Role of Stem Cells and Antioxidant on Modulation of Body Defense Mechanism in Lipopolysaccharide-Induced Acute Lung Injury in Rats

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Abstract

Background: Acute lung injury (ALI) and its more severe form, the acute respiratory distress syndrome (ARDS), are syndromes of acute respiratory failure that result from acute pulmonary edema and inflammation. Current treatment remains primarily supportive with lung-protective ventilation and a fluid conservative strategy. Despite improvements in critical care and mechanical ventilation protocols, the mortality rate for patients with ALI is still 30% to 40%.

Aim of Work: This study was designed to investigate the effect of stem cells and antioxidant treatment on lipopolysaccharide (LPS)-induced acute lung injury in rats, and to explore possible underlying mechanisms.

Methods: Thirty male adult albino rats were randomly divided into 5 experimental groups:

Group 1: Normal rats injected with vehicle solution phosphate buffered saline (PBS)

Group 2: LPS group, the rats were injected with 10mg/kg of LPS in sterile PBS i.p.

Group 3: LPS + N-acetylcysteine (NAC), the rats were injected i.p. with 10mg/kg of LPS and treated with NAC 4.8g/l of drinking water 2 days before LPS injection and maintained until the day of study.

Group 4: LPS + Mesenchymal stem cells (MSCs), the rats of this group were injected i.p. with 10mg/kg of LPS and 3 hours later were given 5X10⁵ BMDMSC in 0.1mL PBS.

Group 5: LPS+NAC+MSCs, rats of this group were injected i.p. with 10mg/kg of LPS and after 3 hours, the rats were given 5X10⁵ bone marrow derived mesenchymal cells (BMDMSC) in 0.1mL PBS and NAC 4.8g/l of drinking water for 2 days before the LPS procedure and was maintained until the day of study. 24 hours after LPS injection, broncho-alveolar lavage fluid (BALF) was obtained, analyzed for protein, cellular content and percent of neutrophils.

Lungs were excised for determination of their water content, inflammatory markers, apoptosis and growth factors.

Results: Our results showed that lung injury parameters, including the wet/dry weight ratio and protein, cellular content and percent of neutrophils in BALF, were significantly higher in the LPS group than in the PBS group (p<0.05). In the LPS group, significant higher MDA level, NF-κB, IL10, and fas1 expression and MPO (myloperoxidase) activity in lung homogenates were observed when compared with the PBS group (p<0.05). There was a marked decrease in keratinocytes growth factor (KGF) expression in lung tissues in response to LPS challenge (p<0.05, LPS group vs PBS group). Stem cells and NAC pretreatment, significantly attenuated LPS-induced inflammation, oxidative stress and apoptosis in treated rats. Stem cells and NAC significantly increased KGF and IL10 expression. Either stem cell therapy or NAC treatment improved epithelial barrier function with significant decrease in protein, cellular content and percent neutrophil in BAL compared with LPS group. Combined therapies induced a significantly greater improvement as compared to each therapy alone.

Conclusion: Stem cells and NAC pretreatment attenuates LPS-induced lung injury in rats. This beneficial effects may involves, inhibition of neutrophilic recruitment, inflammation, oxidative stress, apoptosis, and possibly through activation of lung expression of keratinocytes growth factor.

Key Words: Acute lung injury – Lipopolysaccharide (LPS) – Neutrophils – Stem cells – NAC and KGF.

Introduction

ACUTE lung injury (ALI) and its more severe form, the acute respiratory distress syndrome (ARDS), are syndromes of acute respiratory failure that result from acute pulmonary edema and inflammation [1]. The development of ALI/ARDS is associated with several clinical disorders including direct pulmonary injury from pneumonia and aspiration as well as indirect pulmonary injury from trauma, sepsis, and other disorders such as acute pancreatitis and drug overdose [2].

Because the most common cause of ALI in humans is sepsis, infusion of gram-negative bacterial endotoxin has been used as a model of sepsis-related lung injury [3]. Endotoxemia in several
animal species causes an acute systemic inflammatory response associated with lung edema, altered pulmonary function, deposition of extracellular matrix, and accumulation of inflammatory cells in the lungs [4].

Inflammatory stimuli from microbial pathogens, such as endotoxin [lipopolysaccharide (LPS)], are well recognized for their ability to induce pulmonary inflammation [8], and experimental administration of LPS, either systemically or intratracheally, has been used to induce pulmonary inflammation in animal models of ALI [6]. Endotoxin is a biologically active component of the gram-negative bacterial cell wall that exists as complexes of LPS and protein. Low-dose exposure to LPS activates macrophages [7] and polymorphonuclear cells, presumably aimed at eliminating the toxic agent, whereas higher doses cause tissue injury [8].

Current treatment remains primarily supportive with lung-protective ventilation and a fluid conservative strategy [9]. Despite improvements in critical care and mechanical ventilation protocols, the mortality rate for patients with ALI is still 30% to 40% [10]. Pharmacologic therapies that reduce the severity of lung injury in preclinical models have not yet been translated to effective clinical treatment options. Consequently, further research in translational therapies is needed [11].

