Effect of Green Peas Protein Hydrolysate on Antihyperlipidemia and Antinephrotoxicity of Gentamicin-Induced Wistar Rats

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Abstract- Green peas protein hydrolysate is used in the treatment of kidney disease. Nephrotoxicity of gentamicin (GM) is known to be involved in kidney changes, dyslipidemia, and hematology. The aim of the present study is to evaluate the effects of three doses of green peas protein hydrolysate bromelain (GPPHB) as antinephrotoxicity and antihyperlipidemia on gentamicin (GM)-induced Wistar rats as an effort to find an effective dose for kidney diseases therapy. Nephrotoxicity was induced in female Wistar rats through intraperitoneal administration of GM 80 mg/kg/day for seven days. Nine treatment groups (n=6) were monitored to determine the effects of the concurrent administration of green peas protein hydrolysate at a dose of 50, 100, and 200 mg/kg/day given with GM toward kidney damage using serum creatinine and blood urea nitrogen as indicators. The parameters for nephrotoxicity of GM were kidney organ index (OI), histopathological: cloudy swelling tubular degeneration, nucleus necrosis, and hyaline cast; hematology profiles; and for hyperlipidemia were total cholesterol, low-density lipoprotein (LDL), and triglyceride. After 28 days of treatments, GPPHB at the dose of 50 mg/kgBW has a good effect on kidney OI, whereas, at a dose of 200 mg/kgBW, GPPHB significantly protects rat kidneys from GM-induced histopathological changes. The GPPHB dose of 100 and 200 mg/kgBW significantly lowered the total cholesterol, LDL, and triglyceride levels but had no significant effect on hematology profiles. Three doses of GPPHB had antihyperlipidemia and antinephrotoxicity effects in GM-induced nephrotoxicity Wistar rats.

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Keywords: Green peas protein hydrolysate; Bromelain; Antihyperlipidemia; Hematology; Histopathological changes

Introduction

Extensive use of protein hydrolysate therapy has been widely reported. Green peas protein hydrolysate is used in the treatment of certain diseases such as hypertension, chronic kidney disease (CKD), and diseases involving altered kidney function (1,2). Previous studies have shown that green peas protein hydrolysates hydrolyzed using bromelain (GPPHB) at a dose of 100 mg/kgBW improved the renal function parameters of Cisplatin-induced rats (3).

In the present study, the effects of three doses of GPPHB on gentamicin (GM)-induced nephrotoxicity in rats were evaluated. GM-induced nephrotoxicity is known to cause elevated levels of serum and urea creatinine, tubular necrosis, and glomerular congestion,as well as decreased glomerular filtration rates (4,5). Although GM has an effective role in the treatment of gram-negative bacteria, the administration of GM has been limited because of increasing chances of acute renal failure in 10%-20% of patients treated with this drug (6,7).

Renal diseases are associated with a variety of hemopoietic changes. Anemia parallels the degree of renal impairment failure, but the exact relationship between them remained unclear. The most important cause of anemia is a failure of renal erythropoietin secretion (8).

The nephrotoxicity of GM is known to be involved in kidney changes, hyperlipidemia activity, and

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hematology, and thus, the effects of GPPHB needed to be evaluated.

The purpose of the present study is to evaluate the effect of GPPHB at doses of 50, 100, and 200 mg/kg/day on the antinephrotoxicity (kidney organ index (OI), kidney histopathology and hematology) and antihyperlipidemia in GM-induced Wistar rats.

Materials and Methods

Green peas (Pisum sativum L.) were obtained from Maica leaf, Magelang Plantation, Central Java, Indonesia. Bromelain enzyme was obtained from pineapple stems (Ananas sativus) from Subang, North Bandung, Indonesia. Gentamycin (80 mg) for injection/intraperitoneal was purchased from a local drug store.

Subject

Fifty-six healthy male Wistar rats (five-six weeks) weighing 148-190 g were obtained from the School of Life Sciences and Technology, Bandung Institute of Technology, Indonesia.

