THE JOURNAL OF TROPICAL LIFE SCIENCE
VOL. 4, NO. 2, pp. 101-105 , May , 2014

Dose Optimization of Calcusol™ and Calcium Oxalate Monohydrate (COM) on Primary Renal Epithelial Cells Cultures of Mice (*Mus musculus*)

Ahmad Soni[1], Sasmito Djati[1], Sri Widyarti[1]*.

[1]Department of Biology, Faculty of Mathematics and Natural Sciences Universitas Brawijaya, Malang.

ABSTRACT

Kidney stones are one of the urologic diseases that have plagued mankind for centuries. The main constituents of stones in the kidney are calcium oxalate monohydrate (COM) crystals. Nowadays, there are varieties of drugs and treatments that can be made to minimize the grievances due to kidney stone disease. The treatment can be done either by using chemicals or traditional medicine. Calcusol™ is one of the popular herbal products that have been used by Indonesian people in curing the kidney stone disease. The main constituent that was contained in Calcusol™ is an extract of the tempuyung leaves (*Sonchus arvensis* L.), which is expected could cure the kidney stone disease. This study used primary cultured renal epithelial cells of mice to determine the optimal dose of Calcusol™ and the optimal dose of COM. The primary Kidney epithelial cell were treated with Calcusol™ and COM at various doses. The analysis of the cell death either by necrosis or apoptosis pathways was analyzed by flow cytometric analysis. The results that were obtained is the percentage of cell death that is then analyzed by using a complete randomized design (CRD) One Way Anova. Based on the results that were obtained, it is known that the optimal dose of Calcusol™ in vitro were ranging from 75 ppm to 100 ppm, whereas the optimal dose of COM suggested for 500 ppm.

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

INTRODUCTION

Kidney stones are the diseases that can be caused by many factors such as epidemiological, biochemical metabolism in the body and also genetic factors [1]. Nowadays, there are a variety of drugs and treatments that required could cure the kidney stone disease. The treatment can be done either by using chemicals or by using traditional medicine. One of the popular herbal products that have been used by the people of Indonesia in curing the kidney stone disease is called Calcusol™. The main part of Calcusol™ is an extract of tempuyung leaves (*Sonchus arvensis* L.), which is believed to cure the kidney stone disease. The study that was conducted suggests that the active substance in the extract of tempuyung leaves (*Sonchus arvensis* L.) dissolved 0,28 mg Ca in $CaCO_3$ within 24 hours [2]. The data indicates that the extract of tempuyung leaves that contained in Calcusol™ can reduce crystal deposition in the kidney.

Major part of kidney stones is called Calcium Oxalate Monohydrate (COM) as the main part. COM microcrystals aggregation and attachment to the renal epithelial cells is required because of the adhesion of COM crystal surfaces that were mediated by anionic molecules. The adhesion that occurs in the COM crystal surface is determined by the composition and the structure of calcium oxalate crystal itself [3]. Meanwhile, the presence of renal tubular damage can lead to increased adhesion of COM crystals in the kidneys [4, 5]. The binding of COM crystals in kidney cells is regulated by physiological signals [6]. In addition, the binding of COM crystals was also influenced by the length of time of exposure and the concentration of COM that was given [7]. Furthermore, this study will be discussing the optimization of Calcusol™ and COM dose in vitro.

*Corresponding author:
Sri Widyarti
Biology Department, Brawijaya University, Malang 65145, Indonesia
E-mail: swid@ub.ac.id

MATERIALS AND METHODS

Experimental Animals

Experimental animals that were used in this study are mice (*Mus musculus*) strains Balb/C, which has a range of 2-5 months of age. The animal then acclimated for one week prior to surgery.

Medium Preparation

For 100 ml of medium, the amount of material used is DMEM F12 HAM 1.56 g, and then Sodium Bicarbonate added as much as 0.12 g. The medium was made in the Laminar Air Flow to minimize contaminants, and then the medium materials mixed in the order that was already mentioned, and then diluted with DI (Di-ionized water) to ¾ d volume and adjust pH at 7.2 and then added DI until 1 volume. Then added 2 % Penicillin - Streptomycin and 10 % FBS (Fetal Bovine Serum) on stock medium that was created earlier. 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 of Calcusol™ with a dose of 1 to 5 with a predetermined dose.

