

Protective effect of SIRT3 on acute lung injury by increasing manganese superoxide dismutase-mediated antioxidation

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Abstract. Prolonged exposure to hyperoxia results in acute lung injury (ALI). Pulmonary damage caused by oxygen toxicity occurs due to the generation of reactive oxygen species and subsequent formation of more potent oxidants. The present study demonstrated that sirtuin 3 (SIRT3) may attenuate hyperoxia-induced ALI due to its potential antioxidative effect. In the present study, a hyperoxia-induced acute lung injury mouse model, reverse transcription-quantitative polymerase chain reaction, western blotting, retroviral mediated gene over-expression and knockdown assays revealed that the expression of SIRT3 in the lung tissue of mice with hyperoxia-induced ALI was decreased and overexpression of SIRT3 may significantly reduce hyperoxia-induced ALI, as reflected by decreases in protein concentration, infiltrated neutrophils in bronchoalveolar lavage (BAL) fluid and wet/dry ratio of lung tissues. Furthermore, overexpression of SIRT3 increased the protein levels and enzymatic activity of manganese superoxide dismutase (MnSOD), and inhibited oxidative stress in the lungs of ALI mice. Additionally, the current study demonstrated that SIRT3 promoted the expression of MnSOD, and this regulation was crucial for the protective effect of SIRT3 on hyperoxia-induced ALI. In summary, the results of the current study indicated that SIRT3 overexpression may effectively ameliorate hyperoxia-induced ALI in mice, which indicates a potential application for SIRT3-based gene therapy to treat clinical adult respiratory distress syndrome.

Introduction

Supplemental oxygen is a common lifesaving strategy used in neonatal intensive care units (1). Oxygen therapy utilizing supraphysiological concentrations of oxygen (hyperoxia) is routinely administered during mechanical ventilation for the

management of severe respiratory distress (2-4). However, oxygen therapy can also cause oxygen toxicity, including acute lung injury (ALI) (5,6). Additionally, exposure to high concentrations of oxygen may induce diffuse pulmonary injuries, vascular leakage, excessive inflammation and pulmonary edema (7,8). Hyperoxia-induced damage to lung tissues is attributed to the generation of reactive oxygen species (ROS) and the subsequent formation of more potent oxidants (9-11). Therefore, excessive levels of ROS and the resultant oxidative damage have an important role during the process of hyperoxia-induced ALI (12,13). As the understanding of the mechanism of hyperoxia-induced ALI is incomplete, effective therapies have not yet been developed.

Sirtuins are protein deacetylases that hydrolyze one oxidized nicotinamide adenine dinucleotide (NAD⁺) for each lysine residue that they deacetylate. Thus, their activity is associated with cellular energy levels (14,15). Sirtuins were initially investigated as mediators of the increased lifespan that is associated with calorie restriction in yeast (16,17); however, recent studies indicate that they are involved in a variety of functions, including genomic stability, tumorigenesis, inflammation and metabolic diseases (18). In mammals, sirtuins are comprised of seven proteins (SIRT1-7), which have different subcellular localizations. SIRT1 and 2 are present in the nucleus and cytoplasm, SIRT3-5 are located in the mitochondria, and SIRT6 and 7 are located in the nucleus (19). The majority of studies have focused on SIRT1 and 2, and the investigation of other SIRTs has been less extensive.

SIRT3 is an important mitochondrial protein. It controls various aspects of mitochondrial function by deacetylating various mitochondrial matrix proteins, including antioxidant effectors and proteins involved in the electron transport chain, therefore acting as a tumor suppressor by limiting the production of ROS. SIRT3 is important for mitochondrial function, by limiting oxidative stress and reducing ROS production, which results in a decrease in mitochondrial membrane potential (20). A previous study demonstrated that SIRT3 enhanced the expression of certain antioxidant proteins, including mitochondrial superoxide dismutase (SOD) (21).

SODs are enzymes that alternately catalyze the dismutation, or partitioning, of the toxic superoxide radical into either ordinary molecular oxygen or hydrogen peroxide. Superoxide is produced as a by-product of oxygen metabolism and causes various types of cell or tissue damage. Thus, SOD is a major antioxidant defense in almost all living cells that are exposed

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to oxygen. The following four isoforms of SOD are present in mammalian cells: Manganese superoxide dismutase (MnSOD), copper-zinc superoxide dismutase (CuZnSOD), extracellular SOD (ecSOD) and glutathione peroxidase 1 (GPx1). Studies using exogenous MnSOD or genetically altered mice over-expressing MnSOD demonstrated that MnSOD inactivates mitochondrial ROS and ameliorates hyperoxia-induced ALI (22). Additionally, CuZnSOD, which is evenly distributed intracellularly, is present in the nucleus and lysosomes. A previous study demonstrated that CuZnSOD was expressed in the alveolar epithelium, mesenchymal cells, fibroblasts and vascular endothelial cells of rat lungs (23). By contrast, ecSOD is primarily located in the extracellular matrix and expressed in the bronchial epithelium, alveolar epithelium and alveolar macrophages (24).

Based on the evidence discussed, we hypothesized that SIRT3 may have pharmacological effects on hyperoxia-induced ALI and that the potential antioxidative mechanism may be caused by regulating the expression of SOD in mice. Therefore, the aim of the current study was to investigate whether SIRT3 was able to inhibit the oxidative damage observed during hyperoxia-induced ALI by increasing the expression of SOD.

