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Mitochondrial and Redox Mechanisms of Leflunomide-Induced Hepatotoxicity Following Repeated Exposure

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Abstract

Leflunomide is a disease-modifying antirheumatic drug used for inflammatory arthritis. However, aminotransferase elevations and cases of drug-induced liver injury have been reported during therapy. Although leflunomide or its active metabolite has shown antioxidant or protective effects in acute and extrahepatic models, whether repeated hepatic exposure produces a redox–mitochondrial injury phenotype remains insufficiently defined. This study investigated the effects of repeated oral leflunomide administration on hepatic injury markers, oxidative stress, and mitochondrial function in male BALB/c mice. Animals received leflunomide at 5, 10, or 20 mg/kg by gavage for 28 days. Plasma ALT, AST, and LDH were measured. Hepatic oxidative status was assessed by reactive oxygen species formation, lipid peroxidation, reduced glutathione content, ferric-reducing antioxidant power, and the activities of superoxide dismutase and catalase. Liver mitochondria were isolated to evaluate dehydrogenase activity, membrane potential, and calcium-triggered swelling. Repeated leflunomide exposure reduced body weight gain at 10 and 20 mg/kg without altering the liver weight index. ALT and AST were increased in treated animals, whereas LDH remained unchanged. Leflunomide also shifted the hepatic redox profile toward oxidative stress, with increased ROS formation and lipid peroxidation, GSH depletion, reduced antioxidant capacity, and lower SOD and CAT activities. These alterations were accompanied by mitochondrial dysfunction, including reduced dehydrogenase activity, loss of mitochondrial membrane potential, and enhanced mitochondrial swelling. These findings indicate that repeated leflunomide exposure induces a hepatic injury phenotype characterized by oxidative stress and mitochondrial impairment. Sustained liver exposure may reveal mitochondrial liabilities not evident in acute or extrahepatic experimental settings.

Keywords: Leflunomide; Hepatotoxicity; Oxidative stress; Mitochondrial dysfunction; Drug-induced liver injury.

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1. Introduction

Drug-induced liver injury (DILI) remains an important challenge in clinical pharmacotherapy and drug development. Its presentation is variable and can range from mild, transient elevations in liver enzymes to acute liver failure, liver transplantation, or death (1,

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2). This variability makes DILI difficult to predict, diagnose, and manage in routine practice. It may also lead to treatment interruption, repeated laboratory monitoring, hospitalization, and exclusion of other causes of liver disease. DILI is also a major concern during drug development, because severe hepatotoxicity may be missed in premarketing studies when events are rare, and patient susceptibility is heterogeneous. Therefore, liver safety signals may become evident only after wider clinical use, leading to regulatory restrictions, boxed warnings, drug approval failures, or the withdrawal of otherwise effective medications from the market (2, 3). Identifying hepatotoxic potential and defining the underlying mechanisms of liver injury are therefore essential for safer drug use and better risk assessment.

Leflunomide is a widely prescribed oral disease-modifying antirheumatic drug (DMARD) for rheumatoid and psoriatic arthritis (4, 5). Leflunomide is metabolized to teriflunomide (A77 1726), which in turn inhibits the dihydro-orotate dehydrogenase (DHODH) enzyme and thereby suppresses *de novo* synthesis of pyrimidines. Consequently, the proliferation of activated T and B lymphocytes is effectively limited (4, 5). Although this mechanism provides therapeutic benefit, the metabolite's prolonged half-life, driven by strong plasma protein binding and enterohepatic recirculation, creates sustained systemic exposure that can potentiate both intended immunomodulatory actions and adverse hepatic effects (6). Clinically, leflunomide is associated with a spectrum of liver abnormalities, ranging from mild and transient aminotransferase elevations to serious hepatocellular injury, mixed-pattern DILI, cholestasis, and rare cases of acute liver failure. These risks are accentuated in patients with pre-existing liver disease, alcohol use, or interacting medications (7-9).

