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CORRELATION BETWEEN NEPHRIN EXPRESSION, TUBULAR INJURY, AND SERUM CREATININE LEVEL IN KIDNEY FAILURE MODEL WITH 5/6 SUBTOTAL NEPHRECTOMY IN MICE

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ABSTRACT

Background: *Chronic kidney diseases (CKD) is characterized by glomerulosclerosis, tubular injury, and proteinuria. Neprin is the one of the most important protein involved in glomerular filtration but the mechanism of nephrin expression in chronic kidney failure is not well understood.*
Objective: *We aims to elucidate the correlation between nephrin expression with tubular injury and serum creatinine level.*
Methods: *We performed 5/6 subtotal nephrectomy (SN) in male strain Swiss mice to induce CKD. Sham operation was performed to control group (SO) (n=8). Mice were sacrificed in day 7 (SN7; n=8) and day 28 (SN28; n=8) after operation. We measure creatinine serum level to assess renal function. Tubular injury score was quantified using Periodic Acid Schiff (PAS) staining. Reverse transcriptase PCR (RT-PCR) was carried out to examine Nephrin mRNA expression.*
Results: *5/6 subtotal nephrectomy induced an increased of serum creatinine level in SN7 and SN28 ($p<0,01$ vs SO), followed by an increased of tubular injury score in SN7 and SN28 ($p<0,01$ vs SO). We confirmed reduction of nephrin expression in SN28 ($p<0.01$ vs SO). There was a negative correlation between nephrin and tubular injury ($r=0.719$, $p<0.01$) and the positive correlation between tubular injury and serum creatinine level ($r=0.891$, $p<0.01$). However, we did not find any significant correlation between nephrin expression and serum creatinine level.*
Conclusion:
Nephrin expression downregulation might represent renal function disturbtion in CKD.

Keywords: *nephrin, 5/6 subtotal nephrectomy, remnant kidney, mice, serum creatinine, tubular injury, Sham Operation.*

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INTRODUCTION

Kidney failure enhances the decrement of the renal function and marked by the reduction of Glomerular Filtration Rate (GFR). This alteration was followed by reduction of nephron significantly[1]. Indonesian nephrologist association declared from 2007 until 2012, there is an increase of the new patients who used hemodialyzer, and it reaches 28.782[2].

The 5/6 subtotal nephrectomy (remnant kidney model) has been widely used as a pathogenic mechanism of chronic kidney disease and has been reliance on studies of renal diseases' progressivity. The remnant kidney model is performed by unilateral nephrectomy and followed by contralateral renal ablation. Hypertrophy and hyperplasia are the hallmarks of the remnant kidney model. Shortly, after performing renal contralateral renal ablation various changes develop [3]. Remnant kidney model demonstrates the histopathological features, for example: decreased of podocyte density, segmental proliferation of parietal epithelial cell, tubule cell reflux, and lost of brush border at proximal tubules [4].

Podocyte integrity is maintained by nephrin. Nephrin is located in the slit diaphragm of podocyte specifically [5]. Renal tubules cells destruction is caused by excessive inflammation process that which lead into tissue edema & tubules cells injury, tubule blood circulation distubances, direct contact with toxic agents like drugs, radiocontrast agents, myoglobin, radiation, or tubules obstruction that are caused by *casts*, cellular debris, or crystal sedimentation [6]. Until now, nephrin role toward kidney disease are not well understood. So, this research purpose is to elucidate the correlation between nephrin, creatinine serum level, and renal tubular injury in kidney-failure-model-mice with 5/6 subtotal nephrectomy.

MATERIALS AND METHODS

Preparation of Experimental Animal

We obtained the animals from Unit Pengembangan Hewan Penelitian (UPHP), Universitas Gadjah Mada. We performed quasi-experimental research using 24 male Swiss background mice, 4 months-old (30-40 grams). The mice were divided into 3 groups: (1) SO (*Sham Operation*) group; (2) SN7 (7 days post-subtotal nephrectomy) group; (3) SN28 (28 days post-subtotal nephrectomy) group. Mice were maintained in the Anatomy Laboratorium, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada in the temperature 21°C, 50-60% humidity, and 12 hours light: dark cycle. Mice were fed with standard chows and water *ad libitum*.

5/6 Subtotal Nephrectomy (SN) procedure

We performed the SN procedure in two days. The first day, we anesthetized the mice with intraperitoneal injection of 0,1mL/10grams Body Weight (BW) of sodium pentobarbital. We opened right Flank's region, visualized the kidney, then removed the renal capsule and cut the kidney after renal pedicle ligation (uninephrectomy procedure). In day 2, we did the same procedure for the left kidney, however, we ablated the superior and inferior poles of the left kidney. Bleeding was stopped with electro cauter of the kidney. Mice were kept alive and sacrificed in day7 (SN7 group) and day28 (SN28 group). We committed the same procedure as the SN group without any removing kidney for Sham Operation (SO) group.

