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# Remedial effects of exogenous surfactant in *Pseudomonas aeruginosa* LPS-induced acute lung injury in rat model

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**Abstract**
The present study was planned to evaluate the effects of exogenous surfactant in acute lung injury induced by *Pseudomonas aeruginosa* lipopolysaccharide. Fifty adult male Wistar rats were obtained from faculty of veterinary medicine, Zagazig University, Egypt, during March 2017 and assigned to five equal groups. Animals in the second, third, fourth and fifth groups received a single dose of 0.2 mL of 0.9% normal saline, 0.2 mL of survanta, LPS (5 µg/kg bw), and both survanta and LPS respectively. Exogenous surfactant administration significantly reduced the serum levels of IL-1$_{\beta}$, IL-6, IFNγ, and TNFα from 333.32±14.02, 273.55±10.22, 404.05±10.45 and 364.27±17.26 in LPS group to 179.77±17.05, 185.92±12.78, 300.69±7.81 and 262.08±10.97 respectively. Also, it significantly reduced the values of integrated density of NF-KB p65 pulmonary expression from 348.02±9.30 in LPS group to 167.40±7.29. The pulmonary lesions were markedly ameliorated in the surfactant treated group when compared to the LPS group.

**Keywords:** *P. aeruginosa* LPS, lung injury, surfactant, NF-KB p65, interleukins, rat

## 1. Introduction
ALI represents a major challenge in acute care medicine and commonly resulted in potentially lethal hypoxemic and/or ventilatory respiratory failure [1-2]. Multiple factors can cause ALI, but recently *P. aeruginosa,* a multidrug resistant opportunistic bacterium, became a leading cause of ALI particularly in nosocomial pneumonia, which associated with high mortality rates [3-4]. Even more, *P. aeruginosa* is now listed as one of the greatest threat to human health and one of the priority pathogen to which a new antibiotic is critically needed [5]. The pathogenic effect of *P. aeruginosa*, on the pulmonary tissue, is attributed to multiple virulence determinants including LPS, phospholipase C, exotoxin A, proteases, alginate, and others [6-7]. *P. aureginosa* LPS is an identical gram-negative bacterial LPS [8], having a crucial role in the virulence and host response to infection [9], and considered as oxidative stress inducer in the pulmonary tissue [10]. The mechanism by which *P. aureginosa* LPS stimulates the innate immune response is activation of the inflammatory cells through a receptor complex including Toll-like receptor 4 (TLR4), then activate transcription factors principally NF-κB and proinflammatory gene expression, which increase the production of inflammatory cytokines specially TNFα, IFNγ, IL-1, IL-6, and IL-10 [11-13] . Furthermore, *P. aureginosa* LPS enhance the neutrophilic migration to the lung [14], and exaggerate mucin synthesis in epithelial cells of airways [15]. Histologically, the nature of pneumonic lesions induced by LPS depends on LPS dose, animal species and its immune condition [16-17]. In general, the lesions of acute *P. aeruginosa* lung infection include vascular congestion, polymorphonuclear cell infiltration, alveolar and interstitial edema, and thickening of the alveolar and interlobular septa, besides various degenerative and necrotic changes [12, 17-18], while chronic *P. aeruginosa* lung infection characterized by a significant leukocytic cell infiltrations, progressive damage to the pulmonary tissue that may ultimately ends with respiratory insufficiency [19-20]. This insufficiency resulted from the lung damage plus disturbances in the quantity and function of the pulmonary surfactant system [21]. The current strategies for treatment of respiratory insufficiency caused by inflammatory lung conditions depend mainly on preventing or at least reduction of the inflammation-induced damage, enhancing the reparative process in the pulmonary tissue and improving the respiratory function [22-23]. Many studies hypothesized that exogenous surfactant administration could be considered an effectual treatment strategy for respiratory insufficiency induced by bacterial pneumonia particularly those associated with

