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A N I N T E R N A T I O N A L J O U R N A L

Hepatoprotective Effect of Quercetin Pretreatment Against Paracetamol-Induced Liver Damage and Partial Hepatectomy in Rats

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ABSTRACT

Quercetin has potent antioxidant action and a hepatoprotective role. The aim of this study was to evaluate the hepatoprotective action of quercetin pretreatment in paracetamol-induced liver damage (PILD) and structural injury resulting from partial hepatectomy (PH). In the first model, Wistar rats received oral quercetin (50mg/kg/day) during 8 days. On the 8th day, 3g/kg paracetamol were added. In the second model, the same quercetin dose was given during 7 days and rats were submitted to PH on the 8th day. Blood samples were obtained for determination of enzyme levels. Liver, heart, kidney and lung tissue were also collected for assessment of quercetin biodistribution and/or histological analyses. The results obtained after PILD were more pronounced at 24 hours, as reflected by the reduction of serum ALT levels and by the lower concentration of quercetin in liver at this time point. Quercetin also had a protective effect in groups submitted to PH, as shown by decreased ALT levels after 18 hours, and of AST levels after 18 and 36h. The reduction in serum AST and ALT levels suggest that treatment with quercetin is useful as a preoperative pharmacologic measure and for prevention of liver damage caused by drugs.

Key-words: quercetin, paracetamol, partial hepatectomy, ALT, AST.

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INTRODUCTION

Quercetin, a flavonoid compound with potent antioxidant action and a protective role in various diseases, has been widely studied[1]. A significant number of chemical properties and pharmacological effects have been attributed to quercetin in animal models, such as reduction of serum cholesterol levels[2] and anti-inflammatory action in lung injury. Rat models of various liver disorders have shown a hepatoprotective role of quercetin, which has been associated with increased survival in cirrhosis[3] and protection against induced liver damage[4,5]. In addition, there is evidence that quercetin is effective for cancer treatment and that it provides protection against cardiovascular, renal, and liver disease[6].

Quercetin scavenges reactive oxygen species (ROS), inhibits xanthine oxidase as well as lipid peroxidation, in addition to scavenging and stabilizing iron[7]. The superoxide anion and the hydroxyl radical are involved in liver damage resulting from lipid peroxidation and interstitial matrix degradation[8].

Bona et al.[9] report that a significant increase in serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) after inhalation of chloroform in rats was reversed with quercetin administration, supporting the notion that quercetin has antioxidant and hepatoprotective effects. Also, it has been shown that the use of quercetin significantly reduces the biochemical alterations caused by cirrhosis, increasing survival in animals with secondary biliary cirrhosis. Nevertheless, fewer studies have investigated whether pretreatment with quercetin might be useful to prevent injury. Behling et al.[6] have suggested that oral intake of quercetin may protect the liver against ischemia and reperfusion injury.

Toxic hepatitis is a common condition in clinical practice, and accounts for about 0.2% of all hospital admissions and 2-3% of admissions due to adverse drug effects[10]. In the U.S. and U.K., it is the main cause of fulminant liver failure, especially in the presence of accidental or intentional overdosing[11].

Liver regeneration after partial hepatectomy has been broadly investigated, especially with the rat model proposed by Higgins & Anderson[12].

Considering the studies in which quercetin has liver protective action, few of them refer to the pretreatment . We believe that as quercetin can reverse liver damage, there is a chance to avoid them with pretreatment. The objective of the present study was to evaluate the hepatoprotective action of quercetin pretreatment in two experimental rat models of liver damage: paracetamol-induced damage and structural injury resulting from partial hepatectomy.

MATERIAL AND METHODS

The study protocol was approved by the Animal Research Ethics Committee at the Catholic University of Campinas.

Materials

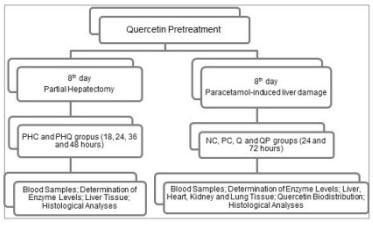
Reagents were acquired from the following sources: quercetin, AllChemistry; paracetamol, Pharma Nostra; enzyme assay kit, LaborLab. All other reagents were analytical grade.