Bromelain Preparation. For bromelain production, a solution obtained from pineapple stem (Ananas sativus) was filtered and centrifuged at 4000 rpm for 10 min. The protein concentration of bromelain was determined using the Bradford method (9) with tryptophan as standard (10). The total specific activity of the enzyme and hydrolysate protein content was measured using Kunitz's method and the BSA curve, respectively (11).

Protein hydrolysate preparation

Protein hydrolysates were prepared as in the previous study with modifications (3,12). Dry seeds (500 g) of green peas were mashed, sieved through a 120-mesh sieve, and dissolved in 2000 mL of water. Bromelain 10% (w/v) was added to each solution (13) and then left for 72 hours (14) on a stirrer at room temperature (25° C-30°C) (15). After 72 hours, the solution was transferred to a tube and centrifuged at 6000 g for 10 min. The supernatant was filtered using filter paper. SDS-PAGE was used to separate and determine the molecular weight of the protein hydrolysates (16).

***In vivo* test in GM-induced Wistar rats**

Nephrotoxicity was induced in Wistar rats by intraperitoneal administration of GM 80 mg/kg/day for seven days. GM administered at a dose of 40 mg/kgBW/day or more is proven to induce renal cortical necrosis with renal dysfunction (17). The 54 male Wistar rats were divided into nine treatment groups:

Group 1: Gentamycin 7d and GPPHB 50 mg/kgBW 28d

Group 2: Gentamycin 7d and GPPHB 100 mg/kgBW 28d

Group 3: Gentamycin 7d and GPPHB 200 mg/kgBW 28d

Group 4: Negative (normal) Control: Aquadest

Group 5: Positive Control Gentamycin 7d

Group 6: Comparison Control Gentamycin 7d and Simvastatin 10 mg/kgBW 28d

Group 7: Comparison Control Gentamycin 7d and Fenofibrate 300 mg/kgBW 28d

Group 8: Comparison Control Gentamycin 7d and Ketosteril 630 mg/kgBW 28d

Group 9: Comparison Control Gentamycin 7d and vitamin E (d-α-tocopherol) 200 IU/kgBW 28d

This animal experiment has been approved by the Ethical Committee of Universitas Kristen Maranatha (185/KEP FK UKM-RSI /III/2018).

Sample collection

Bodyweight (BW) and OI

Each rat was weighed every day to determine the dosage and volume of the GPPHB given each day. OI values were obtained by dividing the organ weight (OW) of each rat with its BW.

Histopathological Analysis of Right Renal Organ of Male Wistar rats

All the rats were sacrificed on day 35 and the right kidneys of GM-induced Wistar rats. Histopathological slides of the kidneys were made using hematoxylin-eosin (HE) staining. The kidneys were weighed, soaked in a solution of 1% formalin, HE-stained, and analyzed under a light microscope with 100- and 400-times magnifications. The semi-quantitative interpretation was conducted through the scoring system.

Histopathology parameters of GM-Induced rat kidneys

The parameters used for assessment are a modification of the Suhita study in Indonesia (18). Observed histopathological damage of the kidneys included cloudy swelling tubular degeneration, nucleus necrosis, and hyaline cast. Following a comparison of damage of each group, the resulting interpretation was converted into median scores and the total obtained. From each group, three renal histopathological slides were measured, and three parameters observed in five

fields of view. The results were recorded in the form of scores ranging from 0-2. The histopathological slides were read from five viewing fields with parameters and scores: tubular degeneration (cloudy swelling): 0 none, 1 focal, 2 diffused; nucleus necrosis: 0 none, 1 focal, 2 diffused; hyaline cast: 0 none, 1 focal, 2 diffused.

Hematology profile

Blood was withdrawn from the tail vein and measured immediately for hematological profiles using hematoanalyzer devices with colorimetric principles.

