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Therapeutic Effects of Rivastigmine in induced Cytokine Storm in Mice: Dose Standardization

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Abstract

Background: A cytokine storm release syndrome is a serious clinical systemic hyperinflammatory response that can be triggered by a variety of factors such as infections and certain drugs. It is a severe clinical condition that complicates infectious diseases, e.g., COVID-19, and non-infectious diseases, e.g., autoimmune diseases and cancer, and may lead to death. Objectives: Evaluation of the effect of Rivastigmine (RA) in Cytokine storm-induced in mice by lipopolysaccharide (LPS) and to explore their impact on Interleukine-1 β, Interleukine-6, Transforming growth factor-β and Tumor Necrosis Factor-α levels and Histopathological changes in Lungs. Methods: Animal: Swiss albino male mice were divided equally and randomly into 6groups (n=10): •Group AH: Healthy control group received no induction and no treatment. •Group LPS: induced by (LPS inducted 5 mg /kg), without treatment. •Group DMSO: Induced and treated with DMSO 1%. • Group CAR: treated with Rivastigmine 0.5 mg/kg. Results: Administration of LPS to Swiss albino mice caused a significant elevation of IL-1β, IL-6, TNF- α, and TGF-β levels and tissue histopathological changes when compared to the healthy control group. A highly significant histopathological improvement (P<0.001) in the tissue sections of the lung (hematoxylin & eosin) stain in comparison with those in the induced-non-treated group. Conclusion: Rivastigmine was found to be effective in attenuating and treating the induced cytokine storm by suppressing IL1β, IL-6, TNF-α, and TGF-β levels were comparable with Methylprednisolone.

Keywords:

AH: apparently healthy, LPS: Lipopolysaccharide, DMSO 1% Dimethyl sulfoxide 1%. RA: Rivastigmine, MP: methylprednisolone, RMPA: Rivastigmine, and methylprednisolone.

Introduction

Cytokine release syndrome most pathological life-threatening systemic inflammatory condition. It refers to the pathological process in which immune cells, especially macrophages, are activated by a bacterial or viral infection, overexpress proinflammatory cytokines, and cause severe inflammation. These cytokines include TNF-α, IL-1β, IL-6, G-CSF, GM-CSF, IFN-γ, and chemokine's such as IL8, MCP-1, MIP-1, CCL5, and CXCL10. The inability to inhibit this inflammatory reaction causes severe pathological damage to the infected organs and induces multiple organ failures like lungs, liver, kidneys, and testes, leading to the death of the patient. The inflammatory storm caused by a sudden increase in cytokines levels is an important cause of acute respiratory distress syndrome (ARDS) and respiratory failure in coronavirus disease 2019 (SARS-CoV-2) patients [1]. In addition to acute infection, the cytokine storm is also commonly seen in autoimmune diseases such as rheumatoid and systemic lupus erythematous, as well as diseases such as tumors and AIDS [2]. Therefore, treatment approaches that suppress the cytokine storm are tremendously important for reducing the body's excessive inflammatory response.

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For example, anti-inflammatory drugs such as glucocorticoids e.g. methylprednisolone and dexamethasone were widely used to treat SARS in 2003 and SARS-CoV-2 in 2019 and can inhibit inflammation and reduce the clinical symptoms of infected persons. Research done in the past years pointed out that the novel function of cholinergic transmission, by this cholinergic transmission can modulate and inhibit various aspects of the immune function, whether innate or adaptive immunity. Cholinergic transmission affects immune cell proliferation, cytokines production, T helper differentiation, and antigen presentation [3].

The circuit came to be dubbed the "cholinergic anti-inflammatory pathway". It was found that this effect was mediated by acetylcholine (ACh) stimulation of nicotinic receptors on splenic macrophages. Subsequent work identified the $\alpha 7$ nicotinic ACh receptor ($\alpha 7$nAChR) as the crucial target for attenuation of pro-inflammatory cytokine release from macrophages and dendritic cells [4]. The $\alpha 7$ nicotinic acetylcholine receptor was designated for anti-inflammatory activity and has shown promise in preclinical models of inflammatory disorders by various components of the immune cholinergic system, specifically the immune suppressive effects of $\alpha 7$ activation [5]. This activation can be accomplished either by direct stimulation or indirectly, by inhibition of AChE. Thus, the presence of the immune cholinergic system can pave the way for novel immunomodulatory agents, or for the broadening of the use of known cholinergic agents. Rivastigmine, a cholinesterase inhibitor, and alpha 7n Ach receptors agonist, effectively treats Alzheimer's disease symptoms [6]. Unlike other ChE-Is, Rivastigmine is a dual inhibitor with a longer duration of action than selective inhibitors and can also delay the development of amyloidogenic proteins. In this context, the ability of Rivastigmine to regulate the production of pro-inflammatory cytokines storm was investigated [7].

