Structural Effects of Vitamin E on Proximal Tubule and Interstitium in a Rat Model of Cyclosporin A Nephrotoxicity

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Abstract: The aim of this study was to investigate whether the antioxidant vitamin E provided morphological protection in the nephrotoxicity caused by Cyclosporin A (CsA). Twenty-five Sprague-Dawley male rats were divided into five groups, each consisting of five rats. One of them was the control group and four of them were the experiment group. CsA (5 mg kg⁻¹ day⁻¹) to the Group II, III and V, CsA plus vitamin E (5 mg kg⁻¹ day⁻¹) to Group IV were administered intraperitoneally (i.p.) for 8 weeks. The kidney specimens of the Group I, II and IV were taken at the end of the 8th week. The kidney specimens of the Group III were taken at the end of 14th week after they had been kept for reversibility for 6 weeks. As for the kidney specimens of the Group V, they were administered vitamin E (5 mg kg⁻¹ day⁻¹) for 6 weeks after the administration of CsA (5 mg kg⁻¹ day⁻¹) for 8 weeks and then were taken at the end of the 14th week. All of these kidney specimens were processed for light and electron microscopical examination. In the Group II, infiltration foci and increase of interstitial connective tissue were observed at the surrounding of vessels, especially in the corticomedullary region. The most obvious changes were encountered in the proximal tubules. These changes were seen as degeneration and regeneration. While the degeneration was seen as the thickening of basement membrane, loss of brush border, vacuolization, dilation of the smooth endoplasmic reticulum, increase in lysosomes in number and size, the proliferation of some of the scattered epithelial cells of the tubules formed the regeneration areas by causing the appearance of new tubules. No obvious regression was seen in the Group III and more or less the same changes as in the Group II were observed. As vitamin E inhibited the oxidative damage of CsA, the least damage occurred in the Group IV. Because of the release of CsA accumulated in tissues later giving it into the organism, more damage was observed in the Group V compared to Group IV. Therefore, using CsA and vitamin E simultaneously may keep the nephrotoxicity caused by CsA at a minimal level.

Key words: Cyclosporin A, vitamin E, kidney, proximal tubule, interstitium

INTRODUCTION

Cyclosporin A (CsA) is the most effective and widely used as the first-line immunosuppressant in solid organ transplantation[1]. CsA which improves quality of life and survival of transplant patients by preventing graft rejections, was extracted from a fungi called Tolypocladium inflatum Gams by Borel et al.[2] for the first time. Although the mechanism of action of CsA is not well known, the drug acts in early phases of the activation of the immune system. CsA is able to block the biosynthesis of some lymphokines produced by T lymphocytes, particularly CsA blocks the interleukin-2 (IL-2) synthesis at transcriptional level. The initial mechanism of action of CsA in T lymphocytes is unknown and several hypotheses have been suggested. Some authors propose that CsA bonds to cytoplasmic proteins, such as cyclophilin, which can inhibit signal mechanisms from the cytoplasm to the cellular nucleus. Finally, CsA can exert its action through direct influences on the nucleus; the binding of CsA to nuclear receptors can induce the synthesis of lymphokines and several cytotoxic enzymes.[1,2]

The drug is highly metabolized in the endoplasmic reticulum of liver cells by hydroxylation and n-demethylation reactions of mixed function oxidases and excreted in the bile.[3,4]. For the drug, the liver is the major depot, followed by the pancreas, fat, blood, heart, lung, kidney and neural and muscular tissue.[5]

The side effects of the CsA are nephrotoxicity, hepatotoxicity, neurotoxicity, hypertension, gingival hypertrophy, hypertrichosis, hyperuricemia, thrombocytopenia, anemia and elevated erythrocyte sedimentation rate.[6,7]. Nephrotoxicity is the most important of all[8] and was declared by Calne for the first time[9].

