



# Expression of Bcl-2 protein and incidence of apoptosis of parietal layer epithelium cell glomerulus of kidneys in male rats (*Rattus norvegicus wistar*) on application of glutamin nephroprotective that are exposed to nephrotoxic modality of cisplatin chemotherapy

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## Abstract

Cisplatin or (SP-4-2)-diamminedichloroplatinum (II) is one of the most potential platinum derivatives and is widely used for the treatment of various solid cancers such as testes, ovarian, head and neck, bladder, lung, and cervical cancers; melanoma; and lymphoma. The proapoptotic mechanism produced by cisplatin is quite effective in treating neoplastic cells. Cisplatin therapy is a non-target therapy. The cancer cell to which cisplatin is targeted inhibits several antiapoptotic regulators, so that cancer cells immediately start apoptosis. Increased apoptosis causes decreased Bcl-2 protein expression. This research is aimed at analyzing the effect of intravenous glutamine on the expression of Bcl-2 protein in the incidence of apoptosis in parietal layer epithelial cells of the glomerulus of male rats exposed to cisplatin. Glomerular epithelial cells are investigated as a marker of damage to the glomerulus. This study adopted an experimental design with "The Randomized Post Test Only Control Group Design" with a total sample size of 30 male rats that were randomly divided into three groups (randomized). Each group consisted of 10 male rats. Group P0 was a control without any injection, only standard diet; P1 group was injected intraperitoneally with a dose of 20 mg/kg of cisplatin on seventh day; and group P2 was injected intravenously with a dose of 100 mg/kgBW of glutamine for seven days then injected intraperitoneally with 20 mg/kg of cisplatin on seventh day. There was no significant effect but moderate correlation change with  $p > 0.05$  administration of intravenous glutamine on the expression of Bcl-2 proteins in the parietal layer epithelial cell glomerulus the incidence of apoptosis male rats exposed to cisplatin.

**Keywords:** glutamine, Bcl-2, apoptosis, cisplatin

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## INTRODUCTION

The kidney is a filtration organ in humans that functions to filter and remove metabolic waste products out through urine. This organ is divided into four compartments namely Bowman capsule, essence, tubules, and blood vessels (Yang et al., 2016). The Bowman capsule compartment contains the glomerulus which contains afferent and efferent blood vessels. In the inner vessel wall, there is a layer of endothelial cells that play a role in supporting the glomerular filtration function. On the glomerular outer wall, there is a visceral epithelial layer consisting of podocyte cells and parietal

epithelium attached to the inner wall of the Bowman capsule (Yang et al., 2016).

In cases of acute or chronic kidney failure, the glomerulus filtration rate decreases; this is one of the reasons for damage to glomerular endothelial cells accompanied by damage to the visceral and parietal epithelial lining. The glomerular filtration rate (GFR) can be calculated by measuring creatinine levels in urine. The higher the creatinine level, the lower the filtration

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rate. Therefore, high creatinine levels can be used as an indication of kidney failure (Bastard et al., 2019).

The number of patients with chronic kidney failure in Indonesia tended to show an increase of 1.8 per ml from 2013 to 2018 (Balitbangkes, 2018). This increase indicates that the kidney is an organ that is prone to neoplastic and non-neoplastic damage. Neoplastic causes can be observed from kidney malignancies such as renal cell carcinoma, including clear cell carcinoma, papillary renal cell carcinoma, and chromophobe renal cell carcinoma (Verratti et al., 2019). By contrast, non-neoplastic damage is seen from damage to filtration function without malignancies, such as renal failure due to hypertension (Judd and Calhoun, 2015), diabetes mellitus (Karner-Hutuleac, 2012), and nephrotoxic effects of drugs (Qu et al., 2018). One nephrotoxic effect arises from the use of long-term chemotherapy (Alibakhshi et al., 2018).

Nephrotoxic prevalence in the use of chemotherapy for bone cancer treatment shows a diagnosis of bone metastasis; the average age is 67 years, and 24% of the patients show renal insufficiency (RI). The 5-year prevalence is 43% for RI and 71% for chronic kidney disease (CKD) among RI patients. Nearly half (46%) of CKD patients received intravenous bisphosphonate (IV BP) within 12 months after eGFR confirmation, and 13% of these patients received at least one other nephrotoxic agent during that period (Hernandez et al., 2015). In Indonesia, the prevalence is 34.1% of patients experiencing nephrotoxic effects from cisplatin chemotherapy (Prasaja et al., 2015).