A growing body of experimental and clinical evidence highlights mitochondrial dysfunction and oxidative stress as unifying mechanisms underlying diverse DILI phenotypes (10, 11). Excessive mitochondrial reactive oxygen species (ROS) generation can trigger inner membrane depolarization, disturbances in ATP synthesis, opening of the mitochondrial permeability transition pore (mPT), and activation of cell death pathways (12, 13). In parallel, mitochondrial ROS drives redox-sensitive inflammatory signaling, including NF-κB and NLRP3 inflammasome activation, further amplifying hepatocellular injury (14, 15). This mechanistic framework has been increasingly investigated in DILI and may provide a compelling rationale for the mechanistic investigation of leflunomide hepatotoxicity.

Paradoxically, the literature surrounding leflunomide's redox biology includes reports of antioxidant or protective effects in certain extrahepatic or acute injury models. Previous research on the protective properties of leflunomide has often been associated with upregulation of antioxidant enzymes or suppression of inflammatory mediators (16-19). In contrast, hepatic experimental studies suggest a more injurious profile for leflunomide. Leflunomide and its active metabolite have been shown to impair mitochondrial function in human hepatic cells, supporting a possible role for mitochondrial toxicity in leflunomide-associated hepatotoxicity (20). In mice, leflunomide-induced liver injury has also been linked to activation of TLR4–PI3K/mTOR/NF-κB signaling, accompanied by increased liver enzymes and necro-inflammatory changes (21). Clinical registry data further confirm that leflunomide can cause DILI in humans, although these data describe the clinical phenotype rather than the underlying redox or mitochondrial mechanisms (8). Differences in dose, exposure duration, tissue-specific metabolic demand, and inflammatory context likely account for these conflicting observations. Chronic hepatic exposure, where oxidative metabolism is high and mitochondrial burden is substantial, may unmask injurious redox–mitochondrial liabilities not observed in short-term or extrahepatic models.

Given these uncertainties, clarifying how sustained leflunomide exposure affects hepatic redox balance and mitochondrial function is critical. Biomarkers reflecting oxidative stress (ROS, lipid peroxidation, GSH depletion, antioxidant capacity), mitochondrial function (dehydrogenase activity, membrane potential, and swelling), and hepatocellular integrity (ALT/AST) provide a framework for

defining the mechanistic trajectory from redox imbalance to hepatic injury. The present study was therefore designed to investigate whether chronic, dose-dependent oral administration of leflunomide induces a coordinated oxidative and mitochondrial injury phenotype in the mouse liver. By examining biochemical and mitochondrial endpoints after 28 days of exposure, this work aims to provide clues to the discrepancies in the literature and a coherent mechanistic explanation for leflunomide-associated hepatotoxicity *in vivo*.

2. Materials and Methods

2.1. Reagents

Leflunomide was obtained from Arena Life Science (Iran). 2′,7′-Dichlorofluorescein diacetate (DCFH-DA), rhodamine 123 (Rh 123), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), 2,4,6-tripyridyl-s-triazine, D-mannitol, malondialdehyde, reduced glutathione (GSH), ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), sucrose, trichloroacetic acid, sodium citrate, fatty acid-free bovine serum albumin fraction V, and 3-(N-morpholino)propane sulfonic acid (MOPS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethylenediaminetetraacetic acid (EDTA), n-butanol, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 5,5-bis-dithio-nitrobenzoic acid (DTNB), meta-phosphoric acid, and 2-amino-2-hydroxymethyl-propane-1,3-diol hydrochloride (Tris-HCl) were supplied by Merck (Darmstadt, Germany). Kits for the quantification of plasma biomarkers of hepatic injury were procured from Pars Azmoon® (Tehran, Iran).

2.2. Animals

Male BALB/c mice (n=24; 23±2 g) were obtained from Shiraz University of Medical Sciences, Shiraz, Iran. Animals were maintained at an environmental temperature of 23±1 ºC, 42±3% relative humidity, and adequate ventilation. During experiments, mice had free access to tap water and standard pellet chow. The institutional laboratory animal care and use committee at Shiraz University of Medical Sciences approved all animal experiments (Code: IR.SUMS.AEC.1403.091).

2.3. Experimental setup

Animals were randomly assigned to the leflunomide group (model group, n=18, 6 animals/dose) and the control group (n=6). Leflunomide-treated animals received 5, 10, and 20 mg/kg of the drug by gavage for 28 consecutive days (22). On day 29, animals were deeply anesthetized with thiopental (100 mg/kg, i.p.). Blood samples (1 mL from the inferior vena cava) were transferred to sodium citrate-coated tubes, and the plasma was prepared (3000 g, 15 min, 4 °C). Liver samples were also excised and used to assess markers of oxidative stress and to isolate mitochondria.

