

Therapeutic Potential of a novel SIRT1 Activator "CWR tripeptide" in LPS-Induced Acute Lung Injury: An evidence from Biochemical and Histopathological studies

Arsh Tanvir

GITAM University

Venkata Sai Konda

GITAM University <https://orcid.org/0009-0005-4157-3213>

Rahul Kumar.

GITAM University <https://orcid.org/0009-0000-1260-3261>

Konchada Tarun

GITAM University

Rahul Kumar

rkumar2@gitam.edu

GITAM University <https://orcid.org/0000-0002-3603-7572>

Research Article

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Abstract

Acute lung injury (ALI) is a life-threatening condition marked by pulmonary inflammation and edema, with limited therapeutic options. SIRT1 protect against ALI, upon activation using SRT1720 and resveratrol, but exhibits limited bioavailability and specificity. We investigated the effectiveness of SIRT1 activation using tripeptide CWR in a lipopolysaccharide (LPS)-induced murine model of ALI.

Pulmonary edema was determined by wet-to-dry lungs ratio. Hematoxylin and eosin (H&E) staining used to assess histopathological changes, while oxidative stress and mitochondrial integrity was quantified using respective biochemical assays for catalase, superoxide dismutase (SOD), lipid peroxidation and citrate synthase. Additionally, qPCR was performed to determine the expression levels of key pro-inflammatory cytokines and neutrophils infiltration was assessed by immunostaining. We observed that LPS administration resulted in more than 3 folds increase in the pulmonary damage and fluid accumulation, which was attenuated upon CWR treatment. LPS mediated neutrophils infiltration, release of inflammatory cytokines such as TNF α , IL-6, CCL3, CCL10 and mitochondrial impairment was alleviated through CWR administration. The study for the first time reported the protective effect of CWR in the murine model of ALI and lays the foundation for the future preclinical and clinical studies to explore its therapeutic potential.

Introduction

Acute Lung Injury (ALI) is a severe life-threatening condition characterized by intense pulmonary inflammation, alveolar capillary disruption, infiltration of immune cells and fluid accumulation (1), often triggered by direct or indirect insults, such as bacterial infections, sepsis, trauma, or exposure to endotoxins like lipopolysaccharide (LPS) (2). The resulting cascade of inflammatory cytokines, reactive oxygen species (ROS), and endothelial dysfunction results in acute respiratory distress syndrome (ARDS). The Large Observational Study to Understand the Global Impact of Severe Acute Respiratory Failure (LUNG SAFE) estimated the period prevalence of ARDS as 10.4% among ICU admissions from 50 different countries (4). Although, supportive care for ARDS had improved over the last few decades, effective pharmaceutical interventions are still not available, particularly due to the complex pathophysiology and patient heterogeneity.

Several clinical and *in vivo* studies reported an increase in the expression of inflammatory cytokines and chemokines such as tumour necrosis factor- α (TNF α) (5), Interleukin-6 (IL-6) (6, 7) and C-X-C Motif Chemokine Ligand 3 (CCL3) (8, 9), and IL-1 β (10). Furthermore, various studies reported that pathogenesis of ALI depends on TNF α and IL-1 β mediated neutrophils infiltration into murine (11–14) and human lungs (15). Subsequently, infiltrated neutrophils start generating ROS in a NADPH oxidase 2 (NOX 2) dependent manner (16). The resultant accumulation of oxidative stress inflicts severe mitochondrial damage (17) and lipid peroxidation that compromises the integrity of the alveolar-capillary membrane as revealed by increased edema, thickening of hyaline membranes, and hypoxemia, with peak effects at 4–24 hours after LPS instillation in rodent (18).

Emerging avenues in ALI research focus on the identification of cellular and molecular pathways that regulate inflammation and oxidative stress (19, 20). Among them, Sirtuin 1 (SIRT1), a NAD⁺-dependent deacetylase, has garnered considerable attention due to its critical role in modulating inflammatory responses, mitochondrial function, and oxidative stress (21–23). Specifically, SIRT1 inhibits the activity of pro-inflammatory transcription factors such as NF- κ B (24, 25), and downregulates the expression of inflammatory cytokines, including TNF- α , IL-6, and IL-1 β (26, 27). Moreover, SIRT1 plays a key role in maintaining cellular homeostasis, enhancing antioxidant defences such as superoxide dismutase (SOD), and prevents mitochondrial damage by reducing the loss of citrate synthase activity (28), making it a promising target for treating inflammatory lung diseases, including ALI (28, 29). SIRT1 activators such as resveratrol and SRT1720 exhibits protective effect against LPS induced lung injury models (31–35), but translational potential is limited by low oral bioavailability (36, 37).

