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The effects of quercetin on liver regeneration after liver resection in rats

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The aim of the present study was to assess the influence of quercetine (QE) on liver regeneration after partial hepatectomy (PH) in rats. A total of 24 male Wistar albino rats were divided into three groups: sham-operated (SH), PH and PH+QE; each group contain 8 animals. The rats in QE-treated groups were given QE (15 mg/kg body weight) once a day i.p., for 7 days starting 3 days prior to hepatectomy operation. At 7 days after resection, liver samples were collected. The malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione (GSH) levels were estimated in liver homogenates. Moreover, histopathological examination, mitotic index (MI), proliferating cell nuclear antigen labelling, proliferation index (PI), transferase-mediated dUTP nick end-labelling assay, apoptotic index (AI) were evaluated at 7 days after hepatectomy. As a result, QE significantly increased MI, PI, and significantly decreased AI in PH rats. Additionally, QE remarkably inhibited the elevation of MDA, restored impaired antioxidant SOD activity and GSH level, and also attenuated hepatic vacuolar degeneration and sinusoidal congestion. These results suggested that QE treatment had a beneficial effect on liver regenerative capacity of the remnant liver tissue after hepatectomy, probably due to its antioxidative, antiapoptotic and proliferative property. (Folia Morphol 2016; 75, 2: 179–187)

Key words: quercetine, partial hepatectomy, oxidative stress, immunohistochemistry, apoptosis, rat

INTRODUCTION

The liver is a unique organ with regards to its high capacity of regeneration after injury or ablation [42]. Despite the removal of 70% of its mass after partial hepatectomy (PH), the residual liver can expand to nearly its original weight in order to compensate for its lost tissues and function [35, 36]. This remarkable capacity enables liver transplantation in clinical practice. Regardless, hepatic resection imposes a large burden on patients with liver disorder and healthy donors. To minimize the risk of liver transplantation for a donor, a method for safe promotion of liver regeneration after PH is required.

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Oxidative stress, involving reactive oxygen species and antioxidant enzymes, which arises during intraoperative and postoperative process, is an important adverse factor for liver regeneration [19, 26]. Reducing reactive oxygen species production and preventing oxidative stress in the remnant liver are regarded as important methods to accelerate liver regeneration [14, 40].

Protection against oxidative stress may enhance liver regeneration after resection, as shown in a study using vitamins C and E [45]. Aside from these directly acting antioxidants, which after oxidation can act as pro-oxidants, indirect antioxidants exist. These are able to activate the aforementioned transcription factor Nrf2 and subsequently many cytoprotective proteins [27, 50]. The indirectly acting antioxidants have very low prooxidative effects [8].

Flavonoids are phenolic phytochemicals; they are important constituents of the nonenergetic part of the human diet and are thought to promote optimal health, partly via their antioxidant effects in protecting cellular components against reactive oxygen species (ROS) [17]. Quercetin (3,5,7,3'4'-pentahydroxy flavon) is one of the most widely distributed flavonoids, present in fruit, vegetables, and many other dietary sources [43]. This compound was reported to scavenge superoxide in ischaemia-reperfusion injury [23], to protect against oxidative stress induced by UV light [11], spontaneous hypertension [9], secondary biliary cirrhosis [44], and bacterial lipopolysaccharide [53], and to inhibit angiogenesis [24], carcinogenesis, [54] and portal hypertensive gastropathy [38]. At doses of 50 and 80 mg/kg, quercetin significantly lowered plasma thiobarbituric acid reactive substances and lipid hydroperoxides when given to rats with streptozotocin-induced diabetes for 45 days [33, 39]. A very recent report indicates that quercetin is also able to partially prevent serum nitric oxide increases in streptozotocin-treated rats [7]. The liver is the main organ of oxidative and detoxifying processes, as well as free radical reactions; in many diseases, biomarkers of oxidative stress are elevated in the liver at an early stage [47].

However, there has been no report about the effects of quercetine (QE) on regenerative capacity after hepatectomy. Therefore, our study presents the effects of QE on liver regeneration of rats which have undergone PH by performing biochemical and histological methods.

