Role of non-neuronal cholinergic system in the early stage response of epithelial-mesenchymal transformation related markers in A549 cells induced by coal particles

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OBJECTIVE: This study was aimed to investigate the role of non-neuronal cholinergic system (NNCS) in the early stage response of epithelial-mesenchymal transformation (EMT) related markers in human lung adenocarcinoma A549 cells induced by coal particles.

METHODS: A549 cells were exposed to different concentrations of GBW11110K, GBW11126D and exogenous acetylcholinesterase (AChE) (the exposure doses were determined according to the results of CCK-8 experiment, and the doses that had no significant effects on cell viability were selected) for 24 h. After exposure, the indexes of oxidative stress (SOD and MDA), inflammatory factors (IL-6 and TNF-α), EMT marker proteins (E-cadherin and vimentin), AChE enzymatic activity and mRNA expression levels of different types of acetylcholine receptors (CHRM3, CHRM5, CHRNA5, CHRNA7, CHRNA9 and CHRNB2) were determined.

RESULTS: GBW11110K and GBW11126D exposure could lead to the following injury effects: the levels of oxidative stress and inflammatory factors changed to a certain extent (SOD decreased gradually, while MDA, IL-6 and TNF-α increased). The protein level of E-cadherin decreased while the vimentin level increased (P<0.05), suggesting the occurrence of EMT. The AChE enzymatic activity decreased gradually. The expression of acetylcholine receptor mRNA changed as follows (GBW11110K/GBW11126D: CHRM3 (↑↑), CHRM5 (↓↓), CHRNA5 (↓↓), CHRNA7 (↓↓), CHRNA9 (–), CHRN2 (–)). The addition of exogenous AChE recombinant protein could antagonize the damage effects caused by the coal particles to a certain extent.

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Conclusion: The coal particle exposure could induce the change of oxidative stress response, inflammatory response and EMT related markers, down-regulate the AChE enzymatic activity, and interfere the mRNA expression levels of AChRs in A549 cells. The addition of exogenous AChE recombinant protein could reverse the above effects to a certain extent.

1. Introduction

Pneumoconiosis is a group of heterogeneous occupational interstitial lung diseases caused by the inhalation of mineral dust in the lungs, which leads to lung dysfunction (Wood and Yates, 2020). The pathological characteristics of the disease are chronic pulmonary inflammation and fibrosis. Inflammation can promote pulmonary fibrosis, and then lead to pneumoconiosis. The International Labour Organization and the World Health Organization seeks to eradicate pneumoconiosis by 2030. The disease is still widespread worldwide and has maintained a relatively high incidence rate in recent years. For example, in 2019, the incident cases, the age-standardised incidence rate and the age-standardised mortality rate of coal worker’s pneumoconiosis were 7153 (95% uncertainty interval 5870, 8717), 0.09 (0.07, 0.11) per 105, and 0.04 (0.03, 0.05) per 105 globally (Wang et al., 2021). It remains a severe global public health issue due to the lack of prevention of dust in the workplace, the failure of diagnosis of the disease at the early stages, and limited effective treatments for the disease (Qi et al., 2021). Strengthen comprehensive health management and actively carry out comprehensive clinical treatments, including symptomatic treatment, complication treatment, comorbidity treatment and rehabilitation treatment, so as to reduce the suffering of patients, delay the progress of disease, improve the quality of life and social participation, increase the survival income and prolong the life of patients. The commonly used drug treatments for pneumoconiosis mainly include antiasthmatic treatment, expectorant treatment, and antitussive treatment. Notably, the antiasthmatic treatment plan includes anticholinergic drugs, such as short-acting muscarinic antagonists - ipratropium bromide and long-acting muscarinic antagonists - tiotropium bromide (Consensus of Chinese experts on pneumoconiosis treatment, 2018).

Numerous researchers have studied the pathogenesis of pneumoconiosis for more than a century and put forward many theories, such as mechanical stimulation theory, chemical poisoning theory, surface activation theory, immunity theory, and comprehensive action theory. But so far, it is still unable to completely reveal the pathological process of the disease (Qi et al., 2021). One of the first descriptions of epithelial-mesenchymal transformation (EMT) in fibrosis was made by the team of Eric Neilson in 2002 (Nashville, TN, USA) (Iwano et al., 2002). Here, we focus on how coal dust exposure causes pulmonary fibrosis. Briefly, when the coal dust enters the body, it can interact with alveolar epithelial cells, making them lose polarity, down-regulate the expression of epithelial cell markers (e.g. E-cadherin), while up-regulate the expression of mesenchymal cell markers (e.g. vimentin), and synthesize more a-smooth muscle actin. This process is called EMT (Mahmood et al., 2021; Phan et al., 2021). Then, it leads to the accumulation and proliferation of a large number of stromal cells (mainly refers to fibroblasts and myofibroblasts), the deposition of extracellular matrix increases, and eventually aggravates pulmonary fibrosis (Lachat et al., 2021; Pei et al., 2022).

