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The biochemical basis of antioxidant therapy in critical illness

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During critical illness free radical production may increase as a result of, for example, sepsis or tissue trauma. In addition, because of a potential for increased losses, and the possibility of inadequate nutrition, the antioxidant defences of the body may become compromised. Thus, the delicate balance between free radicals and antioxidants may be disturbed. Various nutritional and pharmacological strategies to enhance antioxidant defences have been proposed, which aim either to maintain or enhance endogenous antioxidant stores or to provide alternative antioxidant agents. Trace elements and amino acids are particularly important, and their synergistic role in the maintenance of the body's antioxidant defence network will be discussed.

Critical illness: Free radical production: Antioxidant therapy: Sepsis

Free radicals and antioxidants are terms that are in common parlance. However, free radicals are usually considered to be deleterious, and it is often forgotten that free radicals and other reactive species perform many essential functions. During critical illness patients are exposed to many factors that may lead to depletion of antioxidant defences and increases in free radical production. Nutritional provision of antioxidants and their precursors enables maintenance of whole-body antioxidant defences without jeopardising the essential benefits of free radical production.

Free radicals, reactive oxygen species and reactive nitrogen species

Free radicals are chemicals that contain unpaired electrons, which make them very reactive and able to damage many cellular and extracellular components. Some examples of free radicals are superoxide, lipid peroxides, hydroxyl radicals and NO. 'Reactive oxygen species' (ROS) and 'reactive nitrogen species' are terms that are frequently used interchangeably with 'free radicals', although this usage may not be strictly correct because they may not contain unpaired electrons. ROS and reactive nitrogen species may be formed from, or give rise to, free radicals and some antioxidants (e.g. glutathione) may be active against more than one class.

Why do all these harmful compounds have to be dealt with?

From an evolutionary perspective, these compounds have to be dealt with as a consequence of living in aerobic environments. The O_2 content of the atmosphere started increasing 2500×10^6 years ago and is now 21%; at times it has been as high as 35%. In order to survive in hostile environments in which reactive chemicals are constantly being formed, organisms have harnessed O_2 for energy metabolism and other functions, and have developed elaborate and synergistic antioxidant defences to cope with these chemicals. Free radicals, ROS and reactive nitrogen species are always present and antioxidant defences are always active. O_2 is used in the mitochondrial respiratory chain, which is so important in ATP generation and is particularly vulnerable during critical illness. As a consequence of using O_2, the mitochondrial respiratory chain is one of the major sites of free radical production in the body. In order to even deliver O_2 to cells, O_2 has to be transported bound to Hb in a way that also leads to the production of substantial quantities of ROS. Maintenance of vascular tone requires the reactive nitrogen species NO, and the reaction of this NO with ROS formed by Hb and O_2 transport may generate yet more reactive species such as peroxynitrite, even in healthy individuals. Yet, in critical illness there is another, extremely important, function that is triggered and massively increases whole-body

Abbreviations: ROS, reactive oxygen species; SOD, superoxide dismutase.
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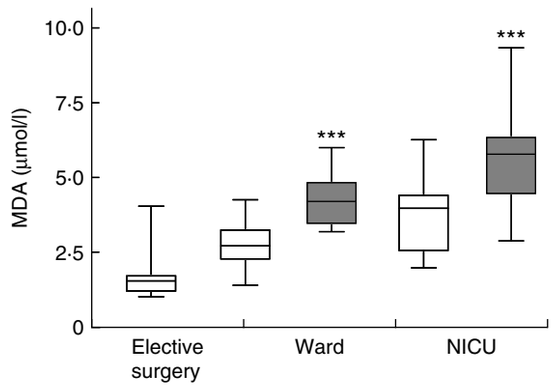

Fig. 1. A comparison of plasma malondialdehyde (MDA; lipid peroxidation marker) concentrations for infants undergoing elective surgery, stable after having undergone a major operation (ward) or in the neonatal intensive care unit (NICU). Patients are further divided into those receiving enteral nutrition (□) and those receiving total parenteral nutrition (■). Values are medians represented by the horizontal bar within the box and interquartile ranges represented by upper and lower limits of the box and ranges represented by vertical bars. Mean values were significantly different from those for patients receiving enteral nutrition: ***$P<0{\cdot}001$. Mean values for the elective surgery, ward and NICU patient groups were significantly different ($P<0{\cdot}001$). (Redrawn from Basu *et al.* 1999.)

production of reactive species, i.e. bacterial killing. Activated leucocytes produce a vast array of reactive compounds that are indispensable for the phagocytosis and killing of pathogens, and these reactive compounds can very easily damage host tissues if their reactivity is not checked by antioxidants.