MATERIALS AND METHODS

Animals and treatment

In this study, 24 healthy male Sprague Dawley rats, weighing 300–350 g and averaging 12 weeks old were utilised. Food and tap water were available ad libitum. In the windowless animal quarter automatic temperature (21 ± 1°C) and lighting controls (12 h light/12 h dark cycle) was performed. Humidity ranged from 55% to 60%. All animals received human care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health. The study was approved by the Institutional Animal Ethical Committee of the University of Trakya, Edirne, Turkey (Protocol no: TUHDYEK-2011/89).

Experimental groups

Twenty-four Sprague Dawley adult rats are enrolled in this study, and divided into three groups as sham-operated (SH), PH and PH+QE; each group contain 8 animals. SH group, in which rats underwent anaesthesia, laparotomy, and exposure of the portal triad without hepatectomy; PH group, in which rats were subjected to 70% PH; PH+QE group, in which rats were given QE (in a dose of 15 mg/kg body weight) once a day orally for 7 days starting 3 days prior to hepatectomy operation. At the same time, the rats in the SH and PH groups received an equivalent volume of vehicle as control. At 7 days after resection, liver samples were collected.

Experimental protocols

Rats underwent 70% PH under intraperitoneally (i.p.) ketamine (90 mg/kg) and xylazine (10 mg/kg) anaesthesia in the midmorning using the method of Higgins and Anderson [18]. Briefly, the liver was exposed through a 1–2-cm midline abdominal incision and the two anterior lobes were exteriorised, the vascular pedicles were ligated, and the lobes were excised. After 7 days of PH, the animals were decapitated. Liver tissue samples were obtained for biochemical and histopathological investigation. A standard portion of the median liver lobe was used for histological evaluation, and the rest was rapidly frozen in liquid nitrogen for biochemical investigation.

Biochemical procedures

At the end of the experiment, the harvested liver tissue samples were quickly washed in cold saline and stored at –70°C.

Measurement of tissue malondialdehyde level

Liver tissue samples were frozen at –70°C and irrigated well with a solution of sodium chloride (NaCl) (0.9%). By admixing it with potassium chloride (KCl) (1.5%), homogenisation at a ratio of 1:10 was achieved. The DIA × 9000 Homogeniser (Heidolph Instruments, Germany) was used to homogenise the tissue samples. The lipid peroxide level in the centrifuged tissue homogenates was measured according to the method described by Ohkawa et al. [41]. The reaction product was assayed spectrophotometrically (Shimadzu UV-1700, Japan) at 532 nm. The lipid peroxide level was expressed as the nanomole (nmol) of malondialdehyde (MDA) per milligram of liver tissue protein. Protein levels were measured according to the method described by Lowry et al. [32].

Measurement of tissue superoxide dismutase activity

Superoxide dismutase (SOD) activity was determined according to the method of Sun et al. [49]. This method is based on the inhibition of nitroblue tetrazolium (NBT) reduction by the xanthine oxidase as a superoxide generator. Activity was assessed in the ethanol phase of the lysate after 1.0 mL ethanol/ /chloroform mixture (5/3, v/v) was added to the same volume of sample and centrifuged. One unit of SOD was defined as the enzyme amount causing 50% inhibition in the NBT reduction rate. SOD activity was also expressed as units per milligram protein (Sigma-Aldrich, 19160 SOD determination kit, St. Louis MO, USA).

Measurement of tissue glutathione level

Liver glutathione (GSH) content was determined as described by Ellman [10]. Briefly, liver homogenates (500 μL) were precipitated by adding 500 μL 4% sulfosalicylic acid and centrifugation at 2,858 9 g for 10 min. The supernatant (900 μL) was mixed with 100 μL 0.004% 5,50-dithiobis-2-nitrobenzoic acid (DTNB). After standing for 10 min at room temperature, the absorbance of chromophoric products (2-nitro-5-thiobenzoic acid) was determined at 412 nm using a spectrophotometry. GSH content was calculated using a standard curve (Sigma-Aldrich, Glutathione Assay Kit, St. Louis MO, USA).

Histopathologic evaluation

At the end of the surgical procedure, the liver specimens were individually immersed in Bouin's solution, dehydrated in alcohol and embedded in parafin and 5-μm-thick sections were cut and stained with haematoxylin and eosin (H&E). Liver pathological changes were observed under microscope and mitotic index was determined. In randomly selected region, mitotic hepatocytes were counted, and mitotic index (MI) was determined by random evaluation of at least 1000 hepatocytes and was expressed as a percentage (number of cells with mitosis/total hepatocytes × 100). The section of each animal was counted at least three times.

