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A Modified Goldblatt 2K1C Kidney Arterial Ligation Method and Salt Consumption: Which one is best for Hypertensive Mouse Model?

Astrid Feinisa Khairani[1,7]*, Muhammad Afif Auliya[2], Mas Rizky Anggun Adipurna Syamsunarno[3,7], Yunisa Pamela[1,7], Kurnia Wahyudi[4,7], Nandina Oktavia[5,7] and Mohammad Iqbal[6,7]

[1]Divison of Cell Biology, Department of Biomedical Sciences,
Faculty of Medicine, Universitas Padjadjaran, Bandung, Indonesia.
[2]Undergraduate Program, Faculty of Medicine, Universitas Padjadjaran, Bandung, Indonesia.
[3]Divison of Biochemistry and Biology Molecular, Department of Biomedical Sciences,
Faculty of Medicine, Universitas Padjadjaran, Bandung, Indonesia.
[4]Department of Public Health, Universitas Padjadjaran, Bandung, Indonesia.
[5]Divison of Anatomy, Department of Biomedical Sciences,
Faculty of Medicine, Universitas Padjadjaran, Bandung, Indonesia.
[6]Department of Cardiology and Vascular Medicine Dr. Hasan Sadikin Hospital,
Universitas Padjadjaran, Bandung, Indonesia.
[7]Research Center of Health System, Faculty of Medicine,
Universitas Padjadjaran, Bandung, Indonesia.
*Corresponding author E-mail: astrid.khairani@unpad.ac.id

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The Goldblatt method is one of several methods that can be used in making mouse model of hypertension. The principle of the Goldblatt method itself aims to cause renovascular hypertension by constricting renal blood vessels that results in hypoxic injury and consequently affects the RAAS (Renin-Angiotensin-Aldosterone System). This study aims to determine whether a modification to the Goldblatt method and an additional high salt consumption could be a more effective method in developing a mouse model of hypertension. This study was done on DDY strain mice with 4 weeks of age and 25-30 grams of weight. A simple ligation to constrict renal blood vessel was used as modification from the original Goldblatt 2K1C (two-kidney, one-clip) method. A total of 32 mice were randomized to receive either a modified Goldblatt, modified Goldblatt with salt diet, sham surgery only, or sham with salt diet. Blood pressure was measured at baseline, first week after intervention, and second week after intervention. The data was then analysed by repeated ANOVA method. Hematoxylin and eosin staining was used to analyse histology appearance. This study showed that using a high salt diet only-method generated a hypertensive state faster compared to the modified Goldblatt method, while the modified Goldblatt method produced a steadier increase of blood pressure. Statistically, there was a significant difference of blood pressure between the sham and salt diet group compared to the other three groups at the first week after intervention ($p<0,05$). The resulting blood pressure from all the methods used in this study was influenced by time. From all four interventions, it is concluded that the modified Goldblatt 2K1C arterial ligation method with an additional high salt consumption had an effect on mice blood pressure. It is prospective to use the salt intervention method for study of hypertension with a short-time period because its acute effect on the rise of diastolic blood pressure was more rapid than the other three groups. On the other hand, the ligation group produced a steadier increase in diastolic blood pressure, therefore might be effective to be used for study of hypertension in a long-time period.

Keywords: *Goldblatt 2K1C*, hypertension, mouse model, salt consumption.

Hypertension kills almost 8 million people annually and nearly 1 billion of population have hypertension worldwide.[1] Hypertension is a common disease with a high prevalence not only in developing countries but also in developed countries. Research on the pathophysiology of hypertension in human is complex and multifactorial, making it a challenging task.[2] Using animal subjects in research of hypertension is an effort to simplify the study of etiology, pathophysiology, complication, and treatment of hypertension.