Animals

Eighty sixty-day old male Wistar rats (weight: 230g ±10g) were obtained from the Life Sciences Center Animal Facility (Pontifícia Universidade Católica de Campinas, SP, Brazil). During all experiments, the animals were maintained in rooms with temperature control (23±1°C) under a 12-hour light/dark cycle. They were fed commercial chow (Nuvilab) and water *ad libitum*.

Paracetamol-induced liver damage

Fourthy animals were divided into two groups: quercetin and control (Figure 1). The quercetin group was further divided into four subgroups (five animals in each subgroup): quercetin 24h (Q24) and quercetin 72h (Q72), receiving oral suspension of quercetin through a gastric tube (50 mg/kg/day) during eight days and euthanized with ketamine 24h or 72h after the last administration of quercetin respectively; and quercetin and paracetamol 24h (QP24) and quercetin and paracetamol 72h (QP72), receiving oral suspension of quercetin through a gastric tube (50 mg/kg/day) during eight days plus 3g/kg of paracetamol with the last dose of quercetin. These animals were also euthanized with ketamine.



The control group was also subdivided into four subgroups: negative control 24h (NC24) and negative control 72h (NC72); these groups received saline solution for eight days and were euthanized with ketamine 24 or 72 hours after the last administration of saline solution respectively; and positive control 24h (PC24) or positive control 72h (PC72), which received saline during eight days, with paracetamol (3 g/kg) added on the last day and were euthanized with ketamine.

Blood samples were obtained by cardiac puncture of the left ventricle for determination of ALT and AST levels. Liver tissue samples were obtained for histological processing. Additionally, liver, heart, lung, and kidney samples were obtained for analysis of quercetin biodistribution.

Paracetamol dose was determined as described by Oyagbemi and Odetola[12]. Quercetin dose was based on Vidhya & Indira[4] and on Kawai et al.[13]. Both were administered via a gastric tube. Quercetin was dissolved in physiological saline for a total volume of 1 mL.

Partial hepatectomy

Fourthy animals were divided into a quercetin and a control group (Figure 1). Each was further subdivided into four subgroups. The quercetin group received 50 mg quercetin/ kg/day suspended in saline for a volume of 1mL and administered via a gastric tube during seven days. After that, the rats underwent partial hepatectomy. After 18, 24, 36 or 48h the animals received an intraperitoneal injection of vincristine sulfate (groups PHQ18, PHQ24, PHQ36, PHQ48 respectively). After 2h, the animals were anesthetized for removal of the lobes remaining from partial hepatectomy and for cardiac puncture, followed by euthanasia with ketamine.

The control group received 1 mL saline solution via gastric tube during seven days. The animals were then submitted to partial hepatectomy. After 18, 24, 36 or 48h the animals received an intraperitoneal injection of vincristine sulfate (groups PHC18, PHC24, PHC36, PHC48 respectively). After 2h the animals were anesthetized for removal of the lobes remaining after partial hepatectomy and for cardiac puncture, followed by euthanasia with ketamine.

Partial hepatectomy was carried out as described by Higgins and Anderson[12]. Samples of the remaining lobes were obtained 18, 24, 36 or 48 hours after surgery and were used for histological and histochemical analyses.

Histological processing

Liver tissue fragments were cut into 7 μ-thick slices and stained with hematoxylin-eosin (HE), picrosirius red (PR), or periodic acid-Schiff (PAS). Images were digitally captured using a photomicroscope (Nikon Eclipse E200TM) coupled to a camera (Nikon Colpix 4500TM). The following aspects were taken into consideration to analyze hepatoprotective activity: type and intensity of liver damage, and presence of steatosis, inflammatory infiltrate, fibrosis, and necrosis. To evaluate necrosis, the number of damaged centrilobular veins (clv) was determined (clv) in HE-stained slides (a section of 2 liver fragments from different lobules) and the results were expressed in vcl/cm^2. TPS Dig® 1.30 software was used for counting. To evaluate fibrosis, 5 micrographs of the centrilobular region and 5 micrographs of the portal region from slides stained in Picrosirius Red (a total of 50 micrographs per group) randomly obtained in 480x magnification were analyzed. AreaMed® software was used to measure the fibrotic area (collagen fibers). To evaluate the distribution of glycogen and other 1,2-glycols, qualitative assessment of PAS-stained slides was performed, comparing treated animals to negative and positive control groups. Results were shown in the micrographs.

