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THE EFFECT OF CYCLOSPORIN A (CsA) ON ANTIOXIDANT STATUS IN THE MALE WISTAR RAT

by

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A thesis submitted to the Faculty of Graduate and Postdoctoral Studies of the University of Ottawa in partial fulfillment of the requirements for the degree of Master of Science

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ABSTRACT

Cyclosporin A (CsA) is the most commonly used and successful immunosuppressant drug available for the treatment of autoimmune diseases and patients undergoing transplantation. However, cytotoxic effects have been found in several organs, particularily the testis and kidney of CsA treated patients. Toxic effects include mildly to severely reduced organ function as well as permanent structural damage.

Studies suggest that one aspect in the mechanism of CsA toxicity may be lipid peroxidation, an oxidative process initiated by oxyradicals, which can lead to structural and functional disruptions. Cellular defense strategies against oxidative stress include the presence of antioxidants to neutralize generated oxyradicals. These antioxidants can exist in the aqueous environment of the cell, in the case of glutathione (GSH) or in the lipid phase, as with vitamin E. Vitamin E (of which the most bioactive form is α-tocopherol) is the major if not the only lipid soluble antioxidant in biological systems. Thus antioxidant utilization potentially is an excellent index of oxidative stress.

These studies assessed the effects of two doses of CsA (10 mg/kg/day and 20 mg/kg/day for 14 days) on antioxidant status in numerous tissues, and on the function and structure of testis and kidney in male Wistar rats. Animals were placed on either vitamin E sufficient or deficient diets and injected subcutaneously with either CsA or vehicle. Vitamin E levels in various tissues were measured using the very sensitive technique of gas chomatography and mass spectrometry (GC-MS) to determine the effect of CsA on vitamin E status and the protective extent of vitamin E on CsA toxicity. Oxidative damage was also assessed by measuring levels of GSH and protein sulfhydryl (PSH) content using colorimetric methods. In addition, frozen sections of kidney and testes were subjected to standard Hematoxylin and Eosin staining to examine these tissues for structural alterations following CsA treatment.

Results show that on the low dose of CsA (10 mg/kg/day) vitamin E levels were reduced in the kidney and the testis in both the vitamin E sufficient and deficient

treatment groups. No other tissues examined displayed a tocopherol-lowering effect by CsA. All tissues from animals placed on a vitamin E deficient diet displayed reduced α-tocopherol concentrations. Morphological examination of the kidney showed no evident structural alterations at the light microscopic level with the low dose of CsA. Vitamin E deficiency alone had no effect on renal morphology. The testis however showed degeneration of the seminiferous tubules (ST) after exposure to either CsA treatment or vitamin E deficiency. Leydig cells were damaged by CsA treatment while vitamin E defiency had no apparent effect. A combination of CsA and a vitamin E deficient diet exacerbated the degeneration of the ST. While not statistically significant, the plasma testosterone levels tended to be reduced in CsA treated animals and in vitamin E deficiency, in agreement with the morphological changes that were seen.

The antioxidant profile of tissues treated with a high dose of CsA (20 mg/kg/day) differed from the profile obtained on the low dose. A tocopherol lowering effect of CsA was apparent in the kidney and testis of only vitamin E sufficient animals. Vitamin E deficient animals were resistant to decreases in α-tocopherol by CsA. Vitamin E deficient animals treated with CsA showed increases of GSH in kidney, liver and brain and reductions of GSH in testis and muscle. With the exception of a reduction in GSH in muscle, vitamin E sufficient animals showed no changes in GSH with CsA treatment. Vitamin E deficiency alone had no effect on GSH concentrations. Vitamin E deficient animals treated with CsA showed increases of PSH in the brain and the liver, no change in the muscle and decreased levels in the kidney and testis. Vitamin E sufficient animals treated with CsA showed increases in PSH for the liver only. Degeneration of ST was similar in pattern to that obtained with the low dose of CsA. However, the extent of damage to ST did not seem to be as extreme as in the low dose study. Plasma testosterone levels were reduced in CsA treated and vitamin E deficient animals, however these results were not statistically significant. Plasma luteinizing hormone (LH) and follicle stimulating hormone (FSH) both increased with CsA treatment, but only FSH increases for the vitamin E sufficient animals

were significant. Finally, vitamin E concentrations in animals recovering from the higher dose of CsA rebounded to control levels after 14 days.

In conclusion, this is the first *in vivo* study to examine the effect of CsA on antioxidant status in any animal model. The ability of CsA to reduce vitamin E, GSH or PSH concentrations in some tissues indicates that CsA has a free radical component. The effect is most likely linked to the metabolism of CsA by cytochrome P-450 enzymes. CsA exerts its strongest tocopherol-lowering effect in the testis and the kidney, the two tissues noted for exhibiting CsA toxicity. This study also suggests that vitamin E's major role in the testis is to quench free radicals generated by steroidigenic P-450 enzymes.

Acknowledgements

This thesis would never have been written without the love, support and advice from my best friend, my wife Kim. I thank my son Liam for the extra motivation and joy that he has brought to my life. Thank you both for believing in me. I will love you always.

I wish to thank Dr. Graham Burton for giving me the chance to pursue this degree. Without your generosity none of this would have been possible. You provided me with a wonderful learning experience that will benefit me always. I want to thank everyone that was a part of that great lab, especially Dr. Phil Dutton, Dave Lindsay, Dr. Prabhat Arya, Dr. Brady Clarke, Dr. Janusz and Malgosia Daroszewski, Ewa Pietrzak, Ula Wronska, Dr. Ewa Lustyk, Tracey Neville, Dr. Andrew Moore, and Guy Boisvert. Thank you for all your help and your friendship.

To my mom and dad, Sandra and Richard Hodgkinson, and my brother Bradley, thank you for always believing that I could do it and for always being there when I needed you. I cannot understate the contribution my 'other' family has made to this work, you made me welcome and never judged me, always giving me encouragement. So to mom, dad and Brad; to mom Nichols, Kevin, Ginnette, Michel, Francois and Joseph; Ann, Keith, Breanna, Roberta and Jon... I love you all.

To Dr. Gordon Kinson, thank you for opening some doors, this study started with you, and you were right !

To Dr. Anthony Krantis, thank you for standing by me when things looked bleak, I will be eternally grateful. You are like a father, never giving up on 'your' students.

Warmest thanks to Donna Mulder, without your special help with this manuscript all would have been lost.

And a very special thank you to Dr. Kiriti Sarkar. You were my friend, I shall miss you.

Brent

Dedication

To my loving wife Kim and my son Liam

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CHAPTER 1

1.1 General Overview

Cyclosporin A (CsA) was discovered in an extract from a Norwegian fungus in 1976 (J. F. Borel, et al., 1976). Animal studies showed CsA to be a powerful immunosuppressant drug that exhibited a low level of myelotoxicity and with no other observable side effects (J. F. Borel, 1976, Borel, et al., 1976, J. F. Borel, et al., 1977) . Subsequently, CsA was found to be as effective an immunosuppressant in humans as in animal models (R. Y. Calne, et al., 1978, R. Y. Calne, et al., 1979). The discovery of this property of CsA provided the impetus for its use in transplant patients, enabling the earliest heart-lung transplant patients to survive for years rather than days as compared with traditional therapies (T. Beveridge, 1992). In two-year followup studies of transplant patients receiving CsA, graft and patient survival exceeded 80% as compared to 52% on the traditional regimen of azathioprine and prednisone. Although many analogues and potential substitutes have been prepared or discovered since (e.g. FK506 and cyclosporin G) (J. Mason, 1992, P. Hytiroglou, et al., 1993, A. J. Krentz, et al., 1993), CsA is the best studied and is still the most effective immunosuppressant drug (B. D. Kahan, 1993).

An attractive feature of CsA is its ability to selectively target T lymphocytes while leaving the rest of the immune system functionally intact. CsA blocks the transmission of a cell signal to the T-cell nucleus, initiated at an antigen receptor on the cell surface, thus preventing nuclear transcription of important lymphokines that initiate a full-blown humoral response (S. L. Schreiber and G. R. Crabtree, 1992).

In contrast to early animal studies, CsA has been found to produce adverse reactions in human subjects (R.Y. Calne, et al., 1978, R.Y. Calne, et al., 1979, C. Stiller, 1983). The major side effect of CsA treatment is the problem of nephrotoxicity, which depresses renal function in nearly all treated patients (S. Thiru, 1989). Acute toxicity produces reversible renal impairment with normal function returning as quickly as 24 hours after withdrawal of CsA. Chronic toxicity, however, can lead to irreversible renal failure, the

onset of which is unpredictable. More recently, the liver, the major site of CsA metabolism (J. C. Kolars, et al., 1992), the adrenal gland (Z. Jie and Z. Bing-Yan, 1994), the central nervous system (CNS) (M. R. Cilio, et al., 1993) and the testis (L. Seethalakshmi, et al., 1987) have also been found to display CsA toxicity. In the liver, adrenal gland, and CNS of animal models, concentrations of CsA usually exceed therapeutic levels before toxicity occurs. The testis however, is particularly sensitive to CsA administration at or below therapeutic levels (J. Rajfer, et al., 1987, Seethalakshmi, et al., 1987).

The cytotoxic mechanism(s) of CsA is not well understood and may vary in different tissues. A compelling theory involves the production of superoxide ($\bullet O_2^-$) during CsA metabolism by cytochrome P-450 enzymes (F. Serino, et al., 1991, F. Serino, et al., 1993) or by CsA inhibition of these enzymes (L. Seethalakshmi, et al., 1992). While not a strong oxidizing agent itself, $\bullet O_2^-$ can be a precursor to highly reactive oxygen-derived radicals (oxy-radicals) such as the hydroxyl radical ($\bullet OH$). Reaction of polyunsaturated fatty acids (PUFA) with molecules such as $\bullet OH$ can result in lipid peroxidation (H. W. Gardner, 1989, B. Halliwell and J. M. C. Gutteridge, 1989). Interestingly, the kidney shows evidence of lipid peroxidation concurrent with CsA treatment (R. J. Walker, et al., 1990) and the testis has been found to undergo marked morphological and functional changes with CsA that are dose responsive (L. Cavallini, et al., 1990). In addition to PUFA, thiol groups (SH) of proteins (E. R. Stadtman, 1992) and DNA and RNA nucleotides (Halliwell and Gutteridge, 1989), are susceptible to oxy-radical attack.

Cells have developed a number of antioxidant strategies to combat the deleterious effects of oxidative stress (for a review see: Halliwell and Gutteridge, 1989). The first line of defence is the rapid conversion of superoxide to hydrogen peroxide (H_2O_2) via superoxide dismutase (SOD) and the chelation or removal of transition metals that would react with superoxide and H_2O_2 to form products such as $\bullet OH$. In the event of the formation of hydroperoxides, enzymes such as glutathione peroxidase and catalase can reduce the hydroperoxides to stable end products. Finally, to control the formation and

propagation of oxy-radicals such as •OH, a second form of defence exists. This involves the direct reaction of oxyradicals (eg. resulting from •OH) with chain-breaking antioxidants, which are thereby converted into stable and usually unreactive radicals. These antioxidants can exist in the aqueous environment of the cell in the case of ascorbate (vitamin C) (A. Bendich, et al., 1986) and glutathione (A. Meister and M. E. Anderson, 1983), or in the lipid phase as with vitamin E (of which the most active form is α-tocopherol (G. W. Burton and K. U. Ingold, 1981)).

The possibility exists that under certain pathological conditions, such as the introduction of xenobiotics, oxyradical formation can exceed the capabilities of these cellular defence mechanisms. Excessive generation of oxyradicals or the reduced availability of cellular antioxidants within a cell may result in permanent injury or death of that cell.

1.2 Hypothesis :

If metabolism of CsA involves a significant free radical component then we expect antioxidant levels to decline. As CsA is a hydrophobic molecule, there may be a specific effect on vitamin E, the only major lipid soluble antioxidant present in mammalian tissues.

1.3 Cyclosporin A (CsA)

In 1976 an extract of the Norwegian fungus, *Topycladium inflatum*, was shown to depress antibody production in mice (Borel, et al., 1976). Isolation of the active component revealed a hydrophobic cyclic peptide 11 amino acids in length with a molecular weight of 1203 (figure 1.1) (R. M. Wenger, 1989) and was named Cyclosporin A (CsA). Xray crystallography showed that CsA contained the novel amino acid (4R)-4[(E)-2-butenyl]-4N-dimethyl-L-threonine (MeBmt, designated as amino acid R1). It was later established (using deletion, replacement and antibody studies) that this amino acid was essential for the immunosuppressive activity of the compound (Wenger, 1989).

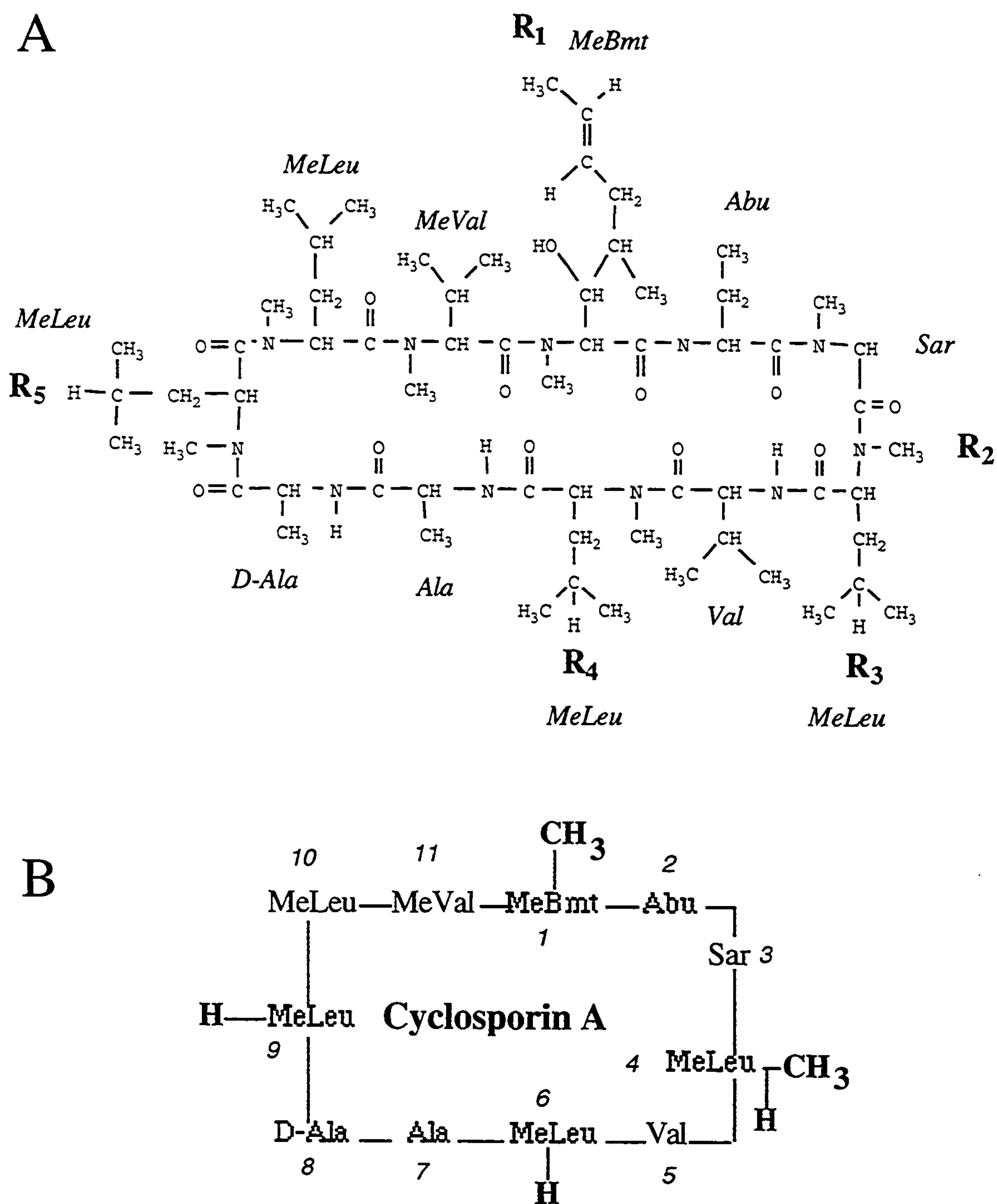


Figure 1.1: **(A)** Structure of cyclosporin A. **(B)** A simplified version to be used hereafter to discuss P-450 metabolism (see section 1.6.3 figure 1.3). R_1, R_4, R_6 and R_9 are sites most commonly altered by P-450 enzyme metabolism (A)

1.4 Efficacy of CsA

The initial studies on the immunosuppressive activity of CsA performed by Borel and co-workers (Borel, 1976, Borel, et al., 1976, Borel, et al., 1977) showed that this compound could effectively depress the level of antibody titre in animals previously sensitized to an antigen, if it was given at the time of, or no later than one day after a second challenge by the same antigen (Borel, et al., 1976). In looking at graft rejection, the study found that dosing with CsA at days 0 to 4 after the grafting procedure significantly prolonged the survival of the graft at doses that were less toxic than those of traditional therapy (eg. azathioprine). Finally, CsA was found to strongly suppress autoimmune conditions such as experimental allergic encephalomyelitis in rats (C. Bolton and M. L. Cuzner, 1980).

The major concern with using other immunosuppressive drugs is their tendency to damage haematopoietic tissues. CsA given in doses causing strong immunosuppression in mice (200-250 mg/kg/day for 4 days) did not lower total leukocyte or red blood cell numbers or cause a reduction in spleen weight. However, lymphocytes were significantly reduced in number at these CsA concentrations. Unlike other immunosuppressive agents, CsA displayed a specificity for inhibiting lymphocyte activity while having a very low cytotoxicity. This specificity was found to be targeted to a subset of lymphocytes, the T cells (Borel, et al., 1977). Moreover, CsA has been shown to inhibit primary and secondary humoral responses in mice after antigenic challenge, a T cell-mediated process. However, CsA was not found to inhibit antibody synthesis to lipopolysaccharide antigen challenge, which is believed to be a predominantly B cell mediated response (ibid).

In addition to these findings, studies including renal allografts in dogs (R. Y. Calne and D. J. G. White, 1977), rhesus monkeys (J. C. C. Borleffs, et al., 1982) and rabbits (C. J. Green and A. C. Allison, 1978) and cardiac transplants in pigs (R. Y. Calne, et al., 1978) and rats (A. J. Kostakis, et al., 1977), also showed CsA to be a powerful

immunosuppressant with no obvious side effects. These observations led to the implementation of a pilot study in humans in 1978 (Calne, et al., 1978). In humans, the therapeutic dose of CsA (initially 25 mg/kg/day for more than 10 days) was found to be much lower than that given in most experiments with rats, but was comparable to the amount found to be effective in the studies with pigs (Calne, et al., 1978), dogs (Calne and White, 1977) and rabbits (Green and Allison, 1978). While effective immunosuppression was generally obtained, complications ranged from mild hirsutism in female participants, and gynecomastia in males, to transient hepatotoxicity with consistent depressed renal function in all patients. These effects were unexpected based on the results of the animal studies.

Further studies in human transplantation patients showed similar findings (Calne, et al., 1979, Stiller, 1983). That is, impressive immunosuppressive effects, but with a disturbing frequency of acute nephrotoxicity and hormonal irregularities. Combination therapy of steroids and CsA, as well as tailoring the level of CsA given to each individual, brought these acute toxic effects under control. It was found that withdrawal of the drug led to improved renal function in most patients within 24 hours. However, withdrawal or insufficient dosage of CsA increased the incidence of rejection episodes to a high level and decreased graft survival rates (C. R. Stiller and G. Opelz, 1991, P. S. Almond, et al., 1992)

An additional problem was the unpredictable appearance of chronic nephrotoxicity, a condition that led to an irreversible decline in kidney function, eventually resulting in total renal failure. With time, most physicians and researchers were able to construct a therapeutic dosage regime that minimized the degree of acute toxicity and the occurence of chronic CsA toxicity. It was established that this treatment had to be specifically tailored for each individual (ie., the therapeutic and toxic levels of the drug appeared to be different for every individual).

Follow-up studies of up to 10 years (Almond, et al., 1992) showed that long term application of CsA and careful monitoring usually limited further functional degeneration to that experienced in the first posttransplant year. Exceptions to this were

patients who had poor pre-transplant renal function. Nonetheless, there was always a small but significant population of CsA recipients that developed end stage renal failure (Thiru, 1989, Almond, et al., 1992, G. Thiel, et al., 1992).

Since these original studies, the numbers and types of transplantation procedures has increased exponentially, with vast improvement in graft survival and quality of life of the patient. Virtually all tissues are now successfully transplanted and maintained with the aid of CsA treatment (A. Haverich, 1992, B. Speck, et al., 1992, Thiel, et al., 1992, S. P. Dunn, et al., 1994) Various autoimmune diseases also respond to CsA treatment (G. Feutren, 1992, M. Lombard, et al., 1993). However, in spite of all the studies performed in the past twenty years very few have been able to shed any light on the important aspect of CsA therapy that may limit its application : the mechanism of CsA toxicity. In fact, until recently even the mechanism by which CsA exerts its immunosuppressive action was not known.

1.5 Mechanism of immunosuppressive action by CsA

The method of immunosuppression by CsA is unique in biological systems because it inhibits T lymphocytes from responding to foreign antigens while having no direct impact on the functioning of other components of the immune system (i.e., cellular immune function). The effect of the drug is to block the intracellular signalling in the T cell at some point in the pathway and prevent the induction of lymphokine genes (most notably interleukin-2 or IL-2) (A. Roa, 1991) by the T helper cell (A. L. DeFranco, 1991).

Studies have shown that the point of action of CsA must be very early in the signalling pathway as little or no suppression was found for stimulated cells that were given CsA more than 3 hours after antigen presentation. CsA did not inhibit the binding of the antigen, did not affect the function of G proteins or inositol triphosphate action and did not affect the entry of Ca^{2+} into the cell. Therefore CsA had to act at a point after the signal had been internalized but before DNA transcription.

One important advance was the discovery of a CsA binding protein called cyclophilin (R. E. Handschumacher, et al., 1984). This protein was discovered to be a cis-trans isomerase, catalyzing the interconversion of the cis and trans isomers of the peptidyl-prolyl bonds in peptide and protein substrates, accelerating their folding into the proper conformation (A. Galat, 1993).

Initially it was believed that the binding of CsA to this protein, inhibiting its function, could explain the reduced response of T cells to a signal, ie. proteins responsible for some portion of DNA transcription may be inactive due to improper folding. However, there were four problems with this theory. Firstly, cyclophilin is a ubiquitous protein (B. Ryffel, et al., 1991), making it difficult to account for the specificity of CsA for inhibiting T cell response. Secondly, FK506, another immunosuppressant drug, has similar selective actions for T cells as does CsA. It also has a ubiquitous binding protein, FK binding protein (FKBP) which is itself a cis-trans isomerase. This posed the question as to whether a cell would require both of these proteins in order to respond normally to antigenic challenge. It was found that only one active cis-trans isomerase was required for normal cell function (DeFranco, 1991). Further, it was discovered that these isomerases only increased the rate of protein folding but were not essential for the adoption of the proper protein structure (D. Ringe, 1991). Therefore the inhibition of the activity of one or both isomerases was of no consequense to the targeted T lymphocyte. Finally, concentrations of CsA required for immunosuppression are far lower than that required to completely saturate binding of cyclophilin ie. functioning cyclophilin is still present in cells exposed to immunosuppressive concentrations of CsA (Schreiber and Crabtree, 1992).

In 1991, two independent laboratories discovered the target of CsA (W. M. Flanagan, et al., 1991, J. Liu, et al., 1991). The CsA-cyclophilin adduct binds to calcineurin, a serine-threonine protein phosphatase, and inactivates it (see figure 1.2). This disruption prevents a series of intracellular events from instructing a B subunit of NF-AT (nuclear factor of activated T cells, a protein specific to activated T cells) to translocate to the cell

nucleus (J. N. Jain, et al., 1993, P. G. Mccaffrey, et al., 1993). This subunit is pre-made and awaits the signal produced by the docking of an antigen to the cell receptor. Under normal conditions the B subunit enters the nucleus and binds to the second component of NF-AT, an A subunit, which is synthesized only after cell stimulation by the same antigenic challenge. The A and B subunits form a complete transcription promoter protein that aids in the transcription of certain genes essential for a maximum humoral response such as the IL-2 gene.

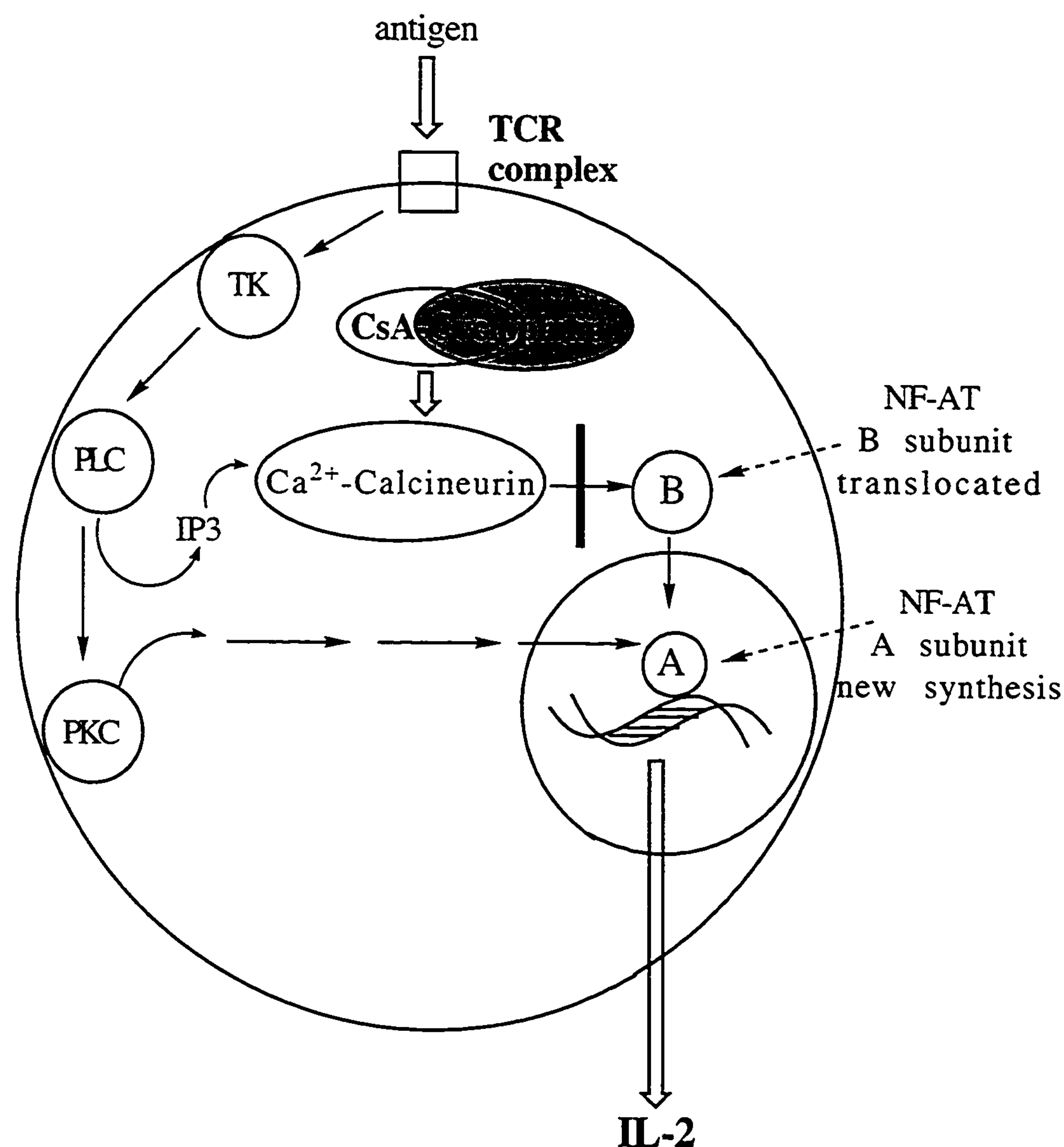


Figure 1.2: Mechanism of CsA immunosuppressive action on activated T lymphocytes. An antigen is presented to the T cell receptor (TCR) which stimulates the activity of tyrosine kinase (TK), which in turn stimulates phospholipase C (PLC) activity. PLC activates the production of inositol triphosphate (IP_3) and stimulates phosphokinase C (PKC). PKC stimulates the synthesis of the A subunit of the nuclear factor of activated T cells (NF-AT). IP_3 causes release of Ca^{2+} from intracellular stores which activates calcineurin activity. Ordinarily calcineurin would dephosphorylate target protein(s) leading to the translocation, to the nucleus, of the preformed B subunit of NF-AT. Together the A and B subunits of NF-AT stimulate the transcription of genes, including the IL-2 gene. The CsA-cyclophilin complex binds with calcineurin inhibiting its phosphatase activity.

1.6 Pharmacokinetics of CsA

The elucidation of the probable mechanism of CsA action did little to aid in the development of a dosing regimen to provide for effective immunosuppression while avoiding toxic or rejection episodes. Studies have revealed that this "therapeutic window" is quite narrow and varies from individual to individual (B. D. Kahan, 1985, J. Grevel and B. D. Kahan, 1989, J. Woo, 1994). The reasons for this variability are so numerous that effective immunosuppressive therapy with minimal side effects can only be accomplished with a tailoring of the therapy to each individual patient. Therefore both the immunosuppressive and the toxic nature of CsA are inherently related to its metabolism and pharmacokinetic profile.

CsA can be administered in various ways. In experimental animals, dosing techniques vary from oral gavage to intraperitoneal, intramuscular and subcutaneous injections. In humans the primary mode of application is oral administration of CsA dissolved in olive oil, usually mixed in orange juice or chocolate milk, or by intravenous injection of CsA dissolved in castor oil.

1.6.1 Absorption of CsA

The fate of CsA administered by oral dosing

In rats, it is estimated that it takes approximately three times more CsA given orally to provide the same plasma concentration as a dose given intravenously (R. Wassef, et al., 1985). The bioavailability of CsA administered orally is, on average, about 30 percent of the administered dose. CsA is absorbed in the upper portion of the small intestine and this absorption is dependent upon various conditions in the gut. Most important is the availability of bile salts to solubilize CsA, a prerequisite for maximum absorption (J. Grevel and B.D.Kahan, 1989). Therefore, any hepatic disorders or obstructions to bile flow can affect CsA absorption. Further, the ingestion of food along with CsA results in increased absorption due to stimulation of bile flow and longer transit time with the presence of a

meal. CsA is then transported into the systemic circulation. Peak plasma levels are usually seen by about 3-4 hours with this dosing technique. It is speculated that the low bioavailabilty of CsA via oral administration is due to the variable absorption of CsA by the intestine (Grevel and Kahan, 1989, V. F. J. Quesniaux, 1989). To this end, new microemulsion formulations to improve CsA bioavailability have been tried with some success (J. M. Kovarik, et al., 1994, E. A. Mueller, et al., 1994).

The efficacy of subcutaneous injections of CsA

In laboratory rats, 43-77% of CsA administered by subcutaneous injection (s.c.) is bioavailable and provides the most stable release of CsA into the circulation (Wassef, et al., 1985). Once entered into the circulation, CsA is incorporated into red blood cells and plasma lipoproteins and distributed to the various tissues. This method of dosing also provides for higher plasma and tissue levels than those obtained by oral dosing.

1.6.2 Distribution of CsA

CsA distribution in whole blood is contained mainly in the erythrocytes (50-70%) (W. Niederberger, et al., 1983, Kahan, 1985, Grevel and Kahan, 1989, Quesniaux, 1989). This strong affinity of red blood cells for CsA is believed to be due to the presence of a binding protein similar to cyclophilin. Of the CsA in the plasma, 80% is incorporated into lipoproteins. Of this, approximately 50-60% of CsA is found associated with high density lipoprotein (HDL). A further 25% is bound to low density lipoprotein (LDL) and approximately 2% to very low density lipoproteins (VLDL). The remainder of the plasma CsA is unbound (Niederberger, et al., 1983, Kahan, 1985, Grevel and Kahan, 1989, Quesniaux, 1989). There is no correlation between CsA immunosuppressive or toxic action and free CsA concentrations. Unlike many drugs, the activity of CsA appears to be associated entirely with the bound CsA fraction in blood and tissue.

Examination of the tissue distribution of CsA reveals that the major sites of deposition are the liver, fat, kidneys, adrenals and the testes (Niederberger, et al., 1983, Kahan, 1985, Grevel and Kahan, 1989, Quesniaux, 1989). Evidence for the deposition of CsA in neural tissue, especially the brain is under debate, with some studies indicating that CsA can cross the blood-brain barrier (B. D. Kahan, et al., 1983, K. Nooter, et al., 1984) while other studies show no evidence for this (K. Atkinson, et al., 1983, Niederberger, et al., 1983, M. Ried, et al., 1983, G. L. Lensmeyer, et al., 1988), despite the ubiquitous presence of cyclophilin in brain and neural tissue (Ryffel, et al., 1991).

1.6.3 Metabolism of CsA

The liver is the major site of CsA metabolism and eventually over 90 % of the CsA reaches this organ to be converted into more soluble forms for elimination in the urine or bile. The half-life of CsA is between 6-9 hours in human subjects. Less than 2% of the parent molecule is found in excreted bile or urine (Grevel and Kahan, 1989, Quesniaux, 1989). There is evidence, however, that CsA metabolism also occurs in the kidney (T. G. Rosano, et al., 1986, Lensmeyer, et al., 1988). In both the liver and the kidney, CsA conversion occurs via the P-450 3A enzyme family of mixed function oxidases (MFO) (Grevel and Kahan, 1989, Quesniaux, 1989). The first step is usually the hydroxylation of amino acid 1 (MeBmt) at one of two positions or the formation of a tetrahydrofuran group at this position. Another option is the demethylation of amino acid 4 (MeLeu). Finally, the primary metabolites may undergo a second step in which further demethylations and/or hydroxylations occur (see figure 1.3). The cyclic structure of the peptide is retained throughout these manipulations.

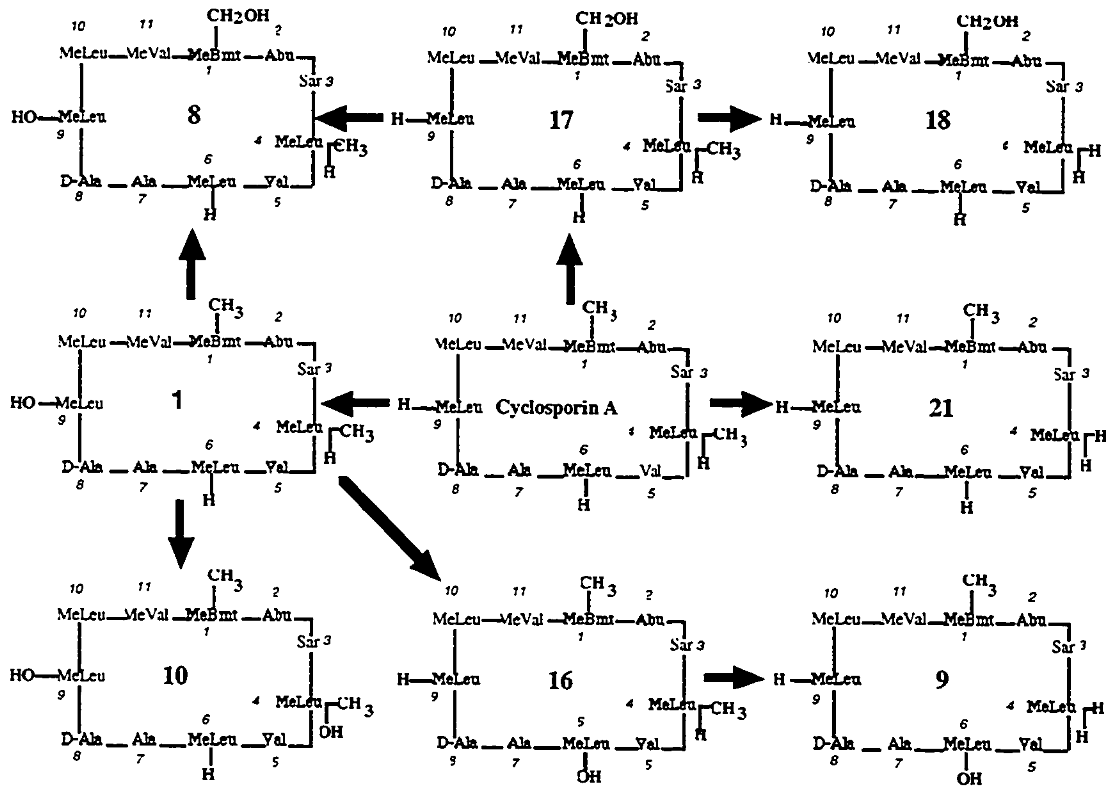


Figure 1.3: **Metabolism of CsA by cytochrome P-450.**

Primary metabolism of CsA by cytochrome P-450 enzymes is either by hydroxylation of amino acid 1 (metabolite 17) or amino acid 9 (metabolite 1), or by demethylation of amino acid 4 (metabolite 21). Secondary metabolism occurs by further demethylation (metabolite 18) or hydroxylation (metabolites 8, 10 and 16) of these primary metabolites. Metabolite 9 is generated by the further demethylation of a secondary metabolite (16).

