Anti-inflammatory and Anti-apoptotic Potentials of Apigenin against Liver Injury Induced by Ischemia-Reperfusion in Rats

Original Article

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ABSTRACT

Apigenin is a dietary flavonoid that exists copiously in several herbs and vegetables. It exhibits anti-inflammatory, anti-mutagenesis, anti-proliferative and antioxidant properties. The present work aimed to investigate some of mechanisms underlying protective potential of apigenin in hepatic ischemia-reperfusion injury. Rats were divided into four groups; sham-operated, sham-operated pretreated with apigenin (25 mg/kg, p.o.), ischemia/reperfusion (I/R) (30 min ischemia and 1 h reperfusion) and I/R pretreated with apigenin. Compared with I/R group, pretreatment with apigenin markedly reduced transaminases levels and ameliorated tissue histopathological changes. Apigenin significantly reduced high mobility group box 1 (HMGB1) expression and suppressed liver tumor necrosis factor- α (TNF-α), nuclear factor κB (NF-κB) and myeloperoxidase (MPO) activity. Moreover, apigenin restored reduced glutathione (GSH), decreased liver lipid peroxidation, and boosted glutathione peroxidase (GPx) activity in addition to attenuation of apoptosis by increasing Bcl-2/Bax ratio. It may thus be concluded that inhibition of HMGBI by apigenin plays a role towards its antioxidant, anti-inflammatory as well as anti-apoptotic properties which are involved in conferring its hepato-protective properties

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1. INTRODUCTION

In liver surgery, prolonged periods of interrupting blood supply with subsequent lacking of nutrients and oxygen may occur during transplantation, removal of liver tumors, vascular reconstruction and trauma[1,2,3]. Shortly after blood flow restoration, there is an upsurge in injury instigated by ischemia, exacerbating the global damage, which is mainly attributed to the excessive triggering of innate immune response[4,5,6].

The response in reperfusion comprises two successive phases. Immediately after reperfusion, sudden re-oxygenation provokes reactive oxygen species (ROS) generation which induce oxidative imbalance and stimulate Kupffer cells (KC) in the liver[7,8]. Meanwhile, levels of nitric oxide (NO) are reduced and there is an imbalance between endothelin-1 and NO production, leading to vasoconstriction and entrapment of platelets and neutrophils[9]. Hepatocyte injury is then promoted through necrosis and apoptosis[10,11,12]. In the late phase of reperfusion there is a further activation and recruitment of neutrophils, T helper cells, and platelets to the liver. Permanence of theses inflammatory cells in the constricted sinusoids results in inflammatory damage and microvasculature collapse[13]. Activation of KC, neutrophils, and platelets results in a massive local production of ROS as well as a cascade of inflammatory events including release of pro-inflammatory cytokines such as interleukin-2 (IL-2), interleukin-1 (IL-1), tumor necrosis factor-alpha (TNF-α) and damage associated molecular patterns (DAMPs) such as high mobility group box-1 (HMGB1)[14]. HMGB1 then creates a positive feedback circuit causing the release of extra chemokines and cytokines. Thus, under stress, HMGB1 endures a continuing inflammatory state[15].

Apigenin, naturally is present as a dimer, biapigenin, isolated mostly from the flowers and buds of Hypericum perforatum[16]. It abundantly exists in common vegetables and fruits as grapefruit, parsley, plant-derived beverages, oranges, tea, onions, wheat sprout and chamomile[17]. Apigenin exerts anti-inflammatory, anti-mutagenesis, anti-proliferative and antioxidant activities[18,19,20]. It has been known as a cancer chemopreventive agent. It is a cell cycle inhibitor[21], having low intrinsic toxicity on normal versus cancer cells[22]. Apigenin activates nuclear factor erythroid-related factor-2 (Nrf2), that induces the transcriptional activity of the antioxidant response element (ARE) which is crucial for induction of genes encoding many cytoprotective enzymes as heme oxygenase-1(HO-1) and glutathione peroxidase (GPx)[23,24,25,26].

The aim of the current investigation was to evaluate the possible hepatoprotective activity of apigenin against hepatic injury associated with I/R. It is also extended to gain convincing insights into its putative underlying mechanism.

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2. MATERIAL AND METHODS

2.1. Animals

Adult male Wistar albino rats, each weighing 200±20 g, were purchased from the National Research Centre in Cairo and housed at a temperature of (23 ± 2oC) and a relative humidity of (60 ± 10%). They were kept on a standard pellet chow and allowed water ad libitum. The Ethical Committee for animal Experimentation at the Faculty of Pharmacy, Cairo University approved the study (Permit number: 1273).

