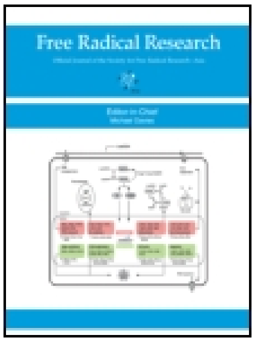

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Chemical Reactivity of the Carbon-centered Free Radicals and Ferrous Iron in Coals: Role of Bioavailable Fe^{2+} in Coal Workers' Pneumoconiosis

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Striking differences in the prevalence of coal workers' pneumoconiosis (CWP) exist between different coal mine regions. The major factors responsible for the observed regional differences in CWP have not yet been identified. In the present study, chemical reactivity of the carbon-centered free radicals in coals and lung tissues, as well as ferrous iron in the coals, were studied by ESR techniques. The ESR spectra clearly demonstrated the presence of at least two types of carbon-centered free radical species, which might respectively attribute to the macromolecular phase and the molecular phase of coal. Grinding produced free radicals in coals. Exposure of freshly ground coal to air for 28 h induced a slight increase of free radicals for most of the coals, and a slight decrease after 4 months' exposure. The lung tissue samples of coal workers deceased of CWP showed similar ESR spectra as coal samples, and these radicals were highly stable in the lung. After incubation of coals with glutathione, hydrogen peroxide, sodium formate or oxygen, the coal sample from the Gardanne mine which has never induced CWP, and thus is the least hazardous coal, showed the most significant change in the carbon-centered free radical concentration. No significant changes were observed among other coals reported to induce CWP. On the other hand, we found that the coals released different amounts of Fe^{2+} in an acidic medium. Interestingly, the prevalence of CWP correlates positively with the released Fe^{2+} content in these coals and with the amount of oxygen radicals produced by the interaction of Fe^{2+} with O_2 in the acidified coal filtrates. Our studies indicate that the carbon-centered free radicals may not be biologically relevant to coal dust-induced pneumoconiosis, whereas the acid soluble Fe^{2+}, which may be dissolved in the phagolysosomes of macrophages, can then lead to Fe^{2+}-induced oxidative stress and eventual CWP development.

Keywords: Pneumoconiosis, coal, free radical, ESR, lung tissue, iron, coal rank

Abbreviations: CWP: coal workers' pneumoconiosis; NSCWP: National Study of CWP; GSH: glutathione; AMs: alveolar macrophages; CERCHAR: Centre d'Etudes et de Recherches des Charbonnage; ESR: electron spin resonance; SP: strong pitch; DPPH: 2,2′-diphenyl-1-picryl-hydrazyl; DMPO: 5,5′-dimethyl-1-pyrroline N-oxide; PMF: progressive massive fibrosis

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INTRODUCTION

Coal workers' pneumoconiosis (CWP) implicate a limited fibrosis, often associated with emphysema and chronic bronchitis. Epidemiological studies of the relationship between the prevalence of CWP and environmental measurements have consistently revealed that the predominant adverse exposure factor is respirable mixed coal dusts.[1] It has been shown in the USA, Great Britain, France, and Germany that the prevalence and severity of CWP differed remarkably between different coal mines despite comparable exposures to respirable dust.[2–5] For example, the first round of the US National Study of CWP (NSCWP), which was completed in 1971, examined a total of 9076 miners from 29 bituminous and 2 anthracite mines.[6] The average exposure concentration in USA mines during that period was $3\,mg/m^3$.[7] It was found that 41% of Eastern Pennsylvania anthracite miners had simple pneumoconiosis and a further 14% had progressive massive fibrosis (PMF), but the comparable figures for bituminous miners in Colorado and Utah were 4% and 0.4%, respectively.[7] In France, coal miners of Provence never had CWP (0%) and the prevalence of CWP in coal miners of Nord Pas de Calais was 24%.[4] It has been found that quartz was a minor contributor to CWP development in general.[8–10] Moreover, pneumoconiosis was also found in coal trimmers who shoveled coal that contained little or no silica and in graphite and carbon electrode workers who were exposed to carbonaceous materials free of silica.[11–13]

Coal rank was found to play a role: risk of developing CWP increases with the coal rank.[14] Coal rank is defined as the extent to which the organic materials have matured during geological time ongoing from peat to anthracite. Coal rank can be roughly estimated by the carbon content in the coal, molar ratio of carbon/hydrogen (C/H), heat value, volatile materials, or moisture.[15] A correlation between coal rank and cell cytotoxicity has not yet been established in biological studies.[16–19] Since coal rank is only an indicator of the carbonization of organic materials and is not an active compound, chemically speaking, which can induce CWP, other compound(s) or a complex interaction between the different particles may exert a significant role in the observed regional differences in CWP.