Materials and methods

Animals. Eighty adult pathogen-free female C57BL/6 mice (6-8-weeks-old, weight 20 ± 5 g) were provided by the SLRC Laboratory (Shanghai, China). Animals were raised under standard conditions and were provided with water and food *ad libitum*, with a 12 h day/night cycle, and were acclimatized to their environment for at least one week prior to the initiation of the experiments. The study was approved the Ethics Committee of Shengli Oilfield Central Hospital. Animal care and handling were performed in accordance with the National Institutes of Health guidelines.

Retrovirus preparation and infection. Retroviral vectors containing either the SIRT3 gene or SIRT3 small interfering RNA (siRNA) were constructed according to the sequence information from NCBI (NCBI reference sequence: NM_022433.2). RNAi design software (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was utilized for the sequence design. The sequence for the SIRT3 siRNA was: 5'-GCAUAGAUUUAUGACAG-3'. Retrovirus vectors (SIRT3 overexpression and SIRT3 siRNA) were transfected into 293 cells using calcium phosphate precipitation (25). After 24 h, the medium was changed to DMEM containing 10% FBS. The cells were cultured for another 24 h, and then the supernatant containing the lentivirus was harvested.

Animal model of hyperoxia-induced ALI. Mice were randomly divided into the following four groups: Control (n=20; mice subjected to normal air containing 21% O₂; Ctr group); hyperoxia-induced ALI model group (n=20; mice subjected to 90% O₂; Hyper group); vector-carrying retrovirus (vector-RV)-treated ALI group (n=20; mice subjected to 90% O₂ and received lentivirus containing only vector via tail vein injection; Hyper + vector group) and SIRT3-overexpressing retrovirus (SIRT3-RV)-treated ALI group (n=20; mice subjected to 90% O₂ and received lentivirus

containing SIRT3 via tail vein injection; Hyper + SIRT3 group). The mice were treated with vector or SIRT3-RV 3 days after exposure to 90% O₂.

To induce hyperoxia-induced ALI, mice were allowed to roam free under normobaric pressures in chambers under 90% O₂ or normal air containing 21% O₂. O₂ mixtures or normal air were delivered through the chamber at 3 l/min and allowed to vent through a distal ventilation port to maintain normobaric pressures. After 6 days of exposure, mice were terminally anesthetized with ketamine intraperitoneally (80 mg/kg body weight). Subsequently, under sterile conditions, thoraxes were opened, and blood was sampled by cardiac puncture. Simultaneously, three bronchoalveolar lavage (BAL) procedures were performed, each with 1 ml of normal saline. Fluid and blood were centrifuged (2,000 x g, for 10 min at 4°C) and the supernatant and plasma were stored for further processing. Survival of mice following ALI induction and group-specific treatments were assessed, and the cumulative survival curve was produced using the Kaplan-Meier method.

Bronchoalveolar lavage fluid (BALF) collection and determination of cytokine and protein concentration. At the end of the procedure, the right lungs were ligated at the right main bronchus and the BALF was collected from the left lung through a tracheal cannula with 5 ml of sterile PBS. The collected BALF was centrifuged at 300 x g for 10 min at 4°C, and the supernatants were stored at -70°C. The protein concentration in the BALF supernatants was determined by the Bradford method (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with bovine serum albumin (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) used as a reference standard.

BAL cell counts. The trachea was exposed via a midline incision and the lungs were gently lavaged via a tracheal cannula with three aliquots of 1 ml PBS (0.5 M, pH 7.4). The volume of the recovered lavage fluid was recorded, and cell counts were determined using a hemocytometer. Differential counts were performed on cells stained with Wright-Giemsa stain as previously described (26).

Histopathological grading of hyperoxia-induced ALI. Histopathological evaluation was performed on paraffin-embedded tissues as described previously (27). Prior to removal, the lungs were rinsed with PBS and then instilled with 1 ml of buffered formalin through an angiocatheter inserted in the trachea. The lungs were then paraffin embedded, and these paraffin blocks were sliced into 5 µm sections. Five random 5 µm thick paraffin-embedded tissue sections from five different mice lungs taken at day 6 of ALI treatment were stained with hematoxylin and eosin (H&E). The histopathology analysis was performed using a conventional light microscope (Olympus BX51; Olympus Corporation, Tokyo, Japan) and images were captured using a Nikon DXM1200C digital camera (Nikon Corporation, Tokyo, Japan).

To assess the severity of the lung injury, a semi-quantitative histological index was used. Five sections were randomly selected from each group of mice, and five fields were examined per section. The lung histopathological changes were scored on a scale of 0-5 according to the degree of congestion, lung edema, infiltration of inflammatory cells and hemorrhage

in lung tissues (28). A score of 0 indicated no injury in lung tissues, 1 indicated modest injury, 2 indicated intermediate injury, 3 indicated widespread injury and 4 indicated severe injury. The overall score of hyperoxia-induced ALI was generated based on the summation of all scores, and the mean + standard error of the mean (SEM) of the scores were calculated for the lungs of the normal air controls.

Cell culture. A549 cells, a tumor cell line derived from a human lung carcinoma with properties of type II alveolar epithelial cells, and 293 cells were purchased from Cell Resource Center of Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc.) containing 10% heat-inactivated fetal calf serum (Gibco/Invitrogen; Thermo Fisher Scientific, Inc.), and cultured in a 5% CO₂-95% air chamber at 37°C. For the delivery of Ritrovirus into A549 cells, the cells were resuspended with virus solution, and then the plates were centrifuged at 1,500 x g for 120 min at 4°C.

