Innovations in Urology

Investig Clin Urol 2020;61:310-315.
https://doi.org/10.4111/icu.2020.61.3.310
pISSN 2466-0493 • eISSN 2466-054X

INVESTIGATIVE AND CLINICAL UROLOGY
ICUROLOGY

Effect of bisphosphonates on the crystallization of stone-forming salts in synthetic urine

Larisa Kovacevic[1], Hong Lu[1], Natalija Kovacevic[1,2], Yegappan Lakshmanan[1]

[1]Department of Pediatric Urology, Children's Hospital of Michigan, Detroit, MI, [2]Vattikuti Urology Institute, Henry Ford Hospital, Detroit, MI, USA

Purpose: We investigated the inhibitory effect of bisphosphonates (BPs) on the crystallization of calcium oxalate monohydrate (COM), calcium phosphate (CaP), and magnesium ammonium phosphate (MAP) in synthetic urine, aiming to see 1) which specific BPs work best on a particular type of crystal and 2) what is the lowest concentration of BPs that inhibits crystal formation.

Materials and Methods: Crystals from synthetic urine were exposed to different concentrations of BPs. Urinary turbidity was used as a marker of crystallization and was measured by spectrophotometry by use of a validated method in our laboratory. The percent inhibitory activity (IA) was calculated by using the formula: $(a\text{-}b)/a\times100$, where a is baseline maximal turbidity and b is maximal turbidity with various concentrations of medication. Potassium citrate and magnesium citrate were used as positive controls.

Results: At the lowest dose of 0.001 mg/mL, risedronate induced the highest IA of 37% on CaP, whereas ibandronate had the strongest IA on COM (24%). To initiate the inhibition of MAP crystallization, risedronate required a two-fold higher concentration (0.002 mg/mL) to reach 30% IA, whereas etidronate required a four-fold higher concentration (0.004 mg/mL) to reach 42% IA.

Conclusions: BPs are good inhibitors of crystallization in synthetic urine, with risedronate and ibandronate being the most potent. At a low clinically acceptable dose, their highest inhibitory action was on CaP and COM crystals. Higher doses were needed to prevent MAP crystallization. Further investigation of the use of BPs in kidney stone prevention is warranted.

Keywords: Crystallization; Urine; Urolithiasis

INTRODUCTION

Bisphosphonates (BPs) are synthetic analogues of pyrophosphate. Their action on osteoclasts leads to inhibition of bone resorption [1]. Therefore, BPs have been used in the treatment of various diseases including resistant hypercalcemia, metabolic bone diseases, and osteoporosis [2]. The inhibitory effect of pyrophosphate on the precipitation of calcium phosphate (CaP) was initially demonstrated by Fleisch et al. in 1968 [3]. Since then, other investigators have shown that BPs inhibit urine crystallization of calcium oxalate (CaOx) and CaP by forming soluble aggregates with calcium for which they have high affinity [4]. BPs have been shown to work on all three phases of crystallization: nucleation, growth, and aggregation of CaOx and CaP crystals [4]. They also act on calcium ion transport at the cell membranes [5] through GTP-binding protein. Therefore, it has been hypothesized that BPs may be useful in the prevention of kidney stones.

We previously validated a simple and inexpensive *in vitro* crystallization assay for measuring turbidity by spectrophotometry in synthetic urine [6]. Using this method we

Received: 14 November, 2019 • **Accepted:** 1 January, 2020
Corresponding Author: Larisa Kovacevic https://orcid.org/0000-0001-7694-7071
Department of Pediatric Urology, Children's Hospital of Michigan, 3901 Beaubien Blvd, Detroit, Michigan 48201, USA
TEL: +1-313-745-5588, FAX: +1-313-993-8738, E-mail: lkovacev@dmc.org

investigated the inhibitory effect of various BPs on the crystallization of calcium oxalate monohydrate (COM), CaP, and magnesium ammonium phosphate (MAP) in synthetic urine. We aimed to see 1) which BPs work best on a particular type of crystal and 2) what is the lowest concentration of BPs that can inhibit crystal formation.