At inflammatory sites, tissue damage and repair often occur. The initial injury and acute inflammatory response can result in apoptosis and necrosis of parenchymal cells, including endothelial and epithelial cells [12]. The repair process may require replacement of these dead cells that are not able to divide with bone marrow derived progenitor cells (BMPCs) that are capable of differentiating in endothelial or epithelial directions [13].

Cell-based therapy with mesenchymal stem cells (MSCs) is one attractive new therapeutic approach. MSCs have the capacity to secrete multiple paracrine factors that could be able to regulate endothelial and epithelial permeability, decrease inflammation, enhance tissue repair, and inhibit bacterial growth [9].

Oxidative stress is thought to play an important role in the pathogenesis of lung disease not only through direct injurious effects, but also by involvement in the molecular mechanisms that control lung inflammation [14]. Recent in vitro experiments have shown that thiol antioxidants such as N-acetylcysteine (NAC) block the release of these inflammatory mediators from epithelial cells and macrophages by a mechanism involving increasing intracellular Glutathione (GSH) and decreasing NF-xB activation [15]. The sulfhydryl (thiol) group (SH) of cysteine serves as a proton donor and is responsible for the biological activity of glutathione. Glutathione has multiple functions it is the major endogenous antioxidant produced by the cells [16].

**Aim of the study**

To study the protective role of two pathways, first cell-based therapy with mesenchymal stem cells (MSCs) which is one attractive new therapeutic approach, second the use of N-acetylcystein as a precursor of antioxidant cellular enzymes glutathione which is protective against free radicals that are highly reactive with the cell membrane and DNA on acute rat lung injury by lipopolysaccharide and if there is a synergistic correlation between both or not?

**Material and Methods**

**Experimental animal and groups.**

Thirty male albino rats, of an average weight 100-120gm, 6-8 weeks old, were included in this study which was carried out in March 2012. The rats were supplied by the Animal House Unit of Kasr Al-Ainy, Faculty of Medicine, Cairo University, housed in cages at room temperature with normal light& dark cycle. They were fed the standard rat chow diet and had free access to water. This study was carried out in the Physiology and Biochemistry Departments, Faculty of Medicine, Cairo University. The rats were divided into 5 groups, 6 rats each:

- **Group 1:** PBS group, served as normal control, these rats were injected intraperitoneal with 1 ml of phosphate buffered saline (PBS) as a vehicle solution.
- **Group 2:** LPS group, the rats of this group were injected intraperitoneally with 10mg/kg of LPS in 1ml of sterile PBS (pH 7.4) to induce endotoxemia [17]. LPS (extracted from Escherichia coli 0111: B4) was purchased from Sigma (St Louis, MO).
- **Group 3:** LPS+NAC, the rats of this group were injected intraperitoneally with 10mg/kg of LPS in 1ml PBS and treated with NAC administered orally (in water) for 2 days before the LPS injection and was maintained until the day of study. The NAC dose was 4.8g/l of drinking water [18].
- **Group 4:** LPS+ MSCs, the rats of this group were injected intraperitoneally with 10mg/kg of LPS in 1ml PBS and 3 hours later were subsequently anesthetized by isoflurane inhalation.
(Baxter Pharmaceuticals, Deerfield, IL), and given $5 \times 10^3$ BMDMSC (bone marrow derived mesenchymal cells) in 0.1mL PBS. The retro-orbital vein was used for intravenous administration. Former studies have shown that BMDMSCs at this dose exert anti-inflammatory effects in vivo [19].

Group 5: LPS+NAC+MSCs, rats of this group were injected intraperitoneally with 10mg/kg of LPS in 1ml of PBS and after 3 hours, the rats were anesthetized by isofluorane inhalation and given $5 \times 10^5$ BMDMSC in 0.1ml LPBS in the retro orbital vein and pretreatment with NAC 4.8g/l of drinking water was administered orally (in water) for 2 days before the LPS injection and maintained until the day of study.

Preparation of BM-derived MSC:

Bone marrow was harvested by flushing the tibiae and femurs of 6-week-old female white albino rats with Dulbecco’s modified Eagle’s medium (DMEM, GIBCO/BRL) supplemented with 10% fetal bovine serum (GIBCO/BRL). Nucleated cells were isolated by a density gradient [Ficoll/Paque (Pharmacia)] and resuspended in complete culture medium supplemented with 1% penicillin-streptomycin (GIBCO/BRL). Cells were incubated at 37°C in 5% humidified CO$_2$ for 12-14 days as primary culture or upon formation of large colonies.

When large colonies developed (80-90% confluence), cultures were washed twice with phosphate buffer saline (PBS) and the cells were trypsinized with 0.25% trypsin in 1mM EDTA (GIBCO/BRL) for 5min at 37°C. After centrifugation, cells were resuspended with serum-supplemented medium and incubated in 50cm$^2$ culture flask (Falcon). The resulting cultures were referred to as first-passage cultures [20]. MSC in culture were characterized by their adhesiveness and fusiform shape [21]. We also detected CD29 gene expression by RT-PCR as a marker of MSC [22,23].

Rats were killed 24 hours after LPS injection and broncho-alveolar lavage fluid (BALF) was obtained, lungs were excised for determination of their water content, inflammatory markers, oxidative stress condition, apoptosis and growth factors.