Kumar *et al* developed a novel tripeptide based activator of SIRT1 i.e., CWR, using structure based drug designing, that activates the purified SIRT1 with an EC_{1.5} value of 62.5 μ M (21). Later, it was found that the CWR peptide enhances SIRT1 activity through formation of covalent bond with C1' carbon of the ribose ring in OAADPr and exhibits highly specific nature, with no effect on SIRT2, SIRT3, SIRT5, or SIRT6 (38). In this study, we investigated the therapeutic potential of CWR peptide against LPS-induced murine model of ALI and aim to provide critical insights into the translational potential. Findings obtained from the current study could pave the way for future pre-clinical and clinical studies to determine clinical applications for patients suffering from ALI and related respiratory conditions.

Methods

2.1 Treatment Plans

The C57Bl/6 mice, aged 6–8 weeks, were housed under standard conditions (25 $\pm$ 2°C, 60 $\pm$ 5% RH, 12:12 h light-dark circle). The mice had free access to standard diet and water, and prior approval was obtained from Institutional Animal Ethics Committee (IAEC), in accordance with Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) for all the experimental protocols involving mice. The ALI model was established via intraperitoneal (i.p.) administration of LPS, and mice were divided into three groups with 8–10 mice each group as follows:

- • Group I: Saline i.p. for 7 days
- • Group II: LPS i.p. (1mg/kg) for 7 days.
- • Group III: CWR peptide i.p. (5mg/kg) for 7 days and LPS i.p. (1mg/kg) for next 7 days.

The detailed description plain is described in Fig. 1A.

2.2 Pulmonary Edema

Lungs tissue samples were dried for 2–3 days at 50–60°. Following edema was evaluated by calculating wet-to-dry weight ratio.

W/D ratio = Wet Weight ÷ Dry weight

2.3 ROS Determination

Catalase assay was performed using the method described by Weydert CJ *et al.* with slight modifications (39). Briefly, 30 mM H₂O₂ solution was incubated with tissue homogenate as the enzyme source and the absorbance was immediately recorded at 240 nm using a UV-Vis spectrophotometer. The absorbance was measured over a time span of 5 minutes and changes in the concentration of H₂O₂ was calculated using its molar extinction coefficient (43.6 M⁻¹cm⁻¹). Enzyme activity was calculated as the number of units of the enzyme required to decompose 1 μmol of H₂O₂ per minute under the given experimental conditions.

Malondialdehyde (MDA), one of the end products of lipid peroxidation was quantified using method described by Reitznerová *et al* (40). Briefly, proteins in lung tissue homogenates were precipitated in 2.5% w/v trichloroacetic acid (TCA), and centrifugation at 4000 rpm for 5 mins. followed by the treatment with freshly prepared thiobarbituric acid (TBA) (1.5mg/ml) and incubation at 100°C for 1 hour to yield pink colored MDA-TBA adduct. The samples were cooled down and absorbance was measured at 532 nm in a 96-well plate. MDA levels were calculated using the molar extinction coefficient ($\epsilon = 1.56 \times 10^5$ M⁻¹ cm⁻¹) and results were normalized as nanomoles of MDA per milligram of protein to quantify oxidative stress-induced lipid peroxidation(40).

The SOD activity was determined on the basis of its ability to scavenge free radicals generated by hydroxylamine. The reaction mixture consisted of 50mM Na₂CO₃ buffer (pH 10.2), 1.2 mM NBT, 0.1 mM EDTA and 1mM hydroxylamine. The lung tissue homogenate was added and the absorbance was recorded using kinetic scan for 10 mins at 560 nm to measure the formation of formazan (41). The enzyme activity of SOD was calculated as percentage of inhibition of NBT reduction, and normalised to total tissue weight.

