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UROLOGY - ORIGINAL PAPER

Theoretical and laboratory investigations of the effects of hydroxyproline ingestion on the metabolic and physicochemical risk factors for calcium oxalate kidney stone formation in a small group of healthy subjects

Allen Rodgers[1] · Phindile Cele[1] · Neil Ravenscroft[1] · Cesarina Edmonds-Smith[1] · Graham Jackson[1]

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Abstract

Purpose Dietary hydroxyproline may be involved in the endogenous synthesis of oxalate. Glycolate, produced during the metabolism of hydroxyproline, may exert physicochemical effects on urinary calcium by virtue of its dihydroxycarboxylic acid structure. The aim of this study was to investigate these possible stone-risk scenarios.

Methods We modelled the effect of different glycolic acid concentrations on ionized calcium (iCa^{2+}) and relative supersaturation (RSS) of calcium oxalate (CaOx) using the program JESS. Thereafter, three healthy white males and two healthy black males ingested 30 g gelatin for 3 days. 24-h urines were collected at baseline and after completion of the protocol. Urines were analysed for physicochemical risk factors and for iCa^{2+} and glycolic acid. Speciation concentrations and RSS values were calculated.

Results Theoretical modelling showed that binding between calcium and glycolate does not occur and that iCa^{2+} and RSS CaOx are unaffected. However, after ingestion of hydroxyproline, iCa^{2+} decreased significantly. Urinary pH and glycolate increased significantly. Oxalate excretion and RSS CaOx did not change

Conclusions We attribute the decrease in iCa^{2+} to increases in the concentrations of several Ca–phosphate species, the formation of which is due to the increase in pH. We speculate that the absence of an increase in oxalate excretion despite an increase in glycolate excretion may be due to the mixed racial composition of our test group in which some pathways may be preferred to others. Our findings alert stone researchers to the importance of measuring urinary pH in their workup of subjects and to select racially homogenous groups for investigation.

Keywords Endogenous production of oxalate · Endogenous production of glycolate · Hydroxyproline ingestion · Modelling glycolate–calcium binding · Racial dependence of hydroxyproline metabolism

Introduction

Endogenous production of oxalate is a potential contributor towards hyperoxaluria and calcium oxalate (CaOx) stone formation. Characterization of the metabolic pathways involved in its production is, therefore, important. While many details are known, uncertainty remains regarding the synthesis of glyoxylate, which is widely regarded as the principal precursor of oxalate. Once formed, it can convert to glycine via enzymatic activity of alanine glyoxylate aminotransferase, or to glycolate via glycolate reductase or to oxalate via lactate dehydrogenase [1]. Insights into these pathways and the extent to which they may be influenced by dietary factors are of interest. As such, further investigation of these pathways is warranted.

In a relatively recent study by Knight and co-workers, dietary hydroxyproline (in the form of gelatin), a potential precursor of glyoxylate, increased urinary oxalate and glycolate in a group of healthy subjects [1]. The authors concluded that hydroxyproline metabolism is likely to be a significant source of glyoxylate production, but that a more definitive picture of its contribution to daily glycolate and oxalate synthesis is required. In the only subsequent study

✉ Allen Rodgers
allen.rodgers@uct.ac.za

[1] Department of Chemistry, University of Cape Town, Rondebosch, 7701, Cape Town, South Africa

in which the urinary response of humans to ingestion of hydroxyproline was investigated, Akiduki et al. also concluded that the metabolic significance of hydroxyproline is still unclear and that further investigation was warranted [2]. As such, we adopted this challenge as an objective of the present study. A highly simplified version of the metabolic pathways for oxalate synthesis, based on those published by Frederick et al. [3] and Robijn et al. [4], is shown in Fig. 1.

While glycolate has been investigated in the metabolic production of oxalate and the concomitant risk of calcium oxalate renal stone formation, its potential physicochemical influence on the latter has been ignored. Its relevance in this regard relates to its chemical structure since it is derived from a dihydroxycarboxylic acid and, therefore, has two potential binding sites for calcium. These might affect thermodynamic and/or kinetic factors associated with CaOx stone formation. Exploration of this possibility provided further motivation for the present study.

Methods

Declaration

Parts of this work were presented as a poster at the American Urological Association (AUA) annual meeting in 2014 and were published as an abstract [5].