After partial hepatectomy, mitotic index and apoptosis were assessed.

Determination of enzyme activity

ALT and AST activity was determined in blood serum using LaborLab™ kits and a UV-Vis Varian™ spectrophotometer.

Biodistribution of quercetin following paracetamol overdose

Liver, heart, lung, and kidney tissue samples were macerated at a ratio of 200 mg of tissue to 0.2 ml of ethanol p.a. (Merck™). The suspension was then centrifuged for 5 minutes (2260 g). The process was repeated 5 times for maximum quercetin extraction. The supernatant was concentrated in an incubator at 50°C with continuous nitrogen flow. Samples were re-suspended in 0.5 ml ethanol and filtered with a 45 μm-filter membrane. Twenty μl of each sample were directly injected in a high-performance liquid chromatography (HPLC) system (Varian Prostar

320/210). Ethanol p.a. was used as mobile-phase solvent and elution flow rate was 1 ml/min. The chromatographic peaks obtained were quantified by spectrophotometry (370 nm)[14]. The analytical curve was constructed by analyzing the following standards of quercetin: 0.001 mg/ml, 0.002 mg/ml, 0.006 mg/ml, 0.01 mg/ml and 0.02 mg/ml. A Microsorb-MV 100-S C18 250x4.6 column was employed[13].

Analysis of results

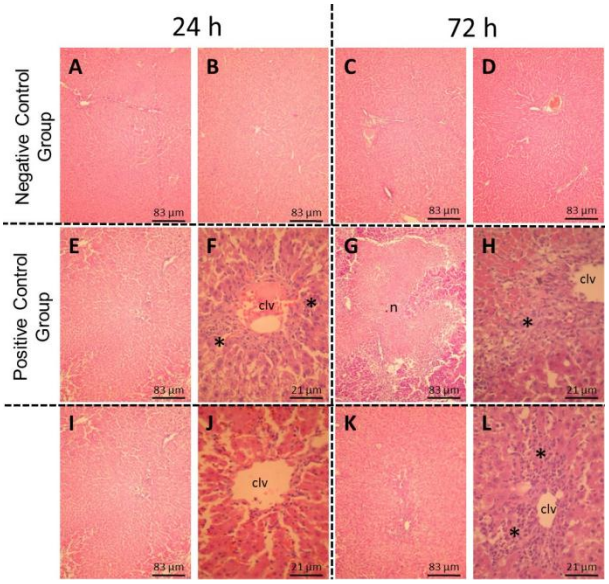
To evaluate necrosis, the number of centrilobular lesions was determined in slides stained with HE using TPS Dig software version 1.30. The results were expressed as centrilobular vein/cm^2 (CLV/cm^2). For assessment of fibrosis, five micrographs of the centrilobular region (CR) and five random micrographs of a portal region were analyzed in PR-stained slides, from a total pool of fifty micrographs per group (X480). The AreaMed^{TM} software was used to measure fibrosis area. Distribution of glycogen and other 1,2-glycols was analyzed in PAS-stained slides (semi-quantitative analyses). Mitotic and apoptotic figures were counted in 20 fields per section of liver tissue stained with HE and PAS.

Statistical analyses of morphometry, AST and ALT, and quercetin biodistribution were performed in Graph Pad PRISM^{TM} 3.0 software. Analysis of variance (ANOVA) followed by Bonferroni's test ($p < 0.05$) were used for group comparisons.