Several animals can be used as models of hypertension, one of them being mouse. The methods for making such animal models vary, depending on the target of study. Based on past research, *Goldblatt* method is often used for developing rat models, but rarely used in mouse because of its smaller size.[3] The principle of *Goldblatt* method is producing renovascular hypertension by constricting renal blood vessels that results in hypoxic injury and later affects the RAAS (Renin-Angiotensin-Aldosterone System).[2]

The original *Goldblatt* method used silver clips to constrict renal blood vessel.[4] In this study, the method was modified by using a simple ligation to constrict renal blood vessel. To achieve hypertension state more rapidly, high salt consumption was also added to the method by using combined salt diet that contains sodium (0,22 mmol/gram) and potassium (0,20 mmol/gram).[5] This study aims to determine whether the modified *Goldblatt* and high salt consumption method is more effective in making mouse model of hypertension. It is prospective that this research could be a future reference for determining an effective method for developing mouse model of hypertension

MATERIAL AND METHODS

Research Subject and Design

The research design used is experimental laboratory analysis study. The subjects used in this study were DDY strain mouse with 4 weeks of age and 25 – 30 grams of weight, obtained from PT. Bio Farma (Bandung, Indonesia). All mouse adapted to the local air for 3 days after pickup. All mouse were placed in proper cage and given food and drink.

This research was done in Animal Laboratory of Biochemistry and Molecular Biology, Biomedical Sciences Department, Faculty of Medicine Universitas Padjadjaran, Jatinangor and Pharmacology Laboratory of Faculty of Pharmacy Universitas Padjadjaran, Jatinangor.

This study was performed based on 3R principle and was approved by The Research Ethics Committee of Universitas Padjadjaran with the ethical clearance number of 596/UN6.KEP/EC/2018.

Intervention

The mice were divided into four groups based on intervention. The mouse models of hypertension were established using the modified Goldblatt two-kidney, one-clip (2K1C) method through ligation of left renal artery. Following anaesthesia, a ligation on left renal artery was done on group I and II, while sham opening on the left side of each samples was done without left renal artery ligation on group III and IV. High salt solution (Sodium 0,22 mmol/gram and Potassium 0,20 mmol/gram) were given to group II and IV.[5,6]

Histology Analysis

A total of 12 mice were prepared for kidney dissection. The mice were perfused with formaldehyde 4% to fixate both normal (right) and ligated renal artery (left kidney). Afterward, the kidneys were dissected and soaked in a formaldehyde 4% solution. The kidneys were blocked in paraffin, cut transversally with a thickness of 5 µm, and then stained with Hematoxylin and Eosin (H&E) in the Laboratory of Cell Biology, Department of Biomedical Sciences, Faculty of Medicine, Universitas Padjadjaran, Bandung.

The kidney samples were observed under a light microscope using a camera (OptiLab Advance Plus) and images were taken using OptiLab Viewer Plus 3 software. The histological images of the kidneys were processed using ImageJ software version 1.51j8. A total of 1-3 sampling sections were selected from one slide of the mice kidney samples in each group. In each section, images from three cross-sectional areas were taken randomly. The images of the cross-sectional area were taken using 10 × objective magnification.

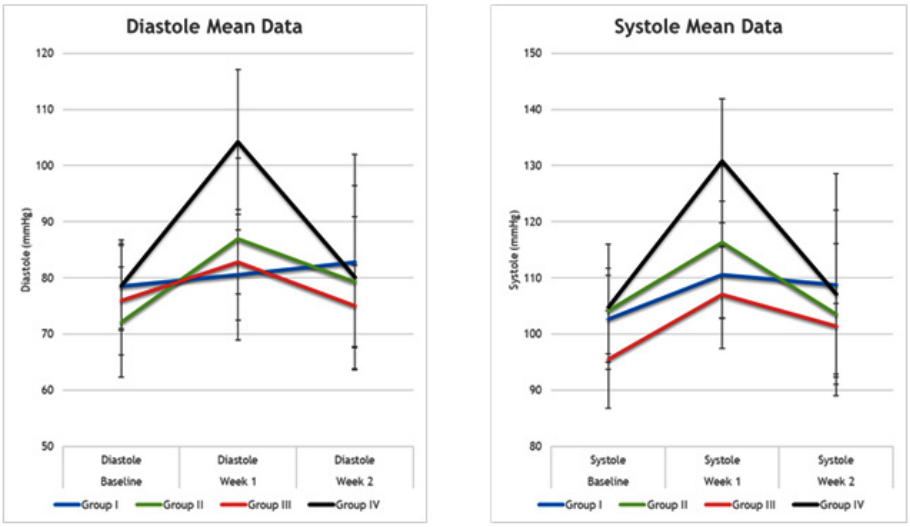
Blood Pressure Measurement

Blood pressure of the mice was measured without anaesthesia by using "The CODA High