Broncho-alveolar lavage fluid (BALF):

The animals were anesthetized 24 hours after LPS injection with an intraperitoneal injection of sodium pentobarbital (50mg/kg). The rats were euthanized by exsanguination and a thoracotomy was performed to expose the lungs and the trachea; a cannula was inserted into the trachea and secured using a silk suture. Then 12ml of 37°C of phosphate-buffered saline (0.9% NaCl-50mM phosphate, pH 7.4) injected into the lungs via a syringe and the tracheal cannula. Infusion was allowed to remain for 30sec, retrieved and then reinstituted for a total of three washes with the same solution. An average of 9.0ml BALF was recovered from each rat [24].

BALF collected was then centrifuged at 800g, and supernatant was stored at −70°C immediately and was used for detection of protein. Protein was measured in the BALF from all experimental groups, as a marker of endothelial and epithelial permeability, by the Bio-Rad protein assay kit. Total cell counts were determined using a haemocytometer. Differential cell counts were determined on BALF smear slides that were stained with Hemacolor (EMD Chemicals). Percent of neutrophils was calculated as the ratio of neutrophils x 100 divided by the total number of cells in the BAL fluid sample.

Lung water content estimation:

Lungs were dissected immediately after euthanasia. One lung from each animal was washed with normal saline to remove residual blood and weighed to obtain the wet lung weight (W) and then dried in a drying oven at 80°C for 12 hours and weighed again to obtain the dry lung weight (D). The values obtained were used to determine the W/D ratio [25].

Inflammatory, oxidative stress, apoptotic markers and keratinocyte growth factor in lung tissues:

The other lung was collected as frozen tissue and used to quantify MDA, an index for lung oxidative stress, Fas 1, an apoptotic factor, MPO, an index for neutrophil infiltration, IL-10, an anti-inflammatory cytokine which represent a major feature of ALI [26], nuclear factor kappa B (NFκB), a central regulator of inflammatory and immune responses [27] and keratinocyte growth factor (KGF) production. KGF is an epithelial-specific growth factor secreted by fibroblasts and vascular smooth muscle cells. It has recently been discovered to be the principal mitogen for alveolar type II cells [28]

Biochemical measurements:

Measurement of MDA:

To measure the MDA concentration [29], 100mg of lung tissue in 1mL PBS, pH 7.0 was homogenized with micropestle in microtube. 20% TCA was added to lung homogenate to precipitate the protein, and centrifuged. Supernatants were collected and thiobarbituric acid (TBA) solution was
added to the supernatants. After boiling for 10 minutes in water bath, the absorbance was measured. Concentration of MDA in supernatants of lung homogenate was calculated using the standard curve.

**Determination of myeloperoxidase (MPO) activity in rat lung tissue:**

Assaying of MPO activity was described by Mizutani et al. [30]. Briefly, lung tissue (0.25gm) were homogenized in 10ml of homogenization buffer pH (4.7) [0.1mol/L NaCl, 0.02mol/L NaPO4 and 0.015mol/L sodium ethylenediamine tetracetic acid (EDTA)], centrifuge at 260 x g for 10minutes and the pellet underwent (dissolved) hypotonic lysis (0.2% NaCl) solution followed 1minutes later by addition an equal volume of solution containing (1.6% NaCl and 5% glucose). After further centrifugation, the pellet was then suspended in resuspen-sion buffer pH 5.4 (0.05mol/L NaPO4 containing 0.5% hexadecyl trimethy lammonium bromide) and rehomogenized. One milliliter aliquots of the suspension were Freeze and thawed three cycles in liquid nitrogen, then centrifuged for 15 minutes at 3000g. the pellet was discarded. MPO activity was assayed by measuring the change in optical density at 450nm using tetramethylbenzidine, as substrate (1.5mmol/L) and H2O2 (0.5mmol/L). Results were expressed as MPO relative units/100 mg tissue. One unit of MPO activity was defined as the quantity of enzyme degrading one mmol peroxide at 25°C. The activity of purified known human neutrophil MPO was used as the standard (Sigma Chemical Co, Egypt).

Gene expression of KGF, NFKB, IL-10 & fasl by real time PCR:

**RNA-Isolation and reverse transcription:**

RNA-Isolation was conducted by means of the Qiagen Tissue Mini Kit (Qiagen, Valencia, CA, USA). In the beginning the frozen lung tissue was homogenised under liquid nitrogen using a mortar and pestle and than the cells were lysed and the RNA released by centrifugation of the cell-homogenate through a biopolymer shredder (Qiashredder, Qiagen, Valencia, CA,USA). The quality and yield of the RNA was determined by spectrophotometry at 260nm and the integrity examined by agarose gel electrophoresis with ethidium-bromide staining. The Quantification of the total RNA was performed using a Nano Drop®ND-1000 UV-Vis Spectrophotometer (NanoDropTechnologies). cDNA was generated from 5µg of total RNA extracted with 1µl (20pmol) antisense primer and 0.8µl superscript AMV reverse transcriptase for 60min at 37°C.