2.4. Reactive oxygen species (ROS) formation

Hepatic ROS formation was measured using 2′,7′-dichlorofluorescein diacetate (DCFH-DA) as previously described (23, 24). Liver tissue (200 mg) was homogenized in 5 mL of ice-cold Tris-HCl buffer (40 mM, pH 7.4). An aliquot of the homogenate (100 µL) was then mixed with 1 mL of Tris-HCl buffer containing DCFH-DA at a final concentration of 10 µM. Samples were incubated for 10 min at 37 °C in the dark. Fluorescence intensity was measured using a FLUOstar Omega® multifunctional fluorimeter at λ-ex=485 nm and λ-em=525 nm (23, 24).

2.5. Lipid peroxidation

DHepatic lipid peroxidation was assessed using the thiobarbituric acid reactive substances (TBARS) assay as previously described (23). Briefly, 500 µL of liver homogenate (10% w/v in 40 mM Tris-HCl buffer) was mixed with 4 mL of TBARS reagent. The reagent contained 0.375% w/v thiobarbituric acid, 50% w/v trichloroacetic acid, and 1% w/v meta-phosphoric acid at pH 2, prepared at a 1:1:3 volume ratio. Samples were vortexed for 1 min and heated in a boiling water bath at

100 °C for 45 min. After cooling, 2 mL of n-butanol was added. Samples were mixed and centrifuged at 10,000 g for 20 min at 4 °C. The absorbance of the n-butanol phase was then measured at 532 nm (23, 24).

2.6. Total antioxidant capacity of the liver tissue

The ferric-reducing antioxidant power (FRAP) assay measured the liver's total antioxidant capacity in leflunomide-treated animals (23). The FRAP reagent was freshly prepared by mixing 10 volumes of acetate buffer (300 mM, pH 3.6), one volume of TPTZ solution (10 mM in 40 mM HCl), and one volume of $FeCl_3 \cdot 6H_2O$ solution (20 mM). Liver homogenate (100 µL) was added to 900 µL of FRAP reagent and incubated for 5 min at 37 °C in the dark. Absorbance was then measured at 595 nm (23).

2.7. Hepatic glutathione content

Liver tissue GSH levels were determined by utilizing Ellman's reagent (DTNB) (23). Briefly, 1 mL of the tissue homogenate was treated with 200 µL of trichloroacetic acid (50% w-v; 4 °C). After thorough mixing and centrifugation (16000 g, 4 °C, 10 min), the supernatant was then mixed with 1 mL of Tris-HCl buffer (pH=8.9; 4 °C) and 100 µL of 40 mM DTNB (dissolved in pure methanol). Subsequently, the absorbance of the resulting color was measured at λ=412 nm using an EPOCH® plate reader (23, 25).

2.8. Hepatic antioxidant enzymes activity

The activities of key hepatic antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT), were assessed using commercial kits according to the manufacturer's instructions. Specifically, SOD and CAT activities were quantified using ZellBio assay kits (ZellBio GmbH, Germany) according to the supplier's protocols. Enzyme activities were measured spectrophotometrically, with results expressed in standard activity units as defined by the kit protocols.

2.9. Liver mitochondria isolation

Liver mitochondria were isolated by differential centrifugation as previously described (23). Fresh liver tissue was washed and minced in ice-cold isolation buffer containing 220 mM sucrose, 70 mM mannitol, 2 mM HEPES, 0.5 mM EGTA, and 0.1% BSA, pH 7.4. The minced tissue was homogenized in fresh isolation buffer at a ratio of 5 mL buffer per 1 g liver tissue. The homogenate was first centrifuged at 1,000 g for 20 min at 4 °C to remove nuclei, cell debris, and unbroken cells. The resulting supernatant was then centrifuged at 10,000 g for 20 min at 4 °C to pellet the mitochondrial fraction. This washing step was repeated three times using fresh isolation buffer. The final mitochondrial pellet was resuspended in buffer containing 70 mM mannitol, 220 mM sucrose, 2 mM HEPES, and 0.5 mM EGTA, pH 7.4. For mitochondrial membrane potential assessment, mitochondria were resuspended in assay buffer containing 220 mM sucrose, 68 mM mannitol, 10 mM KCl, 5 mM KH_2PO_4, 10 mM HEPES, 2 mM $MgCl_2$, and 50 µM EGTA, pH 7.2. For mitochondrial swelling assessment, mitochondria were resuspended in swelling buffer containing 125 mM sucrose, 65 mM KCl, and 10 mM HEPES, pH 7.2. (23).