Recent studies have shown that the non-neuronal cholinergic system (NNCS) is closely related to EMT (Chen et al., 2019a,b; Feng et al., 2018). Our previous results showed that the median values ($P_{50}$, $P_{75}$) of serum acetylcholinesterase (AChE) enzymatic activity in the people in the control group, CWP phase I subgroup, and phase II subgroup were 1.00 (0.70, 1.22), 0.94 (0.79, 1.11), and 0.70 (0.53, 1.08), respectively (Hao et al., 2019a). Further, we measured the level of AChE enzymatic activity in the serum of patients with silicosis and reached a similar conclusion (the data was published in patent CN107245512A, Xu et al., 2018). These results suggest that NNCS may play an important role in the pathological process of pneumoconiosis (the hypothesis of this study).

In this study, lung adenocarcinoma A549 cells derived from human type II alveolar epithelial cells were used as an in vitro cell model to systematically study the toxic effects (oxidative stress response, inflammatory response, the early stage response of EMT related markers, effect on the NNCS) caused by coal particle exposure. At the same time, the potential antagonistic effects of exogenous AChE recombinant protein on the above injury effects were also studied.

2. Materials and methods

2.1. Preparing coal particles using coal reference materials

Since the exogenous substances with particle size less than 5 μm can enter the lower respiratory tract, the purchased coal standard reference materials – GBW11110K and GBW11126D (National Sharing Platform for Reference Materials, China) were ground thoroughly with an agate mortar and screened with a mesh with a pore size of 5 μm. The particle size in the prepared coal particles was observed under the scanning electron microscope (S-3400N, Hitachi, Japan). The content of free silica was determined by pyrophosphoric acid method. The particle size and stability were detected by laser particle size analyzer (Mastersize 2000, UK). Please refer to supplementary materials for characterization details.

Before the cell exposure experiment, the pulverized coal was irradiated under the ultraviolet lamp for 24 h to achieve the purpose of disinfection. The medium containing a certain concentration of the prepared coal particles was freshly prepared.

2.2. Cell culture

The A549 cells were purchased from Cell Resource Center, Shanghai Academy of Life Sciences, Chinese Academy of Sciences. It was routinely cultured in dulbecco’s modified eagle’s medium (DMEM, GIBCO, USA) basic medium containing 10% fetal bovine serum (FBS, Biological Industries, Israel) and 1% streptomycin-penicillin solution (GIBCO, USA) at 37 °C in a carbon dioxide incubator with a volume fraction of 5% (HERACELL 240i, Thermo Fisher Scientific, USA).

When the cell density reached about 80%, 0.25% trypsin-EDTA (100 U/mL:100 μg/mL, Solarbio Science & Technology Co., Ltd., China) solution with phenol red was used for passage. The cells were subcultured three times and then used in formal exposure experiments.

2.3. Cell viability

CCK8 method was used to detect the viability of A549 cells after exposure to the coal particles and exogenous AChE recombinant protein by CCK8 kit (BB-4202, Bestbio Co., Ltd., China). The cells with good growth conditions in the logarithmic growth phase were digested with 0.25% trypsin-EDTA solution with phenol red. The cell suspension was prepared and seeded into a 96-well-plate at the density of 5000 cells/well. In the culture wells of the control and exposure groups, 100 μl of cell suspension was added. After 24 h, the culture medium was sucked and discarded. Subsequently, 100 μl of complete culture medium was added into the culture wells of the blank group and the control group. The fresh prepared complete medium (100 μl) containing the prepared coal particles (0, 1, 2, 4, 8, 16 and 32 μg/cm²) and/or the exogenous AChE recombinant protein (0, 0.02, 1 and 5 U/ml) (Sigma-Aldrich, USA) was added into the culture wells of the exposure groups respectively (n = 6), and then cultured in the incubator for 24 h. Finally, The absorbance
values were measured on a multi-detection microplate reader (VICTOR Nivo, PerkinElmer, USA) at 450 nm.

