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Leflunomide-induced liver injury in mice: Involvement of TLR4 mediated activation of PI3K/mTOR/NFκB pathway

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ABSTRACT

Aims: Leflunomide is a disease modifying anti-rheumatic drug (DMARD) beneficial in refractory cases of rheumatoid arthritis. Since leflunomide approval, hepatotoxicity and instructions of liver function monitoring have been recommended. The current work aimed to explore the possible role of inflammation in leflunomide-induced hepatotoxicity with a focus on the TLR4-mediated stimulation of PI3K/mTOR/NFκB pathway.

Main methods: Forty-eight male albino mice were allocated into four different groups (n; 12 mice/group). Group (i): normal mice, Group (ii–iv) mice received escalating dosed/s of leflunomide (2.5, 5 or 10 mg/kg, p.o.) every 48 h for eight weeks. At the end of the study, mice were sacrificed, and blood samples were collected for determination of liver enzymes. Liver samples were collected; (1) formalin-fixed for histologic examination, (2) frozen for PI3K and mTOR genes PCR assays.

Key findings: Results indicated a significant elevation of liver enzymes in leflunomide-treated mice (10 mg/kg); AST and ALT activities were 218.17 ± 6.83 U/L and 99.83 ± 9.82 U/L versus 130.5 ± 12.79 U/L and 44.72 ± 3.58 U/L in the vehicle group. Additionally, histopathological examination revealed higher necro-inflammatory scores in leflunomide-treated mice. Immunohistochemistry indicated dose-dependent increased staining of TLR4 and caspase 3. Furthermore, leflunomide-treated mice (5 or 10 mg/kg) showed greater staining for NFκB compared to vehicle control. RT-PCR results revealed upregulations in genes expressing PI3K and mTOR by leflunomide.

Significance: The current study highlights the possible role of TLR4-PI3K/mTOR/NFκB in the pathogenesis of leflunomide-induced hepatic injury. A better understanding of mechanisms of leflunomide-induced hepatotoxicity may be of translational implication for the predictive, preventive and therapeutic purposes.

1. Introduction

Leflunomide is a disease-modifying antirheumatic drug (DMARD). In 1998, leflunomide has been approved and launched as an alternative or supportive therapy in refractory cases of rheumatoid arthritis [1,2]. Leflunomide is a prodrug almost entirely activated in the gut mucosa and liver into its active metabolite teriflunomide (compound A-771726) [3]. The immunosuppressant role of leflunomide is mediated through inhibition of dihydroorotate dehydrogenase enzyme leading to inhibition of de novo synthesis of pyrimidine nucleotides [1,3].

Leflunomide-induced elevation of hepatic enzymes was repeatedly reported [1,4–10]. Van Roon and colleagues reported that 8.9% of

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Table 1
Effect of leflunomide on serum liver enzyme activities in mice.

	AST U/L	ALT U/L
Vehicle control	130.5 ± 12.79	44.72 ± 3.58
Leflunomide 2.5 mg/kg	153.83 ± 10.76	66.83 ± 4.66
Leflunomide 5 mg/kg	157 ± 10.53	66.75 ± 1.98
Leflunomide 10 mg/kg	218.17 ± 6.83[a]	99.83 ± 9.82[a]

ALT: alanine aminotransferase and AST: aspartate aminotransferase. Data are mean ± SEM and analyzed using one-way ANOVA followed by Bonferroni's multiple-comparison's test at $P < 0.05$.

[a] Compared to the vehicle group.

patients suffer 2nd–3rd-grade hepatotoxicity, which is based on elevated liver enzymes while clinically asymptomatic [8]. Accordingly, clinicians were recommended regular monitoring of patients' hepatic enzymes during the first six months of therapy [11–14].

Additionally, leflunomide-associated serious and fatal hepatotoxicity has been repeatedly reported [8,10,15]. In a review of 129 clinical reports, European Medicines Evaluation Agency stated 60% fatality in 15 cases with hepatic failure [10]. According to the Data Bank for rheumatic diseases, Leflunomide-associated hepatic disturbances requesting hospitalization occurs in a rate of 2/1000/year [16]. In 2010, the FDA labeled leflunomide with a black box warning [17,18]. Thus, leflunomide usage is contraindicated in patients with hepatic impairment or concomitantly used with potentially hepatotoxic drugs [19,20].

The pathogenic mechanisms underlying leflunomide-induced hepatotoxicity are not fully elucidated. Oxidative stress was reported as a possible mechanism of pathogenesis of leflunomide-induced hepatotoxicity [21,22]. Moreover, mitochondrial stress and cellular energetic derangement are also described as a pathogenic mechanism [23]. Endoplasmic reticulum stress and accumulation of unfolded proteins were also reported as a player in leflunomide-induced hepatotoxicity [24]. Leflunomide-induced acute hepatitis was characterized as metabolic idiosyncrasy in a patient possesses *CYP2C9*3* allele which mandates a slow CYP2C9 enzyme activity, that leflunomide/teriflunomide may be metabolic substrates [25].

Toll-like receptors (TLR) are type I transmembrane pattern recognition receptors [26]. TLR4 can be activated by bacterial lipopolysaccharides (LPS) as well as debris of tissue damage to enhance immune responses [26,27]. Activation of NFκB is a major contributor to TLR-mediated inflammatory responses through the release of a variety of cytokines and chemokines [26]. PI3K/Akt/mTOR is a downstream signaling pathway of TLR4, which controls cell proliferation and survival [28,29]. Inhibiting PI3K/Akt/mTOR signaling pathway protects against acute liver failure in rats treated with d-galactosamine/lipopolysaccharide [30].

Inflammation has been implicated as a triggering mechanism in drug-induced liver injury [31–33]. Also, TLR4 plays a role in regulating the transcription genes of inflammation [34]. Based on this context, the current animal study was conducted to evaluate leflunomide-induced dose-dependent hepatotoxicity from the inflammatory perspective with a focus on the role of TLR4-mediated PI3K/mTOR/NFκB signaling pathway.

