Superoxide Anion, the Main Species of ROS in the Development of ARDS Induced by Oleic Acid

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It is believed that reactive oxygen species (ROS) play a very important role in the pathogenesis of acute respiratory distress syndrome (ARDS), but the mechanism has not been so clear, owing to the absence of direct measurable (experimental) data. In majority of the medical studies on free radicals, the analysis of ROS has generally been done by the way of measuring their secondary and breakdown products. In our study, we used electron spin resonance (ESR), a sensitive and accurate technique to detect ROS directly and also used some other sensitive techniques including ultra-weak luminescence and chemical luminescence to identify the species and relative amount of ROS. Furthermore, superoxide dismutase (SOD) was pre-administrated in ARDS rats to verify the results from the above studies and explore the possibility of the clinical application of SOD in ARDS. The spectra of ESR showed that the concentration of ROS increased at 10 min and reached a summit at 30 min after injection of oleic acid (OA), then dropped gradually. The scavenging effects of different scavenging agents on ROS by the analysis of ultra-weak luminescence proved that superoxide anion was the main species of ROS in the development of OA-induced ARDS. Moreover, the results of quantified measure of superoxide anion by chemical luminescence also showed the accordant tendency exhibited in ESR measurement. The pre-treatment of SOD might distinctly inhibit the production of superoxide anion, obviously improve the blood gas status, lung wet/dry ratio and lung/body ratio in ARDS rats. It is suggested that ROS may play a key role in the initiation phase of ARDS, while superoxide anion may be a leading actor in this process and SOD could effectively protect rats from ARDS. These results may provide helpful information for the treatment and prevention of ARDS.

Keywords: Reactive oxygen species; Acute respiratory distress syndrome; Electron spin resonance; Ultra-weak luminescence; Superoxide dismutase

INTRODUCTION

It is well known that acute respiratory distress syndrome (ARDS) is a serious disease with high mortality despite extensive supportive treatments. Although much effort was made for elucidating the pathogenesis of ARDS in the past decades, the precise mechanisms still remain obscure.[1,2] Reactive oxygen species (ROS) are believed to play a pivotal role in the pathogenesis of ARDS, while preventing the damage by ROS would be a key measure for the treatment of ARDS.[3,4] Therefore, understanding the mechanism by which ROS generated and damaged lungs should be very important. Although ROS has been studied in the research of ARDS for many years, because of its high reactivity and short life, detailed and direct data could not be obtained. So, ROS is generally analyzed by measuring their secondary or end products, such as H_2O_2, oxidized proteins or peroxidized lipids, i.e. MDA.[5,6]

In this study, we used oleic acid (OA) to establish a stable rat ARDS model, which was close to the early stage of clinical ARDS,[7,8] and used electron spin resonance (ESR) spectroscopy to evaluate ROS. ESR is an effective method for studying short life-span free radicals such as superoxide anions, hydroxyl radicals, etc. Moreover, we also used some other sensitive methods such as ultra-weak luminescence analysis and chemical luminescence to identify the species and the relative quantity of ROS. To verify the above results from another point of view,

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SOD was also administered before OA injection in rats. The purpose of this study is to ascertain the metabolic rule and species of ROS during the pathogenesis of ARDS. We hope that it may provide helpful information, for the future, in the treatment and prevention of ARDS.

MATERIALS AND METHODS

Animal Model and Experimental Group Conformation

Adult pathogen-free male Sprague–Dawley rats weighing 200–240 g were purchased from the Experimental Animal Center of Beijing Medical University (Beijing, China), and allowed to acclimate upon arrival for 3 days before experimentation. Animals were fed rodent chow and water *ad libitum*. All experimental protocols were approved by the Animal Care and Use Committee.

Animals were randomly divided into normal control, ARDS and SOD pre-treatment groups. ARDS group was also divided into 6 subgroups according to the different observing times such as 10 min, 30 min, 1, 2, 4 and 6 h after the injection of OA. OA (0.15 ml/kg) was administered into the femoral vein to prepare rat ARDS models and then anesthetized with urethane (1.0 g/kg i.p.). SOD (7.5 mg/kg) was first administered into the femoral vein of the SOD group animals and then (1 min later) injected with OA. The normal control rats were only administered intravenously the same volume of saline. Each group had 5 rats. The ARDS model was verified by clinical blood gas analysis and pathology observation.

