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Use of Antioxidants to Prevent Cyclosporine A Toxicity

Jinhwa Lee
Dept. of Clinical Lab Science, Dongseo University, Busan 617-716, Korea

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Cyclosporine A (CsA) is a potent immunosuppressor that is widely used in transplant surgery and the treatment of several autoimmune diseases. However, major side effects of CsA such as nephrotoxicity, hepatotoxicity, neurotoxicity and cardiovascular diseases have substantially limited its usage. Although molecular mechanisms underlying these adverse effects are not clearly understood, there is some evidence that suggests involvement of reactive oxygen species (ROS). In parallel, protective effects of various antioxidants have been demonstrated by many research groups. Extensive studies of CsA-induced nephrotoxcity have confirmed that the antioxidants can restore the damaged function and structure of kidney. Subsequently, there have appeared numerous reports to demonstrate the positive antioxidant effects on liver and other organ damages by CsA. It may be timely to review the ideas to envisage the relationship between ROS and the CsA-induced toxicity. This review is comprised of a brief description of the immunosuppressive action and the secondary effects of CsA, and a synopsis of reports regarding the antioxidant treatments against the ROS-linked CsA toxicity. A plethora of recent reports suggest that antioxidants can help reduce many CsA's adverse effects and therefore might help develop more effective CsA treatment regimens.

***Key words*:** Cylcosporine A, Immunosuppressant, Toxicity, Antioxidant, ROS

INTRODUCTION

Cyclosporine A (CsA) has promoted organ transplantation greatly because of its potent immunosuppressive activity. CsA treatment during transplantation prevents allograft rejection and increases patient survival (Calne *et al*., 1978; Langford *et al*., 1988a, 1988b; Taylor *et al*., 2005). Other than the use for transplantation, CsA also has been introduced for the treatment of autoimmune diseases such as psoriasis and rheumatoid arthritis (Rezzani, 2004; Fraser *et al*., 2003). CsA is a lipophilic compound of cyclic endecapeptide that is originally from the fungus Tolypocladium inflatum (Behforouz and Wenger, 1988). Together with other immunosuppressive chemicals FK506 and rapamycin, CsA blocks the synthesis of interleukin 2 (IL2) at transcriptional level (Ho *et al*., 1996).

The efficient use of CsA as an immunosuppresant has been limited by its side effects such as nephrotoxicity, hepatotoxicity, and neurotoxicity. Among the toxic effects, most serious ones include the nephrotoxicity and hepatotoxicity (Mason, 1990; de Mattos *et al*., 2000; Herrero *et al*., 2000). Moreover, long term treatment of CsA causes chronic nephrotoxicity to the all recipient patients (Nankivell *et al*., 2003). The acute CsA-induced nephrotoxicity involves renal vasoconstriction and renal dysfunction, both of which are reversible on CsA withdrawal. The renal vasoconstriction appears to be induced by the release of imbalanced vasoactive substances. The chronic toxicity involves more serious structural damages such as arteriolopathy and tubulointerstitial fibrosis that are characterized by irreversible damage to the tissues and renal failure (Mourad *et al*., 1998; Myers *et al*., 1988; Young *et al*., 1995a, 1995b). Alteration in calcium homeostasis leading to increased contraction of smooth muscle cells is a cause to the renal toxicity as well as others. However, these effects are related to the generation of reactive oxygen species (ROS). ROS can attack and damage all types of macromolecules and they have been attributed, at least partly, to CsA toxicity. Recent studies show evidence that many antioxidant substances can alleviate the CsA toxicity, shedding a light on this most widely used fault-ridden immunosuppressant for a better use. This review will discuss the CsA's beneficiary action and the adverse action, and also summarize the recent reports on antioxidant effects on CsA treatment.

Correspondence to: Jinhwa Lee, Department of Clinical Laboratory Science, Division of Health Science, Dongseo University, Jurea 2-dong, Sasang-gu, Busan 617-716, Korea
E-mail: jinhwa2000@gdsu.dongseo.ac.kr

Cyclosporine A actions. CsA suppresses immune responses mainly by inhibiting production of immune reac-

tive cytokines such as IL2. Intracellular interaction of CsA involves its receptor protein peptidylprolyl cis-trans isomerase (PPIase) cyclophilin and the protein phosphatase 2B calcineurin, inhibiting both the PPIase activity of cyclophilin and calcineurin phosphatase activity (Erlanger, 1992). Since calcineurin activity is essential for the dephosphorylation and activation of the nuclear factor of activation of T cells (NFAT), cytokines that are regulated by NFAT are consequently down regulated by CsA (Shaw *et al*., 1995). NFAT is a transcription factor that activates the transcription of cytokines that promote the growth and proliferation of T- and B-cells. IL2 that is produced by T lymphocytes in response to antigenic or mitogenic stimulation is also necessary for the proliferation and differentiation of many immune cells including activated T lymphocytes, natural killer cells, lymphokine-activated killer cells, B lymphocytes and macrophages (Suthanthiran *et al*., 1996).

