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Protective effect of Huperzine A in induced cytokine storm in mice

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Abstract

Background: Life-threatening systemic inflammatory syndromes can be caused by various infections, autoimmune and cancerous diseases, genetic disorders, and certain therapeutic approaches. Such situations are frequently referred to as "cytokine storms." If detected too late and treated insufficiently, cytokine storm and the ensuing systemic reaction can proceed from vague clinical symptoms to multi-organ failure. TNF-α, IL-1β, and IL-6 are the three main participants in the interplay of the cytokine storm and the most major proinflammatory cytokines of the innate immune response. Objective: evaluate Huperzine A protective effect on cytokine storm initiated by lipopolysaccharides in Swiss Albino mice. Method: Five groups, each consisting of ten mice (n=10), were created by randomly selecting 50 male Swiss albino mice. The Control group was neither induced nor treated; the Model group was injected with a single intraperitoneal dose (5 mg/kg) of Lipopolysaccharides solution and left untreated. The Vehicle group receive1% Dimethyl sulfoxide solution, the Methylprednisolone group received 50 mg/kg/day Methylprednisolone solution, and the Huperzine A group received 0.2mg/kg/day Huperzine A solution; all use administered by single intraperitoneal injection for three sequential days then induced by single intraperitoneal injection of Lipopolysaccharides at (5 mg/kg) dose. Results: After administering LPS, the non-treated groups' IL-1β, IL-6, and TNF-α serum levels were distinctly elevated ($p < 0.001$) with histopathological changes in the lung compared to the control group. Conclusion: Huperzine A demonstrates a protective effect against cytokine storm induced in Swiss Albino mice using LPS by suppressing serum levels of IL-1β, IL-6, and TNF-α and improving the lung histopathological changes.

Keywords:

Huperzine A, Methylprednisolone, lipopolysaccharides, cytokine storm, DMSO 1%.

Introduction

Various treatments, infections, malignancies, autoimmune disorders, and monogenic disorders can bring on the potentially lethal systemic inflammatory state known as a cytokine storm. It is identified by acute overproduction, uncontrolled pro-inflammatory cytokine release, and immune-cell hyper activation [1].

This occurrence has been linked to the recently identified SARS-CoV-2 coronavirus causing the coronavirus disease 2019 (COVID-19). The prognosis and mortality rate of COVID-19 patients is thought to be worsened by cytokine storms[2].

The innate immune system's neutrophils, macrophages, and natural killer cells have been linked to the pathophysiology of cytokine storms. By using PRRs to identify pathogens, these cells trigger a sequence of activation processes that cause the release of several pro-inflammatory cytokines and activate the adaptive immune system [3].

Depending on the underlying cause and treatments, cytokine storms might start at different times and last longer [4]. Pro-inflammatory cytokines, like TNF-α, IL-1β, and IL-6, play a key role in inflammation [5].

During cell injury, infection, and inflammation, Monocytes, macrophages, and non-immune cells such as fibroblasts and endothelial cells are the main producers of IL-1β [6]. Interleukin IL-6, which possesses pleiotropic and redundant activity, aids in

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the host's defense against short-term environmental stress; however, it has also been demonstrated that dysregulated continuous IL-6 production plays a pathogenic role in many autoimmune and chronic inflammatory illnesses [7]. One of the most significant innate immune system cytokines that promote inflammation is TNF-α. Monocytes and macrophages mostly produce it, though T and B lymphocytes can also release it [8]. Additionally, it has a significant role in both acute and long-term systemic inflammatory reactions and in enhancing the production of other cytokines and chemokines. Cytokine release syndrome can be brought on by dysregulated TNF- signaling [9].

