Jurnal Farmasi Galenika (*Galenika Journal of Pharmacy*) (e-Journal) 2023; 9 (1): 55 – 70
ISSN: 2442-8744 (electronic); 2442-7284 (printed)
Journal Homepage: https://bestjournal.untad.ac.id/index.php/Galenika
DOI: 10.22487/j24428744.2023.v9.i1.15992

Vitamin C Elicits Protection against Cylophosphamide-induced Nephrotoxicity in Rat Animal Model

(*Vitamin C Memproteksi terhadap Nefrotoksisitas yang Diinduksi Siklofosfamid pada Hewan Model Tikus*)

Dwi Yulianti Alifah[1], Yulia Yusrini Djabir[2*], Prihantono Prihantono[3]

[1]*Postgraduate Program, Faculty of Pharmacy, Hasanuddin University, Makassar, Indonesia.*
[2*]*Laboratory of Clinical Pharmacy, Faculty of Pharmacy, Hasanuddin University, Makassar, Indonesia.*
[3]*Department of Surgery, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia.*
[*]*E-mail: dwialifah17@gmail.com*

Article Info:
Received: 09August 2022
in revised form: 27 December 2022
Accepted: 27 Januari 2023
Available Online: 27 Januari 2023

Keywords:
Creatinine
Urea
Nephrotoxicity
Urine protein
Cyclophosphamide
Vitamin C

Corresponding Author:
Yulia Yusrini
Department of Pharmacy
Faculty of Pharmacy
Hasanuddin University
Makassar
90245
Indonesia
email: dwialifah17@gmail.com

ABSTRACT

Background: Cyclophosphamide (CPD) causes renal cell toxicity due to its toxic metabolites. **Objectives:** This study aimed to evaluate the nephroprotective effect of vitamin C at 125, 250, and 500 mg/kg doses based on the biomarker level of urea, creatinine, urinalysis and renal histopathology. **Material and Methods:** The experimental animals consisted of 25 rats that were divided into 5 treatment groups: healthy control, placebo (water for injection + CPD 250mg/kg), and 3 vitamin C treatment groups (125, 250, or 500 mg/kg + CPD 250mg/kg). **Results:** The results of blood biomarker, urine analysis, and histopathological analysis showed that CPD-induced nephrotoxicity was characterized by an increase in urea levels from 21.79 mg/l to 156.65 mg/l, creatinine from 0.375 to 0.717 mg/l, urine protein from 0 to 2.7, with histopathological damage scores from mild to severe (scores 1-3). In the treatment groups, the average damage score was 1-2 (mild score). However, of the three doses used, only the 500 mg/kg dose had significantly improved biomarkers compared to the placebo group, including the urea, creatinine, and urine protein levels, as well as histopathological scores ($p<0.05$). **Conclusions:** Vitamin C at a dose of 250 mg/kg was able to prevent the increase of urea, creatinine, and urine protein levels. However, a higher dose (500 mg/kg) was required to provide optimal protection against renal structural damage caused by cyclophosphamide.

How to cite (APA 6th Style):
Alifah, D. Y., Djabir, Y. Y., Prihantono, P. (2023). Vitamin C elicits protection against cylophosphamide-induced nephrotoxicity in rat animal model. *Jurnal Farmasi Galenika: Galenika Journal of Pharmacy (e-Journal), 9*(1), 55-70. doi:10.22487/j24428744.2023.v9.i1.15992

ABSTRAK

Siklofosfamid (SFD) menyebabkan toksisitas sel ginjal karena metabolitnya bersifat toksik. Penelitian ini bertujuan untuk mengevaluasi efek nefrotoksisitas vitamin C dengan dosis 125 mg/kgBB, 250 mg/kgBB, dan 500 mg/kgBB berdasarkan kadar biomarker ureum dan kreatinin, parameter urinalisis dan analisis histopatologi ginjal. Hewan uji terdiri dari 25 ekor tikus (*Rattus norvegicus*) yang dibagi kedalam 5 kelompok perlakuan uji, kelompok 1 (kontrol sehat), kelompok 2 (*water for injection* + SFD), kelompok 3 (vitamin C 125 mg/kgBB + SFD), kelompok 4 (vitamin C 250 mg/kgBB + SFD) dan kelompok 5 (vitamin C 500 mg/kgBB + SFD). Hasil dari pemeriksaan biomarker darah, analisis urin dan analisis histopatologi menunjukkan bahwa SFD memiliki efek nefrotoksisitas yang ditandai dengan peningkatan kadar ureum dari 21,79mg/l menjadi 156,65 mg/l, kreatinin dari 0,375 menjadi 0,717 mg/dl, protein urin dari 0 menjadi 2,7, dengan skor kerusakan histopatologi dari ringan hingga parah (skor 1-3). Pada kelompok 3, 4 dan 5 skor kerusakan rata-rata 1-2 (skor ringan). Namun dari ketiga dosis yang digunakan, hanya kelompok 5 yang memberikan perbedaan signifikan dari kelompok 2 baik dari kadar ureum, kreatinin, Protein urin maupun skor histopatologinya ($p<0.05$). Disimpulkan bahwa vitamin C dosis 250 mg/kgBB mampu mencegah peningkatan kadar ureum, kreatinin, protein urin, namun dibutuhkan dosis yang lebih tinggi (500 mg/kg) untuk memberikan perlindungan yang optimal terhadap kerusakan struktur ginjal akibat siklofosfamid.

Kata kunci: Kreatinin, Ureum, Nefrotoksisitas, Protein Urin, Siklofosfamid, Vitamin C.

INTRODUCTION

Cancer treatment is very complex because in addition to have anticancer activity, this class of drugs is also destructive to normal body cells. Cyclophosphamide (CPD) is a cytotoxic drug used for treating, prolonging lifespan, or alleviating cancer symptoms (palliative). Following the metabolism by liver enzymes, the active metabolite of cyclophosphamide forms covalent bonds with DNA and proteins, leading to cellular death. The cytotoxic effect of cyclophosphamide is not cell cycle specific since cyclophosphamide can cause DNA damage in all phases of cell cycle. In addition, several active metabolites of cyclophosphamide have been shown to trigger apoptosis by suppressing the synthesis of antioxidant glutathione (GSH) in the cells (Panigrahy *et al*., 2011).

