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Evaluation of ischemia/reperfusion injury in streptozotocin-induced diabetic rats and its amelioration by hazelnut

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Beneficial effects of hazelnuts on human health are well known, and recent research on heart health has generated more interest. However, studies based on its oxidative damage preventive effect are not well explained in patients with diabetes mellitus (DM). Therefore, the aim of this study was to investigate the protective effect of hazelnut against oxidative stress-induced damage in hepatic ischemia/reperfusion (I/R) injury in rats induced by streptozotocin (STZ). Rats were divided into four groups (Control, DM, DM+I/R, DH; n=6 in each). The I/R model was created four weeks after DM was induced in rats. In four weeks until the I/R model was created, the DM+I/R+Hazelnut group was fed with pellet feed (2 g/100 g/day) prepared with hazelnut. At the end of experiment, glutathione (GSH) and malondialdehyde (MDA) levels in liver tissue, tumor necrosis factor-α (TNF-α), and interleukin 6 (IL-6) levels were measured in serum. The results showed that TNF-α, IL-6, and MDA levels increased significantly in the DM+I/R group, while this increase was less in the DM+I/R+Hazelnut group. It is concluded that hepatic I/R damage caused increased inflammatory response and oxidative stress in diabetic rats, and this response was decreased/modulated by the pre-treatment with hazelnuts.

Keywords: Ischemia/reperfusion injury, Animal model, Diabetes mellitus, Hazelnut, Oxidative stress, Inflammatory response

Diabetes mellitus (DM) is a chronic metabolic disease caused by an insufficiency in insulin secretion, effect or both, and scharacterised by hyperglycemia[1]. The prevalence of DM, which has increased four times during the last 35 years worldwide is 224 million now, and according to the World Health Organization (WHO) which will reach 360 million by the year 2025[2,3]. Vascular complications such as the increased risk of ischemic heart disease, atherosclerosis, and nephropathy, occurring in the advanced stages of diabetes are among the most serious health problems in the world due to the high prevalence of the disease[3,4]. Ischemic diseases are frequently diagnosed in the clinic and cause vital damage to organs and their functions[5]. Ischemia/reperfusion (I/R) injury, which is characterised by deficient oxygen supply and subsequent restoration of blood flow, is associated with oxidative stress and inflammation. I/R can cause irreversible damage to tissues[1,6]. The reactive oxygen species (ROS) generated at the onset of reperfusion induces irreversible injury that results in either direct cellular damage (e.g., apoptosis, membrane disturbances) or indirect damage through cellular signalling that stimulates inflammatory responses[7,8]. The liver consists of unstable cells that are very susceptible to I/R damage[9]. Although I/R pathogenesis is complex, ROS have the greatest effect on its pathobiology. While ROS is generally known as a toxic byproduct created by metabolism and the initiator of macromolecular damage in the body, it plays important roles in signalling. Low levels of ROS contribute to normal cellular functions, including signal transduction and counterbalanced by antioxidants. It has been recognised that the effects of

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ROS on cell viability and functions are directly related to pathologies observed in the liver[1,6,10]. Although DM increases I/R sensitivity, its relevant mechanism has not been fully elucidated. In addition to hyperglycemia, increased oxidative stress and abnormalities in the immune response are thought to increase I/R injury.

Prooxidants are enhanced while a decrease in antioxidants causes oxidative stress, which plays important role(s) in liver damage. DM also contributes oxidative stress by causing free radical production and reduced antioxidant protection. In addition to being a stable metabolite of the lipid peroxidation cascade, malondialdehyde (MDA) is used as a marker for oxidative stress in studies that aimed to demonstrate oxidative damage by determining MDA levels in liver tissues and the effect of hazelnut on this damage[1,11]. In DM, the related inflammatory damage causes an abnormal immune response due to decrease in the number of leukocytes. In addition, proinflammatory cytokines such as interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) can activate neutrophils and are over-produced in DM and reperfusion damage. Thus, the response of the liver to I/R damage is further induced by inflammation that occurs in DM, and the accumulation of neutrophils in the liver causes parenchymal damage[1,6].

