Original
ISSN 2413-0516

The Protective Effect of Ramelteon and in Combination with Dexamethasone on the Lipopolysaccharide-Induced Cytokine Storm in Mice

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(Submitted: 06 August 2023 – Revised version received: 22 August 2023 – Accepted: 06 September 2023 – Published Online: 29 October 2023)

Abstract
Cytokine Storm Syndrome (CSs) is a potentially life threatening condition, characterized by robust elevated; of circulating pro-inflammatory cytokines; occurring after a hyperactive immune system. Is a well-known as a worldwide health problem, leading to multi-organ failure and death.
Objectives: This study was carried out to investigate the protective role and probability of additive or synergistic anti-inflammatory activity; of ramelteon and in combination with dexamethasone on the lipopolysaccharide (LPS) induced "Cytokine Storm" on mice model and its potential regulatory mechanism(s).
Methods: Sixty Swiss albino male mice of; (25 ± 5 grams; 8–12 weeks) had free access to food and water. After 2 weeks of adaptation, mice; randomly separated in five groups ($n = 12$): Group I, mice received (0.9% N/S i.p.); Group II, mice received (5 mg/kg i.p.) LPS only. Group III, mice received (2.5 mg/kg, i.p.) dexamethasone, Group IV, mice received (100 mg/kg i.p.) ramelteon, Group V, mice received half dose of dexamethasone+ ramelteon combination (1.25 mg/kg i.p +50 mg/kg i.p). For systematic inflammatory stimulation mimicking "cytokine storm" LPS; *E. coli* O55:B5 (5 mg/kg i.p.) was induced within one hr. After 48 h the effects of interventional agents and vehicle or LPS challenge; on lung, heart, liver, kidney histopathological changes, and levels of inflammatory cytokines: (IL-6, IL8, IL-1β, and TNF-α) in the serum were detected.
Results: IL-1β, IL-6, IL8 and TNF-α elevated serum levels significantly reduced ($P < 0.001$) in all treatment group. Additionally, they ameliorated the histopathological changes induced by (LPS) and improving macroscopic scores ($P < 0.001$).
Conclusion: In conclusion, ramelteon treatment had a diverse protective effects against "Cytokine Storm" with a mechanism based on attenuation serum levels of inflammatory cytokines (IL-1β, IL-6, IL-8 and TNF-α) and through reduction of histopathological damage during endotoxemia induced via LPS challenge on male mice model. RAM/DEX combination had superior advantage than an agent use alone probably via synergistic anti-inflammatory activity.
Keywords: Cytokine storm, inflammation, lipopolysaccharide, ramelteon, dexamethasone

Introduction

Cytokine Storms (CS) are potentially fatal "hyperinflammatory-states" that share the underpinnings of persistent immune cell activation and uninhibited over cytokine production) "hypercytokinemia".[1] Such a profuse upsurge in cytokines, chemokines, interferons and other growth factors; initiating a "severe hyper inflammatory illness", which owing to its severity, can be life threatening; subsequently of multi-organ failure, or, at best, prime "irreversible tissue damage" owing to the fibrosis.[2] Cytokine storms linked with various pathologies: of infectious and non-infectious diseases[3] such as graft versus-host illness.[4] Infections of numerous relevant agents, as viruses[5] bacteria,[6] fungi,[7] autoimmune diseases,[8] or even acute pancreatitis,[9] a distinct mention should be made of the cytokine storm concomitant with SARS-CoV-2 infection that instigated so many deaths in the COVID-19 pandemic,[10] hence accounting for 19.7% of global mortality worldwide.[11] In the specific case of an infectious process; associated with cytokine storm; inflammation arises when "macrophage-like cells" of the innate immune-system identify pathogen-derived stimuli, pathogen-associated molecular pattern; (PAMPS),[12] by pattern recognition receptor (PRR) that express TLR and NOD as well as NOD-like receptors (NLR) that each recognize a particular class of (microbial PAMP) including: endotoxins primarily lipopolysaccharide (LPS) on the exterior membrane of gram-negative bacteria. Additional stimuli will come from self-molecular structures resultant from damage; due to cells and tissues, which well known as damage-associated molecular patterns (DAMPS).[13] The whole process will generate a cascade of pro-inflammatory cytokine over production; that regulating the duration and intensity of the immune-response to the pathogen.[14,15] Consequently, the (CSS) is result from an exaggerated production, of soluble pro-inflammatory and profibrotic mediators (especially IL-6, IL-8, IL-1β, and TNF-α), through (NF-κB signaling-pathway).[16] Together with a deviant immunopathological feedback concerning; a lack of synchronization between the innate and adaptive immune-system with an overactivation of the innate immunity. The central cellular actors being macrophages, monocytes, dendritic cells, neutrophils, and T-lymphocytes[17,18] as well as, a condition of hyperinflammation in multi-organs, was prompted; mainly disturbing the lung, often leading to acute respiratory distress syndrome and/or acute lung injury. Furthermore, an injury in pulmonary, cardiovascular, and renal tissues could occurred.[19,20] Currently, no effective treatment exists for the (CSS). One of the candidate compounds with a potential for the treatment of the cytokine storm-induced "inflammatory process" is ramelteon (RAM). Based on the capability of melatonin in the management of the (deadly inflammatory phenomenon) and the

predicted dual protective role of melatonin against both the initiation and exacerbation of "inflammation and oxidative stress" in various variables, hence, ramelteon (RAM) was considered in this regard for its capability to control an induced hyperinflammatory disorders. (RAM), as a synthetic selective melatonergic agonist drug, for melatonin MT1 and MT2 receptors, being involved in the circadian rhythms maintenance that regulating the normal sleep-wake cycle,[21] used clinically in the management of chronic insomnia.[22] (RAM) has no potential for drug addiction or dependence because it has been exposed to avoid the distraction associated with neurotransmitter receptor-drugs.[23]

Materials and Methods

Animals

Sixty Swiss Albino male mice of (25 ± 5 grams) aged (8–10 weeks); were used in the experiments. (The animals; were gained from the Animal House Facility, National Center for Drug-Quality Control and Research in Baghdad, Iraq). And kept; in the animal house of the college of pharmacy/Al Nahrain University; in a standard plastic cages at 22°C ± 2°C; relative humidity and the air of the room was changed continuously by using ventilating vacuum under a 12 hours light-dark cycle. All the animals were allowable free access, to food and tap water (*ad libitum*). Mice were allowed (two weeks) to acclimate to the animal house environment before commencement of our experiment. (All animal care and experiments were approved by the Animal Ethical Committee at Al-Nahrain University, College of Pharmacy, under the issue number; PH-Nah 4).