2.10. Mitochondrial dehydrogenases activity

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used as a colorimetric method to determine hepatic mitochondrial dehydrogenase activity (26). For this purpose, a mitochondrial suspension (0.5 mg protein/ml) was incubated with 0.4% MTT (37 °C, 30 min, in the dark). Then, the formazan crystals were dissolved in dimethyl sulfoxide (1000 µL). Samples were centrifuged (1 min, 1000 g), and the absorbance of the supernatant was measured at λ=570 nm (26).

2.11. Mitochondrial depolarization

Mitochondrial uptake of rhodamine 123 has been used to estimate mitochondrial depolarization (23). Briefly, the mitochondrial fractions (0.5 mg protein/mL) were incubated with 10 µM rhodamine 123 in the assay buffer (15 minutes in the dark). Afterward, samples were centrifuged (16,000 g, 1 min, 4 ºC), and the fluorescence intensity of the supernatant was assessed at $\lambda_{excitation}$=485 nm and $\lambda_{emission}$=525 nm using a fluorometer (23).

2.12. Mitochondrial swelling

Mitochondrial permeabilization was evaluated using light-scattering analysis (23). For this purpose, 100 µL of isolated mitochondria was mixed with 900 µL of a pre-warmed (30 ºC) swelling buffer. Then, 10 µM of calcium (Ca^{2+}) was added to trigger mitochondrial permeabilization. Absorbance at λ=540 nm was then monitored for 30 minutes. The change in absorbance (ΔOD) from the beginning to the end of the measurement period was used to quantify mitochondrial swelling amplitude (23).

2.13. Organ weight index

The liver weight index was determined as Organ weight index = [Wet organ weight (g)/Whole body weight (g)] × 100.

2.14. Statistical analysis

Data are presented as mean ± SD. Normality was assessed for each dataset using the Kolmogorov–Smirnov test. Comparisons among experimental groups were performed using one-way analysis of variance (ANOVA), followed by Tukey's multiple-comparison post hoc test. A P value < 0.05 was considered statistically significant.

3. Results

Chronic administration of leflunomide resulted in a dose-dependent reduction in body weight gain compared with untreated controls. Animals in the control group exhibited the greatest weight gain during the dosing period, whereas those receiving 5 mg/kg maintained a similar trajectory, without reaching statistical significance relative to controls. In contrast, weight gain was significantly reduced at the 10 mg/kg dose. It remained comparably suppressed in the 20 mg/kg group, indicating a plateau of the effect at higher exposures (Fig. 1). Post hoc analysis confirmed that the 10 and 20 mg/kg groups differed significantly from controls. In contrast, the 5 mg/kg group did not. No mortality or overt signs of systemic toxicity were observed during the experimental period.

In contrast to body weight dynamics, liver weight index (liver weight normalized to body weight) remained stable across all treatment groups. Mean liver indices in the 5, 10, and 20 mg/kg groups did not differ significantly from controls. Variance remained narrow within each group (Figure 1). These findings

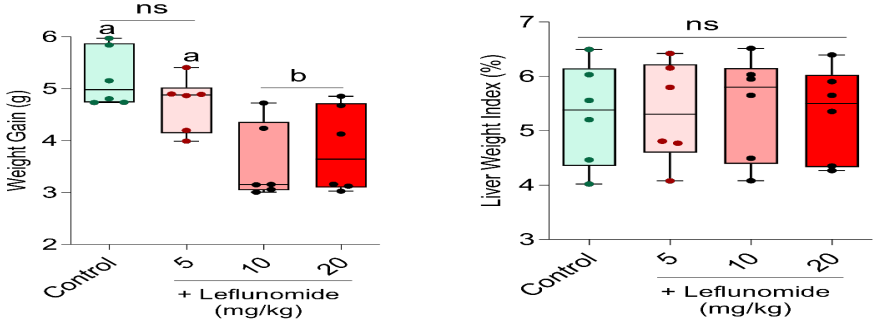

Figure 1. Animal weight gain and liver weight index in leflunomide-treated mice. Data sets with different alphabetical superscripts are significantly different (P<0.05). ns: not significant.

