The potential anti-inflammatory effect of tetrahydrobiopterin administration in renal mass reduction-induced chronic renal failure in rats

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ABSTRACT

Objectives: To investigate the impact of tetrahydrobiopterin (BH4) supplementation on the markers of inflammation, and on the histological picture of the kidney in chronic renal failure C-reactive protein (CRF) induced in rats by subtotal nephrectomy (SNx).

Methods: This study was performed at the Faculty of Medicine, King Saud University, Riyadh, Saudi Arabia during the period from December 2005 to January 2007. Chronic renal failure was induced by 5/6 SNx in 20 male Wister rats, and another 10 rats were sham operated by flank incision and served as controls. Ten SNx rats received 10mg kg-1 BH4 intraperitoneally daily for 4 weeks. Plasma C-reactive protein (CRP), interlukin-6 (IL-6), malondialdehyde (MDA), and kidney functions were measured in all rats. Histopathological examination of the kidney tissues was also performed.

Results: Untreated CRF rats showed significant elevation of plasma CRP, IL-6, and MDA levels, and significant decrease in plasma albumin and total protein levels, tubuloglomerular fibrosis and, interstitial tubular infiltration with inflammatory cells in comparison with the sham-operated rats. Tetrahydrobiopterin treatment decreased CRP, IL-6, and MDA levels, and decreased tubuloglomerular fibrosis and interstitial inflammation in treated CRF rats.

Conclusions: Supplementation with exogenous BH4 decreased markers of inflammation and protected the kidney against post-renal mass reduction histologic damage. Restoration of intracellular BH4 balance could normalize nitrous oxide production. Therefore, BH4 might be a promising strategy in attenuating inflammation in CRF. This may decrease endothelial dysfunction and limit the associated cardiovascular morbidity and mortality of this disease.

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Persistent inflammation usually recognized by elevated serum levels of C-reactive protein (CRP), either alone or in combination with low protein intake, plays a significant role in uremia patients.[1,2] This is not unexpected because both serum albumin and CRP participate in opposite ways in the same acute phase process.[3] Accelerated inflammation may cause the nutritional state to deteriorate more in these patients.[4] C-reactive protein as one of the acute phase reactants is an exquisitely sensitive systemic marker of inflammation and tissue damage, and via its binding to lipids and lipoproteins and its capacity to activate the classic complement pathway, it has the potential to contribute to atherogenesis.[5] Elevated CRP is widely considered as a marker of the underlying inflammatory process in end stage renal disease (ESRD).[6] Numerous studies have shown that elevated CRP predicts both all-cause, and cardiovascular mortality in chronic kidney disease (CKD),[7] as well as in ESRD patients treated with hemodialysis (HD)[8] or peritoneal dialysis (PD).[9] Tetrahydrobiopterin (BH4), a substance belonging to the pteridine group, is very important for the role it plays in the metabolism of some amino acids, because it acts as a coenzyme for phenylalanine hydroxylase (PH), tyrosine hydroxylase, and other enzymes.[10] In addition, it acts as an essential cofactor for all isoforms of nitric oxide synthase (NOS).[11] Endothelial BH4 availability appears to be a key requirement for maintaining normal endothelial function.[12,13] In the uremic state, BH4 metabolism is adversely affected.[4,14] In the absence of BH4 activation, plasma levels of active oxygen radical (O^-) might be generated in large quantities, due to the oxidase activity of NOS, which activates an inflammatory reaction.[15-17] In inflammatory conditions, inflammatory

cytokines may interfere with the normal biosynthesis of BH4 in the cells and leads to the accumulation of one of its intermediates called neopterin, which can be detected in the plasma and serves as a marker of inflammation, for example, in acute coronary syndrome.[12,13] Serum levels of CRP appear to reflect generation of proinflammatory cytokines (interlukin-1 [IL-1], interlukin-6 [IL-6], and tumor necrosis-alpha [TNF-α]), which also have been reported to be increased in chronic renal failure (CRF) patients.[18] Inflammation is considered a major contributing factor to atherosclerosis both in the general population and in patients on dialysis,[19-21] hence, measurement of biomarkers that reflect inflammation could enhance risk evaluation.[22] High levels of proinflammatory cytokines may cause muscle wasting by stimulating protein catabolism.[23] Furthermore, inflammatory cytokines may lead to expression of inducible NO synthase, which can produce NO at high rates and in parallel with superoxide radicals.[24] Nitrous oxide and superoxide can combine to form peroxynitrite, a radical with potentially deleterious effects on the vascular wall. Peroxynitrite formation also results in chemical inactivation of NO, which will lead to further reduction of activity of NO beyond inhibition.[25] Because inflammatory and pro-thrombotic markers predict change in kidney function,[26] interventions that reduce inflammation have been suggested to confer not only cardiovascular, but also renal benefits. In this regard, the present study aimed at investigating the impact of BH4 supplementation in the CRF model, induced in rats by subtotal nephrectomy (SNx) operation, on CRP and IL-6 (as markers of inflammation), on plasma protein concentration and albumin levels, on kidney functions, and on the histopathological changes in kidney tissues.