According to recent research, increasing parasympathetic response, whether achieved through the direct triggering of the vagus nerve or using acetylcholine esterase inhibitors (AChEIs), modifies the immune system's response and regulates inflammation through CAP [10, 11]. The parasympathetic nervous system controls inflammation through the cholinergic anti-inflammatory pathway [12]. Later, it was discovered that the "cholinergic anti-inflammatory pathway" key target for preventing dendritic cells and macrophages from producing pro inflammatory cytokines was the 7a nicotinic ACh receptor (7nAChR). Acetylcholine then activates the 7 nicotinic acetylcholine receptors (7 nAChRs) found on immune cells such as macrophages, dendritic cells, lymphocytes, neutrophils, and microglia [13]. Acetylcholine inhibits the NF-kB pathway by upregulating the expression of IRAK-M, which can inhibit the phosphorylation of IκB [14]. When 7nAChRs are activated, JAK2 is recruited, and STAT3 is phosphorylated. STAT3 then moves to the nucleus and binds to DNA rather than NF-B, limiting the synthesis of TNF-α and other cytokines [15]. Additionally, acetylcholine blocks macrophage NLRP3 inflammasome pathways, which reduces the production of IL1β [16].

In this context, the capacity of huperzine A to regulate a produced hyperinflammatory cytokine storm was investigated. Huperzine A is a competitive and reversible acetylcholinesterase inhibitor [17]. That was extracted from the dried herb of Chinese clubmoss Huperzia serrata (family Lycopodiaceae) [18]. In traditional Chinese medicine, the extracts or pure ingredients from Huperzia serrata have been used for thousands of years to treat various conditions, including schizophrenia, inflammation, edema, organophosphate poisoning, pain, and memory loss [19]. Huperzine A was approved for use in Alzheimer's treatment in China.

Furthermore, it's a recognized dietary supplement for improving memory in the US. Huperzine A has attracted considerable interest in clinical use because it possesses higher selectivity for acetylcholinesterase than peripheral butyrylcholinesterase. Consequently, it may provoke lower toxicity and have fewer patient side effects [20].

MATERIAL AND METHODS

Animals

Fifty pathogen-free male Swiss Albino mice (age 7-8 weeks and weighing 25-30 gm) were housed in wood chip-bedded plastic cages. Throughout the study, the animals were kept in the animal house at AL-Nahrain University/College of Medicine in a particular, non-pathogenic environment with appropriate food and water in a 12-hour light-dark cycle and a temperature regulated to (15°C -21°C). The mice were allowed to adjust to their new environment a week before the work began[21].

Chemicals and drugs

Huperzine A and Methylprednisolone were obtained as a powder from Hangzhou Hyper Chemicals Limited Company (china). LPS lyophilized powder from Sigma Aldrich Chemical Company (USA).

Chem-lab NV supplied dimethyl sulfoxide (DMSO) in Belgium. Chloroform 99% provided by Loba Chemie Pvt. Ltd, India. Mouse IL-1β, IL-6, and TNF-α ELISA kits were obtained from SUN LONG Biological Technology Co. Ltd, China.

Induction of Cytokine storm

The induction of cytokine storm was done by single IP injection from LPS solution that was previously prepared by thoroughly mixing 10 mg of LPS lyophilized powder with 10 ml of normal saline in a sterile glass bottle for 15 minutes until completely dissolved.

Preparation of drugs

Huperzine A and Methylprednisolone solution was prepared by being dissolved in 1%DMSO and diluted with distal water to the desired volume.

Experimental design

Five groups of ten mice (n=10) were created by randomly selecting 50 male Swiss albino mice. AH (control) group, apparently healthy mice, were neither induced nor treated; LPS (model) group was induced by a single IP injection of LPS solution at (5 mg/kg) dose and left without treatment; DMSO (vehicle) group receive1% Dimethyl sulfoxide solution by single daily IP injection for three sequential days then injected by LPS solution at 5mg/kg dose as a single dose on day three, MA group received Methylprednisolone solution at (50 mg/kg) dose by single daily IP injection for three sequential days then injected by LPS solution at 5mg/kg dose as a single dose on day three, HA group received Huperzine A solution at (0.2mg/kg) dose by single daily IP injection for three sequential days then injected by LPS solution at 5mg/kg dose as a single dose on day three.