MATERIALS AND METHODS

1. Reagents

All reagents and BPs were obtained from Sigma (St. Louis, MO, USA).

2. Synthetic urine preparation

Synthetic urine was made by using a modified version of the method previously described by Ebisuno et al. [7] and was formulated to contain components present in normal urine. The composition of synthetic urine consisted of (mg/mL) the following: $CaCl_2 \cdot H_2O$ (0.65), $MgCl_2 \cdot H_2O$ (0.65), NaCl (4.6), Na_2SO_4 (2.3), Na_3- citrate $\cdot 2H_2O$ (0.65), Na_2- oxalate (0.02), KH_2PO_4 (2.8), KCl (1.6), NH_4Cl (1.0), urea (25), and creatinine (1.1), with a pH of 5.7. The composition of the synthetic urine was modified depending on the desired type of crystal.

3. The effect of BPs on COM crystallization in synthetic urine

Spectrophotometric measurement of turbidity was used to assess the effect of BPs on COM crystallization in synthetic urine. For this purpose, we used synthetic urine with a high concentration of calcium and without sodium oxalate. Therefore, we added $CaCl_2 \cdot H_2O$ (1.47) to the synthetic urine to reach a final calcium concentration of 10 mmol/L. In 1.5-mL microcentrifuge tubes we mixed 1 mL of synthetic urine and 125 μL of various concentrations of BPs from 0.001 to 2.5 mg/mL of synthetic urine. The solution was incubated at 37℃ for 10 minutes. To induce crystallization, sodium oxalate was added to reach a final concentration of 10 mmol/L. The solution was mixed well and incubated at 37℃ for 10 minutes. The turbidity was measured by spectrophotometry at 660 nm immediately after vortexing.

4. The effect of BPs on CaP crystallization in synthetic urine

Synthetic urine without Na-oxalate from which $MgCl_2$ was removed was used. In 1.5-mL microcentrifuge tubes we mixed 1 mL of synthetic urine and 125 μL of various concentrations (0.001 to 2.5 mg/mL) of BPs. The solution was mixed thoroughly and incubated at 37℃ for 10 minutes. Then 300 IU jack bean urease was added. The solution was mixed well again and incubated at 37℃ for 10 minutes. Turbidity was measured by spectrophotometry at 660 nm immediately after vortexing.

5. The effect of BPs on MAP crystallization in synthetic urine

Synthetic urine without Na-oxalate from which $CaCl_2$ was removed was used. In 1.5-mL microcentrifuge tubes we mixed 1 mL of synthetic urine and 125 μL of various concentrations (0.001 to 2.5 mg/mL) of BPs. The solution was incubated at 37℃ for 10 minutes, and 300 IU jack bean urease was added. The solution was mixed well and incubated at 37℃ for 10 minutes. The turbidity was measured by spectrophotometry at 660 nm immediately after vortexing.

The percent inhibitory activity (IA) was calculated by using the formula: $(a\text{-}b)/a \times 100$, where a is baseline maximal turbidity and b is maximal turbidity with various concentrations of medication.

RESULTS

The range of effective doses of the various BPs that resulted in inhibition of crystallization of COM, CaP, and MAP in synthetic urine (expressed as IA) is presented in Table 1. The lowest dose at which we noticed an inhibitory effect was 0.001 mg/mL for alendronate, risedronate, and ibandronate. At this dose, alendronate had a similar low inhibitory effect on both COM (IA, 8%) and CaP (IA, 10%). At the same dose of 0.001 mg/mL, risedronate showed the highest IA for CaP (37%) and also prevented the crystalliza-

Table 1. Range of effective doses of various bisphosphonates that resulted in inhibition of crystallization of COM, CaP, and MAP in synthetic urine (expressed as IA)

Medication	Range of effective dose (mg/mL)	Type of crystal	Range of IA (%)
Etidronate	0.004–0.3	COM	36–65
	0.021–0.3	CaP	29–68
	0.004–0.3	MAP	42–71
Alendronate	0.001–0.625	COM	8–73
	0.001–0.039	CaP	10–63
	0.039–0.625	MAP	39–94
Risedronate	0.001–2.5	COM	18–67
	0.001–0.625	CaP	37–97
	0.002–2.5	MAP	30–98
Ibandronate	0.0012–1.25	COM	24–77
	0.0012–0.078	CaP	17–69
	0.005–1.25	MAP	11–91

COM, calcium oxalate monohydrate; CaP, calcium phosphate; MAP, magnesium ammonium phosphate; IA, inhibitory activity.

