

Bioavailability and catalytic properties of copper and iron for Fenton chemistry in human cerebrospinal fluid

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A breakdown in homeostasis of redox-active metals represents an important factor for neurodegeneration. We have used EPR spectroscopy and BMPO spin-trap to investigate the catalytic properties and ligand modulation of redox activity of copper and iron in human cerebrospinal fluid (CSF). In contrast to iron, copper supplementation provoked a statistically significant increase in hydroxyl free radical generation in CSF treated with H₂O₂. However, in a binary copper/iron containing Fenton system, iron catalytically activated copper. The chelator EDTA, which represents a model of physiological metal ligands, completely prevented copper's redox activity in CSF, while iron chelation led to a significant increase in hydroxyl radical generation, indicating that copper and iron do not only have diverse catalytic properties in the CSF but also that their redox activities are differently modulated by ligands. The application of DDC reduced hydroxyl radical generation in the CSF containing catalytically active metals (free Cu²⁺ or Fe³⁺-EDTA complex). We conclude that chelators, such as DDC, are capable of preventing the pro-oxidative activity of both metals and may be suitable for reducing hydroxyl radical formation in certain pathophysiological settings.

Keywords: copper, iron, Fenton reaction, cerebrospinal fluid, diethyldithiocarbamate

Introduction

A low level of antioxidants and low regenerative capacity in combination with high oxygen consumption together

result in brain tissue being susceptible to oxidative damage.¹ The most detrimental pro-oxidative process is the Fenton reaction allowing H₂O₂-mediated oxidation of redox-active metals such as iron and copper producing highly reactive HO• radical.^{2,3} In contrast to the cytosol

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Abbreviations: EPR, electron paramagnetic resonance; BMPO, 5-tert-butoxycarbonyl 5-methyl-1-pyrroline N-oxide; CSF, cerebrospinal fluid; EDTA, ethylenediaminetetraacetic acid; DDC, diethyldithiocarbamate, ALS, amyotrophic lateral sclerosis

and other body fluids, CSF contains catalytically active iron and copper as well as small molecular weight ligands which may catalyse the formation of HO[•]. CSF is poor in antioxidants, transferrin and ceruloplasmin which normally bind and isolate transition metals. Nervous tissue spontaneously generates and releases H₂O₂ into the CSF where, even under physiological conditions, its concentration can reach up to 1 mM, while in neurodegeneration H₂O₂ production is promoted via several different pathways.⁴⁻⁶ Such characteristics make CSF highly susceptible to Fenton chemistry.

The levels of copper and iron in brain tissue increase with age.⁷ Therefore, a breakdown in metal homeostasis could represent a key factor in the promotion of oxidative stress⁸ related to a variety of age-related neurodegenerative diseases.⁹ Extensive data in the literature show that copper and/or iron play a significant role in the development of Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS) and other neurodegenerative diseases.^{8,10} Alzheimer's disease is characterised by the appearance of amyloid plaques,¹¹ which could be described as metallic sinks as unusually high concentrations of iron (1 mM) and copper (0.4 mM) have been found within them.¹ Amyloid β-peptide is capable of reducing iron and copper to their oxidatively active forms (Fe²⁺ and Cu⁺) which promote the Fenton reaction.¹² In Parkinson's disease, iron deposits have been observed in substantia nigra.¹³ The cells that contain neuromelanin, which has been postulated to be an iron-storage molecule, are most likely to be lost first due to oxidative damage.¹⁴ In addition, it is known that neuromelanin primarily consists of products of dopamine redox chemistry, the most abundant neurotransmitter.¹⁵ Dopamine coordinates metals such as Cu²⁺ and Fe³⁺,¹⁶ reduces the oxidation state of metals and subsequently causes H₂O₂ production,¹⁷ setting up the conditions for Fenton chemistry. It has also been shown in several MRI studies that the motor cortex of ALS patients contains regions of accumulated ferric ions.¹⁸ About 20% of familial ALS cases are characterised by mutations resulting in misfolded Cu,Zn-superoxide dismutase which may form copper-rich deposits in the brain.¹⁹ The increased level of copper observed in the CSF of Parkinson's disease, Alzheimer's disease⁴ and ALS patients¹⁹ demonstrates that this metal leaks from brain deposits into the CSF. This appears not to be the case for iron since its oxidised form (Fe³⁺), preferentially produced under physiological pH values, readily forms insoluble complexes with hydroxyl and phosphate ions present in the CSF. It