2.4. Determination of oxidative stress indicators (SOD and MDA) and inflammatory factors (IL-6 and TNF-α)

The density of A549 cell suspension was adjusted to 2×10^5 cells/ml and inoculated into 6-well plates. The exposure experiment was carried out when the cell density increased to about 60%. A volume of 2 ml complete culture medium was added into the culture wells of the control group, and the same volume of the complete culture medium containing a certain concentration of the coal particles and/or the exogenous AChE recombinant protein was added into the culture wells of the experimental groups. Then, the 6-well-plates were placed in the incubator for 24 h. After exposure, the cell morphology of each exposure group was observed under light microscope. The supernatant was collected into a precooled centrifuge tube. The levels of IL-6 and TNF-α were measured by the Human IL-6 ELISA Kit and the Human TNF-α ELISA Kit (E-EL-H6156, E-EL-H0109c, Elabscience Biotechnology (Wuhan) Co., Ltd, China), respectively. Then, the 6-well-plates were lightly washed twice with the precooled PBS solution (Hyclone, USA), and the cells were scraped off with a cell scraper and transferred to a clean centrifuge tube. The cells were lysed with a cell ultrasonic crusher. After cryogenic centrifugation, the supernatant was transferred to a clean centrifuge tube. The levels of SOD and MDA in cell lysate were measured by the superoxide dismutase (SOD) assay kit and malondialdehyde (MDA) assay kit (WST-1 method) (TBA method) (A001-3-1, A003-1-2, Nanjing Jiancheng Bioengineering Institute, China), respectively. The protein concentration in the cell lysate was determined by BCA Protein Quantitation Assay (KeyGen Biotech Co., Ltd., China).

2.5. Western blot

After exposure, the culture medium was sucked and discarded, washed twice with precooled PBS, and the cells were scraped off with a cell scraper and transferred to a centrifuge tube to make the cell suspension. The total protein was extracted with the Whole Cell Lysis Assay (KGP250, KeyGen Biotech Co., Ltd.), and the protein concentration was determined by the BCA method. After SDS-PAGE electrophoresis, it was wet-transferred to the PVDF membrane. After blocking with TBST containing 5% skimmed milk for 1 h, the primary antibody (anti-β-actin antibody, anti-E-cadherin primary antibody, anti-Vimentin primary antibody, ProteinTech, USA) was incubated overnight (the dilution ratio was 1:1000 and the incubation temperature was 4°C). The next day, the secondary antibody (ProteinTech, USA) was incubated for 1 h (the dilution ratio was 1:2000 and the incubation temperature was 4°C). After that, the membrane was washed with TBST solution 3 times. Finally, the chemiluminescence imaging system (iBright CL750, Thermo Fisher Scientific, USA) was used for exposure, and the gray value was measured by Image J v1.8.0 software. The ratio of the gray value of the target protein to the gray value of the internal reference protein was calculated as the relative expression of the target protein.

2.6. AChE enzymatic activity-modified Ellman method

The AChE enzymatic activity in the supernatant of A549 cell lysate was determined by the modified Ellman method. Briefly, in each well of the clean 96-well-plate, 80 μl Na2HPO4 solution (20.0 mmol/L), 10 μl DTNB (12.5 mmol/L), 2 μl ISO-OMPA (10.0 mmol/L), 50 μl double distilled water and the sample to be tested were successively added and incubated in dark for 30 min. Subsequently, 50 μl ATCh solution (2.5 mmol/L) was added to each well by using a 12-channel micro-pipette. The OD value was measured every 2 min at the wavelength of 412 nm of the Multimode Plate Reader (VICTOR Nivo, PerkinElmer, USA) for 6 consecutive times. Taking the measurement time as the independent variable and OD value as the dependent variable, the scatter diagram was drawn and fitted linearly. The calculated slope value was the relative expression of the AChE enzymatic activity in the samples. The AChE enzymatic activity was expressed as specific activity units (the amount of enzyme that can convert 1 μmol of substrate in 1 min is 1 international unit). The above results were standardized with total protein concentration.

2.7. Total RNA extraction, reverse transcription and RT-qPCR

After exposure, the culture medium was sucked and discarded, and washed twice with precooled PBS. The total RNA of A549 cells was extracted by RNAsimpleTotal RNA Kit (DP419, TIANGEN Biotech (Beijing) Co., Ltd.), the quality and integrity of the extracted total RNA was detected by ultra micro ultraviolet spectrophotometer (Nanodropone, Thermo Fisher Scientific, USA). Then, the total RNA was reverse transcribed by FastKing gDNA Dispelling RT SuperMix Kit (KR118, TIANGEN Biotech (Beijing) Co., Ltd.), and the genes were amplified by SuperReal PreMix Plus Kit (SYBR Green) (FP205, TIANGEN Biotech (Beijing) Co., Ltd.). The relative expression of mRNAs was calculated based on the 2^-ΔΔCt method with β-actin serving as the internal reference. The primers sequences and other relevant information of the reference gene and the target genes were shown in supplemental files (Table S1). The primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd., China.