Western blotting. The levels of SIRT3 and MnSOD protein in lung tissue were measured using western blot analysis. The lung tissues of treated and control mice, and differentially treated cells, were homogenized, washed with PBS, incubated in lysis buffer for 30 min at 4°C, and a mixture of protease inhibitors was added (Sigma-Aldrich; Merck Millipore) to obtain extracts of tissue or cell proteins. The protein concentration in the supernatant was determined using the Bradford assay. Briefly, total protein (50 µg) was loaded into each lane. The proteins were transferred onto polyvinylidene fluoride membranes following 10% SDS-PAGE, and the membranes were blocked with 5% non-fat milk for 1 h at room temperature. Subsequently, membranes were incubated with primary antibodies overnight at 4°C, and the membranes were washed, incubated with secondary antibodies for 1 h at room temperature, and visualized by enhanced chemiluminescence reagent (Thermo Fisher Scientific, Inc.). The following antibodies were used: Rabbit anti-mouse SIRT3 polyclonal antibody (cat. no. ab86671; Abcam, Cambridge, UK); rabbit anti-mouse MnSOD polyclonal antibody (cat. no. PA1-125; Thermo Fisher Scientific, Inc.) and rabbit anti-mouse β-actin antibody (cat. no. ab189073; Abcam). Horseradish peroxidase-conjugated goat anti-rabbit IgG (cat. no. ab6721; Abcam) was used as secondary antibody. The dilutions for all antibodies were 1:1,000. ImageJ version 1.46r software (National Institutes of Health, Bethesda, MD, USA) was used for densitometric analysis. The experiment was repeated three times.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA from treated lung tissues and cells were isolated using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA (0.4 µg) was then treated with RNase-free Dnase (1 U/sample, Sigma-Aldrich; Merck Millipore), and cDNA was generated using random hexamer primers provided in the RevertAid First-Strand cDNA synthesis kit (Applied Biosystems; Thermo Fisher Scientific, Inc., Cat no. K1621). The products of RT reaction were diluted and used as templates in subsequent qPCR or stored at -20°C. qPCR analysis was performed using a sequence detection system (7900HT Fast Real-Time PCR

system; Applied Biosystems; Thermo Fisher Scientific, Inc.). Specifically, diluted cDNA sample was amplified using the SYBR Green PCR kit (Invitrogen; Thermo Fisher Scientific, Inc.). Thermal cycling was initiated with an initial denaturation step of 5 min at 95°C, followed by 40 cycles of 95°C for 10 sec and 60°C for 30 sec. The number of replicates per sample was 40 and the 2^{-ΔΔC_q} method was used to analyze the results (29). The following mouse-specific primer pairs were used: β-actin, 5'-GGCCAGGTCATCACTATTG-3' (forward) and 5'-GAGGTCTTTACGGATGTCAAC-3' (reverse); CuZnSOD, 5'-GACAAACCTGAGCCCTAAG-3' (forward) and 5'-CGACCTTGCTCCTTATTG-3' (reverse); MnSOD, 5'-ATGTCTGTGGGAGTCCAA-3' (forward) and 5'-TGAAGGTAGTAAGCGTGCTC-3' (reverse); ecSOD, 5'-ATTTTCAGTCTGGAGGGCT-3' (forward), 5'-CACGAA GTTGCCAAAGTC-3' (reverse); GPx1, 5'-GACTACACC GAGATGAACGAT-3' (forward) and 5'-CACTTCGCACTT CTCAAACA-3' (reverse); SIRT3, 5'-CATCGACGGGCTTGA GAGA-3' (forward) and 5'-GGTCCCGTGGGCTTCAAC-3' (reverse). The Primer Express 3.0 software (<https://www.thermofisher.com/order/catalog/product/4363991>) was used to design the qPCR primers.

Lung wet/dry (W/D) ratio. The mice (40 in total) were anesthetized using sodium pentobarbital (intraperitoneally, 40 mg/kg) and sacrificed via exsanguination 6 days after ALI induction. Right lungs were removed and the wet weights were obtained. Subsequently, the lungs were dried at 80°C and weighed again 3 days after drying. The W/D ratio was calculated to assess tissue edema. The W/D ratio was calculated as follows: (wet weight-dry weight)/dry weight (30).

Measurement of oxidized/reduced glutathione (GSH) ratio. The ratio of reduced GSH and oxidized GSH (GSSG) was determined in lung tissue homogenates from treated ALI mice by reaction with 5,5'-dithiobis-2-nitrobenzoic acid, using the Glutathione Assay kit (Merck Millipore) according to the manufacturer's instructions.

Lipid peroxidation. The lung tissues were immediately flash frozen in liquid nitrogen at time of harvest and stored at -80°C to prevent auto-oxidation. Lipid peroxidation, a well-defined mechanism of cellular damage, was assessed by measuring the level of 8-isoprostane, an indicator of oxidative stress; 8-isoprostane levels were determined using an 8-isoprostane ELISA kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's instructions.

Measurement of tissue SOD activity. The BIOXYTECH® SOD-525 assay kit (OXIS Health Products, Inc., Portland, OR, USA) was used according to the manufacturer's instructions to measure SOD activity. Tissue SOD activity was determined by spectrophotometric detection of formazan production at 550 nm, as a result of inhibition of nitroblue tetrazolium reduction, with xanthine-xanthine oxidase used as a superoxide generator, as described previously (31).

Survival study in mice with hyperoxia-induced ALI. Mice were randomly divided into four groups (n=10 per group) as mentioned above. The survival rates were recorded at the

indicated time points (day 1, 3, 5, 7, 9, 11, 13 and 15 after treatment).

