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Limitation of apoptotic changes and crystal deposition by Tutukon following hyperoxaluria-induced tubular cell injury in rat model

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Abstract This study aimed at evaluating the protective effects of a herbal medication (Tutukon) on the hyperoxaluria induced apoptotic changes and crystal deposition in renal tubular epithelium in rat model. 60 male wistar rats were divided into three different groups (each group n: 20). In Group I severe hyperoxaluria was induced by ethylene glycol (EG) (0.75 %) administration for 28 days. In Group II, in addition to hyperoxaluria induction, animals were treated with Tutukon for 28 days. Group III animals constituted the controls without any specific medication and/or intervention. While the presence and degree of crystal deposition in the tubular lumen were examined histopathologically under light microscopy, tubular apoptotic changes were evaluated using immunohistochemical staining for cysteine-aspartic acid protease-3 (Caspase-3) and tumor necrosis factor alpha (TNF-α) positivity on days 14 and 28, respectively. Evaluation of apoptotic changes by Caspase-3 positivity showed that while the majority of animals undergoing EG only showed evident apoptotic changes (n: 9), Tutukon application demonstrated a significant limitation with limited or no apoptosis (n: 7) in these animals. Similar data were noted for TNF alpha expression; while apoptotic changes were evident in 8 (80 %) in Group I animals, limited changes were noted in Tutukon Group (n: 2). Regarding crystal deposition despite evident changes in Group I (9 animals), like

apoptotic alterations, it was again significantly limited in animals receiving Tutukon (4 animals). Renal tubular crystal deposition and apoptotic changes induced by hyperoxaluria play a role in the pathogenesis of urolithiasis and the limitation of these changes might be instituted by Tutukon as a result of its antioxidant and antiinflammatory effects.

Keywords Herbal medication · Apoptotic · Changes · Crystal deposition · Antioxidant effects

Introduction

Hyperoxaluria is the main risk factor for human idiopathic calcium oxalate (CaOx) stone formation, and the induction of hyperoxaluria is a powerful driving force in stone formation and the development of CaOx urolithiasis [1]. Both clinical and experimental studies suggest that renal tubular epithelium is the major target for oxalate-induced injury and sustained hyperoxaluria, along with crystal–cell interaction may result in injury to the renal tubular cells [2, 3]. Recent studies on LLC-PK1 cells have shown well that a high oxalate concentration together with CaOx crystal formation/deposition may induce renal tubular cell damage and/or dysfunction producing a variety of changes in renal tubular cell morphology and function, including increased cellular proliferation and, at elevated concentrations which may express as cell apoptosis [4–6].

The etiopathogenesis of cell injury during hyperoxaluric diet involves induced lipid peroxidation in tubular cells, which usually leads to the functional impairment of cellular components by reactive oxygen species (ROS) formation due to oxidative stress [4, 7, 8]. Apart from possible hyperoxaluria-induced tubular ischemia, which gives rise to ROS production as a result of lipid peroxidation, studies have

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clearly demonstrated that oxalate ions themselves may also be responsible tubular alterations as a result of their direct toxic effects [4, 5, 9, 10].

Taking the injurious effects of hyperoxaluria causing crystal deposition in renal parenchyma and apoptotic changes in renal tubular epithelium (as a result of ROS production) into account, it may be useful to apply protective agents in an attempt to prevent or at least limit the extent of the pathologic alterations. While verapamil and nifedipine (calcium channel blocking agents) were found to limit the histologic changes as well as crystal deposition induced by certain renal trauma [8, 11, 12], potassium citrate was found to limit stone recurrence after extracorporeal shockwave lithotripsy [7], and allopurinol as well as vitamin E has been used as antioxidant agents to minimize the effects of free oxygen radical formation in certain tissues [11, 13, 14].

In addition to the use of these medical agents; recent data clearly demonstrated that as a promising and effective alternative, herbal medicine has gained an increasing importance in the prevention and metaphylaxis of renal stones [22, 23] as well as to improve the results of lithotripsy [24]. Related with this subject, Tutukon is a medication composed of different herbal ingredients demonstrating certain biological traits among which the antioxidant potential is one of the most important characteristics of these ingredients.

In this present animal study, taking the effective antioxidant potential into account; we aimed to evaluate the possible protective effect of Tutukon on the presence and degree of apoptotic changes as well as crystal formation in renal tubular epithelial cells induced by hyperoxaluric diet in a rat model.

Materials and methods

A total of 60 male wistar white rats, each weighing 250–300 g/each, were included into the study program. Ethical committee approval was obtained from the animal laboratory of the Pendik animal research laboratory. All animals were fed standard chow and kept under normal room conditions, and following a complete physical examination, biochemical evaluation including blood and urine analyses and stool examination was performed in all of them.