Free radical production during critical illness

During critical illness many factors can combine to dramatically increase free radical production and tissue damage. First, during a systemic inflammatory response to a particular cause (infection, bowel perforation, trauma, surgery etc.) bacterial killing will be increased as previously described. In addition, during various forms of critical illness other factors may yet further increase production of these reactive species: elevation of O_2 concentration for respiratory compromise; NO therapy; ischaemia/reperfusion injury and its consequences; parenteral nutrition; impaired kidney function leading to decreased clearance of substances that may be pro-oxidant; fluid and electrolyte imbalances etc. In addition, massive losses of antioxidants may occur as a result of capillary leak, impaired renal reabsorption and other factors. For example, the plasma concentration of a marker of lipid peroxidation and free radical production (malondialdehyde) has been measured in infants before minor surgery, stable infants on the ward recovering from a major operation and critically-ill infants in the neonatal intensive care unit (Basu *et al.* 1999). Some of these infants were receiving enteral nutrition and some were receiving parenteral nutrition. As can be seen in Fig. 1, lipid peroxidation increases in the order elective surgery<major surgery<neonatal intensive care unit, but also increases in each patient group if infants are fed parenterally as compared with enterally.

Antioxidant defences

The antioxidant defences of the body consist of several interacting systems. They can be divided into: antioxidant enzymes; fat-soluble antioxidants; water-soluble antioxidants; non-specific antioxidants. Together, under most circumstances, these various systems protect membranes, intracellular contents, organelles and extracellular fluids from excessive damage by reactive species. Several of these systems can become depleted during excessive free radical production (e.g. during critical illness) and some may be amenable to manipulation, either pharmacologically or nutritionally. The present review will consider each system in turn and whether there is potential for nutritional or pharmacological intervention during critical illness, concentrating on those systems for which interventions have been tried in human critical illness, but also discussing some use in animal models.

Antioxidant enzymes

The major ROS and free radical produced in the bacterial killing process is superoxide, generated by the action of NADPH oxidase. Superoxide is also produced in substantial quantities by the mitochondrial respiratory chain, xanthine oxidase and the metabolism of drugs and endogenous compounds. Superoxide is specifically acted on by the enzyme superoxide dismutase (SOD) in the mitochondria (Mn-SOD), cytosol (Cu,Zn-SOD) and extracellular fluids (Cu,Zn-SOD). SOD is difficult to use therapeutically, although several variants of different liposomal formulations have been used in animal studies. The problem is, as with other enzymes, one of delivery in an active state. A gel consisting of liposomally-encapsulated human recombinant Cu,Zn-SOD has been used for the treatment of a urological disease (Peyronie's disease; Riedl *et al.* 2005), and tracheal installation has been used, with some success, in premature infants at risk from lung sequelae (Davis *et al.* 1997). In the latter paper the authors tempered their positive findings with the following quotes, both of which are pertinent to all studies in which the aim is to modify antioxidant defences: '... supports a critical role of SOD in the prevention of ROS-induced lung injury, caution should be exercised with the use of antioxidants such as SOD, especially in high-risk premature infants. ROS may potentially be toxic, but may also have important cellular functions in many organ systems ...' and 'Although administration of human recombinant Cu,Zn-SOD could potentially interfere with superoxide generation and bacterial killing by neutrophils and macrophages, the incidence of late-onset sepsis and pneumonia were comparable between groups ...'. Much interest has been expressed in the potential of chemical compounds that act like SOD ('SOD mimetics') to have beneficial effects in critical illness (Cuzzocrea *et al.* 2001; Salvemini & Cuzzocrea, 2003; Tuder *et al.* 2003), as well as in the prevention of ageing (Melov *et al.* 2000). However, the same problems may exist to restrict their clinical usage, i.e.

