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Novel therapeutic effects of rifaximin in combination with methylprednisolone for LPS-induced oxidative stress and inflammation in mice: An *in vivo* study

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ABSTRACT

Cytokine-releasing syndrome (CRS) is a special form of systemic inflammatory response syndrome provoked by factors like viral infections and certain immunomodulatory drugs. To elucidate the potential role of rifaximin (RIF) and its combination with methylprednisolone (MP) against the development and progression of CRS in mice. This experiment consists of two parts: protective and therapeutic interventions. The protective experiment: in the induction group, mice received an intraperitoneal injection (IP) of 5 mg/kg lipopolysaccharide (LPS) without intervention. The other group received various drugs before the induction by three days, then observed for an additional two days (50 mg/kg MP, 50 mg/kg RIF, and a combination of 25 mg/kg RIF with 25 mg/kg MP. The second part of the study involves the therapeutic potential; all groups received similar doses of drugs to that received in the prevention groups, except LPS induction was given first, and after one hour, the mice received daily doses of the drugs for five days. At the end of the experiment, blood and tissue samples were obtained. Mice treated with RIF and its combination with MP showed improved serum TNF-α, IL-6, IL-8, IL-1β, INF-γ, MDA, and GSH in both prevention and therapeutic groups. Histopathologically, mice treated with rifaximin and its combination with MP ameliorates the tissue damage in both lung and liver tissues following LPS induction. In conclusion, rifaximin showed protective and therapeutic effects in LPS-induced cytokine storms in mice through anti-inflammatory and antioxidant mechanisms, and its combination with methylprednisolone showed additive/synergistic action.

1. Introduction

Cytokine-releasing syndrome (CRS) is a special form of systemic inflammatory response syndrome (SIRS) provoked by various factors like viral infections and certain immunomodulatory drugs like monoclonal antibodies and adoptive T-cell therapy [1]. SIRS is a special form of immune disturbance characterized by an exaggeration of immune response to various noxious factors, including acute infection, surgery, trauma, ischemia, and malignancy [2]. SIRS promotes the release of acute-phase reactants, which induce extensive changes in body systems, end-organ changes, and failure [3]. Cytokines are specific regulatory proteins that control intercellular communications and signaling, controlling cell differentiation and proliferation and regulating the immune response [4]. Upon detecting exogenous pathogens, the immune system responded with proportional synthesis and release of proinflammatory and anti-inflammatory cytokines to maintain body homeostasis [5]. Sufficient amounts of cytokines are required to eradicate pathogens without developing hyper-inflammation. Disproportionate production of proinflammatory cytokines induces hypercytokinemia and hyperinflammation, causing systemic inflammation and associated multiorgan failure (MOF) [5]. Over-activated immune response and hypercytokinemia are linked with the development of acute lung injury and acute respiratory distress syndrome (ARDS) in COVID-19 [6,7]. Abnormal immune response triggers apoptosis of lymphocytes with the development of lymphopenia, which induces upregulation of B lymphocytes and

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inappropriate production of immunoglobulins [8]. With the upregulation of proinflammatory cytokines and neuroendocrine-immune system interaction, glucocorticoid response is impaired, leading to MOF [5,9].

Hypercytokinemia in the CRS is regarded as a physiological response due to the excessive and uncontrolled release of proinflammatory cytokine from the innate immune system [1]. Proinflammatory cytokines play a vital role against invading pathogens under normal physiological conditions; however, abnormal immune responses with exaggeration of the release of proinflammatory cytokine during some viral infections, mainly SARS-CoV, MERS-CoV, and SARS-CoV-2, as well as other viral infections like hantavirus, H1N1 influenza, and cytomegalovirus [10]. Of note, interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α are the main cytokines involved in the progression of CRS [11]. Of note, unpredictable severe adverse events were observed in the phase I clinical trial of anti-CD28 monoclonal antibody that gives an initial clue concerning CRS. The patients in that trial developed hypotensive shock, pancytopenia, and fibrinolytic failure with significant elevations of proinflammatory and inflammatory cytokines. A rapid increase in the early-phase cytokine TNF-α and IL-1 causes MOF in CRS [12]. Different cytokine types are involved in the development and progression of CRS, like IL-1, IL-6, 1L-8, 1L-10, TNF-α, and interferon (INF)-γ. However, the key pathogenic roles of these cytokines differ according to the underlying causes of CRS. For example, INF-γ is the chief cytokine in hemophagocytic lymphohistiocytosis, IL-1β for still disease, IL-18 for macrophage activation syndrome, and IL-6 for CRS; however, CRS in sepsis is complex and involves various factors [13,14]. In COVID-19, CRS is rapidly developed and correlated with high mortality. IL-1β, IL-2, IL-6, IL-7, monocyte chemoattractant protein-1, and granulocyte-macrophage colony-stimulating factor are the main cytokines triggered during the development of CRS in severe COVID-19. IL-6 is regarded as a prototype cytokine intricate with COVID-19 severity. CRS might develop in COVID-19 due to failure of the viral clearance mechanism with persistent immunological stimulation similar to that of present hemophagocytic lymphohistiocytosis [15].

Methylprednisolone (MP) is a synthetic corticosteroid that acts systemically and shares similar physiological effects with naturally occurring glucocorticoids. The primary clinical application of methylprednisolone is attributed to its anti-inflammatory and immunosuppressive properties within the human body [16]. MP is either used in a large dose in acute flare-up of inflammatory disorders or used as a small dose in chronic conditions. MP is administrated either orally or parentally [17]. The primary indications of MP are to inhibit immune and inflammatory responses during acute and chronic inflammatory disorders [17]. MP undergoes passive diffusion through the cellular membrane and then attaches to the intracellular glucocorticoid receptor. This intricate structure moves into the nucleus, where it engages with certain DNA sequences, leading to either an increase or decrease in the transcription of specific genes. The methylprednisolone-glucocorticoid receptor complex binds to and obstructs the promoter sites of proinflammatory genes [18,19]. It stimulates the production of anti-inflammatory gene products and hinders the production of inflammatory cytokines [20]; this is primarily achieved by impeding the activity of transcription factors, such as nuclear factor-kappa-B (NF-κB) [21,22].

Rifaximin is a broad-spectrum semisynthetic antibiotic derived from the chemical modification of rifamycin. Rifaximin is poorly absorbed from the intestine after oral administration; thus, it has poor bioavailability [23]. The mechanism of action of rifaximin is by binding the β subunit of bacterial RNA polymerase of Gram-positive and Gram-negative bacteria. In addition, rifaximin inhibits bacterial translocation across the intestinal epithelial lining, significantly suppressing the expression of pro-inflammatory cytokines [24,25].

Rifaximin has potent anti-inflammatory effects through modulation of the pregnane X receptor (PXR). Activation of PXR attenuates the expression of nuclear factor kappa B (NF-κB) with subsequent reduction in the expression of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6. Therefore, stimulating PXR by rifaximin reduces inflammatory changes in inflammatory bowel diseases. Evidence from preclinical findings proposed that rifaximin attenuates inflammatory changes in experimental inflammatory bowel diseases. Experimental inflammatory bowel diseases revealed that injury of intestinal epithelial cells increases intestinal permeability and expression of pro-inflammatory cytokines, resulting in systemic inflammation [26]. Rifaximin, via induction of PXR, promotes the repair of the intestinal epithelium and inhibits the expression of pro-inflammatory cytokines. Furthermore, rifaximin prevents intestinal inflammation and barrier injury by modulating gut microbiota, which represses the expression of pro-inflammatory cytokines. These findings illustrate that rifaximin has local and systemic anti-inflammatory effects through activation of PXR and modulation of gut microbiota, respectively [27].

Rifaximin had not been examined previously in the context of cytokine syndrome. Additionally, this is the first study to examine the systemic administration of rifaximin using an intraperitoneal injection in animal models, which was devised to bypass its poor oral bioavailability. Much of the literature focused on its local effects in the intestine; this is the first study to examine its systemic effects. This gap of knowledge is addressed in current work. The present experimental study aimed to elucidate the potential role of rifaximin and its combination with methylprednisolone against the development and progression of CRS in mice.

2. Methods

2.1. Materials

All materials used were pharmaceutical grade purity, 10 % formalin (Roche, Germany), chloroform (Meghmani Finchem Limited, India), 70 % ethanol (AL-Hikmah, Jorden), distilled water (Pioneer, Iraq), Hematoxylin and Eosin stain (BDH, England), LPS (lipopolysaccharide) (Sigma-Aldrich, Germany), normal saline 9 % (Pioneer, Iraq), methylprednisolone and rifaximin powder (Hangzhou hyper chem. Limited, China). ELISA kit for TNF-α, IL-1β, IL-6, IL-8, IFN-γ, malondialdehyde (MDA), and glutathione (GSH) purchased from Sunlong Biotech, China.

