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Influence of Caffeine on Crystallization and Amelioration of Oxidative Stress on *in vitro* Model of Urolithiasis

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ABSTRACT

Urolithiasis is a disease characterized by formation of solid crystals within the urinary tract. Kidney stone formation is still not clear but it is mostly composed of calcium oxalate which can produce free radicals that are toxic to renal tubular cells. Oxidative stress is an important contributory mechanism in cell damage and is associated with a number of disorders. Several studies have shown antioxidative effects of caffeine, proposing its possible role in stopping the formation of calcium oxalate stones in urinary tract. Hence, the aim of this study was to evaluate the toxic effects of calcium oxalate monohydrate crystals (COM) on renal epithelial cell line; Madin-Darby canine kidney cells subtype I (MDCK I) and Epithelial-like pig kidney cell line (LLC-PK1), and to determine possible inhibition of COM that caused oxidative stress by antioxidant treatment with caffeine in different concentrations in a cell culture model of urolithiasis.

Key words: *urolithiasis, oxidative stress, MDCK cells, LLC-PK1 cells, calcium oxalate monohydrate, caffeine*

Introduction

Urolithiasis, defined as the formation of urinary calculi, represents one of the oldest known diseases, and nowadays it is the most common disease of the urinary tract (UT). Although globally occurrence and recurrence rates of kidney stones are constantly increasing, incidence can vary between different regions due to numerous specific risk factors[1,2–6]. Another public health problem associated with urolithiasis is a significant increase of its occurrence in the pediatric population[7–9]. Spivacow et al. showed an important role of metabolic abnormalities in children who suffer from urolithiasis[10]. The most common biochemical abnormality in their research was idiopathic hypercalciuria, and the second most common hypocitraturia, followed by hyperoxaluria[10]. Risk factors associated with increased urolithiasis incidence in both adult and pediatric population are: increased sodium intake, decreased water and calcium intake, increased use of antimicrobials, poor nutrition, sedentary lifestyle, and various pharmacological therapies (e.g. diuretics, anticonvulsants, corticosteroids), metabolic syndrome (decreased citrate excretion, increased urinary calcium and oxalate excretion), along with global warming and higher environmental temperature[10–12]. Calcium stones, calcium oxalate and calcium phosphate are predominant kidney stones accounting for 80% of all urinary calculi, and their rate is higher than other types of kidney stones[2]. Other types of kidney stones are: cystine stones, uric acid stones, struvite or magnesium ammonium phosphate stones, and drug-induced stones. Pathophysiological pathway in kidney stones formation consists of several events one leading to another: urinary supersaturation (e.g. hyperoxaluria), oxidative stress (OS), cell injury and rupture of cell membrane, nucleation, crystal growth, crystal aggregation, crystal-cell interaction (considered as the crucial step in the formation of stone), crystal retention and, finally, stone formation[2].

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Due to the above described pathophysiological mechanism, the kidney stones formation is considered to be a complex process, especially because of numerous types of kidney stones, each with its specific characteristics. Nevertheless, four most common models of stone formation have been identified: stones forming in inner medullary collecting ductus, stones forming in free solution in the renal collecting systems, formation of stones by attachment to the renal papilla surface at sites of Randall's plaque, and formation of stones by fixation to the plugs protruding from the opening of the Bellini ducts[13]. Oxidative stress, induced by calcium oxalate crystals, plays a significant role in calcium oxalate stone formation. Reactive oxygen species (ROS) are produced during interactions between renal cells and crystals, causing injury in renal cells[14,15]. This injury promotes sites for the crystal nucleation, aggregation, retention and stone development and represents a risk factor for the crystallization and crystals deposition in the kidney[15]. Accordingly, numerous studies showed a protective role of antioxidants catechin, vitamin E, L-arginine and selenium, against crystal and oxalate deposition-induced oxidative injury[1,15].