MATERIAL AND METHODS

Rivastigmine, Methylprednisolone, and Lipopolysaccharide were obtained from the USA/ Sigma Aldrich Chemical Company. SUN LONG Biological Technology Co. Ltd, China, provides ELISA kits, and Hematoxylin and Eosin stains from BDH/England.

Animals

In the Al-Nahrain College of Medicine's animal house center, 60 albino adult mice (male) weighing between (25-30 gms) and (25-30 gms) were maintained throughout the study under specific non-pathogen conditions with food and water supplements, a 12-hour light-dark cycle, and a controlled air temperature (15-21 °C).

Cytokine Storm Model Experiment Protocol

Before each usage, the solution of LPS was prepared according to the manufacturer's instructions/Sigma-Aldrich, by dissolving 10 mg of LPS powder in 10 mL normal saline in a glass tube and mixing by vortex for 30 minutes before each use [8], cytokine storm was induced [9, 10], then injecting a single dose (5 mg/kg) of Lipopolysaccharide (LPS) (Escherichia coli, serotype 055: B5, lot 0000133605 / 99%) intraperitoneally [11].

Assessment of pro-inflammatory cytokines and Histopathological Scoring

On day 8 the experimental study was end; light chloroform was used for anesthesia, blood was obtained by puncturing the jugular vein, and blood was centrifuged for 20 minutes at 3000 rounds per minute (rpm). The serum was stored at -20 °C to determine the cytokines levels (IL-1β, IL-6, and TNF-α) by the ELISA test following the directions provided by the manufacturer. The reader was configured for 450 nm within 5 minutes. Lung tissue homogenate was done to investigate the Transforming growth factor beta (TGF-). A lung histopathological study was also done and the Histopathologists examined the tissue samples in a blinded manner, and the results were scored from 0 to 3 (0 = normal; 1 = mild; 2 = moderate; 3 = severe) [12, 13].

Group HA: 10 male mice were apparently healthy.

Group LPS: 10 male mice received 5mg/kg lipopolysaccharide (LPS), and were left without treatment.

Group DMSO: 10 male mice with 5 mg/kg lipopolysaccharide (LPS), and after one hour received Dimethyl sulfoxide 1% for 7 constitutive days.

Group MP: 10 male mice received 5 mg/kg lipopolysaccharide (LPS), and after one hour received methylprednisolone 50 mg/kg for 7 constitutive days.

Group RA: 10 male mice received 5 mg/kg lipopolysaccharide (LPS), and after one hour received Rivastigmine 0.5 mg/kg for 7 constitutive days.

Group CARMP: 10 male mice received intraperitoneally 5 mg/kg lipopolysaccharide (LPS), and after one hour received half doses of Rivastigmine and methylprednisolone for 7 constitutive days.

Experimental and statistical design and analysis

The experiment design utilized in this study is a prospective control randomized design. All data were gained, tabulated, and went into statistical analysis put through using Social Sciences Software Statistical Package (SSPS) statistical software version (20). The result was presented as Means ±SD. One-way analysis of variance (ANOVA) followed by a t-test (2-tail) was used to compare between groups. The level of significance was set at the P values <0.05, P< 0.01, and P<0.001 as significant, highly significant and very high significant respectively [14].

Results

Therapeutic effects of Rivastigmine in cytokine storm

Serum was collected, lung tissue homogenate, and lung histopathological parameter values were quantitatively determined. Swiss albino mice of the LPS group at a dosage of 5 mg result editing in substantial increases in IL-1, IL-6, TNF-α, and TGF-β levels (P 0.001) when compared to the healthy control (AH) group.

Role of Rivastigmine in the Reduction of IL-1β

This study revealed that significant reduction (*P value* <0.001) of IL-1β Levels in the RA group other than observed in MP and RAMP groups in comparison with the induced non-treated LPS group. MP shows no obvious advantage with no significant differences when compared with each of the LPS and DMSO groups, as shown in Table 1, **Figure (1.1)**

Table (1) shows the study group's serum IL-1β level.