Statistical analysis. Data were analyzed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA) and expressed as the mean \pm SEM. Significant differences were assessed by one-way analysis of variance followed by Fisher's least significant difference test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

SIRT3 expression is reduced in hyperoxia-induced ALI. Previous studies have demonstrated that sirtuins are highly conserved class III NAD⁺-dependent deacetylases that target histone proteins (23,32-35). As a member of the sirtuin family, SIRT3 is reported to target mitochondrial proteins for lysine deacetylation and to regulate cellular functions. Evidence indicates that SIRT3 may regulate the mitochondrial adaptive antioxidant response (36-38). Therefore, the present study induced ALI in mice by exposure to high concentrations of O₂ in order to investigate the function of SIRT3 in hyperoxia-induced ALI. The expression of SIRT3 mRNA and protein in the lung tissues of hyperoxia-induced ALI mice and control mice were then measured by RT-qPCR and western blot analysis. Representative histological sections of lung tissues from control mice (Fig. 1A) and hyperoxia-induced ALI mice (Fig. 1B) are presented. It was observed that the lungs from hyperoxia-induced ALI mice exhibited inflammatory infiltrations, edema and thickening of the alveolar walls, which was not observed in the control mice (Fig. 1A and B). The lung injury index that represents the severity of lung injury was significantly increased in the ALI group compared with the control mice ($P < 0.05$; Fig. 1C). To assess the expression of SIRT3, the mRNA and protein levels of SIRT3 in different mice lung tissues were measured. Compared with the controls, SIRT3 mRNA (Fig. 1D) and protein (Fig. 1E and F) expression in the lungs of hyperoxia-induced ALI mice were significantly decreased ($P < 0.05$). These results indicated that SIRT3 may have an important role in hyperoxia-induced ALI.

Retrovirus treatment enhances SIRT3 expression in lung tissue. To determine the effect of a SIRT3-containing retrovirus on the expression of SIRT3 in the lung tissues, hyperoxia-induced ALI mice were treated with SIRT3-RV and vector-RV (retrovirus containing a blank vector as a control) via tail vein injection 3 days after ALI induction and the expression of SIRT3 protein in the differentially treated lung tissues was measured 3 days after injection by western blot analysis. As presented in Fig. 2, SIRT3 was overexpressed in the lung of SIRT3-RV treated ALI mice (Hyper + SIRT3), compared with the control ALI group (Hyper; $P < 0.05$) and the vector-RV-treated ALI group (Hyper + vector; $P < 0.05$; Fig. 2).

Enhanced expression of SIRT3 protects against hyperoxia-induced ALI in mice. To further investigate the function of SIRT3 in hyperoxia-induced ALI, mice were treated with SIRT3-RV via tail vein injection. The effect of SIRT3-RV on lung histopathology is presented in Fig. 3. Treatment with SIRT3-RV reduced visible lung damage caused by exposure to

a high concentration of O₂, compared with untreated ALI mice and vector-treated ALI mice (Fig. 3A-D). The comparison of lung injury scores between groups was consistent with these findings; the Hyper + SIRT3 group demonstrated a lower score than Hyper and Hyper + vector groups (Fig. 3E). Additionally, compared with Hyper and Hyper + vector groups, treatment with SIRT3-RV significantly reduced the concentration of protein in BALF ($P < 0.05$; Fig. 3F), the wet/dry ratio ($P < 0.05$; Fig. 3G), and the number of infiltrated neutrophils ($P < 0.05$; Fig. 3H) in the lung tissue.

SIRT3 overexpression inhibits oxidation and the level of 8-isoprostane in lung tissues. It is known that oxidative stress and lipid peroxidation are involved in hyperoxia-induced ALI. The ratio of reduced/oxidized GSH (GSH/GSSG) reflects the oxidative status of tissues. To further determine the effect of SIRT3 on the hyperoxia-induced oxidative damage of lung tissues, the GSH/GSSG ratio in treated ALI mice was measured in the current study. It was observed that, compared with the control group, there was significant reduction in the ratio of GSH/GSSG in lung tissues of hyperoxia-induced ALI mice (Hyper; $P < 0.05$; Fig. 4A). Additionally, the current study demonstrated that the overexpression of SIRT3 (Hyper + SIRT3) significantly inhibited the reduction of the GSH/GSSG ratio caused by high O₂ exposure, compared with the untreated ALI group (Hyper) and vector-treated ALI group (Hyper + vector; $P < 0.05$; Fig. 4A). 8-Isoprostane is one of the most reliable biomarkers of lipid peroxidation and oxidative stress. Therefore, the present study also measured the level of 8-isoprostane in lungs and demonstrated that treatment with SIRT3-RV significantly reduced the level of 8-isoprostane 3 days after treatment, compared with the untreated ALI group (Hyper) and vector-treated group (Hyper + vector; $P < 0.05$; Fig. 4B). These results indicated that SIRT3 may have a potential antioxidative effect in hyperoxia-induced ALI.

Overexpression of SIRT3 enhances the total enzyme activity of SOD in ALI mice. Animal and human studies have indicated that acute and chronic lung injury due to hyperoxia may be ameliorated by the administration of antioxidants, such as SOD (39-43). Therefore, the enzyme activity of SOD in the lung tissues of differentially treated ALI mice was determined using a photometric assay that measured the autoxidation of a tetracyclic catechol. The current study demonstrated that SOD enzyme activity was significantly decreased in hyperoxia-induced ALI mice (Hyper) after 6 days of O₂ inhalation, compared with the control group ($P < 0.05$; Fig. 5). Overexpression of SIRT3 in the lungs of hyperoxia-induced ALI mice (Hyper + SIRT3) increased the level of SOD enzymatic activity to a level comparable to the healthy controls (Fig. 5).