In addition to the inhibitory effect on IL2, CsA inhibits the production of interleukins 1a and 1b, interleukin6, gamma-interferon and other lymphokines (Olyaei *et al*., 2001). These cytokines together modulate the immune and inflammatory reactions, stimulate the hematopoiesis and also present diverse physiological roles regulating the innate and adaptative immunity (Rezzani, 2004).

Close examination of CsA binding to different cyclophilin family proteins can open its action span into totally new area. For example, cyclophilin D is a component of mitochondrial transition pore complex and thus inhibition of this protein by CsA can prevent apoptosis. Discussing physiological roles of various cyclophilins and inhibitory effect of those individual PPIase isozymes by CsA is beyond the scope of this manuscript. However, the major cytosoilic CsA receptor cyclophilin A (CypA) is viewed as an antioxidant protein by some researchers (Doyle *et al*., 1999; Hong *et al*., 2002). Overexpression of CypA provides extra antioxidant capacity to the cells and protects them from oxidative stress. Therefore, CsA can work as a prooxidant merely by inhibiting CypA's antioxidant activity.

Cyclosporine A complications. CsA treatment accompanies an adverse effect that is characterized by occurrence of considerably broad and serious complications including nephrotoxicity, hepatotoxicity, neurotoxicity, hyperkalemia, hypertension, dyslipidemia, gingival hyperplasia, hypertrichosis, malignancies, and an increased risk of cardiovascular events (de Mattos *et al*., 2000; Herrero *et al*., 2000; Stallone *et al*., 2004). Acute toxic effects are often reversible whereas chronic symptoms may last permanently even after the treatment is discontinued. CsA can induce increased serum bilirubin and transaminases, which usually appear in the first 2 months of treatment and disappear on withdrawal of the drug (Shen *et al*., 1987; Racusen *et al*., 1987; Pickrell *et al*., 1988). In addition to these CsA specific side effects, CsA produces important inhibitory effects on immune defense mechanisms against infections and malignancies. Varied viral, fungal, and bacterial infections as well as an increased frequency of cancers are observed among transplant recipients treated with CsA (Kumar *et al*., 2005; Pascual *et al*., 2002). For example, transplant patients treated with CsA have a greater incidence of viral infections of herpes simplex, herpes zoster and cytomegalovirus, and pneumocystis carinii pneumonia. Besides, these patients have a lower prevalence of bacterial and fungal infections and also a lower incidence of de novo neoplasias (Tugwell *et al*., 1995; Heydendael *et al*., 2003; Lichtiger *et al*., 1994; Weber *et al*., 2001; ten Brinke *et al*., 2004).

The histopathological changes occurring in the liver comprise sinusoidal dilatation, cytoplasmic vacuolization of hepatocytes, cell infiltration (especially in the periportal areas), parenchymal mitosis and moderate hepatocellular necrosis. The mechanisms underlying the hepatic side effects have not been explained despite extensive studies (Romero *et al*., 2001; Diao *et al*., 2002). 10~28% of CsA-treated patients experience some form of neurotoxic adverse event, but only 5% of patients suffer from severe neurotoxicity such as psychoses, hallucinations, blindness, seizures, cerebellar ataxia, motoric weakness, or leukoencephalopathy, and withdrawal of the drug reverses the symptom. Direct effect of CsA on endothelial vascular cells that account for the atherosclerotic process, togethor with the CsA effects on metabolic, inflammatory and coagulatory disorders, has been attributed to the CsA side reaction of cardiovascular risks. It is also suggested that CsA-induced vascular damage predisposes individuals to atherosclerosis and also can be causative to cerebrovascular and cardiovascular diseases (Malyszko *et al*., 1996; Kobashigawa and Kasiske, 1997; Moien-Afshari *et al*., 2003; Boots *et al*., 2004). Detrimental effect of CsA on glucose homeostasis, posttransplant hyperlipidemia, increases in the levels of total homocysteine and a sulfur-containing amino acid intermediate in methionine metabolism, all together can be an independent risk factor for atherosclerosis and peripheral vascular disease (Stallone *et al*., 2004; Hjelmesaeth *et al*., 2005; Arnadottir *et al*., 1996; Marchetti, 2004).

