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Evaluation of Protective and Therapeutics Effects of Baicalein in Rat Kidney Stone Models Induced by Ethylene Glycol and Hydroxy-L-Proline

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What's known on the subject? and What does the study add?

Oxidative stress and inflammation are key factors in the development of urinary stones. Baicalein, a flavonoid known for its antioxidant and anti-inflammatory properties, was investigated in this study for its effectiveness in preventing and treating urinary stones in a rat model.

Abstract

Objective: To explore the effect of baicalein on the development and treatment of renal stones in a rat model induced by ethylene glycol or hydroxyproline.

Materials and Methods: A grand total of 63 rats were split into nine distinct groups, each comprising seven rats: control, baicalein, ethylene glycol (EG), baicalein+ethylene glycol (BE), ethylene glycol+baicalein (EB), dimethyl sulfoxide (DMSO), hydroxy-L-proline (HP), baicalein+hydroxyproline (BH), and hydroxyproline+baicalein (HB) respectively. Urinary stone formation was induced in rats using EG or HP. Rat kidneys were examined by two histologists using light and electron microscopes. Calcium oxalate crystals and cellular changes were examined. Kidney injury molecule-1 (KIM-1), N-acetyl-β-D-glucosaminidase (NAG), neutrophil gelatinase-associated lipocalin (NGAL), osteopontin and interleukin-18 (IL-18) levels were measured as potential biomarkers in both the kidney tissue and blood.

Results: There was no noticeable difference in histological features when the specimens were examined under a light microscope. Kidney stone formation and mitochondrial differences were observed in the EG and HP groups in electron microscopic (EM) examinations; however, EM findings were normal in all preventive and therapeutic groups. Serum IL-18 and KIM-1 levels were significantly lower in the therapeutic group than in the EG and HP groups ($p<0.05$).

Conclusion: Baicalein has both protective and therapeutic roles in the management of kidney stone disease, and its therapeutic efficacy is superior to its protective efficacy. Further clinical studies in humans are needed to provide conclusive results on this issue.

Keywords: Baicalein, ethylene glycol, hydroxyproline, phytochemicals, rat kidney

Introduction

Kidney stone disease (KSD) is a common health issue globally, affecting 10-20% of people at some point in their lives. Each year, this condition leads to hospital admissions for approximately one in every 1000 individuals (1). The precise mechanisms underlying the formation of urinary stones remain unclear. Factors such as precipitation and crystallization of insoluble components like calcium phosphate, oxalate, and uric acid, along with a combination of genetic factors, dietary habits, and environmental influences, are believed to contribute. Dehydration, obesity, high dietary protein and sodium intake, hypercalciuria, alternations in urinary pH, severe climate conditions, and consumption of certain medications can lead to

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urinary stones (2). They develop secondary to Randall's plaques on the papillary surfaces of the kidney. The formation of plaques is promoted by the accumulation of reactive oxygen species (ROS) and oxidative stress. Exposure of the renal epithelium to high levels of urinary crystals causes excessive ROS production, resulting in injury and inflammation (3,4). The appropriate prevention and management of KSD differ according to stone type. Dietary interventions, medications, and surgical approaches have been used to relieve symptoms and minimize complications such as chronic renal failure (5). However, effective treatment of KSD remains an ongoing challenge. Various medicinal plants and their phytochemicals with antispasmodic, diuretic, anti-inflammatory, and antioxidant activities exhibit inhibitory effects on the aggregation of urinary components (6). Baicalein, a prominent flavonoid derived from the roots of Scutellaria baicalensis Georgi, has demonstrated its efficacy as an inhibitor for stones in both in vivo and *in vitro* experiments (5). Baicalein reduced nuclear factor kappa levels (7-10). Baicalein decreases interleukin (IL)-1β and tumor necrosis factor-alpha levels (8) and the expression of inducible nitric oxide synthase and transforming growth factor-beta 1 (9). In addition, baicalein upregulates FOXO proteins, which play important roles in DNA damage repair. It also supports catalase and superoxide dismutase activities (10). Baicalein also has an antihyperuricaemic effect by reducing GLUT9 and URAT1 expression and xanthine oxidase activity (11). However, the preventive and therapeutic effects of baicalein in the management of KSD have not been fully elucidated.