Reagents

PBN was purchased from Sigma Chemical Co., USA. OA was purchased from Golden Dragon Chemical Co., Beijing, China. DETAPAC (diehtylonethiamine-pentaacetic acid), anhydrous alcohol, pyrogallol and ethyl acetate were products of Beijing Chemical Agent Manufactory, China, which were all of analytical reagent level. Superoxide dismutase (SOD) and catalase (CAT) were products of the Institute of Biochemical, Academic Sinica, Shanghai, China. Luminal was a product of Merck-Schuchardt. The SOD assay kit was purchased from Nanjing Jianchen, Co. Deionized water was used in all the aqueous systems.

Samples Collection

The experimental rats were anesthetized with urethane and laid supine, after making a midline cervical incision catheters were inserted from the left carotid into the left ventricle for measuring the left ventricle pressure (LVP) and sampling blood. Then a polythene catheter (0.9 mm out diameter) was inserted from the right jugular vein directly into the pulmonary arterial trunk through the right atrium and ventricle for measuring the mean pulmonary artery pressure (mPAP) and sampling blood by monitoring of polygraph (360, san-ei Instrument Co., Tokyo, Japan). Blood gases were analyzed with a blood gas analyzer (STAT Profile 3 Analyzer, NOVA Biomedical, Walthan, MA). Venous blood sample was collected from the femoral vein. The lungs were dissected for the measurement of ESR, histological observation and lung wet/dry ratio, lung/body ratio measurements.

ESR Measurements

Spin trap agent dispensing: PBN and DETAPAC were dissolved in PBS buffer (pH 7.4) at a final concentration of 100 and 2 mM, respectively. To 2 g of lung tissues 1 ml of spin trap agent and electric homogenate was added. Lung homogenates were centrifuged at 14,000 rpm for 10 min after adding 0.6 ml ethyl acetate. Finally, the ethyl acetate extracts were taken in a test tube and stored in the dark at 0–4°C and left for 2 h measuring the ESR.

The specimen was then transferred into a quartz tube for ESR measurement. The ESR spectra were recorded at room temperature (298 K) by a Varian E-109 spectrometer with measurement conditions such as: X-band; central magnetic field, 3445 G; scan width, 200 G; microwave power, 20 mW; frequency, 100 kHz; modulation amplitude, 2.5 G and time constant, 0.128 s. Before performing the experiment, the ESR spectrometer was standardized with DPPH (Diphenyl-picri-hydrazyl) to make sure that the equipment was in the same condition every time the experiment was performed.

Ultra-weak Luminescence Measurements

In order to find out the kind of ROS in the ESR signals of ARDS, different scavenging agents such as SOD, CAT and anhydrous alcohol were used to measure the ultra-weak luminescence (Ultra-weak Luminescence Analyzer was from the Institute of Biophysics, Academia Sinica). The instrumental parameters were as follows: time, 1200 s; interval, 1.00 s; light source, 7000; high voltage, 980 and stew, 10. OA and normal controls were designed in order to eliminate the influence of OA and antioxidants in normal serum. After 1 min 10 μl of left ventricle blood serum (or OA dilutor liquid) was added to 1 ml of redistilled water and transferred into the luminescence tube to measure the base line running. After that, SOD, CAT and anhydrous alcohol, each 10 μl, were added to the system, respectively, to measure the time-dynamical curve.

TABLE I   Results of blood gas analysis in OA-induced ARDS and control rats ($\bar{x} \pm s$)

Group	n	PaO_2 (mmHg)	CO_2 (mmHg)	pH	O_2 saturation (%)	PaO_2/FIO_2 (mmHg)
Control	5	94.63 ± 5.85	41.58 ± 2.52	7.36 ± 0.03	96.99 ± 0.51	452.79 ± 27.99
10 min	5	70.73 ± 3.92**	50.65 ± 5.03*	7.27 ± 0.06*	90.68 ± 2.75	338.40 ± 18.77**
30 min	5	54.10 ± 2.83**	40.33 ± 1.91	7.20 ± 0.12**	79.83 ± 5.30**	258.85 ± 13.56**
1 h	5	50.34 ± 7.34**	52.96 ± 7.84**	7.26 ± 0.06**	77.76 ± 6.99**	240.87 ± 35.12**
ARDS						
2 h	5	35.08 ± 4.59**	50.25 ± 11.09*	7.23 ± 9.85**	55.80 ± 12.76**	167.86 ± 21.94**
4 h	5	45.30 ± 7.94**	42.30 ± 5.49	7.33 ± 0.09	76.30 ± 8.78**	216.75 ± 37.97**
6 h	5	63.34 ± 5.33**	45.17 ± 9.67	7.36 ± 0.06	90.59 ± 2.88*	303.08 ± 25.51**

*$P < 0.05$, **$P < 0.01$ vs. control; PaO_2/FIO_2 of ARDS diagnostic criterion <200 mmHg.