CWP is considered to be a disease related to the occurrence of oxidative stress in alveolar macrophages (AMs).[20,21] Since the discovery of the paramagnetic resonance property of coal-like materials,[22] coal and carbon are known as "tanks" of free radicals which are carbon-centered. As free radicals have been recognized to be important reactive intermediates in many biochemical processes,[23] we postulated that some of the free radicals in coal dusts might contribute to CWP development by participating in redox cycling of biomolecules such as glutathione (GSH) and hydrogen peroxide (H_2O_2). Five coal samples and fifteen lung tissue samples were investigated in the present study. Using electron spin resonance (ESR), we found that the carbon-centered free radicals in high rank coal had little chemical reactivities to GSH and H_2O_2. In contrast, the ferrous ions (Fe^{2+}) in certain coals that are linked to a high prevalence of CWP can easily be released under acidic conditions and go on to produce oxygen radicals in the acidified coal filtrates. These results are consistent with our observation in US coal samples that the coals from mines with a high prevalence of CWP had a high content of acid soluble Fe^{2+}.[24]

MATERIALS AND METHODS

Materials

The five French coal samples were provided by the mini coal bank of the Centre d'Etudes et de Recherches des Charbonnage de France (CERCHAR). A large amount of coals (at least 250 kg) were freshly collected, ground, and homogenized in accordance with appropriate International Standards for Testing and Materials methods. They were stored under argon as 1 kg aliquots with particle sizes below 3 mm. These five coal samples were from four coal mine regions with prevalences of CWP ranging from 0–24%. The coal ranks of the samples are listed in increasing order as follows: Gardanne (from Provence region), Vouters (Lorraine), Mericourt and Escarpelles (Nord Pas de Calais), and La Mure (Les Alpes). Detailed petrographic analysis of these coal samples and characteristics is described elsewhere.[25,26]

The fifteen lung tissue samples were kindly provided by Dr. A. Wastiaux (CERCHAR, 11 samples) and by Dr. X.H. Chen (Institute of Industrial Hygiene and Occupational Disease of Sichuan Province, P. R. China, 4 samples). The eleven lung tissue samples from France were prepared as follows: 7 g of lung were cut in small pieces and digested for 45 min at room temperature in concentrated sodium hypochlorite (25 min under magnetic stirring). The recovered residue, after centrifugation at 23,000*g*, was washed and centrifuged three times. After resuspending in distilled water, the samples were frozen at – 70°C before lyophilization. The four lung tissue samples from China were first washed in water and then put in a stove at 50°C until dryness. The samples were ground in an agate mortar with pestle.

Measurements of Free Radicals in Coal and Lung Tissue Samples

ESR spectra were taken on a Varian CSE spectrometer operating in the X band mode (9.39 GHz) with a constant microwave power (0.1 mW) lower than the previously reported minimal microwave saturation power of all the coal samples (Jeunet *et al.*, 1989).[30] Unless otherwise stated, the spectra were recorded *in vacuo* ($< 10^{-4}$ torr) at 77 K (liquid nitrogen). The samples were degassed for 1 h and sealed in a tube of quartz just before the ESR measurement. The intensity of the ESR signal was taken as arbitrary units (a.u.) per gram of sample after taking into account the weight and the volume of samples in the ESR cavity. Strong pitch (SP, $g = 2.0029$) or 2,2′-diphenyl-1-picryl-hydrazyl (DPPH, $g = 2.0036$) was used as reference standard for g value calculations. To study the grinding effects, the coal samples were ground either for 5 min in argon or for 3 min in air both in an agate mortar with pestle or for 1 h in air by a mechanical grinding mill. To study the effect of aging in air, the freshly ground coal samples were left in the ESR quartz tubes and the intensities of the carbon-centered free radicals in coals were periodically measured in air at room temperature.

Chemical Reactivities of Free Radicals in Coals with Redox Reagents

The chemical reactivities of the carbon-centered free radicals were measured by their concentration change after various treatments. The chemical treatments of the coal samples were carried out as follows: Approximately 2 g of coals were freshly ground under argon. Exactly 135 mg of the ground coals were immediately taken and suspended in 6 ml of aqueous solution of sodium formate (1 M, Merck, Paris), an oxygen free radical scavenger, or H_2O_2 (50 mM, Merck, Paris), or GSH (10 mM, Merck, Paris) containing 120 µl of 0.1% polyethylene oxide (Merck, Paris). GSH and H_2O_2 are redox reagents that are bioavailable in cells. Addition of polyethylene oxide was to ensure the moistening of the coal samples in aqueous suspensions. Controls were coals suspended in 6 ml of distilled water (O_2 solubility in H_2O: 3.3% v/v at 20°C). The suspensions were shaken for 1 h at 37°C. Then the coal samples were filtered through a 0.65 µm filter (cellulose acetate, Prolabo, Paris), recovered, dried *in vacuo* by lyophilization, and detected by ESR.

Measurements of Acid Soluble Fe^{2+} Content in Coal Samples

Coal samples (2.7 g) ground under argon were suspended in 30 ml of 50 mM HCl for extraction of Fe^{2+} overnight, and were then filtered twice through a 0.65 µm membrane (cellulose acetate) to remove coal particles. Eighteen ml of 5 mM of 2,2′-bipyridine (Sigma, Paris) dissolved in

0.5 M of phosphate buffer solution (PBS, pH 7.4) was added to 2 ml of aqueous coal filtrates. Fe^{2+} in the aqueous coal filtrates was detected by the formation of 2,2′-bipyridine-Fe^{2+} complexes which can be analyzed using a spectrophotometer (520 nm). The quantity of Fe^{2+} was determined by comparison with the standard curve obtained using commercial $FeSO_4 \cdot 7H_2O$ (Sigma) and was presented as % of coal (w/w).