Pro-inflammatory cytokines evaluation

The blood was collected from the jugular vein under light chloroform anesthesia after 48 hours of LPS induction. Subsequently, blood was centrifuged at 3000 rpm for 20 minutes, and at -20°C, the serum was kept. The enzyme-linked immune-sorbent assay (ELISA) was used to quantitatively measure the levels of tumor necrosis factor-alpha (TNF-alpha), interleukin one beta (IL-1β), and interleukin six (IL-6 in the serum. Following the directions from the manufacturer, the reader was prepared to read at 450 nm within 5 minutes.

Histopathological evaluations

All mice were sacrificed and immediately performed an autopsy to obtain lungs using fine scissors and forceps, then preserved in 10 %Formalin solution. Dehydration, paraffin embodiment, and deparaffinization were done on the samples. Lung samples were cut into sections and dyed with Hematoxylin and eosin (H&E).

The histopathological changes were assessed by examining and scoring the slides. Experienced histopathologists tested the tissue sections in a blinded manner, and results were evaluated according to a scoring system ranging from 0-3 (0=normal; 1=mild; 2=moderate; 3=sever).

Statistical analysis

The Statistical Program for the Social Sciences (SPSS) software was used to gather, chart, and analyze all data. The findings were using the mean and standard deviations. A one-way analysis of variance (ANOVA) with a 2-tail (t-test) test was performed comparing groups. For P values of 0.05, 0.01, and 0.001, the significance levels were set to significant, highly significant, and very significant [22].

Results

The effect on pro-inflammatory cytokines

According to the results of the current study, the LPS model and DMSO vehicle groups had significantly higher serum levels of IL-1β, IL-6, and TNF-α than the AH group (P <0.001). However, no significant difference was noticed between the LPS model and DMSO vehicle groups (P >0.05). Table (1) shows the details.

Table (1) shows the study group's serum IL-1β, IL-6, and TNF-α levels.

	Groups mean ± stander Deviation (SD) in (pg/ml)		
Parameters	AH (control)	LPS (induction)	DMSO (vehicle)
IL-1β	7.5±1.7 a	36.1±2.6 b	32.2 ±5.3 b
IL-6	13.8±3 a	77.8±5.1 b	72.9±5.5 b
TNF-α	21.7±5.7 a	87.4±6.5 b	83.7± 5.8 b

Letters are used to express comparison; dissimilar letters signify a substantial difference. A similar letter indicates that there are no notable differences. Huperzine A and Methylprednisolone pre-treatment, in contrast, showed a very high significant reduction in IL-1β, IL-6, and TNF-α levels in the serum post-LPS administration in MA and HA groups (P <0.001) as compared to LPS model group. The MA group IL-1β, IL-6, and TNF-α serum levels were comparable to that of the MA group; Table (2) illustrates these results.

Table (2) shows the study group's serum IL-1β, IL-6, and TNF-α levels.

	Groups mean ± stander Deviation (SD) in (pg/ml)		
Parameters	LPS (induction)	MA (Methylprednisolone)	HA (Huperzine A)
IL-1β	36.1±2.6 b	18±2.6 c	16.7±2.9 c
IL-6	77.8±5.1 b	43±4.5 c	44.6±5.5 c
TNF-α	87.4±6.5 b	25.6±5.1 c	26.5±5.6 c

Letters are used to express comparison; dissimilar letters signify a substantial difference. A similar letter indicates that there are no notable differences.

The effect on the histopathological score

In the current study, the LPS model and DMSO vehicle groups showed significant changes and damage in the lung section, such as intra-alveolar congestion and hemorrhage, proteinaceous material, severe inflammatory cells infiltration, and destruction in the alveolar septa and diffuse alveolar damage with emphysematous changes in compared with the AH group (P <0.001), as shown in figure (1).

