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Influence of walnut on hepatic ischemia-reperfusion injury in streptozotocin-induced diabetic rats

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Nutritional benefits of walnut are well known; however, currently, there is no research showing that walnut can be used as an antioxidant in people with diabetes mellitus (DM). Therefore, the objective of this study was to investigate the protective effects of walnut against oxidative stress in hepatic ischemia-reperfusion (I/R) injury in streptozotocin (STZ) induced diabetic rats. Animals were divided into four-groups (Control, DM, DM+I/R, DM+I/R+Walnut; n=6 each). STZ treatment and I/R procedures were not performed in the control group, other groups were first administered 60-mg/kg STZ intraperitoneally. After 48-h, animals were considered as DM. After four-weeks, DM groups were subjected to 30 min of hepatic-ischemia followed by 45-min reperfusion. DM+I/R+Walnut group was fed pellet-feed mixed with walnut (2 g/100 g/day) until I/R. Other groups were fed only with pellet-feed. At the experimental end, animals decapacitated, blood samples collected to determine serum levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6, malondialdehyde (MDA). Liver samples were collected for histological examinations. Forty-eight-hours after STZ, animals showed significant weight-loss compared to age-matched controls, blood glucose levels were increased ($P< 0.05$). Four-weeks post-STZ, blood glucose also increased significantly. TNF-α, IL-6, MDA substantially increased in the DM+/I/R group ($P< 0.01$), whereas in DM+I/R+Walnut group this increase was lesser ($P< 0.05$). Aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase (LDH) levels were lower in DM+/I/R+Walnut vs. DM+I/R groups; but higher than DM/control groups ($P< 0.05$). Positive-correlation observed between TNF-α and IL-6 (Spearman r:0.793; $P< 0.001$), and moderately-positive-correlation between IL-6 and LDH (Spearman r:0.429; $P< 0.05$). Histopathology revealed disordered hepatic lobules, swelling cells, vacuoles in liver specimens visible in the DM+I/R group, implicating hepatic I/R injury, which improved in DM+I/R+Walnut specimens. We conclude that in diabetic rats, hepatic I/R injury is associated with augmented inflammatory response and oxidative stress, while walnut pre-treatment significantly decreased these responses.

Keywords: Antioxidant, Diabetes mellitus, Histopathology, Inflammatory response, Ischemia-reperfusion injury, Rat, Walnut

Reduced perfusion due to any decrease or complete loss in blood supply to the organ or tissues is referred to as ‘ischemia’. Diminished oxygenation of the tissue due to ischemia for a certain period of time results in tissue damage or necrosis. Reconstruction of tissue blood supply after ischemia is called ‘reperfusion’. However, while meeting the oxygen and other metabolic needs of the tissue, paradoxically this condition causes much more damage in the tissues[1,2], and this condition is called ‘ischemia– reperfusion’ (I/R) injury. The tissue damage during the reperfusion period is especially caused by free oxygen derivatives such as reactive oxygen species (ROS) generated by the oxygen input into the cell, activation of polymorphonuclear leukocytes, and the effects of endothelial and complement systems[3,4]. Cellular structures that are most sensitive to reperfusion injury

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Abbreviations: ALT, Alanine amino transferase; AST, Aspartate amino transferase; DM, Diabetes mellitus; H&E, Hematoxylin and eosin; I/R, Ischemia reperfusion; IL-6, Interleukin-6; IU, International Unit; K, Control; LDH, Lactate dehydrogenase; MDA, Malondialdehyde; TNF-α, Tumor necrosis factor-α; W, Walnut

are membrane lipids, proteins, nucleic acids, and deoxyribonucleic acid molecules[5].

The I/R injury is not limited to the region in which it occurs, but it can also cause varying degrees of damage to other organ systems. Although its mechanism has not been fully elucidated yet, in the I/R injury that takes place in organs (such as lung tissue, kidneys, liver, skeletal muscles, heart, and gastrointestinal tract), it has been shown that damage also occurs in distant organs[6]. The local and systemic effects caused by I/R injury contribute to high morbidity, and mortality in many clinical conditions such as organ transplantation, myocardial infarction, cerebrovascular diseases, major surgical interventions, thrombolytic therapy, hemorrhagic shock and resuscitation[7,8].

