CoQ10 Enhances the Efficacy of Airway Basal Stem Cell Transplantation on Bleomycin-induced Idiopathic Pulmonary Fibrosis in Mice

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Abstract

Background: Recent studies have demonstrated that airway basal stem cells (BCs) transplantation can ameliorate bleomycin-induced idiopathic pulmonary fibrosis (IPF) through lung regeneration promotion. However, BCs under oxidative stress in the alveolar microenvironment are poor in survival, causing unsatisfied efficacy of BCs transplantation. In this study, we investigated whether Coenzyme Q10(CoQ10) counteracts oxidative stress in the alveolar microenvironment, thus improved the efficacy of BCs transplantation for IPF treatment.

Methods: The protective effects of CoQ10 on H₂O₂-induced BCs apoptosis and cytoplasmic reactive oxygen species (ROS) level were tested by flow cytometry *in vitro*. The therapeutic effects of BCs combined with CoQ10 were compared to a single BCs transplantation protocol in IPF treatment after two weeks and were evaluated by parameters including changes of body weight and survival rate, as well as various levels of pulmonary inflammation, α -SMA expression and hydroxyproline (HYP) in IPF mice lung tissues.

Results: CoQ 10 preincubation with BCs (10 mM, 24 h) significantly reduced the late apoptosis of BCs and the number of oxidative stressful BCs as a result of H₂O₂ stimulation (1mM, 6h) *in vitro*. IPF mice models were constructed through bleomycin (5 mg/Kg) intratracheal instillation. Bleomycin-induced IPF mice showed weight loss continuously and mortality increased progressively during modeling. Serious pulmonary inflammatory cell infiltration, collagen fiber proliferation, and collagen protein deposition were observed in lung tissues of IPF mice. Though BCs transplantation alone improved indicators above in bleomycin-induced IPF mice to some extent, the combination with CoQ10 improved the transplantation efficacy and obtained better therapeutic effects.

Conclusion CoQ10 blocked H₂O₂-induced apoptosis of BCs and ROS production *in vitro*, and enhanced the efficacy of BCs transplantation on bleomycin-induced IPF in mice.

Introduction

Idiopathic pulmonary fibrosis (IPF), regarded as a "tumor-like" disease, is caused by lung epithelial cells injury and repair disorder. IPF is more common in the elderly, with a median survival time of nearly 3 years. With the global aging population, the incidence of IPF is increasing yearly. At present, only Pirfenidone and Nintedanib are recommended as first-line therapeutic drugs in IPF treatment. Though they can postpone the IPF disease progress, they cannot realize the radical cure of IPF [1–4]. Recent researches suggest that stem cell transplantation may provide a potential treatment [5–7].

Stem cells hold differentiatonal potentials and can be differentiated into alveolar epithelial cells to repair the damage and fundamentally cure IPF. Therefore, stem cell transplantation, [8] such as mesenchymal stem cells (MSCs) [9–14] and induced pluripotent stem cells (iPSCs) [15–19], were widely used for allotransplantation to repair injury of IPF. Studies have shown that transplanting bone marrow-derived MSCs significantly ameliorated lung injury and pulmonary fibrosis in bleomycin-induced IPF animal

models [10] and the safety of transplanting human MSCs in patients with IPF has also been verified in clinical trials [11]. Studies have shown that MSCs have the homing function. After lung injury, bone marrow-derived MSCs migrate and home to the lungs, decrease lung inflammation and collagen deposition in the bleomycin-induced pulmonary fibrosis animal [9, 12]. Many viewpoints also demonstrated that MSCs play an anti-fibrosis role through paracrine and immune regulation [13–14]. Although injection of MSCs can weaken the lung fiber and lung injury induced by bleomycin, most cell transplantation researches carried out in the early stages of inflammation instead of fibrosis in the late stage. Moreover, the main questions come from the tumorigenicity and multi-directional differentiation potential of MSCs, which means MSCs may not differentiate into type II alveolar epithelial cells, radically curing cure pulmonary fibrosis. iPSCs have been reported to differentiate into alveolar epithelial cells *in vitro* [15–19], however, the tumorigenesis and the ability of iPSCs to differentiate into alveolar epithelial cells *in vivo* have yet to be verified.