Immunohistochemistry

The harvested liver tissues were fixed in Bouin's, embedded in paraffin and sectioned at 5 μm thickness. Immunohistochemical reactions were performed according to the ABC technique described by Hsu et al. [21]. The procedure involved the following steps: (1) endogenous peroxidase activity was inhibited by 3% H_2O_2 in distilled water for 30 min; (2) the sections were washed in distilled water for 10 min; (3) non-specific binding of antibodies was blocked by incubation with normal goat serum (DAKO X 0907, Carpinteria, CA) with phosphate-buffered saline (PBS), diluted 1:4; (4) the sections were incubated with s specific mouse monoclonal anti-proliferating cell nuclear antigen (PCNA) antibody (Cat. # MS-106-B, Thermo LabVision, USA), diluted 1:50 for 1 h at room temperature; (5) the sections were washed in PBS 3 × 3 min; (6) the sections were incubated with biotinylated anti-mouse IgG (DAKO LSAB 2 Kit); (7) the sections were washed in PBS 3 × 3 min; (8) the sections were incubated with ABC complex (DAKO LSAB 2 Kit); (9) the sections were washed in PBS 3 × 3 min; (10) peroxidase was detected with an aminoethylcarbazole substrate kit (AEC kit; Zymed Laboratories); (11) the sections were washed in tap water for 10 min and then dehydrated; (12) the nuclei were stained with haematoxylin; and (13) the sections were mounted in DAKO paramount. All dilutions and thorough washes between steps were performed using phosphate buffered saline unless otherwise specified. All steps were carried out at room temperature unless otherwise specified.

Proliferation index (PI) was issued for determining the proliferative activity. Hepatocytes with particles of red pigment over their nuclei were considered labelled. A ratio of the number of PCNA positive

hepatocytes to the total number of hepatocytes was counted. The section of each animal was counted at least three times.

TUNEL assay

The transferase-mediated dUTP nick end-labelling (TUNEL) method, which detects fragmentation of DNA in the nucleus during apoptotic cell death *in situ*, was employed using an apoptosis detection kit (TdT-Fragel™ DNA Fragmentation Detection Kit, Cat. No. QIA33, Calbiochem, USA). All reagents listed below are from the kit and were prepared following the manufacturer's instructions. Five-μm-thick liver sections were deparaffinised in xylene and rehydrated through a graded ethanol series as described previously. They were then incubated with 20 mg/mL proteinase K for 20 min and rinsed in tris-buffered saline (TBS). Endogenous peroxidase activity was inhibited by incubation with 3% hydrogen peroxide. Sections were then incubated with equilibration buffer for 10–30 min and then TdT-enzyme, in a humidified atmosphere at 37°C, for 90 min. They were subsequently put into pre-warmed working strength stop/wash buffer at room temperature for 10 min and incubated with blocking buffer for 30 min. Each step was separated by thorough washes in TBS. Labelling was revealed using 3,3'-diaminobenzidine (DAB), counter staining was performed using Methyl green, and sections were dehydrated, cleared and mounted.

Apoptotic cells were counted only if they were TUNEL positive and displayed hallmark characteristics of apoptosis. An apoptotic index (AI) was calculated for each sample by counting the number of positively stained hepatocyte nuclei divided by the total number of hepatocytes and expressed as percentage.

Statistical analysis

All statistical analyses were carried out using SPSS statistical software (SPSS for windows, version 11.0). All data were presented as means ± standard deviations (SD). Differences in measured parameters among the three groups were analysed with a nonparametric test (Kruskal-Wallis). Dual comparisons between groups exhibiting significant values were evaluated with a Mann-Whitney *U*-test. These differences were considered significant when probability was less than 0.05.

