Cordyceps sinensis extract suppresses hypoxia-induced proliferation of rat pulmonary artery smooth muscle cells

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ABSTRACT

Objective: To investigate the effects of a Chinese herb Cordyceps sinensis (C. sinensis) extract on hypoxia-induced proliferation and the underlying mechanisms involved.

Methods: This prospective study was carried out at the Central Laboratory of Yichang Central People's Hospital, Yichang, China from March 2008 to April 2010. The C. sinensis was extracted from the Chinese herb C. sinensis using aqueous alcohol extraction techniques. Forty healthy adult male Sprague Dawley rats were used in the study. The proliferation of pulmonary artery smooth muscle cells (PASMCs) was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and cell viability was determined by trypan blue exclusion. Cell cycles were analyzed using FACSor flow cytometric analysis. The expression of proliferating cell nuclear antigen (PCNA), c-jun, and c-fos in rat PASMCs was determined by immunohistochemistry.

Results: We found an increased proliferation of PASMCs and increased expression of transcription factors, c-jun and c-fos in PASMCs cultured under hypoxic conditions. The C. sinensis extract significantly inhibited hypoxia-induced cell proliferation in a dose-dependent manner. In addition, C. sinensis extract also significantly inhibited the expression of PCNA, c-jun, and c-fos in these PASMCs.

Conclusion: Our results indicated that C. sinensis extract inhibits hypoxia-induced proliferation of rat PASMCs, probably by suppressing the expression of PCNA, c-fos, c-jun, and decreasing the percentage of cells in synthesis phase, second gap phase, and mitotic phase in cell cycle (S+G2/M) phase. Our results therefore, provided novel evidence that C. sinensis extract may be used as a therapeutic reagent in the treatment of hypoxic pulmonary hypertension.

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Pulmonary hypoxia results from diseases characterized by abnormal pulmonary function or respiratory obstructions, such as chronic obstructive pulmonary diseases (COPD), neuromuscular diseases, and interstitial lung disease. Pulmonary hypoxia is a common cause of hypoxic pulmonary hypertension (HPH), which plays a pivotal role in the development and prognosis of pulmonary heart diseases and COPD.\textsuperscript{1,2} Increased pulmonary vasoconstriction and pulmonary vascular structural remodeling (PVSR) are the critical pathological changes that occur during HPH development. Hypoxia-induced abnormal proliferation of pulmonary artery smooth muscle cells (PASMCs) has been suggested to be the key feature of PVSR.\textsuperscript{3,4} However, the molecular mechanisms involved in these processes are not clear. Cordyceps sinensis (C. sinensis) is an herb used as a Chinese herbal medicine. Many studies have indicated that it plays an important role in reducing blood glucose, inhibiting tumor growth, and improving the body’s immune responses.\textsuperscript{5-15} However, whether C. sinensis could be used as a therapeutic option to treat HPH has not been studied. In this study, we investigated the effects of C. sinensis extract on hypoxia-induced proliferation of rat PASMCs, and the gene expression of c-jun and c-fos in these cells. The experiments presented here evaluated the potential role and mechanisms of C. sinensis in regulating the proliferation of PASMCs.

**Methods.** This study was performed in the Central Laboratory of Yichang Central People’s Hospital, Yichang, China from March 2008 to April 2010. Approval from the University Ethical Committee was obtained. The experiment was carried out according to the National Guidelines for Care and Use of Laboratory Animals.

**Extraction of C. sinensis.** A water-based extract from the herb C. sinensis was extracted using aqueous alcohol extraction method. A 50 gms of chopped crude C. sinensis was added to a 400 ml water, and was extracted twice via decoction. The C. sinensis extract was filtered through a filter paper, and was precipitated at 95% ethanol for 24 hours at 4°C. After the supernatant was removed, the precipitate was resuspended in 50 ml water. The dissolved C. sinensis (one g/ml) extract was sterilized by passing through a 0.22 µm membrane filters, and stored at 4°C as a stock before being diluted in Dulbecco modified eagle medium (DMEM) prior to the experiments.