2.4 Assessment of Mitochondrial function

Mitochondrial function was evaluated by performing citrate synthase activity assay, an important enzyme for the overall mitochondrial activity by monitoring the formation of coenzyme A (CoA-SH) during the condensation of acetyl-CoA and oxaloacetate to form citrate (42). Reaction mixture contained phosphate buffer (50 mM, pH 7.4), DTNB (0.4 mM), oxaloacetate (0.1 mM), and tissue homogenate. The reaction was initiated with acetyl-CoA (0.3 mM) and absorbance at 412 nm at regular intervals of 10s for 10 minutes and the specific enzyme activity was calculated on the basis of slope of changes per minute using extinction coefficient of TNB (13.6 M⁻¹ cm⁻¹).

2.5 RNA Isolation and cDNA Synthesis

Total RNA was extracted from lungs tissue using TRIzol reagent as per the standard protocols. Briefly, phase separation was performed using chloroform and RNA was precipitated with isopropanol. 100 ng RNA was used for cDNA preparation using RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific) using following conditions: 25°C for 5 minutes, 42°C for 60 minutes, and held at 4°C. The

cDNA was further treated with RNase1 and stored at -20°C for further use. Expression level of TNF α , IL-6, CCR10 and CCL3 was determined by SYBR™ green Master Mix on a CFX Opus Real-Time PCR System (Bio-Rad). Table 1 shows the list of forward and reverse primers used for the study. The fold change in the transcript level was quantified using $2^{-\Delta\Delta C_t}$ method with GAPDH as the housekeeping gene.

Table 1
List of primers used in this study

Genes	Forward (5'-3')	Reverse (5'-3')
IL-6	GACAAAGCCAGAGTCCTTCAGAGAGA	GGTCTTGGTCCTTAGCCACTCCTT
TNF- α	TCTCATTCTGCTTGTGGC	CACTTGGTGGTTTGCTACG
CXCL10	CAACTGCATCCATATCGATGAC	GATTCCGGATTTCAGACATCTCT
CCL3	CCACTGCCCTTGCTGTTCTT	GCAAAGGGTGCTGGTTTCAA

2.6 Immunofluorescence

Tissue sections were fixed in 4% paraformaldehyde, sequentially washed in xylene, followed by rehydration through graded ethanol to distilled water(43). Next, antigen retrieval was done using 10 mM sodium citrate buffer (pH 6.0). Endogenous peroxidase was blocked with 3% H₂O₂ in methanol (15min), permeabilized in 10% FBS + 0.4% Triton X-100 in PBS (2x, 10min) and blocked in 5% FBS in PBST (30-60min). Thereafter, sections were incubated with polyclonal rabbit anti mice NE IgG (1:1000 in fetal bovine serum, overnight) and FITC conjugated goat anti rabbit IgG (1:5000 in fetal bovine serum, 2 hours). Slides were imaged using confocal microscopy from Olympus FLUOVIEW FV4000 and % area of neutrophil infiltration via image analysis was performed using ImageJ Version 1.54g.

2.7 Haematoxylin & Eosin Staining

Sections were rehydrated through graded ethanol, then immersed in haematoxylin for 5–8 minutes and counterstained with eosin for 30 seconds to stain cytoplasm. Following staining, sections were dehydrated through ascending grades of ethanol, cleared in xylene, air dried, and mounted with a coverslip and examined microscopically to evaluate the histological alterations between groups. Histological damage assessed using a modified semiquantitative scoring method reported by silva et al. (2022)(44). Damage were evaluated within three histological parameters- inflammatory-cell infiltrations, alveolar wall thickening/damage, and overall lung injury on a scale of 1–10. Additionally, infiltration of immune cells was quantified using Fiji software (version ImageJ 1.54g National Institute of Health, USA) (45).

Statistical Analysis

The data is presented as mean $\pm$ SE., and statistically significant differences were determined at $p < 0.05$. Statistical analysis was performed between groups using one-way analysis of variance (Anova) in GraphPad prism software.

Results

3.1 CWR attenuates LPS induced histopathological changes and pulmonary edema in mice lung tissue

Pulmonary structural integrity and inflammatory cell recruitment were assessed via H&E staining. Histological changes across different groups were evaluated using a blinded, semi-quantitative scoring matrix (scale 1–10), which revealed that damaged group displayed severe three histological parameters when compared with saline controls ($p < 0.05$) (Fig. 1B,C). However, the damage was alleviated upon CWR pretreatment, as indicated by the scoring matrix, indicating its protective effect against LPS-induced damage ($p < 0.05$) (Fig. 1B,C). Similarly, we assessed the % of area damaged upon LPS administration and observed that it was prevented in the presence of CWR administration (Fig. 1D). To evaluate the extent of acute alveolar fluid accumulation and microvascular permeability, pulmonary edema was quantified using the lung tissue wet-to-dry (W/D) weight ratio. While LPS administration induced around 3 folds increase in the pulmonary fluid retention compared to controls, co-treatment with the CWR tripeptide demonstrated a reduction in edema (Fig. 1D). Taken together, the overall data suggests that CWR exerted a mitigating effect on LPS-induced pulmonary damage and edema.