Methods. Thirty male Wister rats weighing 280-320g were used and housed in the animal house of the Faculty of Medicine, King Saud University, Kingdom of Saudi Arabia from December 2005 to January 2007. Animals were kept individually in metallic cages under standard laboratory conditions, at adjusted temperature of 25°C and 12 hour light/dark cycles with free access to rat chow, and water ad libitum. The study was conducted in accordance with the standard established guidelines of Laboratory Animals of Collage of Medicine Research Council (CMRC), King Saud University. Chronic renal failure was induced in 20 rats by 5/6 SNx involving right nephrectomy and removal of 2/3 of the left kidney through flank incision under ether anesthesia.[27] The other 10 rats were sham operated by flank incision under ether anesthesia. One week after SNx or sham operation, the rats were allocated into 3 groups (n=10 in each) as follow: Group 1 (Sham): sham operated healthy rats, receiving no treatment, serving as control. Group 2 (SNx): SNx rats receiving no treatment, and Group 3 (SNx+BH4): SNx rats treated with 10 mg kg-1 - (6R)-5, 6, 7, 8-tetrahydrobiopterin daily[28] for 4 weeks. The BH4 was purchased from Schircks Laboratories (Switzerland). Blood samples were collected before surgery and at the end of the study from the retro-orbital venous sinuses under light ether anesthesia into chilled EDETA tubes. Twenty-four hour urine samples were collected before surgery and at the end of the study by keeping rats individually in metabolic cages. At the end of the 4th week, rats were injected intraperitoneally with Ketamin-Xylazin (60 and 7.7mg/kg body weight)[29] and were sacrificed by neck dislocation. The left kidney of the sham operated rats, and the remnant kidney of the SNx rats were separated, washed immediately in iced cold saline and kept in 10% buffered formalin solution for histopathological examination. Blood was centrifuged at 1500 rpm for 15 minutes at 4°C. Plasma was kept under -70°C until biochemical assays. As markers of inflammation, CRP was measured by a commercially available rat CRP ELISA Kit (Alpha Diagnostica International, Inc., San Antonio, Texas, USA), and IL-6 was measured by sandwich ELISA using a commercially available kit (PromoKine, Germany) according to the manufacturers instruction. Plasma level of thiobarbituric acid reactive substances (TBARS), as an index of oxidative stress-induced lipid peroxidation, was measured by a spectrofluorometer and expressed as the amount of MDA according to the method of Yagi.[30] Plasma albumin, total proteins, urea, creatinine, and urinary protein levels were measured by commercial kits from Spinreact, Spain.[31] For evaluation of the effects of the BH4 treatment on renal pathology, kidney biopsies were examined in a blind fashion. Paraffin-embedded 3-µm sections were cut and hematoxylin-eosin staining was performed. Histological analysis was made using the following variables: glomerulosclerosis, tubular atrophy, and interstitial inflammation. The kidney tissue slides were examined under light microscope at 20 x. We used a score out of 17 describing the glomerular, tubular, and interstitial changes. Glomerular changes were classified as normal (N) = 0, periglomerular fibrosis (PG) (presence of fibrous tissue around the glomeruli) = 1, mild fibrosis (MF) (increase in the mesangial matrix with broadening of mesangial areas up to the diameter of 2 mesangial cells) = 1, moderate fibrosis (MOF) in the case of broadening of the mesangial areas by more than the diameter of 2 mesangial cells = 2, severe fibrosis (SF) in the case of mesangial sclerosis corresponding to at least 25% of the glomerular area = 3. Tubular changes were classified as normal (N) = 0, mild atrophy (MA) when microscopic foci of tubular atrophy were found in less than 5% of the tubules = 1, presence of casts (casts) = 1, severe atrophy (SA) diagnosed by the presence of tubular atrophy in large multiple foci occupying more than 5% of the tubular

area = 2. Interstitial changes were described as normal (N) = 0, mild fibrosis (MF) (<5%) = 1, severe fibrosis (SF) (>5%) = 2, mild inflammation (MI) when microscopic foci of plasma cells and lymphocytes infiltration were occupying less than 5% of the kidney = 1, severe inflammation (SI) diagnosed when more than 5% of the kidney was infiltrated = 2.