All of the steps in the absorption, transport and metabolism of CsA have been shown to affect its bioavailability and hence its therapeutic value. This leads to a wide variability among patients in the position of this "therapeutic window" between adequate immunosuppression and potential toxic side reactions. Due to ease of sampling, plasma or serum CsA levels are closely monitored in human patients to attempt to establish an effective dosing regimen. This analysis of trough levels of CsA (ie. the lowest plasma concentration of CsA just prior to the next dose) have been somewhat successful in maintaining an immunosuppressive effect (K. T. Kivisto, 1992). However, tissue concentrations of CsA can exceed levels found in the plasma (Niederberger, et al., 1983, Quesniaux, 1989) and these levels vary dramatically from tissue to tissue. Therefore the predictability of the effects of CsA on each individual tissue is low and dangerous toxic reactions can and do occur frequently and unexpectedly.

1.7 Cytotoxic effects of cyclosporin A

The problem of CsA toxicity has restricted the use of this otherwise potent and effective immunosuppressive drug. The most prevalent side effect of CsA treatment is nephrotoxicity. Nearly all patients receiving CsA experience some degree of decreased renal function (Thiru, 1989). Other toxic reactions have been noted in the liver (S. M. Moochhala, et al., 1991), testis (Seethalakshmi, et al., 1987), adrenal gland (K. Oka, et al., 1993, Jie and Bing-Yan, 1994), and the central nervous system (Cilio, et al., 1993). Examining these lesions may provide some information as to the mechanism of CsA toxicity. To date the toxic effects of CsA have been most widely studied in the kidney through numerous animal studies (Borleffs, et al., 1982, K. Jung, et al., 1987, S. Teraoka, et al., 1989, L. Denicola, et al., 1993). However, the CsA dosages required to produce characteristic toxicity in rat tissues are above the therapeutic range in humans (B. Ryffel, et al., 1986). The rat testis, however, displays a particular sensitivity to CsA, exhibiting functional abnormalities at CsA concentrations deemed therapeutic in humans (Seethalakshmi, et al., 1987).

1.7.1 Nephrotoxicity

Clinical use of CsA has shown that the nephrotoxic action of the drug can manifest itself as not only an acute dose-dependent reversible toxicity but also as chronic toxicity that is irreversible and non-dosage dependent. In kidney transplant recipients the onset of CsA nephrotoxicity is difficult to measure due to the potential overlap of rejection episodes. However, kidney function is altered in all patients receiving CsA (P. H. Whiting, 1990), even those with previously healthy native kidneys (Thiru, 1989, A. Andres, et al., 1992). In animal models, simple administration of CsA to otherwise healthy animals can lead to decreased renal function, although higher concentrations of CsA are usually required than in human subjects (R. Petric, et al., 1990, S. Rosen, et al., 1990).

Acute stage renal toxicity exhibits initially as a decrease in glomerular filtration rate (GFR), a drop in plasma sodium and a rise in plasma creatinine. Removal of the drug or lowering the dose always improves kidney function. In renal transplant recipients however, function seldom returns to the normal range. In these patients, the condition of the kidney, degree of antigenic matching between donor and recipient, and the success of the transplantation procedure can have a marked effect on the vulnerability of the kidney to acute CsA toxicity (M. J. Penny, et al., 1994).

The onset of chronic CsA toxicity cannot be predicted by the previous number of rejection and acute toxic episodes endured by the kidney. Even kidneys exhibiting no previous episodes of rejection and/or toxicity can spontaneously lapse into a progressive form of permanent renal damage that is unresponsive to CsA reduction or other treatments. The general outcome is complete renal failure (Thiru, 1989). The possibility exists that kidneys exposed to CsA all undergo some form of toxic damage that builds irreversibly, becoming evident if the functional residual capacity of the kidney is exceeded (ie. when over 75% of the nephrons are destroyed).

Two general hypotheses, the *vasoconstrictive* and the *tubular toxicity* theories, have been forwarded to explain CsA nephrotoxicity (S. Thiru, 1989).

The *vasoconstriction theory* suggests that CsA has a direct tonic constrictive effect upon the afferent arteriole, reducing GFR, renal blood flow and thus oxygen supply to the vascular tree of the kidney. Countercurrent exchange and high rate of metabolism as a result of active reabsorption of sodium chloride makes the thick ascending limb of the renal medulla very sensitive to changes in local oxygen tension (M. Brezis, et al., 1984). Prolonged periods of ischemia have been shown to produce focal lesions in this area along with the proximal tubule that are coincident with acute renal failure. These areas exhibit mitochondrial swelling and vacuolization of the tubular epithelial cells, resulting in impaired solute management (M. J. Mihatsch, 1992).

The case for initial vasoconstriction has been proposed to be a direct effect by CsA on the renin-angiotensin (RA) system or on an imbalance in prostaglandin-thromboxane synthesis brought about by CsA treatment. Effects of CsA on the RA system are contradictory (N. D. C. Sturrock and A. D. Struthers, 1994). Animal studies have shown an increase in renin release with CsA therapy (C. R. Baxter, et al., 1982) producing hypertension (S. Lustig, et al., 1987, S. Lustig, et al., 1989). However, only one human study has shown an increase in renin activity with CsA treatment (S. Schuler, et al., 1987). In fact most human studies show either no change or even a decrease in plasma renin activity (J. P. Bantle, et al., 1985, M. Bellet, et al., 1985, B. Stanek, et al., 1985, B. Stanek, et al., 1987). Equally unresolved is the argument for CsA effects on prostaglandin production. Animal studies (Petric, et al., 1990) have shown that CsA can inhibit the enzymatic production of prostaglandins such as PGE_2 (important in vasodilation) while increasing the production of thromboxane B_2 (a potent vasoconstrictor) (A. Kawaguchi, et al., 1985). Human studies are equally contentious. Weir et al,.(M. R. Weir, et al., 1989, 1990) showed a rise in thromboxane excretion after CsA administration in healthy subjects and others have found decreased PGE_2 in renal transplant patients receiving CsA (R. A. K. Stahl, et al., 1985). Other studies have failed to show any such changes in groups (J. P. Bantle, et al.,

1987, D. Jorasky, et al., 1987). Moreover, one human study showed an increase in plasma PGE_2 in patients treated with CsA (D. K. Klassen, et al., 1989).

The *tubular toxicity theory* suggests that CsA causes damage directly to some portion of the renal tubule creating a functional deficiency of solute absorption. The possibility that CsA may bind to the tubule brush border membrane and generate toxic metabolites through cytochrome P-450 action has been suggested (see B. D. Kahan, 1989).

The evidence for direct proximal tubular toxicity by CsA is also varied. Numerous human and animal studies have been performed to elucidate its mechanism. Interestingly, CsA concentrations found to be therapeutic with toxic side effects in humans ($\leq$ 10 mg/kg/day), have shown little or no acute or chronic toxic effects in most animal models. Only one animal model, a pathogen-free rabbit, was found to exhibit these same characteristic features of proximal tubular CsA toxicity while receiving concentrations deemed therapeutic in humans (10 mg/kg/day for six months) (J. A. Thliveris, et al., 1994). However, if animals were given pharmacological doses of CsA (ie. $\geq$ 15 mg/kg/day) indicators of both acute and chronic toxicity appear with predictable regularity. For example, in rats given CsA at $\geq$ 25 mg/kg/day for more than 2 weeks, light microscopy revealed proximal tubular lesions consisting of cytoplasmic vacuolizations and microcalcification, while under electron microscopy there was evidence of giant mitochondria, dilated smooth endoplasmic reticulum and enlarged lysosomes (Ryffel, et al., 1986, P. H. Whiting and A. W. Thompson, 1989).

In humans, therapeutic doses of CsA ($\leq$ 10 mg/kg/day) for periods as short as two weeks and as long as several years have been shown, after examination of biopsies, to cause tubular damage similar to that found in animal models (M. J. Mihatsch, et al., 1983, Thiru, 1989). However, long term studies on patients receiving maintenance concentrations of CsA ($\leq$ 4 mg/kg/day) showed that the incidence of decreased renal function and tubular damage plateaued in most subjects after approximately one year of treatment (D. R.

Salomon, 1991, Almond, et al., 1992, J. W. Slaton, et al., 1994). Unfortunately, 10-20% of patients still develop chronic CsA toxicity.

The exact cause of this tubular disruption is unclear. However, studies suggest that CsA toxicity has an oxidative damage component. *In vitro* studies with isolated renal tubule cells indicated that CsA treatment stimulated the production of lipid oxidation products and inhibited glucose uptake (G. Inselmann, et al., 1988, G. Inselmann, et al., 1990). *In vivo* studies on rats given 15 mg/kg/day of CsA for 21 days also showed higher levels of lipid oxidation products in kidney homogenates than did controls (K. Kumano, et al., 1989). There is strong evidence to suggest that the oxidation process may be promoted by a toxic metabolite produced when proximal tubular mixed function oxidases (a family of P-450 enzymes) interact with CsA (Walker, et al., 1990). The apparent sensitivity of the proximal tubule to CsA is believed to be due to the high concentration of these enzymes in the tubular epithelial cells.

Impairment of the absorptive capacity of the proximal tubule by either mechanism will lead to increased solute delivery to the distal tubule, stimulating further tubulo-glomerular feedback and increasing the secretion of powerful vasoconstrictors, such as angiotensin II, to act on the afferent arteriole. The resultant further reduction in GFR and/or oxygen delivery to specific areas of the kidney already exposed to low oxygen tensions would render them hypoxic or ischaemic (Brezis, et al., 1984, Rosen, et al., 1990). Ischaemic renal tissue has been shown to generate oxyradicals (K. J. Johnson, et al., 1988). It is speculated that continued ischemia could lead to the slow, progressive destruction of renal tubules that is evident in chronic CsA nephrotoxicity (Walker, et al., 1990).

1.7.2 Testicular toxicity

In the first human trials, one of the most prevalent side effects of CsA treatment, apart from nephrotoxicity, was the appearance of abnormalities in sexual characteristics (R. Y. Calne, et al., 1978, 1979, Stiller, 1983). Gynecomastia, the growth of fatty tissue in the breast region, was most notable in males, whereas hirsuitism, or growth of facial hair, was common to varying degrees in women. Both of these abnormalities result from imbalances between estrogen and androgen production, indicating that CsA may somehow affect the hypothalamic-pituitary-gonadal axis. Prior to this, no observable changes in the reproductive function of laboratory animals had been noted.

The discovery of hormonal imbalances in human subjects treated with CsA (D. J. Handelsman, et al., 1984) prompted a study using mature, male Sprague Dawley rats (Seethalakshmi, et al., 1987). In this study, each animal was subcutaneously injected with 10, 20 or 40 mg CsA/kg/day for 14 days. It was found that CsA concentrations equal to or greater than 20 mg/kg/day resulted in significant reductions in body and testis weight. Histological examinations of the testes showed no evidence of any structural disruptions at 10 mg/kg/day dosages. However, at higher concentrations there was obvious damage to the structure of the seminiferous tubules, including the degeneration of the primary spermatocytes, spermatids and Sertoli cells. Vacuoles were seen in the primary spermatocytes, and the association between spermatids and the Sertoli cells was lost. In addition there was evidence of pronounced degeneration of the interstitial cells and there were distinct signs of morphological abnormalities to spermatozoa, including fractured head and missing tail pieces. When these animals were tested for fertility it was found that males given 10 mg/kg/day of CsA were able to impregnate 4 of 6 females. At the higher dosages, no females were fertilized (0 of 6 and 0 of 8 for the 20 and 40 mg/kg/day groups respectively). Examination of serum hormone levels revealed increases in luteinizing hormone (LH) and follicle stimulating hormone (FSH) coincident with significant decreases in testosterone. Since LH normally increases testosterone production in Leydig cells, CsA

was believed to have a lowering effect on testosterone production at the level of the Leydig cell. This would reduce the feedback on the pituitary gland increasing LH in an effort to raise testosterone production.

In a study by Rajfer et al. (1987) adult male Sprague Dawley rats were given oral doses of CsA at 7.5, 15 or 30 mg/kg/day. Plasma as well as testicular testosterone was again found to be decreased with these treatments, but plasma levels of LH were also found to decrease. This study suggested that oral dosing of CsA caused a direct inhibitory effect on LH production and/or release, downregulating testosterone production.

Further, with *in vivo* studies (S. C. Sikka, et al., 1988), oral dosing of male Sprague Dawley rats with 30 mg/kg/day of CsA for 4 weeks caused reductions in the activity of the testicular 17α-hydroxylase and $C_{17,20}$ lyase (see figure 1.4). It was concluded that the decrease in these enzyme activities were in response to a decrease in LH stimulation of the testis.

Close examination of Leydig cells after prolonged daily doses of CsA (20 mg/kg/day for 30 days (Cavallini, et al., 1990), revealed the loss of mitochondria and smooth endoplasmic reticulum, causing cell atrophy. Also, there was a concurrent and notable increase in the size of cytoplasmic lipid droplets, reduction in nuclear volume and the surface area of the rough endoplasmic reticulum. These studies suggested that CsA inhibited the steroidigenic capacity of Leydig cells. Whether the effect of CsA toxicity was at the level of the Leydig cell itself or at some point along the hypothalamic-pituitary-gonadal axis remains to be determined

Seethalakshmi et al., (1990) believed that the primary effect of CsA was directly on the testis. In rats dosed subcutaneously with 1 and 2 mg/kg/day for 45 days the activity of Δ^5-3β-hydroxy steroid dehydrogenase and $\Delta^{5,4}$ isomerase (3β-HSD) was inhibited (figure 1.4). Krueger (B. A. Krueger, et al., 1991) showed that subcutaneous injection of 25-40 mg/kg/day of CsA for 6 days markedly reduced the activity of the steroidogenic enzymes, cholesterol side chain cleavage (CSCC, figure 1.5) and

17α-hydroxylase (figure 1.4). An *in vitro* study wherein cultured interstitial cells were incubated with CsA, (Seethalakshmi, et al., 1992) found uncompetitive inhibition of 17α-hydroxylase and competitive inhibition of 17β-hydroxysteroid dehydrogenase (17β-HSD), but no interference with LH binding or with intracellular signalling measured by the accumulation of the second messenger cAMP. The authors concluded that CsA had a direct inhibitory action on these enzymes which are responsible for the conversion of pregnenolone to testosterone.

It has been proposed that renal failure itself can cause Leydig cell dysfunction and impaired spermatogenesis (D. J. Handelsman, 1985). However, the above studies have shown reductions in rat testis function with low CsA dosages (10 mg/kg/day or less) where the animals plasma creatinine levels are normal. This suggests that alterations in the male reproductive system found during CsA treatment cannot be entirely due to nephrotoxicity.

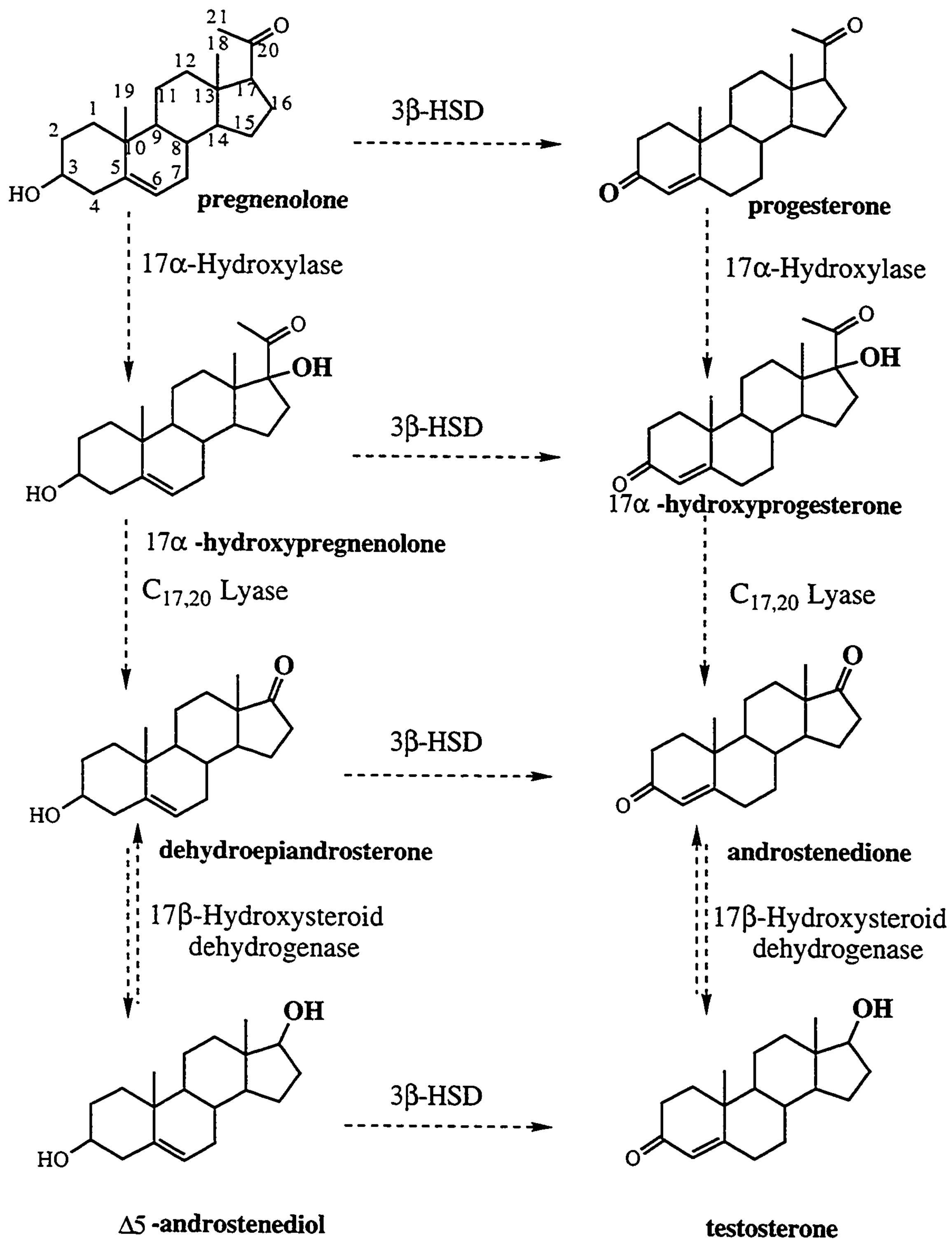


Figure 1.4: Synthesis of androgens from pregnenolone.

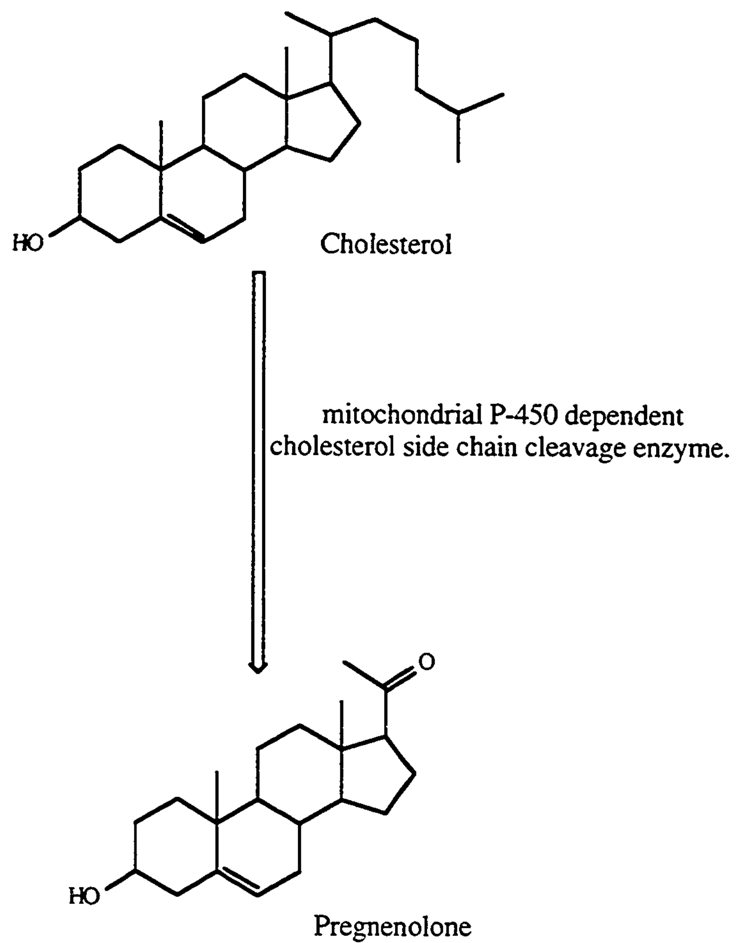


Figure 1.5: Synthesis of pregnenolone from cholesterol.

1.8 Summary

Several theories have been proposed for CsA toxicity, including effects on prostaglandins (see section 1.6.1) and the ability of CsA to inhibit translation of specific mitochondrial P-450 proteins (W. C. Buss, et al., 1988, W. C. Buss, et al., 1989). However, the observation that CsA treatment in rats results in cellular and functional disruption of the testis and deformation of sperm, along with evidence of CsA induced lipid peroxidation in the kidney, suggests a role for free radical oxidation in CsA toxicity.

1.9 Free radical sources

A free radical is defined as *any molecular species that contains one or more unpaired electrons* (B. Halliwell, 1987, Halliwell and Gutteridge, 1989). These radicals can be produced by cells during their normal metabolic activities, such as mitochondrial respiration (G. Loschen, et al., 1974), immune function and prostaglandin and leukotriene synthesis (Halliwell and Gutteridge, 1989), and cytochrome P-450 enzyme activity (T. L. Poulos and R. Raag, 1992).

1.9.1 Normal cellular respiration

Respiring cells require oxygen to act as a terminal electron acceptor in the electron transport chain (ETC). The ETC is a series of multienzyme complexes, located in the inner mitochondrial membrane, that initially accept electrons from a reducing agent such as NADPH and sequentially reduce the next component of the chain, each one lower in energy than the last. This process results in the production of ATP with the eventual formation of water from the four-electron reduction of molecular oxygen by the cytochrome oxidase complex (A. L. Lehninger, 1982). However, some electrons being transported down the ETC may "leak" from enzyme components of the ETC (G. Loschen et al., 1974) and result in the one electron reduction of oxygen, forming the superoxide radical ($\bullet O_2^-$)

(Halliwell and Gutteridge, 1989). It has been shown that superoxide radicals and ultimately hydrogen peroxide (H_2O_2) are routinely produced by mitochondria (Loschen, et al., 1974).

1.9.2 Ischemia and reperfusion

Ischemia followed by reperfusion is a widely studied event (W. J. Mergner, et al., 1977, Brezis, et al., 1984, M. S. Paller, et al., 1984, R. B. Reddy, et al., 1989) in which tissue is denied oxygen (ischemia) in some manner (usually by vessel occlusion) and then replenished with oxygen and nutrients (reperfusion). The lack of oxygen during ischemia prevents oxygen from being the terminal electron acceptor in the ETC. This effectively prevents the further production of ATP. To obtain energy and maintain homeostasis the cell turns to depleting its remaining adenine nucleotide pool (ATP, ADP, AMP and adenosine), generating high concentrations of an end product, hypoxanthine (R. B. Jennings, 1990). As energy sources disappear, calcium begins to flow more freely into the cytosol, activating proteases. Proteolysis converts xanthine dehydrogenase into an uncommon enzyme called xanthine oxidase (J. McCord, 1988), and/or the deactivation of cytochrome oxidase of the ETC (J. T. Flaherty and M. L. Weisfeldt, 1988). Upon reperfusion, damage to the ETC makes it unable to cope with the large influx of oxygen and subsequently electrons normally transferred through the ETC leak onto oxygen to form $\bullet O_2^-$ (Flaherty and Weisfeldt, 1988). Xanthine oxidase utilizes the flow of oxygen to convert hypoxanthine to xanthine generating $\bullet O_2^-$ as a byproduct.

1.9.3 P-450 enzymes

Superoxide is also believed to be formed as a by-product of the controlled oxidation of organic substrates catalyzed by the family of P-450 enzymes (Poulos and Raag, 1992). P-450 enzymes are monoxygenases that are involved in stepwise, one-electron ($1e^-$) reductions of substrates using reducing equivalents from sources such as NADPH (see figure 1.6).

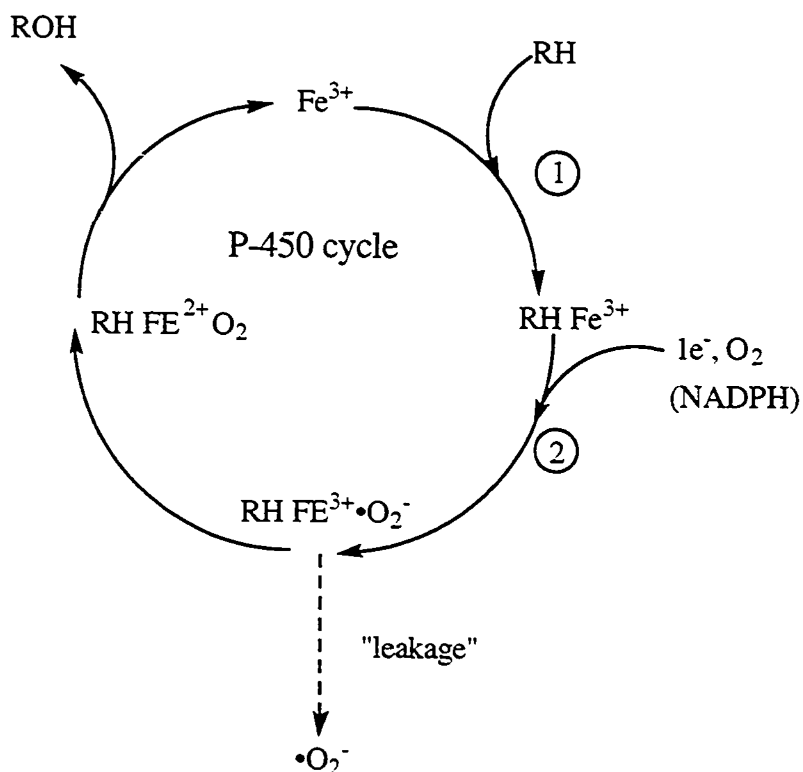


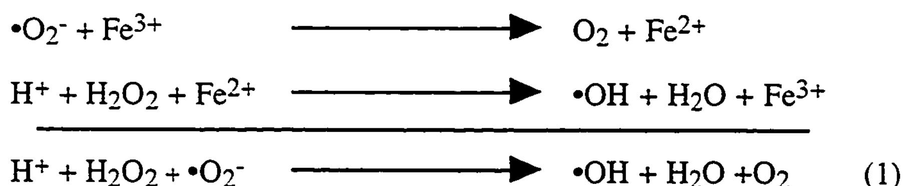
Figure 1.6: Superoxide production from P-450 enzymes.
The first step (1) is the incorporation of the substrate (RH) into the active site (containing Fe^{3+}). This is followed by a $1e^-$ reduction of the heme iron and the binding of molecular oxygen (step 2). It is at this step that any uncoupling of the electron transfer from NADPH to the heme can result in the reduction of O_2 to $\bullet O_2^-$.

CsA has been shown to be a substrate for a P-450 isoform (P450 3A , see 1.6.3) found in the liver and kidney (Kolars, et al., 1992, A. Fahr, 1993), whereas in the testis, CsA can inhibit the activity of numerous cytochrome P-450 enzymes responsible for testosterone production (Rajfer, et al., 1987, Seethalakshmi, et al., 1992) (see section 1.7.2).

1.10 Lipid peroxidation

Although not inherently toxic to cells due to its low oxidizing ability, the superoxide radical's toxic nature is derived from its ability to generate more reactive free radical species. First, $\bullet O_2^-$ can spontaneously dismutate to H_2O_2 and O_2 at a rate constant of ~$2x10^5$M/s. The enzyme superoxide dismutase (SOD) can also perform this function at

a much higher rate (2×10^9 M/s). SOD is present in two forms: 1) a copper (Cu), zinc (Zn) containing enzyme present in the cytoplasm, and 2) a manganese (Mn) containing enzyme found in the mitochondria. The H_2O_2 formed is also a weak oxidizing agent. However, via the Haber-Weiss reaction (Halliwell and Gutteridge, 1989), H_2O_2 can react with intracellular transition metals, (such as the reduced form of iron or Fe^{2+}), to form the highly reactive hydroxyl radical (•OH, reaction 1). Available $•O_2^-$ can then reduce iron in the oxidized state, Fe^{3+} thus regenerating Fe^{2+} for further reaction with H_2O_2 .



The formation of the highly reactive hydroxyl radical is termed the *initiation* phase of a free radical chain reaction (see Figure 1.7). The hydroxyl radical continues the reaction by abstracting a hydrogen atom from a neighboring PUFA to form a carbon-centered radical (R•), which in turn reacts with oxygen to form a peroxyl radical (ROO•).

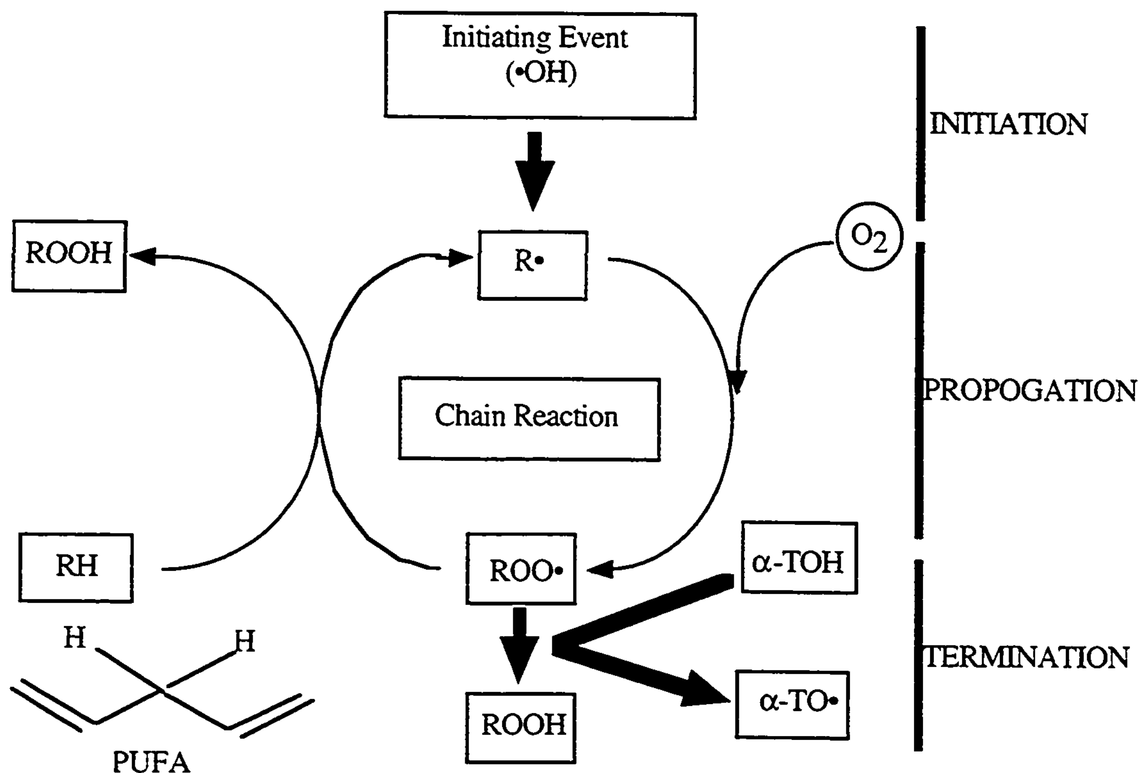
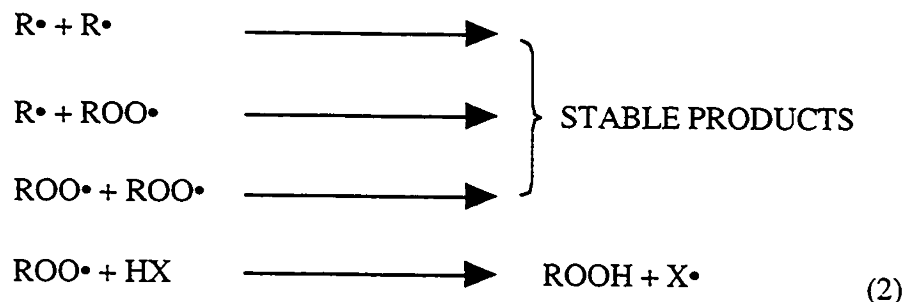


Figure 1.7 : Diagramatic representation of the three phases of the free radical chain reaction underlying lipid peroxidation. RH, R•, ROO•, ROOH, represent PUFA, a carbon centered free radical, a peroxyl radical, and a hydroperoxide respectively. α-TOH and α-TO• are α-tocopherol and α-tocopheroxyl radical respectively. Adapted from Burton and Traber (G. W. Burton and M. G. Traber, 1990)

This *propagation* phase continues as the peroxyl radical reacts with PUFA hydrogens to form a hydroperoxide (ROOH) and a new carbon-centered radical (figure 1.7). *Termination* of the reaction can only occur when the radical species reacts with another radical or with a hydrogen donor to form stable reaction products (figure 1.7 and Reaction 2).



1.11 Free radical repair concept

Biological systems have devised ways in which to protect themselves from total oxidation. The concept that reactive oxygen is less toxic to biological molecules when other protective molecules are present was born from experiments involving the modification of DNA and proteins by ionizing radiation in bacteriophage (P. Howard-Flanders, 1960), and bacterial cell types (T. Alper and P. Howard-Flanders, 1956). In these experiments, the susceptibility of certain biological molecules to ionizing radiation was reduced when proteins, sulfhydryl compounds, or whole cell constituents where also present.

1.12 Mechanism of antioxidation

The effectiveness of "protective" compounds was determined to be due to their ability to interfere with the development and/or progression of oxyradical-mediated chain reaction. These molecules are termed *antioxidants* which can be divided into two classes, depending on their mode of action. *Preventive antioxidants* are species that reduce the rate of free radical chain *initiation* whereas *chain breaking antioxidants* actually react with the radical species to terminate the *propagation* phase of autoxidation (G. W. Burton and K. U. Ingold, 1986).

1.13 Vitamin E

1.13.1 Effects on reproductive function - the discovery of a new vitamin

In 1923, Evans and Bishop (1923), found that female rats on synthetic diets became sterile yet exhibited every sign of sexual normality including the ability to ovulate, copulate and become pregnant. However at some point during gestation the fetuses were reabsorbed. Manipulation of the diet to improve known vitamin and nutrient levels (e.g., increased levels of vitamin B or modified fat intake) had no effect, whereas the introduction of fresh green lettuce leaves or wheat germ oil completely reversed the sterility. The conclusion, therefore, was that there existed a new dietary constituent, dubbed factor X, necessary for successful reproductive function in female rats. In 1924, Sure (B. Sure, 1924) confirmed the existence of an essential vitamin, necessary for reproductive function in rats, that was distinct from B, C or D and logically named it vitamin 'E'. Further studies determined that vitamin E deficiency also correlated with marked testicular atrophy in male rats (K. E. Mason, 1926, H. A. Mattill and M. M. Clayton, 1926, K. E. Mason, 1930). Through isolation and purification from plant oils it was discovered that vitamin E is a mixture of a number of structurally related phenolic compounds (A. T. Diplock, 1985) of which the most biologically active form is α-tocopherol (Burton and Ingold, 1981)

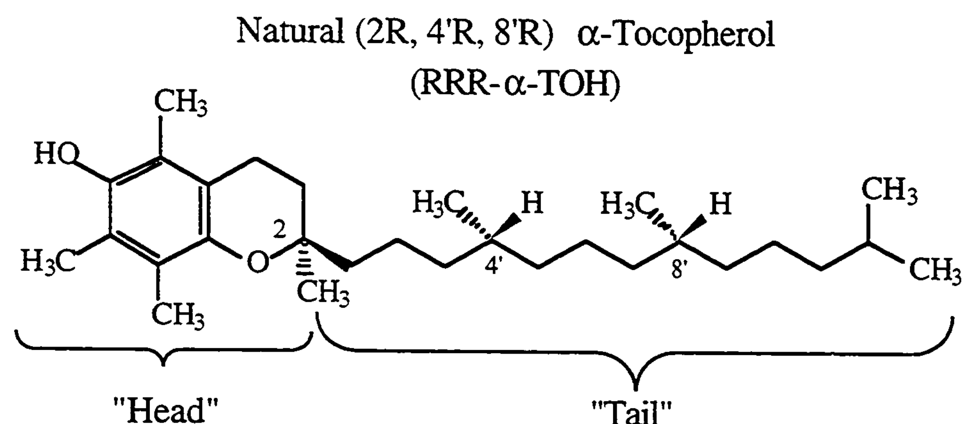
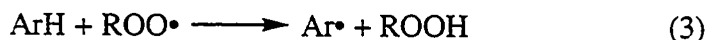


Figure 1.8 : RRR-α-tocopherol (adapted from Burton and Traber (1990)

In addition to reproductive dysfunction, studies on vitamin E deficiency uncovered evidence of nervous system degeneration (encephalomalacia in growing chicks) and nutritional muscular dystrophy in rabbits and guinea pigs (M. L. Scott, 1978), cardiovascular disorders in chicks, pigs and rabbits, hepatic necrosis and accumulation of lipopigments in rats (see Diplock, 1985). In humans, vitamin E deficiency is rare with patients exhibiting the neurologic abnormalities observed in vitamin E deficient animals (A. Mauro, et al., 1991). The cause of vitamin E deficiency in man is not usually one of poor diet but rather of malabsorption of the vitamin E (R. J. Sokol, 1989). Conditions such as abetalipoproteinemia (H. J. Kayden, et al., 1983), chronic cholestasis (R. J. Sokol, et al., 1984), and isolated vitamin E deficiency syndrome (M. G. Traber, et al., 1990) all impair the absorption and/or distribution of vitamin E to the tissues.

