ORIGINAL ARTICLE

Diacerein ameliorates acetaminophen hepatotoxicity in rats via inhibiting HMGB1/TLR4/NF-κB and upregulating PPAR-γ signal

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Abstract

Background Acetaminophen (APAP) is a worldwide antipyretic as well as an analgesic medication. It has been extensively utilized during the outbreak of coronavirus 2019 (COVID-19). APAP misuse would lead to liver injury. Diacerein (DIA), an anthraquinone derivative, has antioxidant and inflammatory properties. Hence, this study attempted to evaluate the impact of DIA treatment on liver injury induced by APAP and its influence on nuclear factor-κB (NF-κB) /toll-like receptor 4 (TLR4)/high mobility group box-1(HMGB-1) signaling as well as the expression of peroxisome proliferator-activated receptor-gamma (PPAR-γ) expression.

Methods Male albino rats received 25 as well as 50 mg/kg/day DIA orally for seven days. One hour after the last administration, rats received APAP (1gm/kg, orally). For histopathological analysis, liver tissues and blood were collected, immunohistochemical (IHC) assay, biochemical assay, as well as quantitative real-time polymerase chain reaction (qRT-PCR).

Results DIA markedly reduced liver injury markers and ameliorated histopathological changes. Moreover, DIA dose-dependently alleviated oxidative stress status caused by APAP administration along with inflammatory markers, including the level of interleukin-1 beta (IL-1β), myeloperoxidase (MPO), tumor necrosis factor-alpha (TNF-α), and interleukin 6 (IL-6). Furthermore, DIA downregulated protein levels as well as mRNA of HMGB-1, TLR4, NF-κB p65 expression, and enhanced PPAR-γ expression. Moreover, DIA ameliorated apoptotic (Bax) and caspase-3 expressions and increased the anti-apoptotic (Bcl2) expression.

Conclusions This study demonstrated that DIA exerts anti-apoptotic, anti-inflammatory, and antioxidant properties against liver injury induced by APAP that is attributed to inhibition of the HMGB1/TLR4/NF-κB pathway, besides upregulation of the expression of PPAR-γ.

Keywords Diacerein (DIA) · Acetaminophen (APAP) · Hepatotoxicity · HMGB1/TLR4/NF-κB signaling · PPAR-γ · Bax/Bcl2/caspase-3

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Introduction

Acetaminophen (APAP) is widely utilized as an over-the-counter drug for treating inflammation, fever, as well as pain [1]. APAP is currently the main antipyretic and analgesic drug recommended by several studies over non-steroidal anti-inflammatory drugs (NSAIDs) used to manage fever and pain associated with the pandemic infection of coronavirus 2019 (COVID-19). This assumption could cause a possible misuse of acetaminophen, which is considered the major cause behind drug-induced liver injury (DILI) worldwide. [1–3]. Although *N*-acetylcysteine (NAC) is the current drug used to protect against liver injury induced by APAP, it is only effective when given an early period of APAP ingestion and associated with

adverse effects such as nausea, vomiting, allergic reactions, and headaches [4]. It is pivotal to find new therapeutic targets and effective agents.

Inflammation and elevated oxidative stress significantly contribute to APAP hepatotoxicity, resulting in hepatocyte necrosis and apoptosis [3, 5]. Consequently, necrotic hepatocytes induce damage-associated molecular patterns (DAMPs like high mobility group box 1(HMGB1 that is determined by toll-like receptor 4 (TLR, and activate the innate immune system and release excessive inflammatory mediators, including cytokines including nuclear factor-κB (NF-κB), tumor necrosis factor-alpha (TNF-α), as well as interleukin 1 beta (IL-1β), which eventually leads to severe hepatic injury [4, 6, 7]. Peroxisome proliferator-activated receptor-γ (PPAR-γ), which belongs to the family of the nuclear hormone receptors, was proven to enhance the antioxidant and anti-inflammatory gene transcription [8], modulate TLR4 activation [9], and regulate NF-κB-induced inflammation [10].

Diacerein (DIA), anthraquinone derivatives, is an anti-inflammatory, analgesic, and antipyretic drug used clinically to treat osteoarthritis. Furthermore, rhein, the active metabolite of DIA, was demonstrated to induce several pharmacological activities, such as anti-inflammatory, antioxidant, and antitumor [11]. DIA is an IL-1β inhibitor which evidenced to markedly protected against inflammatory damage via its ability to inhibit inflammatory cytokines like IL-1β and TNF-α [12], in addition to down-regulating the TLR4/NF-κB-mediated signaling pathway activation, followed by suppressing the secretion of inflammatory cytokines [13]. It has been proven that DIA has a hepatoprotective impact against liver ischemia and reperfusion (I/R) via its antioxidant and anti-inflammatory activities [13]. However, its effect on liver injury induced by APAP remains unclear. Hence, this study attempted to examine the potential protective impact of DIA on hepatotoxicity induced by APAP and explore its impact on HMGB-1/TLR4/NF-κB, Bax/Bcl2/caspase-3 signaling pathway, and PPAR-γ expression.

Materials and methods

Chemicals and drugs

The chemicals utilized were highly analytical and purchased from Sigma-Aldrich (St. Louis, MO, USA). Paracetamol (Paramol ®) tablets (500 mg) were obtained from Misr Co. for Pharmaceutical Industries (Cairo, Egypt). They were dissolved in saline. DIA powder was purchased from Eva Pharma Company (Cairo, Egypt) and dissolved in 1% carboxymethyl cellulose (CMC).

Animals

Twenty-four male albino rats, with nearly 150 to 180 g, were purchased from the animal facility of The Nile Co. for Pharmaceuticals and Chemical Industries (Cairo, Egypt). Animals were kept in a controlled environment at a temperature between 20 to 25 °C, 55% humidity, as well as a 12- h light/dark cycle. They were supplied with free drinking and given a regular diet. One week prior to the experiment, animals were acclimatized. The experiment was carried out based on The Public Health Guide for the care and use of laboratory animals, as well as the Faculty of Pharmacy's Animal Ethics Committee, Al-Azhar University, Egypt.

Experimental design

The animals were randomized into four groups (n = 6 rats per group).