disturbances in the pulmonary surfactant system [21,24-26]. This hypothesis based on many evidence including the biophysical functions, anti-inflammatory, immunomodulatory and antimicrobial properties of surfactant [27-29]. Pulmonary surfactant is a phospholipid-protein complex composed of 90% phospholipid and 10% surfactant-specific proteins secreted by type II pneumocytes [30-31]. Basically, surfactant maintains alveolar stability through reducing the alveolar surface tension, thus preventing the collapse of alveoli during expiration and allows gas exchange and enables normal breathing [27]. The anti-inflammatory properties of surfactant related to its ability to inhibit cytokines secretion from alveolar macrophages [32-33], nitric oxide release [34], neutrophil chemotaxis [35], regulation of lymphocytes proliferation and activation within the lungs [36-37], and oxidative burst response [38]. The surfactant antibacterial effects were multifactorial, as surfactant inhibit growth, viability, respiration and cause autolysis to many bacterial species [39]. The surfactant proteins (SPs) A and D act as an agglutinating agent to a wide variety of numerous pulmonary pathogens including *p. aeruginosa* [40], and modulate the way of the response of alveolar macrophage to bacterial infection through acceleration of chemotaxis and regulation of oxygen radicals [41-43], and stimulating phagocytosis [44]. Exogenous surfactant therapy used routinely in neonatal intensive care and in premature infants with ARDS [29, 45]. The first use of exogenous surfactant in the treatment of lung injury in animals was in the middle 1980s [46-47], while in adult humans were in late 1980s [48]. Since then, a continuous debate regarding whether exogenous surfactant is a well-tolerated beneficial therapy improve oxygenation, reduce atelectasis, stabilize pulmonary fluid system, ameliorate lung injury and lowered mortality rates [47,49-54], or whether exogenous surfactant had no beneficial therapeutic effects at all as a therapy in lung injury [55,-56]. Other studies reported that exogenous surfactant had promising therapeutic effects in experimental animal models only, but had no positive effects in human studies [57]. These conflicting results might be related to several factors that could affect the exogenous surfactant therapeutic efficacy including the severity of lung injury, timing of treatment, method of administration, dosing, and types of surfactant [58]. Since, *P. aeruginosa* is resistant to a wide range of antibiotics, finding a new antibiotic to it is a critical need and the efficacy of exogenous surfactant as a therapy in acute lung injury remains unclear, the present study tried to investigate its possible beneficial effects in *P. aeruginosa* LPS-induced lung injury.

## 2. Materials and methods

**2.1 Animals:** Fifty adult male albino Wistar rats (Lab animal house, Faculty of Veterinary Medicine, Zagazig University, Egypt), weighing 150-200 gm, were used in this study. They fed with a standard pellet ration (El-Nasr Chemical Company, Cairo, Egypt) and get free accesses to water add libitum. All the experimental procedures were done during March 2017 in the department of pathology, faculty of veterinary medicine, Zagazig university, Egypt and were in accordance with the Ethical Norms on Animal Care and Use approved by Zagazig University, Egypt.

**2.2 Surfactant:**The used surfactant in this experiment was Survanta (Abbott Laboratories, Colum-bus, OH 43229, USA). It was supplied in single-use vial containing 4 ml of intratracheal natural bovine surfactant suspension providing 25 mg/ml phospholipids, 0.1-1.0 mg/ml surfactant-associated proteins B and C (SP-B and SP-C), 1.4-3.5 mg/ml free fatty acids, and 0.5-1.75 mg/ml triglycerides suspended in 0.9% sodium chloride solution. It was used as supplied.

**2.3 Experimental design:** Animals were randomly divided into five equal groups and all reagents were administered via intratracheal instillation. Rats in the first group were negative controls. Animals in group 2 and 3 were positive controls received 0.2 ml of 0.9% normal saline and 0.2 ml of survanta (providing 5 mg/mL phospholipids), for each animal respectively. Animals in group 4 were exposed to 0.2 ml of normal saline containing *P. aeruginosa* 10 LPS at a dose of 5 µg/kg bw, (Sigma-Aldrich, product number; L9143). Animals in group 5 were exposed to simultaneous administration of both 0.2 mL of normal saline containing *P. aeruginosa* 10 LPS at a dose of 5 µg/kg bw and 0.2 ml of survanta. Intratracheal instillations were done according to the method described by [59], with few modifications. First the rats were anaesthetized by intraperitoneal injection of Ketamine (80 mg/kg bw) and Xylazine (12 mg/kg bw) [60], (Kahira Pharmaceuticals & Chemical Industries, Cairo-Egypt), then the animals were fixed in a supine position at angle 70◦ on a glasses board, then intratracheal instillations of the regents were done using 3-gauge intravenous plastic needles fixed on insulin syringes followed by 0.3 ml air bolus, to speed up the distribution of reagents to all parts of the lungs. Post-installation, the animals were placed vertically, then rotated for 1 minute for equal distribution of the instilled materials within the pulmonary tissue. All animals were sacrificed 24 hours after instillation and blood samples were collected in nonheparinized tubes.