Theoretical model

With respect to our objective of investigating glycolate's possible influence on thermodynamic risk factors, we initially adopted a theoretical approach in which we modelled the effect of different glycolic acid concentrations on the concentration of urinary Ca^{2+} and on the relative supersaturation (RSS) of the calcium-containing urinary salts calcium oxalate monohydrate (COM) and calcium hydrogen phosphate dihydrate, commonly known as brushite (Bru). For our urine model, we used literature values for the urinary parameters of healthy individuals [6]. The modelling itself was achieved using the speciation program JESS [7]. Modelling was performed at 1×, 10× and 100× the physiological concentration of glycolic acid (4.0×10^{-4} M). We calculated the latter value from the glycolate excretion value reported by Niederwieser et al. [8] for which we assumed a 24-h urinary volume of 1500 ml.

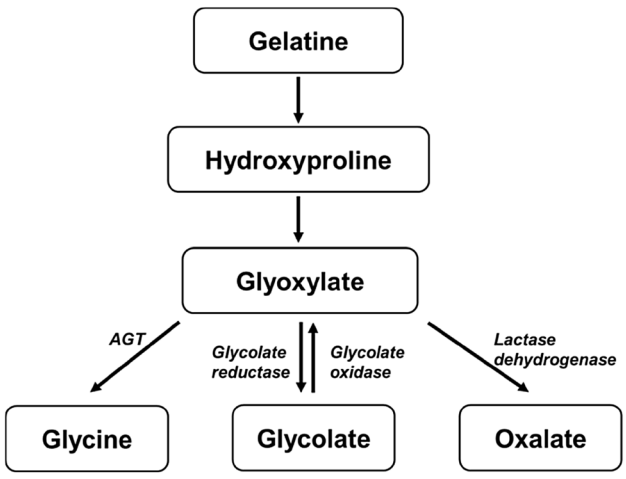

Fig. 1 Simplified metabolic cycle diagram for endogenous production of oxalate from dietary gelatin

Human model

Subjects and urine collections

Five healthy subjects (aged 22–26 years, three white males, two black males) with no history of kidney disease were recruited from the postgraduate student cohort of the University of Cape Town. Subjects provided 24-h urine collections at baseline and after completing the hydroxyproline trial described below. Samples were collected in plastic bottles without preservative. Each was tested for the presence of blood and infection (Combur 10 test strip, Boehringer Mannheim, Mannheim, Germany) and was discarded if the test was positive. Urines were then filtered through a 0.75-µm pre-filter and a 0.45-µm cellulose nitrate filter before analysis, to remove cellular debris and proteinaceous material.

Hydroxyproline trial

Each subject ingested a low (15 g/day) and a high (30 g/day) dose of Sheridan's (Bovine) Gelatine [Libstar Operations (Pty) Ltd, South Africa]. Doses were divided into three equal portions (3 × 5 g and 3 × 10 g, respectively) for ingestion at breakfast, lunch and dinner. Prior to ingestion, doses were dissolved in 100 ml of warm water having a low mineral content. Each protocol lasted 3 days with a washout period of 4 days between them. Subjects maintained their free unselected diets throughout the experiment.

Urine analysis

Urines were analysed for pH, Cit, oxalate, Ca, Mg, Na, K, creatinine, phosphate, urate and chloride using routine procedures, as described elsewhere [9]. Ionized calcium (iCa^{2+}) was measured using a calcium ion-selective electrode (Metrohm AG, Switzerland) stored in 0.01 M $CaCl_2$ and a Ca reference electrode stored in 3 M KCl. Urinary glycolic acid was determined by a chromotropic acid–sulphuric acid calorimetric assay [8]. The concentrations of iCa^{2+} and various chemical species were calculated in each urine at

both doses. RSS of COM and Bru were calculated for the post-high-dose samples only. Calculations were performed using JESS.

CaOx upper limit of metastability

The CaOx metastable limit (MSL) of each urine was measured according to the method of Ryall et al. [10]. This parameter provides a measure of how readily CaOx crystallization can be induced [10]. Briefly, aliquots of filtered urine (pre-filter, 0.45 µm) were dosed with 100 µl of increasing concentrations of sodium oxalate (Na_2Ox) from 15 to 195 mM at 1-min time intervals and were then incubated in an oven at 37 °C for 30 min. UV absorption in each aliquot was measured at 620 nm in a Specord 40 Spectrophotometer (Analytik Jena, Germany) The exogenous sodium oxalate concentration at which spontaneous crystallisation occurred (indicated by a sudden increase in absorption) was determined and was interpreted as a measure of the urine's CaOx MSL.