2.2. Drugs and chemicals

Dimethyl sulfoxide (DMSO) and apigenin were purchased from Sigma–Aldrich Chemicals (St. Louis, MO, USA).

2.3. Induction of hepatic I/R injury

After anesthetizing the animals with thiopental sodium (50 mg/kg, i.p)[27], an upper abdominal midline incision was made to expose the common hepatic artery and portal vein. These were then clamped for 30 min to induce hepatic ischemia, then reperfusion was allowed for 1h[28] and designed as "ischemia/reperfusion" (I/R) rats.

2.4. Experimental design

The study was carried out both on I/R rats and on sham-operated animals where rats were exposed to laparotomy but without undergoing ischemia/ reperfusion.

Four groups of 10 rats each were allocated as follows

Groups 1and 2: Sham-operated rats. Animals were given either 0.1% v/v DMSO (1 ml/kg) or apigenin (25 mg/kg) respectively orally for 5 days before being sham-operated.

Groups 3 and 4: I/R rats. Animals were treated as above with either DMSO or apigenin for 5 days before being subjected to I/R.

Blood samples were then collected from the retro-orbital sinus for plasma preparation before subjecting the animals to euthanasia by decapitation under anesthesia. The liver of all animals was removed, and divided into four segments.

One segment was used to prepare a 10% homogenate in ice-cold saline, one was used for histological examination, one for Western blot analysis, and one for quantitative real time PCR (qRT PCR).

2.5. Biochemical measurements

2.5.1 Determination of relevant parameters in plasma

The separated plasma was used to assess liver functions by determination of the enzymes, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) using suitable kits (Biodiagnostic Co, Cairo, Egypt).

2.5.2. Determination of relevant parameters in liver tissue

The 10 % liver homogenate prepared in ice-cold saline was used to estimate liver contents of nuclear factor κB (NF-κB), TNF-α, myeloperoxidase (MPO) activity and oxidative stress biomarkers. NF-κB and TNF-α were determined using rat specific ELISA kits (Wuhan Eiaab science, Wuhan, China) and (RandD, USA), while MPO was measured as described by *Bradley et al*[29]. The method depends on the oxidation of dianisidine by MPO resulting in the formation of a compound exhibiting an increased absorbance at 460 nm. The selected oxidative stress biomarkers in the liver were glutathione peroxidase (GPx), reduced glutathione (GSH) and thiobarbituric acid reactive substances (TBARS). GPx was measured using a specific kit obtained from Oxis Research (Oxford, USA), while GSH and TBARS were measured colorimetrically in the homogenate using specific kits obtained from Biodiagnostic Co (Cairo, Egypt) as described by Beutler *et al*[30] and Ohkawa and Ohishi[31], respectively.

Since the above parameters had to be represented per mg protein liver tissue, the protein content of the homogenate was determined according to the method described by Bradford[32].

Western Blotting analysis of HMGB1

20 µg protein concentration of each sample was first loaded on a polyacrylamide gel then transferred onto polyvinylidene difluoride membranes (Thermo Fischer Scientific, MA, USA). A blocking buffer consisting of 20 mM Tris-Cl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20 and 3% bovine serum albumin (BSA) was then added to the membranes for 1 hour to prevent nonspecific binding of the antibodies before incubating them overnight at 4 °C with HMGB1 or with Beta actin (β- actin) primary antibodies (Thermo Fisher Scientific, MA, USA). Secondary antibodies conjugated to horse-radish peroxidase (Thermo Fisher Scientific, MA, USA) were then added to the membrane for 1 h at 37 °C followed by the chemiluminescent substrate (ClarityTM Western ECL substrate - BIO-RAD, USA). Band intensity was then analyzed by Chemi Doc ™ imaging system with Image Lab ™ software version 5.1 (Bio-Rad Laboratories Inc., Hercules, CA, USA). The results were normalized to β-actin.