One of the chemotherapy materials used is cisplatin. Its wide use in benign and malignant neoplasms makes it the first choice in cancer therapy. However, recent research demonstrates that cisplatin causes damage to tubular epithelial cells and glomerulus. Cisplatin causes apoptosis in neoplastic cells, but normal cells can be affected by similar apoptosis (Gómez-Sierra et al., 2018).

The increase in the incidence of apoptosis in normal cells can be evaluated with proapoptotic and antiapoptotic proteins. Commonly known proapoptotic markers are Bcl-2-associated x protein (Bax), cysteine-aspartate protease (caspase 12), Apaf-1, and procaspase 9, whereas antiapoptotic proteins such as Bcl-2 (Portt et al., 2011). Decreased expression of the Bcl-2 protein from the normal threshold in the administration of chemotherapy shows that there has been damage to the epithelial cells of the glomerular visceral and parietal layers. Examination of Bcl-2 protein expression is done through immunohistochemical staining with the Bcl-2 antigen (Portt et al., 2011). Nephrotoxic effects above are needed materials that can reduce the level of damage called nephroprotective substances. These materials can be synthesized chemically or from natural materials such as glutamine, zingerone, L-theanine, and virgin coconut oil (Alibakhshi

et al., 2018, Altıkkaynak et al., 2018, Famurewa et al., 2017).

From the description above, the use of chemotherapy cannot be avoided causing damage to the kidneys. Thus, cancer patients who get chemotherapy are at risk of chronic kidney failure. Then, further research is needed on the right combination of therapies for chemotherapy and nephroprotective substances so that nephrotoxic side effects can be suppressed. Glutamine is a solution that is being developed because it inhibits the nephrotoxic effects of cisplatin by inhibiting OCT2 receptors, p53 protein, caspase 3, and TNF- $\alpha$ . The combination of glutamine with cisplatin is likely to increase the efficacy and safety of chemotherapy. It is expected that with the use of glutamine as a nephroprotective agent, patients who receive chemotherapy can avoid the risk of damage to the kidneys (Gao et al., 2019).

## MATERIALS AND METHODS

### Animal and treatments

White rat (*Rattus norvegicus*) strain of male Wistar obtained from the experimental animal unit of the Faculty of Medicine, Airlangga University, which related the inclusion and exclusion criteria. Rats are placed in a cage with one cage containing one mouse. Rat food in the form of standard rat food pellets is given *ad libitum*, and drinks in the form of bottled water are given *ad libitum*.

### Chemicals

Glutamine solution was prepared with a dose of 1 g of glutamine diluted in 10 ml of PZ solution. Glutamine is given intravenously to rat tails at a dose of 100 mg/kgBB daily for 6 consecutive days. Cisplatin is given intraperitoneally with a single dose of 20 mg/kgBW Apoptotic Detection Kit POD (11684817910 ROCHE), anti-Bcl-2 antibodies (Santa Cruz).

### Experimental design

Wistar strain male white rats amounted to 30 at the start of the study divided into three groups randomly; the control group (P0) was sacrificed by cervical dislocation after anesthesia with ether. Then, the kidney organs were taken for the preparation of immunohistochemistry.

Group P1 was given a single dose of intraperitoneal cisplatin injection of 20 mg/kgBW on day 7 and then observed for 72 hours. Then, the rats were sacrificed on the 10th day by cervical dislocation after ether anesthesia; then, the kidney was taken for the preparation of immunohistochemistry. P2 groups from day 1 to day 7 were given intravenous injection of glutamine at a dose of 100 mg/kgBW once a day, and on day, seven mice were given a single dose of intraperitoneal injection of 20 mg/kgBW cisplatin. Then, the rats were observed for 72 hours and then sacrificed on the 10th day by means of cervical dislocation after anesthesia with ether; then, the kidney organs were

taken for making immunohistochemical preparations. The observation time on the 10th day after cisplatin injection was apoptotic in parietal capsula Bowman epithelial cells (Tsuruya et al. 2003).

### Histopathological preparation

On the 10th day, rats in groups P0, P1, and P2 were killed by cervical dislocation after anesthesia with ether, and abdominal dissection of the rats was performed to remove the kidney.

Kidney tissue is fixed to 10% formalin for 15–24 hours. After that, dehydration is done using multilevel alcohol to prevent tissue morphology changes (30%, 50%, 70%, 80%, 96%, and absolute) for 60 minutes, successively. Clearing was done using xylol twice every 60 minutes. Then, infiltration with soft paraffin was carried out for 60 minutes at a temperature of 48 degrees.