**2.4 Histopathological techniques:** After sacrifice, the lungs were carefully examined in situ and any detectable gross lesions were reported, then representative specimens were collected from lungs and tracheas, fixed immediately in 10% neutral buffered formalin. The fixed tissue samples were processed by paraffin technique, five-micron thick sections were prepared and stained with Hematoxylin and Eosin, then examined microscopically [61]. The reported lesions were evaluated by using the scoring design; (0) absence of the lesion from all animals within a group, (1) a rare lesion within a group, (2) a lesion occasionally found within a group, (3) a lesion frequently found within a group, (4) a lesion observed in nearly all animals of a group.

**2.5 Nuclear factor kappa detection:** The (NF)-kB p65 expression in the pulmonary tissue in each group was determined using Rabbit polyclonal to NF-kB p65 (phospho S536) antibody (purchased from Abcam distributor; KEMET Medical, 14a El Emam Aly St, Off Orouba St, Ismailia Square, Heliopolis 11341, Egypt). The slides were stained according to manufacturer's instructions. One slide was stained for each animal then images were taken using AmScope microscope digital camera, then one high power field (40x), was selected for each animal in each group (10 images for each group). Because it was too difficult to sharply count the number of immunoreactive cells, the integrated density (IntDen) (summation of signal intensities throughout a determined area) of immunopositive cells was quantified according to the method described by [62-63]. Briefly, by using Adobe Photoshop CC software (Adobe Systems, Inc. 345 Park Ave. San Jose, CA 95110-2704, USA), the images were converted from RGB color to grayscale mode, then the signals were highlighted by reducing the color of the image's

background, and then the signal's color was inverted from black to white. Ultimately, the signal IntDen for the processed images were analyzed by using the open-source ImageJ v1.51k1 software.

**2.6 Cytokines analysis:** The serum levels of IL-1β, IL-6, IFNγ, and TNFα were determined by quantitative sandwich enzyme-linked immunosorbent assay (ELISA) [64], using ELISA kits (Abcam distributor; KEMET Medical, 14a El Emam Aly St, Off Orouba St, Ismailia Square, Heliopolis 11341, Egypt).

**2.7 Statistical analysis:** Statistical analysis of the scores of pulmonary NF-kB p65 immunostaining expression and the serum levels of IL-1β, IL-6, IFNγ, and TNFα were done and the results were expressed as means ± standard error. The statistical analysis was performed using SAS statistical system Package V9.2 [65]. Data were analyzed using one-way analysis of variance (ANOVA) through the general linear models (GLM) procedure, after verifying normality using Kolmogorov-Smirnov test. The comparison of means was carried out with Duncan's multiple range tests.

## 3. Results

**3.1 Clinical and gross findings:** Clinically, no mortalities were reported in any groups. Animals in the control groups were apparently healthy while those in LPS group were tachypneic and apathetic. Animals in group 5 were restless and showed mild tachypnea. Grossly, no detectable gross lesions were reported in the control groups. The lungs in the LPS group showed petechia in the pleural surfaces, appeared dark red with patchy firm consolidated areas and the cut surfaces oozed frothy exudate in the air passages. Similar lesions but with mild severity were observed in group 5.

