

TNFR/TNF- α signaling pathway regulates apoptosis of alveolar macrophages in coal workers' pneumoconiosis

Qing-Zeng Qian¹, Xiang-Ke Cao², Hai-Yan Liu¹, Guo-Ying Zheng¹, Qing-Qiang Qian³ and Fu-Hai Shen¹

¹School of Public Health, North China University of Science and Technology, Tangshan 063000, P.R. China

²College of Life Sciences, North China University of Science and Technology, Tangshan 063000, P.R. China

³Department of Neurology, Tangshan Gongren Hospital Affiliated to North China University of Science and Technology, Tangshan 063000, P.R. China

Correspondence to: Fu-Hai Shen, **email:** fuhai_sh@163.com

Keywords: coal mixture workers, coal workers' pneumoconiosis, cumulative dust exposure, alveolar macrophages, apoptosis

Received: July 27, 2016

Accepted: May 23, 2017

Published: July 01, 2017

Copyright: Qian et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License 3.0 (CC BY 3.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

ABSTRACT

We explored the role of TNFR/TNF- α signaling in apoptosis among alveolar macrophages (AM) and its relevance to the development of coal workers' pneumoconiosis (CWP). Purified alveolar macrophages (AMs) were prepared from bronchoalveolar lavage fluid harvested from 366 CWP patients and 120 healthy subjects enrolled in the study. The purified AMs were then divided into control, SOD, anti-TNFR, TNFR and NF κ B inhibitor groups and analyzed for apoptosis using flow cytometry (sub-diploid peak) and western blotting (Bcl-2, Caspase-3 and Caspase-8 expression). We found that AM apoptosis was higher among CWP patients than the healthy controls. Expression of Bcl-2, Caspase-3 and Caspase-8 was higher in AMs from CWP patients than in those from the controls and correlated with increased AM apoptosis. Univariate and multivariate analyses suggested that CWP grade, initial exposure time, exposure time in years, and CWP onset age were all associated with altered levels of Bcl-2, Caspase-3 and Caspase-8. Inhibition of TNFR/TNF- α signaling using anti-TNFR antibody, SOD or NF κ B inhibition reduced AM apoptosis and decreased Bcl-2, Caspase-3 and Caspase-8 expression. These data suggest inhibition of a TNFR/TNF- α signaling pathway is a potentially effective means of alleviating CWP by inhibiting AM apoptosis.

INTRODUCTION

Although there is increased emphasis on physical health and regular health examinations at work places, occupational disease rates have increased significantly with rapid development of China's coal industry [1–3]. Long-term exposure of mining workers to coal dust contributes to increased risk of chronic occupational lung diseases and irreversible lung damage [4, 5, 6]. Coal workers' pneumoconiosis (CWP) is one of the most common lung diseases associated with different degrees of pulmonary function abnormalities in coal mining workers [7]. It is one of the most serious occupational diseases in China that results in lung fibrosis due to long-term inhalation of coal dust [8, 9]. Previous studies have associated long-term exposure

to coal mine dust with CWP mortality [10]. Stringent enforcement of occupational exposure limits for coal mine dust has resulted in declined prevalence of CWP among underground coal miners [11]. However, miners still working in contemporary conditions continue to develop lung diseases, thereby implying that further restrictions and regulations in occupational dust exposure are necessary [12].

Apoptosis refers to a genetically controlled program of cell death that is involved in elimination of old or unhealthy cells that could be detrimental to organismal health. In recent years, investigation of CWP pathogenesis has revealed that apoptosis of alveolar macrophages (AM) is involved in development of pneumonia and pulmonary fibrosis [13]. The three major signal transduction pathways that regulate AM apoptosis include the mitochondrial pathway, the

endoplasmic reticulum pathway, and the membrane-associated cell death receptor pathway [14–17]. The cell death receptors belonging to the members of the TNFR super-family initiate apoptosis upon binding to their ligands and operate mainly through three basic signal transduction pathways namely, Fas/Fas ligand (FasL), TNFR/TNF- α , and TRAILR/TRAIL [18, 19, 20]. These pathways ultimately modulate critical players of apoptosis including initiator and executioner caspases, P53, Bcl-2 and other apoptotic regulatory proteins [21]. Therefore, blocking the death receptor mediated apoptosis of AM is probably an effective measure to prevent or cure CWP [22]. Therefore, in this study, we explored the role of TNFR/TNF- α signaling pathway in AM apoptosis and association to CWP. We also investigated if inhibition of TNFR/TNF- α signaling could provide a therapeutic basis for the early prevention and treatment of CWP.

RESULTS

Baseline characteristics of CWP patients

In the exposed group, the mean age of the 366 coal mine workers was 47.8 ± 9.2 years (range: 24 to 65 y) and the mean body weight of included subjects was 57.7 ± 10.3 kg (range: 42–86 kg). In the control group, the mean age of the 120 coal mine workers was 48.2 ± 8.4 years (range: 28 to 68 years) and the mean body weight of control subjects was 56.8 ± 10.5 kg (range: 43–85 kg). As shown, there were no significant differences between the exposed and the control group with regard to mean age and body weight (both $P > 0.05$). The other baseline characteristics including smoking status, initial working time, occupational history, current occupation, retirement period and others were all similar between the exposed and the control groups (all $P > 0.05$; Table 1).