Diabetes mellitus (DM) is a chronic disease characterized by hyperglycemia and is primarily caused by insulin deficiency or resistance. In addition, DM is a metabolic disorder that is related to defects in carbohydrate, protein, and fat metabolism, and is usually accompanied by many severe complications such as retinopathy, nephropathy, and neuropathy[9,10]. It is also associated with several metabolic abnormalities such as elevated free fatty acid levels, increased advanced glycation end-products, and augmented superoxide anion productions, which are major factors concerned in the pathogenesis of diabetic complications[11].

Elevated glucose levels provoke non-enzymatic and auto-oxidative glycosylation, promote protein kinase-C activation, which leads to alterations in the levels of inflammatory mediators[12]. In previous experimental studies, oxidative stress produced by high glucose levels has been described as a reason for diabetic inflammation[13,14]. DM induces oxidative stress and augments the inflammatory response stimulated by I/R injury. Oxidative stress and ROS are the key factors in the pathogenesis of diabetic complications[15].

Walnut (*Juglans regia* L.) is a foodstuff that is of high importance because of its nutritional value and its positive effects on human health. It contains oils; 72% consists of polyunsaturated fatty acids (59% linoleic, 13% α-linoleic), 18% monounsaturated fatty acids (oleic acid), and only 10% saturated fatty acids[16]. It is rich in multiple fatty acids, especially in Omega-3. These polyunsaturated fatty acids in the walnut were defined to prevent cardiovascular diseases, by virtue of having anti-inflammatory and anti-hypertensive effects, especially by decreasing the blood fat levels and preventing thrombosis and vascular occlusions[17,18]. Walnut consumption increases the level of melatonin hormone, which is an antioxidant and determines the biological rhythm, as Reither *et al.*[10] reported that owing to the antioxidant content of walnut, it may have the ability to reduce the risk of cancer development, cardiovascular diseases, and/or neurological diseases such as Parkinson's disease and Alzheimer's disease[19].

Although there are several studies in the peer-reviewed literature about the nutritional benefits of walnut, currently there is no research showing that walnut can be used as an antioxidant in people with DM. Therefore, in this study, we aimed to investigate the anti-inflammatory and antioxidant effects of walnut on ischemia-reperfusion injury in diabetic rats and evaluate its protective effects on liver parenchyma.

Materials and Methods

Animals

In this study, 24 male Sprague-Dawley albino rats weighing 250-300 g were used. Rats were housed in a room at a constant temperature of +22°C, with 12-h light/dark cycles, and fed on standard pellet chow and water *ad libitum*. The animals were obtained from the Laboratory of Animal Care Division of the School of Medicine at Marmara University, Istanbul, Turkey. Experiments were performed according to the international ethical standards and approved by the Ethics Committee (Animal Care and Use Committee) of Marmara University, School of Medicine, Istanbul, Turkey.

Study design

Rats (n=24) were equally divided into four groups (n=6 each) as follows: Control group (C), Diabetes mellitus group (DM), Diabetes mellitus + Ischemia/Reperfusion group (DM+I/R), and Diabetes + Ischemia/Reperfusion + Walnut group (DM+I/R+Walnut). Streptozotocin (STZ) treatment and ischemia-reperfusion procedure were not performed in the control group. The other three groups were first administered 60 mg/kg STZ intraperitoneally (IP; Sigma-Aldrich, St. Louis, MO, USA) to create a diabetes model. The body weight was measured using Precision Scales (Northglenn, CO, USA). After 48 h, the blood glucose levels of all these rats reached over 200 mg/dL, and were

considered as diabetic. Blood glucose levels were measured using ACCU-CHEK® Performa glucometer (Amicadeal, Inc., Federal Way, WA, USA). An ischemia-reperfusion model was developed after four-weeks of waiting for the development of chronic diabetes. The DM+I/R+Walnut group was fed with pellet feed mixed with walnut (2 g/100 g/day) until ischemia− reperfusion. All other groups were fed only with pellet feed. In the fourth week, the rats were given 100 mg/kg ketamine for anesthesia. After laparotomy with a midline incision, a 30 min ischemia was created by clamping the vena porta and the hepatic artery. Tissue samples were taken from the liver and blood was drawn from the vena cava inferior, 45 min after the reperfusion was achieved. The hepatic tissue samples were placed in formaldehyde (10%) for histological evaluations. Serum samples thus obtained after centrifugation of blood were stored at −80°C until further use.

