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Light Microscopic and Electron Microscopic Features of Cyclosporine Nephrotoxicity in Rats

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In order to clarify morphologic changes associated with cyclosporine(CS) nephrotoxicity, CS in ethyl alcohol at 25 mg / kg / day i.p. was administered to male Sprague-Dawley rats for periods of 1 to 8 weeks. Mean systolic BP was slightly increased in the CS group at 4 weeks ($p<0.05$), but there was no difference compared to a control group at 8 weeks. Blood urea nitrogen was significantly elevated at 4 weeks and continued to rise($p<0.005$), whereas serum creatinine was elevated at 8 weeks. Microscopic examination of the kidneys from CS-treated rats at one week revealed cytoplasmic vacuolization in all segments of the proximal tubules, tubular inclusion bodies, and peritubular capillary congestion. Ultrastructurally, some vacuoles were neutral fat droplets, while others appeared as single membrane-bound structures due to dilatation of the endoplasmic reticulum. The tubular inclusion bodies were enlarged autolysosomes filled with distorted mitochondrial fragments. At two weeks, tubular regeneration was prominent, in addition to the above mentioned toxic tubulopathy. At four weeks, focal areas of interstitial fibrosis and tubular atrophy associated with cystic dilatation were seen. At 8 weeks, interstitial and intratubular microcalcification were present, in addition to patchy foci of interstitial fibrosis, but vascular lesions were not demonstrated. Although renal tubular changes characterized by vacuolization, inclusion bodies, and microcalcification and interstitial fibrosis are not specific for CS toxicity, these changes are commonly found in both humans and rats at high doses of CS.

Key Words : *Cyclosporine nephrotoxicity, Sprague-Dawley rats, Endoplasmic reticulum, Toxic tubulopathy*

INTRODUCTION

Cyclosporine(CS) is a well-known, effective immunosuppressant which has played an important role on its own or, in combination with steroids, in preventing the rejection of organ transplants and contributed to increased graft survival rates(Kahan, 1982 ; Morris, 1982 ; White and Calne, 1982 ; Borel et al., 1983). However, the clinical application of CS is sometimes limited due to its nephrotoxicity(Calne et al., 1979 ; Mihatsch et al., 1983 ; Mihatsch et al.,1985). The clinical features distinguishing CS toxicity from other causes of graft failure are occasionally not clear, even with morphologic examination. Furthermore, the

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coexistence of rejection makes CS-induced nephrotoxicity difficult to differentiate.

CS nephrotoxicity is characterized by a gradual rise in serum creatinine and urea nitrogen levels, which may also be due to rejection, as well as distinctive tubular alterations, reversible by decreasing the CS dose(Canadian Multicentre Study Group, 1983 ; Flechner et al., 1983, Shell et al., 1983). CS nephrotoxicity has been investigated in different animal models(Farthing et al., 1981 ; Ryffel et al., 1983 ; Simonton et al., 1983 ; Duncan et al., 1986 ; Mihatsch et al., 1986 ; Ryffel et al., 1986 ; Verani, 1986 ; Jackson et al., 1987 ; Gillum et al., 1988). The majority of studies using rodents have failed to exhibit the chronic CS nephrotoxicity seen in humans, but two reports using Sprague-Dawley(SD) rats resulted in renal lesions consistent with chronic CS nephrotoxicity(Jackson et al., 1987 ; Gillum et al., 1988). More recently, a study using rabbits as an experimental model has demonstrated leukocytic infiltration, tubular atrophy, interstitial fibrosis, and arteriolopathy, similar to those seen in chronic CS-induced nephrotoxocity in humans (Thliveris et al., 1991).

This study was performed in order to obtain a comprehensive picture of acute and chronic CS nephrotoxicity, by daily intraperitoneal injections of CS(25 mg/kg) for periods of 1 to 8 weeks in male SD rats. This could be useful to distinguish CS toxicity from other causes of graft failure, especially rejection.