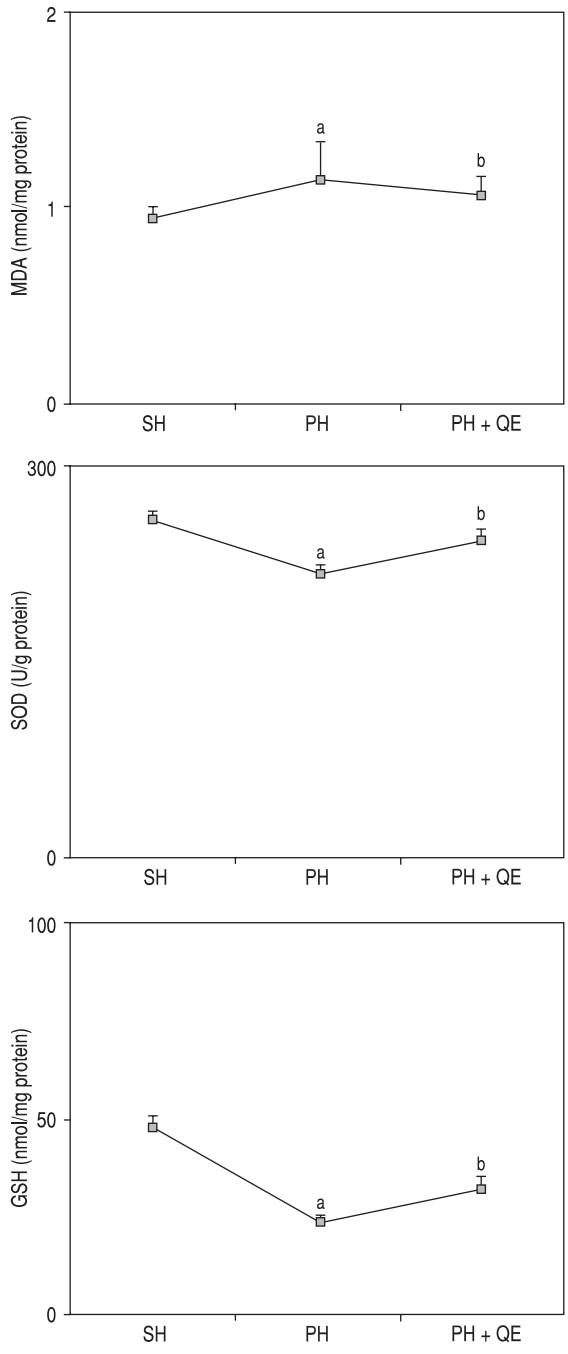

Figure 1. Tissue malondialdehyde (MDA) ($^a p < 0.01$) levels were significantly increased, superoxide dismutase (SOD) activity ($^a p < 0.01$) and glutathione (GSH) levels ($^a p < 0.001$) were significantly decreased in partial hepatectomy (PH) rats in comparison with sham-operated (SH). Quercetin (QE) treatment significantly ($^b p < 0.05$) decreased the elevated tissue MDA levels and increased the reduced SOD activity ($^b p < 0.05$) and GSH level ($^b p < 0.05$) in the liver tissues.

RESULTS

Biochemical findings

Partial hepatectomy significantly increased the tissue MDA level and decreased the antioxidant activity SOD, GSH level. QE treatment significantly decreased the elevated tissue MDA level and increased of reduced SOD activity, and GSH level in the tissues (Fig. 1).

