The effect of indomethacin on hyperoxaluria-induced renal tubular epithelial injury

Hiperoksalürinin neden olduğu renal tübüler epiteliyal hasar üzerine indometazinin etkisi

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Abstract

Objective: The aim of this study was to determine the effect of indomethacin, an anti-inflammatory agent, on apoptosis and crystal deposition developing as a consequence of tubular cell injury induced by hyperoxaluria in an animal model.

Materials and methods: Fifty New Zealand rabbits were divided into 3 groups. The first 2 groups were fed with hyperoxaluric diet and Group 3 was the control group with no supplementary procedure or treatment. While the animals in Group 1 were given only hyperoxaluric diet, Group 2 animals was applied indomethacin in addition to the hyperoxaluric diet. Animals were sacrificed at the early (7th day) and late (28th day) periods and renal tissue specimens were sent for the pathological analysis of crystal deposition and apoptosis.

Results: The presence and degree of crystal deposition were significantly less in the specimens obtained from indomethacin-treated group during both the early and late periods ($p<0.001$). Similarly, despite the evident tubular apoptosis in group receiving only hyperoxaluric diet; tubular apoptotic changes were limited in animals treated with additional indomethacin ($p<0.001$).

Conclusion: As a result of cell injury developing due to hyperoxaluria, crystal deposition and apoptotic differences occur and the presence of ischemia increases both effects. At this point, in experimental model, indomethacin limits crystal deposition and apoptotic differences in the renal tissue.

Key words: Hyperoxaluria; indomethacin; renal tubular injury.

Özet

Amaç: Bu çalışmanın amacı anti-inflamatuvar bir ajan olan indometazinin, hiperoksalürinin neden olduğu tübüler hücre hasarı sonucu gelişen apopitozis ve kristal birikimi üzerine etkisini hayvan modeli üzerinde belirlemektir.

Gereç ve yöntem: Elli Yeni Zelanda tavşanı 3 gruba ayrılmıştır. İlk 2 grup hiperoksalürik diyetle beslenmiş, Grup 3 ise herhangi bir ilave işlem ya da tedavi uygulanmayan kontrol grubunu oluşturmuştur. Grup 1'deki hayvanlara yalnızca hiperoksalürik diyet verilirken, Grup 2'deki hayvanlara hiperoksalürik diyete ek olarak indometazin de uygulanmıştır. Hayvanlar erken (7. gün) ve geç (28. gün) dönemde kurban edilmiş ve renal doku örnekleri, kristal birikimi ve apopitozis açılarından patolojik inceleme için gönderilmiştir.

Bulgular: Kristal birikiminin mevcudiyeti ve derecesi, indometazinle tedavi edilmiş olan gruptan elde edilen örneklerde hem erken, hem de geç dönemde anlamlı olarak daha azdı ($p<0.001$). Benzer şekilde, hiperoksalürik diyet alan gruptaki bariz tübüler apopitozise karşın, ek indometazinle alan gruptaki tübüler apopitotik değişiklikler sınırlıydı ($p<0.001$).

Sonuç: Hiperoksalüriye bağlı olarak gelişen hücre hasarının bir sonucu olarak, kristal birikimi ve apopitotik değişiklikler ortaya çıkar ve iskeminin bulunması her iki etkiyi de artırır. Bu noktada indometazin, deneysel modelde, renal dokudaki kristal birikimini ve apopitotik değişiklikleri sınırlamaktadır.

Anahtar sözcükler: Hiperoksalüri; indometazin; renal tübüler hasar.

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Renal tubular damage is the most important step for the formation of renal stones.[1,2] It has been shown in numerous experimental studies that oxalate ions form oxidative stress either on their own or in the form of calcium oxalate (CaOx) crystals in the renal tubular cells leading to the formation of free oxygen radicals and reactive oxygen species (ROS) which causes either cell proliferation at lower concentrations or cell death.[1,3] On the other hand, recent studies have shown that this injury may be limited by some antioxidant agents.[4,5] Although the underlying mechanism leading to renal epithelial cell injury in hyperoxaluria is not well established, some authors have focused on the hyperoxaluria-dependent inflammatory changes as a triggering factor for epithelial hazards. ROS are produced during the interactions between the crystals and renal cells, and are responsible for the various cellular responses. CaOx crystals generally form in the renal tubules. Exposure of renal epithelial cells to CaOx crystals results in the increased synthesis of osteopontin, bikunin, heparan sulphate, monocyte chemoattractant protein (MCP)-1, and prostaglandin (PG) E2, which are known to participate in inflammatory processes and in extracellular matrix production.[3]

