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Anomalin attenuates LPS-induced acute lungs injury through inhibition of AP-1 signaling

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ABSTRACT

In the present study, the anomalin was investigated to determine the protective effects and underlying mechanism against LPS-induced acute lung injury in mice. Anomalin administration 30 min after the LPS injection, significantly attenuated the mechanical allodynia, decrease body temperature, and improved the histological changes and inhibited the infiltration of leukocytes. The anomalin treatment markedly inhibited the production of pro-inflammatory mediators such as cytokines (IL-1β, IL-6 and TNF-α) and NO in contrast to the LPS treated groups. Similarly, the anomalin also enhanced the level of anti-oxidant enzymes such as GST, GSH, Catalase and inhibited oxidative stress marker such as MDA. In order to explore the molecular mechanism the effect of anomalin was evaluated for mitogen activated protein kinases (MAPK) in LPS-stimulated RAW264.7 cells. The anomalin treatment significantly attenuated the MAPK proteins such as ERK1/2, JNK and p38 (which is downstream signaling proteins to the MAPKKKs and MAPKKs protein) in the RAW264.7 macrophages using western blot analysis. Furthermore, the western blot analysis showed that anomalin treatment significantly inhibited the activation of the Akt proteins in the RAW264.7 macrophages. The AP-1 served as downstream target for the MAPK pathways and the blocking MAPK pathways is responsible for the inhibition of the AP-1 protein. The AP-1/DNA binding was assessed in the RAW264.7 cells using EMSA. The anomalin treatment significantly restricted the AP-1/DNA binding activity and the decrease in the AP-1/DNA binding activity might be contributed due to the upstream inhibition of the MAPKs signaling.

1. Introduction

Acute lung injury (ALI) is an important clinicopathological conditions associated with higher morbidity and mortality rate [1]. The most severe form of the ALI, acute respiratory distress syndrome (ARDS) trigger the failure of multiple organs and death of the patients characterized by the sepsis, pneumonia, trauma, shock, pancreatitis and multiple transfusions [1]. The most promising feature of the ALI is the infiltration of the immune cells with resultant destruction of the pulmonary parenchyma, neutrophilic infiltration and the release of the pro-inflammatory mediators such as IL-1β, IL-6 and TNF-α [1–3]. Additionally, these inflammatory mediators trigger the infiltration of the neutrophils into the interstitium of the lung and alveolar spaces [1,2]. Once activated, neutrophils tends to enhance the inflammatory process and tissue destruction via synthesizing and releasing proteolytic enzyme and reactive oxygen species [2,4]. In spite of being increasing research over the past decades to precisely understand the underlying pathophysiological mechanism, the mortality associated with the ALI/ARDS still remains very high [2,4]. Lipopolysaccharide (LPS) is an endotoxin and integral component of the cell wall of gram negative bacteria and have been recognized to be associated with the sepsis and inflammation mediated ALI. The LPS is believed to trigger the ALI via several signaling cascades. The LPS interact with Toll like receptor (TLR4), recognize it and induced the activation of intracellular signaling mechanism such as NF-κB and Akt [4]. The activation of these signaling mechanism are associated with induction of their concerned genes involved in inflammatory process. Acute lung injury is associated with the acute respiratory failure and can be developed in peoples of all

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ages [4]. The various factors that contributed to the development of ALI includes pneumonia, sepsis, major trauma, aspiration of gastric and oropharyngeal contents and several other clinicopathological conditions such as acute pancreatitis, blood products and drug over dose [4].

Macrophages are key player of the inflammatory processes and implicated in various inflammatory conditions by regulating the production of the various inflammatory mediators and cytokines [5–7]. Thus, therapies directing against the macrophages mediated inflammation opens new avenues to deal with various inflammatory diseases [5]. Furthermore, the induction of macrophages are influenced by LPS via interaction with the TLR-4 [5,6,8]. Following interaction of LPS with the TLR-4, trigger the activation of various transcriptional factors such as AP-1, which induce the production of the pro-inflammatory mediators following receiving signals from the upstream proteins [5]. The MAP kinase signaling cascade is well established pathway implicated in various conditions including inflammation, classified into several components such as p38, JNK (c-jun-N-terminal kinase), and ERK1/2 (extracellular signal regulated kinases 1/2) [6–8]. The induction of MAPKs family proteins trigger the synthesis of various pro-inflammatory mediators and cytokines. PI3K serve as dual protein and lipid kinase, has been implicated to be regulated by several factors such as growth factors and cytokines [8,9]. The PI3K is heterodimeric complex comprises of regulatory unit p85 (85-kDa) and catalytic unit p110 (110-kDa) [10–12]. Following activation, the PI3K phosphorylates phosphatidylinositol and regulate the synthesis of phosphatidylinositol 3,4,5-triphospahte as the second messenger molecule via acting on the substrate phosphatidylinositol 4,5-bisphosphate [9–12]. The second messenger thus formed induce the activation of downstream signaling mechanism such as Akt. The stimulated Akt then separate from the membrane to alter the activity of concerned genes in the cytoplasm and nucleus [9,11,12]. The role of the PI3K/Akt signaling has been associated with regulating the LPS-induced acute inflammation both in-vitro and in-vivo and PI3K/Akt pathway proved putative target for several anti-inflammatory therapies [3,6,12]. The present study investigated the effect of anomalin, a pyranocoumarin derivative from *Saposhnikovia divericata* on the ALI and the molecular mechanism through which anomalin confer this protection.

2. Materials and methods

2.1. Chemicals and reagents

All the chemicals and reagent in the current study includes dexamethasone, LPS, phosphate buffer saline (PBS), Tween 20, EDTA and phenylemethylesulfonyl fluoride (PMSF) were purchased from the sigma Aldrich (St. Louis, MO, USA). All the antibodies (primary and secondary such as p38, p-p38, ERK, p-ERK, JNK, p-JNK, SP, and β-actin) were obtained from the Santa Cruz Biotechnology (Santa Cruz, CA). However, the primary and secondary antibodies for the detection of the p-Akt and Akt were obtained from the Epitomics (Epitomics Inc., Burlingame, CA). The cytokines such as IL-1β, IL-6 (eBioscience, Inc., San Diego, CA) and TNF-α (Molecular Probes, Inc., OR) were analyzed using ELISA kits.

2.2. Cells and culture medium

RAW 264.7 murine macrophages were purchases from American Type Culture Collection (Manassas, VA, USA). These cells were subcultured and maintained (at 95% air and 5% CO2 humidified environment having temperature of 37 °C) as reported previously [13]. DMEM supplemented with 10% FBS (fetal bovine serum), 100 μg/mL of streptomycin and 100 U/mL of penicillin were added for in-vitro assays and routine subculturing. Almost all the chemical were purchased from the Sigma-Aldrich Co (St. Louis, MO). The samples were dissolved in the DMSO (dimethyl sulfoxide) and final concentration of DMSO was maintained at 0.2% and did not affected the assays performance.