Studies on the Oxidative Activities of the Acidified Coal Filtrates

An experimental protocol was designed to test for the presence and reactivity of oxidants (symbolized here as A*) after interaction of the acidified coal filtrate with O_2 in aqueous media. The reactivity was measured by its ability to react with the formate anion as the target molecule. The principles of the experimental reactions are presented in equations (1) and (2):

$$A^*(\text{i.e. } {}^\bullet OH) + HCO_2^- \rightarrow AH(\text{i.e. } H_2O) + CO_2^{\bullet -} \quad (1)$$

$$DMPO + CO_2^{\bullet -} \rightarrow (DMPO{-}CO_2^{\bullet -}) \quad (2)$$

The carboxylate radical anion ($CO_2^{\bullet -}$), whose lifetime is in the order of 10^{-9} s, can be trapped if formed in solution by a spin trapping agent, 5,5′-dimethyl-1-pyrroline N-oxide (DMPO) (Sigma). This creates a radical adduct (DMPO–$CO_2^{\bullet -}$), whose lifetime is at least 1 h if there are not stronger reducing species in the medium. The quantity of formed A* is evaluated by the intensity of the ESR signal (DMPO–$CO_2^{\bullet -}$). The experiments were carried out as follows: 0.5 ml of acidified coal filtrates were added to 0.5 ml of 4 M sodium formate dissolved in 1 M phosphate buffer solution (pH 7.4). Then 1 mL of 100 mM DMPO solution, which was prepared according to the method of Buettner and Oberley by passing through charcoal,[27] was immediately added. The reaction proceeded at 37°C in the dark as previously reported.[26,28] Aliquots of the reaction were withdrawn 5 min after addition of DMPO. Control experiments utilized 0.5 ml of distilled water instead of 0.5 ml of coal filtrate. The ESR signal intensities presented in this article are in arbitrary units (a.u.). One thousand a.u. corresponds to 4.14×10^{18} radicals per liter.

RESULTS

Study of the Carbon-centered Free Radicals in Coal Samples

This study was essentially carried out on four of the five coal samples and fifteen lung tissue samples. The presence of conduction electrons in the La Mure sample complicated the ESR study, and the exact measurement of radical concentration in this coal sample has not been performed.[29,30]

The ESR spectra obtained in air at room temperature or at 77 K present a relatively broad line (about 7 G). Figure 1 shows an example of the spectrum realized in air at 77 K (liquid nitrogen) from a ground coal sample of Gardanne. This spectrum is not completely symmetric, suggesting the presence of at least two radical species. In order to improve the resolution and sensitivity, most of the spectra were obtained *in vacuo*

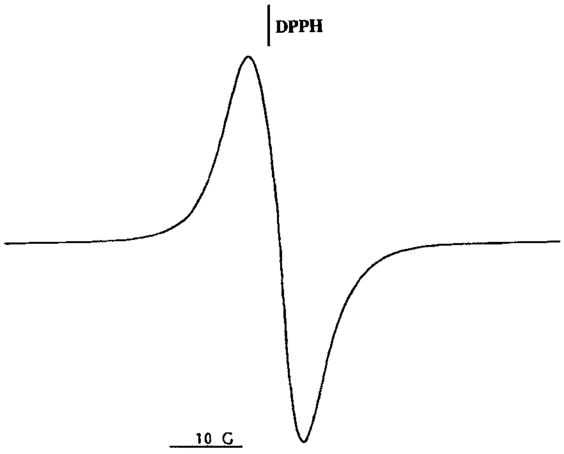

FIGURE 1 ESR spectrum of the coal sample of Gardanne ground in air for 1 h. The spectrum was performed in air at 77 K. Varian CSE instrument, microwave power level: 0.1 mW, time constant: 0.016 s.

($< 10^{-4}$ torr) at 77 K. Under these experimental conditions, we observed an increase in intensity of ESR signals and the presence of two components – a broad one approximately 7 G wide and a narrow one approximately 1 G wide (Figure 2).

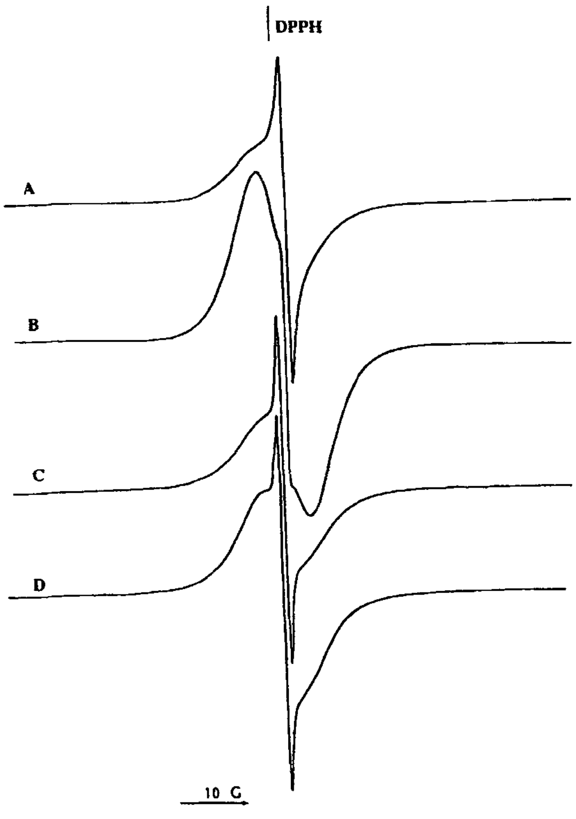

FIGURE 2 ESR spectra of non-ground coal samples. (A) Gardanne, (B) Vouters, (C) Mericourt, (D) Escarpelles. The spectra were performed *in vacuo* at 77 K. See Figure 1 for ESR operational conditions.