Chemicals

Preparation of drugs working solution

For ramelteon (RAM; Hangzhou hyperchem. limited/China, > 98% purity), dexamethasone (DEX; Sigma-Aldrich, St. Louis, MO, USA, >98% purity) working solution, it were prepared by freshly dissolving in suitable solvent Dimethyl formamide DMF (BDH, chemical, Ltd. Poole, England), dimethyl sulfoxide 1% DMSO (Chem-lab NV, Belgium) respectively; The resulting solution then was diluted with a sterile double distilled water (DDW) immediately one hr. before (LPS) administration as single dose (i.p.).

LPS-induced cytokine storm mice model

Preparation of lipopolysaccharide working solution

The stock solution of LPS (Sigma-Aldrich, Co.; *E. coli* serotype O55:B5) prepared by dissolving 10 mg of (LPS) lyophilized powder in 10 mL of 0.9% normal saline (Pioneer/Iraq) in a glass tube and mixed by vortex for 30 minutes before each use. For a dose of (5 mg/kg, i.p). (About 0.1–0.125 ml of the solution according to weight), was injected intraperitoneally for each animal.

Healthy animals must be weighted; prior to each injection to accurately calculate the dosage of LPS. After that, mice were monitored; at the time of injection and every 2 hr. after the injection for 8 hr. for the occurrence and severity of endotoxemia (Figure 1).

Male mice were injected (LPS; 5 mg/kg, body weight); injected intraperitoneally as single dose. (The animals were allocated randomly in to sixth groups (n = 12 per group) as follows:

Group I: Twelve mice will receive an equal volume of (0.9%) of normal saline intraperitoneally (IP) injected into the group II.

Group II: Twelve mice will receive (5 mg/kg) LPS **lipopolysaccharide** only intraperitoneally (according to manufacturer's instructions).

Group III: Twelve mice will receive 2.5 mg/kg **dexamethasone** intraperitoneally (used as standard anti-inflammatory control).Then within one hour will receive (5 mg/kg) LPS lipopolysaccharide intraperitoneally.

Group IV: Twelve mice received 100 mg/kg **ramelteon** intraperitoneally then within one hour will receive (5 mg/kg) LPS lipopolysaccharide intraperitoneally will be injected.

Group V: Twelve mice received combination (1.25 mg/kg **dexamethasone** +50 mg/kg **ramelteon**) then receive (5 mg/kg) LPS lipopolysaccharide intraperitoneally within one hour.

The doses of a number of agents that was used in current study were selected according to prior experimental animal studies.[24-27]

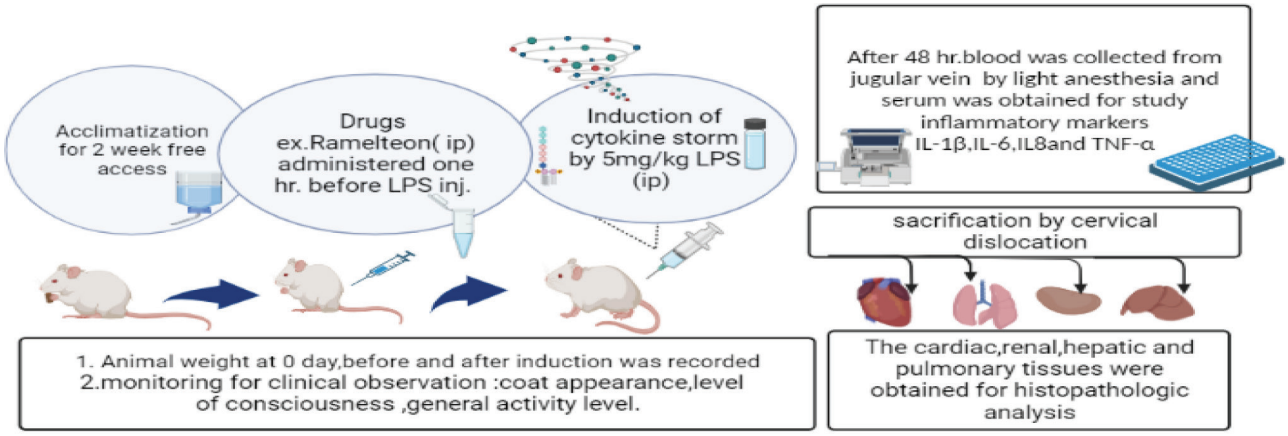

Fig. 1 **Overview of study protocol for cytokine storm induction with lipopolysaccharide on mice, model. This figure designed with the aid of BioRender.com.**

Sample Collection

After Forty-eight hr. Animals were euthanized; (cervical dislocation) by a light anesthesia with diethyl ether (BDH, chemical, Ltd. Poole, England) (A piece of cotton soaked in 'Diethyl ether' and placed in a glass jar), after that, both blood as well as tissue samples were gained as follows:

Blood sample

Blood was collected by jugular vein puncture.[28] The collected blood was incubated; at room temperature for 30 min, to enable clot formation then centrifuged for 15 minutes at 5000 rpm. Serum samples was collected; then reallocated; to a Eppendorf tube (1.5 ml), and kept under −20°C until further analysis.