Superoxide Anion Measurements by Chemical Luminescence

Superoxide anion was generated by the pyrogallic acid system and measured by a luminescence analyzer (WDD-1, The Second Optics Instrument Co., Beijing, China). The reaction system was as follows: luminal, 900 μl (which was diluted by carbonic acid buffer, pH 10, 0.01 mM); 4 M NaOH, 80 μl; pyrogallic acid, 10 μl and serum, 10 μl. After mixing, the sample was transferred into a luminescence tube and measured immediately. Values were expressed in arbitrary units (a.u.).

Assay of SOD Enzymatic Activity in Serum

SOD enzymatic activity was measured before and after injection, which used the commercial SOD assay kit purchased from Nanjing Jianchen, Co., following the manufacturer's instructions.

Lung Histology

Lung tissues of rats were fixed by inflation with a buffered 10% formalin solution for 24 h and embedded in paraffin. Tissues were then sectioned at 3 μm, stained with hematoxylin and eosin (HE), observed and took pictures under a microscope of ×100 magnification.

Statistics

All data were expressed as mean ± SD ($n = 5$). Statistical analyses were performed using one-way ANOVA or Student's t-test by SPSS statistical software package (SPSS for Windows, 10.0). P-values less than 0.05 were considered to be significant.

RESULTS

Blood Gas Analysis

Table I shows that hypoxemia was evident in all rats after injection of OA, especially 2 h after injection.

The changes of PaO_2/FIO_2 corresponded with the diagnostic criterion of ARDS by the Meeting of the American–European Consensus Conference on ARDS,[9] suggesting that the animal model of ARDS was successful.

ESR Spectra of ROS from Lung Tissues

The ESR spectra of the spin trapping adduct of ROS with PBN extracted from the lung tissue is shown in Fig. 1. The peak height of the spectrum represented relative concentration of ROS. The result showed that nearly no ROS signals could be seen in normal lung tissues; whereas after injection of OA, the ROS signal notably increased and reached a summit at 30 min, and then returned to the normal level 6 h after the injection.

Figure 2 shows the comparison of ESR spectra and blood gas analysis results (PaO_2). It could be found that the concentration of ROS increased significantly at 30 min after OA exposure and began to drop at 1 h, whereas the reduction of PaO_2 began from second
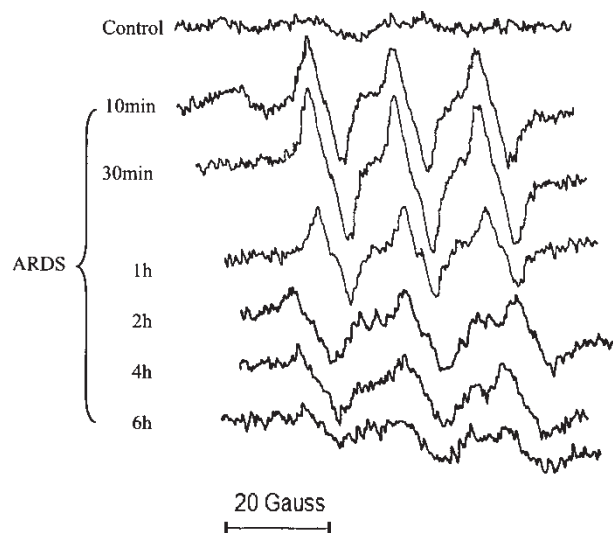