Nowadays, medicinal plants are widely used to treat different medical conditions depending on the worldwide interest for exploitation from natural products. Numerous preclinical (experimental) and clinical reports described the antioxidant properties of nuts. Increasing the level of antioxidants in diabetic patients contributes to the regulation of cellular signals and gene expressions that lead to the inflammatory response, to decrease the inducer effect of ROS on I/R, and thus to the protection of liver[4]. Hazelnut (*Corylus avellana* L.) belongs to the family of Betulaceae, and it consists of various bioactive compounds such as selenium, tocopherol, folate, magnesium, potassium, calcium, protein, natural phytosterols, mono- and poly-unsaturated fatty acids, vitamin B, and phenolic acids such as protocatechuic acid, salicylic acid, syringic acid, vanillic acid, caffeic acid, ferulic acid, o-coumaric acid, and gallic acid. Tannins and polyphenols that are existent in both the shell and the seed of hazelnut are responsible for its antioxidant effect[8,12-16]. Antioxidant compounds in hazelnuts reduce fasting blood sugar levels and total cholesterol by maintaining the prooxidant-antioxidant balance[17]. Considering the properties of hazelnut studies in patients with DM and epidemiological studies, it is hypothesised that hazelnut consumption may decrease the incidence of chronic diseases such as cardiovascular disease, DM, and metabolic syndrome by increasing insulin sensitivity and secretion or by regulating postprandial glycemia[18]. Since the incidence of DM is increasing day by day, there is an urgent need to cure the I/R damages in the livers of diabetic patients. Therefore, the antioxidant, hepatoprotective and anti-inflammatory effects of hazelnut on these damages were investigated in this study by inducing I/R damage in diabetic rats.

Materials and Methods

Reagents

The Tombul hazelnuts (*Corylus avellana* L.) were acquired from Giresun City, Türkiye. Thiobarbituric acid (TBA), KCl, 1,1,3,3 tetraethoxypropane, trichloroacetic acid (TCA), and dithionitro benzene were purchased from Sigma Aldrich. ELISA kits were bought from Bioassay Technology Laboratory, Korain Biotech Co., Ltd. (Shanghai, China). All purchased materials were of analytical grade.

Animals and study design

Male Sprague-Dawley albino rats (n=24) weighing 250-350 g were used, which were housed in a room at a constant temperature of 22±2°C, with 12h light/dark cycles, and fed on a diet enriched with hazelnut pellet chow and water *ad libitum*. The animals were obtained from the Laboratory of Animal Care Division of School of Medicine at Marmara University, Istanbul, Türkiye. 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, Türkiye (Ethical Committee Reference No: 26.2020 mar). The rats (n=24) were equally divided into four groups (n=6 each) as follows: Control (C), Diabetes mellitus (DM), Diabetes mellitus+Ischemia/Reperfusion (DM+I/R), and Diabetes mellitus+Ischemia/ Reperfusion+Hazelnut (DH). Streptozotocin (STZ) treatment and I/R procedure were not performed in the control group. The other three experimental groups were first administered 60 mg/kg STZ intraperitoneally (IP; Sigma Aldrich, St. Louis, MO, USA) to create a diabetes model. The rats' body weight was measured using Precision Scales (Northglenn, CO, USA). After 48h, 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 I/R model was created in the rat groups fed with hazelnut (2 g hazelnut/day) and not fed, 10 days after diabetes formation. All other experimental groups were fed only with pellet feed. In the fourth week, the rats were given 100 mg/kg ketamine for anaesthesia. 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, 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 analysis

Analysis of MDA

The levels of MDA, which is the final product of lipid peroxidation, were measured in tissues and serum. Tissues removed immediately after decapitation were homogenised with 150 mM KCl (10% homogenate), then 1 mL of TBA (thiobarbituric acid) solution was added onto 5 mL of homogenate which was kept at 100°C for 15 min, and then cooled. The absorbance of the resulting colour was read at 532 nm and the results are expressed as nmol MDA/g tissue. 1,1,3,3-tetraethoxypropane was used as the standard solution.

Analysis of GSH

Glutathione (GSH) levels were determined was made according to the Ellman method in 10% homogenates (that was prepared for MDA determination also). After adding 0.1 mL of 20% TCA (trichloroacetic acid) and mixing, it was centrifuged for 10 min, then 0.2 mL of supernatant was taken and 1 mL of 0.3 M Na_2HPO_4 and 50 µL of DTNB (dithionitro benzene) were added. The absorbance of the resulting color was read at a wavelength of 412 nm and the results are expressed in µmol GSH tissue.

