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Review

Exploring cyclosporine A-side effects and the protective role-played by antioxidants: the morphological and immunohistochemical studies

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Summary. Cyclosporine A (CsA) is the immunosuppressor most frequently used in transplant surgery and in the treatment of autoimmune diseases, because of its specific inhibiting effect on the signal transduction pathways of cell T receptor. It has been shown that CsA is able to generate reactive oxygen species and lipid peroxidation, which are directly involved in the CsA nephrotoxicity, hepatotoxicity and cardiotoxicity. So, the use of antioxidants seems to be a useful tool in attempting to reduce CsA adverse effects. The aim of this review is to summarise the general aspect of CsA, the classification of antioxidants, their mechanism of action and their administration for improving CsA side effects.

The protective role of different antioxidants has been evaluated on CsA-induced nephrotoxicity. It has been shown that the antioxidants, improved the morphological renal cytoarchitecture, increased the antioxidant enzyme content, decreased lipid peroxidation and reactive species oxygen (ROS).

The protective role of antioxidants was also found in CsA hepatotoxicity and was related to the increase in antioxidant capacity of hepatic tissue, which was responsible for ameliorating hepatic morphology.

Recently, it has been demonstrated that CsA induces side effects on the heart but the data to this purpose are very few and also the number of results on the protective role played by antioxidants it is very limited.

In conclusion, not only do these observations provide insight into the intricate mechanism of CsA adverse effects, but they also present novel opportunities for the design and development of more effective therapeutic strategies against negative effects.

Key words: Cyclosporine A, Antioxidants, Kidney, Liver, Heart

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General aspects of Cyclosporine A

Cyclosporine A (CsA), a cyclic undecapeptide with potent immunosuppressive activity, was derived from extracts of Topocladium inflatum gams, a member of the Fungi imperfecti family (Fig. 1) (Kahan, 1999).

"*The high treeless plain where this fungus was discovered, Hardarger Vidda, is a land populated by malevolent spirits. One man, Askelad, a Nordic folk hero, used his ingenuity and perseverance to subvert the spirits' schemes and preserve the peace and harmony of the community*" (Kahan, 1999).

Since the early 1980's, CsA has been widely used to prevent rejection of organ transplants and it has increased graft survival and decreased patient mortality (Kahan et al., 1987) and the use of CsA to treat autoimmune diseases has increased during the past 6 years (Bach, 1999). However, several adverse effects have been reported in both transplant and nontransplant settings (autoimmune disorders), including toxicities (nephrotoxicity, hepatoxicity and neurotoxicity), hypertension, dyslipidemia, gingival hyperplasia, hypertrichosis, malignancies and increased risk of cardiovascular events (de Mattos et al., 2000; Olyaei et al., 2001). The molecular mechanism by which CsA causes toxicity is still a matter of debate. However, numerous studies support the hypothesis that CsA-induced toxicity may be the consequence of oxidative stress, i.e. oxidation and cross-linking of cellular thiols and membrane lipid peroxidation (Ichikawa et al., 1994). So, the use of antioxidant agents associated with CsA treatment could be a useful tool in attempting to reduce CsA-induced side effects.

Here, we considered the pharmacological CsA properties underlying its action mechanism and then we focused on the studies which supported, by morphological and immunohistochemical analysis, the improvements of antioxidants in the prevention of CsA-induced adverse effects. Since the literature on antioxidants seems to be confused, we indicated a classification in order to find a single way through the

diverse language used to consider and discuss these substances. Moreover, we reported the role of these substances in human and experimental oxidative stress.

Cyclosporine A: pharmacological properties and mechanism of action

As above reported, CsA was first approved by the US Food and Drug Administration in the early 1980's for use as prophylactic antirejection therapy in patients receiving allogeneic transplants (kidney, liver and heart). It has improved the 1-year graft survival rate with conventional therapy (prednisone and azathioprine) in renal allografts from 50-85% (Cecka and Terasaki, 1991; Li et al., 2004). The mechanism of CsA action has been described by several laboratories (Schreiber and Crabtree, 1992; Rao, 1995). It was thought to block the immune system by inhibiting signalling through the T-cell receptor. The effect of this was to prevent the production of cytokines that would normally stimulate an immune response (Fig. 2) (Nabel, 1999). But this explanation was not necessarily complete. First, when one of these cytokines, interleukin-2 (IL-2), was disrupted in transgenic animals, the effect on immune function was not the same as treatment with CsA (Horak et al., 1995). Second, if another molecule in the proposed signalling pathway (the nuclear factor of activated T-cells, NF-AT) was knocked out, again the effects did not match treatment with CsA (Xanthoudakis et al., 1996). An alternative explanation came from the fact that CsA stimulates the production of transforming growth factor-ß (TGF-ß) (Khanna et al., 1994) (Fig. 3). The TGF-ß induction, due to CsA, was considered to be responsible for: 1) liver and kidney damage, 2) stimulation of cancer cells, 3) suppression of immune response, 4) heart disease.

Regarding CsA metabolism, it is metabolized in the liver by the cytochrome P450-3A4 enzyme system. Because many drugs are metabolized via this system, they may directly influence the rate of metabolism of CsA. These potential drug-drug interactions may enhance the risks of under-immunosuppression (rejection), over-immunosuppression (infection) and toxicity (Sketris et al., 1995). The major route of CsA metabolite excretion is via the biliary system and renal elimination plays a minor role.

Nutritional antioxidants

Antioxidants are a group of substances which present at low concentrations in relation to oxidizable substrates and significantly inhibit or delay oxidative processes while often being oxidized themselves. The applications of antioxidants are widespread in the industry and are in use in preventing polymers from oxidative degradation, rubber and plastic from losing strength, gasoline from auto-oxidation, synthetic and natural pigments from discoloration and as additives to cosmetics, food (especially food with high fat content), beverages and baking products (Vaya and Aviram, 2001). In recent years there has been an increased interest in the application of antioxidants to medical treatment as information is constantly gathered linking the development of human diseases to oxidative stress. The generally accepted hypothesis is that in any biological system an important balance must be maintained between the formation of reactive oxygen and nitrogen species (ROS and RNS, respectively) and their removal (Fig. 4).

Reactive oxygen species

ROS are formed within the body by various physiological processes and damage. The reactive species superoxide ($O2^-$), hydrogen radical ($HO\cdot$), nitrogen oxide ($NO\cdot$), peroxynitrite ($ONOO^-$) and hypochlorous acid (HOCl) are all products of the normal metabolic pathway of the human organs but, under certain conditions, when in excess they can exert harmful compounds. Superoxide, the most important source of initiating radicals "in vivo", is produced in mitochondria during electron chain transfer and it regularly leaks outside of the mitochondria. Furthermore, superoxide is the source of several other ROS, including the $O2^-$ and the very reactive $ONOO^-$ and $HO\cdot$ (McCord, 2000; Benzie, 2000, 2003). A generation of various ROS, therefore, stems from superoxide, and this cannot be totally prevented at any point in the chain of formation. Indeed, even if this were possible, it is becoming increasingly clear that it is not desirable, as many ROS have important roles in physiological processes and systems, including microbial killing, blood pressure control, endothelin function, cell signalling, apoptosis, cell division, and gene transcription (Suzuki et al., 1997; Dalton et al., 1999; Clememt and Pervaiz, 2001). "Anti-oxidation"

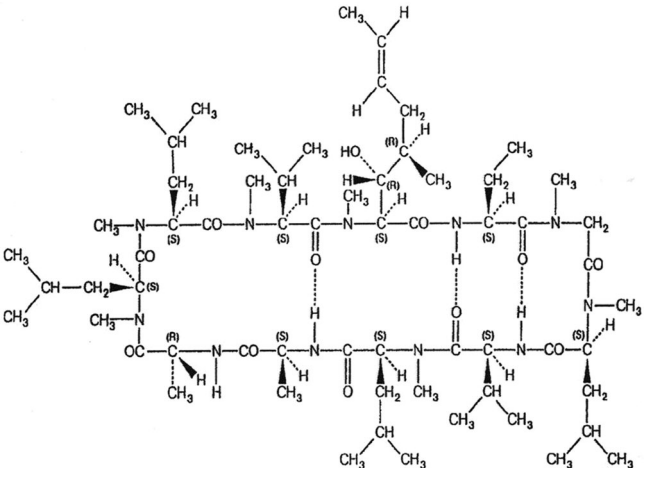


Fig. 1. Chemical structure of Cyclosporine A (from Kahan, Transplantation Proceedings-Elsevier, 1999)

strategies are needed, therefore, to limit damaging interaction between ROS and valuable substrates such as protein thiol groups, DNA bases, and polyunsaturated fatty acids (PUFA). Oxidative damage to these macromolecules can lead to enzyme inactivation, mutation, membrane disruption, mitochondrial dysfunction and cell death. These are the toxic effects of oxygen and they are implicated in ageing and in the development of chronic, inflammatory, degenerative and age-related disease (Finkel and Holbrook, 2000; McCord, 2000) (Fig. 4).

Antioxidant protection

To protect cells and organs against ROS, the body has evolved a highly sophisticated and complex antioxidant protection system. It evolves a variety of components, both endogenous and exogenous antioxidants, which function interactively and synergistically to neutralize free radicals (Fig. 4) (Vaya and Aviram, 2001).