Animals were then divided into three different groups ($n = 20$). While the animals in Group I were given hyperoxaluria-inducing diet of 0.75 % ethylene glycol (EG) in distilled drinking water from day 0 to day 28, animals in Group II received a protective agent (Tutukon—see drug characteristics below) in addition to the induction of hyperoxaluria via a feeding catheter (5 ml/BW–20 ml/day—three times in a day in divided doses) for 28 days

during the study program. Last, Group III included control animals receiving only distilled drinking water during all study phases. During the experimental period, all animals were provided regular rat chow ad libitum. Following a 2-week hyperoxaluric diet in Group I and additional protective agent in Group II and a normal diet in Group III, ten animals in each group were killed on days 14 and 28 after which a bilateral flank incision was performed and the kidneys removed for histopathologic evaluation.

Drug characteristics

Pharmacological group: plant-based fixed-dose combination, Dosage form: 45 ml three times/daily.

Active plant ingredients: Essential oils, flavonoids-quercetin, polysaccharides, rosmarinic acid, boldin, flavone glycosides.

Pathological processing

The kidneys were fixed with 10 % formalin solution, embedded in paraffin blocks, sliced to 4–6 µm thickness, and stained with hematoxylin–eosin (H&E), Periodic acid–Schiff stain (PAS) (Bio-Optica, Milano), and Masson's trichrome stain (MTK) (Bio-Optica, Milano). Immunohistochemical studies were performed using the Bond polymer refine detection method (Leica Biosystems Newcastle Ltd., Newcastle) with diaminobenzidine as the chromogen and hematoxylin as the nuclear counterstain. All immunohistochemical processes were performed using the Leica BOND-MAX automated system (Leica Biosystems, Melbourne). Included antibodies were anti-TNF-alpha antibody (Polyclonal, 1:500 dilution; Novus Biologicals, UK), anti-Caspase-3 antibody (clone JHM62, 1:400 dilution; Leica Biosystems Newcastle Ltd., Newcastle). All antibodies were diluted with Lab Vision™ Antibody Diluent (TA-125-AD, Thermo Scientific™, CA).

Caspase and TNF-α positive immunostaining was graded using a three-point scale: none, score 0; faint immunoreactivity or single positive cell, score 1; scattered moderately intense immunoreactivity or numerous positive cells, score 2; and dense intense immunoreactivity or clusters of positive cells, score 3. By utilizing a specific grading system, tubules demonstrating granular crystallization and/or calcification were evaluated and scored under light microscopy with polarized filter in hematoxylin eosin stain with in double-blinded fashion. The extent of the crystallization was graded as follows: none: score 0, Minimal: single crystal formation in one focus: score 1, Moderate: scattered crystal formations in more than one focus: score 2, Severe: dense crystal formations—numerous crystal clusters: score 3.
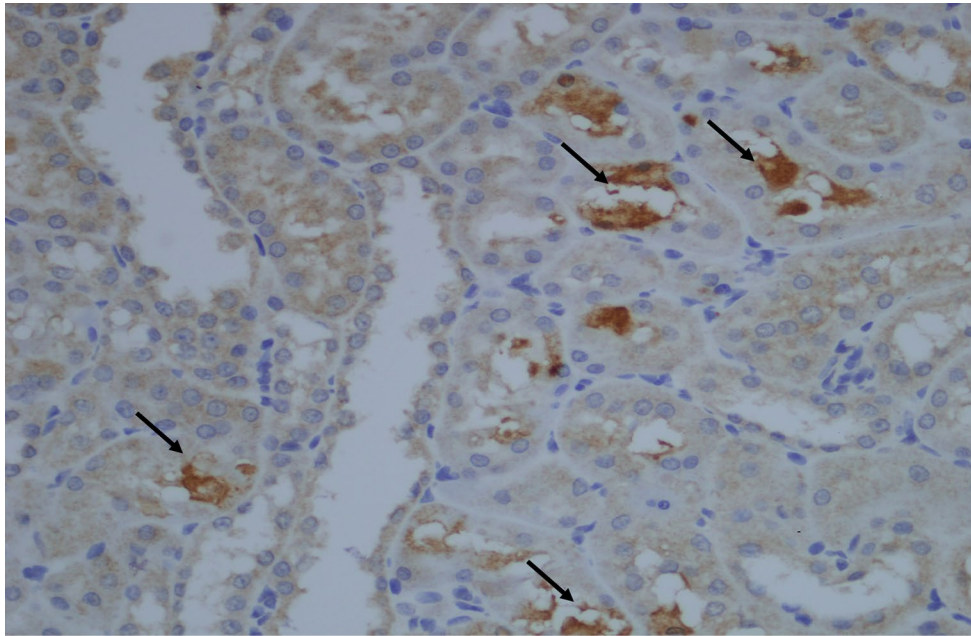
Fig. 1 Evident Caspase-3 expression with immunohistochemistry staining in an animal undergoing hyperoxaluria induction without any protective agent. (Caspase-3, original magnification ×400)

