EFFECT OF CALCUSOL™ TO REDUCE THE CALCIUM CRYSTAL RETENTION IN KIDNEY EPITHELIAL CELLS MODEL OF NEPHROLITHIASIS

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ABSTRACT

Kidney stones is a disease that characterized by a disturbance in the bladder. The main constituent of kidney stones namely Calcium Oxalate Monohydrate (COM) crystals. The presence of a COM crystal adhesion to renal tubular cells, will initiate the internalization which will further lead to the formation of crystals retention in the kidney. In Indonesia, there are many herbal products are considered able to cope the complaints due to the kidney stone disease. One of the herbal product is Calcusol™, which is the main constituent of those herbal product was the leaf extract of tempuyung. This study observed the effectiveness of Calcusol™ in reducing crystals retention that was formed in kidney epithelial cells model of nephrolithiasis. The result showed that Calcusol™ is able to reduce the average number of calcium crystals retention in the renal epithelial cells. It indicate that Calcusol™ has the ability to reduce crystals retention that already formed in renal epithelial cells. Furthermore, the results of this study are expected to be one of the considerations for further research on the potential of overcoming Calcusol™ in kidney stone disease

Keywords *: Calcusol™, COM, kidney stones, tempuyung.*

INTRODUCTION

Kidney stones are one of the urologic disorders that have plagued mankind for centuries. Kidney stone itself is a disease that can be caused by several factors such as epidemiological, biochemical metabolism in the body and also genetic factors (Abbagani et al., 2010). According to Sheng et al. (2004), a major constituent of kidney stones is Calcium Oxalate Monohydrate (COM). COM microcrystals aggregation and attachment to renal epithelial cells is expected because of the COM crystal adhesion to surfaces that mediated by anionic molecules. The binding and internalization of crystals of calcium oxalate monohydrate (COM) by renal tubular epithelial cells may be an important step in the formation of kidney stones. Studies conducted by Lieske et al. (2000) provide new evidence that the binding of COM crystals in kidney cells is regulated by physiological signals. In addition, according to Campos and Schor (2000), binding of COM crystals was also influenced by the length of time of exposure and the concentration of a COM that was given.

Kidney stone disease could be addressed in several ways, including through surgery to remove the stone that clog the kidneys, or a strict diet to reduce the stones. Also, it can also be done by using the drug therapy, including thiazide diuretics, allopurinol, and potassium citrate (Heilberg & Nestor, 2006). In Indonesia, there are many herbal product that expected could reduce the stones that formed in the kidney. One of these product called Calcusol™ with tempuyung extract as a major constituent. Research that was conducted by Sardjito & Ismadi (1964), suggests that the active substance in the tempuyung leaf (*Sonchus arvensis* L.) extracts that was contained in Calcusol™ can dissolve 0.28 mg of Ca in CaCO3 within 24 hours. In addition, the Tempuyung extract can also dissolve 0.54 mg Ca that contained in marble powder, and 12.59 mg Ca that contained in CaCO3. This indicates that the extract of Tempuyung has a great solubility of the CaCO3 powder, either in the form of a fine powder or in the form of grain. This suggest that the extract of tempuyung leaf that contained in Calcusol™ can reduce crystal deposition in the kidney. In addition, Calcusol ™ is widely used traditionally to lower and reduce complaints because of kidney stone disease. However, it still need scientific base to know the effect of Calcusol™ in reducing calcium crystal retention that was occurs in the kidney in patients with kidney stones disease.

MATERIALS AND METHOD

Ethical Clearance

All procedures were performed has been accepted by the Research Ethics Committee of the UB with the number 127-KEP-UB.

Medium Preparation

For 100 ml of medium, the amount of material used is DMEM F12 HAM 1.56 g, which is coupled with sodium bicarbonate as much as 0.12 g. The media made in the laminar air flow to minimize contaminants, and then mixed the medium materials in the order that was already mentioned, and then diluted with DI (Di-ionized water) to ¾ volume and adjust pH at 7.2 and then added DI until one volume. Then added 2 % Penicillin-Streptomycin and 10 % FBS (Fetal Bovine Serum). Medium then sterilized using Millipore membranes with a 0.20 µm diameter pore. After that, the medium divided into several treatments,the

negative control (K-) which is a medium without any additions, medium 1 to medium 5 (M1 - M5) is a medium with the addition Calcusol™ with a dose of one to five with a predetermined dose.