MATERIALS AND METHODS

Experimental protocol

Male SD rats, aged 8 weeks, were treated with CS(50 mg/ml, Sandoz Ltd, Basle) at doses of 25 mg/kg/day dissolved in 20 % ethanol by intraperitoneal injection for periods ranging from 1 to 8 weeks(Table 1). All animals were given free access to standard rat chow and water. Rats were weighed once a week and dosages adjusted accordingly. The control group was injected with 20 % ethanol in volumes equivalent to those used in the experimental group. Systolic BP was recorded in conscious restrained rats by the tail-cuff method(Narco-Biosystem, Houston, Texas) at the begining of the experiment, 4 and 8 weeks. At the end of the appropriate treatment period, 5 ml of blood was drawn for determination of plasma creatinine and blood urea nitrogen. One ml of blood was collected in a heparinized tube for analysis of whole blood CS levels. The rats were killed by ether anesthesia after 1 to 8 weeks and autopsies were performed. The kidneys were removed, sectioned, and subjected to study by light and electron microscopy.

Tissue sections of kidney for light microscopy were fixed in 10 % neutral buffered formalin. Paraffin sections were cut at 3-4 um, then stained with hematoxylin and eosin, PAS, and Masson-trichrome. Tissue sections for electron microscopic examination were fixed in 4 % formaldehyde and 1 % glutaraldehyde, then processed to epoxy resin ; sections were stained with uranyl acetate and lead citrate, and examined with a Hitachi-600 electron microscope.

Analytical techniques

Whole-blood CS levels were analysed using a TDx system with a Cyclosporine Monoclonal Whole Blood Kit(Abbott Diagnostics, Abbott Park, Illinois, U.S.A.). Statistical comparisons were made using the Students' t-test, when appropriate.

Morphologic parameters

The parameters for morphologic evaluation were classified into several patterns, as follows ; 1) toxic tubulopathy, 2) peritubular capillary congestion, 3) striped/diffuse interstitial fibrosis with tubular atrophy, 4) focal/diffuse interstitial infiltrates, 5) arteriolopathy, and 6) glomerulopathy. Toxic tubulopathy included isometric tubular vacuolization, tubular inclusion bodies, and microcalcification. Numbers of cytoplasmic inclusions in proximal tubule cells, and the degree of proximal tubular cell vacuolization were graded as -(normal), +(minimal), ++(mild), +++ (moderate), and ++++(severe). Foci of tubular atrophy and interstitial fibrosis with or without inflammation were evaluated.

Table 1. Experimental protocol.

Weeks	CS group	Control group	Total No. of rats
1	7	3	10
2	7	3	10
4	7	3	10
5	7	3	10
8	7	3	10
Total	35	15	50

RESULTS

Body weight gain was significantly reduced in the CS group compared to a control group. Mean weight gain in the CS group was 97.5 gm at the end of the experimental period, versus a 276.6 gm gain for the control group($p<0.001$)(Fig.1). Mean systolic BP was slightly increased in the CS group(137±7.7, control 122±3.5 mmHg) at 4 weeks ($p<0.05$), but there was no difference when compared to a control group at 8 weeks(119±5.6 mmHg). Blood urea nitrogen was significantly elevated at 4 weeks(43.4±2.4, control 19.1 ±4.3 mg/dl) and continued to rise($p<0.005$), whereas serum creatinine was elevated at 8 weeks(1.1± 0.2, control 0.8±0.2 mg/dl). Whole blood CS levels were increased above 1500 ng/ml at 1 week, and continued to 8 weeks(Table 2).

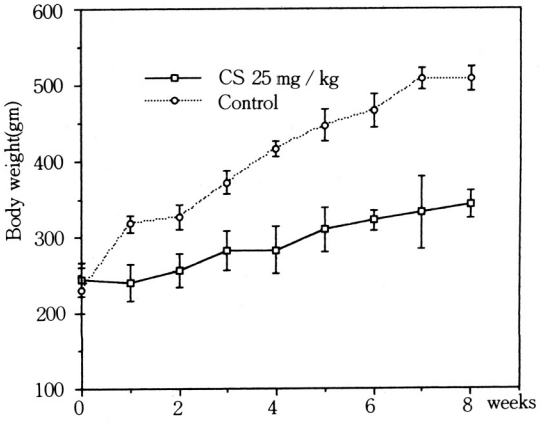

Fig. 1. Body weight of SD rats given CS at 25 mg/kg for 8 weeks.

