Urol Res (2002) 30: 35–47
DOI 10.1007/s00240-001-0228-z

ORIGINAL PAPER

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Calcium oxalate stone disease: role of lipid peroxidation and antioxidants

Received: 4 April 2001 / Accepted: 20 November 2001 / Published online: 15 February 2002

Abstract Membrane injury facilitated the fixation of calcium oxalate crystals and subsequent growth into kidney stones. Oxalate-induced membrane injury was mediated by lipid peroxidation reaction through the generation of oxygen free radicals. In urolithic rat kidney or oxalate exposed cultured cells, both superoxide anion and hydroxyl radicals were generated in excess, causing cellular injury. In hyperoxaluric rat kidney, both superoxide and H_2O_2-generating enzymes such as glycolic acid oxidase (GAO) and xanthine oxidase (XO) were increased, and hydroxyl radical and transition metal ions, iron, and copper were accumulated. The lipid peroxidation products, thiobarbituric acid-reactive substances (TBARS), hydroperoxides, and diene conjugates were excessively released in tissues of urolithic rats and in plasma of rats as well as stone patients. The accumulation of these products was concomitant with the decrease in the antioxidant enzymes, superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and glucose-6 phosphate dehydrogenase (G6PD) as well as radical scavengers, vitamin E, ascorbic acid, reduced glutathione (GSH), and protein thiol. All the above parameters were decreased in urolithic condition, irrespective of the agents used for the induction of urolithiasis. Oxalate binding activity and calcium oxalate crystal deposition were markedly pronounced, along with decreased adenosine triphosphatase (ATPase) activity. Lipid peroxidation positively correlated with cellular oxalate, oxalate binding, γ-glutamyl carboxylase, and calcium level and negatively correlated with GSH, vitamin E, ascorbic acid, and total protein thiol. Antioxidant therapy to urolithic rats with vitamin E, glutathione monoester, methionine, lipoic acid, or fish oil normalised the cellular antioxidant system, enzymes and scavengers, and interrupted membrane lipid and protein peroxidation reaction, ATPase inactivation, and its associated calcium accumulation. Antioxidant therapy prevented calcium oxalate precipitation in the rat kidney and reduced oxalate excretion in stone patients. Similarly, calcium oxalate crystal deposition in vitro to urothelium was prevented by free radical scavengers such as phytic acid and mannitol by protecting the membrane from free radical-mediated damage. All these observations were suggestive of the active involvement of free radical-mediated lipid peroxidation-induced membrane damage in the pathogenesis of calcium oxalate crystal deposition and retention.

Keywords Oxygen free radicals · Lipid peroxidation · Hydroperoxides · Membrane injury · Antioxidants · Calcium · Oxalate deposition

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Introduction

Kidney stone disease is a multifactorial disorder resulting from metabolic abnormalities influencing the composition of body fluids and urine. It affects about 1–3% of the population and the recurrence rate is quite high, about 50% at 10 years and 75% at 15 if untreated [9, 120]. Several factors such as heredity, age and sex, geographical factors, climate, race, and diet have been suggested for the aetiology of stone disease [107]. The major risk factors for recurrence are suggested to be male sex, multiple and lower calyx stones, early onset, familial history, and complications after stone removal [109]. The majority of upper urinary stones are composed of calcium oxalate and calcium phosphate and usually occur in men, while most stones of magnesium and ammonium phosphate occur in the bladder, mostly in women [41, 106].

The principal causative factor for the formation of calcium salt stones is attributed to the supersaturation of precipitating salts. Decreased excretion of substances such as citrate, phosphate, pyrophosphate, or

glycosaminoglycans, which form complexes with calcium or elements such as magnesium and sodium which bind oxalate, also enhance the potential for calcium oxalate crystallisation [60, 19]. Even though urine is supersaturated with calcium and oxalate ions in normal subjects, they do not form stones because of the presence of smaller sized crystals and qualitatively improved inhibitors and/or larger quantities of inhibitors [19, 86]. However, stone formers excrete larger crystals and aggregates [36]. Urine, because of its unique combination of anions and cations and small and macromolecular organic components, is capable of holding ions in solution at levels above saturation conditions in a pure aqueous environment. However, the crystal nucleation process can be influenced by the urine chemistry in the presence of particles of organic matter and cellular debris [33]. These organic molecules serve as the nidus for nucleation. Several mechanisms have been postulated for the formation of stones in the urinary tract, such as extracellular matrix nucleation, absence of inhibitors, and supersaturation of stone-forming ionic species [8, 86]. However, the events leading to attachment of crystals followed by retention of the crystals by renal cells is still not well understood.

Cell injury as the primary event for crystal binding

In order for the renal cell to retain calcium oxalate crystal, the crystals should bind to the cell primarily. In support of this, Randall was the first to show initiation of renal calculi to occur as subepithelial calcified plaques in the renal papilla, both in the interstitium and within the nephronic duct [71]. Carr [18] had observed renal calculi formation following the obstructions of the lymphatic system in the form of small radiographic opacities (Carr bodies) that were found in all the kidneys from stone-forming patients. He observed that the presence of renal cavities with low urodynamic efficacy retain urine for long periods of time, favoring calculus formation. In these studies, it is evident that both molecular adhesion and stagnation of crystals in an anatomically constrained region play a vital role for the pathogenic mechanism in the growth of renal stones. Free calcium oxalate crystals formed within the renal tubule can not grow rapidly enough to block a collecting duct at the rate of normal urinary flow and become a kidney stone, because the time needed for a crystal to grow to a diameter of 200 μm and block the nephron is calculated to be from 90 min to 1500 years [27]. So Finlayson and Reid [28] concluded that, in order to form a stone, the crystals should be attached to the epithelium, and they suggested the fixed particle hypothesis. In support of this, Vermeulen et al. [119] demonstrated crystal retention in the rat renal papilla in experimentally induced crystalluric rat. The renal papilla was found to be the primary nucleation site because of the existence of oxalate and calcium gradient [40]. The evidence for crystal attachment to the epithelial basal lamina in crystalluric rat kidney was presented by the studies of Khan et al. [48]. Mandel et al. [58] observed more crystals' attachment to cells that have lost partial or complete intercellular junctional integrity. They suggested that membrane injury exposes the basolateral or basement membrane crystal-binding molecules, facilitating crystal attachment. Further studies using chemically injured urothelium [29, 47], gentamycin-pretreated rat kidney [104], or damaged epithelium with reduced antiadherent glycosaminoglycan layer [32] were in support of membrane injury as the cause of the enhanced crystal retention. These findings have suggested that membrane injury plays a significant role as a predisposing factor for crystal binding and retention reaction.

