European Review for Medical and Pharmacological Sciences 2013; 17: 403-409

Influence of long-term drinking alcohol on the cytokines in the rats with endogenous and exogenous lung injury

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Abstract. – BACKGROUND: Acute lung injury (ALI) and the acute respiratory distress syndrome (ARDS) are syndromes of acute respiratory failure.

PURPOSE: Exploration of the impacts of long-term drinking alcohol on the cytokines of rats with endogenous and exogenous lung injuries.

MATERIALS AND METHODS: Through giving the model rats long-term drinking alcohol or water, we acquired the changes of the cytokines in the serum and bronchoalveolar lavage fluid (BALF) of these rats with lung injuries due to different incentives.

RESULTS: The partial pressure of oxygen in rats with lung damage after long-term drinking alcohol were significantly lower than those drinking water group ($p < 0.05$); W/D values of groups drinking alcohol were significantly higher than those drinking water ($p < 0.05$); The levels of TNF-alpha, IL-6 and IL-10 in the serum and BALF were significantly higher in the group drinking alcohol ($p < 0.01$). While the cytokine levels in the serum of the rats with exogenous lung injury were higher than those of rats with endogenous lung injury ($p < 0.05$), the cytokines in the BALF of the rats with endogenous lung injury were higher than those with exogenous lung injury ($p < 0.05$).

CONCLUSIONS: Long-term drinking alcohol can aggravate the inflammatory response induced by the exogenous lung injury. The expression of TNF-α, IL-6 and IL-10 are different according to the different ways that lead to the acute lung injury.

Key Words:

Long-term drinking alcohol, acute lung injury, cytokine, tumor necrosis factor-α, interleukin-6, interleukin-10, rat, ELISA.

Introduction

Acute lung injury (ALI) and the acute respiratory distress syndrome (ARDS) are syndromes of acute respiratory failure[1]. The incidence of ALI/ARDS still remains unknown, but it is estimated that 36,000 patients die from these diseases every year in the United States[2]. The mortality rate associated with ARDS has declined from 90% about twenty years ago to 30%~40% at present[3]. However, it is still one of the major causes of pulmonary and nonpulmonary morbidity. The pathophysiological consequences of ALI/ARDS are related to the changed pulmonary capillary permeability and alveolar diffusion capacity, as well as the increased intrapulmonary shunt. It is well established that endothelial injury and increased vascular permeability as a critical feature of ALI/ARDS, and instead of an injurious mechanism, some studies have supported the neutrophils as a defense[4,5].

The ALI/ARDS may occur as a consequence of critical illness of diverse etiologies, including direct injury to lung, such as pneumonia, aspiration, toxic inhalation, near-drowning, or lung contusion; as well as indirect mechanisms, such as sepsis, burn, pancreatitis, gynecological insults (abruption of placenta, amniotic embolism, eclampsia), or massive blood transfusion[4,6]. The alcohol abuse has been proven to be related with the occurrence of the ARDS by several studies[7-9]. There are likely many potential mechanisms by which alcohol abuse increased susceptibility to developing ARDS. But the mechanisms are not yet completely clear, and besides the abnormal oxidant/antioxidant system, the alteration of the cytokine levels may contribute to these diseases.

In this study, we have successfully established the rats with endogenous and exogenous lung injury. Through giving the model rats long-term drinking alcohol or water, we acquired the changes of the cytokines in the serum and bronchoalveolar lavage fluid (BALF) of these rats with lung injuries due to different incentives.

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Materials and Methods

Materials

Thirteen-six male SD rats with the weight of 210-250 g were housed in six different cages at the China Medical University and maintained under temperature (23 ± 2°C) and light cycle (12 h light, 12 h dark)-controlled quarters. Mice were provided food and water ad libitum. The ELISA kits of TNF-α, IL-6 and IL-10 were purchased from the Senxiong Biotech Limited Corporation (Shanghai, China) and the lipopolysaccharide (LPS) was bought from the Sigma Co (St Louis, MO, USA.

Long-term Drinking Alcohol Model

The rats were randomly divided into the group drinking water and the group drinking alcohol (n = 18), according to the previous literature[10]. The rats were first fed for 3 days to get accustomed to the environment. The rats in the group drinking alcohol were fed with 6% alcohol in place of water for three days, then 10% alcohol for four days, and 20% alcohol for continuous five weeks. The rats in the group drinking water were fed with water.