2.3. Animals

In the current study, male albino mice (BALB/c) having weight in the range of 30–35 g and age of 5–6 weeks. All the animals were purchased from the national institute of health, Pakistan (NIH, Pakistan). All the animal activities were performed in the pathogen free environment of Quaid-i-Azam University Islamabad, Pakistan and the animals were provided free access to food and tap water. All the procedures were performed according to the guidelines approved by national institute of health declaration and Quaid-i-Azam university ethical committee (Approval No. BEC-FBS-QAU2018-84). Greater efforts were made to minimize harm to the animals.

2.4. Model and grouping

All the animals were divided into six groups and the animals were randomly assigned to each treated group.

1. Normal control treated with normal saline intraperitoneal (i.p)
2. Negative control treated with LPS (5 mg/kg, i.p)
3. Positive control treated with the dexamethasone (20 mg/kg)
4. Anomalin (1 mg/kg, i.p)
5. Anomalin (10 mg/kg, i.p)
6. Anomalin (50 mg/kg, i.p)

2.5. Effect of anomalin treatment on the LPS-induced fever

The LPS administration is strikingly associated with the elevation of the body temperature and hence, causes pyrexia [14]. The body temperature of the mice were measured before and 24 h after the commencement of the LPS administration. The rectal route was used to assess the body temperature as described previously [14].

2.6. Wet to dry ratio of lung

The effect of anomalin on the wet to dry ratio of the LPS treated lung tissue was perform as described previously. The animals were randomly assigned to various groups (normal control, negative control, positive control and anomalin treated). The lung samples were obtained 24 h after LPS treatment from the all the treated groups. The lung samples were weighed immediately after their isolation and dried in the oven at 70 °C. 72 h following desiccation, the wet to dry ratios were calculated to assess the pulmonary edema [14].

2.4. Effect of anomalin on the survival rate

The survival rate was assessed following the administration of the LPS (i.p) for 24 h as described previously [14]. The animals were treated with the various drugs following the LPS administration. The animals were randomly assigned to the various groups such as normal control, negative control, positive control and anomalin with three different doses (1 mg/kg, 10 mg/kg, and 50 mg/kg). The animal death rate were observed for 24 h after the LPS administration [14].

2.7. Mechanical allodynia

Von Frey filaments were used to assess the mechanical allodynia according to the previously reporting methods [15,16]. Mice were placed in a transparent mesh floor plastic floor to allow the free access of the Von Frey filaments to right hind paw ventral surface of the mice [6,17,18]. Before the assessment of mechanical allodynia, animals were acclimatized to their testing environment for 30 min and calibrated Von Frey filaments (Stoelting, USA) by employing double blinded assignments [17,18]. The Von Frey Filaments were applied five times via increasing the applied force and withdrawal reflex for at least three time was considered as positive response [17,18]. All the groups

(normal control, negative control, positive control and anomalin 1 mg/kg, 10 mg/kg and 50 mg/kg) were treated 40 min after LPS (i.p) administration.

2.8. Effect of anomalin treatment on NO production

The NO is an important mediator involved in the regulation of various physiological and pathological conditions including inflammation [17,18]. The NO production can be used as marker of the inflammatory process and effect of drug on the ongoing inflammatory process [6,16,17]. In order to evaluate the effect of the anomalin against the LPS-induced ALI, the animals were divided into 6 groups as discussed above ($n = 8$). The NO production was assessed 24 h after the LPS challenge in all the treated groups in both plasma and lung tissue. The animals were killed by cervical dislocation and the blood were collected from the cardiac puncture directly [16,17]. The collected blood were centrifuged at 5000 rpm for 5 min at the temperature of the 4 °C. The plasma was separated from the whole blood and was utilized for the determination of the NO as described previously [6,16–18].

2.9. Histological analysis

To assess the protective effect of anomalin on the histological changes of LPS-induced lung injury, H and E staining of the lung tissue was performed. Mice were killed using terminal anesthesia and the lung tissue were removed. The lung tissue was washed with normal saline following removal, fixed in 10% formalin solution, embedded in paraffin following rinsing and dehydration as described previously [18–20]. Lung tissue blocks were sectioned at 4 um thickness, observed with the microscopy (40×) after H and E staining. The histopathological findings were quantified using grading system from 0 to 4. The 0 represent the normal histology, while the grade 4 is assigned to the severe alteration in the lung tissue. The grade 4 is characterized by the pulmonary congestion, inflammation of the interstitium, and the recruitment of the inflammatory cells. The total histological score was calculated by adding the individual score [14,17].

2.10. Effect of anomalin treatment of hematology

The blood complete profile was performed to assess the effect of the anomalin treatment on the hematological parameters. The blood sample was obtained directly from the cardiac puncture and collected in the EDTA containing tubes and were analyzed with the CELL-DYN 4000 hematology analyzer [21]. For the differentials analysis blood smears were prepared and stained [22].

2.11. Effect of anomalin on anti-oxidant enzymes

The effect of anomalin was investigated on the anti-oxidant enzymes such as GSH, GST and Catalase according to the previously reported method [23]. The animals were randomly assigned to 6 experimental groups such as normal control, negative control, positive control and anomalin treated groups (1 mg/kg, 10 mg/kg and 50 mg/kg). The mice lung tissue was obtained 24 h after the LPS administration and the concentration of the anti-oxidant such as GST, GSH and Catalase was estimated. The reduced glutathione concentration was estimated adding 0.1 ml of tissue homogenate and 2.4 ml of the phosphate buffer saline. Finally, 0.5 ml of the DTNB was added to the described mixture to make the volume up to 3 ml. The absorbance was recorded at 412 nm [23,24]. The concentration of the GST was measured as per previously reported method [24]. The concentration of the GST was quantified by its capability to conjugate CDNB and GSH. In order to estimate the concentration of the GSH, 0.1 ml of the mice lung tissue homogenate was mixed with an equal concentration of the CDNB i.e. 0.1 ml. 0.1 M phosphate (pH 6.5) buffer was introduced into the above mixture the above mixture to make the final volume up to 3 ml. The UV-spectrophotometer was used to quantify the GST at 314 nm wave length [23]. Similarly, the quantity of the Catalase in the mice lung tissue was measured as reported previously [24]. The Catalase assay was performed by the rapid addition of the 40 µl of an enzyme extract to the 3 ml of the H_2O_2-phospahte buffer in the experimental cuvette. Finally, the Catalase was quantified using UV–Visible spectroscopy at 240 nm wave length [23,24].