Statistical analysis

For statistical analysis of the obtained data NCSS (Number Cruncher Statistical System) 2007&PASS (Power Analysis and Sample Size) 2008 Statistical Software (Utah, USA) program was used. While evaluating the obtained data, in addition to definitive statistical methods (Mean, Standard deviation, Median, Frequency, Ratio) Mann–Whitney U test was utilized to evaluate the parameters demonstrating abnormal distribution for comparison of the three groups. However, if the number of groups demonstrating abnormal distribution was more than 3, Kruskal–Wallis test was used and last, Mann–Whitney U test was used to identify the group causing difference among the groups. Statistical significance was defined as $p < 0.01$ and $p < 0.05$ levels.

Results

A total of four rats died during the study program due to infection in two and resistance to feeding in the other two animals. Evaluation of tubular apoptotic changes as demonstrated by immuno-histochemical staining of TNF-alpha and Caspase-3 and crystal deposition in renal parenchyma during early as well as late follow-up period demonstrated the following findings.

Evaluation of tubular apoptotic changes

Apoptotic changes were found to be more prominent in tubular cells surrounding the crystals; however, some degree of apoptotic changes could also be found in tubular cells, even when no crystal formation was seen. On the other hand, apoptotic changes were prominent in distal tubular as well as collecting duct cells. Evaluation of apoptotic changes in renal tubular cells with respect to Caspase-3 positivity during early phase (14 days) showed that while number of animals demonstrating moderate to severe apoptotic changes was meaningfully lower in control group, a considerable number of animals undergoing EG only showed moderate to severe degree of apoptotic changes (n: 9, 90 %) (Fig. 1). Tutukon application in these animals, however, demonstrated an evident limitation of apoptotic changes with limited (n: 7, 70 %) degree of changes observed in the majority of the animals (Fig. 2). The mean percentage of apoptotic cells stained by Caspase-3 was higher in Group 1 animals (2.30 ± 0.67) than in animals in Group 2 (1.50 ± 1.08) during this period. The same findings were noted in favor of Tutukon application during late-phase (28 days) evaluation. Mean percentage values were (2.25 ± 0.71) and (1.22 ± 0.83) ($p = 0.021$) in both groups, respectively this period (Table 1). Thus our results indicate that Tutukon application seemed to prevent or at least limit the formation of apoptotic changes
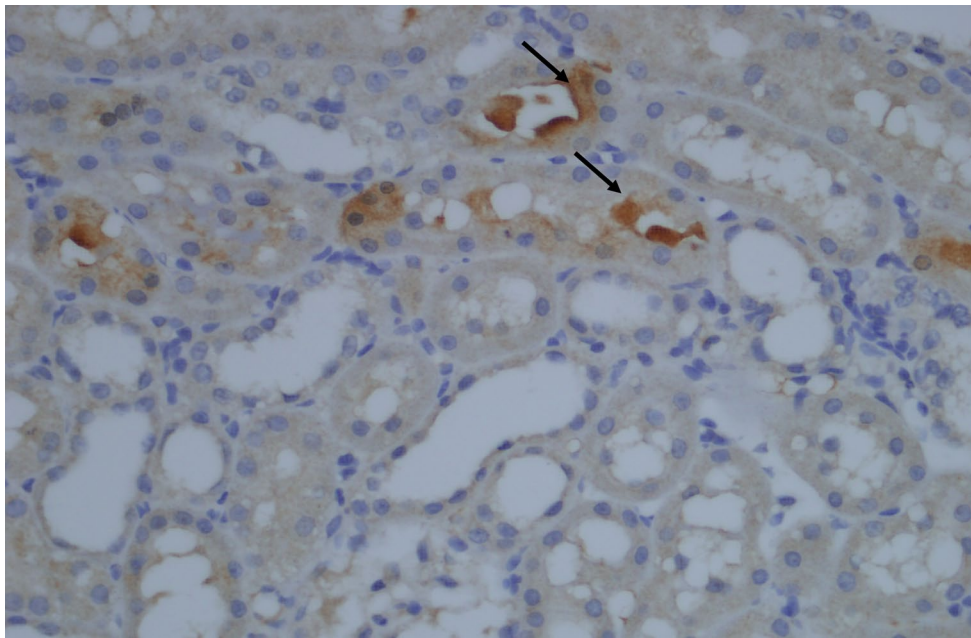
Fig. 2 Minimal Caspase-3 expression with immunohistochemistry staining in an animal undergoing hyperoxaluria induction along with Tutukon application. (Caspase-3, original magnification ×400)