The manifestation of drug-induced nephrotoxicity of CPD include the abnormalities of urinary sediment, electrolyte imbalance, and most commonly, a decrease in the glomerular filtration rate. Cyclophosphamide is an inactive cytostatic and is metabolized in liver cells into active metabolites. During bioactivation, *reactive oxygen species* (ROS) are also formed and can reduce natural antioxidant capacity. Cyclophosphamide causes renal cell toxicity because of its two toxic metabolites. The two active metabolites of cyclophosphamide are mustard phosphoramide and acrolein. Phosphoramide produces antineoplastic effects and acrolein produces free radicals by interacting with the body's antioxidant defense system (Singh *et al.,* 2014).

Previously, it was found that a 150 mg dose of cyclophosphamide was able to induce kidney damage which was characterized by the increase of serum blood urea nitrogen (BUN) and creatinine (Cr), renal tissue damage, and high levels of renal tissue malondialdehyde (MDA) in rats injected with cyclophosphamide (Subramanian *et al.,* 2019). In another study, the toxic effect of cyclophosphamide

was tested with different doses (100 mg, 200 mg, and 250 mg) where the results at a dose of 250 mg/kg on day 7 already showed a toxic effect, while at a low dose it showed a toxic effect on day 7 28. The study suggested monitoring of liver and kidney function with hepatoprotective and nephroprotective administration along with the use of cyclophosphamide (Bhat *et al.,* 2018).

Vitamin C is a very powerful antioxidant compound that can scavenge free radicals and other reactive oxygen species (ROS) (Lee *et al.,* 2001). A previous study reported that a dose of 250 mg/kg of vitamin C was effective in reducing kidney and liver dysfunction in doxorubicin-injected rats (Djabir *et al.,* 2016). It has also been investigated that doses of 50, 100, and 200 mg of vitamin C were able to reduce nephrotoxicity in rats based on the volume of urine and glomerular filtration rate (Lusania, 2000). Accordingly, this study aimed to examine the protective effect of vitamin C against cyclophosphamide-induced nephrotoxicity in rats based on blood biomarker levels, protein urine level, and histopathological examination.

MATERIAL AND METHODS

Materials

The materials used in this study were 70% and 90% alcohol, aquadest, 10% v/v Neutral Formalin Buffer (BNF), diethyl ether, hematoxylin eosin (HE), Cyclophosphamide 1000 mg (Kalbe Farma, Indonesia) , creatinine and urea diagnostic kit (Human®), sodium chloride, paraffin, 100% alcohol reagent (mixture of 90% v/v absolute ethanol, 5% v/v methanol, and 5% v/v isopropanol), Vitamin C powder (Merck), U -120® urine analyzer, sterilized water for injection..

Methods

Animals

The experimental animals consisted of 25 male Wistar rats (*Rattus novergicus*) weighing at 200-250 g. All animals were adapted under laboratory conditions for 7 days and given standard pellets and drink every day. Rats were divided into 5 treatment groups, each group consisted of 5 rats placed in one cage. The ethical clearence was isused by the Faculty of Medicine, Hasanuddin University, with the number of 82/UN4.6.4.5.31/PP36/2021.

Preparation of Vitamin C Solution

The vitamin C doses varied from 125 mg, 250 mg to 500 mg/kg. A dose of 250 mg/kg was found to be able to improve the kidney function of rats given doxorubicin (Djabir, *et al.,* 2016; Lusiana, 2000). For a dose of 125 mg, 3750 mg of ascorbic acid (Vitamin C) powder was weighed and then dissolved using 30 mL of aquadest to form a stock solution of Vitamin C with 125mg/mL concentration. For a 250 mg dose, 7500 mg of ascorbic acid (Vitamin C) powder dissolved in 30 mL of aquadest to get a stock

solution of Vitamin C with a concentration of 250 mg/mL. As for a dose of 500 mg, 1500 mg of ascorbic acid (Vitamin C) powder was dissolved using 30 mL of aquadest to obtain a stock solution of Vitamin C with a concentration of 500 mg/mL.

Cyclophosphamide Dose Calculation

Cyclophosphamide is available in powder form with a dosage strength of 1000 mg reconstituted with 50 mL sterile water for injection. The therapeutic dose of cyclophosphamide for cancer treatment in humans is 40-50 mg/kg, however, the dose applied in this study was based on animal study that produced nephrotoxicity (250 mg/kg or 50 mg/200g) when injected in rats intraperitorially (Bhat, *et al.,* 2018). As much as 1000 mg of cyclophosphamide was dissolved with 50 mL of water for injection. The end result, the concentration of cyclophosphamide solution was 50 mg/2.5 mL with the volume of injection of 2.5 ml per 200 g rats.

Experimental Procedures

In this study, the experimental animals were divided into 5 groups. They are:

1. Group I (n=5) as a healthy control was given only 2.5 ml/200g of water orally.
2. Group II (n=5) as a placebo group was given water orally for 5 days and intraperitorial (i.p.) injection of 250mg/kg cyclophosphamide (CPD) on day 6.
3. Group III (n=5) was given a solution of vitamin C 125 mg/kg orally for 5 days and an i.p. injection of 250 mg/kg (2.5 ml/200 g) CPD on day 6.
4. Group IV (n=5) was given 250 mg/kg Vitamin C orally for 5 days and an i.p. injection of CPD on day 6.
5. Group V (n=5) was given 500 mg/kg Vitamin C orally for 5 days and an i.p. injection of CPD on day 6.

Blood and Urine biomarker analysis

Before treatment (day 0), after 24 hours of CPD injection (day 7), and after 72 hours of CPD injection (day 10), the rat serum was obtained to measure the creatinine and urea levels. A 2 ml of rat blood was placed in the EDTA vacutainer tube, homogenized and then centrifuged at 3000 rpm for 20 minutes to separate serum and blood plasma. Blood samples were analyzed using a Semi-Auto Chemistry Analyzer with a spectrophotometry. In addition to blood samples, fresh urine of rats was also collected before and after treatments, then were analyzed using urinalysis machine (verify U-120). The urinalysis parameters assessed were urine protein and pH.

Histopathological examination

1. Organ Sampling

   After treatment, the rats were euthanized by cervical dislocation and the kidneys were harvested and placed into a container containing 10% BNF for the histopathological analysis

2. Preparation of Kidney Histopathology Preparations

   The rat renal tissue samples were fixed for 48 hours using BNF with the volume 10 times of the tissue volume. The fixed tissue specimens were then cut using a scalpel knife with a thickness of 0.5 – 1 cm. The rest of the specimens were stored in tightly closed bottles containing 10% BNF. The cutting specimens are inserted into the embedding cassette, then processed into the tissue processor. The specimen is removed from the basket and then placed on a mold and filled with paraffin. After the paraffin blocks were set, the mold is sepearated from the basket and it is ready to be sliced using a microtome with a thickness of 4-5 µm. The specimens were then stretched in a water bath with a temperature of about 40^{o}C. then sprinkled with gelatin powder as much as 5 grams for 100mL of distilled water then allowed to stand until completely dissolved. The results of the cuts were taken using a microscope slide and then placed on a heating plate for 2 hours. Staining was done using Mayer's Haematoxylin and Eosin and dried in an oven overnight (Wahyuni *et al.,* 2012).

