

# Intraperitoneal Administration of Apigenin in Liver Ischemia/Reperfusion Injury Protective Effects

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## ABSTRACT

**Background/Aims:** Hepatic injury caused by ischemia/reperfusion (I/R) is a clinical problem associated with major liver surgery. Among other flavonoids, apigenin has shown a promising effect on I/R cases. In this study, we have investigated the effects of apigenin after liver I/R injury in rats. **Materials and Methods:** Forty eight rats were randomized into the following eight groups: (1) Control-sham group: rats subjected to the surgical procedure, except for liver I/R; (2) DMSO group: rats subjected to surgery, except for liver I/R given the apigenin solvent dimethyl-sulfoxide intraperitoneally; (3) C60 group; (4) C120 group; (5) C240 group: rats underwent liver ischemia for 45 min followed by reperfusion for 60 min, 120 min, and 240 min; (6) AP60 group; (7) AP120 group; (8) AP240 group: rats underwent liver ischemia for 45 min, and then given apigenin (5 mg) intraperitoneally followed by reperfusion for 60 min, 120 min, and 240 min. Reverse transcription polymerase chain reaction was performed on liver tissues to measure BCL-2/BAX expression, enzyme-linked immunosorbent assay to measure M30/M65 and ICAM-1. Immunohistochemistry was used to identify M30 biomarker in liver tissues. **Statistical Analysis:** Quantitative variables were tested by Kolmogorov-Smirnov test, repeated measures analysis of variance/Friedman test. Gene levels were assessed by Student's *t*-test/Mann-Whitney U-test. **Results:** BCL-2 levels were significantly higher in I/R apigenin groups than in I/R control groups. BAX levels were lower in the AP240 group than in C240 group. Prolongation of reperfusion resulted in increased activation of M30. ICAM-1 levels were lower in the AP240 group than in C240 group. **Conclusions:** Apigenin seems to inhibit the process of apoptosis and ameliorate the hepatic I/R injury.

**Key Words:** Apigenin, BCL-2, ICAM-1, liver ischaemia/reperfusion injury, M30, M65

Received: 11.06.2015, Accepted: 08.07.2016

**How to cite this article:** Tsaroucha AK, Tsiaousidou A, Ouzounidis N, Tsalkidou E, Lambropoulou M, Giakoustidis D, *et al.* Intraperitoneal administration of apigenin in liver ischemia/reperfusion injury protective effects. Saudi J Gastroenterol 2016;22:415-22.

Hepatic ischemia/reperfusion (I/R) injury is common in major liver surgery including liver transplantation and hepatectomies. Despite improvement in surgical techniques and perioperative management, liver failure remains one of the major complications.<sup>[1]</sup> Liver ischemia and reperfusion involve a variety of mechanisms each of which contributes to the overall concomitant injury. More specifically, I/R injury can be divided into two distinct phases, the initial phase, 2

hours after reperfusion, which is characterized by Kupffer cell activation, reactive oxygen species (ROS) production, and the release of proinflammatory chemokines and cytokines, including tumor necrosis factor alpha (TNF- $\alpha$ ).<sup>[2]</sup> The cytokines upregulate adhesion molecules, such as ICAM-1, and cause polymorphonuclear neutrophils (PMN) to adhere to endothelial cells followed by sequestration of PMNs and further production of ROS.<sup>[3]</sup> The second phase of injury, 6 hours after reperfusion, is characterized by the recruitment and extravasation of polymorphonuclear leukocytes (neutrophils) into the parenchyma, significant ROS production, and the

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	DOI: 10.4103/1319-3767.195556

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release of proteases and additional inflammatory mediators, leading to further liver tissue injury. Incomplete restoration of the blood flow, including heterogeneous distribution in the hepatic microcirculation, would also appear to form a part of the underlying mechanism of I/R-induced liver injury. This major cell injury can lead to either hepatic cell necrosis or apoptosis. These two types of cell death coexist in liver pathology as a result of common pathways and signals.<sup>[4]</sup> The apoptotic changes include blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation, and global mRNA decay and death. There are two pathways through which apoptosis can be initiated. Via the intrinsic pathway, the cell kills itself because of cell stress, whereas via the extrinsic pathway, the cell kills itself because of signals from other cells. In both the pathways, cell death is induced by the activation of caspases.<sup>[5]</sup>