These components can differ in their composition, their physical and chemical properties and in their mechanism and site of action. So, they were classified in different ways according to the above reported data. Instead, we described in Table 1 a scheme of possible classification of antioxidants considering endogenous and exogenous substances.

The reputation of vitamin A, C, E and ß-carotene, as well as the minerals selenium and zinc, as potent antioxidants is well documented. In addition, the body itself produces certain amounts of potent antioxidants, including melatonin (MEL), coenzyme Q10 (CoQ10) and enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and glutathione reductase, which catalyze ROS/RNS quenching reactions. However, their production declines with age. It is understandable that the "diseases of aging" would appear as these levels decline. In recent years, scientists have discovered powerful antioxidant properties of other nutrients and herbals that are classified as polyphenols or "photochemicals" and "photonutrients".

Dietary antioxidants (nutrient-derived antioxidants)

Vitamin C, vitamin E and ß-carotene are among the most widely studied dietary antioxidants. Vitamin C is considered the most important water-soluble antioxidant in extracellular fluids. It is capable of neutralizing ROS in the aqueous phase before lipid peroxidation is initiated. Vitamin E, a major lipid-soluble antioxidant, is the most effective chain-breaking antioxidant within the cell membrane where it protects membrane fatty acids from lipids peroxidation. Vitamin C has been cited as being capable of regenerating vitamin E (Sies et al., 1992). ß-carotene and other carotenoids are also believed to provide antioxidant protection to lipid-rich tissues. Research suggests ß-carotene may work sinergically with vitamin E (Jacob, 1995; Sies and Stahl, 1995). Fruits, vegetables and grains are rich sources of vitamin C, carotenoids and vitamin E (Scalbert and Williamson, 2000).

A number of other dietary antioxidant substances exist beyond the traditional vitamins discussed above. Many plant-derived substances, collectively termed

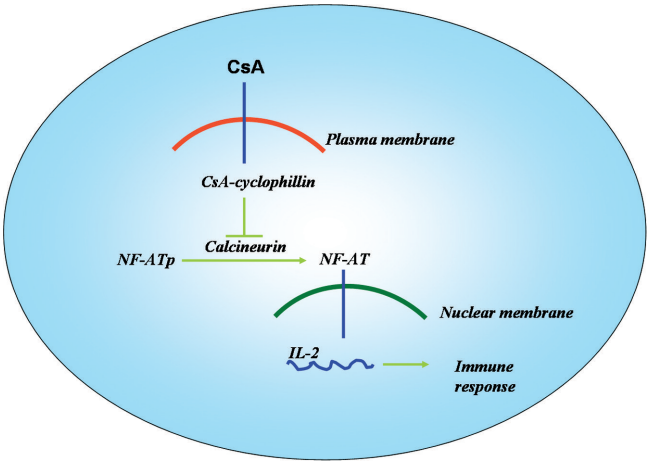


Fig. 2. Main mechanism for the immunosuppressive action of cyclosporine A. Cyclosporine A (CsA), nuclear factor of activated T-cells (NF-AT); phosphate group (p); interleukin-2 (IL-2) (adapted from Nabel, Nature-Nature Publishing Group, 1999).

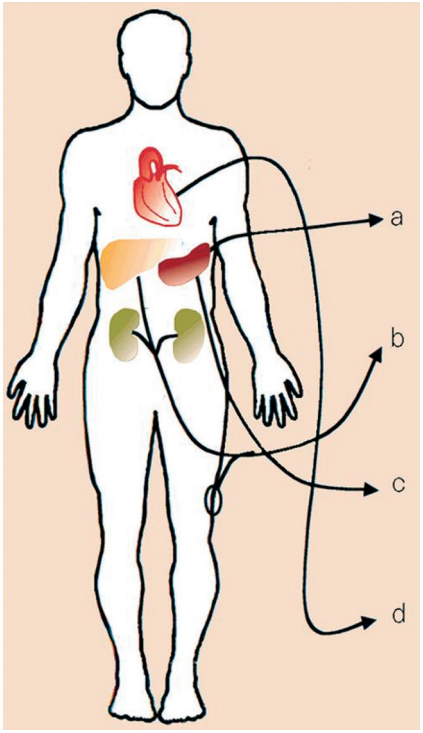


Fig. 3. Cyclosporine A action on production of the transforming growth factor-ß (TGF-ß). TGF-ß then: a, stimulated the growth of existing cancers; b, caused liver and kidney damage; c, suppressed immune responses. d, produced heart disease (from Nabel, Nature- Nature Publishing Group, 1999).

polyphenols, "phytonutrients" or "phytochemicals" are becoming increasingly known for their antioxidant activity. More than 8.000 phenolic compounds are known, of which 2/3 belong to the flavonoid family. They are found in fruits, vegetables, grains, bark, stems, flowers, tea and wine (Middleton, 1998; Nijveldt et al., 2001). These natural products were known for their beneficial effects on health long before flavonoids were isolated as the effective compounds. Research on flavonoids received an added impulse with the discovery of the French paradox, i.e., the low cardiovascular mortality rate observed in the Mediterranean population in association with red wine consumption and a high saturated fat intake. The flavonoids in red wine are responsible, at least in part, for this effect (Formica and Regelson, 1995; Nijveldt et al., 2001). Furthermore, flavonoids have been demonstrated to have anti-inflammatory, anti-allergenic, anti-viral, anti-aging and anti-carcinogenic activity. The broad therapeutic effects of flavonoids have been largely attributed to their antioxidant properties. In addition to an antioxidant effect, flavonoid compounds exert protection against heart disease through the inhibition of cyclooxygenase and lipoxygenase activities in platelets and macrophages (Havsteen, 1983).

Endogenous antioxidants

In addition to dietary antioxidants, the body relies on several endogenous defense mechanisms to help protect against free radical-induced damage. The antioxidant enzymes metabolize oxidative toxic intermediates and require micronutrient cofactors such as selenium, iron, copper, zinc and manganese for optimum catalytic activity. Glutathione, an important water-soluble antioxidant, is synthesized from the amino acids glycine, glutamate and cysteine. Glutathione and vitamin C directly quench ROS and they have a sparing effect upon each other (Jacob, 1995).

Additional physiological antioxidants are listed in Table 1.

Mechanism of action of antioxidants

Two principle mechanisms of action have been proposed for these substances dividing them into primary and secondary antioxidants (Vaya and Aviram, 2001). The first is a chain-breaking mechanism, by which the primary antioxidant donates an electron to the free radical present in the system (e.g., lipid radical). The second mechanism involves removal of ROS/RNS initiators (secondary antioxidants) by quenching chain-initiating catalyst. The present review section will discuss the mechanism of action of primary and secondary antioxidants, and of those compounds which by themselves can act as antioxidants, but under certain conditions they can also prevent other pro-oxidants from initiating free radical reaction acting as co-antioxidants. The effect of some antioxidants on gene expression will also be discussed.

Electron donation

Primary antioxidants are compounds which are able to donate hydrogen atoms rapidly to a lipid radical, forming a new radical, more stable than the initial one. Biological organs contain many PUFA, such as linoleic

Table 1. Scheme of antioxidant classification.

EXOGENOUS ANTIOXIDANTS	ENDOGENOUS ANTIOXIDANTS
Vitamins	Vitamins
Minerals	Minerals
Melatonin	Melatonin
Polyphenols	Enzymes
Thiazines	Coenzyme Q10

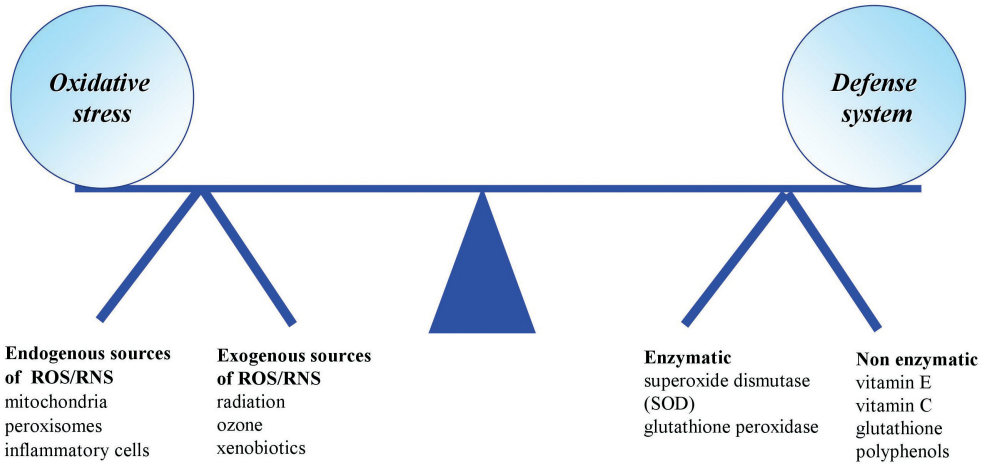


Fig. 4. Oxidation/reduction balance (adapted from Vaya and Aviram, Curr. Med. Chem., Bentham, 2001).

and arachidonic acids, mainly in the forms of esters with phospholipids, triglycerides or with cholesterol. These PUFA can undergo lipid peroxidation which can be interrupted by antioxidants by the donation of electrons. The mechanism of lipid peroxidation is shown in Fig. 5. In the first step, a hydrogen atom is abstracted from the PUFA (represented in Fig. 5 as linoleic acid), by initiators such as enzymes or by ROS generated in the biological system. In the presence of O_2 , a rapid reaction can take place between the fatty acid alkyl radical and the molecular oxygen, forming a peroxyl radical which can then further abstract a hydrogen atom from a new linoleic acid, to form linoleic hydroperoxide and a new linoleic radical. The last two reactions are known as the propagation steps in lipid peroxidation and cause a chain reaction.