These results suggest that the administration of LPS and DMSO led to significant pathological changes and damage in the lung tissues, as evidenced by the observed histological alterations

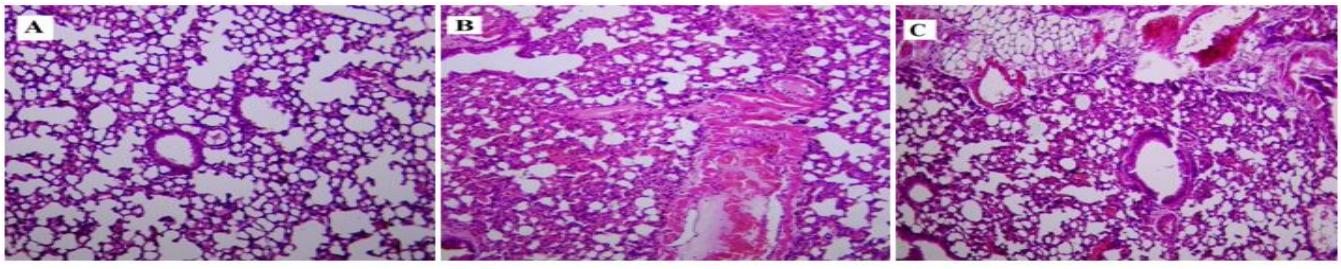

Figure 1: Lung histological section of Control group (A) (x20), LPS group (B) (x20), and DMSO group (C) (x20)

Huperzine A and Methylprednisolone both elicit a significant reduction in histopathological score with mild congestion in alveolar capillaries and mild interstitial inflammatory cells infiltration as compared to the LPS model group (P <0.001), as shown in figure (2). Both Huperzine A and Methylprednisolone demonstrated a beneficial effect in reducing histopathological scores, suggesting a potential protective or therapeutic impact on lung tissue compared to the LPS model group

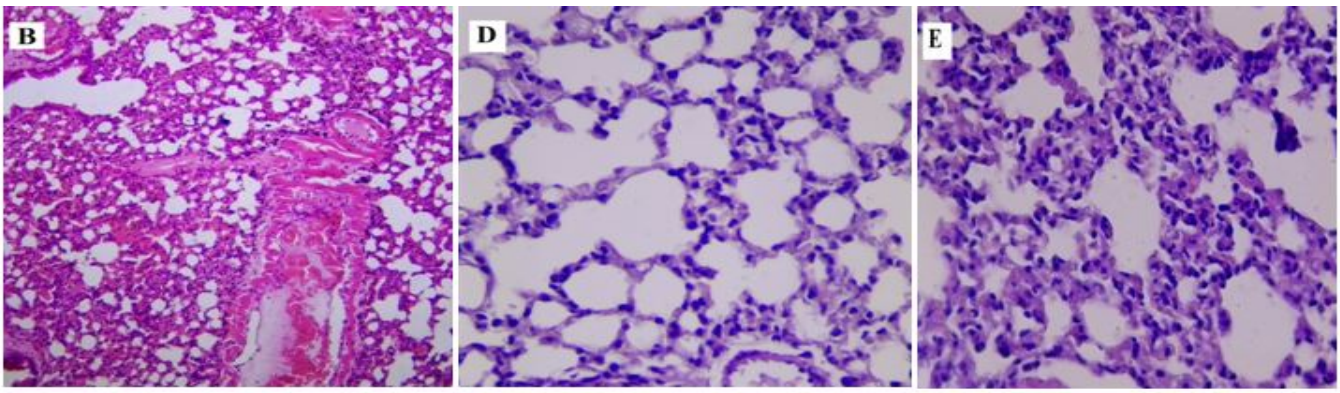

Figure 2: Lung histological section of LPS group (B) (x20), Huperzine A group (D) (x40), and Methylprednisolone group (E) (x40).