SIRT3 enhances the expression of MnSOD but has no effect on the expression of other SODs. Increased production of ROS, including superoxide, hydroxyl radicals and hydrogen peroxide is generally considered essential for enhancing O₂ toxicity (44-47). Hyperoxia-induced injury increases the intracellular production of ROS, which occurs via the mitochondria. Additionally, an increasing number of studies suggest that SIRT3 may regulate the expression of SOD (48-51). Therefore,

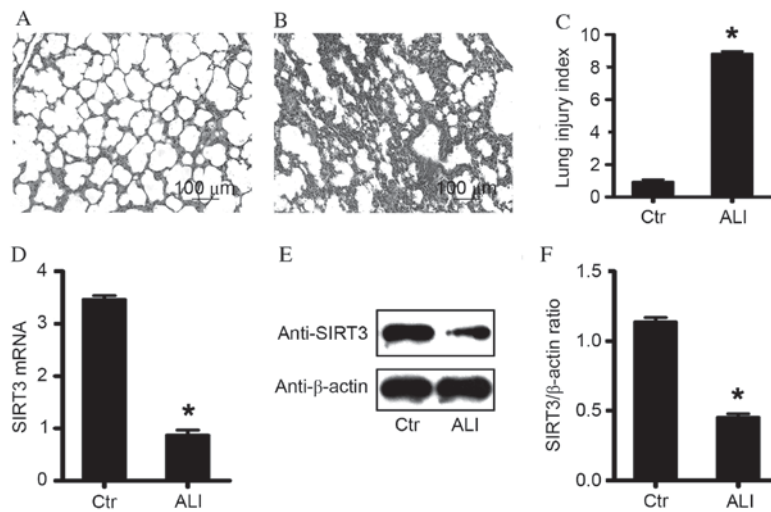


Figure 1. Hyperoxia-induced ALI and expression of SIRT3 in Ctr and ALI mice lung tissues. Hematoxylin and eosin-stained lung sections following 6 days of exposure to (A) normal air and (B) a high concentration of O_2 . (C) The lung injury index of Ctr and hyperoxia-induced ALI mice. (D) Relative level of SIRT3 mRNA in Ctr and hyperoxia-induced ALI mice. (E) Western blot analysis of SIRT3 protein levels. (F) Quantified western blot analysis of SIRT3 protein levels. Data are presented as the mean \pm standard error of the mean. * $P < 0.05$ vs. Ctr. Ctr, control; ALI, acute lung injury; SIRT3, sirtuin 3.

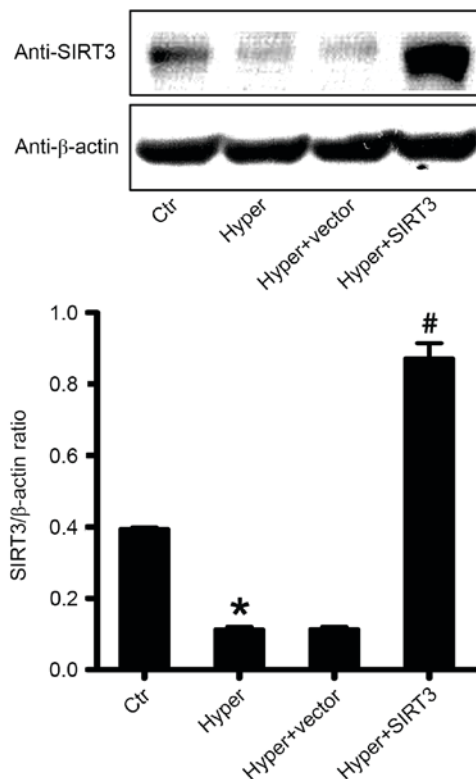


Figure 2. The expression of SIRT3 protein in lung tissues of ALI mice transduced with an RV containing the SIRT3 gene (SIRT3-RV) or vector (vector-RV), and control ALI mice. * $P < 0.05$ vs. Ctr; # $P < 0.05$ vs. hyperoxia-induced ALI group (Hyper) or vector-treated group (Hyper + vector). Data are presented as the mean \pm standard error of the mean. ALI, acute lung injury; RV, retrovirus; SIRT3, sirtuin 3; Ctr, control; Hyper, hyperoxia.

we hypothesized that SIRT3 may reduce hyperoxia-induced ALI by increasing the expression of SODs *in vivo*, as these enzymes scavenge ROS. The expression of MnSOD, CuZnSOD, ecSOD and GPx1 was measured by RT-qPCR in the current study. As presented in Fig. 6, compared with the untreated ALI group (Hyper) and vector-treated ALI group

(Hyper + vector), SIRT3 overexpression (Hyper + SIRT3) significantly increased the expression of MnSOD ($P < 0.05$; Fig. 6A), while the expression of the other SODs investigated, CuZnSOD (Fig. 6B), ecSOD (Fig. 6C) and GPx1 (Fig. 6D), was unchanged. These results indicated that SIRT3 overexpression may increase the expression of MnSOD in the lung tissue of hyperoxia-induced ALI mice and inhibit hyperoxia-induced ALI through the antioxidative effect of MnSOD *in vivo*.

Western blot analysis of MnSOD in mouse lung tissue. To further confirm the effect of SIRT3 on the expression of the MnSOD protein, the current study detected the protein levels of MnSOD in lung tissues using the western blot analysis. As presented in Fig. 7, MnSOD protein levels in the lungs of hyperoxia-induced ALI mice (Hyper) was significantly increased compared with control mice. Treatment with SIRT3-RV increased MnSOD protein levels induced by O_2 exposure compared with the untreated ALI mice (Hyper) and the vector-treated ALI mice (Hyper + vector; $P < 0.05$; Fig. 7).