Primary antioxidants, such as ascorbic acid, polyphenols and tocopherol, can stop chain reactions by donating an electron to the peroxyl radical of the fatty acid, thus stopping the propagation steps. Enzymes such as GPx can also act as antioxidants, by reducing oxidized lipids and phospholipids hydroperoxide (ROOH and PL-OOH) to their corresponding alcohols (ROH, PL-OH) (Thomas et al., 1990).

Any compound which can react with the initiating radical (or inhibit the initiating enzyme), or reduce the oxygen level (without generating ROS), can be considered as secondary antioxidants. Inhibition or progression of lipid peroxidation can also be catalyzed by other mechanisms, e.g., reaction of PUFA with singlet oxygen, energetically exited molecular oxygen, or with metal ions. Thus, PUFA can react with singlet oxygen to form lipid hydroperoxide (Fig. 5, Reaction II). In addition, the presence of transition metal ions, such as copper or iron, in either oxidative state (Cu^{+1}, $Cu^{+2,}$ Fe^{+2}, Fe^{+3}), can re-initiate lipid peroxidation and form species with much higher activity than the starting materials (Fig. 5, Reaction III and IV). Thus, in a reaction catalyzed by metal ions, fatty acids, hydroperoxide can break down to form an alkoxyl radical, which is more reactive than the initial hydroperoxide (Fig. 5, Reaction III). This radical can re-initiate lipid peroxidation or it can be hydrolyzed to form aldehyde molecules.

The oxidation of polyunsaturated lipids results first in the formation of lipid hydroperoxide (ROOH), lipid hydroxide (ROH) and oxysterols, which further degrade to small fragments, such as aldehyde. Exposure of cells to such aldehydes can result in growth inhibition, alteration in enzymatic activities and inhibition of protein synthesis.

Metal chelation

Secondary antioxidants can retard the rate of radical initiation reaction by means of initiators elimination. This can be accomplished by deactivation of high energy species (e.g. singlet oxygen), absorption of UV light, scavenging of oxygen and thus reducing its concentration, chelations of metal catalyzing free radical reaction, or by inhibition of peroxidases, such as NADPH oxidase, xanthine oxidase, dopamine-hydroxylase or lipoxygenases. The vitamins bind directly or indirectly to redox active metals and thus inhibit the production of metal-catalyzed free radicals. The polyphenols, in addition to their ability to donate hydrogen atoms and thus to act as chain-breaking antioxidants, can also chelate transition metal ions and hence inhibit free radical formation (van Acker et al., 1996). Thus, the relative contribution of free radical scavenging or of metal ion chelation to the antioxidative effect is not clear (Terao et al., 1994).

Co-antioxidants

Some Authors (Bowry et al., 1995) observed that while vitamin C alone has little effect in preventing lard oil from oxidation, the combination of ascorbic acid with tocopherol (-TOH) gave rise to a strong synergistic antioxidative effect. These authors concluded that the role of vitamin C was to preserve –TOH from consumption. This behaviour of vitamin C is termed a co-antioxidant effect. Since then, many other compounds have been found to produce a similar co-antioxidant effect with –TOH activity (Fig. 6). In fact, this latter examines the co-antioxidative activity of various radical scavenger compounds, including bilirubin, aminophenol derivates, catechol derivates, various quinols and 6-palmitylascorbate ascorbate (Fig. 6). Like ascorbic acid, all these compounds were also very effective.

The number of known natural amino antioxidants (aniline derivatives) is very limited, compared with the

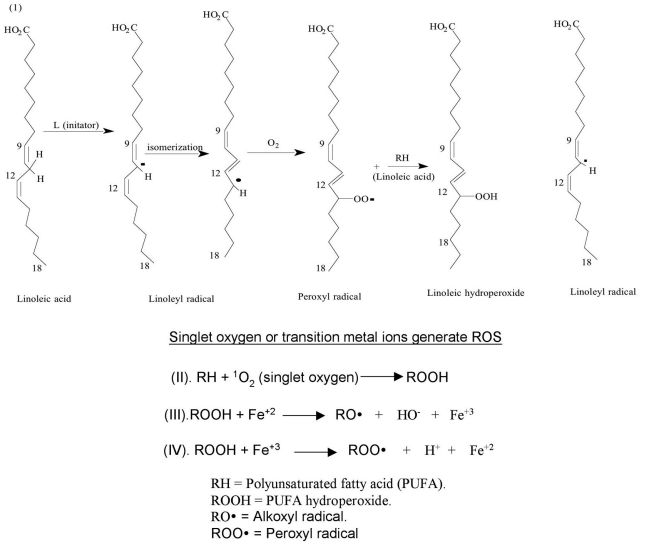


Fig. 5. Mechanism of linoleic acid peroxidation and reactive oxygen species (ROS) formation (from Vaya and Aviram, Curr. Med. Chem., Bentham, 2001).

group of phenols and polyphenols. Comparison of the co-antioxidant activity of phenols with molecules of similar structure, in which part or all the hydroxyl groups are replaced by amine groups (e.g., catechol with 2-aminophenol or 2-aminoaniline), could be of interest, since indications in the literature showed the superiority of aminophenols over phenols as antioxidants (Iwatsuki, et al., 1995).

Gene expression

Antioxidants possess the ability to donate electrons and thereby act, as reducing agents, to chelate metal ions and thereby remove potential radical initiators and to facilitate antioxidant activity by other compounds (co-antioxidants). Antioxidants can also affect directly or indirectly the expression of genes in tissues. A number of genes are regulated by changes in the cellular redox status (Allen and Tresini, 2000). Cellular redox status is determined, among other things, by the type and concentration of ROS/RNS present in the tissue as well as by the type and levels of antioxidants. ROS such as H2O2 (Crawford et al., 1996), UV radiation (Devary et al., 1991), HOCl (Ramana et al., 1998), singlet oxygen (Scharffetter-Kochanek et al., 1993) and also oxidized low-density lipoprotein (LDL) (Suc et al., 1998) were all studied for their ability to stimulate the expression of genes and protein activity, in order to identify redox-sensitive genes. Among the genes affected there are amphiregulin (AP-1), c-fos, c-jun, c-myc and MAP kinase. Oxidative stress was shown to increase Type III and Type IV collagen mRNA, the trascription of collagenase and the expression of ferritin. So, antioxidants may modulate signal transduction pathways and gene expression through their reducing properties, rather than through their ability (under certain conditions) to generate ROS or through their metabolic products. It was shown that only polyphenols with relatively high antioxidant potential were able to induce c-fos mRNA, while those lacking potent antioxidant properties were inactive (Choi and Moore, 1993). Induction of c-fos and c-jun mRNAs by phenolic antioxidants is mediated by an antioxidant response element (ARE) in a specific and dose-dependent manner. Antioxidants, like other signaling molecules, induce specific activity, which depends not only on the structure of the stimulus but also on the target place on the specific tissue or the cell type. When tissue interacts with an antioxidant, the signal formed must then translate into a biological response and, as tissues differ in their composition, the biological effect or response also varies. Antioxidants can thus mediate activities of biological systems.

Role of antioxidants in oxidative stress in human disease

A growing body of animal and epidemiological studies as well as clinical intervention trials suggest that antioxidants may play a pivotal role in preventing or slowing the progression of both heart disease and some forms of cancer (Block et al., 1992; Hennekens and Gaziano, 1993). The latter are reported in Table 2.

Heart disease

While several factors, such as high cholesterol levels, hypertension, cigarette smoking and diabetes are believed to promote atherosclerosis, a growing body of evidence suggests that a critical step in its development is the oxidation of LDL within the arterial wall (Jialal and Fuller, 1993). This theory is supported by several epidemiological studies which link low intake of dietary antioxidants to an increased frequency of heart disease (Hennekens and Gaziano, 1993). Additionally, an inverse relationship between heart disease and plasma antioxidant levels has been reported. Antioxidants have been shown to prevent LDL oxidation “in vitro” and retard the progression of atherosclerosis in animal models (Gey et al., 1987). It has been estimated that dietary increase in antioxidant vitamins may reduce the risk of heart disease by 20-30% (Hennekens and Graziano, 1993).

Table 2. Conditions associated with oxidative damage.

