ASPI | Afarand Scholarly Publishing Institute; Turkey
ISSN: 2008-2630 *Iranian Journal of War & Public Health 2023;15(1):35-42* *10.58209/ijwph.15.1.35*

Protective Effect of Dulaglutide on Lung Injury in Endotoxemia Mouse Model

ARTICLE INFO

Article Type
Original Research

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How to cite this article
Abd Uljaleel A Q, Hassan E S, Mohammad A R, Hadi N R. Protective Effect of Dulaglutide on Lung Injury in Endotoxemia Mouse Model. Iranian Journal of War & Public Health. 2023;15(1):35-42.

ABSTRACT

Aims Endotoxemia is the most common condition in patients treated in critical care units. This study aimed to investigate if dulaglutide may help to protect the lungs during endotoxemia by modulating the inflammatory and oxidative stress pathways. This study is self-funded. All authors contributed to the costs.

Materials & Methods 20 adult male Swiss-albino mice aged 9–12 weeks, weighted 25–35g, were randomized into four equal groups (n=5), sham group (laparotomy without Cecal Ligation and Puncture (CLP), CLP group (laparotomy with CLP), vehicle group (normal saline 2 weeks before CLP), and dulaglutide group (0.6mg/kg twice weekly S.C for 2 weeks before CLP). After 24 hrs of sepsis, lung tissue was harvested and used to assess IL-6, Interleukin-IL-1β, TNF-α, MIF, TLR4, and 8-isoPGF2α, as well as histological examination.

Findings Lung tissue levels of IL-6, IL-1β, TNF-α, MIF, TLR4, and F2-isoprostane were significantly higher in the sepsis group compared to the sham group ($p<0.05$), while dulaglutide group showed significantly lower level in these inflammatory mediators and oxidative stress compared to sepsis group ($p<0.05$). Histologically, all mice in the sepsis group showed a significant lung tissue injury ($p<0.05$), but this injury was significantly reduced in the dulaglutide pre-treated group ($p<0.05$).

Conclusion Dulaglutide can attenuate acute lung injury during CLP-induced endotoxemia in mice through its modulating effects on TLR4 and oxidative stress, downstream signaling pathways, and subsequently decreased lung tissue levels of pro-inflammatory mediators.

Keywords Dulaglutide; Endotoxemia; Toll like Receptor 4; F2-Isoprostane

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Article History
Received: December 27, 2022
Accepted: February 20, 2023
ePublished: March 11, 2023

CITATION LINKS

[1] Protocol for ... [2] Nephroprotective ... [3] Septic ... [4] Risk of acute ... [5] The epidemiology ... [6] Sepsis and ... [7] Neutrophil ... [8] Sepsis and ... [9] Comparison ... [10] Animal ... [11] Protective ... [12] Pathophysiology ... [13] New insights ... [14] Acute lung ... [15] The effects ... [16] Potential ... [17] Protective ... [18] Toll-like ... [19] The role of ... [20] Toll-like ... [21] NF-κB signaling ... [22] Interleukin-6 displays ... [23] Effects of ... [24] Role of ... [25] Pulmonary ... [26] Macrophage ... [27] Functional ... [28] F2-isoprostanes ... [29] Oxidative ... [30] Biomarkers ... [31] Measurement ... [32] Glucagon-like ... [33] Dulaglutide ... [34] Acute ... [35] Neuromuscular ... [36] Dulaglutide exerts ... [37] Peroxisome ... [38] Comparison ... [39] The cardioprotective ... [40] Effects of ... [41] Neuroprotective ... [42] Combination ... [43] Sepsis ... [44] Local and ... [45] A large lung ... [46] Protective ... [47] MIF but ... [48] Macrophage ... [49] Pentamidine ... [50] Alpha-chymotrypsin ... [51] Inflammopharmacology [52] Attenuation ... [53] Expression ... [54] Isoprostanes ... [55] Ginsenoside ... [56] Dexmedetomidine-mediated ... [57] Effect of ... [58] IL-6 inhibitors ... [59] On PAMPs ... [60] Dulaglutide ... [61] Glucagon-like ... [62] Cordycepin ... [63] Oxidative ... [64] Serum 8-iso-PGF2α ... [65] Glucagon-like ... [66] A randomized ... [67] Glucagon-like ... [68] Glucagon-like ... [69] GLP-1 analogue ... [70] Glucagon-like ...