2.12. Estimation of Lipid peroxidation

The lipid peroxidation (LPO) is an important marker for the oxidative stress and have been reported in various inflammatory conditions including ALI/ARDS [25]. The rate of the lipid peroxidation was determined by calculating the level of malondialdehyde (MDA) as described previously [25]. The supernatant from the lung tissue was obtained and the quantification of the thiobarbituric acid reactive substances (TBARS) was performed at 535 nm wave length [25].

2.5. Effect of the anomalin on the pro-inflammatory cytokines

The effect of anomalin on the production of inflammatory cytokines were determined using ELISA assay according to the previously described method [6,20]. The lung tissue samples from all the treated groups such as normal control (treated with normal saline), negative control (treated with LPS only, i.p), positive control (treated with dexamethasone 10 mg/kg) and anomalin (50 mg/kg) were obtained and the level of inflammatory cytokines were determined using commercially available ELISA kits. For the determination of the IL-1β, IL-6 and TNF-α (eBioscience, Inc., San Diego, CA, United States) kits were used as per the instruction of the manufacturer [6,26]. The lung from each treated group were removed, tissue protein extraction was performed using phosphate buffer saline (PBS) having concentration of 100 mg tissue per ml PBS. To this solution 0.4 M NaCl, 0.05% tween 20 and protease inhibitors were incorporated. The samples were than centrifuged for 10 min at 3000 *g* as described previously [6,20,26].

2.13. Western blot analysis

Western blot analysis was performed as described previously [6]. RAW 264.7 macrophages were stimulated with the 1 µg/ml LPS for 18 h following treatment with the 10, 30 or 50 µM concentration of 6 tested stilbene compounds for 2 h to determine the MAPKs and Akt. The RAW macrophages were stimulated for time course experiements with the LPS (1 µg/ml) for 30 min pre-treated with desoxyrhapontigenin for the determination of the p-p38, p38, p-JNK, JNK, p-ERK, ERK, p-Akt and Akt with their respective primary (1:1000) and secondary antibodies (1:5000). For the quantification of the target bands UN-SCAN-IT™ gel 6.1 software (silk scientific Corp, Orem, UT) was used [27].

2.14. Electrophoretic mobility shift assay (EMSA)

To investigate the effect of anomalin on the nuclear translocation as well as the binding interaction of the DNA with AP-1 EMSA was performed as described previously [3,6]. The AP-1 is a downstream target for the MAPK and Akt signaling pathway and translocated into the nucleus following activation. Within the nucleus, AP-1 interact with the TPA response element (TRE) to regulate the expression of the concerned genes. The nuclear protein were extracted using Buffer A (10 mM HEPES pH 7.9, 1 M HEPES, 10 mM KCL, 0.1 mM EDTA, 0.1 mM EGTA with 1 mM DTT, 0.5 mM PMSF, while before the experiment protease inhibitors were added) and Buffer C (20 mM HEPES pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA with 1 mM DTT, 0.5 mM PMSF, and before the initiation of assay protease inhibitors were incorporated) [3,6]. The nuclear proteins extract were incubated for 30 min at room temperature with AP-1 consensus oligonucleotides (Promega, sequence: 5′-AGT TGA GGG GAC TTT CCC AGG C-3′, 5′-AGA GAT TGC CTG ACG

TCA GAG AGC TAG-3′ and 5′-(CGC TTG ATG AGT CAG CCG GAA)-3′). Native polyacrylamide gel (6%) were used to separate the DNA protein complexes from the free oligonucleotides [3]. FLA-3000 apparatus (Fuji) was used to quantify the signals obtained from the dried gel, utilizing BAS reader version 3.14 and Aida version 3.22 software (Amersham Biosciences, Piscataway, NJ). The optimization of the binding conditions was done according to the previously reported methods [3,6].

2.5. Statistical analysis

The results were expressed as means standard deviation from three independent experiments. In order to determine the statistical significance one way analysis of variance (ANOVA) followed by the Dunnett's test was employed (SPPS version 10.0, Chicago, IL). For statistical significance $p < 0.05$ was chosen as criterion.

3. Results

3.1. Inhibitory effect of the anomalin treatment on the body temperature

The LPS (i.p) significantly increased the body temperature 24 h after the administration. The anomalin treatment markedly attenuated the pyrexia dose dependently, however, the dose of 50 mg/kg strikingly reduced the body temperature. Similarly, the positive control treated with the dexamethasone also dramatically attenuated the body temperature compared to the control as shown in the Fig. 1.

3.2. Anomalin attenuates the lung wet/dry ratio

The LPS administration is associated with the significant increase in the pulmonary edema. The wet to dry ratio for all the treated groups were determined 24 h following LPS challenge. The anomalin treatment significantly attenuated by the anomalin dose dependently as shown in figure. Similarly, the positive control treated with the dexamethasone also markedly reduced the wet to dry lung ratio compared to the negative control as shown in the Fig. 2.

3.3. Anomalin treatment improved the survival rate

The LPS was associated with significant death rate 24 h after their administration. However, anomalin administration markedly improved the survival rate in dose dependent fashion and the dose of the 50 mg/kg exhibited significant improvement in the overall survival rate compared to negative control. Furthermore, the positive control treated with the dexamethasone also significantly improved the mortality rate in contrast to the negative control as shown in the Fig. 3.

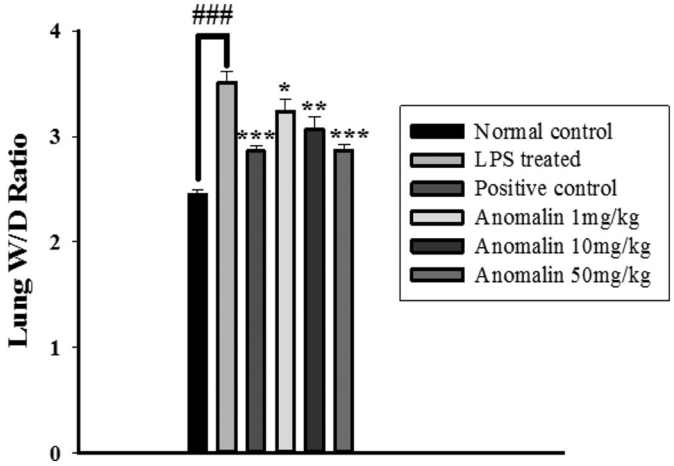

Fig. 2. The effect of the anomalin (1 mg/kg, 10 mg/kg and 50 mg/kg) on wet/dry ratio of lung. The anomalin and dexamethasone was administered after the LPS (i.p) administration and the wet/dry ratio was assessed. The anomalin treatment markedly attenuated the lung weight to dry ratio dose dependently 24 h after the LPS administration. The dexamethasone treated group also exhibited the significant improvement in the lung wet to dry ratio. The data are represented as the means (n = 8) ± SD, $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ indicate significant differences from the CFA-treated group. ###indicates a significant difference vs. negative control group.