Atherosclerosis	Pancreatitis
Cancer	Inflammatory bowel and disease
Pulmonary dysfunction	Parkinson’s disease
Cataracts	Drug reactions
Arthritis and inflammatory diseases	Neonatal lipoprotein oxidation
Diabetes	Skin lesions
Shock, trauma and ischemia	Aging
Renal disease and hemodialysis	
Multiple sclerosis	

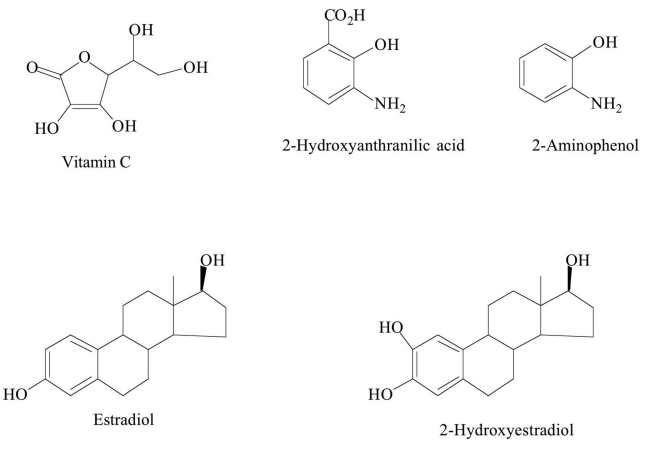


Fig. 6. Structure of some co-antioxidants (adapted from Vaya and Aviram, Curr. Med. Chem., Bentham, 2001).

Cancer

Epidemiological evidence consistently relates low antioxidant intake or low levels of antioxidants with increased cancer risk (Block et al., 1992). Oxidants are capable of stimulating cell division, which is a critical factor in mutagenesis. When a cell with a damaged DNA strand divides, cell metabolism and duplication become deranged. It is believed that antioxidants exert their protective effect by decreasing oxidative damage to DNA and by decreasing abnormal increase in cell division. Additional anticancer activities have been observed from several plant-derived substances (Milner, 1994). Sulfur containing phytochemicals, such as the allyl sulfides found in the allium family (garlic, onion and leeks) and isothyocyanates and sulphoraphane (cabbage, broccoli and cauliflower) have been shown to inhibit various steps in tumour development in animal and "in vitro" studies (Milner, 1994). Indoles, also found in cruciferous vegetables and natural constituents of citrus oils, may also be protective.

Pulmonary disorders

Recent studies suggest that free radicals may be involved in the development of pulmonary disorders such as asthma (Greene, 1995). Cellular damage caused by free radicals is thought to be partly responsible for the bronchial inflammation characteristic of this disease. Vitamin C, vitamin E and ß-carotene supplementation has been associated with improved pulmonary function (Hatch, 1995). Some evidence suggest glutathione or its precursors may be helpful in protecting against pulmonary damage as well (Bland, 1995).

Prevention of CsA-induced injury

Pharmacological intervention on CsA nephrotoxicity

Information on CsA nephrotoxicity in transplant recipients and patients with autoimmune disorders suggests that renal insufficiency is a major problem that may lead to end-stage renal disease requiring dialysis. Consequently, renal function should always be carefully monitored, even in patients whose renal function appears to be stable. In addition, early recognition and pharmacological intervention may be necessary to minimize the rate of allograft loss or chronic renal failure. Overproduction of ROS and lipid peroxidation are pathogenic factors involved in CsA-induced injury and antioxidant agents may be therapeutic.

Regarding thiazine antioxidants (i.e. quinacrine, methylene blue), there were many biochemical and morphological data underlying their protective renal role. In particular, al Khader et al. (1996) showed that quinacrine treatment protected animals against CsA-induced increase in malondialdehyde (MDA) and depletion of glutathione and vitamin E. These beneficial effects of quinacrine against CsA-induced injury were also confirmed by histological studies. The CsA treatment produced prominent changes in proximal tubules (PT) with a different degree of cytoplasmic vacuolation, tubular necrosis, severe periglomerular fibrosis and mild focal mesangial sclerosis with respect to control animals (Figs. 7, 8). Tubular necrosis and vacuolation were present both in the straight and convoluted portions of PT and the distribution was patchy involving single or multiple tubules. The vacuoles were numerous and non-isometric, showing

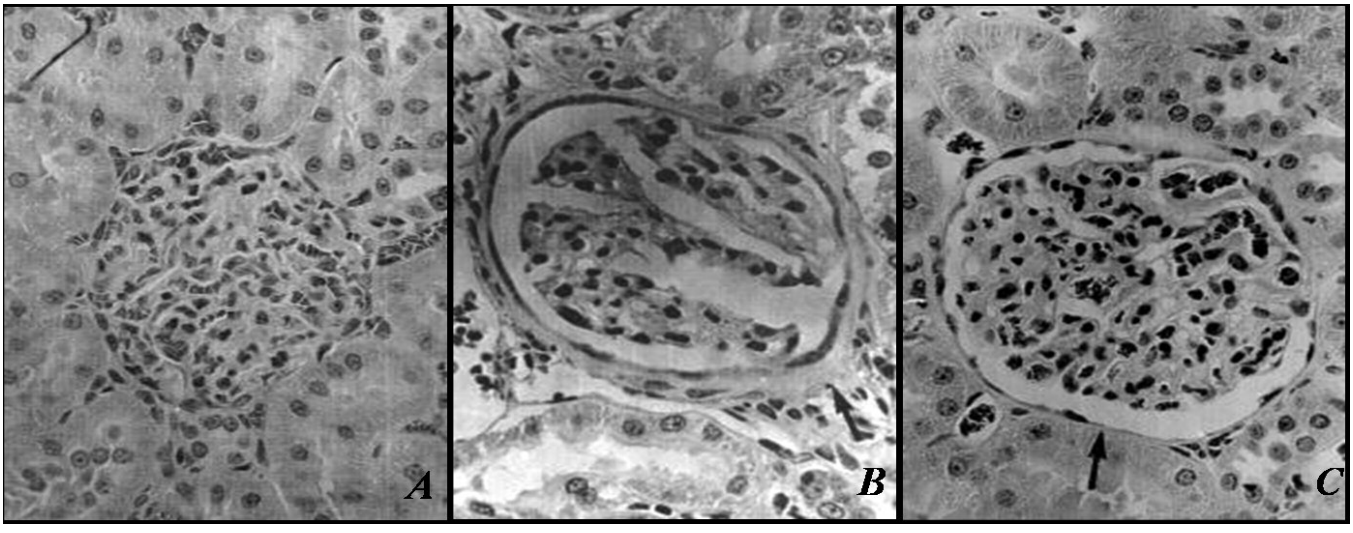


Fig. 7. Light microscopic sections showing renal glomerulus and proximal tubules from the control **(A)**, CsA-treated **(B)** and CsA plus quinacrine-treated **(C)** animals. The cells of control rats appeared to be normal while those of CsA treatment showed severe periglomerular fibrosis (arrow). Quinacrine treatment presented only mild (arrow) periglomerular fibrosis (haematoxylin-eosin; x 400), (from al Khader Transplantation, Lippincott, 1996).

marked variability in the degree of vacuolation between the adjacent cells. Distal tubules (DT) did not show any of these changes. Concomitant administration of quinacrine, as previously reported, attenuated CsA-induced structural changes in tubules (Fig. 8). The treatment of quinacrine prevented these changes to a great extent as well as arteriolar vasoconstriction (Fig. 8). These observations led to the hypothesis that, since quinacrine and CsA are highly protein bound, quinacrine may displace CsA from protein binding sites in blood and tissue and the free form of CsA may be readily excreted without inducing adverse effects.

About methylene blue (MB) antioxidant, which competitively inhibits reduction of molecular oxygen to superoxide by acting as alternative electron acceptor for tissue oxidases (Salaris et al., 1991), we demonstrated that MB combined with CsA preserved cytoarchitecture and enzymatic (NADPH-diaphorase, cytochrome c oxidase) and immunoenzymatic content (inducible nitric oxide synthase-iNOS, endothelial nitric oxide synthase-eNOS) of the renal parenchyma (Rezzani et al., 2001) (Figs. 9, 10). This has been explained as follows: 1) MB acts as an antioxidant by inhibiting xanthine oxidase as well as the allopurinol mechanism of action (Assis et al., 1997), and 2) MB decreases the nitric oxide (NO) production, reducing iNOS activity.