A number of experimental studies have been conducted to test the effect of several pharmacological agents in liver injury inhibition, including the use of flavonoids.<sup>[6]</sup> Flavonoids are a group of plant polyphenols that are abundant in fruits and vegetables, and represent substantial constituents of the nonenergetic part of human diet. In addition to their physiological role in plants, they exhibit an antioxidant activity, and this is why they are known to possess a therapeutic potential in some diseases, including cancer, ischemic heart disease, atherosclerosis, and liver diseases.<sup>[7-12]</sup> Apigenin is a natural flavonoid reported to have several biologic effects such as cancer chemopreventive properties and an antioxidant effect including protection of the endothelium and liver cells against oxidative stress.<sup>[13-18]</sup> It is not clear yet which are the receptors that apigenin binds to liver cells. The mechanism through which apigenin protects living cells against oxidative stress has not been fully investigated yet, however, it has been shown that apigenin upregulates the gene expression of antioxidant enzymes such as superoxide dismutase (SOD) 1, SOD2, and glutathione peroxidase (GPx) 1, suggesting that it attenuates oxidative-induced cell damage in living cells.<sup>[19]</sup> Apigenin is also a Pregnane X receptor (PXR) activator, the expression of which is related to the clearance of toxic substances from the body, but it does not directly bind to PXR suggesting that an alternative mechanism may be responsible for flavonoid-mediated PXR activation in HepG2 liver carcinoma cells.<sup>[20]</sup> In addition, we know that apigenin can inhibit the procedure of apoptosis by leading to lower Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand Receptor-1 (TRAIL-R1) expression level on macrophages.<sup>[21]</sup>

The aim of the present study was to evaluate the possible protective effect of apigenin against I/R induced hepatic injury by measuring the expression of apoptosis controlling genes *BCL-2* (anti-apoptotic) and *BAX* (pro-apoptotic) using the reverse transcription polymerase chain reaction (RT-PCR) method.<sup>[22]</sup> In addition, the M30/M65 ratio was measured

in order to be used in defining the relative proportion of apoptosis versus total cell death. M30 measures caspase-cleaved CK18 produced during apoptosis and M65 measures the levels of both caspase-cleaved and intact CK18, the latter being released from cells undergoing necrosis.<sup>[23,24]</sup> Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) serum levels and the serum levels of the adhesion molecule ICAM-1 were measured to use as indicators of the level of hepatic I/R injury.<sup>[3]</sup>

## MATERIALS AND METHODS

### Animals and experimental protocol

All experiments described here were performed according to the European union guidelines for the ethical treatment of experimental animals. Forty-eight Wistar type rats, aged 14–16 weeks and weighing 220–350 g were used. The animals were living under controlled temperature and lighting conditions. They were housed individually in cages, and were allowed free access to standard rat food and water before and after the experiments. The animals were fasted overnight before the experiments, but were given free access to water. Rats were anesthetized by using diethyl ether and sevoflurane. After the central incision, the ligament attachments connecting the liver, diaphragm, abdominal wall, and neighboring organs were divided. After the organ was carefully isolated, the liver hilus was exposed to find the common hepatic artery and portal vein. These vessels were obstructed using an atraumatic vascular microclip for 45 min to interrupt the blood supply to the liver. Apigenin was administered intraperitoneally dissolved in dimethyl sulfoxide solvent (DMSO).<sup>[25,26]</sup> The exact consistency of the solution was: 5 mg apigenin + 0.2 ml DMSO + 0.3 ml NaCl 0.9%. Reperfusion was followed for 60, 120, and 240 minutes (AP60, AP120, and AP240 groups). The same number of rats was used as control group and were subjected only to ischemia and reperfusion for the respective times (C60, C120, and C240 groups). Other rats were subjected to a sham operation, which was identical to the surgical procedure used for the I/R group rats without clamping (group C) and the rest were sham operated and given the DMSO solvent intraperitoneally (DMSO group). The rats were kept under anesthesia for the same period of time. The animals were euthanized and liver tissues were obtained and stored individually in liquid nitrogen at  $-80^{\circ}\text{C}$  until the end of the experiment in paraben for histologic examinations by light microscopy and immunocytochemistry, and additionally blood was obtained by cardiac puncture, centrifuged, and the serum was kept at  $-80^{\circ}\text{C}$  pending analysis.