Table 1 Evaluation of the percentage of tubular TNF-alpha staining in all groups during early and late follow-up

Grade	Control		Ethylene glycol		Ethylene glycol + Tutukon		p
	n	%	n	%	n	%	
Early (day 14)							
0	4	40	0	0	2	20	
1	5	50	2	20	6	60	
2	1	10	5	50	2	20	
3	0	0	3	30	0	0	
Overall	10	100	10	100	10	100	
Mean ± SD (median grade)	0.70 ± 0.67 (1)		2.10 ± 0.74 (2)		1.00 ± 0.67 (1)		0.002[a,**]
Late (day 28)							
0	1	11.1	0	0	0	0	
1	7	77.8	1	12.5	5	55.6	
2	1	11.1	4	50	3	33.3	
3	0	0	3	37.5	1	11.1	
Overall	9	100	8	100	9	100	
Mean ± SD (median grade)	1.00 ± 0.50 (1)		2.25 ± 0.71 (2)		1.56 ± 0.73 (1)		0.014[a,*]
Median value of grade early vs late							
p	0.252[b]		0.662[b]		0.118[b]		

TNF-α positive immunostaining was graded using a three-point scale: none, score 0; faint immunoreactivity or single positive cell, score 1; scattered moderately intense immunoreactivity or numerous positive cells, score 2; and dense intense immunoreactivity or clusters of positive cells, score 3