3. Examination of Kidney Histopathology

   Observations of histopathological preparations of the kidneys were carried out under a light microscope with a magnification of 10X and 100X to observe morphological changes of the examined specimens. Parameters of kidney damage can be seen in Table 1 (Nurlina *et al.,* 2014).

Table 1. Parameters of Kidney Damage Level (Nurlina *et al.,* 2014)

Score	Glomerulus	Tubules
0	Normal	Normal
1	Edema	Parenchymal degeneration
2	Necrosis*	Hydrophilic degeneration
3	Inflammation around the glomerulus	Tubular cell vacuolization
4	Athrophy	Necrosis**

Edema	:Bowman's space expansion
Necrosis	:*The core disappeared partly
	**There is protein in the tubular lumen
Inflammation in glomerulus	:Multiple adjacent cells and more colored dark
Atrophy	: Core lost, glomerular shrinkage
Parenchymal degeneration	:Tubular cell granules
Hydropic degeneration	:Cells are swollen/filled with water
Vacuolization	:Empty space, cell nucleus disappears

Statistical analysis

The data were analyzed using a parametric test for numerical data. The biomarker data were analyzed with Saphiro-Wilk to test for normality, then analyzed with One-way ANOVA test followed by the Post

hoc Tukey HSD test to determine the significant differences. The results obtained were declared significant if the P value <0.05.

RESULTS AND DISCUSSION

Biomarker analysis

In this study, biomarker examination was carried out after 1x24 hours and 3x24 hours after administration of cyclophosphamide. The results showed that the use of CPD at the dose of 250mg/kg increased the levels of urea and creatinine within 1x24 hours after the administration.

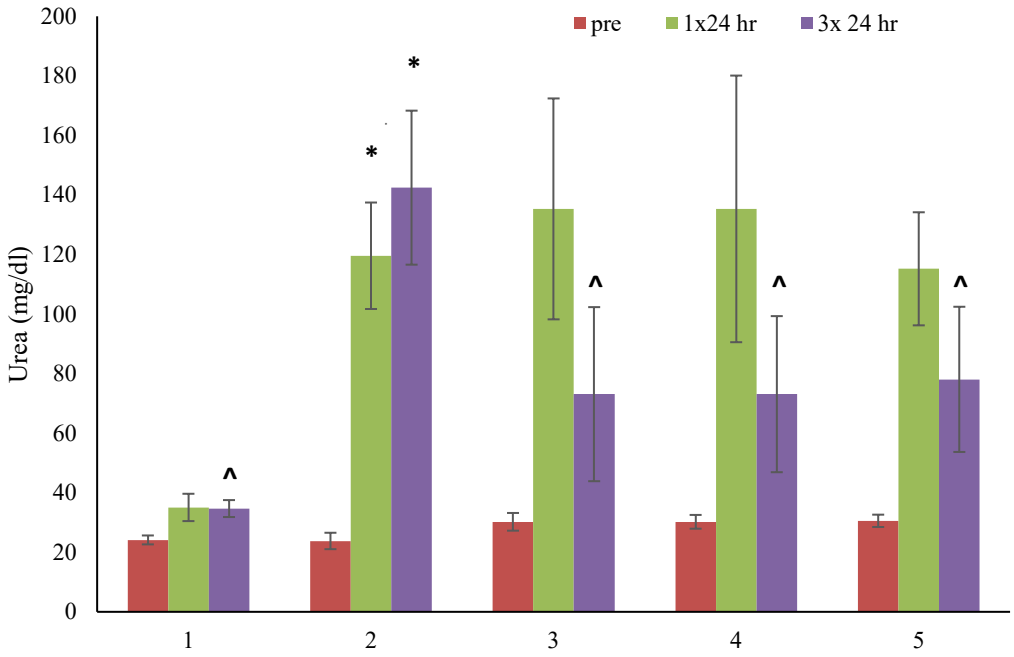

Figure 1. Comparison of urea levels in each group at baseline (blue), 24 hours after CPD injection (red), and after 72 hours CPD injection (green). 1 = Healthy Control, 2 = Placebo + Cyclophosphamide 250mg/kg, 3 = Vitamin C 125mg/kg + Cyclophosphamide 250mg/kg, 4 = Vitamin C 250 mg/kg + Cyclophosphamide 250mg/kg, 5 = Vitamin C 500mg/kg + Cyclophosphamide 250mg/kg. *p<0.05 compared to healthy control and ^p<0.05 compared to placebo group at 72 hours after CPD injection.

Figure 1 shows that at a dose of 250mg/kg of CPD in Group 2, urea levels increased from 21.79 mg/l on the first day to 125.724 mg/dl after administration of CPD 250 mg/kg, and to 156.65 mg/dl after 72 hours from injection. Based on one way ANOVA, Group 1, which is a healthy control, did not show a significant change in the value of urea levels after 6 and 10 days of treatment. The three vitamin C-treated groups, which were also given cyclophosphamide, also experienced an increase in urea levels, but it was significantly lower when compared to the placebo group. This suggests that vitamin C has a nephroprotective effect indicated by the value of urea levels in Groups 3 to 5. Previous study conducted by Djabir et al (2016), rats treated with vitamin C or E were found to improve the kidney function of

rats injected with doxorubicin. This is presumably due to the antioxidant ability of vitamin C in reducing ROS by forming stable ascorbate free radicals, since their molecular structures enable them to resonate making it relatively stable (Farshid *et al.,* 2013).