The role of some antioxidant nutrients (i.e. vitamin E, C) and co-antioxidants or minerals (i.e. selenium) on the renal CsA effects has been extensively studied (Durak et al., 1998; Perez de Lema et al., 1998; Parra Cid et al., 2003). Durak et al. (1998) showed that the level of thiobarbituric-acid (TBARS) was higher and

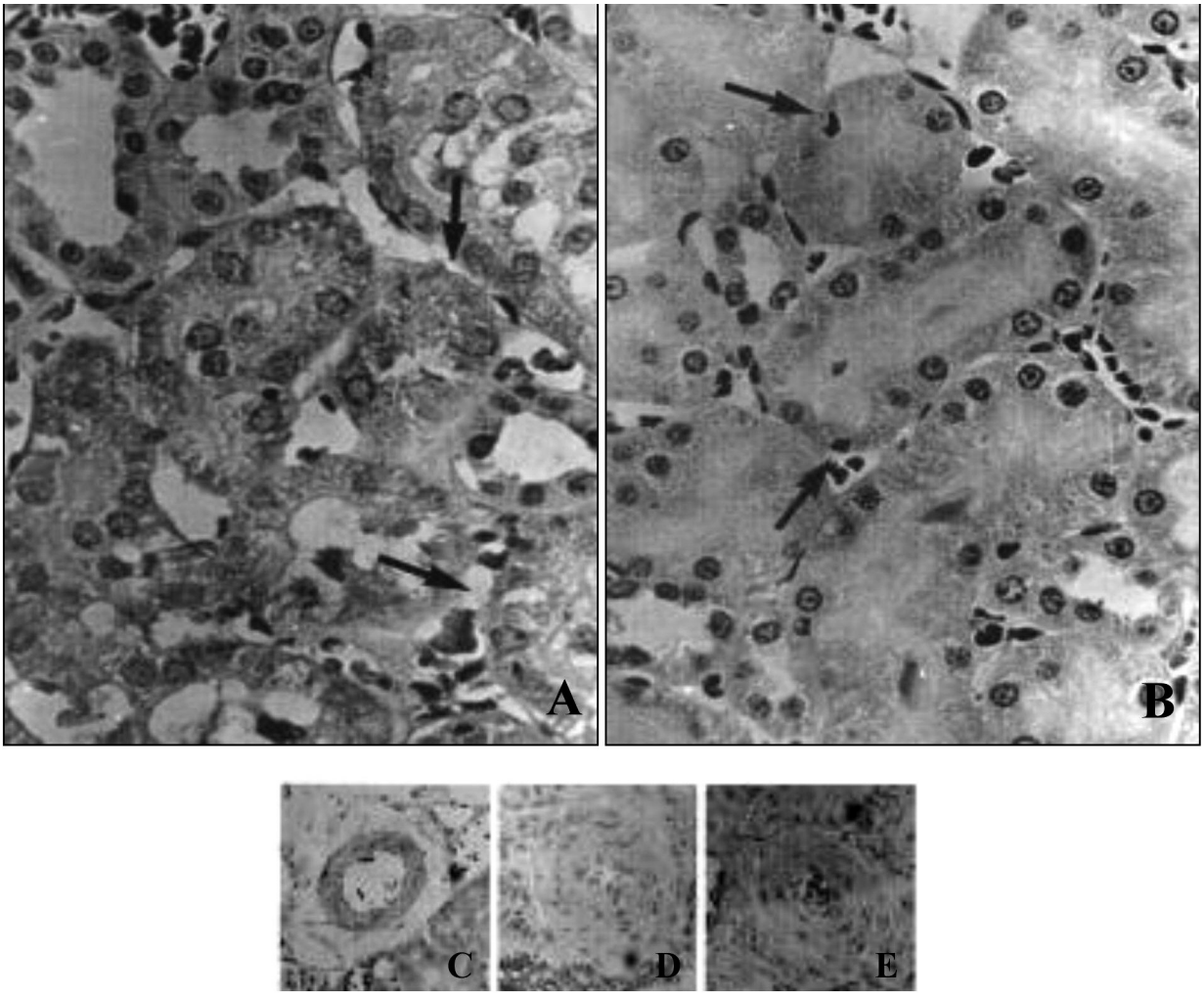
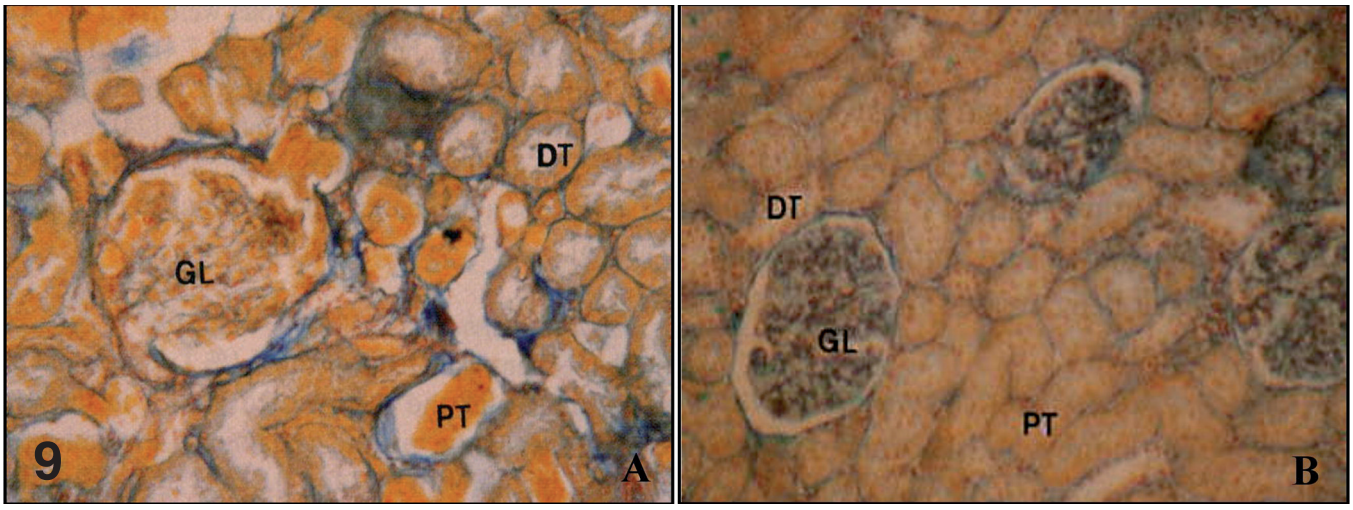


Fig. 8. Proximal tubules from CsA treated kidney **(A)** showing severe necrosis and vacuolations (arrows) while the same tubules from CsA plus quinacrine treated rats **(B)** showed only mild necrosis (arrow). Histological changes in the artery. **(C)** control rat: normal artery; **(D)** CsA treated artery showing severe constriction; **(E)** concomitant administration of quinacrine reduced the arteriolar constriction caused by CsA (haematoxylin-eosin; x400), (from al Khader Transplantation, Lippincott, 1996).



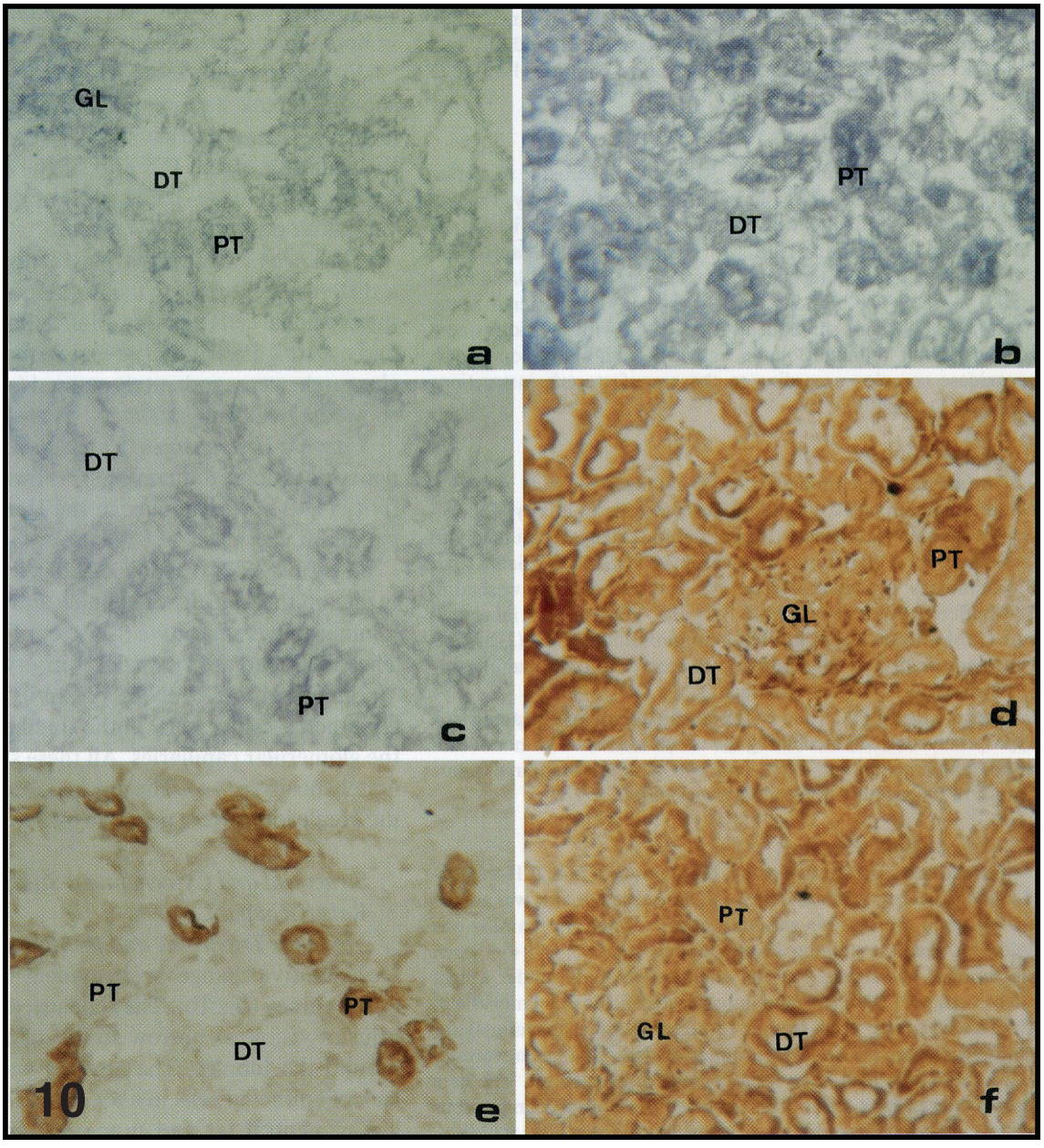


Fig. 9. Photomicrographs showing renal morphology in CsA **(A)** and CsA plus methylene blue treatment **(B)** (Masson's trichrome, x 230). DT: distal tubule; GL: glomeruli; PT: proximal tubule (from Rezzani, Nephron, Karger, 2001).