The objective of this study was to examine the effect of baicalein on stone formation and treatment in an induced rat kidney model using either ethylene glycol (EG) or hydroxyproline (HP).

Material and Methods

Classification of Groups

Sixty-three adult male Wistar albino rats, each weighing between 300 and 350 g, were used in this study. The animals were housed individually in separate cages under controlled environmental conditions, including a 12-hour light-dark cycle, a stable temperature of 22±2 °C, and 45-50% relative humidity. They had free access to standard laboratory food and water. The rats were randomly assigned to one of nine groups (n=7) in each group (Table 1).

For 7 days, baicalein was orally delivered via gavage at a daily dosage of 100 mg/kg. EG and HP were added to the drinking water. The rats were confined to urine collection cages for 12 h to harvest their urine. At the end of the experimental procedure, surgical dissection was performed after anesthetization with ketamine xylazine (90/10 mg/kg), and large amounts of blood were collected from the hearts of the patients. All necessary kidney tissue and blood samples were collected.

The right kidneys were divided into two parts for histological and transmission electron microscopy (TEM) examinations. The left kidneys were used in biochemical studies. For histological examination, tissue sections were submerged in a solution of 10% neutral buffered formalin (NBF). TEM images were fixed in 2.5% glutaraldehyde solution with 0.1 M phosphate buffer (PB), and the left kidneys were stored at -80 °C in polyethylene tubes. Tissue specimens from the left kidney (0.1 g) were collected and suspended in 0.3 mL of phosphate-buffered saline (PBS) at pH 7.4. The samples were then subjected to homogenization using an ultrasonic device (Ika Ultra-Turraz T25, Ika Labortechnik, Germany) operating at 8000 rpm. Finally, the specimens were centrifuged at 3000 rpm and were subjected to centrifugation at

Table 1. Determination of 9 study groups, involving 7 rats each

Groups	Experimental procedure
C	Animals were given saline by gavage
B	100 mg/kg baicalein was dissolved in dimethyl sulfoxide (DMSO) as 33 mg/mL and administered to the animals via intragastric gavage for 7 days
EG	Ethylene glycol was mixed in drinking water at 1% by volume and given for 28 days.
BE (P)	Protective group. 100 mg/kg baicalein was dissolved in DMSO as 33 mg/mL and given to the animals using intragastric gavage for 7 days. Afterwards, ethylene glycol was mixed in drinking water at 1% by volume and administered to animals for 28 days
EB (T)	Therapeutic group. Ethylene glycol was mixed with 1% volume in drinking water and administered to the animals for 28 days. Afterwards, 100 mg/kg baicalein was dissolved in DMSO as 33 mg/mL and administered by gavage for 7 days
D	Animals were given 1 mL of DMSO by intragastric gavage for 7 days
HP	Hydroxy-L-proline was mixed in drinking water at 3% by volume and given to the animals for 28 days
BH (P)	First, 100 mg/kg baicalein was dissolved in DMSO as 33 mg/mL and given to the experimental animals through intragastric gavage for 7 days. Afterwards, hydroxy-L-proline was mixed in drinking water at 3% by volume and given to the experimental animals for 28 days
HB (T)	First, hydroxy-L-proline was mixed in drinking water at 3% by volume and administered to the experimental animals for 28 days. Afterwards, 100 mg/kg baicalein was dissolved in DMSO as 33 mg/mL and given by gavage for 7 days

C: Control group, B: Baicalein group, EG: Ethylene glycol group, BE: Baicalein + ethylene glycol group, EB: Ethylene glycol + baicalein group, D: DMSO group, BH: Baicalein + hydroxyproline group, HB: Hydroxyproline + baicalein group, P: Preventive group, T: Treatment group, DSMO: Dimethyl sulfoxide

4 °C for a duration of 20 min using a Megafuge 1.0 R centrifuge (Heraeus, Hanau, Germany).

Subsequent to the elimination of the supernatant, the residual components were cryopreserved at -80 °C for future analysis using enzyme-linked immunosorbent assay (ELISA).

