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Full Length Research Paper

Protective effect of quercetin against necrosis and apoptosis induced by experimental ischemia and reperfusion in rat liver

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Quercetin is a well-known flavonoid and a strong antioxidant. Quercetin an important flavonoid possesses beneficial effects on health due to its antioxidant function. One mechanism of the antioxidant action of quercetin was involved in scavenging free radicals, such as superoxide radicals generated by xanthine and xanthine oxidase. To study the effect of quercetin on apoptosis and necrosis induced by 1 h ischemia followed by 1.5 h reperfusion. Adult Wistar rats underwent 1 h of partial liver ischemia followed by 1.5 h reperfusion. Eighteen Wistar rats were divided into sham-operated control group (I) (n = 10), ischemia and reperfusion (I/R) group (0.9 % saline (5 ml/kg, orally) for 14 days) (II) (n = 10), and quercetin group (15 mg/kg body weight daily orally for 14 days before inducing ischemia-reperfusion maneuver) (III)(n=10). Apoptotic and necrotic hepatocytes, nitric oxide levels in hepatocytes. Liver injury was assessed by plasma alanine transaminases (ALT), aspartate transaminases (AST), liver histopathology. An ischemic and reperfusion hepatocellular injury occurred as was indicated by increased serum ALT, AST, histopathology. Pretreatment with quercetin significantly decreased serum ALT and AST level and apoptotic and necrotic cells after 1 h ischemia followed by 3 h of reperfusion. Nitric oxide production in hepatocytes was increased twofold by quercetin treatment when compared with I/R group. Histopathology studies showed markedly diminished hepatocellular injury in quercetin -pretreated rats during the hepatic I/R. Thus, it may be concluded that quercetin can significant protection from necrosis and apoptosis in I/R injury with nitric oxide and plasma alanine transaminases (ALT) production and it has anti-ROS effect in ischemic reperfusion.

Key words: Apoptosis, ischemia, reperfusion, quercetin.

INTRODUCTION

Ischemic reperfusion (I/R) injury is a phenomenon whereby cellular damage occurs because of oxygen delivery into tissues (Mühlberger et al., 2009; Piao et al., 2009). This form of injury in the liver was recognized as a clinically important pathological disorder (Carden et al., 1993; Muriel, 2009). Deprivation of oxygen, nutrients, or growth factor may be important for the cause of I/R injury resulting in cell atrophy and apoptosis. The acute injury phase (early phase), which is characterized by liver injury occurring within 1 - 6 h after reperfusion, is associated with Kupffer cell activation, release of the pro inflammatory cytokines, and the generation of reactive oxygen species (ROS) (Carden et al., 1993; Delva et al., 1989). Apoptosis and cell atrophy have been indicated as an important mode of cell death during hepatic I/R injury (Gujral et al., 2001). Previous study showed that the initial peak of apoptosis and necrotic cells was after 1 h ischemia, followed by 30 min reperfusion. Oxidant stress-induced cell killing involves the oxidation of pyridine nucleotides, accumulation of calcium in mitochondria, and

superoxide formation by mitochondria, which ultimately lead to the formation of membrane permeability transition pores and breakdown of the mitochondrial membrane potential (Carden et al., 2000; Curran et al., 1989). Quercetin is regularly consumed by humans as it is the major flavonoid found in human diet (Manach et al., 1998). This flavonoid is reported to decrease capillary fragility, to protect against diabetic cataracts, to possess antiviral and antiallergenic activities, to inhibit platelet aggregation and the oxidation of low density lipoproteins, and to act as an anti-inflammatory agent (Sexton et al., 1997).

Quercetin an important flavonoid possesses beneficial effects on health due to its antioxidant function. One mechanism of the antioxidant action of quercetin involved in scavenging free radicals, such as superoxide radicals generated by xanthine and xanthine oxidase (Peluso, 2006). The effect of quercetin therapy on hepatic ischemia has not yet been completely elucidated. Therefore, it was decided to investigate the role of Protective effect of quercetin against necrosis and apoptosis induced by experimental ischemic and reperfusion in rat liver.

MATERIALS AND METHODS

Preparation of quercetin

Quercetin powder was obtained from Sigma Chemical Company (St. Louis, MO, USA). It was dissolved and diluted with 20% glycerol in 0.9% normal saline, mixed vigorously and stored in a dark bottle at 4ºC. The quercetin solution was freshly prepared each week.