Throughput Non-Invasive Blood Pressure". All samples were measured on the same day every week according to the day the interventions were given.[7]

Statistical Analysis

The quantitative data obtained was analysed using repeated ANOVA method. The results were considered as significant when p value is < 0.05.



Graph 1. Mean of Systole and Diastole Blood Pressure after 3 times of measurement (Baseline, One week following intervention, and two weeks following intervention). Systolic and diastolic blood pressure of each samples was measured at baseline, one week following intervention, and two weeks following intervention. There was no difference among the four groups at baseline and at second week after intervention. There was a significant difference at first week after intervention between sham and salt diet group compared to the other three groups ($p<0.0001$). Blue line represents group I (mice with modified Goldblatt 2K1C ligation on left renal artery) ; green line represents group II (mice with modified Goldblatt 2K1C ligation on left renal artery and salt consumption); red line represents group III (mice with sham surgery without ligation on left renal artery); black line represents group IV: mice with sham surgery without ligation on left renal artery, with salt consumption

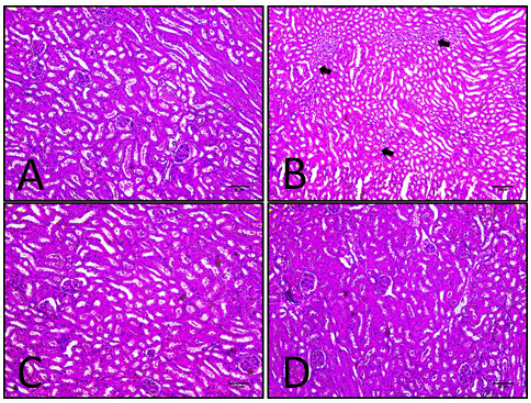


Fig. 1. Representative image of the left kidneys stained with H&E stain at 100x magnification. Histologic appearance from control (A, upper left), arterial ligation (B, upper right), salt and ligation (C, lower left), and salt only (D, lower right). Pointed arrows indicates infiltration of inflammatory cells

RESULTS AND DISCUSSION

From 32 mice enrolled in this study, two of them were dropped out because of death during the data collection period. One excluded sample came from the first group and another was from the second group. Mean blood pressure of the samples is shown on Graph 1.

A total of 32 mice were randomized to receive either ligation on left renal artery, ligation with salt diet, sham only, or sham with salt diet. Blood pressure was measured at baseline, first week after intervention, and second week after intervention. Complete data was available at all time points for 10 mice that received ligation, 10 mice that received ligation with salt diet, 6 mice that received sham, and 6 mice that received sham with salt diet. Mauchly's test indicated that

the assumption of sphericity had been violated, therefore the Huynh-Feldt corrected tests were applied. There was a significant effect of time ($p<0,0001$). Post hoc comparison on systole and diastole indicated that there was no difference among the four groups at baseline and at second week after intervention. There was a significant difference between sham and salt diet group compared to the other three groups at the first week after intervention. From all four groups, it

Table 1. Result of Systolic Post-Hoc Comparison (p<0,05 = significant)

