Effects of different states of oxidative stress on fetal rat alveolar type II epithelial cells *in vitro* and ROS-induced changes in Wnt signaling pathway expression

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Abstract. In the present study, we aimed to investigate the effects of different states of oxidative stress on fetal rat alveolar type II epithelial cells (AEC IIs) cultured *in vitro* as well as reactive oxygen species (ROS)-induced changes in the Wnt signaling pathway expression. Primary AEC II cultures were randomly divided into three oxidative damage groups: a high oxygen fraction (>0.95) group (95% O_2), a low oxygen fraction (0.4) group (40% O_2) and a room air group (21% O_2). Each group was exposed to the different states of oxidative stress for 12, 24 and 48 h. Cell morphological changes were observed using an inverted microscope, the cell survival rate was determined by the MTT assay, and the apoptosis rate was determined using flow cytometry. Wnt5α gene expression was determined using reverse transcriptase-polymerase chain reaction (RT-PCR) and changes in non-phosphorylated β-catenin protein in the cell nucleus were determined using western blot analysis. Compared with the room air group, the survival and apoptosis rates of the low oxygen fraction (0.4) group were not significantly different after 12 and 24 h, while significant differences were observed after 48 h of exposure. However, a significant difference was detected in the high oxygen fraction (>0.95) group at all three time-points. After 12 h, ROS concentration in the high oxygen fraction (>0.95) group was significantly higher compared with the room air group, and continued to rise after 24 h of exposure. Following 12 h of exposure, the expression of the Wnt signaling pathway in the high volume oxygen fraction (>0.95) group was significantly higher, whereas it decreased after 24 h of exposure. The groups exposed to room air showed no significant difference. In conclusion, with an increase in the time of exposure to oxidative stress, oxidative damage was evident after 48 h even with stimulation with low concentrations of oxygen (40%). The Wnt signaling pathway as an early regulatory factor is involved in hyperoxia lung injury, which is prematurely activated by ROS.

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Introduction

Oxygen therapy is an important clinical treatment for hypoxemia (1). However, long exposure to a high concentration of oxygen causes the production of oxygen-free radicals and their derivatives in the lung tissue (2). Once over the body's compensation limit, hyperoxia lung injury can occur (3).

The Wnt signaling pathway is composed of Wnt proteins, receptors and regulatory factors (4). Previous studies have shown that external factors interfere with the expression and normal transduction of this signaling pathway, and thus cause abnormal lung development (5).

An increased number of studies have shown that the repair of lung injury completely depends on the proliferation and differentiation of alveolar type II epithelial cells (AEC IIs). The proliferation and differentiation of AEC IIs not only repairs the normal alveolar structure, but also improves alveolar function. Therefore, AEC IIs are regarded as the stem cells of the alveolae (6).

In the present study, premature rat AEC IIs were exposed *in vitro* to a low oxygen fraction of 0.4 (40% O_2), which is normally used for oxygen therapy in the clinic, and were compared with cells exposed to a high oxygen fraction of >0.95 (95% O_2), and to room air (21% O_2), to investigate the growth and apoptosis of AEC IIs. Moreover, the effect of ROS on the Wnt signaling pathway in the high oxygen fraction (>0.95) (95% O_2) and the room air groups is discussed.

Materials and methods

Reagents. Adult clean-grade Sprague-Dawley rats were purchased from the Experimental Animal Center of Chongqing Medical University (Chongqing, China). Trypsin, DNase I,

Dulbecco's modified Eagle's medium/F12 (DMEM/F12) and fetal calf serum (FCS) were purchased from Gibco (Grand Island, NY, USA). Collagenase I, MTT and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA), while the Annexin V-FITC-labeled apoptosis kit was from Nanjing KeyGen Biotech. Co., Ltd. (Nanjing, China). The DCFH-DA molecular probe was obtained from the Shanghai Beyotime Institute of Biotechnology (Shanghai, China). TRIzol and chloral hydrate were obtained from Tiangen Biotech. Co., Ltd. (Beijing, China). Reverse transcription system kits and PCR kits were purchased from Fermentas (Burlington, ON, Canada), and the BCA protein assay kit was from Pierce Biotechnology, Inc. (Rockford, IL, USA). The study was approved by the ethics committee of Chongqing Medical University.