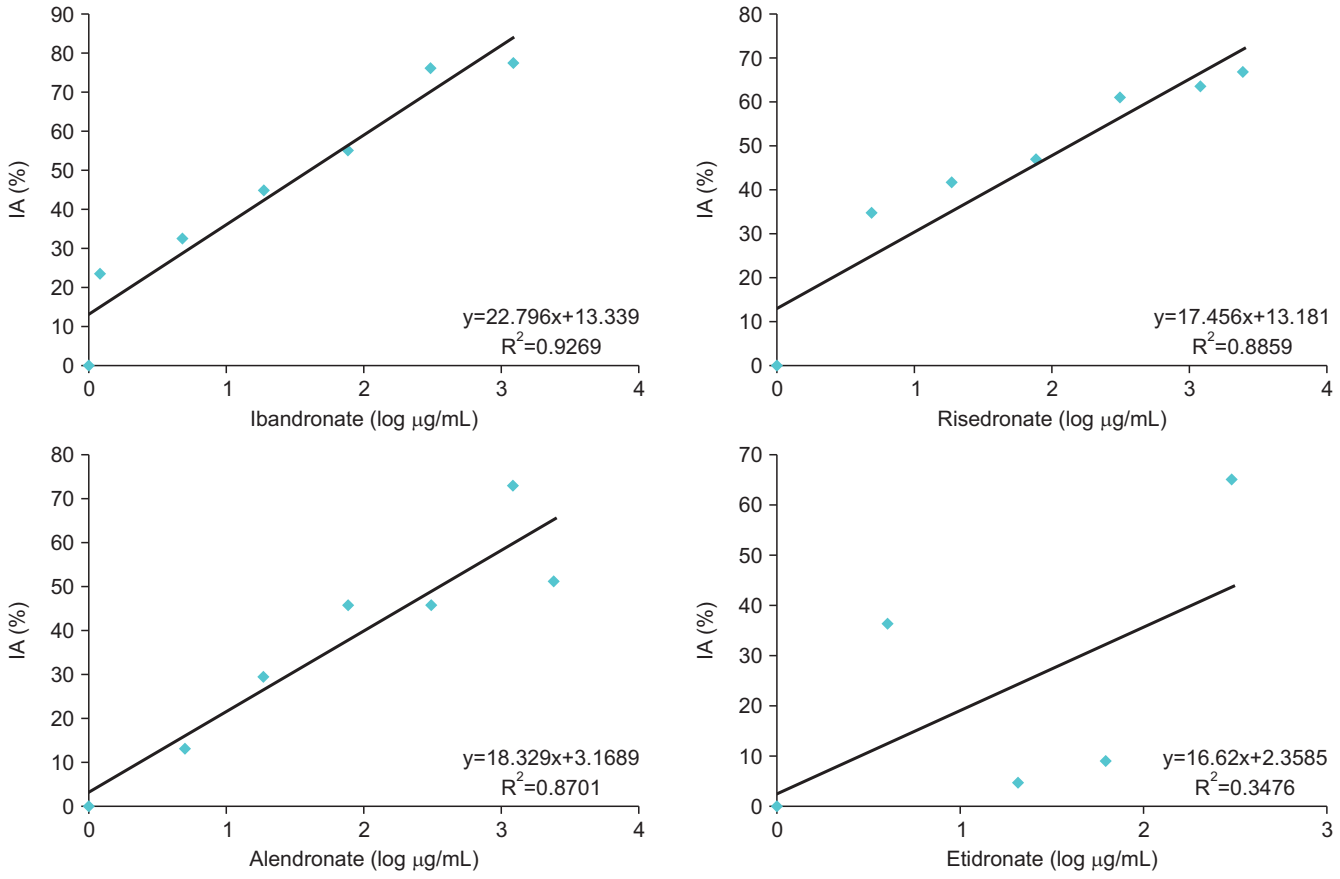

Fig. 1. The inhibitory activity of bisphosphonates on calcium oxalate crystallization in synthetic urine. IA, inhibitory activity.