Outcome measurement

The experiment was performed following the national security protocols of biological laboratories and as follows:

Inflammatory markers: (IL-1β, IL-6, IL8 and TNF-α)

Inflammatory cytokines (IL-1β, IL-6, IL-8, and TNF-α) levels were determined in the collected serum's mice after experiment; employing an Enzyme-Linked Immunosorbent-Assay (ELISA) commercial kits obtained from (myBioSource/USA) consulting to the manufacturer's guidelines, "Sandwich-ELISA" technique; was the assessment methodology employed in these ELISA-kits. The special plates of (ELISA kits) was pre-coated with (IL-8, IL-1β, IL-6, and TNF-α) antibodies respectively. The (IL-8, IL-6, IL-1β, and TNF-α) existing in the samples, were added; and bounded; in to antibodies: then; had been covered on the wells. Then adding the biotinylated (IL-8, IL-1β, IL-6, and TNF-α) antibodies and bounding to IL-6, IL-8, IL-1 β, and TNF-α in the samples. At that, time (Streptavidin-HRP) was added; and bounded; to the Biotinylated IL-8, IL-1β, IL-6, and TNF-α antibody. Throughout a washing–step was performed next to incubation, the (unbound Streptavidin-HRP) had washed-away. Later, adding the 'substrate solution'; and the colour – was established in accordance with the amount of (IL-1β, IL-8, IL-6, and TNF-α). The final reaction was terminated, by adding of (acidic stop solution), with using an ELISA reader (Bio-Tek Instruments. Inc., USA), finally determining the optical density (OD) "spectrophotometrically" usually, at a wavelengths of 450-nm. Contently OD values was correlated; with the of (IL-6, IL-8, IL-1β, and TNF-α) levels. Accordingly, (samples optical density) was compared to the reference curves, for determining the quantities of IL-8, IL-6, IL-1β, and TNF-α.

Histopathological analysis

After Forty-eight hr. of the LPS challenge; scarification process had been done. The cardiac, pulmonary, hepatic and renal tissues slices; had been fixed in 10% of buffered-formalin (Fluka co., Switzerland). The tissue section; was dehydrated, embedded with paraffin cleared, and infiltrated. Subsequently, the tissue-samples were placed; into molds, and waxed till they became (hard). Meanwhile, tissues were partitioned; into 4 μm sections by using a microtome (Sakura, Japan). Hematoxylin and eosin (H&E) stain (Thermo Shandon, U.S.A) was performed; in order to stain the nuclei to blue; owing to its high affinity to nucleic-acids in the cell nucleus, whereas eosin, an acidic-dye, stained the cytoplasm. At least of "ten random areas" were observed. A Zeiss Imager M2 microscope (Carl Zeiss Micro-Imaging) fitted with an Axio-CamHRc CCD camera (Carl Zeiss Microscope) was used; to observe histopathological alterations.

Slides; for all vital organs; were coded, randomized, and assessed by expert pathologists, read blindly; to evaluate for histologic tissue alterations and severity of organ damage according to following variables: edema, congestion, hemorrhage, and inflammatory cellular infiltration or aggregation Table 1.

Assessment of macroscopic tissues score

Histological slices from all sample sections were evaluated and graded according to Steven's technique: (samples assigned to a category showing an ordered progression in severity of tissue damage). The resultant scores were summed to denote the organ injury score.[29]

Statistical Analysis

Experiment data collected were expressed as the quantitative mean ± standard deviation (SD) and was entered; into Microsoft Excel Worksheet and statistically analyzed; by using SPSS (Statistical Package for Social Sciences)-version 23. With One-way analysis of variance (ANOVA) to conclude; statistical significance amongst multiple- groups, followed by Student's *t* test (2-tail) was used; for two groups. Data was only statistically significant, highly significant and very highly significant when *P* value was set less than 0.05, 0.01, and 0.001, respectively.[30]

Results

Quantitative Determination of Inflammatory-Markers

Serum collected from Swiss Albino male mice that received (5 mg/kg, i.p.) LPS lipopolysaccharide after Forty-eight hr. at the end of experiment, revealed; a significant increment of IL-6, IL-8, IL-1β, and TNF-α levels ($P < 0.001$) in the LPS received only (group II) which is about 6-6.5 fold; in comparison with those of the control received (0.9% N/S) of (group I).

Effect of "Ramelteon" on serum IL-1β levels in (LPS) inducing cytokine storm on mice

The current study shown a significant decrease of (serum IL-1β) levels detected in-group III, IV, and their combination group V; approximately to the same degree ($P < 0.001$) for all pre-treated groups; as compared with that of the induced (group II) as presented in (Figure 2).

Effect of "Ramelteon" on serum IL-6 levels in (LPS) inducing cytokine storm on mice

Table 1. **Organ damage scoring system according to Steven's qualitative data of ordinal scales**

Score	Tissue findings
Grade 0	(0% absent damage)
Grade 1	(0–33% mild damage)
Grade 2	(33%–66% moderate damage)
Grade 3	(66–100% severe damage)

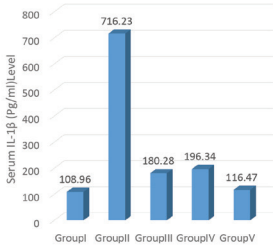

Fig. 2 **Mean of mice serum IL-1β level (pg/mL) in different study groups of the study ($n = 12$). Data are expressed Mean ± SD, Group I: apparently healthy mice received (0.9% N/S), Group II: induced by lipopolysaccharides (LPS) only, Group III: injected with (LPS) and received dexamethasone, Group IV: injected with (LPS) received ramelteon, Group V: injected with (LPS) received (ramelteon + dexamethasone) combination.**