Methods

Isolation and culture of fetal rat AEC IIs. Fetal rat AEC IIs were isolated at the canalicular stage (19-20 days gestation) by modification of a method previously described (7). Briefly, a pregnant rat at 19-20 days of gestation was anesthetized by intraperitoneal injection of 10% chloral hydrate (1 ml/100 g) and fetal rats were extracted following the onset of adequate anesthesia. Fetal lungs were minced and digested with 0.125% trypsin and 10 mg/ml DNase for 20 min at 37˚C. The trypsin reaction was stopped with DMEM/F12 with 10% FCS, and the cell suspension was centrifuged at 1,500 x g for 5 min. Supernatants were removed and cell pellets were resuspended in collagenase and incubated for 15 min at 37˚C. The collagenase reaction was stopped by adding serum followed by centrifugation, and cell pellets were resuspended and plated into 6-well plates for differential adherence to remove fibroblasts. Purified cells were plated in 6-well plates at a seeding density of $1x10^6$ cells/ml and grown to 70-80% confluence over 20-24 h in DMEM/F12 supplemented with 10% fetal bovine serum (FBS). The purity of AEC II cells was found to be >90% by modified Papanicolaou staining, and the cell survival rate was >85% by trypan blue staining.

Hyperoxia model and experimental groups. AEC IIs were inoculated into 6-well plates, grew to 70-80% confluence, and were then randomly divided into three groups: i) the room air group, where cells were placed into an incubator with 5% CO_2 at 37˚C; the low oxygen fraction (0.4) group, where cells were exposed to 40% O_2 with 5% CO_2 at 37˚C; and the high oxygen fraction (>0.95) group, where cells were exposed to 95% O_2 with 5% CO_2 at 37˚C. The last two groups were placed in two modular chambers, and the chambers were flushed with a gas mixture of 40% O_2/5% CO_2/55% N_2 and 95% O_2/5% CO_2, respectively, until equilibrium. The cells were cultured for 12, 24 and 48 h.

Survival rate assessment using MTT assay. The cells were plated in 96-well plates at a seeding density of $1x10^6$ cells/ml. The room air, the low oxygen fraction (0.4) and the high oxygen fraction (>0.95) groups were each seeded into four wells to be cultured for 12, 24 and 48 h. Following exposure to oxygen, the medium was removed from each well, and 20 μl of 5-mg/ml MTT solution and 180 μl of medium were added to each well. The medium was immediately removed after 4 h of exposure, and 150 μl of DMSO were added to each well and mixed for 10 min. A multi-detection microplate reader was used to detect absorbance at 492 nm (A492). Empty wells were used for blanking; the higher the absorbance value, the more the living cells. The formula used to calculate the cell survival rate was: cell survival rate = experimental well A492/control well A492 x100%.

Apoptosis assessment using flow cytometry. Cell apoptosis and death were detected using Annexin V and propidium iodide (PI) staining with an Annexin V-FITC apoptosis detection kit according to the manufacturer's instructions.

Intracellular ROS assessment using flow cytometry. The DCFH-DA molecular probe was used to detect the level of intracellular ROS. AEC IIs were washed with DMEM and cultured with 10 μM DCFH-DA at 37˚C for 20 min. The cells were then harvested, and the deposit was washed twice with ice-cold phosphate-buffered saline (PBS). The fluorescent signal intensity of DCF was detected using flow cytometry (excitation at 488 nm and emission at 610 nm).