tion of COM (18%), but needed a higher dose (0.002 mg/mL) for inhibition of MAP (IA, 30%). Ibandronate at the lowest dose of 0.001 mg/mL reduced the formation of COM crystals (IA, 24%) and CaP crystals (IA, 17%), but required a five-fold dose for the inhibition of MAP crystallization (IA, 11%). The initial inhibitory effect of etidronate was noticed at 0.004 mg/mL, inducing a similar IA for both COM and MAP (36% and 42%, respectively).

The dose-dependent effect of the BPs on each type of crystal is shown in Figs. 1, 2, and 3. With regard to the action of the BPs on a specific type of crystal, ibandronate had the strongest IA on COM (24%), whereas risedronate induced a higher IA of 37% on CaP at the lowest dose of 0.001 mg/mL. To initiate the inhibition of MAP crystallization, risedronate required a two-fold higher concentration (0.002 mg/mL) to reach 30% IA, whereas etidronate required a four-fold higher concentration (0.004 mg/mL) to reach 42% IA.

DISCUSSION

The incidence of urolithiasis is increasing [8] and the current treatment is unsatisfactory, leading to high morbidity and an increased risk of reoccurrence [9,10]. Increasing evidence suggests that stone disease has multi-systemic involvement [11,12] and that vascular theory plays a major role in stone formation [13]. Therefore, new treatment strategies are imperative, especially in patients with severe disease presenting with kidney stone, osteoporosis, and arterial calcifications. Since all these conditions are affected by BPs through their action on calcium transport in cell membranes and on osteoclasts, BPs seem worthy of investigation.

To our knowledge, we are the first to report a comparison of the inhibitory effect of various BPs on the crystallization of three different salts: COM, CaP, and MAP. Using spectrophotometric measurement of urinary turbidity, a method we established in our laboratory, we found that BPs are good inhibitors of crystallization in synthetic urine [6]. Overall, BPs showed the best inhibitory effect on CaP and COM crystallization at clinically acceptable low doses. Higher doses of BPs were needed to prevent MAP crystallization. The difference in the IA of BPs on these three types of crystals is likely due to their high affinity for calcium, although our experiment was not designed to study the exact pathway of action of the BPs. Of all the BPs that we tested, both risedronate and ibandronate showed the strongest IA at the same low dose. This is explained by their high potency