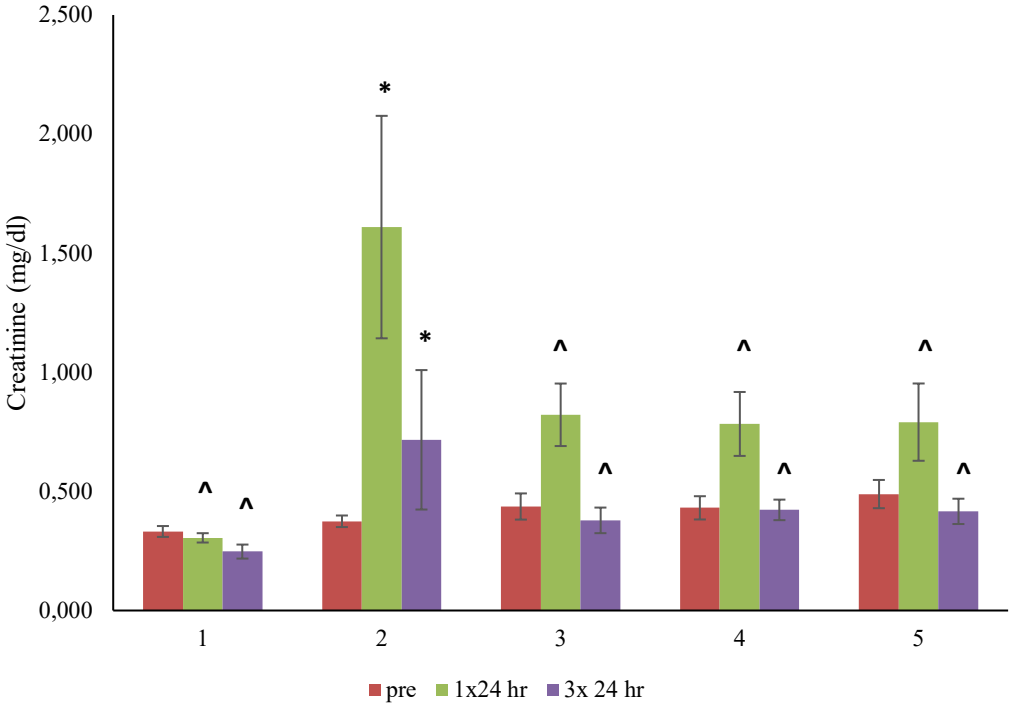

Figure 2. Comparison of creatinine levels at baseline (blue), 24 hours after CPD injection (red), and after 72 hours CPD injection (green). 1 = Healthy Control, 2 = Placebo + Cyclophosphamide 250mg/kg, 3 = Vitamin C 125mg/kg + Cyclophosphamide 250mg/kg, 4 = Vitamin C 250 mg/kg + Cyclophosphamide 250mg/kg, 5 = Vitamin C 500mg/kg + Cyclophosphamide 250mg/kg. *p<0.05 compared to healthy control and ^p<0.05 compared to placebo group at 24 and 72 hours after CPD injection.

In Figure 2, a dose of 250 mg/kg of cyclophosphamide (Group 2) caused an increase in creatinine levels in rats from 0.375 µg/l at baseline to 610 µg/l after 24 hours of CPD administration, and rose to 0.717 µg/l after 72 hours. Based on statistical analysis, group I, which is a healthy control, did not show any significant change in the levels of creatinine after 7 days of treatment. Similarly, the other groups which also received CPD injection (3, 4, 5) but pretreated with vitamin C experienced an increase in creatinine levels, but not as much as group 2 (placebo). Farshid et al (2013) revealed that the administration of Vitamin C 200 mg/kg can attenuate the histopathological changes of the bladder wall caused by cyclophosphamide injection (Farshid *et al.,* 2013).

Table 2. Urinalysis results receiving cyclophosphamide and Vitamin C therapy

Urinalysis Parameters	**Treatment Groups**					**Initial range**
	1	2	3	4	5	
Pro (g/l)	0.1	2.7	0.6	1.0	2.5	0 - 0.3
Ph	7.7	7.8	8.1	7.5	7.5	6 - 8

1 = Healthy Control, 2 = Placebo + Cyclophosphamide 250mg/kg, 3 = Vitamin C 125mg/kg + Cyclophosphamide 250mg/kg, 4 = Vitamin C 250 mg/kg + Cyclophosphamide 250mg/kg, 5 = Vitamin C 500mg/kg + Cyclophosphamide 250mg/kg.

In the group 2 that had no pretreatment of vitamin C, the injection of cyclophosphamide caused an increase in urine protein of 2.7 U/L indicating the occurrence of kidney failure. In the groups 3 and 4, there was also an increase in urine protein found, but it was not as high as the Group 2. This may indicate that the administrations of 125 mg/kg and 250mg/kg of vitamin C reduce the occurrence of kidney failure. Meanwhile, in the group 5, the urine protein was 2.5 U/L (Table 2). This happened may be due to the high dose of vitamin C given. The proteinuria persists in this group indicating that the administration of 500 mg/kg vitamin C was somehow not able to improve kidney dysfunction induced by cyclophosphamide. Meanwhile, the pH parameter of rat urine did not increase or was still in the normal range in all groups. The administration of high dose of vitamin C may harmful to kidney function because it can concentrate the urine and may disturb the process of filtration, reabsorbtion and secretion of renal tubules (Abdullah, 2022). Therefore, the administration of vitamin C seen in group 5 at a dose of 500mg/kg was considered ineffective to reduce the urine protein levels, which was different from that in groups 3 and 4.

Histopathological examination

Table 3. The scoring of histopathological damage found in rats

Group	Sample	Observation Results (score)
I	A	(0)
	B	(0)
	C	(0)
	D	(0)
	E	(0)
II	A	Fibrosis in the glomerulus, 60% more necrotic in the tubules (3)
	B	There are inflammatory cells and bleeding in the glomerulus. In the tubules there is thickening of the basement membrane and 25-60% necrotic damage (2)
	C	There are inflammatory cells and bleeding and thickening of Bowman's capsule in the glomerulus. Thickening of the basement membrane and 25-60% necrotic damage (2).
	D	There are inflammatory cells and bleeding in the glomerulus. Thickening of the basement membrane and 25-60% necrotic damage in the tubules (2).
	E	There are inflammatory cells and bleeding in the glomerulus. There is thickening of the basement membrane and 25-60% necrotic damage (2).