Lipid profile measurement

Blood samples were collected three times on day 0 and day 7 from retro-orbital sinus, then on day 35 from rats' intracardiac vein. The data analyzed statistically is the percentage difference between the average D7 result minus D35, divided by the average D7 result (D7-D35)/D7 (%). Total cholesterol and low-density lipoprotein (LDL) levels were measured three times (D0, D7, and D35) using Cobas Roche 311 with spectrophotometry method, cholesterol oxidase-phenol aminophenazone (CHOD-PAP), while triglyceride levels were measured using enzyme Glycerol-3-phosphate oxidase-phenol aminophenazone (GPO-PAP). The normal value for lipid and hematology profile referred to the Giknis manual, based on the list for 8–16 week-old male rats (19).

Statistical analysis

Values were presented as mean±SD. Data were analyzed using ANOVA, followed by a post-hoc LSD test for multiple comparisons. Differences were considered significant (P<0.05) and highly significant (P<0.01). Histopathology scores of GM-induced kidney rats were analyzed using the Kruskal-Wallis test.

Results

Results of OI

Table 1 reveals the results of the OI. In general, all groups of rats after GM induction had a decrease in BW, except for the negative control group. The BW started to increase on the 21st day. Data can be seen in Table 1.

The results of the ANOVA analyses showed that the OI kidney negative control group had a significant difference compared to the GM control group, where the GM group showed a much greater OI, indicating that the induction of GM caused the OW of the kidney to become heavier, and thus, the harmful effect of GM on kidney OI can be concluded. The GPPHB group at a dose of 50 mg/kgBW had a significant difference as compared to the GM control group, indicating that the GPPHB group's kidney weights were lighter and stayed healthy. ANOVA on heart OI showed that all groups were not significantly different as compared to the positive control and negative control groups; therefore, the induction of GM had no effect on the OI of the heart.

Table 1. Data of OI for kidney and heart

Group	Organ Index (OI)	
	Kidney	**Heart**
1. GPPHB 50	0.38±0.03a	0.36±0.02
2. GPPHB 100	0.42±0.04	0.36±0.04
3. GPPHB 200	0.40±0.03	0.37±0.03
4. Negative C	0.35±0.02a	0.37±0.02
5. Positive/GM C	0.43±0.04	0.36±0.02
6. Simvastatin	0.41±0.04	0.35±0.35
7. Fenofibrate	0.46±0.04	0.35±0.04
8. Ketosteril	0.42±0.04	0.37±0.02
9. Vitamin E	0.43±0.03	0.35±0.03

Note a = significantly different from the data of the positive control group

Result of kidney histopathological examination of GM-Induced rat (Figure 1)

The result of the Kruskal-Wallis test for cloudy swelling tubular degeneration has a value of P>0.05, indicating the absence of significantly different outcomes among all groups, where all obtained a score of 1.

The GM control group showed a picture of severe nucleus necrosis, and almost all of the nucleus disappeared and was given a score of 2 (Figure 1 D). Results of the analysis of nucleus necrosis between the median score of the negative control and the GM control group had very significant differences (P<0.01), thereby proving that the result was valid. The scores of GPPHB 50, 200 mg/kgBW, Ketosteril, and vitamin E were significantly different from the GM control group

(P<0.05).

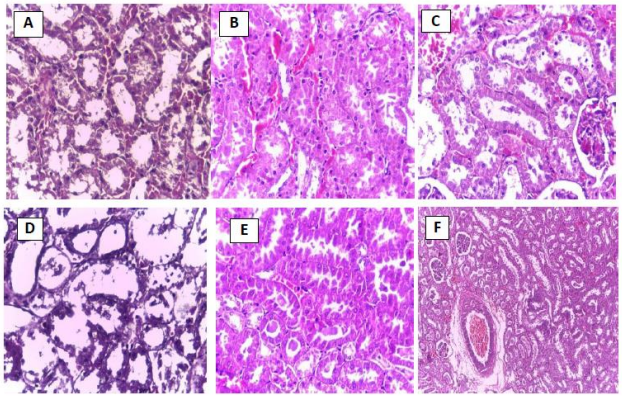

Figure 1. Histopathological of Kidneys of GM-Induced Rat based on 3 Parameters
A. Scores of Cloudy Swelling = 1, Nucleus necrosis = 1 and *Hyaline Cast* = 0,B. Scores of Cloudy Swelling tubules degeneration= 1,C. Scores of Nucleus Necrosis = 1,D. Scores of Nucleus Necrosis = 2,E. Scores of *Hyaline Cast* = 1,F. Scores of *Hyaline Cast* = 2