2. Materials and methods

2.1. Animals

Animal experiments were conducted according to NIH (USA) guidelines for care and use of laboratory animals [35] and were accepted by the Institutional Animal Care and Use Committee, Faculty of Pharmacy, Suez Canal University. The mice used in this study were obtained from Moustafa Rashed Company (Giza, Egypt). Animals were housed at the laboratory animal house of Faculty of Pharmacy under supervision of trained technicians. The study protocol was approved by the Ethical Committee at the faculty of Pharmacy, Suez Canal University in accordance to the ethical guidelines of the 1975 Declaration

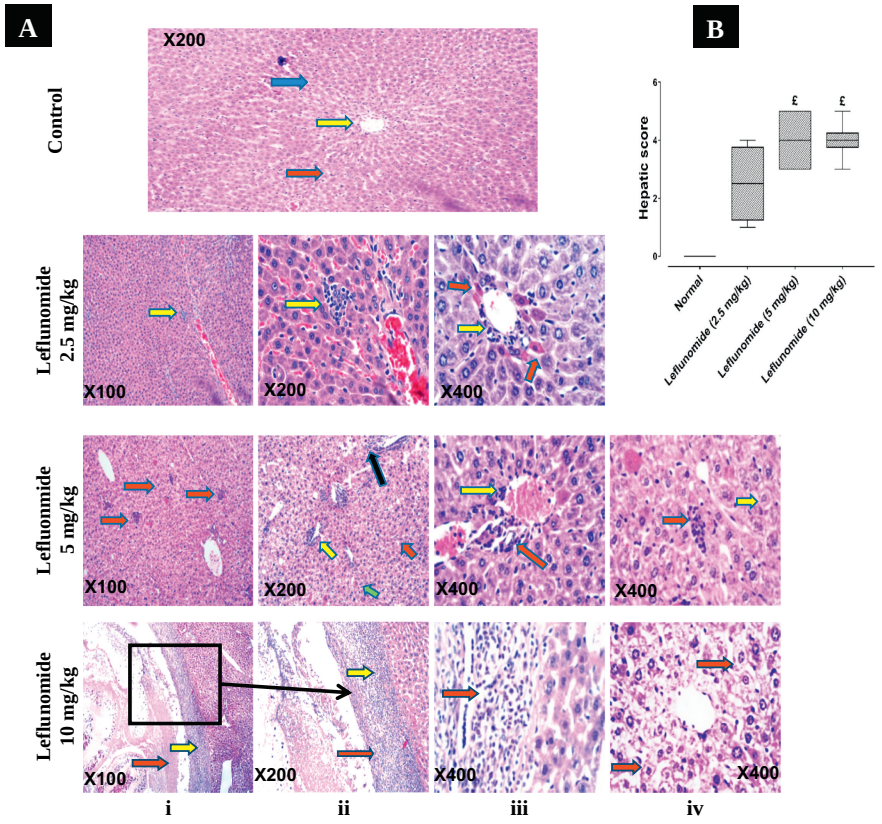

Fig. 1. A: Histopathological images of hepatic specimens treated with leflunomide. Hepatic specimens were stained with (H&E) and examined for inflammatory and apoptotic features using HAI of Ishak scoring system. Control: normal liver histology with average central venule yellow arrow, average blood sinusoids red arrow and hepatocytes blue arrow. Leflunomide 2.5 mg/kg: Images of liver tissue i & ii) showing spotty focal necrosis presented by infiltration of liver parenchyma by a small group of lymphocytes yellow arrows. iii) Portal inflammation yellow arrow and many apoptotic bodies red arrow. Leflunomide 5 mg/ml: Images of liver tissue showing i) marked spotty necrosis red arrows ii) moderate vacuolar degeneration red arrow portal inflammation yellow arrow, piecemeal necrosis black arrow, apoptotic bodies green arrow, iii) dilated portal vein yellow arrow, and portal inflammation red arrow, iv) mild vacuolar degeneration of hepatocytes yellow arrows and spotty necrosis red arrow. Leflunomide 10 mg/kg: images of liver tissue showing i) inflammatory degenerated cyst, a fibrous wall surrounded by mixed acute & chronic inflammatory cells yellow arrow, the cavity contains degenerated liver cells red arrow. ii) Degenerated cyst wall infiltrated by mixed acute & chronic inflammatory cells red arrow, including eosinophils. iii) Inflammatory degenerated cyst wall infiltrated by mixed acute & chronic inflammatory cells including eosinophils, picture of acute hepatitis red arrow. iv) Moderate vacuolar degeneration of hepatocytes red arrows. B: Median histologic score evaluated according to Ishak score. £Compared to the normal group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

