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

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ABSTRACT

الأهداف: دراسة تأثير أحد الأعشاب الصينية وهي خلاصة فطر كورديسيبس سينسيس (cordyceps sinesis) على عملية تكاثر الخلايا التي يحفزها نقص الأوكسجين وما يترتب على ذلك من الآليات الحيوية.

الطريقة: تم إجراء دراسة استطلاعية في المعمل المركزي بمستشفى ييتشانغ الشعبي، ييتشانغ، الصين وذلك خلال الفترة من مارس 2008م إلى إبريل 2010م. لقد تم استخلاص هذه المادة من عشبة كورديسيبس سينسيس الصينية بطريقة الإذابة ثم الاستخلاص من الكحول، وشملت الدراسة 40 جرذياً ذكراً من النوع سبراغو دوللي. لقد تم قياس تكاثر خلايا العضلات الملساء بالشريان الرئوي باستخدام مقايسة إم تي تي وهو اختبار يعتمد على الألوان في قياس نشاط الإنزيمات التي تختزل أملاح إم تي تي إلى صبغات فورمازان (MTT assay)، في حين تم تحديد عدد الخلايا بطريقة عزل زرقة الترايبان وهو عبارة عن تلوين حيوي يقوم بتلوين الخلايا الميتة وعزلها عن الخلايا الحية (trypan blue exclusion). تم تحليل دورة حياة الخلايا عن طريق اختبار مقايسة الخلايا في المجرى باستخدام تصنيف فاك (FACSort flow cytometric analysis)، فيما قام اختبار الكيمياء المناعية (immnohistochemistry) بقياس مولدات الأجسام المضادة الناتجة عن تكاثر الخلايا (PCNA)، وجين سي جن (c–jun)، وبروتين سي فاس (c–fos) وذلك في خلايا العضلات الملساء بالشريان الرئوي.

النتائج: لقد لاحظنا زيادة في نسبة تكاثر خلايا العضلات الملساء بالشريان الرئوي، بالإضافة إلى زيادة في عوامل النسخ (transcription factors)، وسي جن، وسي فاس وذلك في هذه الخلايا التي زُرعت في بيئة ينقص فيها الأوكسجين. لقد قامت خلاصة كورديسيبس سينسيس بتثبيط تكاثر الخلايا التي يحفزها نقص الأوكسجين وذلك اعتماداً على الجرعة المأخوذة من هذه الخلاصة، كما أنها قامت أيضاً بتثبيط إنتاج مولدات الأجسام المضادة الناتجة عن تكاثر الخلايا، وسي جن، وسي فاس في تلك الخلايا.

خاتمة: تشير النتائج بأن خلاصة كورديسيبس سينسيس تقوم بتثبيط تكاثر خلايا العضلات الملساء بالشريان الرئوي والتي يحفزها نقص الأوكسجين وذلك قد يكون بسبب قدرتها على إيقاف إنتاج مولدات الأجسام المضادة الناتجة عن تكاثر الخلايا، وإيقاف إنتاج سي جن، وسي فاس. هذا بالإضافة إلى قدرتها على تقليل نسبة الخلايا في أطوار نمو الخلية التالية: مرحلة بناء DNA، وطور النمو الثاني، وطور الانقسام التفتلي ($S+G_2/M$)، وهكذا فإن النتائج تدل على أن خلاصة كورديسيبس سينسيس قد تُستخدم كعامل مساعد عند علاج ارتفاع ضغط الدم الرئوي (HPH).

Objectives: 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 FACSort 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 ($S+G_2/M$) 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.[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.[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.[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 in 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_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_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 10^4 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_2) or hypoxic (2% O_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 $(OD)_{570}$-OD_{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)

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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 4ºC 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.[16] 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 HMIAS-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.[17,18] 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,[19] using MTT assay, we found that the accumulation of cell mass in PAMSCs cultured under hypoxic conditions was approximately 2-fold higher than that of cells cultured under normoxic conditions. The OD absorbance of rat 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 PAMSCs 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 ($S+G_2/M$) 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 $S+G_2/M$ 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 indictor of active cell proliferation.[20,21] We examined