RESULTS AND DISCUSSION

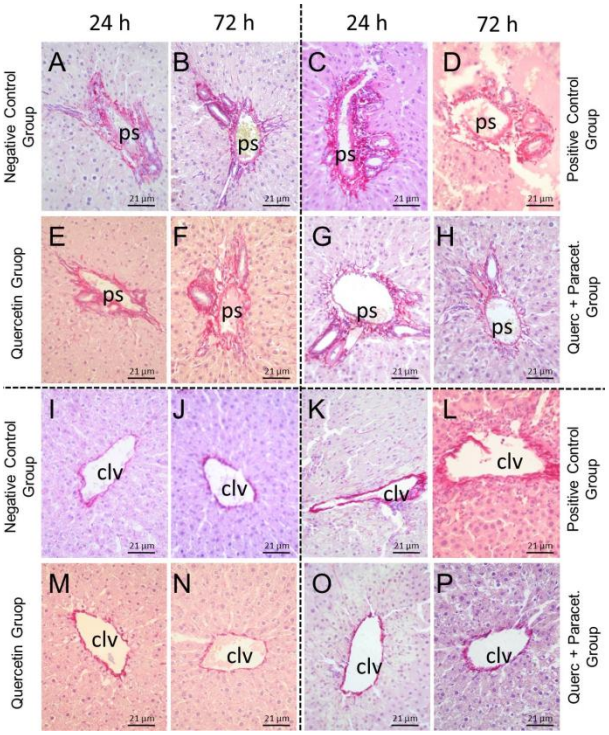
Effects of quercetin on paracetamol-induced liver damage

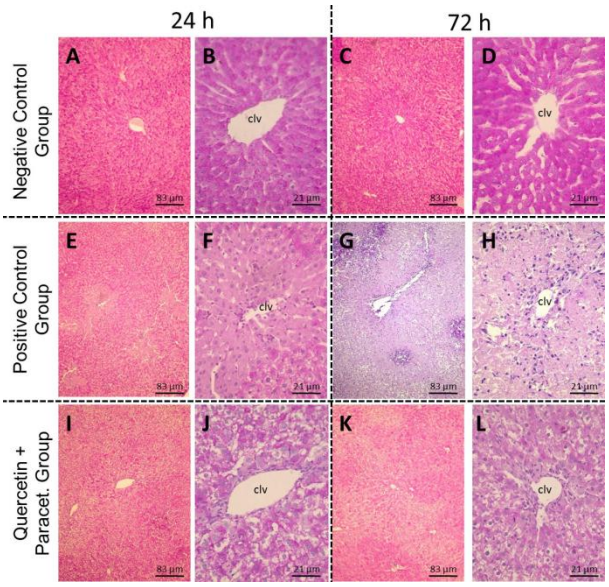
Histological analysis revealed absence of steatosis in the positive paracetamol control groups. However, fibrosis and necrosis with polymorphonuclear infiltrate were present. Necrosis was observed predominantly in the centrilobular region, especially in the PC72 group. Based on this observation, inflammatory foci were quantified in this region (semi-quantitative analyses), whereas fibrosis was quantified in acinar zones 1 and 3 in PR-stained slides. Liver tissue was preserved in groups receiving quercetin (Q24 and Q72) and in negative controls (NC24 and NC72) (Figure 2).



Inflammatory infiltrates with and without necrosis were observed in the positive control (PC) and quercetin-paracetamol (QP) groups, especially in PC24 and PC72: five animals in each of these groups developed centrilobular necrosis, whereas in QP24 and QP72 only two animals developed necrosis. Nevertheless, this difference was not statistically significant. A similar result has been reported by Mandal et al.[14] in arsenite-induced liver fibrosis. In that study, administration of galactosylated liposomal quercetin reduced histological alterations such as focal necrosis, Kupffer cell hyperplasia, and steatosis.

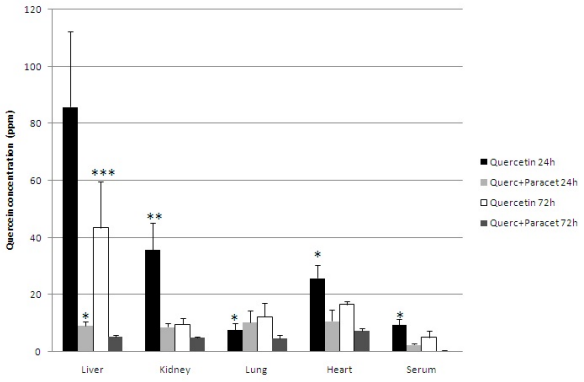
Figure 3 shows collagen fibers present in acinar zones 1 and 3. Collagen deposition was moderate in all groups. There were no significant differences between the groups in terms of fibrosis, even though less collagen deposition was observed in the portal region in QP24 vs. PC24 (Figure 4). Less collagen deposition in the centrilobular region was also observed when QP24 (zones 1 and 3) and QP72 (zone 3) were compared with PC24 and PC72 respectively.