**Isolation of rat PASMCs.** Forty healthy adult male Sprague Dawley (SD) rats (weight; 200-250 g) were maintained in a laboratory at the animal center. Animals were anesthetized using 20% urethane (0.5 ml/100 g body weight) followed by a rapid removal of the lungs and hearts into pre-cooled D-Hanks solutions. Grades III to IV pulmonary arteries (300-700 µm in diameter) were isolated, and the outer membrane was removed. Tissues were cut into pieces, and the PASMCs were purified by digesting the tissues in the presence of 2 ml D-Hanks containing 2 mg collagenase, and 3 mg trypsin for 10 minutes at 37°C. The reaction was terminated by centrifuging at 1000 rpm for 10 minutes, followed by discarding the supernatant. The pellet was further digested in 2 ml DMEM, supplemented with 20% fetal bovine serum (FBS) in the presence of 2 mg collagenase for one hour at 37°C in a humidified 5% CO\textsubscript{2} incubator. The mixture was then pipetted up and down to break clusters into single cells followed by centrifugation at 1000 rpm for 10 minutes. The cell pellet was resuspended in DMEM supplemented with 20% FBS, and kept at 37°C in a humidified 5% CO\textsubscript{2} incubator. Cells were passaged upon reaching 80-90% confluency, and cells at passages between 2-4 were used in our experiments.

**Measurement of cell proliferation and cell viability.** Cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay. Briefly, PASMCs were seeded into a 96-well plates at a density of 10\textsuperscript{4} cells/200 µl/well. Twenty-four hours later, serum-free medium was replaced, and cells were incubated for another 24 hours under either normoxic (21% O\textsubscript{2}) or hypoxic (2% O\textsubscript{2}) conditions in the absence or presence of indicated concentrations of C. sinensis extract for an additional 48 hours. At the end of the incubation, 20 µl of MTT (5 mg/ml) was added to the cultures, and cells were then incubated for an additional 4 hours. After the supernatant was removed, 150 µl of dimethyl sulfoxide (DMSO) was added to dissolve MTT crystals. The plates were then read on a plate reader at a wavelength of 570 nm with a background reading at 630 nm. The relative cell mass were determined as (optical density (OD)\textsubscript{570} - OD\textsubscript{630}) x 100. Cell viability was determined by trypan blue exclusion. The experiment was conducted 6 times for each group and data were expressed as mean ± SD.

**Cell cycle analysis.** Cells treated with or without the C. sinensis extract were trypsinized, and washed in phosphate buffer solution (PBS) followed by the addition of a fixation solution (70% ice-cold ethanol), and were fixed overnight at 4°C. Fixed cells were washed with PBS and then digested with ribonuclease (RNase)
A (0.25 mg/ml) for 30 minutes at room temperature. Cells were permeabilized using 0.1% Triton X-100, and stained with propidium iodide (PI, 100 mg/L) for 30 minutes at 4°C in the dark. Cell cycles were analyzed using FACSort flow cytometry (BD Bioscience, San Jose, CA, USA).

**Immunocytochemistry.** For immunocytochemical analysis, PASMCs in different experimental groups were cultured on cover slides. At the end of the experiments, cells were washed with PBS, and were then fixed in 4% paraformaldehyde for 20 minutes. Immunostaining for proliferating cell nuclear antigen (PCNA), c-fos, or c-jun was performed as described previously. To quantify the percentage of cells expressing the indicated protein, random samples of 200 cells in each slide, with a total of 6 slides for each condition tested were analyzed using an HMIA-2000 (Wuhan Kilo-screen Imagine Technology, Wuhan, China) high-resolution color graphic analysis system. The average integrated optical density (AIOD) value was used to determine the percentage of cells expressing the protein in question. The antibodies for PCNA, c-fos, and c-jun were purchased from Santa Cruz Biotechnologies CA, USA.