2.2. Experimental animals

One hundred male Swiss albino mice are pathogen-free, weighing 25 – 35 g, and are aged 7–8 weeks. Each mouse has been purchased from the Center for Drug Control and Research, Ministry of Health. All animal handling and experimental procedures have been performed strictly per the guidelines for the care and use of laboratory animals by the animal ethics committee at Al-Nahrain University, College of Pharmacy (following AVMA guideline 2020 [28]). Animals were left free in the animal care facility of Al-Nahrain University, College of Pharmacy, a 12-hour light-dark cycle, room temperature 18–22 °C, and 40 % humidity. Animals were acclimatized for seven days in laboratory conditions before the start of the experiments. Regular rodent chows and water were provided ad libitum. The room was well-ventilated with 100 % fresh air.

2.3. Study design

This experiment consists of two parts: protective and therapeutic experiment. The first part of the experiment involves the protective effects of rifaximin against cytokine storm: Group HA (normal control): 10 apparently healthy mice that did not receive any intervention; Group LPS-P (induction): 10 mice received a single dose of intraperitoneal (IP) injection of 5 mg/kg lipopolysaccharide (LPS) and did not receive any intervention for the next seven days; Group MP-LPS: 10 mice received IP injection 50 mg/kg methylprednisolone once daily for three constitutive days [29], one hour after the last dose, received the same induction in

the LPS-P group, then left for two days without treatment. Group RIF-LPS: 10 mice received a 50 mg/kg IP injection of rifaximin for three constitutive days, then after one hour from the last dose, received the same induction in the LPS-P group, then left for two days without treatment; Group RIF-MP-LPS: 10 mice received 25 mg/kg IP injection of rifaximin plus 25 mg/kg methylprednisolone for three constitutive days [29], one hour after the last dose, they received the same induction in the LPS-P group and then left for two days without treatment, as seen in Fig. 1.

The second part of the study involves the therapeutic potential of rifaximin on cytokine storm: Group HA (normal control): 10 apparently healthy male mice that did not receive any intervention; Group LPS-T (induction): 10 mice received a single dose IP injection of 5 mg/kg LPS and did not receive any intervention for seven days; Group LPS-MP: 10 mice received the same induction as in the LPS-T group, after one hour, received IP injection methylprednisolone 50 mg/kg once daily for seven constitutive days [30]; Group LPS-RIF: 10 mice received the same induction as in the LPS-T group, after one hour, received rifaximin 50 mg/kg IP injection once daily for seven constitutive days. Group LPS-RIF-MP: 10 mice received the same induction as in the LPS-T group, after one hour, received an IP injection of 25 mg/kg rifaximin and an IP injection of 25 mg/kg MP once daily for seven constitutive days [30], as seen in Fig. 1. At the end of the experimental phase, the mice were anesthetized intraperitoneally with 80 mg/kg of ketamine and 10 mg/kg of xylazine [31–33]. After complete anesthesia, the mice were euthanized by exsanguination through cardiac puncture, a method appropriate for tissue collection and preservation [28]; blood and tissue samples were collected for further analysis.

2.4. Experimental protocol for cytokine storm induction

A single dose of LPS 5 mg/kg (Escherichia coli, serotype 055: B5, lot 0000133605/99 %) is administered intraperitoneally. The LPS solution was prepared according to the manufacturer's instructions (Sigma-Aldrich, Germany) by dissolving 10 mg of LPS powder in 10 ml normal saline (Pioneer, Iraq) in a glass tube and mixing by vortex for 30 minutes before each use. A cytokine storm was induced [34–37].

2.5. Clinical observations and animal care

All efforts were made to minimize the suffering and the number of animals involved in the experiments. The animal was monitored

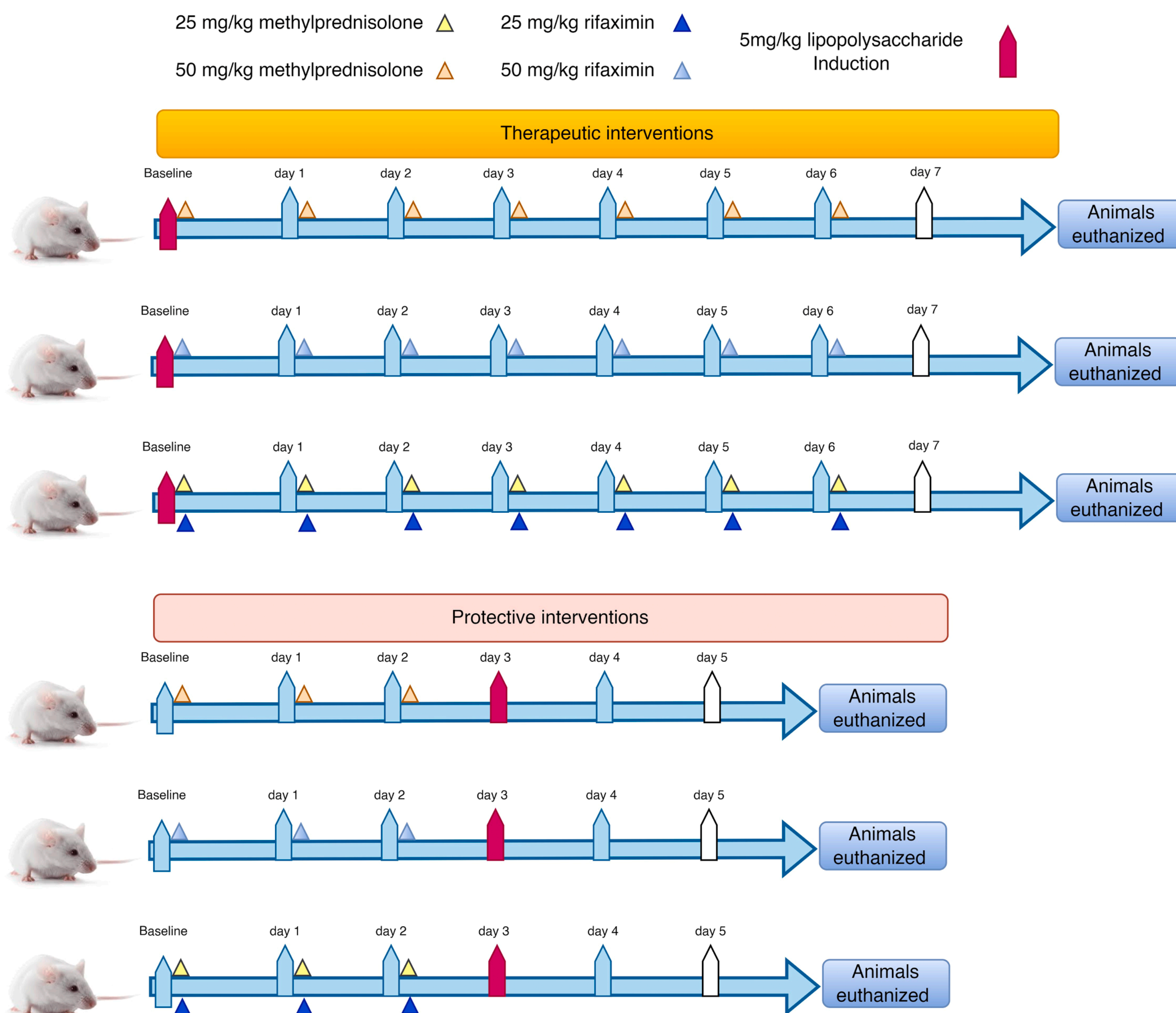

Fig. 1. Flow chart of the study.

immediately after the injection, about 10 minutes later, and the next day. If bleeding occurred, gauze was placed, and pressure was applied. Once the bleeding stopped, the site was cleaned with gauze and water. In case of peritonitis, laceration of internal organs, and/or infection, a veterinarian was consulted to assess whether the animal could continue in the experiment [38].

2.6. *Serum sample collection*

After 48 hours of LPS injection for protective intervention [39], and on day 7 of the therapeutic intervention [40], 1.0 – 1.5 ml of blood was obtained from the jugular vein [41] to determine the inflammatory and oxidative stress markers of all groups in the gel tube, the samples were allowed to clot for 15 min at room temperature. Then, the serum was separated by centrifugation at 3000 rpm for 10 minutes; the serum was deposited at −20℃ for subsequent thawing, and the quantitative determination of the biomarkers was determined in the serum of mice [42,43].