2.8. Statistical analysis

SPSS 26.0 software was used for statistical analysis. The experimental data were expressed as mean ± standard deviation (SD), and were analyzed by homogeneity test of variance, followed by one-way ANOVA and the LSD method. A value of P < 0.05 was considered significant and the significance ABC method was used to mark significance.

3. Results

3.1. Characterization of the prepared coal particles

Table 1 showed the composition of the coal particles. Figure 3a and b showed the coal particles observed under the scanning electron microscope. It could be seen that the particle sizes of these 2 kinds of coal particles were less than 5 μm. The contents of free silica in GBW11110K and GBW11126D were 2.70% and 5.78% respectively. The particle sizes (uniformly dispersed in pure water system) were between 1 and 2 μm. The determination results of zeta potential indicated that the prepared coal particles were relatively stable (Supplementary materials).

3.2. Effects of exposure to the coal particles or the exogenous AChE recombinant protein on the viability of A549 cells

It could be seen that when the coal particles were exposed to A549 cells, the cell viability decreased gradually (Figure 2a and b). Compared with the control group, the cell viability of GBW11110K exposed to 8, 16 and 32 μg/cm² for 24 h decreased significantly by 21.6%, 37.87%, and 41.99%, respectively (P < 0.05). Similarly, the cell viability of GBW11126D exposed to 8, 16 and 32 μg/cm² for 24 h decreased significantly by 14.34%, 25.18%, and 33.33%, respectively (P < 0.05). During the exposure experiment, it could be found that agglomeration was easy to occur when the exposure dose was too high. In the subsequent experiment, doses (0, 1, 2, 4, and 8 μg/cm²) that had no significant or minor effect on cell viability were studied in depth.

Compared with the control group, exposure to the exogenous AChE recombinant protein at concentrations of 0.02, 1 and 5 μg/ml for 24 h had no significant effects on the viability of A549 cells. Therefore, these 3 doses were selected as the exposure doses for the follow-up experiments (Figure 2c).
3.3. Effects of exposure to the coal particles and/or the exogenous AChE recombinant protein on oxidative stress indicators and inflammatory factors in A549 cells

Compared with the control group, there was no significant change in cell morphology in the coal particle exposed groups (data not shown). The levels of SOD in A549 cells in the groups exposed to the coal particles alone decreased significantly, while the levels of MDA, IL-6 and TNF-α increased significantly ($P < 0.05$) (Figure 3a–d). Taking 4 μg/cm² exposure group as an example, when exposed to GBW11110K, SOD, MDA, IL-6 and TNF-α in A549 cells were changed by $-36.9\%$, $+18.4\%$, $+66.6\%$ and $+28.4\%$ of the control groups. When exposed to GBW11126D, SOD, MDA, IL-6 and TNF-α in A549 cells were changed by $-50.8\%$, $+19.2\%$, $+158\%$ and $+25.2\%$ of the control groups. Therefore, combined with the results of the Part 2.2, in the combined exposure group, the exposure dose of the coal particles was selected as 4 μg/cm² (Figure 3a–d).

Compared with the group exposed to the prepared coal particles alone, the levels of SOD in the combined exposure groups of exogenous AChE recombinant protein and the coal particles increased, while the contents of MDA, IL-6 and TNF-α decreased ($P < 0.05$), even though it could not be completely restored to the control levels. For example, the TNF-α content in the combined exposure group of 5 U/ml AChE recombinant protein and 4 μg/cm² GBW11110K was equivalent to 88% of that in the single exposure group of 4 μg/cm² GBW11110K ($P < 0.05$).

3.4. Effects of exposure to the coal particles and/or the exogenous AChE recombinant protein on EMT related markers in A549 cells

Compared with the control group, with the increase of exposure dose, the protein level of intracellular E-cadherin showed a downward trend, while the level of Vimentin showed an upward trend. Specifically, when the exposure concentration of GBW11110K was 8 μg/cm² and the concentration of GBW11126D was 4 and 8 μg/cm², the protein level of E-cadherin significantly decreased by 21.1%, 16.4%, and 19.5%, respectively ($P < 0.05$). When the exposure concentration of GBW11110K was 1, 2, 4 and 8 μg/cm² and the concentration of GBW11126D was 4 and 8 μg/cm², the protein level of Vimentin significantly increased by 84.7%, 99.9%, 113%, 119%, 58.7%, and 79.9%, respectively ($P < 0.05$) (Figure 4a and b).