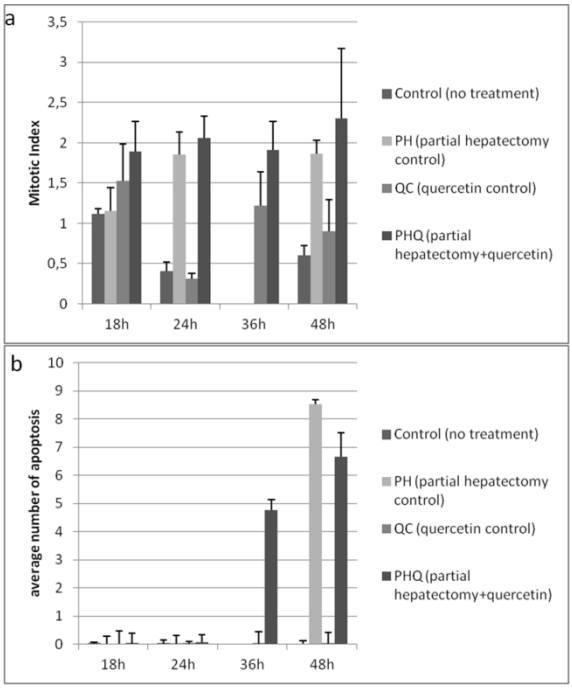
These results may be explained by the small number of collagen fibers observed in the positive control groups (PC). This is in agreement with previous studies[10,15,16,17], which also do not describe fibrosis in association with paracetamol-induced toxic hepatitis. In addition, since the development of fibrosis in liver is progressive and dependent on the activation of hepatic stellate cells, intervals of 24h and 72h are too short to allow observation of significant production of collagen fibers. Other studies with different models of carbon tetrachloride-induced toxic hepatitis[9,18] or a model of methionine-choline deficient diet[19] or studies of arsenite-induced lesions[15] describe attenuation of fibrosis by quercetin. Kawada et al.[20] showed that quercetin and other natural phenolics inhibit stellate cell activation by disturbing signal transduction pathways and cell protein expression, thus interrupting the differentiation of stellate cells into myofibroblasts, and the consequent production of collagen type I and II.

Figure 5 shows that animals treated with a high dose of paracetamol presented a great reduction in glycogen and other 1,2-glycols (PAS-positive inclusions) in the centrilobular region. In the group receiving quercetin, the inclusions were kept, indicating a possible hepatoprotective action of quercetin regarding this aspect. A decrease in liver glycogen resulting from paracetamol action has been reported by Itinose et al.[21] and is related to the irreversible linking of paracetamol to a reactive metabolite or the inhibition of mitochondrial energy metabolism. Even though a quantitative analysis was not carried out, we clearly observed that pretreatment with quercetin inhibited glycogen depletion in zone 3.