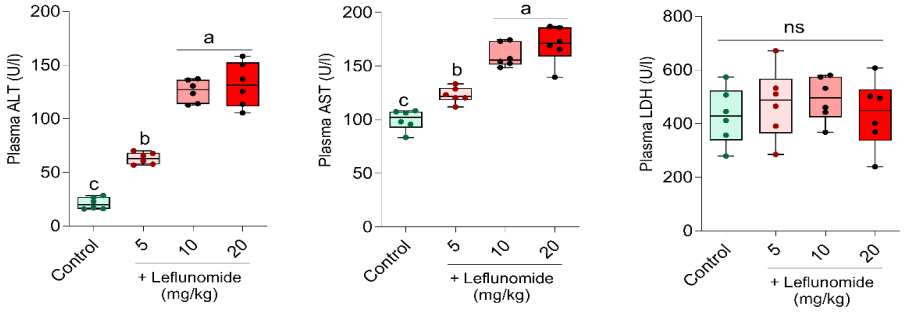

Figure 2. Plasma biochemistry of leflunomide-treated animals. Data sets with different alphabetical superscripts are significantly different (P<0.05). ns: not significant.

indicate that leflunomide did not induce hepatomegaly or liver mass loss sufficient to alter the organ-to-body weight ratio, despite the observed reductions in overall body weight gain at higher doses (Figure 1).

Chronic oral administration of leflunomide for 28 days resulted in a clear dose-dependent pattern of hepatocellular injury. Mice receiving 10 and 20 mg/kg leflunomide showed marked elevations in plasma ALT and AST levels compared with controls. In contrast, the 5 mg/kg group showed a smaller increase (Figure 2). Plasma LDH activity did not differ significantly among the experimental groups (Figure 2).

In parallel with plasma biochemical alterations, hepatic oxidative stress parameters were substantially disturbed (Figure 3). Leflunomide-treated animals showed significant increases in ROS and lipid peroxidation, accompanied by pronounced depletion of reduced glutathione and a marked decline in total antioxidant capacity. Activities of key antioxidant enzymes, including superoxide dismutase and catalase, were also significantly decreased in the mid- and high-dose groups, indicating suppression of endogenous defense mechanisms (Figure 3).

Mitochondrial assessments revealed notable impairment of organellar function (Figure 4). Mitochondrial dehydrogenase activity measured by the MTT assay was mark-

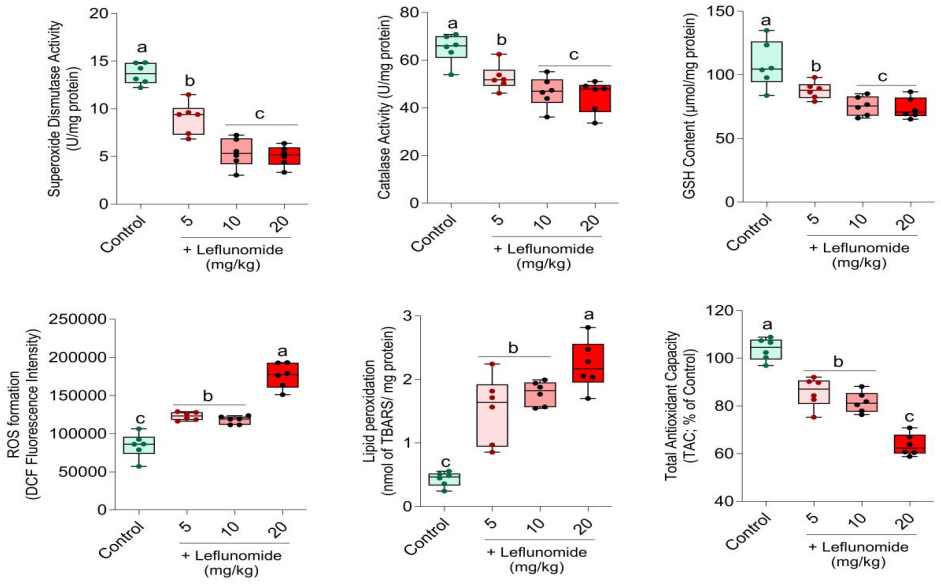

Figure 3. Biomarkers of oxidative stress in the liver of leflunomide-treated mice. Data sets with different alphabetical superscripts are significantly different (P<0.05). ns: not significant.