All results were expressed as Mean ± SD, comparison between several groups was carried out using one way ANOVA test and when the F-value was significant, the post Hoc least significance difference (LSD) was used to locate the significant groups. Comparison between the values taken for the same group before and after the study was carried out using paired sample t-test, $p<0.05$ was considered significant. Statistics were performed by version 10.0 of the computer software program SPPS for windows.

Results. Subtotal nephrectomy caused a picture of CRF (**Table 1**) characterized by a 3.25 folds increase in plasma urea and 2.4 folds increase in creatinine levels, significant increase in urine volume and 24-hour urinary protein loss in the untreated SNx group 2 rats in comparison with the sham operated controls (group 1) ($p<0.05$). The BH4 treatment caused significant decrease in proteinuria in the treated (SNx+BH4) group 3 rats in comparison with the untreated SNx rats ($p<0.05$), but it caused no significant change in plasma urea and creatinine levels in the treated (SNx+BH4) rats ($p>0.05$). Plasma CRP levels increased significantly ($p<0.05$) in SNx rats in comparison with the sham operated group 1 rats (213.60±13.1 versus 167.80±6.8 pg/ml, $p=0.000$). The BH4 treatment significantly decreased CRP level of (SNx+BH4) group 3 rats in comparison with the untreated group 2 rats (173.60±5.05 versus 213.60±13.1 pg/ml), showing no significant difference from the sham-operated group ($p=0.000$) (F=75.96). The IL-6 level increased significantly in untreated SNx group 2 rats in comparison with the control ones (88.37±6.9 versus 39.94±4.4 pg/ml, $p=0.000$), BH4 treatment administered to group 3 rats significantly decreased their IL-6 level in comparison with untreated group 2 rats (57.30±6.8 versus 88.37±6.9 pg/ml, $p=0.000$), but it remained significantly higher than the control value ($p=0.000$, F=175.59). Plasma MDA levels were significantly higher in untreated group 2 SNx rats in comparison with the sham-operated group (6.6±0.96 versus 2.6±0.67 nmol/mL). The CRF rats treated with BH4 for 4 weeks exhibited significantly lower plasma MDA level in comparison with untreated group 2 CRF

Table 1 - Kidney function tests (expressed as mean± SD) of all the studied groups before and at the end of the study.

Parameter	Time	Sham Group 1 n=10	T test	*P*-value	SNx Group 2 n=10	T test	*P* value	SNx + BH4 Group3 n=10	T test	*P*-value	F value	*P*-value
Plasma urea (mg/dl)	Wk 0	40.0 ± 3.2	1.90	0.08	40.78 ± 3.8	32.0*	<0.001	39.98 ± 2.9	44.2*	<0.001	0.16	0.84
	Wk 8	40.8 ± 4.0			132.8 ± 8.9 †‡			128.9 ± 5.7 †‡			620.6	<0.001
Plasma creatinine (mg/dl)	Wk 0	0.45 ± 0.04	1	0.30	0.45 ± 0.4	9.81*	<0.001	0.45 ± 0.4	13.9*	<0.001	0.027	1
	Wk 8	0.45 ± 0.04 §			1.95 ± 0.46 †‡			1.85 ± 0.29 †‡			68.33	<0.001
Urine volume (ml/24h)	Wk 0	14.4 ± 1.4	1.93	0.08	14.4 ± 1.4	10.5*	<0.001	14.3 ± 1.2	16.3*	<0.001	0.004	0.9
	Wk 8	14.7 ± 1.7 §			28.2 ± 3.9 †‡			26.8 ± 2.4 †‡			68.33	<0.001
Urine proteins (mg/24h)	Wk 0	9.7 ± 1.2	1.84	0.09	9.8 ± 1.3	26.6	<0.001	9.5 ± 1.1	17.0*	<0.001	0.15	<0.9
	Wk 8	9.9 ± 1.5§			105.2 ± 11.0 †‡			70.05 ± 12 †‡§			252.6	<0.001