### Measuring the expression of *BCL-2/BAX* genes by reverse transcription polymerase chain reaction method

Real-time RT-PCR was performed in order to determine the relative amounts of RNA transcripts for *BCL-2* and

BAX genes in total RNA isolated from liver tissue in the experimental model of liver ischemia reperfusion.<sup>[27]</sup> Total RNA was extracted from rat liver tissue samples using Trizol reagent, according to the manufacturer's instructions. Reverse transcription was performed using the SuperScript Preamplification System (Invitrogen, Paisley, UK) and random hexamers in a total volume of 20 ml, following DNase treatment (Invitrogen). Using the Light Cycler MX3005P (Stratagene, La Jolla, CA), reactions were carried out using the SYBER Green MM QPCR Brilliant mix (Stratagene), 0.2  $\mu$ M of each primer and 0.5  $\mu$ l of cDNA in a final volume of 25  $\mu$ l. Cycling parameters were as follows: a pre-amplification cycle (denaturation for 10 min at 95°C), 40 cycles of amplification (denaturation for 30 s at 94°C, annealing for 30 s at 60°C, and extension for 30 s at 72°C), and a final extension cycle (denaturation for 60 s at 94°C, annealing for 30 s at 60°C, and extension for 30 s at 95°C followed by dissociation). Results were calculated using MaxPro QPCR Software Version 3 (Stratagene California USA), according to the comparative threshold cycle method. Analysis of relative gene expression data was performed according to the 2-DDCT method<sup>[28]</sup> using  $\beta$ -actin as an endogenous reference (internal control) gene and cDNA from total rat brain RNA extract as a control reference sample, the discrete cosine transform (DCT) value, of which was deducted from all sample values (also used as an intra-assay standard).

### Histology and immunohistochemistry

Liver tissues were fixed in 10% neutral-buffered formalin, processed routinely, embedded in paraffin, sectioned at 4  $\mu$ m, and stained with hematoxylin and eosin (H and E). Liver sections were also stained immunohistochemically with M30 CytoDeath Monoclonal Antibody, 1:50 dilution, (Alexis Biochemical).

### ELISA M30/M65, ICAM-1

The values of ELISA M30 represent the apoptotic cell death because monoclonal antibody M30 is used to trace the apoptotic epithelial cells. The monoclonal antibody M30 is specific for the epitope in caspase-3 broken CK-18 (CK18Asp396; NE M30 neo-epitope), which appears during apoptosis whereas it is not identifiable in live epithelial cells. The quantitative designation of rat ICAM-1 levels was done through the immunoabsorption technique ELISA (rat ICAM-1, ORB 50029 Biorbyt, UK), in blood serum according to the manufacturer's instructions. Blood samples were collected immediately after the end of the reperfusion time according to the protocol.

### Statistical analysis

Statistical analysis of the data was performed using the Statistical Package for the Social Sciences (SPSS), version 19.0 (IBM, NY, USA). The normality of quantitative variables was tested by Kolmogorov–Smirnov test. Levels

of M65, M30, BCL-2, BAX, and AST/ALT were expressed as mean  $\pm$  standard deviation (SD), whereas ICAM-1 levels were expressed as median value and range. Within group differences of normally distributed quantitative variables (M65, M30, BCL-2, and BAX) were examined by one-way repeated measures ANOVA (rmANOVA) and Friedman test (ICAM-1). Because of the small size of the samples, no correction of the significance level in multiple comparisons was performed. Between group differences and the differences of levels of the genes (BCL-2 and BAX) were assessed by Student's *t*-test and Mann–Whitney U-test. All tests were two tailed and statistical significance was considered for *P* values less than 0.05.

## RESULTS

### Histology

There was an evident Kupffer cells activation in groups subjected to liver ischemia and reperfusion. In all groups receiving apigenin, significant inhibition of Kupffer cells activation was noted, with a more intense impact on the apigenin treated 240 min I/R group compared to 240 min I/R group. In all I/R groups, there was vascular congestion, and hepatocytes frequently showed cytoplasmic edema. Nuclear pyknosis was also frequent in both Kupffer and endothelial cells. Increased number of neutrophils was seen in the dilated sinusoids. The typical histopathological findings of I/R-induced liver injury described above were more rarely found in the groups that were pretreated with apigenin [Figures 1 and 2].