Much attention was focussed on oxalate-induced cell injury for the facilitation of crystal adherence. Calcium oxalate crystal itself was found to injure the membrane [34] by interacting with the cell membrane and releasing the cellular contents when they were exposed to culture cells. Similarly, in hyperoxaluria, renal tubular membrane injury was observed with increased excretion of enzymes of epithelial membrane origin. Further crystal deposition led to the detachment of the basement membrane, and membranous cellular degradation products were found to promote crystal formation and aggregation [50] and facilitate its retention [46]. In addition, oxalate was found to produce changes in the activities of several enzymes such as lactate dehydrogenase and pyruvate kinase [43]. These studies suggest that the susceptibility of the tissue to crystal attachments depends on the concentration of oxalate and crystal/oxalate-induced toxicity. The first biochemical evidence that oxalate damages membrane by way of promoting lipid peroxidation (LPO) reaction was presented by this laboratory [93] under hyperoxaluric conditions.

Lipid peroxidation is a degenerative pathway of membrane components mediated through free radicals produced in the cell. The evidence of the involvement of oxalate in free radical-mediated LPO reaction for the membrane injury is further strengthened by the subsequent observations made in several other laboratories [90, 114, 115]. The involvement of free radicals and LPO-mediated biochemical alterations in the processes of stone formation and retention has not been dealt with extensively. This review describes the current developments in the involvement of the cell peroxidation reaction as a consequence of free radicals as one of the predisposing factors of oxalate and calcium oxalate binding for subsequent stone formation and retention.

Free radicals mediated cell injury on renal calcium oxalate retention

Experimental evidence for the accumulation of hydroxyl radicals in urolithiasis

All aerobic cells generate enzymatically or nonenzymatically oxygen-derived free radicals, superoxide

anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^{\cdot}$), hydroxyl radicals ($OH^{\cdot}$), and peroxyl radical ($ROO^{\cdot}$) [35] as part of the many normal biological processes. At the same time, the presence of an abundant antioxidant defense of most cells, again both enzymatic and nonenzymatic, prevent the species from causing cell injury. Under pathological conditions, the rate of formation of partially reduced oxygen species is increased and/or the antioxidant defenses of the cells are weakened, eventually leading to oxidative cell injury [35, 38]. The mechanism of cell injury by partially reduced and thereby activated oxygen species is explained through the action of hydrogen peroxide (H_2O_2).

Ascorbic acid, a precursor of oxalate biosynthesis, has been shown to promote LPO in vitro in tissue nonenzymatically. Ernster [24] was the first to show enhanced ascorbic acid-linked LPO in the presence of iron complexes as well as oxalate [24], showing the prooxidant nature of oxalate. The increased LPO formation by oxalate is probably associated with the generation of oxygen free radicals.

The experimental evidence for the accumulation of hydroxyl radical in both kidney and liver was presented by our laboratory in urolithic rats when induced by feeding either a sodium glycolate-supplemented diet [94, 96] or a B_6-deficient diet [73, 98]. The excessive formation of hydroxyl radical observed in urolithic rat kidney was explained on the basis of inhibition of catalase activity by oxalate in vitro [93]. The inhibition of catalase activity was found to be oxalate concentration-dependent (17% of inhibition at 0.5 mM and 30% inhibition at 1 mM), and the same effect had been suggested for the observed decreased catalase activity in urolithic kidney [14]. In the urolithic kidney, both iron and copper were accumulated, and this process could facilitate the formation of hydroxyl radical [73, 74, 76, 98].

Increased production of superoxide anion

The concentration of superoxide anion can be increased either by inhibition of superoxide dismutase or the increased activity of enzymes which contribute superoxide anion as one of the products. In urolithic kidney, SOD activity decreased [73, 94, 96, 99]. Inhibition of SOD activity can induce accumulation of superoxide anions, which in turn can be directed to form the hydroxyl radical. Enhanced superoxide production was observed during phagocytosis of crystals [23, 25], hyperoxia, xenobiotic metabolism, inflammation [35], and ischaemia reperfusion [69]. Direct evidence of increased production of both superoxide and hydroxyl radicals had been reported in the LLC-PK1 cells as well as MDCK cells when incubated with oxalate [90, 115, 116]. As oxalate inhibits catalase activity directly [93], H_2O_2 can accumulate. Extracellular addition of superoxide dismutase, catalase, desferoxamine, and mannitol inhibited the production of superoxide anion as well as the hydroxyl radical in cultured cells exposed to oxalate, supporting the view that oxalate stress induces the production of oxygen radicals. Under experimental urolithic conditions, the concentration of oxalate in renal cells increased from 0.47 mg/g tissue to 1–1.67 mg/g tissue [10, 78, 79]. This might be sufficient to produce oxidative stress and thus excess hydroxyl radical formation.