was observed in environmental studies that reduced copper transfers an electron to iron which, in turn, participates in the oxidation of organic compounds in atmospheric waters.²⁰ Buettner and Jurkiewicz²¹ have postulated that such processes may be of significance in a biological setting. For example, the production of HO[•] in the CSF of ALS patients^{5,22} appears to be higher than that attributable only to the increased level of catalytically active copper.¹⁹

The aim of this study was to elucidate the differences between the catalytic properties of copper and iron in the Fenton system in CSF. As chelation can modify the redox activity of transition metals, we have also examined the effects of ligands on HO[•] generation in the Fenton system in CSF using EDTA and DDC as model compounds. EPR spectroscopy and spin-trap BMPO were used to quantify the production of HO[•] radicals in the CSF systems containing H₂O₂, iron and copper with or without EDTA and DDC.

Subjects and methods

CSF samples

Lumbar CSF was obtained from a group of 10 patients with neurological disorders unrelated to oxidative stress (2 migraines, 3 tension headaches, 3 lumbar disc herniations and 2 cervical disc herniations). The age range of this group of patients was 32–59 years (6 males and 4 females). The CSF samples were obtained after an overnight bed rest and fasting (at the point when diagnosis took place). CSF not used for clinical evaluations was used for our study. CSF was centrifuged (5000 g, 10 min at 4°C), rapidly frozen and stored at –80°C. All patients received a balanced diet prescribed by a nutritionist without any vitamin supplements. The recruitment and counselling of patients, sample collection and sample handling were all conducted according to the internationally recognised ethical standards (The Helsinki Declaration of 1964, as revised in 1975, 1983, and 1989). Institutional approval for the study was granted by 'The Clinics Ethics Committee' which followed international guidelines.

Fenton system in CSF

FeCl₃ and CuCl₂ (Merck, Darmstadt, Germany) were added to CSF at final concentrations of 10 μM or 100 μM. In all experiments, H₂O₂ (Renal, Budapest, Hungary) was added at a final concentration of 1 mM. The system with no supplemented metals served as a control. The experiments with 100 μM metals

were repeated in the presence of EDTA (Merck; 100 μM final concentration) which was added to the initial solutions of metal salts. The effect of DDC (Merck; 500 μM final concentration) on the production of HO^\bullet was determined in two systems: Cu^{2+} (100 μM) + H_2O_2 (1 mM) and Fe^{3+} -EDTA (100 μM) + H_2O_2 (1 mM). Experiments using the binary catalytic system ($\text{Cu}^{2+}/\text{Fe}^{3+}$) were performed by combining Cu^{2+} (100 μM), Fe^{3+} (300 μM) and H_2O_2 (1 mM) in CSF. All initial solutions were prepared in ultrapure 18 M Ω water. All experiments were performed using CSF from all 10 patients involved in the study. The concentration of spin-trap BMPO (Northwest LSS; Vancouver, WA, USA) in all experiments was 500 mM.