Airway basal stem cells (BCs) are a kind of adult stem cells located in the basal layer of the airway and can self-proliferate and renew. Its specific makers include KRT5 and P63[22]. Several studies have demonstrated that airway basal stem cells have potent regenerative capacity after lung injury [23, 24], and BCs transplantation can ameliorate pulmonary fibrosis [25, 26]. However, cell survival is affected by the microenvironment during transplantation; the efficacy of cell transplantation is a standing challenge. Coenzyme Q10 (CoQ10) or ubiquinone (2,3-dimethoxy-5-methyl-6-polyprenyl-1,4-benzoquinone) is a lipophilic molecule found in the phospholipid bilayer of cellular membranes and is concentrated in the mitochondrial inner membrane. As a mitochondrial electron transport chain component, CoQ10 can make mitochondrial mass, improve mitochondrial function, and inhibit ROS generation [27, 28, 29, 30]. It has been shown that CoQ10 supplementation can reduce DNA double strand damage and increase the life cycle of peripheral blood monocytes [31]; CoQ10 could inhibit D-gal-induced cell aging [32]; CoQ10 protects neural stem cells against hypoxia by enhancing survival signals [33].

At present, no literature report combining with CoQ10 can improve the survival efficiency of transplanted stem cells. The purpose of this study was to investigate the effect of BCs transplantation combining with CoQ10 on bleomycin induced pulmonary fibrosis mice.

Methods

Chemicals and reagents.

Bleomycin was purchased from Hanhui Pharma Co., Ltd (Hangzhou, China). 4% paraformaldehyde was purchased from Wuhan Saiweier Biological Technology Co., Ltd (Wuhan, China). Hydroxyproline assay kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). CoQ10 (purity > 98%, using HPLC) was purchased from Sigma-Aldrich (St. Louis, MO, USA), dissolved in DMSO (St. Louis, MO, USA), and diluted in the culture medium. The final concentration of the DMSO was 0.1%. Annexin V-FITC apoptosis detection kit and total ROS/superoxide detection kit were purchased from Dalian Meilun Biotechnology Co., Ltd (Dalian, China).

Cell culture.

BCs were obtained from the First Affiliated Hospital of Guangzhou Medical University (Guangzhou, China) and maintained with primary lung cells culture medium at 37°C with 5% CO₂ in a humidified atmosphere. The culture medium was changed every 3–4 days. Cells were passaged when 80% confluency and the second to the eighth generation were used for the experiment. MSCs were obtained from Southern Medical University (Guangzhou, China).

Cells groupings and treatments.

Groupings: (1) blank group: human BCs were cultured in medium without CoQ10 and H₂O₂ before measurement, and the other steps were the same; (2) H₂O₂ group: BCs were cultured with 1 mM H₂O₂ for 6 h before measurement; (3) CoQ10 + H₂O₂ group: BCs were incubated with 10 mM CoQ10 for 24 h, and then changed to medium with 1 mM H₂O₂ for 6 h before measurement.

Determination of intracellular ROS levels.

ROS assay kit was used to detect the level of reactive oxygen species in human BCs of each group. Cells were operated according to the instructions of the kit and inspected by flow cytometry (BD Biosciences, NJ) or Nikon microscope (Tokyo, Japan).

Determination of cells apoptosis.

Apoptosis of BCs was detected by flow cytometry. After mixing, PI (10 µL) and annexin V-FITC (5 µL) were added into cell suspension. Incubated in the photophobic environment for 15 min and resuspended every 3 min. Apoptosis detections were conducted within 1 h.

Animals.