**3.2 Histopathological findings:** Concisely, the microscopic examination revealed no apparent histological differences in the pulmonary tissue of the saline-exposed and surfactant-exposed groups compared to the control group (Fig. 1a and b). The animals exposed to LPS showed pronounced inflammatory response while those treated with exogenous surfactant revealed a marked reduction in this response (Fig. 1c and d). Precisely, the LPS-induced histopathological changes involved the alveoli, bronchial tree, pulmonary interstitial tissue, and tracheas. Alveolitis represented by alveolar edema with thickening of the interalveolar septa by extensive leukocytic infiltrations primarily polymorphonuclears was a consistent feature (Fig. 2a), and areas of obliterations either by leukocytic infiltrations and/or hemorrhages were commonly seen (Fig. 2b). Peribronchial lymphoid hyperplasia, vascular congestion besides edema and leukocytic infiltration of the bronchial wall were frequently observed. Alveolar emphysema was observed but was less frequent (Fig. 2c). Interlobular septal thickening with edema, leukocytic infiltrations, and fibrin deposits was evident (Fig. 2d). The rats exposed to *P. aeruginosa* LPS and surfactant showed a moderate inflammatory response compared to the LPS group. The alveolar edema was occasionally seen. Thickening of the alveolar wall by edema and leukocytic infiltrations was markedly reduced (Fig. 3a). The peribronchial lymphoid hyperplasia, leukocytic infiltration of the bronchial wall, and vascular congestion were less prominent. The tracheas of the control groups should no histological changes (Fig. 3b), while those of the LPS exposed group showed purulent tracheitis characterized by mucosal neutrophilic infiltration with a presence of a copious purulent mass in the tracheal lumen (Fig. 3c). The tracheas of the LPS-surfactant treated group showed mild tracheitis in the form of vascular congestion and mild leukocytic infiltration of the tracheal mucosa (Fig. 3d). The PMN infiltration, vascular congestion, hemorrhages, alveolar edema, alveolar emphysema, thickening of the interalveolar septa, peribronchial lymphoid hyperplasia, interlobular septal thickening, and tracheitis scores in all groups were recorded in Table 1.

**Table 1:** Lesion scores in all groups

| Lesion \ Group | Control | Saline | Surfactant | LPS | LPS-surfactant |
|---|---|---|---|---|---|
| PMN infiltration | 0 | 0 | 0 | 4 | 2 |
| Vascular congestion | 0 | 0 | 1 | 4 | 3 |
| Hemorrhages | 0 | 0 | 0 | 3 | 2 |
| Alveolar edema | 0 | 0 | 0 | 3 | 2 |
| Alveolar emphysema | 0 | 0 | 0 | 2 | 1 |
| Interalveolar septal thickening | 0 | 0 | 0 | 4 | 2 |
| Interlobular septal thickening | 0 | 0 | 0 | 2 | 1 |
| Peribronchial lymphoid hyperplasia | 0 | 0 | 0 | 3 | 2 |
| Tracheitis | 0 | 0 | 0 | 3 | 2 |

**3.3 Nuclear factor (NF)-kB p65 immunohistochemical staining expression:** The pulmonary tissues of the LPS group showed a strong NF-kB p65 immunoreactivity (golden brown staining of the epithelial and connective tissue elements) compared to the control groups (control, saline, and surfactant) that showed a Weak NF-kB p65 immunohistochemical expressions fig (4 a,b&c). Marked reduction in the staining intensity of immunoreactive cells was detected in LPS-Surfactant group fig (4d). Statistically, the means of IntDen of (NF)-kB p65-immunoreactive cells in the LPS group were significantly high compared to the control groups. The means of IntDen of (NF)-kB p65-immunoreactive cells in the surfactant treated group were significantly lower than those of LPS group and significantly higher than those of the control groups, Table 2.

**Table 2:** IntDen of (NF)-kB p65-immunoreactive cells in the all groups

| Group | Mean±Std. Error |
|---|---|
| Control | 65.91±2.81$^{c}$ |
| Saline | 67.58±2.08$^{c}$ |
| Surfactant | 64.72±1.98 $^{c}$ |
| LPS | 348.02±9.30$^{a}$ |
| Surf+LPS | 167.40±7.29$^{b}$ |

Means with different superscripts significantly differ ($p<0.05$)

**Serological findings:** A significant increase in the levels of the serum levels of IL-1$_{\beta}$, IL-6, IFNγ, and TNFα was recorded in LPS group compared to the controls (control, saline, and surfactant groups). The cytokine levels in the surfactant treated group (LPS- surfactant group), were significantly low compared to the LPS group, and significantly high compared to the controls, (Table 3).

