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Special Topic for 973 Program

Influence of moxa smoke on mitochondrial transmembrane potential and Bax/Bcl-2 in alveolar type Ⅱ epithelial A549 cells

艾烟对肺泡Ⅱ型上皮细胞 A549 线粒体膜电位及 Bax/Bcl-2 影响的研究

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Abstract

Objective: To investigate the influence of moxibustion products on mitochondrial transmembrane potential (MTP) and mRNA expression of Bax/Bcl-2 in alveolar type Ⅱ epithelial A549 cells, and to further explore influence of moxibustion products on the oxidative damage of A549 cells.

Methods: Smoke and particles generated by moxibustion were collected using the filter box for gas sampling. The moxa smoke extract (MSE) was diluted sequentially to the final concentrations of 0.05 mg/mL, 0.1 mg/mL, 0.2 mg/mL, 0.3 mg/mL and 0.4 mg/mL using the cell culture medium, and A549 cells were then intervened by the above MSE solution. Cell MTP was detected by JC-1 staining. Fluorescence quantitative polymerase chain reaction (PCR) was used to detect Bax/Bcl-2 mRNA expression of A549 cells.

Results: Compared with cells in the normal control group, MTP was significantly decreased in cells of 0.3 mg/mL and 0.4 mg/mL MSE intervention groups ($P<0.01$); while MTP showed no significant changes in cells of 0.05 mg/mL, 0.1 mg/mL and 0.2 mg/mL MSE intervention groups ($P>0.05$); compared with cells in 0.05 mg/mL MSE intervention group, MTP was decreased significantly in cells of 0.1 mg/mL, 0.2 mg/mL, 0.3 mg/mL and 0.4 mg/mL MSE intervention groups ($P<0.05$); compared with cells in 0.1 mg/mL MSE intervention group, MTP was decreased significantly in cells of 0.4 mg/mL MSE intervention group ($P<0.01$). Bax mRNA expression of cells in each concentration of MSE intervention group all showed no significant difference compared to that in the normal control group; Bcl-2 mRNA expression of cells was reduced with the increase of MSE intervention concentration. Wherein, Bcl-2 mRNA expressions of cells in 0.4 mg/mL and 0.3 mg/mL MSE intervention groups were significantly reduced compared with that of cells in the normal control group ($P<0.05$); Bcl-2 mRNA expression of cells in 0.4 mg/mL MSE intervention group was significantly reduced compared to that in 0.05 mg/mL MSE intervention group ($P<0.05$).

Conclusion: Certain higher concentration of moxa smoke could reduce MTP and mRNA expression of the anti-apoptosis gene Bcl-2 in alveolar type Ⅱ epithelial A549 cells. Oxidative damage may be the important mechanism of apoptosis caused by the high concentration of moxa smoke solution, and further studies are necessary on the specific mechanisms.

Keywords: Moxibustion Therapy; Artemisia Argyi; Smoke; Smoke Inhalation Injury; Adverse Effects; Safety; Primary Cell Culture

【摘要】目的：观察艾灸生成物对肺泡Ⅱ型上皮细胞 A549 线粒体膜电位(mitochondrial transmembrane potential, MTP)、Bax/Bcl-2 mRNA 表达的影响，进一步研究艾灸生成物对 A549 细胞的氧化损伤作用。**方法：**采用气体采样滤盒采集艾灸生成的烟气颗粒物，用细胞培养液稀释后依次配制质量浓度为 0.05 mg/mL、0.1 mg/mL、0.2 mg/mL、0.3 mg/mL、0.4 mg/mL 的艾烟提取物(moxa smoke extract, MSE)溶液干预 A549 细胞。采用 JC-1 染色法检测细胞 MTP，荧光定量聚合酶链反应(polymerase chain reaction, PCR)法检测细胞 Bax/Bcl-2 mRNA 的表达。**结果：**与正常对照组细胞相比，0.3 mg/mL 和 0.4 mg/mL MSE 干预组细胞 MTP 显著降低 ($P<0.01$)，0.05 mg/mL、0.1 mg/mL 和 0.2 mg/mL MSE 干预组细胞 MTP 无显著变化($P>0.05$)；与 0.05 mg/mL MSE 干预组细胞相比，0.1 mg/mL、0.2 mg/mL、0.3 mg/mL 和 0.4 mg/mL MSE 干预组细胞 MTP 显著降低(均 $P<0.05$)；与 0.1 mg/mL MSE 干预组细胞