* significant at $p<0.05$
[a] † significant versus wk 0 before SNx or sham surgery at $p<0.05$
‡ significant versus control group 1 at the same time point and $p<0.05$
§ Significant versus subtotal nephrectomy (SNx) group 2 at the same time point and $p<0.05$.
F0 (ANOVA test) between all groups before the start of the study.
F8 (ANOVA test) between all groups at the end of the study
t-value (paired sample t-test comparison between results of the same group before and after the study), wk - week, BH4 - tetrahydrobiopterin

rats (3.08±0.70 versus 6.6±0.96 nmol/l, p=0.000), but it was significantly higher than that of the control rats (p=0.000). The CRF (group 2) rats had significant hypoalbuminemia (2.76±0.33 versus 3.76±0.35) and hypoproteinemia (5.3±0.48 versus 6.7±0.76) (p<0.05) in comparison with the sham operated rats (p<0.05). The BH4 supplementation to group 3 SNx rats caused significant increase (p<0.05) in their plasma albumin (3.50±0.38 versus 2.76±0.33) and total protein levels (5.9±0.2 versus 5.3±0.48) in comparison with that of untreated group 2 rats (p=0.02). Although BH4 caused a full correction of plasma albumin level of treated SNx+BH4 rats in comparison with the sham operated ones (3.50±0.38 versus 3.76±0.35, p=0.12) their total plasma protein level remained significantly lower than that of the control value (5.9±0.2 verus 6.7±0.76, p=0.02). Regarding the histological finding in the kidney tissue, untreated SNx rats (group 2) exhibited periglomerular fibrosis, glomerular expansion with foci of moderate glomerular fibrosis, focal tubular atrophy with intraluminal casts, in addition to, interstitial inflammation with infiltration with lymphocytes and plasma cells in comparison with the sham operated (group 1) rats (**Figures 1 & 2, Table 2**). The BH4 treatment to CRF rats decreased glomerular expansion and fibrosis and showed less tubular fibrosis and interstitial inflammation in group 3 rats in comparison with the untreated group 2 rats (**Figure 3**).

Table 2 - Histopathological findings in the kidney tissue of untreated SNx rats, SNx rats receiving tetrahydrobiopetrin treatment and their sham operated control.

Groups	Glomerular changes					Interstitial changes								Total score
						Atrophy			Fibrosis			Inflammation		
	N 0	PG 1	MF 1	MOF 2	SV 3	N 0	MA 1	SA 2	N 0	MF 1	SF 2	MI 1	SI 2	17
Sham	+					+			+					**0**
SNx		+		+			+ with casts			+			+	**8**
SNx+ BH4			+				casts					+		**3**

N - normal, PG - periglomerular fibrosis, MF - mild fibrosis,
MOF - moderate fibrosis, SF - severe fibrosis, MA - mild atrophy, SA - sever atrophy,
MF - mild fibrosis, SF - sever fibrosis, MI - mild inflammation, SI - severe inflammation.
SNx - subtotal nephrectomy, BH4 - tetrahydrobiopterin

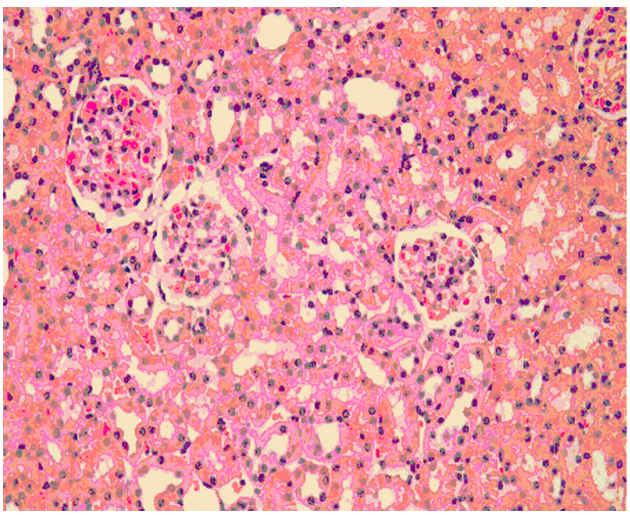

Figure 1 - Microscopic picture of the kidney tissue of control sham operated Group 1 rats showing normal morphology of the glomeruli, tubules and interstitial tissue (hematoxilin and eosin stains, pictures taken with Olympus PM 20 Photomiroscope 20 x).