Fig. 10. Photomicrographs showing: NADPH-diaphorase and cytochrome-c oxidase activity respectively in control **(A, C)**, CsA-treated animals **(B, D)** and CsA plus methylene blue-treated rats **(E, F)** (x 120), (from Rezzani, Nephron, Karger, 2001).

antioxidant defense potential (AOP) lower in the CsA-treated rabbit and the histological examination revealed subcellular damage in renal tissues. Antioxidant vitamin E and C therapy caused full improvement in the enzyme activities, TBARS levels and AOP, but the subcellular damage was partly ameliorated. The other authors above reported evaluated the role of vitamin E and selenium in "in vitro" and "in vivo" experiments suggesting that these latter decreased the synthesis of hydrogen peroxide induced by CsA. Furthermore, preincubation of selenium for 24 h increased GPx activity, suggesting that it exerted its antioxidant effects by modulating the activity of the enzyme.

Several other studies have confirmed the beneficial actions of antioxidants on the deleterious effects of CsA. For example, carvedilol, a vasodilating ß-blocker, reduced elevated levels of TBARS and significantly attenuated renal dysfunction and morphological changes in CsA-treated rats and it was 10- and 1,000-fold more potent than vitamin E (Satyanarayana and Chopra, 2002). Its antioxidant effect was mainly attributed to inhibition of lipid peroxidation by scavenging free radicals and sequestering ferric ions (Tadolini and Franconi, 1998). Kumar et al. (1999) demonstrated that, while allopurinol prevented the lipid peroxidation formation, melatonin (MEL), which is considered to have antioxidant properties in addition to its known hormonal activities, selectively neutralized OH radical, which is considered the most reactive and noxious free radical. Moreover, experimental studies outlined that MEL stimulated antioxidant enzymes such as superoxide dismutase (SOD), MDA and GPx (Reiter et al., 1997; Shin et al., 2002). Considering MEL lipophilicity, ability to pass easily though all biological membranes, least toxicity and potent antioxidant property, this indole may play a very important role as a nephroprotective agent against CsA-induced renal impairment. These data have been confirmed by electron microscopy and morphometry. In fact, Stacchiotti et al. (2002) found that MEL partly prevented apoptosis in proximal tubules and

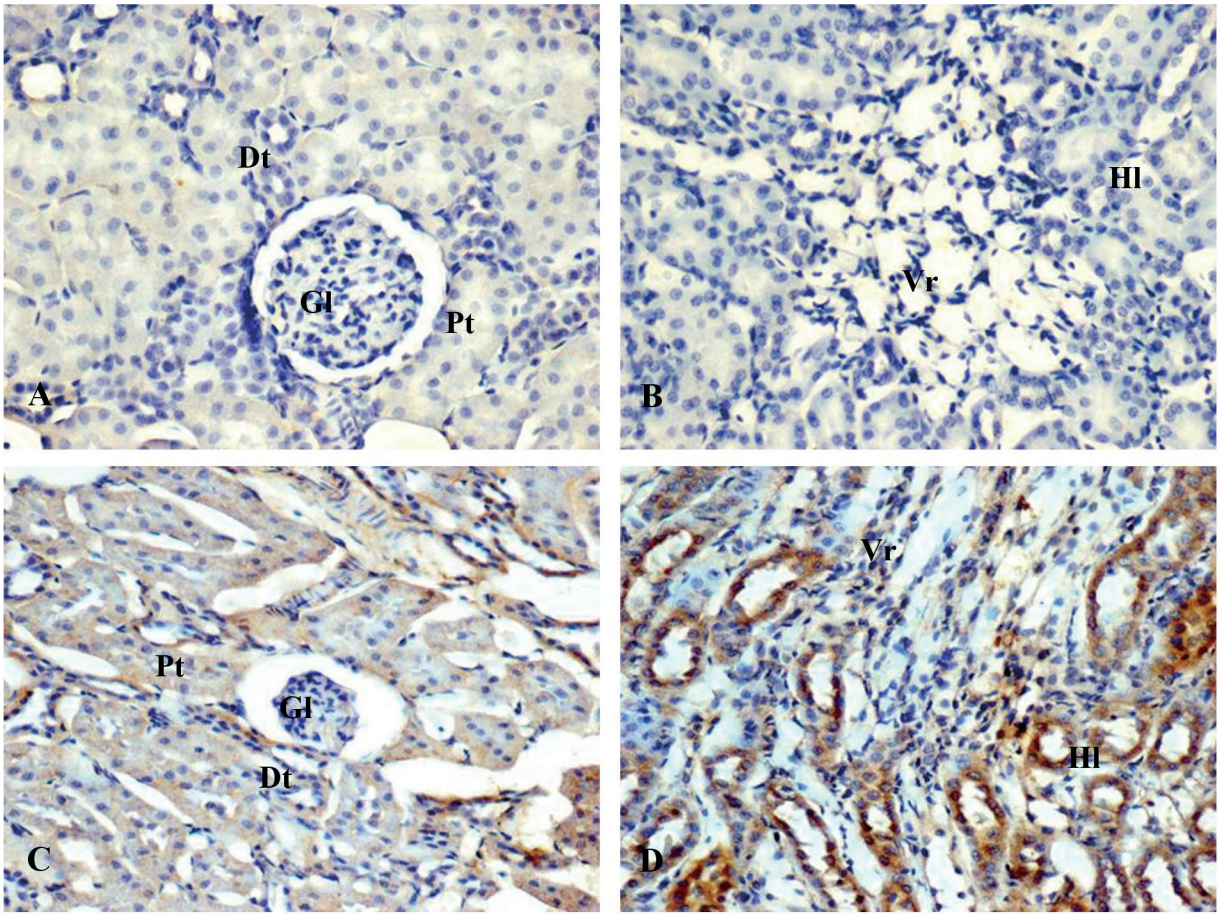


Fig. 11. Immunolocalization of NF-κB in kidney of control **(A, B)** and CsA treated **(C, D)** rat. Brown color indicates immunopositivity (x 200). **A, C:** cortex; **B, D:** medulla. Gl: glomerula; Pt: proximal tubules; Dt: distal tubules; Vr: vasa recta; Hl: Henle loops (from Buffoli, J. Histochem. Cytochem., The Histochemical Society, 2005).

an abnormal formation of mesangial matrix in the glomerula, which were altered after CsA treatment.

Recently, it has been demonstrated that dietary plant polyphenols also showed an important antioxidant role in CsA nephrotoxicity. In particular, the studies of Satyanarayana et al. (2001) were designed to investigate the effects of quercitin, a plant kingdom bioflavonoid with antioxidant properties in CsA-induced renal side effects. They showed that quercitin markedly reduced elevated levels of TBARS and significantly attenuated renal dysfunction and morphological changes in CsA-treated animals. It is likely that the inhibitory effect of quercitin on cyclooxygenase and lipoxygenase enzymes of the arachidonic acid pathway and the subsequent decrease in the production of mediators such as leukotrienes and prostaglandins (Alcaraz and Ferrandiz, 1987; Kim et al., 1998) play a role in improving renal dysfunction caused by CsA. Recently, different authors (Anjaneyulu et al., 2003; Mohamadin et al., 2005) studied the antioxidant properties of catechin, that is a flavonoid present in green tea, black tea and other plant foods (Cook and Samman, 1996) and known to have effects on human health, serving to protect against congestive heart failure and cancer. Their results underlined significantly improved CsA-induce renal dysfunction and changed oxidative markers. The antioxidant action of catechin has been speculated to be due to angiotensin converting enzyme (ACE) inhibition (Hara et al., 1987) properties, along with hypotensive (Hara and Tonooka, 1990) activity. This could be one of the reasons for which catechin has its beneficial effect by inhibiting angiotensin formation and direct vasorelaxation effect along with antioxidant properties.