Quantitative real time PCR analysis of Bax and Bcl-2 gene expression

A specific extraction kit (Qiagen, Germantown, MD, USA) was used to extract total RNA from liver homogenate, which was then quantified spectrophotometrically at 260 nm. Equal amounts of extracted RNA were then reverse transcribed into cDNA using high capacity cDNA reverse transcription kit (Thermo Fischer Scientific, MA, USA). To assess the gene expression of Bax and Bcl-2, qRT-PCR was performed using an ABI PRISM 7500 fast sequence detection system (Applied Biosystems, CA, USA). The sequences of PCR primer pairs used were the following. For Bax, F:5'- CCCTGTGCACTAAAGTG-CCC -3′, R: 5′- TTCTTCACGATGGTGAGCG-3′, for Bcl-2 F:5'-CTACGAGTG-GGATGCTGGAGG-3′,

R:5′ GTCAGATGGACACATGGTG -3′ and for β-actin F: 5′-TGCTGGTGCTGAGTATGTCG-3′, R: 5′-TTGAGAGCAATGCCAGCC-3′.

The relative expression of target genes was obtained using the 2−ΔΔCT formula as described by Livak and Schmittgen[33], using β-actin as a housekeeping gene.

2.6. Histopathological evaluation

Transverse 4µm sections of liver specimens fixed in 10% formalin were stained with hematoxylin and eosin (H and E) and examined under a light microscope for detection of histo-pathological changes.

2.7. Statistical Analysis

The biochemical results were represented as means ± standard error (SEM) then subjected to one way analysis of variance (ANOVA) followed by Tukey-Kramer test for multiple comparisons. Statistical analysis was done using GraphPad Prism software (version 6; GraphPad Software, Inc., San Diego, CA, USA).

3. RESULTS

3.1. Effect of apigenin on aminotransferases activities

A significant increase in the plasma aminotransferase levels of rats subjected to I/R was shown, reaching 2.68 fold the sham-operated control group for AST and 1.45 fold the sham-operated control group for ALT. Treatment with apigenin effectively guarded against this rise (Fig.1 A and B).

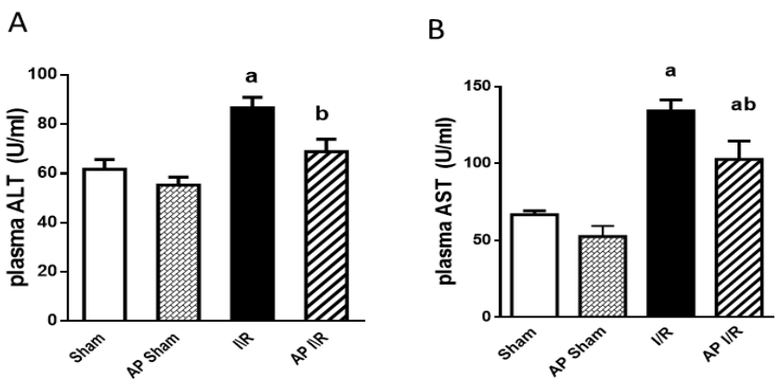

Fig.1: Changes in ALT (A) and AST (B) in the plasma of rats exposed to hepatic I/R injury after treatment with apigenin (AP).
Results are presented as means ± SEM of 8 rats and analyzed statistically using one-way ANOVA followed by Tukey-Kramer test for multiple comparisons. Statistically significant differences (< 0.05) from sham-operated rats and from the I/R rats are denoted by a and b respectively.

3.2. Effect of apigenin on liver histopathology:

The liver sections of sham-operated rats pretreated with DMSO or apigenin showed no histological changes of the portal area and surrounding hepatocytes. However, subjecting the rats to I/R led to dilation of the central and portal veins as well as infiltration of inflammatory cells within the tissue surrounding the bile duct. Pretreatment with apigenin led to protection against these changes and resulted in no histological changes (Fig.2).

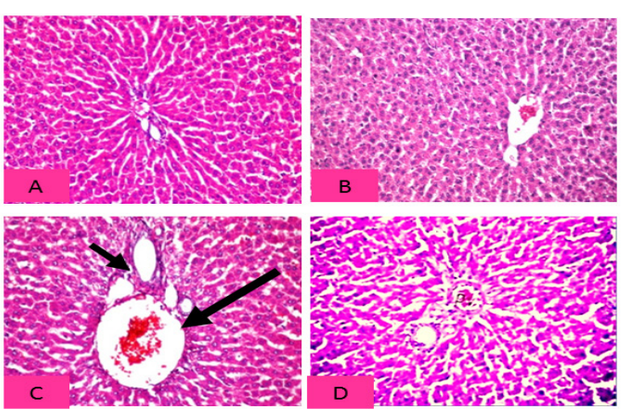

Fig.2: Hepatic histological changes in rats exposed to I/R after treatment with apigenin (AP). Liver sections of sham-operated control rats pretreated with DMSO (A) or apigenin (B) showed no histological changes of the portal area and surrounding hepatocytes. Dilation of the central and portal veins (large arrow) as well as infiltration of inflammatory cells within the tissue surrounding the bile duct (small arrow) in rat exposed to I/R (C). Normal histological hepatocellular architecture in I/R rats pretreated with apigenin (D) (HandE x 400). The livers of 4 rats from each group were used for detection of histopathological changes.

