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Analysis of coal tar pitch and smoke extract components and their cytotoxicity on human bronchial epithelial cells

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ABSTRACT

Coal tar pitch and its smoke are considered hazardous by-products and common pollutant generated from coal industry processing. In this study, coal tar pitch and its smoke extracts were characterized by gas chromatography/mass spectrometry (GC/MS) with dimethylsulfoxide. We identified only 0.3025% of components in the total coal tar pitch using GC/MS. Among 18 identified compounds, polycyclic aromatic hydrocarbons (PAHs) has the highest relative abundance (0.19%). The remaining components were composed of monocyclic aromatic hydrocarbons, heterocyclic compounds and alkenes. In contrast, among 38 coal tar pitch smoke extract constituents that have been profiled, 87.91% were PAHs, and the remaining 12.09% were composed of monocyclic aromatic hydrocarbons, heterocyclic compounds and alkenes. The cytotoxic effect of coal tar pitch and its smoke extracts on BEAS-2B cells were also evaluated by MTT assay. BEAS-2B cells exposed to coal tar pitch showed a non dose-dependent U-shaped cytotoxicity with a dosage for maximal inhibitory of 3.75 mg/L. In contrast, BEAS-2B cells exposed to coal tar pitch smoke extracts showed a dose dependent cytotoxicity with a LC_{50} of 8.64 mg/L. Our study demonstrated the significant different composition and cytotoxicity of coal tar pitch and its extracts, suggesting two different underlying mechanisms that are pending future investigation.

1. Introduction

Coal currently is and will be one of the main energy resources in China. The dominant uses of coal are in thermoelectricity generation and coke production industry. With the development of steelmaking and coking, the yield of coke has been rapidly increasing recently in China. In 1999, more than one third of the total coke production in the world was produced in China, thus making China one of the major coke producing countries in the world [1]. In the coking process, the main by-product is coal tar which accounts for 3–4% of the total raw material of coal. It is estimated that annual production of coal tar is about 12 million tons in the world, of which, approximately 5 million tons are made in China. Coal tar pitch is the waste residue generated from the processing of distilling coal tar and it accounts for about 54–56% of total processed coal tar. The main applications of coal tar pitch include the production of carbon electrode adhesive and impregnate, waterproof and anti-corrosion coatings and road-construction materials.

The composition of coal tar pitch is complicated; there are some differences on physical properties, but not chemical compositions between coal tar pitch and coal tar. The main chemical composition of coal tar pitch is polycyclic aromatic hydrocarbons (PAHs), such as anthracene, phenanthrene and pyrene [2]. The composition of coal tar pitch differs from process to process, mainly due to different raw materials and treatment used during production. Coal tar pitch is toxic for human and animals on the aspects of carcinogenesis, teratogenesis, and mutagenesis [3,4]. Because of its broad industry application, it has long been regarded as one of the major environment pollutant in China.

Initial recognition of coal tar pitch's toxic effect was from studies of polycyclic aromatic hydrocarbons and their typical chemical, benzo[a]pyrene. Infact, the direct contact with coal tar pitch is common in our daily life because it is widely used in waterproofing coating, anticorrosion coating and road-building materials. Through environmental pollution, coal tar pitch could also affect animal's health [5].

Smoke extracts of coal tar pitch were usually used as toxicant in carcinogenesis, teratogenesis, and mutagenesis studies [6,7]. The coal tar pitch was also directly used in some animal experiments [8]. Coal tar pitch and smoke extracts are different in nature despite that they were considered as the same in many studies. Firstly, when coal tar pitch is heated to smoke, its ingredients are decomposed and/or condensed. And new compounds could be produced that cause the different chemical composition between coal tar

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pitch and its smoke extracts [9]. Secondly, smoke of coal tar pitch is absorbed directly through respiratory tract and skin compared with coal tar pitch, which affect worker's health indirectly through contamination of water [10,11] and soil [5]. Nevertheless, much is still unclear on their composition and it hinders the development of effective remediation methods for their toxic effects.

This study was conducted to characterize coal tar pitch and the smoke samples using GC/MS, in the aim to investigate their cytotoxicity in vitro. The coal tar pitch was obtained from coking plant of Anyang Steel Company (China), and the smoke extract was collected by heating up the coal tar pitch. Both the coal tar pitch and its smoke extract were dissolved in dimethylsulfoxide (DMSO). Their main ingredients were determined by gas chromatography/mass spectrometry (GC/MS). The effect of cytotoxicity on BEAS-2B cells was evaluated by MTT assay.