Apoptosis status of AM in CWP patients

Figure 1 shows the representative images of control and CWP patient alveolar macrophages (AM). The mature normal AM from control subjects showed increased cell volume, integral cell membrane, round or oval shaped cell-centric nucleus and were free of intracellular dust particles. The apoptotic AM cells from CWP patients showed marginalized chromatin and fragmented nucleus with massive apoptotic bodies typical of apoptosis.

Comparison of AM apoptosis among different subgroups of CWP patients

The apoptotic AM cells were analyzed by flow cytometry and the sub-diploid peak representing the AM cells undergoing apoptosis is shown in Figure 2. Our analysis showed that AM cells from CWP stage I and stage II patient groups showed significantly higher

apoptosis than those from normal controls (both $P < 0.05$). Further, apoptosis in AM cells from CWP stage I patients was significantly enhanced compared to those from CWP stage II patients ($P < 0.05$). Table 2 shows the apoptotic index of subjects in the exposed group stratified by age, smoking, initial exposure time, exposure years, and CWP onset age. Except for the subgroup representing different exposure years ($P < 0.05$), there was no significant difference in apoptosis between other subgroups (all $P > 0.05$).

Analysis of the expression of key apoptotic regulatory proteins in AM from CWP patients

As shown in Table 3, we observed that the expression of key apoptotic regulatory proteins, Bcl-2, Caspase-3 and Caspase-8 was significantly enhanced in the exposed group compared to those in the control group (all $P < 0.05$). Besides, there were positive correlation between elevated expression of Bcl-2, Caspase-3 and Caspase-8 and enhanced AM apoptosis (all $P < 0.05$).

Furthermore, we conducted stratified analyses investigating the association between the expression levels of Bcl-2, Caspase-3 and Caspase-8 with factors like age, smoking, CWP stages, initial exposure time, exposure years, and CWP onset age (Table 4). Our data showed significant association between the expression levels of Bcl-2, Caspase-3 and Caspase-8 and different subgroups based on age, smoking, initial exposure time, exposure years, and CWP onset age (all $P < 0.05$). In addition, multivariate analysis indicated that altered expression levels of Bcl-2, Caspase-3 and Caspase-8 significantly correlated with CWP stages. Besides, initial exposure time, exposure years, and CWP onset age correlated with the expression levels of Caspase-3. Also, Caspase-8 expression levels were associated with exposure years, whereas expression levels of Bcl-2 were influenced by CWP onset age (all $P < 0.05$).

Regulatory role of TNFR/TNF- α signal pathway in AM apoptosis from CWP patients

Next, we investigated the role of TNFR/TNF- α signaling pathway in regulating AM apoptosis. We observed that the apoptotic index of AM in the SOD group (14.23 ± 2.01) was significantly lower than that in the control group (23.47 ± 2.68) and the TNFR group (18.33 ± 1.80). Furthermore, the apoptotic index of AM in the anti-TNFR group (14.00 ± 1.45) and NF- κ B inhibitor group (13.52 ± 1.77) were also significantly reduced when compared to the TNFR group (all $P < 0.05$).

Consequently, the expression level of Bcl-2, Caspase-3 and Caspase-8 were significantly decreased when TNFR/TNF- α signaling pathway was blocked SOD and was least compared to all other 4 groups (all $P < 0.05$). In addition, blocking the TNFR/TNF- α signal

Table 1: Comparisons in smoking rate, pulmonary function and pulmonary function indexes between the case group and the control group

Group	Exposed group (n = 366)	Control group (n = 120)	χ^2/t	P
Age (year)	47.80 ± 9.20 (24~65)	48.20 ± 8.40 (28~68)	0.422	0.673
Weight (kg)	57.70 ± 10.30 (42~86)	56.80 ± 10.50 (43~85)	0.409	0.827
Smoking distribution				
Non-smoking	165 (45.08%)	57 (47.50%)	0.462	0.645
Smoking	201 (54.92%)	63 (52.50%)		
Initial working time	21.10 ± 4.16	22.00 ± 5.50	1.890	0.059
Work years	23.50 ± 5.85	24.34 ± 6.78	1.311	0.191
Retirement period	3.83 ± 1.11	4.05 ± 1.06	1.905	0.057

Note: Initial working time, the starting age of workers exposed to dust; work years, the length of the time employed; retirement period, the duration after stopping dust exposure of workers exposed to dust.

pathway with anti-TNFR antibody or NFkB inhibitor significantly reduced expression levels of Bcl-2, Caspase-3 and Caspase-8 compared with the control group and the TNFR group (all $P < 0.05$). The data are shown in Figure 3 and Table 5.

DISCUSSION

CWP is a result of long-term inhalation of coal mine and silica dust that results in impaired pulmonary function and lung diseases, thereby representing a relevant occupational hazard for coal miners [8]. Despite dust control and reduction technology being utilized in the recent decades, the morbidity of CWP has shown increased incidence [23]. Since the mechanistic details of CWP pathogenesis are not clear, the prevention and treatment of CWP has been a challenge. Hence, it is very important to explore mechanisms that promote CWP in order to obtain greater understanding of this disease and identify effective treatment methods. *In vivo* and *in vitro* studies have shown

that AM plays an important role in CWP [24, 25]. The dust particles that enter the alveoli are phagocytosed by the AM cells that results in their activation and caspase-dependent apoptosis. In the present study, we investigated the role of TNFR/TNF- α signaling pathway in the pathogenesis of CWP and explored if it represented a relevant strategy to inhibit CWP.