Table 1. Composition and proportion of the coal particles used in this study.

| Reference material No. | Total sulfur (%) | Ash content (%) | Volatile matter (%) | Carbon (%) | Vacuum relative density (20 °C) | Calorific value (%) | Free silica (%) |
|------------------------|-----------------|-----------------|--------------------|------------|---------------------------------|--------------------|---------------|
| GBW11110K             | 4.31            | 42.25           | 16.85              | 45.39      | 1.84                            | 17.96              | 2.70          |
| GBW11126D             | 0.16            | 14.05           | 6.22               | 80.75      | 2.02                            | 26.99              | 5.78          |

Figure 1. Characterization of the prepared coal particles observed under a scanning electron microscope ($\times 3.00k$). (a) GBW11110K. (b) GBW11126D.

Figure 2. Effects on the viability of A549 cells at 24h. Values were expressed as mean ± SD, $n = 6$. *$P < 0.05$ vs control (a) GBW11110K. (b) GBW11126D. (c) Exogenous AChE recombinant protein.
Figure 3. The expression levels of oxidative stress indicators and inflammatory factors in A549 cells after exposure to the prepared coal particles and/or the exogenous AChE recombinant protein. Values were expressed as mean ± SD (n = 3). If there is the same superscript letter between different groups, it means that there is no significant difference between groups. Conversely, it means that the differences are statistically significant. (a) SOD (U/mg prot). (b) MDA (pmol/mg prot). (c) IL-6 (ng/L). (d) TNF-α (ng/L).
Compared with the single exposure group of 4 μg/cm² GBW11110K and GBW11126D, the level of E-cadherin protein in the combined exposure group increased to a certain extent while the level of Vimentin protein showed a certain degree of downward trend with the increase of the concentration of exogenous AChE recombinant protein (Figure 4c and d).

For example, compared with 4 μg/cm² GBW11110K exposure group, the level of E-cadherin protein in cells in the AChE recombinant protein (1, 5 U/ml) and 4 μg/cm² GBW11110K combined exposure group was significantly increased by 28.9% and 58.6%, respectively (all \( P < 0.05 \)). The level of Vimentin protein in cells in the combined exposure group of AChE recombinant protein (0.02, 1, 5 U/ml) and 4 μg/cm² GBW11110K...
significantly declined respectively by 28.7%, 37.3%, and 39.1%, compared with the single GBW11110K exposure group (all \( P < 0.05 \)).

3.5. Effects of exposure to the coal particles and/or the exogenous AChE recombinant protein on the AChE enzymatic activity in A549 cells

Compared with the control group, the AChE enzymatic activity in A549 cells in the prepared coal particles alone groups decreased significantly (\( P < 0.05 \)). For GBW11110K, the AChE enzymatic activity in A549 cells in 8 \( \mu \)g/cm\(^2\) group decreased by 40.0% significantly (\( P < 0.05 \)). For GBW11126D, the AChE enzymatic activity in A549 cells in 4 \( \mu \)g/cm\(^2\) group decreased by 56.6% significantly (\( P < 0.05 \)) (Figure 5a). When the different concentrations of exogenous AChE recombinant protein were added, the AChE enzymatic activity in A549 cells raised with the increase of the concentration of exogenous AChE recombinant protein (Figure 5b). Specifically, the AChE enzymatic activity increased from 9.36 U to 18.43 U (GBW11110K), from 7.33 U to 12.76 U (GBW11126D), after exposed to 5 U/ml exogenous AChE recombinant protein and the prepared coal particles.

3.6. Effects of exposure to the coal particles and/or the exogenous AChE recombinant protein on the mRNA expression of the different subtypes of acetylcholine receptors (AChRs) in A549 cells

It could be seen from Figure 5 that with the increase of exposure dose of the prepared coal particles, the relative mRNA expression of the

![Figure 5](image-url)