Determination of hydroxyproline in gelatin

The hydroxyproline (HYP) content in the gelatin used in the present study was determined by microwave-assisted mild methanolysis followed by silylation and chromatographic analysis [11]. *Trans*-4-hydroxy-L-proline (Sigma-Aldrich, H54409) was used as the standard. Microwaving was performed at 120 W at 121 °C for 5 min. Sweeley's silylating reagent was used for sialylation of sample and standard. Derivatised samples were analysed using an Agilent 8720 gas chromatograph/5977E with a 30-m HP-5-ms capillary column. Hydroxyproline content was found to be 9.6%, which compares favourably with the value of 9.2% in the sample used by Knight and co-workers [1].

Statistical analysis

Statistical analyses were conducted using GraphPad InStat 3.06 (GraphPad Software Inc., La Jolla, USA). Mean values and standard errors of the mean (SEM) are reported for the urine data. Significant differences were assessed using a *t* test and *P* values less than 0.05 were regarded as significant.

Results

Theoretical modelling

Results are given in Table 1. These show that theoretical values for Ca^{2+} concentrations and for RSS of both salts remain constant at the relatively low concentrations of glycolate but tend to decrease at a glycolate concentration which is 100-fold greater than its baseline value. It is, therefore, concluded that glycolate will not affect urinary thermodynamic properties such as RSS and MSL in physiologically derived human urine.

Table 1 Theoretical modelling of the effects of glycolate concentrations on Ca^{2+} concentration and RSS of Ca-containing urinary salts

Glycolate concentration	Ca^{2+} concentration (mmols. dm^{-3})	RSS COM	RSS bru
Baseline	1.14	3.61	1.88
2×baseline	1.14	3.61	1.88
5×baseline	1.14	3.61	1.88
10×baseline	1.15	3.59	1.88
100×baseline	1.21	3.37	1.85

Baseline: 4×10^{-4} mols.dm^{-3}

Urine composition

Mean urinary parameters at baseline and after ingestion of low (15 g/day) and high (30 g/day) doses of hydroxyproline are given in Table 2. The excretion of three urinary components changed after both low and high doses of hydroxyproline, relative to baseline values. These were magnesium and glycolate, both of which increased significantly, and iCa^{2+} which decreased significantly. Urinary pH showed an increasing trend after the gelatin low dose and reached significance after the high dose. Three components changed after ingestion of the low dose (K, creatine and Cl) but these were not sustained after the high dose. We do not regard these as clinically significant. Importantly, oxalate excretion did not change after ingestion of either dosage. Finally, despite the significant decrease in iCa^{2+}, RSS of COM showed only a weak tendency to decrease. However, RSS of Bru did not change,

Urinary Ca speciation

The urinary calcium species after ingestion of hydroxyproline is shown in Fig. 2. Comparison of each species at baseline, low dosage and high dosage shows an increase in the concentrations of several Ca–phosphate (Ca–Phos) species (particularly $[Ca_2H_2(PO_4)_2]$) and a concomitant decrease in the concentration of Ca^{2+} and all other complexes. The absence of any Ca–glycolate complexes is noteworthy.

CaOx metastable limit and crystallization kinetics

The CaOx MSL for each urine showed an increasing (but non-significant) trend with increasing doses of

Table 2 Mean measured urinary parameters at baseline and after low and high doses of hydroxyproline