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比较, 0.4 mg/mL MSE 干预组细胞 MTP 显著降低(P＜0.01)。各浓度 MSE 干预组细胞 Bax mRNA 的表达与正常对照组相比均无显著性差异; 细胞内 Bcl-2 mRNA 表达随 MSE 干预浓度的增加而减少, 其中 0.4 mg/mL 和 0.3 mg/mL MSE 干预组细胞与正常对照组细胞 Bcl-2 mRNA 的表达相比显著降低(P＜0.05), 0.4 mg/mL MSE 干预组细胞 Bcl-2 mRNA 的表达与 0.05 mg/mL MSE 干预组细胞内 Bcl-2 mRNA 的表达相比显著降低(P＜0.05)。**结论：**艾烟升高到一定浓度时可以降低肺泡 II 型上皮细胞 A549 细胞内 MTP, 减少凋亡抑制基因 Bcl-2 mRNA 的表达, 氧化损伤可能是高浓度艾烟溶液引起细胞凋亡的重要机制, 具体机制仍需进一步研究。

【关键词】灸法; 艾叶; 烟; 烟雾吸入损伤; 不良反应; 安全性; 原代细胞培养

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With the clinical popularity of moxibustion therapy (a therapeutic method of traditional Chinese medicine) and improvement of people's health awareness, the safety of moxa smoke attracts more and more attentions of doctors and patients[1]. It has been reported that moxa smoke could inhibit microorganism for air disinfection and anti-infection, but ingredients of phenol naphthalene and polycyclic aromatic hydrocarbon in the moxa smoke may be harmful to humans[2]. Since moxa smoke enters into the body primarily through the respiratory tract, studies on the influence and effects of moxa smoke on the respiratory tract cells are important to reveal the safety of moxa smoke. We previously found, 0.4 g/L (mass concentration) of moxa smoke solution could increase reactive oxygen species (ROS) levels and reduce super oxide dismutase (SOD) activity in A549 cells after being challenged with moxa smoke[3], which indicated that a certain concentration of moxa smoke showed oxidative damages on respiratory tract cells. Based on these findings, in this study, we further investigated the pathways of oxidative damage, to reveal the mechanism of moxa smoke intervention on apoptosis.

1 Materials and Methods

1.1 Experimental materials

Moxa sticks (3:1 in the grade, 18 mm in diameter, 200 mm in length, 22 g in weight, and water content ≤13%) were bought from Li Shi-zhen Qi Ai Products Factory (China). Type Ⅱ alveolar epithelial cell line (A549) was from Cell Resource Center 000of China Academy of Sciences Shanghai Institutes for Biological Sciences (Shanghai, China).

1.2 Main instruments and reagents

1.2.1 Main reagents

Fetal bovine serum (FBS) and penicillin/streptomycin (Thermo Fisher Biochemical Products Co., Ltd., China), Trypsin-ethylene diamine tetraacetic acid (EDTA) (0.25% Trypsin and 0.02% EDTA, BIOWEST Company, France), NaCl, KCl, $Na_2HPO_4{\cdot}12H_2O$, KH_2PO_4, isopropanol (Sinopharm Chemical Reagent Co., Ltd., China), mitochondrial transmembrane potential (MTP) detection kit (JC-1), cell lysis buffer (Beyotime Institute of Biotechnology, China), diethy pyrocarbonate (DEPC)-treated water (Sangon Biotech Shanghai Co., Ltd., China), TRIZOL (Invitrogen, USA), chloroform (Shanghai Leimeng Technology Co., Ltd., China), iScript™ cDNA synthesis kit and iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Inc., USA).