TNF-α Tumor necrosis factor alpha

* Statistically significant, ** Significance is high

* $p < 0.05$, ** $p < 0.01$

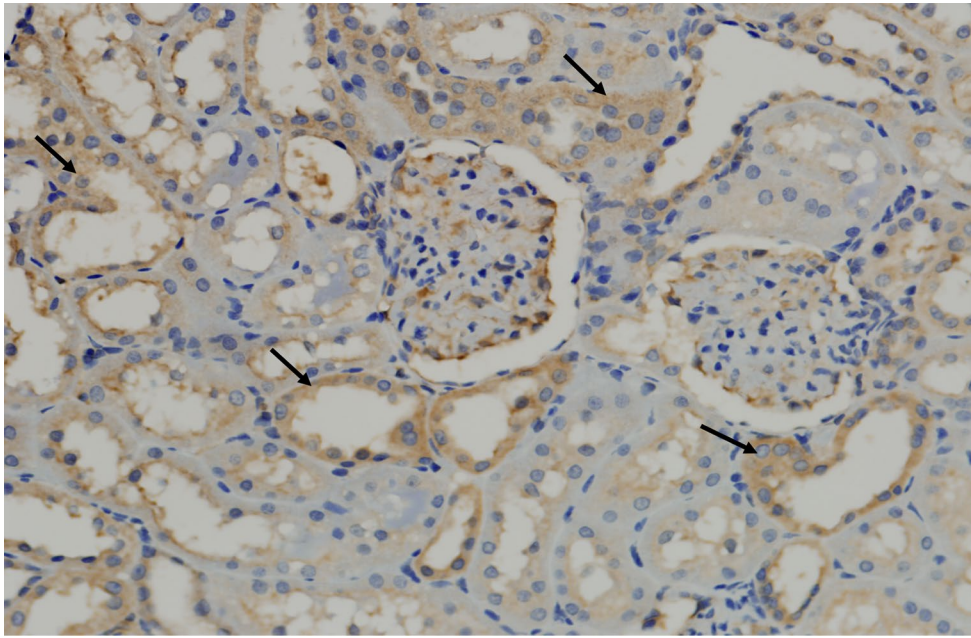[a] Kruskal Wallis Test

[b] Mann Whitney *U* Test

Fig. 3 Evident TNF- alpha expression with immunohistochemistry staining in an animal undergoing hyperoxaluria induction without any protective agent. (TNF-alpha, original magnification ×400)
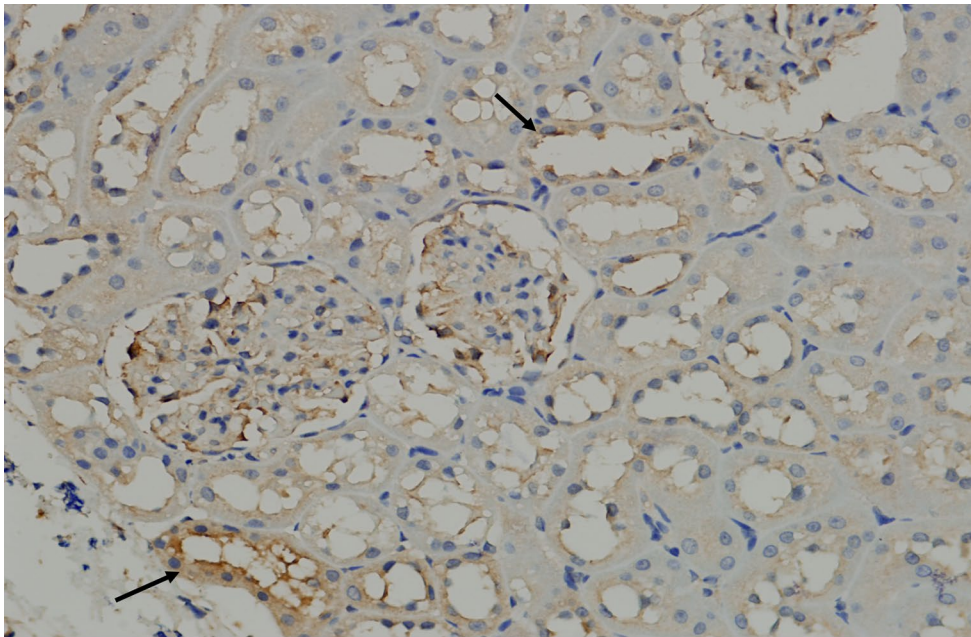

Fig. 4 Minimal TNF-alpha expression with immunohistochemistry staining in an animal undergoing hyperoxaluria induction along with Tutukon application. (TNF-alpha, original magnification ×400)

in renal tubular epithelium during both the early (day 14) and late period (day 28). However, while the significance was meaningful during late evaluation period ($p = 0.021$), it was near to statistical significance but not meaningfully significant during early-phase evaluation ($p = 0.079$).

On the other hand again, TNF alpha expression after hyperoxaluria induction in study group animals showed evident apoptotic chances during early phase (14 days) with moderate to severe apoptotic changes in 8 (80 %) animals (Fig. 3). But these changes were found to be limited again

Table 2 Evaluation of tubular expression of Caspase-3 in all groups during early and late follow-up

Grade	Control		Ethylene glycol		Ethylene glycol + Tutukon		p
	n	%	n	%	n	%	
Early (day 14)							
0	5	50	0	0	3	30	
1	5	50	1	10	0	0	
2	0	0	5	50	6	60	
3	0	0	4	40	1	10	
Overall	10	100	10	100	10	100	
Mean ± SD (median grade)	0.50 ± 0.53 (0.5)		2.30 ± 0.67 (2)		1.50 ± 1.08 (2)		0.001[a],**
Late (day 28)							
0	3	33.3	0	0	2	22.2	
1	6	66.7	1	12.5	3	33.3	
2	0	0	4	50	4	44.4	
3	0	0	3	37.5	0	0	
Overall	9	100	8	100	9	100	
Mean ± SD (median grade)	0.67 ± 0.50 (1)		2.25 ± 0.71 (2)		1.22 ± 0.83 (1)		0.007[a],**
Median value of grade early vs late							
p	0.475[b]		0.883[b]		0.421[b]		

Caspase, positive immunostaining was graded using a three-point scale: none, score 0; faint immunoreactivity or single positive cell, score 1; scattered moderately intense immunoreactivity or numerous positive cells. score 2; and dense intense immunoreactivity or clusters of positive cells, score 3

** Significance is high

** $p < 0.01$

[a] Kruskal Wallis Test

[b] Mann Whitney U Test

in both control group (n: 1, 10 %) and in animals receiving Tutukon (n: 2, 20 %) (Fig. 4) (Table 2). The mean scores of apoptotic cells stained by TNF-alpha during this period was significantly higher in Group 1 animals (2.10 ± 0.74) than in animals in Group 2 (1.00 ± 0.67) ($p = 0.005$) during 14-day evaluation. The same findings were again obtained during late-phase (28 days) evaluation (Table 2).

Evaluation of crystal deposition

Examination of renal parenchymal tissue specimens from rats on an hyperoxaluric diet for 14 days revealed various degrees of crystal deposition, mainly in the tubular lumen. Apart from a slight degree of crystal deposition, no notable histologic alteration could be demonstrated in the interstitial areas. Crystal deposition was widespread in these animals both during early and late follow-up examination (Fig. 5). While severe crystal deposition was observed in 3 (30 %) rats, moderate and minimal deposition have been observed in 6 (60 %) and 1 (10 %) animals, respectively, during early phase (14 days). The overall mean crystal deposition value was 2.20 ± 0.63 in this group. Evaluation of the animals receiving Tutukon after hyperoxaluria induction, however, demonstrated the protective effect of