The GM control group showed many features of the hyaline cast (score of 6) (Figure 1 D). Analysis of the results of the median hyaline cast score of the negative control and the GM group revealed a very significant difference (P<0.01). The GM group showed a significant among of hyaline deposits, a sign that severe kidney damage happened. The score of GPPHB 200 mg/kgBB was significantly different from the GM group (P<0.01) as depicted by almost no hyaline cast, giving rise to the assumption that the administration of GPPHB 200 mg/kgBB had an improved effect of renal tubular epithelial cells, as demonstrated by the decrease in the hyaline cast score. The results score of GPPHB 50, 100 mg/kgBB (score of 2) and Ketosteril were significantly different from the GM control group (score of 6) (P<0.05). The administration of a GPPHB dose of 200 mg/kgBB had a hyaline cast score of 0, and the score was the same as the negative control group (score of 0), which was not given any treatment.

Result of hematology profiles (Table 2)

All treatments on GM-induced rats had WBC, Hb, RBC, and MCV mean levels that had no significant differences. The mean of platelet, MCH, and MCHC levels between the negative control and GM groups had significantly different results. The mean level of platelets showed that GPPHB at a dose of 100 and 200 mg/kgBW could increase platelet levels. The comparison groups, Ketosteril, and Vitamin E also had increased platelet levels but not as good as the negative controls (Table 3). Only the negative control group had a normal value of platelets. Analysis of MCH means levels demonstrated that all treatment groups were still in the normal range value of MCH (17.1-20.4 pg/cell). All treatments did not show a significant effect on the MCHC level; however, all results were lower than the normal value (32.9-37.5 g/dL). Only the negative control and Vitamin E groups showed normal values of MCHC. The results of the analysis of mean hematocrit levels showed low results, and all groups exhibited a decrease compared with D7, although results still in a normal range value (39.6%-52.5%), as illustrated in Table 3.

Tabel 2. Total number of median score of histopathological parameters of kidneys of GM-induced rat

Groups (3 slides)		Cloudy Swelling	Nucleus Necrosis	Hyaline Cast
1.	GPPHB 50	3	1	2
2.	GPPHB 100	3	0	2
3.	GPPHB 200	3	1	0
4.	Negative C	3	0	0
5.	Positive/GM	3	4	6
6.	Simvastatin	3	1	2
7.	Fenofibrate	3	4	4
8.	Ketosteril	3	3	5
9.	Vitamin E	3	0	5

Table 3. Hematology profile of GM-induced Rats After Treatment

Groups	WBC (10^3/mm^3)		Hb (g/dL)		RBC (x10^6/mm^3)		Platelet (10^3/mm^3)	
	D7	D35	D7	D35	D7	D35	D7	D35
1	8.1±0,31	7.0±0,51	13.4±0,13	12.1±0,23	6.7±0,05[a]	6.5±0,08	546,0±59,07	489.3±54.11[c]
2	7.7±0,53	7.20±0,37	13.7±0,14	11.6±0,40	7.6±0,08	6.2±0,03	475,6±57,37	548.0±96.97[c]
3	8.6±0,40	7,9±0,65	14.0±0,16	13.5±0,20	8.3±0,07	7.0±0,07	397.0±33,79	525.1±53.98[c]
4	8.1±0,31	9.2±1,19	14.1±0,21	13.5±0,08	7.8±0,08	5.8±0,09[bc]	466,5±22,30	647.8±75.39[b]
5	10.4±0,43[a]	7.1±0,50	14.3±0,14	13.7±0,12	8.6±0,05	7.1±0,07[c]	420,6±49,53	474.5±52.24
6	8.9±0,57	6.7±0,39	14.4±0,45	13.3±0,08	7.4±0,12	6.5±0,05	560.5±36,86	556.2±15.27[b]
7	7.1±0,40[a]	6.4±0,59	12.9±0,17	12.6±0,16	6.6±0,08	6.3±0,06[b]	492.1±100,55	466.1±.18.37
8	8.4±0,53	8.1±0,33	13.8±0,19	13.1±0,08	7.6±0,06	6.6±0,06[b]	479.8±52,30	547.7±62.49[c]
9	5.0±0,59[a]	6.1±0,30	11.3±0,26	12.7±0,26	5.5±0,07	5.7±0,08[b]	477.3±84,69	609.7±97.88[c]
Sig	**	NS	NS	NS	NS	NS	NS	**
Normal value		1.96-8.25		13.7-17.6		7.27-9.65		638-1177