2.7. *Measurements of biomarkers (IL-1β, IL-6, IL-8, TNF-α, IFN-γ GSH, and MDA)*

Quantitative determination of the biomarkers in the serum of mice was detected by enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's directions (Sunlong, China). This ELISA kit uses the Sandwich-ELISA method for protein quantification. The kit's strip plate has been pre-coated with an antibody specific to biomarkers. Standards or samples are added to the appropriate wells and combined with the specific antibody. Then, a Horseradish Peroxidase (HRP)-conjugated antibody specific to biomarkers is added to each well and incubated. Free components are washed away. The TMB substrate solution is added to each well. Only those wells that contain biomarkers and HRP-conjugated antibodies will appear blue and then turn yellow after adding the stop solution. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm. The OD value is proportional to the concentration of biomarkers, ensuring accurate calculation of the concentration of proteins in the samples by comparing the OD of the samples to the standard curve.

2.8. *Histopathological examination*

All animals were euthanized (as mentioned in Section 2.3) at the end of the experiment. The vital organs (the lung and liver) were dissected and prepared using the formalin fixed paraffin embedded method to be sent for histopathological study and changes after induction of cytokine storm and treatments.

A. Chemical fixatives of the tissue: Organ samples of the mice (liver and lung) were kept in formalin (4 % formaldehyde in phosphate-buffered saline) to preserve tissue from degradation and to keep the building of the cell and sub-cellular components such as cell organelles (e.g., nucleus) [44].
B. Dehydration: This approach involves moving the samples via serial ethanol concentrations using a manual method: 70, 80, 90, and 100 percent ethanol for two hours, respectively.
C. Clearing: Two steps were used to mask the transparency of the tissue, remove the fats, and ensure that adequate water dehydration from the tissue was carried out, using xylol for 2 hours.
D. Embedding: In this process, paraffin wax is used at the melting point (57℃), and tissue is incorporated into a bath of paraffin wax for three hours to achieve wax filtration of the tissue. To be ready for cutting, the tissue is poured into blocks of pure wax. Then, give paraffin time to solidify overnight in a fridge. Sectioning: Each block was cut by a rotary microtome into serial segments. A suitable segment with the selected micrometer thickness (5–6) was spread out on the slide. To expand the segment, the 10 percent ethanol injection between the slide and section was very important; then, each slide was moved to a 40℃ drying oven for 24 hrs.
E. Staining: hematoxylin dye was dissolved in liquid alcohol and alum with the help of gentle fire and water. In a 500 ml boiling flask, the two solutions were mixed and brought to a boil quickly. Then mercuric oxide was added, and the mixture was instantly cooled by immersing the flask in cold water. When mercuric oxide was added, the solution assumed dark purple; the solution was transferred to an appropriate storage bottle. The eosin was prepared by dissolving 1 g of eosin with 70 % alcohol in 100 ml [45]. The staining of eosin and hematoxylin was done as follows:

For (10–15) minutes, sections were dewaxed in xylene.

In ethanol alcohol, the sections were rehydrated using a processing decreasing concentration of ethanol (99 percent, 90 percent, and 70 percent), then passed to the distilled water.

Hematoxylin stained the sections for 10 minutes and then transferred them to water. The section was divided into acid alcohol (100 ml of 70 % ethanol alcohol and 1 ml of HCl) as one dip.

By using flowing tap water, the bluing was accomplished.

Eosin stained the slides with (a few dips).

The parts were dehydrated with a rising ethanol alcohol concentration (70 %, 90 percent, and 99 percent).

Xylene clearing was conducted for 10 minutes.

The slides were enclosed by coverslips and surrounded by balsamic Canadian Histopathologists using a Zeiss Imager M2 microscope (Carl Zeiss Micro-Imaging) fitted with an Axio-CamHRc CCD camera (Carl Zeiss Microscope) to observe histopathological changes [46].

2.9. *Scoring of histopathological changes in liver*

Assessed the whole structure of the liver lung at 100x and 400x amplification; the damage score depends on four features: 1 indicates congestion, 2 indicates edema, 3 indicates infiltration by polymorphonuclear leukocytes, and 4 indicates necrosis. The summation of these scores was calculated and appointed as the total score at 400x amplification in 10 selected areas of the prepared slide [47].

2.10. *Ethical consideration*

The study was approved by the Research Ethical Committee of the College of Medicine, Al-Nahrain University, approval number (UNCOMIRB35902024), data (4 December 2022), following the American Veterinary Association Guidelines (AVMA) [28].

2.11. *Sample size calculation*

The software program G.Power was employed to calculate the sample size [48,49]. A post hoc sample size was done with an effect size of 0.42 and an alpha level of 0.05, 80 % power, F-family tests with a total sample size of 100, and 10 animals in each group.

2.12. *Statistical analysis*

The Kolmogorov-Smirnova test of normality was performed, and all variables followed normal distribution except for histopathological score. Ordinary one-way ANOVA with post hoc Tukey test is used to analyze normally distributed variables. In contrast, the Kruskal-Wallis test with The Two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli (correct for multiple comparisons by controlling the False Discovery Rate) was used for pair-wise comparison of not normally distributed variables. The significance level was defined by p-value $\leq$0.05 (alpha level). All analyses used GraphPad Prism version 10.2.0 for Windows, GraphPad Software, and Boston, Massachusetts, USA [50].

3. Results

3.1. Evaluation of protective effects of studied drugs

Comparison among the studied groups was made regarding the levels of TNF-α, IL6, IL8, IL1β, and IFN-γ, MDA, and GSH, in addition to histopathological pictures of ↱vital organs (lung, liver) in Swiss Albino mice, in which cytokine storm induced ↱by LPS after treatment with the studied drugs ↱↱[methylprednisolone (MP), and rifaximin (RIF)] to assess their protective effectiveness.↱

The serum levels of TNF-α, IL6, IL8, IL1β, IFN-γ, and MDA were ↱significantly elevated in the induction group compared to the control group, ↱indicating the severity of the cytokine storm. The serum level of ↱GSH was significantly higher in the induction group, suggesting a potential mechanism ↱of the protective effects of the studied drugs. These findings, presented in Figs. 2 and 3, are important in understanding the protective effects of the ↱drugs in the context of the cytokine storm.↱

The serum levels of TNF-α, IL6, IL8, IL1β, and IFN-γ were significantly lower in RIF, MP, and their combination than those in the induction group. RIF alone showed significantly higher TNF-α, IL6, IL8, IL1β, and IFN-γ levels than the MP-LPS group; simultaneously, RIF combined with MP showed significant differences compared to MP-LPS, as seen in Fig. 2.

he serum level of MDA was significantly lower in the RIF, MP, and their combination than in the induction group. The serum level of GSH was significantly higher in the RIF, MP, and their combination than in the induction group, as seen in Fig. 3.

MDA levels in the RIF-LPS group were statistically higher than those in the MP-LPS group, while those in the RIF-MP-LP group were significantly lower than those in the MP-LPS group (Fig. 3A).

GSH levels were significantly lower in the RIF-LPS group than in the MP-LPS group, and there was a statistical difference between the RIF-MP-LPS and the MP-LPS group, as seen in Fig. 3B.

3.2. Evaluation of therapeutic effects of studied drugs

Comparison among the studied groups was done in the levels of TNF-α, IL6, IL8, IL1β, IFN-γ, MDA, and GSH; and histopathological pictures for vital organs ↱↱(lung, liver) in Swiss Albino mice in which cytokine storm induced by ↱LPS then treated with the studied drugs ↱↱(MP and RIF) to assess its ↱therapeutic effectiveness, as seen in Figs. 4 and 5.