Detection of Wnt5α expression using reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was extracted from AEC IIs using a total RNA isolation system kit (Fermentas) for the detection of Wnt5α mRNA expression by RT-PCR. cDNA was synthesized with the reverse transcription system kit. The following primers were used: Wnt5α (amplicon length, 364 bp), 5'-CCCACTCCCAGGACCCACATA-3' and 5'-CTTTCACCAGGATACCACCCA-3'; β-actin (amplicon length, 293 bp), 5'-ACCCACACTGTGCCCATCTATG-3' and 5'-CATCGGAACCGCTCATTGCCGA-3'. PCR was performed as follows: holding for 3 min at 94˚C followed by amplification of cDNA for 30 cycles with melting for 40 sec at 94˚C, annealing for 40 sec at 60˚C and extension for 60 sec at 72˚C. The relative gene expression was determined using the β-actin gene as an endogenous internal standard with a Bio-Rad Quantity One software (Bio-Rad, Hercules, CA, USA).

Non-phosphorylated β-catenin protein assay using western blot analysis. The cells were washed once with ice-cold PBS at the appropriate time, and lysed in 0.10 ml lysis buffer [20 mM Tris/HCl, pH 7.4, 100 mM NaCl, 10 mM sodium pyrophosphate, 5 mM EDTA, 50 mM NaF, 1 mM sodium vanadate, 0.1% (w/v) SDS, 10% (w/v) glycerol, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate] containing 1 μM leupeptin, 0.1 μM aprotinin, 1 mM phenylmethanesulfonyl fluoride and 1 μM pepstatin]. Protein concentration was calculated using a BCA protein assay kit. Protein (60 μg) was loaded onto a 10% SDS/polyacrylamide gel and electrophoretically transferred to nitrocellulose membranes (Pall Corporation, East Hill, NY, USA), analyzed with antibodies according to the manufacturer's instructions, and visualized with peroxidase and an enhanced chemiluminescence system (ECL kit; Pierce Biotechnology, Inc.).

Statistical analysis. Data were presented as the means ± standard deviation (SD). $P<0.05$ was considered to indicate a statistically significant difference. Repeated measurement data analysis of variance was used to test the significant differences

Table I. Survival and apoptosis rates of AEC IIs in each group at different exposure times.

	Survival rate (%)			Apoptosis rate (%)		
Group	12 h	24 h	48 h	12 h	24 h	48 h
Air	100.00±2.013	87.09±3.851[a]	71.17±1.655[a]	4.35±1.516	5.67±0.301	8.17±1.479
40% O_2	93.87±0.389	87.73±4.505	65.74±1.479[c]	4.46±2.110	12.24±1.256	28.33±6.189[c]
95% O_2	67.64±1.929[b,c]	51.86±6.724[b,c]	37.99±1.215[b,c]	14.76±2.265[b,c]	25.28±8.353[b,c]	28.78±6.681[c]

P<0.05, [a]compared with the room air group at 12 h of exposure; [b]compared with the low oxygen fraction (0.4) group at the same time of exposure; [c]compared with the room air group at the same time of exposure to the different states of oxidative stress.