Then, a block was formed in hard paraffin on the mold and allowed to stand for a day. The next day, it was placed on the holder and cut 4–5  $\mu\text{m}$  thick with a rotary microtome. After that, the object glass was mounted with poly-L-lysine coated. The glass of the object produced by paraffin block was immersed in xylol for 5 minutes twice. After that, rehydration was done using gradual alcohol (absolute, 96%, 80%, 70%, 50%, and 30%) for 5 minutes. Then, sample was rinsed in dH<sub>2</sub>O for 5 minutes.

### Immunohistochemistry assay Bcl-2

The slides were washed using PBS pH 7.4 once for 5 minutes. Thereafter was endogenous peroxide blocking used 3% H<sub>2</sub>O<sub>2</sub> for 20 minutes. Washed using PBS pH 7.4 three times, each for 5 minutes. Unspecific protein blocking used 5% FBS containing 0.25% Triton X-100. Washed using PBS pH 7.4 three times, each for 5 minutes. Incubation using primary antibodies (monoclonal anti-Bcl-2) 60 minute at 25°C. Washed using PBS pH 7.4 three times, each for 5 minutes. Incubation using an anti-mouse biotin conjugated antibody for 20 minutes at room temperature. Washed using PBS pH 7.4 three times, each for 5 minutes. Incubation used SA-HRP (Strep-Avidin Horse Radix Peroxidase) for 40 minutes. Washed using PBS pH 7.4 three times, each for 5 minutes. Drops with diaminobenzidine and incubation for 3 minutes. Washed using PBS pH 7.4 three times, each for 5 minutes. Counterstaining using Mayer Hematoxylin incubated for 10 minutes and washing using tap water. Rinsed using dH<sub>2</sub>O and air-dried. The mounting was done using the lid and cover with a glass cover. Looked under a light microscope at 400 $\times$  magnification.

Actually, there is similarity between the IHC process for Bcl-2 protein and caspase 12; the difference is only in the incubation period of primary antibodies. Caspase 2 needed 24 hours, and Bcl-2 only needed 60 minutes. The first or primary antibody will be shown in brown and blue for a cell that is not shown in Bcl-2. The glomerular

**Table 1.** Descriptive statistic of express Bcl-2 protein and apoptotic cell

Variable	Glomerular epithelial expressing Bcl-2 protein	Apoptotic cell
	Average $\pm$ SD	Average $\pm$ SD
P0	5.3 $\pm$ 0.71	23.1 $\pm$ 3.3
P1	6.4 $\pm$ 0.37	28.1 $\pm$ 4.3
P2	6.3 $\pm$ 0.21	27.2 $\pm$ 4.05

**Table 2.** Mann-Whitney U test for the Bcl-2 protein between P0 and P1 group

Test	Score
Mann-Whitney U	5.000
Wilcoxon W	60.000
Z	-3.411
Asymp. Sig. (two-tailed)	0.001
Exact Sig. [2*(one-tailed sig.)]	0.000 <sup>b</sup>

**Table 3.** Mann-Whitney U test for the Bcl-2 protein between P1 and P2 group

Test	Score
Mann-Whitney U	48.000
Wilcoxon W	103.000
Z	-0.154
Asymp. Sig. (two-tailed)	0.878
Exact Sig. [2*(one-tailed sig.)]	0.912 <sup>b</sup>

epithelial cells was counted in teen place in every slide: three in the upper pole, four in the middle pole, and three in the lower pole.

### Statistical analysis

All dependent variables used in this study are variables with a ratio data scale, so it is necessary to do a normality test with the Shapiro-Wilk test ( $\alpha = 0.05$ ) and homogeneity test with the Levene test ( $\alpha = 0.05$ ). If the normality test results show a normal distribution, then a different test with ANOVA ( $\alpha = 0.05$ ), and if there are differences followed by the *least significant difference* (LSD) ( $\alpha = 0.05$ ). If the results of the normality test show an abnormal and homogeneous or normal and non-homogeneous distribution, then a different test is performed with the Kruskal–Wallis test ( $\alpha = 0.05$ ), and if there is a difference then followed by the T-independent test or the Mann–Whitney test ( $\alpha = 0.05$ ).

