.3	35.3±3.1
	Versus control	0.003*	0.003*	0.008*	0.010*	0.002*
	Versus KD	0.03*	0.010*	0.003*	0.011*	0.003*
	Versus ATO	0.004*	0.021*	0.002*	0.011*	0.015*
ATO	Mean ±SD	60.9±15.6	12.3±3.2	75.5±12.8	16.7±2.9	21.7±1.5
	Versus control	0.03*	0.02*	0.04*	0.954	0.161
	Versus KD	0.002*	0.001*	0.005*	0.001*	0.001*
	Versus EODKD	0.004*	0.021*	0.002*	0.011*	0.015*

*= p-value significant

The serum cholesterol levels of the KD and EODKD groups were lower than those of the control group by 26 and 18%, respectively, while the levels of the KD group were 11% lower than those of the EODKD group. With ATO, the levels were 38% lower than those of the control group (Table 5).

There was an increase in ALT levels in the KD and EODKD groups as compared to the control group by 250% and 143%, respectively. For the KD group, the level was 32% higher compared to the EODKD group. The levels in ATO and control groups were similar (Table 5).

As compared to the control, the KD and EODKD groups had higher levels of AST by 267% and 134%, respectively. ATO and control groups did not differ in terms of AST levels. The KD group's level was 36% higher compared to the EODKD group's level (Table 5).

Increase oxidative stress in the KD and EODKD groups

In the KD and EODKD groups, there was a 740% (7-fold) and a 520% (5-fold) increase as compared to the control group. MDA levels were 23% higher in the KD group than the EODKD group. The levels in the ATO and control groups were similar (Table 6).

As compared with the control group, TBARS levels in the KD and EODKD groups exhibited 175% and 120% increase, respectively. The level of both in the KD group was 20% higher than in the EODKD group, while levels in the ATO and control groups were similar (Table 6).

SOD levels in the KD and EODKD groups decreased by 46% and 23%, respectively, compared with the control. SOD in the KD group was 44% lower than in the EODKD group. Levels in the ATO and control groups were similar (Table 6).

In the KD and EODKD groups, GSH levels fell 48% and 34%, respectively, in comparison to the control group. In the KD group, GSH levels dropped 28% less than in the EODKD group. In the control group, levels were essentially identical (Table 6).

Increase expression of NLRP3 and CD11b in the KD or EODKD groups

As compared to the control group, the expression level of CD11b in KD and EODKD groups increased 540% (>5 fold) and 340% (> 3-fold), respectively. The level in the KD group was 31% higher compared to the EODKD group. The levels in ATO and control groups were similar (Fig. 10, Table 7).

Table 6. Oxidative/antioxidative markers in the studied groups.

Group		MDA (nmol/mg protein)	TBARS (nmol/mg protein)	SOD (U/mg protein)	GSH (U/mg protein)
Control	Mean ±SD	5.0±1.0	20.0±8.0	84.0±7.3	144.0±12.0
KD	Mean ±SD	42.3±3.5	55.0±5.6	45.0±6.0	74.7±9.5
	Versus control	0.000*	0.01*	0.005*	0.000*
	Versus EODKD	0.022*	0.550	0.008*	0.002*
	Versus ATO	0.000*	0.002*	0.000*	0.001*
EODKD	Mean ±SD	31.3±2.2	44.3±7.0	64.0±3.1	95.0±8.0
	Versus control	0.000*	0.001*	0.000*	0.000*
	Versus KD	0.024*	0.504	0.007*	0.002*
	Versus ATO	0.021*	0.004*	0.006*	0.004*
ATO	Mean ±SD	8.0±7.0	25.0± 6.0	80.0±3.0	135.3±8.4
	Versus control	0.110	0.983	0.376	0.564
	Versus KD	0.000*	0.003*	0.000*	0.001*
	Versus EODKD	0.011*	0.003*	0.005*	0.002*

*= p-value significant

In the KD and EODKD groups, NLRP3 expression levels were 250% (2.5-fold) and 160% (>1.5-fold), respectively, compared to the control group. In the KD group, the expression levels were 24% higher than in the EODKD group. In the ATO group, the expression levels were like those of the control group (Fig. 10, Table 7).