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CsA through its nephrotoxic effect induces functional and structural changes on the kidneys. The functional changes appear in small doses and may be reversible. These are the changes, such as the decrease in the glomerular filtration rate and renal blood flow and increase in the blood urea nitrogen/creatinine ratio, renal vascular resistance and proximal fractional reabsorption\(^{20,16}\). Regarding the structural changes, they exist mainly in S3 segment of proximal tubules\(^{27}\), appear in high doses and they may not be reversible\(^{20,18}\).

The data of some experimental studies suggest a possible role of free oxygen radicals in CsA toxicity\(^{20,21}\). CsA due to these free oxygen radicals induces an increase in the in vitro and in vivo lipid peroxidation\(^{22,23}\). It has been shown that the deficiency of antioxidant in the diet also enhances lipid peroxidation\(^{24,23}\), whereas the antioxidant plus the diet inhibits lipid peroxidation\(^{23}\). As a result of this, studies of various antioxidants have been started in order to prevent the CsA damage\(^{22,20,24}\). Biochemical changes were examined more in these studies, however structural changes have not been studied yet. Vitamin E is the primary liposoluble antioxidant, which may have an important role in scavenging free oxygen radicals and in stabilizing the cell membranes, thus maintaining its permeability\(^{23}\). That's why, this study was planned to investigate whether the antioxidant vitamin E provided morphological protection by decreasing the oxidative damage of CsA in the nephrotoxicity caused by CsA.

**MATERIALS AND METHODS**

Twenty-five Sprague Dawley male rats, two and half months old, weighing 250-300 g were used in this study. They were fed daily tap water and pellet foods including 21% pure protein under optimum laboratory conditions (temperature: 21°C, humidity: 40-60%, light/dark period: 12 h/12 h, optimum air condition system). The rats were divided into five groups, each consisting of five rats: The Group I was the control group; Group II which was administered CsA (5 mg kg\(^{-1}\) day\(^{-1}\)) for eight weeks; the Group III which was kept for reversibility for 6 weeks after it had been administered CsA (5 mg kg\(^{-1}\) day\(^{-1}\)) for 8 weeks; the Group IV which was administered CsA plus vitamin E (5 mg kg\(^{-1}\) day\(^{-1}\)) for 8 weeks; the Group V which was administered vitamin E-alone (5 mg kg\(^{-1}\) day\(^{-1}\)) for 6 weeks after it had been administered CsA (5 mg kg\(^{-1}\) day\(^{-1}\)) for 8 weeks.

CsA (Sandimmun, Novartis) as concentration consisting of CsA (50 mg)+ cremophor-EL (650 mg) + ethanol (278 mg) mL\(^{-1}\) was administered intraperitoneally (i.p.) 2 mL kg\(^{-1}\) by being diluted at the ratio of 1/20 with 0.9% NaCl. As for vitamin E (Evigen, Aksan), it was administered i.p. 1 mL kg\(^{-1}\) by being diluted in corn oil.

The kidney specimens were obtained from the same areas of the animal kidney tissues in the Group I, II and IV at the end of the 8th week and in the Group III and V at the end of the 14th week.

For light microscopical observation, kidney specimens were embedded in the paraffin blocks after they had been fixed in Bouin's solution. In order to show the histologic structure properties of the specimens, 5 μm of sections were obtained and stained with Hematoxylin + eosin (H+E), periodic acid-Schiff-hemalum (PAS+HIL) and Masson's trichrome.

For electron microscopical observation, kidney specimens were embedded in the araldite blocks after they had undergone the prefixation with 3% gluteraldehyde (dilute in phosphate buffer, pH 7.3) and postfixation with 1% osmium tetroxide (dilute in phosphate buffer, pH 7.3). Ultrathin sections were obtained and stained with uranyl acetate and Reynolds's lead citrate.

**RESULTS**

Results showed that when the kidney specimens obtained from the control group (Group I) were examined as light and electron microscopic, all the histological components were found normal.