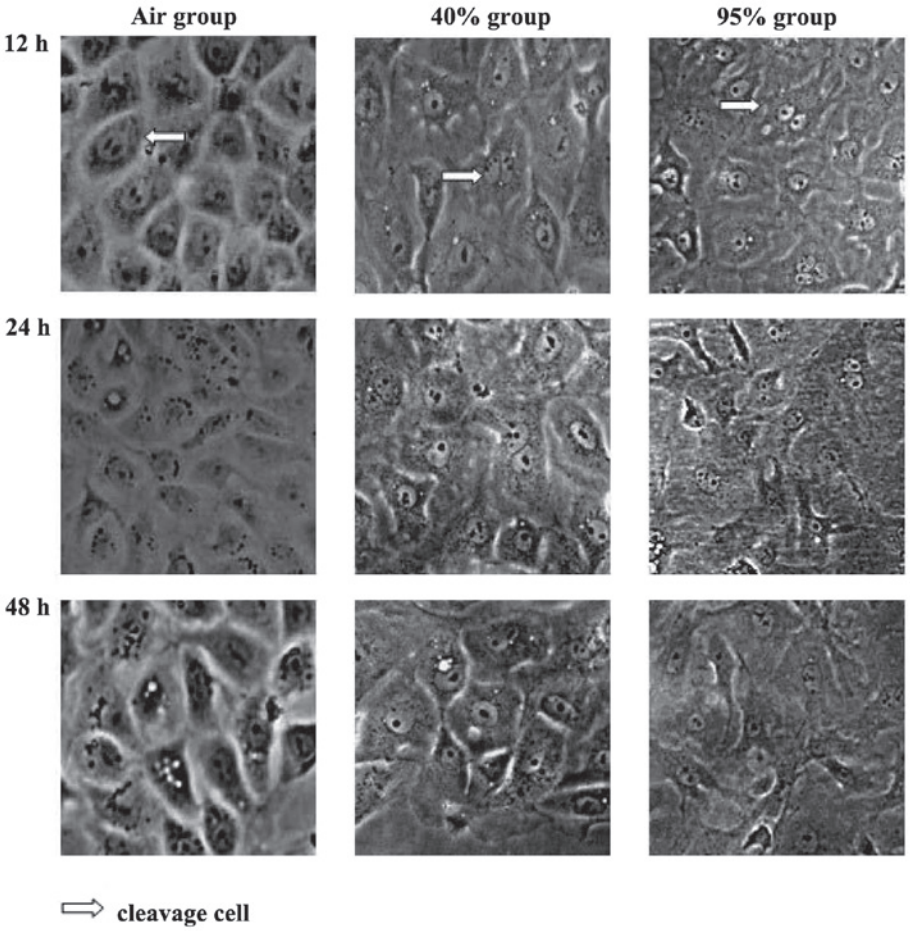

Figure 1. Cell morphology of AEC IIs following exposure to the different states of oxidative stress (magnification, x200).

in measured variables between groups. SPSS 17.0 software was used for all the statistical analyses.

Results

Morphological changes of each group observed using an inverted microscope. AEC IIs in the room air group grew as islands at the early time-points. The nucleus was large and deeply stained. Dividing cells were occasionally observed. As the dutation of exposure increased, the gap between cells increased, the color of the cytoplasm faded, the number of lamellar bodies decreased and vacuoles appeared around the nucleus and cytoplasm.

The above-mentioned structural changes were more significant after 12 and 24 h of exposure in the high oxygen fraction (>0.95) group. Parts of the cells increased or decreased in size and intracellular vacuoles were more obvious, while the number of floating dead cells increased. After 48 h, the majority of adherent cells lost their normal basic structure.

The changes occurred at an intermediate level between the other two groups in the low oxygen fraction (0.4) group over the same period of time (Fig. 1).

Survival and apoptosis rate of each group. Compared with the room air group, the survival rate of the cells in the high oxygen fraction (>0.95) group was significantly lower after 12,

Table II. Reactive oxygen species (ROS) levels in cells in each group after 12 and 24 h of exposure.

	ROS	
Group	12 h	24 h
Air	36.54±2.338	95.57±5.259
95% O_2	321.14±15.976[a]	504.63±30.982[a,b]

P<0.05, [a]compared with the room air group at the same time of exposure; [b]compared with the 95% O_2 group at 12 h of exposure.

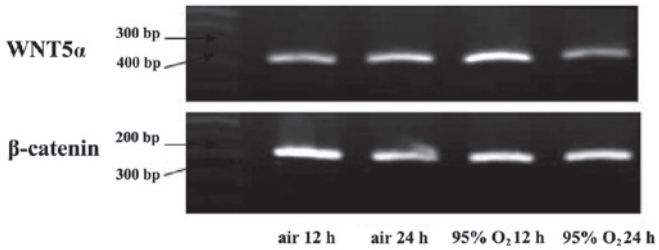

Figure 2. Detection of Wnt5α mRNA expression in each group at various durations of exposure to the different states of oxidative stress using RT-PCR.