Histologic examination

Microscopic examination of kidneys from CS-treated rats at one week revealed cytoplasmic isometric vacuolization and tubular inclusion bodies in all segments of the proximal tubules, and peritubular capillary congestion. At two weeks, tubular regeneration characterized by basophilic epithelium was prominent, in addition to the above mentioned toxic tubulopathy.

Table 2. Effect of CS 25 mg/kg in SD rats.

Group	Weeks	BP(mmHg)	BUN(mg/dl)	Creat.(mg/dl)	CS(ng/ml)
Control (Mean)*		122±3.5	19.1±4.3	0.8±0.1	-
CS	2	-	26.8±2.4	0.6±0.1	>1500
	4	137±7.7**	43.4±2.4	0.6±0.1	>1500
	5	-	41.2±11.6	0.5±0.1	>1500
	8	119±5.6	40.0±4.4	1.1±0.2***	>1500
t-test		$p<0.05$**	$p<0.005$	$p<0.05$***	-

#Mean*: Mean value during experimental periods.

Table 3. Time dependence of renal changes.

Weeks of CS treatment	5days*	1	2	4	5	8
Morphology						
Toxic tubulopathy						
vacuolization	++++	+++	++	++	+	+
inclusion bodies	++	++	++	++	++	++
peritub. cap. congestion	+++	+++	++	++	+	±
regeneration	−	−	++	+	+	±
microcalcification	−	−	±	+	+	++
Chronic nephrotoxicity						
tubular atrophy	−	−	±	+	+	+
interstitial fibrosis	−	−	±	+	+	+
interstitial inflammation	−	−	±	+	+	+
arteriolopathy	−	−	−	−	−	−
Glomerulopathy	−	−	−	−	−	−

*Rats injected with 50 mg/kg/day died at 5 days.

At four weeks, patchy foci of minimal interstitial fibrosis and tubular atrophy associated with cystic dilatation, as well as tubular microcalcification, were seen. At 8 weeks, focal interstitial fibrosis and tubular atrophy associated with intratubular and interstitial calcification were prominent, but any vascular or glomerular lesions were not demonstrated. Sparse interstitial infiltration of mononuclear cells was found at 2 week, increasing in severity at 8 weeks. Tubular eosinophilic inclusion bodies and vacuolization were continuously seen until 8 weeks (Table 3).

Tubular vacuolization : The tubular vacuoles confined to the proximal tubule were of variable size and empty-looking(Fig. 2). Ultrastructurally, some vacuoles were neutral fat droplets, while others appeared as empty, single membrane-bound structures due to dilatation of the endoplasmic reticulum. The brush border was relatively well preserved(Fig. 3).

Tubular inclusion bodies : The tubular inclusion bodies were diffusely present in the proximal tubules. Cytoplasmic inclusion bodies were PAS-positive, variable sized, and some were as large as epithelial cell nuclei(Fig. 4). Ultrastructurally, these inclusion bodies were enlarged, membrane-bound vacuoles filled with disorted mitochondrial fragments, myeloid bodies, and osmiophilic irregularly arranged lamellae. These bodies may have been enlarged autolysosomes(Fig. 5). However, some of them could be classified as giant mitochondria with distorted cristae and highly osmiophilic membrane whorls.

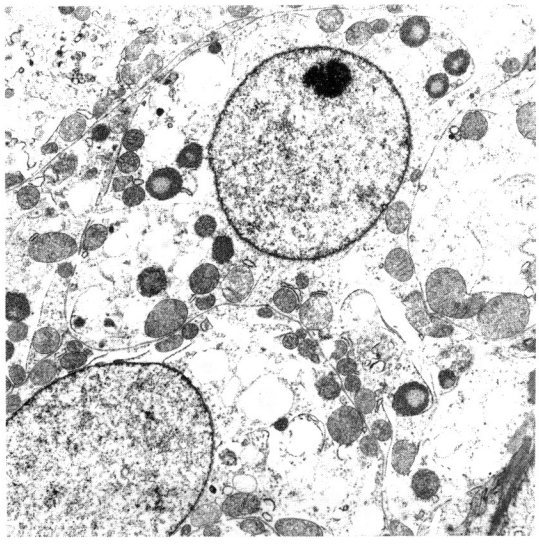

Fig. 3. Ultrastructurally, some vacuoles are neutral fat droplets and others appear as empty, single membrane-bound structures due to dilatation of the endoplasmic reticulum, uranyl acetate and lead citrate, ×4000 (one week, CS group).