TNF-α, IL6, IL8, IL1β, IFN-γ, and MDA serum levels were ↱significantly elevated. GSH was significantly lower in the induction group than in the control group, ↱as seen in Figs. 4 and 5. This indicates

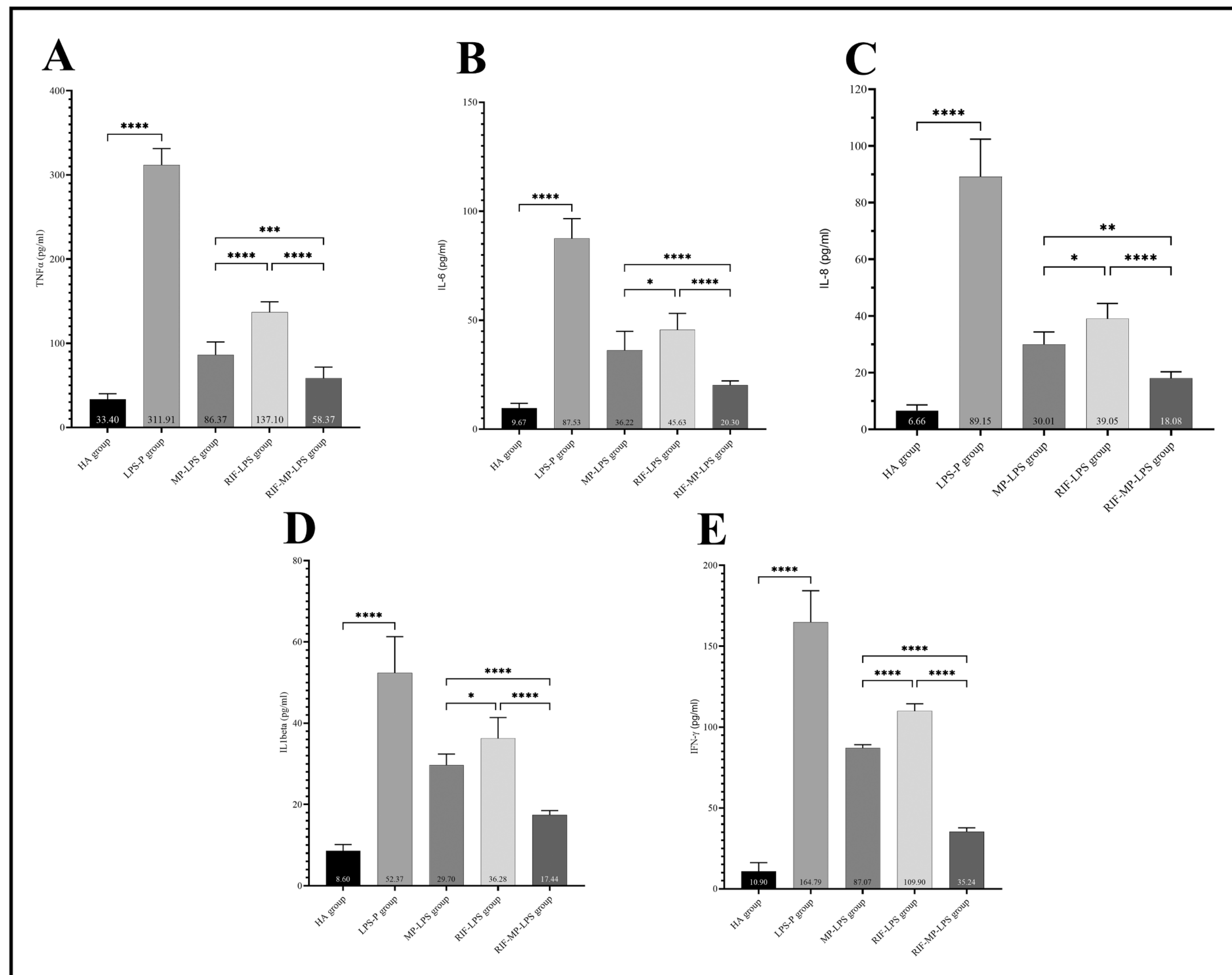

Fig. 2. Protective role of rifaximin and its combination with methylprednisolone on the inflammatory markers in cytokine-releasing syndrome in mice. A) serum TNF-α levels, B) serum IL6 levels, C) serum IL8 levels, D) serum IL1β levels, E) serum IFN-γ levels. Bar represents mean ± standard deviation (one-way ANOVA with post hoc Tukey test). ↱* Indicate p-value ↱↱<0.03, ** indicate p-value <0.002, *** indicate p-value <0.0002, **** indicate ↱p-value < 0.0001, ns indicate p-↱value ≥0.05.↱.

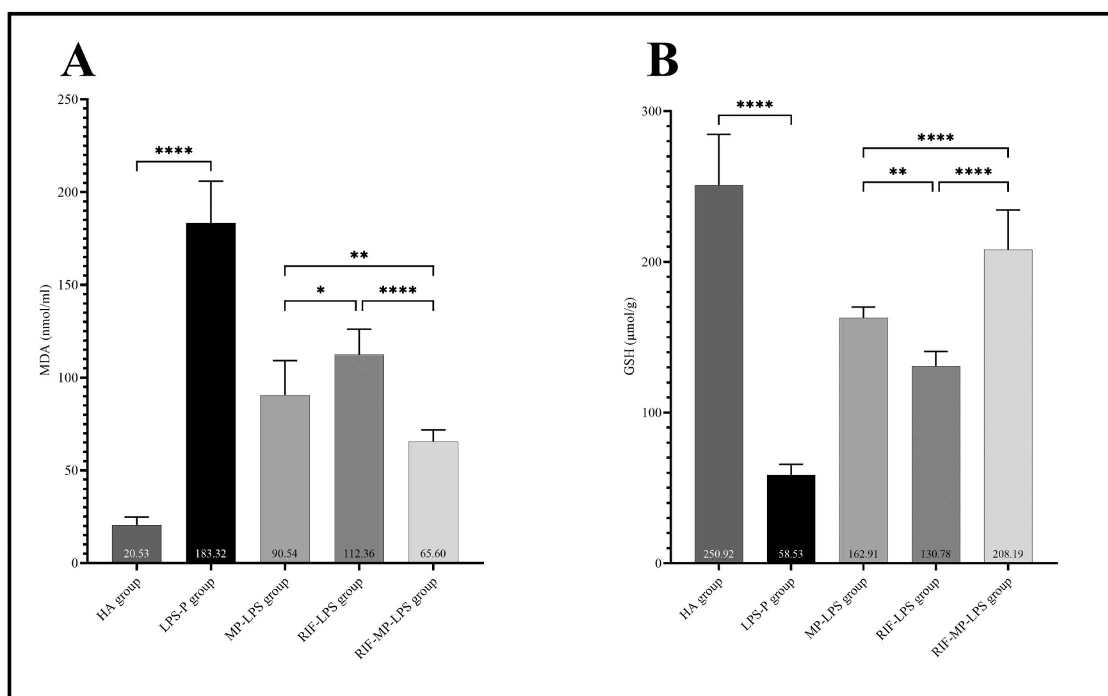

Fig. 3. Protective role of rifaximin and its combination with methylprednisolone on the oxidative stress markers in cytokine-releasing syndrome in mice. A) serum MDA levels, B) serum GSH levels. Bar represents mean ± standard deviation (one-way ANOVA with post hoc Tukey test). * Indicate p-value <0.03, ** indicate p-value <0.002, *** indicate p-value <0.0002, **** indicate p-value < 0.0001, ns indicate p-value ≥0.05.

the severity of the cytokine storm induced by LPS.

RIF alone showed significantly higher IL1β and IFN-γ levels than the LPS-MP group (no difference in IL-8, TNF-α, and IL6); at the same time, RIF combined with MP showed no significant differences compared to LPS-MP in IL1β but significantly lower TNF-α, IL6, IL8, and IFN-γ, as seen in Fig. 4.

RIF alone showed significantly higher MDA levels than MP monotherapy. The combination of RIF with MP showed significantly lower levels than MP monotherapy, as seen in Fig. 5A. RIF monotherapy showed no difference in GSH levels from MP monotherapy. RIF combined with MP showed significantly higher levels from MP monotherapy, as seen in Fig. 5B.

Details about the quantitative data of inflammatory and oxidative stress markers are illustrated in supplementary tables S1 and S2.

3.3. Histopathological examination of liver and lung tissue

3.3.1. Lung tissue

Lung sections of untreated animals showed normal lung architecture with thin interalveolar septa and clear alveoli, alveolar sacs, and normal alveolar septa with regular air sacs. The induction group shows severe acute inflammation with vascular congestion, capillary destruction, thick alveolar walls, and narrow air space with hyaline membrane formation. Mice treated with methylprednisolone, followed by LPS induction, showed mild interstitial inflammatory cell infiltration, mild vascular congestion, and intact alveolar space without rupture. Regarding rifaximin as a protective agent, mice treated with rifaximin + LPS group showed multifocal moderate inflammatory cell infiltration with mild congestion and intact alveolar. In contrast, the mice in the rifaximin + MP + LPS group showed mild vascular congestion and inflammatory cell infiltration with an intact alveolar membrane. Mice treated with LPS induction, followed by methylprednisolone, showed mild to moderate interstitial inflammatory cell infiltration, mild vascular congestion, and intact alveolar space without rupture. Regarding rifaximin as a therapeutic agent, mice treated with LPS + rifaximin group showed multifocal with moderate inflammatory cell infiltration with scarce dilatation and congestion and normal alveoli with intact membrane. In contrast, mice in the LPS + rifaximin + MP group showed mild focal interstitial inflammatory cell infiltration with vascular congestion, dilatation, and destruction of some of the alveoli, as seen in Fig. 6.

