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RESEARCH ARTICLE

Development rat models of nephrotoxicity: A pre-clinical test model to discover nephroprotective agents

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Abstract

Background: The use of drugs, including aminoglycoside drugs and anticancer drugs, is one of the things that can cause toxicity to the kidneys. Therefore, the development of agents that function as nephroprotectors continues to be researched. **Objective:** This study aims to obtain a nephrotoxic experimental animal model for testing nephroprotective agents. **Method:** The treatment was normal group, groups given a single dose of Cisplatin 7 mg/kgBW i.p (Cis), Doxorubicin single dose 25 mg/kgBW i.p (Dox-25), Doxorubicin repeated dose 4 mg/kgBW i.p twice/week for two weeks (Dox-4 repeated dose), and Gentamicin single dose 112 mg/kgBW i.p (Gen). The nephrotoxic effect was assessed by the parameters of kidney weight ratio, Ureum, Creatinine and Albumin levels in rat serum. **Result:** The nephrotoxic effect was indicated by an increase in the renal weight ratio of the Cisplatin; Dox-25, Dox-4 repeated, and Gen groups than normal, respectively. There was an increase in serum BUN levels, an increase in serum creatinine, and a decrease in serum albumin levels compared to the normal group. **Conclusion:** Cisplatin, Doxorubicin and Gentamicin have nephrotoxic effects. The administration of a single dose of cisplatin at 7 mg/kg BW gave the strongest nephrotoxic effect, making it appropriate as a model of acute nephrotoxicity in rats.

Introduction

Nephrotoxicity causes a sustained decline in renal function, resulting in nitrogen (urea and creatinine) retention, non-nitrogenous waste products, cellular volume dysregulation, and the inability of the kidney to regulate fluid and electrolyte homeostasis. Drugs are often associated with nephrotoxicity, including the use of aminoglycoside antibiotics, anthracyclines, amphotericin B, chemotherapeutic drugs such as ifosfamide, doxorubicin and cisplatin, acyclovir, and other chemicals (Perazella & Shirali, 2018; Toale *et al.*, 2021).

In Indonesia, there were also cases of acute renal failure in children since January 2022, with a total of 36 cases in August 2022, which increased again in September with 78 cases (Salman *et al.*, 2023). The Ministry of Health Republic of Indonesia has received reports from 22 provinces in Indonesia of 241 cases with 133 reported deaths. The increase in cases of acute kidney failure in children is due to fever syrups containing ethylene glycol (EG) and diethylene glycol (DEG) that exceed safe levels. The formation of oxalic acid metabolites from ethylene glycol can precipitate into calcium oxalate monohydrate crystals in the lumen of the tubules, triggering the formation of kidney stones and acute kidney injury. In diethylene glycol, the formation of the metabolite 2-hydroxyethoxyacetic acid (HEAA) has a toxic effect resulting from the accumulation of HEAA, causing acidosis and renal organ failure (Salman *et al.*, 2023).

The above shows the importance of obtaining agents or compounds that are nephroprotective and can protect kidney health. Nephroprotectors are compounds that protect the kidneys from exposure to drugs and industrial or environmental chemicals.

Nephroprotector effects are often studied on natural materials that contain a bioactive compound that can provide pharmacological effects and deal with the problem of drug side effects (Negi & Mirza, 2020).

Finding nephroprotective agents and mechanisms to reduce the risk of nephrotoxicity is urgently needed. The design and development of experimental animal models for nephrotoxicity are still being pursued to test the effectiveness of an agent and a nephroprotector against kidney injury. Some drugs are used as nephrotoxic inducers in experimental animals, such as cisplatin, doxorubicin, gentamicin, colistin, and others (Jafari & Elyasi, 2021).

Cisplatin is a first-line chemotherapeutic agent for the treatment of various cancers, including ovarian cancer and breast cancer. Although it has been proven that cisplatin is effective for cancer therapy, its clinical use is limited by the side effects of cisplatin in the form of ototoxicity, peripheral neuropathy, and nephrotoxicity (Brown *et al.*, 2019; Sandhiutami *et al.*, 2019). Approximately 27% to 100 % of patients treated with cisplatin experience nephrotoxicity (McMahon *et al.*, 2020).