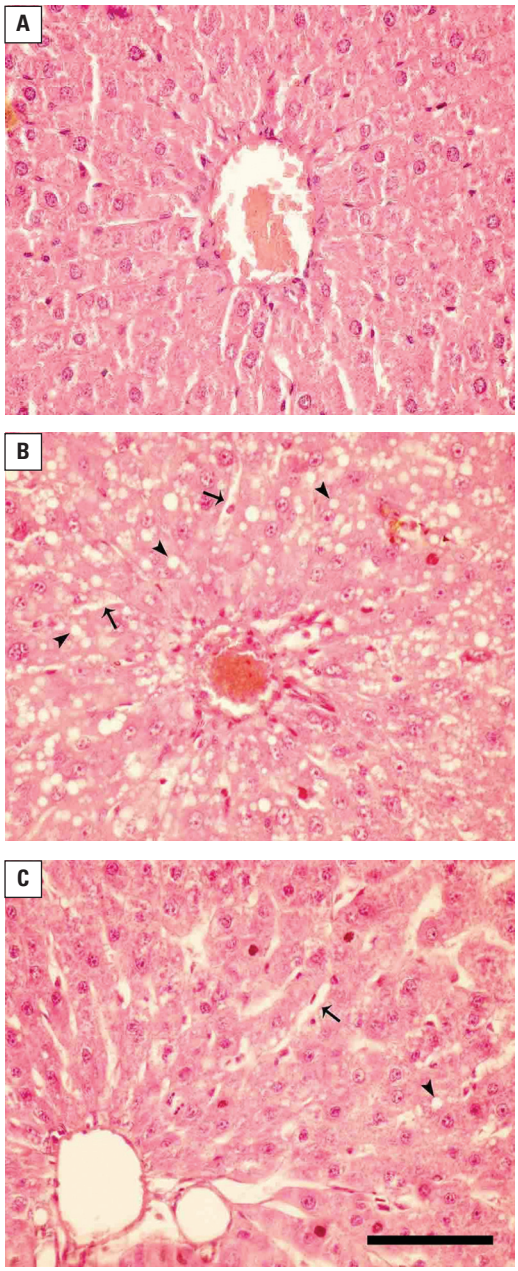

Figure 2. A. In with sham-operated group, fewer mitosis and normal histologic structure were seen in the liver; **B.** In partial hepatectomy group, mitosis and vacuolisation were prevalent in hepatocytes. Sinusoidal congestion was evident; **C.** Treatment of quercetin markedly reduced vacuolisation and sinusoidal congestion in the liver; Arrowhead — vacuoles; Arrow — sinusoidal congestion (H&E, scale bar, 50 μm).

Histopathologic findings and mitotic index

In H&E stained sections, in sham group fewer mitosis and normal histologic structure were seen in the liver (Fig. 2A). In PH group, mitosis and vacuolisation (vacuoles were observed in the cytoplasm of hepatocytes) were prevalent in hepatocytes. Sinusoidal congestion was evident (Fig. 2B). Similarly in PH+QE group mitosis was prevalent in hepatocytes, but vacuolisation and sinusoidal congestion was markedly reduced (Fig. 2C).

The MI was evaluated in H&E stained sections. There was a lower MI in the SH group (1.73 ± 0.09%). MI were significantly higher in PH+QE group (70.02 ± 5.13%) compared with the PH group (51.1 ± 3.88%) (Fig. 3).

Immunohistochemical findings and proliferation index

In SH group, a few PCNA positive cells were observed in the hepatocytes (Fig. 4A). PCNA positive cells were significantly higher in the PH+QE group than in the PH group (Fig. 4B, C).

The proliferation of the hepatocytes was assessed by the immunostaining of PCNA (a marker of cell proliferation) (Fig. 2). Compared with SH rats (2.17 ± ± 0.14), PI (percentage of PCNA positive hepatocytes) was notably increased in PH rats (31.03 ± 2.35), while the index in PH+QE treated rats (59.99 ± 4.71) was significantly higher than that in PH rats (Fig. 2).

TUNEL findings and apoptotic index

The number of TUNEL positive cells in the control group was negligible (Fig. 5A). When liver sections were TUNEL stained, there was a clear increase in the number of positive cells in the PH rats in the liver parenchyma (Fig. 5B). Treatment of QE markedly reduced the number of TUNEL positive cells (Fig. 5C).

The hepatocytes located in the liver parenchyma with nucleus-positive staining and nonstaining plasma were identified as hepatic apoptotic cells by TUNEL assay (Fig. 5). AI (percentage of TUNEL positive hepatocytes) was notably increased in PH rats (8.61 ± 1.3) compared with SH rats (2.20 ± 0.19), while the index in PH+QE treated rats (4.35 ± 0.41) was significantly lower than that in PH rats (Fig. 2).

DISCUSSION

The liver is one of the vital organs of the body with multiple important responsibilities, including metabolism, maintenance of water balance, bile acid production and excretion, detoxification, immune response and so on [48]. In addition, the liver becomes distinguished from other organs mainly by its amasing regenerative ability, which is primarily attributable to the quick re-entry of highly differentiated quiescent hepatic cells into the cell cycle in response