When the kidney specimens obtained from the Group II were examined as light microscopic, infiltration cells and foci were encountered around the vessels, especially in the corticomedullary region more than in the cortex region (Fig. 1). Most of the infiltration cells consisted of mononuclear phagocyter system elements. Besides these cells, some epithelial cells left from scattered tubules, connective tissue cells and active

Fig. 1: Tubular Degeneration (TD) and Tubular Regeneration (TR) areas and Infiltration Focus (IF) around the Vessel (Ve) in the corticomedullary region are seen. H+E, scale bar = 40 μm
epithelial cell masses which formed the new tubules having thick basement membrane were encountered in the infiltration foci. As for in the surrounding of the infiltration foci, proximal tubules in degeneration that have a thick basement membrane and loss of brush border were found (Fig. 1 and 2).

When examined as electron microscopic, extensive vacuolization and an increase in lysosomes number and size were observed in the cytoplasm of the proximal tubular cells around the infiltration area. Also mitochondria of these cells were swollen and their cristae were indistinct. Lateral junctional complexes of these cells got loosened and cytoplasmic sections placing on the basement membrane became rather thinner. In addition, apical microvilli of them were rare and their length got shortened (Fig. 3). It was observed that the basement membrane of proximal tubular cells occasionally got thickened (Fig. 4).

Distal tubular cells placed on a normal basement membrane and thinness in their basement folds were observed. The lateral junctional complexes of these cells rarely got loosened. An increase in collagen fibres and vessels with narrowed lumens were seen in interstitial connective tissue.

When the kidney specimens obtained from the Group III were examined as light and electron microscopic, it was determined that the findings were similar to the ones in the Group II (Fig. 5).

When the kidney specimens obtained from the Group IV administered CsA plus vitamin E were examined
Lateral junctional complexes of these cells were intact. Moreover, no increase in lysosomes was seen in Group IV (Fig. 6).

When the kidney specimens obtained from the Group V administered vitamin E after receiving CsA were examined as light microscopic, infiltration foci were similar to the cyclosporin groups, but compared to them, the infiltration foci, less in number and diameter, usually found around the vessels were encountered.

These infiltration areas had dense PAS positive regions depending on the increase of mucopolysaccharide. In this areas, thickened basement membrane and loss of brush border in the proximal tubules were seen.

When the kidney specimens of Group V were examined as electron microscopic, cytoplasm of some proximal tubular cells which was in the infiltration area had several slightly large vacuoles and increased lysosomes (Fig. 7).

Proximal and distal tubules which were found outside the infiltration areas had a normal appearance and a basement membrane.

**DISCUSSION**

CsA has a limited use area because of its nephrotoxic effect\(^\text{30}\), this effect was shown on people in 1978 by Calne for the first time\(^\text{30}\).

CsA nephrotoxicity could be divided into two groups\(^\text{30}\):
- Functional toxicity without significant morphologic lesions
- Morphological forms of toxicity with tubular and/or vascular-interstitial lesions.

These changes appear after CsA has reached a certain level (200-500 ng mL\(^{-1}\)) in blood\(^\text{30}\).

It has been shown in cell culture studies that CsA has a direct toxic effect on proximal tubular cells by Malhotra and Weenk\(^\text{30}\).

Whiting et al.\(^\text{14}\) suggested that CsA had a primary effect on the straight portion of the proximal tubule. However, Verani\(^\text{15}\) pointed out that CsA had an effect on all segments of proximal tubule. Jackson et al.\(^\text{31}\) reported that CsA had no direct toxic effect on the proximal tubule, but CsA caused minor changes like focal atrophic tubules and did not induce any damage in glomerular or vascular structure.

Tubular lesions are dose dependent; if the dose increases, tubular lesions increase too. After CsA withdrawal, they are reversible\(^\text{30}\). Morphologic lesions encountered in tubular toxicity are found more in corticomedullary region\(^\text{30}\). These lesions are such as:
tubular inclusion bodies, regeneration foci, tubular vacuolization and tubular microcalculifications\(^{[28]}\). These findings are not specific for CsA, but highly characteristic\(^{[23]}\).