**Polymerase chain reaction-real-time PCR**

The relative abundance of mRNA species was assessed using the SYBR Green method on an ABI prism 7500 sequence detector system (Applied Biosystems, Foster City, CA). PCR primers were designed with Gene Runner Software (Hasting Software, Inc., Hasting, NY) from RNA sequences from GenBank. All primer sets had a calculated annealing temperature of 60°C. Quantitative RT-PCR was performed in duplicate in a 25-µl reaction volume consisting of 2X SYBR Green PCR Master Mix (Applied Biosystems), 900nM of each primer and 2-3 µl of cDNA. Amplification conditions were 2min at 50°, 10min at 95° and 40 cycles of denatura-tion for 15 , and annealing/extension at 60° for 10 min. Data from real-time assays were calculated using the v1.7 Sequence Detection Software from PE Biosystems (Foster City, CA). Relative expres-sion of studied gene mRNA was calculated using the comparative Ct method. All values were normal-ized to the beta actin gene and reported as fold change over background levels detected in diseases group [31].

**Beta actin gene primer sequence, forward 5'-TGGTTGTCCCTGTATGCCTCTC-3' reverse 3'-TAATGTCAGGCAGATTTCC-5'**

**Primer sequence of the studied gene.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence, 5'-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>KGF</td>
<td>Reverse TTTTGTTCTTTCTCCAGAA</td>
</tr>
<tr>
<td></td>
<td>Forward GAACAAGGAGAAACATCTTAT</td>
</tr>
<tr>
<td>NFKbeta</td>
<td>Reverse GCCGAAGCTGATGGACACT</td>
</tr>
<tr>
<td></td>
<td>Forward GCAGATCCAGCAACACAAATAC</td>
</tr>
<tr>
<td>IL-10</td>
<td>Reverse GGTCTTTAGCTCCCTGTGA</td>
</tr>
<tr>
<td></td>
<td>Forward GCTATGGTCCTGCTTT</td>
</tr>
<tr>
<td>Fasl</td>
<td>Reverse CAGAGGTGGACGGAGAGGAA</td>
</tr>
<tr>
<td></td>
<td>Forward GCAGCCCTTCAATTCCCAT</td>
</tr>
</tbody>
</table>

**Statistical methods:**

Data were coded and entered using the statistical package SPSS version 16. Data was summarized using mean, standard deviation for the quantitative variable. Comparisons between groups were done using analysis of variance (ANOVA) with multiple comparisons post hoc test in quantitative variables [26]. p-values less than 0.05 were considered as statistically significant.

**Results**

**Effect of LPS injection and MSCs and NAC treatment on lung wet to dry ratio:**

Table (1) and Fig.(1) show that ALI induced by LPS injection resulted in a significant increase in lung wet to dry ratio as compared with control
rat lungs. MSCs treatment after 3 hours of LPS induced ALI significantly improved severity of lung injury as evidenced by reduction in lung wet-to-dry ratio in treated rats group as compared to LPS untreated groups \( (p<0.05) \). Also treating injured lungs with NAC induced a significant reduction in lung water compared to LPS group \( (p<0.05) \). In rats treated with both MSCs and NAC, lung improvement was more marked with wet to dry ratio after 24 hours of LPS injection significantly decreased from values recorded in rats of MSC treatment or NAC treatment \( (p<0.05) \).

**Effect of LPS injection and MSCs and NAC treatment on the protein and cellular content of BAL fluid:**

Concentration of total protein was assayed on collected BAL fluid to evaluate the integrity of the alveolar-capillary membrane barrier and assess pulmonary vascular leakage as a marker for ALI. (Table 1), (Fig. 2) illustrates that the protein content was significantly increased in BAL fluid 24 hours after LPS injection compared to control rats \( (p<0.05) \). Whereas treatment with MSCs or NAC alone partially reduced protein level, treatment with MSCs-NAC restored these lung injury indicators to levels not significantly different from control rats \( (p<0.05) \).

The total inflammatory cell count in the BALF was significantly increased 24 hours following administration of LPS, mainly attributable to an increase in neutrophils (around 84% of total cells in LPS/10% in PBS group) (Table 1, Figs. 3, 4). Treatment of animals with MSCs alone significantly reduced the total cell and neutrophil percent in BALF. Treatment with MSCs-and NAC further significantly reduced the BALF cell and neutrophil percent compared to the LPS group \( (p<0.001) \) for total cells and neutrophils counts compared to LPS) and the values become insignificantly changed compared to PBS control rats.

**Table (1): Effects of n acetyl cysteine (NAC) and mesenchymal stem cells (MSCs) treatment on lung wet/dry ratio (W/D), bronchoalveolar lavage fluid protein (BAL ptn), total cell count (BAL cells), percent neutrophil of the cell count (% neutrophil) after 24 hours of lipopolysaccharide (LPS) induced acute lung injury in male rats.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Lung w/d weight</th>
<th>BAL protein ng/ml</th>
<th>BAL cells x10⁷/ml</th>
<th>%neutrophil</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS (Control)</td>
<td>4.00±0.32</td>
<td>58.55±2.49</td>
<td>122.67±1.75</td>
<td>10.38±0.23</td>
</tr>
<tr>
<td>LPS</td>
<td>9.22±0.28*</td>
<td>120.97±4.92*</td>
<td>851.83±6.05*</td>
<td>84.24±0.49*</td>
</tr>
<tr>
<td>LPS+NAC</td>
<td>8.02±0.33*#</td>
<td>93.62±6.72*#</td>
<td>135.00±3.03*#</td>
<td>33.14±0.14*#</td>
</tr>
<tr>
<td>LPS+MSC</td>
<td>8.03±0.36*#</td>
<td>79.18±6.12*#</td>
<td>132.5±2.88*#</td>
<td>28.82±0.2*#</td>
</tr>
<tr>
<td>LPS+NAC+MSC</td>
<td>5.97±0.33 *#$@</td>
<td>67.03±3.61*#</td>
<td>127.50±1.87#$</td>
<td>15.12±0.28#$</td>
</tr>
</tbody>
</table>