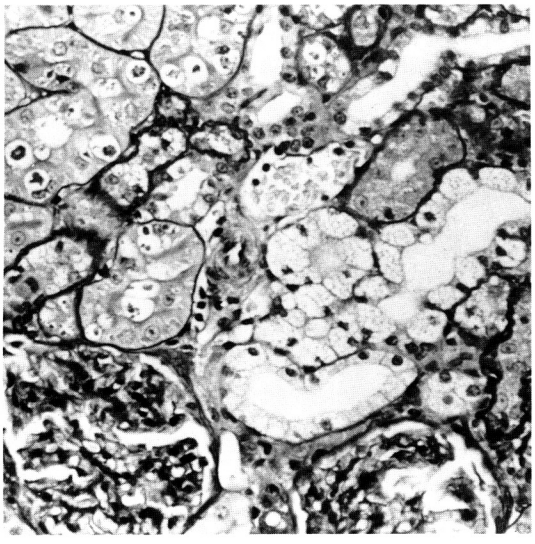

Fig. 2. Isometric tubular vacuolization and PAS-positive inclusion bodies in tubular epithelial cells, PAS, ×100 (one week, CS group).

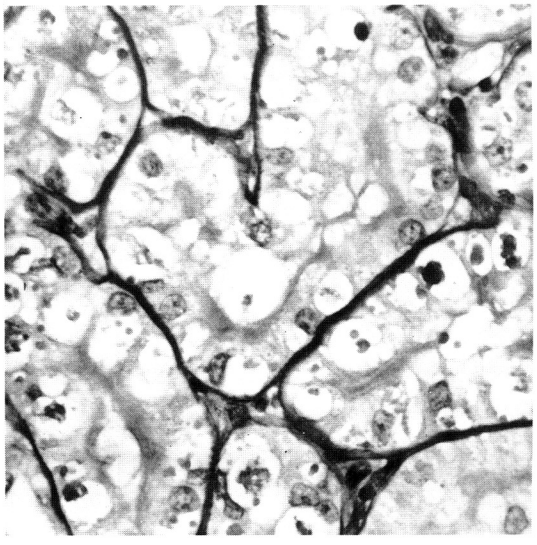

Fig. 4. PAS-positive inclusion bodies in proximal tubular epithelial cells, PAS, ×400 (two weeks, CS group).

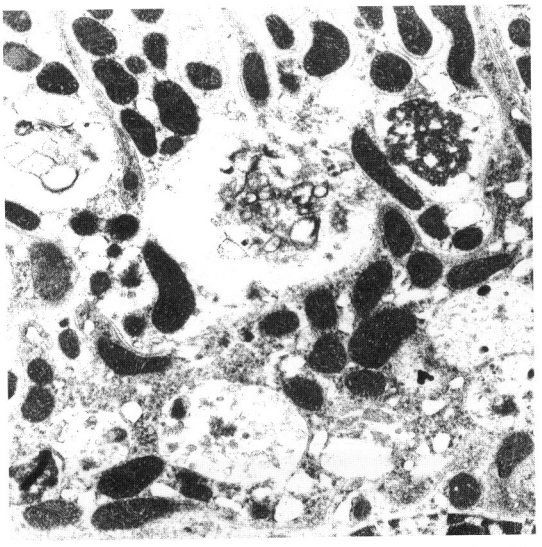

Fig. 5. A proximal tubular cell displays intact mitochondria admixed with autolysosomes filled with distorted cristae and osmiophilic lamellae, uranyl acetate and lead citrate, × 8000 (two weeks, CS group).