EPR spectroscopy

EPR spectra were recorded at room temperature using a Varian E104-A EPR spectrometer operating at X-band (9.572 GHz) using the following settings: modulation amplitude, 2 G; modulation frequency, 100 kHz; and microwave power, 10 mW. The spectra were recorded using EW software (Scientific Software, Bloomington, IL, USA). In order to maintain a constant level of oxygen, samples were

drawn into 10-cm long gas-permeable Teflon tubes (wall thickness 0.025 mm and internal diameter 0.6 mm; Zeus Industries, Raritan, NJ, USA), which were placed in quartz capillaries. Computer simulations of EPR spectra were performed using the WINEPR SimFonia computer program (Bruker Analytische Messtechnik GmbH, Karlsruhe, Germany) in order to identify BMPO adducts formed in the system and to determine the intensity of EPR spectra. Simulation parameters for the spectrum of the BMPO adduct with HO^\bullet (BMPO/OH) were: diastereomer 1 (81.6%): $a^N = 13.56$; $a^{H\beta} = 12.3$; $a^{H\gamma} = 0.66$; diastereomer 2 (18.4%): $a^N = 13.47$; $a^{H\beta} = 15.31$; $a^{H\gamma} = 0.62$.

Simulation parameters for the signal of BMPO adduct with carbon-centred radical (BMPO/C) were: $a^N = 15.2$ G; $a^{H\beta} = 21.6$ G.²³

Statistical analysis

Statistical analysis was carried out using Statistica v.6.0 (StatSoft Inc.; Tulsa, OK, USA). The results are presented as mean values (of 10 experiments) \pm SD. Statistical significance was determined using a non-parametric two-tailed Mann–Whitney test to compare data pairs ($P < 0.05$).

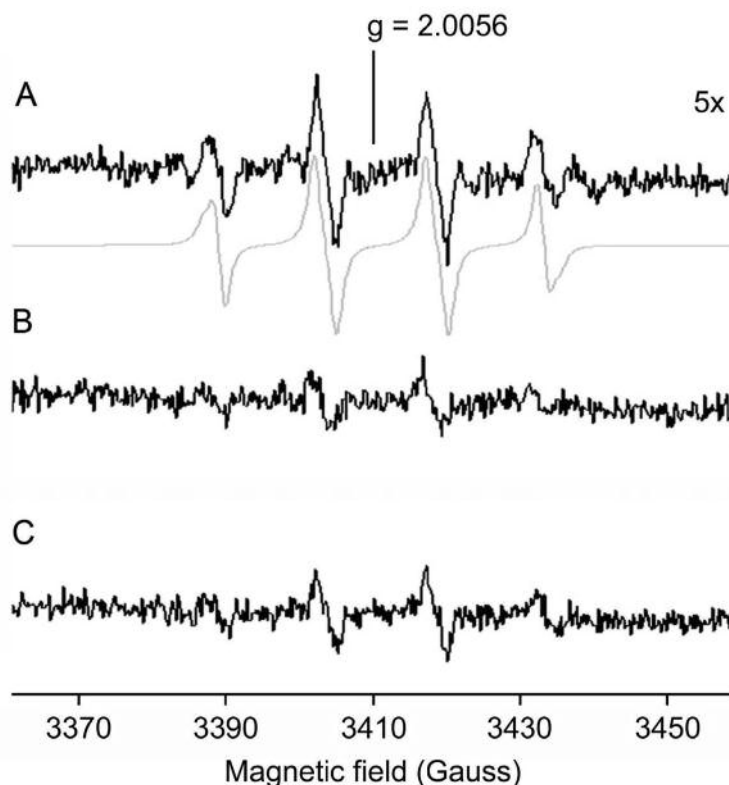


Figure 1 Characteristic EPR spectra of BMPO adducts in the CSF with: (A) Cu^{2+} (10 μM) and H_2O_2 (1 mM); (B) H_2O_2 (1 mM); (C) Fe^{3+} (10 μM) and H_2O_2 (1 mM). Grey: the spectral simulation of BMPO/OH signal. The centre of the signal of BMPO/OH is at $g = 2.0056$. Each spectrum represents an accumulation of five spectra obtained using different CSF samples

Results

Characteristic EPR signals (5 accumulations) of BMPO adducts obtained in CSF after the addition of 1 mM H₂O₂ with or without pre-added 10 μM redox-active metals is shown in Figure 1. The spectral simulation of BMPO/OH adduct was used to identify the presence of HO• in the systems. The addition of iron did not lead to increased generation of HO•. However, supplementation of copper provoked a statistically significant increase in HO• production when compared to CSF with only H₂O₂ added.