All data were analyzed using the Statistical Package for Social Sciences version 13.0 for Windows (SPSS Inc, Chicago, IL, USA). Data are expressed as mean ± standard deviation (mean ± SD). Differences among treatment groups for MTT, cell viability, cell cycle data, and AIOD values for the expression of c-jun and c-fos were analyzed using a q test, whereas a Chi-square test was used to compare the positive percentage of PCNA among treatment groups. A *p* < 0.05 was considered significant.

**Results. Identification of rat PASMCs.** Rat PASMCs in culture were oval shaped, with nuclei located in the middle of the cells. Cells grew in multiple layers and exhibited hill-and-valley morphology (Figure 1a). Immunostaining of α-actin demonstrated a filament-like distribution in the cytosol along the longitudinal axis of the cells, which is a phenotypic characteristic of PASMCs (Figure 1b). The *C. sinensis* inhibits hypoxia-induced proliferation in rat PASMCs. As hypoxia-induced cell proliferation and PVSR are important factors for HPH development, using MTT assay, we found that the accumulation of cell mass in PASMCs cultured under hypoxic conditions was approximately 2-fold higher than that of cells cultured under normoxic conditions. The OD absorbance of PASMCs in chronic hypoxia group was 0.4672 ± 0.0600, while the OD in normoxia group was 0.2297 ± 0.0321 (*p* = 0.0052). Interestingly, we found that treatment with *C. sinensis* extract significantly inhibited the accumulation of cell mass in PASMCs cultured under hypoxic conditions in a dose-dependent manner, and a significant effect was detected at 10 mg/ml of *C. sinensis* extract (Figure 2a). As we did not find significant changes in cell viability between normoxic and hypoxic conditions (both were over 90% [Figure 2b]), we assumed that the effect of *C. sinensis* on cell accumulation resulted from its inhibition of cell proliferation, but not cell viability. To further confirm the increased proliferation of PASMCs under hypoxic conditions, we analyzed the cell cycle of these cells. The cell population in the synthesis phase, second gap phase and mitotic phase in cell cycle (S+G2/M) in hypoxic conditions was 29.88 ± 4.47%, and this was significantly higher than that of the cells cultured under normoxic condition (13.65 ± 2.72%), which suggests that increased DNA synthesis occurs under hypoxic cultures. After treatment with *C. sinensis* extract, the cell population in the S+G2/M phase was 21.81 ± 3.11%, which was significantly lower than that of the cells cultured in hypoxic condition without *C. sinensis*, and this finding indicates that *C. sinensis* extract inhibited the hypoxia-induced cell proliferation. Thus, the addition of *C. sinensis* extract significantly suppressed hypoxia-induced DNA synthesis (Figure 2c). The PCNA, which was expressed in the nucleus during DNA synthesis is an indicator of active cell proliferation. We examined...
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Figure 2 - *Cordyceps sinensis* (CS) inhibits hypoxia-induced proliferation of rat pulmonary artery smooth muscle cells. a) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. \( p = 0.0314 \) and \( **p=0.0056 \) determined by q test. b) cell viability. c) cell cycle analysis. \( *p=0.0068 \) determined by q test. (d [i, ii, iii] & e) hypoxia-induced increased proliferating cell nuclear antigen expression suppressed by *Cordyceps sinensis*, d [i, ii, iii] 400x magnification. e) quantification of proliferating cell nuclear antigen immunostaining. \( **p=0.0072 \) determined by Chi-square test. OD - optical density, S+G2M - synthesis phase, second gap phase and mitotic phase in cell cycle

Figure 3 - *Cordyceps sinensis* inhibits hypoxia-induced expression of c-fos. a [i, ii, iii]) immunostaining of c-fos (400x magnifications), b) quantification of c-fos expression in pulmonary artery smooth muscle cells. \( **p=0.0081 \) determined by q test.