Caffeine, also known as 1,3,7-trimethylxanthine, C8H10N4O2, is one of the most commonly consumed active compounds worldwide. The main sources of dietary caffeine are coffee, soft drinks, tea, caffeinated beverages and chocolate products[16]. It exhibits pharmacologic and physiologic impact in renal, cardiovascular and muscular system[17]. Caffeine is a natural alkaloid methylxanthine. It causes toxic or preventive effect in some physiological and pathological conditions, depending on numerous factors, for instance concomitant drug consumption, prior chronic exposure, genetic-enzymatic axes, and dose of caffeine. To date no evidence was found that usual daily dose of caffeine, such as 3 or 4 cups of coffee, increases the risk of nephropathy in healthy people[18]. On the other hand, it is well known that frequent caffeine consumption may enhance mood, cognitive function and alertness. The main mechanism of action of caffeine includes activation of ryanodine receptors and inhibition of phosphodiesterase enzyme and adenosine receptors, causing specific effects on organs[19,20]. It has been shown that consuming caffeinated beverages reduces the risk of developing kidney stone disease. On the other hand, its protective role and mechanisms behind it are not fully elucidated and remain unknown[21]. The aim of this study was to evaluate the effects of caffeine on formation of crystals and its antioxidative effects in an *in vitro* model of urolithiasis.

Materials and Methods

Madine-Darby canine kidney cells subtype I (MDCKI) and LLC-PK1 were sub-cultivated in 10 cm dishes in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic solution at 37 °C in a humidified atmosphere of 5% CO_2 in air. For the experiments of CaOX toxicity cells were cultivated in DMEM with antibiotic/antimycotic solution but without FBS. These cells were used as an *in vitro* model of collector (MDCKI) and proximal (LLC-PK1) tubules. These models have previously been established and are widely used[1].

To assess crystallization of CaOX crystals and protective effects of caffeine on it, cells were sub-cultivated in a 6 well dish to 90% of confluency. CaOX crystals were prepared according to previously established protocol by Peerapen at al.[20] After achieving confluency, CaOX crystals were added at a concentration of 8mM and caffeine in different concentrations (0,1, 1 and 10 mM). The cells were observed under light microscopy and crystals were counted in three fields of view after one, three and twenty-four hours with an inverse light microscope. Counting was done in triplicates and the results are shown as an average with error bars.

To determine total glutathione (GSH), cells were treated according to the previously described protocol, and after 24 hours of treatment with CaOX crystals and caffeine, cells were detached from the surface of the dish using rubber cell scraper. From these cells GSH was determined using a commercial kit according to manufacturer's protocol (Gluthatione Assay Kit, Sigma-Aldrich, Saint Louis, MO, USA). Three controls were used; cells treated only with caffeine, cells treated only with CaOX crystals and untreated cells. Results are shown as a percentage according to untreated cells.

Results

To show the effects of caffeine on formation of CaOx crystals in MDCKI and LLC-PK1 cell lines cells were cultivated to 90% confluency. Furthermore, CaOx and caffeine were added and formed crystals were counted after 1, 3 and 24 hours, according to previously described protocol. Results are graphically shown in Figure 1, as number of crystals counted in three fields of view. As a control, cells treated only with 8 mM CaOx crystals were used, and they showed a decreasing number of crystals in later counting (3 and 24 hours) compared to number of crystals after 1 hour, although a highest decrease of crystals was observed in MDCKI cell line after 24 hours.

Comparing the number of crystals in MDCKI cell line concentrations of 0.1 mM and 1 mM of caffeine after 1 hour showed no significant change of crystal numbers compared to control. Significantly less crystals were present at all time points in cells treated with higher concentration of caffeine (10 mM), and concentration of 1 mM caffeine showed less crystals after 3 and 24 hours compared to the control. In LLC-PK1 cells treated with 1- and 10-mM caffeine significantly less crystals were measured at all time points but in the cells treated with lowest concentration of caffeine (0.1 mM) no significant difference in crystal numbers was observed (Figure 1). To evaluate redox tone in these cell lines GSH levels were measured in both cell lines after treatment of cells with CaOx crystals and different concentrations of caffeine. Three controls were used, untreated cells, cells treated only with CaOx and cells treated only with caffeine. There was no signifi-