Fifty male C57/B6 mice (weighing from 20 to 30 g) were purchased from Laboratory Animal Center of Sun Yat-sen University (SYXK 2016 – 0112). The animals were acclimated to the laboratory for at least 7 days before experiments. The animal care and use complied with the Provisions and General Recommendation of the Chinese Experimental Animals Administration Legislation. Animal experiment protocol was approved by Laboratory Animal Center of Sun Yat-sen University.

Animals' groupings and treatments.

Mice were randomly divided into five groups (n = 10): blank group, model group, BCs group, MSCs group, and CoQ10 + BCs group. On day 0, bleomycin (5 mg/kg) was infused into the trachea to induce pulmonary injury and fibrosis. On day 7, 1×10⁶ cells (BCs or MSCs) were resuspended with 50 µL PBS and then transplanted into lung tissue of anesthetized mice through endotracheal intubation. The cells of CoQ10 + BCs group were incubated with 10 mM CoQ10 for 24 h before transplantation and resuspended

into 50 μ L PBS containing 10 mM CoQ10, and the other steps were the same. The body weight of mice was recorded on day 7, 14 and 21 respectively. Lung tissues were extracted on day 21.

Histological examination of lung tissue.

The lungs were perfused with 4% paraformaldehyde overnight at 4 °C and performed standard paraffin embedding protocols containing dehydrated with gradient ethanol and embedded with wax. Slicer (Leica, Germany) was used to cut the lung tissues into slices with a thickness of 5–7 μ m. The slices were placed on a slide coated by poly-lysine and then stored at room temperature until further use. H&E staining and Masson staining were performed according to the standard protocols. The degree of pulmonary fibers was evaluated through Ashcroft scoring standard. The blue part of Masson staining was quantified by Image J (collagen fibers).

Measurement of lung hydroxyproline.

To evaluate the extent of pulmonary fibrosis, the total collagen contents were measured using a hydroxyproline assay kit according to the manufacturer's instructions.

Immunofluorescence staining of pulmonary α -SMA.

The sections were reconstituted in citric acid buffer (Boster Biological Technology Co., Ltd, California, USA) at 120°C. The nonspecific antigens were blocked with 1% BSA for 20 min. Sections were incubated with primary antibody of α -SMA (dilution 1:200, SAB) overnight at 4 °C, and then washed in PBS 3 times. Then sections were incubated with the secondary antibody of donkey anti-rabbit IgG (H + L) (dilution 1:400, Invitrogen) at room temperature for 1 h. Sections were washed in PBS 3 times and then incubated with DAPI (Beijing Solarbio Co., Ltd., Beijing, China) for 5 min, followed by washed in PBS 5 times. Slides were stored in a dark place at 4°C, and staining of pulmonary α -SMA was recorded with a fluorescence microscope.

Statistical analysis.

The data were analyzed by Graphpad Prism 8.0 software (La Jolla, CA, USA). The survival curve was drawn using the Kaplan Meier method. All data were expressed as mean $\pm$ standard deviation (SD). T-test was used for analysis between two independent sample groups and single-factor analysis was used for comparison between multiple groups of data. $P < 0.05$ was considered statistically significant.

Results

Identification of BCs.

Previous studies demonstrated that KRT5 and P63 were two characteristic markers of BCs. In the present study, expressions of KRT5 and P63 in BCs were stained by immunofluorescence staining (Fig. 1). Results showed that KRT5 was mainly expressed in the cell membrane and cytoplasm, while P63 was

mainly expressed in the nucleus of BCs. Most cells have KRT5 and P63 double-positive staining, indicating that the BCS cells cultured in the present study had stable and consistent phenotypes. 3–6 passages of BCs were used in subsequent experiments.

Incubation with CoQ10 reduces H₂O₂-induced production of ROS in BCs.