Serum Creatinine Level

After the due date, the blood was taken from the retro-orbital vein. Afterwards, we obtained the serum through centrifugation at 10.000 rpm for 10 minutes. The creatinine level was assessed in the clinical pathology laboratory, Faculty of Medicine, Public Health, and Nusing, Universitas Gadjah Mada.

Kidney harvesting

Termination was carried out with euthanasia procedure using lethal dose of ketamine. Then mice were dissected and perfused with NaCl 0.9% through apex cordis. To reduce the intracardiac pressure, we opened the right atrium. The kidney was harvested then put into RNA Later for assessing mRNA expression and fixated the kidney in 4% paraformaldehyde for histological examination.

Histopathological Quantification

Four micrometers of the tissues were cut and stained with Periodic Acid-Schiff (PAS) in Pathological anatomy Laboratory. This staining was performed in order to assess tubular injury. Quantification of tubular injury was performed under microscope using 400x magnification. We captured 10–15 random fields for quantification of tubular injury score.

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Tubular injury score was assessed based on histopathological changes such as tubular dilatation, brush border loss, tubular atrophy, nucleus condensation, intraluminal cast, and leukocyte infiltration [7]. Each slide was assessed based on the criteria and divided into score 0 until score 4. Score 0 was given if there is no changing at all (normal), score 1 if there were tubules destruction less than 25%, score 2 if there are tubules destruction between 25 until 50%, score 3 if there are tubules destruction between 50 until 75%, and score 4 if there are tubules destruction more than 75%. Tubular injury score quantification was performed by 3 slide-observers that blinded, then we made average tubular injury score from 3 observers.

Nephrin mRNA Expression

Nephrin mRNA expression was examined using Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). Afterwards, we performed densitometry analysis to analyze nephrin band intensity using ImageJ software with GAPDH as *house-keeping gene*. As much as 3 µL cDNA was used for RT-PCR with addition of these following reagents: (Primers, dNTP, 10×ExTaq buffer, *enzyme*). We prepared RT-PCR mixture for each cDNA that consist of: forward primer Nephrin 0,6 µL; reverse primer Nephrin 0,6 µL; PCR water 8,3 µL; Taq master mix 12,5 µL (last added). The condition for RT-PCR are denaturation step for 10 second at 94^0C, annealing step for 20 second at 60^0C, extension step for 1 minutes at 72^0C with 35 cycles. The amplicon was kept in 4^0C. Nephrin expression intensity was analized using ImageJ software. Analysis score was compared with GADPH expression intensity, so we earned the nephrin expression result.

RESULT

Creatinine serum level in SO group were 0.74 mg/dL; SN7 group 1.28 mg/dL; and SN28 group 1.48 mg/dL respectively. There was significant difference between SO group vs SN7, SO vs SN28, dan SN7 vs SN28 ($p<0.01$). Tubular injury score in SO group was 0.522; SN7 group 2.04; dan SN28 group 3.28 (Fig. 1). There was significant difference between SO vs SN7 and SO vs SN28 ($p< 0.01$). There was significant difference between SN7 vs SN28 ($P< 0.05$). Nephrin mRNA expression was significantly different between SO group and SN28 group. The SN28 group had lower Nephrin mRNA expression ($p<0.01$) compared to SO and SN7 group ($p< 0.05$). There high significant positive correlation between tubular injury and creatinine serum level. There was intermediate no-significant negative correlation between nephrin expression and serum creatinine level. There was high significant negative correlation between nephrin expression and tubular injury score.

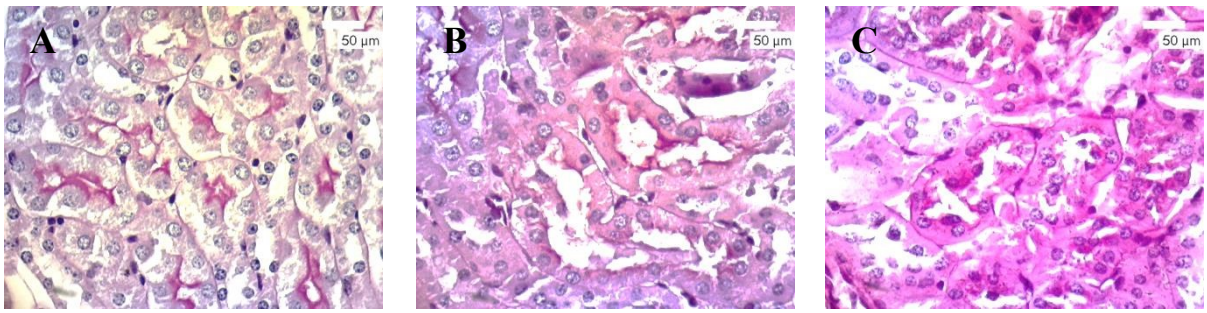

Figure 1. Histopathological tubular injury. **(A)** Tubular injury in SO group**. (B)** Tubular injury in SN7 group. **(C)** Tubular injury in SN28 group