III	A	The tuft is pulled and accompanied by inflammation in the glomerulus, thickening of the capsule occurs 25-60% damage is found in tubules.
	B	Tuft is pulled and accompanied by inflammation and thickening of the capsule in the glomerulus. 25-60% damage and widening of the membrane is found in the tubules (2).
	C	Tuft is pulled with inflammation in the glomerulus. 25-60% damage is found in the tubules (2).
	D	At the glomerulus, tuft is pulled with inflammation and thickening of the capsule. In the tubules there are 25-60% damages and membrane widening occurs (2).
	E	Red cells in the glomerulus and 25% inflammatory cells damage is found in the tubules (1).
IV	A	Tuft in the glomerulus is pulled, the basement membrane thickens in the tubules (2)
	B	Glomerulus capsule thickens and 25% damage is found in the tubules (1)
	C	At the glomerulus, tuft is attracted. In the tubules, the basement membrane is thickened (2)
	D	Light bleeding in the glomerulus. 25% necrotic damage is found in the tubules 25% (1)
	E	Light bleeding in the glomerulus. In the tubules there is 25 % necrotic damage (1)
V	A	Light bleeding in the glomerulus, in the tubules minor damage less than 25% (1)
	B	There are inflammatory cells in the glomerulus. in the tubules minor damage less 25% (1)
	C	(0)
	D	(0)
	E	thickening of the capsule in the glomerulus. In the tubules light bleeding and minor damage is less than 25% (1)

1 = Healthy Control, 2 = Placebo + Cyclophosphamide 250mg/kg, 3 = Vitamin C 125mg/kg + Cyclophosphamide 250mg/kg, 4 = Vitamin C 250 mg/kg + Cyclophosphamide 250mg/kg, 5 = Vitamin C 500mg/kg + Cyclophosphamide 250mg/kg**.** The degree of damage to heart muscle cells is normal (0), normal (1), edema (2) necrosisx, and (3) inflammation around the glomerulus.

The group 1 (healthy controls) showed mormal histological structure of the rat kidney. No significant damage was found in the structure of the rat kidney (Figure 3). Figure 4 is the microscopic pictures of renal structure of group 2, that only received placebo and cyclophosphamide 250 mg/kg. In contrast to controls, in group 2 given cyclophosphamide and placebo, showed a mild to moderate damage (Table 3). The tissue damage changes were spread and visible at 40X magnification, including moderate histopathological changes, such as fibrosis, inflammatory cells and bleeding, thickening of the basement membrane, and necrotic damage.

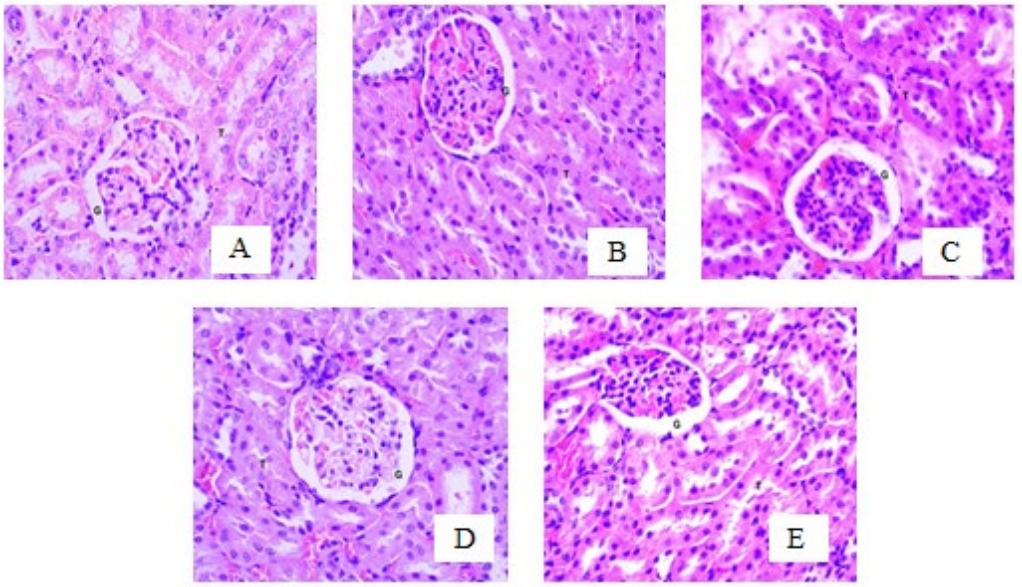

Figure 3. Histological microscopic view of group 1 kidney displays renal cells with normal structure at 40X magnification.

The administration of cyclophosphamide often causes complications in cancer patients. Due to nephrotoxicity, cyclophosphamide causes renal cell toxicity due to its two toxic metabolites. The two active metabolites of cyclophosphamide are mustard phosphoramide and acrolein. Phosphoramide produces antineoplastic effects and acroleinin produces free radicals by interacting with the body's antioxidant defense system (Singh *et al.,* 2014). Bhat et al investigation showed harmful effects of cyclophosphamide on kidneys. The pathology ranges from mild infiltration of inflammatory cells to necrosis, and even cytolysis was seen in the kidneys (Bhat *et al.,* 2018).

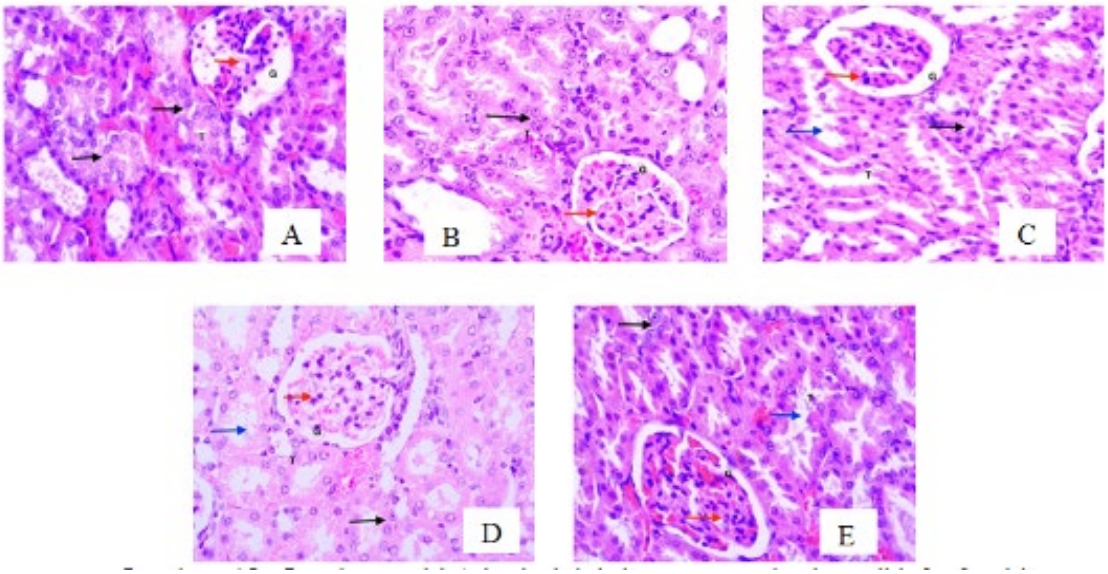

Figure 4. Histological picture of kidneys with 250mg/kg cyclophosphamide showing renal cells with the structure at 40X magnification; A = Glomerulus (G) with fibrosis (red), More than 60% of necrotic (black) in Tubules (T); B = Moderate

damage. Glomerulus (G) contains inflammatory and bleeding cells (red). Tubules (T) with thickening of the basement membrane (blue) and 25-60% necrotic damage (black); C = Glomerulus (G) with inflammatory cells and bleeding (red) and thickening of Bowman's capsule (white). Tubular (T) basement membrane thickening (blue) and 25-60% necrotic damage (black); D = Moderate damage. Glomerulus (G) contains inflammatory and bleeding cells (red). Tubules (T) thickening of the basement membrane (blue) and 25-60% necrotic damage (black), E = Glomerulus (G) with inflammatory cells and bleeding (red).