Isolation of Kidney Cells

Mice (*Mus musculus*), which has acclimated for one week then killed by neck dislocation. Subsequently, the mice were dissected and then kidney organ was isolated. The kidneys were then perfused by using sterile PBS with 2 % penicillin - streptomycin 10x additions, then the kidneys were washed with PBS with 2 % penicillin - streptomycin 10x additions. Furthermore, the kidney squeezed using the base of sterile syringe at a petri dish containing sterile PBS, which had been added 2% penicillin-streptomycin 10 xs. Then, homogenates transferred into polypro-pylene tubes. Centrifugation was performed at room temperature at 1000 rpm for 10 minutes twice, to maximize the results obtained. Pellets were then re-suspended with 1 ml of DMEM medium that had been added 10 % FBS and 2 % penicillin-streptomycin 10 xs. Subsequently isola-ted cells were cultured in a culture flask. The 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 three days. Once confluent made the passage of cell culture to obtain a monolayer of cells. If the monolayer cells were obtained, the cells were then transferred to the 48 well plated cultures.

Calcusol™Dose Optimization

Sub-cultured cells were then transferred to 48 well plated that had been given complete DMEM F12 HAM medium. Cells were then incubated in a CO_2 incubator and then observed every day. After the cells undergo approximately 80 % confluent, the cells then treated by administering of Calcusol™ at various doses. The doses that were used in this treatment are 0 ppm, 5 ppm, 50 ppm, 75 ppm, 100 ppm, 200 ppm, 300 ppm, 400 ppm and 500 ppm. Cells were then incubated for 24 hours in a CO_2 incubator. After the incubation, then the analysis of cell death performed by using flow cytometry.

COM Dose optimization

Sub-cultured cells were then transferred to 48 well plated that had been given complete DMEM F12 HAM medium. Cells were then incubated in a CO_2 incubator and then observed every day. After the cells undergo approximately 80 % confluent, the cells then treated by administering of COM (Calcium Oxalate Monohydrate) at various doses. The doses that were used in this treatment are 0 ppm, 250 ppm, 500 ppm, 750 ppm, 1000 ppm, 1250 ppm, 1500 ppm, and 2000 ppm. Cells were then incubated for 24 hours in a CO_2 incubator. After the incubation, then the analysis of cell death performed by using flow cytometry.

Cell Death Examination by FlowCytometry Analysis

The effects of treated epithelial cells were harvested using trypsin-EDTA 20 %. Cells culture in 48 well plated was incubated with trypsin-EDTA 20 % for 10 minutes. Then do pipetting with complete medium at 2x volume. Furthermore, cells were transferred to micro tube for centrifugation. The centrifugation performed at a speed of 2,500 rpm for 5 min at 40 C. The supernatant was discarded, and the pellets were given PI antibody and Annexin V-FITC labeled.

Each tube containing the cells was then given 50 mL of antibody and then pipetting. Besides, do incubation for 15 min at room temperature and in dark conditions. Then each tube was given 300 mL of PBS and then an examination of cell death performed by flow cytometric analysis.

RESULTS AND DISCUSSION

Based on the results of the flow cytometric data analysis, it is showed the percentage of cell death through apoptosis or necrosis pathway after treatment with Calcusol™ at various doses. The percentage of cell death is shown in Figure 1.

Based on Figure 1a, it can be shown that the percentage of cell death in control treatment through necrosis is 3.65 ± 0.8 % , while the percentage of cell death through apoptosis pathway is 6.66 ± 0.8 %. Furthermore, the percentage of cell death at a dose of 5 ppm through necrosis is 1.89 ± 3.24% , while the percentage of cell death through apoptosis is 8.70 ± 3.24%; then the percentage of cell death at dose of 50 ppm that were through necrosis is 5.38 ± 7.13%, while the percentage of cell death through apoptosis pathway is 8.31 ± 7.13%. At the dose of 75 ppm, the percentage of cell death through necrosis is 7.69 ± 0.06 %, while the percentage of cell death through apoptosis pathway that is 32.56 ± 0.06 %. Therefore at a dose of 100 ppm, the percentage of cell death through necrosis is 8.24 ± 3.88 %, while the percentage of cell death through apoptosis pathway is 70.50 ± 3.88 %. The percentage of cell death through necrosis at doses of 200 ppm, 300 ppm and 400 ppm respectively are 9.30 ± 8.45 %; 12:27 ± 2.27%, and 8.11 ± 1.64 %, while the percentage of cell death through the apoptotic pathway in a row is 69.79 ± 8.45 %, 62.13 ± 2.27%, and 76.32 ± 1.64 %. Based on Figure 1 above, it can be seen that after a dose of 100 ppm, there was no increase in the percentage of cell death significantly. Increasing the percentage of significant cell death occurred precisely at a dose of 75 ppm and 100 ppm. Based on these results, it can be seen that a dose of 100 ppm is the optimal dose for use in further laboratory test.