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interference with essential free radical-dependent processes such as bacterial killing. One of these compounds, M-40403, is currently in a phase-2 trial for the prevention of pain (Di Napoli & Papa, 2005). The reaction of SOD with superoxide generates H_2O_2, which is itself a ROS that can damage cellular components and give rise to other reactive compounds. This H_2O_2 is detoxified by two enzyme systems, catalase and glutathione peroxidase. Catalase decomposes H_2O_2 to water and O_2 and contains Fe at its catalytic centre, whereas glutathione peroxidase detoxifies H_2O_2 by reacting it with the co-substrate glutathione in the reduced form to yield oxidised glutathione (i.e. two glutathione molecules connected by a disulphide bond) plus water. This oxidised glutathione is then regenerated by the enzyme glutathione reductase, using NADPH from the pentose phosphate pathway. Although it would appear that catalase and glutathione peroxidase have identical functions, they have differing locations and reactivities; catalase is mainly localised in peroxisomes, and it therefore acts mainly on H_2O_2 generated either in the cytosol or in the peroxisome itself. Apart from heart mitochondria, which do appear to possess a catalase activity, mitochondria lack catalase and so rely on glutathione peroxidase to decompose H_2O_2 generated by Mn-SOD. In addition, glutathione peroxidase is found in the cytosol. However, glutathione peroxidase has two other characteristics that differ from catalase: it is active towards lipid hydroperoxides and so protects cell membranes; it is active against low concentrations of H_2O_2, whereas catalase has a high K_m and so has a high rate of H_2O_2 destruction at high concentrations. Administration of catalase is not a therapeutic option because the Fe present in catalase preparations would be likely to behave as a pro-oxidant. However, nutritional intervention to maintain glutathione peroxidase activity is a potentially-important therapeutic option in critical illness. These aspects will be considered later in the present article.

Just as there are chemical compounds that mimic SOD activity, there are compounds that mimic glutathione peroxidase. Ebselen is a Se-containing glutathione peroxidase mimetic that has been used clinically in the area of neuro-intensive care (Saito *et al.* 1998; Yamaguchi *et al.* 1998; Ogawa *et al.* 1999). Recently, a mitochondrially-targetted glutathione peroxidase mimetic, 2-[4-(4-triphenylphosphoniobutoxy) phenyl]-1,2-benzisoselenazol)-3(2H)-one iodide ('MitoPeroxidase'), has been synthesised (Filipovska *et al.* 2005).

Fat-soluble antioxidants

Many ROS are produced in proximity to membranes, and as polyunsaturated lipids in membranes are particularly vulnerable to peroxidative damage that can propagate from lipid to lipid, it is important that membranes are protected from the effects of these compounds. Vitamin E, of which the major biological isomer is α-tocopherol, is particularly important in protecting membranes because it can interrupt this process, i.e. it is a chain-breaking antioxidant. In this process the relatively stable α-tocopheryl radical can be formed, which can be recycled by vitamin C or by ubiquinone (Beyer, 1994). Studies that have aimed to determine whether vitamin E status is compromised during critical illness have yielded equivocal results; a number of studies have shown that vitamin E is depleted, whereas others have not supported this finding. These results may, however, depend on how the plasma vitamin E concentration is expressed, i.e. as absolute plasma vitamin E concentration, or as plasma vitamin E concentration : plasma cholesterol, which is thought to be more physiologically relevant because most of the vitamin E is carried in plasma lipoproteins and its absolute concentration will vary simply as a function of lipoprotein concentration independently of alterations in vitamin E supply and demand. For example, a study by (Quasim *et al.* 2003) has found that vitamin E concentration is decreased in critical illness, whereas when corrected for plasma cholesterol there is in fact an increase in vitamin E concentration. α-Tocopherol can be oxidised to α-tocopheronic acid, which then yields α-tocopheronolactone (Simon *et al.* 1956), which can be detected in urine and has been suggested to be a marker of oxidative stress (Pope *et al.* 2002). This compound is increased in infants with sepsis compared with controls, suggesting that vitamin E consumption is increased during critical illness (G Panagou, K Mills, M Chowdhury, A Pierro, S Eaton and DPR Muller, unpublished results). In addition, tocopherols, especially γ-tocopherol, can be nitrated *in vivo* by reactive nitrogen species, which are known to be elevated in critical illness (Christen *et al.* 1997; Morton *et al.* 2002). Whether either of these two routes quantitatively depletes the vitamin E pool is unknown. There have been few trials of vitamin E in critical illness, partly because the poor solubility of vitamin E in aqueous solutions makes it difficult to administer intravenously, and in those trials in which vitamin E has been used in critical illness it has frequently also been administered in combination with vitamin C (Nathens *et al.* 2002; Crimi *et al.* 2004).