Biochemical Analyses

The quantification of biomarkers in tissue homogenates and serum samples was conducted using an ELISA using a commercially procured rat kit (YL Biont, Shanghai, China). The detection ranges and sensitivities for each biomarker were as follows: 0.05-10 ng/mL and 0.01 ng/mL for IL-18, 0.2-60 U/L and 0.11 U/L for kidney injury molecule (KIM-1), 0.3-90 ng/mL and 0.15 ng/mL for neutrophil gelatinase-associated lipocalin (NGAL), 2-600 pg/mL and 1.02 pg/mL for N-acetyl-β-D-glucosaminidase (NAG), and 0.5-200 ng/mL and 0.25 ng/mL for osteopontin (OPN). For all biomarkers, the coefficients of variation (CV) within and between assays were 8% and 10%, respectively.

Pathologic Assessment

In preparation for histopathological analysis, all tissue specimens were fixed in 10% NBF solution for 24 h. Following the washing process, the specimens were dehydrated using a series of increasing ethanol concentrations (70%, 80%, 90%, and 96%). The samples were immersed in paraffin and xylene for embedding. Sections of tissue, measuring 4-5 µm in thickness, were cut and colored using two staining methods: hematoxylin-eosin and Von Kossa. The latter technique was used to detect calcium deposits. Two independent pathologists who were unaware of the group assignment performed the histopathological evaluations using binocular light microscopy. To prepare samples for TEM examination, the specimens were promptly fixed in a solution containing 2.5% glutaraldehyde and 0.1 M PB and maintained at 4 °C for 24 h. Following this, the specimens underwent a series of three 15-min washing cycles using PB. The specimens were subjected to an additional fixation step using a mixture of 1% osmium tetroxide dissolved in 0.1 M phosphate buffer (PB), which was applied for 2 h at ambient temperature. Following this procedure, the specimens were rinsed three times with PB. The specimens were subjected to dehydration using a series of ethyl alcohol solutions with gradually increasing concentrations (30%, 50%, 70%, 90%, 96%, and 100%) at different time intervals and propylene oxide twice for 30 min. The experimental protocol involved immersing the specimens in an equivalue mixture of araldite and propylene oxide and maintaining them at 37 °C for 2 h. Subsequently, the samples were exposed to pure araldite for a longer period of time. Polymerization of the embedded samples occurred over the course of the following two days. An ultramicrotome (Leica Ultracut R; Leica, Wetzlar, Germany) was used to produce thin sections of the samples. TEM (JEM-1220 at 80 kV, JEOL, Tokyo, Japan) was used to obtain images of acetate-lead citrate sections at high magnification.

Statistical Analyses

SPSS v21 was used for statistical analyses. The suitability of the variables for normal distribution on a group basis was evaluated using the Shapiro-Wilk test. To compare biomarker levels among the study groups, analysis of variance was conducted. The Bonferroni test was used to conduct pairwise comparisons among the groups. Statistical significance was determined by a p-value 0.05.

Ethical Statement

In line with the 8th edition of the Guide for the Care and Use of Laboratory Animals, all animal care activities and associated experiments were conducted in accordance with the established guidelines. All experimental procedures were approved by the Eskişehir Osmangazi University Animal Experiments Local Ethics Committee (date: 06.09.2017, decision no: 619).

Results

The analysis of kidney tissue biomarkers revealed significant differences across all study groups (Table 2, $p<0.001$). Subsequent analysis showed that IL-18 levels were notably reduced in the group treated with ethylene glycol and baicalein (EB) compared with the group receiving only ethylene glycol (EG). This difference was statistically significant ($p=0.019$). Moreover, IL-18 levels were reduced in both the hydroxyproline + baicalein (HB) and baicalein + hydroxyproline (BH) groups compared with the HP group ($p<0.001$). Moreover, the HB group exhibited significantly reduced IL-18 levels compared with the BH group ($p<0.001$). The BE (baicalein + ethylene glycol) group exhibited significantly lower KIM-1 levels than the EG group ($p<0.001$). Additionally, both the HB and BH groups showed lower KIM-1 levels than the HP group, with p-values of <0.001 and 0.028, respectively. Moreover, the HB group exhibited significantly lower KIM-1 concentrations than the BH group, with a statistical significance of $p<0.001$. The HB and BH groups exhibited markedly reduced NAG levels compared with the HP group ($p<0.001$). Moreover, a statistically significant decrease was noted in the HB group compared with the BH group ($p=0.001$). Statistical analysis revealed a significant reduction in NGAL levels for both the BE ($p=0.005$) and EB ($p=0.028$) groups compared with the EG group. NGAL measurements were found to be significantly lower in both the HB and BH groups compared with the HP group ($p<0.001$ and $p=0.001$, respectively). Moreover, NGAL levels were markedly reduced in the HB group compared with the BH group ($p<0.001$).