https://doi.org/10.4111/icu.2020.61.3.310

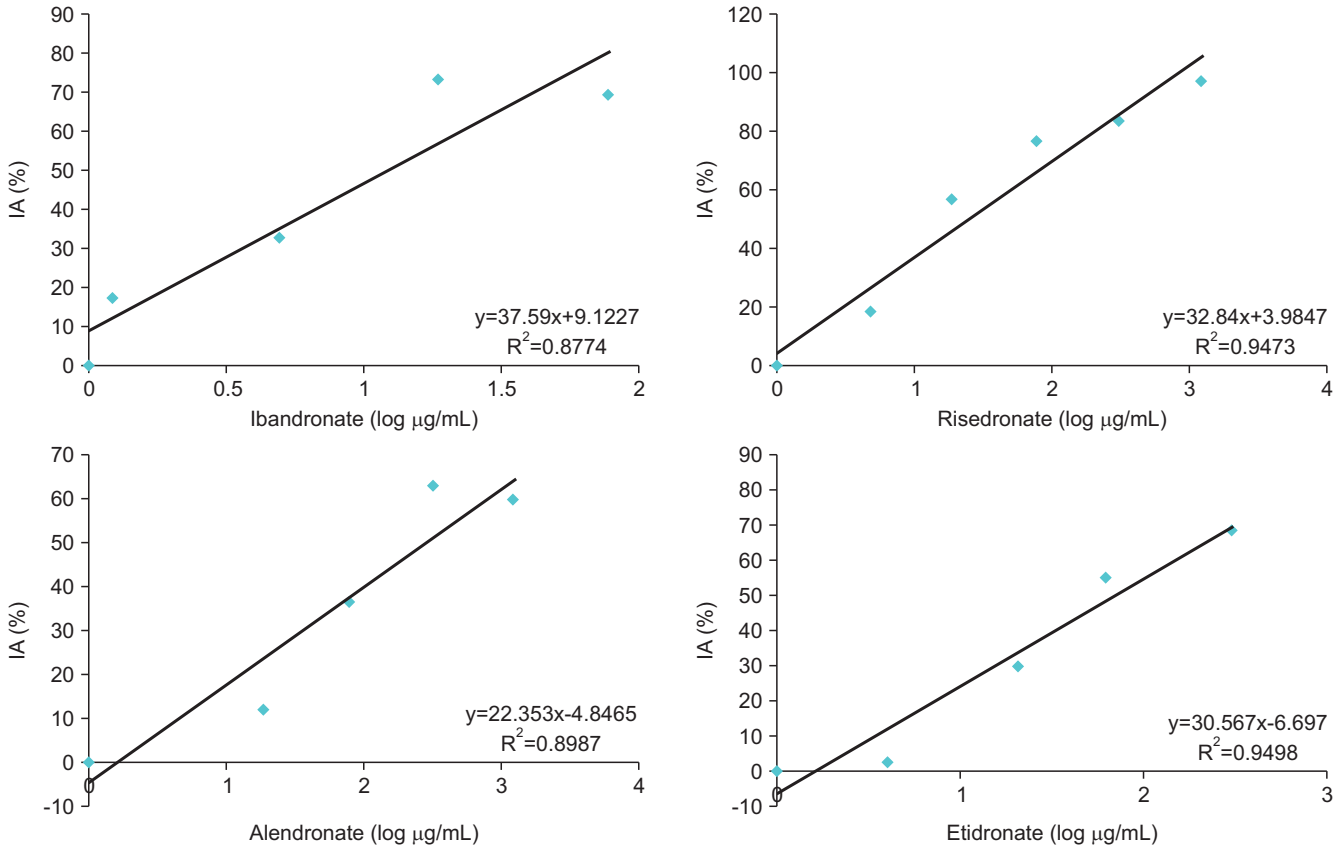

Fig. 2. The inhibitory activity of bisphosphonates on calcium phosphate crystallization in synthetic urine. IA, inhibitory activity.

compared with the other BPs that we investigated. Higher IA was seen with higher concentrations of BPs. However, this has poor clinical significance since these concentrations cannot be safely reached in human urine.

The effect of BPs on stone formation has been reported in cell cultures [14] and in animal models [15]. Senzaki et al. [14] showed that alendronate inhibits CaP microlith formation in cell cultures. BPs decrease urinary calcium excretion in animal models [15]. Kawamura et al. [16] showed that etidronate can suppress the formation of CaOx renal stones induced by synthetic vitamin D and ethylene glycol in rats. They speculated that etidronate may inhibit stone formation by affecting the nidus formation, aggregation, and crystal growth of CaOx [16]. The main advantage of our work compared with the research mentioned above is that rather than looking at a single drug and a single crystal we studied the inhibitory effect of various BPs on all three types of crystals. This allowed a comparison between various BPs and different crystals.

There are only a few reports in the literature describing the role of BPs in patients with kidney stone. Small case reports have shown that etidronate [17], alendronate [18,19], and pamidronate [20] prevent hypercalciuria and reduce the risk of forming calcium stones [21,22]. In addition, treatment with these two drugs leads to normalization of markers of bone resorption [20]. Due to their strong inhibitory effect on bone resorption, BPs are currently indicated in patients with low bone density (osteopenia or osteoporosis), hypercalcemia, hypercalciuria, and urolithiasis [2,18,20]. BPs have been shown to prevent kidney stone formation after prolonged bed rest in adult patients [23]. Their use in pediatrics is restricted to recurrent extremity fractures and reduced bone mass [24]. The drugs are well tolerated and have few adverse effects [24]. However, animal studies have reported teratogenic effects at very high doses of BPs not used in clinical practice [25].