2. Materials and methods

2.1. Materials

2.1.1. Medium temperature coal tar pitch

Medium temperature coal tar pitch was obtained from coking plant in Anyang Iron and Steel Company (China), and was stored at room temperature.

2.1.2. Preparation of coal tar pitch DMSO solution

The coal tar pitch was crushed and grinded into powder with diameter of 10–20 μm in grinding bowl at 0 °C. Then the fine powder was weighed carefully and DMSO solution was added into the powder until it was completely dissolved to make a solution of 1.5 mg/mL. Physical observation shows that the sample is a clear dark brown solution, with no visible particles.

2.1.3. Preparation of coal tar pitch smoke extracts DMSO solution

The fine powder of coal tar pitch with diameter of 10–20 μm was put into a beaker and placed in an exhaust hood with a flat-panel heater. The smoke of coal tar pitch was generated at 400 °C, and collected using a dust sampler with nitrocellulose filter membrane. The flow rate was 20 L/min, the duration of sampling was 40 min each for three times. After sampling, the filter membrane were weighed, cut into pieces and dissolved into 350 mL dichloromethane in flask with plug by supersonic vibrating for 40 min. Then the solution was filtered by sand core funnel to produce crude extracts, further dried in baking oven at 45 °C. When it was completely dried, smoke extracts were dissolved into DMSO with a final concentration of 2.0 mg/mL.

2.1.4. Cell line

BEAS-2B, a SV40 hybrid (Ad12 SV40) transformed human bronchial epithelial cell line, was kindly provided by Professor Weidong Wu (Center for Environmental Medicine, Asthma and Lung Biology, University of North Carolina, Chapel Hill, NC, USA). BEAS-2B cells were cultured in standard media (RPMI 1640 medium containing 10% fetal bovine serum) in 25 cm^2 flasks at 37 °C, in a 5% CO_2, 95% air incubator. When the adherent monolayer cells reached 90% confluent in the flask, the medium was discarded with pipette. The cells were rinsed with cold 1× PBS and detached with 0.25% trypsin. The cells were inoculated and incubated at the density of 2×10^5 cells per mL and splitting was conducted every six days.

2.2. Methods

2.2.1. The instruments and analytical conditions

Gas chromatographic analyses of coal tar pitch were carried out using an Agilent Model 7890A gas chromatograph and 5975C mass spectrometry (GC/MS). The separations were performed using a fused-silica capillary column (HP-5MS) with length of 30 m and I.D. 0.25 mm and D.F 0.25 μm. The temperatures were set from 60 °C to 260 °C at an ascending rate of 2 °C/min, and held for 30 min at 260 °C. Then the ascending rate was set at 10 °C/min until it reached to 280 °C and kept at this level. Helium was used as the carrier gas (constant flow rate at 1.0 mL/min) with split ratio of 10:1. The sample injection volume was 1 μL. The MS was operated in a full scan mode. The scanning scope was between 40 and 500 m/z with an inlet line temperature of 280 °C, ion source temperature of 230 °C and electron ionization (EI) mode of 70 eV.

2.2.2. Analysis methods

Qualitative analysis: The unknown compounds in the samples were identified using the library software of the GC/MS by comparing unknown spectra with the NIST library of known spectra based on their retention time.

Quantitative analysis: The quantity of identified compounds was determined based on the area under the peak of the chromatographs.

2.2.3. Cytotoxic test by MTT assay

The cytotoxic effect of coal tar pitch was assessed by MTT assay according to the method used by Mosmann [12]. In brief, monolayer BEAS-2B cells were harvested by adding 0.25% trypsin and suspended in RPMI 1640. The cell suspension was placed into 96-well plate at the density of 5×10^3 per well in standard media (RPMI 1640 medium containing 10% fetal bovine serum) at a final volume of 200 μL and cultured at 37 °C, 5% CO_2, 95% air incubator for 24 h. On the next day, the wells were added with different dosages of coal tar pitch or its smoke extracts. Every eight wells were treated with same dose. Zero dosage was set as control. After 24 h exposure, MTT (with the final concentration of 0.5 mg/mL) was added to the wells. Cells were incubated for 4 h, followed by addition of 150 μL of DMSO solution for each well. The formazone crystals are completely dissolved in solution by through mixing. The reaction mixture in each well of the 96-well plate was measured using the ELISA reader MultiScan MK3 at wavelength of 492 nm with reference at 570 nm. The viability percentage of the cells was calculated by comparing the values of the controls and the exposed cells. The Half lethal concentration (LC_{50}) and the Maximal dosage of cytotoxicity were calculated according to standard procedure.