SIRT3 enhances the expression of MnSOD *in vitro*. To further investigate the role SIRT3 may have in hyperoxia-induced ALI injury, the current study increased or decreased the expression of SIRT3 in human A549 cells using a retrovirus overexpressing SIRT3 or SIRT3 siRNA, respectively, followed by western blot analysis performed 24 h after cell transfection. As presented in Fig. 8A, compared with the control, overexpression of SIRT3 significantly increased the expression of MnSOD *in vitro* ($P < 0.05$). The present study also demonstrated that the activity of SODs was significantly increased by overexpression of SIRT3, compared with the control ($P < 0.05$; Fig. 8B). These results are consistent with the observations from the *in vivo* studies.

SIRT3 overexpression improves survival following hyperoxia-induced ALI. To evaluate the long-term beneficial effect of SIRT3 on hyperoxia-induced ALI, the survival rate was compared between treated (Hyper + SIRT3) and control

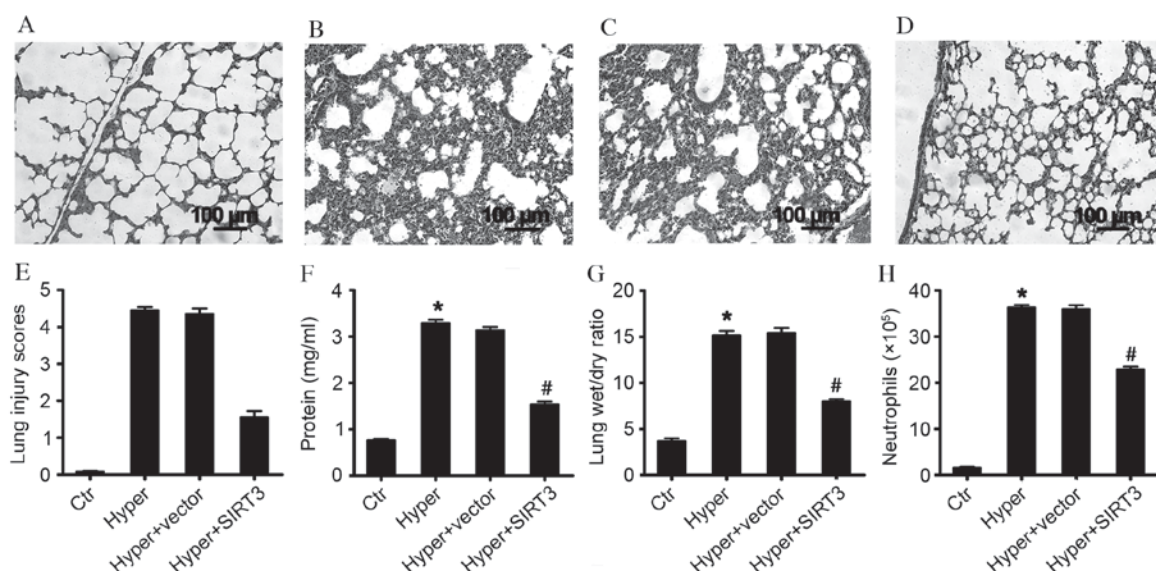


Figure 3. SIRT3 overexpression inhibits hyperoxia-induced ALI. Hematoxylin and eosin-stained lung sections following the different treatments in (A) normal air exposed controls, (B) high concentration O₂ exposed group, (C) vector-treated group and (D) SIRT3 overexpression group. (E) The lung injury scores of differentially treated mice. (F) Protein concentration in BALF from differentially treated mice. (G) The wet/dry ratio of lung tissues from differentially treated mice. (H) Number of infiltrated neutrophils in BALF from differentially treated mice. *P<0.05 vs. Ctr; #P<0.05 vs. hyperoxia-induced ALI group (Hyper) or vector-treated group (Hyper + vector). Data are presented as the mean ± standard error of the mean. ALI, acute lung injury; BALF, bronchoalveolar lavage fluid; Ctr, control; Hyper, hyperoxia; SIRT3, sirtuin 3.

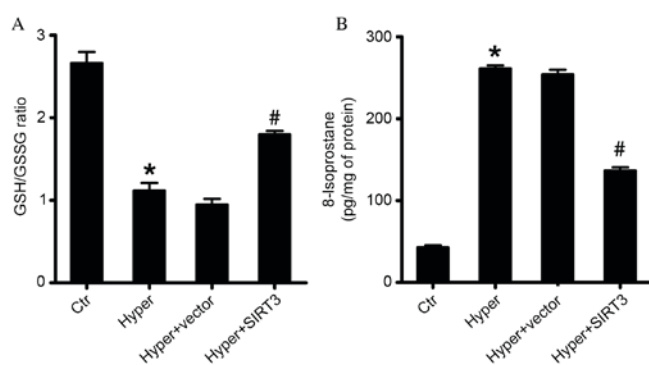


Figure 4. Ratio of GSH/GSSG and 8-isoprostane protein levels in lung tissues of mice from different treatment groups. (A) Ratios of GSH/GSSG in BALF of mice from different treatment groups. (B) 8-Isoprostane concentration in BALF of mice from different treatment groups. *P<0.05 vs. Ctr; #P<0.05 vs. hyperoxia-induced acute lung injury group (Hyper) or vector-treated group (Hyper + vector). Data are presented as the mean ± standard error of the mean. BALF, bronchoalveolar lavage fluid; GSH, reduced glutathione; GSSG, oxidized glutathione; Ctr, control; Hyper, hyperoxia; SIRT3, sirtuin 3.