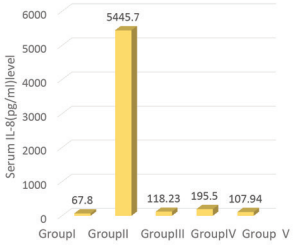

Fig. 4 **Mean mice serum IL-8 level (pg/mL) in different study groups of the study ($n = 12$). Data are expressed Mean ± SD, Group I: apparently healthy mice received (0.9% N/S), Group II: induced by lipopolysaccharides (LPS) only, Group III: injected with (LPS) and received dexamethasone, Group IV: injected with (LPS) received ramelteon, Group V: injected with (LPS) received (ramelteon + dexamethasone) combination.**

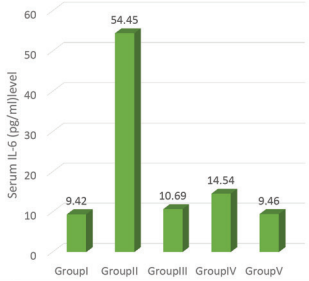

Fig. 3 **Mean mice serum IL-6 level (pg/mL) in different study groups of the study ($n = 12$). Data are expressed Mean ± SD, Group I: apparently healthy received mice (0.9% N/S), Group II: induced by lipopolysaccharides (LPS) only, Group III: injected with (LPS) and received dexamethasone, Group IV: injected with (LPS) received ramelteon, Group V: injected with (LPS) received (ramelteon + dexamethasone) combination.**

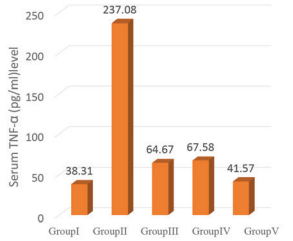

Fig. 5 **Mean mice serum level TNF-α (pg/mL) in different study groups of the study ($n = 12$). Data are expressed Mean ± SD, Group I: apparently healthy mice received (0.9% N/S), Group II: induced by lipopolysaccharides (LPS) only, Group III: injected with (LPS) and received dexamethasone, Group IV: injected with (LPS) received ramelteon, Group V: injected with (LPS) received (ramelteon + dexamethasone) combination.**

Figure 3 demonstrated a significant decrease in (serum IL-6) levels in-group III, IV, and their combination group V ($P < 0.001$) as compared with that of induced; non-treated (group II). A half-dose combination of both (RAM.) and (DEX.) showed; an evident advantage when compared with that of either of them alone, as shown in (Figure 3).

Effect of "Ramelteon" on serum IL-8 levels in LPS inducing cytokine storm on mice

Figure 4 showed data; expose a significant decrease in the (serum of IL-8) levels observed in-group III, IV, and group V. As compared with those of the induced non-treated, (group II). ($P < 0.001$). A half-dose combination of both (RAM.) and (DEX.) Showed a noticeable advantage when compared with that of (RAM.) alone and at full dose, while non-significant differences when as compared with pre-treated (DEX.) group at full dose (Figure 4).

Effect of "Ramelteon" on serum TNF-α level in LPS induced cytokine storm on mice

Figure 5 shows data demonstrate a significant decrease in the (serum TNF-α) levels observed in-group III, IV, and group V ($P < 0.001$) as compared with those of the non-treated (group II) ($P < 0.001$).It is worth noting that half-doses of both (RAM.) and (DEX.) combination revealed an evident advantage when compared with that of either of them alone and at full dose (Figure 5).

Histopathological Analysis

Histopathological outcome for all interventional agents; were shown in (Figures 6-9), representing lung, kidney, and liver, in addition to cardiac tissue sections respectively and as follows:

Discussion

The cytokine storm syndromes (CSS) had captured the attention of the scientific and the public community alike; whereas the overall notion of uncontrolled excessive or release of proinflammatory cytokines is well known, the concept of a cytokine storm and the biological significances of cytokine overproduction are not clearly defined.[31] To overcome this critical health problem, the proposal advocated by our team for the anticipation and control of hyperinflammatory state-associated "cytokine storms" upon LPS-stimulation. With no standard solution is available_new anti-inflammatory drugs with novel mechanisms of action are needed. Hence, ramelteon was studied in this respect lipopolysaccharide as most often contagious-factors eliciting; a local immune-reaction and it is a dynamic constituent of the external envelop of gram-negative bacteria.[32,33] The interaction of (LPS) with mammalian-cells could stimulate; the secretion of pro-inflammatory cytokines, which subsequently, leads to tissue destruction and multiple