During oxalate synthesis oxidases, GAO and XO release hydrogen peroxide and/or superoxide anions as end products. These enzyme activities were elevated in liver and XO and LDH in kidney [3, 73, 94, 96, 98] under urolithic conditions, suggesting that oxalate stress leads to increased production of both H_2O and superoxide anions. This is well supported by the observation of higher activity of XO in ischaemic-reperfused calculus kidney than in control ischaemic-reperfused kidney, a condition in which excessive oxalate formation was present [102]. Xanthine oxidase was first documented as a biological source of $O_2^{\cdot-}$ [42] and a key mediator of cell injury. In normal tissues, the enzyme exists as a dehydrogenase, utilising NAD+ as coenzyme instead of O_2 as the electron acceptor. Xanthine dehydrogenase can be converted to XO irreversibly by oxidation of its critical sulphydryl groups and proteolysis [68]. As hyperoxaluria depletes antioxidants, the conversion of XDH to XO is favoured. The further increased XO in the presence of excess free iron under urolithic conditions can effectively generate OH radicals [16]. On the whole, both the superoxide anion and the hydroxyl radical are produced in excess under oxalate stress conditions.

Consequences of free radical-membrane interaction

Lipid peroxidation

Irrespective of the nature of urolithic agents used to induce either hyperoxaluria or calcium oxalate crystal deposition in experimental rats by administration of sodium oxalate [93], feeding sodium glycolate either in the diet or drinking water [14, 94, 96], vitamin B_6-deficient diet [73, 74, 76, 98], ethylene glycol (EG) [15, 114], ammonium oxalate [1], and dehydroascorbic acid [97], enhanced LPO products that were always commonly observed in kidney, liver, and plasma (Table 1). When the animals were treated with a glutathione-depleting agent such as buthionine sulfoximine or nephrotoxin, and cyclosporine along with a calculi-producing agent, still higher LPO products were released [15, 65] (Table 2). The basal levels of LPO products, thiobarbituric reactive substances (TBARS), hydroperoxides, diene conjugates, and lipofuscin were elevated. The susceptibility of renal tissue for LPO in the presence of promoters like nicotinamide adenine dinucleotide phosphate, reduced (NADPH), ascorbate, t-butyl hydroperoxide, iron, copper, and oxalate was enhanced 3–25-fold [72]. Oxalate or calcium oxalate and not calcium chloride stimulated lipid peroxidation. The release of LPO products was significantly higher in ischaemia followed by reperfused urolithic kidney than in control

Table 1. Status of hydroxyl radical, LPO products, pro-oxidants, calcium, oxalate and crystal deposition, antioxidant enzymes, and scavengers in tissues of urolithic rats induced by feeding different urolithic agents with and without antioxidant supplementation. *K* kidney, *L* liver, *P* plasma, *M* mitochondria, *Mi* microsomes, *B* blood, *CG** Calcium gluconate, *EPA* eicosapentaenoic acid, *Met* methionine, *Sod gly* sodium glycolate

Parameters		B_6 deficiency						B_6 deficiency + Vit E/Met			Sod Gly		Sod Gly + citrate		Sod Gly + Met		CG*	CG + EPA
		K	L	P	M	Mi	B	K	L	P	K	L	K	L	K	L		
OH and LPO products	$OH^{\cdot}$	↑	↑	–	↑	↑	–	→	→	→	↑	↑	↑	↑	→	→	–	–
	Basal	↑	↑	↑	↑	↑	↑	→	→	→	↑	↑	↑	↑	→	→		
	Enzymic/non-enzymic	↑	↑	↑	↑	↑	↑	→	→	→	↑	↑	↑	↑	→	→		
	OOH	↑	↑	–	↑	↑	–	→	→	→	↑	↑	↑	↑	→	→		
	Diene conjugates	↑	↑	↑	↑	↑	–	→	→	→	↑	↑	↑	↑	→	→		
	Lipofuschin	↑	↑	↑	↑	↑	–	→	→	→	↑	↑	↑	↑	→	→		
Pro oxidants	Fe^{2+}	↑	↑	↑	↑	↑	–	↔	↔	→	→	→	→	→	→	→	–	–
	Cu^{2+}	↑	↑	↑	↑	↑	–	↔	↔	→	–	–	–	–	–	–		
	XO	↑	–	↑	–	–	–	↔	↔	–	↑	↑	↑	↑	↔	↔		
	GAO	↑	↑	↑	–	–	–	–	↔	–	–	↑	–	↑	–	↔		
Ca^{2+}, oxalate, and crystal deposition	Calcium	↑	↑	↑	↑	↑	–	→	→	→	↑	↑	–	–	–	–	–	–
	Oxalate	↑	↑	–	↑	↑	–	→	→	↑	↑	↑	↑	↑	↑	↑	–	–
	CaOx deposition	++	–	–	–	–	–	–	–	–	++	–	$	–	$	–	++	$
Antioxidant, enzymes	SOD	→	↓	↔	–	–	–	↓	↓	–	↓	↑	↓	↓	→	↔	–	–
	Catalase	↓	↓	↔	–	–	–	↑	↑	–	↓	↓	↓	↓	→	→		
	GPx	↓	↓	↔	–	–	–	↑	↑	–	↓	↓	↓	↓	→	→		
	G6PD	↓	↓	–	–	–	–	→	→	–	↓	↓	↓	↓	→	→		
Scavengers	TSH	↓	↓	–	–	–	–	↑	↑	–	↓	↓	↓	↓	→	→	–	–
	GSH	↓	↓	–	–	–	–	↑	↑	→	↓	↓	↓	↓	→	→		
	Vitamin E	↓	↓	–	–	–	–	→	→	↑	↓	↓	↓	↓	→	→		
	Ascorbic acid	↓	↓	–	–	–	–	→	→	→	↓	↓	↓	↓	→	→		
References	–	72, 73	74, 99	75, 76	74	74	99	100	100	99	14	93	94	94	96	95	88	88

↑, increased; ↓, decreased; →, normalised; ↔, partially normalised; ++, deposition; $, no deposition