3.4. Mechanical allodynia results

The anomalin treatment markedly attenuated the mechanical allodynia dose dependently compared to the negative control. Similarly, the positive control treated with the dexamethasone also strikingly inhibited allodynic responses in contrast to the negative control group as shown Supplementary 1.

3.5. Inhibitory effect of the anomalin on the NO production

The inhibitory effect of the anomalin on the LPS-induced lung injury was evaluated. The LPS administration significantly enhanced the NO production in both plasma and lung tissue. The anomalin treatment significantly decreased the production of NO in both plasma and tissue compared to the negative control as shown in Fig. 4. Similarly, the dexamethasone treatment also markedly attenuated the NO production in plasma and tissue as shown in the Fig. 4.

3.6. Effect of the anomalin on the histological changes

The H and E staining was performed to evaluate the effect of

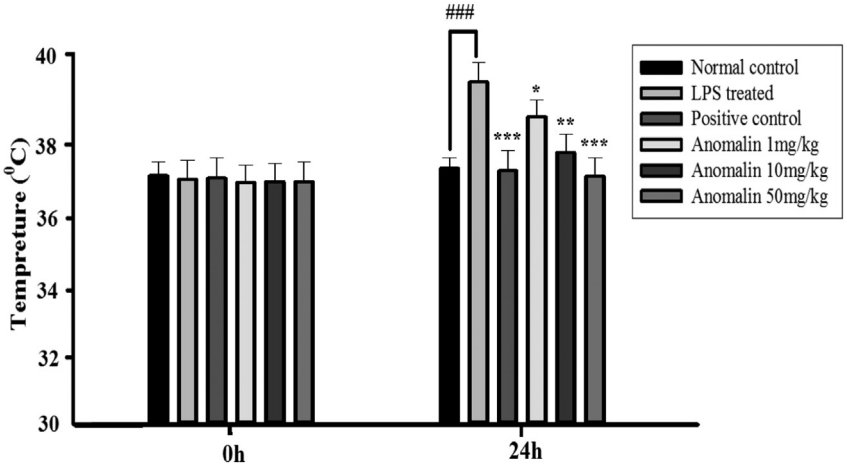

Fig. 1. The effect of the anomalin treatment on the body temperature. The anomalin (1 mg/kg, 10 mg/kg, 50 mg/kg) dose dependently attenuated the body temperature. Similarly, the dexamethasone treated group also markedly decreased the body temperature. The data are reported as the means ($n = 8$) ± SD, $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ indicate significant differences from the CFA-treated group. ###indicates a significant difference vs. negative control group.

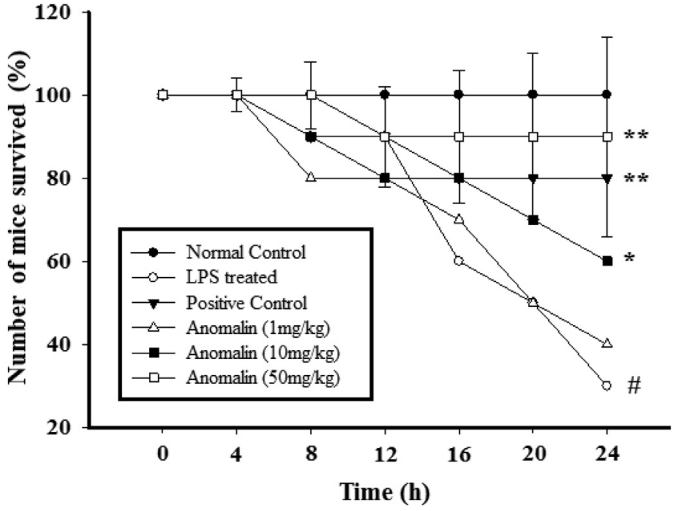

Fig. 3. Effect of anomalin LPS-induced mortality in mice (n = 8). The LPS administration significantly confer the mortality rate in the treated animals. However, the anomalin treatment markedly improved the survival rate dose dependently compared to the negative control. Similarly, the dexamethasone also improved the survival rate following LPS administration.

anomalin treatment on the LPS-induced lung injury as reported previously [14]. The anomalin treatment significantly attenuated the LPS-induced pathological changes dose dependently compared to the negative control. Similarly, the positive control administered with the dexamethasone (10 mg/kg) also markedly reversed the pathological changes compared to the negative control evident from Fig. 5.

3.7. Effect of anomalin on the blood complete profile

The LPS being an integral component of the gram negative bacteria and have been implicated in the eliciting strong inflamamtory reactions [28]. The inflamamtory reaction is cahraterized by the enahncemnt of imune cells such as neutrophils and monocytes etc. [28]. The anomalin treatment significantly reversed the LPS-induced hemotological changes in a dose dependent nature and the dose of the 50 mg/kg exhibited maximum response. Similarly, the positive control treated with the dexamethasone also marekdly attenauted the hematological parameters (Table 1).

3.8. Effect of anomalin on GSH, GST and Catalase Concentrations

The ALI/ARDS is strongly established with the modulation of anti-oxidant defense mechanism [29]. The LPS administration significantly comapromised the anti-oxidant mechanism 24 h after adminsitration. However, the anomalin administration significantly reverse the balanced and marekdly increased the level of anti-oxidant enzymes dose dependently compared to the negative control group only tretaed with the LPS as shown in Fig. 6. Simialrly, the dexamethasone also reversed the balance and dramatically increased the production of the anti-oxidant enzymes in mice lung tissue as depicted in the Fig. 6.

3.9. Anomalin Abrogates LPS-induced pulmonary lipid peroxidation (MDA level)

The anti-oxidant potenatil of the anomalin was confirmed from the LPO assay as describe dpreviouly [30,31]. The animals were reandomly divided into 6 groups ($n = 8$) (normal control, negative control, positive control and anomalin (1 mg/kg, 10 mg/kg and 50 mg/kg)). The LPS adminsitration markedly elevated the level of the MDA 24 h folloing their administration. However, the anomalin administraation significantly reversed the oxidative stress marker dose dependently in contrast to the LPS treated group. Similarly, the dexamethasone administration also drastically attenuated the oxidative stress maker as shown in the Fig. 6.

3.10. Inhibitory effect of the anomalin on the production of the pro-inflammatory cytokines

The animals were randomly assigned to the 4 groups ($n = 8$) such as normal control, negative control, positive control and anomalin treated. The LPS administration significantly enhanced the level of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α in the lung tissue. However, the anomalin treatment significantly attenuated the production of the pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α using ELISA assay as described previously [19]. Similarly, the dexamethasone treatment also markedly inhibited the production of the inflammatory mediators compared to the negative control as shown in the Fig. 7.