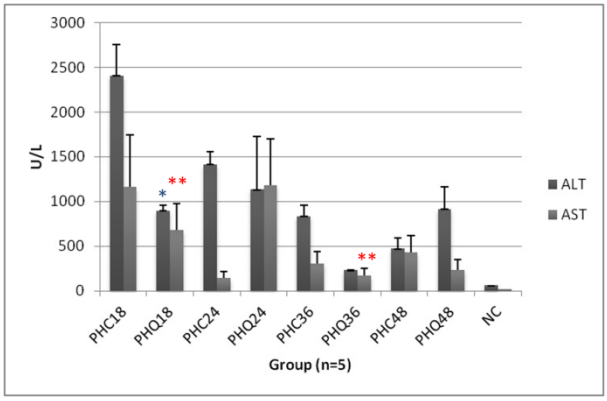


Regarding AST and ALT, the high dose of paracetamol administered to positive control groups (PC) led to an increase in AST and ALT levels. The groups receiving pretreatment with quercetin had a significant reduction in serum ALT and a reduction in serum AST levels vs. PC groups (Figure 6). Yousef et al. [22] administered quercetin suspended in saline for 15 days (20 mg/kg) before a toxic paracetamol dose and also observed a reduction in the serum levels of AST and ALT. Vidhya and Indira [4] and Chen[5] describe a significant reduction in these enzymes with the use of quercetin in a rat model of ethanol-induced hepatic lesion. The same was observed by David et

al. [23] in an experimental model with thioacetamide. A recent work by El-Shafey et al. [24], in which oral quercetin was given to rats (15 mg/kg/day) during 21 days prior to a toxic dose of paracetamol, focused on many of the parameters assessed by us in the present study. The results of that study were significant for AST, ALT, and alkaline phosphatase, showing that quercetin reversed paracetamol-induced damage. It is possible that the extended period of quercetin administration in the study by El-Shafey et al. [24] may explain the broader hepatoprotection and higher number of significant parameters as compared to the present study.No significant alterations were detected in alkaline phosphatase levels when the groups were compared. Some authors describe an increase in this enzyme in cases of paracetamol-induced liver damage[12,22] . In PC groups, this finding is in agreement with histologic results showing absence of changes in biliary ducts using light microscopy. According to Matos and Martins[10], this suggests that paracetamol-induced hepatotoxicity is cytolytic rather than cholestatic.



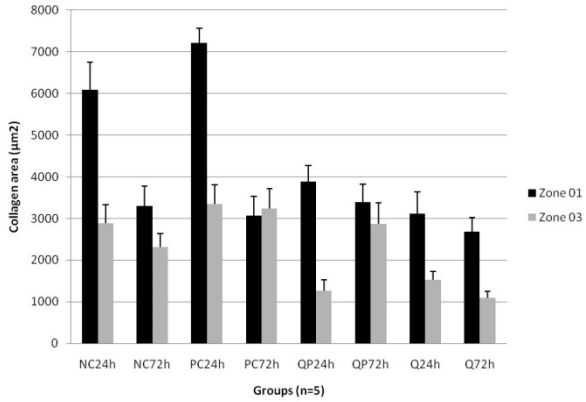
An influence of paracetamol overdose on the biodistribution of quercetin was confirmed (Figura 6). There was a reduction in the concentration of quercetin in liver, with significant differences between Q24 and QP24. This suggests that quercetin was transformed into an unidentified metabolite, probably with ineffective antioxidant action. This result agrees with those obtained for ALT as well as with histologic results showing that the antioxidant activity of quercetin at the dose employed plays an important hepatoprotective role.



Given the role of liver in metabolism and detoxification, the highest concentrations of quercetin were observed in liver up to 72h after the last administration of the drug when compared to other tissues analyzed (Figure 7). There are few reports in the literature regarding the biodistribution of quercetin. In pigs, Bieger et al.[25] observed that the administration of a single quercetin dose of 25 mg/kg produced concentrations of 6.2 nmol/g in liver, 2.84 nmol/g in kidney, 1.48 mmol/l in plasma after 90 minutes. Similar results regarding biodistribution were also obtained by De Boer et al.[26] in rats treated with quercetin for 11 weeks and in pigs treated for 3 days. The present study suggests that a higher concentration of quercetin in liver and kidney, especially at 24h, might be explained by function, since both tissues/organs are highly vascularized.