Biochemical analyses

Serum malondialdehyde (MDA) levels were measured spectrophotometrically by using the thiobarbituric acid reactive substance (TBARS) method. To assess the serum levels of tumor necrosis factor-α (TNF-α) and IL-6, double sandwich enzyme-linked immune sorbent assay (ELISA) kits were used (R&D Systems, Minneapolis, MN, USA). Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) were analyzed utilizing diagnostic kits (BD Biosciences, Franklin Lakes, NJ, USA) and using automated biochemical equipment (Abbott C8000; Abbott Laboratories, Chicago, IL, USA).

Histopathological evaluations

Tissue specimens were fixed in 10% formaldehyde and then embedded in paraffin blocks. The paraffin sections (4-5 μm) were stained with hematoxylin & eosin (H&E) and examined under a photomicroscope (Olympus BX51, Tokyo, Japan) by the qualified-independent histologist in a blinded manner.

Statistical analyses

SPSS statistics 25 software (IBM Corp., Chicago, IL, USA), MedCalc version 15.8 software, and InStat3 GraphPad software were used for all statistical analyses. Kruskal-Wallis test (nonparametric ANOVA) and Post-hoc test (Dunn's Multiple Comparison test) were used to compare nonparametric data in the independent groups with more than two groups (since the number of subjects in each group was <30). Correlation Matrix analysis and Spearman correlation analysis in nonparametric data were used to investigate the relationship between all the independent variables.

Results

Body weight and blood glucose level

The bodyweight and blood glucose levels were measured at the beginning and 48 h after STZ injection and were found to be similar at the beginning of the study among all the groups. Forty-eight hours after the STZ injection, animals showed a significant weight loss compared to the age-matched controls, and the average blood glucose levels were significantly increased. And 4-weeks after the STZ injection, the average blood glucose levels were found to be significantly increased (Table 1).

Serum TNF-α levels

Results obtained from the determination of TNF-α levels of the experimental groups are shown in (Table 2 & Fig. 1). TNF-α levels in the DM+I/R+ Walnut group of animals were found to be significantly lower than the DM and/or DM+I/R groups ($P< 0.05$). There was no significant difference in TNF-α levels between the DM+I/R+Walnut and control groups ($P> 0.05$). Although TNF-α levels were relatively higher in the DM group as compared to DM+I/R group, the difference was not statistically significant ($P> 0.05$) (Fig. 1 & Table 2).

Serum IL-6 levels

As shown in (Table 2 & Fig. 1), serum IL-6 levels in the DM+I/R+Walnut group were found to be significantly lower than in both the DM and

Table 1 — Body weight and blood glucose levels in various groups of rat specimen

Animal Groups	Body Weight (g)		Blood Glucose (mg/dL)		
	Initial	48 h Later	Initial	48 h Later	4 Weeks Later
Control	288.1 ± 12.05	290.1 ± 12.41	90.2 ± 5.36	98.2 ± 14.02	96.2 ± 13.05
DM	287.8 ± 14.63	257.6 ± 13.97	96.4 ± 5.57	325.1 ± 21.03[a]	388.2 ± 32.08
DM+I/R	294.6 ± 14.63	260.2 ± 11.65	94.1 ± 4.52	318.4 ± 32.14[b]	415.2 ± 15.13
DM+I/R+ Walnut	298.1 ± 11.45	266.1 ± 10.22	92.6 ± 4.21	341,2 ± 21.05[c]	350.1 ± 27.09