Table 2. Status of LPO, pro-oxidants, calcium, oxalate and crystal deposition, antioxidant enzymes, and scavengers in tissues of urolithic rats induced by treatment with nephrotoxin along with a calculi producing diet with and without antioxidant supplementation. *K* kidney, *L* liver, *RM* rat mitochondria, *A** ascorbic acid, *M** mannitol, *P** phytic acid, *E.G* ethylene glycol, *AmOx* ammonium oxalate, *BSO* buthionine sulfoximine, *Vit.E* vitamin E, *CPD* calculi producing diet, *CsA* cyclosporin A, *GME* glutathione mono ester, *Uro** urothelium, *IR* ischaemic reperfusion

Parameters		EG		BSO + EG		EG + GME		BSO + EG + GME		AmOx		AmOx + CsA		AmOx + Vit.E		AmOx + CsA + Vit.E		IR	IR + CPD	Uro*	Uro* + H_2O_2	Uro* + A* M* P*	Uro* + Citrate
		K	RM	K	RM	K	RM	K	RM	K	L	K	L	K	L	K	L	K	K				
LPO	Basal	↑	↑	↑	↑	→	→	→	→	↑	↑	↑	↑	→	→	→	→	↑	↑	–	–	–	–
	Enzyme/ nonenzyme	↑	↑	↑	↑	→	→	→	→	↑	↑	↑	↑	→	→	→	→	↑	↑				
Pro oxidants	Fe^{2+}	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	↑	↑	–	–	–	–
	XO	↑	↔	↑	↔	↑	↔	↔	→	↑	↑	↑	↑	→	→	↔	↔	↑	↑				
	GAO	↑	↔	↑	↔	↔	↔	↔	→	↔	↑	↔	↑	↔	↔	↔	↔						
Ca^{2+}, Oxalate and crystal deposition	Calcium	↑	↑	↑	↑	→	→	→	→	↑	–	↑	–	→	–	↔	↑	↑	↑	–	–	–	–
	Oxalate	↑	↑	↑	↑	→	→	→	→	↑	↑	↑	↑	↔	↔	↔	↔	↑	↑	–	–	–	–
	CaOx deposition	++	–	++	–	$	–	+	–	++	–	++	–	$	–	$	–	$	$	COD	COD↑	↓	–
Antioxidant enzymes	SOD	↓	↓	↓	↓	→	→	↔	→	–	–	–	–	–	–	–	–	↓	↓	–	–	–	–
	Catalase	↓	–	↓	–	→	→	→	→	–	–	–	–	–	–	–	–	↓	↓				
	GPx	↓	–	↓	–	→	↔	↔	→	↓	↓	↓	↓	→	→	↔	→	↓	↓				
	G6PD	–	–	–	–	–	–	–	–	↓	↓	↓	↓	→	→	↔	↔	↓	↓				
	GST	→	–	↓	–	→	↔	↓	↔	↓	↓	↓	↓	→	→	↔	→	↓	↓				
Scavengers	TSH	↓	↓	–	↓	→	–	↔	–	↓	↓	↓	↓	↑	→	→	→	↓	↓	–	–	–	–
	PSH	↓	↓	↓	↓	→	→	↔	→	↓	↓	↓	↓	↑	→	→	→	–	–				
	GSH	↓	↓	↓	↓	→	→	↔	→	↓	↓	↓	↓	↑	→	→	→	↓	↓				
	Vitamin E	↓	–	↓	–	↔	–	→	↔	↓	↓	↓	↓	↑	↔	↑	↔	↓	↓				
	Ascorbic acid	↓	–	↓	–	↔	–	→	↔	↓	↓	↓	↓	→	→	→	↔	↓	↓				
References	65, 115	65	65	66, 67	67	66, 67	66	67	2	3	3, 1	3, 1	2, 1, 92	3, 1	2, 1, 92	3, 1	3,1	102	102	31	31	31	31

↑, increased; ↓, decreased; →, normalised, ↔, partially normalised; –, no effect; + +, deposition; $, no deposition

ischaemia-reperfused kidney, suggesting that oxalate plays a significant role in the induction of LPO through the generation of free radicals [42]. Similarly, the RBC of stone patients were found to be osmotically fragile, and the RBC membranes not only showed elevated basal levels of LPO products but also released an excess of it in presence of promoters, suggesting that the RBC of stone formers are prone to LPO reaction because of the loss of some protecting molecules [7]. The plasma of stone patients and urolithic rat showed significant accumulation of lipofuscin pigment, a conjugate product of LPO reaction, suggesting that a free radical-mediated LPO mechanism is highly operative in the stone formers [4, 7, 75] (Table 3).

Lipid peroxidation was found to correlate positively with the tissue concentration of oxalate and calcium and to correlate negatively with the antioxidant system (Table 4). Both oxalate and calcium accumulated and the enzymatic and nonenzymatic LPO in subcellular fractions such as mitochondria, microsomes, and nuclei in both kidney and liver of B_6-deficient diet-fed rats were also increased [73, 98]. Among the subcellular fractions, microsomal and mitochondrial fractions showed significant accumulation of iron and copper [74, 76]. The elevated release of LPO products in this fraction is due to relatively large amounts of polyunsaturated fatty acids in their phospholipids [87]. A subtle change in the composition of fatty acids could damage the membrane, and this is often sufficient to increase greatly the susceptibility of the membrane to oxidative damage. In the chemically damaged urothelium, a direct relationship between the extent of membrane damage and the oxalate salt deposits was observed [47]. Similarly, when buthionine sulfoximine treatment was combined with ethylene glycol, there was more deposition of crystals in the tubules damaged with denudation and shedding of epithelial lining of the tubules [65]. Similar observations were also seen in the cyclosporin-treated ammonium oxalate-fed rats [1] and ethylene glycol-fed rats [46]. Studies by Thamilselvan et al. [114] also revealed that oxidative damage was more related to the hyperoxaluria and crystalluria rather than to tissue crystal deposits. However, the process of membrane damage by way of production of thiobarbituric acid-reactive substances was greatly increased with progressive crystal deposition. Elevated tissue LPO was manifested with increased excretion of TBARS in urine [114]. These observations strengthen the view that oxalate, together with calcium oxalate, exerts more effect on the production of lipid peroxides, as shown in vitro studies [14, 93].