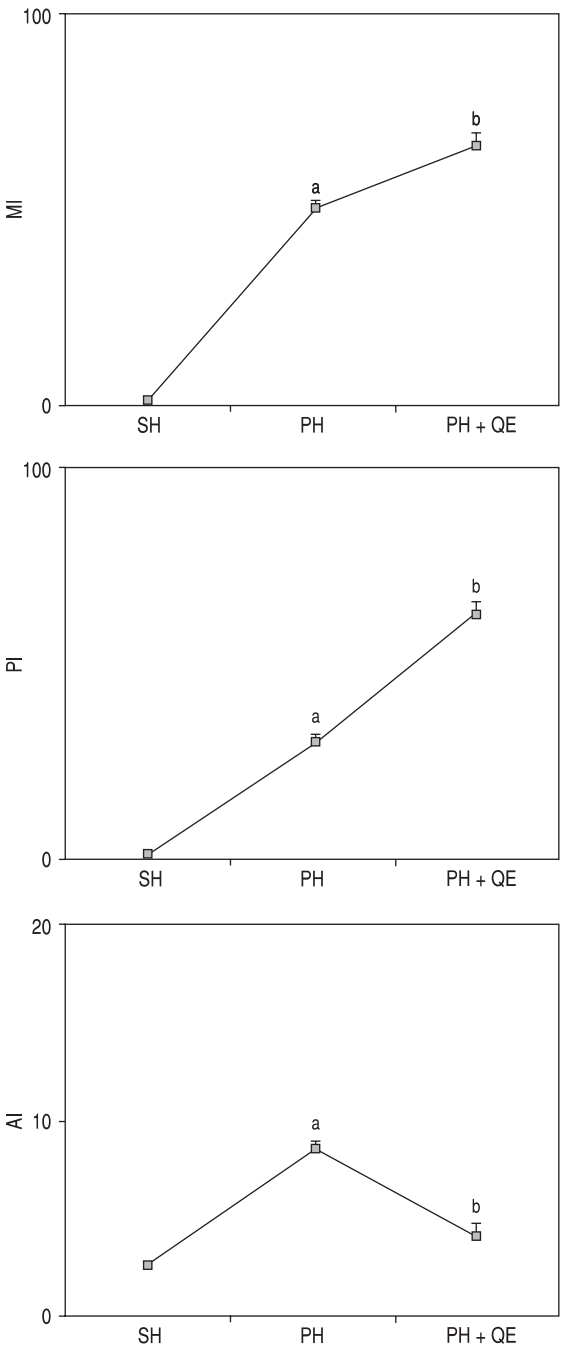

Figure 3. Mitotic index (MI) ($^{a}p < 0.00001$), proliferation index (PI) ($^{a}p < 0.0001$) and apoptotic index (AI) ($^{a}p < 0.001$) were significantly increased in partial hepatectomy (PH) rats in comparison with sham-operated (SH). Quercetin (QE) treatment significantly increased the MI ($^{b}p < 0.05$), PI ($^{b}p < 0.01$) and significantly decreased AI ($^{b}p < 0.01$) compared with PH rats in the liver tissues.