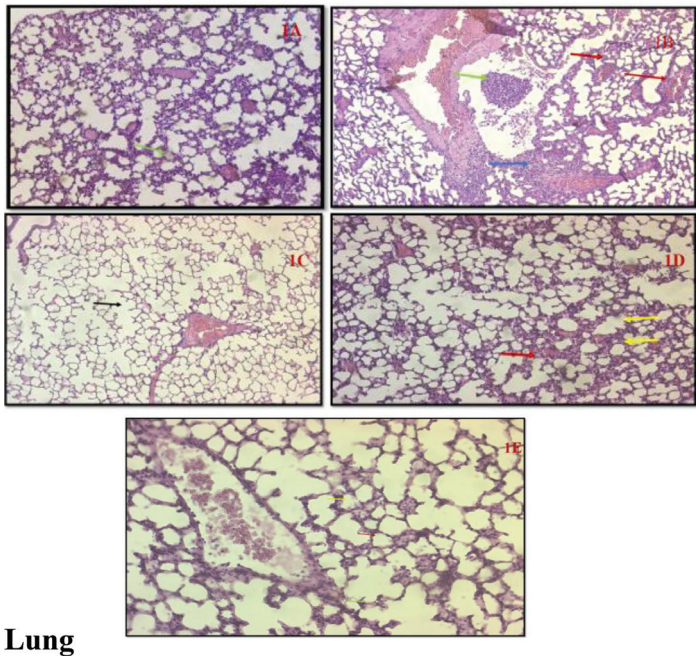

Fig. 6 **Histopathological study findings in the lung-tissue (H&E). (1A) Lung-tissue section treated with 0.9% normal saline as control group I (10x) showed normal histology of the lung. Comprising; of alveoli surrounded by normal alveolar septae (green arrow) and no inflammatory cell infiltration (score 0 no tissue damage). (1B) Lung tissue section of LPS-injected group II (20x) showed marked vascular congestion (red arrow), heavy infiltration of inflammatory cells (blue arrow), and intra-alveolar suppurative inflammation rich in neutrophils (green arrow) diffuses alveolar damage (score 3 sever tissue damage). (1C) Lung tissue section of LPS-injected and pretreated with dexamethasone group III (10x) showed reduced inflammatory cell infiltration in some areas and focal dispersed destruction of alveolar (black arrow) (score 2 moderate tissue damage). (1D) Lung tissue section of LPS-injected and pretreated with ramelteon group IV (10x) showed reduced inflammatory cell infiltration, moderate inflammation, edema in some areas (yellow arrow), moderate congestion (red arrow) (score 2 tissue damage). (1E) Lung-tissue section LPS-injected and pretreated with (ramelteon and dexamethasone) combination group V (20X) showed mild inflammation (yellow arrow) thin alveolar walls, nearly normal appearance with mild edema (red arrow) (score 1 mild tissue damage).**

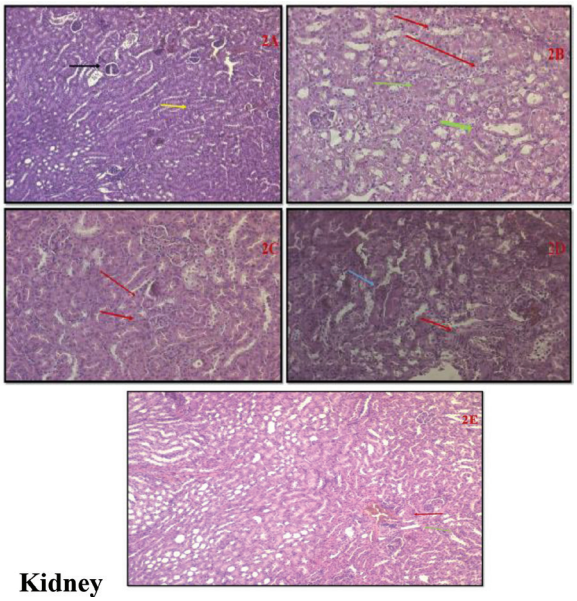

Fig. 7 **Histopathological study findings in the kidney tissue (H&E). (2A) kidney tissue section treated with 0.9% normal saline as control group I (10x) showed normal histological appearance, consist glomeruli (black arrow), proximal and distal Convoluted tubules (yellow arrow) and no inflammatory cell infiltration(score 0 no tissue damage). (2B) Kidney section of LPS-injected group II (40x) showed tubular epithelial swelling and vacuolar degeneration and cast formation, (red arrow), dilated and congested vessels (green arrow). (Score 3 sever tissue damage) (2C) Kidney tissue section of LPS-injected and dexamethasone group III (20x) showed moderate congested and dilated glomerular capillaries (red arrow), moderate degenerative changes of renal tubules, inflammatory cell infiltration reduced in certain areas (score 2 moderate tissue damage). (2D) Kidney tissue section of LPS-injected and pretreated with ramelteon group IV (20x) showed moderate welling and degenerative changes (blue arrow), moderate congestion (red arrow), (score 2 moderate tissue damage). (2E) kidney-tissue section LPS-injected and pretreated with (ramelteon and dexamethasone) combination group V (20X) showed mild focal inflammation (green arrow), mild vascular congestion (red arrow), mild tissue changes (score 1 mild tissue damage).**

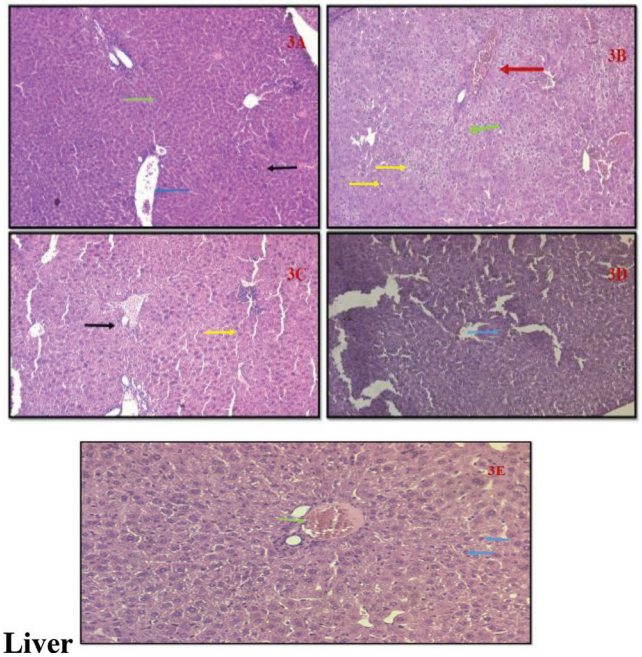

Fig. 8 **Histopathological study findings in the liver tissue (H&E). (3A) liver tissue section treated with 0.9% normal saline group I (10x) as control showed normal histology of the liver consisting of normal hepatocyte architecture (green arrow), central vein (blue arrow) portal area (black arrow) (score 0 no tissue damage). (3B) hepatic tissue section of LPS-injected only group II (20x) displayed central vein congestion and dilatation (red arrow), infiltration of inflammatory cells (yellow arrow), and hydropic degeneration of hepatocyte (green arrow) (score 3 sever tissue damage). (3C) Liver tissue section of LPS-injected and dexamethasone group III (10x) showed hydropic swelling of hepatocyte (Black arrow), moderate congestion (yellow arrow), (score 2 moderate tissue damage). (3D) liver tissue section of LPS-injected and pretreated with ramelteon group IV (10x) showed moderate swelling (blue arrow) and degeneration, moderate congestion. (Score 2 moderate tissue damage). (3E) liver-tissue section LPS-injected and pretreated with (ramelteon and dexamethasone) combination group V (20X) showed mild inflammatory cell infiltration (black arrow), mild vascular congestion (red arrow) (score 1 mild tissue damage).**