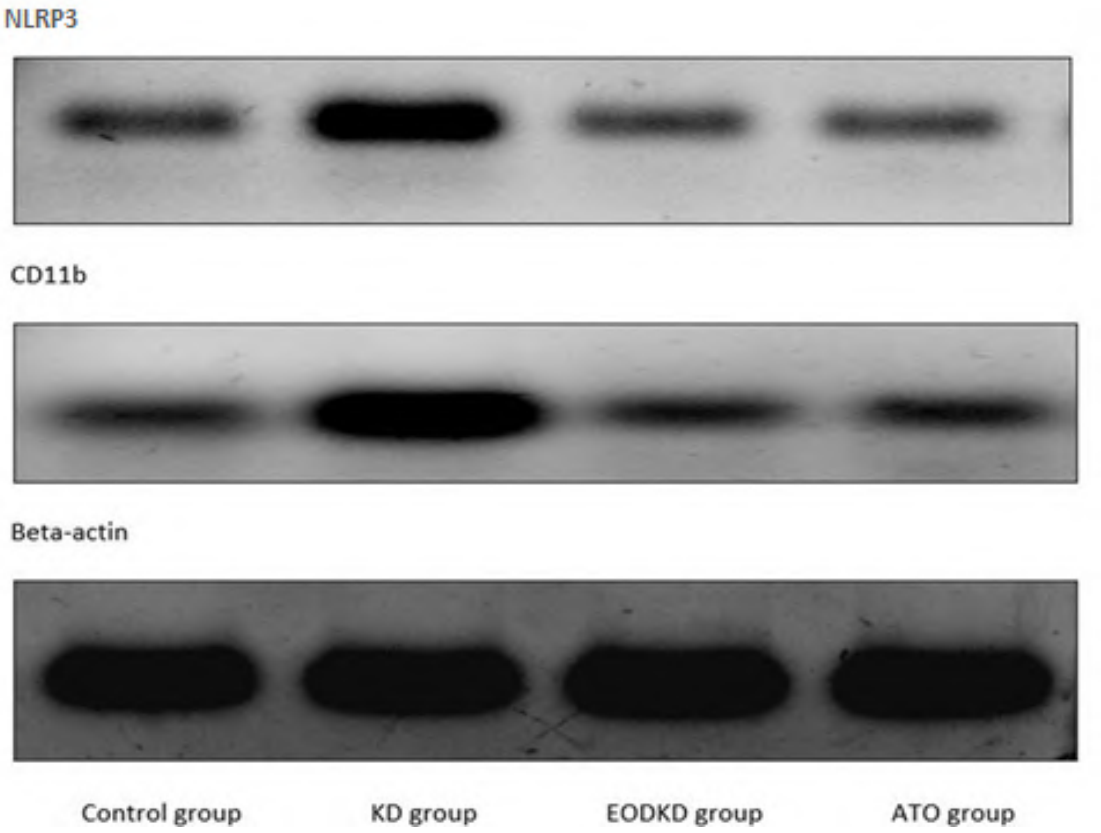


Fig. 10.- Expression levels of NLRP3 and CD11b by Western blot. B-actin was used as a control. The expression of both genes increased in the KD group, while it is decreased in EODKD with more decrease in the ATO group

Table 7. CD11b and NLRP3 levels among the different groups.

Group		CD11b level	NLRP3 level
Control	Mean ±SD	1.0±0.02	1.5±0.2
KD	Mean ±SD	6.4±0.8	5.3±0.4
	Versus control	0.001*	0.001*
	Versus EODKD	0.011*	0.021*
	Versus ATO	0.001*	0.001*
EODKD	Mean ±SD	4.4±0.5	4.0±0.3
	Versus control	0.008*	0.004*
	Versus KD	0.011*	0.021*
	Versus ATO	0.030*	0.003*
ATO	Mean ±SD	2.1±0.01	1.8±0.1
	Versus control	0.251	0.089
	Versus KD	0.001*	0.001*
	Versus EODKD	0.030*	0.003*

*= p-value significant

DISCUSSION

At the end of this study, both KD and EODKD groups had lost weight by 14% and 8%, respectively. This weight loss may be the result of modulating resting energy expenditure by these diets (Krieger et al., 2006), or by reducing insulin levels (Westman et al., 2007), or by loss of appetite consequence to ketosis (Sumithran et al., 2013). Researchers suggest that KDs are responsible for weight loss because they promote ketogenesis, which may enhance mitochondrial fat oxidation and bioenergetic signalling (Miller et al., 2020). Increased gluconeogenesis (energy demand) caused by carbohydrate restriction is also a contributing factor (Fine and Feinman, 2004). Still others believe the diets were metabolically effective (Feinman and Fine, 2007). Kump and Booth (2005) found that KD-fed animals showed a 17% increase in energy expenditure compared with standard chow-fed mice, concluding that KD caused an increase in thermogenesis independent of physical activity. In a study by Frommelt et al. (2014), rats fed a KD for 4 weeks exhibited higher levels of gross energy in urine than rats fed standard chow.

After 22 weeks of KD in mice, there is no weight loss (Ellenbroek et al., 2014). According to other reports, after 18 weeks of KD, the mice's weight returned to baseline and then slowly increased (Douris et al., 2015). We were unable to verify both findings in our study due to its relative shortness.

The relative liver weight of the KD and EODKD groups decreased by 75% and 57%, respectively, compared to the control group. This may be caused by the depletion of liver glycogen (Bian et al., 2014). Many studies have shown that weight loss influences liver health and liver fat percentage. A 5% weight loss decreased hepatic fat by 28-40% (Thomas et al., 2006; Kantartzis et al., 2009).

Both KD and EODKD result in structural liver damage (apoptosis, cytoplasmic vacuolation, inflammatory cellular infiltration, congestion of the portal & central veins and hepatic sinusoids). The biochemical liver markers (ALT and AST) were elevated. The elevation of these markers indicates severe liver damage (Monteiro et al., 2016). ALT and AST were elevated more than two-fold in the KD group and more than one-fold in the EODKD group (being higher in the KD group by 32% and 36%). The medium protein content of KD and EODKD may contribute to the described injury pattern (Garbow et al., 2011).