3.2. CWR administration prevents NETs infiltration in lungs

Since ALI is associated with the infiltration of neutrophils into the lung's parenchyma, we next examined the extent of recruitment by immunohistochemistry using antibody against NE, with DAPI being used for counterstaining. We observed the presence of NE-FITC positive signals as granular, thread-like structures predominantly localized within the alveolar septa and luminal space (Fig. 2A,B). Digital quantification of the NE-FITC signal area using threshold-adjusted ImageJ analysis confirmed three folds increase in the NE-FITC fluorescence intensity in the LPS treated mice relative to controls ($P < 0.05$), that was reversed upon treatment with CWR. The presence of lower NE-FITC signal in the alveolar regions reveals that CWR exerts a protective effect against LPS mediated neutrophils infiltration into lungs parenchyma.

3.3 CWR protects against LPS induced oxidative stress and improves overall mitochondrial activity

As mentioned earlier, ALI is accompanied by the accumulation of ROS and mitochondrial impairment, we next examined whether CWR pretreatment confers protective effect against ALI in the murine model. Oxidative stress was examined by quantifying of the enzymatic activity of antioxidants, such as catalase and SOD activity, along with the estimation of peroxidation within lung tissue homogenates. Surprisingly, we did not observe any significant effect of CWR pretreatment on the catalase, SOD and lipid peroxidation (Fig. 3A-C). Mice treated with LPS have exhibited 50% impairment in the activity of citrate synthase activity when compared to saline controls, that was significantly rescued upon treatment with CWR (Fig. 3D). Despite lacking statistical significance this restoration points toward the tripeptide's potential efficacy in preserving mitochondrial respiratory dynamics during acute pulmonary inflammation.

3.4. Transcriptional Regulation of Pro-Inflammatory Cytokines and Chemokines

qPCR was performed using the RNA isolated from the lungs to understand the modulatory effect of CWR on the transcript levels of inflammatory cytokines and chemokines. The results revealed that LPS treated mice exhibits overexpression of TNF α and IL6 by 3 and 4 folds respectively, that was restored in the presence of tripeptide (Fig. 4A,B). To our surprise, we did not find any effect of CWR on the expression profile of chemokines such as CCL3 and CCL10 (Fig. 4C,D). Although the results are non-significant, but their trend in the graph suggests the moderate protective effect of CWR which mitigated the inflammatory load in lung tissue.

Discussion

In the current study, we explored the therapeutic role of CWR tripeptide against ALI and related pathophysiological features. We employed LPS-induced ALI model, which is considered as efficacious experimental model for the study of oxidative stress, mitochondrial dysfunction, and inflammatory lung damage (46). The main outcomes of the study are; 1) CWR confers protective effect against ALI-associated pulmonary damage and fluid accumulation, 2) Neutrophils infiltration observed during ALI was prevented by CWR and 3) CWR attenuated ALI dependent mitochondrial impairment and inflammation.

The experimental model used in the current study demonstrated the hallmark pathological features of ARDS that includes edema, thickening of alveolar wall, and infiltration of inflammatory cells including neutrophils (47). Lungs W/D weight ratio was quantified to determine the fluid accumulation, and we observed that CWR pretreatment was able to resolve the ALI-associated edema. Moreover, CWR was able to attenuate LPS-induced alveolar wall thickening and damage as observed by 10-point scale system (44). The findings were concomitant with the previous literature, where SIRT1 activation using resveratrol alleviated the severity of ALI via regulation of Transforming growth factor- β (TGF- β), NLRP3 inflammasome and caveolin-1 (31–35).