Apoptosis is a systematic program of cell death which is initiated by specific signaling pathways in response to either external or intracellular stimuli and executed by sequential activation of specific apoptosis regulatory proteins like caspases [26, 27]. Cellular apoptosis is an important physiological mechanism in multicellular organisms, which is critical for maintaining body's normal development and homeostasis and is involved in organ development, tissue repair and immune regulation [28, 29]. In the present study, we observed that alveolar macrophages showed typical characteristics of apoptosis like chromatin marginalization and nuclear fragmentation into massive intracellular apoptotic bodies [30]. Our study

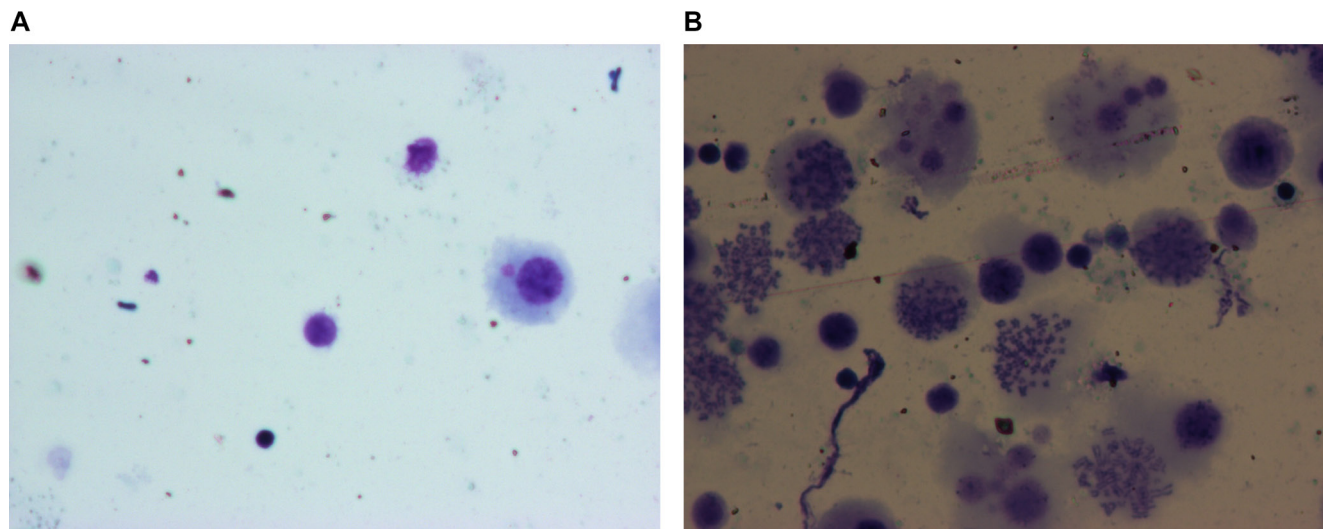


Figure 1: Representative H&E stained photographs of alveolar macrophages undergoing apoptosis as observed under light microscope (1000 \times). (A) Alveolar macrophages from normal controls; (B) Apoptotic alveolar macrophages from CWP patients.

Table 2: Comparisons of alveolar macrophages apoptosis among different subgroups based on age, smoking, initial working time, occupational time, and working age for occurrence in the exposed group

Group	N	Apoptotic index	χ^2/t or <i>F</i>	<i>P</i>
Age				
< 40	156	27.22 ± 5.47	0.597	0.551
≥ 40	210	26.88 ± 5.25		
Smoking (duration time, years)				
0	165	26.52 ± 5.40	1.876	0.155
< 30	88	26.28 ± 5.07		
≥ 30	113	27.62 ± 5.89		
Initial working time (years)				
< 20	144	26.69 ± 5.44	0.791	0.429
≥ 20	222	27.18 ± 6.00		
Work years (years)				
< 20	170	23.02 ± 5.12	12.290	< 0.001
≥ 20	196	29.98 ± 6.12		
Working age for occurrence				
< 10	130	27.33 ± 5.85	1.403	0.161
≥ 10	136	26.48 ± 5.48		

Table 3: Comparisons of the expression levels of Bcl-2, Caspase-3 and Caspase-8 in the exposed group

Group	Case group	Control group	χ^2/t	<i>P</i>
Bcl-2	0.291 ± 0.044	0.211 ± 0.038	17.850	< 0.001
Caspase-3	0.338 ± 0.050	0.267 ± 0.040	14.140	< 0.001
Caspase-8	0.237 ± 0.041	0.219 ± 0.034	3.137	0.002

Note: these results were not influenced following the adjustment for cofounders such as age, smoking history and other baseline factors.

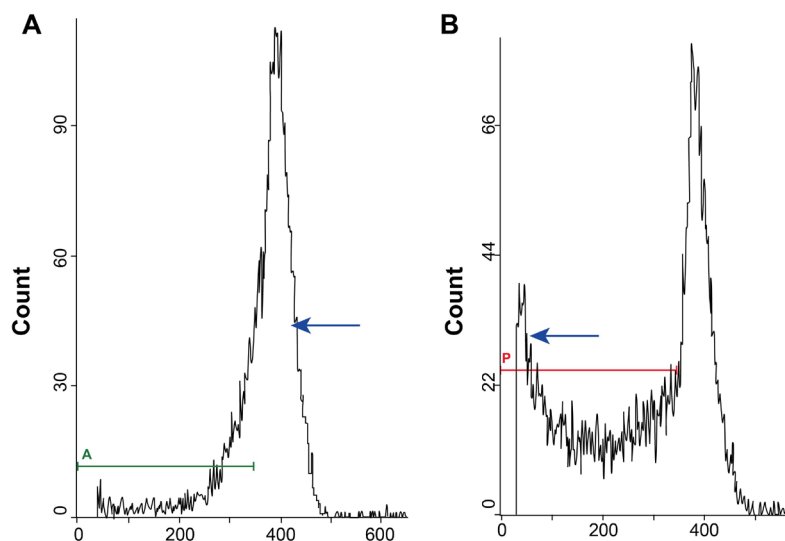


Figure 2: Flow cytometry analysis of apoptotic alveolar macrophages cells showing the sub-diploid (sub-G0/G1) peak. (A) FACS plots showing normal peaks for control alveolar macrophages; (B) FACS plot showing sub-diploid peak representing alveolar macrophages undergoing apoptosis.