An initial examination of the Gardanne (A), Mericourt (C), and Escarpelles (D) spectra confirmed the presence of at least two types of radical species while the spectrum of the Vouters (B) sample was more deformed by the broad signal. The total carbon-centered free radical concentrations in coals increased as coal rank increases (Table I). The g values of the narrow signal (g_n) in different samples varied between 2.0028 and 2.0027 and those of the broad signal (g_b) ranged from 2.0028 to 2.0033. Gardanne presented the most asymmetric signal with a g factor difference of 0.0005 between the narrow and the broad signals. The spectrum of La Mure is presented in Figure 3 and this spectrum seems to include a narrow peak much more intense than the broad one with a $g_b = 2.0032$ and $g_n = 2.0031$.

Effects of Grinding and Aging on the Carbon-centered Free Radicals

Grinding in air or in argon by an agate mortar with pestle or by a mechanical grinding mill produced free radicals in the coals. In accordance with the results previously reported,[26,31] we found that the coal samples were homogeneous and the measurements were reproducible even with 45 mg of coal samples. Table I shows an example of grinding in argon resulting in a total radical increase of 33% in the coal sample of Escarpelles and 25% in that of Gardanne in comparison with non-ground coal samples. This increase was much lower in the Vouters and Mericourt samples

TABLE I Evolution of total carbon-centered free radical concentration and intensity of narrow signals in coals after grinding in argon

Coal	Total radical concentration/g (a.u.)[a]			Intensity of narrow signal/g (a.u.)[b]		
	Non-grinding	Grinding in argon	% increase	Non-grinding	Grinding in argon	% increase
Gardanne	35.6	44.5	25	23.5	26.7	13.9
Vouters	69.6	72.3	3.9	12.2	13.8	13.5
Mericourt	78.5	81.9	4.2	49.3	58.1	17.7
Escarpelles	115.5	153.6	33	56.1	59.6	6.2

[a] Total carbon-centered free radical concentration per gram of coal was obtained by double integration of the ESR spectra.
[b] Intensity of narrow signal was obtained by measuring the height of narrow peak. Please note that the arbitraiy units from narrow peak were not comparable with the ones from total.

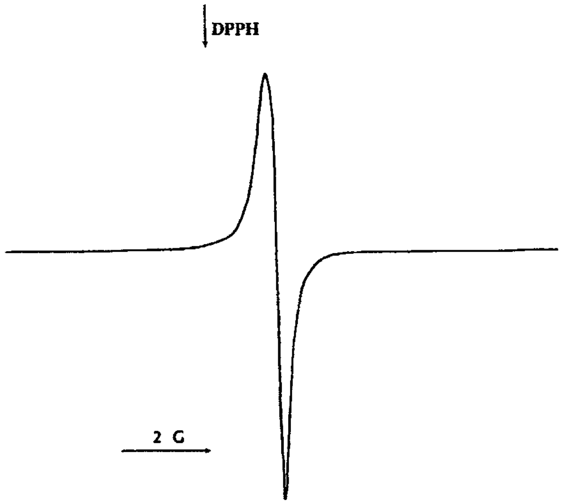

FIGURE 3 ESR spectrum of the non-ground coal sample of La Mure. The spectrum was performed *in vacuo* at 77 K.

(only 3.9% and 4.2%, respectively). The increase of radicals was more significant in the narrow line than in the broad signal. It seems in these two coals that grinding induces free radical species responsible for the narrow component.

With respect to the aging effect, all coal samples were freshly ground in air for 3 min before ESR measurement and then left in air for aging at room temperature. Figure 4 shows that the Gardanne coal sample showed an increase of 5% in radical concentration after 2 h of aging in air and 21% after 28 h. A less significant increase in free radical concentration was observed in the Escarpelles coal. For the two other coal samples the concentrations were approximately constant during the same time period. Exposure to air for 4 months led to a slight decrease of the total radical concentration for all samples.

Effects of Chemical Treatments on the Carbon-centered Free Radicals

For the samples of Vouters, Mericourt and Escarpelles, there is no significant change in free radical concentration following different chemical treatments (Table II). Polyethylene oxide alone