	IL-1β RT		P value (sig≤0.05)				
	Group	M±SD	LPS	DMSO	MP	RA	RAMP
1	AH	11.52±3.77	0.00	0.00	0.56	0.20	0.48
2	LPS	35.77±13.52		0.78	0.00	0.00	0.00
3	DMSO	37.22±16.20			0.00	0.00	0.00
4	MP	14.26±4.44				0.49	0.09
5	RA	17.55±5.78					0.57
6	RAMP	14.84±3.00					

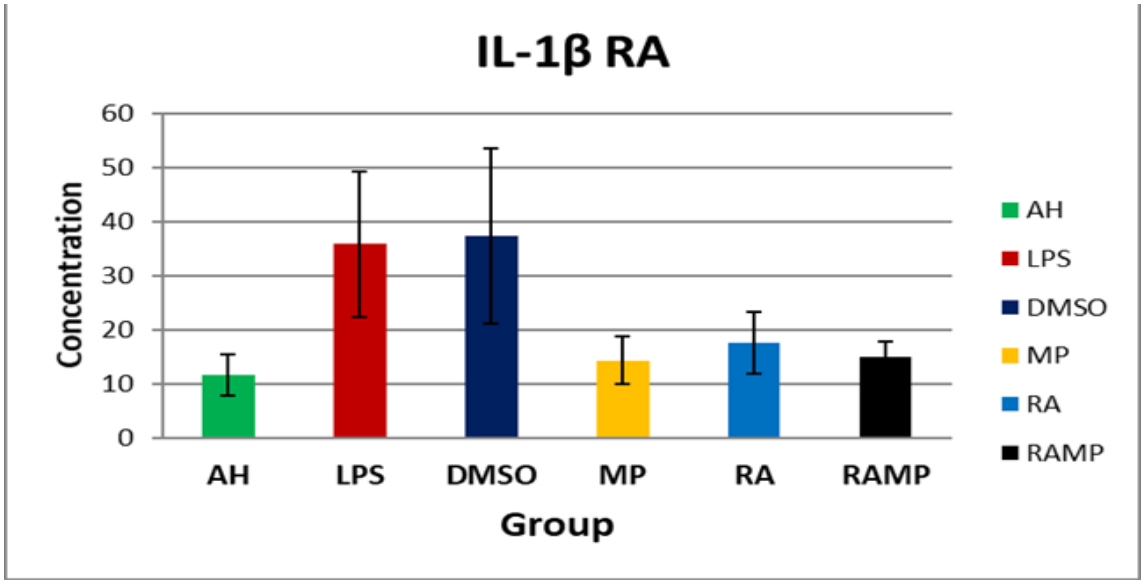

Figure 1: AH: Apparently healthy, LPS: induced by LPS, DMSO: DMSO 1%. RA: Rivastigmine, MP: Methylprednisolone, RAMP: Rivastigmine plus Methylprednisolone.

Role of Rivastigmine in the Reduction of IL6

Regarding to serum level of IL-6 was tabulated and represented as M±SD in pg./ml n=10, then statistically analyzed.

As shown in Table (2) and Figure 2 that showed LPS group showed significant elevations of serum IL-6 (72.97±24.52) in comparison with group AH (16.67±4.83). Rivastigmine, Methylprednisolone, and Rivastigmine plus Methylprednisolone showed a highly significant reduction in IL-6 levels as follows:

(15±2.96, 18.49±4.01, and 16±4.53) respectively, compared with the LPS and DMSO P value <0.001.

Table (2) shows the study group's serum IL-6 level.

	IL-6 RA		P value (sig≤0.05)				
	Group	M±SD	LPS	DMSO	MP	RA	RAMP
1	AH	16.67±4.83	0.00	0.00	0.69	0.71	0.88
2	LPS	72.97±24.52		0.00	0.00	0.00	0.00
3	DMSO	41.72±9.58			0.00	0.00	0.00
4	MP	18.49±4.01				0.44	0.58
5	RA	15±2.96					0.82
6	RAMP	16±4.53					