The stained BCs were photographed by a fluorescence microscope. Results showed that the fluorescence intensity of ROS was significantly increased after incubation with 1 mM H₂O₂ for 6 h, which was remarkably reduced by CoQ10 pretreatment (Fig. 2A and 2B). Moreover, intracellular ROS production in BCs after CoQ10 and H₂O₂ pretreatment was recorded by flow cytometry (Fig. 2C). Results showed that the number of ROS-positive cells in the H₂O₂ group increased significantly than that in the normal control group, which was remarkably blocked by pretreatment with CoQ10 (Fig. 2D).

Incubation with CoQ10 reduces H₂O₂-induced apoptosis of BCs

H₂O₂-induced apoptosis and necrosis of BCs were measured by flow cytometry (Fig. 2E and 2F). The proportion of BCs undergoing apoptosis and necrosis in the H₂O₂ group increased to 24.35%, as compared with the proportion of apoptotic and necrotic BCs was 11.45% in the normal control group. Moreover, the proportion of BCs undergoing apoptosis and necrosis in the CoQ10 + H₂O₂ group was decreased significantly as compared to that in the H₂O₂ group, indicating that CoQ10 pretreatment reduced H₂O₂-induced apoptosis of BCs.

Transplanting BCs combined with CoQ10 alleviated the symptoms of bleomycin-induced IPF in C57/B6 mice.

After mice were infused with bleomycin (5 mg/kg, in 50 μ L PBS) into the trachea to induce pulmonary injury and fibrosis, cells (BCs or MSCs) were transplanted into lung tissue of anesthetized mice through endotracheal intubation on day 7. The changes in bodyweight of mice during the modeling period were recorded (Fig. 3A). Results showed that bodyweight of mice in all of bleomycin treatment groups, including the model group and three groups with cell transplantation, was decreased weekly, while in the normal control group, was increased during the modeling period. Among them, the mortality of mice in the CoQ10 + BCs group (20%) was lower than that in the BCs group (30%) at the endpoint of modeling (on day 21), while mice in the normal control group did not die during whole 3 weeks (Fig. 3B).

The hydroxyproline (HYP) content in lung tissue of model group mice was increased remarkably to 1.52 folds as compared to that in normal control group animals. Moreover, bleomycin-induced increase in HYP was significantly inhibited after transplantation of MSCs, BCs alone or combined with CoQ10 (Fig. 4).

Similarly, the accumulation of α -SMA in lung tissue of each group was detected by immunofluorescence staining. Results showed the deposition of α -SMA was increased remarkably after modeling as compared to that in normal control group animals, which were significantly alleviated by transplantation of MSCs, BCs alone, or combined with CoQ10 (Fig. 5).

The H&E staining pictures showed that rare exudation in the alveolar cavity and inflammatory cells infiltration was founded in the lung tissue of mice in normal control group, while the lung tissue structure of the model group mice was completely destroyed (Fig. 6A). In the bleomycin-induced mice, the lung intervals were significantly thickened, the capillary morphology was unobvious and the alveolar structures were extremely difficult to be distinguished. Also, a large amount of fibrous exudate was deposited, and some regions of the lung tissue were consolidated. On the contrary, the exudation degree in the alveolar cavity and inflammatory cells infiltration in three kinds of cell transplantation groups were significantly weakened and the lesions were also reduced.

In Masson staining sections as shown in Fig. 6B, there was no fibrous tissue hyperplasia in the lung tissue of normal control group animals. However, collagen fibers that dyed blue were significantly increased around small bronchus and lung interstitium in bleomycin-injured model group animals, especially distributed in the basement membrane of bronchial branches and severely damaged bronchus. On the contrary, collagen fibers deposition was significantly reduced in all three cell transplantation groups, especially in the BCs + CoQ10 group animals ($P < 0.01$).