### Immunohistochemistry of M30

The stain for M30 demonstrated a significant activation of the gene in I/R groups compared to the sham operation group. Prolongation of reperfusion resulted in an increased activation of M30. I/R groups pretreated with

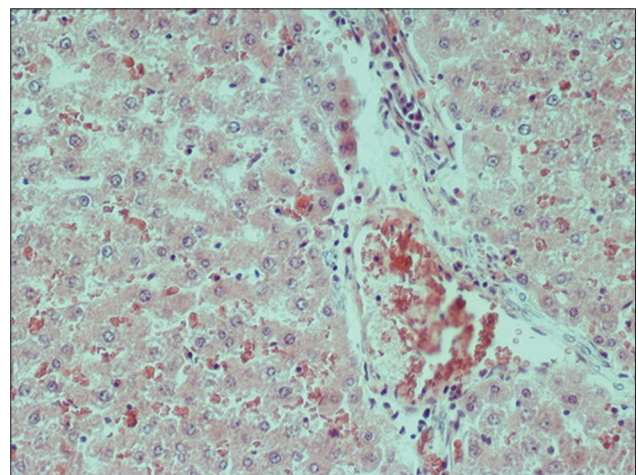
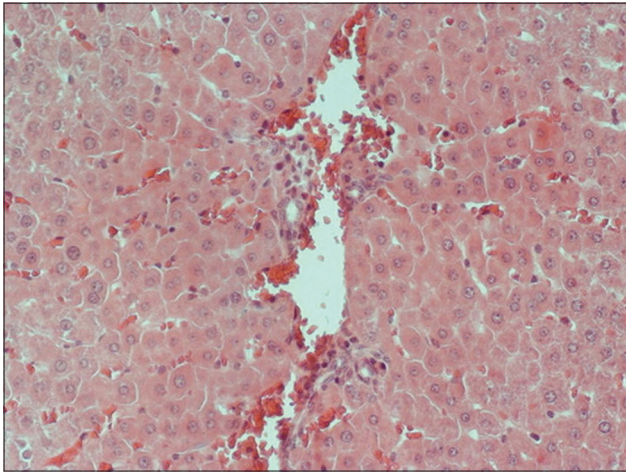


Figure 1: Liver tissue after 240 min I/R 240  $\times$  200

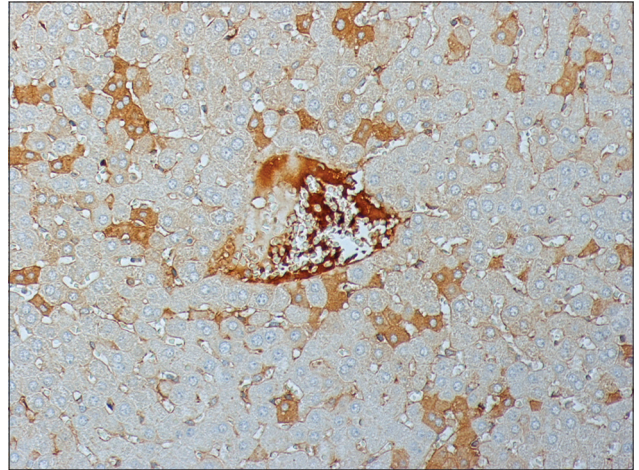


apigenin and subjected to different periods of reperfusion exhibited reduced expression of M30 compared to the respective nontreated animals' groups [Figures 3-8]. This

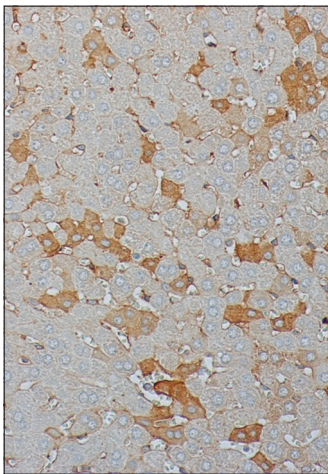
suggests an inhibitory effect of apigenin on liver cell apoptosis.