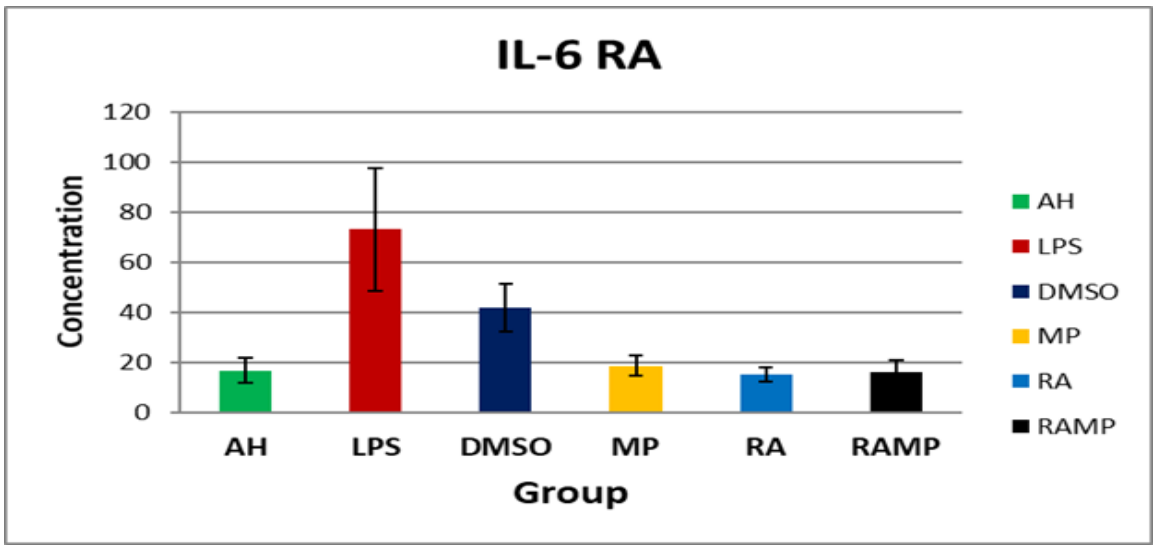

Figure 2: AH: Apparently healthy, LPS: induced by LPS, DMSO: DMSO 1%. RA: Rivastigmine, MP: Methylprednisolone, RAMP: Rivastigmine plus Methylprednisolone.

Regarding serum level of TNF-α tabulated and represent as M±SD in pg./ml n=10, then statistically analyzed. As shown in Table (3) and Figure 3 that showed.

LPS group produced highly significant elevations of serum TNF-α (144.42±30.14) and *P* value <0.001 in comparison with AH.

RA, MP, and RAMP groups (41.35±11.04, 48.36±15.31, and 47.95±11.05) P value <0.001 showed a highly significant reduction in TNF-α compared with the LPS and DMSO groups.

Table (3) shows the study group's serum TNF-α level.

	TNF-alpha RA		P value (sig≤0.05)				
	Group	M±SD	LPS	DMSO	MP	RA	RAMP
1	AH	0.31±0.01	0.00	0.00	0.00	0.001	0.00
2	LPS	144.42±30.14		0.00	0.00	0.00	0.00
3	DMSO	203.02±45.68			0.00	0.00	0.00
4	MP	48.36±15.31				0.56	0.97
5	RA	41.35±11.04					0.59
6	RAMP	47.95±11.05					

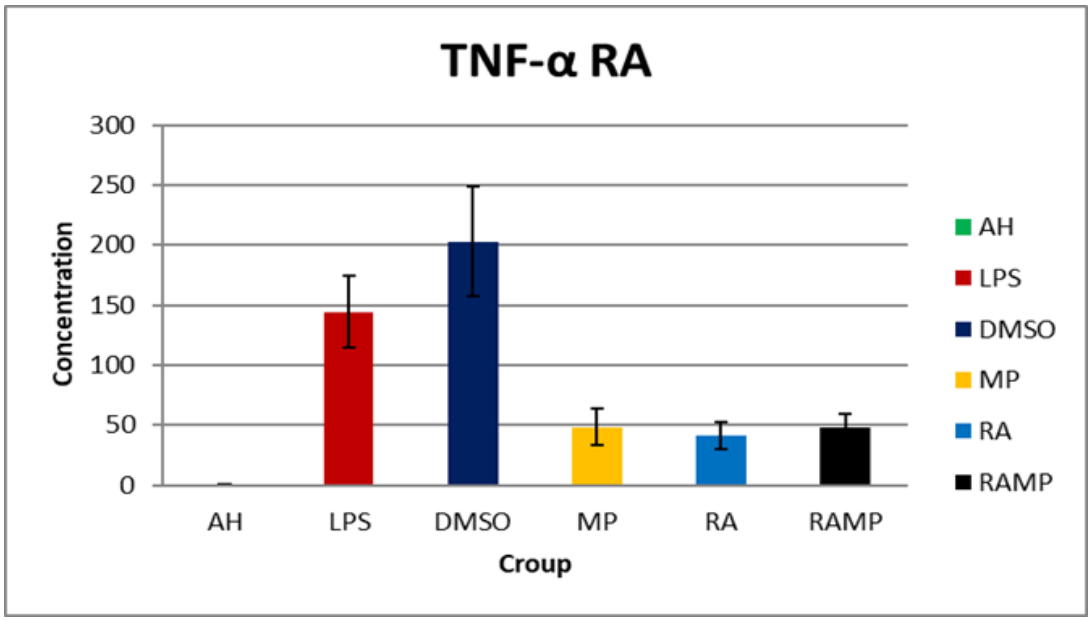

Figure 3: AH: Apparently healthy, LPS: induced by LPS, DMSO: DMSO 1%. RA: Rivastigmine, MP: Methylprednisolone, RAMP: Rivastigmine plus Methylprednisolone.