Pairwise Comparisons

Measure: MEASURE_1

Time	(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
						Lower Bound	Upper Bound
1	Ligate	Ligate and salt	-1.444	4.073	.726	-9.817	6.928
		Sham	7.167	4.554	.128	-2.194	16.528
		Sham and salt	-2.167	4.554	.638	-11.528	7.194
	Ligate and salt	Ligate	1.444	4.073	.726	-6.928	9.817
		Sham	8.611	4.554	.070	-.750	17.972
		Sham and salt	-.722	4.554	.875	-10.083	8.639
	Sham	Ligate	-7.167	4.554	.128	-16.528	2.194
		Ligate and salt	-8.611	4.554	.070	-17.972	.750
		Sham and salt	-9.333	4.989	.073	-19.588	.921
	Sham and salt	Ligate	2.167	4.554	.638	-7.194	11.528
		Ligate and salt	.722	4.554	.875	-8.639	10.083
		Sham	9.333	4.989	.073	-.921	19.588
2	Ligate	Ligate and salt	-5.778	5.497	.303	-17.076	5.521
		Sham	3.556	6.146	.568	-9.077	16.188
		Sham and salt	-20.278*	6.146	.003	-32.910	-7.645
	Ligate and salt	Ligate	5.778	5.497	.303	-5.521	17.076
		Sham	9.333	6.146	.141	-3.299	21.966
		Sham and salt	-14.500*	6.146	.026	-27.132	-1.868
	Sham	Ligate	-3.556	6.146	.568	-16.188	9.077
		Ligate and salt	-9.333	6.146	.141	-21.966	3.299
		Sham and salt	-23.833*	6.732	.002	-37.671	-9.995
	Sham and salt	Ligate	20.278*	6.146	.003	7.645	32.910
		Ligate and salt	14.500*	6.146	.026	1.868	27.132
		Sham	23.833*	6.732	.002	9.995	37.671
3	Ligate	Ligate and salt	5.222	7.070	.467	-9.310	19.754
		Sham	7.444	7.904	.355	-8.803	23.692
		Sham and salt	1.611	7.904	.840	-14.636	17.859
	Ligate and salt	Ligate	-5.222	7.070	.467	-19.754	9.310
		Sham	2.222	7.904	.781	-14.025	18.470
		Sham and salt	-3.611	7.904	.652	-19.859	12.636
	Sham	Ligate	-7.444	7.904	.355	-23.692	8.803
		Ligate and salt	-2.222	7.904	.781	-18.470	14.025
		Sham and salt	-5.833	8.659	.506	-23.632	11.965
	Sham and salt	Ligate	-1.611	7.904	.840	-17.859	14.636
		Ligate and salt	3.611	7.904	.652	-12.636	19.859
		Sham	5.833	8.659	.506	-11.965	23.632

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

a. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

is concluded that the sham and salt intervention method might be effective for study of hypertension with a short-time period, while the ligation group was steadier and might be used for a long-time period study.

Using mouse as a model of hypertension should mimic the clinical manifestation of hypertension in human because mouse and human share about 98% DNA.[8] Based on the Seventh Report of the Joint National Committee on

Table 2. Result of Diastolic Post-Hoc Comparison (p<0.05 = significant)

Pairwise Comparisons

Measure: MEASURE_1

Time	(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
						Lower Bound	Upper Bound
1	Ligate	Ligate and salt	6.444	4.168	.134	-2.123	15.012
		Sham	2.556	4.660	.588	-7.023	12.134
		Sham and salt	-.111	4.660	.981	-9.690	9.467
	Ligate and salt	Ligate	-6.444	4.168	.134	-15.012	2.123
		Sham	-3.889	4.660	.412	-13.467	5.690
		Sham and salt	-6.556	4.660	.171	-16.134	3.023
	Sham	Ligate	-2.556	4.660	.588	-12.134	7.023
		Ligate and salt	3.889	4.660	.412	-5.690	13.467
		Sham and salt	-2.667	5.105	.606	-13.159	7.826
	Sham and salt	Ligate	.111	4.660	.981	-9.467	9.690
		Ligate and salt	6.556	4.660	.171	-3.023	16.134
		Sham	2.667	5.105	.606	-7.826	13.159
2	Ligate	Ligate and salt	-6.333	5.660	.273	-17.969	5.302
		Sham	-2.278	6.329	.722	-15.286	10.731
		Sham and salt	-23.611*	6.329	.001	-36.620	-10.603
	Ligate and salt	Ligate	6.333	5.660	.273	-5.302	17.969
		Sham	4.056	6.329	.527	-8.953	17.064
		Sham and salt	-17.278*	6.329	.011	-30.286	-4.269
	Sham	Ligate	2.278	6.329	.722	-10.731	15.286
		Ligate and salt	-4.056	6.329	.527	-17.064	8.953
		Sham and salt	-21.333*	6.933	.005	-35.584	-7.083
	Sham and salt	Ligate	23.611*	6.329	.001	10.603	36.620
		Ligate and salt	17.278*	6.329	.011	4.269	30.286
		Sham	21.333*	6.933	.005	7.083	35.584
3	Ligate	Ligate and salt	3.556	6.934	.612	-10.698	17.809
		Sham	7.778	7.753	.325	-8.158	23.714
		Sham and salt	2.611	7.753	.739	-13.325	18.547
	Ligate and salt	Ligate	-3.556	6.934	.612	-17.809	10.698
		Sham	4.222	7.753	.591	-11.714	20.158
		Sham and salt	-.944	7.753	.904	-16.881	14.992
	Sham	Ligate	-7.778	7.753	.325	-23.714	8.158
		Ligate and salt	-4.222	7.753	.591	-20.158	11.714
		Sham and salt	-5.167	8.493	.548	-22.624	12.291
	Sham and salt	Ligate	-2.611	7.753	.739	-18.547	13.325
		Ligate and salt	.944	7.753	.904	-14.992	16.881
		Sham	5.167	8.493	.548	-12.291	22.624