The other chemotherapy is doxorubicin, a member of the anthracycline class of antibiotics that are widely and efficiently used to treat many malignancies, including leukaemia and tumours. Doxorubicin use can also result in nephrotoxicity, which is characterised by glomerulosclerosis, interstitial fibrosis, albuminuria, increased serum creatinine, decreased glomerular filtration rate, potassium and magnesium, and renal histological abnormalities (Amarasiri *et al.*, 2022).

Another drug that can be nephrotoxic is the aminoglycoside class drug gentamicin. Gentamicin causes tubular damage through necrosis of tubular epithelial cells and causes oxidative stress by forming superoxide anion and hydroxyl radicals, resulting in cell death in the kidney (Huang *et al.*, 2020).

However, there is very limited scientifically based available literature that reports the ideal nephrotoxicity animal models for testing agents that can provide nephroprotection effects. Therefore, it was considered appropriate to scientifically examine nephrotoxic animal models using nephrotoxic drugs, namely cisplatin, doxorubicin, and gentamicin. This study aims to compare nephrotoxic animal models induced with cisplatin, doxorubicin, and gentamicin by assessing markers of kidney damage, namely kidney weight ratio, blood urea nitrogen, creatinine, and serum albumin levels.

Methods

Chemical and drugs

Doxorubicin (Global Onkolab Farma), cisplatin (Mylan N.V, U.K), gentamicin (PT Indofarma (Persero) Tbk), albumin, creatinine, blood urea nitrogen reagent kit (BIOLABO), sodium carboxymethyl cellulose (CMC) (Brataco Chemical), aquadest (Brataco Chemical).

In vivo design for an experimental animal nephrotoxicity model

In this research, 25 male Wistar rats (200-250 grams), each aged eight weeks, were acclimatised for one week. Wistar rats were obtained from the National Institute of Research and Development, Ministry of Health, Republic of Indonesia. In vivo experiments were carried out after receiving approval from the Ethics Committee for Health Research, Faculty of Medicine, Universitas Indonesia, with the number: KET-187/UN2.F1/ETIK/PPM.00.02/2022. The study was performed at the Department of Pharmacology and Toxicology, Faculty of Pharmacy, Pancasila University, South Jakarta, Indonesia. Rats respectively were each divided into five groups (n = 5). Five major groups, i.e. the normal control group received sodium carboxymethyl cellulose (CMC), the group was induced with cisplatin single dose 7 mg/kgBB i.p (cis); the group was induced with doxorubicin single dose 25 mg/kgBB i.p (dox-25); the group was induced with doxorubicin repeated dose 4 mg/kgBB i.p 2x/week for two weeks (dox-4 repeated dose) and the group were induced with gentamicin single dose 112 mg/ kgBB i.p (gen). Two days after induction, rats were euthanised, and blood was taken for measurement of BUN, creatinine, and albumin levels in rat serum. Kidney organs were weighed to obtain the kidney weight ratio.

$$\text{Kidney weight ratio (\%)} = \left(\frac{\text{Kidney weight}}{\text{Body weight of rats}}\right) \text{x } 100\%$$

Measurement of blood ureum nitrogen

Measurement of blood urea nitrogen levels using enzymatic and colorimetric methods with the specific working principle of urease hydrolysing urea in ammonium ions and carbon dioxide. Ammonium ions then form a colour complex with chloride and salicylate to become blue-green. The test procedure follows the protocol provided in the kit. The test solution was prepared by mixing 5 µL of blood serum sample with 1 mL of R1 (salicylate 31 mmol/L, nitroprussiate 1.67 mmol/L) +R2 (urease ≥15 KUI/L), then waited for four minutes. Next, 1 mL of base solution (sodium hypochlorite 7 mmol/L and sodium hydroxide 62 mmol/L) was added, mixed, then allowed to stand for

eight minutes. After that, the absorbance was read on a UV-VIS spectrophotometer at a wavelength of 600 nm.