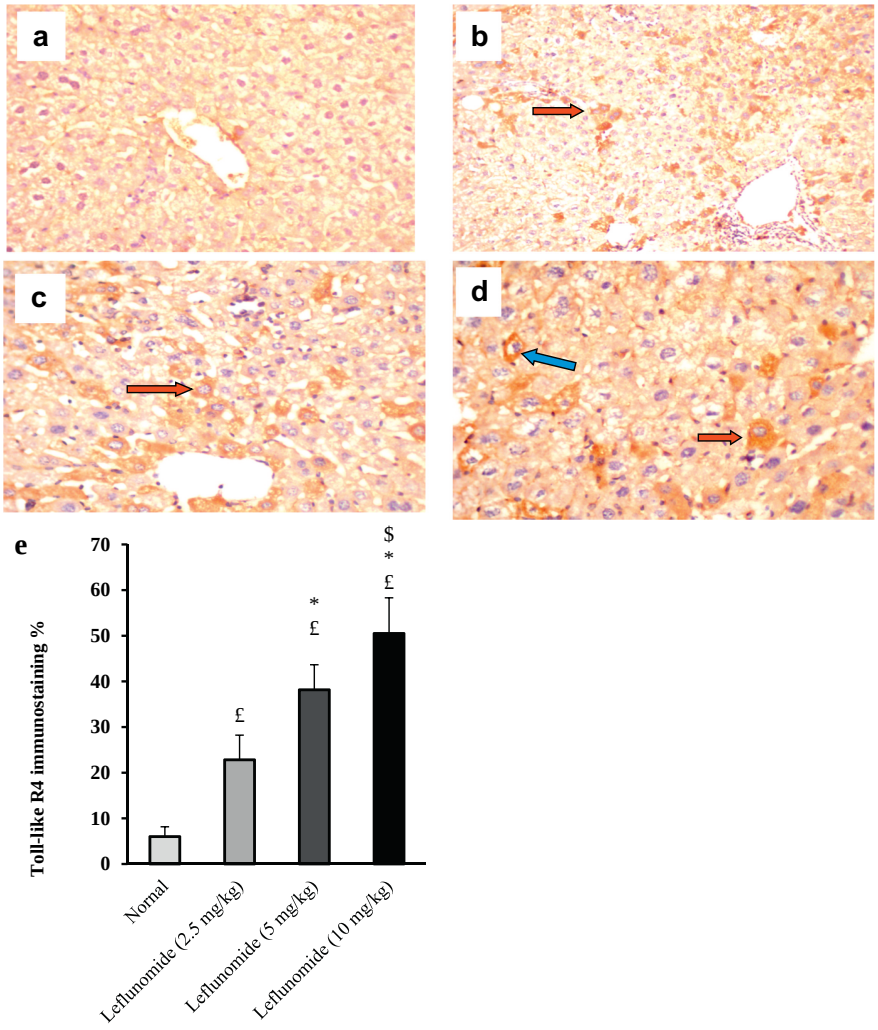

Fig. 2. Effect of leflunomide (2.5, 5 or 10 mg/kg) on hepatic immunostaining for Toll-like receptor-4. Images show different degrees of immunohistochemical staining in hepatic specimens from (a) mice treated with water. (b & c) Mice treated with leflunomide (2.5 and 5 mg/kg respectively) showing positive cytoplasmic immunostaining (red arrows). (d) Mice treated with leflunomide (10 mg/kg) shows positive cytoplasmic (red arrow) and membranous (blue arrow) immunostaining. (e) Column chart for percent of immunostained areas in liver specimens. Data are mean ± SD, analyzed using one-way ANOVA at $P < 0.05$.
£ compared to normal.
* Compared to leflunomide (2.5 mg/kg).
$ Compared to leflunomide (5 mg/kg). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of Helsinki.

2.2. Chemicals

Leflunomide was a gift from Sigma Pharmaceutical Company (Quesna, Egypt). It was suspended in 1% carboxymethylcellulose (CMC) solution and given to mice by oral gavage.

2.3. Justification of the selected doses of leflunomide

The doses of leflunomide for different therapeutic applications have been searched in literature to select the best doses to be used in this study. Leflunomide immunosuppressant dose ranges (0.63–10 mg/kg) were given for one week in mice and successfully prolonged graft survival compared with controls, while using a dose of 5 mg/kg for 3 weeks produced indefinite graft survival in 50% of animals [36]. Further, leflunomide doses used seeking antitumor potential were tested before in mice at doses equal 3, 10 and 30 mg/kg, given by oral gavage for 14 days. Therefore, the utilized doses of leflunomide in the current study (2.5, 5 and 10 mg/kg every 48 h) are in the reported range of mice doses in the literature [37]. In comparison to human doses, the current doses of leflunomide (2.5, 5 and 10 mg/kg) equal to 12.1, 24.2 and 48.4 mg/48 h. So, the corresponding daily dose of a 60-kg human equal (6.05, 12.1 and 24.2 mg). It is known that leflunomide is commonly used at 10 or 20 mg tablets in rheumatoid arthritis patients as maintenance doses. Hence, the currently utilized doses in mice are within the range of therapeutic human doses.

2.4. Experimental design

Forty-eight male albino mice were allocated into 4 different groups (n: 12/group) and assigned as follow:

Group (i): normal mice received CMC solution (12 ml/kg, p.o.).
Group (ii) mice received leflunomide (2.5 mg/kg, p.o.).
Group (iii) mice received leflunomide (5 mg/kg, p.o.).
Group (iv) mice received leflunomide (10 mg/kg, p.o.).

For all groups, drug or vehicle administration was every 48 h and continued for 8 weeks in a volume equals 12 ml/kg.

2.5. Blood and tissue sample collection

At the end of the study, blood was collected from anaesthetized mice (80 mg/kg ketamine HCL, i.p.) [38,39]. Blood samples were centrifuged (1500 × *g* for 7 min/room temperature) and sera were collected and stored at −20 °C till further assayed. Serum samples were tested for liver enzyme activities (alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and albumin were measured by spectrophotometry employing commercial kits purchased from Bio-diagnostics (Cairo, Egypt).

Then, liver biopsies were taken, one part from which was fixed in neutral formalin 10% (v/v) then paraffin embedded for further histopathology and immunohistochemistry. Another part of each liver biopsy was processed for qRT-PCR, western blotting and ELISA assays.