The study groups exhibited notable variations in serum biomarker levels (Table 3). Subsequent analysis demonstrated a statistically significant reduction in IL-18 concentrations in the EB cohort compared with the EG group ($p<0.001$). Furthermore, both the BH and HB groups exhibited markedly decreased IL-18 levels compared with the HP group ($p<0.001$). Moreover, the EB group exhibited markedly reduced IL-18 levels compared with the BE group, with statistical significance ($p=0.001$). The analysis revealed statistically significant differences in KIM-1 levels between the groups. Specifically, the EB group exhibited lower KIM-1 levels than the EG group ($p=0.013$), whereas a similar trend was observed between the HB and HP groups ($p=0.033$). The EB group exhibited a notably lower level of OPN expression than the EG group, with statistical significance ($p<0.001$). Regarding other serum biomarkers, no substantial variations were detected between the therapeutic and preventive groups or between the EG and HP groups.

Upon microscopic evaluation, the kidney tissue specimens exhibited no substantial variation across the experimental groups (Figure 1). In addition, no calcium precipitates were identified in any group using von Kossa staining. Transmission electron microscopy (TEM) of the control group revealed normal, healthy kidney tissue without the presence of calcium crystals. In contrast, the EG group exhibited heterochromatic nuclei, peripheral chromatin condensation, swelling of the outer nuclear membrane, and increased numbers of vesicles and lysosomes. Noticeable gaps were observed near the nucleus and mitochondria, along with mitochondrial cristae loss and potential sand-like structures. The HP group exhibited abnormal mitochondrial structures, severe thickening of basement membranes, endothelial cell wall changes in glomerular capillaries, chromatin condensation, and irregular podocyte structures, along with increased lysosome and vacuole counts. Potential kidney stone-like formations were also observed. However, no such mitochondrial, nuclear, or basement membrane abnormalities were detected in the BE, EB, BH, or HB groups (Figure 2).

Discussion

The primary outcome of this study was the demonstration of baicalein's protective and therapeutic effects in the treatment of KSD. This was most evident in the results of the TEM examinations. Sand-like structures and indicators of renal damage were identified in the kidneys of rats treated with ethylene glycol and hydroxyproline. However, these structures were absent in rats that received baicalein, both as a preventive and therapeutic measure, resulting in healthier kidney tissue. Serum analysis revealed a significant reduction in IL-18 and KIM-1 levels in the EB group compared with the EG group. Similarly, compared with the HP group, the HB group exhibited notably lower IL-18 and KIM-1 levels. As the EB and HB groups were therapeutic, the serum IL-18 and KIM-1 levels could serve as biomarkers for monitoring the effectiveness of KSD treatment.

Ethylene glycol (EG) and hydroxyproline (HP) were used in a manner consistent with previous studies (12-14). In alignment with the existing literature, TEM analysis revealed calcifications and notable mitochondrial and cellular alterations in the EG

Table 2. Marker levels measured in the kidney tissues, p≤0.05 is accepted as statistically significant

	C	B	EG	BE (P)	EB (T)	D	HP	BH (P)	HB (T)	p-value
IL-18	0.46	0.48	0.63	0.58	0.38	0.53	1.57	0.95	0.45	0.000
KIM-1	0.0044	0.0042	0.0053	0.0034	0.0045	0.0036	0.0097	0.0083	0.0032	0.000
NAG	0.097	0.05	0.06	0.052	0.044	0.058	1.17	0.1	0.055	0.000
NGAL	0.028	0.029	0.033	0.019	0.02	0.02	0.058	0.04	0.023	0.000
OPN	0.52	0.057	0.09	0.08	0.035	0.06	0.1	0.04	0.06	0.000