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

a. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC 7), hypertension in human is defined as systolic blood pressure over 140 mmHg and/or diastolic blood pressure over 80 mmHg, while a systolic blood pressure between 120 and 139 and a diastolic blood pressure between 80-89 categorized as pre-hypertension.[9] Graph 1 shows that at the first week after intervention the fourth group achieved a diastolic hypertensive state, and the second group achieved a diastolic pre-hypertensive state. The first group achieved a prehypertensive state at the second week after intervention.

DISCUSSION

There was a rise in blood pressure at the first week after intervention which then decreased at the second week after intervention in the second, third, and fourth group (ligation and salt diet; sham; sham and salt diet group respectively), but not in the first group (ligation group). It may be concluded that the resulting blood pressure in this study was time-dependent. The ligation group was steadier than the other intervention groups, while the salt group attained a hypertensive state more rapidly.

A possible cause of a faster hypertensive state reached by the high salt intervention group is a high concentration of salt in the kidney induced high reabsorption of water to stabilize the osmolarity that resulted in an increased of blood volume. A higher blood volume caused the cardiac output to increase and raised the blood pressure. On the other hand, after some period of time the salt will be removed through urine physiologically. This might explain why on the second week after intervention the blood pressure returned to normal.[10]

Renovascular hypertension cause renal ischemia that stimulate recruitment of leukocyte and higher production of reactive oxygen species. Renal ischemia also cause activation of Renin-Angiotensin-Aldosterone-System to happen and stimulates the release of Angiotensin that later on activated by Angiotensin Converting Enzyme into Angiotensin II.[2,11] Angiotensin II also considered as inflammatory factor that cause inflammation on renal tissue.[12] The inflammatory mechanism was started to appear as infiltration of inflammatory cells on renal tissue as seen on Figure 1. Mice in group II (mice with modified Goldblatt 2K1C ligation on left renal artery and salt consumption) showed some area of infiltration of inflammatory cells. Meanwhile histologic appearance of the other groups were still appeared normal condition.

Based on past research, the use of arterial ligation method was considered to be inconsistent and unstable.[13] Nevertheless, several other studies have achieved a hypertensive state using this method after 1 week of research.[13,14]

Being a preliminary study, there were several limitations of this study. Such limitations were the uncontrolled volume of water intake by the mice and physiology of the mice that allowed them to adapt to high salt water consumption.

CONCLUSION

The resulting blood pressure of all methods used in this study was influenced by time. From all four interventions, it is concluded that the modified Goldblatt 2K1C arterial ligation method with an additional high salt consumption had an effect on mice blood pressure. It is prospective to use the salt intervention group for study of hypertension with a short-time period because its acute effect on the rise of diastolic blood pressure was more rapid than the other three groups. Meanwhile, the ligation group produced a steadier increase in diastolic blood pressure therefore possible to be used for study of hypertension with a long-time period.

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