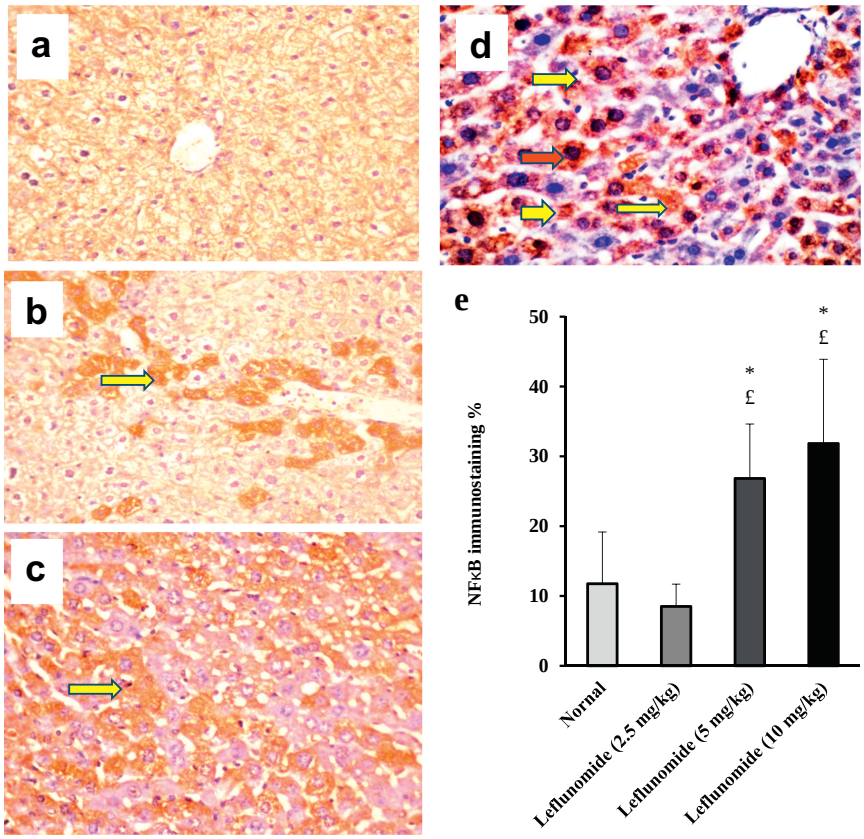

Fig. 3. Effect of leflunomide (2.5, 5 or10 mg/kg) on hepatic immunostaining for NFκB. Images show different degrees of immunohistochemical staining in hepatic specimens. a) Mice treated with saline. (b & c) Mice treated with leflunomide (2.5 and 5 mg/kg) show mild and moderate cytoplasmic staining of hepatocytes respectively (NFκB x 400), (d) Mice treated with leflunomide (10 mg/kg) show strong cytoplasmic staining (yellow arrows) and nuclear staining (red arrow) (NFκB x 400). (e) Column chart for percent of immunostained areas in liver specimens. Data are mean ± SD, analyzed using one-way ANOVA at $P < 0.05$.
£ compared to the normal group.
* Compared to leflunomide (2.5 mg/kg) group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.6. Histopathological and immunohistochemical analysis

From each paraffin-embedded block, three sections (~3–4 μm each) were prepared for routine hematoxylin and eosin (H&E) stain, and TLR4, NFκB, caspase 3 and proliferating cell nuclear antigen (PCNA) immunostaining. All histological staining was performed in accordance with conventional procedures. Sections stained by H&E were examined to detect HAI and fibrosis and other pathological changes, the existence of inflammation and proliferation in liver tissue was examined with an immunohistochemical study for TLR4 (Roche, Mannheim, Germany), NFκB (Roche, Mannheim, Germany), PCNA (1:500, GTX100539, GeneTex) and rabbit polyclonal antibody to caspase 3 (1:500, GTX110543, GeneTex).

Intrahepatic levels of these markers were examined by an immunohistochemical assay using an image analyzer. TLR4 and caspase 3 expressions were detected as the nuclear and cytoplasmic brown coloration of hepatocytes, with various intensity. Positivity of the cells for PCNA expression was defined as only distinct nuclear (S phase) immunostaining with skin tissue as a positive control. The intensity of staining (nuclear): +1 (weak): light red, +2 (moderate): red, +3 (strong): deep to dark red [40]. Only cells with nuclear staining (+2 or +3) with no cytoplasmic staining are scored as "S phase". Positive cells for NFκB expression was defined as only distinct nuclear immunostaining, which is interpreted as activated NFκB, the lymphocytes in sections were used as positive internal controls, which showed positive nuclear staining in all runs (Olympus BX50 F4 Microscope with Olympus DP26 Camera. Olympus Optical Co. LTD. Japan)

2.7. Semiquantitative evaluation of the liver histological status (necro-inflammation)

According to Ishak modified histological activity index (HAI) (Modified Knodell) scoring system which grades necro-inflammation on a scale of 0 to 18 including 0 to 4 for portal necrosis, 0 to 4 for piecemeal necrosis, 0 to 4 for focal lytic necrosis, apoptosis, and focal inflammation, and 0 to 6 for confluent necrosis. To measure the severity of hepatic histopathological changes, the scores of the four necro-inflammatory categories are combined to produce a final score (0–18) that can be interpreted as: minimal (1–3), mild (4–8), moderate (9–12), severe (13–18) [41].

2.8. Real time-polymerase chain reaction for TGF-β and PI3K/mTOR pathway genes

Total RNA was extracted from tissue specimens in RNAlater stabilizing reagent using RNAeasy FFPE kit (Qiagen, catalog number 73504) following the manufacturer's instructions. Assessment of RNA purity and concentration were done via NanoDrop ND-1000 spectrophotometer (NanoDrop Tech., Inc. Wilmington, DE, USA). Complementary DNA (cDNA) was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, P/N 4368814) as described in previous work [42]. Expression profiling of TGF-β, PI3K and mTOR genes were carried out using Real-Time Polymerase Chain Reaction (qRT-PCR) technology following the guidelines of the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE). All reactions were run in triplicate in StepOne Plus Real Time-PCR system using ready-made primers Taqman® assays (Applied Biosystems, assay ID Mm01282781_m1 for PIK3R1 [43,44], Mm01331626_m1 for AKT1, Mm00444968_m1 for mTOR) compared to the endogenous control GAPDH (assay ID Mm99999915_g1). Appropriate controls were included in the reaction [45]. Fold change of mRNA expression for each specimen was estimated using LIVAC method [46].

2.9. Measuring liver PI3K, mTOR, and TGFβ

Liver tissue lysates were prepared using RIPA buffer (Sigma-Aldrich) containing phosphatase and protease inhibitors. The produced