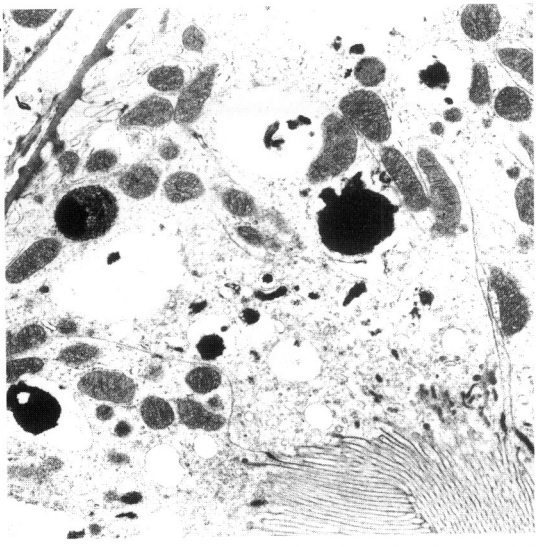

Fig. 6. Electron-dense material deposits in the degenerated mitochondria, suggestive of early microcalcification, uranyl acetate and lead citrate, ×6000 (one week, CS group).

Tubular regeneration : Basophilic, regenerating tubules were focally found in the subcapsular region. Most of these were proximal tubules. The nuclei showed coarse chromatin and prominent nucleoli. Few mitotic figures were also seen. The cytoplasm contained small lipid vacuoles.

Tubular microcalcification : Ultrastructurally, foci of calcification were initially found in degenerated mitochondria of the proximal tubules at 1 week(Fig. 6). Light microscopically, tubular microcalcification was found along the cortico-medullay junction at 2 weeks (Fig. 7). These calcified foci were usually small, but sometimes quite large. The tubular lumen was completely occluded by calcified casts at 8 weeks.

Tubular atrophy : There were patchy foci of tubular atrophy in the subcapsular region, involving small clusters of tubules(Fig. 8). Tubular atrophy was surrounded by interstitial fibrosis and inflammation(Fig. 9). These atrophic cells revealed clear cytoplasm with lipid droplets, decreased numbers of organelles, prominent basolateral infolding, and thickened tubular basement membranes. The brush border was inconspicuous.

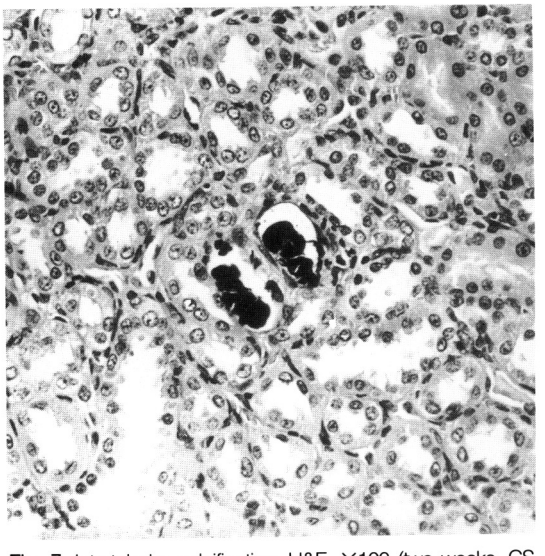

Fig. 7. Intratubular calcification, H&E, ×100 (two weeks, CS group).

DISCUSSION

Cyclosporine(CS) nephrotoxicity in humans can be classified according to time and clinical setting into three different types(Mihatsch et al., 1985) : 1) interactive toxicity characterized by diffuse interstitial fibrosis seen in patients with prolonged oligo-anuria ; 2) acute toxicity with either normal renal tissue, or peritubular

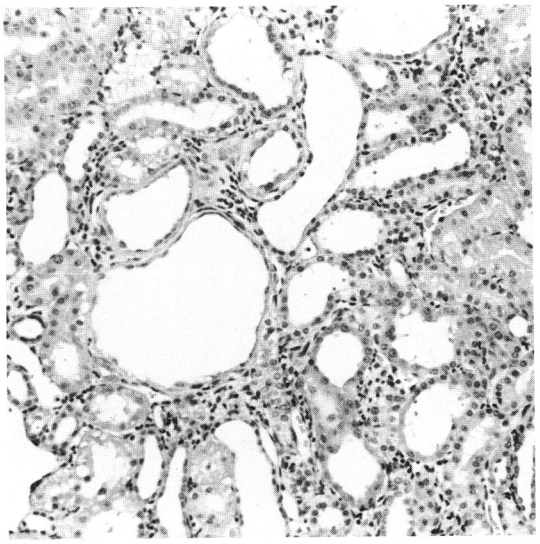

Fig. 8. Patchy foci of tubular atrophy associated with cystic dilatation and minimal interstitial fibrosis, H&E, ×100 (four weeks, CS group).