In order to distinguish the effects of copper and iron, experiments were repeated using a higher final cation concentration (100 μM) (Fig. 2). Even at the high concentration, Fe³⁺ supplementation did not provoke any significant effects. However, 100 μM Cu²⁺ led to a further increase in HO• production. The spectral simulation represents a combination of signals of BMPO/OH and BMPO/C adducts. The carbon-centred radical could be generated by the reactions of HO• with proteins or carbonate anions present in CSF (HO• + CO₃²⁻ → OH⁻ + CO₃^{•-}).

The chelating agent EDTA caused opposing effects on HO• generation in the copper- and the iron-based Fenton systems (Fig. 3A,B). EDTA completely prevented the activity of copper in the Fenton system as no BMPO/OH adduct signals were observed. In contrast, EDTA-mediated chelation of iron (Fe³⁺-EDTA) led to a significant increase in the generation of HO• when compared with the Fenton system containing iron (Fe³⁺). DDC was a very efficient

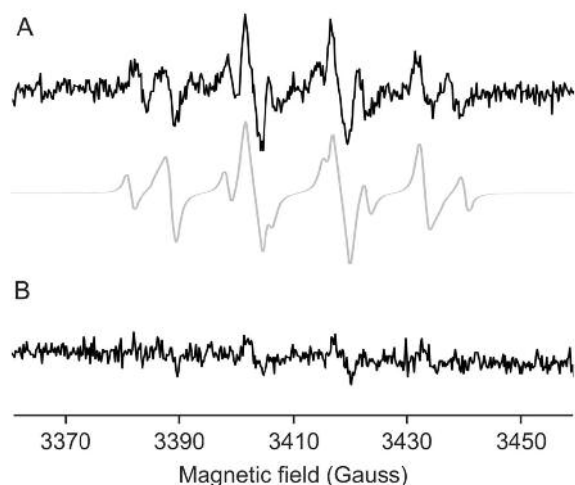


Figure 2 Characteristic EPR spectra of BMPO adducts in the CSF with: (A) Cu²⁺ (100 μM) and H₂O₂ (1 mM); (B) Fe³⁺ (100 μM) and H₂O₂ (1 mM). Grey: the simulation of spectrum composing of BMPO/OH and BMPO/C signals

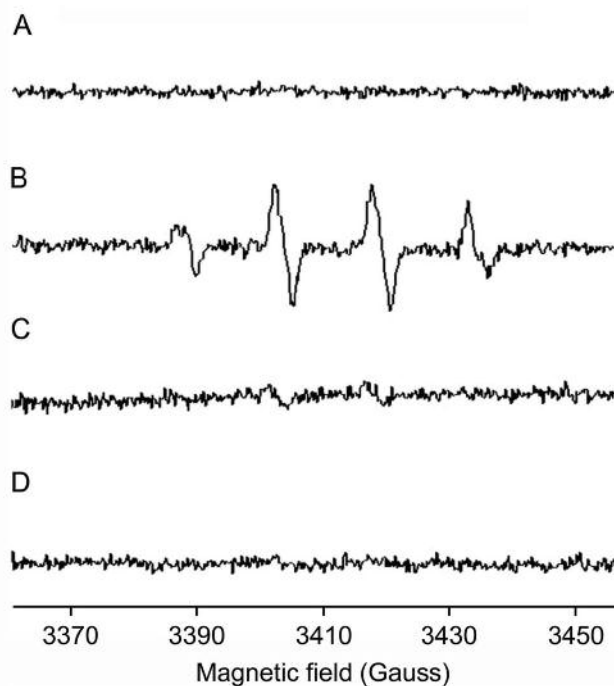


Figure 3 Ligand modulation of the catalytic activity of metals. Characteristic EPR spectra of BMPO adducts obtained in the CSF with: (A) Cu²⁺-EDTA (100 μM) and H₂O₂ (1 mM); (B) Fe³⁺-EDTA (100 μM) and H₂O₂ (1 mM); (C) Cu²⁺ (100 μM), DDC (500 μM) and H₂O₂ (1 mM); (D) Fe³⁺-EDTA (100 μM), DDC (500 μM) and H₂O₂ (1 mM)

antioxidant as it prevented the generation of HO• in the CSF with pre-added Cu²⁺ or Fe³⁺-EDTA (Fig. 3C,D).