In QP24 and QP72, we observed that the concentration of quercetin decreased significantly in all tissues (except lung) and especially in liver and plasma. The reduction of quercetin concentrations in different organs and in serum was probably triggered by the neutralization of free radicals released with the high dose of paracetamol, curtailing the hepatotoxic effect. It should be considered that the administration of toxic doses of paracetamol causes saturation of glucuronidation and sulfation pathways, with the P-450 cytochrome pathway undertaking an important role in the biotransformation of substances, promoting the formation of N-acetyl-p-benzoquinone imine (NAPQI). Thus, the liver reserve of glutathione is depleted, increasing the

reaction with sulfhydric groups of liver proteins, interrupting the flow of mitochondrial calcium and leading to necrosis of liver cells[27]. In the presence of changes in mitochondrial metabolism, hepatotoxicity may also occur through ROS formation, e.g. superoxide anion (O_2), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-), reactive nitrogen species, e.g. nitric oxide and peroxynitrite ($ONOO^-$), and products of peroxidation reaction[28,29]. The reaction is amplified through activation of Kupffer cells and the production of cytokines and free radicals, which leads to centrilobular apoptosis and necrosis in zone 3. Necrosis takes place in this location because zone 3 cells have the highest concentration of cytochrome P-450 and also because this is where the drug is converted into active metabolites [27,28,29,30,31,32]. Dahlin et al[33], Moore et al.[34], and Padma et al.[35] investigated the action of quercetin on the toxicity of lindane, a substance affecting the homeostasis of glutathione and causing alterations in P-450 cytochrome P450 monooxygenase enzymes. Those authors confirmed the efficacy of quercetin, attributed to a potent antioxidant activity.

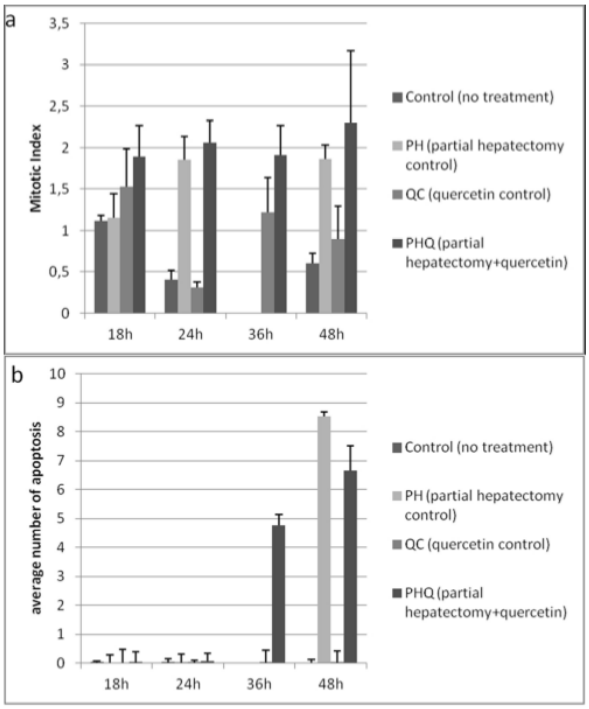


We hypothesize that our overall results were not remarkable/significant for the 72h time point because treatment was interrupted and the amount of quercetin in the liver was not sufficient to reflect a hepatoprotective effect. Terminal elimination half-life was about 11 hours in a study by Graefe et al [36].

The comparison of QP24 vs. Q24 allows us to formulate some hypotheses regarding the mechanisms of action of quercetin. Considering the effect of toxic doses of paracetamol mentioned earlier on glucuronidation and sulfation and formation of NAPQI, the first hypothesis would be that quercetin may react directly with reactive NAPQI. The second hypothesis suggests that NAPQI reduction by paracetamol might occur with the formation of oxidized quercetin metabolites. To elucidate this process, additional studies should be performed using quantitative LC/MS analyses to characterize the quercetin metabolite produced under these circumstances and which could explain the depletion of quercetin.