The proximal tubules were the structures where most changes were seen in the kidney tissue. These changes were both degeneration and regeneration in tubules in the Group II and III. Both of these tubule types were encountered in infiltration areas or nearby in the corticomedullary junction\(^{[1,34]}\). It has been shown that these infiltration area cells consist of mononuclear cells\(^{[12,36,37]}\).

Weinberg\(^{[38]}\) proved that the last part of proximal tubule (S segment) was rich in mixed function oxidases which have a duty in CsA metabolism. As for the S, segments of the proximal tubules, they are found in the cortex near the medulla\(^{[39]}\). Therefore, the degeneration in proximal tubules will start in this area.

Since CsA reaches kidney through blood, it shows its effect mainly on the proximal tubules close to vessels. CsA enters the kidney by means of renal artery and passes into glomerular capillaries with afferent arteriole, then into Bowman’s space and the first part of proximal tubule, respectively. As the concentration of CsA increases through reabsorption, it causes a toxic effect and as a result, this effect induces loss of brush border in tubules and vacuolization in cytoplasm. Mehring \textit{et al}.,\(^{[40]}\) and Zoja \textit{et al}.,\(^{[41]}\) suggested that narrowing and endothelial damage occurred in peritubular capillaries and arterioles found in interstitial connective tissue with CsA effect. Therefore, insufficient supply and damage may have occurred in tubule cells.

Dieperink \textit{et al}.,\(^{[11,14]}\) suggested that the damage in proximal tubules caused the direct vasoconstrictory effect of CsA on the afferent arteriole; it also led to decrease in ultrafiltrate pressure. Consequently, proximal fractional reabsorption increases and tubular flow rates and end proximal delivery decreases. Due to nephron heterogeneity, varying tubular hypoperfusion and focal tubular collapse occur. These collapsed tubules degenerate and so does the tubular basement membrane, while peritubular interstitial fibrosis develops.

Peritubular interstitial fibrosis was also reported by other investigator\(^{[13,35,43]}\).

It has been demonstrated that CsA through toxic effect on renal mitochondria reduced oxidative phosphorylation capacity\(^{[72]}\); thus, adequate energy could not be produced in the mitochondria. This event could explain the reason why mitochondrial disorders occurred in proximal tubular cells.

In the electron microscopic examination of effected proximal tubular cells, a great number of vacuolizations and dilated smooth endoplasmic reticulum were seen. We thought that because of CsA’s entering into cytoplasm, the cell developed smooth endoplasmic reticulum, with the aim of detoxification, owing to CsA’s continually entering into the cell, disorder appeared in the organel and, as a result, vacuolizations occurred in cytoplasm.

Proximal tubular vacuolization is encountered almost in all studies and the authors suggest that this is due to dilation of smooth endoplasmic reticulum\(^{[10,12,13,34,35,36,41]}\).

Smooth endoplasmic reticulum gradually undergoes loss and mitochondrion does not produce adequate energy in this cell. We think that because necessary functions are not carried out, the cell will degenerate. In the meantime, in order to destroy the various waste, an increase in lysosomes will occur. Lysosomes whose size and number increased have been called tubular inclusion bodies\(^{[34,44]}\) and rarely giant mitochondria have been commented as tubular inclusion bodies by some authors\(^{[29]}\).

Olbricht \textit{et al}.,\(^{[13]}\) suggested that cathepsin B and L, lysosomal proteolytic enzymes, increased after administration of CsA and the proteins occurring due to the cell organel damage were disposed in this way.

A few scattered proximal tubular cells seen among the degenerated and scattered proximal tubules will newly form cell groups of tubules. In order to protect themselves, a thick basement membrane separates newly formed tubule cell groups from interstitial connective tissue.

Renal proximal tubular cells which belong to stable cells group have an ability to reconstitute the tissue they originate by the effect of appropriate stimulants. They are normally considered to be in G\(_0\) phase of growing cycle, but in case of extreme damage in tissues such factors as epithelial growth factor emerging from scattered tubular cells are stimulated by binding to the surface receptors of other undamaged cells and then they can drive into G\(_1\) phase. As a result, the chain of events leading the cell into growing cycle are activated\(^{[49]}\). We think that as a consequence of this mechanism epithelial cells which belong to proximal tubule are divided and proliferated. This process causes new tubule forms.