1.13.2 Antioxidant property of vitamin E

The major protective role of vitamin E *in vivo* is believed to be its role as an inhibitor of lipid peroxidation (Scott, 1978, Burton and Ingold, 1981, Diplock, 1985). The "tail" anchors it in the lipid membrane (B. Perly, et al., 1985) leaving the "head" region exposed to effectively interact with peroxy radicals faster than the neighboring PUFA (Burton and Traber, 1990)(see figure 1.8). One equivalent of α-tocopherol (ArH, reaction 3) has been shown to quench two equivalents of peroxy radicals (reactions 3 and 4).



$$ArH + ROO\bullet \longrightarrow Ar\bullet + ROOH \quad (3)$$

$$Ar\bullet + ROO\bullet \text{ (or } RO\bullet, R\bullet) \longrightarrow \text{stable products} \quad (4)$$

The importance of vitamin E as a free radical scavenger cannot be overstated as it has been shown that vitamin E is the major, if not the only, lipid soluble antioxidant present in plasma and tissue (G. W. Burton, et al., 1982, G. W. Burton, et al., 1983, K. U. Ingold, et al., 1987).

In *in vitro* studies, vitamin E showed a protective effect against lipid peroxidation in myocardial (D. R. Janero and B. Burghardt, 1989, D. R. Janero and B. Burghardt, 1989, K. D. Massey and K. P. Burton, 1989), renal and hepatic (D. J. Kornbrust and R. D. Mavis, 1979, H. Maio-Lin, et al., 1989) phospholipid membrane preparations in rats and rabbits. Further, axonal degeneration of peripheral nerves was evident in rats fed vitamin E deficient diets for 40 weeks (M. A. Goss-Sampson, et al., 1988).

Direct *in vivo* evidence of vitamin E inhibition of lipid peroxidation in humans is rare. However, it has been shown that the exhalation of pentane, a peroxidation product of ω-6 fatty acids, was reduced in vitamin E supplemented subjects (M. Lemoyne, et al., 1987, M. Lemoyne, et al., 1988). In rats fed vitamin E deficient diets, lipid peroxidation *in vivo* was monitored by both the exhalation of pentane (J. E. Downey, et al., 1978) and ethane (D. G. Hafeman and W. G. Hoekstra, 1977), an autoxidation product of ω-3 unsaturated fatty acids. Addition of vitamin E to the diets of these animals returned pentane and ethane emissions to normal levels.

Plasma membrane proteins are also known to be a target of free radical oxidation (R. E. Pacifici and K. J. A. Davies, 1990, Stadtman, 1992, S. Gebicki and J. M. Gebicki, 1993). The sulfhydryl groups of plasma proteins are so susceptible to oxidation that in *in vitro* experiments they are utilized during oxyradical attack before vitamin E (D. D. M. Wayner, et al., 1987). However, *in vivo* addition of vitamin E to rats has a protective effect on membrane proteins isolated from liver submitochondrial particles subjected to a radical generating system (R. T. Dean and K. H. Cheeseman, 1987).

1.13.3 Other properties of vitamin E

There have been reports that vitamin E may have other activities *in vivo* that are unrelated to its antioxidant activity but may have an equally powerful protective effect on stressed cells. Studies have shown that vitamin E can inhibit the release of arachidonic acid by platelet phospholipase A_2 (C. E. Douglas, et al., 1986), and in fact can enhance the

synthesis of a powerful vasodilator, prostacyclin, or PGI_2, (D. D. Pyke and A. C. Chan, 1990) while inhibiting the release of an antagonist to prostacyclin, thromboxane or TXA_2. These activities suggest that vitamin E could have an important role in controlling blood flow.

Further, in ischemic tissue neutrophils migrate to the site of tissue damage and mediate an inflammatory response partially through the production of leukotrienes generated via the metabolism of arachidonate by 5-lipoxygenase. The production of leukotrienes by this enzyme has been shown to be inversely proportional to the vitamin E status of the animal (A. C. Chan, et al., 1989).

In other studies it has been shown that vitamin E may have actions in cell signalling pathways. In vitro studies have shown that α-tocopherol can reduce the activity of protein kinase C in bovine brain (C. W. Mahoney and A. Azzi, 1988) and rat aortic smooth muscle (D. Boscoboinik, et al., 1991, D. Boscoboinik, et al., 1991) thus inhibiting cell division and proliferation.

1.14 Glutathione and Vitamin E

The function of vitamin E to protect PUFA from oxidation is extraordinary in light of the low ratio of α-tocopherol to PUFA in membranes. Estimates suggest ratios of PUFA to α-tocopherol in the order of 1000 : 1 or greater (J. L. Buttriss and A. T. Diplock, 1988). This large molar excess of PUFA to α-tocopherol suggests that for vitamin E to be protective some regeneration of the tocopheroxyl radical may occur at the cellular level.

The ubiquitous tripeptide glutathione (L-g-glutamyl-L-cysteinylglycine, GSH) has been suggested as a candidate for α-tocopherol regeneration. In *in vitro* oxyradical generating systems in platelets (D. D. Gibson, et al., 1985) and liver microsomes (C. C. Reddy, et al., 1982) α-tocopherol utilization was inversely proportional to GSH content. However, since GSH is water soluble and resides primarily in the cytosol, and

vitamin E is lipophilic and incorporates into biomembranes, the ability of GSH to regenerate vitamin E *in vivo* remains questionable.

Therefore, the primary role of GSH in cell systems may be as a substrate for several antioxidant enzymes such as the glutathione peroxidases. Glutathione peroxidases are comprised of two important selenium (Se) dependent enzymes (J. Rotruck, et al., 1973). Cytosolic glutathione peroxidase (GPx), which is present in both the cell cytosol and mitochondrial matrix of all tissues (G. C. Mills, 1957), and phospholipid hydroperoxide glutathione peroxidase (PH GPx), an interfacial monomeric protein (F. Ursini, et al., 1985). GPx is able to reduce cytosolic hydroperoxides using glutathione as the specific coenzyme (B. Mannervik, 1985, Ursini, et al., 1985) while PH GPx is capable of reducing esterified membrane phospholipid hydroperoxides using GSH as a coenzyme (F. Ursini, et al., 1982)

The proposed mechanism of peroxide reduction by GPx's (L. Flohe, et al., 1973, Mannervik, 1985) involves the reduction of the selenium atom on the peroxidase by the thiol group of GSH. The reduced selenocysteine in turn reduces the peroxide to either water, in the case of hydrogen peroxide (reaction 5), or to the corresponding alcohol and water in the case of the organic hydroperoxide (ROOH, reaction 6). Both reactions result in the formation of a disulfide between two oxidized glutathione molecules (GSSG).

$$H_2O_2 + 2GSH \longrightarrow GSSG + 2H_2O \qquad (5)$$

or

$$ROOH + 2GSH \longrightarrow GSSG + ROH + H_2O \qquad (6)$$

where R = aromatic or aliphatic group.

The GSSG produced is reduced back to GSH by glutathione reductase (GSHr) with the cofactor NADPH obtained from the oxidation of glucose-6-phosphate and/or 6-phospho-gluconate.(Meister and Anderson, 1983).

It has long been established that there is a relationship between GPx's and α-tocopherol (A. L. Tappel, 1974, G. F. Combs, et al., 1975, W. G. Hoekstra, 1975).

Through GPx reduction of peroxides there is a decrease in the conversion of these peroxides into more harmful free radicals, ultimately having a sparing effect on vitamin E. GPx activity is dependent on dietary Se but a deficiency in the mineral can be overcome by an excess of vitamin E (Tappel, 1974, Combs, et al., 1975, Hoekstra, 1975). Further, it has been shown that feeding excess α-tocopherol reduces GPx activity (Halliwell and Gutteridge, 1989) and that a Se deficiency causes an increased migration of α-tocopherol to the mitochondrial membranes (Buttriss and Diplock, 1988).

1.15 Summary : CsA and Vitamin E:

CsA has become the most successful and widely used immunosuppressive drug to date. However, the problem of tissue toxicity in numerous organs persists, with little being known as to the mechanism. Structural and functional changes in the testis and kidney, along with evidence for lipid peroxidation in the kidney of animals treated with CsA, suggests the possibility that oxygen free radical generation is a component of CsA toxicity. This is the first *in vivo* study to use changes in vitamin E levels in various tissues of rats dosed with CsA as an indicator of the presence of oxygen free radicals.

CHAPTER 2

2.1 Study 1 : Therapeutic levels of CsA

The first study was designed to measure the effects of therapeutic concentrations of CsA (corresponding to 10 mg/kg/day for 14 days in humans) on tissue vitamin E and plasma testosterone concentrations in a male Wistar rat model as a function of dietary vitamin E concentrations. Tissues shown to exhibit CsA toxicity (liver, kidney, testis, adrenal gland, and brain) were analyzed for vitamin E concentrations assuming that CsA toxicity has a free radical component. The weights of kidney and testis were measured at the end of the study and, in addition a morphological examination by hematoxylin and eosin staining and light microscopy was carried out to determine the overall health of these organs. Measurement of plasma testosterone was carried out as a gauge of testis function.

Based on previous studies (Seethalakshmi, et al., 1987, Sikka, et al., 1988), we can expect to see no change in kidney performance but some evidence of disruption in testis function at the concentration of CsA used in this study.

2.1.1 Experimental procedure

At the beginning of the experiment 24 weanling male Wistar rats weighing 50-65 g were divided into 2 groups of 12 animals each and housed 2 per cage. Experimental procedures were carried out following the guidelines set out by the Canadian Council on Animal Care. Experiments were started after 5 days of acclimatization, at which time the rats weighed an average of 95-100 g. The first group, E^+, which served as a control, was fed the vitamin E sufficient diet *ad libitum* throughout the study. The second group, E^-, was fed the vitamin E deficient diet *ad libitum* for the same time period. Both groups were provided tap water *ad libitum*. Rats were weighed at days 0, 14, and 28.

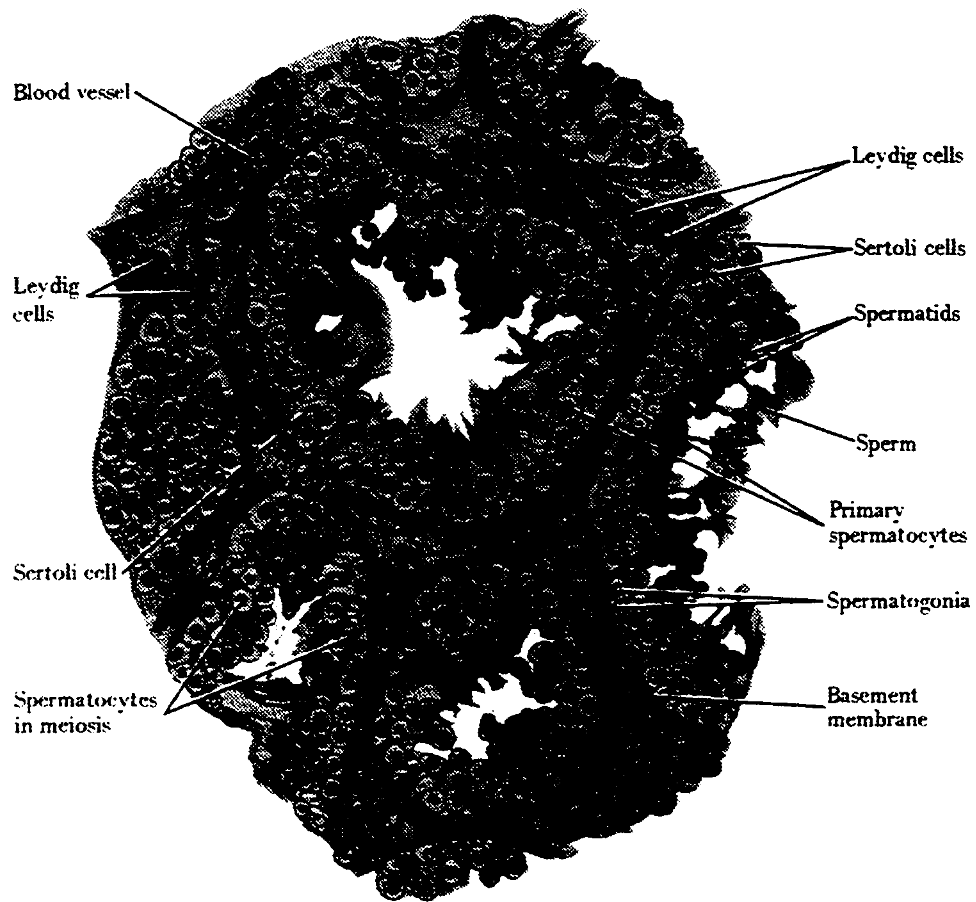
At day 14 the two groups were each split into two equal subgroups. One sub-group from each of the E^+ and E^- groups was injected daily, subcutaneously, with 10 mg/kg body weight CsA, (denoted subgroups E^+/CsA and E^-/CsA, respectively), in a vehicle

of olive oil and ethanol (9:1). The remaining two groups were injected with an equal volume of vehicle only (subgroups E^+/Veh and E^-/Veh, respectively). At day 28 of the study, all rats from each group were weighed and anesthetized with halothane. The abdominal cavity was exposed by a midline incision and up to 5 mL of blood was collected by cardiac puncture. The aorta was then cut to sacrifice the animal.

Plasma was separated from whole blood for analysis of circulating testosterone. Briefly, blood was collected into evacuated containers (Vacutainers) coated with disodium ethylenediaminetetraacetate (EDTA). The blood was then spun at 4°C, at 2000 rpm for 4 minutes, the plasma was removed and stored at -80°C.

Testes, kidneys, adrenals, liver and brain were harvested from all animals in each group, weighed then immediately placed on dry ice and stored at -80°C for later analysis of vitamin E. A testis and a kidney from two animals in each group were fixed overnight at room temperature in 10 % buffered formalin, pH 7.0, and washed in 0.16 M sodium phosphate-buffered saline (PBS), pH 7.2. Tissue was then cryoprotected with 10 % sucrose in 0.16 M phosphate buffer, pH 7.2, overnight at 4°C, then sectioned at -22°C at a thickness of 20 micrometers, placed on glass slides, stained with hematoxylin and eosin, coverslipped with permount (Sigma Chemical Company, St. Louis Mo. USA) and examined by light microscopy. An illustration of a hematoxylin and eosin stained section of rat testis is provided in Figure 2.1(M. H. Ross, et al., 1989). A flow diagram of the experimental design for study 1 is shown in Figure 2.2.

FIGURE 2.1: A schematic illustration of a typical cross section through the testis showing many seminiferous tubules which contain Sertoli and spermatogenic cells. The interstitium between the tubules contains Leydig cells, the testosterone producing cells of the testis. The seminiferous epithelium consists of essentially four concentric layers of cells constituting generations of cells at various phases of their development. The most immature spermatogenic cells, the spermatogonia, are located adjacent to the basement membrane. These cells proliferate and undergo differentiation as they move towards the tubular lumen through a spermatocyte and spermatid phase to eventually form sperm. This process is known as spermatogenesis. Derived from stem cells, spermatogonial cells undergo a series of mitotic divisions, the last of which produces spermatocytes which divide meiotically to form spermatids. As haploid cells, spermatids undergo morphological differentiation, referred to as spermiogenesis as they are transformed ultimately into sperm. The seminiferous epithelium also contains a nonproliferating population of cells composed of a single cell type, the Sertoli cell. These cells are columnar in shape with processes that surround adjacent spermatogenic cells and fill the spaces between them. With the light microscope, the extent and morphology of these processes are poorly defined in hematoxylin and eosin stained preparations. A good demarcating feature of these cells is the triangular shaped nucleus which may be seen in the basal portion of the cell near the basement membrane. Another unique feature of these cells is the tripartite structure within the nucleus which consists of an RNA-containing nucleolus flanked by a pair of DNA-containing bodies called karyosomes. (Taken from M. H. Ross et al., 1989)


Blood vessel
Leydig cells
Sertoli cells
Spermatids
Sperm
Primary spermatocytes
Spermatogonia
Basement membrane
Leydig cells
Sertoli cell
Spermatocytes in meiosis

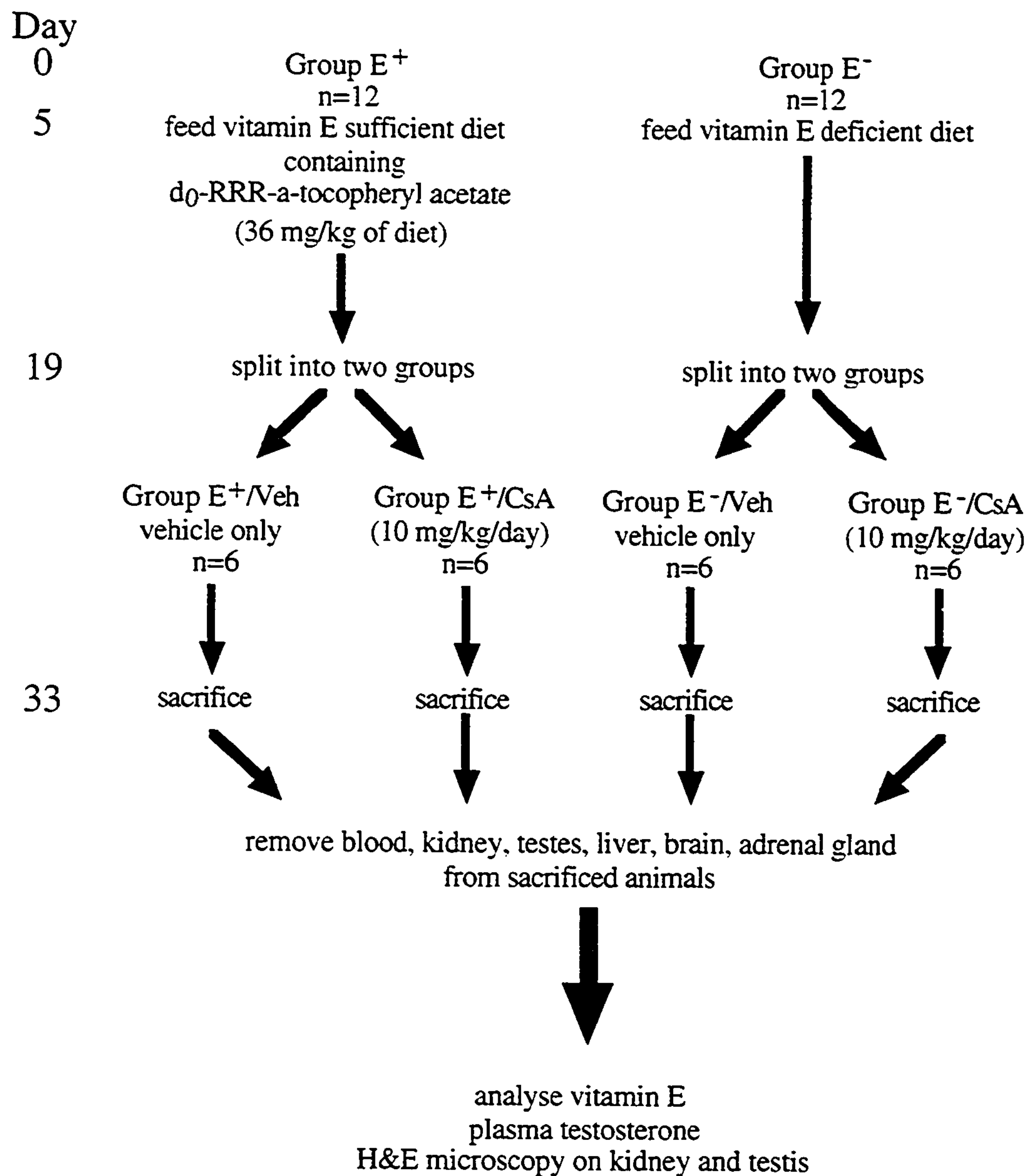


Figure 2.2 : Experimental design for study 1 examining the effect of therapeutic doses of CsA in the presence and absence of dietary vitamin E. E^+/Veh= vitamin E sufficient diet/vehicle (9:1 olive oil:ethanol; 1 mL/kg s.c). E^+/CsA= vitamin E sufficient diet/10 mg/kg CsA in vehicle s.c. E^-/Veh= vitamin E deficient diet/vehicle (9:1 olive oil:ethanol; 1 mL/kg s.c). E^-/CsA= vitamin E deficient diet /10 mg/kg CsA in vehicle (1 mL/kg s.c). Each group consisted of 6 weanling male Wistar rats weighing 50-65 g.

2.1.2 Preparation of tissue homogenate

Weighed samples (1-1.5 g testis, liver, and kidney; 0.06-0.09 g adrenal; 1.5-1.8 g brain) were homogenized with 10 volumes of 50 mM phosphate buffer pH 7.4 using a Brinkmann PT10/35 homogenizer and the pre-weighed vial containing the homogenate was reweighed to determine the concentration of the homogenate in g tissue/g homogenate.

2.1.3 Analysis of vitamin E (α-tocopherol)

An aliquot of the original homogenate was taken in sufficient volume (usually 1 mL) to analyze 0.2- 0.3 g of total tissue. The vitamin E was extracted from the homogenate, purified and analyzed using the methods of Burton (G. W. Burton, et al., 1985) and Ingold (K. U. Ingold, et al., 1987). Briefly, a known quantity of deuterated internal standard (usually ~50μL of a 0.157 mM d_9-α-tocopherol solution (see figure 2.3) in heptane, i.e. ~7.87 nmol sample) was added to the homogenate, followed by 1 mL of 10 % SDS and the mixture vortexed briefly. Next, 4 mL of 95 % ethanol was added and again vortexed for a few seconds. Finally, 2 mL of HPLC grade heptane was added and vortexed for one minute to extract the tocopherol into the organic layer. The mixture was centrifuged at approximately 1000 x g for 6 minutes to separate the aqueous and organic layers. The organic layer was removed and blown down to dryness under a stream of nitrogen. The extract was redissolved in 600 μL of heptane and passed through a high performance liquid chromatography (HPLC) column (Varian model 5000 HPLC equipped with a 250 mm x 4 mm I.D. Hibar LiChrosorb Si 60, 5 mm particle size column) eluted with a mobile phase of 90 % heptane/10 % methyl tert butyl ether at a flow rate of 2 mL/min. Peaks were detected with a Varian fluorescence detector equipped with a deuterium lamp, an interference excitation filter (220 nm), and a 2 mm thick Schott UG-1 glass band detection filter (λ_{max} ca. 358 nm). α-Tocopherol was collected automatically off the column using a peak-sensing device connected to a fraction collector. Samples containing α-tocopherol were blown down to dryness under a stream of nitrogen and the extract redissolved in 60 μL of heptane for

analysis by gas chromatography-mass spectrometry (GC-MS). A 1 mL sample was injected onto a Hewlett Packard Ultra 1 12m x 0.2mm I.D. x 0.33 mm (film thickness) fused silica capillary column maintained at a temperature of 280°C in a Hewlett Packard 5890 A Series gas chromatograph (GC). The Hewlett Packard 5970 series Mass Spectrometer connected to the GC was set to detect 430 (d_0) and 439 (d_9) α-tocopherol parent molecular ions. The relative proportions (%) of d_0-α-tocopherol and d_9-α-tocopherol were measured to determine the quantity of endogenous α-tocopherol ($d_0\alpha$-T) in the tissue sample (expressed in nmol/g tissue) using the following equation :

$$d_0\alpha\text{–T} = \frac{(\%d_0 / \%d_9) \times \text{nmol } d_9 \text{ standard added / sample volume(mL)}}{\text{sample concentration (g tissue / mL)}}$$

2.1.4 Analysis of plasma testosterone

Testosterone initially was analyzed at the Ottawa General Hospital diagnostic laboratory using a "coat-a-count" radioimmunoassay (RIA) ^{125}I labelled single antibody kit from Diagnostics Products Corporation. However this was not very satisfactory due to the large intra-group variation in the data rendering changes in testosterone levels between groups statistically insignificant. Therefore, for the second study (using pharmacological levels of CsA) a GC-MS determination for tissue and plasma testosterone was performed (see section 3.1.4)

2.1.5 Statistical analysis

All data were analyzed for significance using an independent 2 way annalysis of variance (ANOVA) from the STATVIEW 4.0 statistics package (Abacus concepts, Berkeley, California).

2.2 Materials

Weanling male Wistar rats were obtained from Charles River, Quebec, Canada. CsA (100 mg/mL in 9:1 olive oil : ethanol diluted to 10 mg/mL with vehicle) and the vehicle (9:1 olive oil : ethanol) were obtained from Sandoz Canada Inc. (Dorval, Quebec, Canada). The vitamin E deficient diet (Dyets, Bethlehem, PA, USA) was a modified version of the AIN-76 rat and mouse diet (J. G. Bieri et al., 1977,J. G. Bieri, 1980). For control animals the diet was repleted with 0.036 g d_0-RRR-α-tocopheryl acetate/kg diet (Table 1). HPLC grade heptane, methyl tert butyl ether, 95% ethanol and sodium dodecyl sulfate (SDS), were obtained from BDH Chemicals (St. Laurent, Quebec, Canada). The internal standard, d_9-ambo-α-tocopherol, was originally prepared in this laboratory as described by Hughes et. al. (L. Hughes, et al., 1990). Monobasic potassium phosphate and tris(hydroxymethyl) aminomethane (TRIS) were obtained from Anachemia Chemicals Ltd. (Montreal, Quebec, Canada). L-Ascorbic acid was obtained from Eastman Kodak Co. (New York, N.Y. USA).

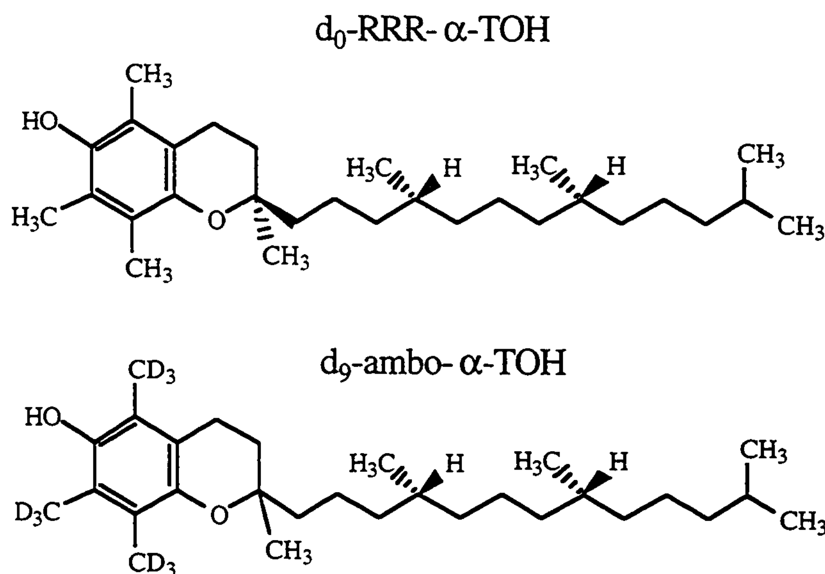


Figure 2.3: Unlabelled and deuterated forms of α-tocopherol. (A) natural stereoisomer (RRR) of α-tocopherol (B) α-tocopherol internal standard with 9 deuterium atoms on the chroman head

Table 2.1 : The composition of the vitamin E deficient and sufficient diets.

Vitamin E Deficient Diet (E^-)

INGREDIENT	grams/kg
Vitamin Free Casein	200
DL-Methionine	3
Cornstarch	350
Sucrose	200
Dyetrose	100
Cellulose	50
Tocopherol Stripped Corn Oil*	50
Salt mix #2000	35
Vitamin mix #319501 (vit E omitted)	10
Choline Bitartrate	2
Green Dye	0.2

*Tocopherol Stripped Corn Oil stabilized with 0.02% Bis-hydroxy toluene (BHT)

Vitamin E Sufficient Diet (containing 0.036 g/kg d_0-RRR-α-Tocopheryl Acetate) (E^+)

Diet is the same as the vitamin E deficient diet except that it contained 199.764 g/kg sucrose, 0.036 g/kg d_0-RRR-α-tocopheryl acetate and was distinguished by the addition of 0.2 g/kg blue dye.

2.3 Results : Effect of 10 mg/kg CsA on body and tissue weights

Feeding weanling male Wistar rats a vitamin E deficient diet for 28 days together with subcutaneous injections of CsA at 10 mg / kg body weight / day for the final 14 days had no observable effects on the overall health of the animals. The body and organ weights of all groups of rats were similar to the dietary and treatment control group (E^+/Veh). In general, the rats gained approximately 200 g in body weight in 28 days (Table 2.2). At the end of the experimental period, the testis weighed about 1.4 g and the kidney 1.2 g (Table 2.3)

2.4 Results : Effect of 10 mg/kg CsA on kidney and testis morphology

The kidneys of the CsA treated rats, with or without vitamin E deficiency, showed no evident morphological changes in the renal tubules of tissue slices stained with hematoxylin and eosin (H and E) and examined by light microscopy (data not shown).

The histological appearance of the H and E stained testes from control (vehicle treated) rats fed a vitamin E sufficient diet showed a normal complement of spermatogenic cells in the seminiferous tubules and Leydig cells in the interstitium between the tubules (figure 2.4A). In the 10 mg/kg CsA treated group fed a vitamin E sufficient diet a number of vacuoles were seen in the spermatocytes (figure 2.4B). In addition, degeneration was evident in the Leydig cells .

The testes of vehicle treated control rats fed a vitamin E deficient diet also displayed seminiferous tubules wherein spermatocytes had vacuolar spaces (figure 2.4C). However, the interstitial (Leydig) cells appeared normal. The administration of 10 mg/kg CsA to rats fed a vitamin E deficient diet affected tubular architecture markedly. Compared to the vehicle treated, vitamin E deficient control group (figure 2.4C), the tubules in the testes of these rats displayed a loss in the orientation of the developmental phases (figure 2.4D) and contained primarily spermatocytes and spermatogonia, with a marked reduction

of spermatids and sperm. As for the CsA treated, vitamin E sufficient group (2.3B), interstitial cell degeneration was observed.

Table 2.2: Whole body weights of male Wistar rats following treatment with vehicle (Veh; 9:1 olive oil:ethanol; 1 ml/kg/day s.c.) or cyclosporin A (CsA; 10 mg / kg / day s.c.) for 14 days. Treatment groups include thosed placed on vitamin E sufficient diets with either vehicle or CsA treatment (E^+/Veh or E^+/CsA) or on a vitamin E deficient diet with vehicle or CsA treatment (E^-/Veh or E^-/CsA). Values are obtained from 6 animals per group expressed as means ± SEM .

Treatment Groups	Body weight (g)		
	day 0	day 14	day 28
E^+/Veh	97.5 ± 5.7	216.0 ± 6.7	318.3 ± 15.1
E^+/CsA	102.3 ± 3.9	217.7 ± 11.3	295.9 ± 13.4
E^-/Veh	98.8 ± 7.4	213.1 ± 11.8	328.3 ± 18.3
E^-/CsA	101.4 ± 2.6	216.9 ± 7.6	300.3 ± 14.5

Table 2.3: Wet weights of testis and kidney of male Wistar rats following treatment with vehicle (Veh) or CsA (10 mg / kg / day s.c.) for 14 days. Treatment groups include thosed placed on vitamin E sufficient diets with either vehicle or CsA treatment (E^+/Veh or E^+/CsA) or on a vitamin E deficient diet with vehicle or CsA treatment (E^-/Veh or E^-/CsA). Values are obtained from 6 animals per group expressed as means ± SEM .

Treatment Groups	Tissue weight (g)	
	testis	kidney
E^+/Veh	1.4 ± 0.1	1.2 ± 0.1
E^+/CsA	1.4 ± 0.1	1.2 ± 0.1
E^-/Veh	1.4 ± 0.2	1.3 ± 0.0
E^-/CsA	1.5 ± 0.2	1.3 ± 0.1

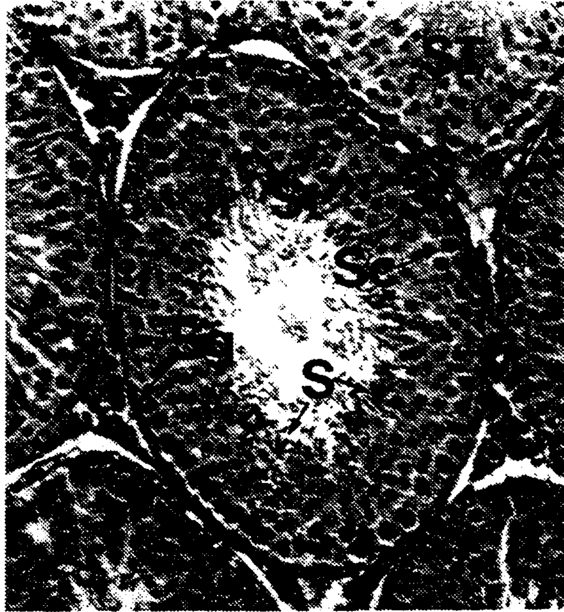
FIGURE 2.4 A-D: Light micrographs of H and E stained sections of testis from a rat treated with vehicle (Veh; 9:1 olive oil:ethanol; 1 ml/kg/day s.c.) or cyclosporin A (CsA; 10 mg/kg/day s.c) and placed on either a vitamin E sufficient or deficient diet for 14 days. Control sections are from Veh treated rats either on a vitamin E sufficient (A) or deficient (C) diet. Comparative sections of testis were taken from CsA treated rats either on a vitamin E sufficient (B) or deficient (D) diet.

(A) Seminiferous tubules (ST) from a vitamin E sufficient vehicle control testis distinctly showing the four layers of spermatogenic cells including a layer of spermatogonia (Sg), spermatocytes (Sc), spermatids (St) and sperm (S). Interstitium between the tubules contains Leydig cells (L).

(B) Vacuolar spaces (V) have formed in spermatocytes (Sc) of a seminiferous tubule from vitamin E sufficient CsA treated testis. Spermatid (St) and sperm (S) layers appear normal. Note the degeneration of Leydig cells (L).

(C) A seminiferous tubule of a vitamin E deficient Veh treated control testis also shows the presence of vacuoles (V) in spermatocytes (Sc). Spermatids (St), sperm (S) and Leydig cells (L) appear normal.