Control group; animals were administrated 1% CMC and saline for seven days. APAP group; rats were given a single APPA dose (1 g/kg, orally) [14] on the seventh day of the trial. DIA 25 + APPA and DIA 50 + APPA groups; rats received diacerein (25 and 50 mg/kg, orally) [15] respectively for a week, and subsequently were given APPA (1 g/kg, orally) on the last day of the experiment. Twenty-four hours after APAP exposure, in order to evaluate aspartate aminotransferase (AST), serum alanine aminotransferase (ALT), total bilirubin (TBB), lactate dehydrogenase (LDH), and blood samples were obtained. Afterward, rats were sacrificed, livers were dissected, washed in ice-cooled saline, and dried. Each dissected liver was subdivided into three sections: the first was preserved in a formalin solution of 10% for immunohistochemical and histopathological analysis. With regard to the second section, it was promptly frozen in liquid nitrogen and kept at − 80 °C for the analysis of gene expression. Finally, the third section was homogenized in saline for subsequent biochemical analysis.

Assessment of liver functions

Serum of rats used for assay of ALT, AST, and TBB using colorimetric kits (Abcam, UK), while LDH assayed using Randox (UK) kit following the instructions of the manufacturer.

Histopathological examination

The samples from the liver were preserved in a formalin of 10%, fixed in paraffin, dissected into 5-μm thickness sections, and stained with eosin and hematoxylin [16].

Assessment of lipid peroxidation and biomarkers of oxidative stress

The determination of lipid peroxidation in hepatic tissue homogenate was performed by measuring malondialdehyde (MDA) content using a commercial kit (Bio-diagnostic Co., Egypt). Diminished glutathione (GSH) level as well as glutathione peroxidase activity (GPx), superoxide dismutase (SOD), and catalase (CAT) activities were performed utilizing kits purchased from (Bio-diagnostic Co., Egypt). It should be noted that the procedures were carried out, and parameters were determined following the protocol of the manufacturer.

Enzyme-linked immunosorbent assay (ELISA) for hepatic HMGB1, TLR4, IL-1β, IL-6, TNF-α, and MPO levels

TLR4 and HMGB1 protein levels were determined using an ELISA kit (My BioSource, USA). IL-6, IL-1β, TNF-α, and MPO were quantified in hepatic tissue homogenate utilizing ELISA kits purchased from (RandD Systems, Inc, USA), (San Diego, California, USA), (Cloud-clone corp., USA), and (My BioSource, USA) respectively. Measurement of sample concentration as well as the measurement of all parameters was performed in accordance with the protocol of the manufacturer.

IHC assay for hepatic NF-κB (p65), PPAR-γ, Bax, caspase-3, and Bcl2 protein expressions

Paraffined blocks of hepatic tissue were sectioned and deparaffinized with xylene and subsequently rehydrated with alleviated concentrations of alcohol. The sections were left for 10 min in 3% H_2O_2 followed by 30 min in 0.1% trypsin at 37 °C, for antigen retrieval. They were subsequently incubated overnight at 4 °C with antibodies of polyclonal rabbit against Bcl2, NF-κB (p65), Bax, PPAR-γ, and caspase-3 (1:1000 dilution; Abcam, Cambridge, UK). Sections were treated at 37 °C for 30 min with biotinylated goat anti-rabbit (Invitrogen), the secondary antibody, and subsequently stained with 3, 3-diaminobenzidine and eventually counterstained with hematoxylin. Afterward,

image analysis was carried out via ImageJ software (version 1.48) to assess the area percent (A%).

qRT-PCR analysis of HMGB-1 and TLR4 mRNA expression

Following the instructions of the manufacturer, the isolation of total RNA from the liver was performed utilizing a commercial kit (QIAzole and Qiagen RNeasy Mini Kits). The purity and the concentration of the isolated RNA were evaluated using the Nanodrop (ND-1000 USA). The primers used for assessment were obtained from (Invitrogen Life Technologies, Carlsbad, CA, USA). The primers' sequences utilized are like the following: HMGB-1 forward AGGCTGACAAGGCTCGTTATG; reverse TGTCATCCGCAGCAGTGTTG, TLR4 forward GGGGGGTATTTGACACACTCTA; reverse TCCTTTGGATGTCTCTATGCGA, β-actin forward GGAGATTACTGCCCTGGCTCCTAGC; reverse GGCCGGACTCATCGTACTCCTGCTT.

qRT-PCR was carried out utilizing QuantiTect® SYBR® Green RT-PCR master mix Kit (Qiagen, Hilden, Germany). Following a 10-min hot start at 95 °C, samples at 95 °C were exposed to 15 s of denaturation followed by 1 min of annealing for 45 cycles at 54–58 °C. Melting curve analysis was done from 60 °C to 95 °C, with temperature increments of 0.5 °C every 10 s to detect any nonspecific products. The Ct value was calculated using the thermal cycler's (Veriti®) software package, in which the ΔCt value denoted the number of the cycle when the fluorescence curve passed the baseline score. Based on the $2^{-\Delta\Delta Ct}$ method, the target genes' relative expression of mRNA was determined via normalizing the control group and the housekeeping gene -actin.

Statistics

Statistical analysis of the experimental results was performed utilizing the GraphPad Prism (version 5.0, GraphPad Software Inc., San Diego, California) and expressed in the form of mean ± S.E.M. The one-way ANOVA was utilized to analyze data, whereas Tukey's post hoc tests were utilized for making comparisons. $P < 0.05$ was deemed significant.

Results

Impact of DIA on hepatic functions and histopathological changes in rats treated with APAP

As depicted in Table 1, administration of APAP significantly elevated ALT, AST, TBB, and LDH serum levels by 151%, 111.4%, 224%, and 132%, respectively, than controls.