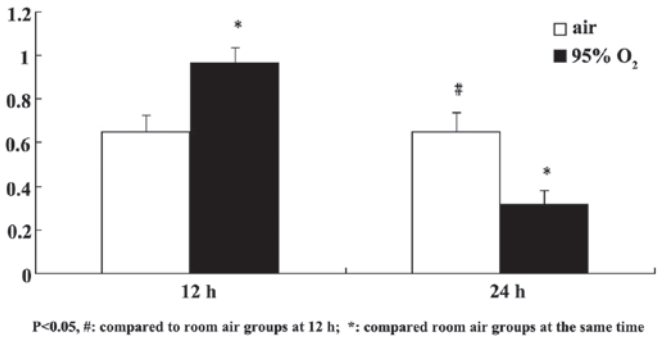

Figure 3. Wnt5α mRNA expression in each group at various durations of exposure to the different states of oxidative stress.

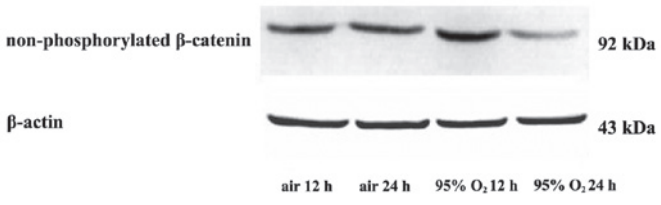

Figure 4. Detection of β-catenin nuclear protein expression in each group at various durations of exposure to the different states of oxidative stress using western blot analysis.

24 and 48 h (P<0.05). Additionally, the rate of apoptosis was significantly higher in the high oxygen fraction (>0.95) group when compared with the room air group after 12, 24 and 48 h of exposure.

Compared with the room air group, the survival rate of the cells in the low oxygen fraction (0.4) group was not significantly different after 12 and 24 h, while it was significantly

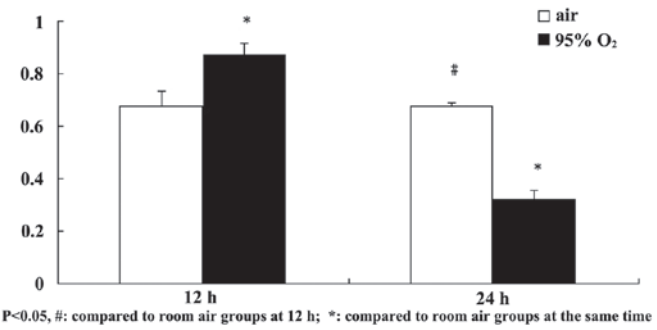

Figure 5. β-catenin nuclear protein expression in each group at different durations of oxygen exposure.

lower after 48 h of exposure (P< 0.05). Similarly, the apoptosis rate of the low oxygen fraction (0.4) group was not significantly different from the room air group at 12 and 24 h, while it was significantly higher after 48 h of exposure (Table I).

Intracellular ROS of each group. After 12 h, the ROS content of the cells in the high oxygen fraction (>0.95) group was significantly higher compared with that of cells in the room air group, which continued to rise after 24 h of exposure (Table II).

Wnt5α mRNA and non-phosphorylated β-catenin protein expression. Wnt5α mRNA and non-phosphorylated β-catenin protein expression reached their maximum and were significantly higher in the cells of the high oxygen fraction (>0.95) group compared with the cells of the room air group after 12 h of exposure. By contrast, after 24 h, the Wnt5α mRNA and non-phosphorylated β-catenin protein expression decreased in the room air and high oxygen fraction (>0.95) groups, and was significantly lower in the high oxygen fraction (>0.95) group compared with the room air group (P<0.05) (Figs. 2-5).