(D) Vacuoles (V) in tubule spermatocytes of a vitamin E deficient CsA treated testis appear greatly enlarged. Note the spermatocytes (Sc) extending to the tubular lumen as well as the loss of layer distinction and phases of differentiation being much less defined compared to the Veh treated control in C. Also note the absence of sperm in this tubule and few discernible spermatids (St) as well as a cluster of undefined cells (*). 200x.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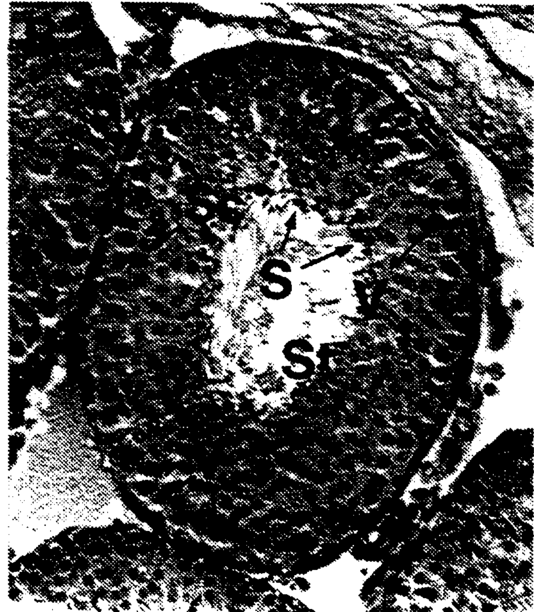
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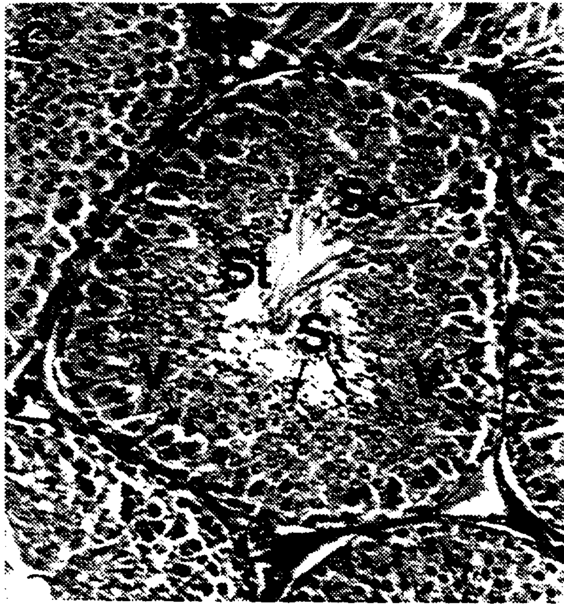
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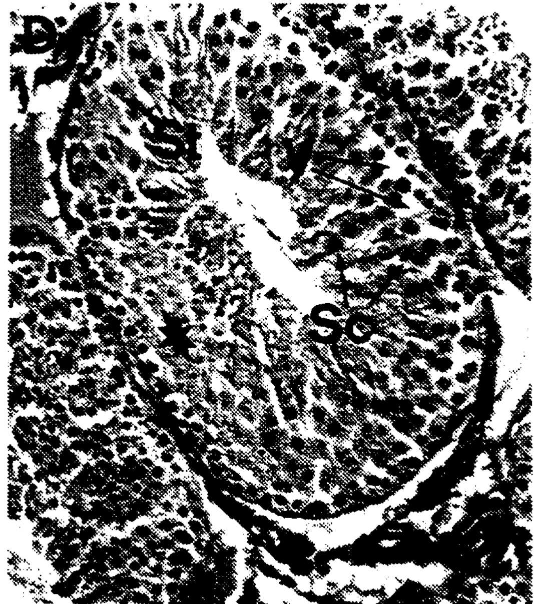
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2.5 Results : Effect of 10 mg/kg CsA on tissue vitamin E

Vitamin E deficient diet groups (E^-/Veh and E^-/CsA) showed significant reductions in tocopherol content of all 5 tissues examined as compared to animals on vitamin E sufficient diets (E^+/Veh and E^+/CsA, respectively, $p<0.05$) (figure 2.5). When vitamin E sufficient (E^+/CsA) or deficient (E^-/CsA) animals were treated with CsA, there was a statistically significant decrease in the tissue level of tocopherol in the testes and kidneys ($p<0.05$). This decrease was not seen in the liver, brain or adrenal gland.

2.6 Results : Effect of 10 mg/kg CsA on plasma testosterone

Figure 2.6 indicates that a deficiency of vitamin E coincides with a statistically significant reduction in plasma testosterone as compared to control (4.3 ± 1.4 nmol/mL for E^-/Veh vs 11.6 ± 4.9 nmol/mL for E^+/Veh, $p<0.05$). Treatment of vitamin E deficient animals with cyclosporin causes a further drop in plasma testosterone (E^-/CsA, 2.4 ± 1.0 nmol/mL), however this result is not statistically significant. CsA had no effect on plasma testosterone levels in vitamin E sufficient animals (E^+/CsA, 11.3 ± 2.9 nmol/mL).

Figure 2.5: α-Tocopherol levels in the kidney, testis, liver, brain and adrenal gland of male Wistar rats following treatment with vehicle (Veh; 9:1 olive oil:ethanol; 1 ml/kg/day s.c.) or Cyclosporin A (CsA; 10 mg/kg/day s.c.) for 14 days. Treatment groups listed in the legend include those placed on a vitamin E sufficient diet with either Veh or CsA treatment (E^+/Veh or E^+/CsA respectively) or a vitamin E deficient diet with either Veh or CsA treatment (E^-/Veh or E^-/CsA respectively). Values obtained from 6 animals per group are expressed as mean ± SEM. Asterisks and obelisks denote statistical significance ($p<0.05$) when compared to the E^+/Veh (†) or the E^-/Veh (*) group.

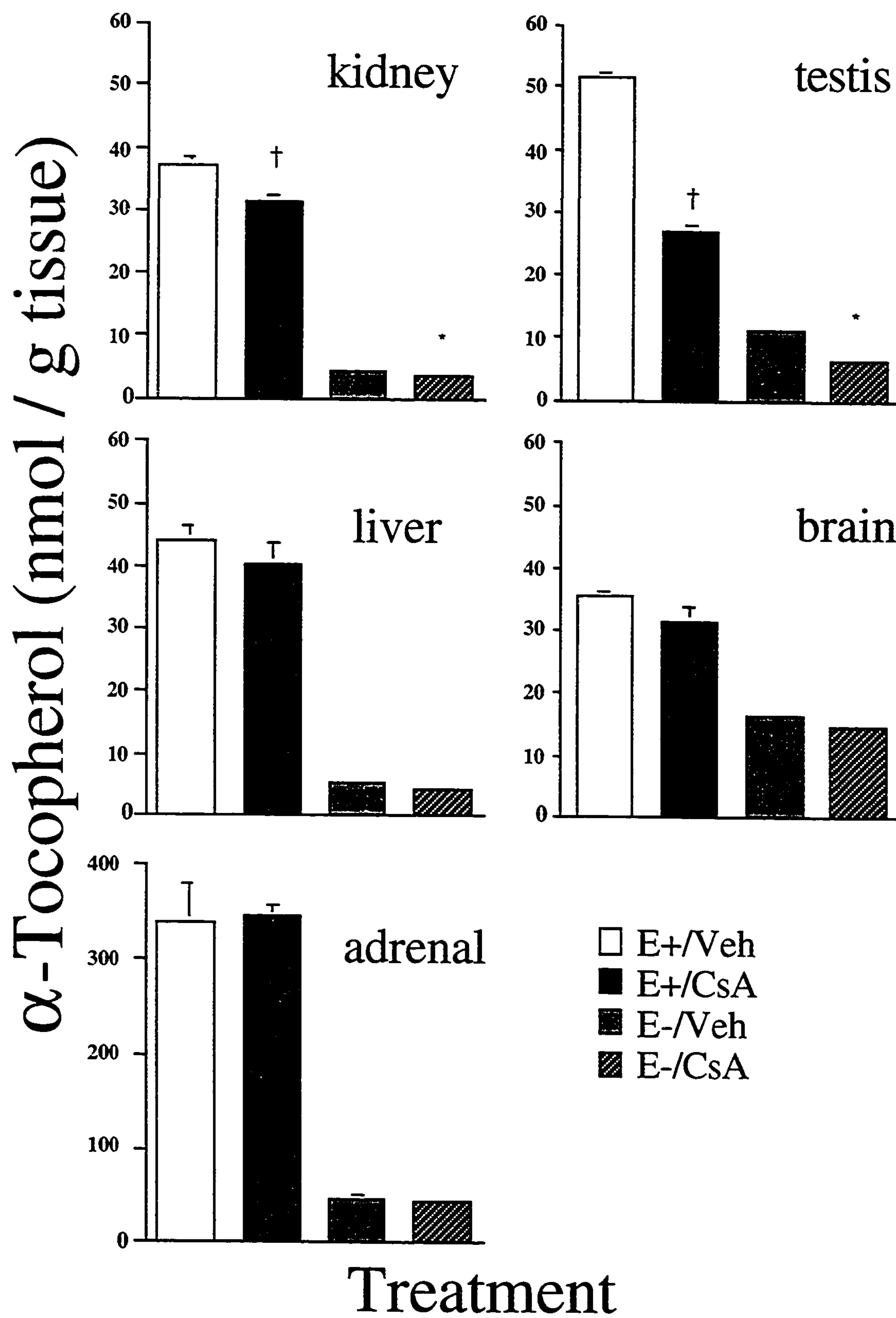
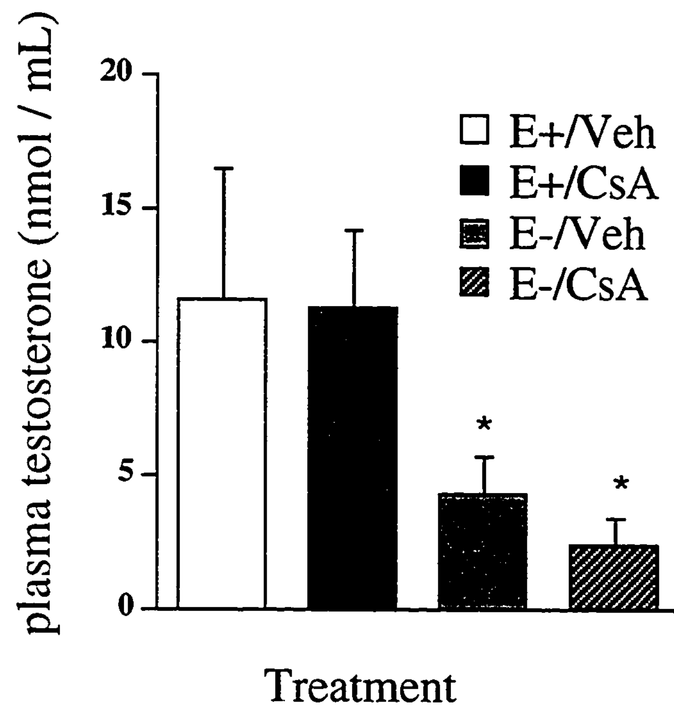

α-Tocopherol (nmol / g tissue)
kidney
testis
liver
brain
adrenal
E+/Veh
E+/CsA
E-/Veh
E-/CsA
Treatment

Figure 2.6: Plasma Testosterone levels following treatment with vehicle (Veh; 9:1 olive oil:ethanol; 1 ml/kg/day s.c.) or Cyclosporin A (CsA; 10 mg/kg/day s.c.) for 14 days. Treatment groups listed in the legend include those placed on a vitamin E sufficient diet with either Veh or CsA treatment (E^+/Veh or E^+/CsA respectively) or a vitamin E deficient diet with either Veh or CsA treatment (E^-/Veh or E^-/CsA respectively). Values obtained from 6 animals per group are expressed as mean ± SEM. Asterisks denote statistical significance ($p<0.05$) compared to respective Vitamin E sufficient animals.


plasma testosterone (nmol / mL)
20
15
10
5
0
E+/Veh
E+/CsA
E-/Veh
E-/CsA
*
*
Treatment

2.7 Discussion

In this study we have shown that subcutaneous injections of cyclosporin A (CsA) at a dosage of 10 mg / kg body weight per day for 14 days can lead to significant reductions in tissue vitamin E (α-tocopherol) concentrations for both kidney and testes. Further, morphological examination of the kidney showed no gross evidence of structural alterations at this dosage of CsA. When vitamin E was measured in the liver, brain and adrenal gland, no tocopherol lowering effect of CsA was observed. CsA imparted no effects on overall animal health as determined by tissue and whole body weight measurements. However, in animals placed on a vitamin E deficient diet for 28 days the testis from both E^-/Veh and E^-/CsA treatment groups showed evidence of seminiferous tubule cell disruption, coincident with reduced plasma testosterone. Further, animals from the E^-/CsA treatment group also showed signs of interstitial cell disruption

2.7.1 Overall health of animals during CsA treatment

Body and tissue weights were unchanged as compared to dietary controls. This result agrees with previous finding in rats in which similar dosages of CsA were given (Seethalakshmi, et al., 1987). In human clinical trials, however, there are frequent reports of bloating, nausea, vomiting, or anorexia that may be attributed to the manner in which CsA is given (Kahan, 1989). Oral suspensions of the drug may be unpalatable and other modes of application such as gelatin capsules are being formulated (Kovarik, et al., 1994, Mueller, et al., 1994). In our study the use of subcutaneous injections rule out the possibility of any weight losses due to dietary alterations. A vitamin E deficient diet for four weeks also did not cause reductions in whole body or tissue weight.

2.7.2 CsA and tissue vitamin E

The tocopherol lowering effect evident in the testis and kidney of CsA treated animals fed either a vitamin E sufficient (E^+/CsA) or deficient diet (E^-/CsA) as

compared to their dietary controls (E^+/Veh and E^-/Veh respectively) suggests the formation of oxy radicals during CsA treatment. Vitamin E concentration can be an indicator of oxidative stress in tissue because it is the major if not the only lipid soluble antioxidant in biological membranes (Burton, et al., 1982, Burton, et al., 1983, Ingold, et al., 1987). It is termed a chain breaking antioxidant because it can quench the propagation of previously formed oxyradicals, (usually peroxy radicals) preventing the oxidation of membrane polyunsaturated fatty acids (PUFA). Therefore the utilisation of vitamin E is directly related to the extent of exposure of lipid membranes to oxy radicals.

In this study, feeding rats a vitamin E deficient diet (E^-/Veh) for 28 days caused significant reductions in tissue vitamin E (compared to E^+/Veh) in all tissues examined. The relative extent of α-tocopherol loss : liver = kidney (12 % of control) > adrenal (13 % of control) > testis (22 % of control) > brain (47 % of control) agrees with a previous report (Ingold, et al., 1987). The observation that CsA at this concentration has no tocopherol lowering effect on liver and adrenal gland indicates that either CsA has no free radical generating component in these tissues or that available vitamin E is spared or regenerated via some other mechanism. The first premise is unlikely given that the liver is the major site of CsA metabolism (by P-450 enzymes, L. M. Forrester, et al., 1992) and the adrenal gland is rich in P-450 enzymes that may recognise CsA as a substrate. *In vitro*, rat liver microsomes, when incubated with CsA, generated high levels of malondialdehyde (MDA), a lipid oxidation product, compared to controls (Serino, et al., 1993). MDA production was inhibited when catalase, superoxide dismutase, α-tocopherol, or reduced glutathione was added to the incubation (Serino, et al., 1993). The authors proposed that CsA is able to uncouple electron transfer to the P-450 enzyme, generating superoxide, while acting as the enzymes' substrate. Similarly, long term use (over one year) of CsA (Oka, et al., 1993) was able to reduce cortisol production in the adrenal gland which was not ameliorated by adrenocorticotropic hormone (ACTH).

There is *in vitro* evidence that vitamin E is spared by plasma proteins (Wayner, et al., 1987) or spared/regenerated by GSH (Tappel, 1974) and/or vitamin C (J. E. Packer, et al., 1979, T. Doba, et al., 1985). GSH is a substrate for phospholipid glutathione peroxidase which destroys lipid peroxides, preventing the formation of oxyradicals such as •OH, thus sparing vitamin E. However, the possibility that GSH or vitamin C, both water soluble antioxidants, could approach close enough to the biomembrane in order to regenerate vitamin E has been disputed. There is recent evidence in guinea pig that vitamin C can regenerate vitamin E *in vivo* (MSc. thesis, University of Ottawa, Ewa Piertzak, unpublished results). Finally, rapid turnover of vitamin E could be accommodated by the liver of animals on vitamin E sufficient diets because any new α-tocopherol absorbed in the gut first passes through the liver to be repackaged in lipoproteins for transport to systemic sites (G. W. Burton, et al., 1993).

The smallest relative depletion of vitamin E in this study was found to be in brain tissue. This has been previously reported (Ingold, et al., 1987). Numerous studies have shown a reluctance for CsA to cross the blood brain barrier (Atkinson, et al., 1983, Niederberger, et al., 1983, Ried, et al., 1983, Lensmeyer, et al., 1988). These observations, combined with our data, suggest that CsA would be unlikely to incorporate sufficiently into brain tissue to exert any toxic effect in this tissue. However, if CsA does cross the blood-brain barrier, as some studies suggest (Kahan, et al., 1983, Nooter, et al., 1984), slow turnover of vitamin E in CsA treated animals may again result from a regeneration or sparing effect by some other mechanism.

The kidney showed a significant loss of α-tocopherol in animals fed vitamin E deficient diets. The reduction of vitamin E in correlation with CsA treatment is consistent with CsA toxicity in the kidney having a free radical component. Morphological findings however, fail to show any tissue disruption in conjunction with CsA treatment. This finding agrees with previous studies (Kumano, et al., 1989, Walker, et al., 1990) in which toxic side

effects of CsA appear at higher doses. It is apparent that tissue vitamin E concentrations can detect a toxic reaction to CsA before functional and morphological changes become evident.

As in the kidney, the testis appears to be both sensitive to a tocopherol deficient diet and the application of CsA. Again, the observation that CsA has a direct tocopherol lowering effect in the testis is consistent with CsA toxicity having a free radical component. The morphological findings of study 1 in the testis indicate that CsA treatment at 10 mg/kg/day for 14 days and/or vitamin E deficiency results in marked alterations to tubular architecture. Disruption to both Leydig and spermatogenic cells were observed in the vitamin E deficient, CsA treated group. Vitamin E deficiency only was observed to cause disruptions to the spermatogenic cells only. Vitamin E sufficient animals displyed none of the structural disruptions of vitamin E deficient animals irrespective of CsA treatment. These findings are consistent with a previous study (Seethalakshmi, et al., 1987) carried out in adult male rats, treated with 20 mg/kg/day. Since both vitamin E deficiency and CsA treatment have been shown here to disrupt the morphology of the testis (and that a combination of the two treatments exacerbates the damage), suggesting CsA has a free radical component, it can be concluded that CsA treatment in effect produces a vitamin E deficiency and a vitamin E deficiency can make CsA doses more toxic.

The feeding of a vitamin E deficient diet to rats for 28 days, in this study, also caused morphological disruptions to the seminiferous tubules of the testis. A deficiency in vitamin E has long been known to lead to reduced reproductive ability in animals (Evans and Bishop, 1923, Mason, 1926, Mattill and Clayton, 1926, Mason, 1930, K. E. Mason, 1954). In rats, females on a vitamin E deficient diet for 2 - 3 months are able to conceive but the fetuses fail to develop and are resorbed (Evans and Bishop, 1923). In males a vitamin E deficiency causes destruction of the germinal epithelium of the testis producing malformed, nonfunctional sperm, leading to infertility (Mason, 1926). Functional analysis of the testis in the present study, reveals that a lowering of plasma testosterone coincides with vitamin E deficiency but not with CsA treatment. However, the variability in

the results was too great to permit assignment of statistical significance, even with evidence of significant disruption to Leydig cells in the CsA/vitamin E deficient group.

Although vitamin E has been known to be essential to reproductive function for many years (Evans and Bishop, 1923), the exact mechanism of its protective action is speculative. In a study by Carpenter and Howard (1974) it was shown that vitamin E deficiency lowered the activity of the hepatic P450 mixed oxygenase system. Vitamin E was suggested to have an effect on P450 activity that was not related to its antioxidant capability. The evidence suggested a role of vitamin E in regulating P450 transcription. Numerous enzymes that are responsible for testosterone production are P-450 monoxygenases (cholesterol side chain cleavage enzyme and 17 α-hydroxylase for example, see section 1.7.2). The observation in this study that vitamin E deficiency presents similar morphological disruptions as CsA treatment, suggests that CsA generates radicals and that vitamin E primary role in the testis is as an antioxidant.

Therefore, this study supports the idea that CsA has the ability to generate free radicals in the kidney and testes. In the testes CsA might uncouple electron transfer to P-450 enzymes by acting as an inhibitor of its own metabolism. It has been shown that P-450 degrades if placed in an NADPH rich environment without a substrate for the enzyme to act on (Carpenter and Howard, 1974). This would 'leak' electrons onto ambient oxygen molecules, forming superoxide ($\bullet O_2^-$). In the kidney, CsA metabolism by cytochrome P-450 3A enzymes in the proximal tubule may also generate $\bullet O_2^-$ (Serino, et al., 1991).

We believe that our finding of decreased concentrations of vitamin E in these tissues is the first *in vivo* evidence of the existence consistent with the excessive oxyradical formation in the testis during CsA administration and may be a major component of this drug's toxic actions. However, the observation that 10 mg/kg/day of CsA for 14 days failed to significantly reduce plasma testosterone levels contradicts a previous study and needs to be examined further. Also, CsA's ability to generate free radicals in other tissues was not

supported by correspondingly significant declines of vitamin E in this study, suggesting that radical generation does not occur to any great extent in these tissues.

CHAPTER 3

3.1 Study 2 : Pharmacologic levels of CsA

The goal was not to repeat the pilot study (study 1) using the same four study groups but rather to examine the effects of pharmacological doses of CsA on antioxidant status. In this study the dosage of cyclosporin was increased from 10 to 20 mg / kg body weight / day and more animals were used per group (i.e. 8 vs. 6). In the pilot study there was a detectable but statistically insignificant decrease in testosterone levels as a result of CsA treatment in vitamin E deficient rats. It was hypothesized that an increased dosage of CsA may enhance the decline in the levels of this hormone. Measurement of plasma testosterone was carried out using gas chromatography-mass spectrometry. After the 14th and final day of injections half the rats were sacrificed and the other half were maintained on their respective diets for a further 14 days. Maintaining some animals on the same diet after injections had ceased was done to determine the extent of recovery from acute CsA toxicity of vitamin E deficient versus sufficient animals. The apparent protective effect of vitamin E seen in the first study prompted the examination of both GSH and protein sulfhydryl (PSH) in tissues of animals sacrificed after the last day of injections (ie. no recovery period).

3.1.1 Experimental procedure

Weanling male Wistar rats (~3 weeks of age and 50 g in weight) were housed two per cage and placed on one of two diets. One group was fed a vitamin E sufficient diet (E^+, n = 32), containing 36 mg of d_0-RRR-α-tocopheryl acetate / kg of food per day. The other group was fed a vitamin E deficient diet (E^-, n = 32). These diets were used for the full study period of 33 and 47 days (see figure 3.1). At day 19 of the experiment the two groups were each split into 2 equal subgroups of 16 rats each. In the E^+ group, 16 animals were injected subcutaneously with cyclosporin A (CsA) at 20 mg / kg

body weight per day (E^+/CsA). The rats in the other E^+ subgroup were injected subcutaneously with a volume of the vehicle (Veh, 9:1 olive oil:ethanol) equivalent to 1 mL / kg body weight (E^+/Veh). Similarly, in the E^- group animals in one subgroup were injected subcutaneously daily with cyclosporin A at 20 mg / kg body weight per day (E^-/CsA). The other subgroup of animals were injected subcutaneously daily with vehicle (E^-/Veh). Injections of vehicle or cyclosporin A were continued daily from day 19 up to and including day 33 (i.e. for 14 days).

All animals were weighed every second day. Experimental procedures were carried out following the guidelines set out by the Canadian Council on Animal Care.

After the last injection on day 33 blood was collected from eight animals as described earlier (designated 2 week animals : $2E^+$/Veh, $2E^+$/CsA, $2E^-$/Veh, $2E^-$/CsA). Tissues and whole blood were harvested as described before (see section 2.1.1). The remaining eight animals from each group were kept on their respective diets for a further 14 days (total 47 days : designated 4 week animals : $4E^+$/Veh, $4E^+$/CsA, $4E^-$/Veh, $4E^-$/CsA).

Prior to exsanguination, cardiac puncture was performed on all rats from each group (i.e., 2 and 4 week), and approximately 5 mL of whole blood collected, separated into plasma and red blood cells, and stored at -80°C as before (see section 2.1.1).

Testes, kidneys, liver, adrenals, skeletal muscle, fat and brain were removed from all animals in each group, placed on dry ice and then stored at -80°C for later analysis. The liver, kidney, testis, adrenal, brain, fat, plasma and muscle were examined for vitamin E content in all groups (2 and 4 week treatment groups). Tissue glutathione and total protein sulfhydryl content were determined for liver, kidney, testis, adrenal, brain, and muscle in animals sacrificed after cessation of injections (2 week animals). Histological evidence of tissue damage in fixed testes tissue samples was sought using light microscopy using H and E staining as before (section 2.1.1). Serum was analysed for testosterone, leutenizing hormone (LH) and follicle stimulating hormone (FSH) in all animals.

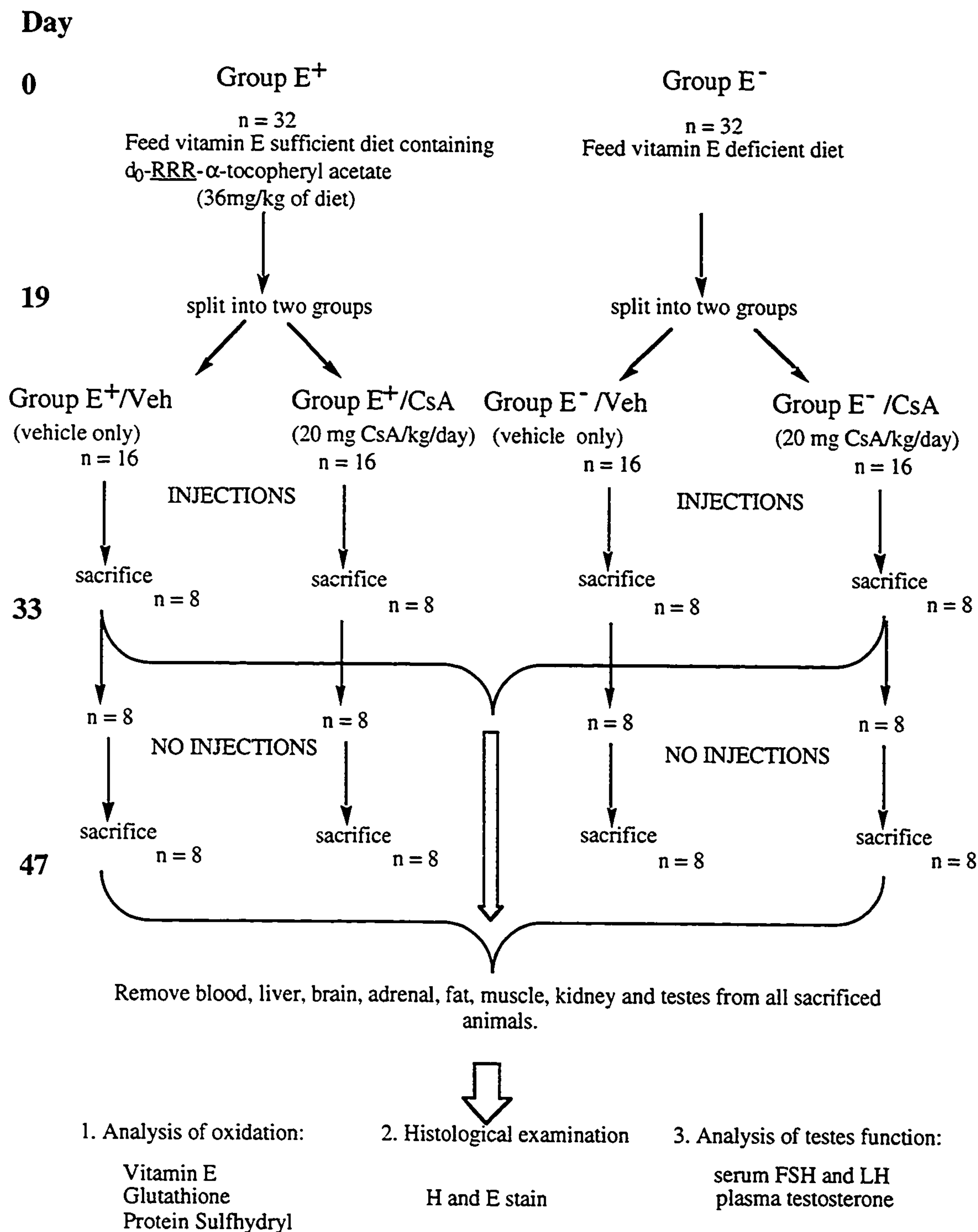


Figure 3.1 : Experimental design for study 2 to determine the effect of pharmacological doses of CsA in the presence and absence of dietary vitamin E. E^+/Veh= vitamin E sufficient diet/vehicle (9:1 olive oil:ethanol; 1 mL/kg s.c). E^+/CsA= vitamin E sufficient diet/10 mg/kg CsA in vehicle s.c. E^-/Veh= vitamin E deficient diet/vehicle (9:1 olive oil:ethanol; 1 mL/kg s.c). E^-/CsA= vitamin E deficient diet /10 mg/kg CsA in vehicle s.c. Each group consisted of 8 weanling male Wistar rats weighing 50-65 g.

Analysis of vitamin E (α-tocopherol) :

Weighed samples (0.3-0.6 g for liver, kidney, and testis; 0.1-0.3 g muscle and brain and 0.03-0.06 g for adrenal) were homogenized with 10 volumes of 50 mM phosphate buffer, pH 7.4, using a Brinkmann PT10/35 homogenizer and directly extracted for vitamin E. The vitamin E was extracted from the homogenate, purified and analyzed using the methods of Burton and Ingold as described in section 2.1.3 with the following modifications.

α-Tocopherol in fat was extracted using an alkaline hydrolysis procedure as follows: to 0.05-0.12 g of fat was added 300-500 µL of saturated aqueous potassium hydroxide (KOH), 2 mL of distilled water, 2 mL 95% ethanol, 100 mg sodium ascorbate and 25 µL of d_9-α-tocopherol standard (0.157 µM) in a 15 mL glass vial equipped with a teflon-lined screw cap. The mixture was heated at 75°C for 30 minutes. The solution was allowed to cool before 1 mL of distilled water and 2 mL of n-heptane were added to extract the α-tocopherol. The heptane fraction was blown to dryness under nitrogen, redissolved in 600 µL of n-heptane and injected onto the HPLC (see section 2.1.3).

After collection from the HPLC the samples were silylated for better peak shape, sensitivity and resolution of concentrated samples (i.e. less tailing of the peaks indicative of sample retention on the GC column) and injected onto the GC-MS column. To silylate the samples, 100 µL of silylation grade pyridine and 50 µL of N, O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) was added to the sample vials. The solution was then heated at 60°C for 20 minutes, cooled and blown to dryness under nitrogen and redissolved in 60 µL of heptane for injection onto the GC-MS. The 5970 mass spectrometer was set to detect 502 (d_0) and 511 (d_9) parent molecular ions. The relative proportions of d_0-α-tocopherol to d_9-α-tocopherol were measured to determine the quantity of endogenous α-tocopherol (d_0-α-T) in the tissue sample (expressed in nmol/g tissue) as per the following calculation:

$$d_0\text{-}\alpha T = \frac{(\%d_0 / \%d_9) \times \text{nmol } d_9 \text{ standard added})}{\text{sample weight (g tissue / mL)}}$$

3.1.3 Tissue sulfhydryl and glutathione analysis :

Tissue (weighing ~ 200 mg) was homogenized with 3 mL of 50 mM phosphate buffer, pH 7.4, using a Brinkmann PT10/35 homogenizer. Samples were then spun at 16,000 rpm for 20 minutes at 4°C. One milliliter aliquots of supernatant were stored at -80°C for analysis of glutathione (GSH), protein sulfhydryl (PSH), and total tissue sulfhydryl content (TSH) using the method by Sedlack (1968). To express these values as nmol/mg protein, the total protein concentration of the tissue was determined using the Bradford assay.

Determination of total protein in sample supernatant :

50 µL of the prepared supernatant was immediately diluted 20 fold by making the volume of the aliquot up to 1 mL with distilled water. Five microlitres of the diluted supernatant was treated with 200 µL of Coomasie blue stain (Biorad protein stain) and 800µL of distilled water and left to stand at room temperature for 10 minutes. The absorbance of the mixture was then read at 595 nm. A standard protein concentration curve plotting absorbance versus concentration (µg/mL) was performed using bovine serum albumin at concentrations of 2.5, 5, 7.5, and 10 µg/mL by reacting 5, 10, 15 and 20 µL of a 0.5 mg/mL protein solution with 0.8 mL of distilled water mixed with 200 µL of the Coomasie blue stain, standing for 10 minutes at room temperature and then reading the absorbance at 595 nm. Absorbances for the unknown samples were determined from the standard curve using the linear least squares fit.

Total SH (TSH) content of the supernatant :

To account for the absorbance of the solution buffer (6 M guanidine HCl, 50 mM Tris HCl pH 8.2) a designated "blank" (O) cuvette was filled with 950 µL of solution buffer, in order to zero the spectrophotometer.

As a control for background absorption of dithiobisnitrobenzoic acid (DTNB), a control vial (**C**) was filled with 950 µL of the solution buffer and 1 mM DTNB solution only.

A solution of 950 mL of solution buffer with 50 mL of supernatant, prepared above, was read as a background cuvette (**B**) to control for absorption of the unreacted sample. The sample (**S**) was prepared by adding 50 mL of the prepared supernatant to a solution of 950 mL of 6 M guanidine HCl, 50 mM Tris HCl pH 8.2 and 1 mM DTNB. All vials were left to sit at room temperature for 30 minutes. After using the blank cuvette to zero the spectrophotometer at 412 nm, the control, background and sample cuvettes were read at 412 nm for absorbance.

Absorbance due to thiol groups was determined by the equation:

Absorbance = Sample (S) - Blank (B) - control (C)
due to SH in supernatant reacting with DTNB.

Next the concentration, **c**, of total thiol groups in the cytosol was determined from:

A= e c l where

A = absorbance at 412 nm

e = 13,600 M^{-1} cm^{-1}

c = concentration (M)

l = light path length through the sample (1 cm)

GSH content of tissue cytosolic fraction :

For determination of glutathione content in the prepared supernatant 300 μL of the supernatant was reacted with 15 μL of 100 % trichloro acetic acid (TCA, final concentration ~ 5 %) to precipitate cytosolic proteins. The sample was then centrifuged at 35,000 x g at 4°C for 8 minutes. 250 μL of the supernatant was then reacted with 25 μL of 25 % NaOH (final concentration ~ 2.5 %). Finally 100 μL of this solution was added to 900 μL of 0.1 mM DTNB, 2M Tris HCl, pH 8.2. This sample was measured for absorbance at 412 nm against a blank of 900 μL of 0.1 mM DTNB, 2M Tris HCl, pH 8.2. The concentration, **c**, of GSH was determined by :

A= e c l where

A = absorbance at 412 nm

e = 13,600 M^{-1} cm^{-1}

c = concentration (M)

l = light path length through the sample (1 cm)

Determination of protein sulfhydryl content :

Protein sulfhydryl content is simply obtained by the subtraction of GSH (in mmol / g tissue) from total SH (TSH) content of the supernatant. The values are expressed in either mmol / g tissue or in mmol / mg protein.

3.1.4 GC-MS analysis of plasma testosterone

The procedure used was adapted from that of S. A. Wudy et al. (1992). Plasma (0.2 mL) was incubated with 100 μL trideutero-testosterone (d_3-testosterone, 1.58 nmol/mL) for 30 min at 37°C. The solution was then extracted with 2 x 1 mL of a dichloromethane : hexane (1:2) solution. This solution was vortexed for 1 minute and the organic extract was centrifuged at 3000 rpm for 6 minutes to sediment out any remaining

solids. The supernatant was collected and evaporated under N_2. The sample was redissolved in 200 µL of cyclohexane : ethanol (9:1).

A Sephadex LH20 column (20 µm pore size, 1 mL of resin packed into pasteur pipette, 0.5 cm i.d. x 4 cm) was preconditioned by passage of 5 mL of cyclohexane : ethanol (9:1) mobile phase. The impure testosterone sample (200 mL) was placed on the column and a total of 5 mL of mobile phase was passed through it. The first 1 mL of eluent was discarded and the next 4 mL was collected and evaporated under a stream of N_2. The residue was derivatized by adding 100 µL acetonitrile and 20 µL heptafluorobutyric anhydride (HFBA) and left standing at room temperature for 1 hour. The solvent was then evaporated under a stream of N_2 and the residue redissolved in 50 µL ethyl acetate. One µL of this solution was injected onto a GC-MS operating under the following conditions:

The injection port was set at 300°C and programmed for splitless injection (i.e. the entire sample was injected onto the column). The column was a Hewlett Packard Ultra 1 (cross-linked methyl silicone gum) of dimensions 12 m x 0.2 mm (I.D.) with a 0.33 mm film thickness.

At the time of injection the column was set at 50°C for 6 minutes and then increased at 30°C /minute until reaching 230°C and held there for 2 minutes. The column temperature was then increased at 3°C/minute to 290°C and held there for 10 minutes. The mass spectrometer was set for single ion monitoring (SIM) for ions 680 and 683 (corresponding to d_0 and d_3 testosterone-HFBA derivatives respectively).

3.1.5 Plasma FSH and LH

Plasma levels of FSH and LH were determined independently using radioimmunoassay (RIA) at the diagnostic laboratories of the Ottawa General Hospital.

3.2 Materials :

All materials were obtained from the same sources as in the pilot study (see section 2.2) with the following additions: HPLC grade pyridine and N, O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS), bovine

serum albumin (BSA), guanidine HCl, dithiobisnitrobenzoic acid (DTNB), trichloroacetic acid (TCA), acetonitrile, cyclohexane, heptafluorobutyric anhydride (HFBA) and d_0- and d_3-testosterone were all obtained from Sigma Chemical Company. (St. Louis, Mo). The Bradford protein assay was obtained as a kit from Biorad (Bio-Rad Protein Assay Kit II, Hercules, CA, USA).