this medication on the presence as well as severity of crystal formation. While severe crystal deposition was noted in 1 (10 %) rat, moderate and minimal deposition of crystal were present in 3 (30 %) and 6 (60 %) (Fig. 6) rats, respectively. Again, the mean value of crystal deposition was significantly lower in this group when compared with animals receiving EG only 1.50 ± 0.71 ($p = 0.031$). Last but not least, no evident crystal deposition has been observed in control group animals as expected (with a mean value for crystal deposition of 0.10 ± 0.32 (Table 3). The same findings were noted during late (28 days) evaluation indicating the limitation of crystal deposition by Tutukon after hyperoxaluria induction ($p = 0.001$). The degree of crystal deposition in all groups is given in Table 3.

Discussion

Experimental studies have well demonstrated that both CaOx crystals and/or oxalate ions forming after hyperoxaluric diet may play a critical role in the formation of urinary stones. Induction of hyperoxaluria has been found to be associated with cellular injury and necrosis in renal tubular epithelial cells [1, 3, 15]. Demonstration of
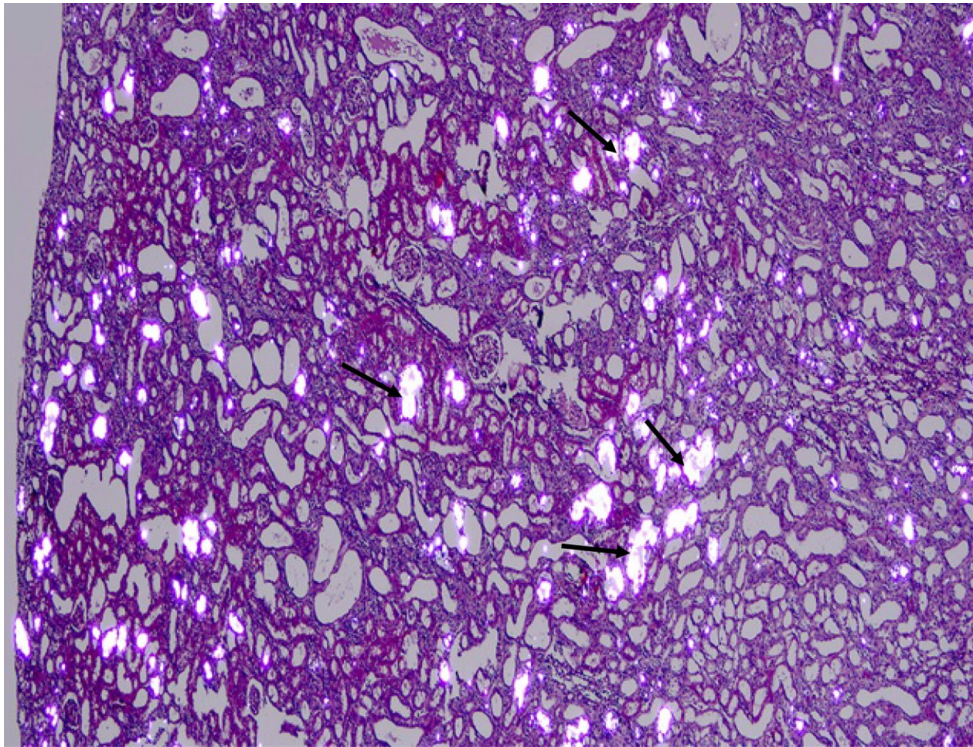
Fig. 5 Evident crystal formation (multiple areas with dense deposition) in an animal undergoing hyperoxaluria induction without any protective agent. (H&E, olympus polarizer filter, original magnification ×400)
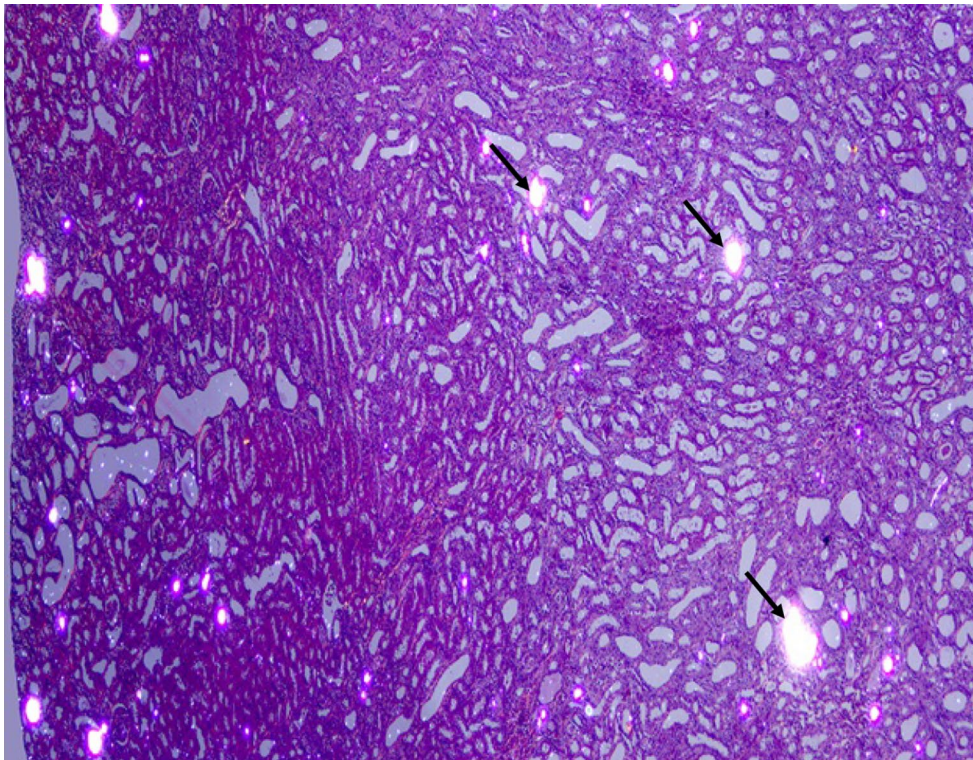