3.3. Effect of apigenin on inflammatory biomarkers:

Subjecting rats to I/R induced a profound spike in HMGB1 reaching 12.74 fold the sham-operated control group and showed a statistically significant rise in MPO activity as well as TNF-α and NF-κB contents. However, apigenin (25 mg/kg; p.o) impeded the noxious effect of I/R on hepatic HMGB1, NF-κB and TNF-α by lessening their liver contents to be 26.65%, 52.81% and 51.84%, respectively. It also blunted the activity of MPO to 53.50% (Fig 3 A, B, Cand D).

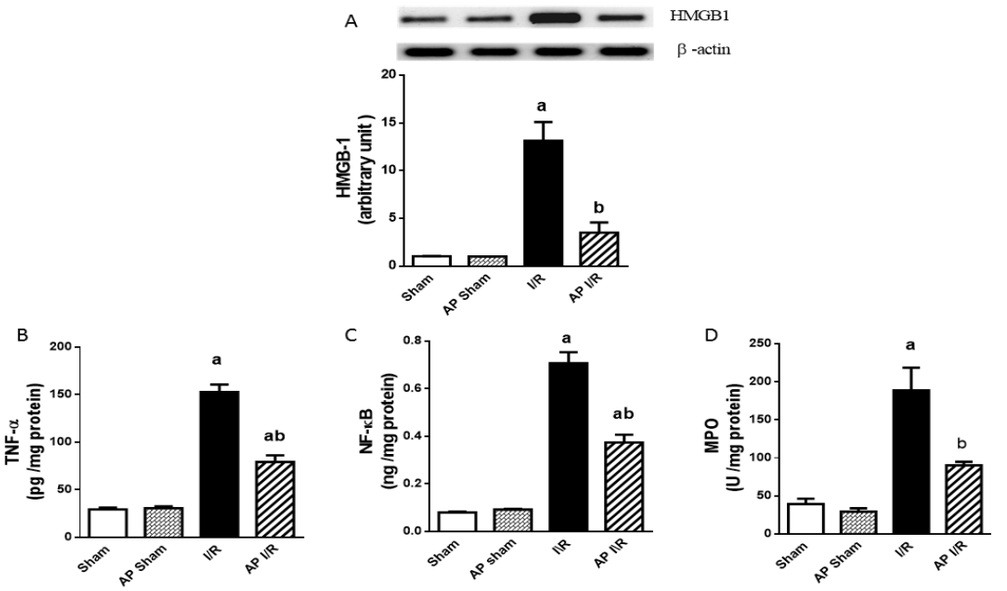

Fig.3: Changes in hepatic HMGB1 (A), TNF-α (B), NF-κB (C) and MPO activity (D) in rats exposed to hepatic I/R injury after treatment with apigenin (AP). Results are presented as means ± SEM of 8 rats and analyzed statistically using one-way ANOVA followed by Tukey-Kramer test for multiple comparisons. Statistically significant differences (< 0.05) from sham-operated rats and from the I/R rats are denoted by a and b respectively.

3.4. Effect of apigenin on oxidative stress biomarkers:

The deterioration in oxidative status following I/R was strongly evident by the obvious decline in both liver GSH content and GPx activity as well as the marked elevation in TBARS content that reached 1.79 fold the sham-operated control group. Apigenin efficiently diminished hepatic TBARS content to 66.33%, restored hepatic GSH content to 278.95 % and enhanced the hepatic GPx activity to 278.95 %. (Fig 4 A, B and C).