Table 1 Impact of DIA on hepatic functions in rats treated with APAP

Parameters	Control	APAP	DIA 25 + APAP	DIA 50 + APAP
ALT (U/L)	25.92 ± 0.55	65.10 ± 1.35[a]	38.98 ± 0.80[a,b]	30.78 ± 1.06[a,b,c]
AST (U/L)	40.25 ± 0.61	85.14 ± 1.22[a]	50.64 ± 0.57[a,b]	24.67 ± 0.71[a,b,c]
TBB (U/L)	17.45 ± 0.59	56.93 ± 0.85[a]	30.06 ± 0.34[a,b]	24.67 ± 0.71[a,b,c]
LDH (U/L)	303.00 ± 1.38	704.00 ± 1.46[a]	440.00 ± 2.30[a,b]	410.00 ± 1.36[a,b,c]

Data are expressed in the form of means ± S.E.M. (n = 6 per group), and one-way ANOVA was utilized for data analysis, followed by Tukey post hoc test. ([a]), ([b]), ([c]): $P < 0.05$ compared the controls, APAP, DIA 25 + APAP groups respectively

APAP acetaminophen, *DIA* diacerein, *ALT* alanine transaminase, *AST* aspartate transaminase, *TBB* total bilirubin
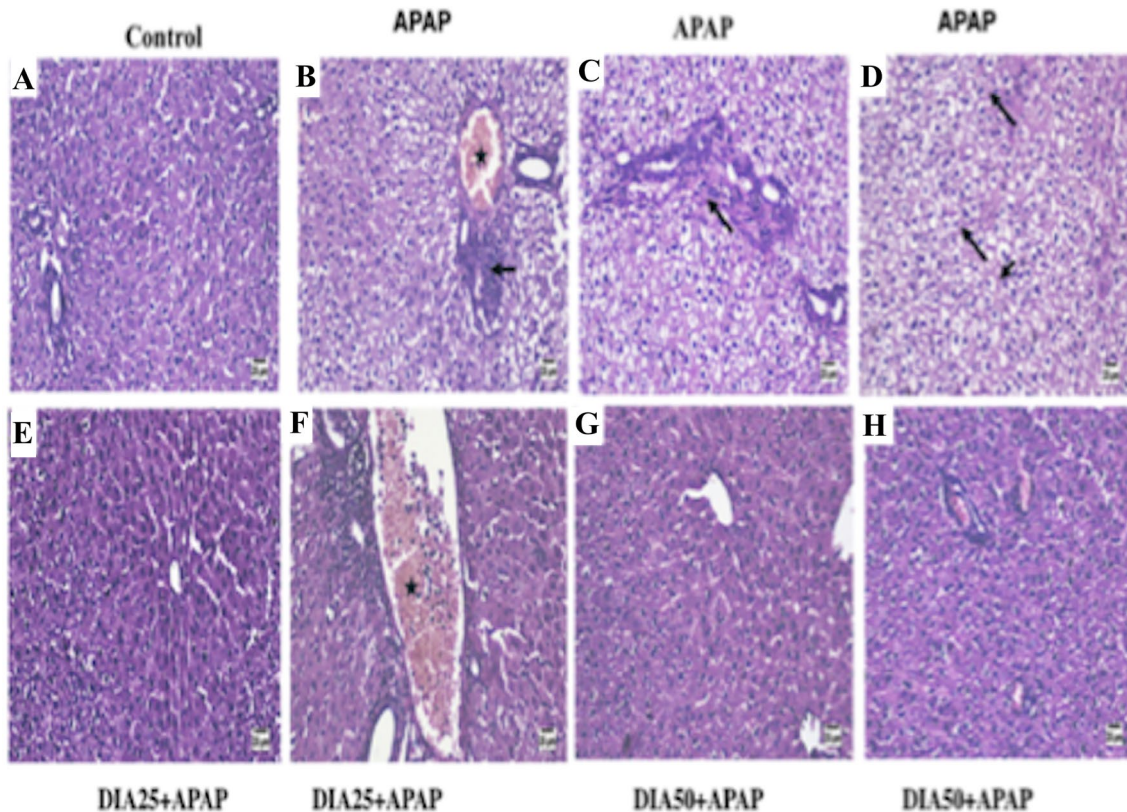

Fig. 1 Effect of DIA pretreatment on histopathological changes in APAP-treated rats. Representative photomicrographs of **A** control sections show normal hepatocytes and portal area while APAP sections **B**–**D** show hepatocytes' diffuse vacuolation along with infiltration of portal mononuclear inflammatory cells (arrow), congestion (star) (**B**), portal fibroplasia (arrow) (**C**), and necrosis (**D**). The DIA 25 + APAP (**E** and **F**) sections display periportal hepatocellular vacuolation (**E**), congestion (star), and portal mononuclear inflammatory cells infiltration (**F**). The DIA 50 + APAP sections (**G** and **H**) show apparently normal hepatocytes normal portal area (**G**) with mild vacuolation in some hepatocytes (**H**). Magnifications: × 200 for all slides

However, DIA (25 and 50 mg/kg) pre-treatment resulted in a substantial decline in AST, ALT, TBB, and LDH serum levels by (40%, 40.5%, 47%, and 37.5%) and (53%, 71%, 57%, and 42%), respectively, in a dose-related manner than the group treated with APAP. These results demonstrated that DIA induces a protective impact against liver damage induced by APAP.

According to microscopic examination in Fig. 1, the histology of control rats' H&E-stained liver sections was normal (Fig. 1A). The dissected liver sections from the rats treated with APAP demonstrated diffuse hepatocellular vacuolation and congestion. In addition, the portal areas contained mononuclear inflammatory cells infiltration (Fig. 1B). Furthermore, portal fibroplasia was noticed along with the detected inflammatory reaction (Fig. 1C) and necrosis (Fig. 1D). However, pretreatment with DIA (25 and 50 mg/kg) ameliorated APAP-induced histopathological changes (Fig. 1E–H).

Table 2 Impact of DIA on the parameters of hepatic oxidative stress in rats treated with APAP

Parameters	Control	APAP	DIA 25 + APAP	DIA 50 + APAP
MDA (nmol/g. tissue)	0.41 ± 0.31	3.56 ± 0.18^a	$1.22 \pm 0.08^{a,b}$	$0.78 \pm 0.01^{b,c}$
GSH (mmol/g. tissue)	101.40 ± 0.43	64.99 ± 1.16^a	$85.82 \pm 1.12^{a,b}$	$97.31 \pm 0.62^{a,b,c}$
GPx (U/g. tissue)	152.90 ± 1.10	103.10 ± 0.77^a	$141.30 \pm 1.03^{a,b}$	$148.5 \pm 0.43^{a,b,c}$
SOD (U/g. tissue)	162.60 ± 1.10	95.90 ± 0.70^a	$137.5 \pm 0.91^{a,b}$	$152.1 \pm 0.90^{a,b,c}$
CAT (U/g. tissue)	199.80 ± 2.60	105.30 ± 1.50^a	$139.10 \pm 0.80^{a,b}$	$160 .40 \pm 1.00^{a,b,c}$