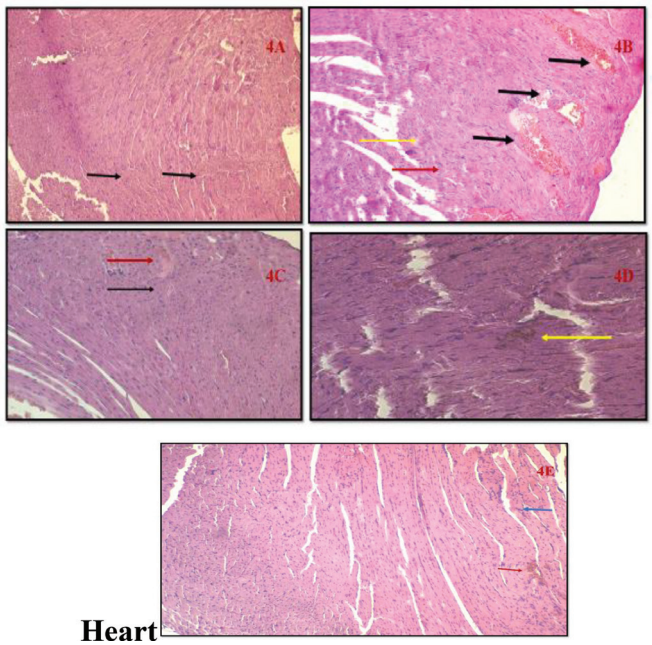

Fig. 9 **Histopathological study findings in the heart tissues (H&E). (4A) Heart tissue section treated with 0.9% normal saline group I (10x) as control. Presented with normal histology of the heart comprising of normal cardiac myocyte architecture (Black arrow) (score 0 no tissue damage). (4B) Heart tissue section of LPS-injected group II (20x) showed abnormal architecture of myocardial cells, dilated and marked congested vessels (Black arrow), heavy inflammatory cell infiltration (yellow arrow), and edema (red arrow) (score 3 sever tissue damage). (4C) Heart tissue section of LPS-injected and dexamethasone group III (20x) showed hypertrophy of cardiac muscle cell (Black arrow), dilated and congested vessels, and moderate tissue changes (red arrow), (score 2 sever tissue damage). (4D) heart tissue section of LPS-injected and ramelteon group IV (10x) showed reduced inflammatory cell infiltration, moderate congestion (yellow arrow), moderate cardiac changes (score 2 tissue damage). (4E) heart-tissue section LPS-injected and pretreated with (ramelteon and dexamethasone) combination group V (20X) showed mild congestion (red arrow), normal cardiac myocyte appearance (blue arrow), mild tissue changes (score 1 tissue damage).**

organ-failure.[34] The primary "cellular receptor" that detects and interacts with (LPS) is toll-like receptor 4 (TLR4) that expressed by 'pattern recognition receptor' (PRR),[35] (TLR4) commonly expressed; in several cell types, including macrophages, dendritic cells, and neutrophils. These receptors were characterized by their unique ability to detect distinctive pathological molecular-patterns, including: lipoproteins, lipopolysaccharide (LPS), bacterial DNA and double-stranded RNA.[36] Thus TLR4 was consider the primary receptor known to interact with (LPS).[37] Following the 'LPS-TLR4 interaction'; a pronounced production of pro-inflammatory-mediators involving: cytokines/chemokines such as interleukin (IL)-1β, tumor necrosis factor-α (TNF-α), IL-8, and IL-6 could occurred. Moreover an imbalance between oxidative and anti-oxidant substances will generate "oxidative stress" was known to arise.[38,39] These consequently, provoking an infiltration of various over activated immune-cells and including macrophages, neutrophils, and CD8+ T as well as B-lymphocytes.[40] Furthermore, (NF-κB) could stimulated by contagious endotoxins (ex: LPS), cytokines/chemokines and oxidative stress. The activation of the NF-κB signaling-pathway was central to the "pathophysiology" of the hyper-inflammatory response. The functional importance of (NF-κB) other transcriptional factors in inflammation was based on its capability to regulate the promoters; of multiple-inflammatory genes, including: (IL-1β, IL-6, IL-8 and TNF-α, as well as iNOS).[41] Consequently, further investigation is warranted to the protective role of ramelteon as anti-cytokine storm agent with the focusing on main proinflammatory cytokines: TNF-α, IL-1β, IL-6; principally chemokine: IL-8, during endotoxemia associated cytokine storm.

The current study results declared that the of serum of pro-inflammatory cytokine: (IL-6, IL8 IL-1β, and TNF-α) levels was significantly; elevated for group II (LPS-administered group II) ($P < 0.001$) when compared with those of the (untreated group I). This finding is consistent with the previous study, which found that there was a significant elevation in the level of (IL-1β, IL-6, IL8 and TNF-α) in LPS-administered mice model in both 24hours and 48 hours periods post-LPS-induced cytokine storm syndrome.[42-45]

Dexamethasone (DEX) a well-known, synthetic glucocorticoid that holds potent long lasting; anti-inflammatory properties. According to prior reports; (DEX.) could manipulate and inactivate the nuclear factor-κB (NF-κB) signaling-pathways in addition to other transcriptional factors[46] therefore, (DEX) was used as "standard anti-inflammatory control" while the secretion of IL-6, IL8,TNF-α, and IL-1β, as dominant mediators of the systemic hyperinflammatory-responses to lipopolysaccharide, were studied in this regard. Even though large dose and repeated administration of corticosteroid (Cs) treatment associated with serious side effect profile ex: steroid-resistant disorders, and nonspecific reactions;[47] hence it have negative impacts on quality of life. That is why numerous physicians and investigators are now focusing on "steroid-sparing" usages; supporting lessen these effects. This another purpose mandatory to practice novel pharmacologic combination that reducing (Cs) side effect through lowering dose; and possibly via diverse mechanism of action (s).