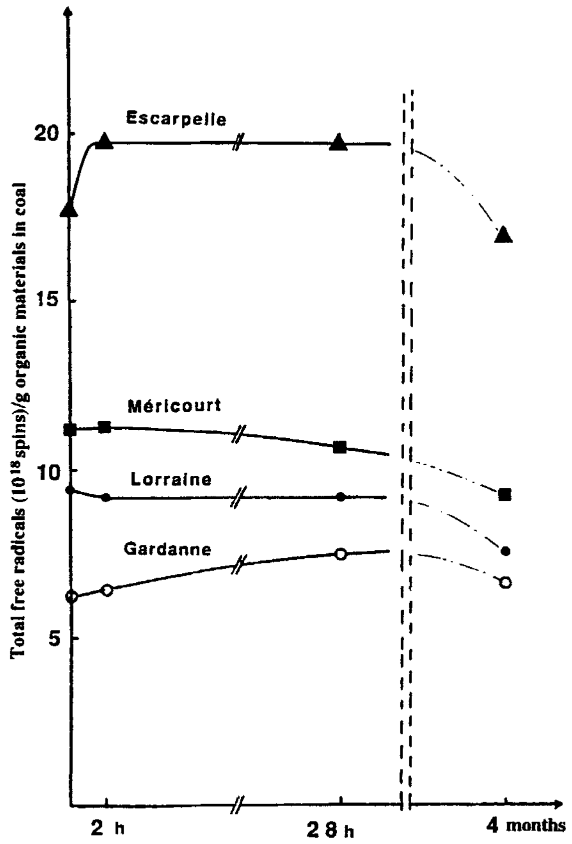

FIGURE 4 Evolution of the intensity of total carbon-centered free radicals in coal samples exposed to air as a function of time. The coal samples were ground in air for 3 min and then left in air for aging. The ESR spectra were performed in air at room temperature and the spin concentration was determined by comparison with the standard solution of DPPH.

TABLE II Evolution (%) of the carbon-centered free radical concentration in coals after different chemical treatments[a]

Coal	H_2O	HCOONa (2 M)	GSH (10 mM)	H_2O_2 (50 mM)
Gardanne	22	36	36	63
Vouters	2	−7	−6	2
Mericourt	4	−5	0	−5
Escarpelles	−2	10	1	7

[a] The percentage of evolution was calculated based on the intensity of the same sample without treatments and was an average of two experiments. The coal samples were ground in argon.

had no effect on any measurements of free radicals. In the case of the Gardanne coal sample, however, which is the least hazardous coal (0% prevalence of CWP), the treatments induced a significant increase of the carbon-centered free radicals either by O_2 dissolved in distilled water (22%), sodium formate (36%), GSH (36%), or H_2O_2 (63%). In contrast, other coals linked to the prevalence of CWP showed no significant changes after those treatments (Table II). Is it possible that the carbon-centered free radicals in these CWP-linked coals are indirectly involved in forming oxygen radicals through catalytic reactions? To test this hypothesis, the spin trapping agent, DMPO (50 mM), was then mixed with the coal (45 mg) and H_2O_2 (5 mM) in a total volume of 2 ml. We found that the levels of DMPO–$OH^\bullet$ were the same as in the control (DMPO + H_2O_2 without coal) for all coal samples except for the one from the Vouters coal mine. We have previously reported that the Vouters coal sample forms DMPO–$CO_2^{\bullet-}$ due to the interaction of the water soluble $FeSO_4$ in the coal with dissolved O_2 in aqueous media.[26,31] These results indicate that the carbon-centered free radicals are essentially inactive.

Study of the Carbon-centered Free Radicals in Lung Tissue Samples

Type of disease, age, sex, mining tenure of the workers, and the concentration of free radicals in the lung tissues are presented in Table III. A few characteristic spectra of lung tissue samples are presented in Figure 5. The spectrum of sample 824 (A) corresponds to a case of pure silicosis in a female worker who made scouring powder. The intensity of this spectrum was much weaker than those obtained from the other lung tissue samples containing coal. Regarding the intensities of the broad and the narrow signals, samples 930, 1536, 1792 and 1981 have a more intense narrow signal compared to the others. As an example, the spectrum of sample 930 (B) clearly showed comparable intensities between narrow and broad signals, whereas the broad signal of the other samples, such as the sample of 1750 (C), is more prominent than the narrow one (Figure 5). The *g* values corresponding to broad and narrow signals in these lung tissues were similar to those of the coals. The *g* values of narrow signals ranged between 2.0027 and 2.0030 and the broad signals between 2.0029 and 2.0035. It is noteworthy that

TABLE III Concentration of carbon-centered free radicals in lung tissue samples of patients with pneumoconiosis or silicosis[a]

Code	Disease	Age (years)	Sex	Mining tenure (years)	Total radical per g coal	Narrow peak/g coal
824	Pure silicosis	22	F	4	0.77	8
1305	Pneumoconiosis	37	M	14	19.5	76
930	Pneumoconiosis	35	M	19	10.9	110
1536	Pneumoconiosis	26	M	7	19.3	188
1723	Pneumoconiosis	?[b]	M	6	7.3	30
1750	Pneumoconiosis	31	M	6	10.1	57
1792	Pneumoconiosis	38	M	22	11.4	146
1939	Tumoral pneumoconiosis	?	M	?	3.8	13
1945	Pulmonary sclerosis	46	M	1.5	10.9	63
1981	Pneumoconiosis	44	M	8	6.9	59
1883	Pneumoconiosis	?	M	?	8.2	42
D1	Silicosis	?	M	?	0.3	1.4
A6	Anthracosis	?	M	?	14.1	37.6
M10	Anthraco-silicosis	?	M	?	0.4	1.8
K1	Anthraco-silicosis	?	M	?	0.2	0.9

[a] See Table I legend for details of quantitation of total and narrow signals.
[b] Age or mining tenure are not known.

no correlation exists between the concentration of the carbon-centered free radicals and the mining tenure of coal workers (Table III).