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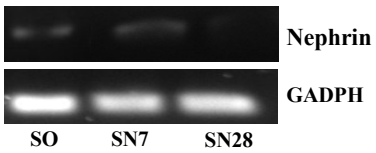

Figure 2. Densitometry result of nephrin expression and GADPH band using electrophoresis.

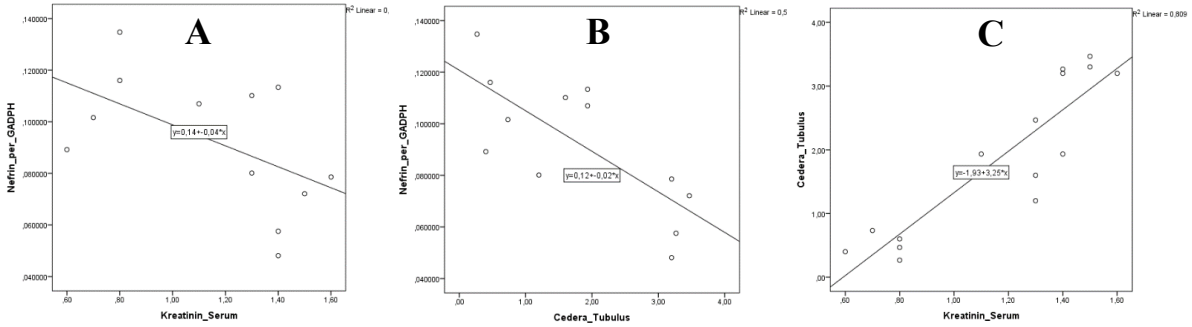

Figure 3. Correlation. **(A)** Tubular Injury with Serum Creatinine. **(B)** Expression of Nephrine with Serum Creatinine. **(C)** Expression of Nephrine with Tubular injury.

Table 1. Average ± standard deviation of serum creatinine level, tubular injury score, and nephrin expression in each treatment group

Group	Average ± Standard Deviation		
	Nephrin expression	Tubular injury score	Serum creatinine level
A (SO)	0.1103 ± 0.019	0.5222 ± 0.1838	0.74 ± 0.089
B (5/6 SN 7 days)	0.1026 ± 0.015	2.0445 ± 0.4305**	1.28 ± 0.109**
C (5/6 SN 28 days)	0.0640 ± 0.0138**	3.2858 ± 0.3074**	1.48 ± 0.083**

** $p<0.01$ vs SO

DISCUSSION

Remnant kidney model induces CKD after 7 days and 28 days with diffrerent degree severity. Chronic kidney failure was confirmed through augmentation of serum creatinine level followed by increasing tubular injury score. Nephrin expression was significantly decreased in SO group with SN28 group and SN7 group with SN28 group. There was significant negative correlation between nephrin expression and tubular injury score. The 5/6 Subtotal nephrectomy stimulates the development of the hypertrophy and hyperplasia of nephron as the compensation of decrement renal function [8,9,10]. It followed by histopathological changes which is shown as glomerulosclerosis, tubulointerstitial injury, renal dysfunction, and the most dangerous is uremic syndrome can occured in mice with 5/6 subtotal nephrectomy [10,11]. There was strong negative correlation between tubulointerstitial destruction with renal function disturbance in some glomerular immune diseases like membranous nephropathy, mesangioproliferative glomerulonephritis, focal segmental glomerulosclerosis (FSGS), type 1 mesangiocapillar glomerulonephritis, lupus nephritis, and other glomerular non-immune diseases like diabetic nephropathy[3]. In this research, 5/6 subtotal nephrectomy in mice can induce the occurence of

kidney failure that confirmed with increasing severity tubular injury and followed by decreasing nephrin expression due to longer treatment.