3.3 Results : Effect of 20 mg/kg CsA on body and tissue weights

In comparison to the previous study where rats were given 10 mg/kg/day of CsA, treatment of weanling male Wistar rats with 20 mg/kg/day of CsA for 14 days (E^+/CsA and E^-/CsA) caused a significant reduction in weight gain compared to dietary controls (E^+/Veh and E^-/Veh respectively, figure 3.2A and B, $p<0.05$). Compared to control group animals, the rate of weight gain in CsA treated groups was less in the two weeks following CsA treatment (see figures 3.2B and 3.3). However, vehicle treated animals also exhibited a reduced growth rate in the two weeks following injections of vehicle (figure 3.3). Figure 3.3 shows that during the first 19 days on diets only all groups showed similar weight gain (approximately 140 g). However, over the next 14 days (to day 33), CsA treated groups (E^+/CsA and E^-/CsA), showed significant reductions in weight gain in comparison with their dietary controls (E^+/Veh and E^-/Veh respectively, $p<0.05$). Although animals in the control groups (E^+/Veh and E^-/Veh) left for a further 14 days on diets only (to day 47), showed significant reductions in weight gain, the CsA treated groups over this same time period still showed significantly lower growth rates than the controls after the CsA injections were stopped. Vitamin E deficiency or supplementation had no observable effects on growth rate during or after CsA treatment (figures 3.2B and 3.3).

Values in table 3.1 show that two weeks of CsA treatment at 20 mg/kg/day for 14 days reduced final testis weight of E^+/CsA and E^-/CsA groups, a difference that was not recovered when CsA treatment was stopped. In the kidney, organ weight was significantly reduced in the E^-/CsA treatment group compared its treatment (E^-/Veh) and dietary (E^+/CsA) control after the 14 day recovery period. Vitamin E deficiency and sufficiency had no effect on CsA induced organ weight loss with the exception of kidney in the 4 week group.

3.4 Results : Effect of 20 mg/kg CsA on serum FSH and LH

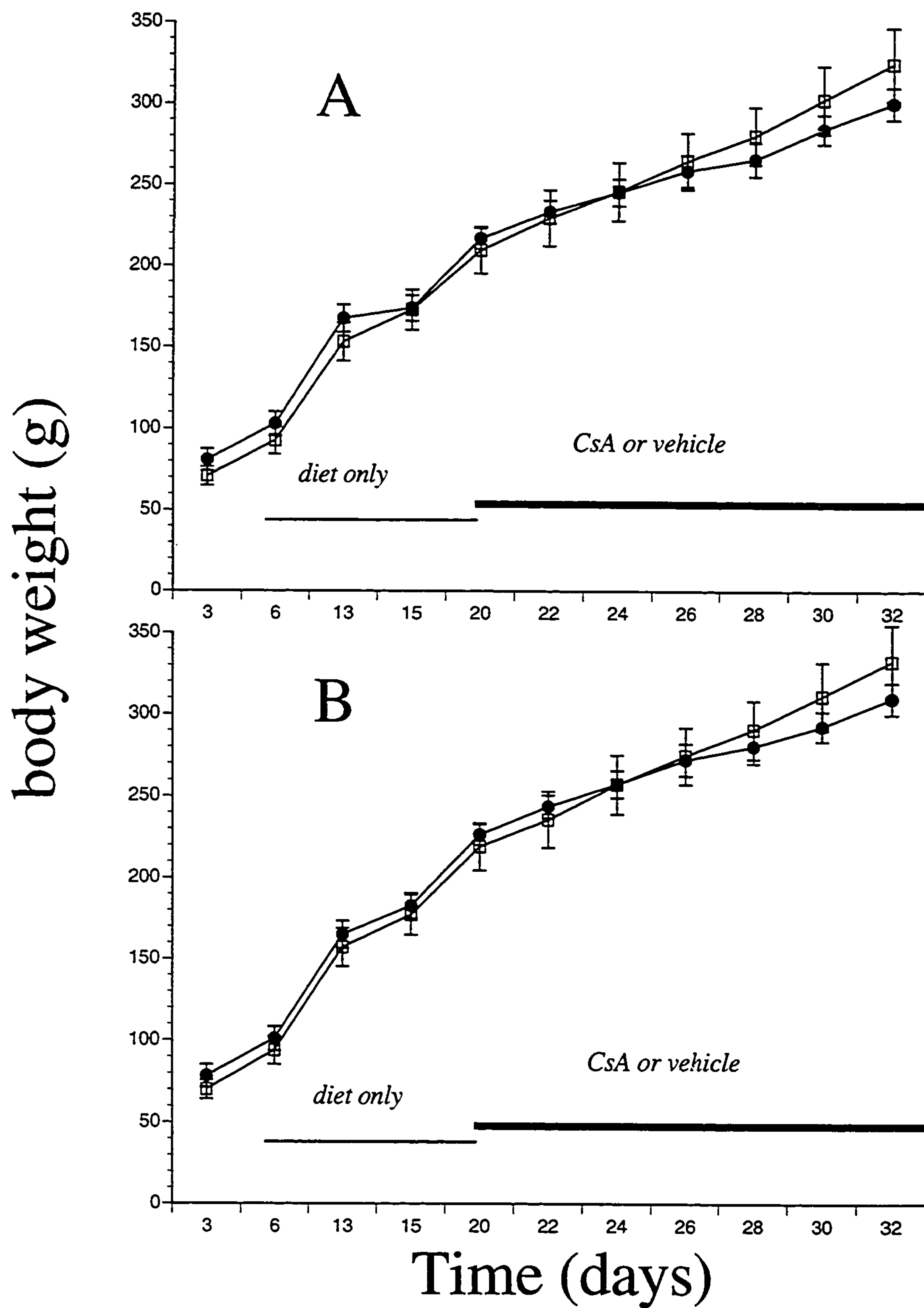
Animals sacrificed after the 14 days of CsA or vehicle treatment (see figure 3.4) showed increases in both FSH and LH with CsA treatment as compared to their vehicle controls, however only the FSH increase in the E^+/CsA group was significant ($p<0.001$). Vitamin E sufficiency or deficiency had no effect on plasma FSH and LH levels.

3.5 Results : Effect of 20 mg/kg CsA on tissue and plasma vitamin E

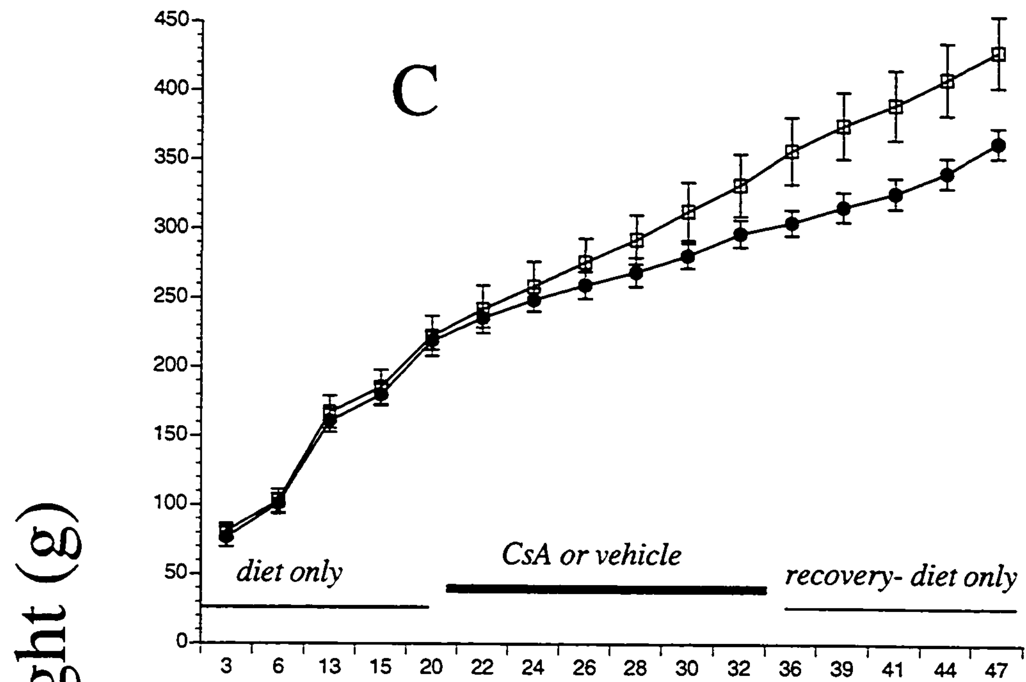
Rats sacrificed after CsA treatment of 20 mg/kg/day for 14 days (figure 3.5 and 3.7A) revealed significant reductions in vitamin E in the kidney, testes and brain of the E^+/CsA group as compared to its vehicle control (E^+/Veh). The liver showed no changes in vitamin E concentration with CsA treatment when compared to vehicle control. Fat, adrenal gland and muscle all showed increases in vitamin E in the E^+/CsA group as compared to E^+/Veh. Vitamin E increased in the E^-/CsA group for brain, adrenal gland, fat, and plasma as compared to E^-/Veh. All tissues and plasma exhibited significant reductions in vitamin E when fed vitamin E deficient diets (E^-/Veh and E^-/CsA), as compared to their treatment counterparts fed vitamin E sufficent diets (E^+/Veh and E^+/CsA respectively).

In animals allowed 14 days to recover from CsA treatment, vitamin E concentrations were significantly higher in the E^-/CsA group for kidney and fat tissue as compared to the E^-/Veh treatment group (figure 3.6). Plasma vitamin E concentration in the E^+/CsA group and the E^-/CsA group were significantly higher and lower, respectively, compared to their vehicle controls (figure 3.7B). The testis, brain, liver, and adrenal gland exhibited no differences in vitamin E concentration between the CsA treated groups (E^+/CsA and E^-/CsA) and their dietary control groups (E^+/Veh and E^-/Veh respectively) after recovery. All tissues and plasma exhibited significant reductions in vitamin E when fed deficient diets as compared to animals fed vitamin E sufficient diets.

Figure 3.2: Body weights of male Wistar rats given (A) vitamin E sufficient diets and treated with vehicle (Veh; 9:1 olive oil:ethanol; 1 ml/kg/day s.c.), or Cyclosporin A (CsA; 20 mg/kg/day s.c.) for 14 days. (B) vitamin E deficient diets treated with vehicle (Veh; 9:1 olive oil:ethanol; 1 ml/kg/day s.c.), or Cyclosporin A (CsA; 20 mg/kg/day s.c.) for 14 days. (C) vitamin E sufficient diets and treated with vehicle (Veh; 9:1 olive oil:ethanol; 1 ml/kg/day s.c.), or Cyclosporin A (CsA; 20 mg/kg/day s.c.) for 14 days with an additional 14 day recovery period without treatment. (D) vitamin E deficient diets treated with vehicle (Veh; 9:1 olive oil:ethanol; 1 ml/kg/day s.c.), or Cyclosporin A (CsA; 20 mg/kg/day s.c.) for 14 days and with an additional 14 day recovery period without treatment. Values obtained from 8 animals per group are expressed as mean ± SEM. After day 28 for all graphs (A-D) body weights of CsA treated animals (black circles) were significantly lower ($p<0.05$) compared to their vehicle treated diet control counterparts (squares).



A
B
body weight (g)
Time (days)
diet only
CsA or vehicle
0
50
100
150
200
250
300
350
3
6
13
15
20
22
24
26
28
30
32


C
450
400
350
300
250
200
150
100
50
0
diet only
CsA or vehicle
recovery- diet only
3 6 13 15 20 22 24 26 28 30 32 36 39 41 44 47
body weight (g)

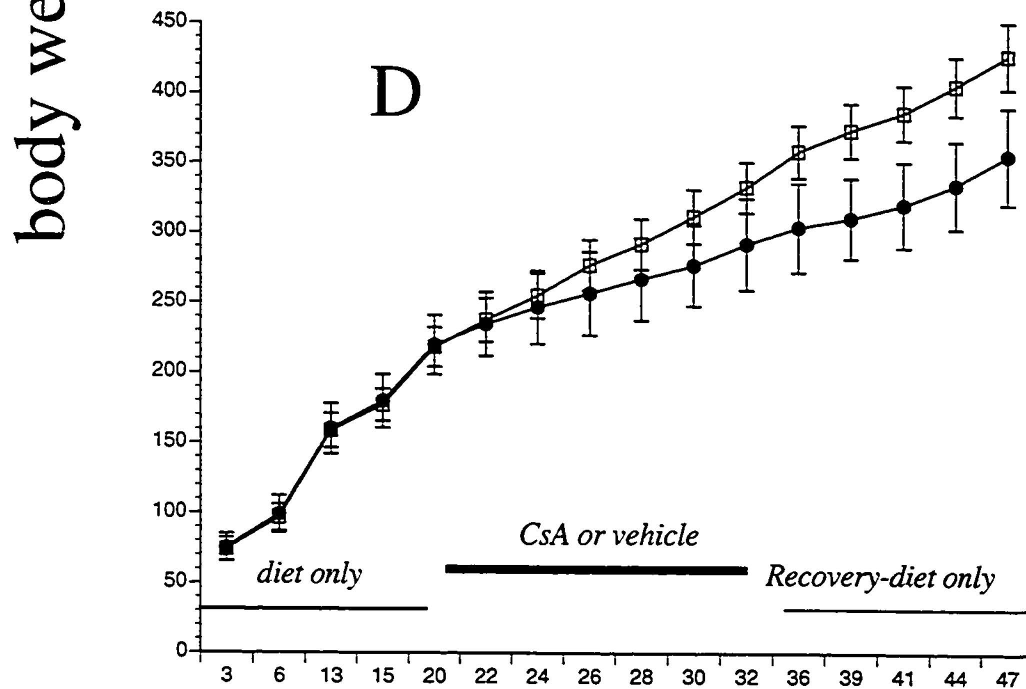
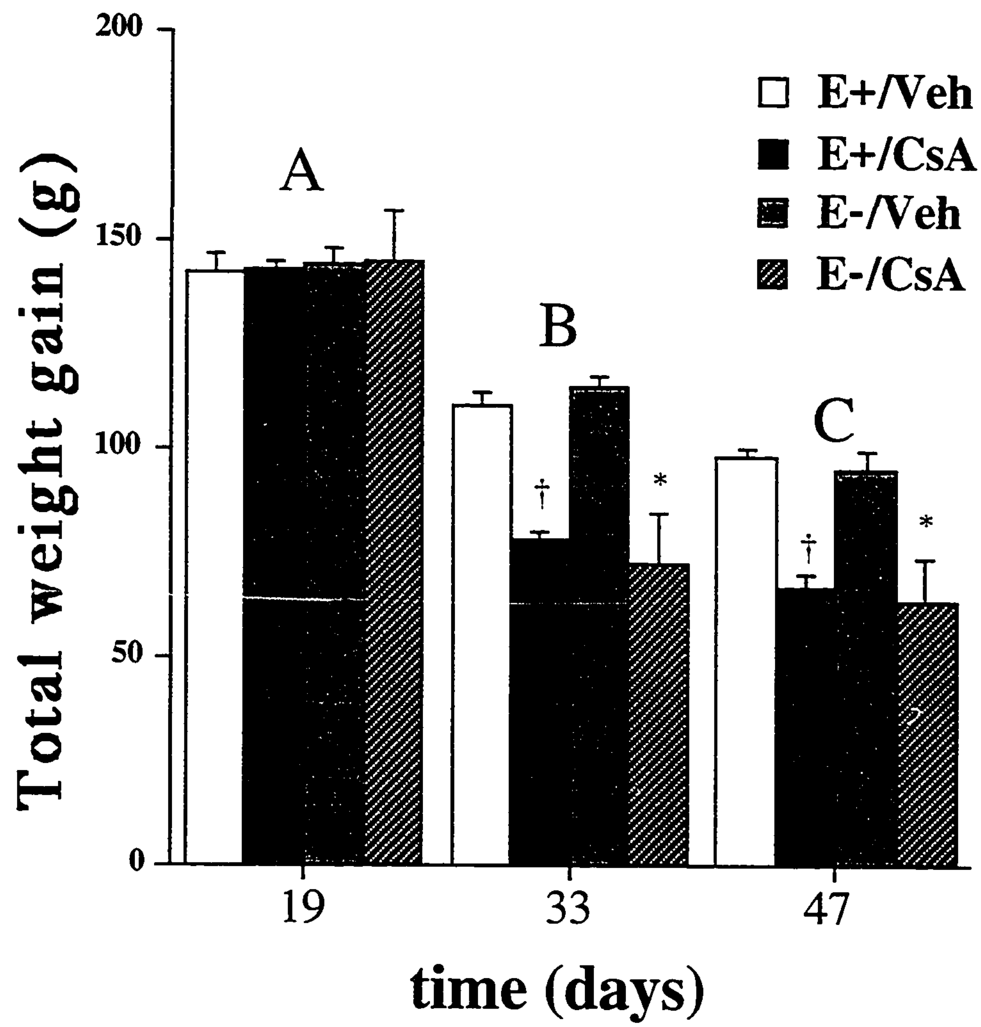

D
450
400
350
300
250
200
150
100
50
0
diet only
CsA or vehicle
Recovery-diet only
3 6 13 15 20 22 24 26 28 30 32 36 39 41 44 47
Time (days)

Figure 3.3: Total weight gain in male Wistar rats (from 4 week group, see figure 3.2B) during (A) the first 19 days on diets only followed by (B) 14 days of treatment with vehicle (Veh; 9:1 olive oil:ethanol; 1 ml/kg/day s.c.) or Cyclosporin A (CsA; 20 mg/kg/day s.c.) to day 33, and (C) a further 14 days of recovery without injections of CsA. Treatment groups listed in the legend include those placed on a vitamin E sufficient diet with either Veh or CsA treatment (E^+/Veh or E^+/CsA respectively) or a vitamin E deficient diet with either Veh or CsA treatment (E^-/Veh or E^-/CsA respectively). Values obtained from 8 animals per group are expressed as mean ± SEM. Obelisks and asterisks denote statistical significance ($p<0.05$) when compared to the E^+/Veh (†) or the E^-/Veh (*) group.



Total weight gain (g)
200
150
100
50
0
A
B
C
E+/Veh
E+/CsA
E-/Veh
E-/CsA
†
*
19
33
47
time (days)

Table 3.1: Wet weights of testis and kidney organs following treatment with vehicle (Veh; 9:1 olive oil:ethanol; 1 ml/kg/day s.c.) or Cyclosporin A (CsA; 20 mg/kg/day s.c.) for 14 days (2 week tissue weight) and after an additional 14 days of recovery without treatment (4 week tissue weight). Treatment groups include those placed on a vitamin E sufficient diet with either Veh or CsA treatment (E^+/Veh or E^+/CsA respectively) or a vitamin E deficient diet with either Veh or CsA treatment (E^-/Veh or E^-/CsA respectively). Values obtained from 8 animals per group are expressed as mean ± SEM. Obelisks and asterisks denote statistical significance ($p<0.05$) when compared to the E^+/Veh (†) or the E^-/Veh (*) group, respectively.

Treatment Groups	2 week tissue weight (g) (day 33)		4 week tissue weight (g) (day 47)	
	testis	**kidney**	**testis**	**kidney**
E^+/Veh	1.52 ± 0.04	1.26 ± 0.05	1.77 ± 0.07	1.55 ± 0.05
E^+/CsA	1.33 ± 0.02†	1.22 ± 0.05	1.55 ± 0.02†	1.54 ± 0.06
E^-/Veh	1.54 ± 0.04	1.28 ± 0.03	1.75 ± 0.04	1.58 ± 0.04
E^-/CsA	1.36 ± 0.02*	1.34 ± 0.04	1.52 ± 0.04*	1.36 ± 0.03*

Figure 3.4: Plasma levels of follicle stimulating hormone (FSH; A) and luteinizing hormone (LH; B) following treatment with vehicle (Veh; 9:1 olive oil:ethanol; 1 ml/kg/day s.c.) or Cyclosporin A (CsA; 20 mg/kg/day s.c.) for 14 days. Treatment groups listed in the legend include those placed on a vitamin E sufficient diet with either Veh or CsA treatment (E^+/Veh or E^+/CsA respectively) or a vitamin E deficient diet with either Veh or CsA treatment (E^-/Veh or E^-/CsA respectively). Values obtained from 8 animals per group are expressed as mean ± SEM. Obelisks and asterisks denote statistical significance ($p<0.05$) when compared to the E^+/Veh (†) or the E^-/Veh (*) group, respectively.

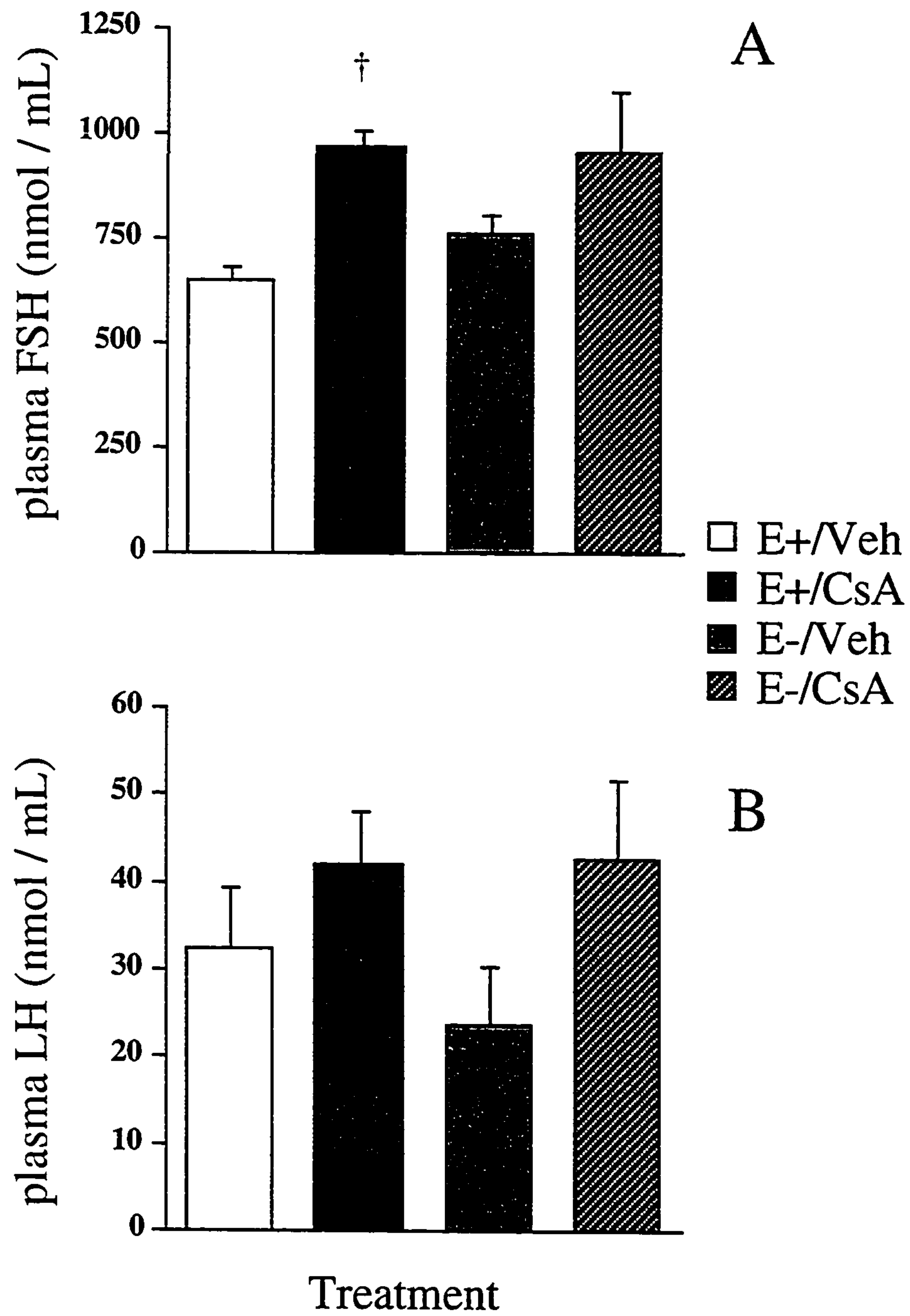
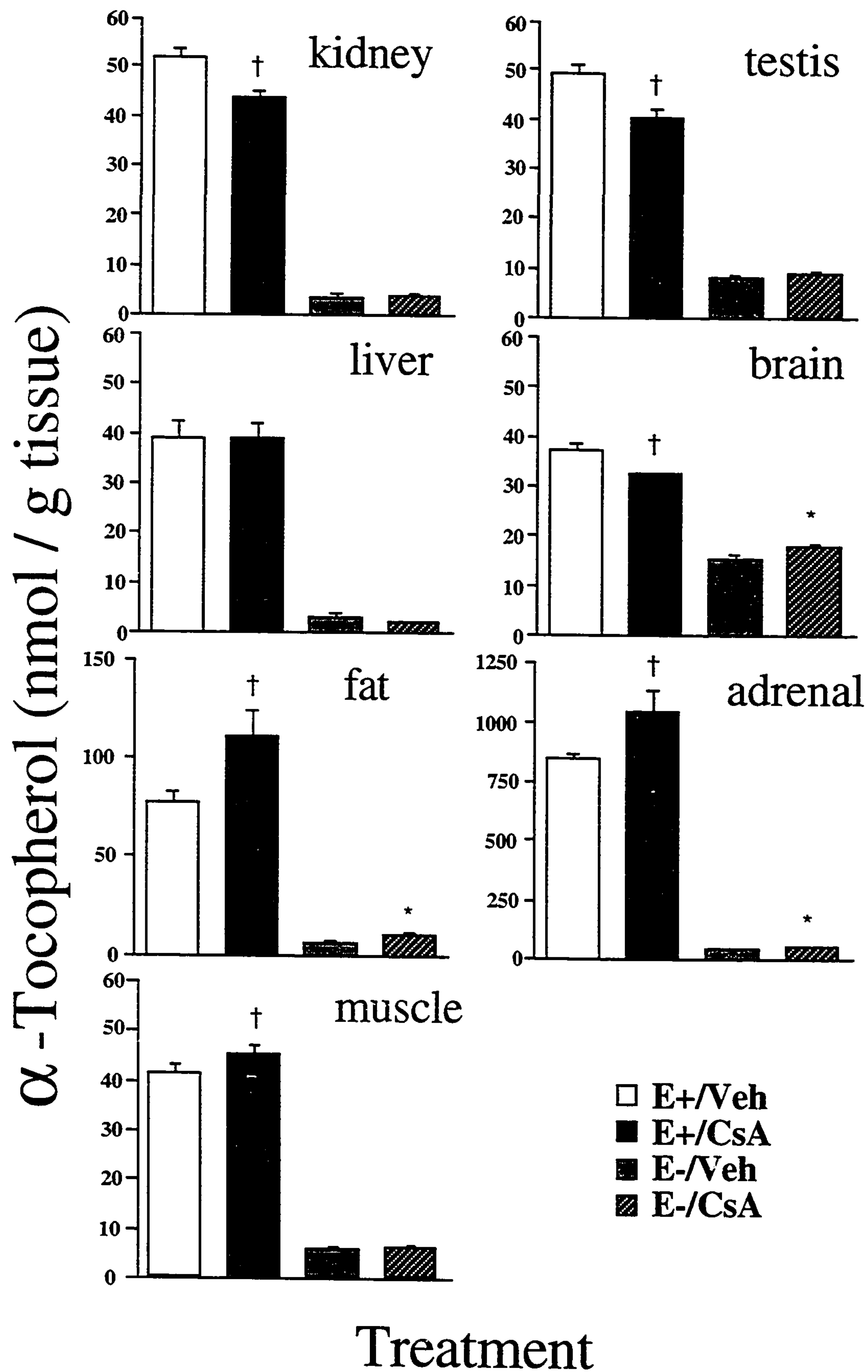

A
B
plasma FSH (nmol / mL)
plasma LH (nmol / mL)
1250
1000
750
500
250
0
60
50
40
30
20
10
0
†
E+/Veh
E+/CsA
E-/Veh
E-/CsA
Treatment

Figure 3.5: α-Tocopherol levels in kidney, testis, liver, brain, fat, adrenal gland and muscle of male Wistar rats following treatment with vehicle (Veh; 9:1 olive oil:ethanol; 1 ml/kg/day s.c.) or Cyclosporin A (CsA; 20 mg/kg/day s.c.) for 14 days. Treatment groups listed in the legend include those placed on a vitamin E sufficient diet with either Veh or CsA treatment (E^+/Veh or E^+/CsA respectively) or a vitamin E deficient diet with either Veh or CsA treatment (E^-/Veh or E^-/CsA respectively). Values obtained from 8 animals per group are expressed as mean ± SEM. Obelisks and asterisks denote statistical significance ($p<0.05$) when compared to the E^+/Veh (†) or the E^-/Veh (*) group, respectively.



α -Tocopherol (nmol / g tissue)
kidney
testis
liver
brain
fat
adrenal
muscle
E+/Veh
E+/CsA
E-/Veh
E-/CsA
Treatment

3.6 Results : Effect of 20 mg/kg CsA on tissue glutathione (GSH)

Glutathione, an important antioxidant molecule known to be the principal substrate for glutathione peroxidases was measured to determine its possible involvement in the free radical component of CsA toxicity. Treatment of weanling male Wistar rats with 20 mg/kg/day of CsA for 14 days in combination with a vitamin E deficient diet (E^-/CsA) significantly increased GSH content (figure 3.8) as compared to vehicle controls (E^-/Veh) in the liver, brain and kidney, and compared to E^+/CsA in the liver and kidney. CsA treated animals sufficient in vitamin E (E^+/CsA) showed no significant changes in GSH concentrations for the kidney, testis, liver and brain. Testis GSH decreased significantly in the E^-/CsA group as compared to its diet control and muscle showed significant decreases in GSH content for both CsA treated groups as compared to vehicle controls.

3.7 Results : Effect of 20 mg/kg CsA on tissue protein sulfhydryl (PSH)

Examination of protein sulfhydryl (PSH) content (an indicator of protein oxidation) revealed treatment with CsA at 20 mg/kg/day for 14 days in vitamin E sufficient rats (E^+/CsA) had no modulating effect on PSH in the kidney, testes, brain or muscle compared to control (E^+/Veh) (figure 3.9). However, PSH was increased in E^+ rats treated with CsA (E^+/CsA) in the liver as compared to control (E^+/Veh). In vitamin deficient rats treated with CsA (E^-/CsA) at 20 mg/kg/day for 14 days, liver and brain displayed increases in PSH content compared to controls (E^-/Veh). The kidney, testes, and muscle of E^-/CsA groups showed reduced PSH concentrations compared to E^-/Veh groups.

Figure 3.6: α-Tocopherol levels in kidney, testis, brain, liver, adrenal gland and fat of male Wistar rats following treatment with vehicle (Veh; 9:1 olive oil:ethanol; 1 ml/kg/day s.c.) or Cyclosporin A (CsA; 20 mg/kg/day s.c.) for 14 days with an additional 14 day recovery period without treatment. Treatment groups listed in the legend include those placed on a vitamin E sufficient diet with either Veh or CsA treatment (E^+/Veh or E^+/CsA respectively) or a vitamin E deficient diet with either Veh or CsA treatment (E^-/Veh or E^-/CsA respectively). Values obtained from 8 animals per group are expressed as mean ± SEM. Obelisks or asterisks denote statistical significance ($p<0.05$) when compared to the E^+/Veh (†) or the E^-/Veh (*) group, respectively.

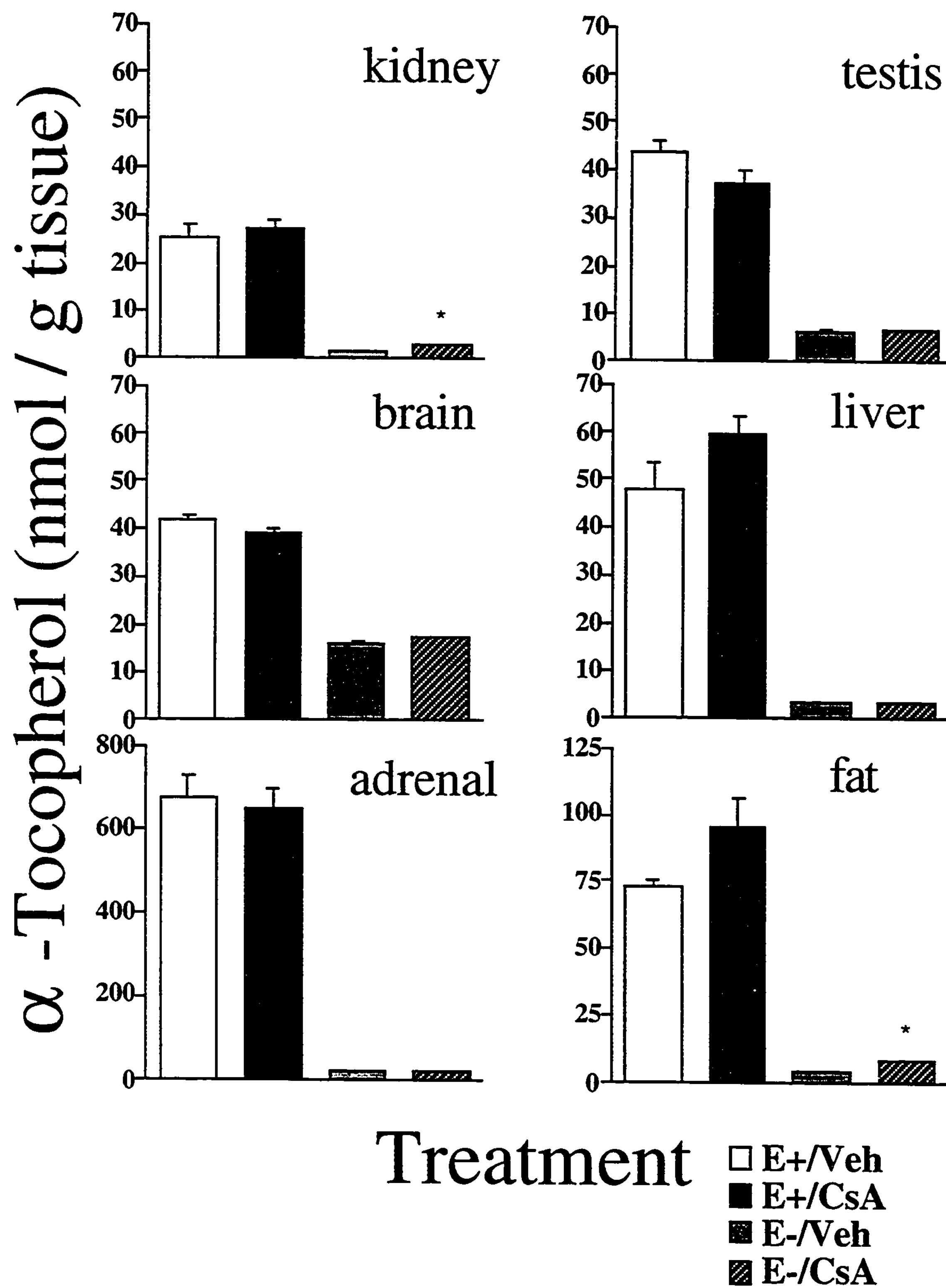
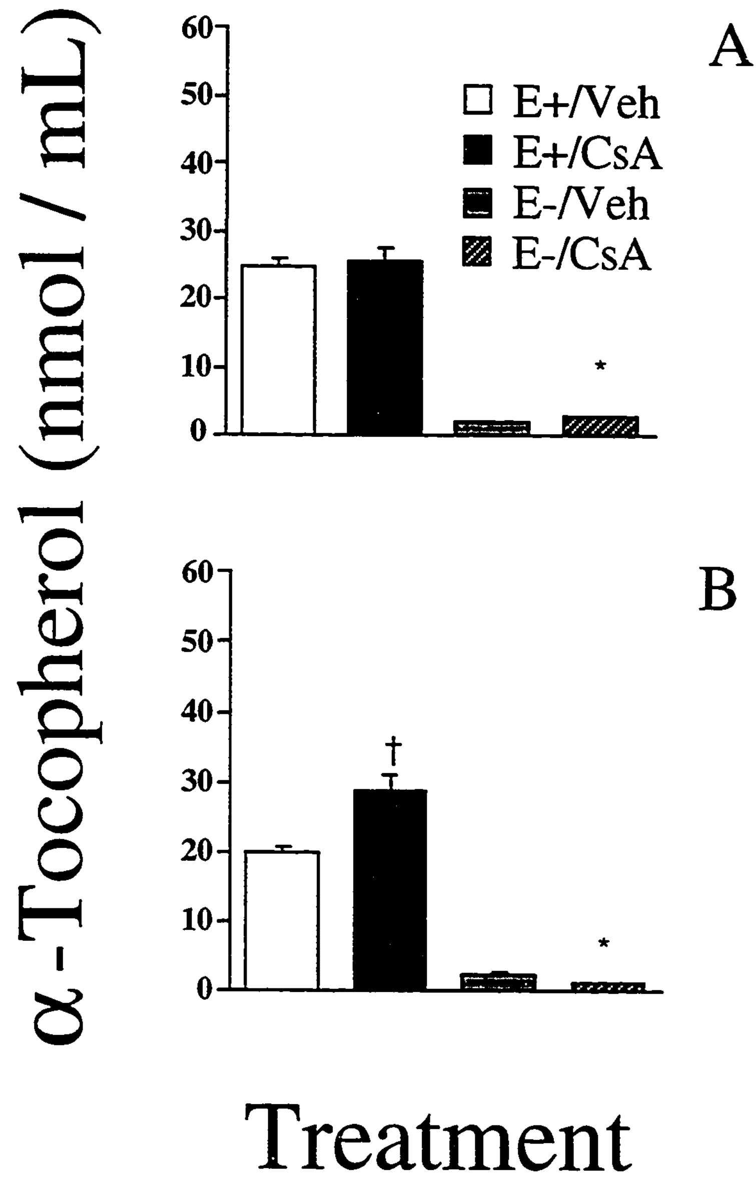
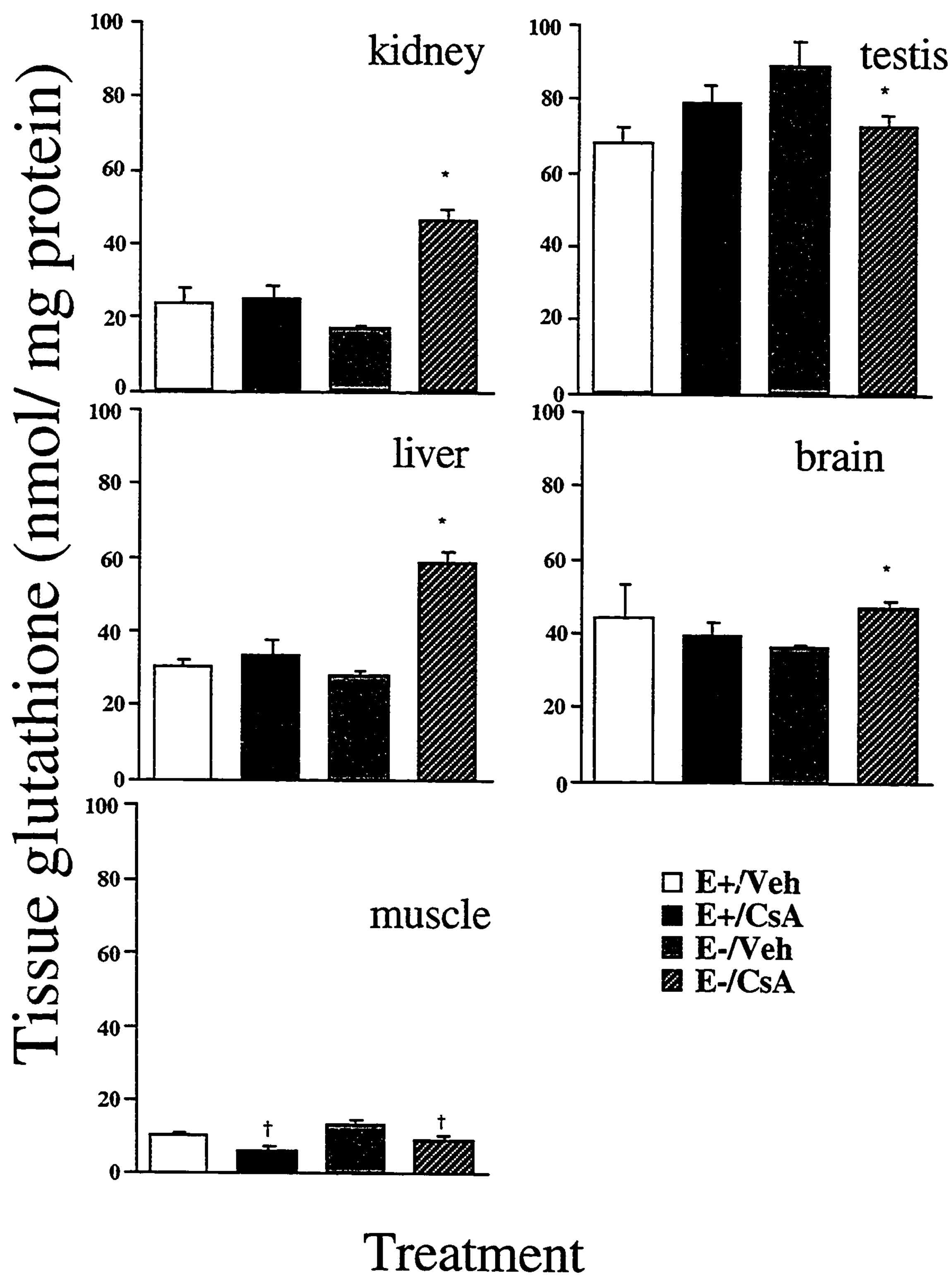