The intensities of BMPO/OH signals obtained in the systems with 100 μM metals and/or 1 mM H₂O₂ are shown in Figure 4. In addition, the intensity of the BMPO/OH signal obtained in the binary system which simulated the real copper/iron ratio in CSF (approximately 1:3),²⁴ containing Cu²⁺ (100 μM), Fe³⁺ (300 μM) and H₂O₂ (1 mM) is presented (spectrum not shown). Iron, which by itself does not provoke increased generation of HO•, catalytically activated copper in the CSF as the level of HO• produced in the binary copper/iron system was significantly higher than in the CSF supplemented only with copper (*P* = 0.011).

Discussion

The sole addition of H₂O₂ to CSF provoked weak HO• generation. This represents an inevitable consequence of the presence of redox-active metals in normal CSF (copper 0.25 μM, iron 0.8 μM).²⁴ Such baseline Fenton system activity may have some constructive

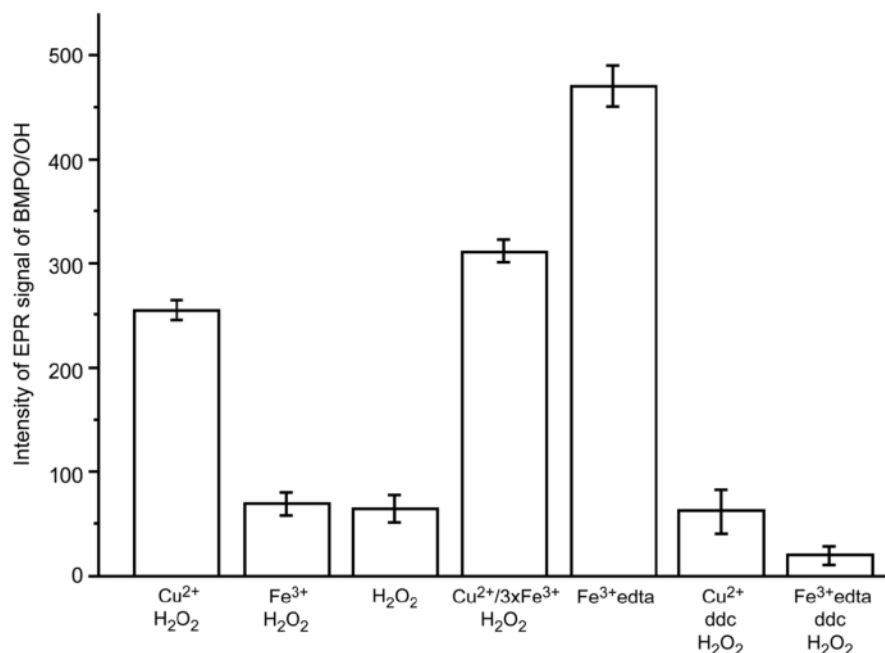
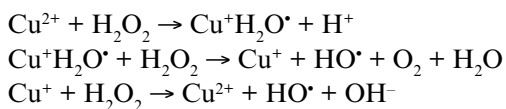
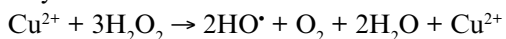


Figure 4 Relative generation of BMPO/OH adducts in the CSF. The intensities were established using the spectral simulations of corresponding EPR signals obtained in 10 different CSF samples.