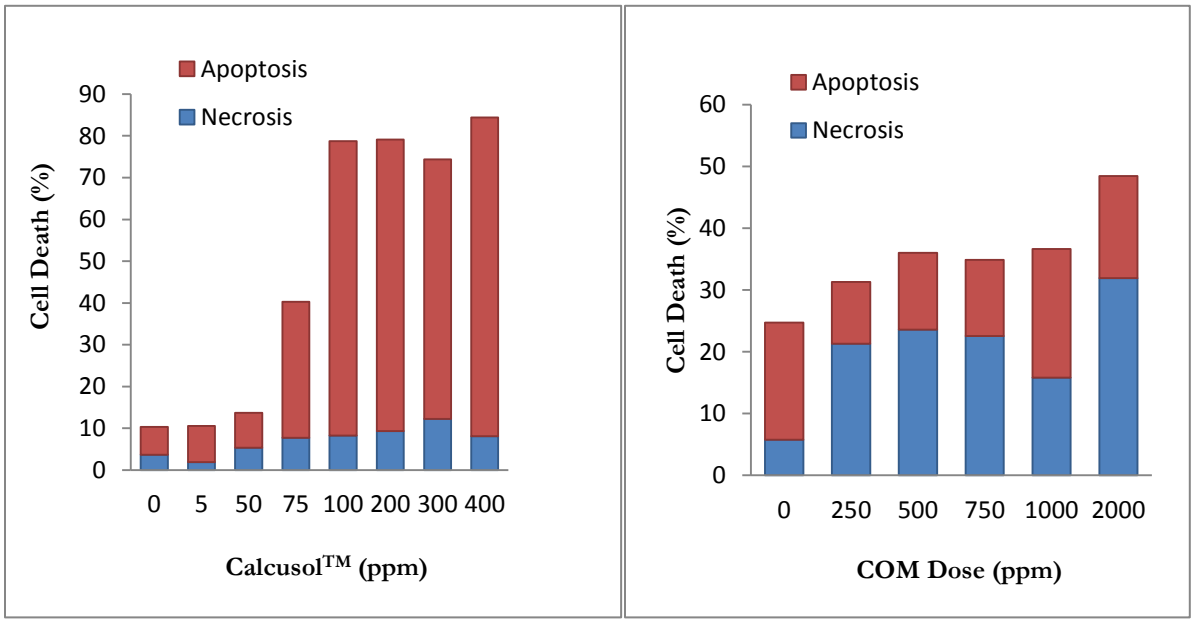

Figure 1. a) The percentage of cell death after treatment of Calcusol™ in several doses. b) The percentage of cell death after administration of Calcium Oxalate Monohydrate (COM) in various doses.

The percentage of cell death after administration of COM will be shown in Figure 1b. Based on Figure 1b, it can be shown that the percentage of cell death in the control treatment through necrosis is 5.79 ± 3.08 %, while the percentage of cell death through apoptosis pathway is 18.97 ± 3.08 %. Moreover, percentage of cell death at a dose of 250 ppm

through necrosis is 21.34 ± 1.23 %, while the percentage of cell death through apoptosis pathway is 9.96 ± 1.23 %. Then, at a dose of 500 ppm, the percentage of cell death through necrosis is 23.60 ± 3.61 %, while the percentage of cell death through apoptosis pathway is 12.43 ± 3.61 %; at a dose of 750 ppm. The percentage of cell death through necrosis is 22.53 ± 4.65 %, while the percentage of cell death through apoptosis pathway is 12.36 ± 4.65 %. The percentage of cell death through necrosis of the treatment dose of 1000 ppm and 2000 ppm respectively is 15.81 ± 4.12 % and 31.93 ± 1.61 %, while the percentage of cell death through apoptosis in a row is 20, 85 ± 4.12 % and 16.50 ± 1.61 %. Based on these data and confirmed by analyzing the deposition of calcium crystals by using Von Kossa staining (data not shown) it indicates that the optimal dose of COM to initiate internalization of calcium crystal formation in primary cultured renal epithelial cells of mice is at a dose of 500 ppm.