FIGURE 1   The ESR spectra of ROS trapped with PBN extracted from the lung tissue in ARDS and control rats. ESR measurements conditions are listed in the "Materials and Methods" section. The peak altitude represented the relative content of ROS.
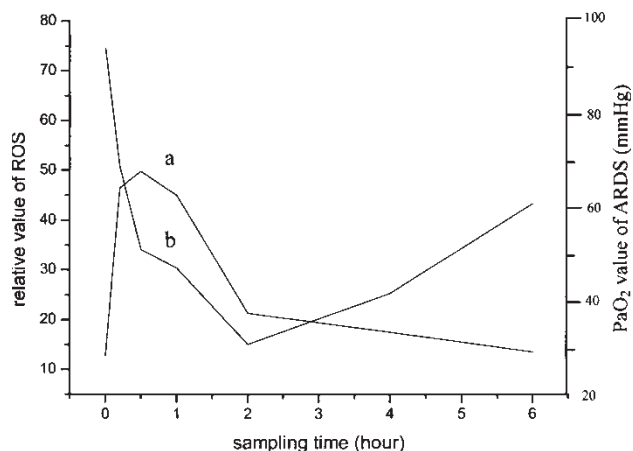
FIGURE 2 Comparison of relative value between ESR spectra and PaO$_2$ (mmHg). a, ESR relative altitude; b, PaO$_2$ values. It showed that ESR signal reached its peak at 30 min and began to drop at 1 h after OA injection, whereas the value of PaO$_2$ decreased mostly at 2 h.

hour. The result suggested that the change of ROS was earlier than that of pathophysiology.

Measurement of ROS and their Time-dynamical Curve by Ultra-weak Luminescence Technique

ROS releases chemiluminescence. Figure 3 shows the dynamic curves of serum from different rats. It could be found that OA and normal serum had little chemiluminescence and the curve of the group after 30 min of OA injection was the highest one of all curves.

SOD is the specific scavenger of superoxide anion, CAT is specific to hydrogen peroxide while ethanol is specific to hydroxyl radicals. In order to determine the species of ROS responsible for the increasing chemiluminescence, the effects of different scavenging agents on the chemiluminescence were detected

(Fig. 4). After addition of SOD to the system, the ROS curve descended, especially at the initiation phase, which suggested that the superoxide anion might be the primary species of ROS in the pathogenesis of ARDS.

Quantificational Measurement of Superoxide Anion by Chemical Luminescence Technique

Figure 4 indicates that the main ROS in the lung tissue of ARDS rats induced by OA was superoxide anion, so we used chemical luminescence to measure the relative amount of ROS. The results given in Table II show that there was a significant increase in the production of superoxide anion after OA injection, especially at 30 min, which corresponded with results of ESR spectra and ultra-weak chemiluminescence. In addition, the results also indicate that the left ventricle blood had the highest amount of ROS than the other samples which suggested that the sample from left ventricle blood might reflect the actual status of ROS in the lung better (Fig. 5).

Results of SOD Enzymatic Activity Assay

After injection, SOD activity decreased from 139.22 ± 2.51 (NU/ml) to 130.74 ± 1.16 (10 min after injection), 127.78 ± 1.14 (30 min after injection), then descended quickly as time elapsed, suggesting that SOD cannot last longer *in vivo* because of its short half-life. But SOD could still prevent ARDS since superoxide anions were mainly produced in the early phase of ARDS.

Blood Gas Analysis after SOD Pre-treatment

Table III shows that PaO$_2$ and PaO$_2$/FIO$_2$ in SOD pre-treatment group is significantly higher than that
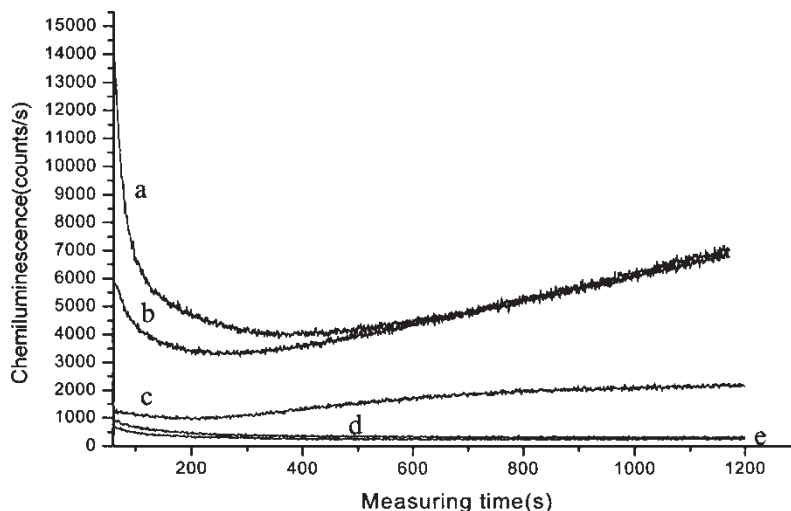