Blood ureum nitrogen = $\frac{\text{Atest}}{\text{Astd}}$ × std conc. (40 mg/dL)

Measurement of serum creatinine

Measurement of serum creatinine levels using the Jaffe method with the principle of a colorimetric reaction of creatinine with the picric base to form a red-orange colour and then the absorbance is measured. One hundred µL of blood serum sample was mixed with 1000 µL of reagent mixture R1 (disodium phosphate 6.4 mmol/L, sodium hydroxide 150 mmol/L) + R2 (sodium dodecyl sulphate 0.75 mmol/L, picric acid 4.0 mmol/L), then read the absorbance at 30 seconds (A1) and 120 seconds after the first absorbance (A2) on a UV-VIS spectrophotometer at λ 500 nm. The absorbance results were entered into the formula:

Creatinine Serum = $\frac{(\text{A2}-\text{A1})\text{test}}{(\text{A2}-\text{A1})\text{std}}$ ×std conc. (2 mg/dL)

Measurement of serum albumin levels

Measurement of serum albumin levels using the bromocresol green (BCG) dye binding method with the principle of green-blue color changes that occur due to the presence of bromocresol green binding to albumin in a pH 4.2 buffer solution. Five µL of blood plasma sample was taken and 1000 µL of R1 (succinic acid 83 mmol/L, bromocresol green (BCG) 167 µmol/L, sodium hydroxide 50 mmol/L, polyoxyethylene mono lauryl ether 1.00 g/L) waited for one minute and then measured on a UV-VIS spectrophotometer at λ 630nm until the test absorbance and standard absorbance were obtained, then entered the formula:

Albumin = $\frac{\text{Atest}}{\text{Astd}}$ × std conc. (5 g/dL)

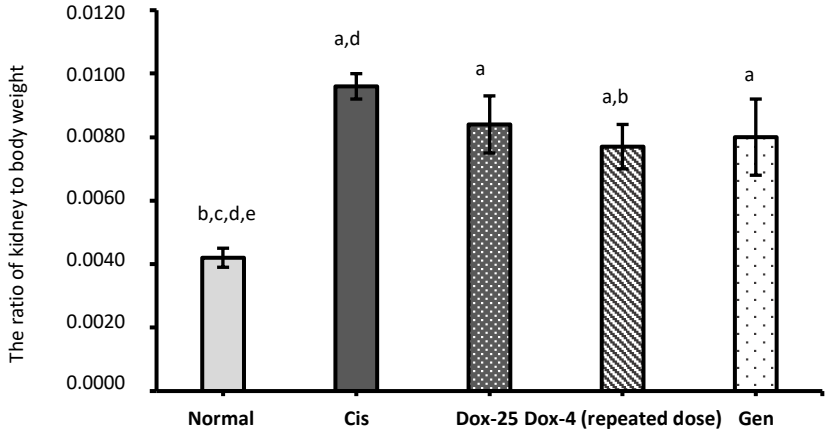
Statistical analysis

The data were presented in the mean of analysis of the sample (n = 5) ± standard error (SE). Data were statistically analysed with the one-way analysis of variance (ANOVA) followed by the least significant differences (LSD) test to determine significant differences between test groups ($p < 0.05$).

Results

The ratio of kidney weight to body weight of rats

The kidney weight ratio describes the toxicity that occurs in the kidney organ. The results of the kidney weight ratio calculation are shown in Figure 1**.**



[a]$p < 0.05$ sig. differences versus the normal control group, [b]$p < 0.05$ sig. differences versus cisplatin, [c]$p < 0.05$ sig. differences versus dox-25, [d]$p < 0.05$ sig. differences versus dox-4 repeated dose, [e]$p < 0.05$ sig. differences versus gentamicin.