Data were expressed in the form of means $\pm$ S.E.M. (n = 6 per group), and then data analysis was performed utilizing the one-way ANOVA and then the Tukey post hoc test. (^{a}), (^{b}), (^{c}): P < 0.05, compared to controls, APAP, DIA 25 + APAP groups, respectively

Impact of DIA on hepatic oxidative stress in rats treated with APAP

As depicted in Table 2, administration of a single APAP dose induced a dramatic increase in the level of hepatic MDA reaching 768%, as well as marked depletion of GSH levels and activities of GPx, SOD, and CAT by 36%, 33%,41%, and 47% respectively, than the controls. Pretreatment with DIA (25 and 50 mg/kg) induced a substantial decline in the level of MDA reaching 66% and 78%, respectively, besides inhibition of the levels of GSH and GPx, SOD, and CAT activities by ([32%, 37%, 43.4%, and 32.4%] and [50%, 44%,58%, and 52%], respectively) in a dose-depended manner compared with animals treated with APAP. These findings verified that DIA ameliorates the oxidative damage induced by APAP and anti-oxidant conditions of the liver tissue.

Effect of DIA on inflammation and neutrophils infiltration in APAP-treated in rats

As depicted in Fig. 2A, B, C, D, administration of APAP markedly increased TNF-α, IL-1β, and IL-6 hepatic levels by 128%, 181%, and 143%, respectively than controls. In contrast, pretreatment with DIA (25 and 50 mg/kg) markedly reduced these changes by ([33%, 30%, and 29%) and (47%, 50%, and 45%] respectively) in a dose-depended manner than animals treated with APAP. These findings revealed that DIA could alleviate the inflammation induced by APAP.

In Fig. 2D, neutrophil infiltration was assessed via the evaluation of MPO. Hepatic MPO level was increased in APAP-treated rats by 188% than controls. In comparison to rats treated with APAP, DIA pretreatment (25 and 50 mg/kg) reduced the increased MPO level by 39% and 52%, respectively. These findings verified that DIA could improve neutrophil infiltration in APAP-induced liver injury in a dose-dependent manner.

DIA impact on HMGB1/TLR4/ NF-κB (p65) signaling in rats treated with APAP

As demonstrated in Fig. 3A and B, administration of APAP substantially increased the mRNA expression of TLR4 HMGB1 by 2.7 and 1.7-fold, respectively, in comparison to controls. Consequently, APAP elevated HMGB1 and TLR4 protein levels by 117.5% and 214%, respectively, compared to control rats (Fig. 3C and D). DIA pretreatment (25 and 50 mg/kg) considerably downregulated the mRNA expression of TLR4 and HMGB1 by ([25% and 26%] and [43% and 39%], respectively) as well as decreased the protein levels of both by ([22% and 33%] and [34% and 48%] respectively), compared to the APAP-treated rats.

In Fig. 4, the IHC examination of NF-κB (p65) expression displayed a strong positive reaction in the APAP-treated rats compared to the control group (Fig. 4B). Pretreatment with DIA (25 and 50 mg/kg) substantially decreased NF-κB (p65) expression in a dose-depended manner (Fig. 4C and D) compared to APAP-treated rats.

DIA effect on PPAR-γ expression in rats treated with APAP

As depicted in Fig. 5, immunostaining of PPAR-γ expression displayed weak expression in APAP- treated rats' liver tissues than controls (Fig. 5B). In contrast, pretreatment with DIA (25 and 50 mg/kg) displayed a strong positive reaction in a dose-depended manner in comparison to animals treated with rat APAP (Fig. 5C and D).

DIA impact on apoptosis in rats treated with APAP

As displayed in Fig. 6A, B, C, D, and E, the IHC examination of Bax, active caspase-3, and Bcl2 in the liver tissue sections demonstrated an elevated expression of both Bax
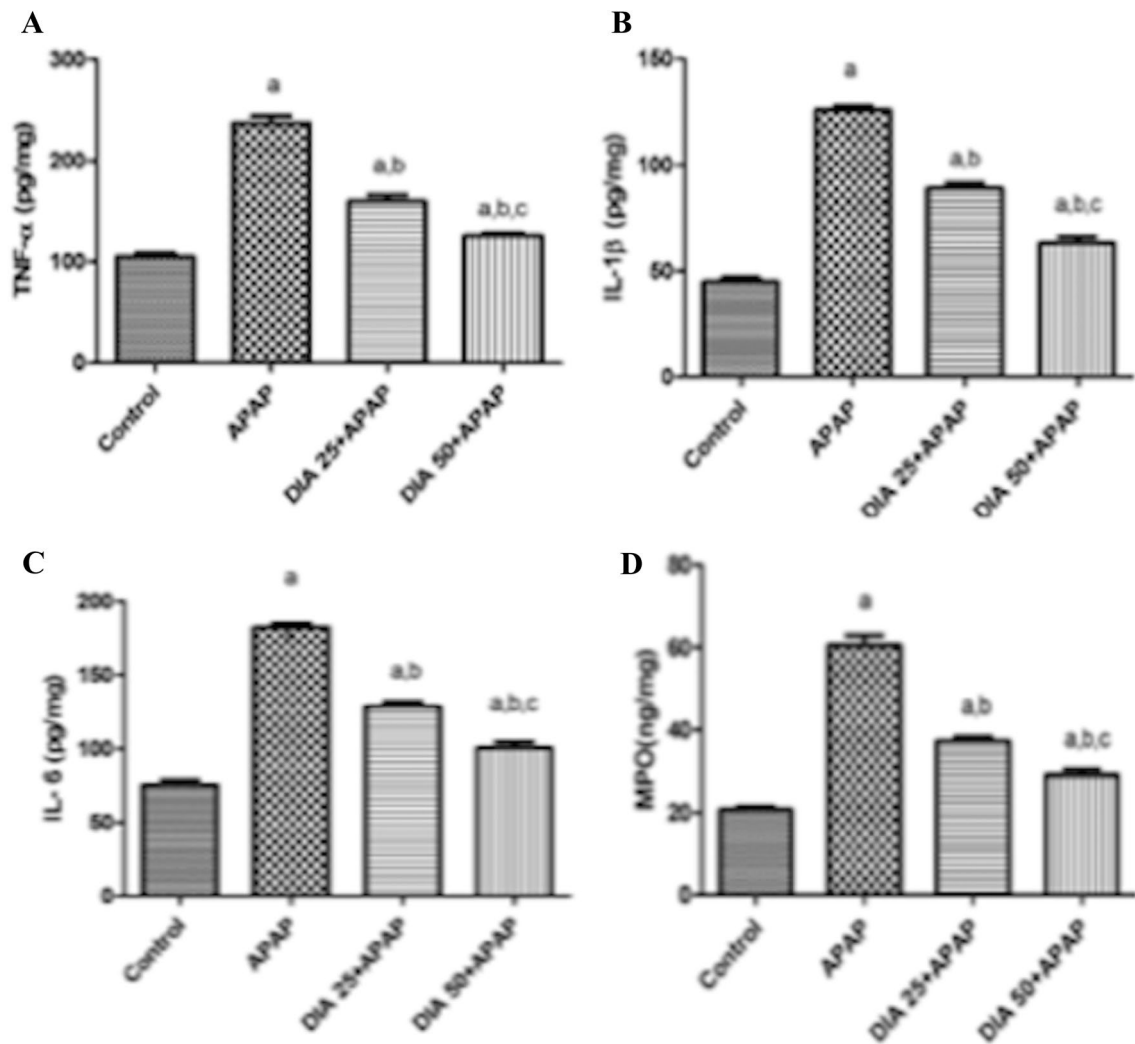
A