FIGURE 3 The time-dynamical curve of ROS by chemiluminescence technique in lung of ARDS rats at 10 and 30 min after OA injection. a, 30 min after OA injection; b, 10 min after OA injection; c, normal serum; d, reagent control; e, OA control.
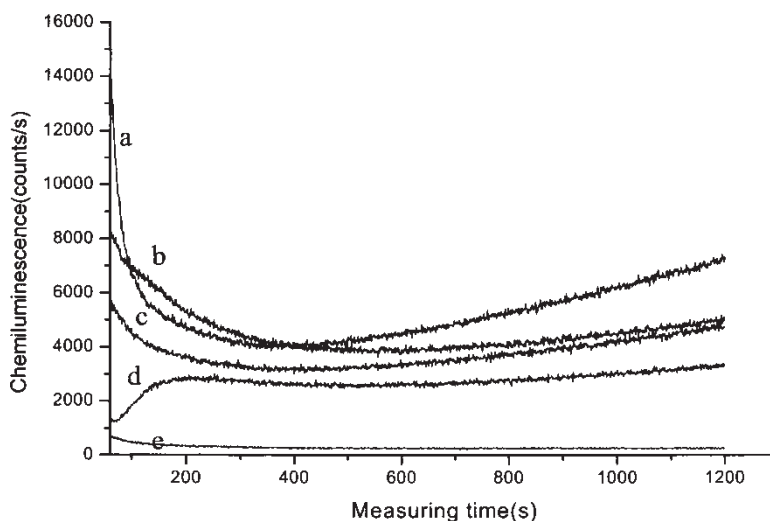
FIGURE 4 The effect of different ROS scavengers on the chemiluminescence of the serum from ARDS rats after OA injection. a, 30 min after OA injection; b, OA + CAT; c, OA + ethanol; d, OA + SOD; e, control group. It showed that ROS of OA + SOD group was the lowest in all experimental groups except the control, which suggested superoxide anion might be the primary species of ROS in the pathogenesis of ARDS.

of the ARDS group ($P < 0.01$), suggesting SOD can effectively protect rats from ARDS.

ESR Spectra of ROS after SOD Pre-treatment

Figure 6 shows that nearly no ROS signals could be seen in the SOD pre-treatment group, which also suggested that superoxide anion might be the primary species of ROS in the pathogenesis of ARDS and SOD could have a reliable scavenging effect.

Lung Wet/dry Ratio, Lung/body Ratio after SOD Pre-treatment

The results given in Table IV show that the lung wet/dry weight and lung/body ratio in the SOD pre-treatment group were all obviously decreased compared to the ARDS group rats, which accorded with the same tendency exhibited in blood-gas analysis.

Histopathologic Observation

Figure 7 shows the histopathologic images of lung sections from the control, ARDS and SOD
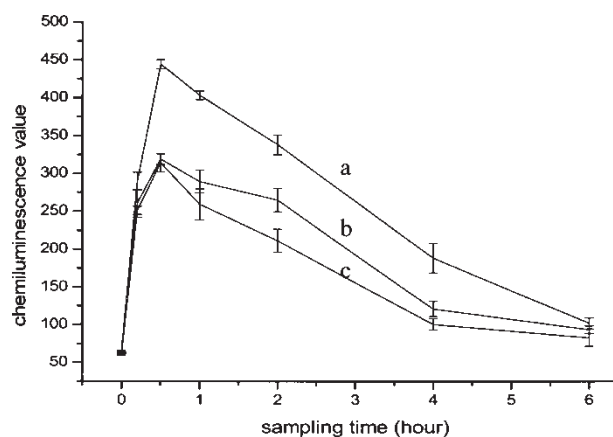

FIGURE 5 The contents of superoxide anion measured by chemical luminescence in serum from a, left ventricle blood; b, pulmonary artery blood; c, femoral vein blood. It showed that left ventricle blood had the highest amount of ROS than other samples.

pre-treatment groups. It could be seen that the lung tissue of SOD pre-treatment group only had a little pulmonary hemorrhage and pulmonary interstitial edema, which was less damaged than that of ARDS group.