Vales are expressed as means ± standard errors of mean (SEM) (n=6 rats in each group)

[a,b,c] $P< 0.001$compared to I^0

Table 2 — Determination of the levels of biochemical parameters and its comparison between the experimental groups

	Control (n=6)	DM (n=6)	DM+IR (n=6)	DM+I/R+Walnut (n=6)	*P*-Value
TNF-α (pg/mL)	169 ± 30	361 ± 73	456 ± 249	161 ± 8	[a]0.0006
	165 (138-222)	345 (279-481)	346 (257-877)	162 (152-171)	
IL-6 (pg/mL)	5.16 ± 0.46	8.44 ± 1.18	10.56 ± 1.63	4.48 ± 0.79	[a]0.0002
	5.1 (4.4-5.6)	8.0 (7.7-10.8)	10.7 (8.6-12.3)	4.35 (3.58-5.7)	
MDA (nmol/mL)	1.22 ± 0.21	1.51 ± 0.46	2.1 ± 0.27	1.37 ± 0.67	[a]0.0340
	1.15 (1.0-1.5)	1.15 (1.2-2.4)	1.83 (0.9-1.9)	1.3 (0.7-2.6)	
AST (IU/L)	113 ± 10	154 ± 14	491 ± 18.1	362 ± 19.8	[a]0.0010
	114 (101-125)	151 (138-173)	410 (378-499)	129 (92-182)	
ALT (IU/L)	47 ± 43	85 ± 16	133 ± 9	95 ± 5	[a]0.0398
	43 (21-55)	83 (90-103)	124 (114-149)	90 (78-108)	
LDH (IU/L)	1,450 ± 455	1,499 ± 329	2,448 ± 376	1,587 ± 743	[a]0.0283
	1,350 (1,193-1,838)	1,327 (1,239-1,929)	2,232 (1,975-2,827)	1,264 (957-2,924)	

[a]*P*-Value from Kruskal-Wallis test (non-parametric ANOVA) and Post-hoc test (Dunn's Multiple Comparison test). If the *P*-value obtained by ANOVA test was found to be <0.05, multiple comparison tests were performed to determine the *P*-value between the groups (as also shown in Figs. 1 & 2). For each panel, upper values are Mean ± SD and the lower values are Median (Range, *i.e.*, Minimum-Maximum values)

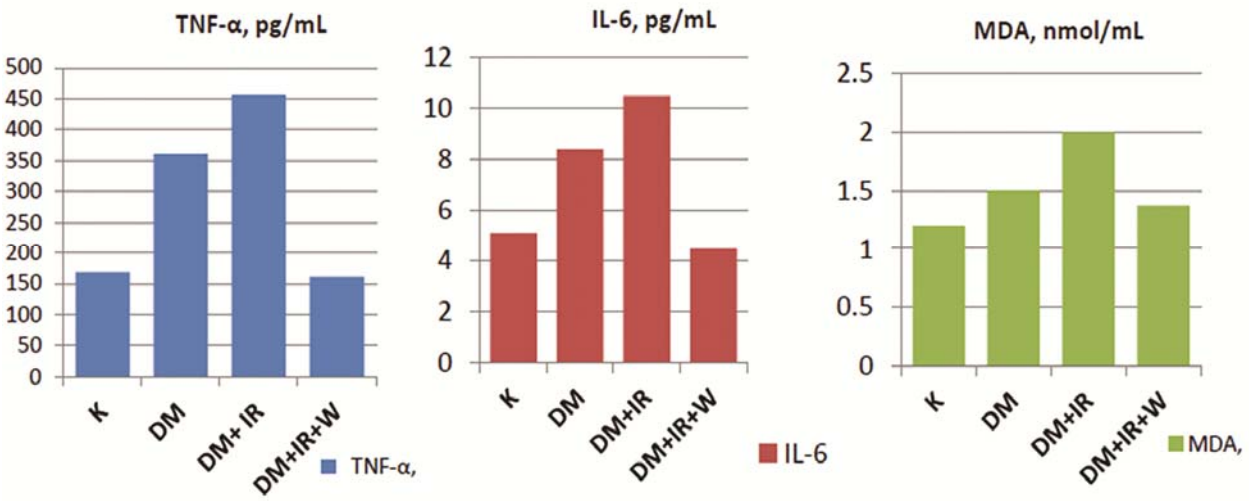

Fig. 1 — Results on the mean serum values of biochemical parameters (TNF-α, IL-6, MDA levels) in all the experimental groups of rats