Ubiquinone, which has a crucial role in the mitochondrial electron transport chain, is also found in other membranes where, in the reduced form, it can act as an antioxidant in concert with vitamin E (Beyer, 1994). There does not, however, appear to be a particular rationale for ubiquinone supplementation during critical illness.

Carotenoids (e.g. lycopene, β-carotene) are important antioxidants in plants and may have some antioxidant function in man, although it is likely that β-carotene is more important as a vitamin A precursor. There are drastic decreases in circulating carotenoids during critical illness (Quasim *et al.* 2003), but there are no studies that have examined a therapeutic role in critical illness.

Water-soluble antioxidants

Vitamin C (ascorbic acid) is one of the most important extracellular antioxidants, working in conjunction with vitamin E as described earlier. It is also an essential cofactor for several enzymes. It cannot be synthesised within the body, so the diet must contain adequate vitamin C to maintain whole-body levels. Vitamin C appears to be dramatically decreased in critical illness, and there are lower levels in patients with multiple organ failure than in critically-ill patients without multiple organ failure (Borrelli *et al.* 1996; Fig. 2). Vitamin C has been used in

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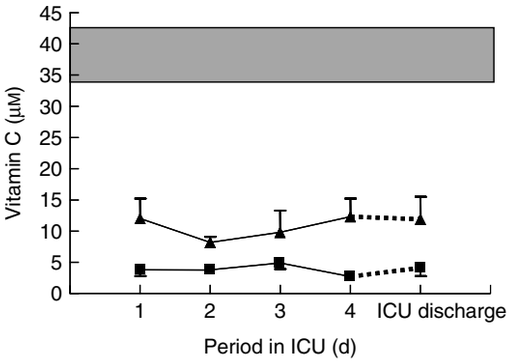

Fig. 2. Vitamin C (ascorbate) concentration in the plasma of critically-ill patients with (■) and without (▲) multiple-organ failure, and a normal healthy adult range for plasma vitamin C (▒). ICU, intensive care unit. Values are means with their standard errors represented by vertical bars. (Data redrawn from Borrelli *et al.* 1996 and Johnston & Cox, 2001.)

several studies in patients in intensive care units, both alone and in conjunction with vitamin E.

The other main water-soluble antioxidant is glutathione. This tripeptide, γ-glutamate-cysteinyl-glycine, is a co-substrate of the enzyme glutathione peroxidase, which is essential for detoxification of H_2O_2 and lipid peroxides as described earlier. Additionally, glutathione may become depleted during oxidative stress by reaction with HClO produced as part of the bacterial killing process (Pullar *et al.* 2001), and can react with high levels of NO to form *S*-nitroso-glutathione. Lipid peroxidation occurs extensively during sepsis, and lipid peroxidation products such as 4-hydroxynonenal can be conjugated and excreted, leading to loss of hepatic glutathione (Wang & Ballatori, 1998; Laurent *et al.* 1999). Glutathione is thought to be particularly important in the protection of mitochondrial respiratory chain complexes (Bolanos *et al.* 1996; Clementi *et al.* 1998), and it has recently been shown for adult patients with sepsis that complex I activity is positively correlated with mitochondrial glutathione content and is lower in non-survivors than in survivors (Brealey *et al.* 2002). The liver is crucial to maintaining whole-body glutathione levels, and during critical illness or sepsis hepatic glutathione efflux is increased, which can result in lower hepatic glutathione levels (Lauterburg *et al.* 1984; Sugino *et al.* 1989; Jaeschke, 1992; Ookhtens *et al.* 1994; Minamiyama *et al.* 1996). The demand for cysteine is increased during sepsis (Malmezat *et al.* 1998), partly to maintain glutathione levels and also because many acute-phase proteins have a high cysteine content. Flux through the trans-sulphuration pathway is increased (Malmezat *et al.* 2000*b*), but the increase in cysteine synthesis is not sufficient to account for the increased cysteine flux, and muscle protein catabolism is necessary to provide cysteine. In rats with sepsis protein breakdown and increased trans-sulfuration together provide enough cysteine for increased glutathione synthesis (Malmezat *et al.* 2000*a*). However, in infants and children with sepsis increased protein breakdown and increased plasma cysteine flux are not sufficient to maintain whole-blood glutathione synthesis, which is decreased by 60% (Lyons *et al.* 2001). This decrease could be a result of inadequate supply of intracellular glutamate, glycine or ATP. Extrahepatic glutathione stores can also be depleted because of inadequate supply of precursors, e.g. in muscle post-surgically (Luo *et al.* 1996) or during sepsis (Brealey *et al.* 2002).