Discussion

Accumulating evidence suggested that stem cells-based therapies may be used for lung regeneration and modulation of inflammatory and fibrotic processes [8]. In the present study, we observed that administration of MSCs, BCs, or BCs combining with CoQ10 into lung tissue of mice 7 days after bleomycin instillation, ameliorated the histopathological characteristics of lung tissues and prevented pulmonary fibrosis development. Cells transplantation reduced the degree of pulmonary fibrosis as compared with the model group. Slices staining showed that both inflammatory cell infiltration and collagen fiber content in cells transplanted animals was significantly reduced as compared with the animals in the model group, as well as pulmonary α -SMA. The hydroxyproline content in the BCs + CoQ10 group was lower than that in the BCs group. Among them, the mortality rate of the mice in the BCs + CoQ10 group (20%) was lower than that in the BCs group (30%). Furthermore, our results showed that CoQ10 could inhibit H_2O_2 -induced ROS production in BCs and cell apoptosis. CoQ10 played a protective role to BCs, which indicates that CoQ10 may improve the survival rate of transplanted cells in the pulmonary microenvironment.

Previous studies reported that stem cells have differentiation potential and can differentiate into tissue-resident lung cells to repair pulmonary damage. Therefore, stem cells therapy is expected to become a radical cure for IPF. Among all cells with clinical potential, MSCs are easy to obtain and handle. However, it is widely recognized that transplanted MSCs perform repair function mainly through the paracrine or immunomodulatory mechanism, with no evidence showing that they can reconstitute lung structure for regeneration purposes [9]. Adult stem cells are undifferentiated cells existing in a differentiated tissue that can self-proliferate, renew and can be directed to differentiate into mature functional cells of that tissue. Such cells are relatively easy to obtain and can be obtained directly from the patient, and then be cultured and proliferated *in vitro*. It avoids the immune rejection problems caused by allogeneic cell

transplantation. Theoretically, the tumorigenic risk of adult stem cells is low, and there are few ethical controversies. Hematopoietic stem cells are mature adult stem cells that are transplanted to treat hematological diseases [20], and limbal stem cells are also mature adult stem cells that are applied in clinical practice [21]. BCs are hematopoietic stem cells located in the lungs. Studies have shown that BCs can migrate to the damaged site after alveolar injury, differentiate into alveolar epithelial cells, and then repair the damage. Zuo et al. found that transplantation of BCs could alleviate bleomycin-induced IPF in mice. Moreover, results proved that the transplanted BCs could differentiate into functional alveolar epithelial cells to repair the damage [25, 26].

Cotreatments by different factors and drugs can influence the biological activity of transplanted cells *ex vivo* and *in vivo*, thereby improving their reparative efficacy for applications in current regenerative medicine. But some factors could activate fibroblasts, which worsening pulmonary fibrosis symptoms [35]. In the present study, CoQ10 was designed to improve the transplantation efficiency of BCs by making cells better adapt to the microenvironment with high oxidative stress levels. Cells were pretreated with CoQ10 before transplantation to improve cell survival signal. On the other hand, transplanting cells combining with CoQ10 alleviated oxidative stress levels in the microenvironment for the survival of cells after transplantation.

However, CoQ10 may also have a pro-differentiation effect which needs further verification, and the expression of cells survival signal after pretreatment needs further exploration. We cannot rule out that the role of BCs is to promote the differentiation of endogenous adult stem cells into alveolar epithelial cells through indirect paracrine, which is still an area that needs to be studied in more detail. At last, the survival rate of transplanted cells in each group also needs further verification.

Conclusions

CoQ10 blocks H₂O₂-induced apoptosis of BCs and ROS production *in vitro*, and enhances the efficacy of BCs transplantation on bleomycin-induced IPF in mice.

Abbreviations

IPF: idiopathic pulmonary fibrosis; BC: airway basal stem cells; MSCs: mesenchymal stem cells; iPSCs: induced pluripotent stem cells; CoQ10: Coenzyme Q10; HYP: hydroxyproline.

Declarations

Availability of data and materials

All the data obtained in from the present study are available from the corresponding author under reasonable request.