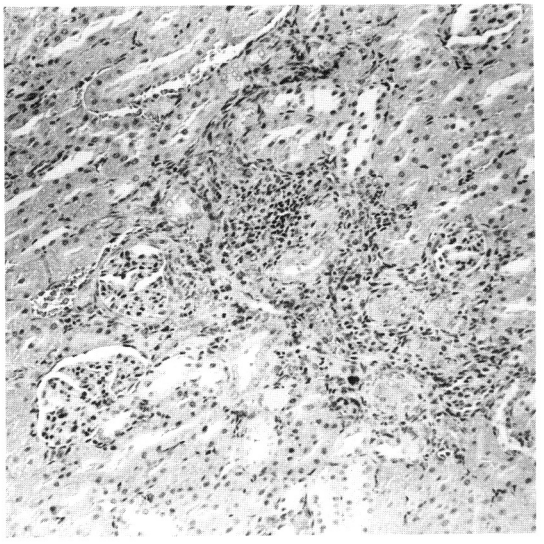

Fig. 9. Stripe form of interstitial fibrosis with sparse infiltration of mononuclear cells, H&E, ×40 (eight weeks, CS group).

capillary congestion or CS-tubulopathy ; and 3) chronic toxicity with either CS-arteriolopathy or striped form of interstitial fibrosis with tubular atrophy, or both.

There is now a consensus that CS nephrotoxicity is the result of alterations in intrarenal hemodynamics(Thiel, 1986). The most favored hypothesis of the nephrotoxicity of CS is the following(Thiel, 1986) : The primary site of action of CS is the preglomerular arteriole, where vascular tone is increased according to the dose of CS. The resulting glomerular hypoperfusion is compensated for by the mechanisms of autoregulation, to which an activation of glomerular prostaglandin(PG) production and of the activity of the renin angiotensin system contribute. After longer periods of CS exposure the glomerular PG synthesis seems to become exhausted for unknown reasons. The resulting possible disequilibrium between reduced PG production and probably unaltered angiotensin II production could diminish the autoregulatory response and lead to a fall in the filtration rate. In addition, morphological alterations to the proximal tubules are an expression of nephrotoxicity which does not seem to be accompanied by any loss of function. Tubular toxicity and arteriolar toxicity are two separate events, the first of minor, the second of major importance.

Acute tubular toxicity, consisting of cytoplasmic vacuolization with the appearance of inclusion bodies, seen in humans can easily be reproduced in rats. Most previous studies(Duncan et al., 1986 ; Mihatsch et al., 1986 ; Verani R, 1986 ; Gillum et al., 1988) have shown that the vacuolization is the result of dilatation of the endoplasmic reticulum(ER) and the inclusion bodies are enlarged lysosomes, which often contain mitochondrion fragments. In this study, some vacuoles were of neutral fat droplets, while others appeared as empty, single membrane-bound structures due to dilatation of the ER. Cytoplasmic inclusion bodies in this study were also considered as enlarged autolysosomes. According to Mihatsch et al.(1986), microcalcification and tubular regeneration are less constant findings. These changes are dose dependent and develop a few days after administration of CS, and are almost completely reversible. Evidence of tubular regeneration and microcalcification were observed at two weeks in this study.