Nutritional and therapeutic modulation of the glutathione system in critical illness

Although intravenous glutathione has been shown to have beneficial effects on lipid peroxidation in a study of patients with sepsis (Ortolani *et al.* 2000), oral or intravenous delivery of glutathione is not thought to be very effective, because glutathione is not directly taken up into cells but is hydrolysed extracellularly before it is taken up. More interest has been shown in the administration of glutathione esters such as glutathione-monoethyl ester, which can be taken up directly by cells, although there are potential problems with contamination and/or plasma esterases. There have been no studies of the use of glutathione esters in human subjects and there are no pharmaceutical preparations. However, administration of glutathione could be effective by providing the amino acids required for glutathione synthesis, and many studies have been performed with the aim of boosting glutathione levels by providing the constituent amino acids or their precursors.

For glutathione synthesis extracellular glutamine is a better source of intracellular glutamate than extracellular glutamate, so that depletion of hepatocyte glutathione during oxidation can be counteracted by glutamine but not by glutamate (Markley *et al.* 2002). During sepsis glutamine is mobilised from muscle and lung, and net glutamine utilisation exceeds production such that glutamine may become a 'conditionally essential' nutrient (Lacey & Wilmore, 1990) that is limiting for glutathione synthesis. However, the actual flux of glutamine into glutathione and whether this flux is altered during sepsis is unknown. Under conditions in which the lack of intracellular glutamate may limit glutathione synthesis glutamine can help maintain glutathione levels, e.g. paracetamol overdose (Hong *et al.* 1992), parenteral nutrition (Denno *et al.* 1996) or after surgery (Flaring *et al.* 2003), although oral glutamine fails to increase glutathione levels in healthy individuals (Valencia *et al.* 2002). There is some evidence to support the beneficial effects of glutamine during critical illness, as suggested by a recent meta-analysis (Avenell, 2006), although whether these effects are mediated via the glutathione system is not known.

Cysteine supply is often compromised during sepsis, and several drugs have been proposed to increase cysteine availability for glutathione synthesis. The 5-oxoproline analogue L-2-oxothiozalidine-4-carboxylate generates intracellular cysteine and can increase glutathione levels (Bernard *et al.* 1997; Moberly *et al.* 1998) and prevent sepsis-related cardiac dysfunction (Moberly *et al.* 1998; Poon *et al.* 1998). Similarly, *N*-acetyl-cysteine, which can be taken up by cells and hydrolysed to cysteine, can increase blood and lung glutathione levels in patients with acute respiratory distress syndrome (Bernard *et al.* 1997) or pulmonary fibrosis (Meyer *et al.* 1995), and although

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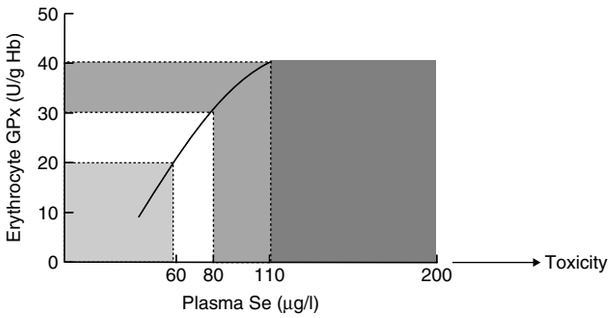

Fig. 3. Erythrocyte glutathione peroxidase (GPx) activity as a function of plasma selenium levels. (▒), 'Deficient'; (▒), 'sub-maximal'; (■), 'maximal'.

lung glutathione levels are unaffected in controls (Meyer *et al.* 1995), blood glutathione levels are increased (Roes *et al.* 2002). Lipoic acid has also been suggested to maintain glutathione levels by allowing cystine reduction to cysteine and thereby increasing intracellular cysteine levels (Han *et al.* 1997). Additionally, lipoic acid may also help to recycle oxidised glutathione to reduced glutathione (Porras *et al.* 2002).