Fig. 6 Limited crystal deposition in an animal undergoing hyperoxaluria induction along with Tutukon application. (H&E, olympus polarizer filter, original magnification ×400)

Table 3 Evaluation of tubular crystal deposition in all groups

Grade	Control		Ethylene glycol		Ethylene glycol + Tutukon		p
	n	%	n	%	n	%	
Early (day 14)							
0	9	90	0	0	0	0	
1	1	10	1	10	6	60	
2	0	0	6	60	3	30	
3	0	0	3	30	1	10	
Overall	10	100	10	100	10	100	
Mean ± SD (median grade)	0.10 ± 0.32 (0)		2.20 ± 0.63 (2)		1.50 ± 0.71 (1)		0.001[a,**]
Late (day 28)							
0	9	100	0	0	0	0	
1	0	0	0	0	3	33.3	
2	0	0	0	0	5	55.6	
3	0	0	8	100	1	11.1	
Overall	9	100	8	100	9	100	
Mean ± SD (median grade)	0.00 ± 0.00 (0)		3.00 ± 0.00 (3)		1.78 ± 0.67 (2)		0.001[a,**]
Median value of grade early vs late							
p	0.343[b]		0.004[b,**]		0.321[b]		

The extent of the crystallization was graded as follows: none: score 0, Minimal: single crystal formation in one focus: score 1, Moderate: scattered crystal formations in more than one focus: score 2, Severe: dense crystal formations—numerous crystal clusters: score 3

** Significance is high

** $p < 0.01$

[a] Kruskal–Wallis Test

[b] Mann–Whitney U Test

enzymuria, proteinuria as well as membranuria, following an hyperoxaluric diet in animal models also supports this hypothesis to a meaningful extent [2]. Observation of tubular injury even in the absence of crystalluria suggests that the damage is not solely produced by CaOx crystals, but oxalate ions themselves may also be toxic to the renal tubular cells [16].

Regarding the mechanism of tubular cell injury, in addition to the original study performed by Khan et al. [15], other groups were also able to demonstrate the functional impairment of cellular components originates from oxidative stress which causes ROS formation [4, 7, 8]. Scheid et al. [6] demonstrated that among the various mono-and dicarboxylates examined in their study, high urinary oxalate level was the most common causative factor for increased free radical production and subsequent cell death. Results of some well-conducted animal studies led the authors to propose that the interaction of oxalate ions with renal epithelial cells may initiate a programmed sequence of events which may in turn lead to cell proliferation or cell death [4, 5, 9, 10]. Last but not least, Koul et al. [4] were able to show that there may be a causal link between the oxalate-induced increase in C-myc gene expression and oxalate-induced increase in cellular proliferation. All these observations indicate that, as a distinctive form of cell death, apoptosis could also be responsible for renal tubular injury induced by hyperoxaluria. To support this proposal, we were able to demonstrate renal tubular cell apoptotic changes in rabbit model where the degree and extent of apoptosis were found to be time dependent [17]. After observing the injurious effects of hyperoxaluria causing crystal deposition and apoptotic changes in renal tubular epithelium, physicians began to search for agents to prevent or at least minimize the extent of these pathologic alterations [18].