role in the maintenance of redox equilibrium (e.g. by removing excess H₂O₂). Free copper as well as weakly chelated iron, in the presence of H₂O₂, provoke an increase in the production of HO• in CSF. As we added the oxidised form of copper (Cu²⁺), it had to be reduced to the cuprous form (Cu⁺) in order to participate in the Fenton mechanism. Ascorbate, glutathione and other reducing agents present in the CSF may be responsible for this. In addition, Cu²⁺ is susceptible to H₂O₂-mediated reduction via the previously proposed reactions:²⁵



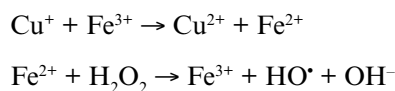
Summary:



This mechanism could account for significant deleterious effects of the Cu²⁺ + H₂O₂ system in biological structures compared to the corresponding system containing iron.^{26,27} The best illustration of the reactivity of free copper is the fact that its intracellular concentration under normal conditions is restricted to only one ion per cell.²⁸

Iron supplementation did not provoke HO• generation. Under physiological conditions, iron is only slightly soluble as it tends to form precipitates with ions such as OH⁻ and inorganic phosphates.²⁹

However, iron and copper can be catalytically interlinked in CSF. It has been previously shown that Cu⁺ can reduce Fe³⁺.^{20,25} Therefore, iron could facilitate copper-induced HO• generation via the reactions:



This we observed in the binary catalytic system in the CSF. HO• generated in CSF can react with proteins and carbonate ions forming carbon-centred radicals which represent very dangerous reactive species due to their long life-time and specificity for the enzyme active centres containing metals (e.g. those of superoxide dismutase or glutathione peroxidase).³⁰

Iron itself becomes redox-active in the CSF when partially chelated.³¹ This was illustrated by using EDTA as a model of physiological low molecular weight iron complexes.³¹ Various compounds present in CSF may, in a similar way to EDTA, bind and stabilise iron *in vivo*. It is known that ADP and ATP complex Fe³⁺ and promote its Fenton-related reactivity.³² Nitric oxide (NO) is also capable of increasing iron solubility via the formation of DNIC (dinitrosyl iron complexes). The sum of nitrites and nitrates, as end products of NO, is increased in Parkinson's disease, Alzheimer's disease⁴ and ALS,³³ which correlates with higher NO production during

neurodegeneration. The opposite effects of complex formation on copper reactivity could be explained by the fact that Cu^{2+} reduction is presumed to proceed by reducing agent polarisation,³⁴ a process that cannot proceed if only a single labile position remains at the metal centre.³⁵

Therapeutic strategies using metal chelators have had some success in altering the progression of symptoms of Alzheimer's disease, Parkinson's disease and other neurodegenerative diseases.³⁶ Our results indicate that copper and iron require different approaches to be developed in order to prevent the Fenton reaction-mediated oxidative stress both in CSF and brain tissue. The high reactivity of free copper requires application of a strong and efficient chelator. On the other hand, the main characteristics of an iron sequestrant should be its ability to compete with metal ligands naturally present in CSF. We demonstrated that DDC was able to inhibit the pro-oxidative activity of both free copper and weakly chelated iron. In addition, we have recently shown that DDC inhibits the *in vitro* generation of HO^\bullet in the Fenton system with Fe^{2+} .³⁷ *In vivo* Fe -DDC₂ complexes bind NO thus forming MNIC and DNIC.³⁸ In this way, DDC may remove iron and any excess NO from the CSF or nervous tissue during neurodegeneration. Such permissive roles of DDC open the possibility for an application of its pharmacological form (disulfiram) in the treatment of neurodegenerative disorders. However, the effects of reducing agents such as ascorbate, urate, glutathione and dopamine present in CSF on the catalytic properties of copper and iron should be further examined.