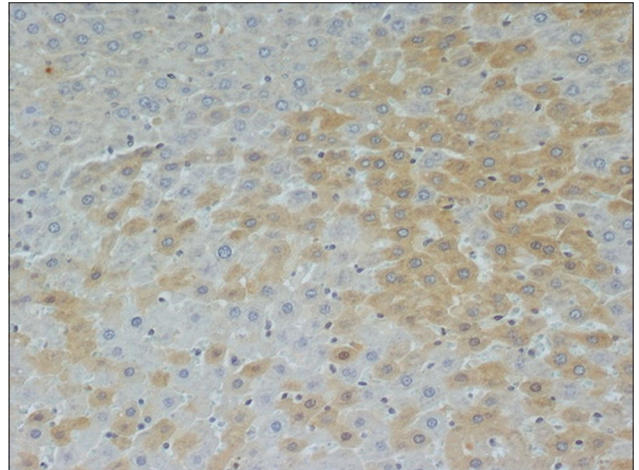
**Figure 2:** Apigenin treated liver after 240 min I/R 240 × 200



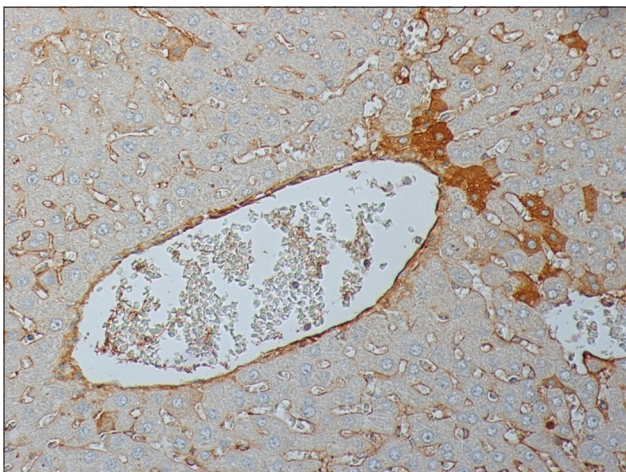
**Figure 3:** Liver tissue after 60 min I/R stained for M30 240 × 200



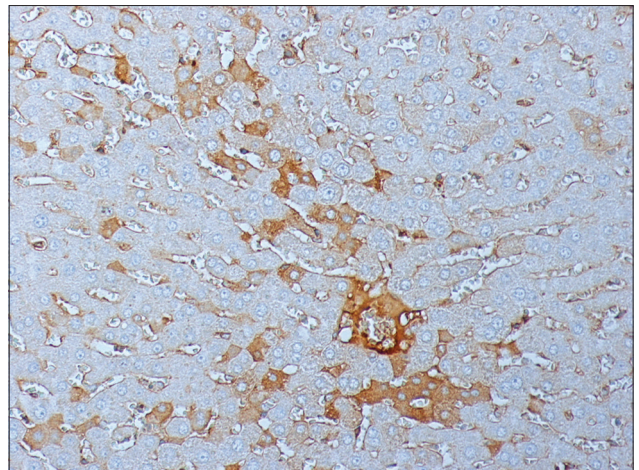
**Figure 4:** Liver tissue after 120 min I/R stained for M30 240 × 200



**Figure 5:** Liver tissue after 240 min I/R stained for M30 240 × 200



**Figure 6:** Apigenin treated liver after 60 min I/R stained for M30 240 × 200



**Figure 7:** Apigenin treated liver after 120 min I/R stained for M30 240 × 200



**Real time polymerase chain reaction**

*Regulation of expression of BCL-2 and BAX by Apigenin*

Liver ischaemia and reperfusion for 60 min resulted in increased expression of BCL-2 compared to the downregulated BCL-2 expression after 120 and 240 min of reperfusion. This can be explained by the enhanced apoptotic activity after 120 min of reperfusion. Administration of apigenin resulted in upregulation of the expression of BCL-2 in groups subjected to 60 min, 120 min, and 240 min of reperfusion compared to the nontreated groups subjected to 60 min, 120 min, and 240 min of reperfusion, respectively [Figure 9]. The increase in the levels of BCL-2 expression was even higher between treated and nontreated groups of 240 min of reperfusion. Sham and DMSO groups showed no difference [Figure 10].

Expression of BAX in liver I/R, sham, and DMSO groups showed no statistically significant differences between all groups. Comparison of the apigenin treated groups with the nontreated groups showed no differences in 60 min

and 120 min of reperfusion. After 240 min of reperfusion, the apigenin treated group demonstrated a statistically significant decrease in the expression of proapoptotic BAX compared to the 240 min nontreated group [Figure 11].