2.2.4. Statistical analysis

Factorial ANOVA was conducted on each dependent measure using SPSS version 12.0 for Windows (SPSS, Chicago, IL). Data were expressed as mean ± SEM. Pearson Goodness of fit Chi-Square was used to decide whether to accept the "symmetric S-curve". It was considered that no heterogeneity factor is used in the calculation of confidence limits. Results were considered significant when $P < 0.05$.

3. Results

3.1. Determination of coal tar pitch components

Fig. 1 shows the GC/MS total ions current chromatogram of coal tar pitch dissolved in DMSO. The total ion current chromatogram of coal tar pitch contains large group of peaks, suggesting the presence of unknown components. It was however challenging to identify all components in the sample spectra due to limitation of the mass spectrum database. As a result, only 0.3025% of the total compounds in coal tar pitch were identified. The identified compounds and their content level are listed in Table 1.

Total 18 compounds were identified in coal tar pitch DMSO solution, including seven PAHs, one monocyclic aromatic hydrocarbon, six heterocyclic compounds and four alkenes. Using

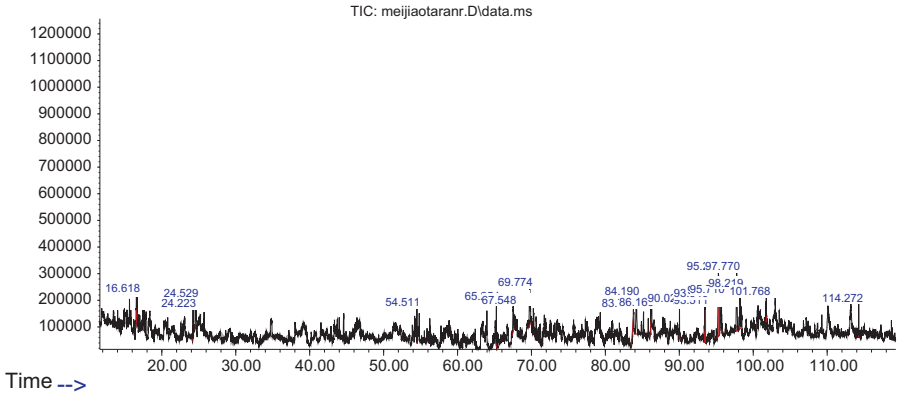

Fig. 1. The total ion current of the coal tar pitch in DMSO solution. The component identified by GC–MS show the retention time. The name of identified component was listed in Table 1.

Table 1
Chemical constituents of the coal tar pitch in DMSO solution.

Retention time	Compound name	Rate (%)
9.527	L-Alanine, 3-sulfo-aminomethanesulfonic acid	0.0603
24.22	N,N′-Bis(2-chloroethyl)oxamide	0.0048
24.527	Pyrimidine-4,6(3H,5H)-dione, 2-butylthio-	0.0024
54.512	Thiophene-2-carboxamide, 3-chloroacetylamino-5-(4-fluorophenyl)-	0.0037
65.257	Spiro[benzofuran-2(3H),1′-[2]cyclohexene]-3,4′-dione, 7-chloro-4,6-dimethoxy-6′-methyl-2′-(methylthio)	0.0077
67.55	Fluoranthene	0.0155
69.774	Pyrene	0.0236
84.19	Triphenylene	0.0260
90.024	N-(2,6-dichlorophenyl)-N-[(2Z)-3-methyl-1,3-thiazinan-2-ylidene]amine	0.0040
93.456	2,5-Dibromo-3,4-dinitrothiophene	0.0057
93.51	cis-7-Ethoxycarbonyl-bicyclo(4,3,0)non-3,7-diene	0.0080
93.541	Copper, bis(4-chloro-3,5-cyclohexadiene-1,2-dione 2-oximato-N2,O1)-	0.0049
95.288	Benz[e]acephenanthrylene	0.0763
95.396	Benzo[e]pyrene	0.0097
95.711	3,5,6-Trimethyl-p-quinone, 2-(2,5-dioxotetrahydrofuran-3-yl)thio-	0.0079
97.766	Perylene	0.0371
101.768	Benz[j]aceanthrylene, 3-methyl-	0.0022
114.275	2-Diphenylethenylsilyloxybut-3-yne	0.0027