The advantages of our study are 1) the well-controlled environment (synthetic urine), 2) the simplicity and practicability of the method, and 3) the ability to study any type of crystal. The major study limitation is the difficulty of converting the BP doses we tested to clinically applicable doses. However, considering the pharmacokinetics of the BPs and taking into account average urinary output, we can conclude that the lowest tested doses are at an acceptable clinical level. Another study limitation is the inability to study the adverse effects of BPs in order to balance dose efficiency

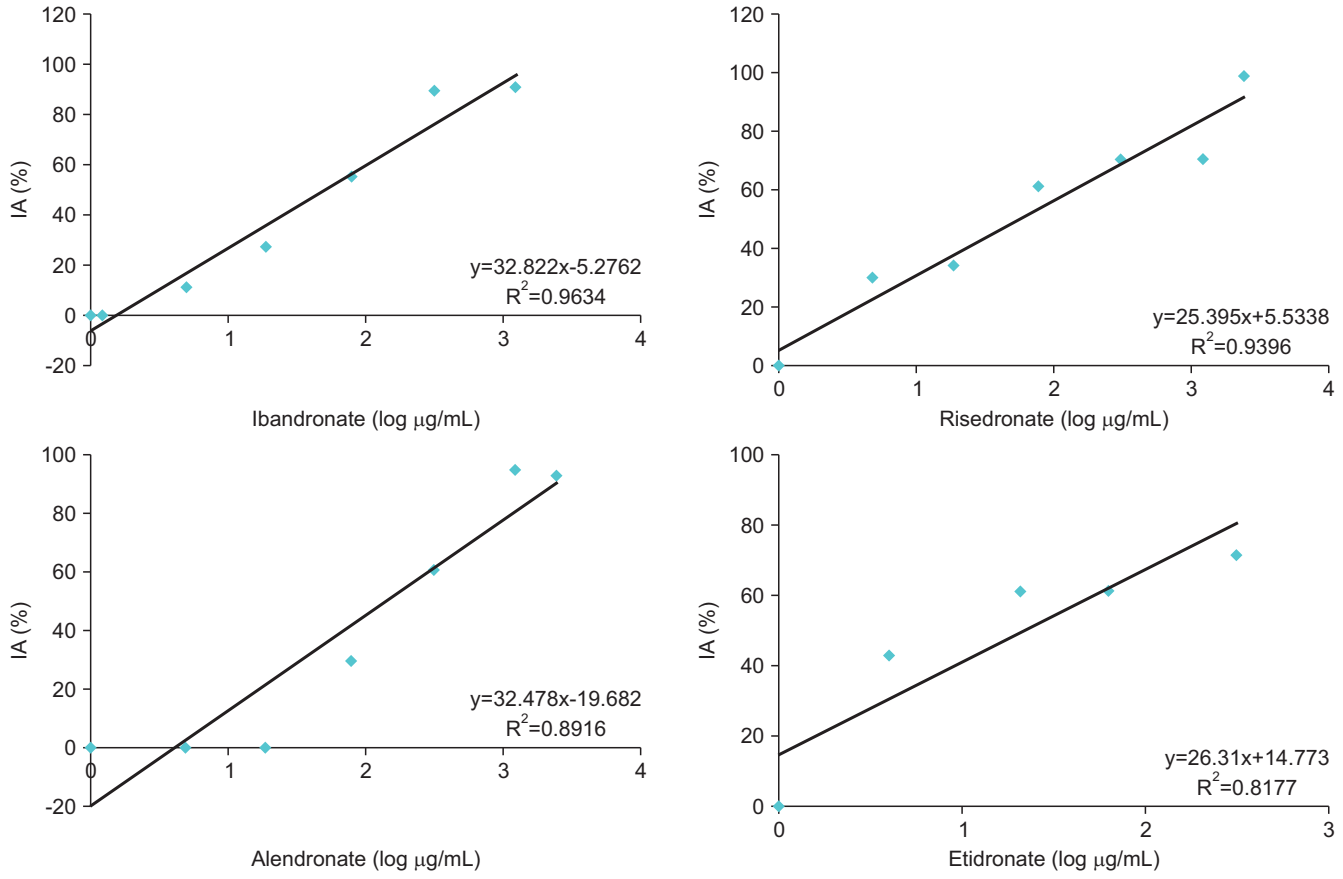

Fig. 3. The inhibitory activity of bisphosphonates on magnesium ammonium phosphate crystallization in synthetic urine. IA, inhibitory activity.

and potential harm.

CONCLUSIONS

BPs are good inhibitors of crystallization in synthetic urine, with risedronate and ibandronate being the most potent agents. At a low clinically acceptable dose, the highest inhibitory action was on CaP and COM crystals, whereas higher doses were requited to prevent MAP crystallization. Further investigation of the use of BPs in kidney stone prevention is warranted.

CONFLICTS OF INTEREST

The authors have nothing to disclose.

AUTHORS' CONTRIBUTIONS