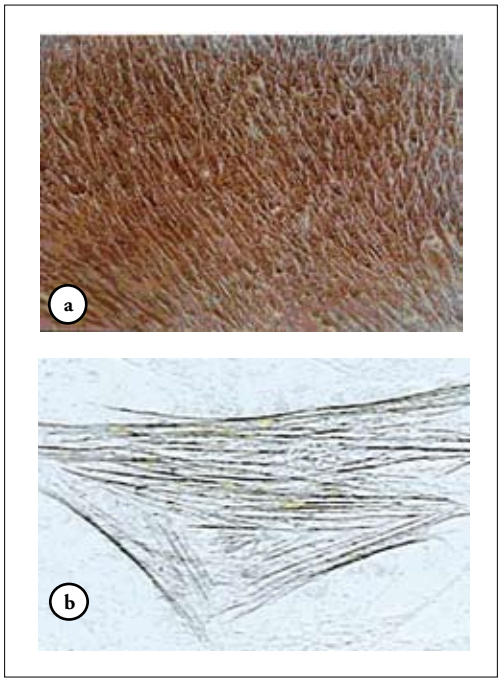

Figure 1 - Identification of rat pulmonary artery smooth muscle cells (PASMCs). **a**) hill-valley morphology. Cells exhibited typical hill-valley morphology due to the stacking growth of cells (200 x magnifications), **b**) immunostaining of α-actin (yellow in color) in PASMCs demonstrated a filament-like distribution in the cytosol along the longitudinal axis of the cells (400 x magnifications).