area normalization method to calculate the relative content of each component, the results showed that polycyclic aromatic hydrocarbon was the main identified components in coal tar pitch, accounted for 0.19% of total components. While the monocyclic aromatic hydrocarbons, heterocyclic compounds, and alkenes only accounted for 0.0027%, 0.0314%, and 0.078% respectively. The ingredients of polycyclic aromatic hydrocarbon were benz[e]acephenanthrylene, perylene, triphenylene, pyrene, fluoranthene, 3-methyl-benz[j]aceanthrylene and benzo[e]pyrene. Hetero-cyclic compounds, such as pyridine, furan, thiophene and thiazine were also identified.

3.2. Determination of components of coal tar pitch smoke extract

Most constituents of coal tar pitch smoke extracts were identified (Fig. 2 and Table 2). According to their structural characteristics, the identified compound in coal tar pitch smoke extracts could be divided into four groups: PAHs (28 compounds), the mono-

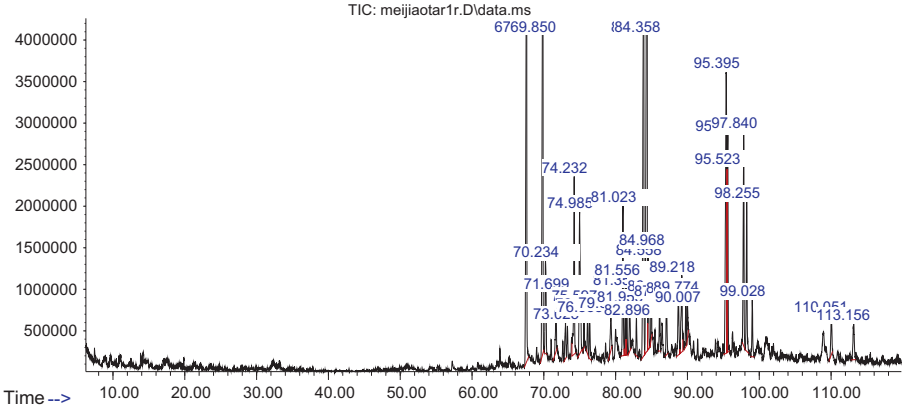

Fig. 2. The total ion current of the smoke extracts of coal tar pitch in DMSO solution. The component identified by GC–MS shows the retention time. The name of identified component was listed in Table 2.

Table 2
Chemical constituents of the extracts of coal tar pitch smoke in DMSO solution.