	Baseline (mean ± SE) $n=5$	Low dose (mean ± SE) $n=5$	High dose (mean ± SE) $n=5$	p value baseline vs low dose	p value baseline vs high dose
pH	5.94 ± 0.14	6.10 ± 0.10	6.32 ± 0.04	0.071	0.0004
Volume (l)	1.052 ± 0.238	1.216 ± 0.116	1.028 ± 0.220	0.203	0.873
Cit	2.15 ± 0.50	2.38 ± 0.13	2.01 ± 0.36	0.348	0.625
Ox	0.224 ± 0.065	0.254 ± 0.030	0.238 ± 0.058	0.376	0.729
Ca	2.39 ± 0.56	3.09 ± 0.59	2.83 ± 0.21	0.0.091	0.139
Mg	2.03 ± 0.21	3.70 ± 0.28	3.40 ± 0.41	< 0.0001	0.0002
Na	119 ± 17	126 ± 6	108 ± 15	0.411	0.310
K	31 ± 4	45 ± 4	26 ± 4	0.0005	0.083
Urate	2.54 ± 0.56	3.34 ± 0.54	2.42 ± 0.32	0.051	0.688
Creat	13.52 ± 1.64	17.12 ± 2.94	12.18 ± 1.07	0.044	0.165
Phos	21.82 ± 4.30	23.38 ± 3.25	18.98 ± 3.68	0.536	0.292
Cl	125 ± 26	157 ± 15	117 ± 19	0.044	0.594
iCa	1.895 ± 0.168	1.035 ± 0.163	0.757 ± 0.101	< 0.0001	< 0.0001
Glycolic acid	18.25 ± 6.40	95.23 ± 19.26	111.65 ± 9.007	< 0.0001	< 0.0001
RSS COM	3.75 ± 0.56	nc	2.77 ± 0.82	nc	0.058
RSS Bru	1.92 ± 0.34	nc	1.95 ± 0.35	nc	0.094

All excretions expressed as mmol/24 h, except glycolic acid mg/24 h

iCa ionized (unbound calcium), *RSS* relative supersaturation, *COM* calcium oxalate monohydrate, *Bru* brushite, *nc* not calculated as our theoretical calculations showed that changes in RSS values can only be expected at very high levels of hydroxyproline

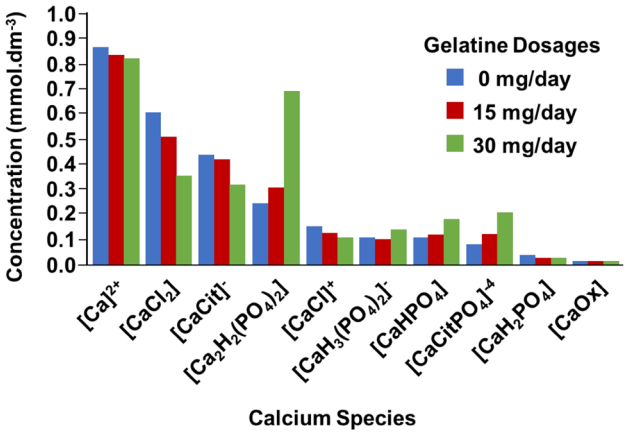

Fig. 2 Calcium speciation calculated by JESS at baseline and after low and high doses of hydroxyproline

hydroxyproline. This is shown in Fig. 3 for one such urine which is representative of the others.

Discussion

Our theoretical modelling predicts that reduction of Ca^{2+} and RSS of CaOx and Bru in urine under normal and moderately elevated glycolate levels does not occur. This is possibly not surprising since the calcium-binding capacity (K) of glycolate is 27 × weaker than that of citrate, its main competitor for Ca complexation in urine (log K = 1.84 and 3.27, respectively) [7]. Therefore, for glycolate to compete effectively with citrate for binding to calcium, its concentration would have to be 27 times greater than that of citrate. Since citrate's concentration in normal urine is about 2×10^{-3} M, the effective concentration of glycolate would have to be about 54×10^{-3} M, i.e. about 135 × greater than its physiological concentration (4×10^{-4} M). This is consistent with our theoretical modelling which demonstrated an effect only when glycolate concentration was set at 100× its normal value (Table 1).

We, therefore, conclude that the decrease in iCa^{2+} which we observed in our subjects after ingestion of hydroxyproline cannot be attributed to the binding of Ca to glycolate. This is confirmed by the absence of any Ca–glycolate complexes in the JESS-generated calcium speciation for the urine data which we measured in our subjects (Fig. 2). However, we can account for the decrease in iCa^{2+} by attributing it to increases in the concentrations of several Ca–Phos species, particularly $[Ca_2H_2(PO_4)_2]$, which utilize free Ca^{2+} in their formation (Fig. 2). Formation of these Ca–Phos complexes can themselves be attributed to the increasing trend in pH [12] which we observed after the low dose and which became statistically significant after the high does (Table 3). Although several studies have previously investigated the physiological effects of gelatin or pure hydroxyproline in animals [13–16] and humans [1, 2, 17, 18], urinary pH was