TABLE II Amount of superoxide anion measured by chemical luminescence from the serum of ARDS rats after OA injection (a.u., $\bar{x} \pm s$)

Group	n	PAB	LVB	FVB
Control	5	63.54 ± 2.30	64.58 ± 1.57	61.34 ± 2.35
10 min	5	260.44 ± 18.54[**##]	289.86 ± 12.06[**]	250.96 ± 5.28[**##]
30 min	5	319.46 ± 6.99[**##]	444.54 ± 6.16[**]	314.44 ± 11.86[**##]
1 h	5	289.66 ± 15.18[**##ΔΔ]	403.32 ± 6.00[**]	259.40 ± 20.44[**##]
2 h	5	264.88 ± 15.34[**##ΔΔ]	338.34 ± 13.22[**]	211.38 ± 11.33[**##]
4 h	5	120.96 ± 10.18[**##Δ]	188.60 ± 19.53[**]	100.36 ± 7.52[**##]
6 h	5	93.44 ± 5.30[**]	101.82 ± 7.52[**]	82.78 ± 11.42[**##]

[**]$P < 0.01$ vs. control group; [##]$P < 0.01$ vs. LVB in the same time point; [Δ]$P < 0.05$, [ΔΔ]$P < 0.01$, PA vs. FVB. PAB, pulmonary artery blood; LVB, left ventricle blood; FVB, fermoral vein blood.

TABLE III   Results of blood gas analysis in ARDS and SOD pre-treatment rats ($\bar{x} \pm s$)

Blood gas analysis	Control	ARDS (2 h)	SOD
PaO_2 (mmHg)	94.63 ± 5.85	35.08 ± 4.59**	66.80 ± 3.94**#
CO_2 (mmHg)	41.58 ± 2.52	50.25 ± 11.09*	48.76 ± 9.05
PH	7.36 ± 0.04	7.23 ± 0.10**	7.36 ± 0.05#
O_2 saturation (%)	97.16 ± 1.51	55.80 ± 12.76**	91.74 ± 1.53#
PaO_2/FIO_2 (mmHg)	460.76 ± 24.38	167.86 ± 21.94**	319.62 ± 18.84**#

*$P < 0.05$, **$P < 0.01$ vs. control; #$P < 0.01$ vs. ARDS (2 h). Blood samples for analysis in ARDS and SOD pre-treatment group were all taken at the same time point (2 h).
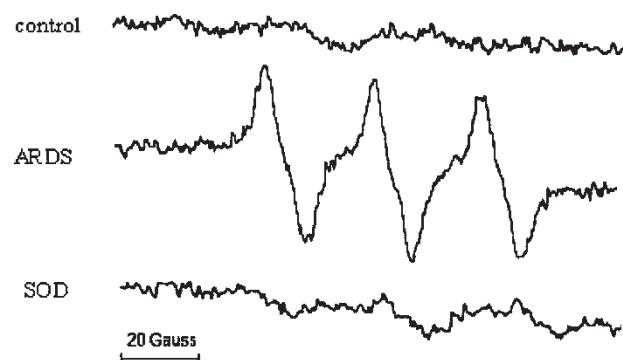
FIGURE 6   The ESR spectra of ROS in lungs of rat groups. It could be seen that nearly no ROS signals were produced in SOD pre-treatment group (samples for ARDS and SOD group were all taken at 30 min after OA injection), which implied that SOD could effectively extinguish the production of superoxide anion.

DISCUSSION

The ARDS is a very common critical condition characterized by pulmonary alveolar epithelial and endothelial injury, acute inflammation and edema, which might lead to acute respiratory failure. There are various risk factors, such as sepsis, shock, multiple trauma and aspiration of irritant gases, etc. There is much evidence of massive oxidative stress in ARDS,[10] and neutrophils from ARDS patients appear to spontaneously release increased amounts of ROS.[11] So it is widely believed that the generation and release of ROS by activated neutrophils might play a very important role in the pathogenesis of ARDS.[12,13] In addition, it appears that the antioxidant system is severely compromised during the course of the disease. Although many progresses have been made in recent years, the pathogenesis still remains unclear, and

the treatment of antioxidants is the absence of focalization.

Our previous study[14,15] showed that the development of ARDS could be inhibited effectively by a preliminary administration of antioxidants, suggesting that the production of ROS might be an early event in the pathogenesis of ARDS.