Likewise, in the current study, the DEX-pretreated mice demonstrated significantly decrease in the of pro-inflammatory cytokine (TNF-α, IL-8, IL-1β, and IL-6) levels; if compared with the untreated LPS (group II) ($P < 0.001$). On the other hand, ramelteon pre-treated group's serum level of the pro-inflammatory cytokine: (TNF-α, IL-8 IL-1β, and IL-6) was dramatically reduced; in comparison to untreated (group II) (Figures 2-5). This outcome is consistent; with a previous study was done on mice that found the treatment with ramelteon showed a significantly reduced level of IL-1β as well as MCP-1 in the brain vessels). Liu and his colleagues explained; (Ramelteon's protective effect) via ameliorating (LPS-induced oxidative stress), and (hyperpermeability) of the BBB; through activating the Nrf2 signaling-pathway.[48] Meanwhile, Yang et al., investigation has showed that the treatment with ramelteon; significantly lowering the level of IL-6, IL-1β, as well as TNF-α in LPS-induced "human pulmonary microvascular endothelial-cells" (HPMECs) an in-vitro model of ALI.[49] Moreover, (RAM) attenuated serum level of IL-8, at sub-MIC concentration, in a previous study was done by Zhou et al, in which they investigated the effects of melatonin receptor agonist (ramelteon) on human gingival fibroblasts (HGFs) were stimulated; with (Pg-LPS)-induced inflammation.[50] This might be attributed to the RAM-anti-inflammatory actions, as well as potent free radical-scavenging ability are partly through melatonin receptors[51] furthermore, Kandezi et al. revealed that ramelteon-treatment displayed anti-apoptotic action through the inhibitory effect on (JNK/Bcl-2-Beclin-1) and/or (JNK/Bcl-2/Bax) signaling-pathways and exhibited antioxidant properties via suppressing-ROS,[52] which further confirming the present study finding.

Regarding the pretreatment with novel combination, including the half doses (50%) of both (dexamethasone and ramelteon) (group V); results showed; it was able to suppress "cytokine storm" via highly significant reduced levels of inflammatory cytokine (TNF-α, IL-8, and IL-1β, and IL-6) if compared with the untreated LPS (group II) ($P < 0.001$). During endotoxemia, LPS induced multiorgan–impairment; there was numerous histological variations; have been detected in current study. Lung injuries are recognized due to exposure to drugs or chemicals; Lung tissue section treated with (0.9%) normal saline as control (group I); showing normal histological appearance of the lung tissues; comprising of the alveoli bounded by normal alveolar septae with no infiltration of inflammatory cells (Figure 6(1A)). All at once, LPS-induced group II (cytokine storm model group) showed diffuse damage, alveolar destruction, marked vascular congestion and was associated with massive inflammatory cell infiltration, grading as score 3-sever tissue damage (Figure 6(1B)). In this regard, Lung tissue section of (dexamethasone and LPS-injected) group III (10x) showed reduced infiltration of inflammatory cells in some areas and (focal dispersed-destruction) of alveolar tissue (Figure 6(1C)); (score 2 moderate tissue damage). This consistent with previous studied reports showed that (DEX) has been used to treat inflammatory airway diseases and ALI.[53] On the other hand, (Lung parenchymal structural changes) found restored in the animal pretreated with (RAM) and showed reduced inflammatory cell infiltration in some areas: inflammation of alveolar wall and moderate to mild edema, reduced congestion (Figure 6 (1D)), score 2 moderate tissue damage (Figure 10).

In contrast, RAM./DEX. combination pretreated (group V) displayed reduced inflammatory cell infiltration, mild congestion and mild edema grading score 1 tissue damage, proving that the "novel combination" can reduce the

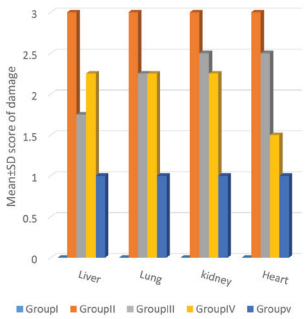

Fig. 10 **The total score of damage of the pulmonary, renal, hepatic and cardiac tissues. Illustrations; LPS-injected (group II) produces: a very high significant degree score of damage ($P < 0.001$) compare with the apparently healthy mice received; 0.9% N/S (group I). While the pre-treated groups: (III, IV, V) with ramelteon, dexamethasone and their combined (ramelteon + dexamethasone) one hour before receiving LPS; displays highly significant ($P < 0.01$), significantly ($P < 0.05$), and very highly significant ($P < 0.001$) improvement respectively in histopathological changes comparing with LPS-injected only (group II).**

tissue damage induced by the cytokine storm (Figure 6(1E)). The "lung tissue protection" evoked by (50 mg/kg, 1.25 mg/kg) of ramelteon/dexamethasone combination; against LPS-induced changes in alveolar tissues was superior to that of Ram (100 mg/kg) and the standard (DEX) (2.5 mg/kg) when used alone.

"Kidney" was the second most vital organ in the body, which is affected; by the cytokine storm related hyperinflammation. (The section of renal tissue nominated from (group I) treated with (0.9%) normal saline; displayed typical histological appearance, consisting of glomeruli, proximal and distal convoluted tubules, with no interstitial inflammatory cell infiltration or aggregation (Figure 7(2A)). In difference, LPS-injected only (group II) exhibited congestion and dilation of glomerular capillaries and blood vessels, destruction of renal tubules with the existence of hyaline cast inside renal tubules besides heavy inflammatory cells infiltration. The tissue damage is scored 3 (Figure 7(2B)). Regarding the (RAM) pretreated (group IV), presented with a significant reduction in deterioration of histopathological alterations that organized from mild to moderate changes as compared to the (LPS) group. While (DEX) pretreated group reducing renal histopathological alteration to (moderate changes only) scored 2.