Introduction

According to World Health Organization estimates, sepsis affects 30 million or more people each year, resulting in 6 million deaths worldwide and 1 million in newborns [1]. Endotoxemia is defined as a systemic response to inflammation as a result of infection with gram-negative and gram-positive bacteria. In intensive care units, it was considered the major disease that can occur in patients [2]. It is a series of combined effects related to infectious agents, microbiologic poisons, the response of the patient to inflammation, and endogenous mediator's motivation that can cause severe hypotension, Acute Respiratory Distress Syndrome (ARDS), Acute Lung Injury (ALI), and systemic multi-organ [3].

The lungs are among the major organs that are most susceptible to damage from sepsis. Acute lung injury or acute respiratory distress syndrome evolves in more than 50% of septic patients [4]. It is manifested by tachypnea, hypoxia, and metabolic acidosis [5]. Some patients will need intubation and mechanical ventilation due to fatigue that occurs in respiratory muscles [6]. Infiltration of neutrophils in the lungs is considered a main pathophysiological feature of ALI. Unrestrained emigration of neutrophils into the lungs leads to excessive production of myeloperoxidase, reactive oxygen species, chemokines, cytokines, nitric oxide, and neutrophils. The extracellular traps result in uncontrolled inflammation, impairment of lung function, and death [7].

Cytokine-associated lung injury leads to increased alveolar and capillary endothelium permeability and non-cardiac pulmonary edema that damages oxygenation and ventilation [8].

Induction of sepsis in animals occurs either by injection of Lipopolysaccharide (LPS) or live bacteria systemically or by generating an endogenous infection by Cecal Ligation and Puncture process (CLP) [9], which results in lung injury due to peritonitis [10]. CLP causes the deliverance of LPS, a gram-negative bacteria element, which is a basic regulator of bacterial infection pathogenesis and is involved in endotoxic crises [11]. Lung injury secondary to CLP matures during 18-24 hours and is distinguished by hypoxemia, neutrophilic inflammation, and edema in interstitial spaces and alveoli [9].

Immunological processes are stimulated in response to the entry of pathogens. The antigen-presenting cells are activated to cause up regulation of many cells, such as macrophages, monocytes, endothelial and dendritic cells [12]. Dysregulation of the patient's immune system during sepsis makes the pro-inflammatory signaling quickens dysfunction of vascular endothelium. After that, they support entry of additional inflammatory cells as macrophages, monocytes, lymphocytes, and neutrophils to produce the so-called vicious pro-inflammatory ring. In acute systemic inflammation, increased permeability of lung microvasculature is characterized as a main injury resulting by sepsis during the development of ARDS, and so plasma exudate fills the alveoli [13]. Furthermore, besides the damage of alveolar epithelial cells, as a result of apoptosis and necrosis encouraged by sepsis, the high exudates that fill alveoli, cause alveolar edema and then the hyaline membrane is developed [14]. In severe inflammation, as in sepsis, the stimulation of inflammatory process by pathogen results in cytokines storm such as Interleukin 1, Interleukin 8, Interleukin 6 (IL-6), Tumour Necrosis Factor- Alpha (TNF-α) to amplify the response to inflammation [15].

Toll like Receptor family (TLR) is one of the Pattern Recognition Receptors (PRRs) which have an essential role in stimulating intracellular signal pathways and provoking pro-inflammatory cytokines after recognition of exogenous and endogenous ligands [16]. The most imperative among this family is TLR4; it can spot LPS and thus stimulates immune response [17, 18] by translocation of Nuclear Factor-κB (NF-κB), which in turn stimulates pulmonary cytokines such as IL-6, IL-1β, and TNF-alpha inducing ALI [19]. The severity of the inflammatory response is linked with the expression level of TLR4. The harm of microarchitecture and damage to alveolar epithelium and vascular endothelium tissue seem to be attached to the TLR4 gene [20].

TNF-α as an inflammatory mediator can also initiate comparable mechanisms resulting in nuclear translocation of NF-κB and thus increase production of cytokines [21].

IL-6 is a main pro-inflammatory mediator in sepsis as it initiates acute phase response [22] and is considered an early marker of lung injury by its levels in broncho-alveolar fluid, plasma, and pulmonary tissues [23] also its prognostic factor for continued mechanical ventilation, morbidity and death in ARDS [24].