The correlation of LPO with various parameters is listed in Table 4.

A negative correlation was observed between LPO and antioxidant enzymes or scavengers such as total thiol, protein thiol, ascorbic acid, vitamin E, and GSH. Similarly, the tissue enzymes, alanine transaminase (ALT), aspartate transaminase (AST), and glutathione-s-transferase (GST) negatively correlated with LPO. In contrast, oxalate binding activity and oxalate-synthesising enzymes positively correlated with LPO. All these reactions were in favour of the accumulation of oxalate and calcium, which positively correlated with LPO.

Vitamin K-dependent γ-glutamyl carboxylase, a microsomal enzyme, synthesises γ-carboxy glutamic acid (GLA) during post-translational modification of proteins [39]. Under stone-forming conditions, the activity of γ-glutamyl carboxylase positively correlated with LPO [5]. This increased activity of the enzyme is suggested to be due to stimulation of the enzyme by oxalate, calcium oxalate, and the increased availability of endogenous carboxylase substrate and LPO [5]. Carboxy glutamic acid is known to bind calcium effectively and facilitate calcium accumulation. Further oxidised lipids produced as a consequence of LPO in the cell membrane also act as calcium ionophores [103], leading to calcium accumulation. Oxalate also can facilitate calcium accumulation under this condition by forming its salt.

Antioxidant enzymes and scavengers in urolithiasis

In urolithic rats induced by different agents such as feeding vitamin B_6-deficient diet, sodium glycolate, ammonium oxalate, or ethylene glycol, the antioxidant enzymes, SOD, catalase, GPx, G6PD, and glutathione -S- transferase activities were decreased. Furthermore, the concentrations of free radical scavengers, vitamin E, ascorbic acid, GSH, and protein thiol also decreased [1, 3, 14, 15, 65, 73, 74, 76, 94, 96, 97, 98, 102] (Table 1, Table 2). Similar to experimental urolithic rats, stone patients also showed enhanced plasma LPO products, TBARS, hydro peroxides, and lipofuscin, and depletion of antioxidant scavengers [4, 7, 75] (Table 3).

The excessive concentration of H_2O_2 and hydroxyl ion radical present under urolithic conditions are attributed for the inactivation of SOD by interacting with its metal ion [26]. Reduced nicotinamide adenine dinucleotide phosphate is essential to keep catalase enzyme in the active form [51]. As G6PD activity is decreased in urolithic kidney, the catalase enzyme exists in the inactive form. In addition, catalase activity is sensitive to oxalate concentration [93]. Glutathione peroxidase activity is GSH-dependent, and it is known to utilise GSH as a major source of reducing power for the removal of H_2O_2 and hydroperoxides. So decreased GPx activity due to decreased availability of GSH thus can lead to accumulation of H_2O_2 and hydroperoxides. Both GSH and NADPH are required to keep its active site and selenocysteine from oxidation, and GSH deficiency or oxidative stress results in loss of activity [21].

Protein damage by thiol oxidation and calcium accumulation

Depletion of cellular GSH results in protein thiol oxidation. Protein oxidation and its underlying mechanism,

Table 3. Oxygen free radicals, LPO and antioxidant enzymes, and scavengers in urolithic rat kidney and oxalate exposed cell cultures and kidney stone patients treated with and without antioxidants

Parameters		Oxalate[a]	Oxalate + vitamin E + mannitol	Oxalate	COM crystals	+ SOD0 + catalase	Stone patients		Stone patients + vitamin E treated
		Kidney	Kidney	MDCK[a]/ LLCPK$_1$*	MDCK/ LLCPK$_1$	MDCK/ LLCPK$_1$	RBC	Plasma	RBC/plasma
Oxy free radicals and LPO products	$O_2^{\cdot-}$	–	–	↑	↑	→	–	–	–
	$OH^{\cdot}$	–	–	↑	↑	→	–	↑	–
	Basal	↑	→	↑	↑	→	↑	↑	→
	Enzyme/non-enzyme	↑	→	–	–	–	↑	↑	–
	$OOH^{\cdot}$	–	–	↑	↑	→	–	↑	–
	Diene conjugates	–	–	–	–	–	–	↑	–
	Lipofuscin	–	–	–	–	–	–	↑	–
Ca^{2+}, oxalate and crystal deposition	Oxalate	↑	↓	–	–	–	–	–	–
	Ca.Ox deposition	++	$	–	–	–	–	–	–
Antioxidant enzymes	SOD	↓	→	–	–	–	↓	–	→
	Catalase	↓	→	–	–	–		–	–
	GPx	↓	→	–	–	–	↓	–	→
	G6PD	–	–	–	–	–	–	–	–
	GST	–	–	–	–	–	–	–	–
Scavengers	TSH	–	–	–	–	–	–	–	↔
	PSH	–	–	–	–	–	–	–	–
	GSH	↓	→	–	–	–	↓	↓	→
	Vitamin E	↓	→	–	–	–	–	↓	→
	Ascorbic acid	↓	→	–	–	–	–	↓	→
References	–	93, 113	113	115, 90	115, 90	115, 90	4, 7	4, 75	4

↑, increased; ↓, decreased; →, normalised; ↔, partially normalised; $, no deposition; ++, deposition

[a] Most of the studies described were performed with high oxalate administration or with cells derived from early parts in the nephron. These cells are not used to encounter crystals or high oxalate. The oxalate concentrations used are high: It is therefore difficult to assess the physiological relevance of these results