Animals

Thirty adult Wistar albino rats with 8 weeks old and weighing 250 ± 10 g, they obtained from animal facility of pasture institute of Iran rats were housed in temperature controlled rooms (25°C) with constant humidity (40 - 70%) and 12 h / 12 h light/ dark cycle prior to use in experimental protocols. All animals were treated in accordance with the Principles of Laboratory Animal Care. The experimental protocol has been approved by the Animal Ethical Committee in accordance with the guide lines for the care and use of laboratory animals prepared by Tabriz medical University. All Rats were fed a standard diet and water. The daily intake of animal water was monitored at least one week prior to start of treatments in order to determine the amount of water needed per experimental animal. The hepatic I/R protocols were performed as described in a previous study (Mahesh et al., 2004; Gujral et al., 2001) After the induction of anesthesia (urethane 10 mg/kg i.p.), the liver of each rat was exposed through a midline laparotomy. Complete ischemia of the median and left hepatic lobes was produced by clamping the left branches of the portal vein and the hepatic artery for 1 h. The right hepatic lobe was per fused to prevent intestinal congestion. After the period of ischemia, the ligatures around the left branches of the portal vein and hepatic artery were removed. To accurately evaluate the blood flow of the median and left hepatic lobes after ischemia, the right branches of the portal vein and the hepatic artery were ligated to prevent shunting to the right lobe after reperfusion and per fused for 1.5 h. The wound was closed with 2 - 0 silk suture.

Control-operated animals were similarly prepared except that no ligature was placed to obstruct the blood flow to the left and median hepatic lobes. Instead, the blood flow to the right lobe of the liver was occluded. In all groups rats were sacrificed after 1 h ischemia followed by 1.5 h reperfusion. A total of 30 Wistar rats were equally divided into three groups (n = 10 each group). Group I (control group) and Group II (ischemia and reperfusion group) were given 0.9% saline (5 ml/kg, orally) for 14 days. Group III was treated with quercetin (15 mg/kg orally) for 14 days before induced ischemia-reperfusion maneuver.

Blood samples

Blood samples were obtained from the right ventricle via left anterior thoracotomy at the time of sacrifice. Blood was collected in a sterile syringe without anticoagulant and centrifuged at 2000 g to separate the serum. The serum samples were stored at -20℃ until use for AST and ALT assays.

Measurement of NO and liver function enzymes

NO was measured as method that described by Hibbs et al. (1998). Serum was used for the assay of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) measured by using estimated by Merck assay kits (Merck Ltd,) according to the manufacture's instructions.

Light microscopy

Serial slices of liver tissues were prepared from rats in each group and stained with hematoxyline-eosin (H&E) and then observed under a light microscope at ×320 magnifications.

TUNEL analysis of apoptosis

The *in-situ* DNA fragmentation was visualized by TUNEL method (khaki et al., 2008). Briefly, dewaxed tissue sections were predigested with 20 mg/ml proteinase K for 20 min and incubated in phosphate buffered saline solution (PBS) containing 3% H_2O_2 for 10 min to block the endogenous peroxidase activity. The sections were incubated with the TUNEL reaction mixture, fluorescein-dUTP (in situ Cell Death Detection, POD kit, Roche, Germany), for 60 min at 37℃. The slides were then rinsed three times with PBS and incubated with secondary antifluorescein-POD-conjugate for 30 min. After washing three times in PBS, diaminobenzidine-H_2O_2 (DAB, Roche, Germany) chromogenic reaction was added on sections and was counterstained with hematoxylin. As a control for method specificity, the step using the TUNEL reaction mixture was omitted in negative control serial sections, and nucleotide mixture in reaction buffer was used instead. Apoptotic cells were quantified by counting the number of TUNEL stained nuclei per cross section. Cross sections of 100 specimens were assessed and the mean number of TUNEL positive cells per cross- section was calculated.

Table 1. Effect of quercetin on ALT, AST, NO, necrosis and apoptosis induced by experimental ischemia and reperfusion in rat liver.

Groups (n=10)	ALT(IU/L)	AST(IU/L)	NO (pmol)	Necrosis (percentage in 100 cross sections)	Apoptosis (percentage in 100 cross sections)
Group I	73.13 ± 8.20	48.02 .± 9.52	9.22 ± 1.16	1.00 ± 1.11	1.30 ± 0.01
Group II	1300.16±20.11**	700.10 ± 54.12**	6.11 ± 1.52	17.34 ± 3.1*	7.50 ± 0.02*
Group III	170.16 ± 10.28*	99.10 ± 22.11	20.10 ± 10.51*	10.54 ± 7.1*	2.72 ± 0.32

Group I (control group)
Group II (ischemia and reperfusion group)
Group III was treated with quercetin (15 mg/kg orally)
Values are mean ± SE
*Significant different at P < 0.05 level, (compared with the control group).
**Significant different at P < 0.01 level, (compared with the control group).