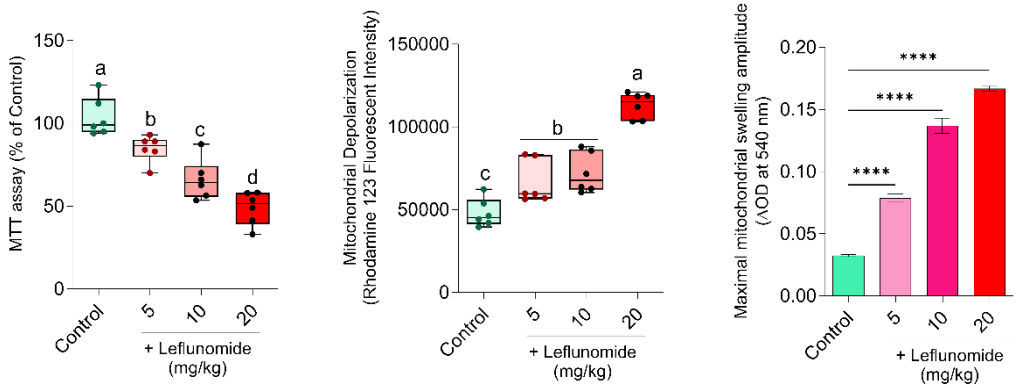

Figure 4. Mitochondrial indices of functionality in leflunomide-treated animals. Data are presented as mean ± SD. Different alphabetical superscripts indicate significant differences among groups for MTT activity and mitochondrial depolarization. For mitochondrial swelling, asterisks indicate significant differences between the indicated groups. $P < 0.05$ was considered significant.

edly decreased, mitochondrial membrane potential showed significant depolarization, and mitochondrial swelling was prominently increased, consistent with induction of mitochondrial permeability transition (Figure 4). These findings are consistent with leflunomide-associated impairment of hepatic mitochondrial bioenergetics and membrane stability.

4. Discussion

Drug-induced liver injury (DILI) remains a major obstacle for both clinical therapeutics and drug development, accounting for a substantial proportion of acute liver failure cases, post-marketing safety warnings, and drug withdrawals (27). Leflunomide is an effective disease-modifying antirheumatic drug used in rheumatoid and psoriatic arthritis, but hepatotoxicity remains an important safety concern during treatment (5, 28). Clinical studies and registry data show that leflunomide can cause aminotransferase elevations and, less commonly, clinically apparent DILI with severe outcomes in some patients (7, 8). This concern is further strengthened by the pharmacokinetics of its active metabolite, teriflunomide. Teriflunomide is highly protein-bound, undergoes enterohepatic recirculation, and has a long elimination half-life, which can sustain systemic and hepatic exposure after repeated dosing (29). Experimental work also supports a hepatic mechanism for this toxicity. Leflunomide and A77 1726 have been shown to impair mitochondrial function in human hepatic cells, and inhibition of hepatic CYP activity or bile acid transport can worsen leflunomide-induced hepatotoxicity in experimental models (20, 30). In mice, leflunomide-induced liver injury has also been linked to TLR4–PI3K/mTOR/NF-κB signaling and inflammatory activation (21). Based on this background, the present study examined whether repeated oral leflunomide exposure produces a redox–mitochondrial pattern of liver injury. Our findings show that 28-day treatment increased plasma ALT and AST, disturbed hepatic antioxidant defenses, increased ROS formation and lipid peroxidation, and impaired mitochondrial function in the mouse liver.