B

C

D

Fig. 2 Effect of DIA on hepatic TNF-α (**A**) IL-1β (**B**), IL-6 (**C**), and MPO (**D**) levels in APAP-treated rats. Results are expressed in the form of means ± S.E.M. (n = 6 per group), whereas data analysis was performed using one-way ANOVA and then the Tukey post hoc test. (^{a}), (^{b}), (^{c}): $P < 0.05$, as compared to the control group, APAP, DIA 25 + APAP groups respectively

and caspase-3 accompanied by a weak expression of Bcl2 in the APAP-treated group. Pretreatment with DIA (25 and 50 mg/kg) showed a decreased expression of both caspase-3 and Bax accompanied by elevated Bcl-2 expression in a dose-dependent manner than APAP-treated rats. The present IHC analysis results suggest the ability of DIA to suppress apoptosis triggered by APAP in the rats' liver tissues.

Discussion

Hepatoxicity triggered by an acute overdose of APAP is considered the main cause of DILI [2]. Several researchers illustrated that DIA has anti-inflammatory and antioxidant properties [12, 14, 15]. Studies have shown that DIA protects testicular injury, nephrotoxicity, and hepatotoxicity

[13]. Hence, the present study investigated the hepatoprotective DIA impact against liver injury induced by APAP, denoting modulation of TLR4/ HMGB-1 as well as PPAR-γ signaling pathways.

Rats that received acute APAP overdose exhibited marked hepatocellular injury in rats verified by the significant increase in the liver index as well as serum levels of AST, ALT, TBB, and LDH. These enzymes are reliable indicators for liver injury, and assessing their serum levels helps identify hepatocellular damage. The histopathological analysis further confirmed the APAP-induced liver injury [14]. Pretreatment with DIA (25 and 50 mg/kg) markedly and dose-dependently protect against APAP-induced liver injury through a reduction in liver function markers, together with alleviating histopathological changes of hepatic tissue, which is in agreement with [10, 15].
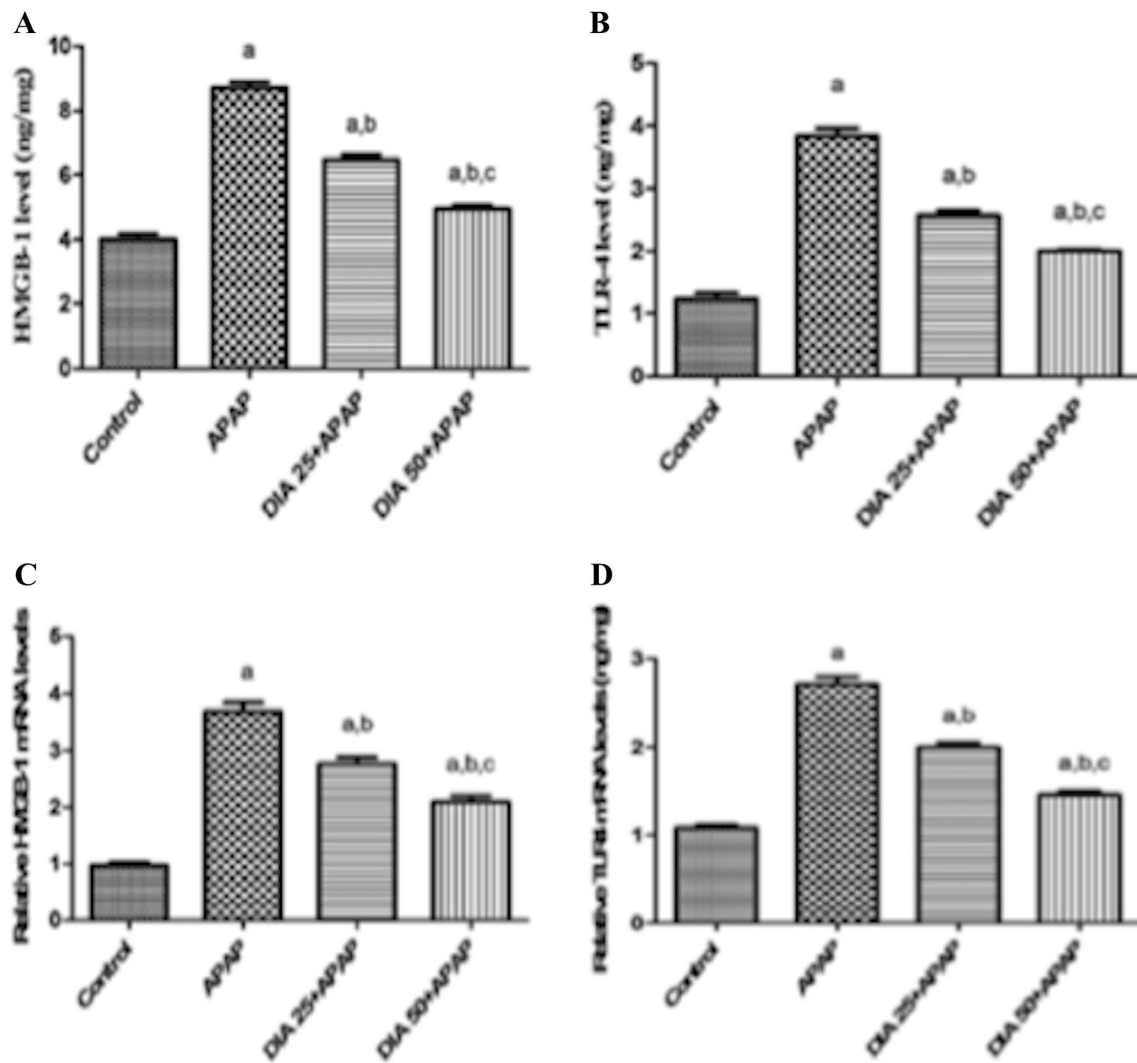
Fig. 3 DIA effect on HMGB-1 (**A**) and TLR4 (**B**) gene expression, and their content of protein (**C** and **D**), in APAP-treated rats' liver tissues. Gene expression quantitation was performed utilizing real-time PCR. The mRNA levels were normalized to that of β-actin in each group and then expressed as relative quantification in comparison to controls. Gene values in the control cohort were defined as 1. ([a]), ([b]), ([c]): $P < 0.05$, compared to the control group, APAP, DIA 25 + APAP groups, respectively. Data are expressed as means ± S.E.M. (n = 6 per group), and data analysis was carried out utilizing the one-way ANOVA and then the Tukey post hoc test