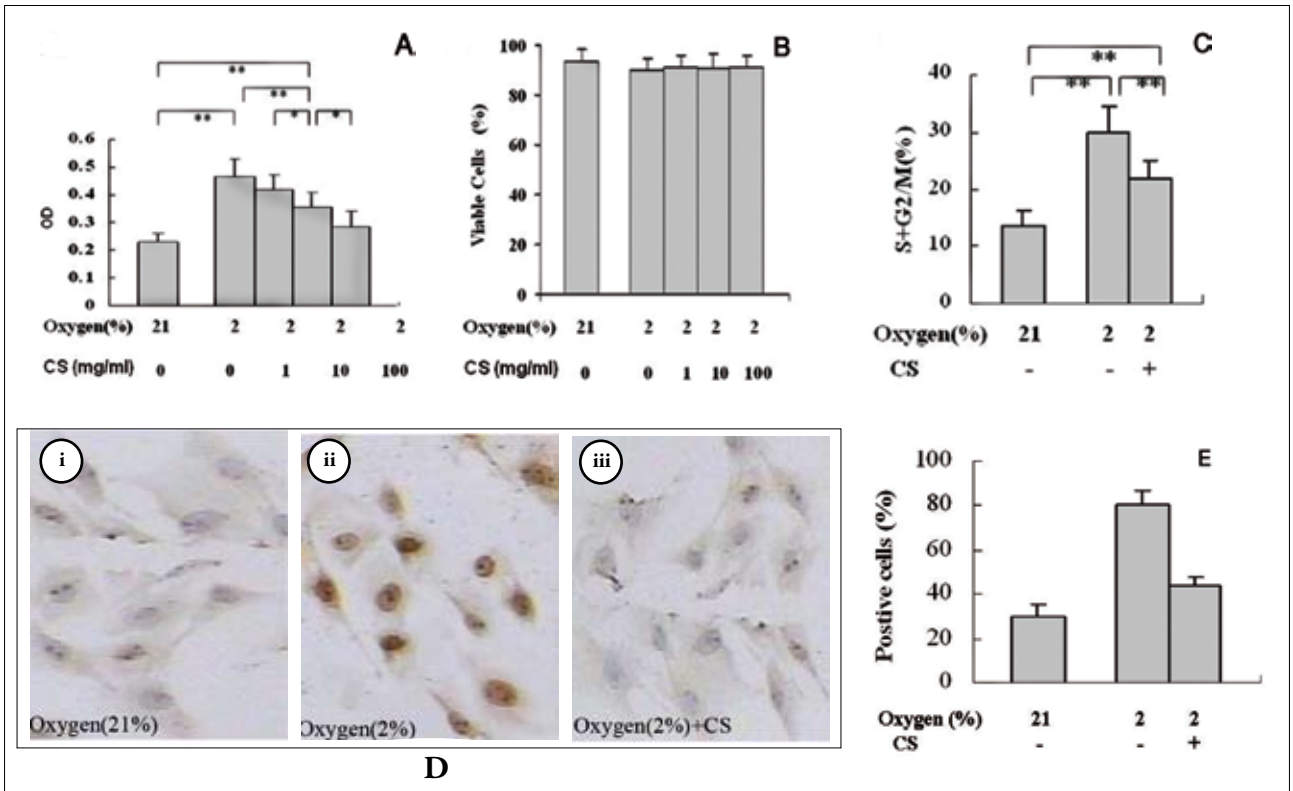

Figure 2 - *Cordyceps sinensis* (*CS*) inhibits hypoxia-induced proliferation of rat pulmonary artery smooth muscle cells. **a**) 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) assay. **p* =0.0314 and ***p*=0.0056 determined by q test. **b**) cell viability. **c**) cell cycle analysis. ***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, $S+G_2M$ - synthesis phase, second gap phase and mitotic phase in cell cycle

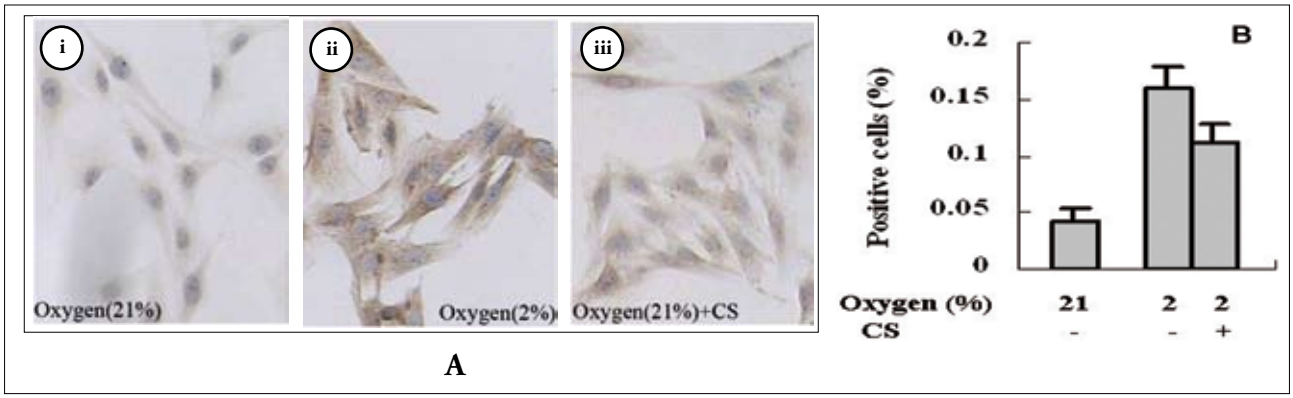

Figure 3 - *Cordyceps sinensis* inhibits hypoxia-induced expression of c-fos. **a** [**i, ii, iii**]) immunostaining of c-fos (400x magnifications), **b**) quantification of c-fos expression in pulmonary artery smooth muscle cells. ***p*=0.0081 determined by q test.

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

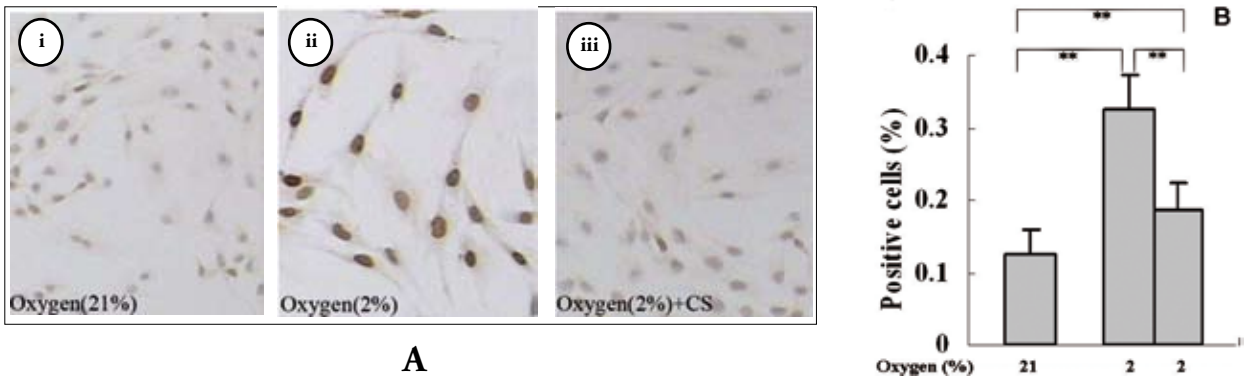

Figure 4 - *Cordyceps sinensis* inhibits hypoxia-induced expression of c-jun. **a** [**i, ii, iii**]) immunostaining of c-jun (200 x magnifications). **b**) quantification of c-jun expression in pulmonary artery smooth muscle cells. ***p*=0.0058 determined by q test.