Among the agents used so far, verapamil and nifedipine (calcium channel blockers) were found to limit histologic changes and crystal deposition to a certain extent [12, 19, 20]. Potassium citrate again was found to limit stone recurrence after extracorporeal shockwave lithotripsy [7], and as highly potent antioxidant agents, allopurinol and vitamin E have been used to minimize free oxygen radical-induced alterations in certain tissues by inhibiting ROS formation [11, 13, 14, 21].

On the other hand, in addition to the use of these medical agents, recently published data clearly demonstrated that as a promising and effective alternative, herbal medicine may have a certain role in the prevention

and metaphylaxis of renal stones [22, 23]. Phytotherapy applied in a complementary fashion for the conservative treatment of urolithiasis has been found to improve the results of lithotripsy and increase the efficacy of metaphylactic management of urinary tract stones [24]. Evaluation of the available data in the literature so far did show that majority of these phytotherapeutic agents have diuretic, anti-inflammatory, antioxidant, vasodilating, and spasmolytic effects [25, 26].

The active ingredients responsible for such specific effects have been found to be essential oils, flavonoids, saponins, xanthine derivatives and glycosides [25, 27–29]. Among the phytotherapeutic agents applied so far, Phyllanthus niruri has been found to be promising in the prevention of stone formation [25, 30] due to its potentially active compounds of genus *Phyllanthus*, alkaloids, flavonoids, lactones, steroids, terpenoids, lignans, and tannins. In addition to its well-demonstrated antispasmodic [26] and analgesic effects [25], this herbal agent has also been shown to inhibit CaOx endocytosis by renal tubular cells. Supporting this hypothesis again in a small clinical trial this agent appeared to reduce urinary calcium excretion amongst hypercalciuric stone formers [31]. Among the other herbal agents used to limit crystal formation and eventual stone formation, a Moroccan herb *Hernaria hirsuta* and *green tea* [22, 30] have also been found to be promising in the limitation of CaOx crystallization.

Taking the reported effects of such antioxidant as well as anti-inflammatory agents in the prevention of ischemia-induced renal tubular epithelial injury into account, in this present study we aimed to evaluate the possible limitation of tubular apoptotic changes and eventual crystal formation resulting from hyperoxaluric state using a potent antioxidant and anti-inflammatory herbal agent (Tutukon) in rat model.

Tutukon (Grand Medical, Spain) is a medication composed of different herbal ingredients demonstrating certain biological effects in different tissues (Fig. 1). Among these effects, the antioxidant potential is one of the most important characteristics of this drug due to its active ingredients. Published data show that among the 8 different ingredients of this herbal agent, alkaloids, rosmarinic acid, flavonoids [27], apigenin, luteolin [28], ekvizetonin saponin [29], and essential oils [32] are well known for their potent antioxidant as well as anti-inflammatory effects. Regarding the mechanism of action, studies have clearly shown that the above-mentioned active ingredients may reduce the permeability of kidney capillaries, dilate kidney blood vessels and ureters, restore the tubular epithelial function, induce an osmotic effect, inhibit synthesis and activation of inflammatory mediators, and thus prevent inflammatory alterations as well as crystal formation and growth [22, 24–26, 33].

Our results clearly demonstrated significant crystal formation/deposition together with apparent renal tubular cell apoptosis in animals undergoing hyperoxaluria induction by EG during the early phase of hyperoxaluric state (day 14). Quantitative examination of the cell apoptosis and crystal deposition indicated a significant difference between these animals and the ones receiving the preventive agent (Tutukon). As mentioned above, ischemia-induced oxidative stress in renal tubules and the inflammatory changes are the major underlying causes for tubular cell apoptosis and subsequent crystal formation/deposition. Our results again demonstrated that Tutukon with its potent ingredients: alkaloids, rosmarinic acid, flavonoids [27] apigenin, luteolin [28], ekvizetonin, saponin [29], and essential oils [32], may be protective enough in the limitation of such alterations causing crystal formation at least in experimental animal models. Treatment of the animals with Tutukon provided a significant protection from calcium oxalate deposition and apoptosis in tubular cells. However, it is clear that the dosage of the herbal agent used in rat model in this study does not match those used clinically in humans.

Conclusions

In the light of our findings and the reported literature data, it is clear that both hyperoxaluria itself and CaOx crystal formation are injurious to renal epithelial cells. Tubular cell apoptosis as a result of free radical formation causing lipid peroxidation is the main causative factor for these alterations. As a potent antioxidant medication, Tutukon was found to limit both crystal deposition and apoptotic changes in this present study indicating that the clinical application of such antioxidant and anti-inflammatory agents might limit the formation of urinary calculi, particularly in recurrent stone formers. However, we believe that further clinical and research studies are certainly needed to support the above-mentioned preventive effects of this drug in CaOx crystal formation.

Conflict of interest We have no conflict of interest.

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