Table 4: Comparisons of the expression levels of Bcl-2, Caspase-3 and Caspase-8 among different subgroups based on age, smoking, initial working time, occupational time, and working age for occurrence in the exposed group

Group	N	Bcl-2	Caspase-3	Caspase-8
Age				
< 40	156	0.356 ± 0.047	0.354 ± 0.047	0.032 ± 0.045
≥ 40	210	0.310 ± 0.035*	0.322 ± 0.042*	0.038 ± 0.043
Smoking (duration time, years)				
0	165	0.345 ± 0.054	0.401 ± 0.054	0.376 ± 0.050
< 30	88	0.336 ± 0.048	0.367 ± 0.041*	0.382 ± 0.056
≥ 30	113	0.342 ± 0.043	0.375 ± 0.050*	0.367 ± 0.048
Initial working time (years)				
< 20	144	0.288 ± 0.043	0.376 ± 0.055	0.256 ± 0.035
≥ 20	222	0.231 ± 0.034*	0.345 ± 0.036*	0.218 ± 0.040*
Working years (years)				
< 20	170	0.288 ± 0.060	0.377 ± 0.062	0.236 ± 0.056
≥ 20	196	0.243 ± 0.051*	0.332 ± 0.054*	0.228 ± 0.047
Working age for occurrence				
< 10	130	0.227 ± 0.057	0.376 ± 0.067	0.321 ± 0.046
≥ 10	136	0.234 ± 0.048	0.337 ± 0.068*	0.327 ± 0.050

Note: * $P < 0.05$ when compared between two subgroups.

indicated that AM cells from CWP patients were prone to excessive apoptosis than that of the normal controls. Although mechanisms regulating CWP are not clear, AM apoptosis has been postulated to be responsible for the pathological development of pulmonary fibrosis [31]. Specifically, apoptosis of AM cells results in production

of large amounts of inflammatory cytokines and fibrogenic factors that potentially play an important role in the pathogenesis and development of CWP [32]. Apoptosis includes signal transduction, sequential activation of apoptotic genes, execution of apoptosis and subsequent removal of apoptotic cells [33]. We demonstrated that

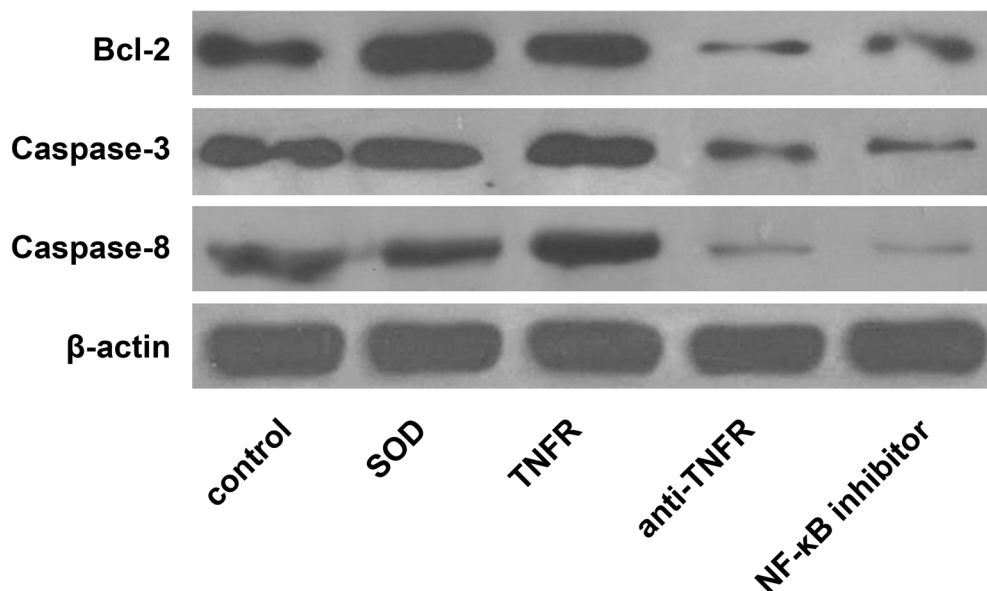


Figure 3: Western blot analyses of apoptosis regulatory proteins among the 5 experimental groups of alveolar macrophages. The 5 experimental groups are as follows: A, control group; B, SOD group; C, TNFR group; D, anti-TNFR group; E, NF-kB inhibitor group.