Attention has also been paid to the protective effects of tea polyphenols (TP) as inhibitors of the transforming growth factor-ß1 (TGF-ß1) expression in the rat model of CsA-induced nephrotoxicity. TGF-ß1 promotes fibronectin and collagen synthesis (Khanna et al., 1999). The mechanisms by which TP inhibits the TGF-ß1 expression in CsA-induced nephrotoxicity may be the following: 1) TP increases tissue bioactivity of SOD and GPx which can inactivate ROS such as O_2^-. Because O_2^-

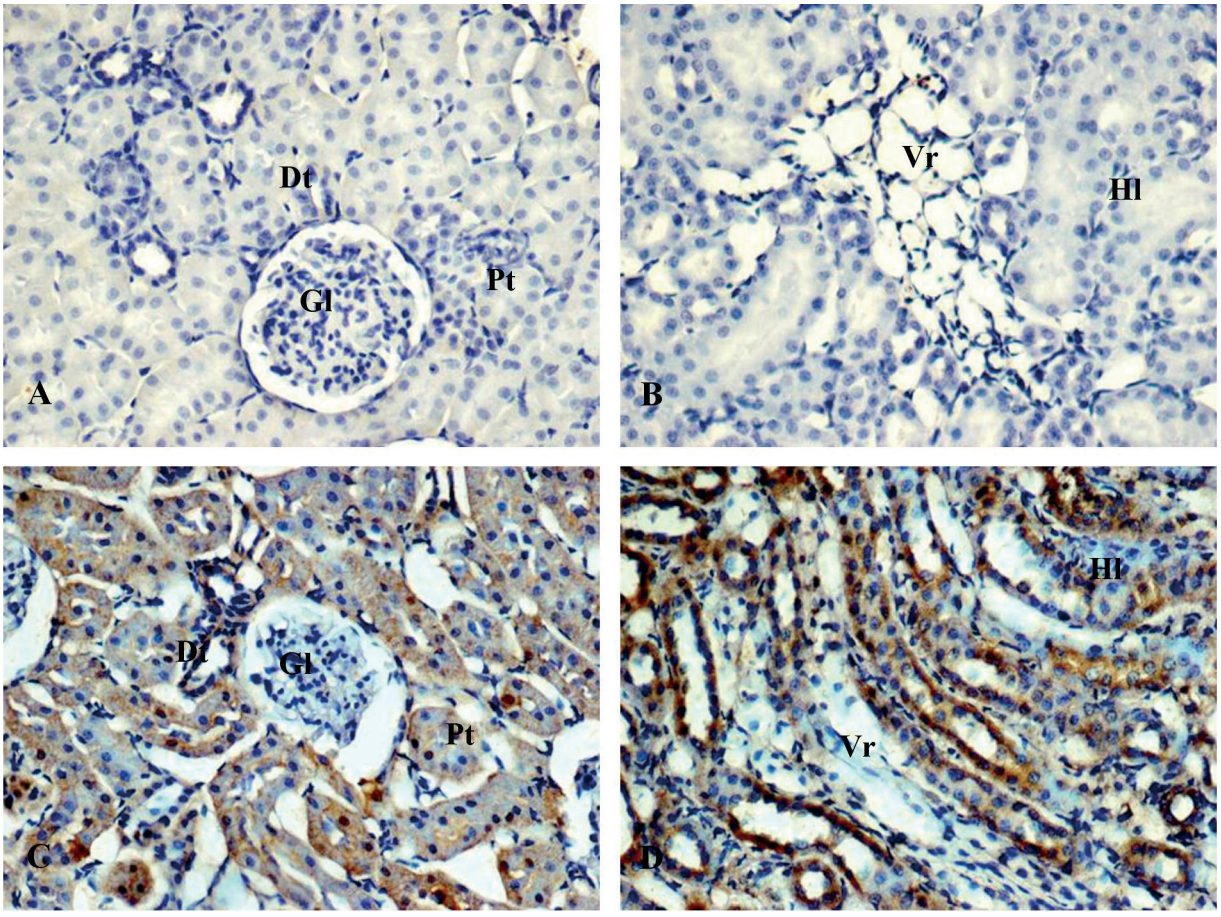


Fig. 12. Immunolocalization of iNOS in kidney of control **(A, B)** and CsA treated **(C, D)** rat. Brown color indicates immunopositivity (x 200). **A, C:** cortex; **B, D:** medulla. Gl: glomerula; Pt: proximal tubules; Dt: distal tubules; Vr: vasa recta; Hl: Henle loops (from Buffoli, J. Histochem. Cytochem., The Histochemical Society, 2005).

can react with NO by forming $ONOO_2^-$ to loose bioactivity of nitric oxide (NO), TP may reduce NO loss and maintain its physical function (Shi et al., 2001). Moreover, in the experiment of long CsA treatment, N-nitro-L-arginine-methyl ester (L-NAME) as the NO synthase inhibitor, strikingly worsened the glomerular filtration rate and the CsA-induced fibrosis and up-regulated expression of TGF-ß1, plasminogen activator inhibitor-1 (PAI-1); L-arginine, as the substrate for NO synthase, had the contrary beneficial effect (Shihab et al., 2000). TP may inhibit the synthesis of tromboxane A2 (TXA2) and leukotriene (LT) which are mediated by CsA-induced lipid peroxidation.

On the line of these researches, we showed that provinol (PV), which is a polyphenol compound, prevents the development of CsA-induced nephrotoxicity. In detail, CsA produced a significant increase in systolic blood pressure, it did not affect urinary output, but caused a significant decrease in creatinine clearance. These side effects were associated with an increase in conjugated dienes, which are lipid peroxidation products, iNOS and nuclear factor (NF)-κB, which are involved in oxidant damage (Buffoli et al., 2005). However, PV prevented these adverse effects through a protective mechanism that involved reduction of both oxidative stress and increased iNOS and NF-κB expression induced by CsA (Figs. 11, 12). Other authors (Chander et al., 2005) demonstrated that resveratol, a polyphenol found in grapes and grape wine (Gao et al., 2002) significantly improved renal dysfunction, tissue and urine total nitric oxide levels, renal oxidative stress and prevented the alterations in renal morphology.

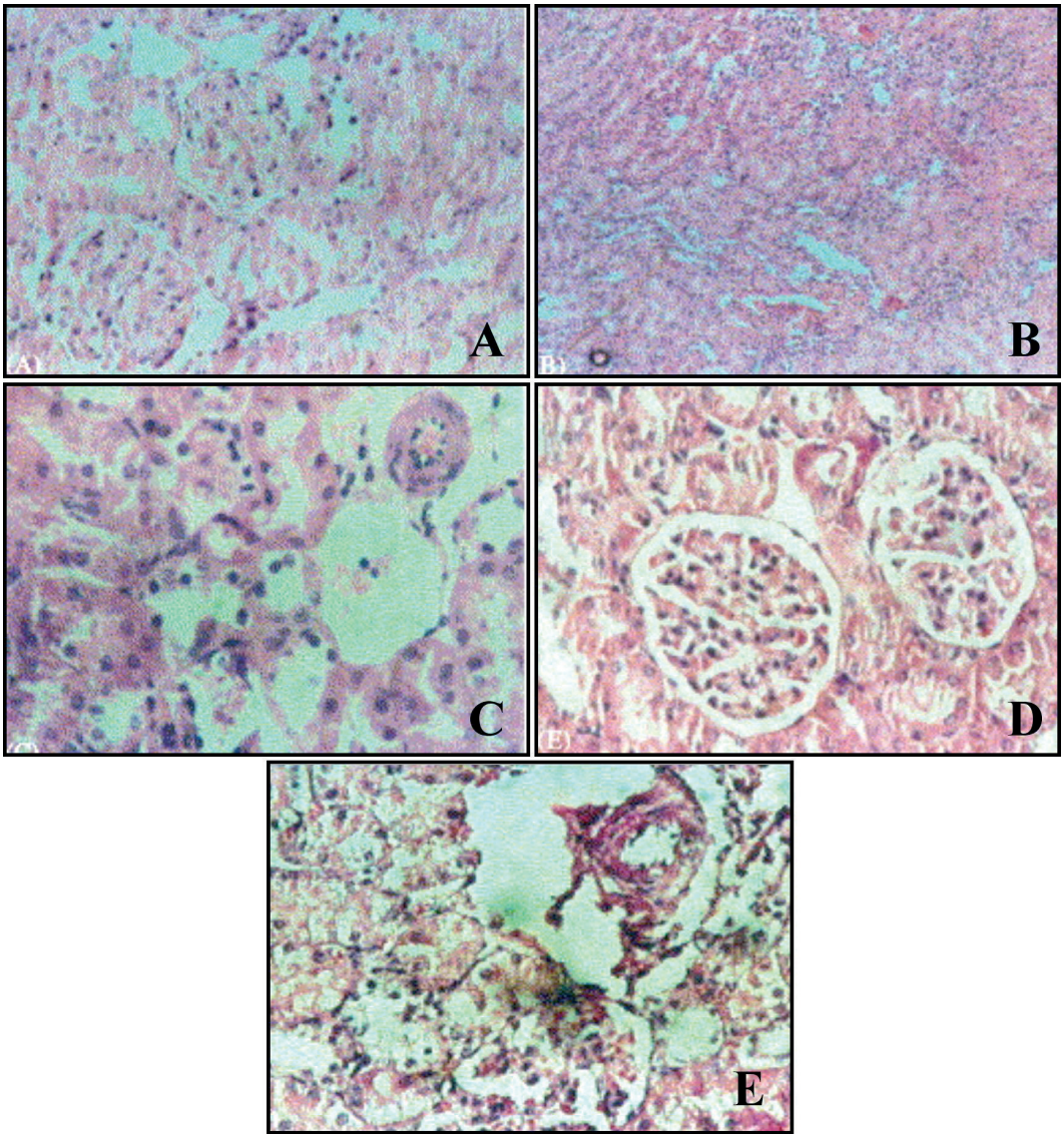


Fig. 13. Hematoxylin and eosin stained sections of rat kidneys: **A.** renal cortex of rat treated with vehicle (olive oil). **B.** Interstitial fibrosis of stripped pattern and tubular atrophy in cortex of CsA-treated rats. **C.** Arteriole with marked luminal narrowing and pronounced intimal thickening in CsA rats. **D.** Renal cortex of rats treated with resveratrol that prevented the development of CsA-induced alterations. **E.** Renal cortex of rats treated with resveratrol and L-NAME along with CsA, showing interstitial fibrosis and arteriolopathy similar to CsA nephropathy (adapted from Chander, Toxicology, Elsevier, 2005).