α -Tocopherol (nmol / g tissue)
kidney
testis
brain
liver
adrenal
fat
Treatment
E+/Veh
E+/CsA
E-/Veh
E-/CsA

Figure 3.7: Plasma α-tocopherol levels in male Wistar rats following treatment with vehicle (Veh; 9:1 olive oil:ethanol; 1 ml/kg/day s.c.) or Cyclosporin A (CsA; 20 mg/kg/day s.c.) for 14 days (A; 2 week plasma) or with an additional 14 day recovery period without treatment (B; 4 week plasma). Treatment groups listed in the legend include those placed on a vitamin E sufficient diet with either Veh or CsA treatment (E^+/Veh or E^+/CsA respectively) or a vitamin E deficient diet with either Veh or CsA treatment (E^-/Veh or E^-/CsA respectively). Values obtained from 8 animals per group are expressed as mean ± SEM. Obelisks and asterisks denote statistical significance ($p<0.05$) when compared to the E^+/Veh (†) or the E^-/Veh (*) group, respectively.



α-Tocopherol (nmol / mL)
A
B
E+/Veh
E+/CsA
E-/Veh
E-/CsA
60
50
40
30
20
10
0
*
†
Treatment

Figure 3.8: Glutathione levels in kidney, testis, liver, brain and muscle of male Wistar rats following treatment with vehicle (Veh; 9:1 olive oil:ethanol; 1 ml/kg/day s.c.) or Cyclosporin A (CsA; 20 mg/kg/day s.c.) for 14 days. Treatment groups listed in the legend include those placed on a vitamin E sufficient diet with either Veh or CsA treatment (E^+/Veh or E^+/CsA respectively) or a vitamin E deficient diet with either Veh or CsA treatment (E^-/Veh or E^-/CsA respectively). Values obtained from 8 animals per group are expressed as mean ± SEM. Obelisks or asterisks denote statistical significance ($p<0.05$) when compared to the E^+/Veh (†) or the E^-/Veh (*) group, respectively.


Tissue glutathione (nmol/ mg protein)
kidney
testis
liver
brain
muscle
Treatment
E+/Veh
E+/CsA
E-/Veh
E-/CsA
0
20
40
60
80
100

3.8 Results : Effect of 20 mg/kg CsA on testis morphology

H and E stained sections of testis from control (vehicle treated) rats fed a vitamin E sufficient diet showed morphologically normal seminiferous tubules with spermatogenic cell types and interstitial (Leydig) cells (figure 3.10A). CsA treatment of rats at 20 mg/kg/day fed a vitamin E sufficient diet for 14 days resulted in the formation of vacuoles in spermatocytes and degeneration of Leydig cells (figure 3.10B) as was observed in the 10 mg/kg/day CsA treated testes of study 1 (see figure 2.4B). However, a further finding of vacuolar spaces between spermatids was only observed in testis from rats treated with the higher dose of CsA.

As in study 1, the seminiferous tubules of testes from vehicle treated control rats fed a vitamin E deficient diet also showed evidence of vacuole formation in spermatocytes, with histologically normal Leydig cells (figure 3.10C). More severe damage to tubular structure was observed in testes of 20 mg/kg/day CsA treated rats fed a vitamin E deficient diet. Exaggerated vacuole formation resulted in the separation of spermatogenic cells between and within the different layers and reduction in thickness of the spermatocyte and spermatid layers (figure 3.10D). Compared to the vehicle treated, vitamin E deficient control group in Figure 3.10C, there was a loss of interstitial cells.

At higher magnification the vacuoles observed in the spermatocytes and between spermatids in the testes of 20 mg/kg/day CsA treated rats fed a vitamin E sufficient diet (figure 3.11B) or vitamin E deficient diet (figure 3.12B) could be seen to be associated with a separation of these cells from Sertoli cells when compared to the vehicle control counterparts (figure 3.11A and 3.12A respectively).

The histological appearance of stained testes from rats treated with vehicle or 20 mg/kg/day CsA for 14 days and allowed an additional 14 days to recover on a vitamin E sufficient diet was similar to their counterparts in the non recovery (14 day treatment) group (not shown). Similar morphological findings were also observed in the vehicle treated, vitamin E deficient animals allowed a 2-week recovery period compared to its counterpart in

the non recovery group (not shown). However, the most severe disruption to tubular architecture was observed in the CsA treated, vitamin E deficient recovery group. Compared to the non recovery counterpart (figure 3.13A), vacuolar spaces spanned an entire radius of the seminiferous tubule (figure 3.13B). Sertoli cells were no longer discernible and there was an obvious loss of all spermatogenic cell types. As a result, organization of the spermatogenic cell development phases from the basement membrane to the tubular lumen was disrupted. Further, Leydig cells and the interstitium have been disrupted.

3.9 Results : Effect of 20 mg/kg CsA on plasma testosterone

CsA treatment combined with a vitamin E deficient diet (E^-/CsA) reduced plasma testosterone levels significantly as compared to dietary control (E^-/Veh) in the non-recovery group (figure 3.14A). In the recovery group (figure 3.14B) a significant reduction in plasma testosterone was maintained in the E^-/CsA group compared to the E^+/Veh group only. Vitamin E deficiency had no effect on testosterone levels.

Figure 3.9: Protein Thiol (PSH) levels in kidney, testis, liver, brain and muscle of male Wistar rats following treatment with vehicle (Veh; 9:1 olive oil:ethanol; 1 ml/kg/day s.c.) or Cyclosporin A (CsA; 20 mg/kg/day s.c.) for 14 days. Treatment groups listed in the legend include those placed on a vitamin E sufficient diet with either Veh or CsA treatment (E^+/Veh or E^+/CsA respectively) or a vitamin E deficient diet with either Veh or CsA treatment (E^-/Veh or E^-/CsA respectively). Values obtained from 8 animals per group are expressed as mean ± SEM. Obelisks and asterisks denote statistical significance ($p<0.05$) when compared to the E^+/Veh (†) or the E^-/Veh (*) group, respectively.

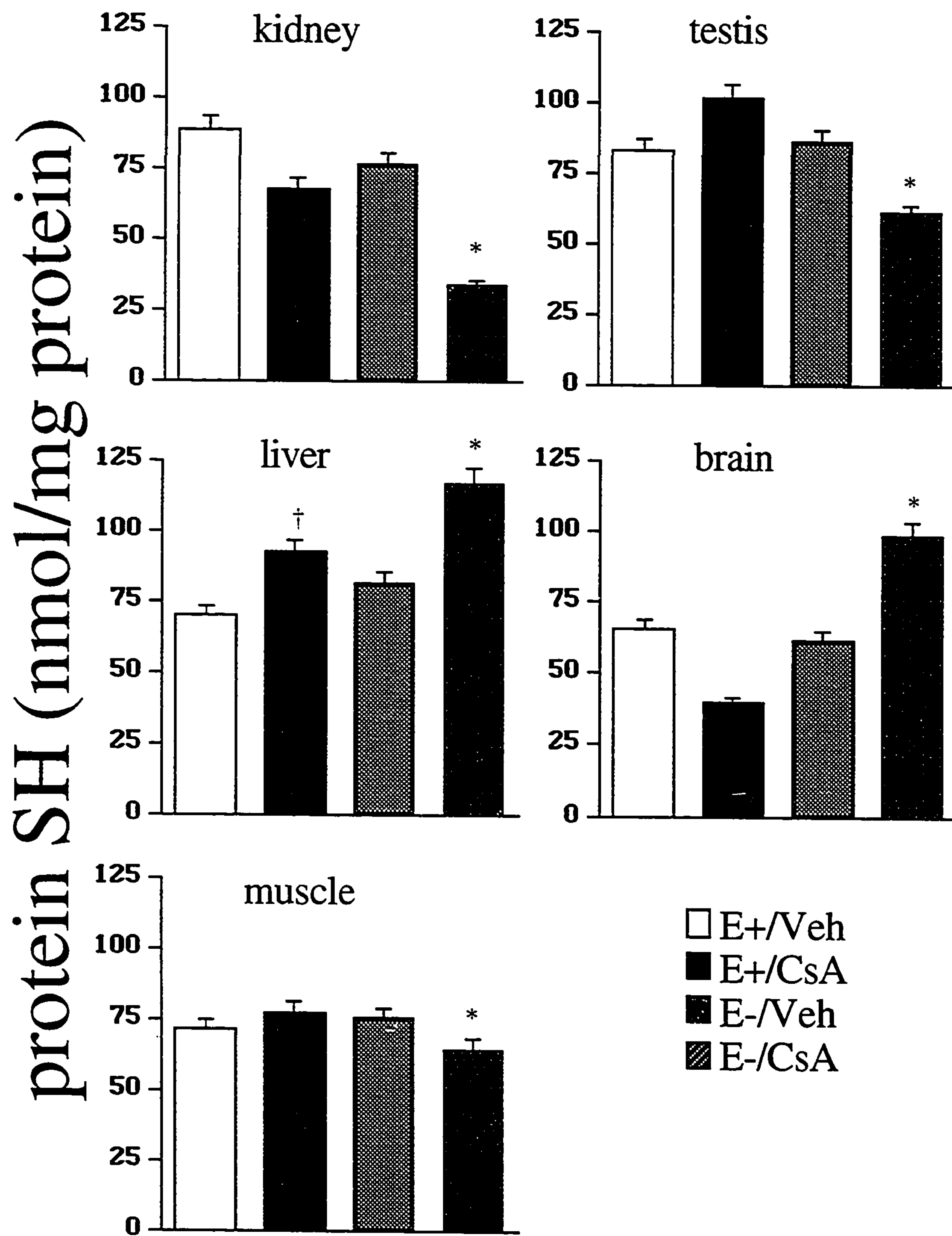

protein SH (nmol/mg protein)
kidney
testis
liver
brain
muscle
125
100
75
50
25
0
*
†
E+/Veh
E+/CsA
E-/Veh
E-/CsA

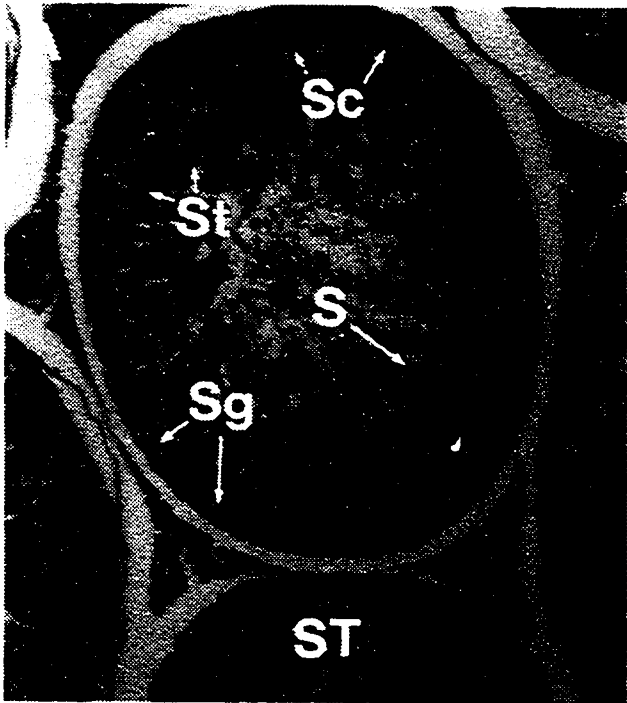
FIGURE 3.10 A-D: Light micrographs of H and E stained sections of testis from a rat placed on either a vitamin E sufficient or deficient diet for 33 days and treated with vehicle (Veh; 9:1 olive oil:ethanol; 1 ml/kg/day s.c.) or cyclosporin A (CsA; 20 mg/kg/day s.c) for the final 14 days (day 19 to 33). Control sections are from Veh treated rats either on a vitamin E sufficient (A) or deficient (C) diet. Comparative sections of testis were taken from CsA treated rats either on a vitamin E sufficient (B) or deficient (D) diet.

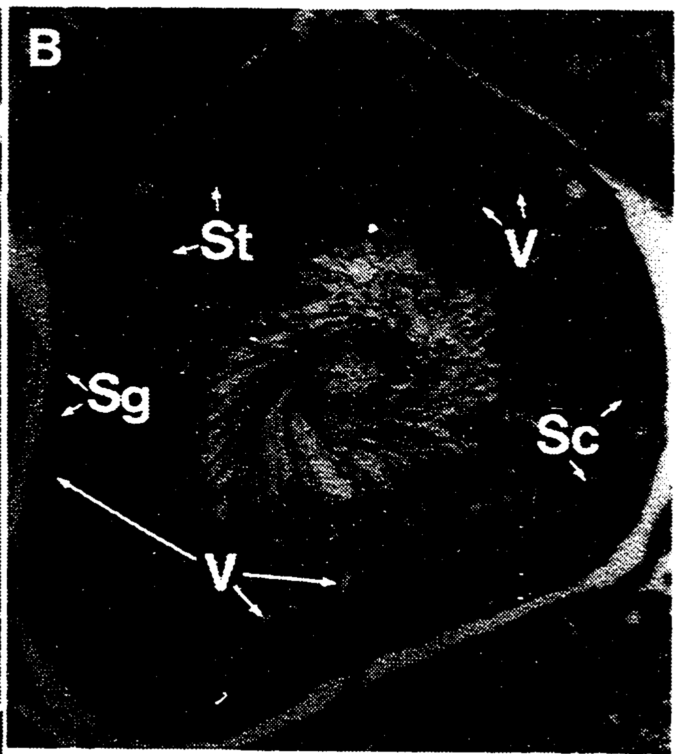
(A) A seminiferous tubule (ST) from a vitamin E sufficient vehicle treated control testis showing distinct layers of spermatogonia (Sg), spermatocytes (Sc), spermatids (St) and sperm (S).

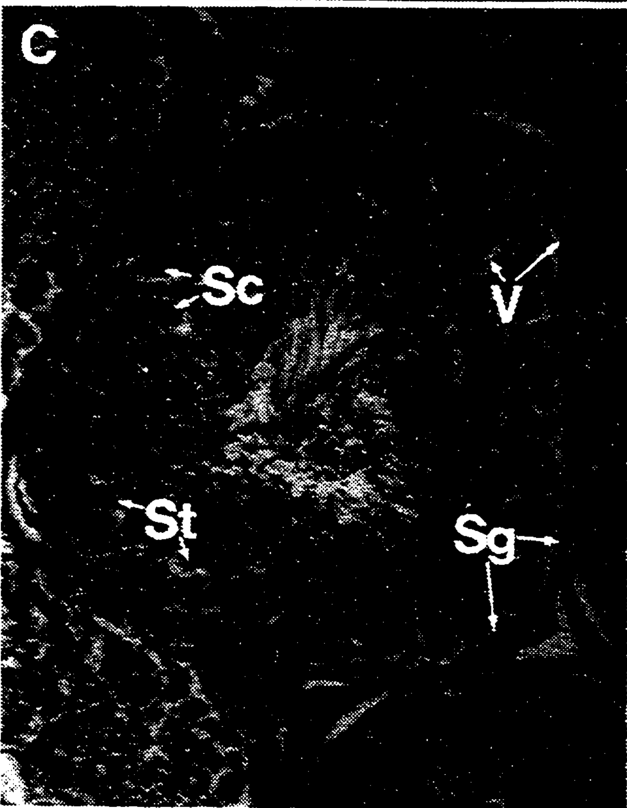
(B) Vacuolar spaces (V) have formed in spermatocytes (Sc) of a seminiferous tubule from a vitamin E sufficient CsA treated testis, separating the layer of spermatocytes from the layer of spermatagonia (Sg) and spermatids (St). Note interstitial cell (L) loss.

(C) As in B, vacuoles (V) in spermatocytes (Sc) of a seminiferous tubule from a vitamin E deficient, vehicle treated control testis separating this layer from the spermatagonia (Sg) and spermatids (St). Leydig cells (L) appear normal.

(D) A seminiferous tubule from a vitamin E deficient, CsA treated testis showing vacuolar spaces (V) not only in spermatocytes (Sc) forming separations between them, but also between spermatids (St). Leydig cell (L) disruption is also evident. 200x.


Sc
St
S
Sg
ST


B
St
V
Sg
Sc
V

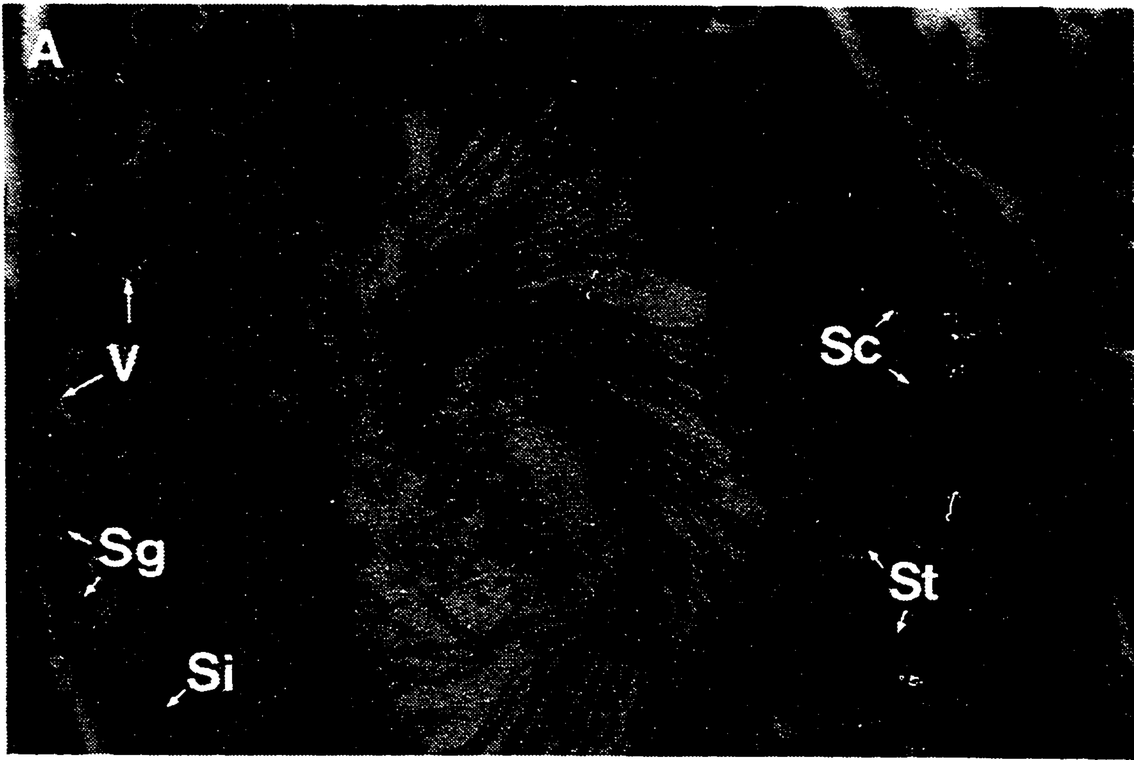

C
Sc
V
St
Sg

D
V
St
Sg
Sc
V

FIGURE 3.11: Photomicrographs of H and E stained sections of testis from rats placed on either a vitamin E sufficient or deficient diet for 33 days and treated with vehicle (Veh; 9:1 olive oil:ethanol; 1 ml/kg/day s.c.) or cyclosporin A (CsA; 20 mg/kg/day s.c) for the final 14 days (day 19 to 33). The control section (A) is from a vehicle treated rat and the comparative section (B) is from a CsA treated rat.

(A) A seminiferous tubule (ST) from a vitamin E sufficient vehicle treated control testis showing distinct layers of spermatogonia (Sg), spermatocytes (Sc), spermatids (St) and sperm (S). A Sertoli cell (Si) is distinguishable in this tubule.

(B) Vacuolar spaces (V) have formed in spermatocytes (Sc) and between spermatids (St), separating these cells from Sertoli cells (Si). 400x.


A
V
Sg
Si
Sc
St

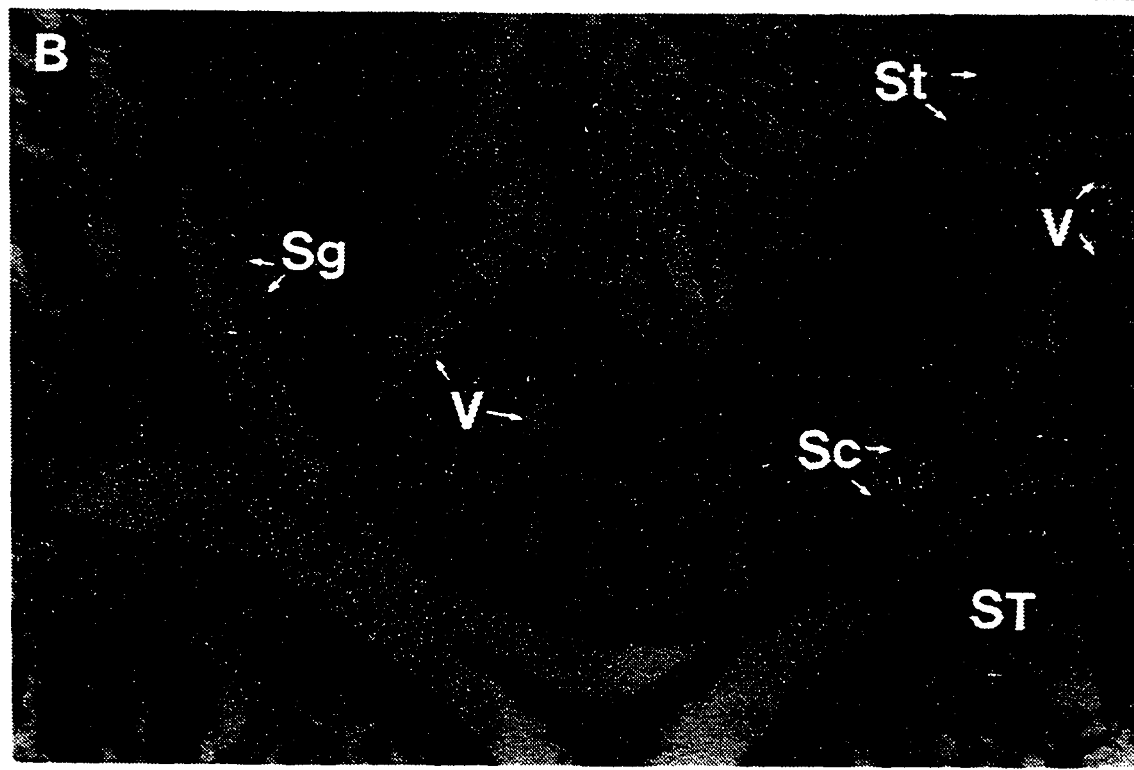
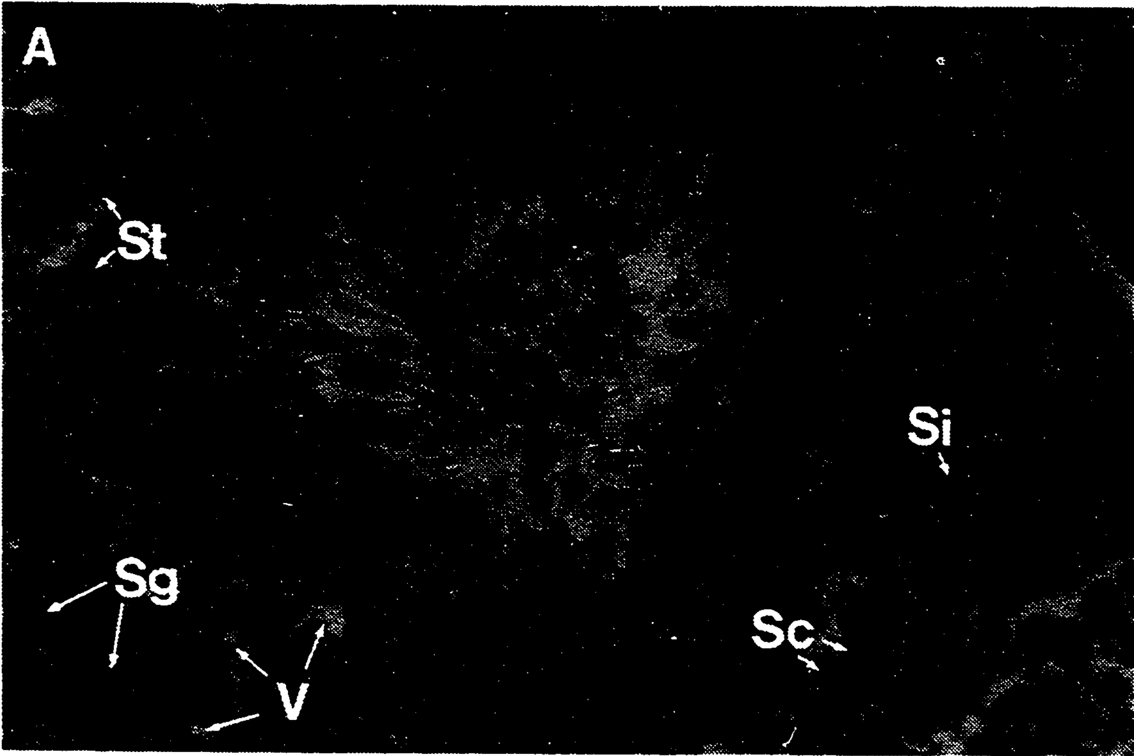

B
St
V
Sg
V
Sc
ST

FIGURE 3.12: Photomicrographs of H and E stained sections of testis from rats placed on either a vitamin E sufficient or deficient diet for 33 days and treated with vehicle (Veh; 9:1 olive oil:ethanol; 1 ml/kg/day s.c.) or cyclosporin A (CsA; 20 mg/kg/day s.c) for the final 14 days (day 19 to 33). The control section (A) is from a vehicle treated rat and the comparative section (B) is from a CsA treated rat.

(A) A seminiferous tubule showing vacuolar spaces (V) between spermatocytes (Sc) and Sertoli cells (Si) and also between spermatids (St) and Sertoli cells. Separation of spermatagonia (Sg) and spermatocytes is evident.

(B) Vacuolar spaces (V) between spermatocytes (Sc) and Sertoli cells (Si) as well as spermatids (St) and Sertoli cells are enlarged with CsA treatment. The separation of the spermatocyte (Sc) layer from the spermatagonial (Sg) and spermatid (St) layers and even within the individual layers is much more obvious. 400x.


A
St
Si
Sg
V
Sc

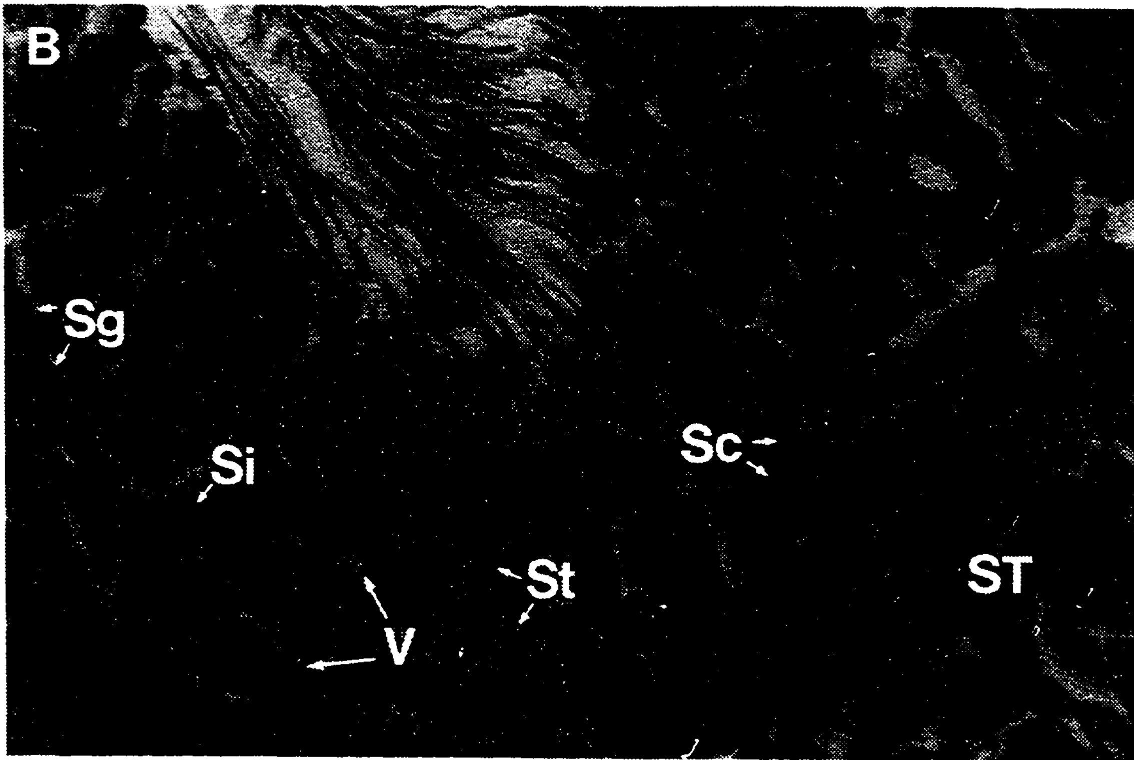
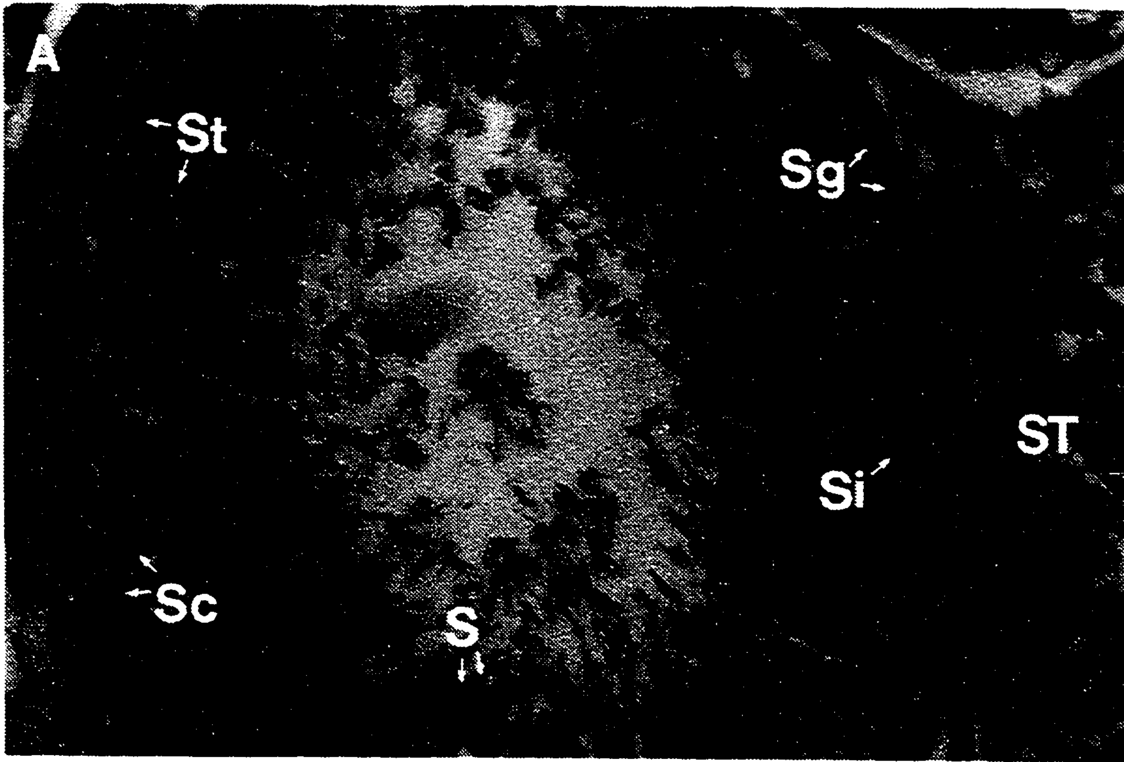
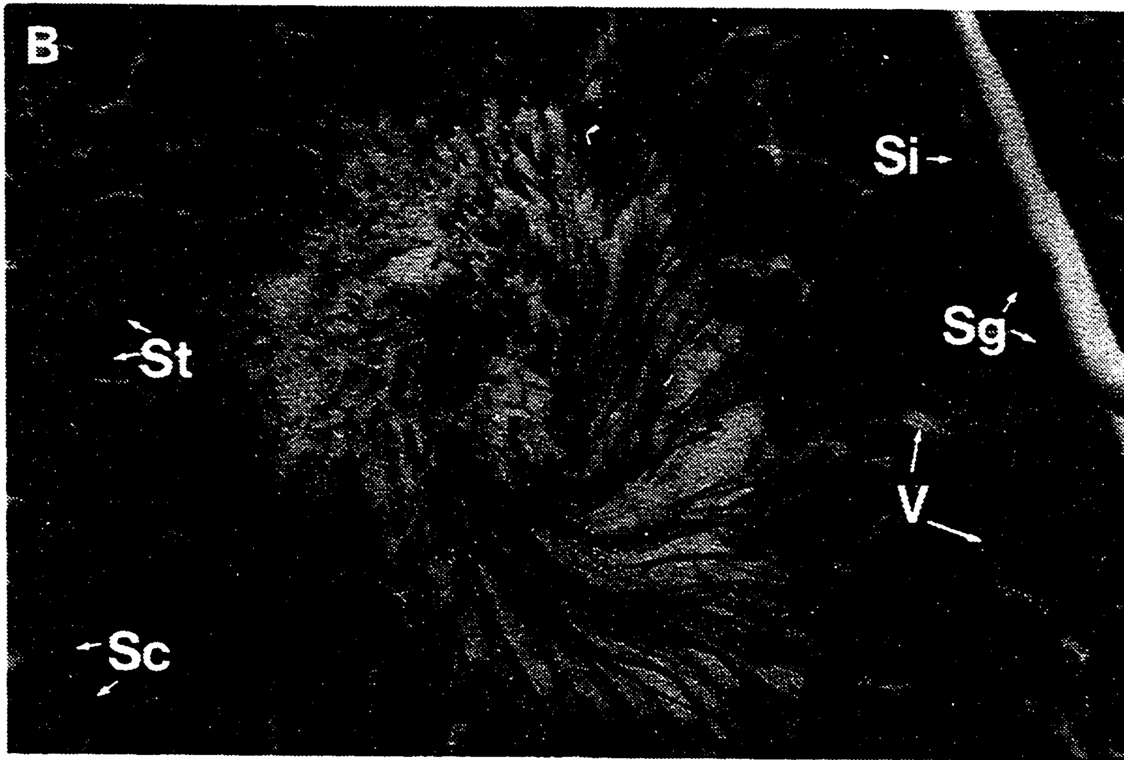

B
Sg
Si
Sc
St
V
ST

FIGURE 3.13: (A) Control photomicrograph of an H and E stained section of testis from a rat placed on a deficient diet for 33 days and treated cyclosporin A (CsA; 20 mg/kg/day s.c) for the final 14 days (day 19 to 33, non recovery animal). As in 3.12B, vacuoles (V) are evident between spermatocytes (Sc) and Sertoli cells (Si) as well as between spermatids (St) and Sertoli cells giving rise to interruptions between and within individual layers. (B) Comparative photomicrograph following the same treatment with an additional 14 day recovery period without treatment on the same diet. Note the large vacuolar spaces (V) which extend from the spermatagonial (Sg) layer to the tubular lumen. 400x.