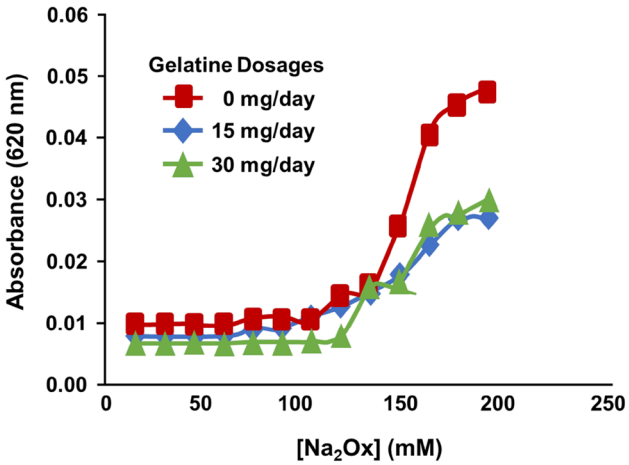

Fig. 3 Plot of absorbance vs concentration of exogenous sodium oxalate solution to determine the calcium oxalate metastable limit

Footnote: The concentration of Na_2Ox at which the absorbance increases suddenly corresponds to the CaOx metastable limit. This occurred in the range 110 – 120 mM for each titration.

Table 3 Comparison of subjects, protocols and results obtained in the present and previous studies

Present study ($n=5$)	Knight et al. [1] ($n=6$)	Akiduki et al. [2] ($n=8^{\phi}$)
Single gender: males Mixed race groups: 3 W, 2B	Mixed genders: 3 M, 3F Race group not reported	Mixed genders: 4 M. 4F Race group not reported
Healthy subjects	Healthy subjects	Healthy subjects
9.6 g HYP per 100 g gelatin	9.2 g HYP per 100 g gelatin	HYP not administered as gelatin
High dose: 30 g gelatin/day (equivalent to 2.88 g HYP per day) for 3 days; three equal doses (10 g) at breakfast, lunch, dinner	30 g gelatin/day (equivalent to 2.76 g HYP per day) for 3 days; single dose at breakfast	High dose: 2.0 g HYP per day for 12 weeks*
Self-selected diet; no dietary restrictions	Self-selected diet; subjects were required to avoid oxalate-rich food and to eat only moderate amounts of protein and calcium	Self-selected diet; subjects were required to avoid oxalate-rich foods
Washout period: 4 days	Washout period: 2 days	na
Gelatin consumed with 100 ml low-mineral-content water	Gelatin consumed with yoghurt & 250 ml bottled water	na
Glycolate excretion relative to self-selected diet: High dose: 11.93 → 81.04 mg/g creat (6.8-fold increase)	Glycolate excretion relative to self-selected diet: 19.9 → 78.3 mg/g creat (3.9-fold increase)	Not reported
Oxalate excretion relative to self-selected diet: high dose:12.89 → 15.21 mg/g creat (1.18-fold increase)	Oxalate excretion relative to self-selected diet: 17.6 → 24.4 mg/g creat (1.39-fold increase)	Oxalate excretion relative to self-selected diet: high dose: 14.08 → 24.64 (1.75-fold increase)

W white subjects, *B* black subjects, *HYP* hydroxyproline, ϕ group receiving highest dose HYP, * max dose, *na* not applicable

reported in only three of them [13, 15, 16]. Buchinsky et al. found in their rat study that urinary pH increased significantly relative to controls after 6-week ingestion of a diet supplemented with hydroxyproline but there were no further increases after 12 weeks and 18 weeks on the same diet [13]. Khan et al. found that urinary pH was generally lower in rats which had been fed hydroxyproline compared to controls, but this was not statistically significant [15]. Neither Khan nor Buchinsky commented in their papers on the pH changes. In the third study, Kaplon et al. did not find any changes in urinary pH in a porcine model involving addition of hydroxyproline to the basal diet of 12 sows [16]. Remarkably, urinary pH was not reported in any of the human studies. It, therefore, seems that our observation in the present study of an increase in urinary pH after gelatin ingestion in humans is the first of its kind. It is not obvious to us the mechanism by which this parameter increased significantly in our subjects, but we speculate that it may be associated with a metabolic process involving hydroxyproline which, as yet has not been identified. Indeed, Knight et al. state that the response of humans to large doses of hydroxyproline is not known [1]. Clearly, the potential effect of hydroxyproline

ingestion on urinary pH in humans is an important metabolic process which needs clarification in future studies. Similarly, we are unable to explain the increase in magnesuria which we observed after ingestion of hydroxyproline. Of the studies conducted in humans, urinary magnesium was reported in only one of them and no effect was observed, save for an increase in its excretion 4 weeks after completion of the trial [2]. No explanation for this was offered by the authors. We speculate that like the increase in urinary pH, the increase in magnesuria may be due to an unknown effect of hydroxylamine. In this case, such an effect may upset magnesium balance by interfering with its renal regulation and reabsorption [19].