The four lung tissue samples from China were classified as anthracosis (A6), silicosis (D1), or anthraco-silicosis (M10, K1), although all the workers were exposed to coal dust. The ESR spectra of the lung tissue samples from Chinese coal workers were similar with those from French coal workers (data not shown). Table III shows that the concentration of free radicals in the sample of anthracosis (sample A6) is comparable with that of French lung tissue samples with pneumoconiosis. The intensities of free radicals in the samples of anthraco-silicosis (M10, K1) or silicosis (D1) are comparable with the one of pure silicosis (sample 824). Since no significant difference either in ESR spectra or in concentration of free radicals was observed between the French lung tissue samples treated with hypochlorite and the Chinese lung tissue samples only washed with water, these results indicate that the carbon-centered free radicals in coals are very stable, even after the hypochlorite treatment.

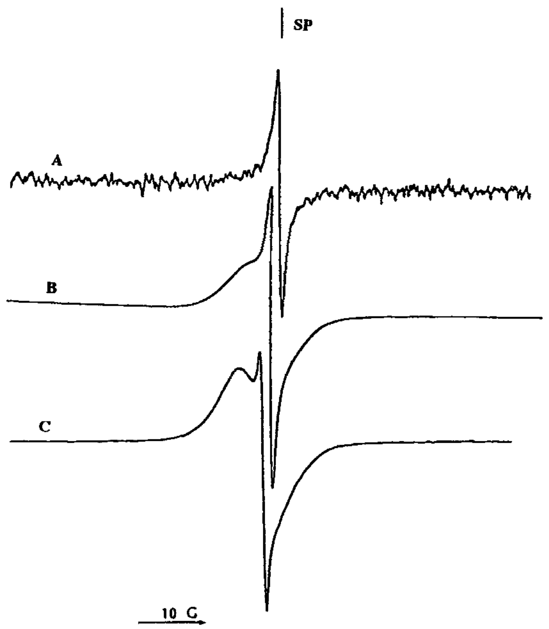

FIGURE 5 ESR spectra of the lung tissue samples. The spectra were performed *in vacuo* at 77 K. (A) Sample 824, (B) Sample 930, (C) Sample 1750. Microwave power level: 0.1 mW, time constant: 0.25 s for sample 824 and 0.128 s for samples 930 and 1750.

Correlation of Acid Soluble Fe^{2+} Content in Coals with the Prevalence of CWP

Table IV shows total Fe^{2+} content in coals as FeO, acid soluble Fe^{2+} content, the DMPO–$CO_2^{\bullet-}$ signal intensities of the acidified coal filtrates, and the prevalence of CWP from the corresponding coal mines. Not all ferrous iron in the coals was acid soluble in 50 mM HCl. The acidified aqueous coal

TABLE IV Coal rank, FeO, acid soluble Fe^{2+} content, and the prevalence of CWP in French coal mines

Coal mine regions	Mine	Coal rank	CWP (%)[a]	FeO (%)[b]	Fe^{2+} (%)[c]	DMPO–$CO_2^{\bullet-}$ (a.u.)[d]
Provence	Gardanne	Sub-bituminous	0	0.29	0[e]	0
Lorraine	Vouters	Bituminous	7	0.68	0.028[f]	255
Nord Pas de Calais	Mericourt	Bituminous	24	1.15	0.25	1670
	Escarpelles	Bituminous		0.65	0.17	1350
Les Alpes	La Mure	Anthracite	12	0.29	0.054	390

[a] Prevalence of category 1 or greater small rounded opacities. Data are from Amoudru[4] by averaging the published prevalences of CWP from years 1980, 1983, and 1984. The prevalence of CWP for the Les Alpes coal mine region was obtained by personel communication. Mericourt and Escarpelles coal mines are from the Nord Pas de Calais region.
[b] Percentage of Fe^{2+} content in coals (w/w) as FeO.
[c] Percentage of acid soluble Fe^{2+} in coals (w/w) was measured after overnight extraction of aqueous coal suspension in 50 mM HCl.
[d] Varian CSE instruments, field set 3380 G, scan range 100 G, microwave power level 10 mW, time constant 1 s. Constants for DMPO–$CO_2^{\bullet-}$: $g = 2.0055$, $a_N = 15.6\,G$, $a_H = 19.0\,G$.
[e] Undetectable.
[f] The coal of Vouters which originally contained 0.058% of water soluble $FeSO_4$ was excluded for calculation of acid soluble Fe^{2+}.

filtrate of Gardanne formed a white precipitate when 2,2′-bipyridine in phosphate buffer (0.5 M, pH 7.4) was added. This was found to be due to the solubilization of large quantities of calcite ($CaCO_3$) in this coal resulting in the precipitation of Ca^{2+} ions by the phosphate.[26] To further confirm that acid soluble Fe^{2+} was not co-precipitated by phosphate, 2,2′-bipyridine without phosphate was added to this aqueous coal suspension before acidification, and no color reaction was observed. This result indicates that the coal of Gardanne did not release Fe^{2+} in an acidic medium. ESR studies on the acidified coal filtrates showed that oxidative activities are related to the content of acid soluble Fe^{2+} in the filtrates. In the presence of *o*-phenanthroline, a specific Fe^{2+} chelator, no ESR signal was observed in the acidified coal filtrates, indicating that Fe^{2+} was essential for the formation of DMPO-$CO_2^{\bullet -}$ radical adduct.