Oxidative stress has been postulated to be directly involved in the development of APAP-induced toxicity by the formation of the reactive metabolite NAPQI that is causing lipid peroxidation, depletion in antioxidants, mitochondrial dysfunction, and ultimately leading to DNA damage and necrotic cell death [7]. GSH significantly contributes to the detoxification of NAPQI as well as scavenging of peroxynitrite in APAP hepatotoxicity. Nevertheless, excessive NAPQI induces prominent depletion of GSH and subsequently results in lipid peroxidation, which is indicated by MDA accumulation [17]. In addition, the decline of GSH levels, APAP decreases the other antioxidants in the body, such as GPx, CAT, and SOD which act as a defense against oxidative stress status [18]. In agreement with previous research, the current study displayed an increase in MDA content with a depletion in hepatic antioxidants GSH, GPX, SOD, and CAT in APAP-treated rats. These findings align with prior reports, which reported that oxidative stress induced by APAP occurs due to utilizing antioxidants to neutralize NAPQI metabolites, which induces severe oxidative stress [19–21]. The current study revealed that DIA (25 and 50 mg/kg) treatment caused a distinct elevation of hepatic GSH, GPX, SOD, and CAT associated with marked depletion of MDA content in a dose-depended manner. These findings are compatible with several studies that reported that DIA neutralizes ROS and has a potent antioxidant capacity in different body tissues [10, 12, 15, 18, 22–24].
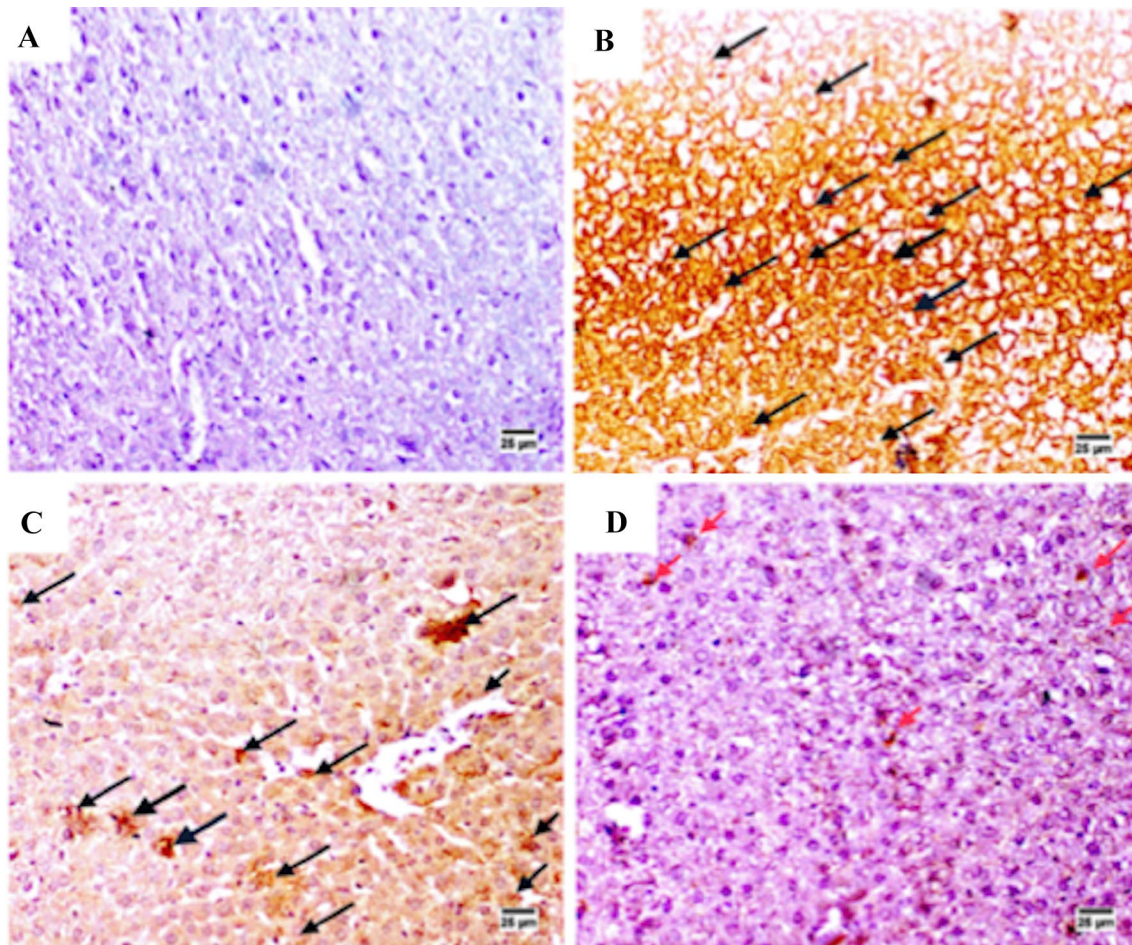
Fig. 4 Representative photomicrograph of immunohistochemical staining of hepatic NF-κB (p65) positive cells in the liver (arrows). Control group (**A**), APAP-treated group (**B**), DIA 25 + APAP-treated group (**C**), DIA 50 + APAP-treated group (**D**) [×200]