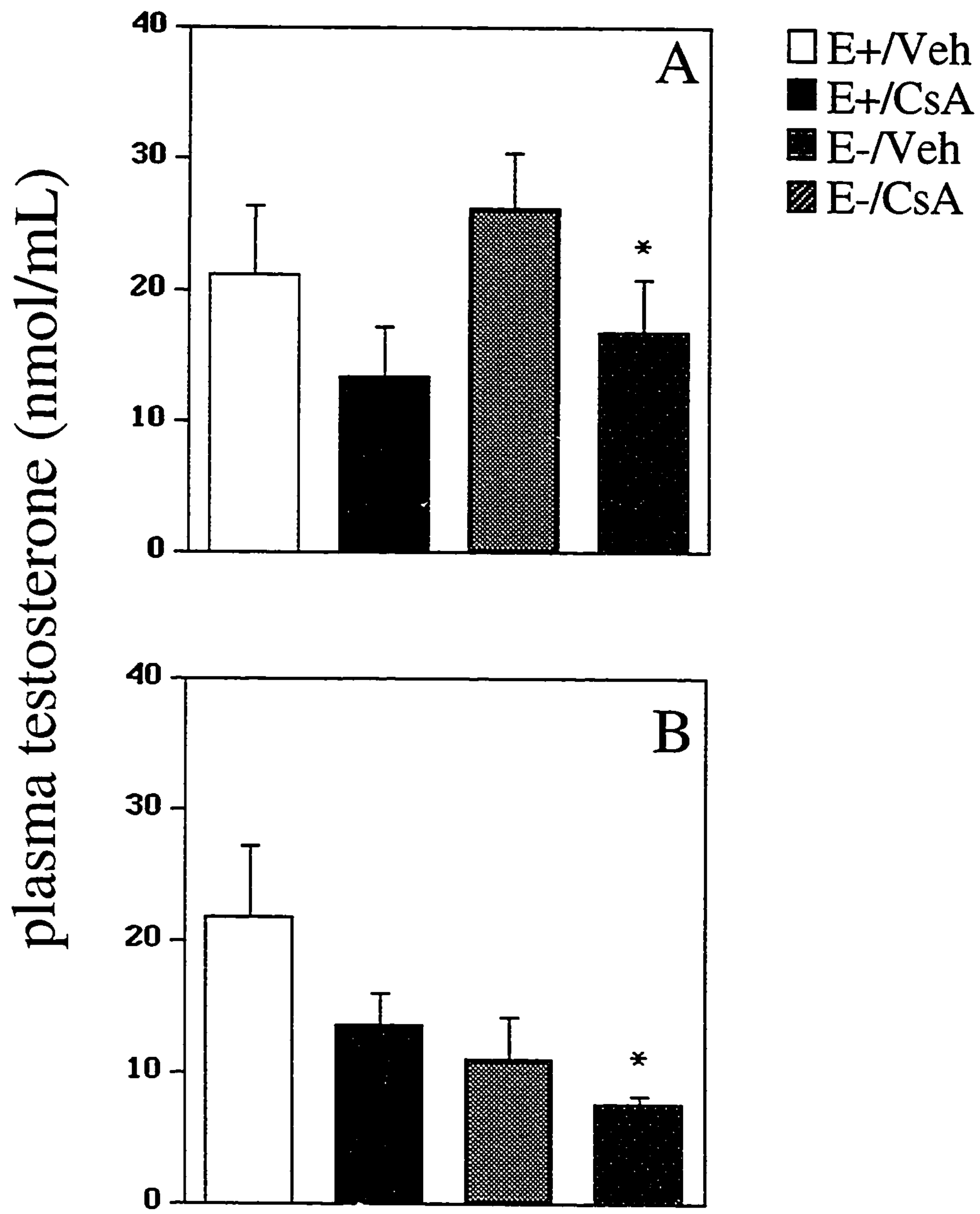


A
St
Sg
ST
Si
Sc
S



B
Si
St
Sg
V
Sc

Figure 3.14: Plasma testosterone levels in male Wistar rats placed (A) on either a vitamin E sufficient or deficient diet for 33 days and treated with vehicle (Veh; 9:1 olive oil:ethanol; 1 ml/kg/day s.c.) or cyclosporin A (CsA; 20 mg/kg/day s.c) for the final 14 days (day 19 to 33) or (B) the same treatment regimen as in (A) followed by a recovery period without injections for a further 14 days (to day 47). Treatment groups listed in the legend include those placed on a vitamin E sufficient diet with either Veh or CsA treatment (E^+/Veh or E^+/CsA respectively) or a vitamin E deficient diet with either Veh or CsA treatment (E^-/Veh or E^-/CsA respectively). Values obtained from 8 animals per group are expressed as mean ± SEM. Obelisks or asterisks denote statistical significance ($p<0.05$) when compared to the E^+/Veh (†) or the E^-/Veh (*) group, respectively.


plasma testosterone (nmol/mL)
A
B
0
10
20
30
40
*
E+/Veh
E+/CsA
E-/Veh
E-/CsA

3.10 Discussion

Administration of CsA at 20 mg/kg/day for 14 days to male Wistar rats showed results which differed from the first study which used lower CsA concentrations (10 mg/kg/day). In the second study CsA administration caused significant reductions in whole body and tissue wet weights. Plasma FSH and LH levels in animals sacrificed after the last day of CsA or vehicle (Veh) treatment (ie. non recovery groups) indicated increases with CsA treatment but only FSH for the E^+/CsA group changed significantly. Plasma testosterone decreased with either CsA treatment (E^+/CsA), vitamin E deficiency (E/Veh) or both (E/CsA). Vitamin E values at 20 mg/kg/day of CsA were higher than controls in several tissues. Only the testis, kidney and brain displayed a tocopherol-lowering effect of CsA, and only in vitamin E sufficient animals. With 14 days of recovery after CsA or vehicle treatment, vitamin E concentrations returned to control levels. The liver, brain and kidney all showed increases in GSH for E/CsA group as compared to its vehicle control (E^-/Veh). Testes and muscle showed losses in GSH when CsA and vitamin E deficiency were combined. The testis, muscle and kidney showed losses in PSH in E/CsA groups while the liver and brain actually demonstrated increases in PSH in one or both CsA treated groups (E^+/CsA or E/CsA). Morphological examination of testis found similar tubular alterations as in the previous study. CsA treated animals on a vitamin E deficient diet (E/CsA) showed the most marked tubular disruptions of any of the treatment groups. This corresponded with the only statistically significant reductions in plasma testosterone.

Previous studies have demonstrated testis and whole body weight reductions in rats subcutaneously injected with 20 mg/kg/day of CsA or greater (Seethalakshmi, et al., 1987, L. Seethalakshmi, et al., 1988). Our results show similar findings with the additional observation that animals first treated with CsA (20 mg/kg/day for 14 days) then allowed to recover from treatment for 14 additional days continue to show slower rates of growth than animals treated with vehicle only. This effect was independent of vitamin E status. Therefore vitamin E was both unable to prevent CsA induced whole body and tissue weight loss at this

concentration of drug, or itself caused weight loss during deficiency at this timepoint. In addition E/CsA animals maintained on a vitamin E deficient diet during the recovery period had lower kidney weights than any other group. Several studies on rats involving oral dosing of CsA resulted in little or no change to whole body or tissue weights (Rajfer, et al., 1987, S. C. Sikka, et al., 1988, Sikka, et al., 1988). However, it has also been shown that in rats the bioavailability of CsA is improved using subcutaneous (s.c.) injections (Wassef, et al., 1985).

In this experiment plasma leutinizing hormone (LH), necessary for testosterone synthesis, and follicle stimulating hormone (FSH), required for sperm maturation, were measured to compare the effects of CsA treatment on the hypothalamic-pituitary-gonadal axis. Previous studies have shown that FSH and LH response was dependent on the dosing method. Rats dosed orally with 7.5, 15 and 30 mg/kg/day of CsA for 4 weeks showed decreases in plasma LH along with increases in plasma FSH (Rajfer, et al., 1987). However, plasma LH was found to increase in rats injected s.c. with 10, 20, or 40 mg/kg/day of CsA for 14 days (Seethalakshmi, et al., 1987). In both of these studies plasma testosterone decreased significantly when CsA concentrations were $\geq$ 10 mg/kg. Therefore, subcutaneous injections of 20 mg/kg/day for 14 days in this study produced results in agreement with the previous study using s.c. injections of CsA at this concentration (Seethalakshmi, et al., 1987). Plasma FSH and LH both showed a tendency to increase in animals treated with CsA, but only FSH in the E^{+}/CsA group showed any statistically significant increase. Further, plasma testosterone decreased only in the E/CsA group as compared to the E^{-}/Veh group. The lack of effect of CsA on lowering plasma testosterone in this study may be the result of several factors. This study shows a tendency for testosterone to decrease with CsA treatment, vitamin E deficiency or a combination of both. While GC-MS is a sensitive technique and testosterone was detected in most samples, the range of values within a group was large with some samples unable to provide a detectable response. However, the trend for testosterone to decrease with CsA treatment in this study,

combined with evidence that CsA inhibits testosterone production at the level of the testis (L. Seethalakshmi, et al., 1989, Seethalakshmi, et al., 1992) could account for the detected rise in LH. The pituitary gland, detecting a decrease in circulating testosterone would release LH to stimulate testosterone production.

Morphological examination of the testis further supports the direct toxic action of CsA on the testis and the ability of vitamin E to protect it. CsA treatment (20 mg/kg/day) in vitamin E sufficient rats showed a degeneration of Leydig cells and vacuolization of the spermatocytes. Removal of dietary vitamin E further exacerbated CsA effects causing vacuolization in all seminiferous tubule (ST) cell types, Leydig cell degeneration and disruption of the cellular organization of the ST. Therefore, vitamin E can reduce or slow the progression of testicular degeneration generated by 20 mg/kg/day of CsA. This is evident in the testis of E/CsA animals allowed to recover from CsA treatment. The continuation of a vitamin E deficient diet during this recovery phase does not allow the ST to recuperate and cell degeneration continues, while the testis of the E^{+}/CsA group showed no worsening of degeneration during the recovery phase (not shown). Testis requirement for vitamin E itself is illustrated in a comparison of the morphology of testis in E^{+}/Veh and E/Veh animals. A vitamin E deficiency for just 28 days in weanling male Wistar rats is sufficient to cause ST degeneration similar to that found in the testis of CsA treated, vitamin E sufficient rats. Vitamin E has been suggested (D. R. Cooper, et al., 1987) to be directly involved in regulating specific steps in germ cell development in the testis. This study shows that vitamin E also has a pivotal role in protecting developing germ cells from oxy radicals, possibly generated under normal conditions by the metabolic processes of steroidogenic P-450 enzymes.

In the first experiment CsA caused effects consistent with generation of free radicals *in vivo* in both the kidney and testis. This was determined by the effect of CsA on vitamin E levels in these tissues. The hypothesis was put forth that CsA at 20 mg/kg/day for

14 days would show an even greater tocopherol lowering effect in testis and kidney while possibly reflecting the role of oxyradicals in CsA toxicity of other tissues.

The vitamin E deficient diet reduced α-tocopherol content in all tissues as compared to vitamin E replete animals. No tocopherol-lowering effect of CsA was seen in testis, kidney, liver or muscle in vitamin E deficient animals (E/CsA). Brain tissue, fat, adrenal gland and plasma actually showed significant increases in α-tocopherol in this treatment group. However, CsA treatment of 20 mg/kg/day for 14 days not only had a tocopherol lowering effect in the kidney and testis of vitamin E sufficient animals (E^+/CsA), as in the previous study, but also in brain tissue. This result provides some evidence that perhaps at this concentration of CsA, the drug is able to cross the blood brain barrier as has been found in other studies (Kahan, et al., 1983, Nooter, et al., 1984). In addition the observation that CsA causes a direct tocopherol lowering effect in brain tissue demonstrates that a component of CsA toxicity in the brain is free radical-mediated. Curiously, CsA treatment in E^+ animals caused α-tocopherol levels to rise significantly compared to E^+/Veh groups in fat, adrenal gland and muscle, suggesting a movement of vitamin E into these tissues from other tissues (i.e. a redistribution). This observation may be in response to an increased level of oxidative stress imposed by pharmacological doses of CsA. This phenomenon has been illustrated in a study where selenium deficiency in rats increased the migration of α-tocopherol to mitochondrial membranes (Buttriss and Diplock, 1988). Selenium is essential for glutathione peroxidase activity (see section 1.14).

CsA at 20 mg/kg/day once again failed to show a tocopherol lowering effect in liver and adrenal gland, suggesting that a very competent antioxidant system is operating in these organs. To examine the hypothesis that the tissues studied may be utilizing other antioxidants in order to spare α-tocopherol, glutathione (GSH) and protein sulfhyryl (PSH) content was measured in various tissues after CsA treatment of 20 mg/kg/day. In *in vitro* experiments involving a free radical generating system, plasma proteins were oxidized before α-tocopherol (Wayner, et al., 1987). GSH is a water soluble antioxidant that is the

major substrate for the family of glutathione peroxidases (Flohe, et al., 1973, Meister and Anderson, 1983, Ursini, et al., 1985), necessary for converting hydroperoxides into harmless products.

The ability of liver to resist free radical oxidation is evident in this study when looking at vitamin E, GSH and PSH levels after CsA treatment. Both E^+/CsA and E^-/CsA groups show no reductions in α-tocopherol due to CsA treatment, while GSH and PSH concentrations increase. It is understandable, then, that very high doses of CsA are required for liver to exhibit CsA toxicity (Kahan, 1989). The assumption can be made that the mobilization of GSH and PSH in liver in response to CsA treatment suggests a free radical component in CsA hepatotoxicity. This would be reasonable since CsA is primarily metabolized by P-450 enzymes in the liver (see section 1.6.3).

The adrenal gland also displayed resistance to CsA toxicity at doses of 10 and 20 mg/kg/day. Vitamin E levels in CsA treated groups remained at control levels in both studies. GSH and PSH determination in the adrenal gland could not be performed as all available tissue was utilized in vitamin E measurements. However, we could expect results similar to those for the liver. Both tissues exhibit very high turnover of vitamin E and have high P-450 activity. The observation that the adrenal gland mobilizes or incorporates additional vitamin E above control levels, indicates that the tissue is responding to some form of oxidative stress coincident with CsA administration.

In the kidney, vitamin E concentrations drop without concurrent reductions in either GSH or PSH in vitamin E replete rats (E^+/CsA). This would indicate that vitamin E is being utilized preferentially to combat CsA free radical generation. Studies show that nephrotoxicity of CsA is evident at this concentration (P. A. Keown, et al., 1986). In vitamin E deficient rats (E/CsA), kidney vitamin E does not differ from control concentrations (E^-/Veh) while PSH levels drop and GSH concentrations rise. This would suggest a mobilization of GSH, to a tissue doubly stressed by vitamin E deficiency combined with

pharmacological doses of CsA, and the preferential utilization of PSH in oxyradical defence.

Muscle tissue shows low levels of GSH in all groups but CsA treatment reduces concentrations further. This is evidence of oxyradical formation by CsA in muscle that is not evident when examining vitamin E concentrations alone.

Static levels of GSH and PSH in testis with a corresponding drop in vitamin E in the E^{+}/CsA treatment group indicates that when vitamin E is in adequate supply it is used preferentially to GSH and PSH. When vitamin E availability is low (E^{+}/CsA) the testis spares α-tocopherol, utilizing GSH and PSH instead. However, it is evident from morphological examinations that CsA is still able to exert a harmful effect on the seminiferous tubules (ST) and Leydig cells.

When animals from all four treatment groups were taken off CsA or vehicle and left on their respective diets for a further 14 days, most tissue α-tocopherol levels returned to control levels. However, as previously stated, vitamin E levels in the E^{-}/CsA treatment group was not sufficient during the recovery period to prevent continued destruction of the ST begun by CsA treatment.

Therefore it is evident from this second study that CsA injected subcutaneously at 20 mg/kg/day for 14 days to male Wistar rats had tocopherol lowering effects only in the kidney and testis of vitamin E sufficient rats. In all other tissues, GSH and PSH mobilization along with other factors were more prevalent, sparing vitamin E. Further, when CsA treatment was combined with vitamin E deficiency, testis and kidney α-tocopherol was also spared. However, examination of the testis morphology showed that defence mechanisms such as GSH and PSH were not sufficient to prevent ST degeneration. Finally, vitamin E deficiency alone showed degenerative changes in the ST indicating that oxyradical generation by such sources as steroidigenic P-450 enzymes must be ongoing.

CHAPTER 4

4.1 Conclusions:

This is the first *in vivo* study to measure vitamin E as an indicator of oxidative stress linked to CsA toxicity. Further this study provides unequivocal proof of the importance of vitamin E in maintaining testicular structure. We believe that CsA toxicity in the testis and other organs is a factor of its metabolism. CsA is extensively metabolized by the cytochrome P-450 enzyme superfamily, which is known to generate oxyradicals under normal operating conditions. The suggestion that the fit of the substrate in the P-450 enzyme can uncouple the transfer of reducing equivalents from a reductase enzyme to the P-450 would allow the electrons to 'leak' out of the reactive site and onto oxygen to form superoxide.

CsA has also been shown to cause vasoconstriction in the kidney, creating an ischemic environment in areas of the kidney. Ischemia, followed by reperfusion has been shown to stimulate lipid peroxidation in the kidney, a process of oxyradical attack on polyunsaturated fatty acids. The ability of CsA to generate oxyradicals in kidney by more than one mechanism may explain why nephrotoxicity is the major problem in CsA immunotherapy.

The testis, the major focus of this study was chosen due to its sensitivity to CsA, even at low concentrations. At 10 mg/kg/day of CsA for 14 days the testis already displayed disruptions to the seminiferous tubules and atrophy of the interstitium, primarily the Leydig cells, that worsened when combined with a vitamin E deficiency. We believe that the uncoupling of the steroidigenic P-450 enzymes found in the Leydig cells by the binding of CsA is a major source of oxygen free radicals in the testis. The demonstration that similar testicular damage can be produced with simply a vitamin E deficiency suggests that oxygen free radicals are generated in the testis under 'normal' metabolic conditions and that a major role of vitamin E in this tissue is as an antioxidant.

In conclusion, the observation that CsA treatment at both 10 and 20 mg/kg/day can reduce vitamin E concentrations *in vivo* selectively in both the testis and the kidney supports the role of oxygen free radicals as a component of CsA toxicity in these tissues. Further, the ability of 20 mg/kg/day of CsA to alter the antioxidant profile in all tissues studied suggests that CsA can generate free radicals in many systems. The ability of a tissue to combat the oxidative stress generated by CsA is a measure of that tissue's susceptibility to CsA toxicity.

The results obtained for testosterone were not satisfactory and it would be important to repeat this analysis, along with analysis of sperm quality, in order to determine how the steroidogenic and spermatogenic capacity of the testis is affected in relation to the degeneration observed after CsA treatment and/or vitamin E deficiency. It would be interesting to determine the protective effect on these parameters of megadoses of vitamin E with the possibility of developing a simple, safe and effective remedy for CsA treated patients.

References

P. S. Almond, K. J. Gillingham, R. Sibley, A. Moss, M. Melin, J. Leventhal, C. Manivel, P. Kyriakides, W. D. Payne, D. L. Dunn, D. E. R. Sutherland, P. F. Gores, J. S. Najarian and A. J. Matas. Renal transplant function after ten years of cyclosporine. *Transplantation* 53: 316-323, 1992.

T. Alper and P. Howard-Flanders. Role of oxygen in modifying radiosensitivity of E. coli B. *Nature* 178: 978-979, 1956.

A. Andres, J. M. Morales, J. Farias, G. Hernandez, M. Gomez, J. Calleja, E. Moreno and J. L. Rodicio. Acute renal failure after liver transplantation in patients treated with cyclosporine. *Transplantation Proceedings* 24: 126-127, 1992.

K. Atkinson, J. Boland, K. Britton and J. Biggs. Blood and tissue distribution of cyclosporine in humans and mice. *Transplantation Proceedings* 15: 2430-2431, 1983.

J. P. Bantle, R. J. Boudreau and T. F. Ferris. Suppression of plasma renin activity by cyclosporine. *American Journal of Medicine* 83: 59-64, 1987.

J. P. Bantle, K. A. Nath, D. E. R. Sutherland, J. S. Najarian and T. F. Ferris. Effects of cyclosporine on the renin-angiotensin-aldosterone system and potassium excretion in renal transplant recipients. *Arch Intern Med* 145: 505-508, 1985.

C. R. Baxter, G. G. Duggin, N. S. Willis, B. M. Hall, J. S. Horvath and D. J. Tiller. Cyclosporin A-induced increases in renin storage and release. *Research Communications in Chemical Pathology and Pharmacology* 37: 305-312, 1982.

M. Bellet, C. Cabrol, P. Sassano, P. Leger, P. Corvol and J. Menard. Systemic hypertension after cardiac transplantation : effect of cyclosporine on the renin angiotensin aldosterone system. *American Journal of Cardiology* 56: 927-931, 1985.

A. Bendich, L. J. Machlin, O. Scandurra, G. W. Burton and D. D. M. Wayner. The antioxidant role of vitamin C. *Adv. in Free Radical Biology and Medicine* 2: 419-444, 1986.

T. Beveridge. Clinical development of cyclosporine. *Transplantation Proceedings* 24: 64-66, 1992.

J. G. Bieri. Second report of the ad hoc committee on standards for nutritional studies. *Journal of Nutrition* 110: 1726, 1980.

J. G. Bieri, Stoewsand, G. S., Briggs, G. M., Phillips, R. W., Woodard, J. C., & Knapka, J. J. Report of the American Institute of Nutrition ad hoc committee on standards for nutritional studies. *Journal of Nutrition* 107: 1340-1348, 1977.

C. Bolton and M. L. Cuzner. Modification of EAE by nonsteroidal anti-inflammatory drugs. In: *The suppression of experimental allergic encephalomyelitis and multiple sclerosis*, edited by A. N. Davison and M. L. Cuzner. London: Academic Press, 1980, p. 189-197.

J. F. Borel. Comparative study of in vitro and in vivo drug effects on cell-mediated cytotoxicity. *Immunology* 31: 631-641, 1976.

J. F. Borel, C. Feurer, H. U. Gubler and H. Stahelin. Biological effects of cyclosporin A : A new antilymphocytic agent. *Agents and Actions* 6: 468-475, 1976.

J. F. Borel, C. Feurer, C. Magnee and H. Stahelin. Effects of the new antilymphocytic peptide cyclosporin A in animals. *Immunology* 32: 1017-1025, 1977.

J. C. C. Borleffs, P. Neuhaus, R. L. Marquet, C. Zuscher and H. Balner. Cyclosporin A and kidney transplantation in rhesus monkeys. In: *Cyclosporin A*, edited by D. J. G. White. Amsterdam: Elsevier Biomedical Press, 1982, p. 329-342.

D. Boscoboinik, A. Szewczyk and A. Azzi. Alpha-tocopherol (vitamin E) regulates vascular smooth muscle cell proliferation and protein kinase C activity. *Arch. Biochem. Biophys.* 286: 264-269, 1991.

D. Boscoboinik, A. Szewczyk, C. Hensey and A. Azzi. Inhibition of cell proliferation by alpha-tocopherol. Role of protein kinase C. *J. Biol. Chem.* 266: 6188-6194, 1991.

M. Brezis, S. Rosen, P. Silva and F. H. Epstein. Renal ischemia : a new perspective. *Kidney Int.* 26: 375, 1984.

G. W. Burton and K. U. Ingold. Autoxidation of biological molecules. 1. The antioxidant activity of vitamin E and related chain-breaking phenolic antioxidants in vitro. *J. Am. Chem. Soc.* 103: 6472-6477, 1981.

G. W. Burton and K. U. Ingold. Vitamin E: Application of the principles of physical organic chemistry to the exploration of its structure and function. *Accounts of Chemical Research* 19: 194-201, 1986.

G. W. Burton, K. U. Ingold, H. Zahalka, P. Dutton, B. Hodgkinson, L. Hughes, D. O. Foster and W. A. Behrens. Biodiscrimination of tocopherols. In: *Vitamin E- Its usefulness in health and in curing disease*, edited by M. Mino. Tokyo: Japan Sci. Soc. Press, 1993, p. 51-61.

G. W. Burton, A. Joyce and K. U. Ingold. First Proof that Vitamin E is a Major Lipid Soluble, Chain-Breaking Antioxidant in Human Blood Plasma. *Lancet* 2: 327-328, 1982.

G. W. Burton, A. Joyce and K. U. Ingold. Is Vitamin E the Only Lipid Soluble, Chain Breaking Antioxidant in Human Blood Plasma and Erythrocyte Membranes? *Arch. Biochem. Biophys.* 221: 281-290, 1983.

G. W. Burton and M. G. Traber. Vitamin E: antioxidant activity, biokinetics and bioavailability. *Ann. Rev. Nutr.* 10: 357-382, 1990.

G. W. Burton, A. Webb and K. U. Ingold. A mild, rapid, and efficient method of lipid extraction for use in determining vitamin E/lipid ratios. *Lipids* 20: 29-39, 1985.

W. C. Buss, J. Stepanek and W. M. Bennett. A new proposal for the mechanism of cyclosporine A nephrotoxicity. *Biochemical Pharmacology* 38: 4085-4093, 1989.

W. C. Buss, J. Stephanek and W. M. Bennett. Proposed mechanism of cyclosporine toxicity : Inhibition of Protein synthesis. *Transplantatioin Proceedings* 20: 863-867, 1988.

J. L. Buttriss and A. T. Diplock. The relationship between α-tocopherol and phospholipid fatty acids in rat liver subcellular membrane fractions. *Biochim. Biophys. Acta* 962: 81-90, 1988.

R. Y. Calne, K. Rolles, S. Thiru, P. McMaster, G. N. Craddock, S. Aziz, D. J. G. White, D. B. Evans, D. C. Dunn, R. G. Henderson and P. Lewis. Cyclosporin A initially as the only immunosuppressant in 34 recipients of cadaveric organs: 32 Kidneys, 2 pancreases, and 2 livers. *The Lancet* : 1033-1036, 1979.

R. Y. Calne, S. Thiru, P. McMaster, G. N. Craddock, D. J. G. White, D. B. Evans, D. C. Dunn, B. D. Pentlow and K. Rolles. Cyclosporin A in patients receiving renal allografts from cadaveric donors. *The Lancet* : 1323-1327, 1978.

R. Y. Calne and D. J. G. White. Cyclosporin A- a powerful immunosuppresant in dogs with renal allografts. *I.R.C.S. Medical Science* 5: 280, 1977.

R. Y. Calne, D. J. G. White, K. Rolles, D. P. Smith and B. M. Herbetson. Prolonged survival of pig orthotopic heart grafts. *The Lancet* i: 1183, 1978.

M. P. Carpenter and C. N. J. Howard. Vitamin E, steroids, and liver microsomal hydroxylations. *The American Journal of Clinical Nutrition* 27: 966-979, 1974.

L. Cavallini, L. K. Malendowicz, G. Mazzocchi, A. S. Belloni and G. G. Nussdorfer. Effects of prolonged cylosporine-A treatment on the Leydig cells of the rat testis. *Virchows Arch B Cell Pathol* 58: 215-220, 1990.

A. C. Chan, K. Tran, D. D. Pyke and W. S. Powell. Effects of dietary vitamin E on the biosynthesis of 5-lipoxygenase products by rat polymorphonuclear leukocytes (PMNL). *Biochim. Biophys. Acta.* 1005: 265-269, 1989.

M. R. Cilio, O. Danhaive, J. F. Gadisseux, J. B. Otte and E. M. Sokal. Unusual cyclosporin related neurological complications in recipients of liver transplants. *Archives of Disease in Childhood* 68: 405-407, 1993.

G. F. Combs, T. Noguchi and M. L. Scott. Mechanisms of action of selenium and vitamin E in protection of biological membranes. *Fed. Proc.* 34: 2090-2095, 1975.

D. R. Cooper, O. R. Kling and M. P. Carpenter. Effect of vitamin E deficiency on serum concentrations of follicle-stimulating hormone and testosterone during testicular maturation and degeneration. *Endocrinology* 120: 83-90, 1987.

R. T. Dean and K. H. Cheeseman. Vitamin E protects proteins against free radical damage in lipid environments. *Biochem. Biophys. Res. Comm.* 148: 1277-1282, 1987.

A. L. DeFranco. Immunosuppressants at work. *Nature* 352: 754-755, 1991.

L. Denicola, S. C. Thomson, L. M. Wead, M. R. Brown and F. B. Gabbai. Arginine feeding modifies cyclosporine nephrotoxicity in rats. *Journal of Clinical Investigation* 92: 1859-1865, 1993.

A. T. Diplock. Vitamin E. In: *Fat soluble vitamins: Their biochemistry and applications*, edited by A. T. Diplock. Lancaster, Pennsylvania: Technomic Publishing Co. Inc., 1985, p. 154-224.

T. Doba, G. W. Burton and K. U. Ingold. Antioxidant and co-antioxidant effect of vitamin C. The effect of vitamin C , either alone or in the presence of vitamin Eor a water-soluble vitamin E analog, upon the peroxidation of aqueous multilamellar phospholipid liposomes. *Biochim. Biophys. Acta* 835: 298-303, 1985.

C. E. Douglas, A. C. Chan and P. C. Choy. Vitamin E inhibits platelet phospholipase A2. *Bioch. Biophys. Acta* 876: 639-645, 1986.

J. E. Downey, D. H. Irving and A. L. Tappel. Effects of dietary antioxidants on in vivo lipid peroxidation in the rat as measured by pentane production. *Lipids* 13: 403-407, 1978.

S. P. Dunn, K. Falkenstein, J. P. Lawrence, R. Meyers, C. D. Vinocur, D. F. Billmire and W. H. Weintraub. Monotherapy with cyclosporine for chronic immunosuppression in pediatric liver transplant recipients. *Transplantation* 57: 544-547, 1994.

H. M. Evans and K. S. Bishop. Existence of a hitherto unknown dietary factor essential for reproduction. *Journal of the American Medical Association* 81: 889-892, 1923.

A. Fahr. Cyclosporin clinical pharmacokinetics. *Clinical Pharmacokinetics* 24: 472-495, 1993.

G. Feutren. Clinical experience with sandimmune (Cyclosporine) in autoimmune diseases. *Transplantation Proceedings* 24: 55-60, 1992.

J. T. Flaherty and M. L. Weisfeldt. Reperfusion Injury. *Free Radic. Biol. Med.* 5: 409-419, 1988.

W. M. Flanagan, B. Corthesy, R. J. Bram and G. R. Crabtree. Nuclear association of a T-cell transcription factor blocked by FK-506 and cyclosporin A. *Nature* 352: 803-807, 1991.

L. Flohe, W. A. Gunzler and H. H. Schock. Glutathione peroxidase: a selenoenzyme. *FEBS Letters* 32: 132-134, 1973.

L. M. Forrester, C. J. Henderson, M. J. Glancey, D. J. Back, B. K. Park, S. E. Ball, N. R. Kitteringham, A. W. Mclaren, J. S. Miles, P. Skett and C. R. Wolf. Relative expression of cytochrome p450 isoenzymes in human liver and association with the metabolism of drugs and xenobiotics. *Biochemical Journal* 281: 359-368, 1992.

A. Galat. Peptidylproline cis-trans-Isomerases - immunophilins. *European Journal of Biochemistry* 216: 689-707, 1993.

H. W. Gardner. Oxygen Radical Chemistry of Polyunsaturated Fatty Acids. *Free Radic. Biol. Med.* 7: 65-86, 1989.

S. Gebicki and J. M. Gebicki. Formation of peroxides in amino acids and proteins exposed to oxygen free radicals. *Biochem. J.* 289: 743-749, 1993.

D. D. Gibson, J. Hawrylko and P. B. McCay. GSH-dependent inhibition of lipid peroxidation : properties of a potent cytosolic system which protects cell membranes. *Lipids* 20: 704-711, 1985.

M. A. Goss-Sampson, C. J. MacEvilly and D. P. R. Muller. Longitudinal studies of the neurobiology of vitamin E and other antioxidant systems, and neurological function in the vitamin E deficient rat. *Journal of the Neurological Sciences* 87: 25-35, 1988.

C. J. Green and A. C. Allison. Extensive prolongation of rabbit kidney allograft survival after short term cyclosporin-A treatment. *The Lancet* : 1182-1183, 1978.

J. Grevel and B. D. Kahan. Pharmacokinetics of cyclosporin A. In: *Cyclosporin-Mode of action and clinical applications*, edited by T. A. W. Dordrecht: Kluwer Academic Publishers, 1989, p. 252-266.

D. G. Hafeman and W. G. Hoekstra. Lipid peroxidation in vivo during vitamin E and selenium deficiency in the rat as monitored by ethane evolution. *J. Nutr.* 107: 666-672, 1977.

B. Halliwell. Oxidants and human disease: some new concepts. *FASEB J.* 1: 358-364, 1987.

B. Halliwell and J. M. C. Gutteridge. *Free Radicals in Biology and Medicine.* Oxford: Clarendon Press, 1989.

D. J. Handelsman. Hypothalamic-pituitary gonadal dysfunction in renal failure, dialysis and renal transplantation. *Endocrine Reviews* 6: 151-182, 1985.

D. J. Handelsman, J. F. W. McDowell, I. D. Caterson, D. J. Tiller, B. M. Hall and J. R. Turtle. Testicular function after renal transplantation: comparison of cyclosporin A with azathioprine and prednisone combination regimes. *Clin. Nephrol* 22: 144, 1984.

R. E. Handschumacher, M. W. Harding, J. Rice and R. J. Drugge. Cyclophilin : A specific cytosolic binding protein for cyclosporin A. *Science* 226: 544-547, 1984.

A. Haverich. Cycloporine in heart transplantation. *Transplantation Proceedings* 24: 82-84, 1992.

W. G. Hoekstra. Biochemical function of selenium and its relation to vitamin E. *Fed. Proc.* 34: 2083-2089, 1975.

P. Howard-Flanders. Effect of oxygen on the radiosensitivity of bacteriophage in the presence of sulphydryl compounds. *Nature* 186: 485-487, 1960.

