EFFECT OF CALCUSOL™ ON CuZnSOD EXPRESSION IN MICE RENAL OF NEPHROLITHIASIS MODEL

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ABSTRACT

This study aimed to determine the effect of traditional medicine, Calcusol™, on number of cells expressing CuZnSOD in mice renal of nephrolithiasis model. Eight weeks old Swiss strain male mice (*Mus musculus*) were divided into five groups: (A) control, (B) nephrolithiasis, (C) Calcusol™, (D) nephrolithiasis & Calcusol™, and (E) nephrolithiasis & Calcusol™ simultaneously. Nephrolithiasis was induced by applying porang tuber (*Amorphophallus muelleri*) flour 0.06 mg/g of body weight during 3 months. Calcusol™ is traditional medicine, made of tempuyung leaves extract with S*accharum lactis* as additional substance. The dosage for Calcusol™ treatment was 3.3 mg/g of body weight. After 3 months treatment, the mice were killed by neck-dislocation, the kidneys were isolated and prepared for paraffin histology. CuZnSOD was analyzed by immunohistochemistry (IHC), using rabbit policlonal antibody anti-SOD1 (Bioss, bs-1079R) as primary antibody. Tissues were observed under Olympus BX51, 400x magnification. Images were documented with *Olympus Digital Camera* DP20. The histology images were analized in Immunoratio software online (URL: http://153.1.200.58:8080/immunoratio/) to receive the percentage of number of cells expressing CuZnSOD. The result showed that Calcusol™ administration could decrease number of cells expressing CuZnSOD in kidney significantly ($P<0.05$). It was supposed that antioxidant content in Calcusol™ could scavenge ROS directly with no induction of CuZnSOD production in cell.

Key words: Calcusol™, CuZnSOD, nephrolithiasis.

INTRODUCTION

Nephrolithiasis is a disease with the formation of stones or crystals in the kidney or in the urinary tract. The formation of kidney stones can be caused by a supersaturation of mineral salts in urine. Crystal sized less than 5 mm can be removed with urine. Accumulation of larger crystals is able to close the urethra, which is able to cause pain in the lower abdomen (Marieb, 2004).

According to data of Cipto Mangunkusumo Hospital, an increase in the number of kidney stone patients occurred in the range of 1997-2002, from 182 to 847 patients. Although it did not threaten the life of patients, the disease could cause pain that was very disturbing. The costs incurred for the care of kidney stone patients was not cheap. In the United States annually spent two billion U.S. dollars to serve patients with kidney stones (Syahputra, 2011).

Some studies suggested that the increase in crystal nucleation in renal tubular cells was mediated by free radicals. A previous research indicates that the administration of porang (*Amorphophallus muelleri*) tuber flour that containing oxalate continuously in mice was able to increase levels of malondialdehyde (MDA) in the kidney (Rosyidah, 2013). The accumulation of free radicals was able to cause oxidative stress in renal tubular cells which could ultimately lead to necrosis. Necrosis made oxalate crystal nucleation going more quickly (Tsujihata, 2007).

To reduce oxidative stress, it requires active compounds that can reduce free radicals, to break the chains of peroxidation in the cell, thus oxalate crystal nucleation can be decreased. The active compounds that reduce free radicals are called antioxidants. Antioxidants that are produced by the body called endogenous antioxidants, while derived from nutrients called exogenous antioxidants. Endogenous antioxidants are usually in the form of enzyme or non-enzyme. One of the antioxidant enzymes that catalyzes the reduction of levels of oxidants is superoxide dismutase (SOD) that reduce superoxide radicals ($O\cdot^-$). While exogenous antioxidants, such as flavonoids which contained in many plants, diminish free radicals directly by providing some electrons then make it non-radical molecules (Halliwell and Gutteridge, 1999).

Calcusol™ is a product of traditional medicine made of *Sonchus arvensis* L. or tempuyung leaves extract with s*accharum lactis* as additional substance. This herbal medicine can be a source of exogenous antioxidant that can overcome oxidative stress in renal cells. *Sonchus arvensis* or tempuyung (local name) contains a variety of bioactive phenolic compounds and flavonoids which act as free radical scavenger (Khan, 2012). A previous study proved that the administration of Calcusol™ to mice orally were able to reduce levels of MDA in the kidney (Rosyidah, 2013). Flavonoid compounds may also stimulate the production of antioxidant enzymes in the body, such as the SOD (Akhlaghi and Bandy, 2009).