Figure 5. AChE enzymatic activity and the mRNA expression of the different subtypes of AchRs in A549 cells after exposure to the prepared coal particles and/or the exogenous AChE recombinant protein for 24h. Values were expressed as mean ± SD, \( n = 3 \). If there is the same superscript letter between different groups, it means that there is no significant difference between groups. Conversely, it means that the differences between groups are statistically significant. (a) The prepared coal particles. (b) The combined exposure of exogenous AChE recombinant protein and the prepared coal particles. (c) GBW11110K. (d) GBW11126D. (e) The different doses of exogenous AChE recombinant protein and 4 \( \mu \)g/cm\(^2\) GBW11110K. (f) The different doses of exogenous AChE recombinant protein and 4 \( \mu \)g/cm\(^2\) GBW11126D.
different subtypes of AChRs in A549 cells changed differently. CHRM3 gradually increased when exposed to these two kinds of prepared coal particles (all \( P < 0.05 \)). The expression of CHRM5, CHRNA5 and CHRNA7 decreased gradually when exposed to these 2 kinds of coal particles (all \( P < 0.05 \)). CHRNA9 gradually increased when exposed to GBW11126D compared with control (\( P < 0.05 \)), while the expression had no significant change after exposure to GBW11110K. The expression of CHRN2B2 had no significant change after exposure to these 2 kinds of coal particles (Figure 5c and d). The expression of CHRM3 in 2, 4 and 8 \( \mu \text{g/cm}^2 \) GBW11110K exposure groups were equivalent to 149%, 218% and 201% of the control group, respectively (\( P < 0.05 \)). Similarly, the expression levels of CHRM3 in 1, 2, 4 and 8 \( \mu \text{g/cm}^2 \) GBW11126D exposure groups were equivalent to 162%, 149%, 154% and 298% of the control group, respectively (all \( P < 0.05 \)).

After treatment with the exogenous AChE recombinant protein, the relative mRNA expressions of CHRM5, CHRNA5 and CHRNA7 were increased compared with the group exposed to the prepared coal particles alone (\( P < 0.05 \)), suggesting that the addition of exogenous AChE recombinant protein could restore coal particle-induced interference effects on the relative expression of the different subtypes of AChRs mRNA to a certain extent. For example, the mRNA expression level of intracellular CHRM5 in A549 cells after exposed to 4 \( \mu \text{g/cm}^2 \) GBW11110K was equivalent to 29% of that in the control group (\( P < 0.05 \)), while the expression levels in 0.02, 1, 5 U/ml exogenous AChE recombinant protein and 4 \( \mu \text{g/cm}^2 \) GBW11110K were equivalent to 179%, 331% and 421% of 4 \( \mu \text{g/cm}^2 \) GBW11110K group (\( P < 0.05 \)) (Figure 5e and f).

4. Discussion

4.1. Pneumoconiosis is a worldwide occupational disease

If you take it for granted that the shadow of pneumoconiosis only appears historically in one or some countries, you are completely wrong. It remains one of the most severe pulmonary fibrotic diseases worldwide. What’s worse, recently, there has been a worldwide resurgence in pneumoconiosis due to occupational mineral dust exposure. For example, in Queensland, Australia, there has been a re-emergence of coal workers’ pneumoconiosis and silicosis. Some coal mining communities have experienced a resurgence of progressive massive fibrosis in the USA and a worldwide epidemic is occurring of accelerated silicosis due to exposure to artificial stone (Blackley et al., 2018; Wood and Yates, 2020). Israel, Turkey and other countries around the world have recently experienced significant outbreaks of silicosis. These outbreaks even have occurred in modern industries, including but not limited to denim jean production, benchtop fabrication and jewelry polishing, where silica has been introduced without recognition and control of the hazard (Barnes et al., 2019).

Given that pneumoconiosis is a preventable but incurable lung disease, a clear understanding of which components are the etiological factors of disease development and may provide information for the determination of new treatment strategies. It is noteworthy to mention that the pneumoconiosis is synergistic effect of coal, silica dust, rock dusting particles, and diesel particulate matter (Zhang et al., 2021). The severity of dust related diseases is well known to be correlated with mass concentration, exposure duration and particle size, especially chemical components (Christian et al., 1979). Different coal types have different compositions, and thus result in varied toxicological effects. For instance, Song et al. reported that the magnitude of the cytotoxic response and cytokine production in epithelial cells and macrophages, as well as the fibroblast response, varied considerably among different types of coals (Song et al., 2022).

4.2. EMT is closely related to the pathogenesis of pneumoconiosis

As mentioned in the introduction, countless researchers have studied the pathogenesis of pneumoconiosis for more than a century and put forward many different theories, but so far they are still unable to fully explain its pathogenesis. It is undeniable that oxidative stress and inflammatory response play an important role in the pathogenesis of pneumoconiosis (He et al., 2022). Frankly speaking, it is far from enough for us to understand the whole (complete/integrated) pathogenesis of pneumoconiosis only by discussing these molecules that have been repeatedly confirmed. Let’s point the spotlight at EMT, which is closely related to pulmonary fibrosis (Chen et al., 2021).