Cell Culture Models of Nephrolithiasis

This experiment use primer epithelial cell culture that isolated from mice kidney. Mice (*Mus musculus*), which has acclimated for one week then killed by neck dislocation. Subsequently, the mice were dissected and then Kidney organs was isolated. The kidneys were then perfused using sterile PBS with 2 % penicillin-streptomycin 10x addition, then the kidneys were washed with PBS with 2 % penicillin-streptomycin 10x addition. Furthermore, the kidney squeezed using the base of sterile syringe at a petri dish containing sterile PBS which had been added 2 % penicillin-streptomycin 10x. Then, homogenates transferred into polypropylene tubes. Centrifugation was performed at room temperature at 1000 rpm for 10 minutes twice. Pellets were then resuspended with 1 ml of DMEM medium. Subsequently isolated cells were cultured in a T25 culture flask. Culture flasks were then incubated in a CO_2 incubator for 24 hours. The cultured cells then observed every day. Replacement of medium was done every 3 days. If the monolayer cells were obtained, the cells were then transferred to the 24 well plate culture.

The subculture cells, then transferred in a 24-well plate culture medium that has given complete DMEM and has been coated with cover slides. The cell culture then placed in an incubator at 37° C with 5% CO_2 to form monolayer. After the cells undergo approximately 80% confluent, the cells are ready to be treated. The cells were treated by using COM on dose 500 ppm (Thongboonkerd *et al.*, 2008 with modifications). After 24 hours, medium was replaced with a medium that has been coupled with Calcusol™ at various doses, ie 0, 75, 100, 200, 300, 400, and 500 ppm. After incubation, cells was stained with Von Kossa staining for the presence of crystals calcium retention. Retention referred to in this research that the amount of calcium oxalate crystals are internalized by cultured renal epithelial cells of mice.

Analysis Calcium Oxalate Crystal Retention Using Von Kossa staining

The cells were fixed with 4 % PFA for 10 -15 minutes and rinsed using sterile distilled water. Coverslip that containing the cells, removed and mounted on glass slides. Epithelial cells were fixed again using ethanol : AAG 10 % :5% for 30 minutes. Fixative solution was subsequently removed and soaked with silver nitrate 0.15 g/L for 30 minutes at 37°C. The cell then washed using sterile distilled water. Then performed a color developing using solution that contained 20 mL of NaOH that was added to 200 mL of 37% formaldehyde. The counterstain staining performed by using eosin. The preparat then air dried overnight. After air dried overnight, preparat were mounted. Calcium crystal deposits then observed using microscope Olympus BX51 at 400x magnification.

The analysis was performed also by categorizing cells that undergo retention of calcium oxalate. The category of slightly is (1-5 sediment), the category of medium (6 to 10 sediment), and category of heavy (more than 10 sediment).

Data Analysis

The data that was obtained in this study are data on cells undergoing internalization of calcium oxalate crystal formation in the cells either with or without the provision Calcusol™. The data were then analyzed using one-way ANOVA to determine the effect of COM and Calcusol™ to internalization of calcium crystal formation and its effect on cell death. If there are known to influence treatment, then it will continue with T test.

RESULTS

Based on the data analysis of calcium deposits in cell culture models of nephrolithiasis after treatment with COM, obtained the results as shown in Figure 1.

Based on Figure 1, it can be seen that the average amount of calcium crystal deposition in the control treatment (K -) is as much as 0.54 ± 0.19 spots/cell , while the average amount of calcium crystal deposition in the treatment Calcusol™ 0 ppm is as much as 1.06 ± 0.48 spots /cell. On the treatment with Calcusol™ 75 ppm , the average amount of calcium crystal deposition is as much as 0.71 ± 0.17 spots/cell, whereas the treatment Calcusol™ 100 ppm, there are decreasing number in the average amount of calcium crystal deposition is as much as 0.62 ± 0.11 spot/cell. Furthermore, the average amount of calcium crystal deposition in the Calcusol™ treatment at a dose of 200 ppm, 300 ppm , 400 ppm and 500 ppm respectively 0.67±0.31 spots/cell; 0.58±0.18 spots/cell; 0.46±0.21 spots/cell, and 0.30±0.1 spots/cell. Based on these results, it can be seen that there are decreasing number in the average amount of calcium crystal deposition in kidney epithelial cell culture models of nephrolithiasis with increasing doses Calcusol™ that were given. This indicates that Calcusol™ can reduce the retention of calcium crystals in cultured renal epithelial cells effectively.