Regarding tissue homogenate level of TGF-β tabulated and represent as M±SD in pg./ml n=10, then statistically

analyzed.

As shown in Table 4 and Figure 4 that showed LPS group produced highly significant elevations of serum TGF-β (1.57±0.27) and P value <0.001 in comparison with group AH (0.43±0.09). Rivastigmine, Methylprednisolone, and Rivastigmine plus Methylprednisolone showed a highly significant reduction in TGF-β levels.

As follows: (0.61±0.15, 0.69±0.18, and 0.66±0.15) respectively, compared with the LPS and DMSO group.

Table (4) shows the study group's Lung Tissue homogenate level.

	TGF-B RT		P value (sig≤0.05)				
	Group	M±SD	LPS	DMSO	MP	RA	RAMP
1	AH	0.43±0.09	0.001	0.00	0.02	0.08	0.03
2	LPS	1.57±0.27		0.00	0.00	0.00	0.00
3	DMSO	1.11±0.29			0.00	0.00	0.00
4	MP	0.69±0.18				0.48	0.78
5	RA	0.61±0.15					0.67
6	RAMP	0.66±0.15					

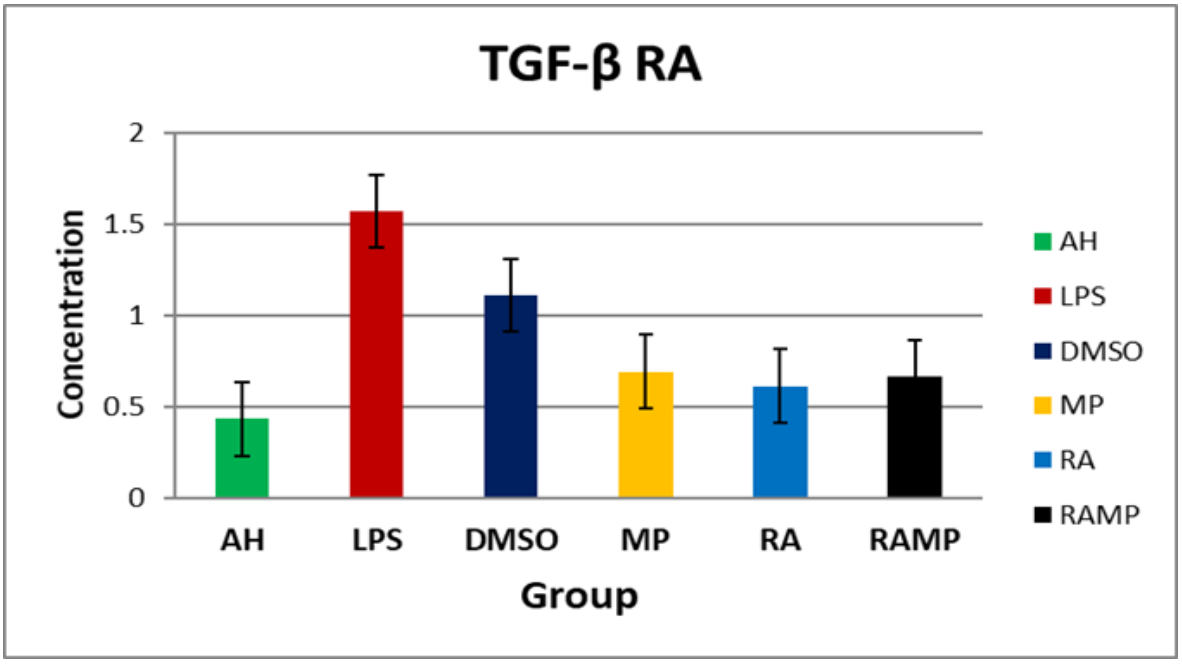

Figure 4: AH: Apparently healthy, LPS: induced by LPS, DMSO: DMSO 1%. RA: Rivastigmine, MP: Methylprednisolone, RAMP: Rivastigmine plus Methylprednisolone.