In animal cells, based on the active side, there are two isoforms of SOD: CuZnSOD and MnSOD. In the human kidney, CuZnSOD activity was higher than the MnSOD (Halliwell and Gutteridge, 1999). Therefore, it is suspected that the antioxidants contained in the Calcusol™ product can affect the number of cells expressing CuZnSOD which is able to affect oxalate crystal nucleation mediated by free radicals. This study was to determine the effect of Calcusol™ on number of cells expressing CuZnSOD enzyme in the mice kidneys of nephrolithiasis model and to determine the significancy of CuZnSOD expression in the cortex and medulla.

MATERIALS AND METHOD

The ethical clearance of this research was approved by Research Ethic Committee of Brawijaya University No. 127-KEP-UB by March 19th 2013. This research was done in two stages, treatment to experimental animals and immunohistochemical analysis. Experimental animals were used Swiss strain male mice (*Mus musculus*) eight weeks old weighing about 25-30 grams in a healthy state.

Mice were obtained from LPPT-UGM Yogyakarta. Before being treated, mice were acclimatized for 7 days. Mice were treated for three months.

Animal Treatment

Treatment groups were (A) control; (B) nephrolithiasis; (C) Calcusol™; (D) nephrolithiasis and Calcusol™; and (E) nephrolithiasis and Calcusol™ simultaneously. Nephrolithiasis was induced by administering porang tuber flour (*Amorphophallus muelleri*) orally with the dosage 0.06 mg/g of body weight (Rosyidah, 2013). Calcusol™ were obtained from PT. Perusahaan Jamu Tradisional (Traditional Herbal Company) DR. SARDJITO Yogyakarta-Indonesia. Dosage of Calcusol™ was 3.3 mg/g of body weight, administered for the last seven days for D group and three months simultaneously with porang administration for E group. After 3 months treatment, the mice were killed by neck-dislocation, the kidneys were isolated and prepared for paraffin histology.

Immunohistochemical Analysis

The slides that had been deparrafinned,washed with PBS 3 x 5 minutes, blocked with peroxidase block (Novolink RE7165) for 5 minutes, washed with PBS 3 x 5 minutes each, incubated with a protein block (Novolink RE7166) for 5 minutes, washed with PBS 3 x 5 minutes, incubated with primary antibody rabbit polyclonal anti-SOD1 (Bioss, bs-1079R) with dilution 1:200 in 1% BSA overnight at 4°C. Washed with PBS 3 x 5 minutes, incubated with Novolink Polymer (RE7168) at room temperature for 30 minutes, washed with PBS 3 x 5 minutes. Incubated with DAB chromogen DAB substrate buffer-Novolink 1:20 (Novolink-RE7171 RE7169) for 5 minutes at room temperature, washed with distilled water until clean. Furthermore, slides were counterstained with hematoxylin for 5 minutes, washed with distilled water until clean, dried, and mounted with entellan. The slides were observed with Olympus BX51 microscope in 400x magnification to determine which cells express the enzyme CuZnSOD. Images of each field were documented with Olympus DP20 digital camera attached to the microscope. The histology images were analized by using Immunoratio software online (URL: http://153.1.200.58:8080/immunoratio/) to receive the percentage of number of cells expressing CuZnSOD.

Analysis Data

This study used a completely randomized design with three replications, using one-way ANOVA test 95% confidence interval for statistical analysis. Tukey HSD test were used to performed for significancy. While the analysis to determine significancy of CuZnSOD expression in the cortex and medulla used independent-samples t test. All data were analyzed with MS. Excel and SPSS 16.0 for Windows.

RESULTS

CuZnSOD expression in the kidneys could be identified by the presence of brown color in the cytosol as a result of immunohistochemistry with anti-SOD1 antibodies. The intensity of brown color indicated the level of CuZnSOD expressed in cell (→ in fig. 3). In nephrolithiasis model (B), CuZnSOD enzyme was expressed in almost every cell in the tissue with a thick brown color. Mice kidney with treatment of control (A), Calcusol™ (C), nephrolithiasis and Calcusol™ (D), and nephrolithiasis and Calcusol™ simultaneously (E) showed lower expression of CuZnSOD than in nephrolithiasis model (B), with a thinner brown color and also less number of cells expressing CuZnSOD.