3.3.2. Liver tissue

The normal liver section in H&E stain revealed portal areas containing elements of the hepatic triad, that is, one or more small branches of the portal vein, a branch of the hepatic artery, and a small bile duct, along with lymphatic vessels and a very small amount of connective tissue. Liver cells are arranged in plates or cords. They radiate from the regions of central venules. LPS induction shows numerous vascular congestion and dilatation with edema, multifocal moderate mixed inflammatory cell infiltrations, and multifocal degeneration of hepatocytes with necrosis of hepatocytes. Mice treated with methylprednisolone, followed by LPS induction, showed vascular congestion, dilatation, and mild mixed inflammatory cell infiltration. Regarding rifaximin as a protective agent, mice treated with rifaximin + LPS group showed severe vascular congestion and dilatation with edema, moderate mixed inflammatory cells infiltration, moderate lobular hepatocyte degeneration with necrosis. In contrast, mice in the rifaximin + MP + LPS group showed mild vascular congestion and dilatation with edema, mild inflammatory cell infiltration, and mild hepatocyte degeneration with mild necrosis. All groups showed a significant reduction in the total liver score compared to the induction group, and all groups showed significantly higher liver scores than the control group. RIF alone or combined with MP showed significantly higher liver scores than the MP-LPS group, as illustrated in Fig. 7.

Regarding rifaximin as a therapeutic agent, mice treated with LPS + rifaximin group showed mild vascular congestion and dilatation with moderate edema, mixed inflammatory cell infiltration, and moderate lobular hepatocyte degeneration with mild necrosis. In contrast, mice in the LPS + rifaximin + MP group showed mild vascular congestion and dilatation with edema, mild inflammatory cell infiltration, and mild hepatocyte degeneration with mild necrosis. All groups showed a

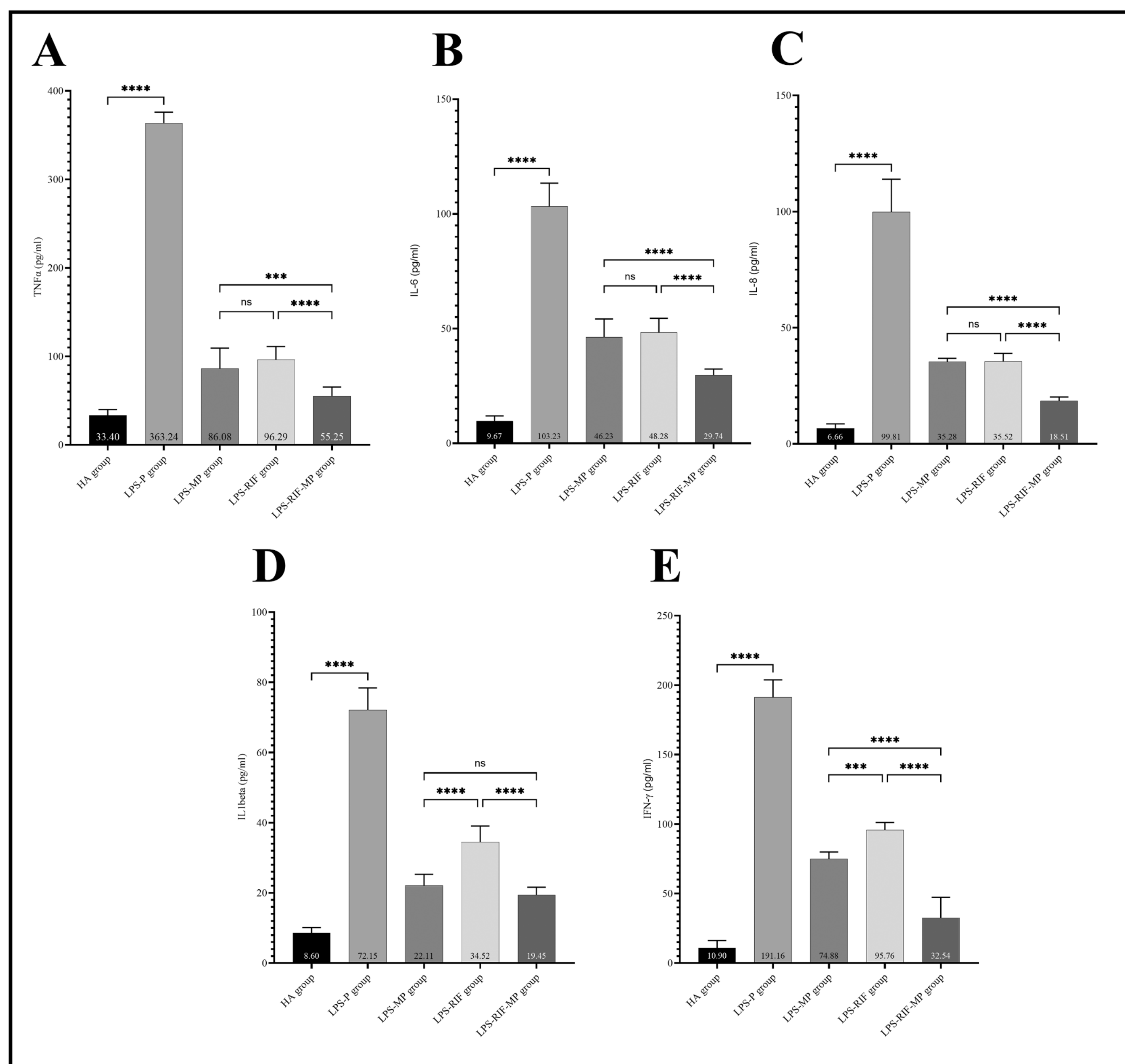

Fig. 4. Therapeutic role of rifaximin and its combination with methylprednisolone on the inflammatory markers in cytokine-releasing syndrome in mice. A) serum TNF-α levels, B) serum IL6 levels, C) serum IL8 levels, D) serum IL1β levels, E) serum IFN-γ levels. Bar represents mean ± standard deviation (one-way ANOVA with post hoc Tukey test). * Indicate p-value <0.03, ** indicate p-value <0.002, *** indicate p-value <0.0002, **** indicate p-value < 0.0001, ns indicate p-value ≥0.05.

significant reduction in the total liver score compared to the induction group and significantly higher liver scores than the control group. RIF alone showed significantly higher liver scores compared to the MP-LPS group. Combined with MP, RIF shows insignificant differences compared to MP-LPS groups, as illustrated in Fig. 7.

4. Discussion

4.1. Cytokine storm induction

LPS, or endotoxin in general, is implicated in developing and progressing different pathophysiological changes by releasing many pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 [51]. In addition, LPS inhibits the expression of anti-inflammatory cytokines such as IL-10 and IL-4 in animal model studies [52]. Upregulation of the cytokine system by LPS may propagate to induce the development of CRS, as in severe bacterial and viral infections [53]. Moreover, abnormal immune response triggers excessive pro-inflammatory cytokine release, leading to multiple organ injury [54]. Of interest is that LPS-induced abnormal inflammatory response may provoke the development of oxidative stress either by increasing the generation of reactive oxygen species (ROS) or by inhibiting endogenous antioxidant enzymes such as GSH [55]. In addition, oxidative stress can exacerbate organ injury through lipid peroxidation that may induce further inflammatory reactions and the progression of CRS [55]. Therefore, exogenous LPS seems to be the best candidate in the induction of the release of pro-inflammatory cytokines and the development of CRS in animal model studies.