DM+I/R groups ($P< 0.05$). However, the IL-6 levels of the DM+I/R group were significantly higher than in the control group ($P< 0.05$). Notably, there was no statistical difference in the IL-6 levels between the DM+I/R+Walnut and control groups ($P> 0.05$).

Serum MDA levels

The MDA levels in various experimental groups were examined and the results thus obtained are shown in (Table 2 and Fig. 1). The MDA levels in the DM+I/R+Walnut group of specimens were significantly lower than the DM and/or DM+I/R groups ($P< 0.05$). There was no significant difference in the levels of MDA between the DM+I/R+Walnut and control groups ($P> 0.05$). Although the MDA levels of DM+I/R group were relatively higher than the DM group of rats, the difference was not found to be statistically significant ($P> 0.05$).

Serum levels of AST, ALT, and LDH

The serum levels of AST, ALT, and LDH were found to be significantly lower in the DM+/I/R +Walnut group as compared to the DM+I/R group; but higher than the DM and control groups of animals ($P< 0.05$) (Fig. 2 and Table 2).

Correlation investigations

According to the correlation matrix assessments, there was a statistically significant good and moderately positive correlation between the TNF-α,

IL-6, and LDH levels. There was also a moderately positive correlation between the IL-6, AST, and LDH levels (Table 3). According to the data obtained through Spearman analysis, there was a statistically significant and positive correlation between the TNF-α and IL-6 levels (Spearman r: 0.793; $P< 0.001$) (Fig. 3A). There was also a moderately positive correlation between the IL-6 and LDH levels (Spearman r: 0.429; $P< 0.05$) (Fig. 3B).

Histopathological findings

Hepatic cells and hepatic microvascular injury were investigated by light microscopy. Utilizing H&E staining, we observed that disordered hepatic lobules, swelling cells and vacuoles in the liver specimens were visible in the DM+I/R group, which implied that the hepatic I/R injury did occur. The phenomenon seems to be improved in the DM+I/R+Walnut group of specimens (Fig. 4).

Discussion

This study demonstrates that the serum TNF-α, IL-6, and MDA levels increased in diabetic rats with hepatic ischemia-reperfusion damage (DM+I/R group), whereas in the diabetic rats fed with walnut

Table 3 — Correlation Matrix results in order to observe the degree of correlation between serum levels of various biochemical parameters in different experimental groups of rats

r	TNF-α	IL-6	MDA	AST	LDH	ALT
TNF-α	1.0000	0.7773	−0.0732	0.1409	0.3973	0.1277
IL-6	0.7773	1.0000	0.1358	0.3945	0.4231	0.2246
MDA	−0.0732	0.1358	1.0000	0.0294	0.1348	0.3323
AST	0.1409	0.3945	0.0294	1.0000	−0.1497	0.1417
LDH	0.3973	0.4231	0.1348	−0.1497	1.0000	0.2488
ALT	0.1277	0.2246	0.3323	0.1417	0.2488	1.0000

Each correlation coefficient (r) was calculated independently regardless of other variables. Results with r < 0.20 are considered statistically insignificant