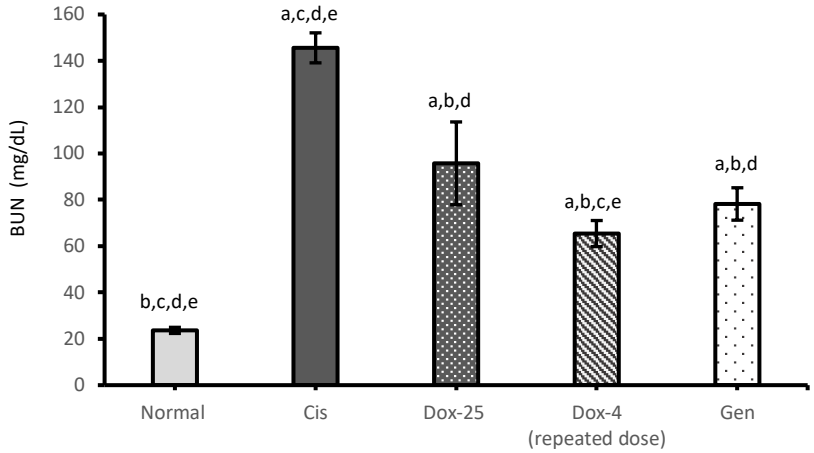
Figure 1: The ratio of kidney weight to body weight in the normal control group; cisplatin single dose 7 mg/kgBB i.p (cis); doxorubicin single dose 25 mg/kgBB i.p (dox-25); doxorubicin repeated dose 4 mg/kgBB i.p 2x/week for two weeks (dox-4 repeated dose) and gentamicin single dose 112 mg/ kgBB i.p (gen). Data represented as mean ± SE (n=5)

The results of measuring the renal weight ratio of the normal and induced groups statistically showed significant differences ($p < 0.05$), indicating damage to the kidneys. In this study, the increase in kidney weight ratio successively occurred in groups induced with cisplatin, dox-25, gentamicin, and repeated doses of

dox-4. LSD test results showed no significant difference ($p > 0.05$) in the renal weight ratio between the groups induced with cisplatin, dox-25, repeated dose dox-4, and gentamicin.

Blood urea nitrogen levels

The measurement of blood urea nitrogen (BUN) can be seen in Figure 2.



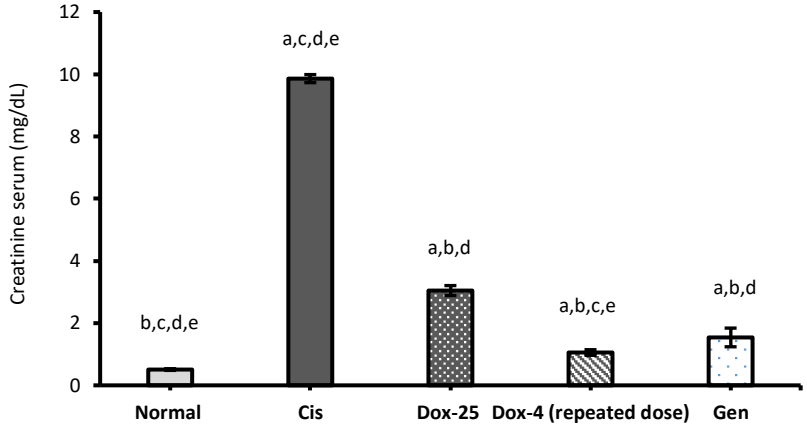
Data represented as mean ± SE (n = 5). [a]$p < 0.05$ sig. differences versus the normal control group, [b]$p < 0.05$ sig. differences versus cisplatin, [c]$p < 0.05$ sig. differences versus dox-25, [d]$p < 0.05$ sig. differences versus dox-4 repeated dose, [e]$p < 0.05$ sig. differences versus gentamicin.

Figure 2: Blood urea nitrogen (BUN) (mg/dL) in the normal control group; cisplatin single dose 7 mg/kgBB i.p (Cis); doxorubicin single dose 25 mg/kgBB i.p (dox-25); doxorubicin repeated dose 4 mg/kgBB i.p 2x/week for two weeks (dox-4 repeated dose) and gentamicin single dose 112 mg/ kgBB i.p (gen)

In this study, the measurement of BUN levels in the groups of rats induced by cisplatin, dox-25, dox-4 repeated and gentamicin compared with the normal group consecutively obtained a significance value in the cisplatin-induced group p (0.016) < α; dox-25-induced group p (0.009) < α; gentamicin-induced group p (0.009) and dox-4 repeated group p (0.009) < α, these four results indicate there is a significant difference between the test group and the normal group.

Creatinine serum levels

The results of the measurement of creatinine levels can be seen in Figure 3.