1.2.2 Main instruments

PM10 gas sampling box (MSP Corporation, USA), 37 mm glass fiber filters (Beijing Safelab Technology Co., Ltd., China), rocker300 vacuum pump (Rocan Scientific Co. Ltd., Taiwan, China), INCUB 5420-1, INCUB 5420-1 constant temperature carbon dioxide incubator (NAPCO, USA), 1300 SERIES A2 superclean bench (Thermo Scientific, USA), SORVALL ST 16R high speed refrigerated centrifuge (Thermo Scientific, USA), 25 cm^2 cell culture flasks (Corning Inc., USA), 5 cm cell culture dishes, 6-well cell culture plates (Santa Cruz Biotechnology, Inc., USA), microscope slides and cover slips (Jiangsu Shitai Experiment Equipment Co., Ltd., China), HOPE-MED8053B and 8050-1 exposure cabinets (Tianjin Hope Industry and Trade Co., Ltd., China), DMIL LED inverted microscope (LEICA, USA), fluorescence microscope (BX53-DP73-U-HGLGPS, OLYMPUS Corporatio, Japan), NANO DROP 2000 spectrophotometer (Thermo Scientific, USA), LightCycler 96 polymerase chain reaction (PCR) instrument (Roche, Switzerland).

1.3 Cell culture

Dulbecco's modified Eagle's medium (DMEM) culture medium, containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin, was filtered with 0.2 μm sterile filter. Recovered A549 cells with 4 mL freshly prepared complete cell culture medium in 25 cm^2 culture flask were incubated at 37 ℃ with 5% CO_2. Cells were ready for passage or seeding in plates when the confluent was about 80%. Cells were washed once with sterile PBS before passage or seeding, then added 2 mL trypsin with EDTA into cell culture flask and digested for 1-2 min in 37 ℃ incubator. Discarded the trypsin and stopped the digestion, by adding cell culture medium containing 10% FBS into the cell culture flask. Cell suspension was prepared by slow pipetting with disposable pipette and performed cell count. Cells were seeded in cell culture plates and observed.

1.4 moxa smoke solution preparation and cell intervention

Controlled the laboratory temperature at (22±2) ℃ and humidity (55±10)%. Moxa stick was lit inside the exposure cabinet. Adjusted the ratio of mixture gas (moxibustion products) and air to 2:5.

The dried and weighed sampling filter was installed on a portable PM10 gas sampling apparatus. Collected smoke and particles and replaced the sampling filter every 5 min. Dried and weighed the filter after sampling. Calculated the weight of the collected smoke and particles and dissolved in dimethylsulfoxide (DMSO) solvent to prepare moxa smoke extract (MSE) solution to a concentration of 250 mg/mL. Prepared MSE solution was stored at －80 ℃ in dark and to be used within 72 h. Thawed MSE was added into DMEM to the final mass concentration of 0.05 mg/mL, 0.1 mg/mL, 0.2 mg/mL, 0.3 mg/mL or 0.4 mg/mL MSE solution. DMEM medium with same amount DMSO without MSE was used as control.

1.5 Detections

1.5.1 Detection of cell MTP

Cell MTP was detected by JC-1 staining method. In brief, cells were washed with cold PBS once, when the intervention was over. Then incubated with 1 mL of DMEM medium and 1 mL of JC-1 staining working solution at 37 ℃ for 20 min. The supernatant was discarded and the cells were washed twice with cold staining buffer. Then 2 mL of DMEM medium was added in for observation. The slides were taken out just before observation and observed under fluorescent microscope using the default channels for the green and red fluorescence.

Eight non-overlapping visual fields were randomly selected for taking pictures in each group. MTP was represented by the average optical density ratio of the red and green fluorescence, which was used for statistical analysis.