Research conception and design: Larisa Kovacevic and Hong Lu. Data acquisition: Larisa Kovacevic and Hong Lu. Data analysis and interpretation: Larisa Kovacevic, Hong Lu, and Natalija Kovacevic. Statistical analysis: Larisa Kovacevic and Hong Lu. Drafting of the manuscript: Larisa Kovacevic and Natalija Kovacevic. Critical revision of the manuscript: Larisa Kovacevic, Hong Lu, Natalija Kovacevic, and Yegappan Lakshmanan. Supervision: Larisa Kovacevic and Yegappan Lakshmanan. Approval of the final manuscript: Larisa Kovacevic, Hong Lu, Natalija Kovacevic, and Yegappan Lakshmanan.

REFERENCES

1. Russell RG, Watts NB, Ebetino FH, Rogers MJ. Mechanisms of action of bisphosphonates: similarities and differences and their potential influence on clinical efficacy. Osteoporos Int 2008;19:733-59.
2. Fleisch H. Bisphosphonates. Pharmacology and use in the treatment of tumour-induced hypercalcaemic and metastatic bone disease. Drugs 1991;42:919-44.
3. Fleisch H, Russell RG, Bisaz S, Mühlbauer R. [Influence of diphosphonates on the deposition and dissolution ofccalcium phosphate in vitro and in vivo]. Helv Physiol Pharmacol Acta 1968;26:CR345-6. German.
4. Wolf JS Jr, Stoller ML. Inhibition of calculi fragment growth by metal-bisphosphonate complexes demonstrated with a new assay measuring the surface activity of urolithiasis inhibitors. J