Histopathological examinations in the therapeutic effect of Rivastigmine in cytokine study

Histopathological changes of lung tissue examined by histopathologist after staining with (H&E). Stain revealed that there is a highly significant difference (P<0.001). In the AH group (Section of the lung showing normal histological structure which consists of alveoli and alveolar space. Score =0) in comparison with LPS-induced group (Section showing congestion, severe inflammatory cells infiltration, destruction in the alveolar septa. score=3). Rivastigmine, Methylprednisolone, and Rivastigmine plus Methylprednisolone groups section showing mild congestion, obvious decreasing thickness in the interalveolar septa, and slightly inflammatory cell infiltration (1-33) % (highly significant differences P<0.001) in comparison with LPS- induced group.

Table (5) showed study groups of lung histopathological score

	Lung -RA		P value (sig≤0.05)				
	Group	Median	LPS	DMSO	MPT	RA	RAMP
1	AH	0	0.00	0.00	0.00	0.00	0.004
2	LPS	3		1	0.00	0.00	0.001
3	DMSO	3			0.00	0.00	0.001
4	MP	1.2				0.16	0.01
5	RA	1					0.07
6	RAMP	0.4					

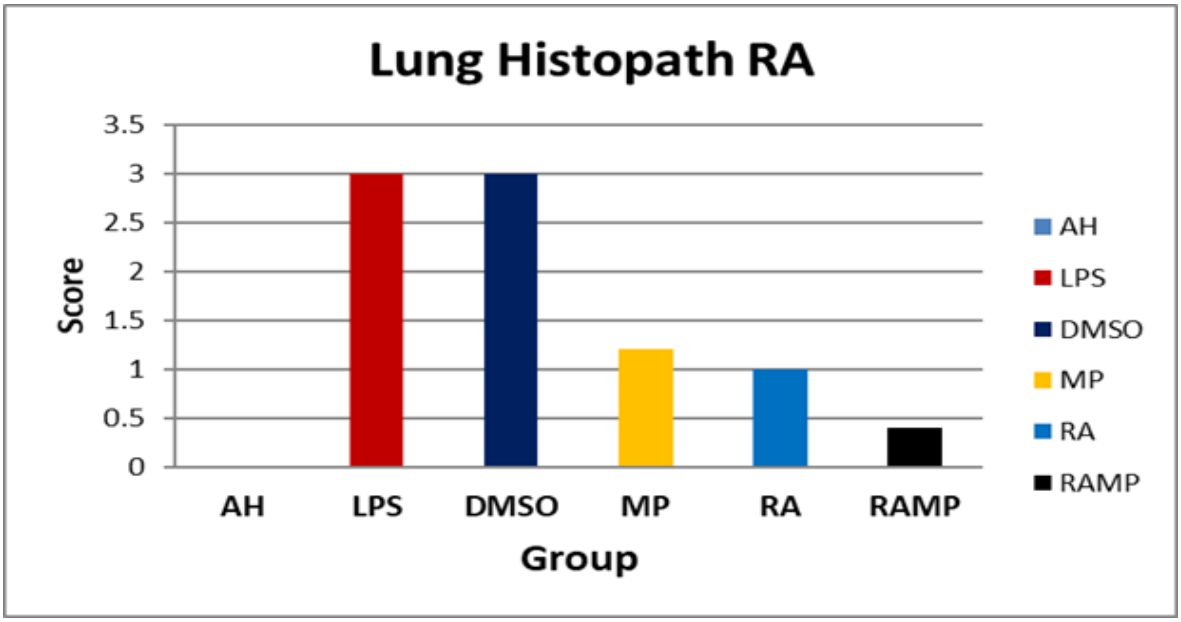

Figure 5: AH: Apparently healthy, LPS: induced by LPS, DMSO: DMSO 1%. RA: Rivastigmine, MP: Methylprednisolone, RAMP: Rivastigmine plus Methylprednisolone.