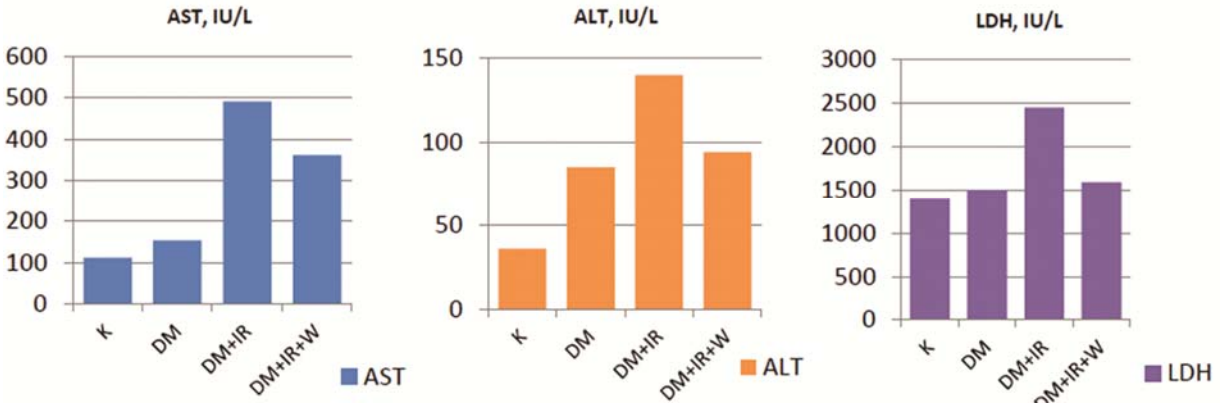

Fig 2 — Results on the mean serum values of AST, ALT and LDH levels in all the experimental groups of rats

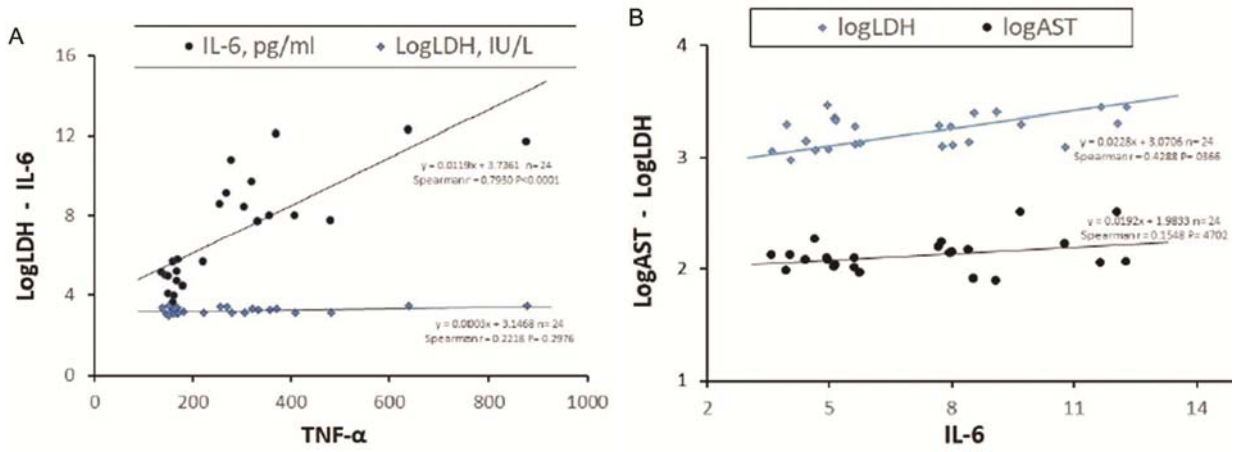

Fig. 3 — (A) Spearman correlation analysis between TNF-α and LDH and IL-6 levels. A positive correlation was found between TNF-α and IL-6 levels; and (B) Spearman correlation analysis between IL-6 and AST and LDH levels. A moderate positive correlation was found between IL-6 and LDH levels