Discussion

Lipopolysaccharides (LPS) are crucial glycolipids that make up the outer membrane of Gram-negative bacteria [23]; they maintain the bacterial cell structural integrity and act as a permeability barrier [24]. The LPS molecule is a potent stimulator of the innate immune system; typically, it's an exogenous agent that unleashes cytokine storm. TLR4, which is present on the surface of immune cells [25, 26], recognizes The LPS molecule; this recognition will lead to a series of intracellular processes to activate NF-KB and MAPK kinase pathways that subsequently lead to an induction of many proinflammatory cytokines and chemokines.

Our results are similar to prior research that showed that LPS significantly raised serum levels of IL-1β, IL-6, and TNF-α at a dose of 5 mg/kg compared to the control group [27, 28]. In contrast to the LPS model group, pre-treatment with 0.2 mg/kg Huperzine A significantly reduced serum IL-1β, IL-6, and TNF-α levels. Zhang *et al*. and Cai *et al*. [29, 30] also found similar results to the present study. Huperzine A exhibited protective effects by inhibiting AChE activity, accumulating acetylcholine, and activating the cholinergic anti-inflammatory pathway [31, 32]. Acetylcholine mainly signals through α7 nAChRs expressed in neural and immune cells [33].

The activation of α7nAChR has no impact on the expression of anti-inflammatory cytokines [34], but it suppresses the production of proinflammatory cytokines [35, 36]. Huperzine A's cholinergic anti-inflammatory properties prevent the translocation of the NF-κB component p65 [37, 38]. Furthermore, LPS can induce the activation of other inflammatory mediators, such as the synthesis of Nitric oxide (NO) and prostaglandin E2 (PGE2) that further contribute to the inflammatory process. Huperzine A suppresses the expression of inducible nitric oxide synthase (iNOS), COX-2, and their related inflammatory mediators NO and PGE2 through the inhibition of p38 and ERK1/2 phosphorylation, which are key players in the MAPK pathway [39]. The common condition known as acute respiratory distress syndrome (ARDS) still has a high fatality rate in critical care medicine. Direct lung injury causes of ARDS include pneumonia and aspiration and extrapulmonary diseases that affect the lung secondarily (such as sepsis and pancreatitis) [40].

When lipopolysaccharides (LPS) are injected intraperitoneally, this causes an excessive formation of reactive oxygen species (ROS) and the release of inflammatory cytokines into the systemic circulation. That leads to indirect lung injury with interstitial edema and vascular endothelium damage. Recently, Li R et al., [41, 42] have shown that the cholinergic anti-inflammatory pathway plays a role in suppressing the inflammatory response in acute lung damage. According to Mohseni-Moghaddam et al. [43], Acetylcholinesterase activity, oxidative stress, and inflammation are all inhibited by huperzine A. Nitrite and malondialdehyde (MDA) levels decreased while catalase and superoxide dismutase (SOD) activities enhanced.

The reduced oxidative stress could decrease caspase-1 activity, NLRP3 overexpression, and IL-1β release. Additionally, prevent ROS-mediated NF-κB pathway activation [44, 45]. One of the first cells to respond to LPS is the endothelial cell, which activates TLR4 to produce proinflammatory cytokines, chemokines, and adhesion molecules like VCAM-1 and ICAM-1. These interactions enable leukocyte recruitment into inflamed tissues, impairing organ function. In addition, LPS induces endothelial cell apoptosis and promotes NO production, aids in vasodilatation, and raises endothelial permeability, which results in endothelial barrier damage . Yang et al. [46] found that huperzine A reduces oxidative stress, inflammatory cytokine production, and

caspase-3 activity to protect against Hepatic Ischemia-Reperfusion injury in mice. Ruan *et al*. mention that Huperzine A suppresses endothelial cell apoptosis and inhibits endothelial cell senescence by increasing endothelial cell proliferation.

CONCLUSION

Huperzine A demonstrates a protective effect against cytokine storm induced in Swiss Albino mice using LPS by suppressing serum levels of IL-1β, IL-6, and TNF-α and improving the lung histopathological changes.

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