https://doi.org/10.4111/icu.2020.61.3.310

Urol 1994;152(5 Pt 1):1609-14.
5. Guilland DF, Fleisch H. The effect of in vivo treatment with EHDP and/or 1,25-DHCC on calcium uptake and release in isolated kidney mitochondria. Biochem Biophys Res Commun 1974;61:906-11.
6. Kovacevic L, Lu H, Lakshmanan Y. Urinary turbidity as a marker of crystallization: is spectrophotometric assessment useful? Int Urol Nephrol 2013;45:1009-15.
7. Ebisuno S, Kohjimoto Y, Nishikawa T, Nishihata M, Inagaki T, Komura T, et al. Effects of etidronate disodium on crystallizations in synthetic urine and calcium oxalate crystal adhesion to Madin-Darby canine kidney (MDCK) cells. Int J Urol 1998;5:582-7.
8. Bowen DK, Tasian GE. Pediatric stone disease. Urol Clin North Am 2018;45:539-50.
9. Scoffone CM, Cracco CM. Pediatric calculi: cause, prevention and medical management. Curr Opin Urol 2018;28:428-32.
10. Vaughan LE, Enders FT, Lieske JC, Pais VM, Rivera ME, Mehta RA, et al. Predictors of symptomatic kidney stone recurrence after the first and subsequent episodes. Mayo Clin Proc 2019;94:202-10.
11. Kovacevic L, Lu H, Caruso JA, Kovacevic N, Lakshmanan Y. Urinary proteomics reveals association between pediatric nephrolithiasis and cardiovascular disease. Int Urol Nephrol 2018;50:1949-54.
12. Sakhaee K, Maalouf NM, Sinnott B. Clinical review. Kidney stones 2012: pathogenesis, diagnosis, and management. J Clin Endocrinol Metab 2012;97:1847-60.
13. Gambaro G, Ferraro PM, Capasso G. Calcium nephrolithiasis, metabolic syndrome and the cardiovascular risk. Nephrol Dial Transplant 2012;27:3008-10.
14. Senzaki H, Yasui T, Okada A, Ito Y, Tozawa K, Kohri K. Alendronate inhibits urinary calcium microlith formation in a three-dimensional culture model. Urol Res 2004;32:223-8.
15. Bushinsky DA, Neumann KJ, Asplin J, Krieger NS. Alendronate decreases urine calcium and supersaturation in genetic hypercalciuric rats. Kidney Int 1999;55:234-43.
16. Kawamura J, Nonomura M, Okada Y, Yoshida O, Takashima M, Itokawa Y. [Effect of etidronate disodium (EHDP) on calcium oxalate renal stones induced by synthetic 1 alpha(OH) vitamin D3 and ethylene glycol in rats]. Hinyokika Kiyo 1985;31:749-62. Japanese.
17. Heilberg IP, Martini LA, Teixeira SH, Szejnfeld VL, Carvalho AB, Lobão R, et al. Effect of etidronate treatment on bone mass of male nephrolithiasis patients with idiopathic hypercalciuria and osteopenia. Nephron 1998;79:430-7.
18. Giusti A, Barone A, Pioli G, Girasole G, Siccardi V, Palummeri E, et al. Alendronate and indapamide alone or in combination in the management of hypercalciuria associated with osteoporosis: a randomized controlled trial of two drugs and three treatments. Nephrol Dial Transplant 2009;24:1472-7.
19. Weisinger JR, Alonzo E, Machado C, Carlini R, Martinis R, Paz-Martínez V, et al. [Role of bones in the physiopathology of idiopathic hypercalciuria: effect of amino-bisphosphonate alendronate]. Medicina (B Aires) 1997;57 Suppl 1:45-8.
20. Arrabal-Polo MA, Arias-Santiago S, de Haro-Muñoz T, Lopez-Ruiz A, Orgaz-Molina J, Gonzalez-Torres S, et al. Effects of aminobisphosphonates and thiazides in patients with osteopenia/osteoporosis, hypercalciuria, and recurring renal calcium lithiasis. Urology 2013;81:731-7.
21. Yasui T, Itoh Y, Okada A, Hamamoto S, Hirose M, Kobayashi T, et al. Alendronate reduces the excretion of risk factors for calcium phosphate stone formation in postmenopausal women with osteoporosis. Urol Int 2009;83:226-9.
22. Watanabe Y, Ohshima H, Mizuno K, Sekiguchi C, Fukunaga M, Kohri K, et al. Intravenous pamidronate prevents femoral bone loss and renal stone formation during 90-day bed rest. J Bone Miner Res 2004;19:1771-8.
23. Prochaska M, Taylor E, Vaidya A, Curhan G. Low bone density and bisphosphonate use and the risk of kidney stones. Clin J Am Soc Nephrol 2017;12:1284-90.
24. Okada A, Ohshima H, Itoh Y, Yasui T, Tozawa K, Kohri K. Risk of renal stone formation induced by long-term bed rest could be decreased by premedication with bisphosphonate and increased by resistive exercise. Int J Urol 2008;15:630-5.
25. Srivastava T, Alon US. The role of bisphosphonates in diseases of childhood. Eur J Pediatr 2003;162:735-51.