Endogenous and Exogenous Lung Injury Model

Six weeks later, two groups were randomly divided into three groups (n = 6) as follows: rats drinking water, rats with endogenous lung injury drinking water, rats with exogenous lung injury drinking water; rats drinking alcohol, rats with endogenous lung injury drinking alcohol, and rats with exogenous lung injury drinking alcohol. In the group with endogenous lung injury, the rats were first anesthetized by the intraperitoneal injection of 1% sodium pentobarbital (50 mg/kg), and then were instilled with LPS (15 mg/kg) using seven-gauge needle through the separated trachea in the neck midline incision. In the group with exogenous lung injury, the rats were intraperitoneally injected with LPS (15 mg/kg).

Specimen Collection

Four hours after administration, the rats were anesthetized by intraperitoneal injection of 1% sodium pentobarbital (50 mg/kg). The abdominal cavity was opened, the abdominal aortic blood was used for the measurement of PaO_2 and the inferior vena cava blood (4 ml) was collected. The specimens of blood were centrifuged with 3000 rpm for 10 minutes under 4°C, and the supernatant was collected and then frozen at –80°C.

The complete lungs were removed from the abdominal cavity, carefully rinsed with ice brine to clear the blood, wiped with dry gauze, and then placed on the ice. The right lower part of the lung was fixed in 10% paraformaldehyde, dehydrated with the gradient alcohol, and embedded with paraffin. Then the paraffin embedded lung tissue was cut into 4 m slides, stained by hematoxylin-eosin, and examined under the light microscope. The wet weight of the right upper part of the lung was measured. After dried by the 80°C oven, the dry weight of the same tissue was measured and the final W/D ratio was calculated.

Ice brine (3 ml) was injected into the left lung tissue with homemade lavage needle lavage. After washed for three times, the lavage was collected, centrifuged at 1000 rpm for 5 minutes under 4°C. The supernatant was collected and stored at –80°C refrigerator.

The Measurement of TNF-α, IL-6 and IL-10 in the Bronchoalveolar Lavage Fluid and Serum

The levels of TNF-α, IL-6 and IL-10 in the serum and bronchoalveolar lavage fluid were measured using a double antibody sandwich ELISA assay according to the manufacturer's protocol (Senxiong Biotech Limited Corporation, Shanghai, China).

Statistical Analysis

SPSS13.0 statistical software was used for the statistical analysis (SPSS Inc., Chicago, IL, USA). Data are expressed as mean ± standard deviation. Analysis of variance (ANOVA) between groups was used to compare the average of each group. Least significant difference method was applied for multiple comparisons. p values ≤ 0.05 were considered to be statistically significant.

Results

Changes of Oxygen Partial Pressure and Lung W/D Ratio Values in Different Groups

There was no difference between the oxygen partial pressure (PaO_2) of the group only drinking alcohol and that of the group only drinking water. The PaO_2 in the exogenous and endogenous lung injury groups drinking alcohol were significantly lower than those in group with the same injury drinking water ($p < 0.05$). Further-

more, the reduction of PaO_2 in the endogenous lung injury group was more significant than those in the exogenous lung injury groups ($p < 0.05$) (Table I).

In addition, no changes of W/D values were found between the groups drinking water and the groups drinking alcohol. The W/D values in the groups with lung injuries increased ($p < 0.05$), and the alcohol enhanced this effect in the groups with lung injuries ($p < 0.05$). There was no significant change between the rats with exogenous lung injury and the rats with endogenous lung injury in the groups drinking alcohol (Table I).

Pathological Observation of the Lung Tissues

The lung structure of the groups only drinking water or alcohol were normal, and there was no alveolar infiltration of inflammatory cells (Figure 1A and 1B). The interstitial of the rats with endogenous lung injury drinking water were thickened and massive infiltration of inflammatory cells and a small amount of red blood cells occurred (Figure 1C). There was also thickened interstitial and fewer infiltration of inflammatory cells in the rats with exogenous lung injury drinking water (Figure 1D).

Alveolar diffuse congestion, alveolar structural damages, the infiltration of various inflammatory cells and the transparent membrane were found in the rats with endogenous lung injury drinking alcohol (Figure 1E). In the rats with exogenous lung injury and drinking alcohol, the interstitial significantly widened, and the alveolar membrane disappeared. There was also the infiltration of massive inflammatory cells (Figure 1F).

The Level of Cytokines in the Serum of Different Groups

The TNF-α, IL-6 and L-10 levels in the serum of the rats only drinking alcohol and the rats with lung injuries were higher than those of the rats only drinking water ($p < 0.01$). The rats with exogenous or endogenous lung injuries and drinking alcohol produced more TNF-α, IL-6 and IL-10 than the rats with the same injuries and drinking water. And this effect was more obvious in the rats with exogenous lung injuries and drinking alcohol ($p < 0.05$) (Table II).