IL-1β has a significant role in pulmonary inflammation caused by bacteria and bacterial products. It is secreted in lungs after induction by LPS during CLP [25].

Macrophage migration inhibitory factor (MIF) is a pro inflammatory cytokine. Immune cells such as macrophage, monocytes, B-cells and T-cells are the main source of this marker. It releases in response to microbial products, hypoxia and proliferative signs [26]. MIF level will be noticeably higher in patients with sepsis, and it can be utilized as a diagnostic marker in this condition [27].

Mitochondria is the main organelle that maintains cellular function through ATP production. Pathological conditions such as sepsis can harm mitochondria, which lead to energy stress and production of reactive oxygen species due to lipid peroxidation [28]. Shifting of the metabolic pathway from aerobic to anaerobic condition that occurs

during sepsis as a result of tissue hypoxia will lead to a reduction in ATP production and accumulation of acid lactate, thus intracellular acidosis occurs [29]. 8-iso-Prostaglandin F2-alpha (8-isoPGF2α), the Lipid peroxidation product of arachidonic acid, is considered the greatest useful oxidative stress biomarker in vivo [30]. It can be counted in all biological tissues and fluids such as Cerebrospinal Fluid (CSF), plasma, urine, pulmonary epithelial lining, and fluid of broncho-alveolar lavage [31].

Approaches that target sepsis advancement and break the linking of the inflammatory net at the correct time to preserve vascular barrier integrity are instantly required [15].

Dulaglutide is a Glucagon-Like Peptide-1 agonist (GLP-1) used for the management of patients with diabetes mellitus type II [32]. It is anti-inflammatory and is demonstrated in a variety of animal species to lower pro-inflammatory cytokine levels. Dulaglutide lowered interleukin-1 beta levels and TNF alpha in the LPS-induced sepsis model in mice [33].

In contrast, despite numerous spans of effort, there is yet a shortage of active pharmacologic approaches against ARDS [34]. Neuromuscular blocking agents are among the most widely used treatments for ARDS, which only act as an adjuvant to avoid lung injury due to ventilation [35]. So there is an insistent requirement to detect particular therapeutic drugs for treating ARDS.

This study aimed to examine if dulaglutide may help in keeping the lungs from polymicrobial sepsis by modulating the pathways of inflammation and oxidative stress.

Materials and Methods

The study was conducted in the Department of Pharmacology and Therapeutics, the Faculty of Medicine, University of Kufa, as well as the Middle Euphrates Centre for Cancer Research in the period between 5 February and 30 August 2022.

Animals

Twenty Swiss albino male mice with an average weight of 25-35 g and mature at 8-12 weeks were acquired from the Faculty of Science, University of Kufa (Animal Resource Center), which were kept at a special temperature of 25°C and humidity of 60-65%, with a 12 h light: 12 h dark cycle.

Study design

In this study, mice were divided into 4 groups of 5 as follows:

- Sham group: mice in this group were anesthetized and exposed to laparotomy surgery without CLP.
- Sepsis group (CLP): mice cecum of this group was ligated and punctured.
- Vehicle group: all mice in this group were given an equal volume of Normal Saline (NS) subcutaneously (S.C) before CLP.
- Dulaglutide pre-treated group: mice in this group received 0.6mg per kg dulaglutide twice weekly S.C. for 2 weeks before CLP [36].

Experimental procedure

Mice were anesthetized intraperitoneally with 1:1 75 mg/kg ketamine and 15 mg/kg xylazine. The cecum was identified after a 1-2cm median abdominal laparotomy. After that, the cecum was ligated just below the ileocecal valve to be punctured twice with a G-20 needle and then returned to its normal position. Then, the abdomen was sutured with 5.0 medical sutures. Mice were monitored every 4 h for 1 day for any signs of illness in their cages with freely reaching for food and drink. The The sham group is the surgical control group, in which mice without CLP undergo anesthesia and laparotomy [37, 38].

Preparation of dulaglutide

Dulaglutide pen was purchased (Eli Lilly and Company, Indianapolis, USA) and diluted with its vehicle (NS) to be administered in a dose of 0.6 mg/kg twice weekly S.C. for 2 weeks before CLP [36].