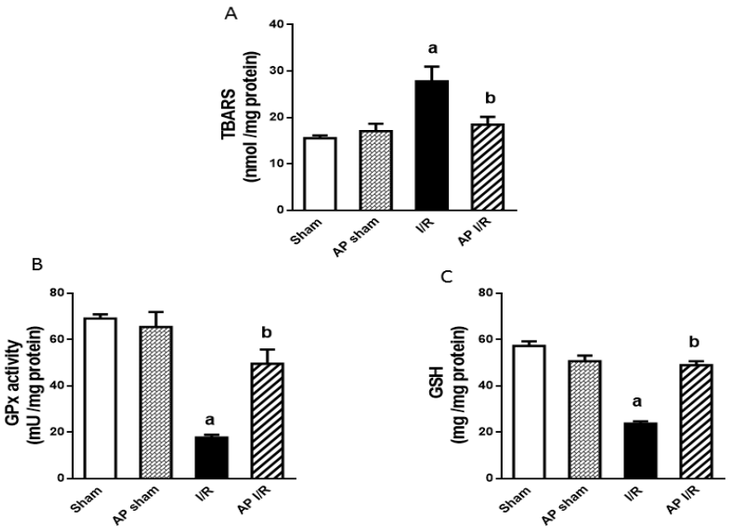

Fig.4: Changes in hepatic TBARS (A), GPx (B) and GSH (C) in rats exposed to hepatic I/R injury after treatment with apigenin (AP).
Results are presented as means ± SEM of 8 rats and analyzed statistically using one-way ANOVA followed by Tukey-Kramer test for multiple comparisons. Statistically significant differences (< 0.05) from sham-operated control rats and from the I/R group are denoted by [a] and [b] respectively.

3.5. Effects of apigenin on apoptotic biomarkers:

I/R injury led to a diminution in the gene expression of Bcl-2, as a measure of anti-apoptotic activity, and a marked rise in Bax, indicating an increase in pro-apoptotic activity (Fig.5). Conversely, apigenin enhanced greatly the gene expression of Bcl-2 to 471.68 %, but dampened that of Bax to19.61 %. (Fig 5 Aand B).

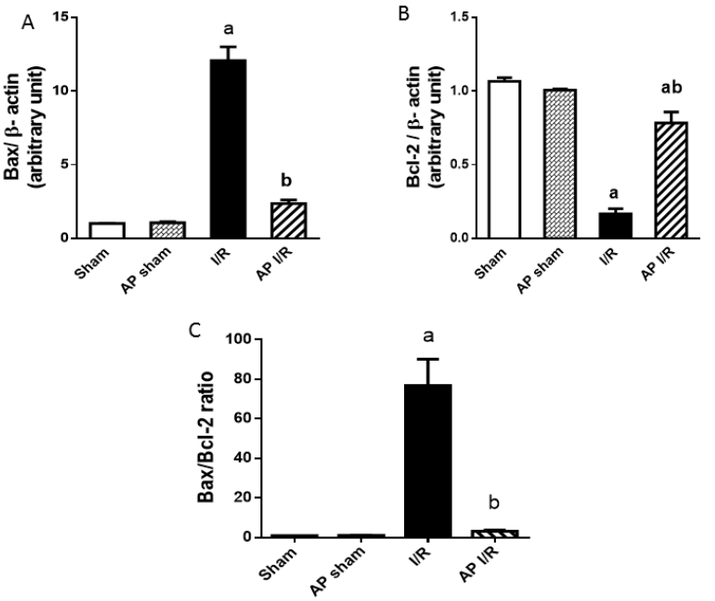

Fig.5: Changes in hepatic mRNA expression of Bax(A), Bcl-2(B) and Bax/Bcl-2 ratio (C) in rats exposed to hepatic I/R injury after treatment with apigenin (AP). Results are presented as means ± SEM of 8 rats and analyzed statistically using one-way ANOVA followed by Tukey-Kramer test for multiple comparisons. Statistically significant differences (< 0.05) from sham-operated control rats and from the I/R group are denoted by [a] and [b] respectively.

4. DISCUSSION

Pretreatment of rats with apigenin for five consecutive days attenuated hepatic damage associated with I/R, as revealed by the preserved structural integrity of the hepatocellular membrane and liver cells architecture in histopathological pictures along with the suppressed plasma activities of liver transaminases (ALT and AST). This is in agreement with *Ali et al.*[34] who reported the protective effect of apigenin on N-nitrosodiethylamine-induced liver injury, where rats treated with various doses of apigenin exhibited a dose-dependent decrease in the levels of ALT and AST enzymes.

Moreover, apigenin restored the elevated transaminases activities following paracetamol-induced hepatotoxicity in mice[35] and rats[36].

The present findings depicted the ability of apigenin to normalize the expression of HMGB1 in liver. This effect was coupled with prominent anti-inflammatory, antioxidant and antiapoptotic activities. The effect of apigenin on HMGB1 may be ascribed to its previously reported capability of up-regulating HO-1[25,26] which prevents the nuclear translocation of HMGB1 and dampens its release[37,38,39]. HMGB1 is a typical DAMP that acts as an alarm in mediating sterile inflammatory response due to I/R damage in multiple tissues as liver[39]. It stimulates monocytes to exhibit an increased capacity for adhesion[40] and release multiple cytokines and inflammatory mediators[41,42,43].