It has also been claimed that CaOx crystals activate the lysosomal enzymes, followed by the development of anti-inflammatory effect together with the increase in the synthesis and expression of the macromolecules.[3] In a recent study, oxaluria-dependent inflammatory responses were defined as the key regulators of development for nephrolithiasis.[6] Last but not least, experimental studies have pointed out that the initial lesion appears to be crystal formation along the brush border of the proximal tubule, with eventual crystal deposition in collecting ducts and papillary interstitium, and eventual tubule obstruction, interstitial inflammation, and fibrosis.[7]

Indomethacin as a potent anti-inflammatory agent is a nonselective inhibitor of cyclooxygenase (COX) 1 and 2 enzymes that participate in prostaglandin synthesis from arachidonic acid. Prostaglandins are hormone-like molecules normally found in the body, where they have a wide variety of effects, some of which lead to pain, fever, and inflammation.[8]

Taking the possible role of inflammatory changes being formed in renal interstitium after hyperoxaluria induction, in this present animal study, we aimed to evaluate the possible effect of indomethacin on the hyperoxaluria-induced crystal deposition and apoptosis in renal tubular epithelial cells.

Material and methods

Fifty New Zealand white male rabbits weighing 2 to 4 kg were studied. The animals were fed a standard ration at room temperature. The animals were divided into 3 groups; the animals in the first 2 groups were fed with hyperoxaluric diet [0.75% ethylene glycol (EG) in distilled drinking water] to induce hyperoxaluria, animals in the last group received no additional diet or procedure. In Group 1**,** animals were fed with EG diet and sacrificed on the 7th (n=10) and 28th (n=10) days. In addition to EG diet, the rabbits in Group 2 were ingested indomethacin 2 mg/kg/day for 7 days, and sacrificed on the 7th (n=10) and 28th (n=10) days. No particular procedure or treatment was applied for Group 3 and animals in this group served as the control (n=10).

Animals were anesthetized with ketamine hydrochloride (25 mg/kg) at the end of the periods designed for the study groups; kidneys were removed with bilateral flank incision, and sent for histopathological evaluation. Histology of the renal tissue and crystal deposition were assessed on the light microscope (10x10, 10x40) subsequent to the performance of haematoxylin–eosin staining. Grading with respect to crystallization and/or calcification was determined in 1 cm^2 area.

According to the grading;[5] (+) means minimal (crystallization and/or calcification in 1-3 tubules), (++) means moderate (crystallization and/or calcification in 4-7 tubules), and (+++) means severe (crystallization and/or calcification in >7 tubules).

Detection and scoring of apoptosis

The in situ detection of renal tubular cells with DNA strand breaks in paraffin-embedded parenchymal sections was achieved by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP in situ nick and labeling (TUNEL) method using an ApopTag kit (Oncor, Gaithersburg, MD, USA). Briefly, after deparaffinization and rehydration, tissue sections were incubated with 200 μg/mL proteinase K (Oncor, Gaithersburg, MD, USA) for 20 min at room temperature, washed in distilled water and then treated with 3% hydrogen peroxide in phosphate buffered salina (PBS) for 10 min at room temperature

Table 1. Degree of crystallization between three groups in the early (7th day) and late (28th day) period (number of specimens with crystallization/total number of specimens)

	Early period (first week)			Late period (first month)		
	+	++	+++	+	++	+++
Group 1 (EG only)	2/10	4/10	3/10	2/10	4/10	4/10
Group 2 (EG + indomethacin)	1/10	3/10	1/10	-	2/10	1/10
Group 3 (control)	-	-	-	-	-	-
p value		0.034			0.015	

EG: Ethylene glycol (hyperoxaluric diet).