the expression of PCNA in PASMCs, and we found that cells cultured under hypoxic conditions demonstrated an increased expression of PCNA, interestingly, addition of *C. sinensis* extract inhibited the hypoxia-induced PCNA expression (Figures 2d & e). The *C. sinensis* inhibits the expression of c-jun and c-fos in rat PASMCs cultured under hypoxic conditions. We next sought to explore the mechanisms underlying hypoxia-induced cell proliferation in PASMCs and the inhibitory effect of *C. sinensis*. The c-jun and c-fos are 2 oncogenes that can be induced by hypoxia and may contribute to increased cell proliferation.22-25 Both c-jun and c-fos are expressed at low levels in PASMCs cultured under normoxic conditions. Upon exposure to hypoxia, the expression of both proteins increased, with a cytosolic distribution...
for c-fos and a nuclear distribution for c-jun. Similar to its inhibitory effect on cell proliferation, the \textit{C. sinensis} extract significantly suppressed the expression of c-jun and c-fos under hypoxic conditions to a level, which was significantly higher than that of cells cultured under normoxic conditions (Figures 3a & b, Figures 4a & b).

**Discussion.** Several lines of evidence suggest that chronic HPH is the key feature in PVSR, which is characterized by the abnormal proliferation of PASMCs. In this study, we successfully collected a water extract from \textit{C. sinensis} and demonstrated that \textit{C. sinensis} extract could inhibit chronic hypoxia-induced cell proliferation in PASMCs. This extract might be a potential therapy and/or prevention for HPH. Further characterization of the single purified component extracted from \textit{C. sinensis} will provide a better understanding of the mechanism that underlies its effect on hypoxia-induced cell proliferation.

The role of hypoxia in cell proliferation is controversial. In some cells, hypoxia inhibits proliferation and induces apoptosis, whereas in other cells it promotes proliferation. Both hypoxia-inducible factor (HIF)-1\(\alpha\) and HIF-2\(\alpha\) are induced under hypoxic conditions, and HIF-1\(\alpha\) is anti-proliferative, and HIF-2\(\alpha\) has been shown to be pro-proliferative. The HIF-1\(\alpha\) is ubiquitously expressed in various cell types, but HIF-2\(\alpha\) exhibits limited expression in endothelial cells.\(^{26}\) The PASMCs express HIF-2\(\alpha\), and it is likely that the induction of HIF-2\(\alpha\) under hypoxia may contribute to increased cell proliferation observed in our study. However, whether the effect of the \textit{C. sinensis} extract on hypoxia-induced PASMC proliferation depends on HIF-2\(\alpha\), or its targets requires further study.

The C-fos is a cellular proto-oncogene that belongs to the immediate early gene family of transcription factors.\(^{27,28}\) Members of the c-fos family dimerize with c-jun to form the activator protein (AP-1) transcription factor, which up-regulates transcription of a diverse range of genes involved in proliferation, differentiation, and a series of pathophysiological processes, such as defense against invasion and cell damage. Both c-jun and c-fos are induced under hypoxic conditions,\(^{20-22}\) and may contribute to an increased rate of proliferation.\(^{23-25}\) In this study, we found an increased proliferation of PASMCs and an increased expression of the transcription factors c-jun and c-fos in PASMCs cultured under hypoxic conditions. Treatment with \textit{C. sinensis} extract significantly inhibited hypoxia-induced cell proliferation, and the expression of c-jun and c-fos in these PASMCs, which suggests that \textit{C. sinensis} inhibits hypoxia-induced cell proliferation in PASMCs, possibly by suppressing the expression of the transcription factors c-jun and/or c-fos. In addition to increased cell proliferation in PASMCs, chronic hypoxia induces other changes in the vessels, including the proliferation of non-muscle cells, increased migration, and matrix protein production of both smooth muscle cells and non-muscle cells.\(^{29,30}\) These changes together leads to the remodeling of the pulmonary arteries, and contributes to the development of HPH. Whether the \textit{C. sinensis} extract has beneficial effects on non-muscle cells is currently under investigation.

In conclusion, our results suggest that the \textit{C. sinensis} extract might be used as a therapeutic reagent to prevent and/or treat hypoxia-induced HPH. Further in vivo studies are needed to test the efficacy of this potential agent.

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