The level of Cytokines in the Bronchoalveolar Lavage Fluid (BALF) of Different Groups

The TNF-α, IL-6 and L-10 levels in the BALF of the rats only drinking alcohol and with lung injuries were higher than those of the rats only drinking water ($p < 0.01$). The rats with exogenous or endogenous lung injuries and drinking alcohol produced more TNF-α, IL-6 and L-10 in the BALF than the rats with the same injuries and drinking water. And this effect is more observed in the rats with endogenous lung injuries and drinking alcohol ($p < 0.05$) (Table III).

Discussions

Heymann C et al[11] found that cytokines like IL-6 and IL-10 in the serum of patients with a history of long-term drinking alcohol were significantly higher than patients with no history of drinking. However, the mechanism the influence of long-term alcohol consumption on the cytokines induced ALI/ARDS resulting through different pathways still remains unclear.

In our study, we first established a rat model of long-term drinking freely, and then produced the internal and external acute lung injury models by intratracheal instillation and intraperitoneal injection of LPS. The results showed that PaO_2 of each group with lung injuries decreased and the W/D values increased. Pathological examination of the

Table I. The level of PaO_2, W/D in different groups ($\bar{x} \pm s$).

Groups	n	PaO_2	W/D values
Group drinking water	6	102 ± 11.97	4.31 ± 0.19
Group with endogenous injury drinking water	6	68 ± 5.72*	5.52 ± 0.25*
Group with exogenous injury drinking alcohol	6	75 ± 4.41*,#,§	5.27 ± 0.23*,#
Group drinking alcohol	6	99 ± 9.18	4.39 ± 0.16
Group with endogenous injury drinking alcohol	6	59 ± 6.22*,#,§	6.47 ± 0.14*,§
Group with exogenous injury drinking alcohol	6	68 ± 5.29*	6.26 ± 0.10*

*Compared with the group drinking water; §Compared with the group with the endogenous injury drinking water $p < 0.05$; #Compared with the group with exogenous injury drinking alcohol $p < 0.05$.

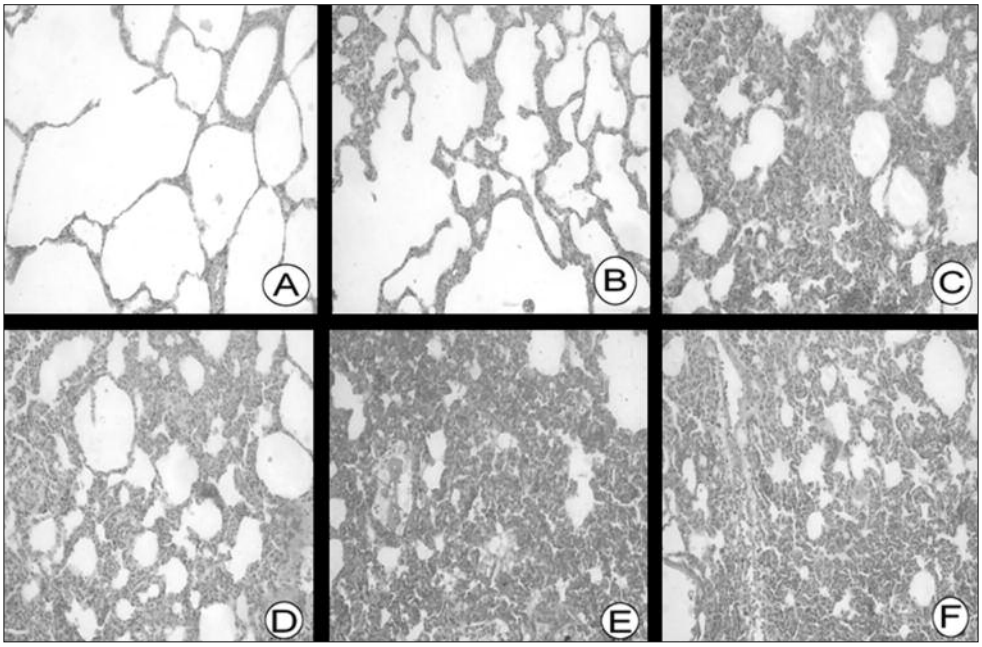

Figure 1. Histological changes of lung tissue in each group (HE×200). ***A-B,*** The lung structures of the group only drinking water and only drinking alcohol are normal. ***C,*** The interstitial of the group with endogenous lung injury and drinking water was thickened, and massive infiltration of inflammatory cells and a small amount of red blood cells occurred. ***D,*** Thickened interstitial and fewer infiltration of inflammatory cells occurred in the group with exogenous lung injury and drinking water. ***E,*** Alveolar diffuse congestion, alveolar structural damages, the infiltration of various inflammatory cells and the transparent membrane were found in the rats with endogenous lung injury drinking alcohol. ***F,*** The interstitial significantly widened and massive inflammatory cells infiltrated in the group with exogenous lung injury and drinking alcohol.