After entering the body, dust particles can transform the cell phenotype from epithelial to interstitial like cells by stimulating alveolar epithelial cells. This process is called EMT. It is not only an important source of fibroblasts, but also can interact with various kinds of immune cells (Luo et al., 2022). For example, TCGA data analysis identified an inverse association between EMT and T-cell infiltration in nonsmall cell lung cancer (NSCLC) (Chae et al., 2018). As an important mechanism for promoting fibrosis, EMT has attracted more and more attention. Briefly, EMT mainly includes the following 4 key steps. Firstly, epithelial cells lose the ability to adhesion. The cell morphology becomes fibroblast like, irregular and pseudopodia appeared. Secondly, the expression level of α-SMA is significantly up-regulated and actin is recombined. Thirdly, the basement membrane of cells is destroyed and the expression level of MMPs is significantly increased. Fourthly, the ability of cell migration increases gradually. The cellular signaling pathways related to EMT mainly include TGF-β/Smad pathway, Ras/Raf/MAPK pathway, NF-κB pathway, Notch signaling pathway, etc.

4.3. NNCS is inextricably linked with the occurrence of EMT

There is no doubt that, according to our current cognition, it is difficult for us to draw the whole story of EMT in one picture. However, if we open any EMT themed book published in recent years, we can see the “figure” of the NNCS. Several studies have shown that the NNCS is closely related to EMT (Chen et al., 2019a,b; Feng et al., 2018).

Although the neuronal cholinergic system is better known and talked about, with the deepening of research, researchers have found that acetylcholine can be synthesized in a variety of tissues or cells and released to extracellular fluid in the form of autocrine and paracrine, acting on acetylcholine receptors (AChRs) and participating in the regulation of diverse physiological and pathophysiological processes such as immune and inflammatory responses, wound healing, cancer development and progression, and cardiovascular, respiratory, digestive, and orthopedic diseases (Chen et al., 2019a,b; Fujii et al., 2017; Mashimo et al., 2021). It is called NNCS (this concept was first proposed by researchers at the 73rd annual meeting of Japanese Pharmacology in 2000) (Wessler and Kirkpatrick, 2008). For example, the expression of a variety of cholinergic system components can be detected in human respiratory epithelial cells, including but not limited to ChAT, high affinity cholines

transporters (ChT1) and VAChT, which play non-neural functions such as cell adhesion, mucus secretion and cell apoptosis (Montalbano et al., 2018).

Next, let’s take a look at how the coal particles make waves in our research.

4.4. Exposure to coal particles can lead to changes in oxidative stress, inflammatory response and EMT related markers, and affect the expression of key molecules of the NNCS in A549 cells

In the initial stage of pneumoconiosis mechanism research, researchers found that with the progress of pneumoconiosis pathological process, the balance of oxidative stress was broken (Liu et al., 2013). At the same time, the body occurs a chronic and progressive inflammatory reaction (Fan et al., 2022). When threatened by environmental factors, the body produces SOD which is the first line of defense against oxidative stress (Pardo and Selman, 2021). It is used to remove free radicals produced when lipid peroxidation occurs in the membrane (Li et al., 2021). Its content is gradually consumed with the increase of dust exposure and...
the extension of time. MDA is the end product of the peroxidation decomposition of polyunsaturated fatty acids in biofilm. It ages tissues by crosslinking nucleic acids and other macromolecules. Its content can be used as one of the quantitative indicators of the damage degree of biofilm. TNF-α is secreted by Th1 cells, which can directly or indirectly promote the development of EMT (Ramundo et al., 2021). IL-6, as an important regulator of TNF-α, is secreted by Th2 cells, which has been shown to predict lung injury prior to pathological changes on X-ray (Peruzzi et al., 2019). Significantly, the results of literature research showed that both oxidative stress and inflammatory reaction are closely related to the pathological process of EMT (Giannoni et al., 2012; Suarez-Carmona et al., 2017).

In this study, with the increase of the coal particle exposure concentration, SOD decreased gradually, while IL-6 TNF-α related markers induced by the coal particles. The content of SOD increased while the contents of IL-6, TNF-α and MDA decreased, which was consistent with the research by other scholars (Li et al., 2022; Qin et al., 2021), indicating that coal particles could break the oxidation and antioxidant balance, induce inflammatory response and then promote the occurrence and development of EMT of A549 cells.

A-cadherin (epithelial cell marker) and vimentin (mesenchymal cell markers) are marker proteins in the process of EMT, which can indicate the occurrence and development of EMT. In this study, with the increase of the coal particle exposure concentration, E-cadherin protein gradually decreased while Vimentin protein gradually increased, suggesting that coal particles could promote the occurrence of EMT in A549 cells. Wu et al. also found the protein expression level of E-cadherin in the lung tissues was decreased, while the protein expression levels of Vimentin showed the opposite trend by constructing a mouse model of pulmonary fibrosis by intratracheal instillation of silica particles (Wu et al., 2021).