Tissue homogenization for IL-6, IL-1β, TNF-α, MIF, TLR4, and F2-isoprostane measurement

The lung was cleaned from Red Blood Cells (RBCs) and clots with a 0.9% sodium chloride solution before being maintained in a deep freeze at -80°C. The lung sections were then homogenized employing an elevated ultrasonic liquid processing in 1:10 [weight/volume] phosphate buffer saline (PBS saltwater with 1% Triton X-100 and 1% protease inhibitor cocktail) [39]. Then the lysates were centrifuged for 10 min at 4°C and 10,000 rpm, and the supernatants were applied to measure IL-6, IL-1β, TNF-α, MIF, TLR4, and F2-isoprostane [40] by Elisa kit for mice (Bioassay®, china), specific for each mediator.

Tissue preparation for histopathology

The other area of the lung tissue sample was immersed in 10% formalin, dehydrated in various alcohols, cleaned in xylene, and embedded in a paraffin block. Tissue slide slices were subsequently cut into 5 mm-thick horizontal lines, stained with Hematoxylin and Eosin (H&E), and then sent to a histopathologist for histological examination [41]. Lung tissue injury was assessed blindly by competent pathologists using four randomly selected areas. The sections were graded using a scale constructed to determine the degree of lung injury.

To assess variation in lung impairment, histological slices were examined for all groups under original magnification from X100 –X400, and results scored as follows [42]:

- Normal texture, without damage (Score 0)
- Score 1, with less than 25% damage (mild)
- Score 2, with 25-50% damage (moderate)
- Score 3, with 50-75% damage (severe)
- Score 4, with 75-100% damage (highly severe)

Statistical analysis

SPSS 26 software was used for the statistical analysis of data. Data were expressed as mean±SEM. To compare different categories, one-way Analysis of Variance (ANOVA) with Bonferroni's post hoc test was used. According to the histopathological

alterations in the lung tissues, the Kruskal-Wallis test was used to determine the statistically significant difference between the groups.

Findings

Effect of dulaglutide on IL-6, IL-1β, TNF-α, MIF, and TLR4

When the CLP and vehicle groups were matched to the sham group, the lung levels of inflammatory cytokines and TLR4 were significantly greater in CLP and vehicle groups ($p<0.05$), while their levels were significantly lower in the dulaglutide group ($p<0.05$; Table 1).

Dulaglutide attenuates oxidative stress (8-iso-PGF2α) in lung tissue

Mice in the CLP group showed a significant increment in lung tissue level of 8-iso-PGF2α than in the sham group. Lung level of 8-iso-PGF2α was decreased significantly in the dulaglutide treatment group compared to CLP one (Table 1).

Table 1) Comparison of mean inflammatory mediators and oxidative stress of lung tissue in different groups

Inflammatory and oxidative stress markers	Sham group	CLP group	Vehicle group (NS)	Dulaglutide group	P-value
IL-6 (pg/ml)	28.176±2.912	48.542± 4.035*	49.16± 3.778**	27.686± 5.418***	*0.0016 **1 ***0.01
IL-1β (ng/L)	137.36±7.99	273.56± 45.266*	311.45±26.206**	141.38± 23.88***	*0.011 **0.897 ***0.014
TNF-α (ng/L)	37.96±3.793	88.16±11.662*	74.27± 7.256**	30.02± 3.259***	*0.00001 **0.701 ***0.00001
MIF (ng/L)	9.18±1.431	33.74± 2.806*	33.04± 3.496**	12.28± 1.381***	*0.00001 **1 ***0.00001
TLR4 (ng/ml)	0.97±0.162	1.84±0.092*	1.81±0.099**	0.79±0.11***	*0.001 **1 ***0.00001
8-iso-PGF2α (pg/ml)	340± 50.635	1219.6±126.655*	1135.8± 78.554**	552± 56.628***	*0.00001 **0.96 ***0.00001

Data were expressed as mean ± standard deviation.
*Comparison between CLP and Sham groups; **Comparison between CLP and Vehicle groups; ***Comparison between CLP and dulaglutide groups

Effect of dulaglutide on lung histopathology

The results indicated a significant improvement in the histopathological features of the lung in the group pretreated with dulaglutide, where the histopathological score of sepsis was significantly reduced compared to the CLP and vehicle groups. Therefore, the dulaglutide-pretreated group showed a mild form of inflammation, summarized by the mild accumulation of macrophages and neutrophils in the alveoli with focal hyperemia and vascular congestion (Diagram 1 and Figure 1).