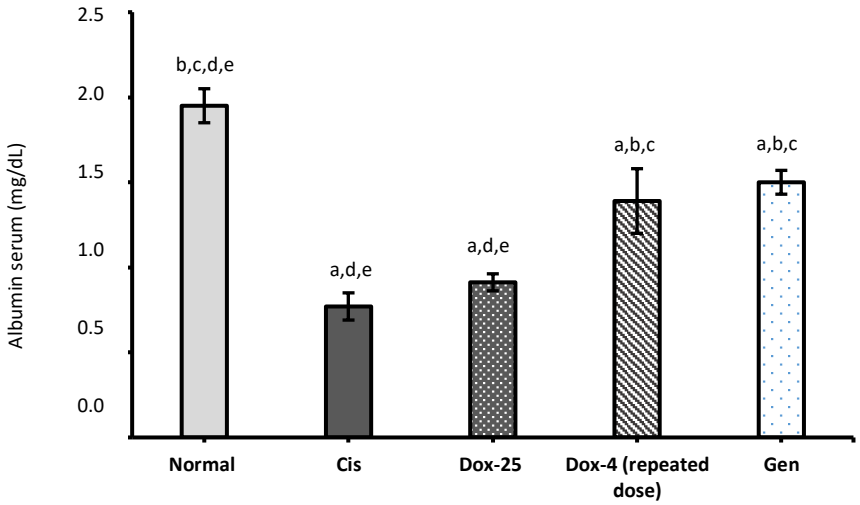
Data represented as mean ± SE (n = 5). [a]$p < 0.05$ sig. differences versus the normal control group, [b]$p < 0.05$ sig. differences versus cisplatin, [c]$p < 0.05$ sig. differences versus dox-25, [d]$p < 0.05$ sig. differences versus dox-4 repeated dose, [e]$p < 0.05$ sig. differences versus gentamicin.

Figure 3: Creatinine serum (mg/dL) in the normal control group, cisplatin single dose 7 mg/kgBB i.p (cis); coxorubicin single dose 25 mg/kgBB i.p (dox-25); doxorubicin repeated dose 4 mg/kgBB i.p 2x/week for two weeks (dox-4 repeated dose) and gentamicin single dose 112 mg/ kgBB i.p (gen)

The results of measuring creatinine levels in normal group rats obtained a significance value of *p* (0.00) <α, indicating a significant difference between the normal group and the cisplatin, dox-25, dox-4 repeated, and gentamicin groups. The results of the measurement of creatinine levels in the groups of rats induced by cisplatin, dox-25, dox-4 repeated, and gentamicin compared with the normal group successively obtained significance values in the cisplatin-induced group; dox-25-induced group; gentamicin-induced group and dox-4 repeated group, these four results show that there are significant differences ($p < 0.05$) between the induced groups and the normal group.

Albumin levels

The results of albumin level measurement can be seen in Figure 4.



Data represented as mean ± SE (n = 5). [a]$p < 0.05$ sig. differences versus the normal control group, [b]$p < 0.05$ sig. differences versus cisplatin, [c]$p < 0.05$ sig. differences versus dox-25, [d]$p < 0.05$ sig. differences versus dox-4 repeated dose, [e]$p < 0.05$ sig. differences versus gentamicin.

Figure 4: Albumin serum (mg/dL) in the normal control group, cisplatin single dose 7 mg/kgBB i.p (cis); doxorubicin single dose 25 mg/kgBB i.p (dox-25); doxorubicin repeated dose 4 mg/kgBB i.p 2x/week for two weeks (dox-4 repeated dose) and gentamicin single dose 112 mg/ kgBB i.p (gen)

The results of measuring plasma albumin levels in the induced group rats obtained a significance value of *p* (0.00) < α, indicating a significant difference between the normal group and the group of rats induced by cisplatin, dox-25, dox-4 repeatedly, and gentamicin. From this study, there is a decrease in albumin levels, or there is a significant difference between the groups of rats induced by cisplatin, dox-25, repeated dox-4 and gentamicin ($p < 0.05$), respectively.

