ORIGINAL ARTICLE

Imbalance between Endothelin-1 and eNOS Expression Associates with Tubular Injury in Mice with 5/6 Subtotal Nephrectomy

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ABSTRACT

Introduction: Chronic Kidney Diseases (CKD) leads to kidney fibrosis which characterized by tubular injury and atrophy with interstitial fibrosis. Endothelin-1 (ET-1) and endothelial Nitrite Oxide Synthase (eNOS) are known to play role in CKD and kidney fibrosis, although their correlation with tubulo-interstitial injury have not been understood clearly. **Methods:** 5/6 Subtotal Nephrectomy (SN) was performed in male Swiss Background mice to induce CKD. Sham operation (SO, n=5) procedure was performed on mice as control. The mice were sacrificed in day 7 (1N, n=5) and day 28 (4N, n=5) after operation. We measured creatinine serum to assess renal function. Tubular injury score was quantified based on Periodic-Acid Schiff (PAS) staining. Prepro-ET-1 and eNOS were quantified using RT-PCR. **Results:** SN_1N and SN_4N groups had significant higher of serum creatinine and tubular injury from SO group. Densitometry analysis of RT-PCR revealed up-regulation of prepro-ET-1 mRNA expression in SN_1N and SN_4N ($p<0.05$ vs SO). Meanwhile, we found a significant increase of eNOS expression in SN_1N, and then it reduced significantly in SN_4N. We found significant parallel correlation between ET-1 and tubular injury expression (r: 0.768;$p<0,05$), meanwhile there were insignificant inverse correlation between eNOS and tubular injury (r: -0.354;$p>0.05$). **Conclusion:** eNOS might play role as a counterbalance in the up regulation of ET-1 in acute condition after SN. However, it failed in chronic condition. These lead to deterioration of renal function and tubular injury. An imbalance between ET-1 and eNOS expression in chronic CKD model might play role in profound renal damage.

Keywords: Chronic kidney disease, Endothelin-1, eNOS, Subtotal nephrectomy, Tubular injury

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INTRODUCTION

Chronic kidney diseases (CKD) is a life-threatening condition and is characterized by progressive and persistent loss of kidney functions. CKD is caused by various etiologies. The two main causes of this condition are diabetes and hypertension. Other causes include glomerulonephritis infection, renal vasculitis, ureteral obstruction, genetic changes, and autoimmune disease (1). The incidence of chronic kidney failure continues to increase globally and is expected to reach epidemic rates in the next decade (2). Limited knowledge about the pathophysiology of this condition continues to encourage scientists to conduct more in-depth research so that it can produce the effective treatment.

Various types of factors have been known to have an important role in the course of chronic kidney failure to end-stage renal failure (2). One of the factors believed to play an important role is the occurrence of tissue hypoxia (3). Severe hypoxic conditions are a consequence of disruption of renal microvasculature and there is a direct relationship between the loss of microvasculature and the development of scar tissue in glomerulus and tubulointerstitial (4). However, there are other mechanisms that can contribute to reduced tissue oxygenation, such as anemia, increased vasoconstriction as a result of excessive production of vasoconstrictor agents (angiotensin II and endothelin-1) or reduced production of vasodilator agents (nitric oxide / NO), decreased capillary rate, increased metabolic activity by injured tubular cells, and increased oxygen diffusion distance due to accumulation of extracellular matrix in tubulointerstitium (3).

Imbalance between vasoconstrictor (ET-1) and vasodilator (eNOS) may induce progression of CKD and promotes renal injury. ET-1 deletion from endothelial cells protects kidney against ischemic/reperfusion (I/R)

injury through reducing inflammation and Reactive Oxigen Species (ROS) production (5). ET-1 transgenic mice also demonstrated spontaneous kidney and pulmonary fibrosis (6). This study investigated the expression of eNOS and ET-1 in the CKD model and correlated it with the tubular injury.

MATERIALS AND METHODS

Experimental Animal Preparation

This research used male Swiss-background mice (3 months old, 20-25 grams) obtained from UPHP, Universitas Gadjah Mada. The mice were divided into 3 groups: Control group (SO), SN_1N group, and SN_4N group. The research variables were serum creatinine level, degree of tubular injury, ET-1 and eNOS expressions as dependent variables, and treatment time from 5/6 subtotal nephrectomy to termination as independent variable.