Neutrophils entry into the lungs plays a pivotal role during the pathogenesis of ALI by further promoting inflammation oxidative stress. Although, CWR was successfully able to prevent neutrophils entry into the lungs parenchyma as shown using immunostaining techniques. We did not find any significant protective effect against lipid peroxidation as well as the enzymatic activity of SOD and catalase. The findings were different from the previous study where resveratrol mediated SIRT1 activation resulted in the decrease of total ROS as determined using oxidized dihydroethidium (48). It could be possibly due to the fact that neutrophils primarily depends upon NOX2 activation for the ROS generation (16) and could be the major source of oxidative stress. Furthermore, CWR administration was able to restore the elevated expression of proinflammatory cytokines, TNF α and IL6, a key step in the pathogenesis of ALI as reported by clinical and *in vivo* studies (6, 7). The results obtained further support the ability of SIRT1 to control inflammation through regulation of various proinflammatory cytokines.

Taken together, the current study demonstrated that CWR mediated SIRT1 activation confers protective effect against ALI pathophysiology and its potential can further be explored as a novel therapeutic intervention. However, the study is limited by the fact that it does not ascertain the specific involvement of SIRT1 and requires more comprehensive insight into the underlying mechanisms to develop combinatorial approaches. Therefore, future studies using tissue specific transgenic and knockout models will be required to specifically ascertain the specific involvement of CWR mediated SIRT1 activation.

Declarations

5. Author's Contribution

Investigation, Methodology and Formal analysis: Arsh Tanvir, Venkata Sai Konda, Rahul kumar, Konchada Tarun.

Writing- Original Draft Preparation: Arsh Tanvir

Conceptualization, Funding Acquisition Writing- Review & Editing: Rahul Kumar*

6. Funding

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7. Conflicts of Interest

Authors have no conflict of interest to declare.