APAP-induced oxidative stress results in hepatocellular necrosis, which elicits immune cell infiltration and a cascade of inflammatory responses [7]. Damage-correlated molecular pattern molecules (DAMPS) such as HMGB1 are released by necrotic hepatocytes, activating pattern recognition receptors (PPRs) such as TLR4 and TLR9 [25]. The stimulation of TLRs signaling releases chemokines and other cytokines like IL-1 β, IL-6, and TNF-α, which attracts inflammatory cells via the family members of TLR as well as correlated pathways as the NF-κB pathway [26]. In APAP-induced hepatotoxicity, the activated TLR4 receptor provokes a series of active mediators that dissociate and translocate NF-κB to the nucleus, a fundamental nuclear transcription factor composed of p50, p52, p65, c-Rel, to commence inflammatory mediators' transcription [23, 27–29]. The knockout or blockade of TLR4 was reported to protect from inflammation in APAP hepatotoxicity. In the current work, HMGB1, TLR4, as well as NF-κB expression (p-65) were reduced in the hepatic tissue of DIA-treated rats in a dose-depended manner. These results proved that DIA ameliorates inflammation by inhibiting the HMGB1/TLR4 pathway after APAP acute-over dose administration in rats. This finding aligns with previous studies in different animal disease models [13, 30].

In addition, TNF-α and IL-1β trigger immune cells and augment inflammatory response, which recruits monocytes and neutrophils in circulation to intensify inflammation and trigger a secondary inflammatory cascade. MPO activity is considered an early marker for inflammatory cell infiltration and predicts the incidence of inflammatory diseases [31].In the same context, this study displayed a marked elevation in pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) levels and MPO levels in the APAP-treated rats [18]. These findings were reversed significantly and dose-dependently by DIA treatment which can be mediated via inhibition of NF-κB (p65). In context, several studies reported the anti-inflammatory effect of DIA in various animal models of inflammation-induced disease [12, 30–32]. In addition, Saleh et al., (1999) illustrated that DIA inhibited MPO levels in the mice model of carrageenan-induced pleurisy [33].
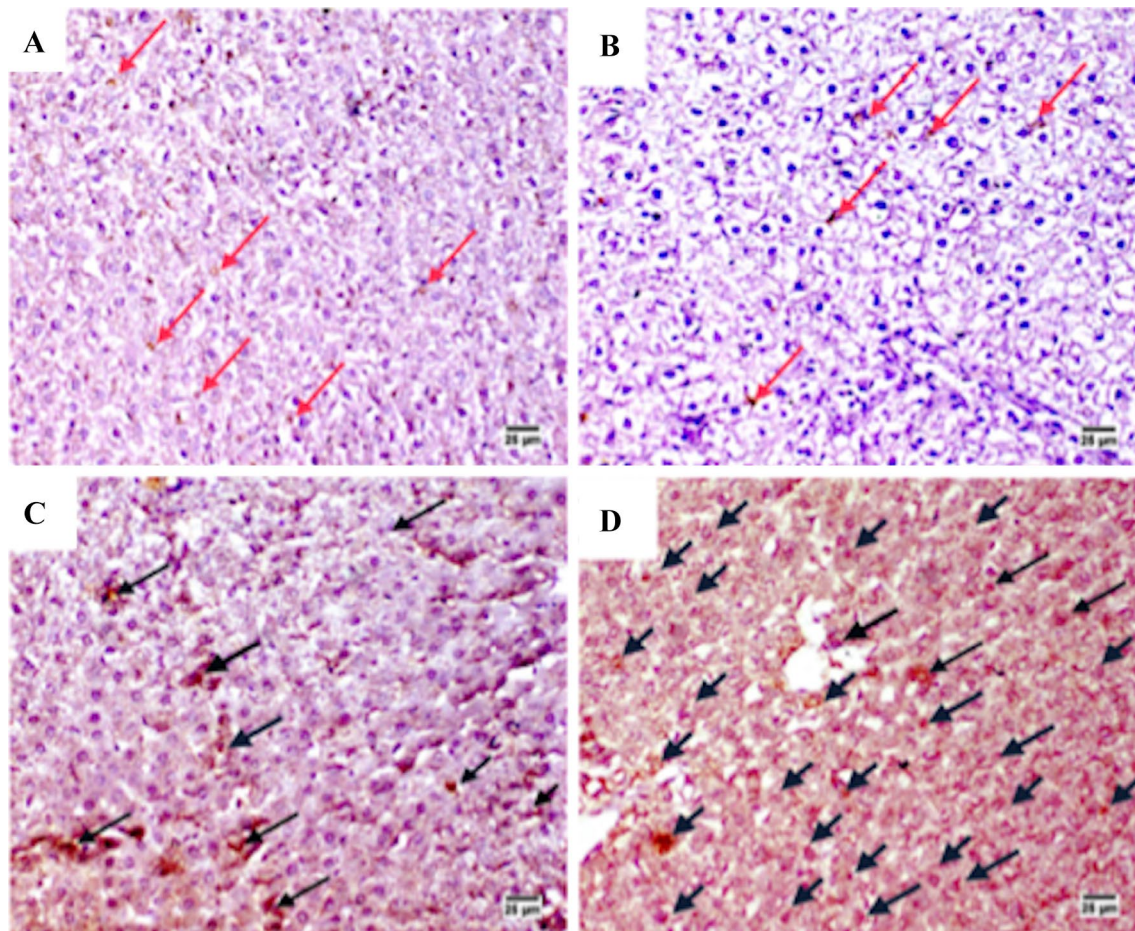
Fig. 5 Representative photomicrograph of immunohistochemical of hepatic PPAR-γ positive cells in the liver (arrows). Control group (**A**), APAP -treated group (**B**), (**C**), DIA 25 + APAP-treated group (**D**), DIA 50 + APAP-treated group [×200]

PPAR-γ contributes to several cellular events, glucose homeostasis, and adipogenesis. It significantly contributes to regulating inflammation as a suppressor of inflammation caused by oxidative stress. This role can be due to the direct inhibitory activity of transcription of pro-inflammatory genes as NF-κB, which is involved in inflammation [34, 35]. The therapeutic effect of PPAR-γ agonist has been determined in APAP-induced hepatotoxicity in rats [36]. Also, agonists of PPAR-γ were manifested as potent TLR4 inhibitors [9]. This study demonstrated that the hepatotoxic impacts of APAP induced a decline in the expression of PPAR-γ [36]. Conversely, DIA treatment showed elevation of PPAR-γ expression associated with the decline in NF-κB (p65) expression in a dose-related manner. These findings are consistent with Wen et al. 2019, who illustrated that Rhein, the active metabolite of diacerein, exerts its anti-inflammatory effect by upregulating the PPAR-γ and downregulating NF-κB expressions in the inflammatory cell model using LPS-stimulated RAW264.7 cell [37].