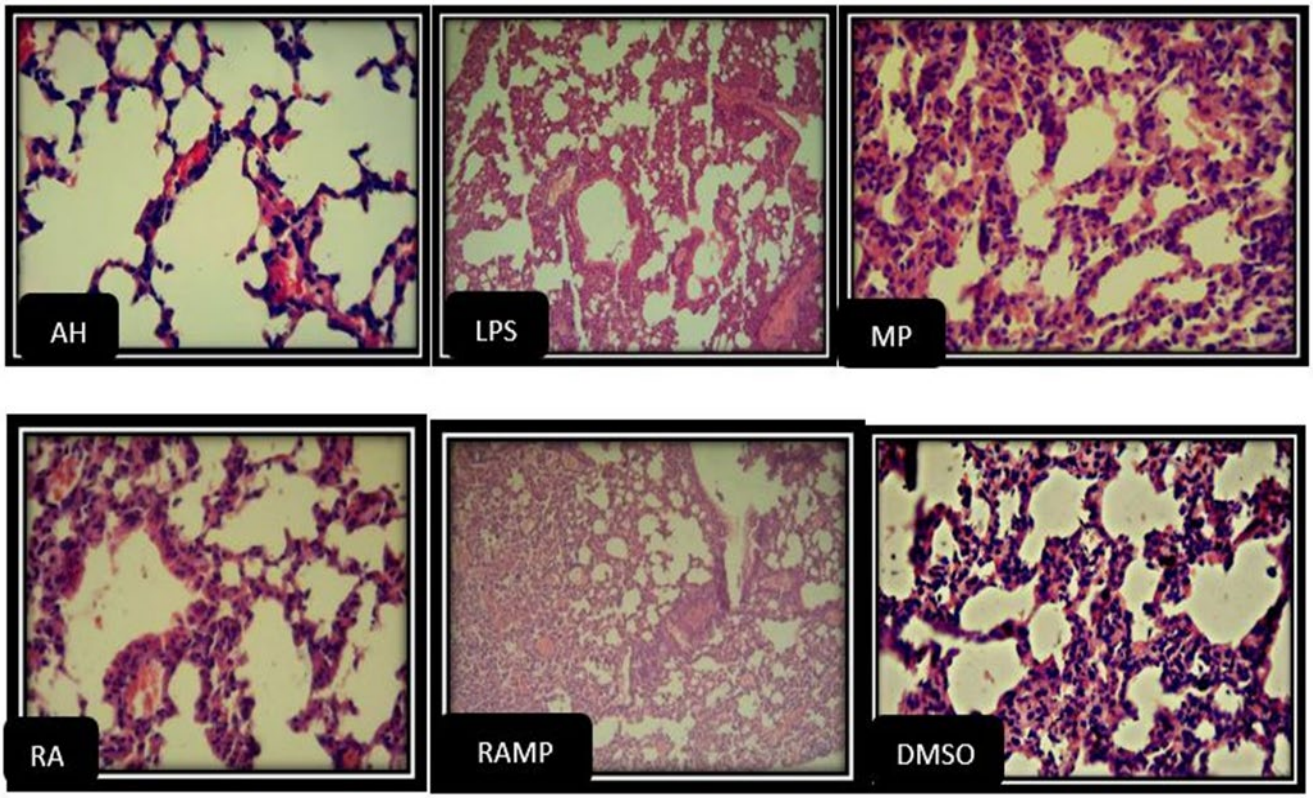

Figure 5: Lung histological section

Discussion

Cytokine storm

Cytokine storm Syndrome is a massive or uncontrolled release of pro-inflammatory cytokines TNF-α, IL- 1β, IL-6, IL-12, interferon (IFN)α, IFN-β, IFN-γ, monocyte chemoattractant protein-1 (MCP-1) and IL-8, and other inflammatory mediators that cause activation and expression of the immune system that is caused by many reasons the most common of these is infection and if not treated leads to Multi-Organ Dysfunction or Multi-Organ Failure that finally leads to death. Many efforts try to decrease or alleviate cytokine storm by any means, but the development of complications may restrict its use. So, investigating a drug with anti-cytokine storm with less complication degree consider a challenge [15].

Attenuated Effect of Rivastigmine on IL-1 β IL-6, and TNF-α Level in LPS-Induced cytokine storm in Mice

Rivastigmine is a carbamate cholinesterase inhibitor (ChEI) with a pseudo-irreversible activity that can inhibit not only acetylcholinesterase (AChE) but also butyrylcholinesterase (BuChE) [16, 17]. Oral administration of Rivastigmine was approved by the US Food and Drug Administration (FDA) in 2000 for the treatment of Alzheimer's disease (AD) [18] Mild to moderate Alzheimer's disease was treated and improved by Rivastigmine. Our study showed that Rivastigmine 0.5 mg/kg (Shifrin, et al. 2013) highly significantly decreased pro-inflammatory cytokine secretion (IL-1β, IL-6, and TNF-α) P< 0.001 as compared to the LPS group, that's was consistent with the previous study showing that Galantamine which is an acetylcholine esterase enzyme inhibitor suppressed the expressions of TNF-α, IL-1β, and IL-6 in the lungs, this suggests that Galantamine suppressed acute inflammatory response during the cholinergic anti-inflammatory pathway of acute acid aspiration-induced ARDS, and also suppressed the inflammation mediator HMGB1 [19]. In previous studies was shown ACh was inhibit pro-inflammatory cytokine production by activated macrophages.