to quench endogenous peroxidase activity. Sections were incubated with TdT and dioxigenin-1, l-dUTP in a humidified chamber at 37°C for 1 h and then treated with antidioxigenin-peroxidase for 30 min at room temperature. Subsequently, sections were exposed to 0.05% substrate for 7 min, washed with distilled water and PBS, and then counterstained for 10 min. Sections were then dehydrated in 100% butanol, cleared in xylene, and mounted with Entellan (Merck Scientific, Fairlacon, NJ, USA). Negative controls were obtained by omitting the TdT enzyme, and the same volume of distilled water was used. The Apoptag kit used during the study contained the positive controls. For the quantification of apoptosis in tubular cells to quantitate the incidence of apoptosis at each time point, the percentage of the number of TUNEL-positive cell nuclei within a renal tubule cross section was calculated after counting 1,000 tubular cells in each sample. Scoring the degree of apoptosis was defined as follows;[5] (+) means limited (apoptosis less than 5%), (++) means moderate (apoptosis ranging from 5% to 10%), and (+++) means evident (apoptosis >10%).

Paired-t and Wilcoxon rank tests were used for the statistical evaluation of the data obtained at the end of the study and $p<0.05$ values were considered statistically significant at 95% confidence level.

Results

Crystal formation

Crystal formation in the renal tubules during the early and late periods were found to be significantly more remarkable in the first 2 groups fed with EG compared with the control group ($p<0.05$). Crystal formation of indomethacin receiving rabbits was also compared with their non-receiving counterparts (only EG group). Indomethacin was found to decrease the presence and degree of crystallization when compared with only EG receiving group in both early and late period ($p=0.034$ and $p=0.015$, respectively) (Table 1). Figures 1 and 2 show the degree of the crystallization in Groups 1 and 2, respectively.

Apoptotic changes

The evaluation of the apoptotic changes during both early and late follow-up phase showed that evident tubular apoptosis could be seen in tissue specimens obtained from animals undergoing hyperoxaluria induction (Groups 1 and 2) (Table 2). Limited or no apoptotic changes were present in control group animals. As compared with Group 1, limited apoptotic changes were seen in indomethacin-treated Group 2 with respect to the mean apoptotic index evaluation both during early as well as late follow-up (Table 2). The severity of the apoptosis was shown in Figures 3 and 4 for Groups 1 and 2, respectively.

Discussion

Hyperoxaluria is being accepted as the main risk factor for the formation of idiopathic CaOx stones as demonstrated in several clinical studies.[1,9] Experimental studies have clearly shown the oxalate ion (and/or CaOx crystals)-induced injury in the renal tubular cells which seems to play a critical role in the formation of urinary calculi.[10,11] The initiation of the oxalate-dependent tubular injury process triggered by the membraneous lipid peroxidation, as a result of formation of free oxygen radicals, cause injury and necrosis in the renal tubular cells.[12] On the other hand, formation of CaOx crystals activate the lisosomal enzymes, followed by the development of anti-inflammatory effect together with the increase in the synthesis and expression of the macromolecules.

Table 2. Mean apoptotic index (min-max) of three groups in the early (7th day) and late (28th day) period

	Early period (first week)	Late period (first month)
Group 1 (EG only)	17.3 (15.2-19.2)	20.9 (20.4-21.8)
Group 2 (EG + indomethacin)	11.2 (9.1-12.0)	14.3 (13.1-17.0)
Group 3 (control)	2.9 (2.4-3.2)	2.4 (1.7-2.6)
p value	<0.001	<0.001

EG: Ethylene glycol (hyperoxaluric diet).