Analysis of cytokines

The blood sample was taken after sacrification and centrifuged to assess the serum levels of TNF-α and IL-6, double sandwich enzyme-linked immune sorbent assay (ELISA) kits were used (R&D Systems, Minneapolis, MN, USA) as per the manufacturer's protocols.

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 analysis

All statistical analysis were determined utilising GraphPad Prism 9.0 program. Outcomes were given as means ± standard error of the mean (SEM). Statistical significance between the groups was analysed by one way analysis of variance (ANOVA) followed by Tukey's post hoc test. Values for $P < 0.05$ were considered statistically significant.

Results

Body weight and blood glucose level

The body weight and blood glucose levels were measured at the beginning and 2 days after STZ injection and were found to be similar at the beginning of the study among all the groups. 48h after the STZ injection, animals showed a significant weight loss compared to the age matched controls (Fig. 1A), and the average blood glucose levels were significantly increased (Fig. 1B) and 28 days after the

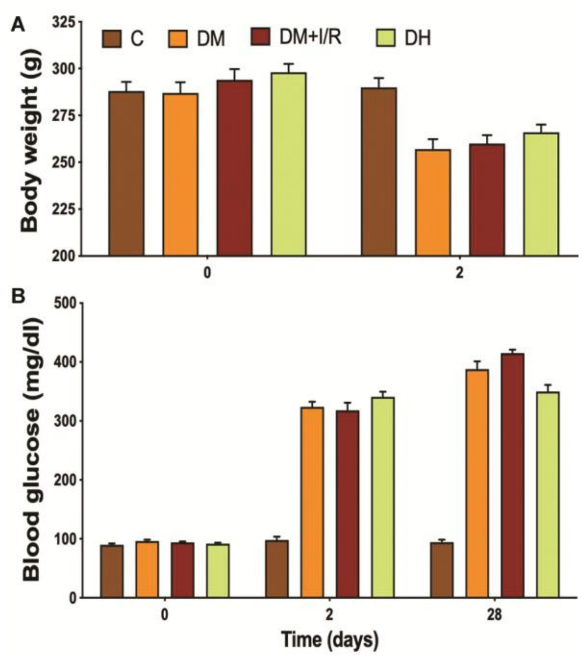

Fig. 1 — Determination of body weight and blood glucose levels in all the experimental groups of rats. [C = Control; DM = Diabetes mellitus; DM+I/R = Diabetes mellitus + Ischemia/Reperfusion injury; DH = DM + I/R + Hazelnut; STZ = Streptozotocin]

STZ injection, the average blood glucose levels were found to be significantly increased (Fig. 1B).

MDA levels

Fig. 2. shows the levels of MDA determined in liver tissues, which showed a significant decrease in the DH group as compared to the DM and DM+I/R groups (P<0.05). The MDA levels in the DH group were nearly equal to the rats in the C group (Fig. 2). In addition, MDA levels in the DM group were significantly higher than those in the C group (P<0.05).

GSH levels

Fig. 3. shows the data on GSH levels determination in liver tissues. There was no significant difference in the GSH levels between the groups in liver tissue (ranged approximately 4-4.5 µmol tissue in liver).

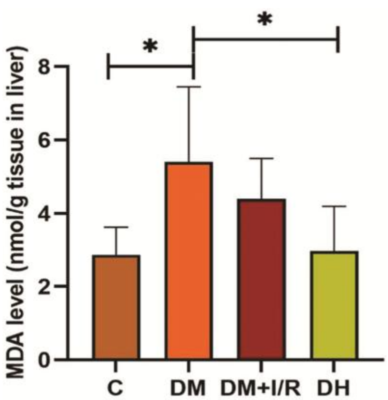

Fig. 2 — Results on the liver tissue MDA levels in all the experimental groups of rats. Each group (n=6) represents mean ± standard error of the mean (SEM). Values are represented statistically when *P<0.05 in comparison to the Control group. [MDA = Malondialdehyde; C = Control; DM = Diabetes mellitus; DM+I/R = Diabetes mellitus + Ischemia/Reperfusion injury, DH = DM + I/R + Hazelnut]