N = 6-Values are represented as mean ± SD.
* : Statistically significant compared to corresponding value in (PBS) group \( (p<0.05) \).
# : Statistically significant compared to corresponding value in (LPS) group \( (p<0.05) \).
$ : Statistically significant compared to corresponding value in LPS+NAC group \( (p<0.05) \).
@ : Statistically significant compared to corresponding value in LPS+MSC group \( (p<0.05) \).
**Role of Stem Cells & Antioxidant on Modulation of Body Defense**

**Effect of LPS injection and MSCs and NAC treatment on lung inflammatory markers:**

Lung expression of NFκB and IL-10 and MPO activity were significantly increased at 24 hours after LPS injection group ($p<0.001$). Stem cell transplant resulted in a significant reduction in the expression of NFκB and MPO activity and a significant increase in the IL-10 expression compared to untreated LPS group ($p<0.001$). Also NAC injection induced a similar significant change in these factors.

Treatment of rats with ALI with both MSCs and NAC returned the values of these parameters toward normal and showed significant changes compared to each treatment separately ($p<0.001$). This suppression of the inflammatory response in the lungs may provide an environment more favorable to normal repair.

**Table (2):** Effects of n-acetyl cysteine (NAC) and mesenchymal stem cells (MSCs) treatment on gene expression of pro-inflammatory cytokine nuclear factor kappa beta (NFκB), marker of neutrophil infiltration myeloperoxidase activity (MPO) and lung expression of anti-inflammatory marker interleukin 10 (IL-10) in lung tissue after 24 hours of lipopolysaccharide (LPS) induced acute lung injury in male rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>PBS (Control)</th>
<th>LPS</th>
<th>LPS+NAC</th>
<th>LPS+MSC</th>
<th>LPS+NAC+MSC</th>
</tr>
</thead>
<tbody>
<tr>
<td>NFκB</td>
<td>0.21±0.02</td>
<td>0.98±0.07*</td>
<td>0.68±0.09**</td>
<td>0.56±0.08*#</td>
<td>0.37±0.04*#$@</td>
</tr>
<tr>
<td>MPO U/100mg</td>
<td>0.52±0.05</td>
<td>2.48±0.32*</td>
<td>1.62±0.33 **#</td>
<td>1.3±0.23 *#</td>
<td>0.78±0.1#$@</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.17±0.08</td>
<td>0.38±0.03 *</td>
<td>0.56±0.06**#</td>
<td>0.60±0.03*#</td>
<td>0.68±0.03*#$@</td>
</tr>
</tbody>
</table>

N = 6

Values are represented as mean ± SD.

* : Statistically significant compared to corresponding value in control group ($p<0.05$).

# : Statistically significant compared to corresponding value in LPS group ($p<0.05$).

$: Statistically significant compared to corresponding value in LPS+NAC group ($p<0.05$).

@: Statistically significant compared to corresponding value in LPS+MSC group ($p<0.05$).
Effect of LPS injection and MSCs and NAC treatment on oxidative stress & apoptosis in lung tissues:

(Table 3) and (Figs. 8,9) show that the levels of oxidative stress marker MDA and apoptotic marker Fas1 were significantly increased in lung tissues after LPS injection compared to PBS group. MSCs injection significantly reduced MDA and Fas 1 as compared to LPS group \((p<0.001)\). NAC treatment significantly reduced both MDA and Fas 1 in lung tissues compared to LPS group \((p<0.001)\). Treatment with both agents together significantly decreased these factors as compared to values recorded in groups treated with each agent alone \((p<0.05)\).

Effect of LPS injection and MSCs and NAC treatment on KGF:

(Table 3) and (Fig. 10) illustrate that LPS injected group of rats showed a significant decrease in expression of KGF in lung tissues as compared to PBS group \((p<0.05)\). Treating rats with MSCs significantly increased the lung expression of KGF as compared to LPS group \((p<0.05)\). NAC treatment also induced a significant increase in KGF expression in lung tissue. Both treatments together induced a significant increase in KGF expression in lung tissues than MSCs or NAC treatment alone \((p<0.05)\).

### Table (3): Effects of n acetyl cysteine (NAC) and mesenchymal stem cells (MSCs) treatment on oxidative stress marker malonaldehyde (MDA), apoptotic factor (FAS1) and keratinocyte growth factor (KGF) after 24 hours of lipopolysaccharide (LPS) induced acute lung injury in male rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>PBS (Control)</th>
<th>LPS</th>
<th>LPS+NAC</th>
<th>LPS+MSC</th>
<th>LPS+NAC+MSC</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA µmol/mg</td>
<td>71.48±4.62</td>
<td>181.97±10.83*</td>
<td>109.35±3.65*#</td>
<td>110.62±4.43*#</td>
<td>98.20±2.91*#$@</td>
</tr>
<tr>
<td>FAS 1</td>
<td>0.07±0.02</td>
<td>0.88±0.04*</td>
<td>0.50±0.07*#</td>
<td>0.50±0.11*#</td>
<td>0.30±0.03*#$@</td>
</tr>
<tr>
<td>KGF</td>
<td>1.74±0.22</td>
<td>0.35±0.11*</td>
<td>0.75±0.08*#</td>
<td>0.91±0.09*</td>
<td>1.41±0.32*#$@</td>
</tr>
</tbody>
</table>

N=6 - Values are represented as mean±SD.