3.11. Effects of anomalin treatment on the MAPKs and Akt proteins

In order to explore further the molecular mechanism of anomalin, the inhibitory effect of anomalin was investigated on the Akt and MAPK

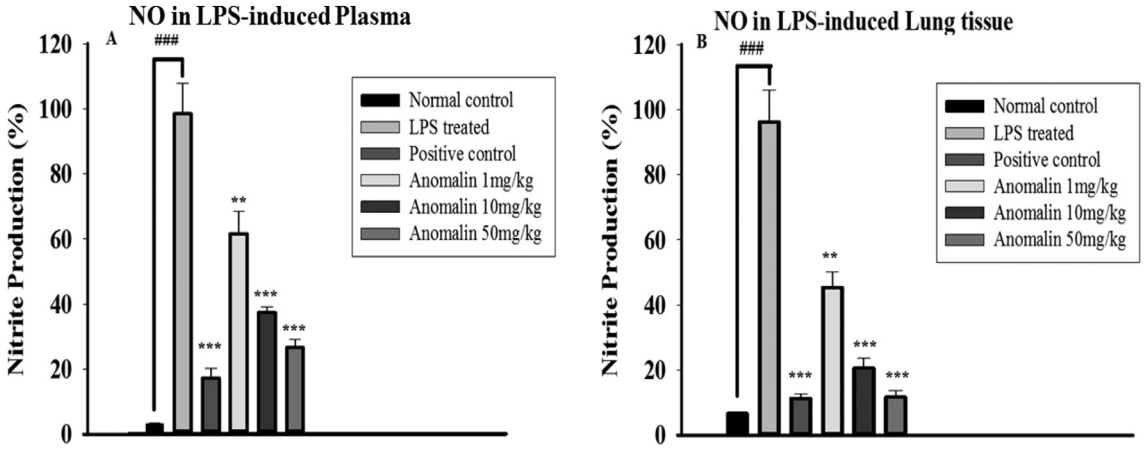

Fig. 4. Effect of the anomalin on the NO production in both plasma and tissue. The anomalin (1 mg/kg, 10 mg/kg and 50 mg/kg, i.p) significantly altered the NO production in both plasma and tissue dose dependently. Similarly, the dexamethasone also markedly decreased the NO production in the both plasma and tissue. The data are reported as the means ($n = 8$) ± SD, $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ indicate significant differences from the CFA-treated group. ###indicates a significant difference vs. negative control group.

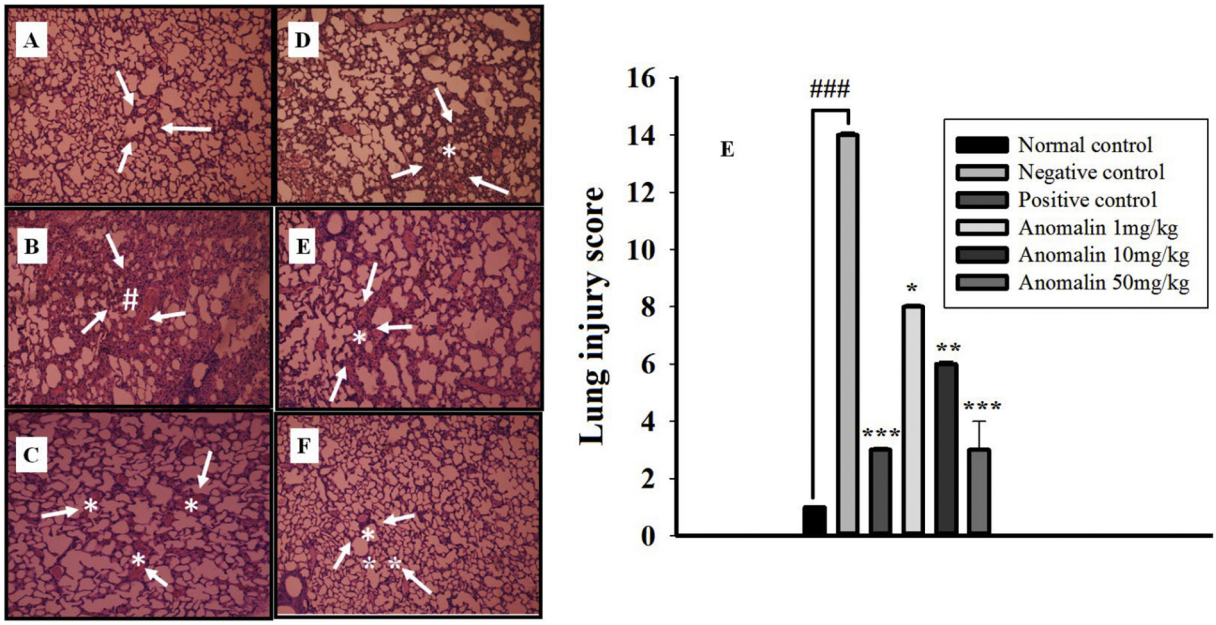

Fig. 5. Effect of the anomalin on the histopathology of LPS-induced lung injury. The anomalin (1 mg/kg, 10 mg/kg, and 50 mg/kg) treatment significantly improved the histopathology changes compared to the negative control. The histological changes were quantified using scoring from 0 (normal) to 4 (severe). These scoring system representing categories such as pulmonary congestion, edema, inflammation of interstitium and the infiltration of the immune cells.

proteins (p38, ERK1/2, and JNK) using Western blot analysis. The phosphorylated and activated form of the Akt was markedly elevated 30 min following treatment with the LPS, however, the anomalin treatment markedly inhibited the p-Akt as shown in Fig. 8. Additionally, the pretreatment with anomalin drastically altered the LPS mediated phosphorylation of the p38, JNK and ERK1/2 30 min following activation as described in Fig. 8.

3.12. Anomalin inhibit DNA/AP-1 interaction

The anomalin was administered to assess the interaction between the AP-1 and DNA binding activity using EMSA. The pretreatment of macrophages with anomalin remarkably attenuated the AP-1 and DNA binding activity following LPS administration as depicted in Fig. 9. However, the anomalin administration inhibited the interaction of AP-1 with its consequent DNA sequences, thus, halting the DNA/AP-1 complexes interaction with the concerned genes to synthesize inflammatory cytokines such as IL-1β, IL-6 and TNF-α.

4. Discussion

The ALI/ARDS is an acute respiratory syndrome and associated with the higher morbidity and mortality in clinical settings [32,33]. The ALI/ARDS is associated with the release of pro-inflammatory mediators and implicated to be related with aggravation of the symptoms [32,33]. Similarly, the LPS-induced ALI has been reported to be highly associated with the elevation of the oxidative stress [32,33]. The LPS, endotoxin of the gram negative bacteria and have been reported as an agent to induced pyrexia. Similarly, the LPS administration trigger the inflammation of the lung tissue as evident from the ALI/ARDS and the lung becomes boggy and the wet weight is significantly increased [32–34]. In the present study anomalin was investigated against the LPS-induced lung injury in mice. Following LPS administration, there was significant increase in the body temperature and wet to dry ratio of the lung. However, the anomalin administration significantly decreased the body temperature and wet to dry ratio of the lung in dose dependent fashion, while the dose of 50 mg/kg exhibited significant activity. Similarly, the dexamethasone treated group also markedly attenuated the pyrexia and wet to dry ratio compared to the negative control.