Figure 1a shows that the percentage of cell death increased with the increasing dose of Calcusol™ that was given. In addition, the percentage of cell death through apoptosis pathway is higher than the percentage of cell death through the necrosis pathway. The high percentage of cells undergoing apoptosis indicates that Calcusol™ can initiate the process of apoptosis, which is required to be able to maximize the regenerating kidney cells that were damaged by the accumulation of calcium oxalate crystals. Furthermore, Figure 1b shows that the percentage of cell death increases with the increasing dose of COM that was given. Unlike the Calcusol™ treatment, the percentage of cell death by the COM treatment through necrosis is higher than through apoptosis pathway. This indicates that the COM is toxic to cells, and it can initiate cell death through necrosis.

Calcium Oxalate Monohydrate (COM) is a toxic compound to the cells. The research earlier, states that the provision of the COM in HK-2 cells can decrease the cell viability and increase the level of LDH, MDA and hydrogen peroxide [8]. Furthermore, COM can increase the concentration of LDH and MDA in LLC-PK1 cells [9]. Meanwhile, administration of COM crystals on NRK-52E cells can influence the changes in mitochondrial membrane potential, increase the expression of cytosolic cytochrome C and caspase 3, which indicates mitochondrial collapse, and they can lead to cell death [10].

From this study, it is known that cell death occurred both in the treatment with COM and Calcusol™. However, the COM treatment causes cell death tends to be through the necrosis pathway, whereas the Calcusol™ treatment causes cell death tends to be through the apoptosis pathway. The data can occur because COM is toxic to the primary culture epithelial cell so that the treatment with COM can be initiated the process of cell death through the necrosis pathway. Meanwhile, on treatment with Calcusol™ could be required to initiate the process of apoptosis. The apoptosis pathway is expected could trigger the regeneration of the cells. With the cell regeneration process, the performance of the organ in this case the kidneys will also be optimal [11]. Apoptosis is a form of programmed cell death and is intended to maintain homeostasis and survival of an organism. One of the signaling cascades that occur in the process of apoptosis is the activation of cysteinyl aspartate protease (caspase) that was available in its inactive form and is called pro-caspase. When activated, caspase can initiate cell death by break down and activate the caspase effectors that responsible for the regulation of apoptosis. Caspases that were involved in the process of apoptosis are caspase-1 and caspase-3. In this case, caspase-3 acts as an initiator of PARP-1 protein to undergo cleavage in response to cellular apoptotic processes [12].

Tempuyung leaves extract 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 tempuyung leaves extract (*Sonchus arvensis* L.) dissolve 0,28 mg Ca in $CaCO_3$ within 24 hours. In addition, the extract can also dissolve 0,54 mg Ca that was contained in marble powder. The result indicates that tempuyung leaves extract has a high solubility to the $CaCO_3$ powder, either in the form of a fine powder or in the form of grain [2]. The result indicates that the tempuyung leaf extract that was contained in Calcusol™ can reduce crystal deposition in the kidney. The solubility of kidney stones by tempuyung presumably through its diuretic effects. Tempuyung leaves extract contain mineral ions in high concentration, especially K^+

and Na^+ that can regulate the electrolyte balance in the body to facilitate discharge of urine [13]. In addition, tempuyung leaf extract also contains many antioxidants that can suppress cell death [14]. Furthermore, an active saponin compounds that were contained in tempuyung leaf extract also has an important role in reducing the retention of calcium crystal deposition so that the natural products that contain saponins also can be used effectively to cure the kidney stone disease [15].