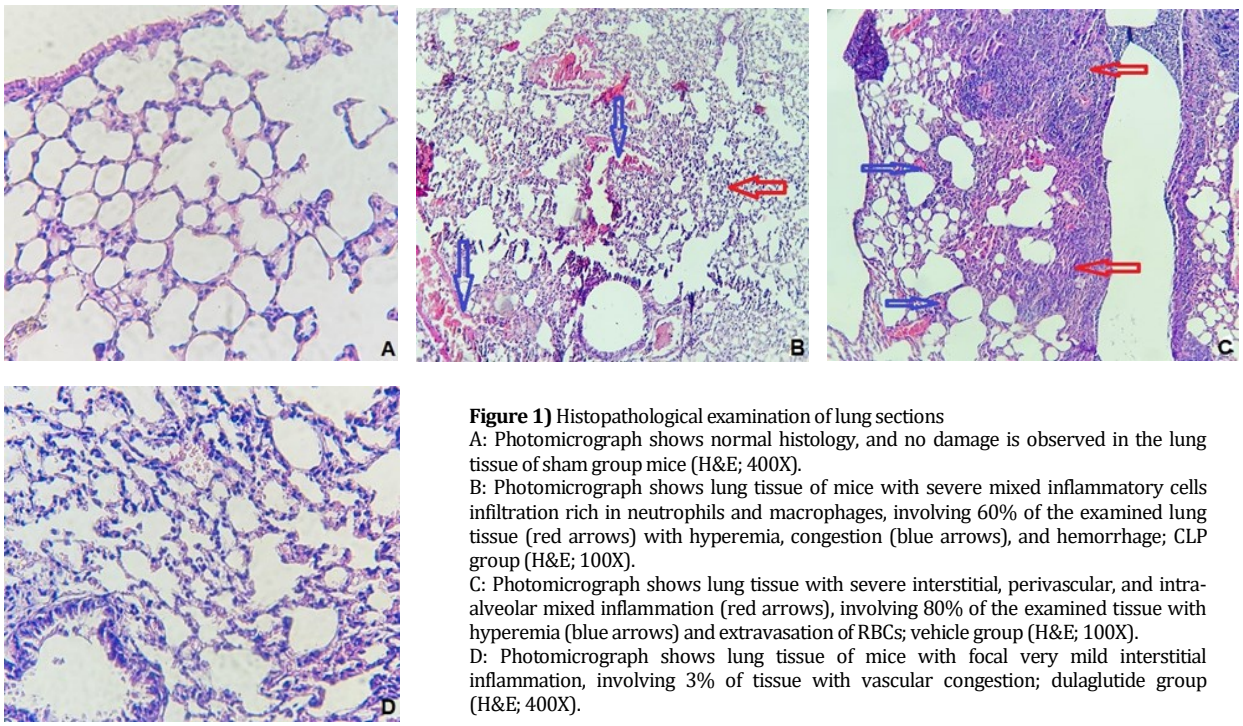

Figure 1) Histopathological examination of lung sections
A: Photomicrograph shows normal histology, and no damage is observed in the lung tissue of sham group mice (H&E; 400X).
B: Photomicrograph shows lung tissue of mice with severe mixed inflammatory cells infiltration rich in neutrophils and macrophages, involving 60% of the examined lung tissue (red arrows) with hyperemia, congestion (blue arrows), and hemorrhage; CLP group (H&E; 100X).
C: Photomicrograph shows lung tissue with severe interstitial, perivascular, and intra-alveolar mixed inflammation (red arrows), involving 80% of the examined tissue with hyperemia (blue arrows) and extravasation of RBCs; vehicle group (H&E; 100X).
D: Photomicrograph shows lung tissue of mice with focal very mild interstitial inflammation, involving 3% of tissue with vascular congestion; dulaglutide group (H&E; 400X).

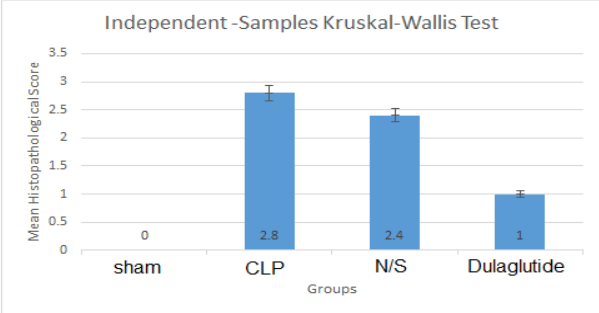

Diagram 1) Mean histopathological score of lung tissue
Sham vs. CLP and vehicle group ($p<0.05$), dulaglutide vs. CLP and vehicle group ($p<0.05$)

Discussion

The studies are still insufficient to determine the role of pharmacological therapies in cases of sepsis [43]. In the current study, for the first time, we estimated the protective effect of dulaglutide on improving lung function following endotoxemia induced by the CLP model in mice.