Nephrotoxicity can lead to end-stage renal disease, limiting the use of CsA. Likewise, kidney dysfunction is the main complication of CsA treatment. The nephrotoxicity is mainly characterized by tubulo-interstitial fibrosis and tubular atrophy. A hypothesis has been proposed that CsA alters the balance between vasodilators and vasoconstrictors in kidney with predominance of vasoconstrictors and vascular smooth muscle cells proliferation in the intima and accumulation of cholesterol esters in macrophages that can be transformed in foam cells in vessel wall with narrowing of vessel lumen (Beckman *et al*., 2002). Several studies indicate that vascular dysfunction induced by CsA results from an increase in vasoconstrictor factors such as endothelin, thromboxane, and angiotensin II and at the same time a

reduction of vasodilator factors such as prostacyclin and nitric oxide (NO) (Parra *et al*., 1998a; Markell *et al*., 1994; Bilchick *et al*., 2004; Baid *et al*., 2001; Halliwell and Gutteridge, 1999). Therefore, an imbalance in the release of vasoactive substances is related to renal vasoconstriction. Decreased glomerular filtration rate and renal plasma flow observed in an early stage is known to be related to afferent arteriolar vasoconstriction (Shen *et al*., 1987). Loss of proximal tubular cells brush border, proximal tubule dilatation, swelling, necrosis, and infiltration of white blood cells in kidney cortex belong to renal tubular toxicities which are considered to be acute (Racusen *et al*., 1987). Chronic CsA nephropathy is characterized by irreversible renal striped vasculointerstitial fibrosis, inflammatory cell infiltrations and hyalinosis of the afferent glomerular arterioles (Nankivell *et al*., 2003; Mourad *et al*., 1998; Myers *et al*., 1988). The damage in the glomerular and arteriolar vessels produces decreased urea and uric acid urinary excretion, along with reduction of fractional excretion of sodium, lithium, potassium and phosphates, and decreased reabsorption of bicarbonate, hyperchloremia and metabolic acidosis (Young *et al*., 1995a, 1995b). Chronic ischemia caused by CsA is believed to be associated with reactive oxygen species and lipid peroxidation.

Oxidative stress. Oxygen molecules with unpaired electrons in the outer orbital are devoid of the kinetic barrier of dioxygen molecule and become highly reactive, and these molecules are called reactive oxygen species (ROS). ROS can oxidatively modify biomolecules of nucleic acid, lipid, sugar, and protein, causing nuclear damage, mitochondrial damage, and endoplasmic reticulum stress (Jones and Hancock, 2000). In order to survive, aerobic cells are equipped with various antioxidant molecules and enzymes. Oxidative stress is the condition when redox balance favors ROS over antioxidants and damaged molecules are accumulated in the cell.

ROS generation in the cell has initially been known for defense system from the respiratory burst of neutrophils and the NADPH oxidase complex (Jones and Hancock, 2000; Hancock *et al*., 2001). Recent findings however showed interesting usage of ROS as key signalling molecules (Fridovich, 1978). As one electron at a time is added to the molecular oxygen, gradual reduction of the dioxygen molecule transforms into the reactive species of superoxide, peroxide, hydroxyl radicals. Superoxide formed by one electron reduction of molecular oxygen can be generated by NADPH oxidase (Deby and Goutier, 1990). Hydrogen peroxide can arise from the dismutation of superoxide molecules, which can occur spontaneously or can be catalyzed by enzymes known as superoxide dismutase (SOD). The formation of hydroxyl radicals catalyzed by metal ions through the Fenton or Haber-Weiss reactions is most fatal because this noxious free radical attacks any molecules that it encounters (Perez de Lema *et al*., 1998).

In aerobically metabolizing organisms, ROS are produced by normal respiratory system. Several defensive mechanisms to counteract the deleterious effects of ROS include low molecular weight compounds (ferritin, glutathione, VtC and VtE) as well as various enzymes (SOD, catalase, glutathione peroxidase, and glutathione reductase). Damages to lipids, proteins, carbohydrates and nucleic acids during normal aerobic metabolism might occur but are constantly repaired or removed. When insufficient antioxidants exist or too much ROS are generated, however, the composed balance is disturbed and the following accumulation of damaged cellular substances becomes toxic and leads to pathogenic states. Excess ROS can be produced by environmental factors such as hypoxia.