C: Control group, B: Baicalein group, EG: Ethylene glycol group, BE: Baicalein + ethylene glycol group, EB: Ethylene glycol + baicalein group, D: DMSO group, BH: Baicalein + hydroxyproline group, HB: Hydroxyproline + baicalein group, P: Preventive group, T: Treatment group, DSMO: Dimethyl sulfoxide, IL-18: Interleukin 18, KIM-1: Kidney Injury Molecule, NAG: N-acetyl-β-D-glucosaminidase NGAL: Neutrophil Gelatinase-Associated Lipocalin, OPN: Osteopontin

Table 3. Marker levels in measured the serum samples, p≤0.05 is accepted as statistically significant

	C	B	EG	BE (P)	EB (T)	D	HP	BH (P)	HB (T)	p-value
IL-18	0.46	0.44	0.52	0.45	0.24	0.41	0.53	0.22	0.27	0.000
KIM-1	0.33	0.33	0.37	0.3	0.27	0.34	0.37	0.3	0.28	0.002
NAG	1.89	1.54	1.93	1.91	1.85	1.9	2.31	2.05	2.32	0.008
NGAL	2.52	2.35	3.47	3.49	3.04	2.62	3.36	3.33	3.17	0.001
OPN	18.7	18.4	21.6	17.8	13.1	18.8	18.9	18.5	17.6	0.004

C: Control group, B: Baicalein group, EG: Ethylene glycol group, BE: Baicalein + ethylene glycol group, EB: Ethylene glycol + baicalein group, D: DMSO group, BH: Baicalein + hydroxyproline group, HB: Hydroxyproline + baicalein group, P: Preventive group, T: Treatment group, DSMO: Dimethyl sulfoxide, IL-18: Interleukin 18, KIM-1: Kidney Injury Molecule, NAG: N-acetyl-β-D-glucosaminidase NGAL: Neutrophil Gelatinase-Associated Lipocalin, OPN: Osteopontin

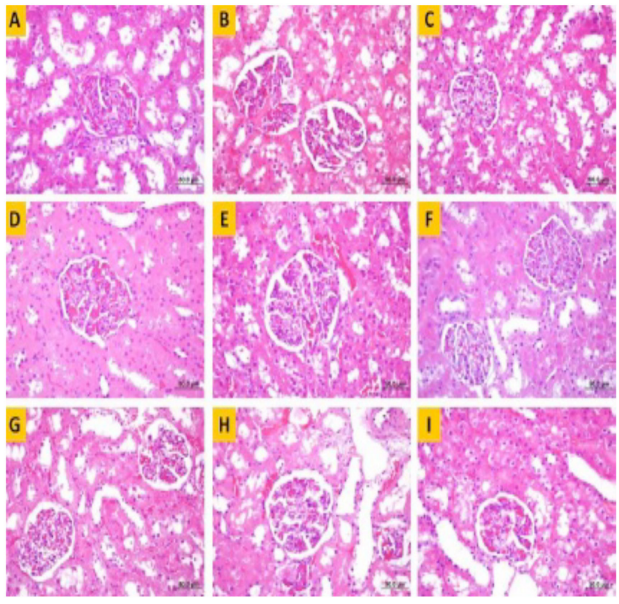

Figure 1: Histologic examinations of kidney sections of the rat groups. A: Control group, B: DMSO Control Group, C: Baicalein group, D: Ethylene glycol group, E: Hydroxy-L-Proline Group, F: Baicalein + Ethylene Glycol Protective Group, G: Baicalein + Hydroxyproline Protective group, H: Ethylene Glycol + Baicalein Therapeutic Group, I: Hydroxyproline + Baicalein Therapeutic Group