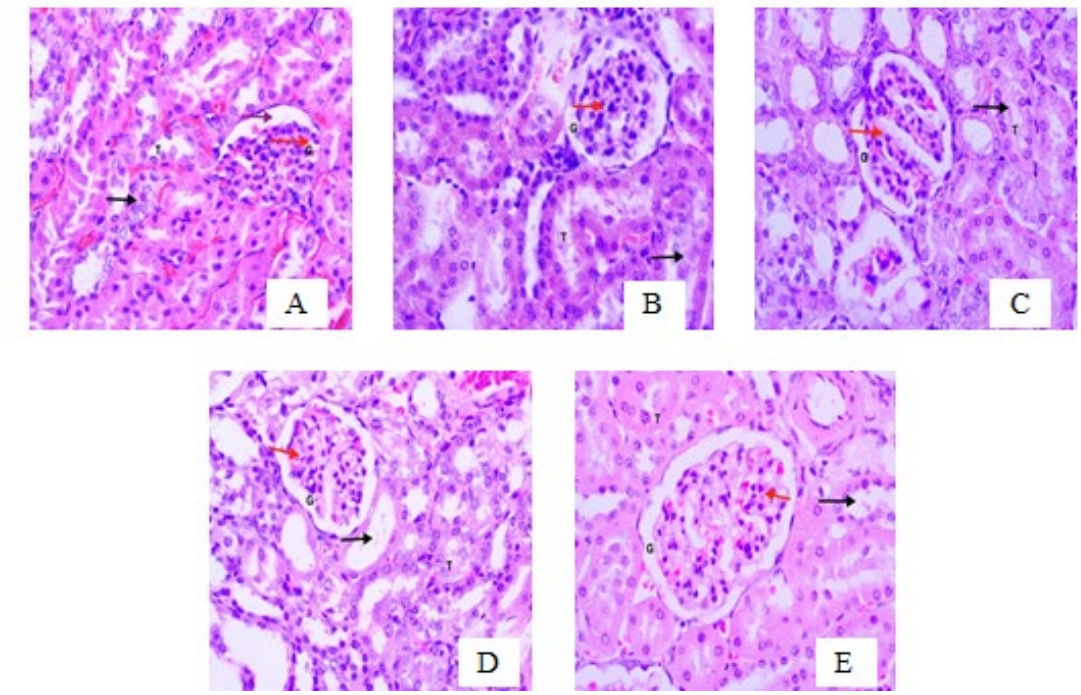

Figure 5. Histological picture of kidneys with the administration of 250 mg/kg cyclophosphamide with 125mg/kg Vitamin C pretreatment showing renal cells at 40X magnification; A = Glomerulus (G) tuft is stretched and accompanied by inflammation (red) and thickening of the capsule (purple). 25-60% damage (black) in Tubulus (T); B = Glomerulus (G) tuft pulled and accompanied by inflammation (red and thickened capsule (white)). Tubulus (T) 25-60% damage (black) and membrane widening (blue); C = Glomerulus (G) tuft is pulled and accompanied by inflammation (red). Tubules (T) damage 25-60% (black); D = Moderate damage. Glomerulus (G) tuft is stretched and accompanied by inflammation (red and thickened capsule (white)). Tubules (T) 25-60% damage (black) and membrane widening (blue); E = Glomerulus (G) there are inflammatory cells (red). Tubules (T) 25% damage (black).

In the group 3 that was given 250mg/kg cyclophosphamide with 125mg/kg Vitamin C pretreatment, the microscopic observation showed a mild to moderate damage at 40X magnification. As seen in Figure 5, the damage include inflammation and thickening of the capsule in the glomerulus, as well as tubulus degeneration.

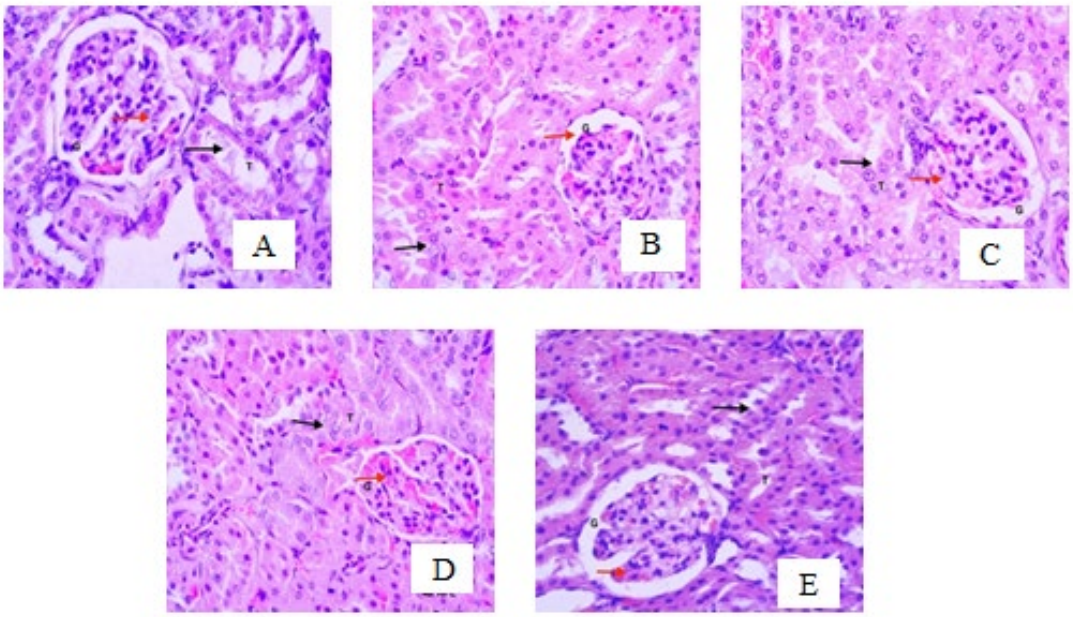

Figure 6. Histological picture of the kidney with the administration of 250mg/kg cyclophosphamide + 250mg Vitamin C shows renal cells with the following structure; A: Glomerulus (G) tuft is pulled (red). Tubule (T) basement membrane thickening (black); B: Glomerulus (G) capsule thickening (red). Tubules (T) 25% damage (black); C: Moderate damage. Glomerulus (G) tuft pulled (red). Tubule (T) basement membrane thickening (black); D: Glomerulus (G) light bleeding (red). Tubule (T) 25% necrotic damage (black), Glomerulus; E = (G) light bleeding (red). Tubules (T) 25% necrotic damage (black) at 40X Magnification.