Notes
a. significantly different from the negative control group
b. significantly different from the positive control group
c. significantly different from the 7th-day data
Sig. ANOVA

Groups	MCH (pg/cell)		MCHC (g/dL)		MCV(μm^3/cell)		Hematocrit (%)	
	D7	D35	D7	D35	D7	D35	D7	D35
1	20.1±2,02	17.6 ±2,23	29.4±0,20	26.2±0,69	68.4±0,20	67.2±0,69	45.5±0,11	43.8±0,32
2	18.1±2,34	18.8 ±2,59	26.9±0,39	27.9±0,50	67,5±0,39	67.3±0,50	51.3±0,32	41.4±0,14[b c]
3	17.0±2,79	17.7±2,12	25.2±0,65	26.1±0,54	67,6±0,65	67,7±0,54	56.0±0,26	47.1±0,28[c]
4	18.1±1,42	23.6±7,37[bc]	26.5±0,33	35.2±0,48[bc]	68.4±0,33	66.9±0,48	53.3±0,30	38.7±0,31[bc]
5	16.6±1,19	18.0±2,88	24.2±0,37	26.4±0,57	68.8±0,37	68.1±0,57	59.0±0,19	48.3±0,29[c]
6	19.5±2,92	20.3±3,14	28.8±0,39	30.1±0,86	67.8±0,39	67.6±0,86	50.0±0,39	44.3±0,25
7	19.8±4,19	21.0±2,61	29.5±0,35	30.4±0,90	67.0±0,35	69.1±0,90	44.0±0,30	43.3±0,26
8	18,5±1,37	20.1±1,89	27.2±0,30	29.7±0,39	67.9±0,30	67.6±0,39	51.3±0,26	44.3±0,62[c]
9	20.7±2,09	22.3±3,14[b]	29.5±0,41	33.8±0,32[b]	71.1±0,41	66.1±0,32	38.7±0,31	37.7±0,27[b]
Sig	NS	**	NS	**	NS	NS	NS	**/?
	Normal value	17.1-20.4		32.9-37.5		48.9-57.9		39.6-52.5

Results of lipid profiles

The total cholesterol levels of all treatment groups at D7 were significantly higher than D0 (P=0.05), indicating that GM induction reduced kidney function, as shown by the increase of total cholesterol level by D7. At D35, the measurements indicated a decrease compared to the results of D7 measurement, except for the GM control group.

The results of the statistical analysis of the percentage difference in total cholesterol levels between D35 and D7 revealed that the negative control group differed significantly from the GM group. The three treatment groups (PHGPB dose of 50, 100, and 200) demonstrated very significant differences with the GM group, although the GM groupalso presented a very significant difference with the Simvastatin comparison group. These results showed that administered PHGPB is able to reduce total cholesterol levels, but the effect is not equivalent when compared to Simvastatin as a standard drug. The results of the analysis are shown in Figure 2.