In addition to oxidative stress, the contribution of apoptosis in the pathogenies of APAP-induced liver injury was confirmed [38]. Several studies emphasized that excessive ROS causes mitochondrial damage, which eventually activates the caspase cascade, leading to apoptosis. Caspase-3 has been proven to contribute to the pathway of hepatocellular apoptosis [33, 34, 39]. In addition, the balance between pro-apoptotic as well as anti-apoptotic Bcl-2 protein BAX displayed a pivotal role in regulating apoptosis during APAP hepatotoxicity [40]. Interestingly, promoting Bcl-2 protein expression can protect from APAP-induced apoptosis [41]. The present work demonstrated that acute dose of APAP caused a marked increase in the activity of caspase-3 as well as the expression of Bax with diminished expression of Bcl-2 [42], that DIA therapy restored these results in a dose-related manner. The anti-apoptotic effect of DIA has been reported previously in different tissues [12, 22, 23, 30, 43, 44].

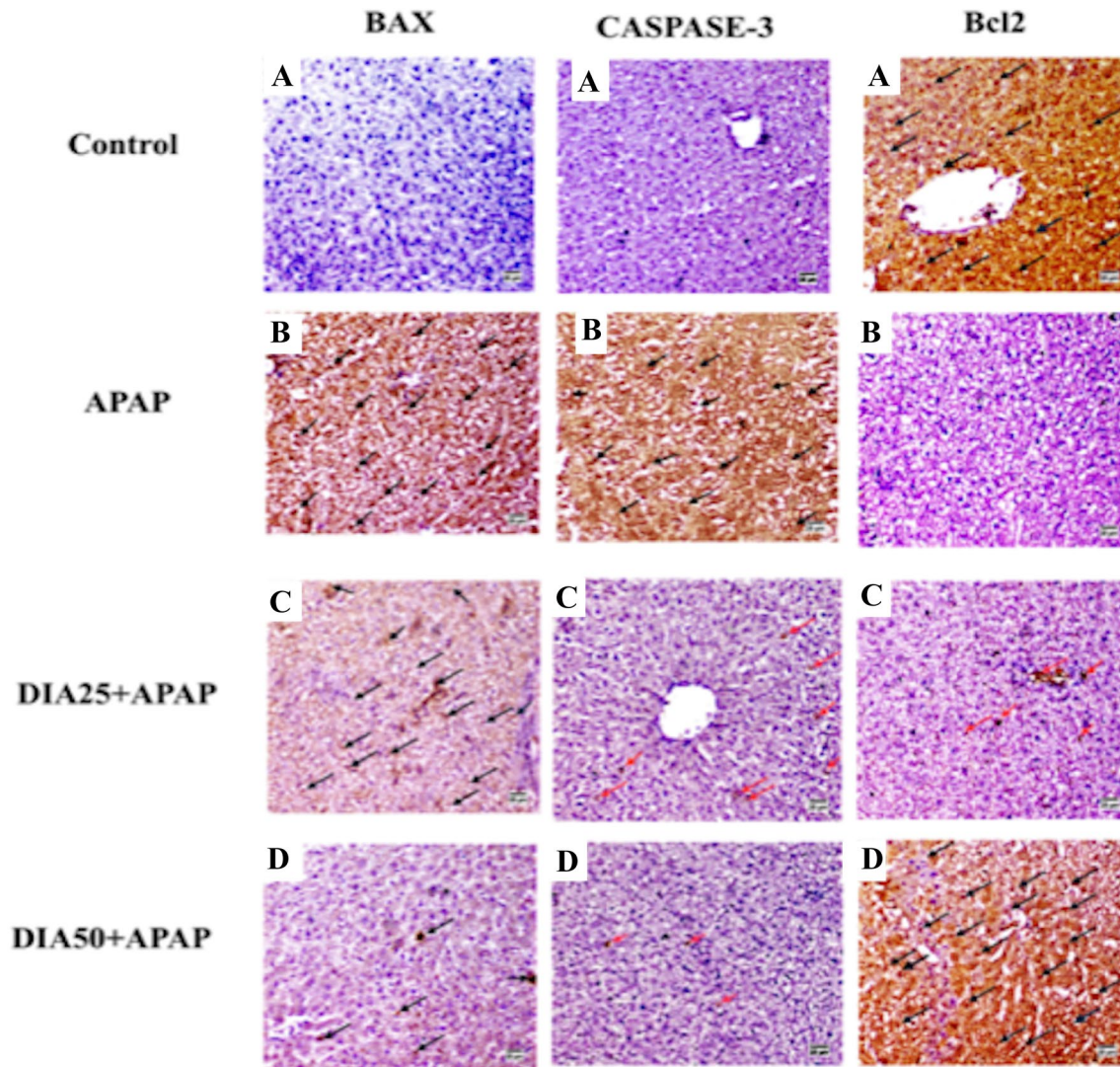The main limitations of our study were the failure to use immunoblotting analysis for NF-κB (p65) expression and

BAX   CASPASE-3   Bcl2

Fig. 6 Representative photomicrograph of immunohistochemical of hepatic BAX, caspase-3, and Bcl2 positive cells in the liver (arrows). Control group (**A**), APAP-treated group (**B**), DIA 25 + APAP-treated group (**C**), and DIA 50 + APAP-treated group (**D**) [×200]

apoptotic markers (procaspase-3 and cleaved caspase-3), along with TUNEL staining. Therefore, further investigation is suggested for these objectives.

Conclusion

DIA dose-dependently protects against liver damage induced by APAP via reducing oxidative stress, inflammatory process, as well as apoptosis. The anti-inflammatory activity attributed to the elevated expression of PPAR-γ, inducing suppression of HMGB-1/TLR4/NF-κB signaling pathway. Therefore, DIA represents an effective hepatoprotective therapy. Further research and clinical implications are needed.

Declarations

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