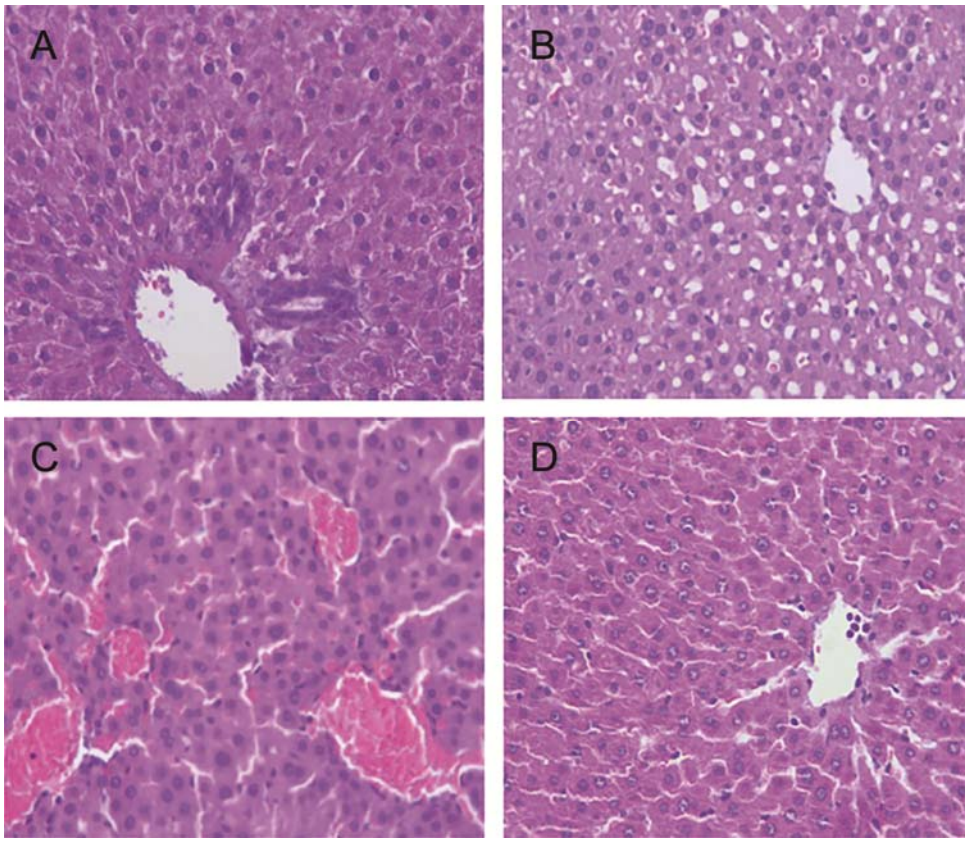

Fig. 4 — Representative H&E stains of rat hepatic tissues (A) Control group sample with normal hepatic tissue structure (H&E; ×40); (B) DM group sample with inflammatory cell infiltration and vacuolization sinus dilatation in the liver (H&E; ×40); (C) DM+I/R group sample with disordered hepatic lobules, swelling cells and vacuoles in the liver specimens were visible (H&E; ×40); and (D) Diabetic and hepatic I/R+Walnut group sample with minimal cell infiltration (H&E; ×40)

(DM+I/R+Walnut group), the increased level was relatively lesser. These results are also supported by histopathological findings and biochemical test results on AST, ALT, and LDH levels in that walnut prevented the alterations which take place due to oxidative stress in diabetic rats.

Diabetes mellitus is one of the diseases that cause the highest mortality and morbidity in the world. Microvascular and macrovascular complications such as diabetic retinopathy, diabetic nephropathy, diabetic neuropathy, ischemic heart disease, stroke, and atherosclerosis are frequently seen in these patients. Oxidative stress plays important role in the emergence of these complications[20]. Oxidative stress is primarily caused by the increase of ROS and/or reactive nitrogen species (RNS) in the organism[21]. The possible sources of oxidative stress in diabetes are the auto-oxidation of glucose, shifts in redox equilibrium, decreased tissue concentrations of antioxidants or reduced glutathione, or inadequate activity of antioxidant enzymes such as superoxide dismutase (SOD) or catalase (CAT)[22,23].

The ROS produced after the I/R injury stimulates the release of pro-inflammatory cytokines thereby activating macrophages. Pro-inflammatory cytokines such as TNF-α and IL-6 play important role(s) in both the local and distant organ damage, causing the activation of polymorphonuclear leukocytes[24]. In our study, we found that inflammatory TNF-α and IL-6 levels were increased in hepatic I/R developed diabetic rats and this increase was accompanied by tissue damage. We also showed this finding with substantial elevation in the biochemical markers of hepatic tissue damage (such as AST, ALT, and LDH) and with the correlation determined between TNF-α, IL-6, LDH, and AST levels. Additionally, histopathological examination of liver tissue of the I/R rats showed tissue damage. Notably, when hepatic I/R developed in the walnut-fed diabetic rats, we observed that this inflammatory response and tissue damage were relatively lesser.