Thus, the initial lesion appears to be crystal formation along the brush border of the proximal tubule, with eventual crystal deposition in collecting ducts and papillary interstitium, and eventual tubule obstruction, interstitial inflammation and fibrosis.[3,13] Finally, it has been well demonstrated that the response of the renal epithelial cells to oxalate ions and/or CaOx crystals was biphasic where mitogenic effect occurred at low concentrations, and toxic effect occurred at high concentrations.[1]

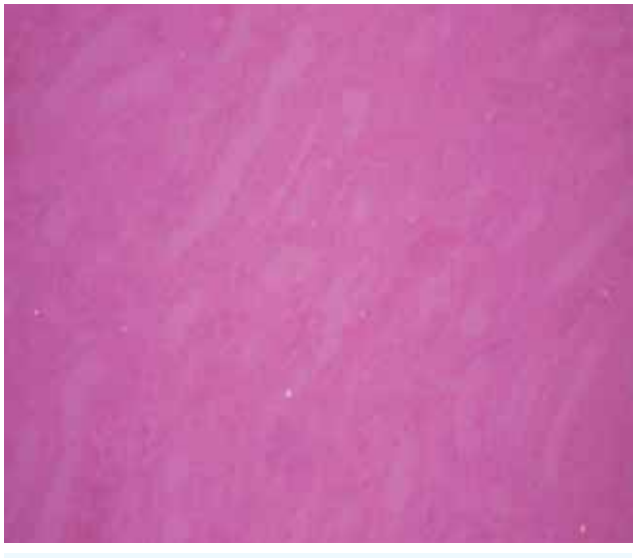

Figure 1 Appearance of dense crystallization on light microscopy of the renal specimens from Group 1 animals receiving only hyperoxaluric diet (0.75% ethylene glycol) (HE, x10).

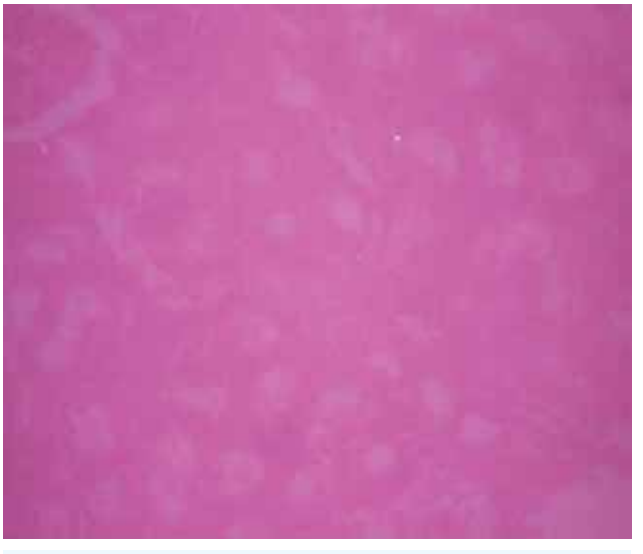

Figure 2 Appearance of crystallization on light microscopy of the renal specimens from Group 2 animals receiving both hyperoxaluric diet (0.75% ethylene glycol) and indomethacin (HE, x10).

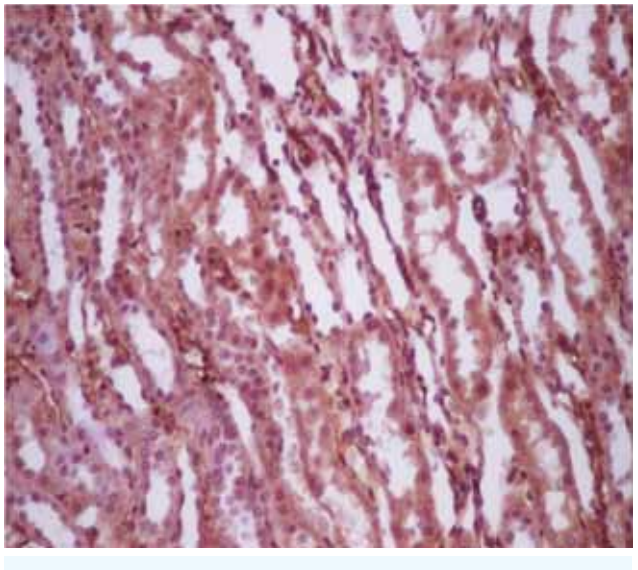

Figure 3 Severe apoptosis in Group 1 animals receiving only hyperoxaluric diet (0.75% ethylene glycol) (TUNEL staining).