Creatinine serum level is the gold standard to assess renal function and it affected by tubular secretion and production, and extrarenal elimination.[12]. Creatinine is excreted by kidney in fixed amount based on muscle mass and free in plasma protein, so it can be a marker to evaluate renal function[13]. Increasing serum creatinine level indicated there was reduction of Glomerular Filtration Rate (GFR)[14]. Creatinine serum level that assessed form 24-hours urine is fragile to the error occurence and tend to not practical[15]. Recommendation for creatinine is better using serum creatinine than clearance creatinine[16]. In this study, creatinine level measured using serum creatinine level. There are factors that can affect to GFR like ages, races, genders, and body sizes[16,17].

Creatinine serum used to assess renal function and affected by tubular secretions, production, and extrarenal elimination[12]. We quantified the increment of creatinine serum in order to confirm renal function in chronic kidney disease model, 5/6 subtotal nephrectomy. Quantification of increasing serum creatinine level was performed to confim the occurence of kidney failure[18]. Remnant kidney model leads to damage of histopathological architecture, such as decrease of podocyte density, segmental proliferation of parietal epithelial cell, tubular cell reflux and lost of brush border at proximal tubule [4].

Increase of creatinine was followed by epithelial tubular cells injury which marked by CD24-positive cells. It consists very least of cytoplasm, mitochondria, and lost of brush border [19]. Dilatation of tubules, lost of brush broder, vascular remodeling, detachment of cells from glomerular basement membrane, infiltration of leukocyte, and capillary edema were the characteristic of tubular injury [7] [20]. Tubular injury score was increase significantly in 5/6 subtotal nephrectomy mice. Glomerulosclerosis may lead to tubular injury in kidney failure disease [21].

Prolong chronic kidney disease reduced mRNA expression of Nephrine. Nephrine has an important role in maintain the integrity of the glomerular. Inactivation of the nephrine genes stimulates severe proteinureia. The alteration of the both mRNA and protein of Nephrine have an important role in pathological process of kidney disease. It affects glomerular membrane permeability [22]. Downregulation of the mRNA Nephrine expression followed by proteinuria and increase of creatinine was not significantly statistic ($p>0.01$). Since creatinine serum is the marker of glomerular injury.

From the previous research, downregulation of nephrine expression was correlates to the progressivity of kidney disease. Progressivity of glomerular injury contributes to the development of tubular injury caused by glomerular injury. During glomerular injury, the integrity of glomerular basement membrane was imperfect. Consequently, the level of nephrine and the other podocyte cytoskeleon was altered. Here, we elucidated that the downregulation of nephrine followed by increase of tubular injury, but this alteration does not followed with increase of creatinine serum [24],[25].

CONCLUSION

Chronic kidney disease induces downregulation of the mRNA Nephrine expression and negatively correlate with the augmentation of tubular injury score. Meanwhile, the tubular injury was positively correlate with serum creatinine level.

REFERENCES:

1. Bargman, J.M. and Scoreecki, K. (2013). 'Chronic Kidney Disease' in Harrison's nephrology and acid-base disorders, 2nd edn, eds Jameson, J. and Loscalzo, J., McGraw-Hill Medical, New York, pp.123-140.
2. Perhimpunan Nefrologi Indonesia. (2012). 5th Report of Indonesian Renal Registry. Pp.1-40.
3. Kliem, V., Johnson, R., Alpers, C., Yoshimura, A., Couser, W., Koch, K. and Floege, J. (1996). Mechanisms involved in the pathogenesis of tubulointerstitial fibrosis in 5/6-nephrectomized rats. *Kidney International*, 49(3), pp.666-678.
4. Qin, W., Xu, Z., Lu, Y., Zeng, C., Zheng, C., Wang, S. and Liu, Z. (2012). Mixed Organic Solvents Induce Renal Injury in Rats. *PLoS ONE*, 7(9), p.e45873
5. Welsh, G. and Saleem, M. (2009). Nephrin -- signature molecule of the glomerular podocyte?. *The Journal of Pathology*, p.n/a-n/a.
6. Alpers, C.E. (2010). 'The Kidney' in Robbins and Cotran Pathologic Basis of Disease, 8th edn, eds Kumar, V., Abbas, A.K., Fausto, N. and Aster, J.C., Elsevier Saunders, Philadelphia, pp.905-969.
7. Wang, Z., Gall, J., Bonegio, R., Havasi, A., Hunt, C., Sherman, M., Schwartz, J. and Borkan, S. (2011). Induction of heat shock protein 70 inhibits ischemic renal injury. *Kidney International*, 79(8), pp.861-870.
8. Purkerson, M.L., Hoffsten P.E. and Klahr, S. (1976). Pathogenesis of the Glomerulopathy Associated with Renal Infarction in Rats. *Kidney Int*, 9, pp.407-417.
9. Olivetti, G., Anversa, P., Rigamonti, W., Vitali-Mazza, L., and Loud, A.V. (1977). Morphometry of the Renal Corpuscle During Normal Postnatal and Compensatory Hypertrophy. *J Cell Biol*, 75, pp.573-580.
10. Shea, S.M., Raskova, J., and Morrison, A.B. (1978). A Stereologic Study of Glomerular Hypertrophy in the Subtotallu Nephrectomized Rat. *Am J Pathol*, 90, pp.201-210.
11. Olson, J.L., Hostetter, T.H., Rennke, H.G., Brenner, B.M. and Venkatachalam, M.A. (1982). Mechanisms of Altered Glomerular Permselectivity and Progressive Sclerosis Following Extreme Ablation of Renal Mass. *Kidney Int*, 41, pp.297-309.
12. Stevens, L., Coresh, J., Greene, T. and Levey, A. (2006). Assessing Kidney Function — Measured and Estimated Glomerular Filtration Rate. *New England Journal of Medicine*, 354(23), pp.2473-2483.
13. Oh, M.S. (2011). 'Evaluation of Renal Function, Water, Electrolytes, and Acid-Base Balance' in Henry's Clinical Diagnosis and Management by Laboratory Methods, 22nd edn, eds McPherson, R.A. and Pincus, M.R., Elsevier Saunders, Philadelphia, pp.174-178.
14. Jones, C., Jones, C., Wilson, I., Knox, T., Levey, A., Spiegelman, D., Gorbach, S., Lente, F. and Stevens, L. (2008). Cystatin C and Creatinine in an HIV Cohort: The Nutrition for Healthy Living Study. *American Journal of Kidney Diseases*, 51(6), pp.914-924.
15. Bragadottir, G., Redfors, B. and Ricksten, S. (2013). Assessing glomerular filtration rate (GFR) in critically ill patients with acute kidney injury - true GFR versus urinary creatinine clearance and estimating equations. *Critical Care*, 17(3), p.R108.
16. Verma, M., Khadapkar, R., Sahu, P. and Das, B. (2006). Comparing age-wise reference intervals for serum creatinine concentration in a "Reality check" of the recommended cut-off. *Indian J Clin Biochem*, 21(2), pp.90-94.
17. Poggio, E. (2005). Performance of the Modification of Diet in Renal Disease and Cockcroft-Gault Equations in the Estimation of GFR in Health and in Chronic Kidney Disease. *Journal of the American Society of Nephrology*, 16(2), pp.459-466.
18. Tremblay, R. (2004). Approach to managing elevated creatinine. *Can Fam Physician*, 50, pp.735-740.

19. Smeets, B., Boor, P., Dijkman, H., Sharma, S., Jirak, P., Mooren, F., Berger, K., Bornemann, J., Gelman, I., Floege, J., van der Vlag, J., Wetzels, J. and Moeller, M. (2013). Proximal tubular cells contain a phenotypically distinct, scattered cell population involved in tubular regeneration. *The Journal of Pathology*, 229(5), pp.645-659.
20. Yamada, K., Miwa, T., Liu, J. Nangaku, M. and Song, W. (2004). Critical Protection from Renal Ischemia Reperfusion Injury by CD55 and CD59. *The Journal of Immunology,* 172, pp.3869–3875.
21. Beck, L.H. and Salant, D.J. (2013). 'Tubulointerstitial Diseases of the Kidney' in Harrison's nephrology and acid-base disorders, 2nd edn, eds Jameson, J. and Loscalzo, J., McGraw-Hill Medical, New York, pp.205-215.
22. Wang, S., Rastaldi, M.P., Patari, A., Ahola, H., Heikilla, E. and Holthofer, H. (2002). Patterns of nephrin and a new proteinuria-associated protein expression in human renal diseases. *Kidney International*, 61, pp. 141–147.
23. Kawachi, H., Koike, H., Kurihara, H., Yaoita, E., Orikasa, M., Shia, M.A., Sakai, T., Yamamoto, T., Salant, D.J. and Shimizu, F. (2000). Cloning of rat nephrin: Expression in developing glomeruli and in proteinuric states. *Kidney Internationa*l, 57, pp.1949–1961.
24. Meyer, T. (2003). Tubular injury in glomerular disease. *Kidney International*, 63(2), pp.774-787.
25. Hussain, S., Romio, L., Saleem, M., Mathieson, P., Serrano, M., Moscat, J., Diaz-Meco, M., Scambler, P. and Koziell, A. (2009). Nephrin Deficiency Activates NF-Â B and Promotes Glomerular Injury. *Journal of the American Society of Nephrology*, 20(8), pp.1733-1743