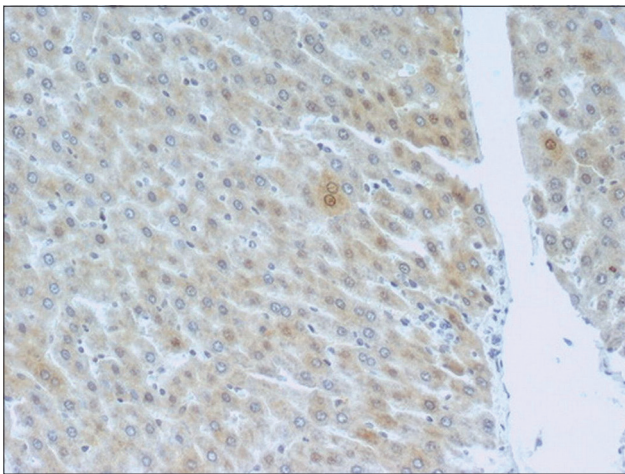
**Enzyme linked immunosorbent assay**

*M30/M65*

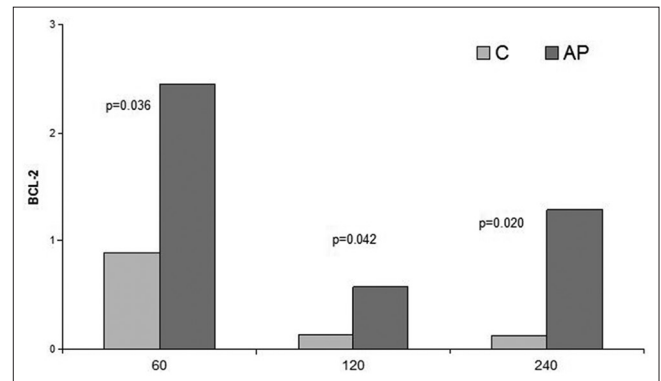
As far as M30 was concerned, there was a slight decrease in its concentration in the apigenin treated 60 min and 240 min groups. However, there was a surprising but not significant rise in its concentration in the 120 min group [Figure 12].

In the case of M65, apigenin treated groups subjected to 60 min, 120 min, and 240 min of reperfusion showed decreased expression compared with the non-treated animals' groups subjected to 60 min, 120 min, and 240 min of reperfusion, respectively [Figure 13].

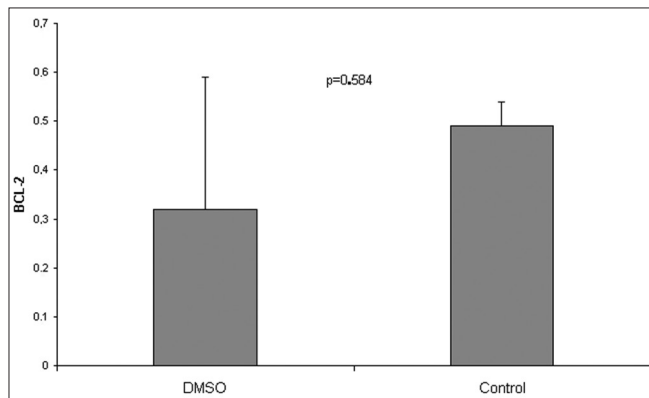
Regarding the M30/65 ratio, that illustrates how apoptosis is correlated to necrosis, there were no statistically significant differences between the treated and nontreated groups. The ratio did not seem to be affected by the reperfusion time either.



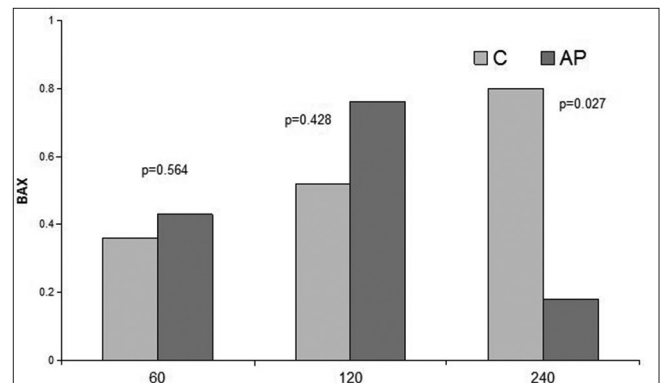
**Figure 8:** Apigenin treated liver after 240 min I/R stained for M30 240 x 200