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Contributions

The authors have made the following declaration about their contributions. Conceived and designed: Huanbin Liu, Yidi Zhang, Jinjun Jiang, Shiyue Li, Yichu Nie and Wenbin Deng. Development and methodology: Yidi Zhang, Jinjun Jiang, Jingxin Zhao, Jian Xu, Yuan Xie, Shiyue Li, Yichu Nie and Wenbin Deng. Acquisition of data: Huanbin Liu, Jingxin Zhao, Jian Xu, Yuan Xie, Weiping Liao, Wei Wang, Shiyue Li and Yichu Nie. Analysis and interpretation of the results: Huanbin Liu, Yidi Zhang, Jinjun Jiang, Shiyue Li, Yichu Nie and Wenbin Deng. Writing, review and revision of the manuscript: Huanbin Liu, Yidi Zhang, Jinjun Jiang, Jingxin Zhao, Jian Xu, Yuan Xie, Shiyue Li, Yichu Nie and Wenbin Deng. Study supervision: Shiyue Li, Yichu Nie and Wenbin Deng. All authors read and approved the final manuscript.

Ethics declarations

Ethics approval and consent to participate

Ethics Committee approval (Approval No. SYSU-IACUC-2020-000066) was obtained from the Institutional Animal Care and Use Committee (Sun Yat-Sen University) to the commencement of the study.

Consent for publication

All the patients signed an informed consent for publication.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Identification of BCs. BCs cell makers, P63 and KRT5, were used for BCs identification by cellular immunofluorescence. Scale bars: 50 μ m.

Figure 2

CoQ10 inhibits BCs apoptosis and ROS level increased causing by H_2O_2 . (A) The cells of each group were stained with DCFH-DA reactive oxygen fluorescent probe. Fluorescence representative pictures of cells in the blank group, H_2O_2 group and CoQ10+ H_2O_2 group were taken with a fluorescence microscope, Scale bars: 200 μm . (B) The cells of each group were stained with DCFH-DA reactive oxygen species fluorescent probe, and the ROS levels of cells in the blank group, H_2O_2 group and CoQ10+ H_2O_2 group were detected by flow cytometry. (C) The cells of each group were stained with Annexin-FITC/PI dye, and

the apoptotic number of cells in the blank group, H2O2 group and CoQ10+H2O2 group was detected by flow cytometry. * P < 0.05, ** P < 0.01, ns: no significant.

Figure 3

Changes in body weight of mice and survival curves. (A) Changes in body weight of mice in each group after bleomycin exposure. (B) The survival rate of mice in each group. The mortality rate on day 21 was 30% (3/10), 30% (3/10), 30% (3/10), 20% (2/10), 0% (0/10) for the Model group, MSC group, BCs group, BCs+CoQ10 group, control group, respectively. Mice survival was higher in the BCs+CoQ10 group than that in other cell transplant groups.

Figure 4

The HYP evaluation of mice lung tissue. The collagen in the mice's lung tissue was evaluated using lung hydroxyproline (HYP). Data represent the mean $\pm$ standard deviation (SD), n=6 per group. *, P < 0.05 vs. control group; #, P < 0.05 vs. model group; ##, P < 0.01 vs. model group; ####, P<0.0001 vs. model group.

Figure 5

Immunofluorescence of α -SMA in the lung section of each group for 21 days after bleomycin exposure treated with three kinds of cells. Scale bars: 100 μ m.

Figure 6

Histopathology of the lung tissues (n=6 for each group). (A) Pulmonary tissue sections were prepared on day 21 and stained with H&E to observe pathologic changes. Scale bars: 100 μ m; (B) the sections were also subjected to Masson's trichrome staining to visualize collagen deposition. Scale bars: 100 μ m; ***, $P < 0.001$ vs. control group; #, $P < 0.05$ vs. model group; ##, $P < 0.01$ vs. model group.