1.5.2 Detection of Bax/Bcl-2 mRNA expression of cells

Extraction of total RNA from cells: Cells were collected at the end of the intervention and lysed with Trizol. Shaked after adding chloroform and collected the upper liquid aqueous phase, followed by adding isopropyl alcohol and discarding the supernatant by centrifugation. RNA was naturally dried at room temperature after washing with 75% ethanol. Precipitated RNA was dissolved in 15 μL of RNase-free H_2O for cDNA reverse transcription or storage in －80 ℃ refrigerator.

cDNA synthesis: RNA concentration was determined using a spectrophotometer followed by reverse transcription of cDNA. Information about primer design and synthesis was shown in Table 1.

Table 1. Primer design and synthesis

ID	Primer name	Sequence (from 5' to 3')
NM_004324.3	Bax F1	AAGCTGAGCGAGTGTCTCAAG
	Bax R1	CAAAGTAGAAAAGGGCGACAAC
NM_000633.2	Bcl-2 F1	GTGGATGACTGAGTACCTGAACC
	Bcl-2 R1	AGACAGCCAGGAGAAATCAAAC
Human 205bP	β-actin F	TGACGTGGACATCCGCAAAG
	β-actin R	CTGGAAGGTGGACAGCGAGG

1.6 Statistical methods

Statistical analyses for all the collected experiment data were performed using the SPSS 18.0 version statistical software. The normal distribution and homogeneity of variance of the data were determined firstly. Statistical results were presented as mean ± standard deviation ($\bar{x} \pm s$), if the data followed a normal distribution. If the data didn't follow the normal distribution, the results were presented as median (min, max). The size of test was set as $\alpha=0.05$, and $P<0.05$ indicated a statistically significance.

2 Results

2.1 Results of cell MTP test

As shown in Figure 1, under fluorescence microscope, the red fluorescence was the main manifestation in the cells of normal and low concentration MSE intervention groups. This indicated that MTP was higher in these cells and JC-1 fluorescent probes were accumulated in the mitochondrial matrix to form polymers and thus showed red fluorescence. With the increase of MSE concentration, MTP was decreased; therefore, JC-1 could not be accumulated in the mitochondrial matrix and existed as monomer, which showed green fluorescence. As we can see in the figure, cell morphologies changed from the long spindle shaped to round and the cell volumes became smaller, after intervention with a high concentration of MSE.

As shown in Table 2, compared with cells in the normal control group, MTP was significantly decreased in cells of 0.3 mg/mL and 0.4 mg/mL MSE intervention groups (both $P<0.01$); MTP in cells of 0.05 mg/mL, 0.1 mg/mL and 0.2 mg/mL MSE intervention groups showed no significant changes (all $P>0.05$); compared to that of 0.05 mg/mL MSE intervention group, MTP was significantly decreased in cells of 0.1 mg/mL, 0.2 mg/mL, 0.3 mg/mL and 0.4 mg/mL MSE intervention groups (all $P<0.05$); compared to that of 0.1 mg/mL MSE intervention group, MTP was significantly decreased in cells of 0.4 mg/mL MSE intervention group ($P<0.01$).