In the present experimental study, IP administration of LPS in mice triggers a significant release of pro-inflammatory cytokines compared

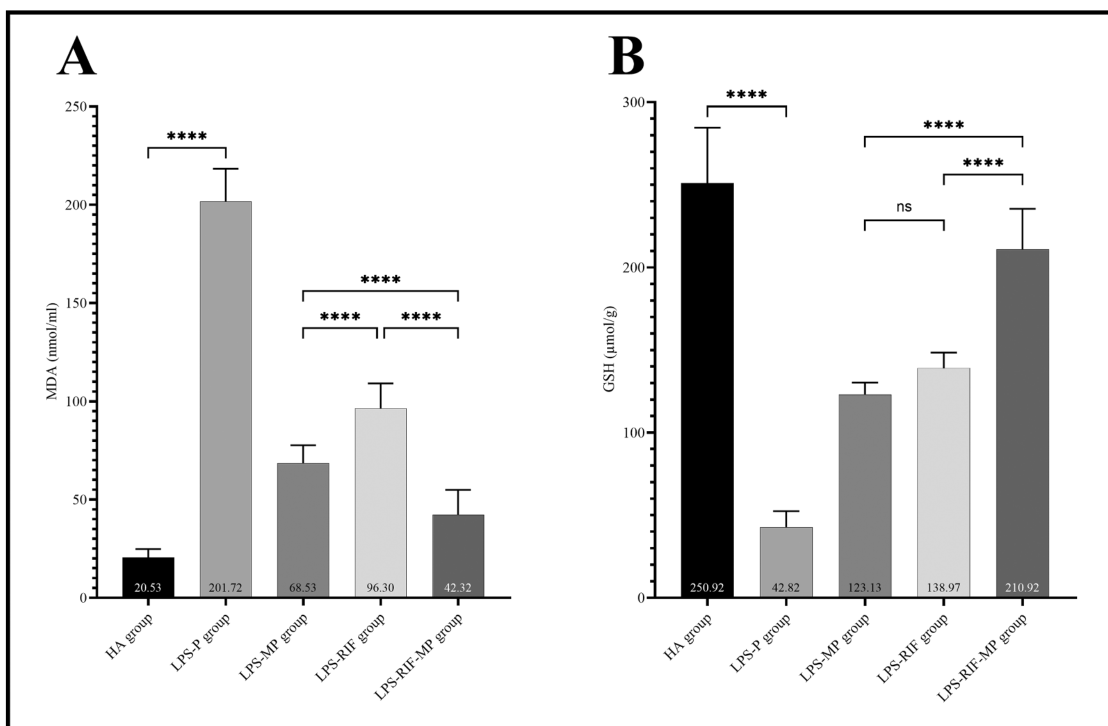

Fig. 5. Therapeutic role of rifaximin and its combination with methylprednisolone on the oxidative stress markers in cytokine-releasing syndrome in mice. A) serum MDA levels, B) serum GSH levels. Bar represents mean ± standard deviation (one-way ANOVA with post hoc Tukey test). * Indicate p-value <0.03, ** indicate p-value <0.002, *** indicate p-value <0.0002, **** indicate p-value < 0.0001, ns indicate p-value ≥0.05.

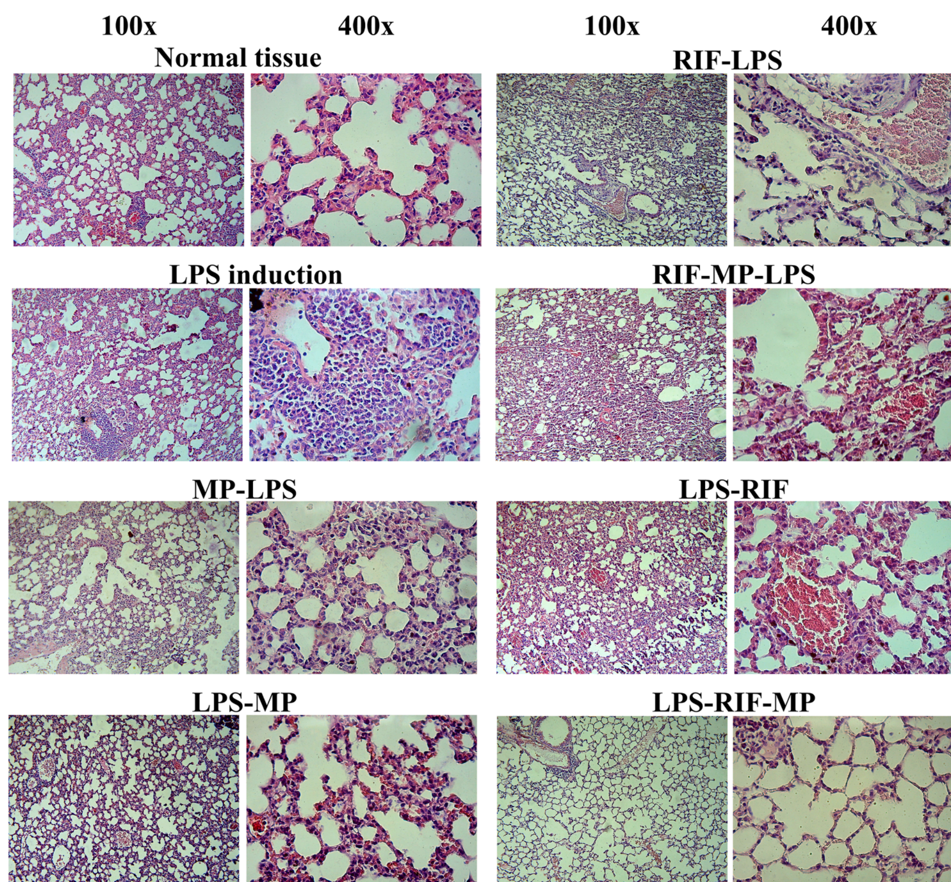

Fig. 6. Effect of various treatment groups on mice lung tissue under the light microscope showing the protective and therapeutic effects of rifaximin, methylprednisolone, and their combination. Magnification: 100x and 400x, H & E stain. *RIF: rifaximin, MP: methylprednisolone, LPS: lipopolysaccharide.*

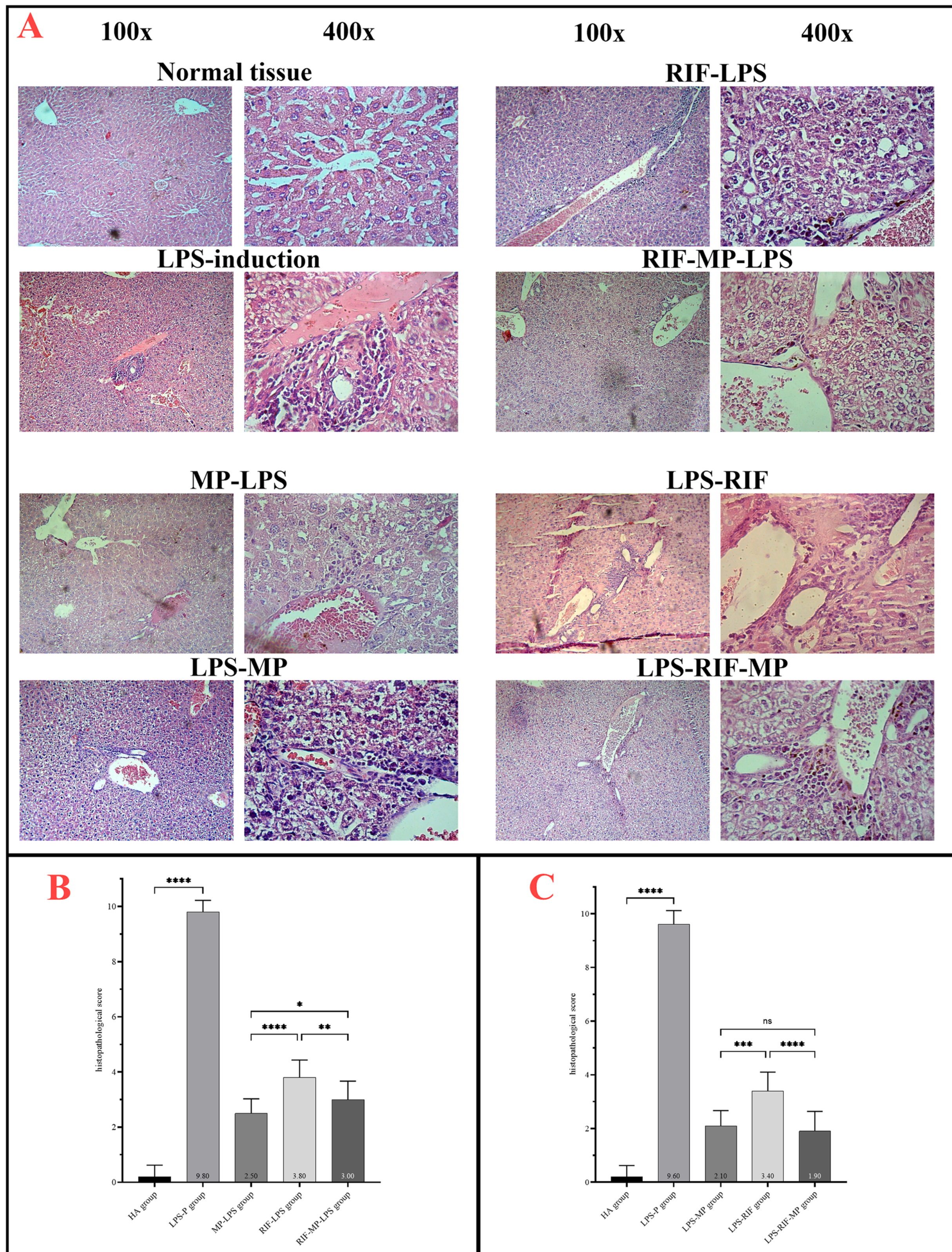

Fig. 7. A) The Effects of various treatment groups on mice liver tissue under the light microscope show the protective and therapeutic effects of rifaximin, methylprednisolone, and their combination. Magnification: 100x and 400x, H & E stain. B) Histopathological score of live tissue showing the protective effects of rifaximin, methylprednisolone, and their combination. C) Histopathological score of live tissue showing the therapeutic effects of rifaximin, methylprednisolone, and their combination. Bar represents mean ± standard deviation. * Indicate p-value <0.03, ** indicate p-value <0.002, *** indicate p-value <0.0002, **** indicate p-value < 0.0001, ns indicate p-value ≥ 0.05.