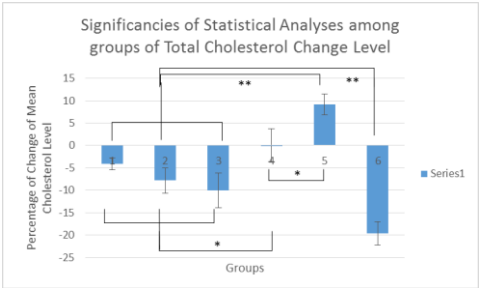

Figure 2. Significancies of statistical analyses among groups of total cholesterol

The result of LDL levels in all treatment groups at D7 significantly increased compared to D0. The results of the D35 measurement indicated a decrease compared

to the results of D7 measurement, except for the negative control group and the GM group.

Results of statistical analysis of the percentage difference of the negative control group LDL levels gave confusing results, depicting an increase in LDL levels, although statistically, the results were still significantly different from GM controls. The three treatment groups (PHGPB dose of 50, 100, and 200) showed very significant differences to the GM group, as well as with the comparison group Simvastatin. These results indicate that administered PHGPB had an effect on reducing LDL levels but not equivalent to the effectiveness of Simvastatin. The analysis result can be seen in Figure 3.

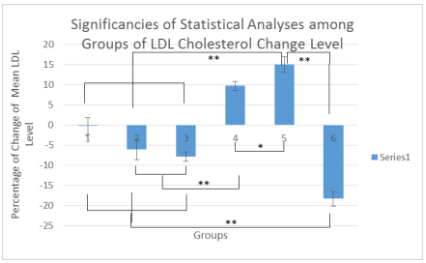

Figure 3. Significancies of statistical analyses among groups of triglyceride

Triglyceride levels from all treatment groups at D7 increased significantly as compared to D0. In the results of the D35 examination, almost all groups showed a decrease compared to the results of the D7 examination, except for the GPHPB 50 and the GM groups.

Results of statistical analysis of the percentage of triglyceride difference between D35 and D7 demonstrate that the negative control group differed significantly from the GM group. The GPPHB 100 and 200 groups showed highly significant differences to the GM group. The GPPHB 50 and 100 group differed significantly from the comparison group, Fenofibrate, indicating that PHGPB treatment is able to reduce triglyceride levels, but its effect is not as effective as Fenofibrate. Data are shown in Figure 4.

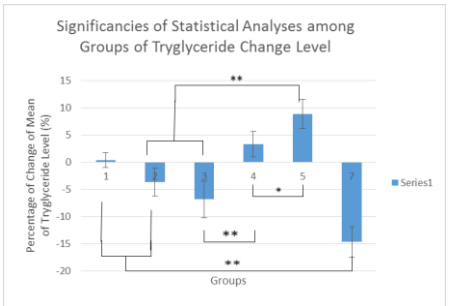

Figure 4. Significancies of statistical analyses among groups of triglyceride

Discussion

Nephrotoxicity caused by GM-induction at a dose of 80 mg/kgBW for seven days was significantly proven by increased urea and creatinine levels (increased by more than 50%). Paired t-tests before and after GM-induction revealed all treatments differed significantly (P<0.01) except for the negative control group (20).

After GM induction, all groups of treatments had a significant decrease in BW, except for the negative control group. On examination of kidney OI, administration of the GPPHB dose of 50 mg/kgBB did not demonstrate significantly different results (0.38±0.03) with the negative control group (0.35±0.02), and the weight was much lighter than the GM group (OI GM: 0.43±0.04). These findings agree with Prairie on the effect of pea protein hydrolysate on CKD in rodent models in that diseased rats weighed less compared to normal rats, but results of kidney weights relative to body weights were to the contrary. Gross examination showed the kidneys of diseased rats were significantly larger than normal rats (3). Hence, the administration of the GPPHB dose of 50 mg/kgBW maintained the weight of the kidney and did not cause enlargement, as in the case of the kidney damage caused by GM.