for c-fos and a nuclear distribution for c-jun. Similar to its inhibitory effect on cell proliferation, the *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 *C. sinensis* and demonstrated that *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 *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)-1α and HIF-2α are induced under hypoxic conditions, and HIF-1α is anti-proliferative, and HIF-2α has been shown to be pro-proliferative. The HIF-1α is ubiquitously expressed in various cell types, but HIF-2α exhibits limited expression in endothelial cells.[26] The PASMCs express HIF-2α, and it is likely that the induction of HIF-2α under hypoxia may contribute to increased cell proliferation observed in our study. However, whether the effect of the *C. sinensis* extract on hypoxia-induced PASMC proliferation depends on HIF-2α, 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 *C. sinensis* extract significantly inhibited hypoxia-induced cell proliferation, and the expression of c-jun and c-fos in these PASMCs, which suggests that *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 *C. sinensis* extract has beneficial effects on non-muscle cells is currently under investigation.

In conclusion, our results suggest that the *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.

References

1. McLaughlin VV, McGoon MD. Pulmonary arterial hypertension. ***Circulation*** 2006; 114: 1417-1431.

2. Hida W, Tun Y, Kikuchi Y, Okabe S, Shirato K. Pulmonary hypertension in patients with chronic obstructive pulmonary disease: recent advances in pathophysiology and management. ***Respirology*** 2002; 7: 3-13.
3. Esteve JM, Launay JM, Kellermann O, Maroteaux L. Functions of serotonin in hypoxic pulmonary vascular remodeling. ***Cell Biochem Biophys*** 2007; 47: 33-44.
4. Stenmark KR, Fagan KA, Frid MG. Hypoxia-induced pulmonary vascular remodeling: cellular and molecular mechanisms. ***Circ Res*** 2006; 99: 675-691.
5. Shin S, Kwon J, Lee S, Kong H, Lee S, Lee CK, et al. Immunostimulatory Effects of *Cordyceps militaris* on Macrophages through the Enhanced Production of Cytokines via the Activation of NF-kappaB. ***Immune Netw*** 2010; 10: 55-63.
6. Xiao G, Miyazato A, Abe Y, Zhang T, Nakamura K, Inden K, et al. Activation of myeloid dendritic cells by deoxynucleic acids from *Cordyceps sinensis* via a Toll-like receptor 9-dependent pathway. ***Cell Immunol*** 2010; 263: 241-250.
7. Hsu CC, Huang YL, Tsai SJ, Sheu CC, Huang BM In vivo and in vitro stimulatory effects of *Cordyceps sinensis* on testosterone production in mouse Leydig cells. ***Life Sci*** 2003; 73: 2127-2136.
8. Choi SB, Park CH, Choi MK, Jun DW, Park S Improvement of insulin resistance and insulin secretion by water extracts of *Cordyceps militaris, Phellinus linteus,* and *Paecilomyces tenuipes* in 90% pancreatectomized rats. ***Biosci Biotechnol Biochem*** 2004; 68: 2257-2264.
9. Yoshikawa N, Nakamura K, Yamaguchi Y, Kagota S, Shinozuka K, Kunitomo M. Antitumour activity of cordycepin in mice. ***Clin Exp Pharmacol Physiol*** 2004; 31 (Suppl 2): S51-S53.
10. Leu SF, Chien CH, Tseng CY, Kuo YM, Huang BM. The in vivo effect of *Cordyceps sinensis* mycelium on plasma corticosterone level in male mouse. ***Biol Pharm Bull*** 2005; 28: 1722-1725.
11. Wu JY, Zhang QX, Leung PH. Inhibitory effects of ethyl acetate extract of *Cordyceps sinensis* mycelium on various cancer cells in culture and B16 melanoma in C57BL/6 mice. ***Phytomedicine*** 2007; 14: 43-49.