One of the most important target structures of the ROS that occurs when the organism develops I/R are

the lipids, and the lipid peroxidation is accepted as the key to I/R injury by some researchers[25]. Free oxygen radicals initiate lipid peroxidation by taking a hydrogen atom from polyunsaturated fatty acids, resulting in the formation of hydroperoxides. As a result of these reactions, the cell membrane loses its fluidity and the membrane integrity deteriorates, which leads to the release of cell fractions into the environment and cell death. On the other hand, these subcellular structures released into the environment trigger inflammatory events and worsen the damage. Malondialdehyde measurement is one of the most common indicators of lipid peroxidation in tissue, and in our study, we found that the MDA levels increased in the hepatic I/R developed diabetic rats and walnut nourishment prevented this increase.

Some enzymatic and non-enzymatic natural antioxidants protect the organism against oxidative stress. Among those, non-enzymatic antioxidants include A, C, E, B1, B2, B12 vitamins, glutathione, alpha –lipoic acid, carotenoids, coenzyme Q10, bioflavonoids, some minerals (*e.g.*, copper, zinc, manganese, and selenium), folic acid, uric acid, and albumin. The enzymatic antioxidants are SOD, CAT, glutathione peroxidase (GSH-PX), and glutathione reductase (GSH-RD)[26]. Under normal physiological conditions, antioxidants affect signal transduction, immune response, and regulation of cell proliferation. Various antioxidants can be used in the treatment and prevention of cancer, diabetic complications, and cardiovascular diseases caused by ROS[27].

Walnut (*Juglans regia* L.) is a food stuff (a functional food) with many positive effects on health[28-32]. Walnuts contains protein (13.6 - 22.3%), fat (56.4 - 70.6%), and ash (2%). Its fat content is consists of 72% of polyunsaturated fatty acid (PUFA, 59% linoleic, 13% α-linoleic), 18% monounsaturated fatty acid (MUFA; oleic acid), and 10% saturated fatty acids[17]. Walnut contains a high percentage of PUFA and Omega-3 fatty acids. Containing both n-6 and n-3 PUFA together is a very important feature of walnut[33]. In addition, it contains numerous antioxidants and minerals such as vitamins A, C, E, B1, B2, folic acid, pantothenic acid, niacin, iron, zinc, copper, magnesium, and phosphorus. Walnut includes δ-tocopherol, phytosterol, and polyphenols, which are also antioxidants and increase the importance of walnut. Furthermore, the presence of melatonin in walnut structure with antioxidant properties and beneficial effects on the cardiovascular system has been found[34]. Considering all these points, walnut is a miraculous antioxidant store, and in our study, the lower I/R damage in the diabetic rats fed with walnuts, the lower TNF-α, IL-6, and MDA levels proved that walnut is a very powerful antioxidant.

As with any biomedical study, there are some potential limitations that are worth mentioning. First, this is an experimental study carrying all potential bias associated with the differences in metabolisms of rats and humans. Second, it would have been better if we could have measured the MDA and glutathione levels in the tissue specimens of various experimental groups, which we could not do due to limited resources.

Thus, we demonstrate that in diabetic rats, hepatic I/R injury is associated with an augmented inflammatory response and oxidative stress, while walnut pre-treatment significantly decreased these responses. Diabetes mellitus is a rapidly growing disease with high morbidity and mortality worldwide, as there are many vascular complications related to oxidative stress in diabetic patients. We suggest that the walnut can be considered as a miraculous antioxidant depot and should be involved in the nutrition and treatment of these patients. We believe that it is important to perform more translational research studies in this exciting area to gain better clinical outcomes for patients with diabetes.

Conflict of interest

All authors declare no conflict of interest.

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