Discussion

Prolonged exposure to oxygen may cause ROS accumulation *in vivo*. A high concentration of ROS may cause irreversible toxicity to DNA and proteins, and speed up the damage and death of the cell (7,8). Previous studies have shown that oxygen toxicity to the lung differs at different oxygen concentrations, patient age and durations of inhalation (9). Oxygen therapy is widely used in acute oxygen deficiency disease (10). However, high oxygen exposure may cause acute damage to the lung (11). Consequently, as long as the blood gas analysis returns to normal, oxygen therapy should be administered for a short time and with a low oxygen concentration (12). There is no clear indication as yet regarding the proper duration of inhalation and oxygen concentration (11,13).

In the present study, 40% O_2, which is a concentration that is commonly used for oxygen therapy in clinical practice, was selected (14). The results showed that the survival and apoptosis rates of the low oxygen fraction (0.4) group were better compared with those of the high oxygen fraction (>0.95) group. However, as the exposure time increased, the degree of oxidative injury to the cells in the low oxygen fraction (0.4) group was also increased. After 48 h, the increase in the apoptosis rate and the decrease in the survival rate of cells in the

low oxygen fraction (0.4) group were statistically significant compared with the room air group. Therefore, as the duration of exposure to oxidative stress increased, oxidative damage also appeared in the cells that were stimulated with a low concentration of oxygen (40%) after 48 h.

Wnt signaling consists of the classical and non-classical pathways. The Wnt/β-catenin signaling pathway is the classical pathway, while the non-classical pathway includes the Wnt/Ca^{2+} and Wnt/planar cell polarity (PCP) pathways. Numerous factors in the Wnt signaling pathway participate in the regulation and control of lung development, and are important in the proliferation and differentiation of cells (15), the formation of lung steric configuration and the development of the distal end of the lung. It has been reported that Wnt/β-catenin plays a critical role in the early stages of lung development (16). β-catenin protein is degraded in the cytoplasm by the accumulation of phosphorylated and non-phosphorylated β-catenin protein in the cytoplasm and its entry into the cell nucleus to activate the late steps of the Wnt pathway. Therefore, detection of the non-phosphorylated β-catenin protein in the cell nucleus provides knowledge regarding the activation status of the Wnt signaling pathway (17). The Wnt5α gene is considered to be involved in the development of embryonic pulmonary parenchyma and lung vessels (18).

As the duration of exposure increased, the ROS levels in the cells of the room air and the high oxygen fraction (>0.95) groups were elevated, and the levels of ROS in the high oxygen fraction (>0.95) group were significantly higher compared with those in the room air group after 12 and 24 h. Wnt5α mRNA and nuclear β-catenin protein expression were higher in the high oxygen fraction (>0.95) group compared with the room air group after 12 h, while they were lower compared with the room air group after 24 h. The present study showed that a higher oxygen fraction stimulates Wnt signaling pathway activation at an early stage in AEC IIs, causing a premature expression of Wnt5α mRNA and accelerated translocation of β-catenin protein into the nucleus. We demonstrated that the Wnt signaling pathway participates as an early factor in the development of hyperoxia-induced lung injury, and that ROS created by AEC IIs after oxidative stress stimulated the premature expression of components of the Wnt pathway.

In most cells (19), the Wnt signaling pathway causes proliferation to some extent, while in our experimental results, prolonged oxidative stress caused an inhibition of Wnt pathway expression. This finding may be due to the fact that the high oxygen fraction caused cell degeneration and death. Some studies have demonstrated that following the treatment of cells with H_2O_2, a lower concentration of ROS at an early stage stimulates the expression of the Wnt signaling pathway. However, the Wnt pathway expression is inhibited with increasing time. These effects may be associated with the intracellular antioxidant system and the interaction of the Wnt signaling pathway itself with ROS. Further studies are needed for the investigation of the underlying mechanism.

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