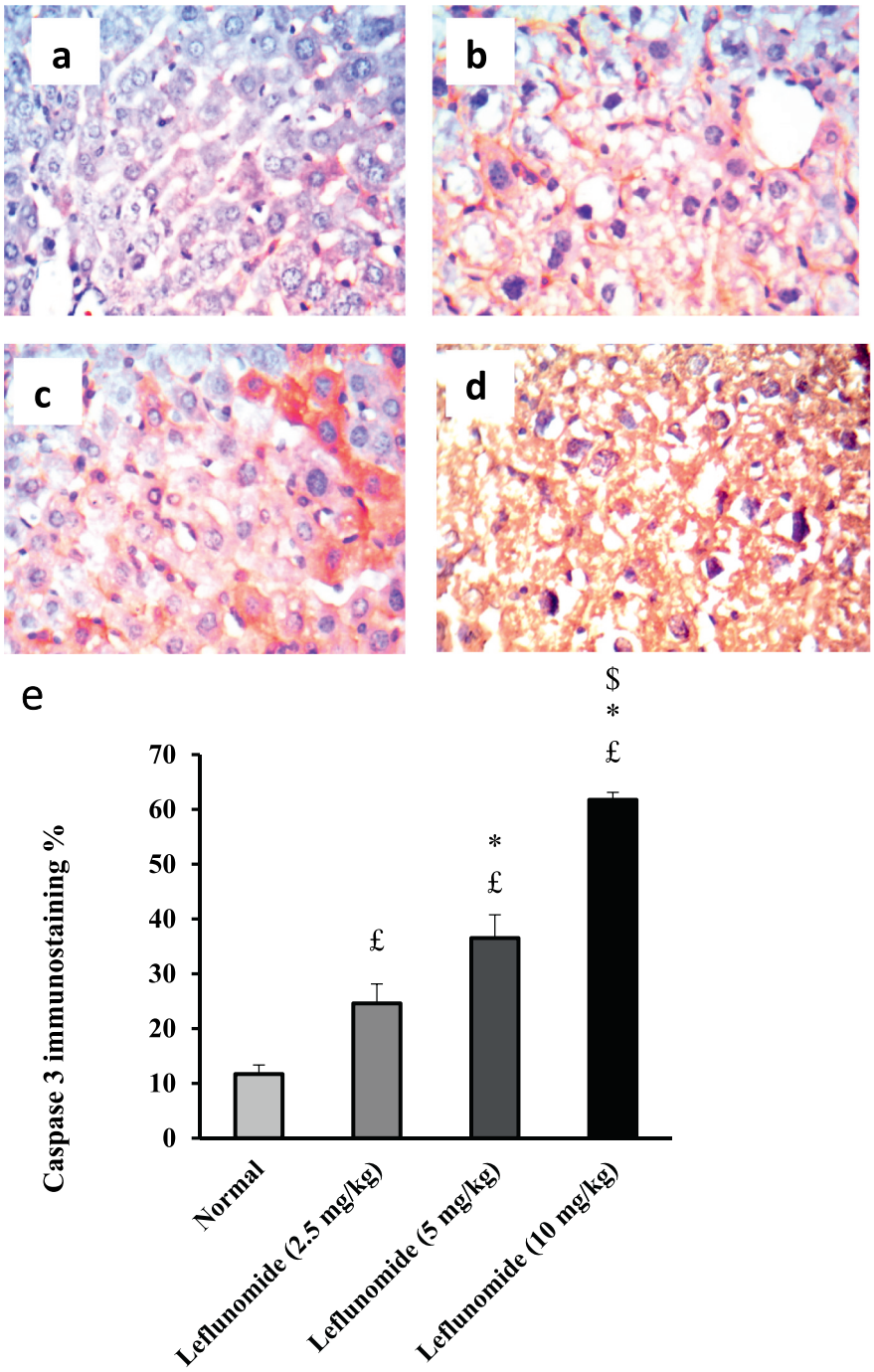

Fig. 4. Effect of leflunomide (2.5, 5 or 10 mg/kg) on hepatic immunostaining for caspase 3. Images show different degrees of immunohistochemical staining for caspase 3 in hepatic specimens (a) Normal mice treated with water showing occasional cytoplasmic positivity$_{\text{yellow arrow}}$ of few hepatocytes. (b, c & d) Hepatic specimens from mice treated with leflunomide (2.5, 5 or 10 mg/kg respectively) showing cytoplasmic positivity$_{\text{yellow arrows}}$ in both b & c figures however showing both cytoplasmic positivity$_{\text{yellow arrow}}$ and nuclear positivity$_{\text{red arrow}}$ positivity (e) Column chart for the percentage of immunestained areas in liver specimens. Data are mean ± SD, analyzed using one-way ANOVA at $P < 0.05$.
£ compared to normal.
* Compared to leflunomide (2.5 mg/kg).
$ Compared to leflunomide (5 mg/kg). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cell lysates were centrifuged, supernatants were collected, and protein concentrations were measured using Bio-Rad protein assay kit (Bio-Rad, CA, USA). The protein samples were denatured, and equal amount of proteins were loaded in each lane of SDS polyacrylamide gel where they are separated by electrophoresis. Then, the separated protein was transferred by electroplating to polyvinyldenefluoride membranes. The blots were blocked and then probed with primary antibodies PI3K (ab86714, abcam), phospho-PI3K (ab182651, abcam), mTOR (ab2732, abcam) and phospho-mTOR (ab109268). The day after, membranes were washed and incubated at room temperature with the appropriate peroxidase-conjugated secondary antibody for 1 h. The blots were visualized using enhanced chemiluminescence. Protein bands were analyzed and quantified using densitometry and actin was used to ensure equal loading and blotting of protein. TGFβ was measured by mouse TGFβ1 ELISA kit (Cat # ab119557, Abcam, USA).

2.10. Statistical analysis

Quantitative data were analyzed using one-way ANOVA and a post hoc test for multiple comparisons. PCR results from the animal groups were compared by non-parametric Dunn's and Kruskal Wallis tests. The bilateral significance level was $\alpha = 0.05$.