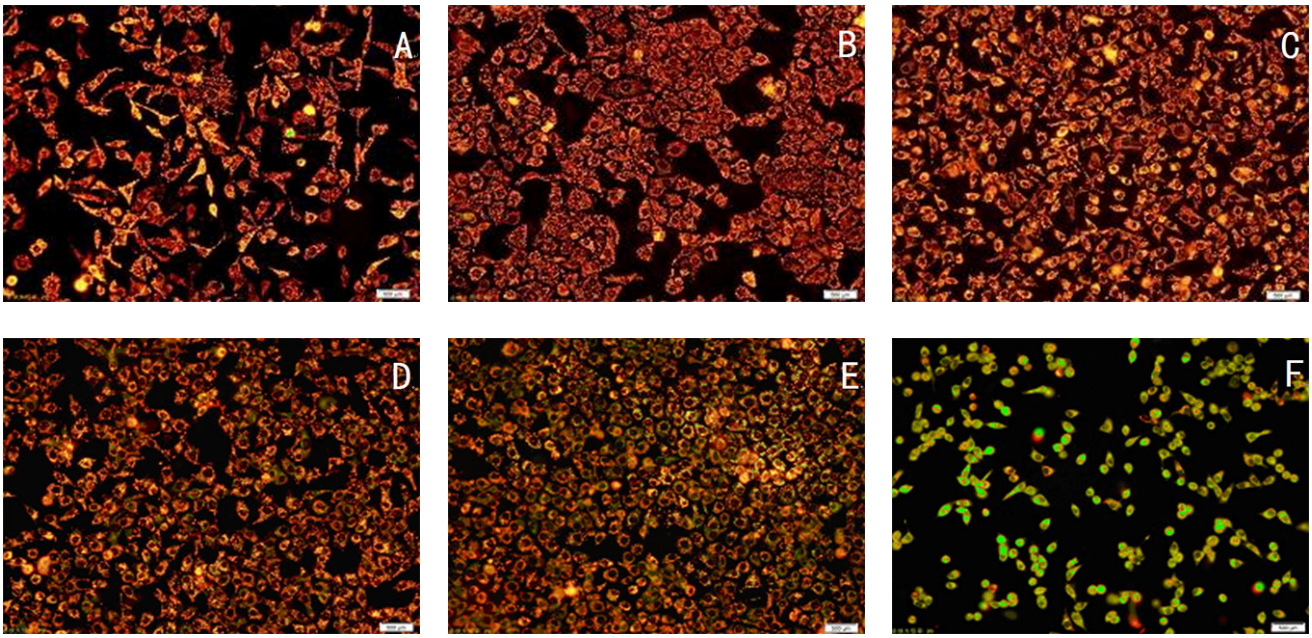

Figure 1. Fluorescence signals of cell MTP after intervention with different concentrations of MSE (Note: A=Normal control group; B=0.05 mg/mL MSE intervention group; C=0.1 mg/mL MSE intervention group; D=0.2 mg/mL MSE intervention group; E=0.3 mg/mL MSE intervention group; F=0.4 mg/mL MSE intervention group)

Table 2. The influence of different concentrations of MSE (with 8 h intervention) on cell MTP ($\bar{x}$ ±s)

Group	n	MTP
Normal control	8	405.33±222.48
0.05 mg/mL MSE intervention	8	3028.42±1160.10
0.1 mg/mL MSE intervention	8	28.40±4.07[1)]
0.2 mg/mL MSE intervention	8	22.98±5.24[1)]
0.3 mg/mL MSE intervention	8	15.86±3.65[1)2)]
0.4 mg/mL MSE intervention	8	1.34±0.12[1)2)3)]

Note: Compared with 0.05 mg/mL MSE intervention group, 1) $P<0.05$; compared with the normal control group, 2) $P<0.01$; compared with 0.1 mg/mL MSE intervention group, 3) $P<0.01$. n=8 indicated that under fluorescence microscope, eight non-overlapping visual fields were selected for statistical analysis

Table 3. Influence of different concentrations of MSE (with 8 h intervention) on Bax/Bcl-2 mRNA expression ($\bar{x}$ ±s)

Group	n	Bax mRNA	Bcl-2 mRNA
Normal control	3	1.04±0.35	1.01±0.14
0.05 mg/mL MSE intervention	3	0.91±0.08	0.80±0.09
0.1 mg/mL MSE intervention	3	0.81±0.08	0.63±0.12
0.2 mg/mL MSE intervention	3	0.69±0.04	0.45±0.12
0.3 mg/mL MSE intervention	3	0.75±0.07	0.27±0.04[1)]
0.4 mg/mL MSE intervention	3	0.70±0.04	0.20±0.05[1)2)]

Note: Compared with the normal control group, 1) $P<0.05$; compared with 0.05 mg/mL MSE intervention group, 2) $P<0.05$. n=3 indicated that the experiment was repeated 3 times

2.2 Test results of Bax/Bcl-2 mRNA expression in cells

As we can see in Table 3, compared to that in normal control group, Bax mRNA expression of cells in each concentration MSE intervention group showed no significant differences; Bcl-2 mRNA expression was decreased with the increase of MSE intervention concentration; Bcl-2 mRNA expression was significantly decreased in cells of 0.4 mg/mL and 0.3 mg/mL MSE intervention groups, compared with that in the normal control group (both $P<0.05$); compared with that in 0.05 mg/mL MSE intervention group, Bcl-2 mRNA expression was significantly decreased in cells of 0.4 mg/mL MSE intervention group ($P<0.05$).