Chronic CS nephrotoxicity in humans, characterized by a striped interstitial fibrosis, tubular atrophy, and arteriolopathy, is difficult to reproduce(Farthing et al., 1981 ; Mihatsch et al., 1986 ; Myers, 1986 ; Thiel, 1986). The strong association in humans between arteriolopathy and tubular atrophy with interstitial fibrosis has led to the proposal that chronic toxicity is the result of arteriolopathy(Thiel, 1986). Based on this

hypothesis, interstitial fibrosis is the consequence of tubular atrophy, which in turn results from destruction of the supporting arterioles(Gillum et al., 1988). According to Jackson et al.(1987) and Gillum et al.(1988), a lesion with many of the features of chronic CS nephrotoxicity in humans, except for arteriolopathy, has been reproduced using male SD rats. In this study, patchy foci of minimal interstitial fibrosis and tubular atrophy associated with cystic dilatation were seen at four and eight weeks, although arteriolar lesions were not identified. Nast et al.(1991) reported that cortical procollagen alpha 1(I) mRNA levels were increased in CS versus control rats at one week($p<0.02$) and four weeks($p<0.02$). In addition, they have commented that the early increase in renal cortical procollagen alpha 1(I) mRNA levels precedes renal morphologic abnormalities, and may represent an important step in the pathogenesis of CS-induced renal cortical fibrosis.

Arteriolar lesions similar to a hypertensive arteriolopathy cannot be induced in experimental animals with CS alone. Spontaneously hypertensive(SH) rats develop arteriolopathy with CS, however, the lesion does not differ from the spontaneously occurring lesion in this strain, and its significance is, therefore, unclear-(Ryffel et al., 1986). The difficulty in developing an experimental model lies in the fact that in rats there are only functional indications of an influence on arterioles, but no progressive destruction of arteriolar tissue(Thliveris et al., 1991). Morphological alterations to the glomeruli or distal nephron segments have not been described. However, hypertrophy of the juxtaglomerular apparatus, which could also be caused by factors other than CS, such as volume depletion, has also been reported(Verpooten et al., 1986 ; Gillum et al., 1988). Vascular or glomerular lesions were not identified in this study.

An interstitial inflammatory infiltrate has been previously reported in Fischer rats treated with 5 to 40mg /kg/day for 2 weeks with i.p. injections of CS(Simonton et al., 1983). Gillum et al.(1988) observed the presence of an infiltrate of mononuclear cells in CS-treated rats, although this was not observed in other studies(Farthing et al., 1981 ; Mihatsch et al., 1986 ; Verani, 1986). Jackson et al.(1987) reported a significant proliferative effect of CS on the cells within the renal interstitium, but they commented that further study is needed before these cells could be identified as either fibroblasts or activated inflammatory cells. Sparse interstitial infiltration of mononuclear cells was observed at two weeks, increasing in severity at 8 weeks in this study. The role of these cells in mediating CS toxicity should be studied.

Verani(1986) reported that the Fischer rats developed seizures, motor weakness at the dose of 100 mg and 50 mg/kg/day and died at 4 to 7 days. Farthing et al.(1981) observed that 4 of 10 Lister Hooded rats treated orally with 50 mg/kg/day of CS died at 12, 15 and 18 days. In this study, six rats injected with 50mg/kg/day died at 3-5 days. Histologically, these rats showed diffuse vacuolization of the proximal tubules. Weight loss has been reported in rats as being related to CS therapy(Farthing et al., 1981 ; Mihatsch et al., 1986 ; Ryffel et al., 1986 ; Gillum et al., 1988). Body weight gain was significantly reduced in the CS group of this study compared to a control group.

According to Jackson et al.(1987), a significant drop in BP occurred after eight doses of 100mg/kg CS, although BP was within the autoregulatory range. It was suggested that a combination of a low sodium diet and CS treatment probably caused volume contraction, which led to the decrease in BP. Gillum et al.(1988) also reported that mean systemic BP was lower in a CS-treated group, but it was statistically insignificant. Ryffel et al.(1986) reported that CS caused BP reduction in older SH rats, although BP was increased in younger rats. In this study, mean systolic BP was slightly increased in the CS group at 4 weeks ($p<0.05$), but there was no difference compared to a control group at 8 weeks. This difference in BP depending on duration of CS therapy needs further study.

Blood urea nitrogen(BUN) was significantly elevated at 4 weeks and continued to rise($p<0.005$), whereas serum creatinine was elevated at 8 weeks. The elevation of BUN and creatinine probably reflects hemodynamically mediated CS toxicity.

In conclusion, CS nephrotoxicity in experimental animals might be caused by combined effects of functional influence on arterioles and tubular toxicity, although there is no unified hypothesis to explain the many aspects of CS nephrotoxicity.

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