8. Acknowledgement

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9. Data availability

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Figures

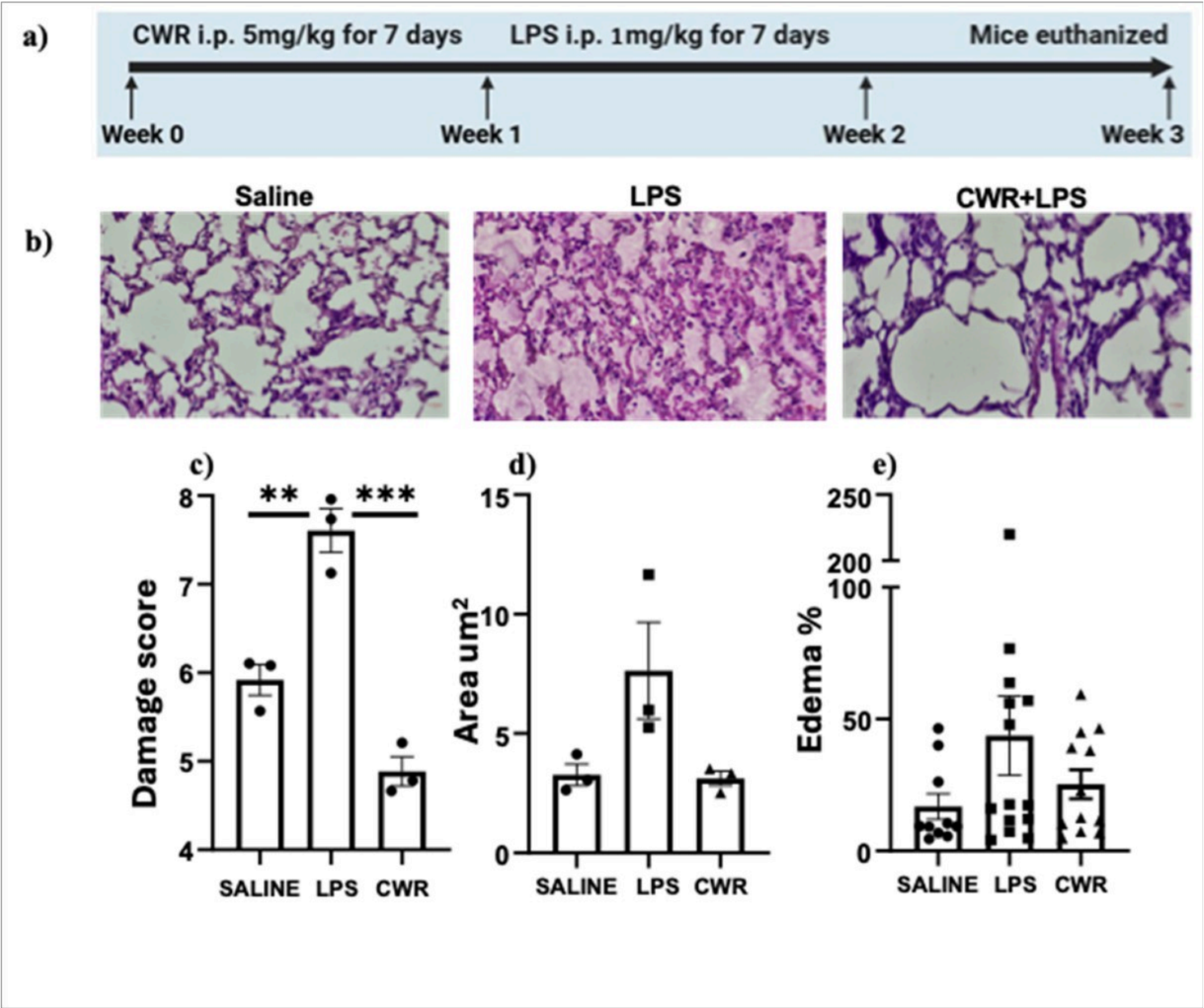


Figure 1

a) Timeline of the experiments and treatments. C57 bl mice were divided into three groups; 1) received only saline from week 0 till week 2, 2) received saline in 1st week followed by LPS in the second week and 3) received CWR peptide in 1st week followed by LPS in the 2nd week **b)** Representative images for hematoxylin and eosin (H&E) staining performed on lungs tissue collected from the mice after **c)** Damage Score calculated using 10 points scale **d)** percentage damage area calculated using image J software, **d)** Percentage edema calculated using dry/wet weight ratio. Measure of central tendency represented as Mean±SEM, N=3 in each group for c-d and N=8-10 for e, data analyzed using One way ANOVA with distribution studied using