Based on the staining that has been done, showed that there are deposits of calcium that is characterized by the presence of black spots on the cell. The observations result of crystals calcium retention are shown in Figure 2. In these figures, it can be seen that there are decreasing num-

ber in the amount of deposition of calcium crystals that was formed on the cells. The decreasing number in the amount of calcium crystal deposition can be seen from the reduced number of black spots on the cell models of nephrolithiasis with increasing the doses Calcusol™ that were given. This is consistent with previous results that Calcusol™ can effectively reduce the amount of calcium crystal deposition in kidney epithelial cell culture models of nephrolithiasis.

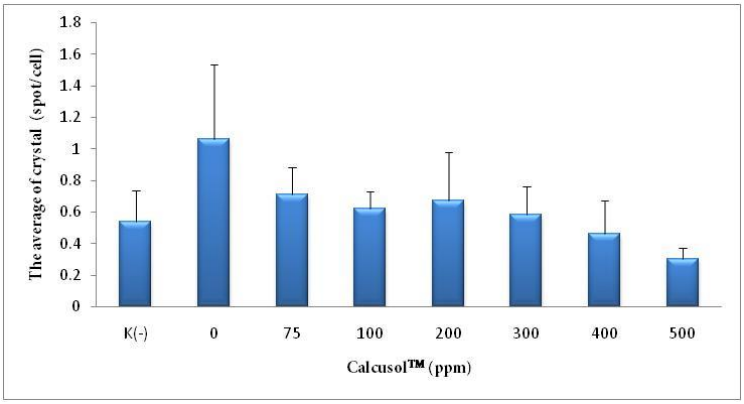

Figure 1. The average of calcium crystal deposition in cell culture models of nephrolithiasis after treatment with Calcusol™ at various doses. There are declining trends in the average amount of calcium crystal deposition with the increasing Calcusol™ dose that was given. This suggests that Calcusol™ can reduce the retention of calcium crystals that form on cultured cells.