to healthy control mice. Besides, LPS led to the development of oxidative stress, as evidenced by increasing MDA serum level (a biomarker of lipid peroxidation) and reduction of antioxidant GSH serum level in LPS-treated mice compared to healthy control mice. The present study's findings are supported by many preclinical studies that observed potential detrimental effects of LPS through induction of the release of the pro-inflammatory cytokines and the development of oxidative stress [54,55]. Therefore, the main objectives of the present study are to modulate the effects of LPS either by inhibiting its effect (preventive) or attenuating its harmful effect (therapeutics).

4.2. Prevention of LPS-induced CRS

4.2.1. Effects of methylprednisolone

MP is a glucocorticoid commonly used to prevent acute and chronic inflammatory and autoimmune disorders [56]. MP prevents LPS-induced vascular stiffness caused by chronic inflammation [57]. In the present study, MP pretreatment reduced TNF-α, IL-6, IL-8, IL-1β, and INF-γ serum levels in LPS-treated mice compared to the LPS-induced CRS model. Also, MP pretreatment reduced MDA serum levels and increased GSH serum levels in LPS-treated mice compared to the LPS-induced CRS model. Furthermore, MP prevents tissue injury in both the lung and liver when administered before LPS in the experimental mice.

MP has strong anti-inflammatory effects by inhibiting the expression of pro-inflammatory genes during acute and chronic inflammatory disorders [56]. In particular, MP attenuates the expression of TNF-α mRNA and its release from immune cells [58]. In addition, MP attenuates the severity of inflammatory reactions by inhibiting the release of IL-6 and IL-8 in patients with severe COVID-19 [59]. Furthermore, MP reduces oxidative stress and lipid peroxidation by inhibiting the accumulation of cholesterol and triglyceride in macrophages in *C57L/6* mice [60]. Moreover, acute but not chronic administration of MP mitigates acute lung injury in rat models by reducing the propagation of lipid peroxidation measured by MDA level and total reactive antioxidant potential [61]. Ultimately, in virtue of its anti-inflammatory and antioxidant effects, MP can prevent the development of CRS.

Recently, COVID-19-induced CRS has gained a great reputation concerning corticosteroid treatment [62]. MP and other corticosteroids prevent the development of acute respiratory failure in severely affected COVID-19 patients by inhibiting the development of hypercytokinemia and CRS [62]. Despite conflicting and controversial findings regarding the use of corticosteroids in COVID-19, however early treatment with corticosteroids can reverse CRS-induced organ injury in severely affected COVID-19 patients with acute lung injury and ARDS [63]. It has been suggested that MP is more effective than IL-6 antagonists in mitigating CRS [64]. It has been observed that MP decreases the CRS-induced central neurological complications more than IL-6 receptor antagonist tocilizumab, which cannot cross BBB [64]. Therefore, these findings indicated that MP could prevent the development and progression of CRS by inhibiting the release of pro-inflammatory cytokines, activating the expression of anti-inflammatory cytokines, and inhibiting oxidative stress.

MP has been shown to reduce the development and progression of acute lung injury and acute liver injury by reducing oxidative stress, hyperinflammation, and the development of CRS [65]. Corticosteroids generally improve lung oxygenation and prevent paraquat-induced acute lung injury in mice [66]. Like other corticosteroids, MP attenuates neutrophil influx into the lung, reduces macrophage activation, and prevents airway fibrosis [67]. Moreover, corticosteroids reduce the development of acute lung injury induced by large-volume ventilation in animal models by reducing the expression of pro-inflammatory cytokines and neutrophil elastase and increasing IL-10 in the bronchial alveolar fluid. In addition, MP prevents alveolar cell apoptosis by downregulating apoptotic signaling such as caspase-3 and Bax and upregulating anti-apoptotic Bcl-2 [68]. MP can prevent acute liver injury induced by oxidative stress and hyperinflammation in LPS-induced CRS. It has been observed that MP has a hepatoprotective effect by preventing liver ischemic-reperfusion injury in mice [69].

Similarly, MP prevents the development of acute and chronic liver failure in mice [70]. However, a high dose of IV but not oral MP may induce acute liver injury in patients with multiple sclerosis [71]. A systematic review and meta-analysis observed that MP could be an effective therapeutic strategy against drug-induced acute liver injury [72]. Therefore, MP has potent anti-inflammatory and antioxidant effects and can prevent CRS-induced acute lung injury and acute liver injury.

4.2.2. Effects of rifaximin

RIF in the present study reduced TNF-α, IL-6, IL-8, IL-1β, and INF-γ serum levels in LPS-treated mice compared to the LPS-induced CRS model. RIF also reduced MDA and increased GSH levels compared to the LPS-induced CRS model. Therefore, RIF can temper the development and progression of CRS induced by LPS in mice. Supporting these findings, RIF was reported to prevent endotoxin-induced expression of the pro-inflammatory cytokines in patients with non-alcoholic fatty liver disease [73]. A randomized controlled clinical trial illustrated that RIF improves liver metabolism in patients with alcoholic hepatitis without significant effects on inflammatory reactions and pro-inflammatory cytokines [74].

Conversely, Patel *et al.* found that RIF regulates gut-barriers and prevents the development of hepatic encephalopathy by reducing the expression of pro-inflammatory cytokines and the development of systemic inflammation [75]. Moreover, RIF has a potent anti-inflammatory effect via activation of the pregnane X receptor, which mediates intestinal epithelial cells' detoxification [76]. In the present study, RIF prevented the development of oxidative stress by reducing MDA and increasing GSH. As a result of the findings of our experiment, many studies have illustrated that RIF has antioxidant effects. Omar et al. confirmed that RIF prevents malathion-induced testicular toxicity in mice by suppressing oxidative through mitophagy modulation [77].

Furthermore, RIF prevents LPS- and iron-overload-induced neurotoxicity in SH-SY5Y by inhibiting the development of oxidative stress [78]. Interestingly, RIF can prevent the development of CRS by inhibiting the development and progression of oxidative stress and regulating the pro-inflammatory/anti-inflammatory axis. It has been suggested that RIF may prevent the development of CRS following gastrointestinal infection by SARS-CoV-2 [79]. Indeed, RIF attenuates the production of pro-inflammatory cytokines from intestinal mucosa, thereby preventing CRS development in patients with Crohn's disease [80]. These observations suggest that RIF could be an effective therapeutic strategy for preventing LPS-induced CRS by suppressing inflammatory and oxidative stress disorders.

Histopathologically, RIF is shown to prevent the development of acute lung injury and acute liver injury, as evident by the findings of the present experimental study. Chen *et al.* illustrated that RIF, through regulation of the gut-lung axis, can attenuate influenza A virus-induced acute lung injury [81]. As RIF is not absorbed from GIT, Kirby et al. confirmed that aerosolized RIF reduces *Pseudomonas aeruginosa*-induced pneumonia and associated acute lung injury in mice [82]. Correspondingly, RIF enhances survival in patients with decompensated liver cirrhosis through modulation of systemic inflammation and oxidative stress [83]. An observational study highlighted that two-year treatment with RIF tempers the severity of hepatic encephalopathy [84].

These studies, coupled with current findings, indicate that RIF alone has protects against acute lung and liver injury. However, the protective effect of RIF against LPS-induced CRS was less effective than that of MP in reducing pro-inflammatory cytokines and oxidative stress disorders in mice. Therefore, combining RIF with MP to produce a more preventive effect against LPS-induced CRS in mice is reasonable.