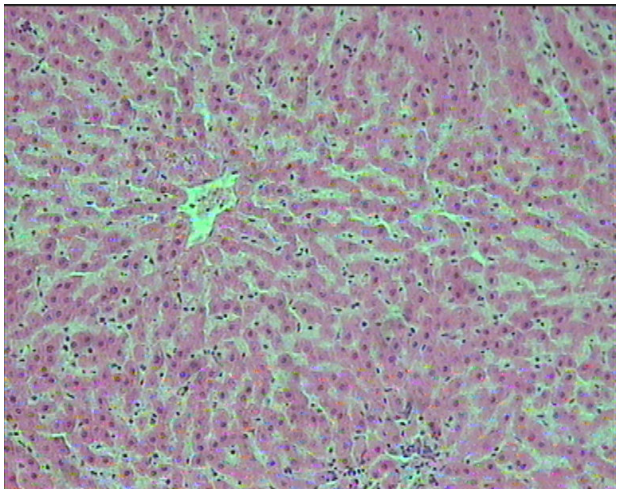

Figure 1. Morphological damage in hepatocyte of rats in sham-operated control group .H&E (x320)

Statistical analysis

All values were expressed as mean ± SD. Differences in mean values were compared by one-way ANOVA test. P < 0.05 was considered as statistically significant.

RESULTS

Effect of 15 mg/kg quercetin on levels of ALT and AST

The ALT and AST levels in the sham-operated control rats were73.13 ± 8.20*IU/L*,and 48.02 ± 9.52*IU/L*, respectively, which increased (P < 0.01)to 1300.16 ± 20.11*IU/L* and 700.10 ± 54.12*IU/L*, respectively, after 1 h ischemia followed by 1.5 h reperfusion. The significant increase in ALT and AST activities that occurred after 1 h ischemia followed by 1.5 h reperfusion in the I/R group was significantly suppressed (P < 0.05) by the administration of 15 mg/kg quercetin (Table 1).

Effect of 15 mg/kg quercetin on the production of NO, necrosis, and apoptosis

In the sham-operated control rats, the NO level remained constant at 9.22 ± 1.16 pmol NO formed/min/mg of protein throughout the experiment. In the I/R rats, NO level decreased to 6.11 ± 1.52 pmol/min/mg of protein after 1 h ischemia followed by 1.5 h reperfusion. This reduction in NO was attenuated by 15 mg/kg quercetin .The percentage of necrotic and percentages of apoptotic cells were 1.00 ± 1.11 and 1.30 ± 0.01, respectively. In the I/R rats, necrotic and apoptotic cells increased to 17.34 ± 3.1 and 7.50 ± 0.02, respectively .The decreases were restored to the level observed in sham-operated control rats by 15 mg/kg quercetin (Table 1).

Effects of 15 mg/kg quercetin on pathological changes of the liver tissue

No pathological damage was observed in any of the rats in sham-operated control group (Figure 1).In I/R-treated rats, the hepatocytes were markedly swollen, with striking vacuolization. In addition, areas of necrosis and cell infiltration were noted (Figure 2). 15 mg/kg quercetin treated rats showed well-preserved liver parenchyma with heaptocytes extending from the central vein. Also quercetin treated rats showed the regular sinusoidal structures and normal morphology, without any signs of congestion (Figure 3)

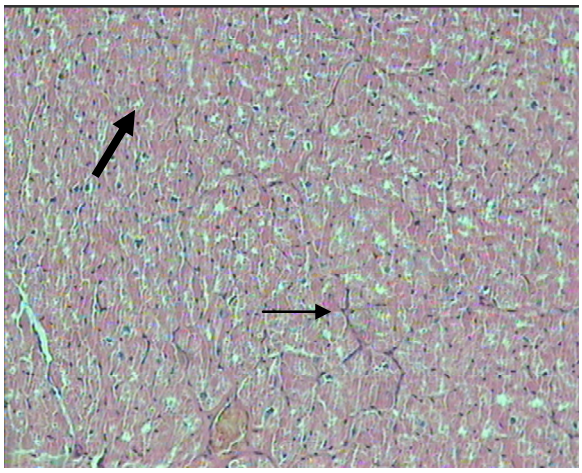

Figure 2. In I/R-treated rats, the hepatocytes were markedly swollen, with striking vacuolization. In addition, areas of necrosis (arrow) and cell infiltration were noted .H&E (x320).