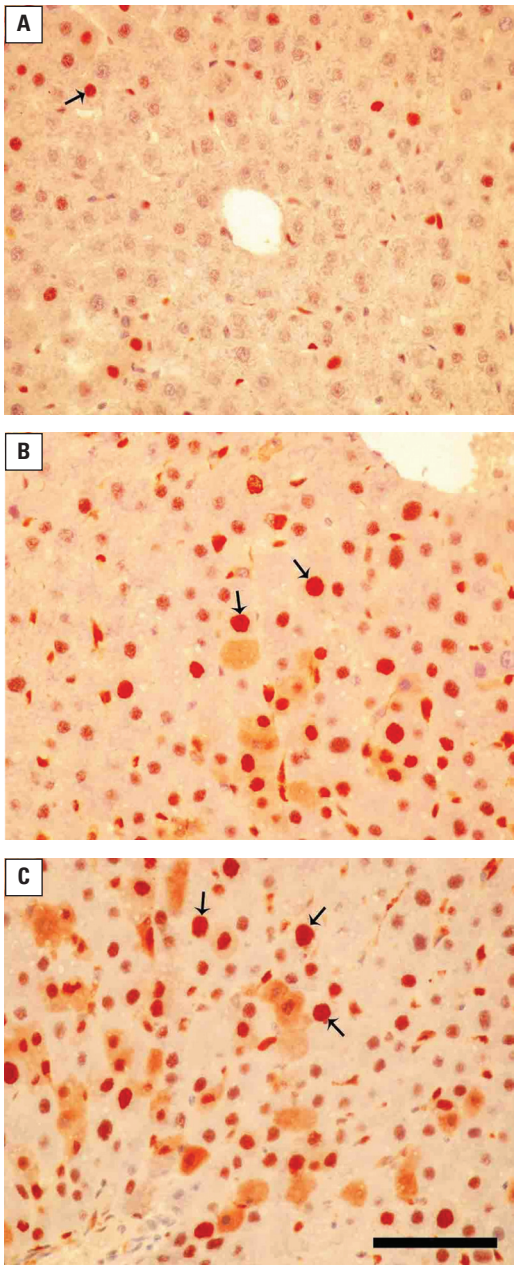

Figure 4. Proliferating cell nuclear antigen (PCNA); **A.** In sham-operated group, a few PCNA positive cells were observed in the hepatocytes; **B.** After partial hepatectomy, the number of PCNA positive cells was markedly increased in the hepatocytes; **C.** Treatment of quercetin significantly increased the number of PCNA positive cells; arrow — PCNA positive cells (immunoperoxidase and haematoxylin counterstain, scale bar, 50 μm).

to liver injury induced by surgical resection (e.g., surgical resection, pathogenic or chemical factors) [12]. Among various liver injury models, PH, resulting in the loss of approximately 70% of the liver volume, is now widely utilised for studying liver regeneration in experimental animals.

Under various conditions tissues and cells undergo oxidative processes that result in the formation of free radicals and the generation of lipid peroxides. Antioxidant properties of QE have been repeatedly

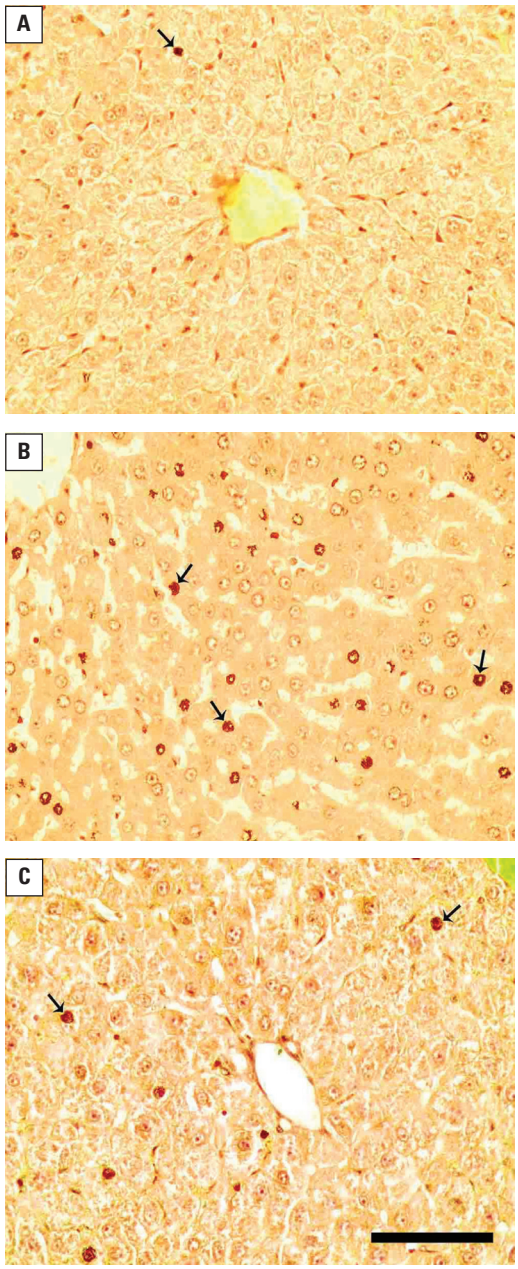

Figure 5. Transferase-mediated dUTP nick end-labelling (TUNEL) detection of apoptotic hepatocytes in sham-operated group (**A**), partial hepatectomy (PH) (**B**), and PH + quercetin (QE) (**C**) groups. Treatment of QE markedly reduced the number of hepatocytes apoptosis. Arrows indicate TUNEL-positive apoptotic hepatocytes (TUNEL, scale bars = 50 μm).

demonstrated in various experimental models [25]. Currently, information is limited regarding the effect of flavonoids on non-tumorigenic cells. In this study, they tested the action of QE on normal cell proliferation using regenerating liver.