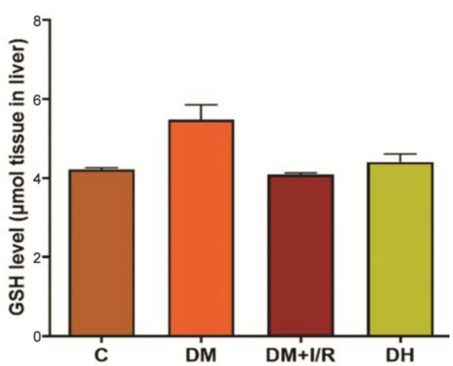

Fig. 3 — Data on the liver tissue glutathione levels in all the experimental groups of rats. [GHS = Glutathione; C = Control; DM = Diabetes mellitus; DM+I/R = Diabetes mellitus + Ischemia/Reperfusion injury; DH = DM + I/R + Hazelnut]

Serum TNF-α and IL-6 levels

Data obtained for the determination of TNF-α levels of the experimental groups are shown in Fig. 4. The TNF-α levels in the DH 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 the TNF-α levels between the DH and control groups (P>0.05). Also, there was no significant difference in the TNF-α levels between the DM and DM+I/R groups (P>0.05; Fig 4). As shown in Fig. 5, serum IL-6 levels in the DH group were found to be significantly lower than in both the DM and DM+I/R groups (P<0.05). However, the IL-6 levels of the DM+IR group were significantly higher than the control group (P<0.05). Notably, the IL-6 levels of the DH group were also significantly lower than the Control group (P<0.05; Fig. 5).

Histopathological findings

Hepatic cells and hepatic microvascular injury were investigated by light microscopy. Utilising H&E

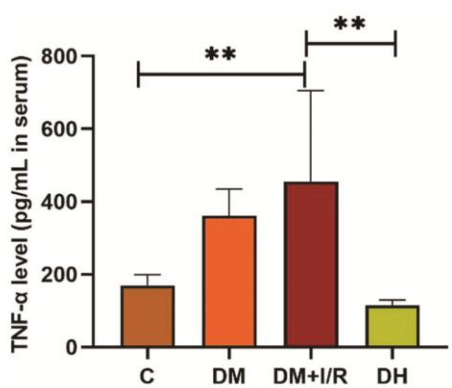

Fig. 4 — Determination of serum levels of TNF-α in all the experimental groups of rats. **P<0.05. [TNF-α = Tumor necrosis factor-alpha; C = Control; DM = Diabetes mellitus; DM+I/R = Diabetes mellitus + Ischemia/Reperfusion injury; DH = DM + I/R + Hazelnut]

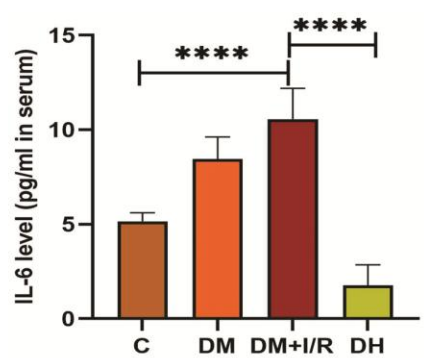

Fig. 5 — Determination of serum levels of IL-6 in all the experimental groups of rats. ***P<0.001. [IL-6 = Interleukin-6; C = Control; DM = Diabetes mellitus; DM+I/R = Diabetes mellitus + Ischemia/Reperfusion injury; DH = DM + I/R + Hazelnut]

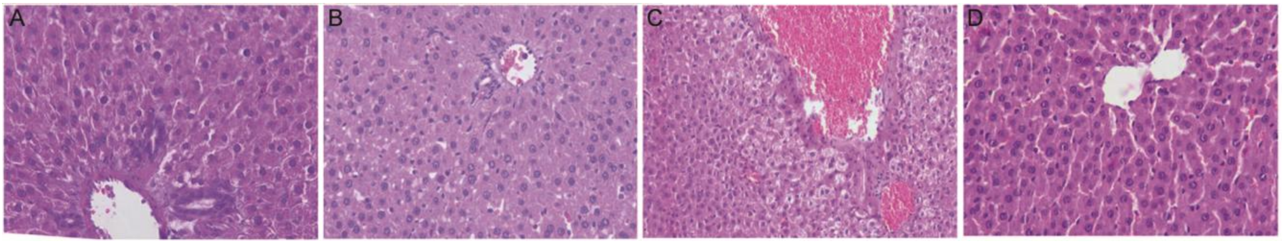

Fig. 6 — Hematoxylin and eosin (H&E) stains of hepatic tissues. (A) Control group sample with normal hepatic tissue structure (H&E;×40); (B) Diabetes mellitus (DM) group sample with inflammatory cell infiltration and vacuolisation sinus dilatation in the liver (H&E; ×40); (C) Diabetes mellitus + ischemia/reperfusion injury (DM+I/R) group sample with disordered hepatic lobules, swelling cells and vacuoles in the liver specimens are visible (H&E; ×40); and (D) DM+I/R + Hazelnut (DH) group sample with minimal cell infiltration and sinusoidal dilatation in the liver (H&E; ×40).