Table 5: Comparisons of the expression levels of Bcl-2, Caspase-3 and Caspase-8 among different subgroups based on the application of different intervention approaches

Protein	Group	SOD treatment	Group	anti-TNFR treatment	Group	NF-kB inhibitor treatment
Bcl-2	A	0.291 ± 0.056	A	0.324 ± 0.045	A	0.350 ± 0.044
	B	0.147 ± 0.034*	C	0.264 ± 0.035*	C	0.265 ± 0.040*
	C	0.245 ± 0.034**	D	0.160 ± 0.033**	E	0.165 ± 0.031**
Caspase-3	A	0.198 ± 0.024	A	0.231 ± 0.050	A	0.231 ± 0.041
	B	0.134 ± 0.030*	C	0.224 ± 0.042	C	0.221 ± 0.038
	C	0.200 ± 0.037**	D	0.154 ± 0.032**	E	0.143 ± 0.030**
Caspase-8	A	0.234 ± 0.050	A	0.238 ± 0.056	A	0.242 ± 0.042
	B	0.153 ± 0.034*	C	0.230 ± 0.043	C	0.195 ± 0.034*
	C	0.213 ± 0.042**	D	0.182 ± 0.040**	E	0.162 ± 0.027**

Note: A, control group; B, SOD group; C, TNFR group; D, anti-TNFR group; E, NF-kB inhibitor group. * $P < 0.05$ when compared with group A; ** $P < 0.05$ when compared with group B or group C.

the AM cells from CWP patients underwent excessive apoptosis than controls as visualized by the sub-diploid (sub-G0/G1) apoptosis peak in flow cytometry assays.

In vivo and *in vitro* studies have shown that AM plays an important role in CWP. AM are the main target cells exposed to dust and increased AM numbers are observed in CWP patients that correspond to excess amount of dust in the lungs [34]. Length of exposure to dust and the onset age are critical determinant factors in the incidence of CWP. Our study demonstrated that the apoptosis index of AM increased with the increase degree of CWP. Further, our data showed that increased length of exposure to dust in the CWP patients resulted in higher expression levels of Bcl-2, Caspase-3 and Caspase-8. Hence, our data suggested that longer coal dust exposure time resulted in higher levels of AM apoptosis, which in turn increased incidence of CWP.

More importantly, we observed the relationship between TNFR/TNF- α signal transduction pathway on AM apoptosis [35] and the higher expression of critical apoptotic proteins in CWP patients, namely Bcl-2, Caspase-3 and Caspase-8. Our data also showed that blocking TNFR/TNF- α mediated AM apoptosis by anti-TNFR antibody resulted in decreased apoptosis and expression of Bcl-2, Caspase-3 and Caspase-8, thereby suggesting that TNFR/TNF- α signal transduction pathway was a key player in AM apoptosis and CWP.

Caspases are a group of cysteine-aspartate proteases that are activated in a step wise manner upon apoptotic signaling [36]. The apoptotic signaling pathways activate initiator Caspases such as Caspases-8, -9, and -10, which then cleave and activate the effector Caspases such as Caspases-3, -6, and -7 that cleave critical proteins required for cellular homeostasis and function resulting eventually in

death of target cells [37–39]. Caspase-8 is an initiator caspase found upstream of the Caspase cascade, whereas Caspase-3 is an important effector caspase that is critical for accomplishing cellular apoptosis [40]. We demonstrated that treating AM cells with anti-TNFR antibody that blocks TNFR/TNF- α signaling apoptotic pathway resulted in decreased apoptosis of AM cells and diminished expression of Bcl-2, Caspase-3 and Caspase-8. This suggested that inhibitors of TNFR/TNF- α signaling may potentially benefit CWP patients therapeutically as it would enhance survival of AM cells and thereby alleviate CWP.

In conclusion, our study demonstrates that constant and excessive exposure to coal dust results in excessive apoptosis of AM due to stimulation of the TNFR/TNF- α apoptotic signaling pathway that leads to enhanced expression of apoptosis related proteins like Bcl-2, Caspase-3 and Caspase-8. Our study also demonstrated that inhibiting TNFR/TNF- α signaling pathway by either anti-TNFR antibody or inhibitors like SOD or NFkB inhibitors results in reduced AM apoptosis suggesting a potential therapeutic target to alleviate CWP.

MATERIALS AND METHODS

Ethical statement

The study was approved by the Ethics committee of Tangshan Gongren Hospital affiliated to North China University of Science and Technology. All subjects or their legal guardians provided written informed consents. Study protocols followed the ethical principles for medical research involving human subjects according to the Helsinki Declaration [41].

Study subjects

For this study, 366 coal mine workers from Kailuan Colliery, China (exposed group) that received treatment in Tangshan Gongren Hospital affiliated to North China University of Science and Technology were recruited as the research subjects. They were diagnosed for pneumoconiosis in accordance with the specific standards of *National Diagnostic Criteria of Pneumoconiosis* [42] and subdivided into CWP stage I ($n = 180$) and CWP stage II ($n = 186$) groups. Subjects were enrolled if (1) they were Han males; (2) they were exposed to dust over 1 year; (3) they had complete physical examination during the past two years; and (4) had complete records of occupational history or could be supplemented via checking working files. Patients with related lung diseases such as pneumonia, lung cancer, and active pulmonary tuberculosis were all excluded from this study. Also, patients diagnosed with pneumoconiosis combined with pulmonary complications, severe heart & lung diseases and infectious diseases were also excluded. In addition, 120 coal mine workers without previous history of any pulmonary diseases were enrolled in the present study as the control group.