In group 4, treatments with 250mg Vit C for 5 days before cyclophosphamide injection showed renal sructure abnormalities, with the score of mild to moderate (Figure 6). The histological changes observed including changes in the structure of the glomerulus, where the tuft was attracted and the presence of hemorrhage. Whereas, in the tubules, the basement membrane was thickened (see Table 3). In group 5, with 500 mg/kg dose of Vitamin C treatment for 5 days showed mild tissue damage in A, B, and E, but in the other 2 rats (C,D), there was no damage found in the renal structure (Figure 7). This may suggest that higher dose was necessary to protect the renal structure.

The two active metabolites of cyclophosphamide are mustard and acrolein phosphoramide. Phosporamide produces antineoplastic effects and acrolein generates free radicals by interacting with the body's antioxidant defense system. These free radicals are very reactive and produce oxidation by sharing enzymes. Acreolin causes cell damage after binding with GSH and reducing its level in cells. Acreolin impairs the glutathione-dependent antioxidant system and promotes free radical formation. These free radicals increase due to damage to the Nitric oxide system in blood vessels. Cyclophosphamide causes nephrotoxicity by alkylation of renal cells by the Cys sulfhydryl groups of acrolein. Acrolein is one of the active metabolites of cyclophosphamide. Renal cell alkylation causes a variable decrease in glomerular filtration rate and tubular dysfunction due to acute kidney failure.

Cyclophosphamide also produces free radicals which cause kidney damage. Various pharmacological interventions can help reduce kidney oxidative stress and kidney failure (Singh *et al.,* 2014).

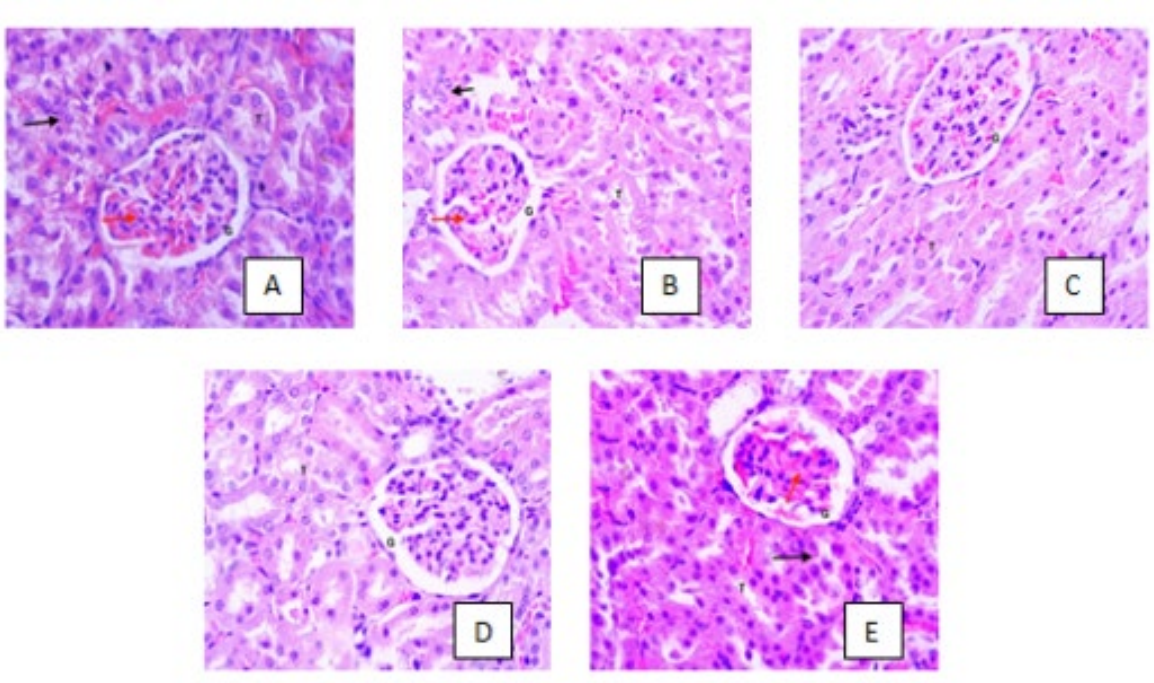

Figure 7. Histological picture of the kidney with the administration of 250mg/kgBB cyclophosphamide + Vitamin C 500mg shows renal cells with normal structure on C and D while in A: Glomerulus (G) light bleeding Tubules (T) minor damage less than 25% (Black), B: Glomerulus (G) contains inflammatory cells. Tubulus (T) less damage 25% (Black), E: Glomerulus (G) thickening of the capsule. Tubules (white) and light bleeding (red)Tubulus (T) 25% less damage (Black). The three pictures show minor damage at 40X magnification.

Vitamin C is an antioxidant compound that is very strong against free radicals and other reactive oxygen species (ROS). Thus, vitamin C has antioxidant potential and can prevent the adverse effects of free radical reactions. In this study, it was found that 250 mg of vitamin C was effective in reducing kidney dysfunction in rats injected with cyclophosphamide.

In this study, administration of cyclophophamide at a dose of 250 mg/kg did not just trigger an increase in renal biomarker levels, but also induced damages to the structure of kidneys, characterized by the tubular necrosis, tubular fibrosis, glomerular congestion and an inflammation that can cause kidney dysfunction. The use of Vitamin C with 3 different doses (125 mg, 250 mg, 500 mg) can reduce creatinine and urea levels, and normalize the urine pH of rats compared to rats given cyclophosphamide only. Histopathological examination showed cyclophosphamide caused damage to renal tissue. This study showed that administration of Vitamin C before cyclophosphamide injection could reduce renal nephrotoxicity. In fact, 2 out of 5 animals in group that was treated with 500 mg/kg of Vitamin C had normal appearance of glomerulus and tubulus.