staining, we observed that disordered hepatic lobules, swelling cells, and vacuoles in the liver specimens were visible in the DM+I/R group, which indicates that the hepatic I/R injury did occur. The phenomenon seems to improve in the DH group of specimens (Fig. 6).

Discussion

Reperfusion is essential to support cell metabolism and to eliminate potentially harmful byproducts of cellular metabolism. When organs are exposed to ischemia, severe tissue damage occurs and liver is one of the organs that is most susceptible to ischemia[19] The I/R injury is a complex process involving multiple intracellular signalling pathways, pathophysiological disorders and is also associated with metabolic diseases that affect the body physiology such as DM[20]. In the I/R injury, microcirculatory disorders caused by ROS production are first detected, while the tissue ischemia and oxidative stress activate protein kinase families. The inflammatory genes such as TNF-α and IL-6 are synthesised, which plays a key role in tissue damage, gets down regulated with the involvement of leukocytes[9]. Although oxidative stress is associated with inflammatory, cardiovascular, and renal diseases, it is also closely related to metabolic diseases such as DM. Within this context, we investigated the fact as to how the use of hazelnut affects oxidative damage in DM associated with I/R injury. Free radicals show their adverse effects on the cells by producing toxic substances such as MDA, which is an indicator of lipid peroxidation induced by oxidative damage. When I/R injury occurs in the STZ mediated diabetic rats, the MDA levels are increased compared to the non diabetic control group. According to our findings, DM especially triggered oxidative damage in the liver. Besides, a significant decrease in the MDA levels in the treatment group suggests that hazelnut is effective in reducing oxidative damage[12]. In previous studies on the antioxidant effect of hazelnut, it has been shown that it is a potential natural antioxidant source by analysing its antioxidant effects[21]. Liver damage after the I/R injury and DM is prevented by hazelnut application, as well as this information is supported by other peer reviewed literature[22-24]. Enzymatic and non-enzymatic antioxidant components in the body against oxidative damage caused by ROS are vitamins A, C, and E, GSH, superoxide dismutase, and catalase. GSH is a non-enzymatic antioxidant found in all animal cells. While GSH levels decreased in serum and tissue specimens potentially due to increased oxidative damage caused by DM and I/R, no significant decrease in the GSH levels was observed in the studies performed in liver tissue[24,25].

Inflammation plays role in the onset and progression of many chronic diseases, including diabetes. TNF-α and IL-6 are inflammatory cytokines that increase in the body due to inflammation[26]. As in our study, the MDA level in the liver tissue was decreased in the DM+I/R and DH groups as compared to the DM group. There was a decrease in the liver tissue in the hazelnut group as compared to the DM group. Also, the TNF-α and IL-6 levels showed a significant decrease after the hazelnut administration.

According to the histopathological assessments, irregular hepatic lobules and vacuoles were observed in the DM+I/R and DM groups, but not in the DH and control groups. The main histological findings in the liver with DM was the 'excessive cell infiltration and vacuolisation'. More severe hepatocyte necrosis, sinus congestion, and hepatocyte ballooning are observed in the diabetic liver exposed to I/R injury. In the control group liver specimens, histopathology findings were normal. In the DM+I/R group, we detected swelling in the cells, congestion, hepatic lobules defect, and cell infiltration. In the DH group, minimal cell infiltration and improvements due to vacuolisation were observed in the liver tissue[11,27]. The histopathological findings demonstrate that treatment with hazelnut effectively benefits for IR induced injury in the diabetic rat liver.

Conclusion

The results of our experimental study suggest that hazelnut is a valuable candidate as an alternative nutrient for the management of DM and I/R injury. Therefore, translational research studies are desirable to further validate our experimental findings in clinical settings.

Ethical Approval

All procedures for experimental protocols of the present study involving animals were performed in accordance with the ethical standards of the institutions of practice at which the studies were conducted (Ethical Committee Reference No: 26.2020mar).

Conflict of interest

The authors declare no conflict of interest.

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