This experimental research reported a significant elevation in IL- 6, TNF-α, IL-1β, MIF, TLR4, and 8-iso-PGF2α in the lung tissue in both sepsis and vehicle groups in comparison with the sham group after sepsis.

IL-6 is one of the most important inflammatory cytokines, which its level is markedly elevated in the majority, if not in all inflammatory situations [44]. IL-1β is a key player in the beginning of inflammatory response in lung diseases such as Chronic Obstructive Pulmonary Disease (COPD) [45]. TNF-α has a very important role in the progression of ALI induced by sepsis [46]. MIF is an innate cytokine that is expressed extensively with an essential role in the prognosis of septic shock [47]. Chao *et al*. found that the blocking of MIF was efficient in reducing mortality in mice with sepsis as it attenuates vascular permeability and leakage [48]. As a pattern recognition receptor (PRR), TLR4 instigates the immune response through recognition of LPS to keep the body from infection. So the high TLR4 activation by LPS results in the extensive release of pro-inflammatory cytokines that may lead to cytokine storm and severe sepsis [49]. NF-κB acts as a main transcription factor of non-specific immune response intermediated by TLR4 [49]. Our findings are consistent with many other studies. In a study conducted by Senousy *et al*. to investigate the protective effects of α-Chymotrypsin on the liver, lungs, and kidneys of septic rats with a CLP model, TLR4 was highly elevated [50].

In Ibrahim *et al*.'s study that dealt with the protective effects of tocilizumab on acute lung injury, IL-6 level obviously increased in the sepsis group compared to the sham group [51]. Additionally, Ali *et al*. stated that the levels of IL-6, IL-1β, and TNF- α were dramatically increased in the sepsis group by checking the anti-inflammatory effects of continentalic acid on mice's lungs [52]. High MIF serum level in the neonate with sepsis was also reported by Chen *et al*. when the expression of cytokines in the serum of septic infants was studied compared to control infants [53].

8-iso-PGF2-α is a biomarker of lipid peroxidation and oxidative stress, and high levels of this marker are associated with a greater risk of pulmonary disease [54]. Chen *et al*.'s study demonstrated that intestinal 8-iso-PGF2-α level increases after ischemia-reperfusion injury-induced sepsis [55].

The findings of this study demonstrated a substantial decrease in TLR4, pro-inflammatory cytokines (IL-6, TNF-α, IL-1β, MIF), and oxidative stress marker 8-iso-PGF2-α in the dulaglutide-treated group compared to the sepsis and vehicle groups. The initiation of inflammation and exaggerated construction of inflammatory elements are the direct manifestations of sepsis on lung tissues. TLR4 activation by PAMPs (e.g., LPS) results in the activation of the TLR4/Myd88 signaling pathway to encourage the phosphorylation of IκB kinase, which is an essential inhibitor of NF-κB. This will induce the dissociation of NF-κB from IκB and NF-κB complex by IκB phosphorylation, then NF-κB further transport and reaches the nucleus to stimulate transcription of inflammatory mediators [56, 57]. In our study, by dulaglutide drug, the activations of the TLR4/NF-κB pathway and inflammation were substantially alleviated, showing the noticeable inhibitory outcome of dulaglutide versus inflammation in lung tissues caused by LPS. Wang *et al*. presented the same outcomes with dulaglutide on LPS-induced cardio-myocytes injury in mice [33]. The exact mechanism under which dulaglutide decreases the production of these cytokines (IL-6, TNF-α, IL-1β, MIF) is poorly understood [58]. In the first period of infection-stimulated acute inflammation, the PAMPs (e.g., LPS) are recognized and react with PPRs (e.g., TLRs) by inflammatory cells [59]. After that, abroad range of inflammatory cytokines is released like TNF-a, IL-1B, and IL-6 [59]. However, the suppression of the TLR4/NF-κB pathway results in the inhibition of these cytokines secretion [33]. Zheng *et al*. also presented that the levels of IL-6, TNF-α, and IL-1β were depressed markedly in treatment with dulaglutide [60]. Unfortunately, there has been no study about the effect of dulaglutide on MIF to the best of our knowledge. Xu *et al*. showed that activation of the GLP-1 receptor improves ALI induced by LPS and results in the inhibition of IL-6 and MIF markers in pulmonary tissues of LPS-challenged mice [61]. Oxidative stress was linked with a variety of lung diseases, such as ALI [62] and COPD [63]. F2-isoprostane is commonly measured to give a figure about oxidative stress occurrence because it is specific and sensitive index to reflect oxidative stress [64]. Indeed, the probable antioxidant effects of dulaglutide and other GLP-1RA may be due to inhibition of arachidonic acid production, which is the main source of Prostaglandins (PGs) [65]. Li *et al*.'s study found that 8-iso-PGF2-a serum level was