**Figure 9:** Comparison of I/R groups to respective apigenin treated I/R groups in terms of BCL-2 levels



**Figure 10:** Comparison of BCL-2 levels between dimethyl sulfoxide and sham operation groups



**Figure 11:** Comparison of I/R groups to respective apigenin treated I/R groups in terms of BAX levels

### ICAM-1

The expression of ICAM-1 increased after 60 min of reperfusion, decreased after 120 min, and increased again after 240 min of reperfusion. Differences did not reach statistically significant levels. Animals treated with apigenin showed decreased mean levels of ICAM-1 expression in all the different reperfusion periods, however, this reduction was statistically significant only for the apigenin treated 240 min group in comparison with the 240 min nontreated group [Figure 14].

### Aspartate aminotransferase/alanine aminotransferase

The liver enzymes' serum levels were elevated for all the I/R groups. The mean values for the 60 min I/R group were AST =  $914 \pm 585.58$  U/L and ALT =  $122.33 \pm 52.78$  U/L, compared to the levels for the apigenin treated 60 min I/R group with means of AST  $224.4 \pm 39.54$  U/L and ALT  $24.6 \pm 3.82$  U/L ( $P = 0.01$  and  $P = 0.0007$ , respectively). For the 120 min I/R group, AST was  $701.6 \pm 286.69$  U/L and ALT  $170.7 \pm 138.12$  U/L; values were significantly higher compared to those of the 120 min apigenin group; AST  $205.25 \pm 31.52$  U/L and ALT  $31 \pm 14.61$  U/L ( $P = 0.0005$  and  $P = 0.014$ , respectively). The results were similar for the 240 min groups with a mean AST of  $997.33 \pm 394.5$  U/L and ALT of  $326.83 \pm 80.62$  U/L for the nontreated group and a mean AST of  $84.5 \pm 15.2$  U/L and ALT of  $14.16 \pm 2.6$  U/L for the apigenin treated group ( $P = 0.0023$  and  $P = 0.00033$ , respectively).

## DISCUSSION

Liver I/R is a clinical phenomenon presenting in several conditions such as in liver surgery and transplantation as well as in hypovolemic shock leading to hepatic cell apoptosis or necrosis.<sup>[1]</sup>

In our study, we have illustrated that the intraperitoneal administration of apigenin resulted in an upregulation of the expression of BCL-2 antiapoptotic proteins in groups subjected to 60 min, 120 min, and 240 min of reperfusion compared to the nontreated groups subjected to 60 min, 120 min, and 240 min of reperfusion. The increase in the levels of BCL-2 expression was remarkably higher between treated and nontreated groups of 240 min of reperfusion. The comparison of the apigenin treated groups with the nontreated groups showed no significant differences in BAX levels after 60 min and 120 min of reperfusion. However, after 240 min of reperfusion, the apigenin treated group demonstrated a statistically significant decrease in the expression of BAX compared to the nontreated group. The protective and antiapoptotic action of apigenin through the mitochondrial pathway has been demonstrated in previous studies.<sup>[29]</sup> Liu *et al.* in 2014 have shown that apigenin has

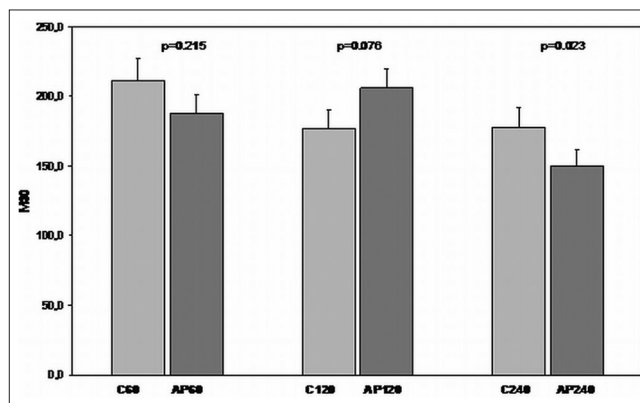


Figure 12: Comparison of I/R groups to respective apigenin treated I/R groups in terms of M30 levels