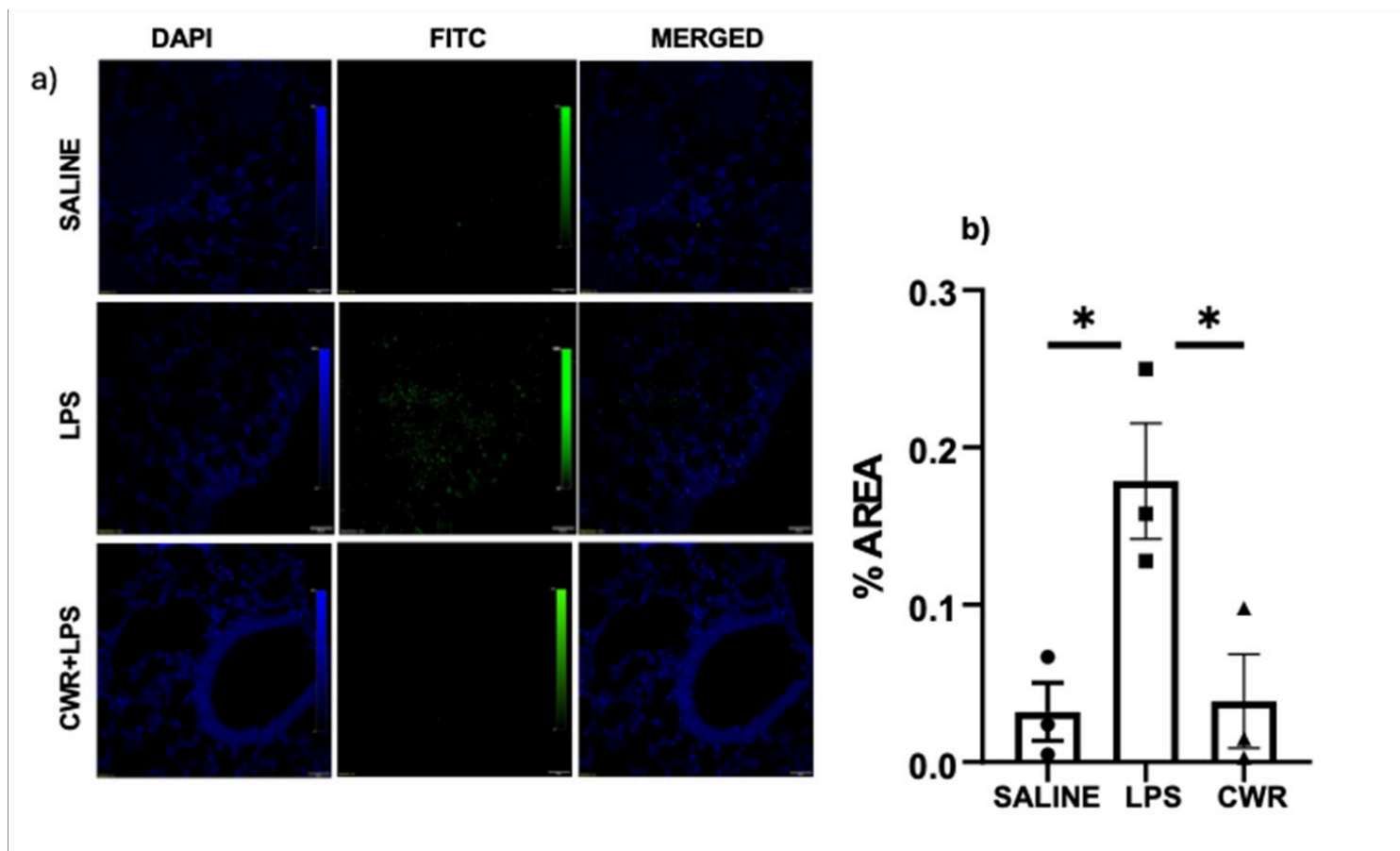


Figure 2

a) Representative images for immunostaining performed using rabbit anti-mouse neutrophils elastase (NE) IgG and FITC conjugated goat anti-rabbit IgG, with DAPI being used for counterstain. **b)** Percentage area calculated using Image J. Measure of central tendency represented as Mean $\pm$ SEM, N=3 in each group, data analyzed using One way ANOVA with distribution studied using