3. Results

3.1. Serum liver enzymes

The current results highlighted significant increases in serum liver

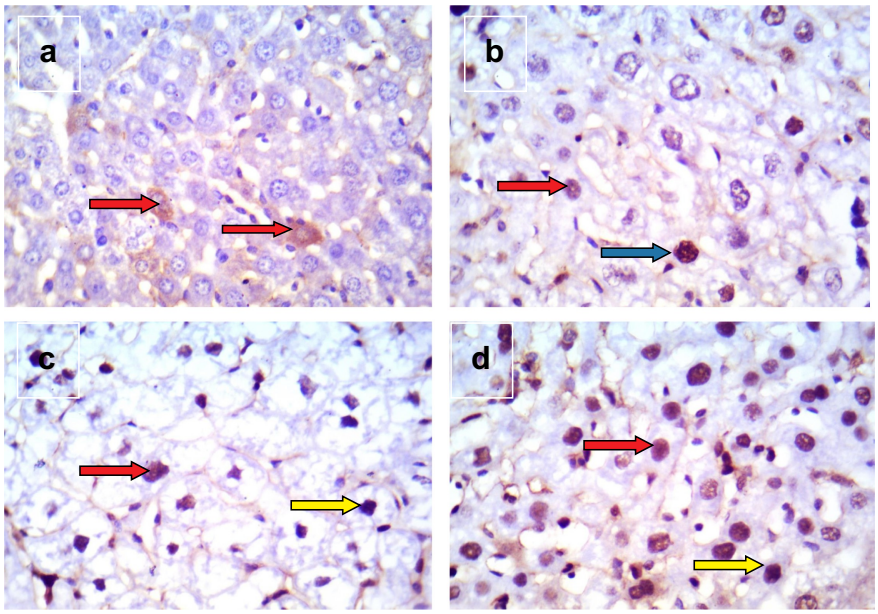

Fig. 5. Effect of leflunomide (2.5, 5 and 10 mg/kg) on hepatic immunostaining for PCNA. (a) A high-power view of normal liver tissue showing unstained nuclei of hepatocytes demonstrating the cells in "G0 phase" of the cell cycle (non-proliferating) with few cells showing granular cytoplasmic staining $_{\text{red arrows}}$ demonstrating the cells in the mitosis phase (PCNA X 400). (b) A high-power view for liver tissues from leflunomide (2.5 mg/kg) showing unstained nuclei of the liver cells demonstrating the cells in "G0 phase" of cell cycle (non-proliferating) with few cells showing light red demonstrating the cells in late "G1 phase" $_{\text{red arrow}}$, few cells showing red staining (+2) "S phase" $_{\text{blue arrow}}$ (PCNA x 400). (c) A high-power view of liver tissues from leflunomide (5 mg/kg) showing few cells with light red nuclear staining $_{\text{red arrow}}$ in late "G1 phase" while others showing darkly stained nuclei $_{\text{yellow arrow}}$ (PCNA x 400). (d) A high-power view for liver tissues from mice treated with leflunomide (10 mg/kg) showing most of the cells showing red staining (+2) "S phase" $_{\text{red arrow}}$, dark stained (+3) $_{\text{yellow arrow}}$ (PCNA x 400). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

AST and ALT activities in animals treated with the largest dose of leflunomide (10 mg/kg) compared to the normal mice as well as mice treated with the lower doses of leflunomide (2.5 or 5 mg/kg) (Table 1).

3.2. Histopathological findings

Liver specimens from normal mice showed preserved liver architecture with average central veins, blood sinusoids & portal tracts, the hepatocytes are within normal limits, with occasional very minimal and focal vacuolar degeneration (insignificant) (Fig. 1A). Histopathologic evaluation of all biopsies treated with dose 2.5 mg/kg revealed that cases showed scattered apoptotic bodies (Fig. 1A.i) with variable grades of portal expansion and spotty necrosis (Fig. 1A.ii & iii).

Cases of dose 5 mg/kg showed marked apoptosis with central veins congestion and variable grades of spotty necrosis (Fig. 1A.i) and portal inflammation (Fig. 1A.ii & iii) with piecemeal necrosis. Some animals showed congested central veins and expanded portal tracts by inflammatory cells with variable grades of spotty necrosis (Fig. 1A.iv).

Four cases of dose 10 mg/kg showed hepatic cystic lesions of inflammatory degeneration cyst formed of fibrous wall surrounded by mixed acute and chronic inflammatory cells including eosinophils, the cystic cavity contains degenerated liver cells and inflammatory cells, the remaining liver tissue showed mild hepatic injury formed of vacuolar degeneration and mild spotty necrosis (Fig. 1A.iv). Fig. 1B shows the median of the total histopathologic score and revealed that the pathologic score in the leflunomide (5 and 10 mg/kg) was significantly greater than the score registered in the normal group or leflunomide (2.5 mg/kg) group.

3.3. Immunohistochemical findings

Some sections of TLR4 showed cytoplasmic staining and others showed cytoplasmic and membranous staining (Fig. 2a–d). Results of TLR4 indicated leflunomide dose-dependent increase of the immunostaining area in hepatic specimens; (Fig. 2e).

According to the image analyzer study, level of NFκB, TLR4, and caspases 3 increased with leflunomide doses. NFκB immunostaining is illustrated in Fig. 3a–e. Images represent the normal and leflunomide (2.5, 5 and 10 mg/kg) treated groups. Staining was mostly cytoplasmic and nuclear; the latter is considered as activated NFκB. Area for immunostaining increased significantly in leflunomide (5 and 10 mg/kg) treated groups compared to the normal group (Fig. 3e).

Immunostaining for caspase 3 is illustrated in Fig. (4a–d), the reaction was predominantly cytoplasmic staining in hepatocytes with normal histology and cytoplasmic and nuclear staining or almost predominantly nuclear staining in cells with apoptosis or chromatin condensation. Immunostaining for caspase 3 demonstrated a dose-dependent increase along with leflunomide doses (Fig. 4e).

Immunostaining for PCNA indicated nuclear and cytoplasmic staining. Only cells exhibiting distinct +2 or +3 nuclear staining and no cytoplasmic staining should be scored as "S phase". The differences in staining patterns detected in our study are deemed to reflect the phases of the cell cycle and demonstrated that cells in "S phase" increase with increased doses of leflunomide (Fig. 5a–d).

3.4. Gene and protein expression of PI3K/mTOR and TGF-β

The gene expression profiling of PI3K and mTOR genes in liver tissues showed significant over-expression of PI3K and TGF-β in all leflunomide-treated groups compared to the normal group. Aberrant activation of mTOR expression was mostly noted in animals receiving a dose of leflunomide (5 mg/kg) (Fig. 6a–c).

Fig. 7A illustrates Western blot analysis for phosphorylated and total forms of PI3K and mTOR proteins in the mice hepatic tissue. Calculation of the relative ratios of phosphorylated proteins to the total value demonstrated higher protein levels in leflunomide-treated animals (Fig. 7B). Additionally, animals treated with leflunomide 10 mg/ml showed significant higher proteins expression from those treated with 2.5 and 5 mg/ml leflunomide.

ELISA was used to measure TGFβ protein levels in hepatic tissue samples from leflunomide-treated and control mice. Fig. 7C shows a significant increase of TGFβ proteins in all groups of leflunomide-treated animals compared to normal.

4. Discussion

DILI represents a major clinical problem with life-threatening sequelae. Therefore, much attention has been paid to studying the potential hepatotoxins, risk factors, and mechanisms of injury involved in DILI. DMARDs are essential therapeutics aim to halting the progression of rheumatoid arthritis. Leflunomide, a DMARD, was marketed in 1998, however, elevated hepatic enzymes, severe hepatitis, and acute liver failure were repeatedly reported [4,47,48], which considered a therapeutic limitation due to noncompliance and therapeutic failure.