ROS, such as superoxide anion, hydroxyl radical and hydrogen peroxide, cause cellular injury through lipid peroxidation which can alter both the structure and function of the pulmonary capillary.[16] Because of the high reactivity and short life, ROS has been generally analyzed by measuring the changes in antioxidases such, as SOD, GSHPx or the products of lipid peroxidation such as MDA, but lacking direct measurable data in ARDS studies.[17]

ESR spin trapping technique provides a sensitive, direct and accurate means of monitoring ROS. Spin trap (such as PBN) could trap the reactive short-lived free radicals to form relatively long-lived free radical products, spin adducts (such as PBN-OOH), which can be easily detected by ESR. Therefore, ESR has been considered as one of the most effective techniques for detecting short life-span free radicals.[18] Green et al. used spin trapping to observe the production of oxygen radicals from stimulated neutrophils, which might be the early application of spin-trapping technique for the study on intact cells.[19] We first used this technique to measure ROS directly in animal model of ARDS induced by OA; the species of ROS were further identified by ultra-weak luminescence and chemical luminescence methods, and we also try to use SOD, the special scavenger of superoxide anion, to prevent ARDS in this work.

According to the results mentioned above, it was showed that the level of ROS in lung tissues rose

TABLE IV   Changes of lung wet/dry ratio and lung/body ratio in ARDS and SOD pre-treatment rats ($\bar{x} \pm s$)

Group	n	Lw	Ld	Lw/Ld	Lw/Bw
Control	5	1.3012 ± 0.1325	0.2540 ± 0.0228	5.1387 ± 0.4896	0.5156 ± 0.0553
ARDS	5	3.5212 ± 0.5833**	0.5130 ± 0.0724*	6.8611 ± 0.5510**	1.3616 ± 0.2456**
SOD	5	2.0364 ± 0.3075**+	0.2931 ± 0.0401+	6.9464 ± 0.3376**	0.9142 ± 0.0277**+

*$P < 0.05$, **$P < 0.01$ (vs. control); +$P < 0.01$ (vs. ARDS group). Samples in ARDS and SOD pre-treatment group were all taken at the same time point (2 h). Lw, lung wet weight; Ld, lung dry weight; Lw/Ld, ratio of lung wet weight to lung dry weight; Lw/Bw, ratio of lung weight to body weight.
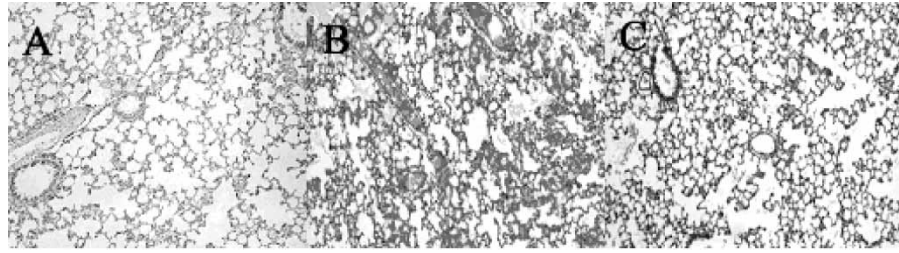
FIGURE 7   Light microscopic photomicrograph of pulmonary histological structure. A: control group. B: ARDS 2h group. Pulmonary hemorrhage, pulmonary interstitial edema, inflammatory cells infiltration, microthrombus formation and epithelium necrosis could be seen. C: SOD pre-treatment group. There was a little pulmonary hemorrhage, pulmonary interstitial edema in the lung after OA injection. H.E. × 100

immediately after injection of OA and reached its peak in 30 min, whereas the blood oxygenic content went down to the lowest point just after 2 h. Analysis of ultra-weak luminescence proved that superoxide anion was the main species of ROS in the development of OA-induced ARDS. Quantified measurement of superoxide anion by chemical luminescence also showed the accordant tendency exhibited in ESR measurement. SOD pre-treatment can effectively extinguish the production of super-oxide anion and inhibit the ill process of ARDS. Our previous study also showed that the desquamation of circulating endothelial cells (CEC) occurred 1 h after OA exposure and the microthrombus occurred 6 h post-exposure to OA in ARDS rats.[14,20] Hence, all these results proved our previous hypothesis that oxidative injury might be the key factor to initiate the ARDS and oxidative injury to pulmonary capillary endothelial cells might be the first step in the pathogenesis of ARDS induced by OA. Superoxide anion was the main species of ROS in the lung of ARDS rats, which may provide a new way for purposeful treatment on ARDS.

All these results might not only improve our understanding of ARDS but also be beneficial to develop new therapeutic methods with correspond-ing antioxidants. We believe that the use of antioxidants in protecting pulmonary capillary endothelial cells from injury of free radicals will be most attractive in the early treatment of ARDS.

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