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References

- Smith MA, Harris PLR, Sayre LM, Perry G. Iron accumulation in Alzheimer disease is a source of redox generated free radicals. *Proc Natl Acad Sci USA* 1997; **94**: 9866–9868.
- Koppenol WH. Fenton chemistry and iron chelation under physiologically relevant conditions: electrochemistry and kinetics. *Chem Res Toxicol* 2006; **19**: 1263–1269.
- Cui K, Luo X, Xu K, Murthy MRV. Role of oxidative stress in neurodegeneration: recent developments in assay methods for oxidative stress and nutraceutical antioxidants. *Prog Neuropsychopharmacol Biol Psychiatry* 2004; **28**: 771–799.
- Boll MC, Alcaraz-Zubeldia M, Montes S, Rios C. Free copper, ferroxidase and SOD1 activities, lipid peroxidation and NO_x content

- in the CSF. A different marker profile in four neurodegenerative diseases. *Neurochem Res* 2008; **33**: 1717–1723.
- Liu D, Wen J, Liu J, Li L. The roles of free radicals in amyotrophic lateral sclerosis: reactive oxygen species and elevated oxidation of protein, DNA, and membrane phospholipids. *FASEB J* 1999; **13**: 2318–2328.
- Huang X, Atwood CS, Hartshorn MA et al. The A β peptide of Alzheimer's disease directly produces hydrogen peroxide through metal ion reduction. *Biochemistry* 1999; **38**: 7609–7616.
- Takahashi S, Takahashi I, Sato H, Kubota Y, Yoshida S, Muramatsu Y. Age-related changes in the concentrations of major and trace elements in the brain of rats and mice. *Biol Trace Elem Res* 2001; **80**: 145–158.
- Valko M, Morris H, Cronin MTD. Metals, toxicity and oxidative stress. *Curr Med Chem* 2005; **12**: 1161–1208.
- Barnham KJ, Masters CL, Bush AI. Neurodegenerative diseases and oxidative stress. *Nat Rev* 2004; **3**: 205–214.
- Cookson MR, Shaw PJ. Oxidative stress and motor neurone disease. *Brain Pathol* 1999; **9**: 165–186.
- Glenner GG, Wong CW. Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem Biophys Res Commun* 1984; **120**: 885–890.
- Nakamura M, Shishido N, Nunomura A et al. Three histidine residues of amyloid- β peptide control the redox activity of copper and iron. *Biochemistry* 2007; **46**: 12737–12743.
- Shapira AHV, Mann VM, Cooper JM, Krige D, Jenner PJ, Marsden CD. Mitochondrial function in Parkinson's disease. *Ann Neurol* 1992; **32**: S116–S124.
- Hirsch E, Graybiel AM, Agid YA. Melanized dopaminergic neurons are differentially susceptible to degeneration in Parkinson's disease. *Nature* 1988; **334**: 345–348.
- Wakamatsu K, Fujikawa K, Zucca FA, Zecca L, Ito S. The structure of neuromelanin as studied by chemical degradative methods. *J Neurochem* 2003; **86**: 1015–1023.
- Gerard C, Chehhal H, Hugel RP. Complexes of iron(III) with ligands of biological interest: dopamine and 8-hydroxyquinoline-5-sulfonic acid. *Polyhedron* 1994; **13**: 591–597.
- Opazo C, Huang X, Cherny RA et al. Metalloenzyme-like activity of Alzheimer's disease β -amyloid. Cu-dependent catalytic conversion of dopamine, cholesterol, and biological reducing agents to neurotoxic H_2O_2 . *J Biol Chem* 2002; **277**: 40302–40308.
- Oba H, Araki T, Ohtomo K et al. Amyotrophic lateral sclerosis: T2 shortening in motor cortex at MR imaging. *Radiology* 1993; **189**: 843–846.
- Ihara Y, Nobukuni K, Takata H, Hayabara T. Oxidative stress and metal content in blood and cerebrospinal fluid of amyotrophic lateral sclerosis patients with and without a Cu,Zn-superoxide dismutase mutation. *Neurol Res* 2005; **27**: 105–108.
- Sedlak DL, Hoigne J. The role of copper and oxalate in the redox cycling of iron in atmospheric waters. *Atmos Environ* 1993; **27**: 2173–2185.
- Buettner GR, Jurkiewicz BA. Catalytic metals, ascorbate and free radicals: combinations to avoid. *Radic Res* 1996; **145**: 532–541.
- Bacic G, Spasojevic I, Secerov B, Mojovic M. Spin-trapping of oxygen free radicals in chemical and biological systems: new traps, radicals and possibilities. *Spectrochim Acta A* 2008; **69**: 1354–1366.
- Teixeira MC, Telo JP, Duarte NF, Sá-Correia I. The herbicide 2,4-dichlorophenoxyacetic acid induces the generation of free-radicals and associated oxidative stress responses in yeast. *Biochem Biophys Res Commun* 2004; **324**: 1101–1107.
- Lentner C. *Geigy Scientific Tables*, vol 1, 8th edn. Basel: Ciba-Geigy, 1981.
- Barb WG, Baxendale JH, George P, Hargrave KR. Reactions of ferrous and ferric ions with hydrogen peroxide. Part 1. – The ferrous ion reaction. *Trans Farad Soc* 1951; **47**: 462–500.
- Gutteridge JMC, Wilkins S. Copper salt-dependent hydroxyl radical formation damage to proteins acting as antioxidants. *Biochim Biophys Acta* 1983; **759**: 38–41.
- Yamamoto K, Kawanishi S. Hydroxyl free radical is not the main active species in site-specific DNA damage induced by copper (II) ion and hydrogen peroxide. *J Biol Chem* 1989; **264**: 15435–15440.
- Rae TD, Schmidt PJ, Pufahl RA, Culotta VC, O'Halloran TV. Undetectable intracellular free copper: the requirement of a copper chaperone for superoxide dismutase. *Science* 1999; **284**: 805–808.
- Chaper E, Mahoney JR, Bryant RG, Eaton JW. Iron-catalyzed hydroxyl radical formation: stringent requirement for free iron