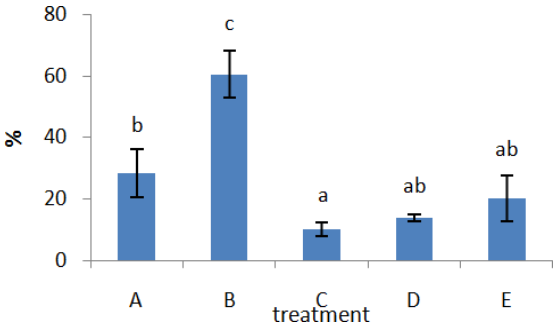

Figure 1. The percentage of number of cells expressing CuZnSOD in the kidneys in each treatment (A) control; (B) neph-rolithiasis; (c) Calcusol™; (D) nephrolithiasis and Calcu-sol™; and (E) nephrolithiasis and Calcusol™ simulta-neously

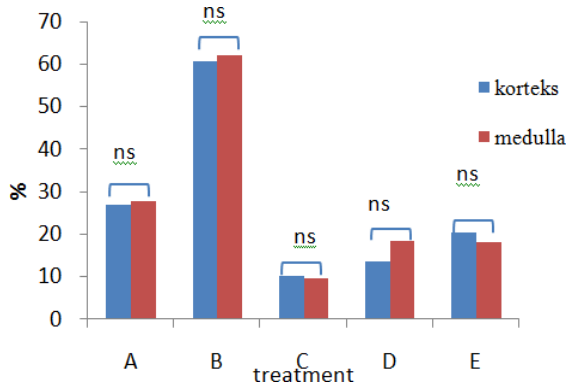

Figure 2. The percentage of number of cells expressing CuZnSOD in the ex and medulla (ns = not significant).

The results of histologic observation of kidney of mice (figure 1) showed that the percentage of number of cells expressing CuZnSOD in the control (A); nephrolithiasis (B); Calcusol™ (C); nephrolithiasis and Calcusol™ (D); and nephrolithiasis and Calcusol™ administration simultaneously (E) respectively were 28.4 ± 7.9%; 60.6 ± 7.7%; 10.1 ± 2.3%; 13.8 ± 1.2% and 20.1 ± 7.4%. According to data, it is known that nephrolithiasis induction by porang administration (B) could increase significantly the number of cells expressing CuZnSOD compared to the (A), (C), (D), and (E) treatment ($P < 0.05$). Calcusol treatment post nephrolithiasis (D) could reduce the number of cells expressing CuZnSOD significantly ($P<0.05$). Calcusol treatment post nephrolithiasis (D) and nephrolithiasis and calcusol administration simulta- neously treatment (E) could decrease the number of cells

expressing CuZnSOD equal to control (A) (P> 0.05). Calcusol (C) were able to decrease the number of cells expressing CuZnSOD significantly compared to the control (A) (P <0.05).