Figure 6 shows the correlation between the acid soluble Fe^{2+} content and the prevalence of CWP in the corresponding French coal mines (correlation coefficient $r = 0.88$). The coal of Gardanne did not release acid soluble Fe^{2+} in 50 mM HCl. Interestingly, this coal is not hazardous to coal workers. In contrast, CWP is very frequent in the workers of the Vouters, La Mure, Escarpelles, and Mericourt mines, and these coal samples are capable of releasing increased amounts of acid soluble Fe^{2+} proportional to the prevalence of CWP.[4]

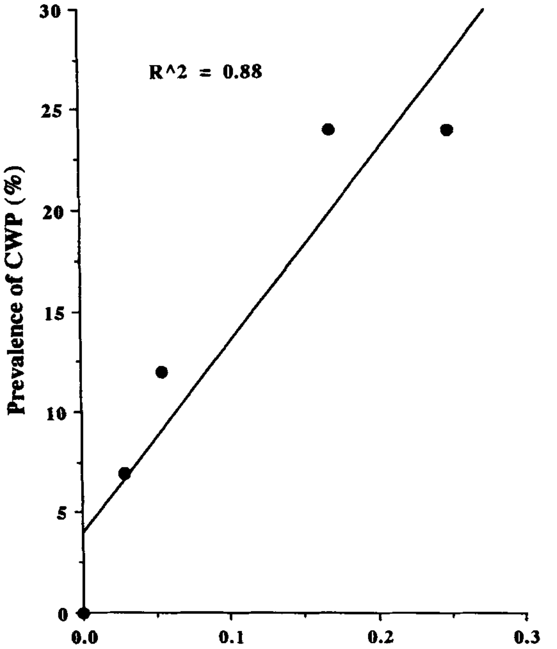

FIGURE 6 Correlation between the prevalence of CWP and acid soluble Fe^{2+} in French coal mines.

DISCUSSION

The field of CWP research is one of the few areas in occupational health in which much epidemiological data are available. This offers a good opportunity to focus on the relationship between epidemiological data and physico-chemical and/or biological characteristics. The objective of this study is to assess whether some physico-chemical parameters play a role in the observed regional differences in the prevalence of CWP.

Since the discovery of the existence of carbon-centered free radicals in coal,[22] Artemov and Reznik suggested that this type of free radical in coal may play a role in the fibrogenicity of coal dust.[32] Dalal *et al.* have shown that the free radicals in anthracite coal are very sensitive to air.[33,34] The only anthracite coal in our studies, the coal of La Mure, did not allow detailed ESR studies because of the conduction electron. The aging studies we have done show that the low rank coal of Gardanne is also very sensitive to air. Although many ESR studies on coal suggest the presence of two peaks, we have demonstrated two types of radical species *in vacuo*. Different interpretations can be found in the literature to explain the existence of this heterogeneity. It has been suggested that coal consists of a macromolecular (MM) phase crosslinked to form a three-dimensional network and a molecular phase situated in the pores of the MM phase.[35] The narrow peak in the spectrum is attributed to the MM phase and the broad peak to the molecular phase.

Another interpretation explains the presence of the two components by the occurrence of three different types of macerals in coals: exinite, vitrinite and inertinite.[36] The broad one results from the paramagnetic center of the vitrinite and the narrow one from the inertinite. Our studies on the four coal samples clearly show that the radicals responsible for narrow signal are much lower in concentration than those giving rise to the broad signal. In comparison with the standard solution of DPPH, the ratio of the narrow and broad radical concentration is estimated to be approximately 1 to 20, which is not quite consistent with the ratio of inertinite/vitrinite (ranged from 1 : 14 to 1 : 4).[25]

Concerning the composition of the two signals, the wings of the broad signal are perfectly symmetric, suggesting that it is generated by a single distinct radical species. The asymmetry of the ESR spectra is probably due to the narrow signal. Minerals in coal such as quartz may contribute to the intensity of the narrow signal, because the signal due to silica radical species with $g = 2.0026$–2.0028 is frequent in many minerals and in coals.[37]

Grinding induced a distinct increase of radicals in the Gardanne and Escarpelles samples. In the case of the Gardanne sample, this increase may be primarily due to oxidation of the surface. In the Escarpelles sample, it is probably due to the creation of new radicals by the mechanical rupture of bonds in a relatively rigid network. To support this interpretation, the oxidation in a simple aerated aqueous medium caused a considerable increase of radicals only in the Gardanne sample (Table II). Since Gardanne coal is not known to induce CWP, the radical species formed by oxidation could not be implicated in the pathogenesis of CWP. Studies on treatments of coal samples with O_2, GSH, and H_2O_2 showed that the carbon-centered free radicals do not have much chemical reactivity in high rank coals, indicating that these types of free radicals are very inert. Examination of lung tissue samples permits us to find the spectrum of the inhaled coal. Since no information such as exposure concentration is available, this does not allow any interpretation of the observed spectral difference as a function of total accumulated dust level. But it evidently appears that the paramagnetic centers presented in coal must have a weak chemical reactivity, because the radicals still exist in the pulmonary medium for many years after inhalation.[35,38] Pure silicosis (sample 824) reveals the existence of different spectrum, weaker than that of coal, which can be confused with the narrow component of the coal spectra. As mentioned earlier, CWP has been reported to occur after inhalation of silica-free carbon. Moreover, Gosset *et al.* have found that the release of TNF-α is not directly linked to the presence of silica in the coal,[39] suggesting that compound(s) other than quartz may contribute to CWP development.