12. Liu WC, Wang SC, Tsai ML, Chen MC, Wang YC, Hong JH,, et al. Protection against radiation-induced bone marrow and intestinal injuries by *Cordyceps sinensis*, a Chinese herbal medicine. Radiat Res 2006; 166: 900-907.
13. Liu WC, Chuang WL, Tsai ML, Hong JH, McBride WH, Chiang CS. *Cordyceps sinensis* health supplement enhances recovery from taxol-induced leukopenia. ***Exp Biol Med (Maywood)*** 2008; 233: 447-455.
14. Ji DB, Ye J, Li CL, Wang YH, Zhao J, Cai SQ. Antiaging effect of *Cordyceps sinensis* extract. ***Phytother Res*** 2009; 23: 116-122.
15. Jin CY, Kim GY, Choi YH. Induction of apoptosis by aqueous extract of *Cordyceps militaris* through activation of caspases and inactivation of Akt in human breast cancer MDA-MB-231 Cells. ***J Microbiol Biotechnol*** 2008; 18: 1997-2003.
16. Venturi A, Piaz FD, Giovannini C, Gramantieri L, Chieco P, Bolondi L. Human hepatocellular carcinoma expresses specific PCNA isoforms: an in vivo and in vitro evaluation. ***Lab Invest*** 2008; 88: 995-1007.
17. Preston IR, Hill NS, Warburton RR, Fanburg BL. Role of 12-lipoxygenase in hypoxia-induced rat pulmonary artery smooth muscle cell proliferation. ***Am J Physiol Lung Cell Mol Physiol*** 2006; 290: L367- L374.
18. Hu J, Xu YJ, Zhang ZX, Tian F. Effect of cigarette smoke extract on proliferation of rat pulmonary artery smooth muscle cells and the relevant roles of protein kinase C. ***Chin Med J (Engl)*** 2007; 120: 1523-1528.
19. Stenmark KR, Fagan KA, Frid MG. Hypoxia-induced pulmonary vascular remodeling: cellular and molecular mechanisms. ***Circ Res*** 2006; 99: 675-691.
20. Moldovan GL, Pfander B, Jentsch S. PCNA, the maestro of the replication fork. ***Cell*** 2007; 129: 665-679.
21. Genovese C, Trani D, Caputi M, Claudio PP. Cell cycle control and beyond: emerging roles for the retinoblastoma gene family. ***Oncogene*** 2006; 25: 5201-5209.
22. Milde-Langosch K. The Fos family of transcription factors and their role in tumourigenesis. ***Eur J Cancer*** 2005; 41: 2449-2461.
23. Shaulian E, Karin M. AP-1 in cell proliferation and survival. ***Oncogene*** 2001; 20: 2390-2400.
24. Eferl R, Wagner EF. AP-1: a double-edged sword in tumorigenesis. ***Nat Rev Cancer*** 2003; 3: 859-868.
25. Zenz R, Eferl R, Scheinecker C, Redlich K, Smolen J, Schonthaler HB, et al. Activator protein 1 (Fos/Jun) functions in inflammatory bone and skin disease. ***Arthritis Res Ther*** 2008; 10: 201.
26. Gordan JD, Bertout JA, Hu CJ, Diehl JA, Simon MC. HIF-2alpha promotes hypoxic cell proliferation by enhancing c-myc transcriptional activity. ***Cancer Cell*** 2007; 11: 335-347.
27. Nakakuki T, Birtwistle MR, Saeki Y, Yumoto N, Ide K, Nagashima T, et al. Ligand-specific c-Fos expression emerges from the spatiotemporal control of ErbB network dynamics. ***Cell*** 2010; 141: 884-896.
28. Takada Y, Ray N, Ikeda E, Kawaguchi T, Kuwahara M, Wagner EF, et al. Fos proteins suppress dextran sulfate sodium-induced colitis through inhibition of NF-kappaB. ***J Immunol*** 2010; 184: 1014-1021.
29. Fredenburgh LE, Liang OD, Macias AA, Polte TR, Liu X, Riascos DF, et al. Absence of cyclooxygenase-2 exacerbates hypoxia-induced pulmonary hypertension and enhances contractility of vascular smooth muscle cells. ***Circulation*** 2008; 117: 2114-222.
30. Aoshima D, Murata T, Hori M, Ozaki H. Time-dependent phenotypic and contractile changes of pulmonary artery in chronic hypoxia-induced pulmonary hypertension. ***J Pharmacol Sci*** 2009; 110: 182-190.