* : Statistically significant compared to corresponding value in control group \((p<0.05)\).
# : Statistically significant compared to corresponding value in LPS group \((p<0.05)\).
$ : Statistically significant compared to corresponding value in LPS+NAC group \((p<0.05)\).
@: Statistically significant compared to corresponding value in LPS+MSC group \((p<0.05)\).
The lung is frequently the first failing organ during the sequential development of multiple organ dysfunction in sepsis. Severe sepsis, when accompanied by acute respiratory distress syndrome, continues to be the leading causes of death in intensive care units, with a mortality that has remained over 40% [33]. As such, the development of novel strategies for the treatment of ALI is critical for the improvement of patient outcome in severe sepsis [34].

It is clear from the results of this study that ALI is the result of the actions of an integrated network of soluble inflammatory mediators and inflammatory cells. The formation of proinflammatory cytokines and oxidative stress appears to play an important role in the tissue injury of ALI.

In agreement with our results, Bhatia and Moochhala [35] and Park et al. [36] confirmed a role of inflammatory mediators in the pathophysiology of acute respiratory distress syndrome and reported that cytokine imbalance in the lungs is a causative factor for lung damage in ALI.

In the present study, administration of MSCs attenuated the severity of acute lung injury. This beneficial effect was primarily demonstrated as reduced lung wet-to-dry ratio, modulation of the inflammatory response in the lung tissues indicated by decreasing the expression of proinflammatory cytokine, NFκB, activity of neutrophil infiltration marker, MPO and increasing the lung expression of antiinflammatory cytokine, IL-10. Tissue injury caused by oxidative stress and apoptosis were attenuated in lung tissues with stimulation of tissue growth and repair as indicated by an increase in KGF levels. The endothelial and epithelial barrier mechanism was protected as observed by decreasing BAL protein and neutrophil at 24h after treatment.

It can be seen from our results that MSCs can limit experimentally-induced lung injury and protect the lung tissues. This protection is multifactorial including modulating the oxidative stress reaction,
inflammatory response, tissue damage and repair and protection of the epithelial and endothelium barrier function.

One theory of tissue repair holds that organ injury is “sensed” by stem cells that migrate to the site of damage and differentiate into organ-specific cells, promoting structural and functional repair [37, 38]. Ortiz et al. [39] found that the donor cells homed to the injured lung and adopted epithelial phenotypes, including that of type II alveolar epithelial cells, or pulmonary capillary endothelial cells. LPS administration induces severe damage to lung parenchymal cells, resulting in apoptosis and necrosis [40]. Because these dead cells are not able to divide, other cells must replace them to repair the tissue and keep the organ homeostasis [12].

However, these results were questioned by multiple groups, who observed only engraftment of leukocyte lineages [39], or low engraftment rates in lung injury models with observed rates of <1% [41-43], and the idea that stem cells simply supply a reservoir of new lung parenchymal cells and therefore hasten repair is not adequate to explain the effect.

Early studies [44, 45], pointed to an important role for paracrine actions of transplanted cells on neovascularization and tissue healing. However, the precise mechanism by which MSCs confer a therapeutic benefit in the model of ALI remains to be clarified.

Recently, the ability of MSCs to restore alveolar epithelial fluid transport and lung fluid balance was demonstrated in a study employing an ex vivo perfused human lung preparation injured by E. coli endotoxin [46]. These studies have ignited much interest in hMSCs as cellular therapy for ALI.

Experimentally and clinically, the ability of the lung epithelium to remove alveolar edema is quantified as alveolar fluid clearance (AFC). Impaired AFC in patients with ALI/ARDS is associated with higher morbidity and mortality [47, 48]. In the alveolar environment, basal AFC is determined predominantly by amiloride-sensitive and insensitive epithelial sodium channels (ENaC) on the apical membrane as well as Na-K ATPase, located on the basolateral membrane, in both alveolar epithelial type I and II cells [49, 50]. MSCs have been showed to partially restore apical membrane protein levels of αENaC, the most significant ENaC isoform, suggesting the beneficial effect of MSCs was mediated in part through increased sodium transport on the alveolar epithelium. In addition to restoring normal sodium uptake across the apical membrane, it is also possible that the beneficial effect of MSCs could also be mediated by improved trafficking of Na-K ATPase subunits to the basolateral membrane [51].

MSCs may also normalize lung fluid balance and AFC through therapeutic effects on the lung endothelium. The integrity of the lung microvascular endothelium is essential to prevent the influx of protein-rich fluid from the plasma as well as inflammatory cells which may further aggravate the ability of the lung epithelium to remove alveolar edema. How do mBMPCs mediate endothelial barrier protection remains unclear [48].