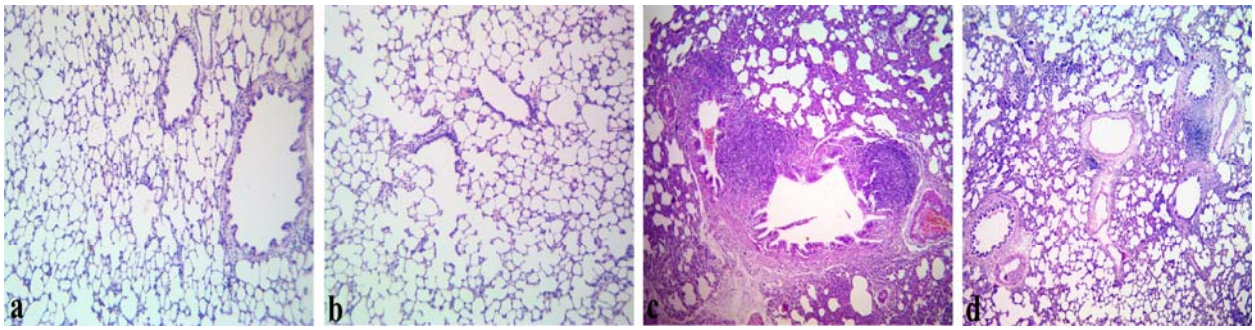


**Fig 1:** Photomicrograph showing pulmonary tissue of a) control, b) surfactant exposed c) LPS-exposed and d) LPS-surfactant treated. [Notice the severe congestion (black arrow), peribronchial lymphoid hyperplasia (black arrowhead), alveolar edema (red arrow) and leukocytic infilteration (red arrowhead) in LPS-group compared to other groups]. H&E stain

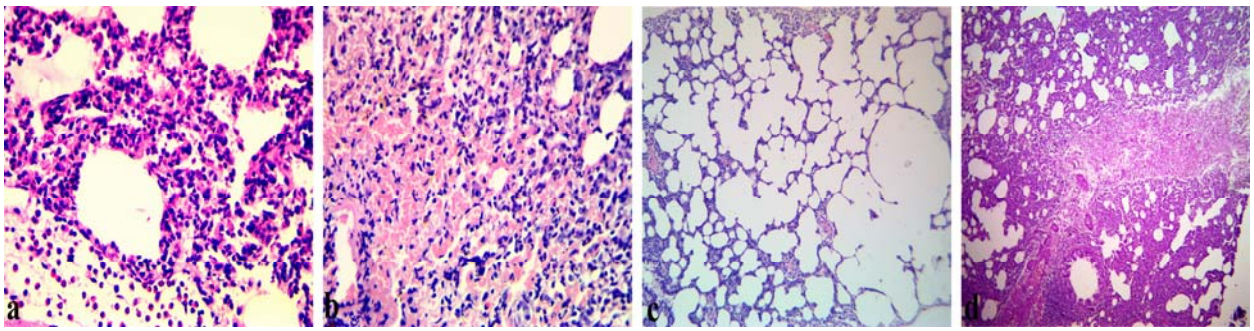


**Fig 2:** Photomicrograph of LPS-exposed animals showing a) alveolalitis [Notice the aleolar edema (red arrow) and interalveolar septal thickening by leukocytes (red arrowhead)], b) alveolar obliteration with leukocytes (black arrow) and RBCs (black arrowhead), c) alveolar emphysema (em), and d) interlobular septal thickening (blue arrow). H&E stain.