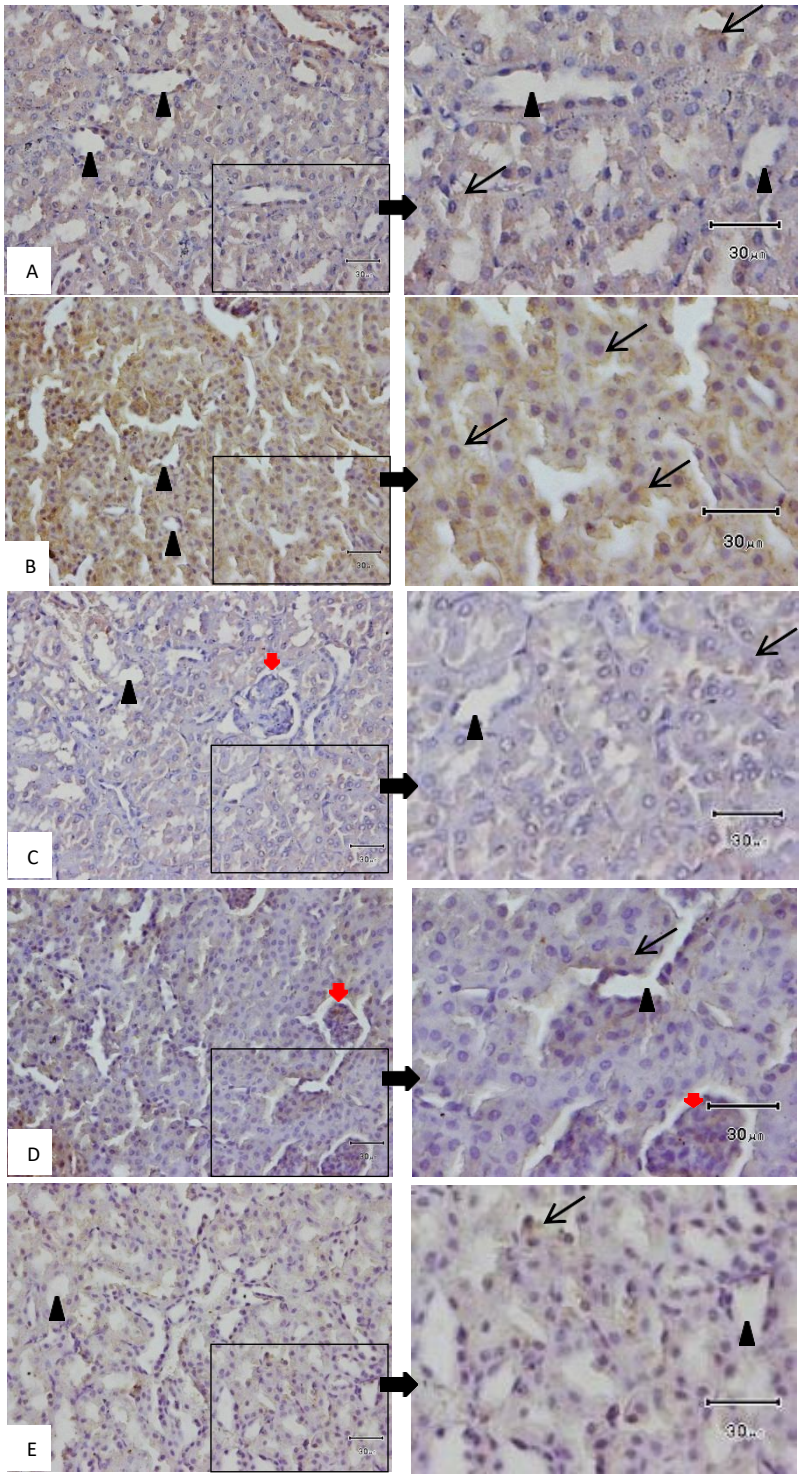

Figure 3. Distribution of CuZnSOD in kidney tissue of each treatment (magnification 400x). The right column shows the zooming 2x of left image: (A) control; (B) nephrolithiasis; (C) Calcusol™; (D) nephrolithiasis and Calcusol™; and (E) nephrolithiasis and Calcusol™ administration simultaneously. (→) indicates the CuZnSOD expression in kidney cells, (▲) tubules, and ( ) glomerulus. Bar scale: 30 µm.