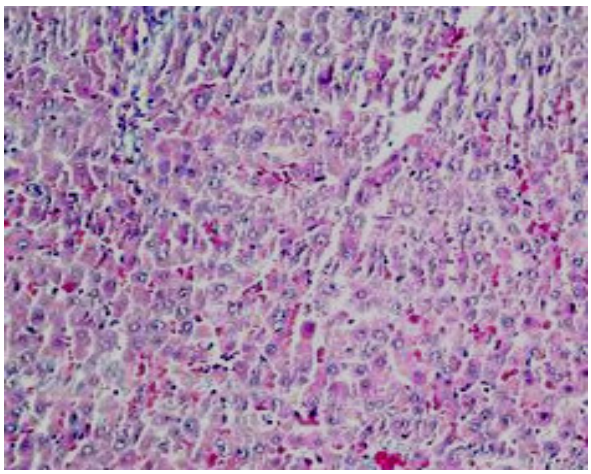

Figure 3. 15 mg/kg Quercetin treated rats showed well-preserved liver parenchyma with hepatocytes extending from the central vein. Also Quercetin -treated rats showed the regular sinusoidal structures and normal morphology, without any signs of congestion H&E (x320).

DISCUSSION

The dietary intake of flavonoids in humans has been estimated to be 16 – 1000 mg/day. Quercetin is regularly consumed by humans as it is the major flavonoid found in the human diet (Manach et al., 1998). This flavonoid is reported to decrease capillary fragility, to protect against diabetic cataracts, to possess antiviral and antiallergenic activities, to inhibit platelet aggregation and the oxidation of low density lipoproteins, and to act as an anti-inflammatory agent (Sexton et al., 1997). Oxidative damage was ascertained by measuring the malondialdehyde levels, reactive oxygen species (ROS) generation, alterations in antioxidant defences and the level of oxidized-LDL. The results indicate that after 1 h ischemia followed by 1.5 h reperfusion, the liver function was improved by 15 mg/kg Quercetin pretreatment and could be protected against liver damage from necrosis and apoptosis induced by I/R injury. Hepatocyte NO production was increased significantly in Quercetin - treated group when compared with sham-operated rats. Nitric oxide, known as an endothelium-derived relaxing factor, is formed from the terminal guanidine nitrogen atom of Quercetin (Hibbs et al., 1988; Moncada et al., 1991; Chen et al., 2001). The family of Bcl-2-related proteins plays a key role in the regulation of apoptosis. Bcl-2, a member of the Bcl-2-related gene, can promote cell survival through protein-protein interactions with other Bcl-2-related protein family members. In this study, we found, increased number of necrotic cells and apoptosis (Khaki et al., 2009; Rajaie et al., 2008). These data are consistent with the previous study finding that over expression of Bcl-2 reduced ischemia/reperfusion injury (Oltvai et al., 1993). In addition, this study provides a new insight into the mechanism by quercetin mediates hepatoprotective. However, attempts to protect against hepatic I/R injury by alleviating the possible detrimental effects of portal venous congestion have not achieved satisfactory results. Previous study showed that apoptosis caused by I/R injury may be due to endonuclease activity or by acting on cell organelles, alternating signal transduction pathways or affecting the intracellular enzymes responsible for proper functioning and survival of the cell. TNF-α may mediate direct toxicity to mitochondria and induce apoptosis or cell death (Carden et al., 1993). The changes observed microscopically in I/R-injured rats showed severe degeneration of cellular architecture. Pretreatment with Quercetin showed considerable prevention in the structural alternations including the distribution of mitochondrial fine structure. Pathological study of the liver revealed regular sinusoidal structures in group III, as opposed to swollen cells with marked vacuolization seen in group II as a result of stimulating action of NO production. The results showed that treatment with quercetin improved the hepatocellular structure. Nitric oxide, known as an endothelium-derived relaxing factor, is formed from the terminal guanidine nitrogen atom of L-arginine by the action of NO synthesis (Moncada et al., 1991). Elevation of cGMP relaxes the smooth muscles in blood vessels including the genitourinary tract, inhibits platelet aggregation and adhesion, and blocks adhesion of white cells

to the blood vessel wall (Oltvai et al., 1993). The results of the present study suggest that treatment quercetin might have a role at a sub cellular level in preventing I/R-induced necrosis and apoptosis. This study also shows that Quercetin protects hepatobiliary function and the structure of liver in hepatic I/R- induced injury.

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