Retention time	Compound name	Rate (%)
63.887	Dibutyl phthalate	0.26
67.596	Fluoranthene	9.4
69.851	Pyrene	9.35
70.236	Naphthalene, 1-phenyl-	2.17
71.014	9-Anthracenecarbonitrile	1.13
71.699	Benzo[b]naphtho[2,3-d]furan	0.62
73.022	Pyrene, 1-methyl-	1.97
73.292	5H-benzo[def]carbazole	0.62
74.231	11H-benzo[b]fluorene	7.28
75.593	9-Phenyl-5H-benzocycloheptene	0.52
76.101	Pyrene, 2-methyl-	0.72
78.61	Naphthacene, 5,12-dihydro	0.23
79.333	o-Terphenyl	0.84
80.003	6,6-Diphenylfulvene	0.49
80.149	Pyrene, 1,3-dimethyl-	0.28
81.027	Benzo[b]naphtho[2,3-d]thiophene	3.4
81.35	Cyclopenta[cd]pyrene	1.12
81.558	Benzo[c]phenanthrene	1.22
81.95	Benz[c]acridine	0.95
83.89	1(2H)-Phenanthrenone, 3,4,9,10-tetrahydro-7-methoxy-	9.24
84.359	Triphenylene	13.07
84.559	11H-benzo[a]carbazole	3.7
84.967	Cyclopenta(cd)pyrene, 3,4-dihydro-	1.87
85.475	7H-benz[de]anthracen-7-one	0.4
86.129	Naphtho[2,1,8,7-klmn]xanthene	1.14
88.738	Triphenylene, 2-methyl-	1.53
89.215	Benz(A)anthracene-7,12-dione	1.86
89.4	Benz[a]anthracene, 1,2,3,4,7,7a,8,9,10,11,11a,12-dodecahydro-	0.22
89.777	2-Propen-1-one, 1-(2-hydroxyphenyl)-3-(4-hydroxyphenyl)-	0.76
90.008	Cyclohexane, hexaethylidene-	0.51
91.424	2,2′-Binaphthalene	0.3
95.396	Benz[e]acephenanthrylene	10.94
95.526	Benzo[e]pyrene	3.84
96.288	Benzo[k]fluoranthene	0.31
97.843	Perylene	4.89
100.937	Benz[j]aceanthrylene, 3-methyl-	0.21
108.949	6-Bromo-2,5-dimethoxy-4-nitroaniline	0.8
110.05	Benzo[ghi]perylene	1.86

cyclic aromatic hydrocarbons (4 compounds), heterocyclic (5 compounds) and alkenes (1 compound). The results showed that the main ingredients of coal tar pitch smoke extracts were polycyclic aromatic hydrocarbon, which accounted for 87.91% of total compounds. The monocyclic aromatic hydrocarbons, heterocyclic compounds, and the alkenes consist of 2.31%, 9.29%, and 0.51% respectively.

The main ingredients of PAHs include triphenylene, benz[e]acephenanthrylene, fluoranthene, pyrene, 11H-benzo[b]fluorine, perylene, benzo[e]pyrene and 1-phenylnaphthalene. Heterocyclic compounds such as acridine, furan, thiophene and carbazole were also identified in the smoke extract.

3.3. The comparison of chemical composition between coal tar pitch and coal tar pitch smoke extract

The chromatogram of total ions current of coal tar pitch and coal tar pitch smoke extracts (Figs. 1 and 2) showed that many small peaks could be seen in the coal tar pitch DMSO solution. In the smoke extracts DMSO solution, the peak changed markedly and converged in the retention time of 60–110 min. The peak area under the retention time within 60–110 min was more than 98%.

The composition of coal tar pitch was identified in only 0.3% of the total mass spectra and a large portion of the ingredients were undetermined by the database search. In contrary to coal tar pitch, the components of coal tar pitch smoke extracts was nearly completely identified with polycyclic aromatic hydrocarbon accounted for 87.91%.

Table 3
The content of identical compound between coal tar pitch and its smoke extracts.

Compound name	Coal tar pitch (%)	Smoke extracts (%)
Benz[e]acephenanthrylene	0.0763	10.94
Perylene	0.0371	4.89
Triphenylene	0.0260	13.07
Pyrene	0.0236	9.35
Fluoranthene	0.0155	9.40
Benzo[e]pyrene	0.0097	3.84
3-Methyl-benz[j]aceanthrylene	0.0022	0.21

By searching mass spectrometry database, we identified 18 compounds from coal tar pitch and 38 compounds from the smoke extracts. As shown in Table 3, seven compounds are the same, but the relative content of the seven compounds is not consistent.

In addition, benzo[α]pyrene was not detected in both samples. The content of benzo[e]pyrene in both samples was very low.

3.4. Effect of coal tar pitch and its smoke extracts on BEAS-2B cell

The coal tar pitch, from 0.059 mg/L to 30 mg/L, was added to culture medium of BEAS-2B cells. With increasing exposure dose of cal tar pitch, cell survival rate dropped first and then increased, showing a U-shaped dose-responsive curve. The maximal dosage of cytotoxic for coal tar pitch was 3.75 mg/L (Fig. 3). We observed a dose-dependent cytotoxic effect of smoke extracts on BEAS-2B cells as shown in Fig. 4. Half lethal concentration (LC_{50}) of coal tar pitch smoke extracts was 8.64 mg/L using Probit method. The statistics accepted the calculation that no heterogeneity factor is used in the calculation of confidence limits and Pearson Goodness of fit χ^2 test, $\chi^2 = 75.56$, $P < 0.001$, and accepted the assumption of the "symmetric S-curve". 95% confidence interval was (6.57–11.48) mg/L.