## RESULTS

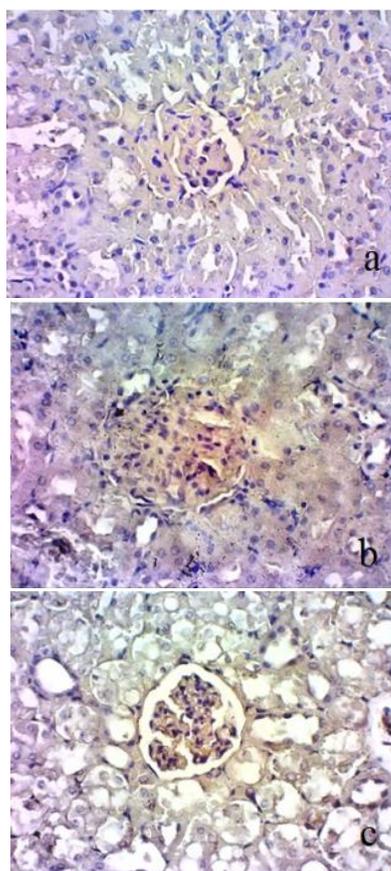
Immunohistochemical examination results of rats with Bcl-2 antibodies showed changes in expression in each group based on descriptive statistic Bcl-2 expression and apoptotic cells in each group (**Table 1**).

Group P0 is a control group without any injection, only standard dietary; P1 group was given intraperitoneal injection of a single dose of 20 mg/kg cisplatin on the seventh day; and group P2 was given an injection of 100 mg/KgBW glutamine intravenous on days 1–7 and an intraperitoneal injection of a single dose of cisplatin 20 mg/kg on the seventh day.

There was a difference in P0 and P1 group of expression of the Bcl-2 protein, following the Mann–Whitney U test. Difference will be stated if Asymp. Sig < 0.05 (**Table 2**). Meanwhile, P1 and P2 did not have a difference because Asymp. Sig > 0.05 (**Table 3**).

**Table 4.** Pearson correlation analysis results

		Score	Group
Score	Pearson's correlation	1	0.428
	Sig. (two-tailed)		0.021
	N	30	29
Group	Pearson's correlation	0.428*	1
	Sig. (two-tailed)	0.021	
	N	29	29



**Fig. 1.** IHC with the Bcl-2 protein. (a) group P0 as the control group, without any injection, only standard dietary; (b) P1 group was given intraperitoneal injection of a single dose of 20 mg/kg cisplatin on the seventh day; and (c) group P2 was given an injection of 100 mg/KgBW glutamine intravenous on days 1–7 and an intraperitoneal injection of a single dose of cisplatin 20 mg/kg on the seventh day

For the apoptotic cell, have same result for expressed Bcl-2 protein, following the Mann–Whitney U test. This indicated a difference between the P0 and P1 groups but no difference for P1 and P2. However, it still has moderate correlation among Bcl-2 expressed in normal cell and the apoptotic cell. This is shown by Pearson's correlation analysis (Table 4).

The results obtained indicated that there was a change in the expression of the Bcl-2 protein and

apoptosis that occurred between the P0 group as a control and P1 with the treatment of a single dose of intraperitoneal injection of 20 mg/kg cisplatin administration. In the P1 group (administration of a single dose of intraperitoneal injection of 20 mg/kg cisplatin) and P2 (treatment of intravenous injection of 100 mg/kgW glutamine for 7 days and a single dose of cisplatin), there were no significant expression changes, but still any changes each group (Fig. 1).

## DISCUSSION

Cisplatin enters the cytoplasm cell by passing through the OCT2 receptor. These receptors are sensitive to cisplatin stimulation, so that if the amount of cisplatin outside the cell increases, the number of these receptors will also increase. An increase in the number of receptors will transport large amounts of cisplatin into the cell and induce apoptosis (Filipski et al., 2009). In P0 and P1, there are significant changes according to the Mann–Whitney U test. These changes are triggered because cisplatin entering the normal cell induces apoptosis.

This is supported by Pearson's correlation test for the number of Bcl-2 expressions, which are antiapoptotic markers with an increasing number of apoptotic cells. Bcl-2 in normal cells is limited in production to keep cells alive according to their time. However, when starting apoptosis induced by cisplatin, the expression will be suppressed by the p53 protein. If glutamine is present, the OCT2 receptor number will be reduced so that the amount of cisplatin that enters the cell is reduced (Kim et al., 2015). The Pearson correlation test indicates that this occurs at P0 and P1, but it is not highly correlated with P2.

Glutamine works by inhibiting the expression and activation of p53 protein, caspase 3, and OCT2 receptors. Glutamine reduces the effects of cisplatin slightly, as evidenced by the decrease in apoptosis with Bcl-2 expression still above normal, even close to the P1 value. Basically, the production of Bcl-2 will be increased if the cell is exposed to damaging substances, with the aim that the cell will survive like a carcinoma cell (Kim et al., 2015).

## CONCLUSION

Glutamine can little decrease the expression of Bcl-2 proteins and decrease apoptosis of parietal layer epithelium cell of glomerulus of kidneys male rats exposed cisplatin.

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