KD and EODKD are associated with vacuolation of hepatocytes. Vacuolation of hepatocytes is defined as microvesicular and macrovesicular steatosis. The macrovesicular steatosis detected in both groups may be ascribed to abnormalities in the delivery, metabolism, synthesis, and export of lipids (Brunt and Tiniakos, 2005). The decrease in the hepatic triglyceride levels, hepatic cholesterol levels, and serum cholesterol levels in EODKD and KD groups supports this assumption. Many studies reported a decrease in these biomarkers in rats fed a KD (Holland et al., 2016). Furthermore, cytoplasmic vacuolation may be attributed to lipid peroxidation as oxidative stress damage cell membranes, as well as membranes of cell organelles leading to an increase in their permeability and disturbance of the concentrations of the ions in the cytoplasm and cell organelles (Panasiuk et al., 2006). Oxidative stress was obviously noticed in KD and EODKD groups (elevation of oxidative markers and flat antioxidant), being higher in the KD group. It is demarcated as the shift between oxidants and antioxidants in favor of oxidants (Bocarsly et al., 2010). Oxidative stress and KDs are controversial issues. Some studies report the oxidative stress and antioxidant effects of KDs (Rhyu et al., 2014). In other studies, researchers have shown that KDs reduce oxidative stress through Nrf2 (nuclear factor-erythroid factor 2-related factor 2) signaling and by suppressing the NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) signaling pathway (Lu et al., 2018).

The periportal areas that are affected by KD and EODKD had inflammation around them. Inflammation in absenteeism of infection is now considered a major mechanism for liver injury (Kubes and Mehal 2012; Hoque et al., 2013). Sterile inflammation is started when the damaged hepatocytes expose the intracellular molecules that are recognized by the innate immune system. Kupffer cells become activated and trigger an inflammatory response through common

pathways involving the NLRP3 inflammasome (Rock et al., 2010). The expression level of NLRP3 in KD and EODKD groups displayed 2.5 and 1.5-fold higher linked to the control group (being 24% higher in the KD group). Activation of the NLRP3 inflammasome results in the production of pro-inflammatory cytokines, chemokines with the subsequent recruitment of neutrophils, and cell death (Broderick et al., 2015). Another possible factor that can trigger sterile inflammation is the sustained supply of high concentrations of fat to periportal hepatocytes (the cells receive more fat than can be oxidized) (Borradaile et al., 2006). The increased apoptosis that observed in KD and EODKD groups could provide other possible explanation of this inflammation (Wang et al., 2008).

Apoptosis was identified in KD and EODKD groups. The outline of the apoptotic signal pathway finally meets a common mechanism driven by caspases. Caspase-3 is the major destroyer of apoptosis, thus helping cell survival (Ma et al., 2014). The value of the caspase 3 is distinctly increased in the KD and EODKD groups.

The caspases mechanism is controlled by the Bcl-2 family (Adams and Cory, 1998). This family is classified into Bcl-2 subfamily, Bax subfamily and Bik and Bid subfamily. The Bcl-2 employs anti-apoptotic activity, while Bax applies proapoptotic activity (Tsujimoto, 1998). The Bax reaction increased with KD ten-fold and with EODKD six-fold. Contrary, the expression of BCL2 decreased in KD and EODKD groups by 30% and 50%. Bcl2/Bax ratio decreased with KD twenty-fold and with EODKD ten-fold. Bcl2/Bax ratio expresses the danger of the cell to apoptosis (Tsujimoto, 1998) as seen in KD and EODKD groups.

The content of collagen fibers in periportal areas increased in the KD group by more than two-fold, and in the EODKD group by more than one-fold (being higher in the KD group by 30%). KD is known to cause hepatic fibrosis in some cases (Brunt and Tiniakos, 2005). The developed liver fibrosis during liver damage causes an irreversible distortion of the normal hepatic architecture (Sokol, 2002). Oxidative stress and chronic inflammation are involved in such progress (Poli, 2000).

The concomitant use of ATO with KD protects liver architecture. Moreover, the biological hepatic markers (AST and ALT) were of their normal levels. ATO provides its protective effect through decreasing the oxidative stress (oxidative/antioxidative markers were matching the control group), normalization expression of NLRP3 (absence of sterile inflammation) and inhibition HMG-CoA reductase (Kogawa et al., 2019), which plays a key role in the production of cholesterol in the body (marked decrease of intrahepatic triglyceride level compared to KD and EODKD groups).

CONCLUSIONS

Both KD and EODKD result in structural liver damage that is accompanied by an elevation in hepatic markers (AST and ALT) along with a decrease in hepatic triglyceride, hepatic cholesterol, and serum cholesterol. KD has a more significant effect. There are several factors contributing to hepatic injury in these diets, including oxidative stress, sterile inflammation, low protein content, and high fat concentrations. ATO in conjunction with KD is beneficial.

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