It has been shown that CsA is able to generate ROS and lipid peroxidation, which appears to be directly related to the pathological outcome. Perez *et al*. using detection method with the fluorescent probe 2,7-dichlorofluorescin diacetate (DCFHDA) showed that 1~10 μM CsA was able to generate the oxidized 2,7-dichlorofluorescein (DCF) signal, interpreted as being H2O2-derived. Similar findings are reported by many researchers, the mechanism of ROS production is still unclear (Ischiropoulos *et al*., 1999; L'Azou *et al*., 1999). It has been suggested that induction of cytochrome P450 (CYP), especially CYP3A, might account for the ROS formed in cellular and animal systems; however, later report suggests that CYPs is not the enzyme responsible for ROS generation by CsA (Watkins, 1990; Krauskopf *et al*., 2002). Several hypotheses to explain the link between CsA treatment and ROS have been proposed but a solid mechanism has not been very successfully established. CsA treatment induces renal perturbation of the vasoconstriction–vasodilation balance to cause tubular hypoxia-reoxygenation; therefore, CsA might cause hopoxia-induced ROS production, at least in the kidney (Inselmann *et al*., 1991). L'Azou *et al*. found that CsA decreased in a dose and time dependent way, the planar cross-sectional area in cultured mesangial cells and isolated kidney glomeruli and increased the synthesis of hydrogen peroxide (Inselmann *et al*., 1994).

CsA increases lipoperoxidation in the rat kidney and liver in vivo has also been observed (Zhong *et al*., 1998; McGrath *et al*., 1997). Haem oxygenase-1, an enzyme responsive to changes in the redox status, varies after treatment with CsA (Rezzani, 2006). It has been proposed that oxidative stress induced by CsA may activate/deactivate transcription factors and affect transcription of genes that are under the regulation. Similarly, ROS generated by CsA may as well activate/deactivate some signalling molecules and influence the downstream transduction system.

Antioxidant effect on cyclosporine A toxicity. Exogenous supplementation of antioxidants leading to ablation of CsA toxicity supports the hypothesis that CsA toxicity is

caused by ROS. In normal physiological condition, ROS can be scavenged by cellular antioxidant system of small molecule antioxidants such as VtC, VtE, glutathione, carotenoids, and thioredoxin as well as enzymes like SOD, peroxidases and catalases. Oxidative stress that is defined as excessive ROS over antioxidant capacity can in turn induce antioxidant enzymes and therefore it is believed that overexpression of the enzymes protect cells from mild oxidative stress by eliminating the extra ROS. However, overexpression of antioxidant production that denotes increased antioxidant capacity does not always result in the enhancement of the antioxidative defense to provide cellular protection. The determinants of the competence of the antioxidant system are considered to possess such aspects as compartmentalization of ROS formation and antioxidant localization, synthesis and transport of antioxidants, the ability to induce the antioxidant defense and cooperation between different antioxidant systems. Here in this section, in vivo and in vitro studies to ask if antioxidants that are exogenously supplemented can protect cells from CsA toxicity will be reviewed from recent studies.

Gingivial hyperplasia is one of important CsA side effects. Proteom of human gingival fibroblasts (HGF) induced by the CsA treatment using proteomic analysis by Jung *et al*. show upregulation of oxidation associated proteins including peroxiredoxins and glutathione-S-transferase. They suggest that overexpression of these antioxidants may play a role in promoting proliferation in the CsA-treated HGF, with reducing cytosolic ROS levels as an antioxidant action (Jung *et al*., 2009). Exogenous antioxidant effects on the CsA nephrotoxicity have been extensively studied 10 years before (Durak *et al*., 1998; Perez de Lema *et al*., 1997; Parra *et al*., 1998b) and reinforced continually with recent reports. Antioxidants can also improve renal function and histological damage produced by CsA administration (A). In cultured hepatocytes and mesenchymal, Vt E addition to the CsA treated cells has been observed to prevent CsA cytotoxicity as well as lipid peroxidation. The same Vt E effect has been reported in vivo using CsA-treated rats with function and structure of kidney intact (Parra Cid *et al*., 2003). Studying the mitochondrial effects of CsA in the porcine renal endothelial cell line LLC-PK1 and the influence of the antioxidant Vitamin E (Vit E), the authors show that Vit E pretreatment inhibits the effects that CsA induced on mitochondrial structure and function in LLC-PK1 cells and avoided apoptosis (de Arriba *et al*., 2009). The same renal proximal tubular LLC-PK1 cells have been used by a different group to study the cytotoxic action of CsA is triggered by oxidative stress (Louie *et al*., 2010). They have shown that glutathione (GSH)-dependent enzyme, glyoxalase I (Gly-I) which plays a key role in cellular detoxification is inactivated and that this inactivation that is fatal to the cell is counteracted by NAC (Louie *et al*., 2010).