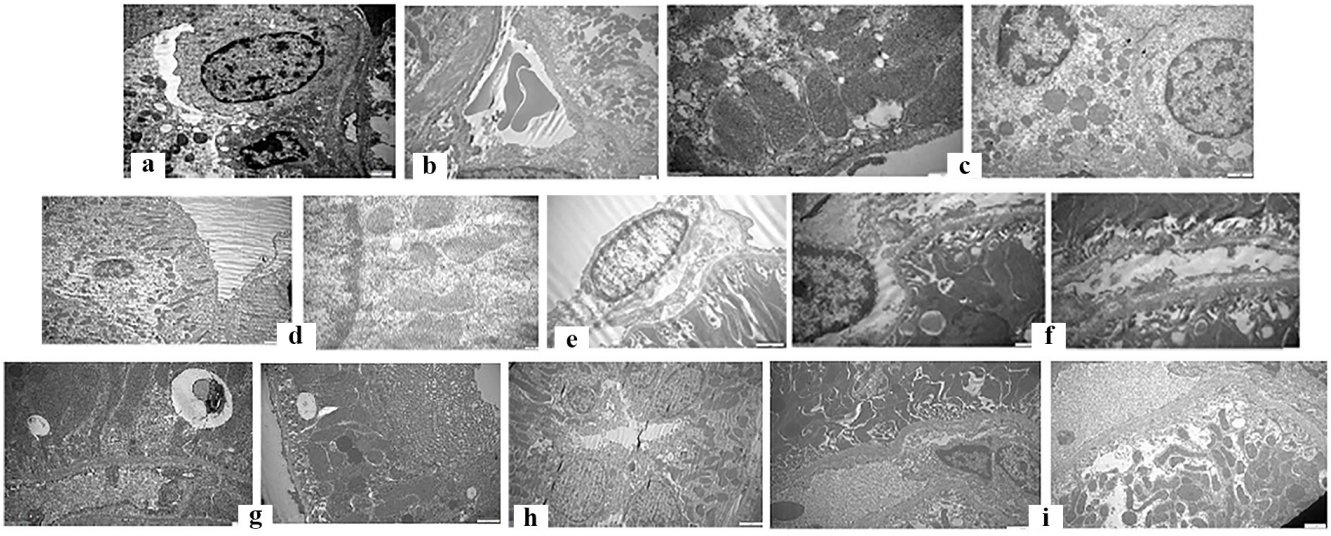

Figure 2. Transmission electron microscopic examinations of kidney sections of the rat groups. (a) Control group (b) Baicalein group (c) Ethylene glycol group (d) Baicalein + Ethylene Glycol Protective Group (e) Ethylene Glycol + Baicalein Therapeutic Group (f) DMSO Control Group (g) Hydroxy-L-Proline Group (h) Baicalein + Hydroxyproline Protective group (i) Hydroxyproline + Baicalein Therapeutic Group

and HP groups. Elevated levels of IL-18, NGAL, KIM-1, and NAG, coupled with reduced OPN levels, are indicative of kidney damage in these groups. The fact that cellular abnormalities were only detectable through TEM might be due to the study being conducted in the early stages of stone formation when such changes are not yet visible under light microscopy (15). Although Wistar rats, which are known for their sensitivity to stone-forming diets, were used, the dose or duration of EG and HP exposure might have been insufficient. Despite this, the cellular and mitochondrial damage observed by TEM underscores the significant impact of hyperoxaluria and calcium oxalate exposure on kidney cells. Biochemical alterations and TEM observations allowed us to evaluate our hypothesis and examine the early stages of stone formation.

According to current research, IL-18, KIM-1, NAG, and NGAL are reliable biomarkers for assessing kidney damage (16). In our study, the levels of these markers were highest in the HP group, in agreement with existing literature, suggesting that the most severe kidney damage occurred in this group.

Animal studies have shown that certain dietary plants hold great promise for managing urinary stones effectively although the clinical application of herbal products remains limited (17). Research indicates that herbal products with anti-inflammatory and antioxidant properties can decrease EG-induced calcium oxalate accumulation, reduce urinary oxalate levels, and mitigate kidney damage (12,18).