Concurrent use of (RAM/DEX) combination pretreated (group V) reducing the histological lesion to mild degenerative alterations of renal epithelial tubules (Figure 7(2E)) score to 1 only. (These findings support the researcher's claims of the renoprotective-effectiveness of the novel combination).

The results of the liver examination revealed that; the hepatic tissue section in (group I) treated with (0.9%) normal saline as control mice displayed normal hepatic cellular architecture, with typically-histological appearance, which comprises of central vein with arrangement of hepatocyte cells as a thread-like in the middle of the sinusoid (Figure 8(3A)) (score 0 no tissue damage). In contrast, (LPS treated group) group II displayed a significant histopathological changes and showed congested & dilated blood vessels and sinusoids, focal inflammatory cells infiltration, hydropic degeneration of hepatocyte (Figure 8(3B)) (score 3 sever tissue damage). The explanations of "Pathogenic mechanisms of liver damage "in current study might be attributed in that LPS act as a potent trigger for pro inflammatory cytokines and could prime; resident macrophages/Kupffer cells, and polymorphonuclear leucocytes, (neutrophils), in addition, to newly "recruited monocytes" for enhancing (ROS-generation) in the hepatocytes. In a response; to additional stimuli, particularly (LPS) via the TLR4 receptor-family, a mediated protein-tyrosine kinase (PTK), protein kinases (PK), and mitogen-activated protein kinase (MAPK) and activation of the NF-κB pathway will occurred.[54] Result in a "multifaceted network of intracellular responses" that ending in inflammation.[55-57] The current study findings of the morphological changes that occurred in the hepatic tissue pre-treatment of DEX. (Group III) showed a reduction in the degenerative area of the tissue with the reduction in inflammatory cell infiltration and improvement in score of damage as compared with (LPS treated group) group II. On the other hand, the current findings was built on previous reports investigated ramelteon antioxidant properties; consequently, it ameliorates cellular damage of brain through the Nrf2 signaling pathway.[58] In terms of antioxidant effects, the current study agree with previous reports where ramelteon has been found to improve liver injury caused by ischemic-shock through the inhibition of oxidative stress.[59]

Heart tissue-section of (LPS-injected) group II revealed abnormal architecture of myocardial cells, dilated and congested vessels, marked inflammatory cell infiltration and interstitial edema (score 3 sever tissue damage). Thus showed a significant histopathological changes when compared with the myocardial tissue section in (group I) treated with (0.9%) normal saline as control mice, that displayed normal cardiac architecture, intact histological appearance of myocyte, as well as clear cells border (Figure 9(4A)) (score 0 no tissue damage). In present study; the administration of 2.5 mg/kg of dexamethasone (group III); one hr. prior to (LPS) induction showing (moderate to severe) histopathologic alteration. This result is somewhat; in agreement with Khodir et al., investigation, in which administration of dexamethasone 1 hr, before (10 mg/kg) LPS treated rats, was a reflecting moderate-myocardial affection on histologic examination.[60] The current study; was demonstrated that pretreatment with (RAM) (group IV) mitigates cardiac-injury and was significantly; improving myocardial score that arranged from (mild to moderate tissue damage); when compared with untreated, LPS-injected (group II) (Figure 9(4D)). These outcomes are agreed with a prior study investigations examining the cardio protective effects of ramelteon during development; of myocardial-ischemia on male Wistar rats' model.[61] They attributed cardio protective effect of ramelteon requires activation of MT2 receptor. Furthermore, it has been shown that the release of reactive oxygen species is critically involved in the signaling cascade of ramelteon-induced cardio protection.

Regarding the pretreatment with combination, including the half doses (50%) of both dexamethasone and ramelteon (group V); results showed; highly significant differences in comparison with dexamethasone in full dose. The novel combination was improved morphological changes, moreover; it reduced histopathologic score to 1 only (mild tissue damage); possibly through their "synergistic anti-inflammatory"; and

antioxidant properties. These results indicated that ramelteon was involved in cardio-protective-properties on LPS-induced heart injury via anti-inflammatory[62] free radical scavenging-activity effects.[63,64]

This study outcome in particular showed that the administration of a potent and highly selective "melatonin receptor agonist" ramelteon (RAM) was significantly-reduced inflammation in the lung, liver, kidney and heart tissues on experimental cytokine storm syndromes (CSS) induced by LPS challenge on mice model, with the positive effects being partly explained; by the involvement of the MT2 receptor. It is worth noting; that (dexamethasone and ramelteon) a novel combination demonstrating significant protective anti-inflammatory activity on vital organ than each drug used alone and at full dose; which might be attributed to significant steroid sparing activity[65] that ramelteon have.

Conclusion and Recommendation

Collectively, current study outcomes spectacle that the melatonin receptor agonist ramelteon (RAM) at a dose 100 mg/kg (ip) for the first time; significantly ameliorated cytokine storm induced mice model by mitigating the pro-inflammatory cytokines/chemokine production; possibly via inactivating NF-κB signaling pathway. Likewise, the protective effects of ramelteon were determined by reduction of histopathological changes induced by LPS and macroscopic scores. Moreover, this is the first evidence that (dexamethasone and ramelteon) combination attenuates LPS-induced lung, liver, and kidney and heart injury possibly through its protective and synergistic anti-inflammatory effects; suggests steroid sparing activity that ramelteon could have; result in superior control of inflammation. Thus, it yields desirable drug efficacy with lower DEX doses. Hence, it could pave the way to be a promising therapeutic agent with anticytokine storm efficacy. Future trainings should elucidate whether (dexamethasone and ramelteon) combination can also be effective in other disease conditions concerning the deleterious actions of inflammatory cytokines events, such as cancer, rheumatoid arthritis, ulcerative colitis and multiple sclerosis.

Conflict of Interest

The authors declare that they have no conflict of interest.

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