Leflunomide is converted to teriflunomide, which inhibits DHODH and limits de novo pyrimidine synthesis in activated lymphocytes (4). This mechanism explains its disease-modifying effect in inflammatory arthritis. However, the long half-life of teriflunomide may also increase the relevance of repeated exposure in toxicity studies (29). Although leflunomide-associated liver injury has been described clinically, the contribution of

redox imbalance and mitochondrial dysfunction to this toxicity remains incompletely defined, particularly after repeated exposure.

At the mechanistic level, a central axis that connects diverse DILI phenotypes is mitochondrial vulnerability to oxidative stress (11, 31). Oxidative stress is a major mechanism in the pathogenesis of DILI. When reactive metabolites, inflammatory signals, or direct drug effects exceed hepatic antioxidant defenses, the redox balance shifts toward ROS accumulation. This can promote lipid peroxidation, protein oxidation, GSH depletion, and impairment of antioxidant enzymes. These changes weaken hepatocyte defenses, creating a setting in which cellular injury can progress (11, 32). Mitochondrial dysfunction is another central mechanism in DILI. Many hepatotoxic drugs impair mitochondrial respiration, decrease ATP production, collapse mitochondrial membrane potential, or trigger mitochondrial permeability transition. These events can shift hepatocytes toward necrotic or apoptotic cell death, depending on the severity and duration of the insult (10, 33). Oxidative stress and mitochondrial dysfunction are also closely connected. Damaged mitochondria are an important source of ROS, while ROS can further injure mitochondrial membranes, respiratory complexes, and mitochondrial DNA. This feed-forward cycle can amplify lipid peroxidation, energy failure, mitochondrial swelling, and hepatocellular injury (10, 34). This mechanistic link provides the rationale for evaluating both hepatic redox markers and mitochondrial endpoints in leflunomide-exposed liver.

Intriguingly, the leflunomide literature is internally discrepant with respect to oxidative stress, with seemingly protective and toxic narratives coexisting. On one hand, several extrahepatic studies reported the antioxidant or cytoprotective effects of leflunomide/teriflunomide in settings such as sepsis-associated intestinal injury, bleomycin-induced pulmonary inflammation/fibrosis, fetal lung endothelial oxidative stress, and ischemia–reperfusion damage (35-39). These studies often suggested that leflunomide exerts protective effects by upregulating SOD/CAT, modulating inflammatory cascades, or inhibiting stress kinases (35-39). On the other hand, hepatic models and clinical observations emphasize redox-mediated mitochondrial injury, innate immune activation, and cholestatic patterns. However, some in vitro discrepancies on the protective properties of the leflunomide metabolite also exist.

In a mouse model, leflunomide has been shown to activate TLR4–PI3K/mTOR/NF-κB signaling (21). This event was associated with lower hepatic antioxidant defenses, increased lipid peroxidation, and ROS formation (21). The present findings are consistent with and extend the previous report by Elshaer *et al*. In that study, it was found that leflunomide-induced liver injury in mice was associated with increased ALT and AST activities and higher necro-inflammatory scores. The authors further showed increased hepatic expression of TLR4 and caspase-3, increased NF-κB immunoreactivity, and upregulation of PI3K and mTOR. These findings suggested that leflunomide hepatotoxicity involves activation of the TLR4–PI3K/mTOR/NF-κB axis and downstream inflammatory and apoptotic responses (21). Our data support the same general hepatotoxic profile, because repeated leflunomide exposure also increased plasma ALT and AST in mice. However, the present study adds a complementary mechanistic layer. Instead of focusing on inflammatory signaling, we evaluated hepatic redox status and isolated mitochondrial function. Leflunomide increased ROS formation and lipid peroxidation, depleted GSH, reduced total antioxidant capacity, and decreased SOD and CAT activities. These changes were accompanied by reduced mitochondrial dehydrogenase activity, loss of mitochondrial membrane potential, and increased mitochondrial swelling. Therefore, the current findings suggest that oxidative stress and mitochondrial impairment may act

upstream of, or in parallel with, the inflammatory signaling described by Elshaer *et al*. Together, these studies indicate that leflunomide-induced liver injury is not limited to a single pathway but rather likely reflects interactions among redox imbalance, mitochondrial dysfunction, inflammatory activation, and cell death signaling.