Effects of quercetin on partial hepatectomy

The analysis of mitotic index and of the number of apoptosis events showed that all groups had similar normal liver hyperplasia, without significant differences (Figure 8). Interestingly, however, apoptosis began in the PHQ group at 36h after PH, whereas in PHC apoptosis events were noted only at 48h. This suggests early onset of apoptosis in the group receiving quercetin. Quantification of mitosis and apoptosis events revealed very small numbers for the control group without partial hepatectomy. In a rat model, Iwao and Tsukamoto[37] injected quercetin (200 mg/kg) intraperitoneally immediately after PH. Those authors found that quercetin inhibited DNA synthesis and induced apoptosis in regenerating liver after PH. Quercetin injection decreased the activity of rate-determining enzymes of DNA synthesis. Conversely, Arash[38] administered quercetin to rats (15 mg/kg orally) for 14 days before inducing ischemia/ reperfusion injury. That investigator found that quercetin might have a role at a sub cellular level in preventing induced necrosis and apoptosis, and that quercetin seems to protect hepatobiliary function and liver structure. According to Liu et al.[39], liver resection leads to portal hyperperfusion injury of the remnant liver. In contrast to normal controls, serum ALT levels were significantly increased in rats subjected to ischemia/reperfusion injury and PH respectively. Surgical manipulation impairs local microcirculation and causes transient focal ischemia. However, it is not possible to establish a direct correlation between the effects of PH and those of ischemia and reperfusion.



According to Gonzales et al.[40] ATP released after PH regulates liver regeneration in rats. Those authors state that extracellular ATP is released immediately after hepatectomy from hepatocytes and Kupffer cells under mechanical stress and promotes liver regeneration in rats. They conclude that ATP is transiently released from the liver in response to immediate hyperpressure following PH. At least both hepatocytes and Kupffer cells contribute to this release, which may be one of the earliest signals triggered by PH. Chávez and Cuellar[41] have produced experimental evidence supporting a role of quercetin as ATPase inhibitor. This could explain the inhibition of liver regeneration by quercetin in earlier studies.

It should be considered that synthesis of DNA in non-parenchymatous cells starts on average 24 hours after the onset of DNA synthesis in hepatocytes. The mitotic peak in Kupffer cells, endothelial cells, and ductal cells has been shown to occur 48-96 hours after partial Hepatectomy [42].

In relation to transaminases, a significant reduction in serum ALT levels occurred in the PHQ18 group as compared to its control the PHC18 group (Figure 9). This suggests hepatoprotection at the 18-hour time point. This finding was not observed at 24 hours, possibly because of quercetin depletion resulting from its antioxidant action, leaving insufficient amounts for significant results after 18 hours. A different result might have been obtained if the administration of quercetin were maintained after PH. This aspect deserves further investigation.

Quercetin administration reduced the serum concentration of AST in PHQ18 and PHQ36 as compared to their control groups. Van-de-Poll et al. [43] state that liver failure is a severe complication of liver surgery and conclude that the inflammatory cascade seems to be initiated early in abdominal surgery; thus, interventions that aim to reduce postoperative inflammation and associated complications should be initiated during or before surgery. In this context, HP could be interpreted as structural damage.

David et al.[23] showed that four days of treatment with quercetin in rats intoxicated with thioacetamide promoted significant reduction in serum levels of AST and ALT, with successful avoidance of morphological alterations. The results obtained suggest that quercetin has a protective effect in rats with thioacetamide-induced lesions. The duration of treatment and the administration route may interfere with the hepatoprotective effect of quercetin. Miltersteiner[3], Bona et al.[9] and David et al.[23] found positive results with intraperitoneal administration of quercetin. Behling et al. [6] and Bona et al.[9] used periods of 28 days and six weeks respectively. It is likely that treatment duration impacts the effect of quercetin, and that periods longer than seven days may be be more effective to produce observable results.

CONCLUSION

The present study provides invaluable insights into the therapeutic efficacy of quercetin in paracetamol-induced toxic liver damage and structural damage caused by PH.

We found evidence of quercetin's hepatoprotective activity in acute paracetamol toxicity, as shown by a reduction in necrosis and inflammation and, most of all, serum levels of ALT. Analysis of quercetin biodistribution revealed that paracetamol led to a significant reduction in quercetin concentration in the organs/tissues studied as well as in serum, suggesting a relationship between antioxidant activity and hepatoprotective action. Treatment with quercetin in PH revealed a mild protective activity, with absence of cellular proliferative action. It seems that different doses of quercetin, as well as different administration routes, even during longer treatment periods,

might enhance this effect.

The reduction in serum AST and ALT levels and the present histologic findings suggest that treatment with quercetin is useful as a preoperative pharmacologic measure and for prevention of liver damage caused by drugs, a topic that might be addressed by future studies.

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