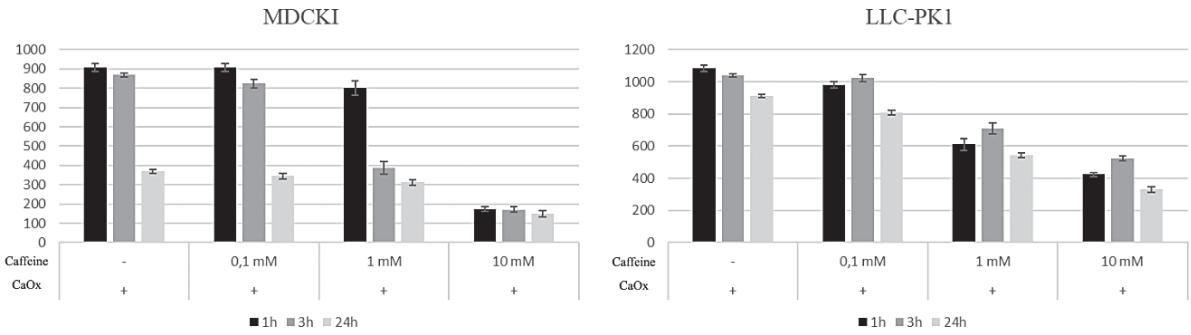

Fig. 1. Number of formed crystals in two cell lines, MDCKI and LLC-PK1. Crystals were counted 1, 3 and 24 hours, after addition of 8 mM CaOX and different concentrations of caffeine. + indicates addition of CaOx, - indicates no caffeine was added, 0,1, 1 and 10 mM indicates amount of caffeine added.

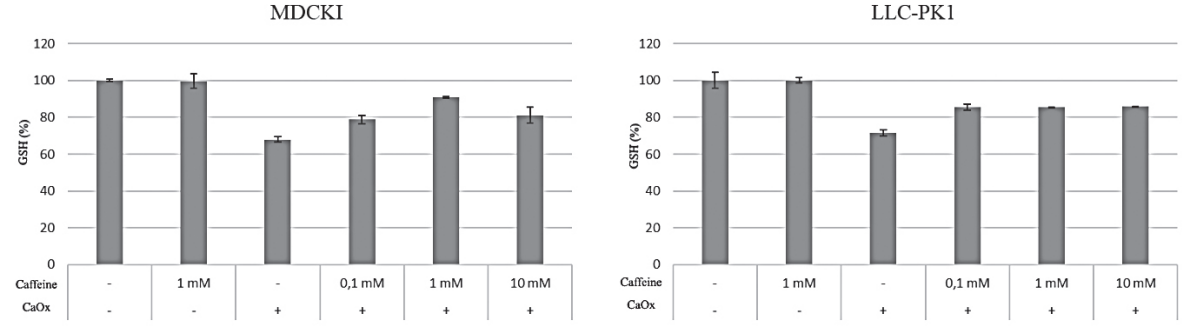

Fig. 2. Effects of caffeine on GSH levels in two cell lines, MDCKI and LLC-PK1. + indicates addition of CaOx or caffeine, - indicates no caffeine or CaOX was added, 0,1, 1 and 10 mM indicates amount of caffeine added.

cant difference between cells treated only with caffeine to untreated cells, but cells treated only with CaOx showed a significant decrease of GSH level compared to untreated cells. In cells treated with caffeine and CaOx levels of GSH were higher than in cells treated only with CaOx. There was no significant difference of GSH levels between LLC-PK1 cells treated with different concentrations of caffeine and CaOx, but in MDCKI cells concentration of 1 mM caffeine showed higher levels of GSH than in cells treated with other concentrations of caffeine and CaOx (Figure 2).

Discussion

Urolithiasis is a multifactorial disease and the most important factor is the supersaturation of urine with salts, including oxalates, which cause damage to epithelial cells[22]. The most frequent form of kidney stone is calcium oxalate which can produce free radicals and its development is a complex process of a few physicochemical events that include: nucleation, expansion, aggregation and supersaturation[2]. Some studies have shown that caffeine induced inhibitory effect on fluid reabsorption along proximal renal tubules causes diuresis and natriuresis[23]. Its effects have been studied in several kidney diseases, but its role in kidney stone disease seems to be insufficiently elucidated[24]. Occurrence of kidney stones may be decreased by certain types of fluids used to increase urine volume[25]. But, caffeine-rich beverages such as regular tea and coffee have been recognized as main sources of oxalate. Moreover, they may increase the urinary excretion of calcium, sodium, magnesium and chloride[17]. According to Massey's research oral doses of coffee and tea can increase urinary calcium excretion[26] while a study by Itoh et al. examined that green tea treatment reduced the urinary oxalate excretion and calcium oxalate deposit formation[27]. After consummation, caffeine is rapidly absorbed from gastrointestinal tract and further distributed to kidneys and numerous other tissues[20,24]. A recent *in vitro* study has demonstrated a significant reduction in the adhesion of calcium oxalate (CaOx) crystals on renal tubular epithelial cells treated with caffeine[20]. Previous retrospective and prospective studies have shown that caffeine has a diuretic effect, i.e. that it increases the excretion of calcium from the urine. After all, three large cohort studies also suggested a preventive role for caffeine in kidney stone disease; a health care study, a health care study of nurses (NHS I), and another group of nurses (NHS II) with a total of more than 200,000 participants. The conclusion of these studies was that the consumption of caffeinated beverages was associated with a lower risk (26–31%) of kidney stone disease[28]. A study by Massey et al. that examined the acute effect of caffeine on urine composition and the risk of developing kidney stones formed from calcium