Al-Qattan \textit{et al}.,\(^{[49]}\) explained that loosening lateral junctional complexes of damaged proximal tubular cells was a kind of paracellular shunt, enabling the fluid transport from the interstitial area to the lumen in case of high blood pressure. We thought that CsA that has an hypertensive effect\(^{[13,18]}\) could cause intercellular enlarging through this mechanism.

In our study no alteration was observed in distal tubule and this finding showed a parallelism to the other studies\(^{[13,14,49]}\).
Interstitial connective tissue changes in vessel surroundings found in corticomedullary region were seen more in the Group II, III. These changes were common infiltration cell foci and narrowed vessels. The increase in capillary wall thickness has been demonstrated by Sund et al. That's why, the tissue may undernourish and renal parenchymal space will occur. As a result of the spaces being filled by connective tissue, an increase in interstitial connective tissue appeared.

Gillham et al. reported that some factors such as IL-1 released from macrophages and T cell forming infiltration cells caused fibroblast proliferation enabling connective tissue to increase. These fibroblast will be responsible for the increased extracellular matrix and collagen fiber synthesis. Also an increase in collagen synthesis was shown by measuring hydroxyproline level in the kidney.

In the investigations that were done for the reversibility by CsA withdrawal, Bertani et al. suggested that almost all of proximal tubular lesions became normal in their study and the drug was reversible. Mihatsch et al. pointed out that tubular vacuolization disappeared completely and tubular inclusion bodies and tubular regeneration persisted slightly, but could not provide a complete reversibility. On the other hand, Dieperink et al. reported that CsA nephrotoxicity still persisted for nine months after drug withdrawal and it could not show a complete reversibility in their study carried out with functional parameters.

In our study, no regression was observed in proximal tubules when the reversibility group was examined. One of these reasons may be because of the fact that CsA accumulated in the tissues and the drug was given steadily in spite of the drug withdrawal or another reason may be because of the fact that adequate time for reversibility was not allocated.

CsA through free oxygen radicals oxidizing phospholipids in cell membranes, convert them into peroxide derivative and cause lipid peroxidation. They have been demonstrated as in vivo and in vitro in many studies. Lipid peroxidation is accepted as a marker of damage free oxygen radicals cause. The deficiency of antioxidant in the diet enhances lipid peroxidation, whereas the antioxidant plus the diet inhibits lipid peroxidation. Vitamin E appears to be the first line of defense against peroxidation of polyunsaturated fatty acids contained in biological membrane phospholipids. Vitamin E, principally in the form of α-tocopherol acts as antioxidant, breaking free radical chain reactions as a result of their ability to transfer a phenolic hydrogen to a peroxyl free radical of a peroxidized polyunsaturated fatty acids. The protective effects of vitamin E have been previously reported for CsA induced nephrotoxicity.

Hence, the disorders seen in the Group II, III may not appear in the Group IV.

Proximal tubules in the Group IV administered vitamin E and CsA simultaneously had almost a normal appearance. Proximal tubular cell groups with thick basement membrane were encountered in the areas where a few infiltration cells situated rarely in vessel surroundings. We thought that the reason why the disorders were less in this group was that the damage CsA caused was inhibited by vitamin E.

In our observations of the Group V administered vitamin E-alone after CsA withdrawal, proximal tubular damage was less than in the groups administered CsA-alone, but it was more than in the Group IV administered CsA plus vitamin E. We thought that the reason might be owing to the drug accumulation in the tissue like in the Group III, the animals' being exposed to CsA for a long time and the damage emerged not being prevented on time as vitamin E could not be used with CsA.

As a result, we think that when CsA needs to be used for a long time and by adding vitamin E to the treatment simultaneously, the nephrotoxicity caused by CsA may be kept at a minimal level.

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REFERENCES