Table I shows the percentage increase in the ratio of kidney to body weight of rats, BUN, serum creatinine, BUN and decrease in serum albumin levels when cisplatin single dose 7 mg/kgBB i.p (cis); doxorubicin single dose 25 mg/kgBB i.p (dox-25); doxorubicin repeated dose 4 mg/kgBB i.p 2x/week for two weeks (dox-4 repeated dose) and gentamicin single dose 112 mg/kgBB i.p (gen) were administered. All four test materials showed significant differences with the normal group ($p < 0.05$). The strongest nephrotoxic effect was produced by cisplatin single dose 7 mg/kgBB i.p (cis); doxorubicin single dose 10 mg/kgBB i.p (dox-25); gentamicin single dose 112 mg/ kgBB i.p (gen) and finally by doxorubicin repeated dose 4 mg/kgBB i.p 2x/week for two weeks (dox-4 repeated dose).

Table I: Percentage increase in kidney weight ratio, BUN level, serum creatinine and albumin serum upon induction of cis; dox-25; dox-4 repeated dose and gen compared to normal group

Groups	Kidney weight ratio	BUN level	Creatinine serum	Albumin serum
Cis	2.29	6.16	19.42	2.53
Dox-25	2.00	4.05	6.00	2.14
Dox-4 repeated dose	1.83	2.77	2.08	1.4
Gen	1.90	3.31	3.03	1.3

Discussion

The development of drugs, herbs, and agents that are nephroprotectors is a very important treatment development because many drugs or chemicals are toxic to the kidneys or nephrotoxic. Pre-clinical trials to obtain nephroprotector compounds require ideal animal models. This study aims to obtain a good nephrotoxic animal model used for testing drugs, herbs, and agents that are nephroprotectors.

Many agents can cause nephrotoxic effects, but the most frequent and common nephrotoxic effects are aminoglycoside antibiotic drugs such as gentamicin and chemotherapy drugs such as cisplatin and doxorubicin. The nephrotoxicity of cisplatin is a side effect that becomes an obstacle in chemotherapy with cisplatin. Cisplatin accumulates in renal tubular cells, causing cell damage and death, which in turn causes acute kidney damage. The side effects of cisplatin are dose-dependent, cisplatin is usually used at high doses to maximise its antineoplastic effects. The nephrotoxicity of cisplatin is characterised by decreased glomerular filtration rate and decreased serum potassium and magnesium. The mechanism of cisplatin nephrotoxicity is due to the accumulation of cisplatin in kidney cells. The biotransformation of cisplatin to toxin is the main mechanism that induces cisplatin nephrotoxicity. The process begins with the formation of glutathione conjugates that are chained by the enzyme glutathione-S-transferase, and then they are broken down by the enzyme gamma-glutamyl transpeptidase into cysteinyl-glycine conjugates. The presence of amino dipeptidase enzyme causes these cysteine-glycine conjugates to be further metabolised into cysteine conjugates. These cysteine conjugates will then be metabolised by the enzyme cysteine-S-conjugate beta-liase to form highly reactive thiol conjugates. It is these thiol conjugates that can induce apoptosis or necrosis in cells (McSweeney *et al.*, 2021). Previous research reported negative control (nephrotoxic animal models using cisplatin), there was a significant increase in creatinine and blood ureum nitrogen levels, an increase in kidney weight ratio, and also a significant decrease in serum albumin levels (Sandhiutami *et al.*, 2019).

In this study, the use of doxorubicin can also have a nephrotoxic effect. Doxorubicin can induce direct damage to the glomerulus and then injury to the interstitial tubules. Doxorubicin causes changes in the glomerular filtration membrane wall, including glomerular endothelial cells, glomerular basement membrane, and podocytes (Hu *et al.*, 2022). The mechanism by which doxorubicin (DOX) damages the kidney may result from the formation of free radicals (An *et al.*, 2020). Increased reactive oxygen species (ROS) production can activate NF-κB and lead to the induction of major pro-inflammatory mediators, including iNOS, TNFα, and COX-2. Further increase in pro-inflammatory mediators will lead to tissue damage and further activation of NF-κB. Inflammation that occurs can trigger injury to podocytes that causes proteinuria and tubular damage until interstitial fibrosis occurs (Arunachalam *et al.*, 2022).