3 Discussion

Mitochondria are organelles involved in cell energy metabolism, capable of modulating intracellular ions, such as calcium and iron, and homeostasis of electrolyte. Mitochondria provide energy for cells by aerobic phosphorylation and maintain the physiological functions of cells. Mitochondria are also the apoptosis regulation center, releasing apoptotic factors when accepting apoptotic signal stimulus, to trigger the mitochondrial apoptotic pathway and induce cell death[4-5]. Mitochondria can produce MTP during respiration. MTP is the potential difference between the 2 sides of the inner mitochondrial membrane, which keeps negative internally and positive externally under normal conditions to maintain the physiological status

of mitochondria. Abnormal changes of MTP are closely related to the apoptosis pathway of mitochondria[6].

Bax and Bcl-2 belong to the oncogene family and together maintain the balance of intracellular apoptosis and anti-apoptosis. The ratio of them determines the probability of apoptosis[7-8]. Other studies found that regulations of apoptosis and mitochondrial functions by Bcl-2 family were closely related[9]. Bcl-2 family proteins are key factors controlled by mitochondria, involved in the mutual regulation between mitochondrial autophagy and apoptosis, and also the regulatory process of chondriokinesis and mitochondrial anastomosis[10-11], and decide the final fate of cells under various stress conditions[12-15].

The results in this study showed that MTP and Bcl-2 mRNA expression were significantly decreased in high concentrations (0.3 mg/mL or 0.4 mg/mL) of MSE intervention groups compared to the normal group. This suggested that apoptosis caused by high concentrations (0.3 mg/mL or 0.4 mg/mL) of MSE was associated with mitochondria and decline of Bcl-2 level of cells. Our previous studies found that moxa smoke contained quinone/semiquinone free radicals[16]. Free radicals contain unpaired electron groups and are active in nature, therefore, easily lead to oxidative damage at high concentration[17]. Free radicals can cause changes in the mitochondrial respiratory chain, including cellular ATP levels, MTP release, expression of apoptotic genes, etc., to generate cytotoxicity[18-19]. Low concentration of MSE (0.05 mg/mL) did not produce negative impact on intracellular ROS, SOD enzymatic activity, MTP level and Bax/Bcl-2 mRNA expression[3]; on the contrary, it could reduce intracellular ROS and elevate MTP. This may be caused by the following factors. Some substances in MSE could clear free radicals; free radicals produced by moxa smoke also could react with each other, thus a part of the free radicals was consumed. Therefore, we presumed, at lower concentrations, moxa smoke solutions may have positive effects on human cells. Studies also showed that high concentrations of MSE (0.3 mg/mL and 0.4 mg/mL) could increase intracellular ROS levels, reduce SOD enzymatic activity, MTP and mRNA expression of anti-apoptosis gene Bcl-2, thus may have some cytotoxicity.

In summary, this study confirmed that the toxic effects of moxa smoke were related with the oxidative damage of free radicals generated by moxa smoke. However, the mechanisms of moxa smoke concentration with ROS, mitochondria and Bcl-2 need to be further studied. Furthermore, to reduce the potential hazards of high concentration of moxa smoke, we should establish clinical monitoring and classification of moxa smoke concentration and prevent patients from oxidative damage. This can reduce the unnecessary psychological burden of both the doctors and patients, and promote the clinical development and application of moxibustion.

Conflict of Interest

The authors declared that there was no potential conflict of interest in this article.

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