CONCLUSIONS

The optimal dose for Calcusol™ that can be used for clinical trials in vitro is in the range of 75 ppm to 100 ppm. While the optimal dose for COM that can be used to initiate the internalization of calcium crystals in the primary culture of renal epithelial cell of the mouse is 500 ppm.

ACKNOWLEDGMENT

We would like to thank PT. Perusahaan Jamu Tradisional DR. Sardjito for the cooperation and funds that support this research.

REFERENCES

1. Abbagani S, Gundimeda SD, Varre S, Ponnala D, Mundluru HP (2010) Kidney Stone Disease: Etiology and Evaluation. International Journal of Applied Biology and Pharmaceutical Technology. 1(1): 175-182.
2. Sardjito, Ismadi M., Sudibjo, Ishom MB (1964). Pemeriksaan Khasiat Decoctum *Sonchus arvensis* Sebagai Anti Calculus Urinariae. Penerbit Lustrum ke III. Universitas Gadjah Mada. Yogyakarta.
3. Sheng X., Jung T, Wesson JA, Ward MD (2004) Adhesion at Calcium Oxalate Crystal Surfaces and the Effect of Urinary Constituents. PNAS. 102(2): 267 – 272.
4. Thongboonkerd V, Semangoen T, Sinchaikul S, Chen ST (2008) Proteomic Analysis of Calcium Oxalate Monohydrate Crystal-Induced Cytotoxicity in Distal Renal Tubular Cells. Journal of Proteome Research. 7(11) : 4689 – 4700.
5. Schepers MSJ, Ballegooijen ESV, Bangma CH, Verkoelen CF (2005) Oxalate is Toxic to Renal Tubular Cells only at Supraphysiologic Concentrations. Kidney International. 68: 1660–1669.
6. Lieske JC, Huang E, Toback FG (2000) Regulation of Renal Epithelial Cell Affinity for Calcium Oxalate Monohydrate Crystals. Am. J. Physiol Renal Physiol. 278: F130 – F137.
7. Campos AH, Schor N (2000) Mechanisms Involved in Calcium Oxalate Endocytosis by Madin-Darby Canine Kidney Cells. Brazilian Journal of Medical and Biological Research. 33: 111-118.
8. Shuo W, Xiao-bo H, Qing-quan X., Hai-yun Y, Kai M, Xiao-feng W (2013) Tea Polyphenol Protects Against HK-2 Cell Injury Induced by Oxalate and Calcium Oxalate Monohydrate Crystals. Journal of Peking University. 45(4): 567-574.
9. Thamilselvan S, Khan SR, Menon M (2003) Oxalate and Calcium Oxalate Mediated Free Radical Toxicity in Renal Epithelial Cells: Effects of Antioxidants. Urol Res. 31: 3-9.
10. Zhai W, Zheng J, Yao X., Peng B, Liu M., Huang J, Wang G, Xu Y (2013) Catechin Prevents the Calcium Oxalate Monohydrate Induced Renal Calcium Crystallization in NRK-52E Cells and the Ethylene Glycol Induced Renal Stone Formation in Rat. BMC Complementary and Alternative Medicine. 13: 1-11.
11. Smaili SS, Hsu YT, Carvalho ACP, Rosenstock TR, Sharpe JC, Youle RJ (2003) Mitochondria, Calcium, and Pro-apoptotic Proteins as Mediators in Cell Death Signaling. Brazilian Journal of Medical and Biological Research. 36: 183-190.
12. Chaitanya GV, Alexander JS, Babu PP (2010) PARP-1 Cleavage Fragments: Signatures of Cell-Death Proteases in Neurodegeneration. Biomed Central. 8(31): 1-11.
13. Chairul SM, Sumarny R, Chairul (2003) Aktivitas Antioksidan Ekstrak Air Daun Tempuyung (*Sonchus arvensis* L.) secara In Vitro. Majalah farmasi Indonesia. 14(4): 208-215.
14. Khan RA (2012) Evaluation of Flavonoids and Diverse AntioxidantActivities of Sonchus arvensis. Chemistry Central Journal. 6: 126-132.
15. Fouada A, Yamina S, Nait MA, Mohammed B, Abdlekrim R (2006) In Vitro and In Vivo Antilisthiasic Effect of Saponin Rich Fraction Isolated from Herniaria hirsuta. J. Bras Nefrol. 28(4): 199-203.