Some previous data also show that leflunomide may not always act as a mitochondrial toxin. In an *in vitro* model of acetaminophen injury, leflunomide and A77 1726 protected immortalized human hepatocytes by inhibiting JNK-mediated mitochondrial permeability transition (40). This finding suggests that leflunomide can affect stress-kinase signaling and mitochondrial responses in a context-dependent manner. However, this result should be interpreted within the limits of that model. Acetaminophen injury in cultured hepatocytes is an acute experimental setting, whereas our study evaluated repeated leflunomide exposure in the intact liver. In vivo, prolonged exposure to teriflunomide, enterohepatic recycling, and sustained hepatic metabolic stress may produce a different outcome. Therefore, the protective effect reported in acute acetaminophen toxicity does not conflict with our findings. Rather, it supports the view that leflunomide-related mitochondrial effects depend on the model, exposure duration, and injury context.

Taken together, previous studies suggest that the hepatic response to leflunomide depends on the exposure context. Protective effects have been reported in some acute or extrahepatic models, whereas hepatic studies indicate liver injury, inflammatory activation, and mitochondrial impairment. However, the redox and mitochondrial consequences of repeated leflunomide exposure in the intact liver remain insufficiently defined. The present study addressed this gap by treating BALB/c mice with oral leflunomide at 5, 10, and 20 mg/kg for 28 days and assessing liver injury, oxidative stress, and mitochondrial function in parallel. Plasma ALT and AST were measured as biochemical markers of hepatocellular injury. Hepatic ROS formation, lipid peroxidation, GSH content, FRAP, SOD, and CAT were evaluated to characterize redox status. Isolated liver mitochondria were used to assess dehydrogenase activity, membrane potential, and swelling. This approach allowed us to determine whether repeated leflunomide exposure produces a coordinated pattern of oxidative stress and mitochondrial dysfunction in the mouse liver.

The present study helps clarify the hepatic response to repeated leflunomide exposure. In contrast to reports of protective effects in some acute or extrahepatic models, 28-day leflunomide administration produced a redox–mitochondrial injury pattern in mouse liver. This pattern was characterized by increased plasma ALT and AST, enhanced ROS formation and lipid peroxidation, depletion of GSH, reduced total antioxidant capacity, and lower SOD and CAT activities. Isolated liver mitochondria also showed reduced dehydrogenase activity, loss of membrane potential, and increased swelling. These findings suggest that oxidative stress and mitochondrial dysfunction are closely linked in leflunomide-induced hepatotoxicity. They also extend previous reports on inflammatory signaling by adding direct evidence of mitochondrial impairment after repeated exposure. Therefore, redox and mitochondrial endpoints may be useful for the mechanistic evaluation of hepatic risk during prolonged leflunomide treatment. Further studies are needed to determine whether these mitochondrial changes precede inflammatory activation and whether targeted protective interventions can prevent them.

5. Conclusion

Repeated oral administration of leflunomide for 28 days produced a coordinated hepatic injury phenotype in male BALB/c mice. Elevations in plasma ALT and AST occurred alongside increased ROS formation

and lipid peroxidation, depletion of GSH, reduced total antioxidant capacity, and suppression of SOD and CAT activities. These redox disturbances were accompanied by reduced mitochondrial dehydrogenase activity, loss of mitochondrial membrane potential, and enhanced calcium-triggered swelling, indicating impaired mitochondrial functional integrity. Collectively, these findings support oxidative stress and mitochondrial dysfunction as closely interconnected components of leflunomide-associated hepatotoxicity during sustained exposure. Further studies incorporating histopathological assessment, inflammatory and cell-death markers, and clinically relevant exposure models are warranted to clarify the temporal sequence and translational significance of these alterations.

Authors contributions

Conceptualization: RH, HN, AJ; Methodology: RH, HN, AA, SG, SM, KY; Investigation: AA, SG, SM, KY; Data curation: AA; Formal analysis: RH, HN, AA; Visualization: AA; Project administration: RH, HN, AJ; Supervision: RH, HN, AJ; Writing—original draft: AA, SG, SM, KY; Writing-review and editing: RH, HN, AJ. All authors reviewed and approved the final manuscript.

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Ethical approval

The experimental protocol was approved by the institutional ethics committee (Ethics code: IR.SUMS.AEC.1403.091).

Conflict of Interest

The authors declare that they have no conflict of interest.

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