Table II. The expression of TNF-α, IL-6 and IL-10 in serum of rats ($\bar{x} \pm s$) (n = 6).

Group	TNF-α (pg/ml)	IL-6 (pg/ml)	IL-10 (pg/ml)
Group drinking water	37.42 ± 1.20	49.98 ± 6.28	50.01 ± 4.17
Group with endogenous lung injury drinking water	75.11 ± 10.66*	243.97 ± 36.40*	159.42 ± 13.95*
Group with exogenous lung injury drinking water	191.63 ± 18.75*,§,#	272.57 ± 45.17*,#	230.84 ± 12.47*,§,#
Group drinking alcohol	50.27 ± 2.28*	177.89 ± 20.46*	90.10 ± 5.32*
Group with endogenous lung injury drinking alcohol	104.39 ± 12.17*,§,#	434.87 ± 62.69*,§,#	208.15 ± 12.16*,§,#
Group with exogenous lung injury drinking alcohol	260.84 ± 21.79*	539.92 ± 72.00*	425.12 ± 35.51*

*Compared with the group drinking water $p < 0.01$; §Compared with the group with the endogenous injury drinking water $p < 0.05$; #Compared with the group with exogenous injury drinking alcohol $p < 0.05$.

Table III. The expression of TNF-α, IL-6 and IL-10 in BALF of rats ($\bar{x} \pm s$) (n = 6).

Group	TNF-α (pg/ml)	IL-6 (pg/ml)	IL-10 (pg/ml)
Group drinking water	24.28 ± 2.27	47.97 ± 2.89	45.63 ± 2.48
Group with endogenous lung injury drinking water	81.03 ± 8.82*	235.16 ± 37.14*	121.5 ± 10.38*
Group with exogenous lung injury drinking water	61.25 ± 12.4*,§,#	229.66 ± 43.90*,#	99.89 ± 4.2*,§,#
Group drinking alcohol	34.65 ± 1.76*	147.29 ± 37.97*	61.49 ± 1.56*
Group with endogenous lung injury drinking alcohol	170.66 ± 18.93*,§,#	390.45 ± 62.57*,§,#	150.26 ± 6.74*,§,#
Group with exogenous lung injury drinking water	140.27 ± 12.76*	302.50 ± 45.82*	125.77 ± 10.73*

*Compared with the group drinking water $p < 0.01$; §Compared with the group with the endogenous injury drinking water $p < 0.05$; #Compared with the group with exogenous injury drinking alcohol $p < 0.05$.

lung tissue demonstrated the manifestations of lung injury: pulmonary interstitial edema; alveolar structure damage, collapse; massive inflammatory cell infiltration; capillary congestion, exudation. All these results suggested that the lung damage models were successfully reproduced.

In the ALI/ARDS, TNF-α can activate signaling pathways which destroy granulocytes and platelets, and further produce reactive oxygen species (ROS) and proteolytic enzymes lipid metabolites. Therefore, TNF-α can reduce the antioxidants GSH level, aggravates the tissue damage[12], increases pulmonary capillary permeability and leads to tissue hemorrhage and edema.

It can be seen from the results that the TNF-α levels in the serum and BALF of the rats only drinking alcohol were higher than those only drinking water. Much acetaldehyde metabolites are produced after long-term drinking alcohol, not only leading to the oxidative shock response, but also resulting in the reduction of the GSH concentration secreted by the alveolar type II epithelial cells and, thus, promotion of the TNF-α production[13]. In addition, acetaldehyde can increase intestinal permeability[14], and cause the shift of the intestinal flora and the intestinal endotoxemia. The endotoxin which can recognize the CD14 and TLR4 (toll-like receptor 4) on the Kupffer cells activates the cells and increases the TNF-α in the blood circulation[15]. Furthermore, the endotoxin can enter the alveoli, increases the TNF-α levels in BALF and thus increase the susceptibility to the inflammatory reaction.