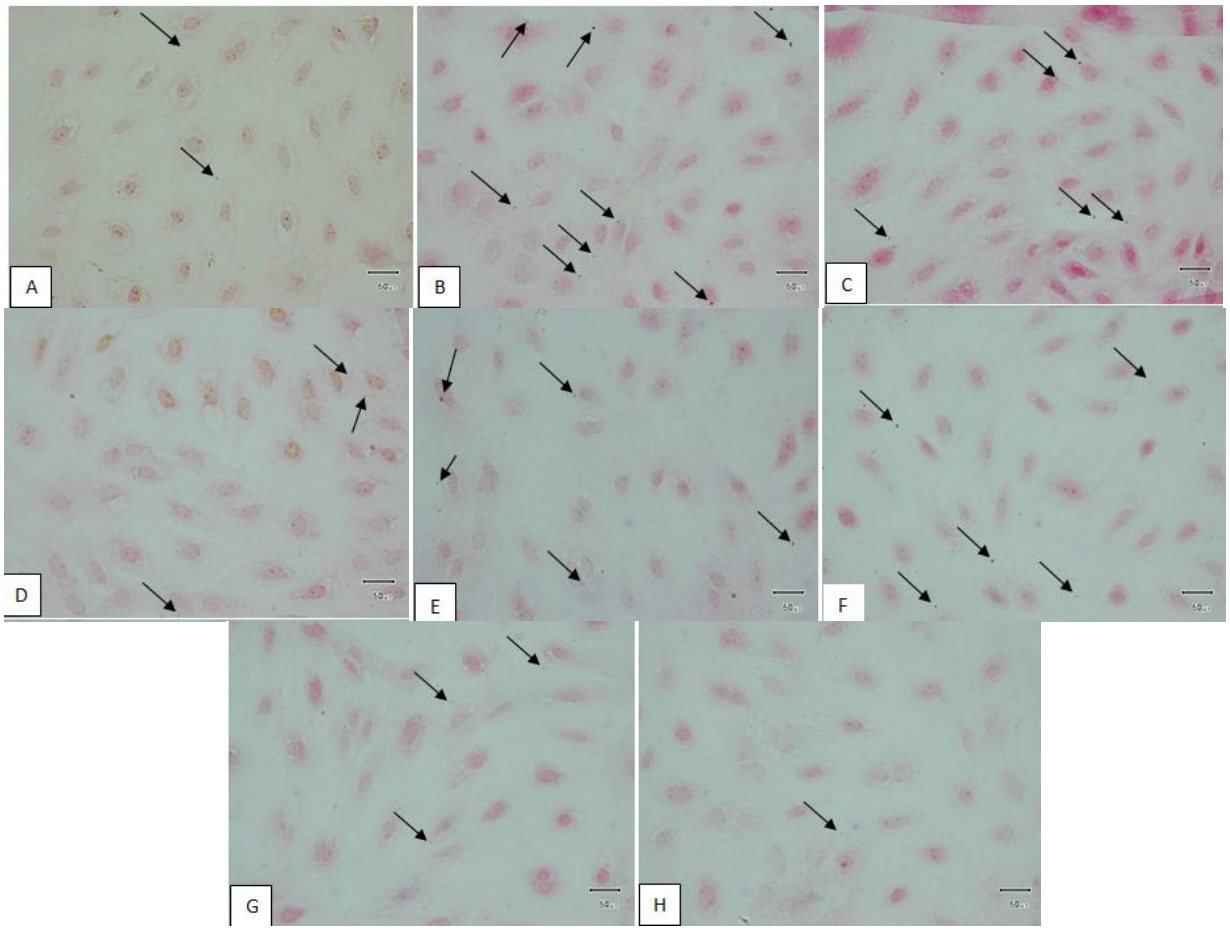

Figure 2. Calcium crystals retention in the renal epithelial cell culture models of nephrolithiasis by Von Kossa staining. (A) negative control, (B) 0 ppm, (C) Calcusol ™ 75 ppm, (D) Calcusol ™ 100 ppm, (E) Calcusol ™ 200 ppm, (F) Calcusol ™ 300 ppm, (G) Calcusol ™ 400 ppm, (H) Calcusol 500 ppm. Calcium deposits indicated by arrows (→). Scale 1: 50 µm

DISCUSSION

The formation of crystals calcium retention in cultured kidney epithelial cells is the result of treatment with Calcium Oxalate Monohydrate (COM). This is due to the interaction between COM crystals to cultured epithelial cells of the kidney. Furthermore, this interaction leads to crystal attachment to cells which may lead to the internalization or not (Schepers et al., 2003). COM is a compound that is toxic to the proximal tubules cells in high doses. Crystals that were formed as a result of treatment with COM will induce inflammation, oxidative stress, and would interfere with the process of DNA synthesis and ultimately will lead to cell death via either necrosis or apoptosis mechanism. This is because the crystals that were formed will be attached to the proximal tubule cells in the kidney and cause cell damage. As a result of this damage then the cells will form hydrogen peroxide compound and eventually the cells will undergo death (Schepers *et al.*, 2005). According Hovda *et al.* (2010), COM crystals have a negative effect on kidney cells. The damage to kidney cells is directly proportional to the number of COM accumulation found in the kidney. The study also mentioned that the internalization of COM positively correlated to the rate of cell death.

Urine supersaturation conditions can trigger nucleation and crystal COM precipitation which is the main constituent of kidney stone (Lieske *et al.*, 1999; Sheng *et al.*, 2005). Calcium crystals that was formed will be attached to the cell membrane and could be internalized by the cells in the kidney tubules, where this process is very crucial in the formation of kidney stones. The process of internalization of calcium crystals themselves by renal tubular cells is identical to the process of endocytosis by cells in other organ systems (Campos and Schor, 2000; Asselman *et al.*, 2002; Hovda *et al.*, 2010). According to Lieske *et al.* (1994), COM crystals will be quickly attach to the surface membrane of renal epithelial cells. Once attached, the COM crystals will be internalized by the cells. When the crystals are attached to the cell, then it will trigger other crystals tend to bind. In this process, is suspected there are positive feedback in the form of the stone in the kidneys, but until now the certain mechanism is still unknown.