Accumulating evidence suggests that release of oxygen radicals can play an important role in diverse pathologies including inflammation, tissue aging, cardiac ischemia, arthritis, cancer, and fibrosis.[21,40–42] It has been proposed that exposure to coal dust can stimulate AMs to release oxidants and to secret inflammatory factors and cytokines.[20] Based on the observation that transition metals, especially iron, are concentrated in the lungs of miners with CWP,[43] Ghio and Quigley postulated that the body's endogenous iron (Fe^{3+}) sequestered by humic-like substances in certain coals may play a role in CWP.[44] They have shown that oxidants generated by coal dusts after exposure to exogenous $FeCl_3$ increased with the concentration of humic-like substances. By studying the ability of different coals to catalyze the generation of $^{\bullet}OH$ from H_2O_2, Dalal *et al.* found that the potential to induce $^{\bullet}OH$ formation and lipid peroxidation by the coal dusts exhibited a good correlation with the available surface iron.[45] However, it has not yet been shown that the levels of humic-like substances or the available surface iron in the coals correlate with the prevalence of CWP.

Although the prevalence of CWP increases roughly as the coal rank increases, the coal of

La Mure which presents the highest coal rank (an anthracite) in our studied samples induces about 12% of CWP (unpublished data, personal communication). In contrast, the coals of Nord Pas de Calais which are in the middle of the coal rank (bituminous coals) induce 24% of CWP.[4] Since coal rank is only an indicator of coal maturation, other chemical substance(s) associated with coal rank may play a role in inducing CWP. We found that the carbon-centered free radical concentration in coals increases as coal rank increases (Table I). However, these types of free radicals in high rank coals may be too stable to play a role in CWP development. Fe^{2+} is known to produce reactive oxygen species in aqueous medium through Fenton or Haber–Weiss reactions. We have recently studied thirty coal samples from Pennsylvania, West Virginia, and Utah coal mine regions. It was found that the coals from Pennylvania with a high prevalence of CWP had high levels of acid soluble Fe^{2+}. The coals from West Virginia with a medium prevalence of CWP had moderate levels of acid soluble Fe^{2+}. The coals from Utah with low prevalence of CWP had little acid soluble Fe^{2+}. Moreover, the coal of Pennsylvania containing acid soluble Fe^{2+} was found to be able to activate the oxidative stress responsive transcription factor activator protein-1 in human tracheal epithelial cells, whereas the coal of Utah had no effect. Also, the coals of Utah contained a large amount of calcite ($CaCO_3$), which can prevent acid solubilization of iron compounds such as siderite ($FeCO_3$) in the coals.[24] Therefore, it is not coincidental that we observed that the coal of Gardanne with no reported CWP had a huge amount of calcite (10% of coal, w/w)[25] but no Fe^{2+} released in an acidic medium.

The role of acid soluble Fe^{2+} in our studies is biologically relevant to coal dust-induced lung injury because of the phagocytic activity of macrophages and the low pH inside phagolysosomes.[20,46] It has been shown that the ability of the macrophages to dissolve inorganic particles is due to the low pH in the phagolysosomes.[47–49] Following inhalation and phagocytosis of coal dusts by AMs, Fe^{2+} in high rank coals with little calcite will be easily released and then become bioavailable. The oxidants subsequently produced by the interaction of Fe^{2+} with O_2 and/or H_2O_2, as we have shown in the present study, may stimulate AMs to release cytokines and other growth factors.[50–52] The coal from Gardanne did not even release acid soluble Fe^{2+} in 50 mM HCl. Again, there has been no reported CWP for the coal workers of the Gardanne coal mine (Provence). In contrast, the other four coal samples each released measurable amounts of acid soluble Fe^{2+}. Epidemiological studies have shown that CWP is very frequent in the coal workers of Nord Pas de Calais (Mericourt and Escarpelles), Les Alpes (La Mure), and Lorraine (Vouters) mines in France.[4] These results suggest that bioavailable Fe^{2+} in coals may be the active compound inducing CWP, at least in the early stages of the disease. Biological responses induced by iron compounds are being investigated.

In the present study, we excluded 0.058% of $FeSO_4$ in the coal of Vouters before measurement of acid soluble Fe^{2+}. The major reason for this consideration is that $FeSO_4$ is highly water soluble (157 g/l). Due to the great solubility of $FeSO_4$ in water, $FeSO_4$ in coal dusts may immediately dissolve in lung medium after inhalation by coal workers and then become unphagocytosable by alveolar macrophages. The dissolved $FeSO_4$ in extracellular medium may not contribute to CWP development, but can inactivate the protective screen afforded by alpha-1-antiprotease.[26,53] Consequently, oxidants produced by $FeSO_4$ with O_2 and/or H_2O_2 might trigger emphysema in these coal workers.[26,31]

In conclusion, our studies show that the carbon-centered free radical in low rank coal such as that of Gardanne, which is not fibrogenic, is more reactive to different chemical treatments than those in high rank coal, indicating that this type of free radical may not be directly involved in the process of CWP. In contrast, Fe^{2+} in certain coals may be solubilized under the acidic conditions inside the phagolysosomes of AMs and thus become bioavailable. These results further strengthen our hypothesis that acid soluble Fe^{2+} may play an important role in coal dust-induced CWP.[24]

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