4. Discussion

The ingredients of coal tar pitch are complicated in nature. The exact composition is still unclear and the reported composition is different from process to process. Most of the researchers analyzed the individual chemicals in each isolated component separated by different solvents [2]. Coal tar pitch could not be completely dissolved in any single known solvent so far, due to its complicated

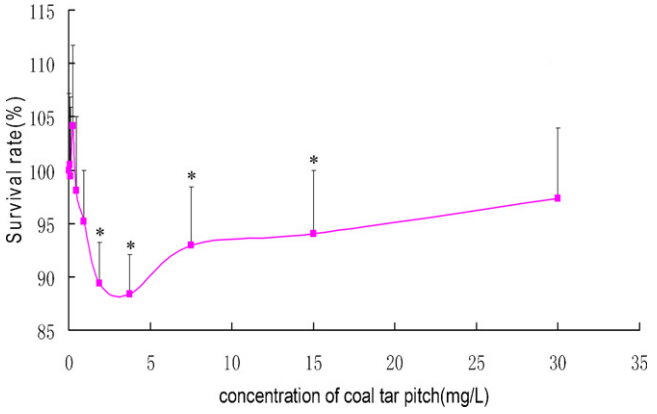

Fig. 3. The MTT survival curve of BEAS-2B cells induced by coal tar pitch ($n = 8$). The BEAS-2B cells were adjusted to 5×10^3/well and cultured at 37 °C, 5% CO_2, 95% air incubator for 24 h. On the next day, the wells were added coal tar pitch with dosages at 0, 0.059, 0.118, 0.235, 0.47, 0.94, 1.88, 3.75, 7.50, 15.00, 30.00 mg/L respectively. Every eight wells shared same dosage. Zero dosage was set as control. After 24 h exposure, the cytotoxicity was tested by MTT assay. Each point represents the means ± SD ($n = 8$). * indicates the $P < 0.05$ compare with the zero group.

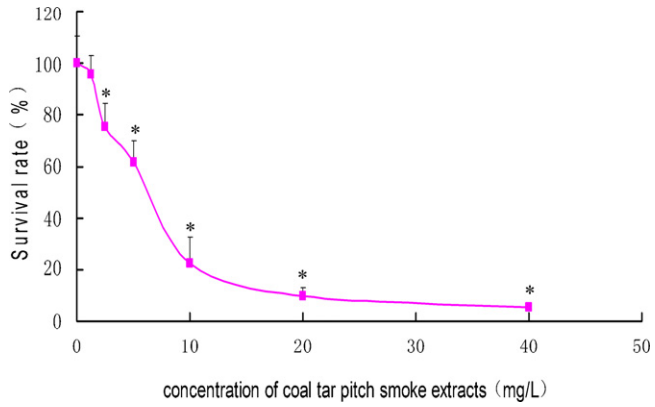

Fig. 4. The MTT survival curve of BEAS-2B cells induced by the extracts of coal tar pitch smoke ($n=8$). The BEAS-2B cells were adjusted to 5×10^3/well and cultured at 37 °C, 5% CO_2, 95% air incubator for 24 h. On the next day, the wells were added coal tar pitch with dosages at 0, 1.25, 2.50, 5.00, 10.00, 20.00, 40.00 mg/L respectively. Every eight wells shared same dosage. Zero dosage was set as control. After 24 h exposure, the cytotoxicity was tested by MTT assay. Each point represents the means ± SD ($n=8$). * indicates the $P<0.05$ compare with the zero group.

multi-phase system containing highly condensed carbon cyclic compounds and heterocyclic compounds.

To find the ideal solvent, we tested ethanol, methanol, acetone, ethyl acetate for their solubility to coal tar pitch. None of them showed good solubility to coal tar pitch. After extensive tests, DMSO was found as an ideal solvent for in vitro cells experiment. When the concentration of coal tar pitch was at 1.5 mg/mL, there were no visible particles in the solution. The solution was used in GC/MS experiments for composition analysis and the in vitro cytotoxicity experiments. Coal tar pitch smoke extracts samples were prepared by traditional methods.