The studies support the role of MSCs sequestration in lungs as a mechanism of lung endothelial barrier protection; however, they have also shown that a humoral mechanism involving secretion by MSCs of paracrine factors such as sphingosine-1-phosphate that contributes to endothelial barrier normalization [52].

In the present study, it was shown that MSCs increased the level of KGF in the lung. In rat lung, KGF improves alveolar fluid transport in part by up-regulating αENaC gene expression [53] and Na-K ATPase activity [54], restores alveolar epithelial and endothelial permeability and enhances resolution of ARDS in pre-clinical models [55].

Aguilar et al. [56] showed that MSCs transfected with a tetracycline inducible KGF construct partially increased surfactant production [57], inhibited apoptosis [58], and increased transcription and/or translation of the major sodium and chloride transport proteins [53].

Other studies indicated that MSCs transfer may be effective against increases in vascular permeability via the modulation of inflammatory process via regulation of proinflammatory cytokines [59].

In the present study, it was shown that LPS injection was associated with increased proinflammatory cytokine, NF-κB with minor increase in the level of the anti-inflammatory IL-10, and that MSCs can have an immunomodulatory effect demonstrated as a significant decrease in the lung expression of NF-κB and increase in the expression of IL-10. This immuno-modulation of MSCs demonstrated in the present work may be an important contributor to the therapeutic effect of stem cell administration.

Normally, the lung alveolar epithelium forms an extremely tight barrier that restricts the move-
ment of proteins and liquid from the interstitium into the alveolar spaces. In ALI, impaired alveolar epithelial function in the lungs is a marker of poor outcome in ALI [60]. Pro-inflammatory cytokines can induce or aggravate the inflammation of endothelial and epithelial cells, leading to their barrier dysfunctions [61].

In agreement with our results, Xu et al. [62] showed that prevention of endotoxin-induced systemic response by bone marrow-derived mesenchymal stem cells in mice was mainly due to their ability to convert the systemic endotoxin response from a proinflammatory to an anti-inflammatory milieu by suppressing generation of proinflammatory mediators without suppressing generation of anti-inflammatory mediators.

Delineating the precise mechanism of the anti-inflammatory effect of MSCs is not clear. Possible mechanisms include generation and secretion of soluble receptors for proinflammatory cytokines, or generation of anti-inflammatory cytokines (e.g., IL-10) directly by MSCs [63].

IL-10 is one of the key cytokines with anti-inflammatory capacities and it has been demonstrated that MSCs can affect the secretion of IL-10 in macrophages or dendritic cells [64,65]. However, it is still controversial if MSCs can secrete therapeutic levels of IL-10 by themselves [66,67].

In agreement with our results, Mao et al. [68] reported that anti-inflammatory cytokine IL-10 levels were dramatically increased in the EPC-treated rats compared with the phosphate buffered saline-treated rats.

On the other hand, Lee et al. [46] reported that, in spite of the beneficial effect of treating the LPS-injured lung lobe with MSCs, the levels of the anti-inflammatory cytokines, IL-1RA and IL-10, were not elevated. Similar findings were reported by Ortiz et al., in bleomycin-injured mouse lungs treated with MSCs 7 days after lung injury induction [69]. These results suggest that the therapeutic benefit of MSCs does not derive primarily from an anti-inflammatory effect.

In the present work, a significantly higher concentration of protein, number of total leukocytes and neutrophils percent than normal were found in BALF samples with increased activity of MPO indicating increased neutrophil infiltration in lungs from rats injected with LPS. Injecting the rats with MSCs significantly decreased BALF protein and cellular content with reduction in the percent of neutrophil with decreased acivity of MPO.

This result confirms the role of endotoxemia as a stimulus for the migration of neutrophils into the lung which causes injury to the alveolar capillary barrier and give an evidence for the ability of MSCs to decrease this neutrophil infiltration and probably the inflammatory cascade induced by ALI, and this could be a mechanism for protection of the alveolar capillary barrier. Recruitment of neutrophils into the lung is an important step in ALI [70].

Neutrophil activation is vital for the host defense, overzealous activation leads to tissue damage by release of cytotoxic and immune cell-activating agents such as proteinases, cationic polypeptides, cytokines, and reactive oxygen species (ROS) [71].

Consistent with these results, MSC treated lungs had significantly fewer neutrophils, the major mediator of lung injury in this model [72].

In agreement with our results, orbital fat-derived stem/stromal cells significantly reduced lipopolysaccharide-induced pulmonary inflammation, which was evidenced by a decrease in total protein concentration and neutrophil counts in alveolar fluid through bronchoalveolar lavage, reduced endothelial and alveolar epithelial permeability as well as neutrophil (Ly6G-expressing cells) and macrophage (CD 68-expressing cells) infiltration [73].

The results of the study performed by Lam et al. [74] showed that protein expression of CD11b and the activity of MPO was significantly suppressed by EPC transplantation, indicating decreased infiltration of PMNs in the lung parenchyma. They suggested that the potent anti-inflammatory paracrine effect of EPCs may contribute to the regional suppression of pulmonary PMN infiltration during ALI [75].