Information regarding baseline characteristics was collected by face to face questionnaires to avoid contradictions or difficulties in obtaining complete information or through telephone interviews with retired workers. The data included (1) demographic characteristics like age, date of birth, gender, weight and height, smoking status, initial exposure time, occupational history, current occupation, and retirement age; (2) dust exposure history, which included initial exposure time, average exposure time, and age of dust removal; (3) pulmonary disease history that included time of initial diagnosis, grade, complications and onset time of complications. Most of the information was obtained from the medical records of the patients from Tangshan Gongren Hospital affiliated to North China University of Science and Technology. Further information was obtained from occupational health examination records and face to face inquiries. Information regarding dust exposure, diagnostic information of pneumoconiosis and other relevant complications was collected from the hospital recording files at Tangshan Gongren Hospital affiliated to North China University of Science and Technology.

Experimental methods

Alveolar macrophage cell culture and grouping

The subjects enrolled for this study underwent large capacity double lung simultaneous irrigation operation under general anesthesia at the Pneumoconiosis rehabilitation center of National Coal Mine Safety Supervision Bureau. After an appropriate amount of bronchoalveolar lavage fluid was harvested, it was filtered through three layers of sterile gauze, followed by centrifugation. The supernatant was discarded and the cell pellets were subsequently washed thrice

in PBS and centrifuged to obtain the AM cell suspension that was diluted in appropriate amounts 10% DMEM medium and cell counts obtained.

The AM cell suspension (5×10^6) was seeded in 6-well culture plates in 2ml DMEM medium containing 10% heat inactivated FBS and placed in a culture box with 84% humidity, 5% CO₂ and 37°C for 2 h. The non-adherent cells were discarded and fresh DMEM medium was added to the adherent cells that represented the purified AM.

After purification, the AM cells were divided into five experimental groups as follows: (1) control group without any treatment; (2) SOD group that received 200U/ml SOD; (3) TNFR group that received 50ng/ml anti-TNF- α antibody; (4) anti-TNFR group that received 50ng/ml anti-TNF- α antibody with 200ng/ml anti-TNFR; and (5) NF κ B inhibitor group.

Furthermore, as for AM culture and harvest, after reagents were added, the cells were incubated for 24 h (84% humidity, 5% CO₂ volume fraction in 37°C). The 5 groups of cells were grown for 24 h at 37°C and 5% CO₂ following which the supernatant was discarded and the adherent cells were trypsinized with 0.25% trypsin for 1 min. Then, the cells in each group were collected, and washed thrice with PBS followed by centrifugation for 10 mins at 1000rpm and the pelleted cells were resuspended and counted. The collected cells were stored for analyzing apoptosis. A portion of the cells were stained with hematoxylin & eosin and observed under the microscope to assess morphological changes associated with apoptosis.

Flow cytometry assay for AM apoptosis detection

The AM cells in the 5 experimental groups were fixed with cold 70% ethanol and analyzed in a flow cytometer, among which the reaction tube was added with 200 μ l binding buffer and 5 μ l propidium iodide. The percent cells in the sub-G₀/G₁ peak represented cells undergoing apoptosis. The sub-G₀/G₁ was identified from the FSC-H versus SSC-H plots.

Western blot analysis of apoptotic signaling proteins

The AM cells from the 5 experimental groups were incubated in protein lysis buffer (Beyotime Institute of Biotechnology) for 30 min at 4°C with intermittent vigorous mixing. After centrifugation at 1000 rpm for 15 min at 4°C, the supernatant was stored at -80°C. The protein amount in the lysate was quantified using the Bradford assay kit (BioRad, Hercules, CA, USA). Then, the protein samples were separated on 10% SDS PAGE and transferred onto PVDF membrane (1.5 h). The initial voltage was 60 V, and elevated at 120 V when the front edge of the bromophenol blue into the separation gel. After blocking, the membrane was incubated with primary

antibody (Anti-human Bcl-2, Caspase-3 and Caspase-8 and anti-human β -actin, dilution ratio of 1:2000, provided by the Santa cruz co., Ltd, CA, USA) for 1h followed by incubation with 1:2000 diluted goat anti-rabbit secondary antibody for 45 min at 37°C. The blot was developed with ECL method (ECL reaction mixture, Santa cruz co., Ltd, CA, USA) and the protein bands were quantified by the image analysis software IPP 6.0.

Statistical analysis

SPSS17.0 software was used for statistical analysis. Continuous variables were presented as mean \pm S.D; categorical variables were presented as frequencies and percentages. Comparisons between continuous variables were analyzed by *t* test and *F* test, whereas Wilcoxon rank-sum test was used for comparisons of non-randomly distributed continuous variables. The chi-square test was used for categorical variables. Correlations were analyzed by Pearson correlation analysis. A *P* value of < 0.05 was considered statistically significant.

ACKNOWLEDGMENTS

We would like to thank our researchers for their hard work and colleagues for their valuable advice.

CONFLICTS OF INTEREST

All authors in our study have no conflicts of interest.