However, up to now, it has not been known whether these agents have similar effects in a model of hepatic resection. The role of the antioxidant defence system, which includes SOD and GSH, is well characterised in the liver [28]. Increased oxidative stress during the early phase of liver regeneration had been observed as a cause of surgery and a reactive response of the reduced organ to compensate for the extra functional load [2, 5, 13]. Hepatic lipid peroxidation peaks at 24 h after PH when GSH content is minimum [1, 14, 16]. It has also been addressed that oxidative stress is reduced before cell division [2, 6, 46]. Increased oxidative stress could diminish the regeneration process. Our results showed that MDA, a biomarker of free radical mediated lipid peroxidation, was increased in regenerating liver after PH, which was consistent with previous studies [5, 30]. In addition, PH also impairs the antioxidative system, such as SOD and GSH, which can offer protection from cell damage by scavenging superoxide anion radical in the upper stream of reactive oxygen metabolism cascade [37]. Impaired antioxidant defences decrease oxidative phosphorylation capabilities to finally impede liver regeneration after PH [55]. However, GSH was increased during the later phase after PH, which may stimulate hepatocytes to shift from G0 to G1 phase of the cell cycle [22]. The lipid peroxidation inhibitory effects of several flavonoids such as luteolin, apigenin, galangin, gardenin D, (+)catechin, QE, rutin (quercetin-3-rhamnosyl glucoside) and biflavonoid ternatin have been previously reported [34]. Our results further showed a remarkable protective effect of QE, as indicated by restoring SOD activity, inhibiting GSH depletion, and alleviation of lipid peroxidation. Accordingly, the enhancement of QE on liver regenerative capacity after PH was probably due to its ability to attenuate liver injury and restore antioxidative activity.

The histological examination of regenerated liver sections revealed that vacuolisation and sinusoidal congestion was only prevalent in hepatocytes of animals, which were applied only hepatectomy in PH group. Parallel to our findings, prevalent vacuolisation was observed in hepatocytes following hepatectomy in a research. Researchers also indicated that vacuolisation had lipid accumulation and that they prevented vacuolisation by means of the organic compound they used [20]. Likewise Toygar et al.'s [52] studies, our findings indicated that vacuolisation was significantly decreased in groups that were treated with QE before the PH. Thus, we think that the protective effects of QE on livers are important following hepatectomy.

Factors that contribute to the initiation and regulation of liver regeneration are very diverse and complex. In general, liver regeneration is regulated by growth-related genes and mediated by different growth factors that are involved in both stimulatory and inhibitory signalling pathways. It has been reported that ROS modulate a variety of signalling pathways that may influence liver regeneration [1, 4]; for example, ROS mediate cell growth arrest and activate proteins inhibiting cell cycle [3, 19]. For this reason, ROS production in the liver remnant may play a key role in the negative control of regeneration. PCNA is an important protein for DNA polymerase and plays a very important role for initiation of cellular proliferation. Expression of this protein is a sign of S-phase of the cell cycle, and this protein can be used as a proliferation marker because it represents the proliferative fraction of cells [15]. Positive cells with PCNA antibodies are very few in normal liver; however, they are found to be increased at 24 h and 48 h after resection [51]. Our study, compared with SH rats, MI and PI were notably increased in PH rats, while the indexes in PH+QE treated rats was significantly higher than that in PH rats. We think that QE stimulates the mitosis of hepatocytes following hepatectomy.

The role for apoptosis in the liver after hepatectomy seems to be controversial. At the peak of regeneration, apoptosis begins. Accompanying cell reproduction, cell apoptosis would eliminate the overgrown cells and rebuilding of the constitution is achieved. A study by Li et al. [31] showed that the apoptosis level after liver regeneration was directly proportional to the regeneration level. In another study, Kirimlioglu et al. [29] showed that increased apoptosis after 70% PH in rats. We couldn't find any study in the literature investigating the effects of QE on apoptosis in PH. In our study, the number of TUNEL positive cells in the control group was negligible. When liver sections were TUNEL stained, there was a clear increase in the number of positive cells in the PH rats in the liver parenchyma. Treatment of QE markedly reduced the number of TUNEL positive cells. In addition, AI was notably increased in PH rats, while the index in PH+QE treated rats was significantly lower than that in PH rats.

CONCLUSIONS

In conclusion, QE has shown a beneficial effect on regenerative capacity of remnant liver tissue, probably due to its antioxidative, antiapoptotic and proliferative property. QE may be an effective pharmacological strategy to enhance regenerative capacity after PH.

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