The TNF-α levels in the serum and BALF of rats with endogenous and exogenous lung injury and long-term drinking alcohol were higher than those of rats with the same injuries and long-term drinking water. While the TNF-α levels in the serum of rats with the exogenous lung injury and drinking alcohol were higher, the TNF-α levels in the BALF of rats with the endogenous lung injury and drinking alcohol increased more significantly. The angiotensin-II (Ang-II) increases after long-term drinking, and activates the nuclear transcription factor (NF-κB) pathway[16]. The administration of LPS can activate monocytes and macrophages through the activation of the NF-κB signaling pathways, and thus produce more TNF-α[17]. More TNF-α would create a vicious cycle and aggravate the damage to the lung tissue. The different TNF-α levels in the serum and BALF in the groups with endogenous and exogenous lung injuries drinking alcohol may due to the different incidence ways. Endogenous lung injury first activates alveolar macrophages and neutrophils, and releases the inflammatory mediators. The occurrence of the inflammatory response can lead to the alveolar edema, fibrin exudation, neutrophil accumulation and pulmonary parenchymal disease[18]. Therefore, TNF-α is mainly expressed in BALF. The TNF-α in the rats with exogenous injury is mainly produced outside the lung and transported to the lungs through the bloodstream, which does damages to the vascular endothelial cells, increases vascular permeability, and induces the interstitial pulmonary edema. Consequently, the TNF-α mainly expresses in peripheral blood.

The IL-6 levels of the rats only drinking alcohol were higher than those of the rats only drinking water. The rats with exogenous or endogenous lung injuries and drinking alcohol produced more IL-6 in the BALF and serum than the rats with the same injuries and drinking water. While IL-6 level in the BALF is higher in the rats with endogenous lung injuries and drinking alcohol, the IL-6 level in the serum is higher in the rats with exogenous lung injuries and drinking alcohol. Long-term drinking can cause liver damage and promote the Kupffer cells to release IL-6[19].

El-Assal et al[20] show that IL-6 plays a protective role in alcohol-induced liver injury by inhibiting alcohol-mediated oxidative stress and mitochondrial damage[20]. In the meanwhile, Il-6 can activate anti-apoptotic gene Bcl-2 (B-cell lymphoma 2) and of Bcl-x (L) and inhibit the cell apoptosis[21]. It was observed that IL-6 gene knockout mice significantly increased sensitivity to alcohol-mediated liver steatosis and apoptosis. Alcohol can inhibit IL-6 anti-apoptotic signal transduction, which induces more production of IL-6 transported to the lungs to maintain its ability of anti-apoptosis[22,23]. The lung damage after long-term alcohol consumption would be aggravated by LPS, and more IL-6 would be generated. The exogenous lung injury after long-term drinking alcohol is caused by extra pulmonary pathways, and, therefore, severe systemic inflammatory response and high IL-6 levels in blood circulation will occur. The endogenous lung injury after long-term drinking alcohol results in the local inflammation, and hence IL-6 levels in BALF were significantly higher than the exogenous lung injury. IL-6 in the lung may activate the expression of the complement and C-reactive protein[24], not only resulting in cell damage, but also activating astrocytes, vascular endothelial cells and lymphocytes, and further leading to ag-

gravation of the inflammatory response. Furthermore, IL-6 also can promote neutrophil release a lot of elastase, which aggravated the injury to the lung tissue.

The IL-10 levels in the serum and BALF of rats with long-term drinking alcohol were higher than those of rats with long-term drinking water. The IL-10 levels in the serum and BALF of rats with endogenous and exogenous lung injury and long-term drinking alcohol were higher than those of rats with the same injuries and long-term drinking water. While the IL-10 levels in the serum of rats with the exogenous lung injury and drinking alcohol were higher, the IL-10 levels in the BALF of rats with the endogenous lung injury and drinking alcohol increased more significantly. TNF-α may be one of the main cytokines that induces the synthesis of IL-10, since TNF-α can increase the mRNA expression of IL-10. IL-10 can inhibit the production of pro-inflammatory cytokines from monocyte macrophages and neutrophils, and inhibit expression of TNF mRNA[25]. The liver damage caused by long-term drinking alcohol can increase the production of TNF-α and IL-10. As an anti-inflammatory cytokines, IL-10 not only can inhibit the cytotoxicity of TNF-α, but also can reduce the liver fibrosis[26]. The generation of IL-10 will increase together with the increment of TNF-α when the body receives the stimulation of LPS. Different expressions of IL-10 may be related with those of TNF-α in different groups.

Conclusions

Long-term drinking alcohol can aggravate the inflammatory response to endogenous and exogenous ALI/ARDS. However, TNF-α, IL-6 and IL-10 expressions will be different because of disparate sites of injury in ALI/ARDS resulting from different pathways. As a result, in the early ALI/ARDS, personalized programs and targeted treatment should be emphasized, which may provide a new idea for the future treatment.

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