Of course, it is not enough to know that coal particles can affect the classic biomarkers of EMT. We would like to know whether the coal particle exposure could affect the expression levels of key molecules of the NNCS. Objectively speaking, the discovery of the NNCS in respiratory system provides a great deal of novel possibilities and paths for the basic research and clinical treatment of chronic airway diseases (such as pneumoconiosis, asthma and COPD) (Hollenhorst and Krasteva-Christ, 2021). Studies have shown that cholinesterase receptors can mediate the pathological process of pulmonary fibrosis. For example, cigarette smoke exposure could up-regulate the expression levels of myofibroblasts markers, which was closely related to the activation of mAChRs in human bronchial fibroblasts (Milara et al., 2013). For another example, aclidinium, an M3 mAChR antagonist, dose-dependently inhibited the transition of human lung fibroblast to a myofibroblast contractile phenotype caused by carbachol and TGF-β1 stimulation and also reduced fibroblast proliferation and migration (Kilić et al., 2020; Milara et al., 2013). The latest research shows that long-acting muscarinic receptor antagonists (LAMAs) are the cornerstone for the treatment of COPD (Cazzola et al., 2021).

In this study, the results showed that with the increase of the coal particle exposure dose, the AChE enzymatic activity in A549 cells decreased significantly, and the relative expression of acetylcholine receptor mRNA also changed. Specifically, compared with the control group, with the increase of the coal particle exposure dose, the relative mRNA expression of CHRM3 and CHRNA9 increased gradually, the relative mRNA expression of CHRM5, CHRNA5 and CHRNA7 decreased gradually, while the relative mRNA expression of CHRNA2 did not change significantly. Overall, the effects of these 2 kinds of coal particles on the NNCS expression levels of different types of cholinesterase receptors were highly similar. Combined with the existing research on the relationship between cholinesterase receptors and pulmonary fibrosis, we can speculate that CHRM3 plays a more important role in coal particle induced pro-EMT effects (of course, this does not mean that the others have no value of attention).

Up to now, we have known that the coal particles can affect the occurrence of EMT in A549 cells, and also known that coal particle exposure affects the expression levels of key molecules of the NNCS. However, what we want to know more is whether the human intervention method of adding exogenous AChE recombinant protein can reverse the changes of EMT related markers caused by coal particle exposure.

4.5. Exogenous AChE recombinant protein can reverse the above damage effects caused by the coal particles to a certain extent

Compared with the coal particle exposure group, the following changes occurred in A549 cells exposed to the exogenous AChE recombinant protein and the coal particles: AChE enzymatic activity increased. The content of SOD increased while the contents of IL-6, TNF-α and MDA decreased. Most importantly, it could reverse the changes of EMT related markers caused by the coal particle exposure to a certain extent. These results suggest that the key molecules of the NNCS plays an important role in counteracting oxidative stress, inflammatory response and EMT related markers induced by the coal particles.

5. Conclusion and perspectives

The coal particle (GBW11110K and GBW11126D) exposure could induce oxidative stress (SOD↑, MDA↓) and inflammatory response (IL-6↑, TNF-α↑), EMT related markers (E-cadherin↓, vimentin↑), down-regulate the AChE enzymatic activity, and interfere the mRNA expression levels of AChRs (CHRM3 (↓), CHRM5 (↓), CHRNA5 (↓↓), CHRNA7 (↓↓), CHRNA9 (↓↓), CHRN11 (↓), CHRN16 (↓), CHRNA2 (↓)). In A549 cells. After exogenous AChE recombinant protein was added, promotion of the expression of oxidative stress indicators and inflammatory factors, EMT related markers, inhibition of AChE enzymatic activity, and expression interference of different types of AChRs, caused by coal particles were antagonized to a certain extent.

Next, we should use in vivo and in vitro experimental methods, combined with molecular biological techniques such as gene silencing and overexpression, to explore and verify the key nodes of EMT antagonistic effect mediated by the NNCS, and further determine the possibility of antagonizing EMT in primary cells and experimental animals (such as rats and mice) by interfering with key molecules of the NNCS.

Declarations

Author contribution statement

Meng-Yu Wu; Xin-Chen Shi: Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.
Jing Shan: Contributed reagents, materials, analysis tools or data; Wrote the paper.
Rui Wang; Yi Wang; Jie Li: Analyzed and interpreted the data; Performed the experiments; Contributed reagents, materials, analysis tools or data.
Da-Nian Tian: Conceived and designed the experiments.
Hai-Ming Xu: Conceived and designed the experiments; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interest’s statement

The authors declare no conflict of interest.
Additional information

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