The proximate analysis showed that GPPHB contained total phenol and tannins. (20) Phenol compounds function as antioxidants to prevent deteriorating kidney function by inhibiting lipid peroxidation (21). Tannin compounds in green peas hydrolysate protein can increase LDL receptor synthesis and activity 3-hydroxy-3-methylglutaryl CoA reductase, resulting in an increase in uptake and LDL degradation, which can further reduce total cholesterol levels (22). Consumption of a sufficient amount of peas in the daily diet has been shown to reduce blood cholesterol levels (23). The lowest total cholesterol, LDL, and triglycerides level were found in the rats administered with GPPHB at a dose of 200 mg/kgBB. The greater the dose of GPPHB, the greater the decrease in lipid profile significance.

Aminoglycosides are ototoxic and nephrotoxic and induce human red cell membrane fragility in a concentration-independent manner. Administering GM can cause fragility of erythrocyte membranes so that erythrocytes are lysed easily (4). The administration of GM caused erythrocyte fragility in the condition of azotemia (3). The administration of GM for several days only caused mild kidney disorders, which are almost always reversible, allowing the body to compensate. Hb levels did not differ significantly, and therefore the

process of homeostasis in the animal's body remained under physiological conditions.

The induction of GM significantly decreased the levels of Hb, Ht, and erythrocytes because of the nephrotoxic condition that decreased kidney function. The results of the group given a GPPHB dose of 200 mg/kgBB did not differ significantly from the comparison control group Ketosteril and Vitamin E. However, the administration of GPPHB did not improve erythropoietin system disorders, which could be due to shorter treatment duration. In the present study, the most effective dose for the hematological profile of GM-induced Wistar rats cannot be ascertained. In normal rats, erythrocytes and hemoglobin levels can fluctuate, and the highest levels occur at the age of six weeks. Male Wistar rats in different age groups can have different hematological profiles and body mass. Animals in the same strain but bred in different places, or having acclimatized to certain areas and conditions, give different hematological profiles (24). According to Nakhjavani *et al*., there is a strong relationship between the ratio of neutrophils/lymphocytes and GFR, although the mechanism underlying changes in white blood cell counts may differ under different conditions. The condition of neutrophilia and lymphopenia can be observed in stressful situations, such as CKD conditions (25).

Consumption of GM in the short-term causes of kidney damage and necrosis of proximal tubules. However, the body can still compensate for the erythropoiesis process because erythropoietin is produced in peritubular kidney interstitial cells, and is also produced by the liver or extrarenal (26).

Cortical or tubules necrosis that occurs due to antibiotics gentamicin cannot be metabolized properly and is retained in epithelial cells proximal tubule after filtrated by the glomerular (17). Nucleus necrosis occurs in severe cell damage, causing the cell nucleus to break and become damaged, processes known as carioexyst and cardiolysis, respectively. GM induction for seven days clearly caused cell damage in histopathological Wistar rat kidneys. Necrosis stimulates cell immunity, such as macrophages and foam cells, to surround the injured kidney. These cells secrete growth factors such as TGF-β1 that stimulate mesangial cells to become mesangioblasts. Mesangioblast cells secreting the extracellular matrix causes glomerulosclerosis and decreased kidney filtration ability. Over time, glomerulosclerosis develops into CKD that interferes with kidney function. Impaired kidney function causes significant changes in lipoprotein metabolism, which can cause severe dyslipidemia and cardiovascular disease (27).

The hyaline cast is commonly found in advanced or chronic kidney damage. The hyaline cast is formed from the deposition of the Tamm-Horsfall mucoprotein, which is secreted by the cells of the renal tubules. The formation of a hyaline cast is influenced by environmental conditions in which precipitation and denaturation of proteins occur (e.g., slow urine flow, high urine salt levels, or low pH) (18).

In the kidney histopathology of all treatment groups, the negative and positive control groups showed mild tubular degeneration. GPPHB at a dose of 100 mg/kgBW showed improvement in the histopathological score of necrosis, while for the hyaline cast, GPPHB was at a dose of 200 mg/kgBB. As a conclusion, three doses of GPPHB had antinephrotoxicity and antihyperlipidemia effects in GM-induced nephrotoxicity Wistar rats.

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