In the present investigation, the injection of LPS induced an oxidative stress manifested by increased formation of MDA and that MSCs decreased oxidative stress in the lung tissue. We can suggest that the antioxidant activity and anti-inflammatory effect of MSCs may contribute to the regional suppression of pulmonary neutrophil infiltration during ALI and this protects the lung tissue against damage induced by oxidative stress and inflammation induced by LPS.

This finding is in agreement with those reported earlier by other laboratories for the prooxidant action of LPS on the brain [71], heart [76], and lung [77], and support the use of antioxidants as adjuncts to the management of sepsis by Gram negative
bacteria with conventional therapeutic agents. In addition, the production of reactive oxygen and nitrogen species is related to apoptosis in alveolar epithelial cells [78].

The potential mechanisms by which MSCs infusion ameliorates ALI may be though improving GSH homeostasis. This may involve increased efflux of Cys and GSH from tissues, increased recycling, and/or increased GSH synthesis. This can be mediated by the secretion of soluble growth factors by MSCs, or by the interaction of MSCs with host cells, or both [79].

For instance, Kim and colleagues [80] have demonstrated that the antioxidant capacity of adipose tissue-derived MSC conditioned medium (ADCM) is comparable to 100µM ascorbic acid. Their study suggests that MSCs actively secrete antioxidant factors which may confer protection in the setting of inflammatory lung diseases.

A candidate growth factor likely responsible for the redox modulatory effect of MSCs is KGF [81]. In the present study it was demonstrated that MSCs increased expression of KGF in lung tissue and so it can be suggested that through its paracrine action, it stimulates the expression of KGF which has multiple protective effects, one of them is modulation of the oxidative stress.

Studies in a murine model of allogenic bone marrow transplant show that subcutaneous infusion of KGF improves hepatic GSH levels [82]. Because 50%-80% of plasma Glutathione GSH is dependent on efflux from the liver [83], increase in hepatic GSH could increase plasma GSH levels. Additionally, elevated levels of proinflammatory cytokines can adversely impact GSH homeostasis. In pulmonary endothelial cells, an increase in TNF-α decreases cellular GSH and increases Glutathione disulfide (GSSG) levels [84]. Thus, MSC-mediated modulation of lung inflammatory process could also be a factor to improved GSH homeostasis.

However, previous studies demonstrated that direct contact of MSCs with endothelial cells (EC:MSC ratio; 1:1 to 1:3) led to increased ROS production resulting in endothelial cell apoptosis and ultimately to capillary degeneration [85].

In the present study, treating LPS-injured lung with NAC induced a partial improvement in the rat condition as manifested by partial reduction of the lung water content, proinflammatory cytokine, NF-κB with decreased protein and neutrophil content in the BAL. NAC significantly reduced the oxidant stress as observed by decreasing MDA level and apoptosis in the lung and significantly increased lung expression of KGF.

Combining NAC pretreatment with MSCs potentiated the therapeutic effect of MSCs injection and had a significant beneficial effect over the MSCs therapy alone.

Recent in vitro experiments have shown that thiol antioxidants such as n-acetylcysteine block the release of the inflammatory mediators from epithelial cells and macrophages by a mechanism involving increasing intracellular GSH and decreasing NF-κB activation [86].

NAC may have additional protective ability to reduce oxyradical-related oxidant processes by directly interfering with the oxidants, up-regulating antioxidant systems such as superoxide dismutase [87], or enhancing the catalytic activity of glutathione peroxidase [88].

NAC can interfere with the inflammatory process at different levels. The inhibitory effect of NAC on activation of the NF-κB is recorded by Schillier et al. [89], another evidence suggested that the activation of NF-κB may also be under the control of oxidant/antioxidant balance [90]. In addition, NAC reduces the recruitment of PMNs into the inflammatory site. This effect of NAC is very likely secondary to the prevention by NAC of endothelial oxidant injury and, hence, preservation of endothelial barrier function [91]. These results suggest that oxidative stress plays an important role in structural, functional and inflammatory responses in the ALI model [92].

Controlling the oxidative stress offers a better environment for the MSCs to induce their beneficial effects and this is not only additive action but it appears to be a synergistic response.

Despite the potential benefits of MSCs in lung regeneration and repair, there is an evidence to suggest a direct role for these cells in the development of parenchymal fibrosis [93,94].

There is also a concern that the defining characteristic of stem cells, unlimited self-renewal, could make them a candidate for malignant change [95]. In addition MSC delivery in a murine model has been associated with sarcoma development within the lung parenchyma [96].

These findings show that inflammatory stimuli in ALI caused by LPS, induce a rapid release of inflammatory cytokines and cells. In addition to the ability of MSCs to sequester and migrate into the inflammatory site and differentiate to become
endothelial and epithelial cells, they play an important paracrine role through modulation of the inflammatory process and protection against oxidative stress and apoptosis and protect the alveolar capillary barrier function. They stimulate the expression of KGF which is involved in lung repair after LPS-induced lung injury. The results from our study indicate that MSCs may have utility for therapeutic use in lung disease. Providing a sufficient antioxidant condition also has a protective role against tissue damage in case of LPS induced ALI and could offer a better environment for MSCs to perform their function and so the combined therapy of MSCs and NACs resulted in more effective protection of lung tissue against injury induced by LPS.

References


Role of Stem Cells & Antioxidant on Modulation of Body Defense