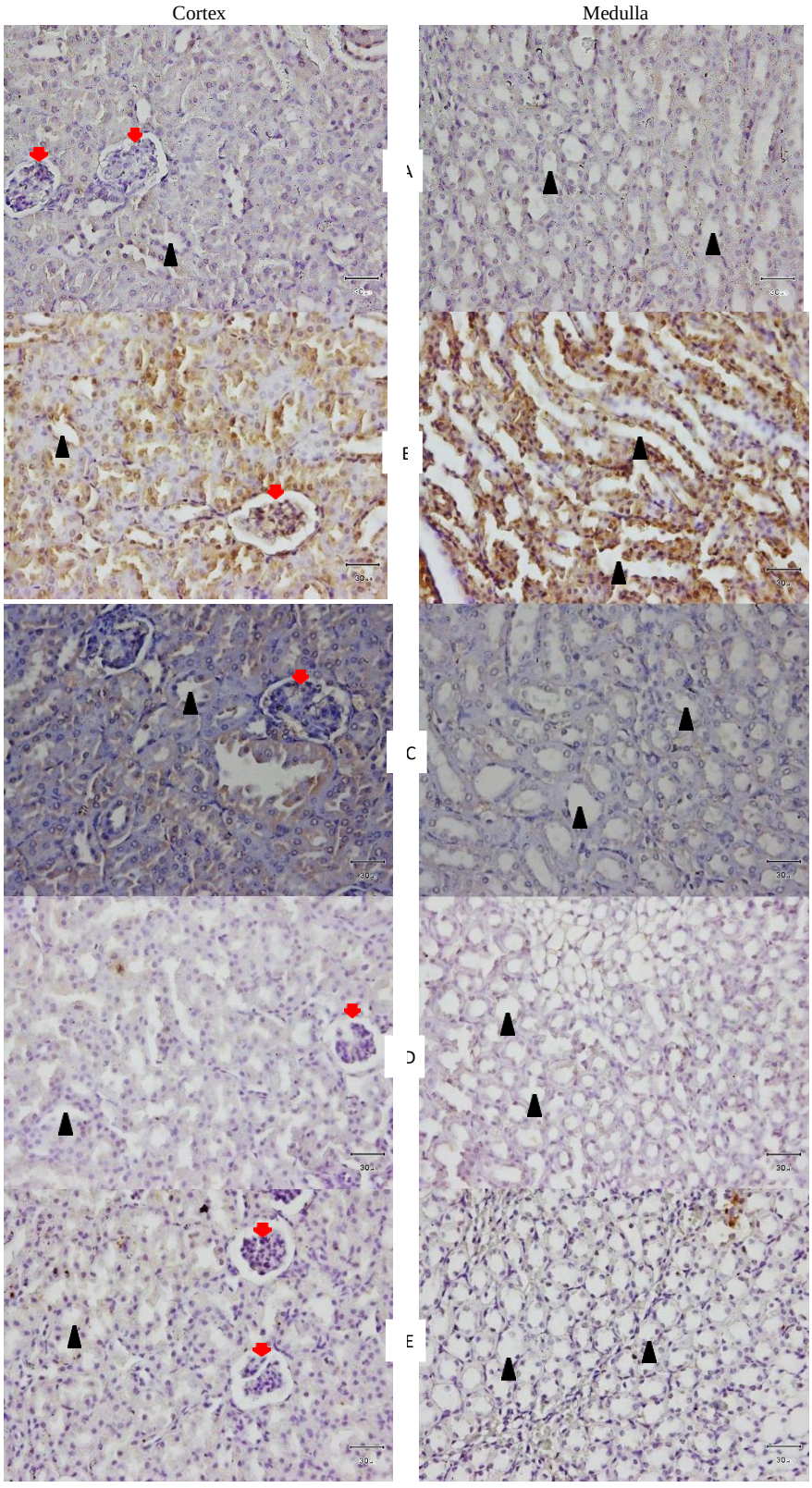

Figure 4. Histology cortex and medulla in each treatment: (A) control; (B) nephrolithiasis; (C) Calcusol™; (D) nephrolithiasis and Calcusol™; and (E) nephrolithiasis and Calcusol™ administration simultaneously. Renal cortex looks more tightly with larger cells and some glomerulus. Renal medulla composed of tubules with smaller cells. (▲) Tubules and (●) glomerulus. The bar scale: 30 µm.

CuZnSOD expression in the kidneys distributed evenly (figure 2). Analysis of independent samples t test found no significancy between the cortex and medulla (P> 0.05).

DISCUSSION

The increase of CuZnSOD and MDA (Rosyidah, 2013) simultaneously after induction of nephrolithiasis indicated that oxidative stress did not suppress the expression of CuZnSOD in the kidney. Oxidative stress in this model was triggered by excessive ROS production. Oxidative stress can occur by two mechanisms: (1) reduction of intracellular antioxidant activity, in this case is the antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase produced in the cell; and (2) the increase of ROS production caused by exposure to high oxygen or the presence of the toxin compounds that were metabolized to produce ROS, and excessive activity [5]. In this study, induction of oxalate porang could increase the production of free radicals that were marked by the MDA (Rosyidah, 2013). However, oxidative stress did not reduce the production of CuZnSOD enzyme in the kidney.