Table 4. Correlation between parameters measured in urolithic rats

S. no.	Parameter	LPO	Oxalate
1	γ-Glutamyl transferase	+0.72	–0.79
2	Oxalate	+0.78	–
3	Calcium Mitochondria	+0.71	+0.73
4	Xanthine oxidase	+0.75	+0.90
5	Lactate dehydrogenase	+0.80	+0.92
6	Aspartate transaminase	–0.62	–0.69
7	Alanine transaminase	–0.73	–0.70
8	Glutathione-s-transferase	–0.51	–0.35
9	Glucose-6-phosphate, dehydrogenase	–0.76	–0.71
10	Glutathione peroxidase	–0.67	–0.68
11	Glutathione reductase	+0.71	+0.84
12	Total thiol	–0.59	–0.49
13	Protein thiol	–0.54	–0.43
14	Nonprotein thiol	–0.67	–0.65
15	Ascorbic acid	–0.63	–0.68
16	Vitamin E	–0.81	–0.77
17	Reduced glutathione	–0.71	–0.86
18	γ-Glutamyl carboxylase	+0.79	–
19	Total ATPase	–0.67	–0.62
20	Ca^{2+}-ATPase	–0.43	–0.42

Statistical analysis was carried out using Student's *t*-test. Pearson correlation coefficient was used to assess to linear correlation between pairs of results obtained from [1, 2, 5, 65, 66]

functional implications, and role as a signal for proteolytic degradation are now fairly well understood [108]. Reactive free radicals damage cells by:

1. Covalent binding of free radicals to membrane enzymes and/or receptors, thereby modifying the activities of membrane components and affecting membrane functions
2. Disturbance of transport processes such as potassium efflux, calcium, sodium influx through covalent binding, thiol group oxidation, or a change in polyunsaturated fatty acid:protein ratios
3. Initiation of LPO of polyunsaturated fatty acids with direct effects on membrane fluidity, cross-linking structure, and inhibition of integrated cellular metabolic processes [63]. These altered functions can take part because protein thiol depletion is another major metabolic reaction observed in urolithic conditions

Protein thiol was found to be significantly reduced in ammonium oxalate-administered rats [1] with or without cyclosporin A treatment (Table 1, Table 2). A positive correlation between ATPase activity and protein thiol content and a negative correlation between ATPase and LPO have been observed under this condition [1] (Table 4). Ca^{2+} ATPase, Mg^{2+} ATPase, and Na^{+}-K^{+} ATPases are lipid-dependent as well as –SH-dependent membrane-bound enzymes. The peroxidative properties of oxygen radicals on unsaturated fatty acids may account for the altered properties of the membrane ATPase. Decreased Ca^{2+} ATPase activity was also noted in the microsomes from the kidneys of patients with nephrolithiasis and active chronic pyelonephritis [30]. The alterations in membrane lipid and protein due to peroxidation can lead to increased permeability of calcium, resulting in loss of enzyme activities [12]. During membrane peroxidation, mitochondrial and microsomal calcium accumulate due to loss of the –SH group of ATPase, which maintains the calcium pump [84], thereby leading to perturbation of cellular calcium homeostasis. The thiol depletion of protein is associated with the depletion of reduced glutathione [11].

There is a negative correlation between GSH and calcium or oxalate, implicating that oxidation of GSH leads to accumulation of calcium and oxalate in the renal cells due to its utilisation to scavenge free radicals. Accumulation of calcium and oxalate is also observed along with depletion of antioxidants in renal ischaemic reperfusion [43]. Increased oxidised glutathione leads to the formation of protein –S-glutathione disulfide, which favours an increase in cytosolic calcium during peroxidation [12]. Administration of the GSH-depleting agent, buthionine sulfoximine (BSO), also results in the accumulation of both calcium and oxalate [65].

A positive correlation between LPO and mitochondrial calcium indicated failure in the calcium pump [66]. At the same time, calcium correlated positively with oxalate. Further oxalate-binding activity correlated positively with TBARS release and negatively with thiol content of the proteins [66]. The oxalate binding was inhibited by GSH and activated by glutathione disulfide (GSSG) [101]. The inhibition of oxalate binding by reduced GSH was due to its competitive inhibition by its dicarboxylic acidic nature. Our results confirmed the importance of mitochondrial protein –SH groups in the reduced state for controlling the oxalate binding or its uptake. The lack of an ability to export GSSG from mitochondria may accentuate mitochondrial susceptibility to protein thiol oxidation, resulting in enhanced oxalate binding and uptake. Furthermore, kidney mitochondria were found to be more vulnerable than liver mitochondria for the membrane effects of oxalate [83]. Urolithic rat kidney mitochondria showed a declined respiratory control ratio [67], suggesting oxalate-induced damage. Additionally, H_2O_2 and XO systems were also capable of altering the ability of mitochondria to sequester and retain calcium [85].

Calcium release from intact mitochondria and microsomes is considered to be a critical event in the genesis of lethal cell injury with oxidative stress. Calcium stress and active oxygen species produced during ischaemic reperfusion are shown to disintegrate and fragment mitochondrial membranes and to denature intramatrical proteins [117], leading to an accumulation of calcium phosphate and calcium hydroxyapatite [59, 117]. A negative correlation between calcium and protein thiol content of the cell, observed in urolithic conditions, supports this [67]. It is true when the reactive oxygen species overwhelms cellular defenses [105]. Renal tubular cells are found to be highly susceptible to injury during periods of exogenous oxidative stress, and an association between depletion of oxidation of GSH pool and the onset of injury has been noted [61].

Antioxidant therapy

If LPO is the major cause of tissue injury, then further experiments should show that prevention of peroxidation by antioxidants prevent cell damage [105]. Supplementation of antioxidants either –SH-generating amino acid methionine [94] or –SH reagents such as GSH monoester [66] and α lipoic acid [111], or cysteine [89] hydroxyl radical scavengers such as mannitol [113] or vitamin E [2, 52, 92, 99] and triterpenes [57] abolished the accumulation of LPO products in tissues under urolithic conditions. This normalisation process has been suggested due to arresting of free radical-mediated reactions during antioxidant therapy in urolithiasis. This is facilitated because of restoration of the levels of prooxidants such as iron, copper, and oxalate and the antioxidant enzymes, SOD, catalase, GPx, free radical scavengers, GSH, ascorbic acid, vitamin E, and protein thiol groups [2, 92, 99, 100]. At the same time, there was no retention of calcium oxalate crystals in the tissue, even though the excretion of oxalate was not reduced in experimental urolithiasis [66, 94, 113]. This was due to the protection of vitamin E against oxalate-induced cell injury. Vitamin E is known to protect against chemically induced cell injury by maintaining cellular protein thiols as a cytoprotective mechanism [70].