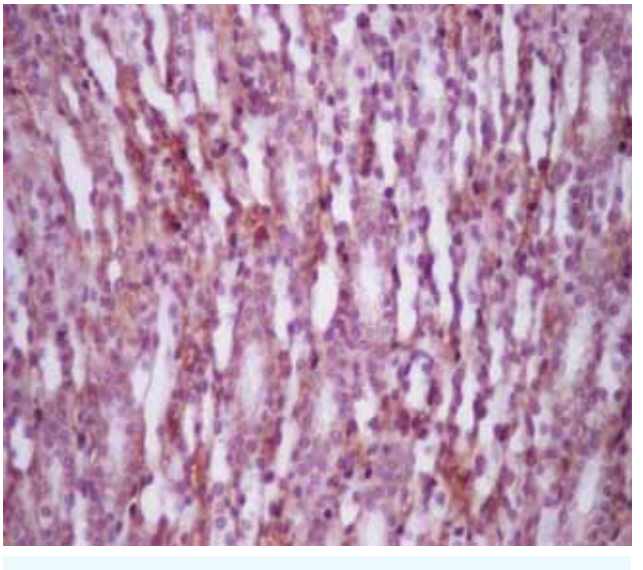

Figure 4 Moderate apoptosis in Group 2 animals receiving both hyperoxaluric diet (0.75% ethylene glycol) and indomethacin (TUNEL staining).

CaOx crystals are agents, free radicals, and ROS that play a critical role at the molecular level in the dependent cell injury. ROS, formed dependent to the oxidative stress, are generally short-lived. Their most important products are malondialdehyde, isoprostanes, and oxidized lipids reacting with lipids, amino acids, proteins, carbohydrates and nucleic acids at the cellular level.[4] In recent clinical studies the urinary level of the mentioned ROS was demonstrated to rise as an indication of the oxidative stress and renal epithelial injury.[14] Thamilselvan et al.[15] determined increase at the levels of urinary lipid peroxidase and LDH in the ethylene glycol-induced hyperoxaluric rats with nephrolithiasis. This effect was proved to decrease with antioxidant vitamin E.[15] Huang et al.[16] demonstrated that the oxidative stress could be decreased, level of glutathione concentration in the renal tissue could be increased significantly, and level of thiobarbituric acid could be decreased with losartan, an angiotensin converting enzyme (ACE) inhibitor.

In numerous animal experiments, tubular ischemia at hyperoxaluric basis was demonstrated to increase the production of ROS. Koul et al.[17] displayed that c-myc gene expression, an apoptotic marker due to oxalate-dependent oxidative stress, increased. In analogous studies, occurrence of cell deaths concurrent with cellular proliferation and concentration as a result of interaction between the oxalate ions and renal tubular epithelium, were stated.[17-19] Sarıca et al.[5] demonstrated that the apoptotic changes in renal tubular cells induced by hyperoxaluria might be limited by allopurinol, an antioxidant agent, and vitamin E.

Although the renal tubular epithelial hazards are caused by direct injury or ROS formation, the eventual inflammation formation is highly expected. In an animal study with experimental CaOX nephrolithiasis, production of chemoattractants leading to migration of monocytes and macrophages which can cause to localized interstitial inflammation and fibrosis to sites of interstitial crystal deposition were shown.[20] Also, several in vitro experiments showed the eventual inflammation and fibrosis formation in crystal deposition areas as a fate of CaOX dependent injury.[21-23]

We investigated the effect of indomethacin, an anti-inflammatory agent, on the injury induced in the renal tubular cells by hyperoxaluria-dependent ischemia and inflammatory changes in the interstitium. In the hyperoxaluric diet treated subjects, crystallization was observed to develop during early (7th day) and late (28th day) periods and to become more severe on the basis of tubular ischemia. While the mean apoptotic index was significantly high in hyperoxaluric diet treated group, indomethacin application was found to decrease tubular crystal deposition and apoptotic alterations significantly.

In the light of these findings, it seems that oxalate ions cause tubular cell injury either by themselves or in the form of CaOX crystals. Taking the ischemia formation and subsequent inflammatory changes into account, anti-inflammatory medication (indomethacin in this study) may limit these changes to some extend. Thus, in experimental conditions, this kind of medication can limit these apoptotic changes which ultimately cause stone formation.

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