Under normal conditions, the presence of calcium crystals that contained in blood won't bound by the epithelial cells of the renal tubules, but it will be excreted along with urine. If there are damage to the epithelial cells in the tubules, then it will initiate the attachment of the crystals by renal tubular epithelial cells. At the cell regeneration process, new epithelial cells would express hyaluronan (HA), Osteopontin (OPN), and CD44 on its membrane. HA itself is a cell surface crystal-binding molecule, so that at the same time the epithelial cells will bind calcium crystals that contained in the blood. OPN is a glycoprotein that plays a role in the formation of kidney stones, but until now the role of OPN itself is very controversial. CD44 is a transmembrane glycoprotein that serves as a surface receptor for HA and OPN. Therefore, it is not surprising that upregulation of CD44 expression was always accompanied by increased expression of ligands HA and OPN, so that all three glycoproteins is thought to have a very important role in the formation of kidney stones (Asselman *et al.*, 2003).

Based on the results that have been obtained, it can be seen that the administration of Calcusol™ in various doses in the cell culture models of nephrolithiasis can reduce the number of crystal retention. In this case, the leaf extract of tempuyung (*Sonchus arvensis* L.) that was contained in Calcusol™ supposedly has the ability to reduce the retention of calcium crystals in cultured renal epithelial cells. The ability of calcium crystals decay in cultured epithelial cells may be due to a potassium compound that contained in the leaf extract Tempuyung. According to Pak *et al.*, (2003) ; Ettinger *et al.*, (1997) ; Helberg & Nestor (2006), restriction of dietary calcium and oxalate as well as in combination with thiazide and potassium citrate treatment able to control hypercalciuria, prevent the increasing levels of oxalate in the urine, reducing the level of saturation of calcium oxalate in the urine, reducing the formation of kidney stones, and can increase the bone density of the spine and femoral neck. Thus, it can be concluded that potassium can reduce the formation of crystals retention in renal tubular cells.

Based on the results that have been obtained, it can be seen that the treatment on the negative control (K-) also found the deposition of calcium crystals. This was reasonable because under normal conditions, the crystals calcium retention will be formed but the number and size is very small. If it was balanced with diet and adequate fluid intake, then the formation of stones in the kidneys could be inhibited. Furthermore, the source of calcium crystals that form on the treatment in K (-) is derived from the culture medium that was used. In the cell culture medium, calcium has a very important role. In this case, calcium is involved in the activation of enzymes, cell attachment on the substrate, cell motility, cell morphology, the process of cell metabolism, signal transduction, and as well as play a role in cell replication process. According to Juliano (2002), in the culture system, calcium has two main roles. First, calcium play a role in mediating signal transduction. Calcium facilitates the binding of protein kinase C in the cell membrane and subsequently activate calmodulin. Calmodulin that was activated regulate the protein kinase and subsequently phosphatase that invol-

ved in cell cycle progression. The second function of calcium in the culture system that facilitates cell attachment to the substrate. In this case, Calcium modulates cadherin function, selectin, and integrins. They're an adhesion molecule that regulates interactions and attachment between cells. In other words, calcium is able to facilitate cell attachment to the substrate and facilitates adhesion between cells so that there will be communication between cells which then affects in the cell movement, shape, and structure of three-dimensional cultured cells.

According to the research that was conducted by Sardjito & Ismadi (1964), tempuyung leaves contain several active compounds, such as saponins, flavonoids, polyphenols, alpha-lactucerol, beta-lactucerol, mannitol, inositol, silica, potassium and taraksaterol. This study also suggests that the active substance in the leaf extract of Tempuyung (*Sonchus arvensis* L.) can dissolve 0.28 mg Ca in $CaCO_3$ within 24 hours. In addition, the extract Tempuyung can also dissolve 0.54 mg Ca that was contained in marble powder, and 12.59 mg Ca that contained in $CaCO_3$. This indicates that Tempuyung extract has a great solubility of the $CaCO_3$ powder, either in the form of a fine powder or in the grain form. This indicates that the leaf extract of Tempuyung that contained in Calcusol™ can reduce crystal deposition in kidney stones. According Chairul (2003), the solubility of kidney stones by tempuyung presumably throughits diuretic effects. Tempuyung leaves contain mineral ions in quite high concentrations, especially K+ and Na+ that able to regulate electrolyte balance in the body to facilitate discharge of urine. In addition, according to Fouada *et al.*, (2006), an active saponin compounds also have an important role in reducing the retention of calcium crystal retention, so that the natural products that contain saponins also can be used effectively to overcome kidney stone disease.

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