Administration of Calcusol™ after induction of nephrolithiasis could reduce the number of cells those expressed CuZnSOD and free radicals in the kidney. It suggested that the antioxidant compounds (such as flavonoids) in Tempuyung extracts contained in Calcusol™ could directly scevenge ROS by giving up electrons and/or protons to free radical molecules (Akhlaghi and Bandy, 2009) without inducing the cells to produce the CuZnSOD enzyme. Flavone, contained in tempuyung leaves, is one of the most powerful flavonoids in reducing ROS produced by the body. The members of flavone are kaempferol, quercetin, and luteolin. Flavonoids reduce free radical molecules to become more stable and less reactive. Flavonoids stabilize ROS via the following reaction (Nijveldt *et al*, 2001).

$$\text{flavonoid(OH)} + R\cdot > \text{flavonoid(O}\cdot\text{)} + RH$$

The ability of the active compounds in reducing free radicals can be determined with IC_{50} values in the DPPH test, which indicates the concentration needed to scavenge 50% DPPH radical. IC_{50} values of methanol fraction of Tempuyung leaves extract in DPPH test was 3.4 ± 0.3 mg/ml (Khan, 2012). The lower the IC_{50} value, the stronger the antioxidant capabilities. The ability of antioxidants rated high if the IC_{50} between 10-20 μg/ml, moderate in the range 21-100 μg/ml, low in the range 101-200 μg/ml, and there is no active compound if IC_{50} is more than 200 μg/ml (Lubis *et al*, 2011). The strength of the active flavonoid compounds are also determined by the location and number of hydroxyl groups that owned the compound (Amic *et al*, 2003).

The pathogenesis of kidney stones occurs through the process of nucleation, crystal growth, crystal aggregation, and attachment of crystals in the renal tubules (Zhai *et al*, 2013). The Increase of ROS production that causes oxidative stress in renal epithelial cells can make cells undergo necrosis. By necrosis, oxalate crystal nucleation can take place more quickly (Tsujihata, 2007). It occurs when cell debrises are more likely to attract calcium or other mineral salts to precipitate through a process called dystrophy calcification (Kumar *et al*, 1992). By reducing free radicals, lipid peroxidation can be inhibited and cell necrosis can be terminated, so that the crystal nucleation can be decreased.

The increase of number of cells expressing CuZnSOD and MDA in synergy does not necessarily indicate that CuZnSOD in high level could reduce superoxide radicals much effectively. This is because hydrogen peroxide (H_2O_2), the result of superoxide convertion by CuZnSOD, can become pro-oxidant through the fenton reaction into the more reactive hydroxyl radical (Goode and Webster, 1993). Hydrogen peroxide should be directly catalyzed by the catalase and/or glutathione peroxidase enzyme into H_2O and O_2, whereas in this study there was no data on the levels of catalase and glutathione peroxidase enzymes which also act as intracellular antioxidants. If ROS decreased these two antioxidant enzymes and other non-enzymatic endogenous antioxidants, then oxidative stress could occur. Superoxide radical can decrease the activity of antioxidant enzymes such as catalase and glutathione peroxidase (Halliwell and Gutteridge, 1999).

Proximal tubule is a functional part of the kidney which is susceptible to oxidative stress, because it contains many mitochondria (the site of oxidative phosphorylation). It shows that tubular cells have a high metabolism activity. Metabolism yields ATP as a source of energy to perform reabsorption of water, ions and glucose for homeostasis. However, when mitochondria membrane potential disrupted, the electron transport chain will produce more ROS (Ozbek, 2012).

Proximal tubules are mostly found in the renal cortex, but its extension in the form of the loop of Henle located in medulla and also acts in the reabsorption process (Bloom and Flawcet, 2002). Medulla which consists of the loop of Henle and collecting tubules is formed by cells that actively metabolize. ATP yielded in the cell are needed for reabsorption and secretion (Patton and Thibodeau, 2000).

Based on the result concluded that Calcusol™ administration could reduce the number of cells expressing CuZnSOD in the kidneys of mice of nephrolithiasis model. There was no significancy found in the number of cells expressing CuZnSOD in the renal cortex and medulla. It was suspected that antioxidant compounds in Calcusol™ could reduce ROS without inducing the cells to produce the CuZnSOD enzyme.

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