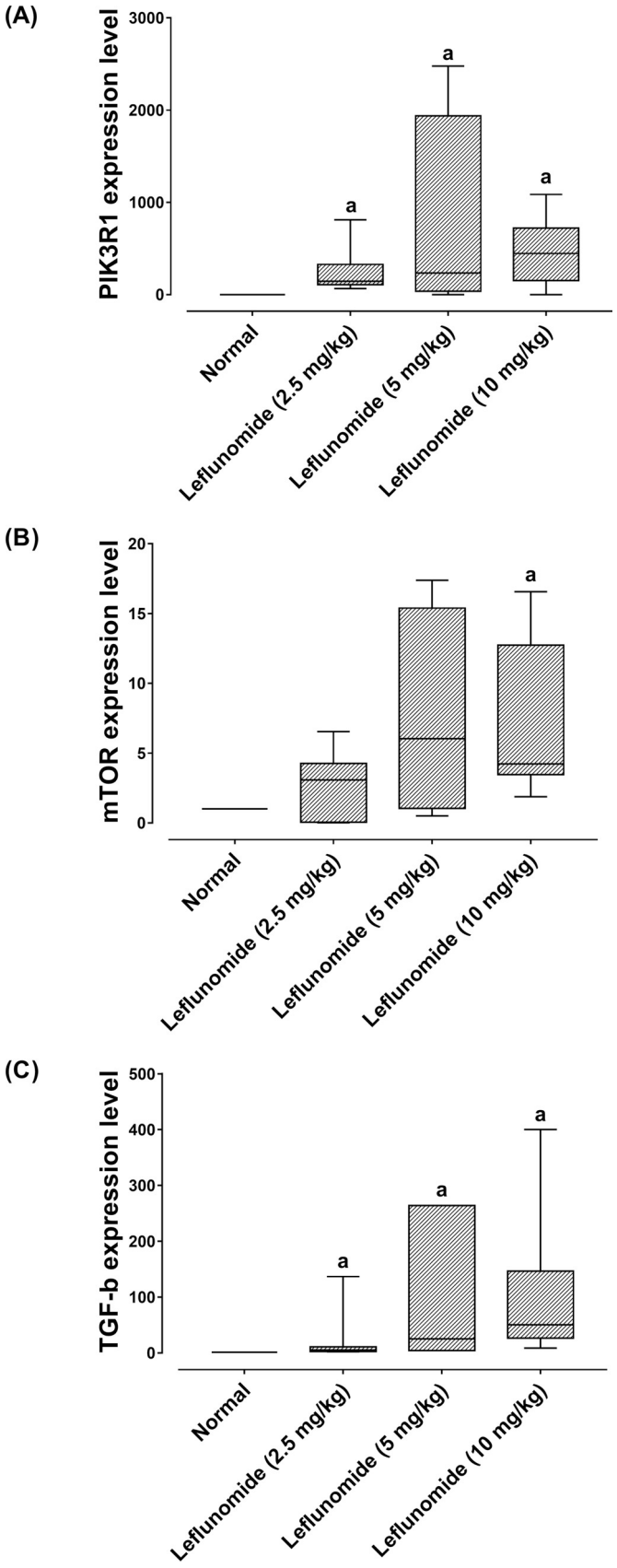

Fig. 6. Real time-polymerase chain reaction for TGF-β and PI3K/mTOR genes. (A) PI3K-α. (B) mTOR (C) TGF-β in each group. Data are medians in boxplots and analysis was done using Kruskal-Wallis ANOVA and a post hoc test. $P < 0.05$ was the accepted level of significance. a: versus normal group.

In the current study, leflunomide induced dose-dependent liver injury, which is detected as; (1) elevated standard enzymatic markers of the liver (ALT and AST) and (2) confirmed by the histopathological assessment of liver necro-inflammatory changes. These data are consistent with reports of experimental and clinical research studies, which state the potential hepatotoxicity of leflunomide [4,11,47,49,50]. Lodhi et al. (2015) used a single dose of leflunomide (10 mg/kg) which induced hepatic injury attributable to oxidative stress and overwhelming of cellular antioxidant mechanisms such as SOD and reduced glutathione [51]. In our model of dose-escalated 8-week exposure to leflunomide, persistent elevation of ALT and/or AST correlated with the histopathologic inflammatory and apoptotic changes. Similarly, reports of other researchers support hepatic apoptotic and fibrotic changes to inflammatory responses [52].

Furthermore, our data showed a dose-related increased expression of TLR4 in the hepatic specimen from leflunomide-treated mice compared to vehicle-treated animals. TLR4 is an intermediary player linking innate to adaptive immune responses in favor of initial enhancement of inflammation. Many studies reported a mechanistic role of TLR4 in acute liver failure [32,53,54]. Furthermore, studies showed that stimulating TLR4 with nontoxic doses of LPS may facilitate the hepatotoxic effects of some drugs [31].

Accordingly, leflunomide-induced liver injury may in part be related to TLR4-mediated NFκB pathway activation as evident from the data presented herein which shows the increased expression of both TLR4 and NFκB in dose-dependent manners. NFκB in its activated form enhances the expression of proinflammatory cytokines and orchestrates an inflammatory to adaptive immune responses [55]. Furthermore, TLR4 plays a pivotal role in the NFκB canonical pathway of activation [34,56]. Normally, NFκB is reserved in an inactive status in the cytoplasm by binding to its inhibitor (IκB), which is regulated by the inhibitor of NFκB kinase [57,58]. However, TLR4 activation and oxidative stress can degrade the IκB and release NFκB and allow transcription of proinflammatory cytokines [55,56,59].

Compatible to findings from previous studies [60–62], NFκB activation was linked to the enhanced PI3K/Akt/mTOR signaling pathway, where a rise in the expression of the phosphorylated form of these markers were observed. Wang et al. (2017) emphasized that PI3K/mTOR are the key proteins involved in NFκB activation with subsequent increased expression and release of various inflammatory mediators [58]. Others concluded that PI3K//mTOR/NFκB pathway has been implicated in various inflammatory diseases and becomes one of the most important means of prediction of its outcome [34].