REFERENCES

1. Xia Y, Liu J, Shi T, Xiang H, Bi Y. Prevalence of pneumoconiosis in Hubei, China from 2008 to 2013. *Int J Environ Res Public Health*. 2014; 11:8612–21.
2. Baur X, Heger M, Köhler D, Kranig A, Letzel S, Schultze-Werninghaus G, Tannappel A, Teschler H, Voshaar T, Bohle MR, Erlinghagen N, Hering KG, Hofmann-Preiss K, et al, and Deutsche Gesellschaft für Arbeitsmedizin und Umweltmedizin. [Diagnostics and expert opinion in the occupational disease No. 4101 silicosis (including coal worker's pneumoconiosis). Guideline (S2; AWMF) of the Deutsche Gesellschaft für Pneumologie und Beatmungsmedizin and the Deutsche Gesellschaft für Arbeitsmedizin und Umweltmedizin]. [Article in German]. *Pneumologie*. 2008; 62:659–84.
3. Sens PM, Almeida CI, Valle LO, Costa LH, Angeli ML. Tuberculosis of the ear, a professional disease? *Braz J Otorhinolaryngol*. 2008; 74:621–7.
4. Kamal A, Malik RN, Martellini T, Cincinelli A. Cancer risk evaluation of brick kiln workers exposed to dust bound PAHs in Punjab province (Pakistan). *Sci Total Environ*. 2014; 493:562–70.
5. Torres Rey CH, Ibanez Pinilla M, Briceno Ayala L, Checa Guerrero DM, Morgan Torres L, Groot de Restrepo H, Varona Uribe M. Underground Coal Mining: Relationship between Coal Dust Levels and Pneumoconiosis, in Two Regions of Colombia, 2014. *Biomed Res Int*. 2015; 2015:647878.
6. Hedmer M, Karlsson JE, Andersson U, Jacobsson H, Nielsen J, Tinnerberg H. Exposure to respirable dust and manganese and prevalence of airways symptoms, among Swedish mild steel welders in the manufacturing industry. *Int Arch Occup Environ Health*. 2014; 87:623–34.
7. Laney AS, Green F, Petsonk E. Pathologic Characteristics Of Coal Workers' Pneumoconiosis By Primary Radiographic Small Opacity Shape. *American Journal of Respiratory & Critical Care Medicine*. 2013.
8. Mo J, Wang L, Au W, Su M. Prevalence of coal workers' pneumoconiosis in China: a systematic analysis of 2001–2011 studies. *Int J Hyg Environ Health*. 2014; 217:46–51.
9. Wang M, Wang S, Song Z, Ji X, Zhang Z, Zhou J, Ni C. Associations of IL-4, IL-4R, and IL-13 gene polymorphisms in coal workers' pneumoconiosis in China: a case-control study. *PLoS One*. 2011; 6:e22624.
10. Miller BG, MacCalman L. Cause-specific mortality in British coal workers and exposure to respirable dust and quartz. *Occup Environ Med*. 2010; 67:270–6.
11. Shen F, Liu H, Yuan J, Han B, Cui K, Ding Y, Fan X, Cao H, Yao S, Suo X, Sun Z, Yun X, Hua Z, et al. Cost-Effectiveness of Coal Workers' Pneumoconiosis Prevention Based on Its Predicted Incidence within the Datong Coal Mine Group in China. *PLoS One*. 2015; 10:e0130958.
12. Najmuddin A, Cohen RA, Petsonk EL, Young B, MacNeill S, Tramma S, Regier M, Abraham JL, Churg A, Green F. Lung pathology in coal workers with rapidly progressive pneumoconiosis emphasizes importance of silica and silicates in addition to carbon. *American Journal of Respiratory & Critical Care Medicine*. 2014.
13. Hoshino A, Hanada S, Yamada H, Mii S, Takahashi M, Mitarai S, Yamamoto K, Manome Y. Mycobacterium tuberculosis escapes from the phagosomes of infected human osteoclasts reprograms osteoclast development via dysregulation of cytokines and chemokines. *Pathog Dis*. 2014; 70:28–39.
14. Radogna F, Dicato M, Diederich M. Cancer-type-specific crosstalk between autophagy, necroptosis and apoptosis as a pharmacological target. *Biochem Pharmacol*. 2015; 94:1–11.
15. Schweickl H, Petzel C, Bolay C, Hiller KA, Buchalla W, Krifka S. 2-Hydroxyethyl methacrylate-induced apoptosis through the ATM- and p53-dependent intrinsic mitochondrial pathway. *Biomaterials*. 2014; 35:2890–904.
16. Fu Z, Zou F, Deng H, Zhou H, Liu L. Estrogen protects SGC7901 cells from endoplasmic reticulum stress-induced apoptosis by the Akt pathway. *Oncol Lett*. 2014; 7:560–4.
17. Lim SC, Parajuli KR, Han SI. The alkyllysophospholipid edelfosine enhances TRAIL-mediated apoptosis in gastric cancer cells through death receptor 5 and the mitochondrial pathway. *Tumour Biol*. 2016; 37:6205–16.