Administration of vitamin E to surgically stone-removed patients rapidly restored the level of antioxidants in the blood and reduced the urinary excretion of oxalate and calcium [4]. Citrate excretion was found to increase gradually, and about 50% normalisation was achieved following 90 days of treatment. This slow recovery may be possibly explained by assuming that normalisation of health requires a balance of a number of antioxidants rather than high levels of a single nutritional supplement. The overall antioxidant status of the biological system may be more important than the levels of any single antioxidant. Improved antioxidant status following dietary intake or supplementation is well documented with decreased LPO [77], decreased protein oxidation, and decreased erythrocyte lysis [77]. Citrate (in the form of potassium sodium citrate) or magnesium citrate is advocated to stone patients, and the reduction of excretion of stone risk factors is attributed to its complex formation with calcium, thereby preventing calcium oxalate complex formation [54]. However, citrate treatment had no effect on free radical-mediated reactions in experimental urolithic rats, on either reducing LPO or improving antioxidant levels, even though it prevented stone formation [81, 95]. Nephrolithiasis, which was readily produced in the control animals, was prevented in the experimental animals by pretreatment with fish oil, and urine calcium excretion was significantly reduced. The urinary calcium and oxalate excretion in the recurrent hypercalciuric stone formers was significantly reduced with fish oil treatment over an 8-week period [20]. All these observations implicated the radical-mediated membrane changes, predisposing a favourable environment for subsequent crystal deposition and then retention. Antioxidants intervene in this process, protect the membrane from injury, and prevent adherence or retention of the crystals. GAGs has an antiadherent property and prevents the development of solid concretions on urothelium. Free radical scavengers such as phytic acid prevent the deposition of crystals [31]. Pretreatment of rats with vitamin A has an inhibitory effect on lithogenesis through its action on tubular cellular repair [88]. Furthermore, vitamin E therapy improves the RBC status of patients undergoing chronic dialysis [121], and mannitol is widely used in acute renal insufficiency [112] and ischaemic acute renal failure [17] as a free radical scavenger.

Effect of hyperoxaluria on calcium oxalate deposition in renal cells

Damaged membrane binds more crystals because of the exposure of more crystal binding sites [50]. Several polyanionic molecules are able to inhibit the binding of calcium oxalate crystals with membranes, suggesting that crystal binds to anionic molecules of the membrane [56]. Phosphatidyl serine has been identified as one of the molecules for the crystal membrane interactions [13]. This is well corroborated with the finding of phospholipid (PL) in the stone matrix of kidney stones [49].

Though several factors are enumerated for the intratubular crystal formation and attachment to the epithelial membrane, very few studies attribute the factors responsible for the intracellular crystal deposition. One of the suggestions is the crystal entry into the cell via internalisation after the adherence of crystals at the tubular membrane. Severe hyperoxaluria induced in rats by the administration of oxalate produces intraluminal calcium oxalate crystals, which appear to attach to the apical membrane of tubular epithelial cells and are subsequently deposited in the interstitium [45, 48]. Most evidence about the role of internalised crystals in renal stone disease are derived from cell culture studies that were performed with renal proximal tubular cells. These cells are specialised in endocytosis but usually are never confronted with crystals. Similarly, when animals are treated with high doses of oxalate, crystals are formed very early in the nephron, where the cells are specialised in taking up the crystals passing by. Although there is no direct evidence for the concept that crystals are formed inside cells, it is speculated that cellular dysfunction in handling the oxalate, calcium, and phosphate can lead to the precipitation of the calcium salts in the cells, as seen in mitochondria and endoplasmic reticulum with calcium phosphate precipitate during ischaemic insult [59, 117] in rat kidney.

Several biochemical changes might take part in the precipitation of calcium oxalate. Phospholipids are known to provide the polar groups needed to bind to the cations such as calcium and magnesium and anions such

as phosphates and oxalates. They provide an environment where cations and anions are prevented from coming together. When the content of phospholipids is decreased, as seen in urolithic kidneys [81, 82], cation and anion charge density on the membrane is expected to decrease and thereby be unable to prevent them from coming together to form their respective salts. The increased concentration of phosphatidic acid, sialic acid, or N-acetyl neuramic acid in urolithic kidney [44, 80, 82] could facilitate provision of a locus of increased concentration of calcium. As the tissue oxalate level is also high under urolithic conditions, one can expect supersaturation of salt-forming ions and initiation of salt deposition encompassing subcellular organelles (Fig. 1). This may be one of the reasons for the inclusion of mitochondria, endoplasmic reticulum, membranous vesicles, and nuclei in the intercrystalline spaces of stones [45]. Among the subcellular organelles, microsomal proteins exhibit maximal COM crystal binding, and peroxidised membranes bind more crystals [6]. Microsomes contain vitamin K-dependent γ-glutamyl carboxylase, and this enzyme is activated by oxalate and calcium oxalate [5]. Both COM adsorption and the calcium oxalate (^{14}C) binding studies showed that enhanced carboxylation of microsomes in urolithic rat kidney was responsible for the observed increase in COM crystal binding through increased formation of γ-glutamyl carboxylated proteins [6]. This is well supported by the observation of increased levels of free and γ glutamyl carboxylated protein in the urine of stone formers [55]. It has been shown that both free and protein-bound γ-glutamic acid exert a marked stimulatory effect on the nucleation rate of calcium oxalate [6].