The absence of any change in the RSS values of COM and Bru in our measured urines confirms the prediction of our theoretical modelling. Furthermore, since MSL embraces thermodynamic (RSS) and kinetic (growth, aggregation) factors, its constancy indicates that ingested hydroxyproline has no effect on these mechanisms of crystal and stone formation.

With respect to potential metabolic effects of hydroxyproline, we found that although urinary glycolate excretion increased significantly after gelatin ingestion relative to baseline, urinary oxalate excretion did not increase (Table 3). This is in conflict with the findings of Knight et al. [1] and Akiduki et al. [2] who both reported significant increases in this urinary parameter. Intriguingly, the magnitude of the increase in our glycolate excretion (mg/creat) is greater than that of Knight and co-workers (6.8 vs 3.9), yet our oxalate excretions did not increase. Glycolate excretion was not reported in the study by Akiduki et al., so its relationship to oxalate excretion is not available for comparison with our results. The greater excretion of glycolate in our study compared to that observed in the study by Knight and co-workers suggests that conversion of glyoxylate to glycolate might have occurred to a greater extent in our subjects, possibly due to an elevated activity of glycolate reductase. Concomitantly, the amount of glyoxylate available for conversion to oxalate in our subjects would have been reduced, leading to an unexpectedly lower excretion of oxalate than that which might have been expected. In addition, or alternatively, the activity of lactate dehydrogenase in our subjects may be diminished relative to those reported in the studies by Knight et al. [1] and Akiduki et al. [2]. Of course, theoretical modelling of the transformation per se of glyoxylate to oxalate would have provided insights into the factors causing these anomalies, but software for doing such modelling does not exist. However, comparison of the demographics, protocols and results of our study with those of the other two studies (Table 3) provides a possible explanation for how these scenarios might have arisen.

An immediately apparent difference relates to the composition of the test groups. In our study, the group was homogenous with respect to gender and heterogeneous with respect to race while the converse is true in the two other studies. The possibility that different gender-based physiological responses to hydroxyproline ingestion are the cause for the different oxalate excretions between those observed in our study and those of the other two is substantiated by other studies which have demonstrated gender-dependent differences in hyperoxaluria frequency [20], glycolate and oxalate excretions [21] and endogenous oxalate production [22]. As such, the mixed gender composition of the groups in the studies by Knight and by Akiduki is a potential source of bias. Equally, the mixed race composition in the present study could also be a conflicting factor since numerous inter-race studies have demonstrated significant differences between black and white subjects regarding their physiological response to various dietary challenges [23, 24]. In retrospect, it would have been insightful to have separated our subjects in the present study into two different race groups, but inter-race differences were not defined as one of our objectives. As such, our findings pertaining to race, albeit that we acknowledge their serendipitous nature, and the hypotheses which we have invoked to explain them provide an intriguing challenge for future research efforts.

We are aware that our study has several shortcomings. Besides the mixed racial composition discussed above, our small study group prevents us from pronouncing definitively on our findings. This is an important limitation. We also recognize that the administration of a controlled diet and measurement of urinary HYP and plasma glycine would have been insightful. However, notwithstanding these shortcomings, we believe that our comprehensive approach involving theoretical modelling, laboratory measurements and metabolic evaluation is unique and that it provides an overview of the role of hydroxyproline ingestion in CaOx nephrolithiasis which will be of interest to stone researchers and clinicians.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

Ethical approval All procedures were performed in accordance with the ethical standards of the University of Cape Town and with the 1964 Helsinki Declaration and its later amendments. The study was approved by the Faculty of Human and Health Sciences Research Ethics Committee of the University of Cape Town.

Informed consent Informed consent was obtained from all individual participants included in the study.

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