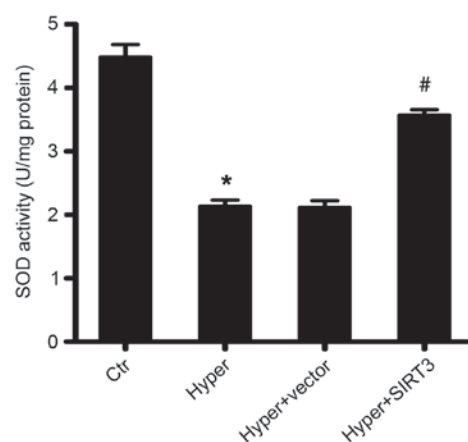


Figure 5. Total SOD enzyme activity in the lung tissues of differentially treated mice. *P<0.05 vs. Ctr; #P<0.05 vs. hyperoxia-induced acute lung injury group (Hyper) or vector-treated group (Hyper + vector). Data are presented as the mean ± standard error of the mean. SOD, superoxide dismutase; Ctr, control; Hyper, hyperoxia; SIRT3, sirtuin 3.

ALI mice (Hyper). The current study demonstrated that the survival rate was significantly improved in SIRT3-RV treated ALI mice compared with the untreated ALI mice (P<0.05) and vector-treated ALI mice (P<0.05; Fig. 9).

Discussion

Hyperoxia is a state of excess oxygen in tissues and organs. Oxygen toxicity occurs when the partial pressure of alveolar oxygen exceeds that which occurs when breathing normal air. With continuous exposure to supraphysiological concentrations of oxygen, a state of hyperoxia develops. Several studies have demonstrated that exposure of lung tissue to high concentration of oxygen causes oxidative stress and lung damage (5,52-54).

Lung oxidative stress results in an oxidant-antioxidant imbalance that can lead to various lung diseases, including adult respiratory distress syndrome. It is well established that oxidant production within the lung can lead to ALI (55-58). The present study demonstrated that SIRT3 overexpression reduced hyperoxia-induced ALI by increasing the expression of MnSOD and inhibiting the oxidative damage caused by hyperoxia induction.

Sirtuins are a family of highly conserved NAD⁺-dependent deacetylases that act as cellular sensors to detect energy availability and modulate metabolic processes. One sirtuin, SIRT3, is central to the control of metabolic processes, and is localized to the mitochondria, where the most damaging oxidants are generated (33,59). Therefore, we hypothesized that SIRT3

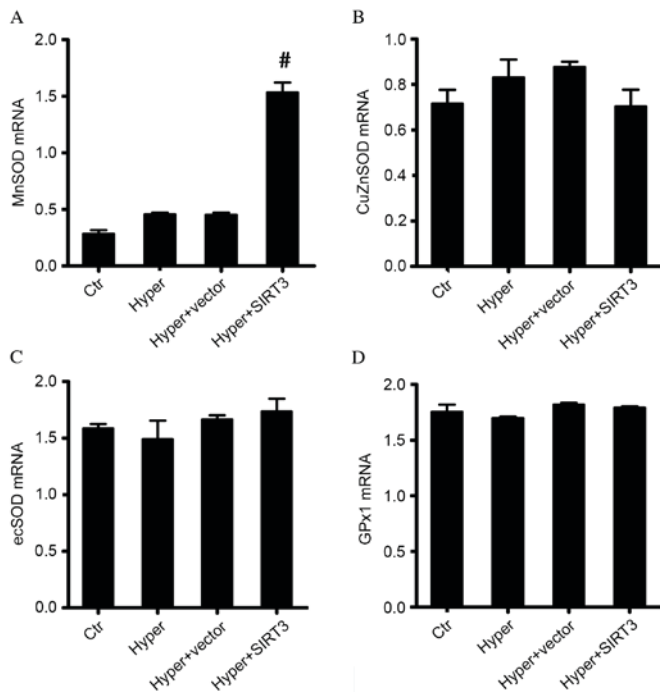


Figure 6. mRNA expression of SOD enzymes in the lung tissues of differentially treated mice. mRNA levels of (A) MnSOD, (B) CuZnSOD, (C) ecSOD and (D) GPx1. $^{\#}P < 0.05$ vs. hyperoxia-induced acute lung injury group (Hyper) or vector-treated group (Hyper + vector). Data are presented as the mean \pm standard error of the mean. SOD, superoxide dismutase; MnSOD, manganese SOD; Ctr, control; Hyper, hyperoxia; SIRT3, sirtuin 3; CuZnSOD, copper-zinc SOD; ecSOD, extracellular SOD; GPx1, glutathione peroxidase 1.

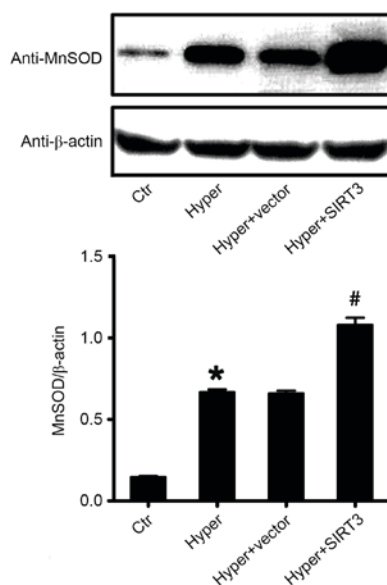


Figure 7. Protein levels of MnSOD in lung tissues of treated mice. MnSOD protein levels in each different treatment group. $^{\ast}P < 0.05$ vs. Ctr; $^{\#}P < 0.05$ vs. hyperoxia-induced acute lung injury group (Hyper) or vector-treated group (Hyper + vector). Data are presented as the mean \pm standard error of the mean. MnSOD, manganese superoxide dismutase; Ctr, control; Hyper, hyperoxia; SIRT3, sirtuin 3.