- coordination site. *J Biol Chem* 1984; **259**: 3620–3624.
30. Michelson AM, Maral J. Carbonate anions; effects on the oxidation of luminal, oxidative hemolysis, γ -irradiation and the reaction of activated oxygen species with enzymes containing various active centres. *Biochimie* 1983; **65**: 95–104.
 31. Engelmann MD, Bobier RT, Hiatt T, Cheng IF. Variability of the Fenton reaction characteristics of the EDTA, DTPA, and citrate complexes of iron. *Biometals* 2003; **16**: 519–527.
 32. Fong KL, McCay PB, Poyer JL. Evidence for superoxide dependent reduction of Fe^{3+} and its role in enzyme generated hydroxyl radical formation. *Chem Biol Interact* 1976; **15**: 77–89.
 33. Nikolic-Kokic A, Stevic Z, Stojanovic S et al. Biotransformation of nitric oxide in the cerebrospinal fluid of amyotrophic lateral sclerosis patients. *Redox Rep* 2005; **10**: 265–270.
 34. Taqui Khan MM, Martell AE. Metal ion and metal chelate catalyzed oxidation of ascorbic acid by molecular oxygen. I Cupric and ferric ion catalyzed oxidation. *J Am Chem Soc* 1967; **89**: 4176–4185.
 35. Ghosh SK, Gould ES. Electron transfer. 98. Copper(II) and vanadium(IV) catalysis of the reduction of peroxide-bound chromium(IV) with ascorbic acid. *Inorg Chem* 1989; **28**: 1948–1951.
 36. Merkofer M, Kissner R, Hider R, Brunk UT, Koppenol WH. Fenton chemistry and iron chelation under physiologically relevant conditions: electrochemistry and kinetics. *Chem Res Toxicol* 2006; **19**: 1263–1269.
 37. Orescanin-Dusic Z, Milovanovic S, Nikolic-Kokic A, Radojicic R, Spasojevic I, Spasic M. Diethyldithiocarbamate potentiates the effects of protamine sulphate in the isolated rat uterus. *Redox Rep* 2009; **14**: 48–54.
 38. Van Faassen E, Vanin AF. (eds) *Radicals for life. The various forms of nitric oxide*, 1st edn. Amsterdam: Elsevier, 2007.