Murine macrophages were activated by lipopolysaccharide (LPS), an endotoxin produced by all gram-negative bacteria and implicated in the pathogenesis of septic shock [20], to secrete TNF-a, IL-1b, and IL-6, all pro-inflammatory cytokines. ACh at the micromolar range significantly reduced this cytokine secretion [21], So the use of Rivastigmine as an AChE inhibitor that decreases the expression of NF-κB by activating α7 nicotinic acetylcholine receptors (α7nAChR) was involved in the anti-inflammatory effect of the cholinergic anti-inflammatory pathway [22]. This pathway inhibited the excessive production of TNF-α, IL-1β, and IL-6 mediated by the nicotinic acetylcholine receptor (nAChR) α7 subunit on macrophages [23, 24]. Mice deficient of the α7 subunit exhibited increased endotoxin-induced TNF production, which explains the role of α7 nAChRs in the inflammatory pathway [25, 26]. Macrophages appear to be very sensitive to acetylcholine, which suggests that any source of acetylcholine, even from non-neuronal sources such as epithelial and endothelial cells [26, 27], could also modulate the activity of adjacent macrophages [28]. Besides TNF, other pro-inflammatory cytokines are inhibited by acetylcholine, such as high mobility group B1 (HMGB1), IL-1β, and IL-6. The treatment of Rivastigmine can increase the antioxidant capacity, increase glutathione reductase and homocysteine, and that's important to reduce the peroxidation product MDA and the inflammatory mediator IL-6 [29, 30].

Effects of Rivastigmine on Lung Fibrosis

Enhancement of parasympathetic domination leads to inhibition of proinflammatory cytokine release and attenuation of tissue lung injury, that's mediated by cholinergic activation via Rivastigmine which downregulates acetylcholinesterase (AChE) improves autonomic influence and reduces inflammatory reactions [31, 32].

Rivastigmine has pleiotropic pharmacological effects through its antioxidant, anti-inflammatory, and anti-apoptotic roles. Our results showed that Rivastigmine significantly less inflammation and distortion of pulmonary architecture, mild congestion, obvious decreasing thickness in the interalveolar septae, and slightly inflammatory cell infiltration (1-33) % (highly significant differences P<0.001) in comparison with LPS-induced group [33]. This effect was mediated through the preserving Ach, which stimulated the nicotinic receptor α7nAChR in the macrophages and reduced the cytokine storm of the macrophages. These results were consistent with a previous study that revealed the important role of the cholinergic anti-inflammatory pathway and activation of α7nAChR as anti-inflammatory effects by inhibiting the release of pro-inflammatory cytokines [34].

Therefore, cholinergic receptors mainly α7nAChR, and related cholinergic agonists like Rivastigmine may affect the pathogenesis of SARS-CoV-2 infection. Cholinergic dysfunction in COVID-19 is due to dysregulation of α7nAChR by SARS-CoV-2 promoting the central sympathetic drive with the development of the sympathetic storm. As well, α7nAChR activators through interaction with diverse signaling pathways can reduce the risk of inflammatory disorders of cytokine storm in COVID-19[35, 36]. In addition, α7nAChR activators may mitigate endothelial dysfunction, oxidative stress, and associated coagulopathy in a cytokine storm. Therefore, α7nAChR activators like Rivastigmine in virtue of its anti-inflammatory and anti-oxidant effects with direct anti-SARS-CoV-2 effect could be effective in the management of cytokine storm in COVID-19 [37, 38].

CONCLUSION

A dose of 0.5 mg/kg of Rivastigmine and its combination with methylprednisolone showed obvious therapeutic effects on cytokine storm induced by LPS in Swiss Albino mice by highly significantly reducing the proinflammatory cytokines IL-1, IL-6, TNF-, and TGF- levels and subsequently protecting the tissue of lungs from damage. Half doses of both Rivastigmine plus Methylprednisolone (RAMP) showed no obvious advantage in comparison with Rivastigmine (RA) alone in induced cytokine storm in mice.

Rivastigmine dramatically suppressed and decrease lung fibrosis by inhibiting and controlling the expression of TGF-β and reducing exudative DAD, desquamation of hyperplastic pneumocystis, fibrosis, and squamous metaplasia in mice with LPS-induced cytokine storm, as seen in the current study. It has the potential to one-day serve as a helpful treatment for ARDS and other forms

of acute lung injury.

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