The ALI/ARDS is exacerbated by the inflammatory mediators and cytokines such as IL-1β, IL-6 and TNF-α and the concentration of these inflammatory mediators are strongly associated with the worse prognosis of ALI [33–35]. The LPS administration markedly enhanced the level of the inflammatory cytokines in the lung tissue. However, anomalin administration significantly inhibited the LPS-induced production of inflammatory mediators and markedly improved the sign and symptoms in animals compared to the LPS treated group. During ALI, the level of oxidative stress markers are highly enhanced and associated with the destruction of the lung tissue [4]. The approaches that target the oxidative stress are reported to have significant impact on the overall improvement of the symptoms associated with the ALI [4,36]. The anomalin administration significantly altered the oxidative stress

Table 1
Effect of anomalin on the complete blood count.

Parameters	WBC count (10^9/L)	LYM (10^9/L)	NEU (10^9/L)	RBC (10^{12}/L)	PLT (10^9/L)
Normal control	4.4 ± 0.17	3.6 ± 0.90	2.01 ± 0.12	4.62 ± 0.19	189 ± 10
LPS treated	8.43 ± 0.11###	6.9 ± 0.17###	6.4 ± 0.08###	4.41 ± 0.13###	135 ± 16###
Dexa 10 mg/kg	4.9 ± 0.21**	3.8 ± 0.11***	2.20 ± 0.12***	4.49 ± 0.30***	183 ± 10***
Anomalin 1 mg/kg	4.90 ± 0.23**	4.90 ± 0.09*	3.01 ± 0.12*	4.34 ± 0.11**	159 ± 22**
Anomalin 10 mg/kg	4.8 ± 0.15**	4.30 ± 0.12**	2.60 ± 0.15**	4.51 ± 0.40***	158 ± 13**
Anomalin 50 mg/kg	4.4 ± 0.10***	3.70 ± 0.15***	2.21 ± 0.90***	4.50 ± 0.09***	182 ± 9***

The data is represented as mean (n = 8) ± SD. (###) denotes comparison to normal group. *p, **p and ***p denotes comparison to LPS-induced group.

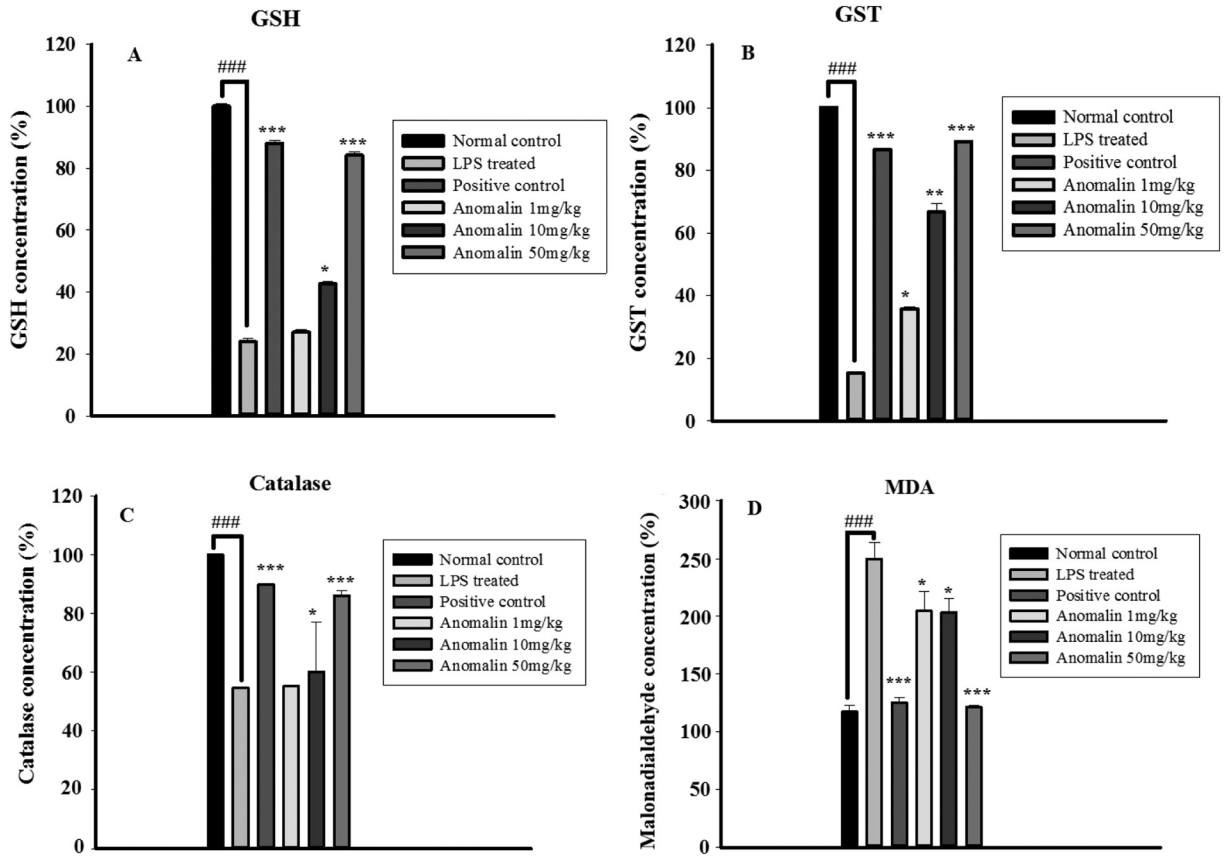

Fig. 6. Effect of anomalin on the anti-oxidant enzymes. The LPS administration significantly compromised the anti-oxidant mechanism. However, the anomalin treatment dose dependently enhanced the anti-oxidant enzymes such as GST, GSH and Catalase compared to the negative control. Similarly, the LPS also markedly enhanced the oxidative stress marker such as MDA, while the anomalin treatment strikingly attenuated the MDA production as described in "Materials and methods" section. The data were represented as the means ($n = 8$) ± SD, $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ indicate significant differences from the CFA-treated group. ###indicates a significant difference vs. negative control group.

markers and significantly enhanced the anti-oxidant mechanism such as GSH, GST and catalase, and improved the symptoms of ALI compared to the negative control. The ALI can better be predicted from the study of the histopathology and the changes can be perceived in a better way [4]. The ALI significantly altered the histology of the lung tissue and trigger the infiltration of the immune cell into the lung tissue [35,36]. In the current study the LPS administration significantly altered the histology of the lung tissue and characterized by increased infiltration of the immune cells, edema and inflammation. The anomalin treated group exhibited reversal of the symptoms compared to the negative control. Similarly, the positive control treated with the dexamethasone also markedly improved the histological markers in contrast to the negative control.