remarkably decreased with dulaglutide in patients with Type 2 Diabetes Mellitus (T2DM) [66]. The overall useful effects of GLP-1 agonist drugs in ALI mammal models and mice model of COPD [61, 67-69] indicated that there are possible ways to repurpose the GLP-1 drugs for the treatment of lung injury.

In the current study, we found that the sepsis and vehicle groups had significantly more lung tissue damage than the sham group. Sepsis and the vehicle group had the highest histopathological damage scores. Histopathological observations in the sepsis and vehicle groups were related to acute and extensive infiltration of inflammatory cells in the alveolar spaces and interstitium, in addition to congestion and extravasation of RBCs. The alveolar wall became thicker with edema and patchy hemorrhage, and also hyaline membrane was formed compared to the sham group. Our findings are consistent with the research of Zhou *et al*., who showed that LPS-challenged mice exhibited severe grades of inflammatory cell penetration, interstitial edema, and within the alveoli in addition to thickening in the septum of alveoli [70]. Dulaglutide pretreatment considerably decreased mononuclear inflammation and another pathological abrasion resulted by the LPS challenge. In the current study, the lung injury score in the CLP group was higher compared to the group treated with dulaglutide. This indicated that dulaglutide pretreatment efficiently attenuated the injury score of the lung. So the histopathological score was significantly reduced in mice receiving the drug. This diminished pathological score in the CLP+ dulaglutide group supported our novel finding that this drug potentially has lung protective effects where it decreased penetration of inflammatory cells into the lungs, hyperemia, edema, and congestion. As far as we know to date, there are no sufficient studies available about the protective effects of dulaglutide on lungs with sepsis. However, many GLP-1 agonists produced the same effects on the lungs as our drug. Zhou *et al*. showed that GLP-1 analog liraglutide effectively decreases histopathological lesions in lungs challenged with LPS and reduced lung injury score by 4 times [70].

This study used dulaglutide in a dose of 0.6 mg/kg for 2 weeks, so further researches with different doses and longer duration may be justified. The protective effect of lung that demonstrated in current study was mediated by TLR4 & NF-κB cascades. Additional researches for other possible signalling pathways are needed. Future studies must investigate diabetic patients with sepsis and treated by this drug.

Conclusion

Dulaglutide can attenuate acute lung injury during CLP-induced endotoxemia in mice through its modulating effects on TLR4 and oxidative stress, downstream signaling pathways, and subsequently decreased lung tissue levels of pro-inflammatory mediators.

Acknowledgments: The researchers would like to thank all the workers in the Department of Pharmacology and Therapeutics, Faculty of Medicine, University of Kufa, for their continuous support in completing the research requirements.

Ethical Permission: The study was authorized by the Bioethics Committee at the University of Kufa, as well as its representation in the Faculty of Medicine (Ethical approval. No. 2933 in 2/2/2022). Committee's recommendations were followed throughout the proceedings of work.

Conflict of Interests: All authors declare that there is no conflict of interest in the study.

Authors' Contribution: Abd Uljaleel AQ (First Author), Introduction Writer/Main Researcher/Discussion Writer (30%); Hassan ES (Second Author), Methodologist/Assistant Researcher/Statistical Analyst (30%); Mohammad AR (Third Author), Assistant Researcher/Discussion Writer (20%); Hadi NR (Fourth Author), Introduction Writer/Assistant Researcher (20%)

Funding: This study is self-funded. All authors contributed to the costs.

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