Little attention has been paid to the role of glycine supply in the maintenance of glutathione levels. However, glycine is known to have beneficial effects in sepsis (Yang *et al.* 2001), and a recent *in vitro* study has shown that glycine enhances glutathione levels when glutamine is low but decreases glutathione levels when glutamine is higher (Wessner *et al.* 2003).

In addition to the maintenance of tissue glutathione levels, in order to ensure optimal functioning of the glutathione system glutathione peroxidase activity should be adequate. The trace element Se is incorporated into the amino acid selenocysteine, which forms the active site of the different glutathione peroxidase isoforms. Se intake has fallen in the UK, so that current daily intake is markedly less than recommendations (Rayman, 2000; Jackson *et al.* 2003). This lowered intake results in submaximal glutathione peroxidase activity (Fig. 3), which may have adverse effects in the general population, e.g. increased cancer risk (Rayman, 2000) and increased risk of cardiovascular events (Blankenberg *et al.* 2003). Critically-ill patients are likely to have additional Se losses such as through exudates, renal therapy etc., so that many patients may have low Se levels (Angstwurm *et al.* 1999; Berger *et al.* 2001; Hardy, 2005). Supplementation of critically-ill patients with Se has been carried out in several trials, and some of these trials have shown either a benefit (Angstwurm *et al.* 1999) or a trend towards benefit. Thus, in meta-analyses Se supplementation (in some trials Se is used with other antioxidants) shows a trend towards lower mortality (relative risk 0·59 (95% CI 0·32, 1·08), $P<0·09$; Heyland *et al.* 2005), although it has been pointed out that several of the included trials were small and of poor quality methodologically, so that the authors of another meta-analysis have recommended a large randomised controlled trial (Avenell *et al.* 2004). Two large multicentre randomised controlled trials of Se and glutamine supplementation in critical illness are now underway, the REDOX study (Critical Care Nutrition, 2006) and the SIGNET trial (Health Services Research Unit, University of Aberdeen, 2006).

Other water-soluble compounds with antioxidant activity

There is a host of other chemicals and proteins with antioxidant activity; however, whether these compounds have any biologically-important antioxidant activity is uncertain. An example of these compounds is the α-amino acid taurine, which is found in high concentration in the heart and in neutrophils. An antioxidant function for taurine in the heart is unproven, but in neutrophils taurine reacts with bactericidal HClO to form taurine-chloramine, which may have further immunomodulatory effects (Schuller-Levis & Park, 2004). Plasma components with antioxidant activity include uric acid (Waring *et al.* 2001), bilirubin (Stocker *et al.* 1987) and albumin (Halliwell, 1988). In a test in which plasma is exposed to an exogenous oxidant challenge, uric acid accounts for 33% of the plasma total antioxidant capacity, with bilirubin, albumin, vitamin C and vitamin E accounting for 2, 43, 9 and 3% of the plasma total antioxidant capacity respectively (Miller *et al.* 1993). Although this type of oxidant challenge is clearly artificial, albumin appears to be an important antioxidant. The reason for this activity is that albumin has a free thiol group that is readily oxidised by reactive species, and the high concentration of albumin in plasma means that this thiol group contributes markedly to plasma free thiols and hence to plasma antioxidant capacity (Quinlan *et al.* 1998, 2004). Some plasma albumin is naturally found in oxidised forms and the proportion in the reduced form (i.e. effective as an antioxidant) can vary (Era *et al.* 1995); for example, in healthy young adults 76% is present in the reduced form, whereas in healthy elderly subjects this percentage is decreased to 46 (Era *et al.* 1995). The redox state is also altered by cardiac surgery, diabetes, renal dysfunction and haemodialysis (Suzuki *et al.* 1992; Hayakawa *et al.* 1997; Soejima *et al.* 2004; Terawaki *et al.* 2004); thus, although it is not known whether there is a change in redox state in critically-ill populations, it is likely to be altered compared with that of healthy young males. Albumin is given to many critically-ill patients as an oncotic support and, although this treatment repletes plasma thiols (Quinlan *et al.* 1998, 2004), most commercial sources of albumin have a decreased proportion of the thiol groups in the reduced form compared with that in the plasma of healthy adult males (Bar-Or *et al.* 2005).

Conclusions

Although there is much data to show that free radical activity is increased in critical illness, and that the levels of many plasma antioxidants are decreased, studies of antioxidant supplementation in critical illness have mostly been carried out on a small scale.

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