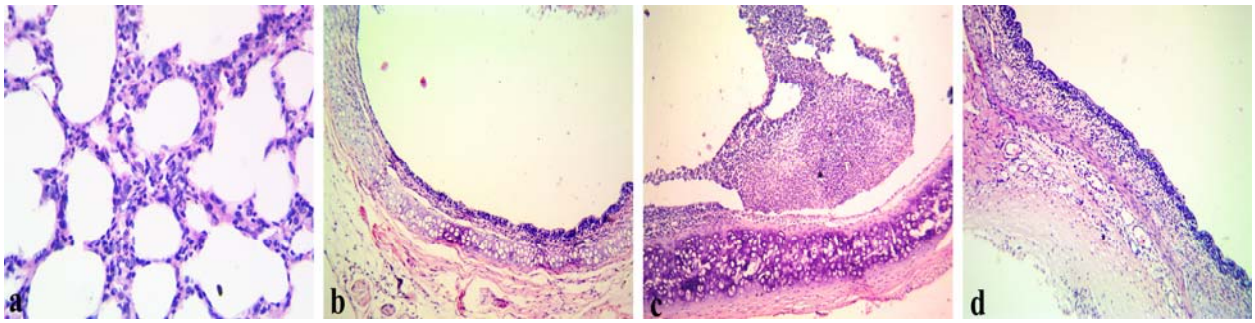


**Fig 3:** Photomicrograph showing a) moderate thickening of the alveolar septa (black arrow), b) trachea of control rat b) purulent tracheitis in LPS-exposed rat [Notice the mucosal neutrophilic infiltration (red arrow) and purulent mass in the tracheal lumen (red arrowhead)], and d) mild tracheitis in LPS-surfactant treated rat [Notice the moderate vascular congestion (black arrow head) and the leukocytic infiltrations in the mucosa (blue arrow)]. H&E stain.

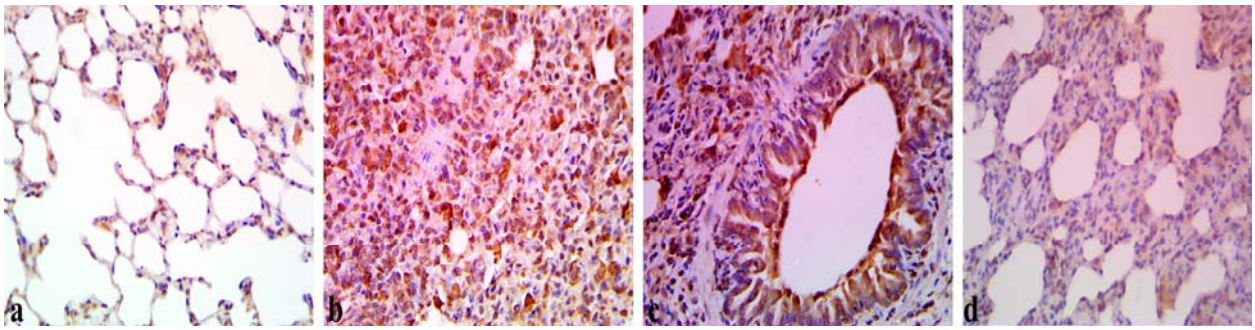


**Fig 4:** Photomicrograph showing Nuclear factor (NF)-Kb p65 immunohistochemical expression in a) control, b&c) LPS-exposed, and d) LPS-surfactant treated rats. [Notice the dark brown staining in connective tissue (black arrows) and epithelial elements (black arrowheads) in immunoreactive cells while negative cells give the blue color (red arrow) of the counterstain].

**Table 3:** Serum levels of IL-1β, IL-6, IFNγ, and TNFα in all groups

| | | Mean±Std. Error |
|---|---|---|
| IL-1β | Control | 79.61±1.79[c] |
| | Saline | 80.66±1.80[c] |
| | Surfactant | 73.94±1.10[c] |
| | LPS | 333.32±14.02[a] |
| | LPS+ Surf | 179.77±17.05b |
| IL-6 | Control | 52.85±1.57c |
| | Saline | 56.28±1.77c |
| | Surfactant | 49.01±0.96c |
| | LPS | 273.55±10.22a |
| | LPS+ Surf | 185.92±12.78b |
| IFNγ | Control | 51.47±1.17c |
| | Saline | 53.20±1.41c |
| | Surfactant | 40.89±1.21c |
| | LPS | 404.05±10.45a |
| | LPS+ Surf | 300.69±7.81b |
| TNFα | Control | 44.99±1.30c |
| | Saline | 50.00±1.97c |
| | Surfactant | 43.40±1.63c |
| | LPS | 364.27±17.26a |
| | LPS+ Surf | 262.08±10.97b |

Means with different superscripts significantly differ ($p<0.05$)