18. Teocchi MA, D'Souza-Li L. Apoptosis through Death Receptors in Temporal Lobe Epilepsy-Associated Hippocampal Sclerosis. *Mediators Inflamm.* 2016; 2016:8290562.
19. Wang L, Liu S, Zhao Y, Liu D, Liu Y, Chen C, Karray S, Shi S, Jin Y. Osteoblast-induced osteoclast apoptosis by fas ligand/FAS pathway is required for maintenance of bone mass. *Cell Death Differ.* 2015; 22:1654–64.
20. Qu J, Zhao M, Teng Y, Zhang Y, Hou K, Jiang Y, Yang X, Shang H, Qu X, Liu Y. Interferon-alpha sensitizes human gastric cancer cells to TRAIL-induced apoptosis via activation of the c-CBL-dependent MAPK/ERK pathway. *Cancer Biol Ther.* 2011; 12:494–502.
21. Song HY, Deng XH, Yuan GY, Hou XF, Zhu ZD, Zhou L, Ren MX. Expression of bcl-2 and p53 in induction of esophageal cancer cell apoptosis by ECRG2 in combination with cisplatin. *Asian Pac J Cancer Prev.* 2014; 15:1397–401.
22. Yao SQ, Zhang XY, Chen ZY. [Regulation role of superoxide dismutase coenzyme on Fas/FasL signal transduction and apoptosis in alveolar macrophages of pneumoconiosis patients]. [Article in Chinese]. *Zhonghua Lao Dong Wei Sheng Zhi Ye Bing Za Zhi.* 2008; 26:271–5.
23. Shen F, Yuan J, Sun Z, Hua Z, Qin T, Yao S, Fan X, Chen W, Liu H, Chen J. Risk identification and prediction of coal workers' pneumoconiosis in Kailuan Colliery Group in China: a historical cohort study. *PLoS One.* 2013; 8:e82181.
24. Wang M, Jin Y, Chen S, Yao S, Zhu L, Duan J, Yuan J. [The study of autophagy in alveolar macrophages of patients with coal workers' pneumoconiosis]. [Article in Chinese]. *Zhonghua Lao Dong Wei Sheng Zhi Ye Bing Za Zhi.* 2015; 33:41–4.
25. Zhang CM, Bai YP, Yan-Qin YU, Chen ZY, Zhang ZH. Significance of apoptosis-related proteins in the occurrence and progression of coal workers' pneumoconiosis. *China Occupational Medicine.* 2011.
26. Campagnolo L, Costanza G, Francesconi A, Arcuri G, Moscatelli I, Orlandi A. Sortilin expression is essential for pro-nerve growth factor-induced apoptosis of rat vascular smooth muscle cells. *PLoS One.* 2014; 9:e84969.
27. Ge Y, Yan D, Deng H, Chen W, An G. Novel Molecular Regulators of Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL)-Induced Apoptosis in NSCLC Cells. *Clin Lab.* 2015; 61:1855–63.
28. Gawaz M, Vogel S. Platelets in tissue repair: control of apoptosis and interactions with regenerative cells. *Blood.* 2013; 122:2550–4.
29. Neumann S, Hasenauer J, Pollak N, Scheurich P. Dominant negative effects of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) receptor 4 on TRAIL receptor 1 signaling by formation of heteromeric complexes. *J Biol Chem.* 2014; 289:16576–87.
30. Soini Y, Eerola AK, Lehto VP, Pääkkö P. Increased numbers of alveolar macrophages with apoptotic bodies predict lung carcinoma. *Apoptosis.* 1998; 261–6.
31. Ueno M, Maeno T, Nomura M, Aoyagi-Ikeda K, Matsui H, Hara K, Tanaka T, Iso T, Suga T, Kurabayashi M. Hypoxia-inducible factor-1alpha mediates TGF-beta-induced PAI-1 production in alveolar macrophages in pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol.* 2011; 300:L740–52.
32. van Berlo D, Wilhelmi V, Boots AW, Hullmann M, Kuhlbusch TA, Bast A, Schins RP, Albrecht C. Apoptotic, inflammatory, and fibrogenic effects of two different types of multi-walled carbon nanotubes in mouse lung. *Arch Toxicol.* 2014; 88:1725–37.
33. Xu X, Wen H, Hu Y, Yu H, Zhang Y, Chen C, Pan X. STAT1-caspase 3 pathway in the apoptotic process associated with steroid-induced necrosis of the femoral head. *J Mol Histol.* 2014; 45:473–85.
34. Davies R, Erdogdu G. Secretion of fibronectin by mineral dust-derived alveolar macrophages and activated peritoneal macrophages. *Exp Lung Res.* 1989; 15:285–97.
35. Tsou HK, Chen HT, Chang CH, Yang WY, Tang CH. Apoptosis signal-regulating kinase 1 is mediated in TNF-alpha-induced CCL2 expression in human synovial fibroblasts. *J Cell Biochem.* 2012; 113:3509–19.
36. Wu H, Che X, Zheng Q, Wu A, Pan K, Shao A, Wu Q, Zhang J, Hong Y. Caspases: a molecular switch node in the crosstalk between autophagy and apoptosis. *Int J Biol Sci.* 2014; 10:1072–83.
37. Samarghandian S, Nezhad MA, Mohammadi G. Role of caspases, Bax and Bcl-2 in chrysin-induced apoptosis in the A549 human lung adenocarcinoma epithelial cells. *Anticancer Agents Med Chem.* 2014; 14:901–9.
38. Kim WH, Song HO, Choi HJ, Bang HI, Choi DY, Park H. Ethyl gallate induces apoptosis of HL-60 cells by promoting the expression of caspases-8, -9, -3, apoptosis-inducing factor and endonuclease G. *Int J Mol Sci.* 2012; 13:11912–22.
39. Inserte J, Cardona M, Poncelas-Nozal M, Hernando V, Vilarrosa U, Aluja D, Parra VM, Sanchis D, Garcia-Dorado D. Studies on the role of apoptosis after transient myocardial ischemia: genetic deletion of the executioner caspases-3 and -7 does not limit infarct size and ventricular remodeling. *Basic Res Cardiol.* 2016; 111:18.
40. Mitupatum T, Aree K, Kittisenachai S, Roytrakul S, Puthong S, Kangsadalampai S, Rojpbulstitt P. Hep88 mAb-mediated paraptosis-like apoptosis in HepG2 cells via downstream upregulation and activation of caspase-3, caspase-8 and caspase-9. *Asian Pac J Cancer Prev.* 2015; 16:1771–9.
41. General Assembly of the World Medical Association. World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects. *J Am Coll Dent.* 2014; 81:14–8.
42. Paolucci V, Sartorelli P. Diagnostic Criteria Of Pneumoconiosis. 2013; 36.