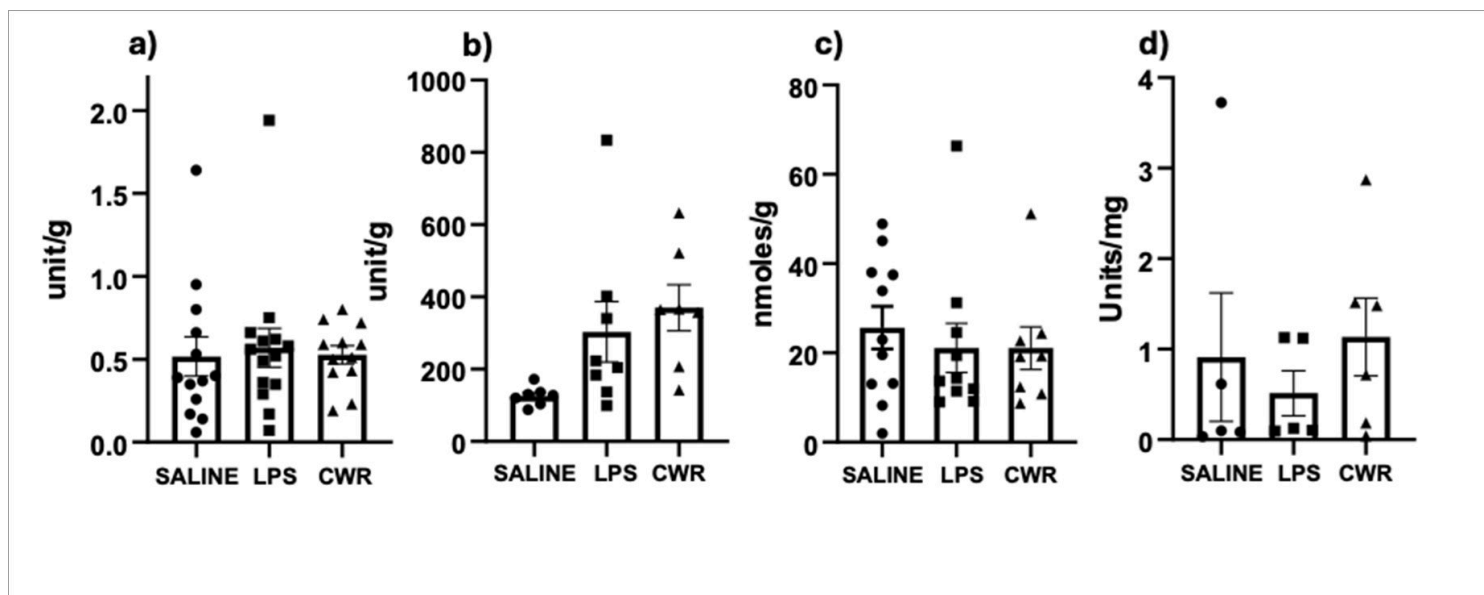


Figure 3

Protective effect of CWR on LPS dependent accumulation of oxidative stress determined by measuring the **a)** catalase activity, **b)** superoxide dismutase (SOD) activity **and c)** malonaldehydes concentration **d)** mitochondrial impairment was determined by measuring the enzymatic activity of citrate synthase. Measure of central tendency represented as Mean±SEM, N=8-10 in each group, data analyzed using One way ANOVA with distribution studied using

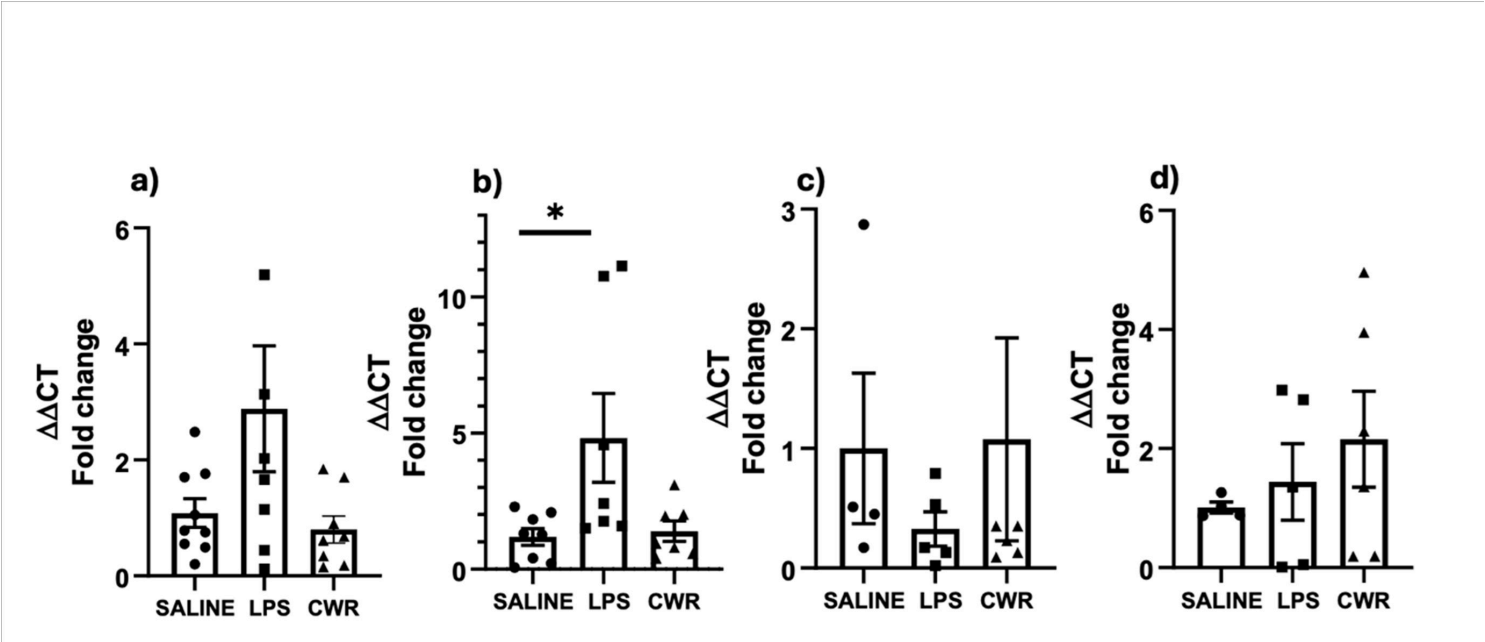


Figure 4

Protective effect of CWR against LPS dependent inflammation was determined by measuring the transcripts level of **a)** TNF α , **b)** IL-6, **c)** CCL3, **d)** CCL10. Measure of central tendency represented as Mean±SEM, N=3 in each group for c-d and N=8-10 for e, data analyzed using One way ANOVA with distribution studied using

Supplementary Files

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