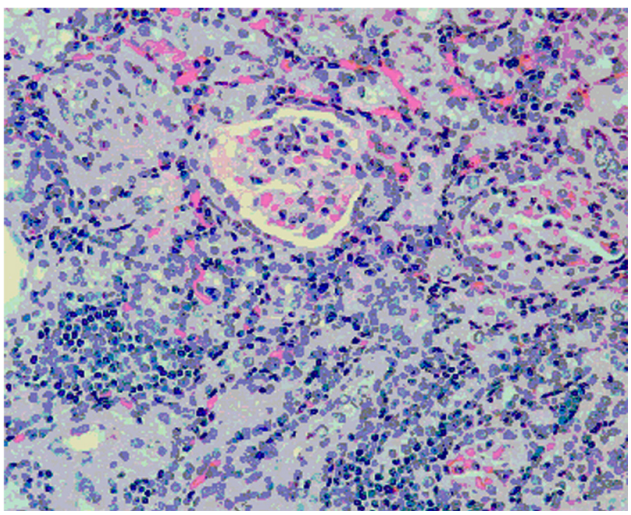

Figure 2 - Microscopic picture of the kidney tissue of untreated subtotal nephrectomy (SNx) Group 2 rats, 4 weeks after SNx, showing glomerulosclerosis, tubular fibrosis with marked interstitial infiltration with lymphocytes and plasma cells (hematoxilin and eosin stains, pictures taken with Olympus PM 20 Photomicroscope 20 x).

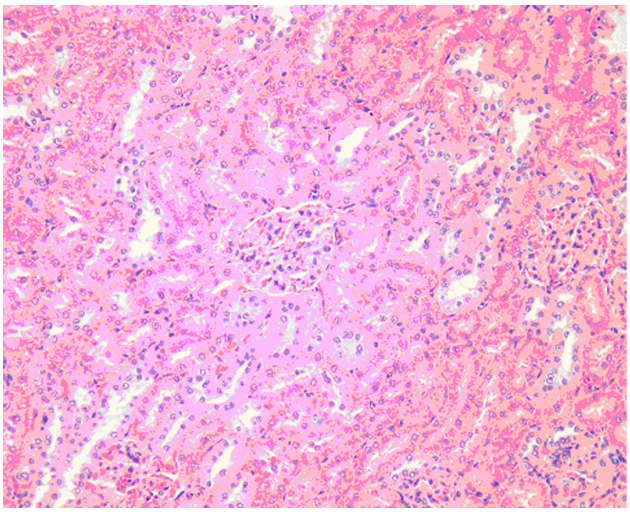

Figure 3 - Section of the kidney tissue of tetrahydrobiopetrin (BH4) treated [subtotal nephrectomy (SNx) +BH4] Group 3 rats showing marked reduction in the interstitial tubular inflammation and the inflammatory cellular infiltration with less tubular fibrosis in comparison to the untreated group.

Discussion. The notion that inflammation is a major contributor to morbidity and mortality in dialysis patients was supported by the demonstration of the elevation of the acute phase reactant CRP and hypoalbuminemia in ESRD and dialysis patients.[2,32] In the current study, CRF induced in rats by SNx operation was presented by significant elevation in plasma creatinine and urea levels, decreased plasma albumin, and total protein levels with increased urinary protein loss. Associated with these signs of renal impairment there was an increase in the plasma CRP and IL-6 levels indicating inflammation, together with increased MDA level indicating oxidative stress. Both CRP and IL-6 have been consistently shown as strong predictors of cardiovascular and all-cause mortality in renal patients,[32] and have been described as markers for atherosclerosis.[33] Recent evidence suggests that CRP may act as a mediator stimulating the release of IL-1, IL-6, and TNF-from monocytes, and vascular smooth muscle cells (VSMCs) and inducing expression of adhesion molecules and plasminogen activator inhibitor type 1 (PAI-1) in the endothelium.[34] Increased oxidative stress marked by high MDA levels, together with increased inflammatory cytokines such as IL-6, may underlie the pathological changes observed in the kidney tissues of the untreated SNx rats in the present study[35] vice tubuloglomerular fibrosis, mild tubular atrophy, and accumulation of tubular casts together with interstitial inflammation and infiltration with lymphocytes and plasma cells. It has been emphasized that tubulointerstitial fibrosis is a main determinant that leads to an irreversible loss of renal function in chronic renal diseases.[36] The assumption that these tubuloglomerular changes may be related to increased levels of IL-6, or other cytokines that were not measured in this study, is supported by the previously reported data on the presence of several cytokines secreted by inflammatory cells that can stimulate fibroblast proliferation and regulate the synthesis of extracellular matrix components,[35] and therefore lead to glomerulosclerosis. The healthy endothelium is a net producer of NO[37] that is believed to act as a key regulator of vascular homeostasis in addition to exerting an important braking effect on inflammation.[38] The BH4, a cofactor required for activity of all NOS isoforms,[11] has antioxidant capabilities.[39] Reduced intracellular BH4 concentration in uremic states[14] could shift production of protective NO to deleterious radical formation.[10] Several biochemical studies have demonstrated that activation of constitutive NOS in the presence of suboptimal levels of BH4 results in uncoupling of oxygen reduction and arginine oxidation, thereby generating reactive oxygen species (ROS);[40-42] this may give an explanation to the elevated lipid peroxidation products (MDA) observed in the untreated CRF rats (group 2) in the current study.