Neutrophils stimulation with HMGB1 increases their adhesive and migratory functions[44]. Furthermore, HMGB1 stimulates neutrophils production of ROS[45] and triggers the activation of NF-κB which further promotes HMGB1 release by activated immune cells[46,47].

The anti-inflammatory effects of apigenin have been revealed clearly in this study by the effective decrease in the liver contents of NF-κB and the pro-inflammatory cytokine, TNF-α. This is in coherent with *Shukla et al.*[48] who reported that apigenin suppressed prostate carcinogenesis via inactivation of NF-κB pathway.

Sundry previous studies documented that apigenin repressed NF-κB activity and consequently inhibited LPS-induced pro-inflammatory cytokines release by human primary monocytes and macrophages[49,50]. Moreover, pretreatment with apigenin decreased NF-κB protein expression and TNF-α level in D-galactosamine/LPS-induced liver injury in rats[51].

Similarly, TNF- α was significantly decreased following apigenin treatment in a mouse model of alcohol-induced liver damage[52] and in different experimental models of inflammatory bowel disease[53,54].

In addition, a significant decrease in MPO activity was observed by apigenin treatment confirming its potent anti-inflammatory effect and harmonizes with *Lampropoulos et al.*[55] where apigenin reduced pancreatic

MPO activity in experimental model of acute pancreatitis. Moreover, administration of apigenin effectively ameliorated neutrophil infiltration as evidenced by the suppression of colonic MPO following acetic acid[56], dextran sulphate sodium and trinitrobenzenesulfonic acid-induced colitis in rats[54].

The hepatoprotective effect of apigenin against various hepatotoxins such as paracetamol[36] and N-nitrosodiethylamine[34] was previously attributed to its powerful antioxidant activity. The authors reported a significant increase in the enzyme antioxidant defense mechanisms along with a reduction of lipid peroxidation in animals treated with apigenin.

Generally, ROS in liver I/R injury enhance the generation of various pro-inflammatory mediators as IL-8, TNF- α, IL-1 and cell adhesion molecules[57,58] along with HMGB1 which ratifies the continuous production ROS by inflammatory cells, especially neutrophils[45]. They induce the expression of many transcription factors such as activator protein-1and NF-κB[59,60] leading eventually to direct cellular injury through DNA damage, protein degradation and lipid peroxidation[61] ROS induce necrosis and apoptosis of hepatocytes.[62,63] Additionally, apoptotic cell death was previously correlated with HMGB1 release during inflammation[64].

The current study revealed that rats pretreated with apigenin showed an improvement in oxidative status as manifested by the significant decrease in hepatic TBARS beside the restored liver GSH content. Apigenin also enhanced the activity of GPX enzyme after I/R injury, endorsing its great antioxidant properties. These findings collaborate with the study of *Singh et al.*[65], in which apigenin inhibited lipid peroxidation in N- nitrosodiethyl amine-induced hepatotoxicity.

Apigenin amended superoxide dismutase (SOD) and GPx activities, as well as the malondialdehyde (MDA) level in rats subjected to spinal cord injury[66]. In addition, the ability of apigenin to boost GSH-dependent enzymes and consequently GSH levels mediated its hepatoprotective effect against liver injury induced by alcohol in mice[35,52].

Additionally, the antiapototic effect of apigenin contributed largely to its hepatoprotective effect following I/R-induced insults in rats as previously reported[67,68]. In the same context, apigenin hampered the process of apoptosis as manifested by the decrease in gene expression of Bax and the increase in that of Bcl-2 in the present study. These results harmonize with, Zhang *et al.*[69] who stated that apigenin attenuated heart injury following lipopolysaccharide (LPS)-induced endotoxemia where, it decreased cleaved caspase-3, cleaved caspase-9, Bax; and increased Bcl-2 mRNA expression. Likewise, Tsaroucha *et al.*[68] accentuated that intraperitoneal injection of apigenin upregulated the expression of Bcl-2 antiapoptotic proteins in rats subjected to 60 min, 120 min, and 240 min of reperfusion after liver ischemia for 45 min.

In conclusion, the present results provide profound insights into the role of apigenin in mitigating hepatocellular injury following I/R. It largely curbed the evoked oxidative stress, inflammatory response and apoptotic cell death throughout the reperfusion time.

CONFLICT OF INTEREST

There are no conflicts of interest.

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