A combination of RIF with MP reduced TNF-α, IL-6, IL-8, IL-1β, and INF-γ serum levels in LPS-treated mice compared to MP-treated and RIF-treated groups. In addition, the RIF and MP combination reduced MDA and increased GSH levels compared to MP-treated and RIF-treated groups. Also, RIF in combination with MP had a protective effect against experimental acute liver injury, which was insignificant compared to MP-treated and RIF-treated groups. These findings suggest that RIF boosts the anti-inflammatory and antioxidant effects of MP by reducing the pro-inflammatory cytokine expression and inhibiting oxidative stress. Jigaranu et al. revealed that a combination of RIF and prednisolone was more effective than prednisolone alone in mitigating the severity of Crohn's disease [85]. Thus, combining RIF and MP led to a more preventive effect than RIF in preventing LPS-induced CRS in mice.

4.3. *Treatment of LPS-induced CRS*

4.3.1. *Effects of MP*

The present study's findings illustrated that MP reduced the toxic effects of LPS-induced CRS in mice by down-regulating pro-inflammatory cytokines and the expression of MD without significantly affecting the GSH compared to the control group. In addition, MP attenuates the progression of acute lung injury and acute liver injury associated with CRS in mice subjected to LPS. In different studies supporting the present study's finding, Bourbon et al. found that treatment with MP reduced systemic inflammatory response induced by cardiopulmonary bypass [86]. A previous experimental study illustrated that administration of MP following experimental spinal cord injury in rats decreased the release of IL-6 and TNF-α by its anti-inflammatory effect [87]. Like other corticosteroids, MP effectively reduces acute lung injury /ARDS in severely affected COVID-19 patients. In addition, MP reduces IL-6 serum levels in COVID-19 patients, suggesting the efficacy of MP in treating CRS [88]. Xian et al. revealed that MP decreases COVID-19 severity through inhibition of IL-6 and the functional activity of ACE2 [59]. MP can be an effective therapeutic strategy in treating cervical myelopathy by inhibiting ischemic reperfusion injury and related spinal cord injury by inhibiting IL-8 mRNA expression [89]. In addition to its anti-inflammatory effect, MP has a potential therapeutic effect against organ injury by decreasing the harmful effect of oxidative stress. Akarsu et al. disclosed that the clinical efficacy of MP against Graves' disease is related to the inhibition of MDA, a biomarker of oxidative stress [90]. Moreover, pulse doses of MP reduce the severity of COVID-19 by inhibiting the propagation of CRS [62]. It has been shown that MP and other corticosteroids effectively treat acute lung injury. Prolonged use of MP reduces mechanical ventilation duration and improves lung oxygenation by anti-inflammatory and antioxidant effects in patients with acute lung injury and ARDS [65]. Furthermore, MP can treat HBV-induced liver injury by reducing inflammatory and oxidative stress [91]. Thus, MP seems effective in treating CRS and associated organ injury.

4.3.2. *Effects of rifaximin*

The present study's findings revealed that the administration of RIF following LPS administration reduced the levels of pro-inflammatory cytokines and MDA, with a significant elevation of GSH serum levels. Besides, RIF reduced acute lung and liver injury severity in mice subjected to LPS. The present study illustrated that the RIF effect was comparable to MP's in reducing TNF-α, IL-6, and IL-8 serum levels. However, the RIF effect was less effective in reducing INF-γ and IL-1β than the MP-treated group. Also, RIF was less effective than the MP-treated group in reducing MDA serum level but was equivalent to mitigating GSH serum level. Similarly, RIF was less effective than MP in treating acute liver injury but comparable to MP against acute lung injury in mice treated with LPS. These findings suggest RIF's potential therapeutic efficacy in treating LPS-induced CRS in mice.

Studies have confirmed that RIF protects against LPS-induced inflammation and oxidative stress [73,92]. RIF mitigates liver fibrosis induced by ethanol by maintaining the integrity of the intestinal barrier by suppressing the detrimental effect of ethanol on the epithelial cell tight junction and apoptosis [92]. Of note, oxidative stress due to gut-derived LPS is implicated in the pathogenesis of non-alcoholic fatty liver disease [55]. Treatment with RIF reduces the circulating level of pro-inflammatory/inflammatory cytokines and endotoxins in patients with non-alcoholic fatty liver disease [73]. It has been reported that treatment with RIF 1100 mg/day for six months reduced pro-inflammatory cytokine levels and liver fat scores in patients with non-alcoholic fatty liver disease [93]. However, a clinical trial showed that RIF 800 mg/day for six weeks was ineffective in the management of non-alcoholic fatty liver disease, which might be due to the low therapeutic dose of RIF and the short duration of treatment [94].

Moreover, RIF alleviates liver injury by modulating gut microbiota and oxidative stress [95]. RIF also reduces the development of acute lung injury and ARDS in severe respiratory viral infections via modulation of the gut-brain axis [81]. These verdicts highlighted the therapeutic efficacy of RIF in treating inflammatory and oxidative stress disorders and associated organ injury against endotoxin-induced inflammatory reactions. Many studies revealed that RIF reduces the absorption of endotoxins from intestines, thereby mitigating systemic inflammation and CRS development [96,97].

On the other hand, the combined effect of RIF plus MP was more effective than the MP-treated group in reducing pro-inflammatory serum levels, the elevation of GSH, and reducing MDA serum levels. Therefore, RIF may have an additive effect on the MP action in mitigating inflammatory and oxidative stress disorders against LPS-induced CRS. Consistent with this finding, a recent study conducted by Jimenez et al. illustrated that RIF could be an adjuvant treatment with corticosteroids in the management of acute and chronic liver failure in patients with alcoholic hepatitis [98]. Remarkably, RIF can be used as a monotherapy in patients with steroid resistance ulcerative colitis [99]. The present study indicated that RIF is an effective preventive and therapeutic measure against LPS-induced CRS in mice.

4.4. *Study limitations*

The Lipopolysaccharide (LPS)-induced paradigm is extensively utilized to investigate Cytokine Release Syndrome (CRS) and neuroinflammation. Nonetheless, it possesses certain shortcomings in comparison to alternative CRS models. Insufficient Specificity: LPS-induced models may not only focus on a specific tissue, resulting in systemic inflammation that can impact other organs. This is beneficial for systemic inflammation but less effective for organ-specific effects [100]. The cytokine profile elicited by LPS may vary from that of alternative CRS models, thus influencing the applicability of the findings to other CRS scenarios [101]. The effects of LPS can differ according on the administered dose, complicating the standardization of research and the comparison of data across investigations [101].

The translational potential of findings from LPS-induced models to human subjects is promising. These models assist in identifying prospective therapeutic targets and assessing the effectiveness of innovative treatments [102]. However, there are several challenges in applying these findings to human patients:

- Species Disparities: The immune systems of mice and humans exhibit substantial differences, impacting the relevance and applicability of the findings.
- Human diseases exhibit multifactorial complexity, rendering them more intricate than the conditions simulated in animal models, hence complicating the replication of precise pathophysiology.
- Variability in Human Populations: Human patients exhibit genetic diversity, and characteristics like as age, sex, and comorbidities might affect treatment outcomes, complicating the generalization of findings from animal models.

5. Conclusions

Rifaximin showed both protective and therapeutic effects in LPS-induced cytokine storms in mice, which are induced by anti-inflammatory and antioxidant pathways. Rifaximin, in combination with methylprednisolone, shows more potent anti-inflammatory and antioxidant effects, which indicates that this combination has synergistic potential in LPS-induced cytokine storms in mice. These findings are further validated histopathologically in lung and liver tissues.

Further studies are required to examine the dose-response effect of RIF, longer duration of LPS induction which will facilitate examining the effect of RIF with or without MP on survival analysis, a different modes of CRS induction like using an anti-CD3 monoclonal antibody, and final dependent molecular mechanism of action.

Ethics approval

The study was approved by the Research Ethical Committee of the College of Medicine, Al-Nahrain University, approval number (UNCOMIRB35902024), data (4 December 2022), following the American Veterinary Association Guidelines (AVMA) [28].

Consent to participate

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CRediT authorship contribution statement

Ahmed R. Abu-Raghif: Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Investigation, Conceptualization. **Marwa Salih Al-↱Naimi:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Data curation, Conceptualization. **Hayder Adnan Fawzi:** Writing – review & editing, Writing – original draft, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxrep.2024.101808.

Data availability

Data will be made available on request.

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