Macrophages are important component of the immune system, and considered the arm of the innate immune system [37]. The RAW 264.7 murine macrophages are commonly employed cell lines to mimic the in vitro ALI. Following challenge with the LPS, the activated RAW 264.7 cells induce the production of the various pro-inflammatory mediators via interaction with the TLR-4 [37]. The interaction of the LPS with the TLR-4 trigger the activation of the several downstream signaling mechanism including MAPKs and Akt [37]. During ALI, the involvement of several signaling mechanism have been implicated including MAPKs and Pi3K/Akt [35,38]. MAPKs proteins critically regulate various physiological and pathological conditions including inflammation, pain, cancer etc. [35,38,39]. The MAPK proteins comprises of three component such as ERK1/2, JNK, and p38. Following the activation by several factors, the TLR4 trigger the phosphorylation and activation of the MAPKs protein (at three stages MAPKKKs, MAPKKs and MAPKs) [35,38,39]. The phosphorylated protein induced the activation of the downstream signaling proteins. The phosphorylated proteins trigger the induction of the inflammatory mediators including cytokines [38–40]. NO is an important mediator of inflammation and the production of NO is directly related with the extent of inflammation. The NO synthesis is regulated by the iNOS gene and served as good predictor of the ongoing inflammatory process [40]. The LPS administration significantly enhanced the production of the NO in both plasma as well as in the lung tissue. However, the anomalin treatment markedly reversed the NO production in plasma and lung tissue relative to the LPS treated group. Additionally, the dexamethasone administration also markedly attenuated the NO production in both the plasma and lung tissue.

The western blot analysis was performed to assess the effect of anomalin on the LPS administration on RAW 264.7 cells. The LPS treatment significantly enhanced the phosphorylation and activation of the MAPK protein in the RAW 264.7 cells as shown in the western blot analysis. However, the anomalin treatment significantly inhibited the phosphorylation of the ERK, JNK and p38 as evident from the figure. Similarly, the effect of anomalin was also investigated on the Akt signaling activation in the RAW 264.7 cells. Furthermore, the LPS activated RAW 264.7 cells significantly enhanced the phosphorylation of the Akt protein as shown in the western blot analysis. The anomalin treatment significantly dephosphorylated the Akt protein in time dependent fashion. The MAPK pathways, regulate the phosphorylation and subsequent activation of the AP-1 proteins [41–43]. The MAPK signaling significantly influence the production of the inflammatory cytokines, growth factors and response of the cells to the environmental stress via activation of the AP-1 proteins [41–43]. The AP-1 served as downstream target for the MAPKs signaling pathways and, blocking of MAPKs pathway upstream is associated with the inhibition of the AP-1

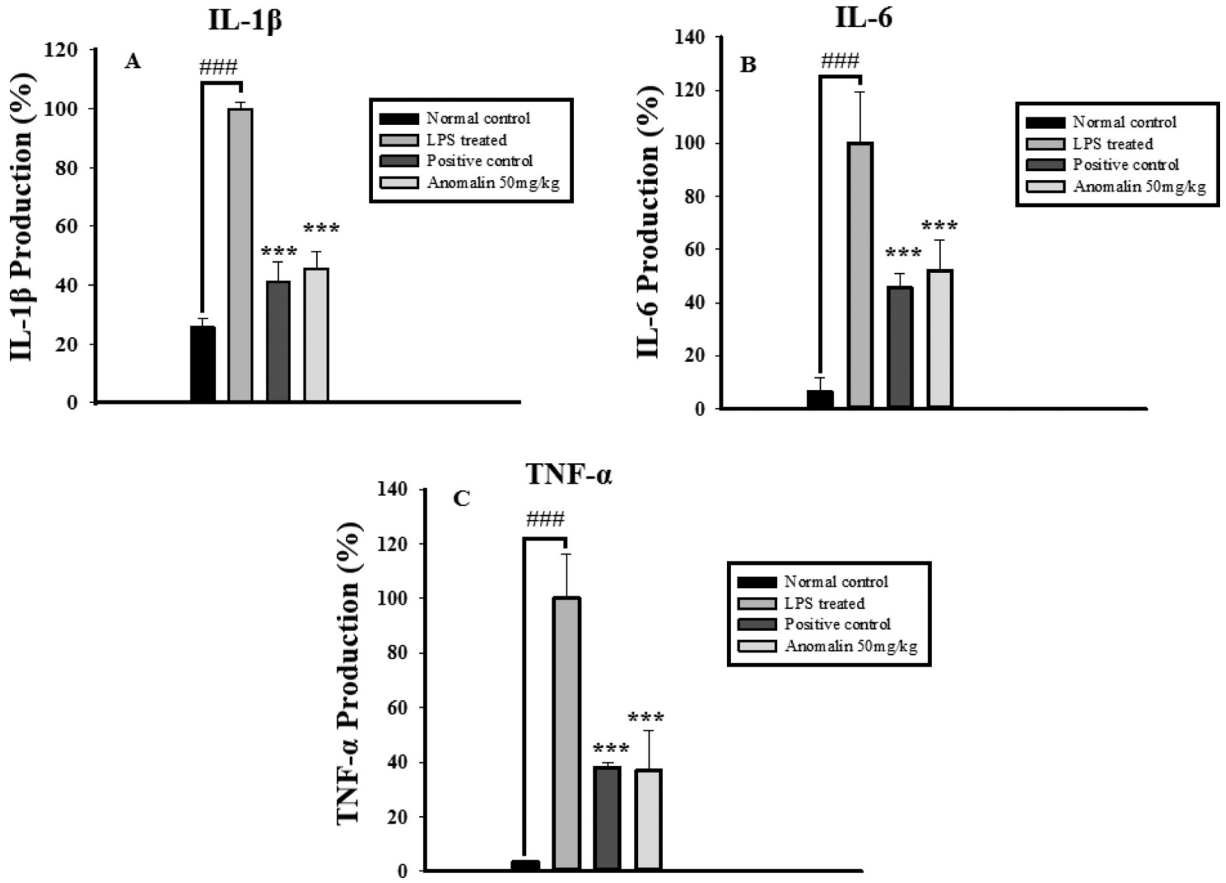

Fig. 7. Effect of anomalin on the production of pro-inflammatory cytokines. The anomalin (50 mg/kg, i.p) treatment significantly inhibited the production of IL-1β (a), IL-6 (b) and TNF-α (c). Similarly, the dexamethasone (10 mg/kg, i.p) also markedly attenuated the pro-inflammatory cytokines in the LPS-induced lung injury. The data were represented as the means (n = 8) ± SD, $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ indicate significant differences from the CFA-treated group. ###indicates a significant difference vs. negative control group.