The study showed that coal tar pitch and its smoke extracts contained polycyclic aromatic hydrocarbons and heterocyclic compounds such as furan, pyridine, carbazole and thiophene, as reported previously by others [13,14]. Our finding is consistent with report from Lazaro et al. [15], who detected the presence of phenyl, alkanes, methylated PAHs, furans, and thiophenes in tar derived from co-pyrolysis of waste lubricating oil and coal. Alkanes, alkenes, and thiophenes have also been found to be presented in asphalt samples [16]. Leonard et al. [2] reported the present of acid tar in numerous organic compounds including saturated and unsaturated aliphatic and cyclic hydrocarbons, polycyclic aromatic hydrocarbons (PAHs), organic acids (sulfonic acids, carboxylic acids, and aromatic acids), phenyl, nitrile, amide, furans, thiophenes, pyrroles, and phthalates.

The compositional variation of coal tar and asphalt are caused by the raw materials, temperature, and processing procedure during the manufacturing process [9,15]. Our study showed that the compositions of coal tar pitch and its smoke extracts are strikingly different. The reasons for such difference might be due to chemical evaporation, decomposition, condensation and replacement of carbon with oxygen, sulfur, and nitrogen in aromatic compound after heating [17].

BEAS-2B cells infected with adenovirus 12 and SV40 hybrid viruses were immortalized via continuous passage selection. The cell line expresses T antigen encoded by exogenous SV40 gene, without any adenovirus DNA sequences. It has normal human respiratory tract epithelial cell morphology and function with the near-diploid karyotype. It has no tumorigenicity in nude mice. BEAS-2B cell is one of the ideal in vitro models to study diseases involving the human bronchial epithelial cell [18].

Similar to benzo[a]pyrene [19], the coal tar pitch smoke extracts inhibited growth rate of BEAS-2B cells in a dose-dependent manner. In contrast, the toxic effects of coal tar pitch on BEAS-2B cell survival rate showed a U-shaped dose–response curve, represented by a initial decreasing followed by a increasing of cell survival rate, indicating a typical "hormesis" phenomenon [20,21]. These two types of toxic effects are caused by the different components between coal tar pitch and its smoke extracts. The main components of coal tar pitch smoke extracts were polycyclic aromatic hydrocarbons, like the effects of the most polycyclic aromatic hydrocarbons, the toxic effects showed the symmetry S-shaped curve, and along with the dose gradually increasing, the cell survival rate was inhibited. The total composition of polycyclic aromatic hydrocarbons accounted for only 0.19% in coal tar pitch DMSO solution where most of the components are unknown. We speculate that some compounds promote and some inhibit cells growth. The cell growth-promoting effect of coal tar pitch at high concentrations was mainly caused by the high concentration of growth-promoting compounds in coal tar pitch. With the reduction in the concentration of coal tar pitch, the concentration of growth-promoting compounds are also reduced and the growth-suppressing compounds takes over the power, and gradually reaches the maximal inhibitory effect. When the inhibitors are diluted, the cells resume normal growth. In this study, we tried to further increase the dose of coal tar pitch to observe its effects on cell growth and found that the BEAS-2B cells growth was significantly inhibited by the increasing doses of DMSO itself. Taking together, we propose that there exists different toxicological mechanisms for coal tar pitch and its smoke extracts on BEAS-2B cells, as evidenced by our GC–MS analysis and modes of dose–response curves.

5. Summary

The components of coal tar pitch and its smoke extracts, along with their cytotoxic effect on BEAS-2B cells, were studied using GC/MS and MTT assay in this study. The results demonstrated the significant different composition of coal tar pitch and its smoke extracts, which correlates well with our findings of different in vitro cytotoxicity response curves for both substances. Most of the coal tar pitch components are unknown, with only 0.3025% components identified in the sample. While almost all of coal tar pitch smoke extract constituents, including PAHs, monocyclic aromatic hydrocarbons, heterocyclic compounds, and alkenes, have been identified. The cytotoxicity of coal tar pitch on BEAS-2B cells showed the U-shaped dose–response, whereas the dose-dependent cytotoxicity effect was observed for coal tar pitch smoke extracts. The results obtained from this work open the avenue to further investigate their different underlying cytotoxicity effects and to develop effective preventive methods.

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