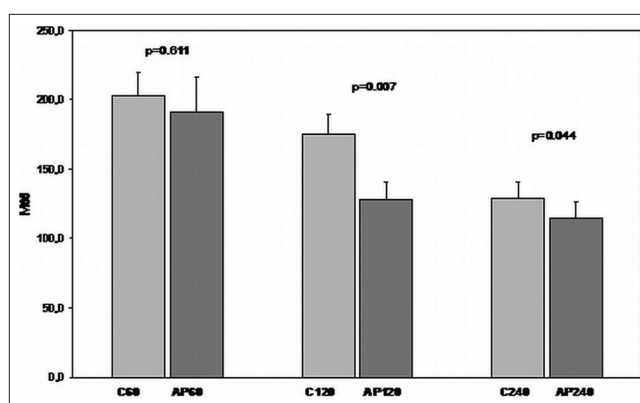


Figure 13: Comparison of I/R groups to respective apigenin treated I/R groups in terms of M65 levels

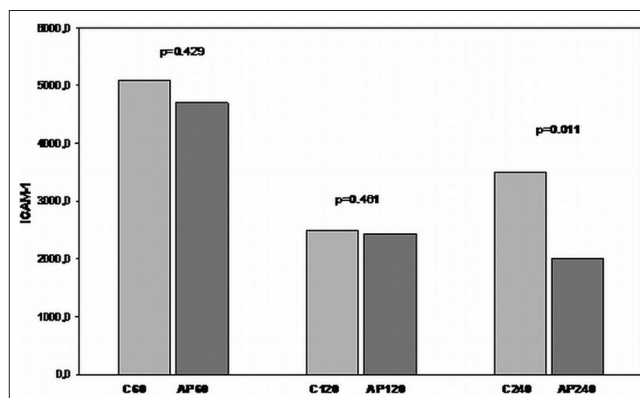


Figure 14: Comparison of I/R groups to respective apigenin treated I/R groups in terms of ICAM-1 levels

neuroprotective effects through the inhibition of oxidative damage and the suppression of apoptosis by demonstrating an increase in BCL-2 levels in the apigenin treated groups.<sup>[30]</sup> Even more specifically in hepatic ischemia-reperfusion injury, Tsalkidou *et al.*, in 2014, have demonstrated that apigenin seems to affect the Fas/FasL mediated pathway of apoptosis, resulting in apoptosis inhibition.<sup>[31]</sup>

In this study, we have also shown that animals treated with apigenin showed remarkably decreased mean levels of ICAM-1 expression in the apigenin treated 240 min group compared to the 240 min nontreated group, suggesting a limitation of the inflammatory process occurring during reperfusion. A similar study in rats by Savvanis *et al.* in 2014, also showed a decrease in the ICAM-1 levels as evidence of the attenuation of the I/R injury by sildenafil.<sup>[3]</sup>

Regarding the M30/65 ratio, that illustrates how apoptosis is correlated to necrosis,<sup>[32]</sup> there were no statistically significant differences between the treated and nontreated groups.

Finally, according to our histology results, in animals pretreated with apigenin before the onset of hepatic ischemia, attenuation of hepatocellular apoptosis and inflammatory infiltration was noted in contrast to the animals of the I/R group, which exhibited a greater degree of necrosis and inflammation under microscopic evaluation. In all groups receiving apigenin, significant inhibition of Kupffer cells activation was noted, with a more intense impact on the apigenin treated 240 min I/R group compared to 240 min I/R group. As previously shown by Lee *et al.* in 2016, the Kupffer cell activation contributes to the inflammatory response, and inhibition of this activation can lead to less hepatocellular damage during I/R injury.<sup>[33]</sup>

Immunohistochemical stain revealed that I/R groups pretreated with apigenin and subjected to different periods of reperfusion exhibited reduced expression of M30 compared with the nontreated animals with respective reperfusion time, suggesting a decrease in apoptosis for the pretreated groups.<sup>[31]</sup>

Finally, the serum liver enzymes values of AST and ALT were statistically significantly lower in all the apigenin treated groups compared to the respective nontreated groups, suggesting that apigenin-induced protection against liver injury in the treated groups. As it has been illustrated before by Yang *et al.* in 2013, apigenin could protect against acute liver damage caused by hepatotoxic agents.<sup>[34]</sup>

These findings suggest that apigenin has potent actions against hepatic I/R injury through suppression of inflammation, oxidative stress, and apoptosis. Further studies are definitely needed to investigate the exact mechanisms through which apigenin acts at a cellular level and to support the potential benefits of pretreatment with this flavonoid.

**Financial support and sponsorship**  
Nil.

**Conflicts of Interest**  
There are no conflicts of interest

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