Baicalein is a key component of traditional Chinese medicine, particularly for treating various liver, kidney, and cardiovascular diseases. The plant *Enhydra fluctuans*, which is commonly used to manage KSD, contains baicalein as a key metabolite. An *in vitro* study evaluating 35 metabolites from *Enhydra fluctuans* extract demonstrated baicalein's ability to inhibit calcium oxalate crystallization (19). Additionally, baicalein has shown anti-inflammatory effects in various studies (20-22). In our study, we also examined its therapeutic efficacy in the EB and HB groups. TEM analysis revealed that the basement membrane in the EB group exhibited a smoother appearance than that in the EG group. Additionally, the HB group displayed healthier mitochondria than those observed in the HP group. The therapeutic potential of baicalein was further demonstrated by the decreased concentrations of IL-18, KIM-1, NAG, and NGAL in the kidney tissues of the HB group compared with the HP group. Moreover, the observed reduction in NGAL levels within the EB group relative to the EG group supports this promising effect. These findings suggest that baicalein may be beneficial for managing KSD and reducing the risk of recurrence. Moreover, the EB group showed significantly reduced serum concentrations of IL-18 and KIM-1 compared with the EG group, whereas the HB group exhibited lower concentrations of IL-18 and KIM-1 than the HP group. These findings suggest that IL-18 and KIM-1 are valuable biomarkers for assessing the effectiveness of baicalein in the management of kidney stone disease. The protective effects of baicalein were evaluated in the BE and BH groups. In contrast to the EG group, in which mitochondrial cristae deterioration was evident, the BE group did not exhibit such damage. Severe mitochondrial abnormalities were also observed in the HP group. In contrast to the HP group, the BH group exhibited histological findings that were within normal parameters. The BH group exhibited reduced concentrations of IL-18, KIM-1, NAG, and NGAL in kidney tissue compared with the HP group. Additionally, the BE group exhibited decreased levels of KIM-1 and NGAL in kidney tissue compared with the EG group. Our results indicate that baicalein prevents the development of kidney stones. In the HB group, the kidney tissue exhibited significantly lower concentrations of IL-18, KIM-1, NAG, and NGAL compared with the BH group. In comparison with the BE group, the EB group exhibited lower serum IL-18 levels. These findings indicate that the therapeutic efficacy of baicalein may exceed its preventive efficacy.

Our investigation's principal limitations encompass the lack of observable stones during light microscopy and the omission of urinary oxalate excretion analysis. Although Wistar rats, which are very sensitive to a stone-forming diet, were used in our study, the application times of ethylene glycol and hydroxyproline may have been insufficient. The application of 3% hydroxyproline may not have created sufficient supersaturation. Stone and crystal structures cannot be seen in histological examination due to the lack of sufficient supersaturation in the groups given ethylene glycol and hydroxyproline, the presence of high amounts of inhibitory substances in the urine of the rats we used, and the insufficient crystallization and adhesion phase due to short urine transit times. Another limitation of our study is that it was an animal experiment, and no clinical research was conducted in humans.

Conclusion

In conclusion, stones, sand-like formations, and indicators of renal damage were identified in the kidneys of rats treated with ethylene glycol and hydroxyproline. However, in the groups in which baicalein was used as both a preventive and therapeutic agent, these structures were absent, and the kidney tissue appeared healthier. Our findings highlight the protective and therapeutic potential of baicalein in managing KSD, reducing stone-induced renal damage, and supporting prior research in this area. These results provide a foundation for future experimental and clinical studies. Further research, particularly in human clinical trials, is essential to explore the potential of baicalein as a medical treatment for urinary tract stones.

Considering the marker levels in kidney and serum, it was determined that the therapeutic effectiveness of baicalein was superior to its protective effectiveness. The treatment groups exhibited significantly reduced levels of IL-18 and KIM-1 compared with the EG and HP groups; thus, these markers measured in serum could be used in the follow-up of urinary system stone treatment.

Ethics

Ethics Committee Approval: All experimental procedures were approved by the Eskişehir Osmangazi University Animal Experiments Local Ethics Committee (date: 06.09.2017, decision no: 619).

Informed Consent: Not necessary.

Footnotes

Authorship Contributions

Surgical and Medical Practices: C.T., Concept: M.C.Ü., B.B., Design: B.B., Data Collection or Processing: E.Y., Analysis or Interpretation: M.Ö., A.Y., Literature Search: E.Y., Writing: E.Y.

Conflict of Interest: No conflict of interest was declared by the authors.

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