may have an important role in hyperoxia-induced ALI. The results of this study demonstrated that lung injury was induced by exposure to a high concentration of O_2 , and reduced SIRT3

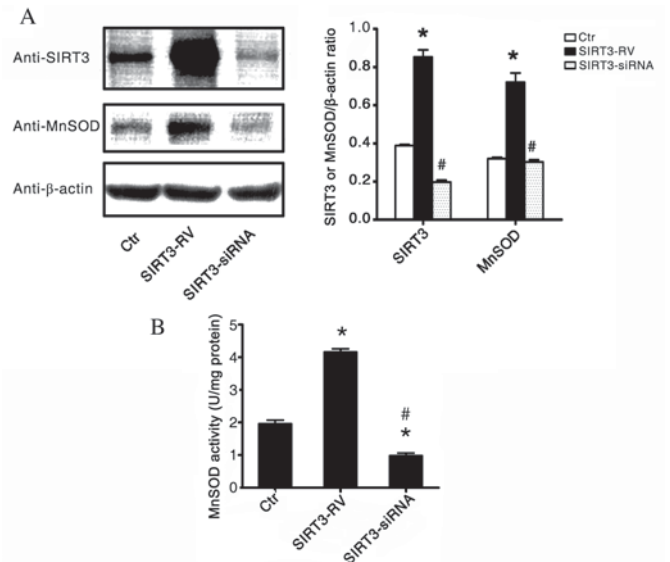


Figure 8. Effect of SIRT3 on the protein expression of MnSOD *in vitro*. (A) The protein expression of SIRT3 and MnSOD in differentially treated human A549 cells. (B) The activity of MnSOD in differentially treated human A549 cells. $^{\ast}P < 0.05$ vs. Ctr; $^{\#}P < 0.05$ vs. SIRT3-RV. Data are presented as the mean \pm standard error of the mean. SIRT3, sirtuin 3; MnSOD, manganese superoxide dismutase; Ctr, control; RV, retrovirus; siRNA, small interfering RNA.

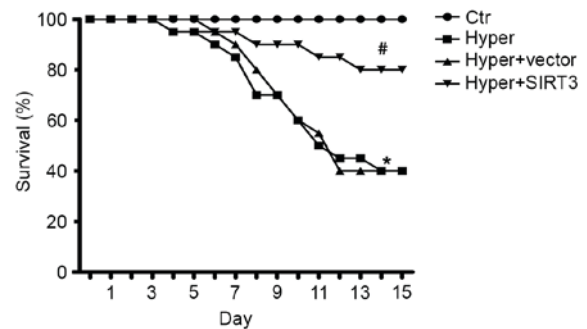


Figure 9. SIRT3 overexpression prolonged the survival of mice with hyperoxia-induced ALI. The Kaplan-Meier survival curves of mice ($n=10$) with the indicated treatments were monitored. $^{\ast}P < 0.05$ vs. Ctr; $^{\#}P < 0.05$ vs. hyperoxia-induced acute lung injury group (Hyper) or vector-treated group (Hyper + vector). Ctr, control; Hyper, hyperoxia; RV, retrovirus; SIRT3, sirtuin 3.

levels was dependent upon hyperoxic exposure (Fig. 1). The data indicated that SIRT3 may have an important role in the process of hyperoxia-induced ALI.

To further investigate the effect of SIRT3 on hyperoxia-induced ALI, the current study treated ALI mice with a retroviral vector containing a SIRT3 gene (SIRT3-RV). The present study demonstrated that SIRT3 overexpression had a beneficial effect on hyperoxia-induced ALI (Fig. 3), including effects on the severity of lung damage (Fig. 3C), protein concentration in BALF (Fig. 3D), the lung wet/dry ratio (Fig. 3E) and the number of infiltrating neutrophils (Fig. 3F). Furthermore, the current study also demonstrated that SIRT3 overexpression increased the total enzyme activity of SODs (Fig. 5). These observations support the model that SIRT3 has a therapeutic effect on hyperoxia-induced ALI due to its antioxidative effect. Previous studies have reported

that SIRT3 deacetylates MnSOD, thereby increasing MnSOD activity (49,60). To explore the mechanism of the antioxidative effect of SIRT3, the mRNA expression of certain antioxidant enzymes that scavenge ROS, including MnSOD, CuZnSOD, ecSOD and GPx1 in the lung tissues of differentially treated mice were measured in the present study. The results demonstrated that SIRT3 overexpression increased the expression of MnSOD (Fig. 6A); however, it did not alter the mRNA levels of the other antioxidant enzymes, CuZnSOD (Fig. 6B), ecSOD (Fig. 6C) and GPx1 (Fig. 6D). These results were consistent with a previous report (61). To confirm the effect of SIRT3 on MnSOD, this effect was investigated *in vitro*. The current study inhibited or overexpressed SIRT3 in human A549 cells, and then detected the expression of SIRT3 and MnSOD proteins by western blot analysis. As presented in Fig. 8, the results demonstrated that SIRT3 significantly increased the expression of the MnSOD protein *in vitro*, compared with the control group. These results support the hypothesis that SIRT3 inhibits hyperoxia-induced ALI by increasing the expression of MnSOD, and thus inhibiting the oxidative damage caused by high concentration O₂ exposure.

In conclusion, the results of the current study demonstrated that SIRT3 inhibited hyperoxia-induced ALI. As a mitochondrial protein, SIRT3 enhanced the expression of MnSOD and reduced the oxidative injury caused by hyperoxic exposure. SIRT3 may be useful as a target for the treatment of hyperoxia-induced ALI due to its potentially antioxidative effect.

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