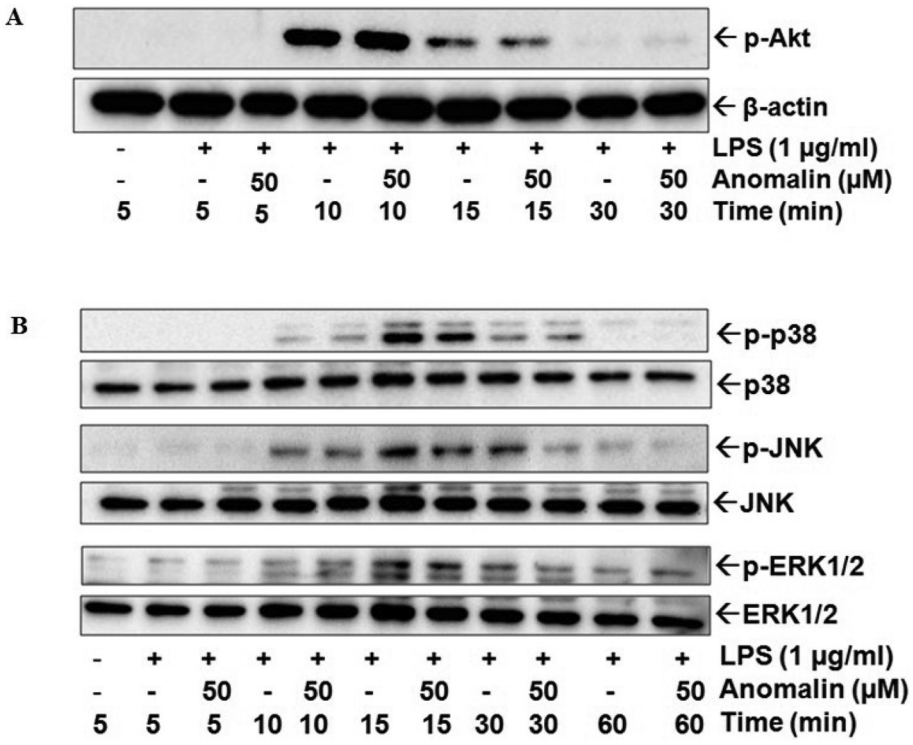

Fig. 8. The expressions of phosphorylated-Akt (A) and phosphorylated MAPKs proteins such as p-p38, phosphor-JNK and p-ERK (B) in the cytosolic extracts were determined by Western blot analysis, as described in the "Materials and methods". The RAW 264.7 cells were pretreated with 50 μM of anomalin for 2 h and treated with LPS (1 μg/ml) for the specified time periods. A representative result from three separate experiments is shown.

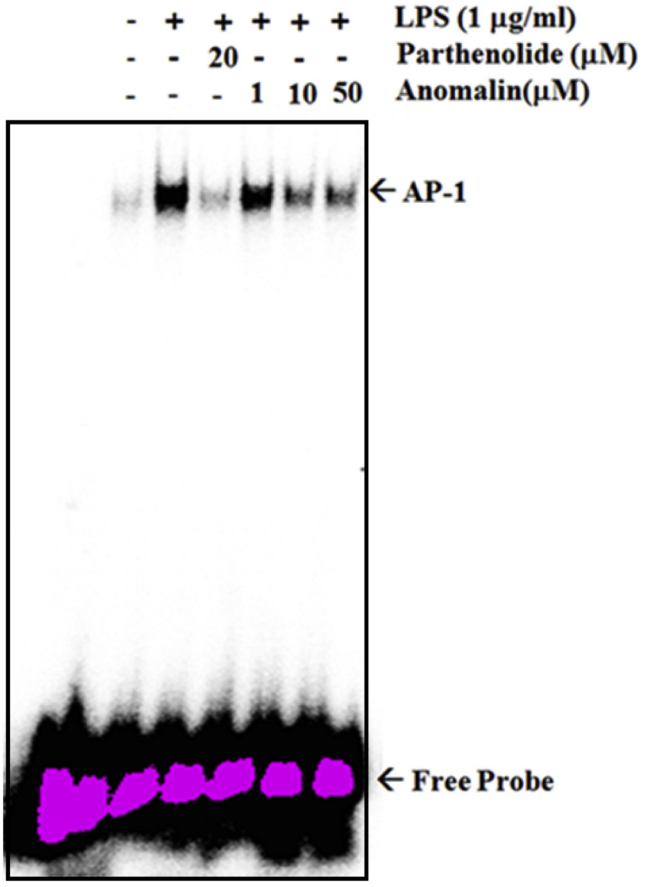

Fig. 9. Effects of anomalin on DNA/AP-1 binding activity. Electrophoretic mobility shift assay (EMSA) was performed as described in the "Material and methods". RAW 264.7 macrophages were pretreated with the indicated concentrations of anomalin for 2 h and stimulated with LPS (1 μg/ml) for 1 h. Five micrograms of nuclear extract was incubated with ^{32}P-labeled oligonucleotide specific to AP-1 and electrophoresed on a 6% PAGE. An EMSA result is represented and AP-1 complexes, nonspecific signals (NS) and excessive probe are indicated by arrows. Parthenolide 20 μM was used as a positive control.

activation [41–43]. In the current study, the DNA binding activity of the AP-1 was determined in RAW 264.7 cells using EMSA to assess the inhibitory effect of the upstream MAPKs protein such as p38, JNK and ERK1/2 on the AP-1 activation. The LPS administration markedly enhanced the DNA/AP-1 interaction. However, the anomalin treatment significantly inhibited the DNA/AP-1 interaction as evident from the Fig. 9.

5. Conclusion

The anomalin exhibited convincing protective activity against the LPS-induced lung injury. The anomalin treatment significantly attenuated the body temperature 24 h after LPS administration. Furthermore, the anomalin significantly reversed the lung edema and NO production. The anomalin administration remarkably improved the histopathology and inhibited the production of the pro-inflammatory cytokines. The mechanism through which the anomalin reversed the LPS-induced lung injury was investigated in the RAW 264.7 cells. The anomalin possibly mediates its protection against the LPS-induced injury through inhibition of the MAPKs (p38, ERK1/2, JNK) and Akt protein. Furthermore, the anomalin treatment also inhibited the downstream MAPKs target such as AP-1 using EMSA. Thus, anomalin exhibited protective activity against LPS-induced ALI via inhibiting the Akt and MAPKs/AP-1 nexus.

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