Antioxidant effect on cardiotoxicity using melatonin that is known to be a potent antioxidant molecule with a capacity to protect tissues from damage caused by oxidative stress has been recently reported (Rezzani *et al*., 2009). CsA-induced cardiotoxicity that accompanies lipid peroxidation and the expression of the isoform of inducible nitric oxide (iNOS) and apoptosis can be reduced by melatonin. The use of CsA for immunosuppression following organ transplantation increases the risk of developing post-transplant lymphoproliferative disorder (PTLD), mainly linked with Epstein-Barr virus infection (Leong *et al*., 2010). Authors first discovered that CsA-induced oxidative stress plays an important role in Epstein-Barr virus (EBV)-related PTLD and further demonstrated that CsA-induced lipid and protein oxidation could be inhibited by Vt E, N-acetyl cysteine, and pyrollidine dithiocarbamate (Chen *et al*., 2008). They also show that CsA exerts direct oxidative stress in EBV-infected as well as non-EBV-infected human B cells.

Several studies of coadministration of natural products with CsA pursuing alleviation of the CsA-induced toxicity have been reported recently. Epicatechin (EC) is a potent antioxidant present in the human diet that are found in tea, apples, and chocolate, Treatment of rats with EC ameliorates the toxicity of CyA by decreasing the lipid peroxidation and enhanced the antioxidants enzyme activities (Al-Malki and Moselhy, 2011). Caffeic acid phenethyl ester (CAPE) is a natural product with potent anti-inflammatory, antitumor, and antioxidant activities. CAPE prevents CsA-induced renal injury, coupled to lipid peroxidation, via inhibition of oxidative process (Gökçe *et al*., 2009). The studies on ellagic acid (EA), a polyphenolic compound against CsA-induced liver injury show the protective effect in rats. By decreasing the levels of thiobarbituric acid reactive substances and hydroperoxides and increasing the levels of enzymic and non-enzymic antioxidants on treatment with EA in the liver, EA might play an important role in protecting CsA-induced oxidative damage in the liver (Pari and Sivasankari, 2008). Another group shows, in the kidney, liver and heart oxidant/antioxidant system, the effects of ellagic acid on CsA-induced alterations (Yüce *et al*., 2008). Nigella sativa oil, used in many types of illnesses, has antioxidant activities to reduce toxicity. The N. sativa oil reduces CsA injury in rat heart, which is evidenced by normalized cardiac histopathology, decrease in lipid peroxidation, improvement in antioxidant enzyme status and cellular protein oxidation (Ebru *et al*., 2008). Hydroxytyrosol, a natural olive oil antioxidant, has been examined for renal histology and haemodynamic alterations induced in rats by CsA treatment. While hydroxytyrosol shows protection from CsA-induced oxidative stress, it is only associated with a mild effect on histological damages and does not affect the altered glomerular function and the hypertension. Therefore, the authors concluded that kidney injury by CsA is only in part dependent on oxidative stress (Capasso *et al*., 2008). Combinationational treatment of antioxidants quer-

cetin and Vt E in attenuating CsA-induced liver in rats has significantly reduced CsA-induced adverse alterations in both liver morphology and function (Mostafavi-Pour *et al*., 2008). Hypothesis that high concentration of nitric oxide (NO) occurring as a result of iNOS induction and peroxynitrite formation is responsible for lipid peroxidation and protein oxidation in CsA- induced cellular damage has been tested using Lipoic acid as an antioxidant. In vovo study showed that Lipoic acid might have a protective effect on nitric oxide mediated cellular abnormalities induced by CsA in rat kidney. against CsA-induced peroxidative changes and cellular damage of the renal tissue of the rat (Amudha *et al*., 2007).

Seaweeds or marine algae are a major Asian diet and Sargassum wightii among them contains sulphated polysaccharides. Sulphated polysaccharides are well known for its antioxidant properties acting as scavengers of free radicals such as superoxide and alkoxy radicals. Experiments of its protective effects on nephrotoxicity and hepatotoxicity in CsA-induced rats have shown that this natural product can be a useful tool in reducing CsA-induced side effects (Josephine *et al*., 2006, 2007, 2008).

CONCLUSIONS

Immunosuppressive activity of CsA is hampered by its severe toxicities that disable functions and structures of some important organs such as liver, kidney and heart. The side effect has been attributed to the increased production of ROS and decreased antioxidant status by CsA in damaged cells of dysfunctional organs. CsA treatment inhibits the expression and activity of antioxidant enzymes like SOD, catalase and glutathione-peroxidase. The exogenous antioxidants including antioxidant natural products have been shown to inhibit the adverse actions of CsA. Thus, antioxidant supplementation with CsA treatment might be beneficiary to prevent severe renal, hepatic, or neurological and cardiovascular side effects in transplant or non-transplant patients.

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