Calcium oxalate crystals formed either through endocytosis or precipitation in the cells, or oxalate, had been shown to initiate induction of several genes such as c-myc, EGR-1, Nur-77, PAI-1, and the PDGF –A chain by inducing oxidative stress [37]. Furthermore, oxalate itself was shown to induce initiation of DNA synthesis [50] and promoted the progression of cells from G_0-G_1 to the S phase of the cell cycle at the concentration of 0.1–0.3 mM in the growth medium. In addition, oxalate exposure caused activation of two lipid signaling cascades, one involving phospholipase A_2 (PLA_2) and another involving ceramide, and these responses were inhibited by antioxidants [91]. These studies suggested that hyperoxaluria or calcium oxalate crystals cause tissue injury and, in response to tissue injury, tissue initiates many biochemical changes, including the recruitment of macrophages and multinucleated giant cells for encapsulation of interstitial crystal [22] and release of modulator proteins such as bikunin [62], which is

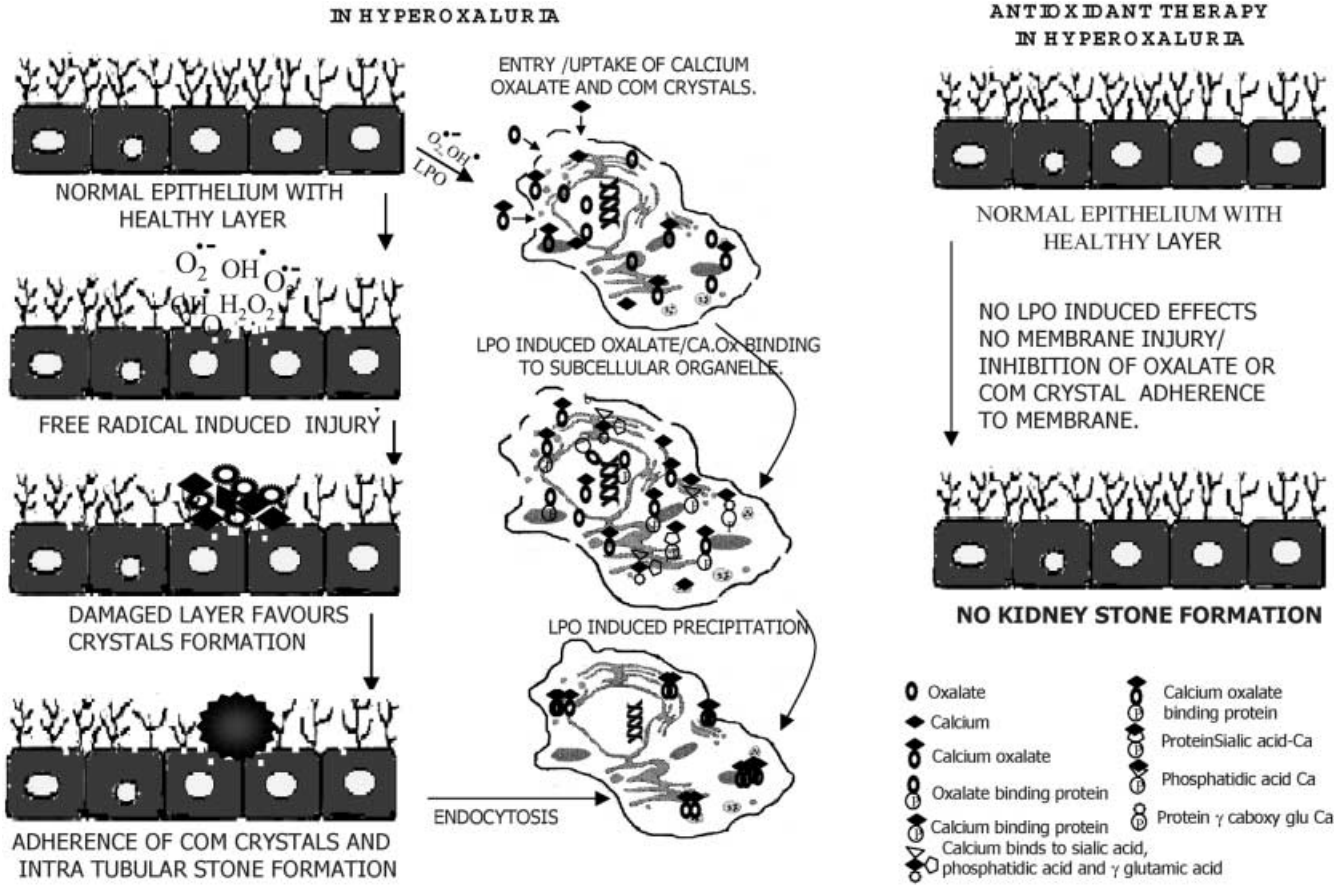

Fig. 1. A hypothetical model depicting calcium oxalate crystal deposition in renal cells by endocytosis and the intracellular process during hyperoxaluric conditions

involved in inflammation-mediated tissue repair [118] and many factors for the proliferation of fibroblast cells, resulting in the accumulation of extracellular matrix.

Conclusion

Knowledge of the mechanisms involved in the retention of calcium oxalate crystals is essential for the successful treatment of stone disease. Membrane injury is considered to be the prime candidate for the binding of oxalate or calcium oxalate crystal in order to be retained in the cell. Among other factors, oxalate-induced free radical-membrane interactions are considered to be significant causative factors of membrane damage. This results in lipid and protein peroxidation with altered biochemical reactions, including depletion of the antioxidant defensive system and failure of the calcium pump. Accumulated calcium and oxalate can be precipitated in the presence of membrane fragments for the formation into stones. Antioxidant therapy prevents calcium oxalate retention by protecting against membrane injury. This could be the key to prevention or successful treatment of stone disease. This may be why the normal epithelium does not favour stone formation, even when the urine is supersaturated with calcium oxalate crystals.

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