Concurrent administration of L-NAME blocked the protective effect of resveratol indicating that it exerted a protective effect by releasing NO (Fig. 13). These latter studies clearly demonstrated the pivotal role of NO in the etiology of CsA nephrotoxicity.

Pharmacological intervention on CsA hepatotoxicity

Cholestasis, hyperbilirubinemia, hypoproteinemis, increased alkaline phosphatase, elevated transaminases and bile salts in the blood, inhibition of protein synthesis and disturbed lipid secretion in both human and experimental animals (Hillebrand et al., 1999; Rezzani, 2004) characterize CsA hepatotoxicity. However, there are not many studies regarding the protective role of antioxidants against CsA hepatotoxicity and prevalently regard the administration of vitamins and amino acids. These observations are reported by Durak et al. (2004) and Hagar (2004). The first authors above cited demonstrated that vitamins E and C combined produced an increase in antioxidant capacity (AOC) of hepatic tissue, which were very low in CsA–treated animals (Table 3). Thus, they established that a decrease in AOC

Table 3.

GROUP	AOC
Control	0.77±0.20
CsA	0.55±0.09
CsA plus vitamins E and C	1.64±0.50
Vitamins E and C	3.26±1.03

Mean ± standard deviation (SD). Values of antioxidant capacity (AOC) (1/nmol.g tissue. h) in rabbit liver tissues (n = 5 for each group).

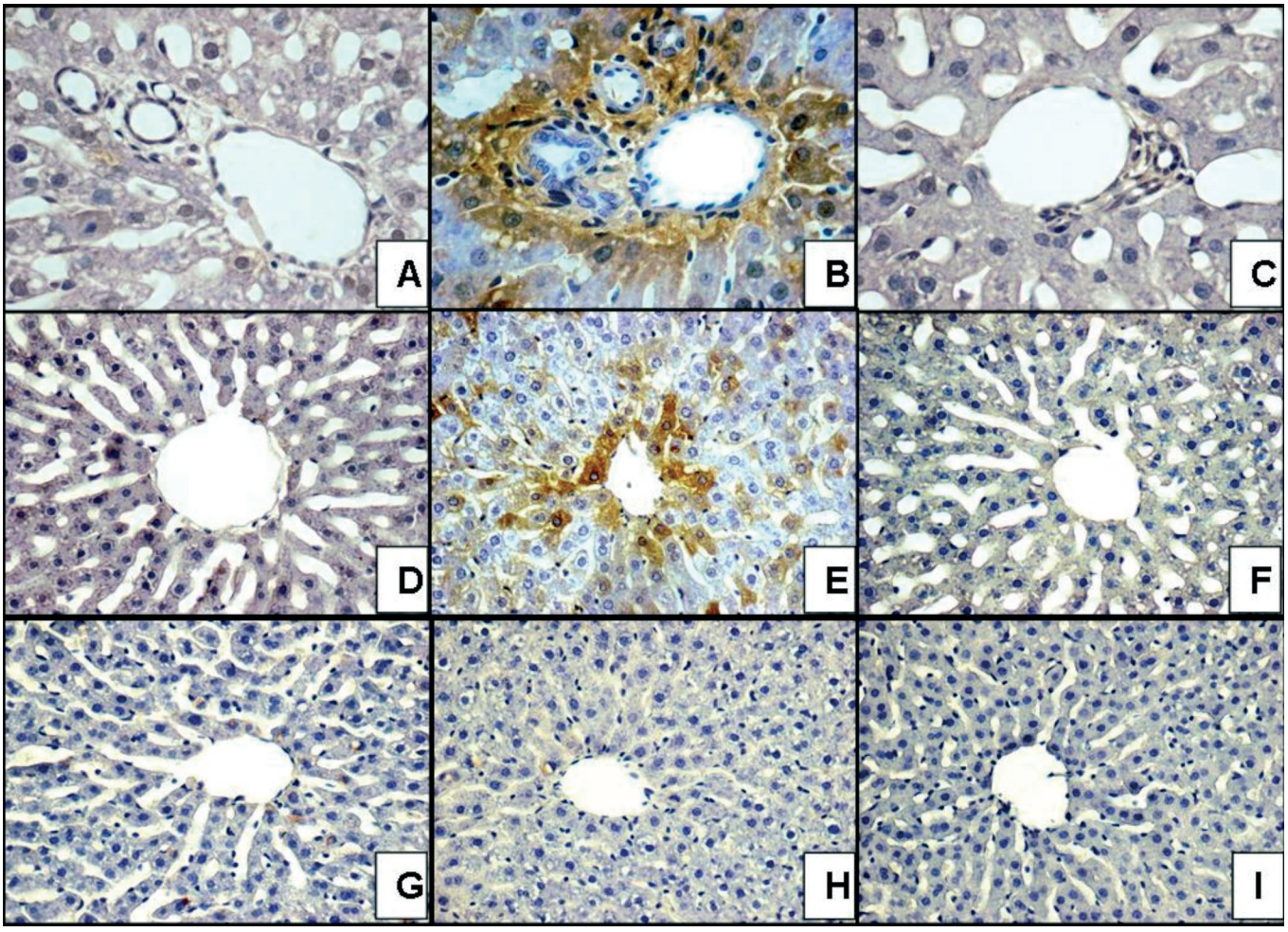


Fig. 14. Photomicrographs of rat liver with metallothionein immunohistochemistry. Control **(A, D)**; CsA-treated **(B, E)**; CsA plus Mel-treated **(C, F)**. **A, B, C:** portal area (x 400); **D, E, F:** centrolobular area. Negative staining for control **(G)**, CsA **(H)** and CsA plus Mel **(I)** treated rats (x 200), (Rezzani, Int. Immunopharm., Elsevier, 2005).

and reduced amounts of some antioxidant substances such as vitamins E, C, A and glutathione etc, may also contribute to the drug impairment. Instead, Hagar (2004) found that taurine (2-aminoethanolsulfonic acid), the major intracellular free ß-amino acid, having various important physiological roles including osmoregulation, bile acid conjugation, modulation of the functioning of the central nervous system and prevention of oxidant-induced injury in many tissues, prevented the CsA-oxidant damage in hepatic tissues. Taurine administration improved hepatic function as indicated by decline of serum transaminases and gamma glutamyl transferase (GGT) levels and elevation of serum total proteins. Moreover, taurine significantly reduced hepatic TBARS and increased glutathione content and catalase and GPx activities in hepatic tissues.

Recently, we showed that MEL has a potent antioxidant action against CsA hepatotoxicity (Rezzani et al., 2005a). CsA induced morphological alterations in hepatic cytoarchitecture, changes in GSH and MDA levels and an increase in stress protein expression. MEL improved these adverse effects reestablishing normal conditions such as normal cytoarchitecture and enzymatic antioxidant content and decreasing the stress protein expression (HsP60, HsP72 and metallothionein) (Fig. 14).

Pharmacological intervention on CsA cardiotoxicity

Few experimental studies have been carried out on myocardial drug effects, even if all findings showed that CsA induced cardiotoxicity. These degenerative changes involved size, shape and organization of the cardiac muscle both in atria and in ventricles (Bianchi et al., 2003; Rezzani, 2004). There is also a very limited number of studies using the antioxidant properties against CsA cardiotoxicity. Oliveira et al. (2004) showed that carvedilol, a ß-adrenergic receptor antagonist with antioxidant properties, was able to improve CsA cardiotoxicity. In particular, carvedilol inhibited mitochondrial-swelling acting on mitochondrial permeability transition (MPT) that was triggered by an enhanced oxidative stress. Recently, Rezzani et al. (2005b) demonstrated that caffeic acid phenethyl ester (CAPE), which is an active component of propolis extracts, protected against degenerative myocardial changes such as fibrosis and cytochrome-c-oxidase decrease. In fact, CAPE improved cardiac cytoarchitecture, decreased levels and the expression of proteins responsible for fibrosis as the matrix metalloproteinase-2 (MMP2) and increased cytochrome-c-oxidase. These protective effects induced a significant ROS decrease and amelioration in myocardial damage.

Concluding remarks

Data gathered from this review provide insight into the mechanism of side effects due to the immunosuppressive drug and related to oxidative stress. Recently, it has been demonstrated that the exogenous and endogenous antioxidants, such as vitamins, melatonin, minerals, thiazines and polyphenols play an important role in the mechanism of CsA action. The scientific results above reported underlined that the ROS are products of the normal metabolic pathway of the human organs, but under certain conditions, when in excess, they can exert harmful compounds and that they are implicated in CsA metabolism. In the future, application of the antioxidants to transplant surgery could help to overcome adverse CsA effects.

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