oxalate confirmed that caffeine consumption may reduce the risk of calcium oxalate stones[29,30].

In the current study, caffeine was shown to have a beneficial effect in reducing the level of oxidative cell damage compared to cells in which there was no caffeine treatment by the level of total glutathione concentration. The expression of total glutathione (tGSH) was increased in samples of cells (MDCKI and LLCPK1) treated with caffeine in proportion to its concentration, thus indicating the existing positive antioxidant activity due to caffeine administration. The highest concentration of caffeine had the greatest protective effect in the MDCKI and LLCPK1 cells model. The protective effects of caffeine on kidney stone formation of COM were evaluated and the results showed that caffeine decreased the number of crystals. In LLC-PK1 cell line a smaller number of crystals depending on time exposure for the same concentrations of caffeine in comparison to MDCKI was observed. In our study a proportional decrease in the number of calcium oxalate crystals was recognized with an increase in the added concentration of caffeine as also shown by Ferraro et al.[19] where a significant association was found between caffeine intake and the risk of developing kidney stones. In the study by Peerapen et al. it has been shown that caffeine did not affect crystal growth and cristallization of calcium oxalate monohidrate (COM), but it has significant role in reducing intracellular calcium storage by increasing calcium-mediated mechanisms of secretion and excretion[20]. Such as annexin A1 translocation from apical membrane into cytoplasm. This causes decreased COM crystal-binding capacity of renal tubular epithelial cells. Therefore, it is considered protective mechanism of caffeine against COM kidney stones formation[17]. Dietary agents play an important role in the formation of urinary calculus, and a change in diet can reduce the risk of stone recurrence. Therefore, new strategies with therapeutic applications of antioxidants to decrease stone recurrence after Shockwawe lithotripsy (SWL) and Ureterorenoscopy (URS) should be considered.

Conclusion

The obtained results suggest that caffeine can lower crystallization causing a significant reduction in the number of crystalline calcium oxalate and has a positive effect by lowering oxidative stress in our *in vitro* models of urolithiasis. It would be interesting for future research to measure caffeine effect on crystal size. More studies are needed to further evaluate the role of caffeine in the formation of kidney stones.

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UTJECAJ KOFEINA NA KRISTALIZACIJU I SMANJENJE OKSIDATIVNOGA STRESA U *IN VITRO* MODELU UROLITIJAZE

S A Ž E T A K

Urolitijaza je bolest koju karakterizira stvaranje čvrstih kristala unutar mokraćnoga sustava. Mehanizam stvaranja bubrežnih kamenaca još je uvijek nejasan, a uglavnom se sastoji od kalcijevoga oksalata koji može proizvesti slobodne radikale toksične za bubrežne tubularne stanice. Oksidativni stres važan je mehanizam koji doprinosi oštećenju stanica i povezan je s nizom poremećaja. Nekoliko je studija pokazalo antioksidativne učinke kofeina, sugerirajući njegovu moguću ulogu u zaustavljanju stvaranja kalcijevih oksalatnih kamenaca u mokraćnim putovima. Stoga je cilj ovoga istraživanja bio procijeniti toksični učinak kristala kalcijevog oksalata monohidrata (COM) na staničnu liniju bubrežnoga epitela, Madin-Darbyjeve pseće stanice bubrega podtipa I (MDCK I) i epitelne stanice bubrega svinje (LLC-PK1), te utvrditi moguće inhibicije COM-a (uzroka oksidativnoga stresa) antioksidativnim tretmanom kofeinom u različitim koncentracijama na modelu stanične kulture urolitijaze.