The BH4 supplementation to CRF (group 3) rats in the present study lead to decreased plasma MDA levels, CRP, and IL-6. This reduction in MDA plasma level may be due to the direct antioxidative effect of BH4.[43] Moreover, BH4 has a strong scavenging activity for ROS,[43] and inhibits their cytotoxicity,[44] which may explain the reduction in oxidative stress manifested by decreased MDA production. Furthermore, BH4 supplementation may lead to correction of the relative BH4 deficiency that may occur in uremia.[4] This would counteract uncoupling of oxygen reduction and arginine oxidation during suboptimal BH4 concentration accordingly, and therefore reduce NO-induced cytotoxicity.[44] Thus, BH4 is not only an important regulator of NOS function,11 but is also an intracellular antioxidant. The BH4 can reverse inflammation-induced impairment of the endothelium,[45] and so decrease the production of inflammatory markers such CRP and IL-6 as observed in this study. An anti-inflammatory role of BH4 supplementation in CRF could be supported by the histological findings in the current study showing reduced interstitial tubular infiltration by the inflammatory cells in the treated SNx+BH4 group 3 rats. Furthermore, the inhibitory effect of BH4 supplementation on CRP and IL-6 level in CRF could further intensify the assumption of an inflammatory action of this pteridine compound. The improvement of CRF-induced hypoproteinemia and hypoalbuminemia in BH4 treated rats may be related in part to decreased

protein loss in urine due to less tubuloglomerular damage, or to increased protein synthesis in the liver, through BH4 reported action as a cofactor to a group of the enzymes involved in the synthesis of some amino acids.[10] This antiproteinuric effect of BH4 in CRF rats is in accordance with the results reported by Podjarny et al.[46] In addition to decreased IL-6 level, correction of the hypoalbuminemia could be another evidence of reduced inflammation in rats treated with BH4, as it was reported that both serum albumin and CRP participate in opposite ways to the acute phase process.[3]

Other than reducing the inflammatory cellular infiltration in the interstitial tissues of the kidney, treatment of CRF rats with BH4 for 4 weeks decreased glomerular expansion and tubular atrophy and fibrosis, which are the fore runners of tubuloglomerular sclerosis and hence CRF.[47] This renoprotective effect of BH4 may be related directly to increases NO production through the action of BH4 as a cofactor for NOS.[14] Or may be an indirect effect, through correcting the intracellular BH4 deficiency, that helps to protect the kidney cells against NOS dysfunction - related cell injury resulting from ROS production instead of NO in conditions of true or relative intracellular BH4 deficiency.[44] Nitrous oxide and ROS are known to be implicated in the development of many pathological states,[48,49] and therefore it is possible that BH4 through its effect in regulating NOS function,[11] antioxidative and free radical scavenging action,[43] in addition to, the proposed anti-inflammatory action could be effective as a supportive measure in decreasing inflammation in chronic renal impairment diseases.

In conclusion, CRF induced in rats by SNx is associated with increased CRP, IL-6, and MDA levels. This was associated with a picture of tubuloglomerular injury and tubulointerstitial infiltration with lymphocytes and plasma cells. The BH4 treatment produced a significant protective effect on the tubuloglomerular structure and reduced inflammation marked by decreased CRP and IL-6 levels. This effect of BH4 may be related to its antioxidant effect presented by decreased levels of the oxidative stress marker, MDA. Restoration of the relative intracellular BH4 deficiency that may occur in uremia by BH4 supplementation is suggested, and may help to prevent the abnormal NOS activity that may lead to oxidative damage of the tissues and induction of inflammatory cellular injury. So we could conclude that BH4 might be a promising strategy in attenuating inflammation in CRF, especially that induced by renal mass reduction. This may decrease endothelial dysfunction and limit the cardiovascular morbidity and mortality associated with persistent inflammation in this disease.

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