CONCLUSION

The administration of vitamin C at a dose of 125 mg/kg to 500 mg/kg can prevent an increase in creatinine and urea levels in rats injected with cyclophosphamide. Protein urine levels was also significantly lower in rats treated with 125 and 250 mg/kg vitamin C, but not with 500 mg/kg dose. However, cyclophosphamide-induced structural damage of the kidneys can only be improved with the 500 mg/kg dose of vitamic C. Further studies on the roles of vitamin C against renal toxicity are tested on an animal model of cancer treated with cyclophosphamid. This study will be important to examine if this result can be translated in clinical setting in cancer patients.

ACKNOWLEDGEMENT

The authors would like to thank drh. Wahyuni for her assistance in histological examination.

CONFLICT OF INTEREST

All authors declare no conflict interest.

REFERENCES

Abraham P, Indirani K, Sugumar E. Effect of cyclophosphamide treatment on selected lysosomal enzymes in the kidney of rats. 2007. Pubmed Vol. 59: 143.

Antunes, Lusaˆnia M. Greggi Antunes, et al. Protective Effects of Vitamin C Against Cisplatin-Induced Nephrotoxicity and Lipid Peroxidation In Adult Rats: A Dose-Dependent Study. Pharmacological Research, Vol. 41, No. 4, 2000: 405-409.

Aroma IO. Free radicals, oxidative stress, and antioxidants in human health and disease. 1998. J Am Oil Chem Soc : 199-212.

Barnett, S., Anthony. The Story of Rats: Their Impact on Us and Our Impact on Them. 2002. Crows Nest NSW: Allen & Unwin.

Bhat N, Kalthur SG, Padmashali S, Monappa V. 2018. Toxic effects of different doses of cyclophosphamide on liver and kidney tissue in Swiss albino mice: a histopathological study. Ethiopian journal of health sciences. 28(6).

Djabir YY, Usmar, Elly Wahyudin, Sukamto, Ika Reskia, Dila Paramitha, Irma Alia. 2016. Roles of Vitamin C and Vitamin E on Doxorubicin – Induced Renal and Liver Toxicities in Rats. Nusantara Medical Science Journal.1:16-20.

Elmore A. Final report of the safety assessment of l-ascorbic acid, calcium ascorbate, magnesium ascorbate, magnesium ascorbyl phosphate, sodium ascorbate, and sodium ascorbyl phosphate as used in cosmetics. 2005. International Journal of Toxicology Vol. 24 : 51-111.

Estakhri R, Hajipour B, Majidi H, Soleimani H.Vitamin E Ameliorates Cyclophosphamide Induced Nephrotoxicity.2013.Life Science Journal : 308-311.

Johnson, M. Labome: Laboratory Mice and Rats. 2014. The World of Laboratories.

Kennedy, Groepper D, Tagen M, Christensen R, Navid F, Gajjar A. Stability of cyclophosphamide in extemporaneous oral suspensions. 2010. Ann Pharmacother Vol. 44 : 295-301.

Kumar S. Free radicals and antioxidants: Human and food system. Adv Appl Sci Res. 011; 2: 129-135.

Kuehnel, W. 2003. Color Atlas of Cytology, Histology, and Microscopic Anatomy. Thiem.

Lawson M, Vasilaras A, De Vries A. Urological implications of cyclophosphamide and ifosfamide. 2008. Scand J Urol Nephrol. Vol. 42(4): 309-317.

Muneb U, Rehman, Mir T, Farrah A, Qamar W, Latieef A, et al. Cyclophosphamide-induced nephrotoxicity, genotoxicity, and damage in kidney genomic DNA of Swiss albino mice: the protective effect of Ellagic acid. 2012.*Molecular and Cellular Biochemistry.* volumes 365: 119–127.

Mescher, AL 2013. Junqueira's Basic Histology Text and Atlas. 13th ed. McGraw-Hill Primis: 851-871.

Naughton CA, . Drug-induced nephrotoxicity. 2008. Am Fam Physician Vol. 78 : 743-750.

Nermin, M, Yusiff. 2018. Vitamin C. Periodontology Department : 1-29.

Nurlina, Estuningsih S, Sugito, Masyitha D. 2014. Intestinal Microbial Stability, Liver and Kidney Histology of Mice After Administration of Pliek Extract u. Indonesian Venterinary Journal : 370-379.

Panigrahy SK, Jatawa S, Tiwari A. 2011. Therapeutic use of cyclophosphamide and its cytotoxic action: A challenge for researchers. J Pharm Res.4(8):2755-7..

Sharp, P., Villano, J. 2012. The Laboratory Rat. CRC Press. US

Sirois, M. 2005. Laboratory Animal Medicine: Principles and Procedures. United States of America: Mosby Inc.

Singh M, Kumar N, Shuaib M, Garg VK, Sharma A. A review on renal protective agents for cyclophosphamide induced nephrotoxicity. World J Pharm Pharmaceut Sci. 2014 Feb 8;3:737-47.

Sloane, E. 2003. Anatomy and Physiology for Beginners. Translation by Palupi Widyastuti. Jakarta: EGC Medical Book : 318-319.

Subramanian R, Pathak H, Ravindran V. 2019. Safety of cyclophosphamide therapy in autoimmune rheumatic diseases. Indian Journal of Rheumatology.14(2):127.

Suckow, MA, Steven, HW, Craig, LF 2006. The Laboratory Rat. 2nd Edition. California (USA): Academic Pr.

Sweetman, SC. 2009. Martindale the Complete Drug Reference. The Pharmaceutical Press: Chicago Vol. 36. 80-81.

Syafhan, NF 2009. TAC (Docetaxel-Doxorubicin-Cyclophosphamide) and FAC (Fluorouracil-Doxorubicin-Cyclophosphamide) regimens in breast cancer patients at Dharmais Cancer Hospital Jakarta Medical Data Analysis 2007-2008. 2009. Jakarta: University of Indonesia

Velisek J, Cejpek K. 2007. Biosynthesis of food constituents: Vitamins. Water-soluble vitamins, part 2—A review. Czech Journal of Food Science : 40-45.

Wahyuni, E, Kumorowati, P, Suardi, S, Yunus M. Guidebook for Pathology Laboratory Work, ed.2. 2012. Maros Veterinary Center. Makassar.

Wistar Institute. 2016. Our History Philadelphia: The Wistar Institute.

Zhang J, Tian Q, Zhou F.. Clinical Pharmacology of Cyclophosphamide and Ifosfamide. Current Drug Therapy. 2016. Vol. 1: 55-85.