TGFβ is an important liver signaling molecule involved in differentiation, maturation and apoptosis within both the physiological and pathological contexts [63]. Persistent hepatic inflammation and unremitting injury leads to hepatic fibrosis which is characterized by activation of hepatic stellate cells into fibrogenic myofibroblast consequences with deposition of extracellular matrix [64]. Although previous studies have reported inhibitory effect of leflunomide on hepatic fibrosis [65], leflunomide cumulative dose has been positively correlated with development of silent liver fibrosis in rheumatoid arthritis patients [66]. TGF-β is significantly involved in hepatic fibrosis both by autocrine and paracrine mechanisms. In response to hepatic injury, TGF-β regulates cross talk between parenchymal, inflammatory and myofibroblast cells. In this context, our results showed a significant increase of hepatic TGF-β in leflunomide treated mice, suggesting that leflunomide-induced hepatic toxicity may be associated with induction of hepatic fibrosis.

Our data also revealed a dose-dependent increase of caspase 3 immunostaining in leflunomide-treated mice compared to vehicle-exposed animals. Activation and nuclear translocation of NFκB also enhance the expression of fatty acid synthetase ligand on hepatocytes, which leads to activation of caspases including caspase3 with a cellular commitment to apoptosis [67]. Furthermore, leflunomide-induced mitochondrial stress and cellular energy depletion can be another pathway of

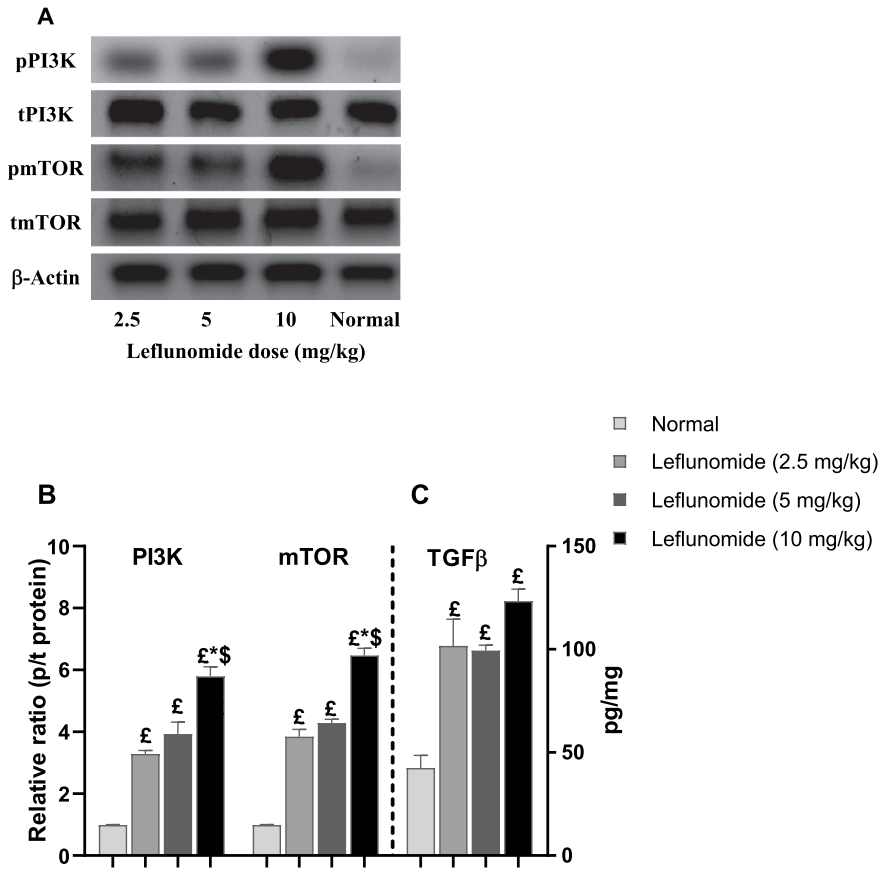

Fig. 7. Determination of TGFβ, PI3K and mTOR total and phosphorylated proteins. A: Western blotting analysis of total and phosphorylated mTOR and PI3K proteins in hepatic tissue isolated from leflunomide treated and control mice. B: A graph presents the relative ratio of densitometric analysis of western blotting of the total/phosphorylated mTOR and PI3K. C: ELISA measurement of TGFβ in hepatic tissue isolated from leflunomide treated and control animals. Data are mean ± SD, analyzed using one-way ANOVA with significance at $p < 0.05$ as significant.
£ compared to normal.
* Compared to leflunomide (2.5 mg/kg).
$ Compared to leflunomide (5 mg/kg).

triggering caspases-mediated apoptosis [68,69].

In contrary, studies reported the therapeutic benefit of leflunomide immune-mediated idiosyncratic acute liver failure [70] and acetaminophen hepatotoxicity [71]. In the previous studies, leflunomide was used as immunosuppressant and anti-inflammatory. The Authors claimed that the effect of leflunomide is attributable to downregulation of TNF-α in the liver through inhibition of NFκB activation [72]. This is not contradictory to our data, in which chronic use of leflunomide is suspected as a trigger of an inflammatory and cytotoxic burden on hepatocytes.

Further, PCNA, a nuclear protein essential for DNA replication and processivity of DNA polymerase. PCNA is a marker of tissue regeneration and is abundant during the S phase of the cell cycle [73]. Our data shows a dose-dependent increase of PCNA protein immunostaining in liver tissue isolated from leflunomide-treated animals. This may be a tissue repair process following hepatic tissue injury after treatment with high doses of leflunomide. Liver regeneration after leflunomide-induced hepatic injury has been addressed in [74]. Interestingly, leflunomide mediates its therapeutic action through inhibition of de novo synthesis of pyrimidine with a cell cycle arrest in S phase and hindering of cell proliferation [32].

To conclude, the present data suggest that leflunomide-induced hepatic injury may partly be mediated through inflammatory mechanisms through TLR4-mediated activation of NFκB by enhancing the PI3K/Akt/mTOR pathways. Further investigation on clinical cases may be required to support animal findings obtained from this work and to check its clinical feasibility.

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Declaration of competing interest

None.

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