## 4. Discussion

Although several clinical trials were made to use the exogenous surfactant as pharmacologic therapy for acute lung injury to lower the mortality rates, its efficacy remains unclear [23]. In this study, we tried to affirm or contradict the hypothesis that exogenous surfactant is a useful therapy in bacterial pneumoniae associated with abnormalities in the amount and function of pulmonary surfactant. Acute lung injury was induced by intratracheal instillation of *P. aeruginosa* LPS in rats. Although rats are not identical in lung physiology and pathology neither to human nor large animals, it was selected as an experimental model because it can provide a valuable information about the pulmonary tissue injury and repair mechanisms [66]. We used *P. aeruginosa* LPS for many reasons; first, *P. aeruginosa* is one of the most common bacteria involved in many pathological pulmonary conditions [13,67]. Second, many studies reported that intratracheal instillation of *P. aeruginosa* LPS associated with pronounced inflammatory response and could be considered a good model for studying the pathogenesis of bacterial pneumonia and acute lung injury [53,68]. Third, pulmonary infection with P. aeruginosa is one of bacterial pneumonia that associated with disturbances in lung physiology particularly endogenous surfactant system [21, 69]. Fourth, there is a current critical need for a new antibiotic for *P. aeruginosa* infections [5]. Our results revealed that intratracheal instillation of P. aeruginosa LPS induced an acute vascular and exudative pulmonary inflammatory response with a pronounced (NF)-kB p65 expression and significant elevations in the serum levels of proinflammatory cytokines (IL-1β, IL-6, IFNγ, and TNFα), compared to the control groups. These pathological changes started by recognition of LPS by TLR4, followed by a complex series of events involving bringing of adaptor molecules that resulted in activation of NF-κB and proinflammatory gene expression, which enhance the production of TNF-α, IFNγ, IL-1, IL-6, and IL-10 with consequent leukocytes recruitment, notably PMN, to the pulmonary tissue [9,70]. The NF-κB activation in the *P aeruginosa*-induced pneumonia is not related only to the bacterial LPS [71], but also to many other stimuli associated with pneumonia including, hypoxemia [72], and high levels of IL-1β, and TNFα [73], and reactive oxygen species [74]. The value of measurement of NF-kB p65 in this study was due to its pivotal role in the pathogenesis of pneumonia and cytokine gene regulation [45,75]. We evaluate the efficacy of exogenous surfactant as a therapy according to its effects on; lung histopathology, (NF)-kB p65 pulmonary expression, and serum levels of IL-1β, IL-6, IFNγ, and TNFα. All the studied lesion scores (PMN infiltration, vascular congestion, hemorrhages, alveolar edema, thickening of the interalveolar septa, peribronchial lymphoid hyperplasia, interlobular septal thickening, and tracheitis) were markedly ameliorated in the surfactant treated group (LPS-Surfactant group) in comparison to LPS group [17,18]. Statistically, the means of IntDen of (NF)-kB p65-immunoreactive cells and the serum levels of IL-1β, IL-6, IFNγ, and TNFα in the LPS-surfactant group were significantly low compared to the LPS group. The amelioration in the lung histopathology, downregulation (NF)-kB p65 expression and lowering the serum levels of IL-1β, IL-6, IFNγ, and TNFα could be due to the role of exogenous surfactant administration. This role could be explained as follow; first, it has been established that the biophysical function of endogenous surfactant was inhibited by pulmonary edema that associated to many forms of lung injury through the leaked plasma proteins in the alveoli [58]. This inhibition could be overcome through exogenous surfactant administration [76-77]. Second, the anti-inflammatory and antibacterial properties of pulmonary surfactant were amplified through the excess amounts of exogenous surfactant.

## 5. Conclusion

In summary, exogenous surfactant administration to *P. aeruginosa* LPS-induced pneumonia in rat model played a valuable role in ameliorating the lung injury, downregulating NF-KB expression and lowering the proinflammatory cytokine response. Despite, these parameters did not come back to normal values either in the histology, serology or NF-KB expression but exogenous surfactant somewhat succeeded in avoiding the LPS-induced progressive lung injury. For all that, exogenous surfactant should be considered in the treatment regimen of patients with acute lung injury for its beneficial therapeutic effects particularly when properly administered.

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