Another inducer used in this study is gentamicin. The most common side effect of gentamicin is nephrotoxic. The form of nephrotoxicity observed is acute. It is characterised by the inability to micturate (no oliguria), elevated serum creatinine, and hyperosmolar urine that develops after several days of administration. Acute toxicity of gentamicin is believed to result in nephrotoxicity by inhibiting protein synthesis in renal cells. This mechanism specifically causes cell necrosis in the proximal tubules, resulting in acute tubular necrosis that can lead to acute renal failure (Petejova *et al.*, 2020). Gentamicin forms a complex with megalin and cubilin, which causes gentamicin to move through the endosomal compartment and accumulate mostly in lysosomes, Golgi bodies, and endoplasmic reticulum. Gentamicin then binds to membrane phospholipids and alters the onset and metabolism of membrane phospholipids, resulting in phospholipids. The binding of gentamicin and phospholipids leads to endocytosis of gentamicin to the cytosol. Gentamicin affects mitochondria through direct and indirect pathways. The direct mechanism is by activating the intrinsic pathway of apoptosis and generating oxidative stress by increasing superoxide anion and hydroxy radicals that contribute to cell death. The indirect mechanism is by inhibiting proteasome degradation, thereby increasing active protease enzymes, namely cathepsins, which can induce cell death. Gentamicin-induced cell death leads to renal tubular lesions and necrosis. This is associated with high levels of urea and creatinine in the blood, which are toxic to the body (Gamaan *et al.*, 2023).

In this study, the evaluation of kidney weight ratio calculation can describe the toxicity that occurs in the kidney organ. Toxicity increases directly proportional to the increase in kidney weight. In this study, rats in the rat group induced by cis, dox-25, dox-24 repeatedly, and gentamicin had an increase in kidney weight compared to the normal group. This is in line with research conducted by Li et al. (2020a) that nephrotoxicity, for example, due to doxorubicin, can increase kidney weight through a decrease in the ability to remove residual metabolite substances in the body, causing increased capillary permeability and glomerular atrophy (Li *et al.*, 2020a).

This increase in renal weight ratio can be attributed to the increase in renal organ weight and weight loss that

occurred in the cis, dox-25, dox-4 repeated dose, and gentamicin-induced groups. Under nephrotoxic conditions, there is also a decrease in urine-concentrating ability and a decrease in papillary hypertonicity, which is also known to be the main cause of weight loss in mice. Therefore, the increase in kidney weight was not due to weight gain but rather due to the enlargement of tubular cells in the kidney exposed to nephrotoxic agents. The increase in kidney weight correlated with the increase in serum creatinine and blood urea nitrogen values (Udupa & Prakash, 2019).

From the results of this study, an increase in urea levels after being given cisplatin, doxorubicin, and gentamicin indicates damage to kidney function experienced by rats. Urea is produced by the liver through the ornithine cycle from ammonia (nitrogen) to form a metabolite called urea to be excreted in the urine, and about 50% of urea is reabsorbed by the tubules. Therefore, urea can be used as one of the parameters of kidney function related to filtration and reabsorption processes. Impaired renal function can be seen by a decrease in the glomerular filtration rate (GFR), which increases blood urea levels (Li *et al.*, 2020b).

The results of this study obtained creatinine levels in negative group rats after being given cisplatin, doxorubicin, and gentamicin, which increased compared to normal rats. These results are in line with research by Altınkaynak and colleagues (2018) that looked at the increase in creatinine levels in the blood after being given nephrotoxic agents, namely doxorubicin (Altinkaynak *et al.*, 2018). Creatinine concentration is inversely proportional to glomerular filtration rate (GFR). The higher the creatinine concentration, the lower the GFR. Doxorubicin can significantly reduce the GFR in rats so that the kidneys are unable to excrete creatinine through the urine, causing high levels in the blood (Naji Ebrahimi Yazd *et al.*, 2018).

The results of measuring plasma albumin levels in this study were in the induced group, and there was a significant decrease compared to the normal group, indicating that there was a decrease in kidney function after being given cisplatin, doxorubicin, and gentamicin. Albumin that escapes the filtration stage is not reabsorbed, so it comes out through urine, and levels in the blood decrease.

Conclusion

In conclusion, we would like to mention that this is the first study to compare the nephrotoxic effects of cisplatin, doxorubicin, and gentamicin to obtain a nephrotoxic experimental animal model for pre-clinical testing of nephroprotectors. In this study, the animal model that can provide the best acute nephrotoxic effect is a single dose of cisplatin 7 mg/kgBB i.p.

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