L. Hughes, M. Slaby, G. W. Burton and K. U. Ingold. Syntheses of alpha- and gamma-tocopherols selectively labelled with deuterium. *Journal of Labelled Compounds and Radiopharmaceuticals* 28: 1049-1057, 1990.

P. Hytiroglou, R. Lee, K. Sharma, N. D. Theise, M. Schwartz, C. Miller and S. N. Thung. FK506 versus cyclosporine as primary immunosuppressive agent for orthotopic liver allograft recipients - histologic and immunopathologic observations. *Transplantation* 56: 1389-1394, 1993.

K. U. Ingold, G. W. Burton, D. O. Foster, L. Hughes, D. A. Lindsay and A. Webb. Biokinetics and discrimination between dietary RRR-and SRR-a-tocopherol in the male rat. *Lipids* 22: 163-172, 1987.

K. U. Ingold, A. C. Webb, D. Witter, G. W. Burton, T. A. Metcalfe and D. P. R. Muller. Vitamin E remains the major lipid-soluble, chain-breaking antioxidant in human plasma even in individuals suffering severe vitamin E deficiency. *Arch. Biochem. Biophys.* 259: 224-225, 1987.

G. Inselmann, M. Blank and K. Baumann. Cyclosporin A induced lipid peroxidation in microsomes and effect on active and passive glucose transport by brush border membrane vesicles of rat kidney. *Research Communications in Chemical Pathology and Pharmacology* 62: 207-220, 1988.

G. Inselmann, J. Hannemann and K. Baumann. Cyclosporin A induced lipid peroxidation and influence on glucose-6-phosphatase in rat hepatic and renal microsomes. *Research Communications in Chemical Pathology and Pharmacology* 68: 189-203, 1990.

J. N. Jain, P. G. Mccaffrey, Z. Miner, T. K. Kerppola, J. N. Lambert, G. L. Verdine, T. Curran and A. Rao. The T-Cell transcription factor NFAT(p) is a substrate for calcineurin and interacts with fos and jun. *Nature* 365: 352-355, 1993.

D. R. Janero and B. Burghardt. Cardiac Membrane Vitamin E and Malondialdehyde Levels in Heart Muscle of Normotensive and Spontaneously-hypertensive Rats. *Lipids* 24: 33-38, 1989.

D. R. Janero and B. Burghardt. Oxidative Injury to Myocardial Membrane: Direct Modulation by Endogenous α-Tocopherol. *J. Mol. Cell Cardiol.* 21: 1111-1124, 1989.

R. B. Jennings. Development of cell injury in sustained acute ischemia. *Circulation, Supplement II* 82: 3-12, 1990.

Z. Jie and Z. Bing-Yan. A study of the toxic effects of cyclosporine on testis and adrenal glands in male rats. *Transplantation Proceedings* 26: 3517, 1994.

K. J. Johnson, A. Rehan and P. A. Ward. The role of oxygen radicals in kidney disease. In: *Oxygen Radicals and Tissue Injury*, edited by B. Halliwell. Bethesda: The Upjohn Company, 1988, p. 115-121.

D. Jorasky, P. Audet, S. Williams, R. Grossman and M. Conrad. Cyclosporine-induced nephrotoxicity: role of prostaglnandins. *Transplantation Proceedings* 19: 1742-1744, 1987.

K. Jung, C. Reinholdt and D. Scholz. Inhibited efficiency of kidney mitochondria isolated from rats treated with cyclosporin A. *Nephron* 45: 43-45, 1987.

B. D. Kahan. Individualization of cyclosporine therapy using pharmacokinetic and pharmacodynamic parameters. *Transplantation* 40: 457-476, 1985.

B. D. Kahan. Cyclosporine. *New England Journal of Medicine* 321: 1725-1738, 1989.

B. D. Kahan. Timeline of immunosuppression. *Transplantation Proceedings* 25: 1-4, 1993.

B. D. Kahan, C. T. Van Buren, M. Boileau, M. Ried and W. D. Payne. Cyclosporin A tissue levels in a cadaveric renal allograft recipient. *Transplantation* 35: 96-99, 1983.

A. Kawaguchi, M. N. Goldman, R. Shapiro, M. L. Foegh, P. W. Ramwell and R. R. Lower. Increase in urinary thromboxane B2 in rats caused by cyclosporine. *Transplantation* 40: 214, 1985.

H. J. Kayden, L. J. Hatam and M. G. Traber. The measurement of nanograms of tocopherol from needle aspiration biopsies of adipose tissue: normal and abetalipoproteinemic subjects. *J. Lipid Res.* 24: 652-656, 1983.

P. A. Keown, C. R. Stiller and A. C. Wallace. Nephrotoxicity of cyclosporin A. In: *Kidney transplant rejection - Diagnosis and treatment*, edited by G. M. Williams, J. F. Burdick and K. Solez. New York and Basel: Marcel Dekker inc., 1986, p. 423-457.

K. T. Kivisto. A review of assay methods for cyclosporin: Clinical implications. *Clin. Pharmacokinet.* 23: 173-190, 1992.

D. K. Klassen, K. Solez and J. F. Burdick. Effects of cyclosporine on human renal allograft renin and prostaglandin production. *Transplantation* 47: 1072-1074, 1989.

J. C. Kolars, P. L. Stetson, B. D. Rush, M. J. Ruwart, P. Schmiedlinren, E. A. Duell, J. J. Voorhees and P. B. Watkins. Cyclosporine metabolism by P450IIIA in rat enterocytes - another determinant of oral bioavailability. *Transplantation* 53: 596-602, 1992.

D. J. Kornbrust and R. D. Mavis. Relative Susceptibility of Microsomes from Lung, Heart, Liver, Kidney, Brain and Testes to Lipid Peroxidation: Correlation with Vitamin E Content. *Lipids* 15: 315-322, 1979.

A. J. Kostakis, D. J. G. White and R. Y. Calne. Prolongation of rat heart allograft survival by cyclosporin A. *IRCS Medical Science* 5: 280, 1977.

J. M. Kovarik, E. A. Mueller, J. B. Vanbree, W. Tetzloff and K. Kutz. Reduced inter- and intraindividual variability in cyclosporine pharmacokinetics from a microemulsion formulation. *Journal of Pharmaceutical Sciences* 83: 444-446, 1994.

A. J. Krentz, B. Dousset, D. Mayer, P. Mcmaster, J. Buckels, R. Cramb, J. M. Smith and M. Nattrass. Metabolic effects of Cyclosporin-A and FK-506 in liver transplant recipients. *Diabetes* 42: 1753-1759, 1993.

B. A. Krueger, G. M. Trakshel, P. M. Sluss and M. D. Maines. Cyclosporin mediated depression of leutinizing hormone receptors and heme biosynthesis in rat testes : a possible mechanism for decrease in serum testosterone. *Endocrinology* 129: 2647-2654, 1991.

K. Kumano, K. Yoshida, M. Iwamura, T. Endo, T. Sakai, K. Nakamura and T. Kuwao. The Role for Reactive Oxygen Species in Cyclosporin A Induced Nephrotoxicity in Rats. *Transplant. Proc.* 21: 941-942, 1989.

A. L. Lehninger. *Principles of Biochemistry*. New York: Worth Publishers Inc., 1982.

M. Lemoyne, A. Van Gossum, R. Kurian and K. N. Jeejeebhoy. Plasma vitamin E and selenium and breath pentane in home parenteral nutrition patients. *Am. J. Clin. Nutr.* 48: 1310-1315, 1988.

M. Lemoyne, A. Van Gossum, R. Kurian, M. Ostro, J. Axler and K. N. Jeejeebhoy. Breath pentane analysis as an index of lipid peroxidation: a functional test of vitamin E status. *Am. J. Clin. Nutr.* 46: 267-272, 1987.

G. L. Lensmeyer, D. A. Wiebe and I. H. Carlson. Deposition of nine metabolites of cyclosporine in human tissues, bile, urine, and whole blood. *Transplantation Proceedings* 20: 614-622, 1988.

J. Liu, J. D. J. Farmer, W. S. Lane, J. FRiedman, I. Weissman and S. L. Schreiber. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes . *Cell* 66: 807-815, 1991.

M. Lombard, B. Portmann, J. Neuberger, R. Williams, N. Tygstrup, L. Ranek, H. Ringlarsen, J. Rodes, M. Navasa, C. Trepo, G. Pape, G. Schou, J. H. Badsberg and P. K. Andersen. Cyclosporin-A treatment in primary biliary cirrhosis - results of a Long-Term placebo controlled trial. *Gastroenterology* 104: 519-526, 1993.

G. Loschen, A. Azzi, C. Richter and L. Flohe. Superoxide radicals as precursors of mitochondrial hydrogen peroxide. *FEBS Letters* 42: 68-72, 1974.

S. Lustig, N. Stern, P. Eggena, M. L. Tuck and D. B. N. Lee. Effect of cyclosporin on blood pressure and renin-aldosterone axis in rats. *American Journal of Physiology* 253: H1596-H1600, 1987.

S. Lustig, N. Stern, M. S. Golub, P. Eggena, J. Barrett and D. N. B. Lee. Experimental cyclosporin hypertension: Characterization of the rat model. *Transplantation Proceedings* 21: 950-951, 1989.

C. W. Mahoney and A. Azzi. Vitamin E inhibits protein kinase C activity. *Biochem. Biophys. Res. Commun.* 154: 694-697, 1988.

H. Maio-Lin, E. N. Frankel, B. E. Leibovitz and A. L. Tappel. Effect of dietary lipids and vitamin E on in vitro lipid peroxidation in rat liver and kidney homogenates. *J. Nutrition* 119: 1574-1582, 1989.

B. Mannervik. The isoenzymes of glutathione transferase. *Adv. Enzymol.* 57: 357-417, 1985.

J. Mason. Cyclosporins past, present, and future. *Transplantation Proceedings* 24: 61-63, 1992.

K. E. Mason. Testicular degeneration in albino rat fed a purified food ration. *J. Exp. Zool.* 45: 159-229, 1926.

K. E. Mason. The specificity of vitamin E for the testes. *J. Exp. Zool.* 55: 101-122, 1930.

K. E. Mason. The tocopherols. Effects of deficiency. A. In animals. In: *The Vitamins*, edited by W. H. J. Sebrell and R. S. Harris. New York: Academic Press, 1954, p. 514-541.

K. D. Massey and K. P. Burton. a-Tocopherol attenuates myocardial membrane-related alterations resulting from ischemia and reperfusion. *Am. J. Physiol.* 256: H1192-H1199, 1989.

H. A. Mattill and M. M. Clayton. Vitamin E and reproduction on synthetic and milk diets. *J. Biol. Chem.* 68: 665-685, 1926.

A. Mauro, L. Orsi, P. Mortara, P. Costa and D. Schiffer. Cerebellar syndrome in adult celiac disease with vitamin E deficiency. *Acta. Neurol. Scand.* 84: 167-170, 1991.

P. G. Mccaffrey, C. Luo, T. K. Kerppola, J. Jain, T. M. Badalian, A. M. Ho, E. Burgeon, W. S. Lane, J. N. Lambert, T. Curran, G. L. Verdine, A. Rao and P. G. Hogan. Isolation of the Cyclosporin-Sensitive T-Cell transcription factor NFATp. *Science* 262: 750-754, 1993.

J. McCord. Free radicals and myocardial ischemia: Overview and outlook. *Free Radical Biology and Medicine* 4: 9-14, 1988.

A. Meister and M. E. Anderson. Glutathione. *Annual Review of Biochemistry* 52: 711-760, 1983.

W. J. Mergner, M. A. Smith and B. F. Trump. Studies on the Pathogenesis of Ischemic Cell Injury: IV. Alteration of Ionic Permeability of Mitochondria from Ischemic Rat Tissue. *Experimental and Molecular Pathology.* 26: 1-12, 1977.

M. J. Mihatsch. Selectivity still has its price - personal experiences with cyclosporine over the last 10 years. *Transplantation Proceedings* 24: 67-70, 1992.

M. J. Mihatsch, G. Thiel, H. P. Spichtin, M. Oberholzer, F. P. Brunner, F. Harder, V. Olivieri, R. Bremer, B. Ryffel, E. Stocklin, J. Torhorst, F. Gudat, U. Zollinger and R. Loertscher. Morphological findings in kidney transplants after treatments with cyclosporine. *Transplantation Proceedings* 15: 2821-2835, 1983.

G. C. Mills. Hemoglobin catabolism : 1. Glutathione peroxidase, an erythrocyte enzyme which protects hemoglobin from oxidative breakdown. *Journal of Biological Chemistry* 229: 189-197, 1957.

S. M. Moochhala, E. J. D. Lee, L. Earnest, J. Y. Y. Wong and S. S. Ngoi. Inhibition of drug metabolism in rat and human liver microsomes by FK-506 and cyclosporine. *Transplantation Proceedings* 23: 2786-2788, 1991.

E. A. Mueller, J. M. Kovarik, J. B. Vanbree, W. Tetzloff, J. Grevel and K. Kutz. Improved dose linearity of cyclosporine pharmacokinetics from a microemulsion formulation. *Pharmaceutical Research* 11: 301-304, 1994.

W. Niederberger, M. Lemaire, G. Maurer, K. Nussbaumer and O. Wagner. Distribution and binding of cyclosporine in blood and tissues. *15* 4 suppl. 1, 1983.

K. Nooter, B. Meershoek, W. Spaans, P. Sonneveld, R. Oostrum and J. Deurloo. Blood and tissue distribution of cyclosporin A after single oral dose in the rat. *Experentia* 40: 559-561, 1984.

K. Oka, H. Shimodaira, T. Hirano, E. Sakurai, T. Tamaki and M. Kozaki. Comparison of adrenal functions in kidney transplant recipients with different Long-Term immunosuppressive treatments - prednisolone and azathioprine versus prednisolone and cyclosporine. *Transplantation* 56: 603-609, 1993.

R. E. Pacifici and K. J. A. Davies. Protein Degredation as an Index of Oxidative Stress. In: *Methods in Enzymology*, edited by L. Packer and A. M. Glazer. Orlando, Fla.: Academic Press, 1990, p. 485-502.

J. E. Packer, T. F. Slater and R. L. Willson. Direct observation of a free radical interaction between vitamin E and vitamin C. *Nature* 278: 737-738, 1979.

M. S. Paller, J. R. Hoidal and T. F. Ferris. Oxygen Free Radicals in Ischemic Acute Renal Failure in the Rat. *J. Clin. Invest.* 74: 1156-1164, 1984.

M. J. Penny, B. J. Nankivell, A. P. Disney, K. Byth and J. R. Chapman. Renal graft thrombosis. A survey of 134 consecutive cases. *Transplantation* 58: 565-569, 1994.

B. Perly, I. C. P. Smith, L. Hughes, G. W. Burton and K. U. Ingold. Estimation of the location of natural α-tocopherol in lipid bilayers by C-NMR spectroscopy. *Biochim. Biophys. Acta* 819: 131-135, 1985.

R. Petric, D. Freeman, C. Wallace, J. McDonald, C. Stiller and P. Keown. Modulation of experimental cyclosporin nephrotoxicity by inhibition of thromboxane synthesis. *Transplantation* 50: 558-563, 1990.

T. L. Poulos and R. Raag. Cytochrome P450cam: crystallography, oxygen activation, and electron transfer. *FASEB J.* 6: 674-679, 1992.

D. D. Pyke and A. C. Chan. Effects of vitamin E on prostacyclin release and lipid composition of the ischemic rat heart. *Arch. Biochem. Biophys.* 277: 429-433, 1990.

V. F. J. Quesniaux. Pharmacology of Cyclosporine (Sandimmune) III. Immunochemistry and Monitoring. *Pharmacological Reviews* 41: 249-258, 1989.

J. Rajfer, S. C. Sikka, C. Lemmi and M. A. Koyle. Cyclosporine inhibits testosterone biosynthesis in the rat testes. *Endocrinology* 121: 586-589, 1987.

C. C. Reddy, R. W. Scholz, C. E. Thomas and E. J. Massaro. Vitamin E dependent reduced glutathione inhibition of rat liver microsomal lipid peroxidation. *Life Science* 31: 571-576, 1982.

R. B. Reddy, R. A. Kloner and K. Przyklenk. Early Treatment With Desferoxamine Limits Myocardial Ischemic/Reperfusion Injury. *Free Radic. Biol. Med.* 7: 45-52, 1989.

M. Ried, S. Gibbons, D. Kwok, C. T. Van Buren, S. Flechner and B. D. Kahan. Cyclosporine levels in human tissue patients treated for one week to one year. *Transplantation Proceedings* 15: 2434-2437, 1983.

D. Ringe. Immunosuppression. Binding by design. *Nature* 351: 185-186, 1991.

A. Roa. Signalling mechanisms in T cells. *Immunology* 10: 495-519, 1991.

T. G. Rosano, B. M. Freed, M. A. Fell and N. Lempert. Cyclosporine metabolites in human blood and renal tissue. *Tranplant. Proc.* 18: 35-40, 1986.

S. Rosen, Z. Greenfeld and M. Brezis. Chronic cyclosporin - induced nephropathy in the rat. *Transplantation* 49: 445-452, 1990.

M. H. Ross, E. J. Reith and L. J. Romrell. *Histology: A text and atlas*. Baltimore: Williams and Wilkins, 1989.

J. Rotruck, A. Pope, H. Ganther, A. Swanson and W. Hoekstra. Selenium: biochemical role as a component of glutathione peroxidase. *Science* 179: 588-590, 1973.

B. Ryffel, H. Siegl, G. Thiel and M. J. Mihatsch. Experimental cyclosporine nephrotoxicity. In: *Kidney Transplant Rejection*, edited by G. M. Williams, J. F. Burdick and K. Solez. New York and Basel: Marcel Dekker Inc., 1986, p. 383-410.

B. Ryffel, G. Woerly, B. Greiner, B. Haendler, M. J. Mihatsch and B. M. J. Foxwell. Distribution of the cyclosporin binding protein cyclophilin in human tissues. *Immunology* 72: 399-404, 1991.

D. R. Salomon. An alternative view minimizing the significance of cyclosporine nephrotoxicity and in favor of enhanced immunosuppression for long-term kidney transplant recipients. *Transplantation Proceedings* 23: 2115-2118, 1991.

S. L. Schreiber and G. R. Crabtree. The mechanism of action of Cyclosporin-A and FK506. *Immunology Today* 13: 136-142, 1992.

S. Schuler, D. Thomas and R. Hetzer. Cyclosporine A-related nephrotoxicity after cardiac transplantation : the role of plasma renin activity. *Transplantation Proceedings* 19: 3898-4001, 1987.

M. L. Scott. Vitamin E. In: *The Fat Soluble Vitamins*, edited by H. F. DeLuca. New York: Plenum Press, 1978, p. 133-210.

J. Sedlak and R. H. Lindsay. Estimation of total, protein-bound, and non protein sulfhydryl groups in tissue with Ellman's reagent. *Analytical Biochemistry* 20: 192-205, 1968.

L. Seethalakshmi, D. A. Diamond, R. K. Malhotra, S. G. Mazanitis, S. Kumar and M. Menon. Cyclosporine- induced testicular dysfunction: A separation of the nephrotoxic component and an assessment of a 60- day recovery period. *Transplantation Proceedings* 20: 1005-1010, 1988.

L. Seethalakshmi, C. Flores, A. A. Carboni, R. Bala, D. A. Diamond and M. Menon. Cyclosporine: Its effects on testicular function and fertility in the prepubertal rat. *Journal of Andrology* 11: 17-24, 1990.

L. Seethalakshmi, C. Flores, R. K. Malhotra, J. D. Pallias, D. Tharakan, R. B. Khauli and M. Menon. The mechanism of cyclosporine's action in the inhibition of testosterone biosynthesis by rat Leydig cells *in vitro*. *Transplantation* 53: 190-195, 1992.

L. Seethalakshmi, M. Menon, R. K. Malhotra and D. A. Diamond. Effect of cyclosporine A on male reproduction in rats. *The Journal of Urology* 138: 991-995, 1987.

L. Seethalakshmi, M. Menon, J. D. Pallias, R. B. Khauli and D. A. Diamond. Cyclosporine: Its harmful effects on testicular function and male fertility. *Transplantation Proceedings* 21: 928-930, 1989.

F. Serino, J. Grevel, K. L. Napoli, B. D. Kahan and H. W. Strobel. Generation of oxygen free radicals during the metabolism of Cyclosporine-A - a Cause-Effect relationship with metabolism inhibition. *Molecular and Cellular Biochemistry* 122: 101-112, 1993.

F. Serino, H. W. Strobel, K. L. Napoli and J. Grevel. Cyclosporine metabolism is related to microsomal peroxidation through generation of oxygen radicals at the level of the cytochrome P-450 system. In: *Oxidative Damage and Repair*, edited by K. Davies Pergammon Press, 1991, p. 747-756.

S. C. Sikka, S. Bhasin, D. C. Coy, M. A. Koyle, R. S. Swerdloff and J. Rajfer. Effects of cyclosporine on the hypothalamic-pituitary-gonadal axis in the male rat : mechanism of action. *Endocrinology* 123: 1069-1074, 1988.

S. C. Sikka, M. A. Koyle, R. S. Swerdloff and J. Rajfer. Reversibility of cyclosporin-induced hypoandrogenism in rats. *Transplantation* 45: 784-787, 1988.

J. W. Slaton, K. A. Kropp, J. S. Jhunjhunwala and S. H. Selman. Cyclosporine versus azathioprine - a 5-Year followup of 200 consecutive cadaver renal transplant recipients. *Journal of Urology* 151: 582-585, 1994.

R. J. Sokol. Vitamin E and neurological function in man. *Free Radicals in Biology and Medicine* 6: 189-207, 1989.

R. J. Sokol, J. Heubi, S. T. Iannaccone, K. E. Bove and W. F. Balistreri. Vitamin E deficiency with normal serum vitamin E concentrations in children with chronic cholestasis. *New Engl. J. Med.* 310: 1209-1212, 1984.

B. Speck, A. Gratwohl, A. Tichelli and C. Nissen. Cyclosporine in bone marrow transplantation. *Transplantation Proceedings* 24: 88-90, 1992.

E. R. Stadtman. Protein oxidation and aging. *Science* 257: 1220-1224, 1992.

R. A. K. Stahl, L. Kanz and S. Kudelka. Cyclosporine and renal prostaglandin E2 production. *Ann. Intern. Med.* 103: 474, 1985.

B. Stanek, J. Kovarik, W. Buschbeck, K. Silberbauer and W. Pinggera. Renin angiotensin aldosterone system in cyclosporine A treated renal allograft recipients. *Nephron* 41: 124, 1985.

B. Stanek, J. Kovarik, S. Rasoul-Rockenschaub and K. Silberbauer. Renin-angiotensin-aldosterone system and vasopressin in cyclosporine treated renal allograft recipients. *Clinical Nephrology* 28: 186-189, 1987.

C. Stiller. The Canadian trial of cyclosporine: Cyclosporine therapy compared to standard immunosuppression in renal transplants: An exploration of nephrotoxicity. *Transplantation Proceedings* 15: 2479-2484, 1983.

C. R. Stiller and G. Opelz. Should cyclosporine be continued indefinitely? *Transplantation Proceedings* 23: 36-40, 1991.

N. D. C. Sturrock and A. D. Struthers. Hormonal and other mechanisms involved in the pathogenesis of Cyclosporin-Induced nephrotoxicity and hypertension in man. *Clinical Science* 86: 1-9, 1994.

B. Sure. Dietary requirements for reproduction. II. The existence of a specific vitamin for reproduction. *Journal Biological Chemistry* 58: 693-709, 1924.

A. L. Tappel. Selenium-glutathione peroxidase and vitamin E. *Am. J. Clin. Nutr.* 27: 960-965, 1974.

S. Teraoka, K. Takahashi, K. Tanabe, Y. Yamaguchi, T. Kawai, T. Tojinbara, I. Nakajima, Y. Nakagawa, H. Fujikawa, T. Hayashi, S. Oba, T. Yagisawa, H. Honda, S. Fuchinoue, H. Toma, T. Agishi and K. Ota. Improvement in renal blood flow and kidney function by modulation of prostaglandin metabolism in cyclosporine-treated animals. *Transplantation Proceedings* 21: 937-940, 1989.

G. Thiel, T. Fellmann, J. Rosman, A. Bock, J. Landmann and M. Mihatsch. Long-Term safety profile of sandimmune in renal transplantation. *Transplantation Proceedings* 24: 71-77, 1992.

S. Thiru. Patholgical effects of cyclosporin A in clinical practice. In: *Cyclosporin-Mode of action and clinical applications*, edited by A. W. Thompson. Dordrecht: Kluwer Academic Publishers, 1989, p. 324-364.

J. A. Thliveris, R. W. Yatscoff and M. J. Mihatsch. Chronic Cyclosporine-Induced nephrotoxicity - a rabbit model. *Transplantation* 57: 774-776, 1994.

M. G. Traber, R. J. Sokol, G. W. Burton, K. U. Ingold and A. M. Papas. Impaired ability of patients with familial isolated vitamin E deficiency to incorporate α-tocopherol into lipoproteins secreted by the liver. *J. Clin. Invest.* 85: 397-407, 1990.

F. Ursini, M. Maiorino and C. Gregolin. The selenoenzyme phospholipid hydroperoxide glutathione peroxidase. *Biochimica et Biophysica Acta* 839: 62-70, 1985.

F. Ursini, M. Maiorino, M. Valente, L. Ferri and C. Gregolin. Purification from pig liver of a protein which protects liposomes and biomembranes from peroxidative degredation and exhibits glutathione peroxidase activity on phosphatidylcholine hydroperoxides. *Biochimica et Biophysica Acta* 710: 197-211, 1982.

R. J. Walker, V. A. Lazzaro, G. G. Duggin, J. S. Horvath and D. J. Tiller. Evidence that alterations in renal metabolism and lipid peroxidation may contribute to cyclosporin nephrotoxicity. *Transplantation* 50: 487-492, 1990.

R. Wassef, Z. Cohen and B. Langer. Pharmacokinetic profiles of cyclosporine in rats. *Transplantation* 40: 489-493, 1985.

D. D. M. Wayner, G. W. Burton, K. U. Ingold, L. R. C. Barclay and S. J. Locke. The relative contributions of vitamin E, urate, ascorbate and proteins to the total peroxyl radical-trapping antioxidant activity of human blood plasma. *Biochimica et Biophysica Acta* 924: 408-419, 1987.

M. R. Weir, D. K. Klassen, S. Y. Shen, D. Sullivan, E. U. Buddemeyer and B. S. Handwerger. Acute effects of intravenous cyclosporine on renal function in healthy humans. *Transplantation Proceedings* 21: 915-917, 1989.

M. R. Weir, D. K. Klassen, S. Y. Shen, D. Sullivan, E. U. Buddemeyer and B. S. Handwerger. Acute effects of intravenous cyclosporine on blood pressure, renal hemodynamics and urine prostaglandin production of healthy humans. *Transplantation* 49: 41-47, 1990.

R. M. Wenger. Pharmacology of cyclosporin (Sandimmune) Part II. Chemistry. *Pharmacological Reviews* 41: 243-247, 1989.

P. H. Whiting. Mechanisms underlying cyclosporin A nephrotoxicity. *Toxicology Letters* 53: 69-73, 1990.

P. H. Whiting and A. W. Thompson. Pathological effects of cyclosporin A in experimental models. In: *Cyclosporin-Mode of action and clinical applications*, edited by A. W. Thompson. Dordrecht: Kluwer Academic Publishers, 1989, p. 303-323.

J. Woo. Therapeutic monitoring of cyclosporine. *Annals of Clinical and Laboratory Science* 24: 60-68, 1994.

S. A. Wudy, Wachter, U. A., Homoki, J., Teller, W. M., & Shackelton, C. H. L. Androgen metabolism assessment by routine gas chromatography/mass spectrometry profiling of plasma steroids: part 1 unconjugated steroids. *Steroids* 57: 319-324, 1992.

APPENDIX

<u>Study 1: CsA (10 mg/kg for 14 days s.c)</u>

Table A1: Effect of CsA (10 mg/kg for 14 days s.c) versus control (vehicle only) on tissue vitamin E content in male Wistar rats given either a vitamin E sufficient or deficient diet.

Tissue	Treatment Groups (nmol/g tissue)			
	E^{+}/Veh	E^{+}/CsA	E^{-}/Veh	E^{-}/CsA
Liver	44.0 ± 2.4	40.2 ± 3.6	5.3 ± 0.6	4.5 ± 0.3
Kidney	37.2 ± 1.5	31.4 ± 1.1†	4.6 ± 0.2	3.6 ± 0.1*
Testis	51.4 ± 1.2	27.1 ± 0.9*	11.5 ± 0.5	6.5 ± 0.2*
Adrenal	340.5 ± 42.1	346.8 ± 11.4	43.4 ± 6.5	42.6 ± 5.3
Brain	35.3 ± 1.0	31.4 ± 2.1	16.5 ± 0.7	14.5 ± 0.5

* $P < 0.01$ as compared to same group on diet treated with vehicle

† $P < 0.05$ as compared to same group on diet treated with vehicle

Values are expressed as means ± SEM for 8 animals per group.

Table A2 : Effects of CsA (10 mg/kg/day for 14 days) and vitamin E deficiency on plasma levels of testosterone (nmol/mL) in male Wistar rats[a].

	Treatment Groups (nmol/mL)			
	E^+/Veh	**E^+/CsA**	**E^-/Veh**	**E^-/CsA**
Plasma testostosterone	11.6 ± 4.9	11.3 ± 2.9	4.3 ± 1.4	2.4 ± 1.0

Study 2: CsA (20 mg/kg for 14 days s.c)

Table A3: Effect of CsA (20 mg/kg for 14 days s.c) on serum FSH and LH content in male Wistar rats at day 33 (given either a vitamin E sufficient or deficient diet).

Treatment Groups (nmol/mL)

	E^+/Veh	E^+/CsA	E^-/Veh	E^-/CsA
FSH	648.45 ± 30.9	971.21 ± 35.9*	764.86 ± 42.2	956.1 ± 140.9
LH	32.28 ± 6.9	42.08 ± 6.0	23.73 ± 6.5	42.5 ± 9.0

* $P < 0.01$ as compared to same group on diet treated with vehicle

Values are expressed as means ± SEM for 8 animals per group.

Table A4: Effect of CsA (20 mg/kg for 14 days s.c) on tissue vitamin E content in male Wistar rats at day 33 (given either a vitamin E sufficient or deficient diet).

Tissue	Treatment Groups (nmol/g)			
	E^+/Veh	E^+/CsA	E^-/Veh	E^-/CsA
Liver	39.2 ± 3.4	39.0 ± 3.4	3.1 ± 0.6	2.1 ± 0.2
Kidney	51.8 ± 1.7	43.4 ± 1.7 *	3.6 ± 0.6	3.8 ± 0.3
Testis	49.3 ± 2.0	40.3 ± 1.7 †	8.2 ± 0.3	9.0 ± 0.3
Adrenal	839.1 ± 26.3	1040.1 ± 85.8 †	41.6 ± 1.3	60.4 ± 4.7†
Muscle	41.3 ± 1.7	45.4 ± 1.5†	6.1 ± 0.3	6.4 ± 0.3
Fat	77.4 ± 5.7	111.3 ± 12.3†	7.4 ± 0.9	11.1 ± 0.9†
Brain	37.6 ± 1.1	32.4 ± 0.7†	15.6 ± 0.7	17.6 ± 0.6†

* $P < 0.01$ as compared to same group on diet treated with vehicle

† $P < 0.05$ as compared to same group on diet treated with vehicle

Values are expressed as means ± SEM for 8 animals per group.

Table A5: Effect of CsA (20 mg/kg for 14 days s.c + 14 days recovery) on tissue vitamin E content in male Wistar rats at day 47 (given either a vitamin E sufficient or deficient diet).

Tissue	Treatment Groups (nmol/g)			
	E^+/Veh	E^+/CsA	E^-/Veh	E^-/CsA
Liver	47.8 ± 5.9	59.5 ± 3.5	3.0 ± 0.2	3.2 ± 0.3
Kidney	25.3 ± 2.4	27.0 ± 2.4	1.5 ± 0.2	2.8 ± 0.2 *
Testis	44.0 ± 2.3	37.6 ± 2.3	6.2 ± 0.4	6.8 ± 0.2
Adrenal	677.5 ± 51.7	648.2 ± 47.5	22.6 ± 1.8	25.2 ± 2.4
Fat	72.2 ± 3.1	95.2 ± 11.2	4.5 ± 0.2	8.4 ± 0.7†
Brain	41.9 ± 1.3	39.0 ± 0.8	15.6 ± 0.9	17.1 ± 0.3
Plasma	20.1 ± 0.7	28.9 ± 2.0	2.4 ± 0.3	1.2 ± 0.1

* $P < 0.01$ as compared to same group on diet treated with vehicle

† $P < 0.05$ as compared to same group on diet treated with vehicle

Values are expressed as means ± SEM for 8 animals per group.

Table A6: Effect of CsA (20 mg/kg for 14 days s.c) on total tissue thiol content in male Wistar rats at day 33 (fed either a vitamin E sufficient or deficient diet).

Tissue	Treatment Groups (nmol/mg protein)			
	E^+/Veh	E^+/CsA	E^-/Veh	E^-/CsA
Liver	100.7 ± 4.1	126.0 ± 8.3†	109.4 ± 4.6	175.4 ± 8.6*
Kidney	112.5 ± 8.2	86.8 ± 4.7	93.3 ± 6.4	80.6 ± 6.3
Testis	150.4 ± 12.8	180.4 ± 10.5	175.2 ± 11.1	134.0 ± 4.2*
Muscle	81.9 ± 6.9	83.4 ± 1.3	88.9 ± 3.2	74.0 ± 3.0†
Brain	110.3 ± 15.4	79.5 ± 9.8	97.6 ± 4.5	145.3 ± 11.2*

* $P < 0.01$ as compared to same group on diet treated with vehicle

† $P < 0.05$ as compared to same group on diet treated with vehicle

Values are expressed as means ± SEM for 8 animals per group.

Table A7: Effect of CsA (20 mg/kg for 14 days s.c) on tissue glutathione content in male Wistar rats at day 47 (given either a vitamin E sufficient or deficient diet).

Tissue	Treatment Groups (nmol/mg protein)			
	E^+/Veh	E^+/CsA	E^-/Veh	E^-/CsA
Liver	30.6 ± 2.0	33.7 ± 3.9	28.2 ± 1.3	58.7 ± 2.9*
Kidney	23.6 ± 4.5	25.0 ± 3.3	17.2 ± 1.0	46.6 ± 3.1*
Testis	67.8 ± 4.9	78.8 ± 4.9	89.0 ± 6.8	73.1 ± 2.9†
Muscle	10.3 ± 0.9	6.1 ± 1.5†	13.5 ± 1.2	9.1 ± 1.2†
Brain	44.5 ± 8.7	39.7 ± 3.1	36.3 ± 0.8	46.9 ± 2.3*

* $P < 0.01$ as compared to same group on diet treated with vehicle

† $P < 0.05$ as compared to same group on diet treated with vehicle

Values are expressed as means ± SEM for 8 animals per group.

Table A8: Effect of CsA (20 mg/kg for 14 days s.c) on tissue protein thiol content in male Wistar rats at day 33 (fed either a vitamin E sufficient or deficient diet).

Tissue	Treatment Groups (nmol/mg protein)			
	E^+/Veh	E^+/CsA	E^-/Veh	E^-/CsA
Liver	70.2 ± 3.0	92.3 ± 5.2*	81.3 ± 4.2	116.7 ± 6.5*
Kidney	88.8 ± 9.0	68.1 ± 8.2	79.7 ± 6.5	33.9 ± 6.0*
Testis	82.6 ± 9.4	101.6 ± 6.1	86.2 ± 4.7	60.9 ± 2.3*
Muscle	71.5 ± 6.2	77.3 ± 2.4	75.5 ± 2.5	64.9 ± 3.3
Brain	65.8 ± 10.1	39.8 ± 7.5	61.3 ± 4.3	98.5 ± 9.2*

* $P < 0.01$ as compared to same group on diet treated with vehicle

† $P < 0.05$ as compared to same group on diet treated with vehicle

Values are expressed as means ± SEM for 8 animals per group.

Table A9: Effect of CsA (20 mg/kg for 14 days s.c) on tissue vitamin E content in male Wistar rats at day 33 (given either a vitamin E sufficient or deficient diet).

	Treatment Groups (nmol/mL)			
	E^+/Veh	E^+/CsA	E^-/Veh	E^-/CsA
Plasma testostosterone	21.2 ± 5.1	13.3 ± 3.9	26.2 ± 4.1	16.8 ± 4.0

Table A10: Effect of CsA (20 mg/kg for 14 days s.c + 14 days recovery) on tissue vitamin E content in male Wistar rats at day 47 (given either a vitamin E sufficient or deficient diet).

	Treatment Groups (nmol/mL)			
	E^+/Veh	E^+/CsA	E^-/Veh	E^-/CsA
Plasma testostosterone	21.7 ± 5.5	13.5 ± 2.4	11.0 ± 3.2	7.6 ± 0.6