Induction of chronic renal failure in mice with subtotal 5/6 nephrectomy model

Swiss mice (n = 15) were anesthetized using intraperitoneal natrium pentobarbital (somnophetnyl, 0.1 ml/10 gram body weight) injection. To obtain CKD model, we used 5/6 subtotal nephrectomy procedure (5/6 SN) for 1N and 4N groups. Right lumbar (flank) region was opened, and then the kidneys and hilum were visualized. The renal pediculus in the hilum were ligated using thread No. 7 and the right kidney was removed (uninephrectomy). After suturing the peritoneal layer and skin, antiseptic solution was applied on the surgical area, and mice are left until conscious. The second stage was done by opening the left flank region, and renal pediculus was ligated using a non-traumatic vascular clamp. The superior and inferior poles of the kidney were taken and the middle part of the kidney was left behind. The total number of kidneys taken was 5/6 of bilateral kidneys. After suturing the peritoneal layer and skin, antiseptic solution was applied on the surgical area, and mice are left until conscious. In the Sham Operation (SO) group, only an incision was made on the skin and the peritoneal layer of the mice, then the kidneys were shown out and re-inserted without nephrectomy.

Serum creatinine examination and sacrifice

Before the sacrifice, 1 mL of blood was taken from the Retro-Orbital vein using a capillary tube for serum creatinine quantification. Sacrifice was done with Na-penthobarbital injection for anasthetizing the mice. In deep anasthetize condition, thorax and abdomen were opened, and then a neddle no. 26 was inserted to cardiac apex for perfusion using NaCl 0.9% solution, then right atrium was cut. After 10 minutes of perfusion, kidneys were harvested and cut in half. A half kidney was kept in Normal Buffer Formaline (NBF) solution for paraffin making, meanwhile the other was kept in RNA preservation solution (Ambion, 7021) for RNA extraction. In the sub-total nephrectomy groups, the left kidney was divided by two, so that one half of the kidney was used for paraffin, and another half for protein and RNA. For histology, the kidneys were fixed with a 4% PFA solution (Paraformaldehyde) in PBS followed by tissue processing to make paraffin blocks. Blood from three groups of mice that were taken was measured for serum creatinine. The examination was conducted at the Clinical Pathology Laboratory, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, Yogyakarta.

Tubular injury and histological examination

Paraffin blocks were cut and colored with Periodic-Acid Schiff (PAS) to be examined for renal tubular injury. This procedure was carried out at the Anatomical Pathology Laboratory, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, Yogyakarta. Tubular injury was obtained by observing 16 randomized, non-repetitive, per specimen fields for each preparat. Then the average was calculated from each group. The tubular epithelial injury was observed with a microscope at 400x magnification. A value of 0 indicates a normal histological appearance of the kidney. Values 1, 2, 3, and 4 show signs of tubular injury involving regions less than 25%, 25-50%, 50-75% and more than 75% in the field of view, respectively. The tubular injury was indicated by tubular dilatation, intraluminal cast formation, tubular atrophy and loss of brush border.

Prepro-ET-1 and eNOS expression measurements

Examination of ET-1, eNOS, and GAPDH mRNA expression were done using RT-PCR (Reverse Transcriptase-Polymerase Chain Reactions). Kidney tissue was extracted using Trizol RNA solution (GENEzolTM; Cat. No. GZR100). RNA concentration was quantified using nanodrop. The cDNA was synthesized using 5xRT-*buffer* (Toyobo, TRT-101), random primer (TAKARA®, 3801), dNTP (TAKARA®, 4030), ReverTra-Ace (Toyobo®; TRT-101). Reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out to examine the following genes: ppET-1 5′-TTCCCGTGATCTTCTCTCTGC-3′ (forward) and 5′-CTGCACTCCATTCTCAGCTCC-3′ (reverse), eNOS 5′-GTGGAGCTGAGGCTTTAGAGC-3′ (forward) and 5′-TTTCCTTAGGAAGAGGGAGGG-3′ (reverse), and GAPDH 5′-AACTTTGGCATTGTGGAAGG-3′ (forward) and 5′-GGATGCAGGGATGTTCT-3′ (reverse). Then a 35 cycle PCR was carried out with a denaturation condition of 94° C for 10 seconds, annealing at 60° C for 30 seconds and an extension of 72° C for the 1-minute final extension phase ending with a 72° C condition for 10 minutes.

Statistical analysis

Data were analyzed using one-way ANOVA test for normally distributed data and Kruskal-Wallis for data which was not normally distributed. The value of $p<0.05$ was considered statistically significant. Statistical

analyses were accomplished using SPSS Software version 23 (SPSS Inc., Chicago).

RESULTS

Serum Creatinine Measurement and Tubular Injury Scoring

CKD model demonstrated higher creatinine level, as shown by significant increase of creatinine level in SN_1N and SN_4N groups compared to SO group. The average of creatine level in SO, SN_1N, and SN_4N groups were shown in Fig. 1. Tubular injury was demonstrated in SN_1N and SN_4N groups, as shown by tubular dilatation, brush border loss and intraluminal cast formation. There were significant difference in tubular injury score between SO group and both SN_1N and SN_4N groups. SN_4N group represented the fully destruction of renal architecture.

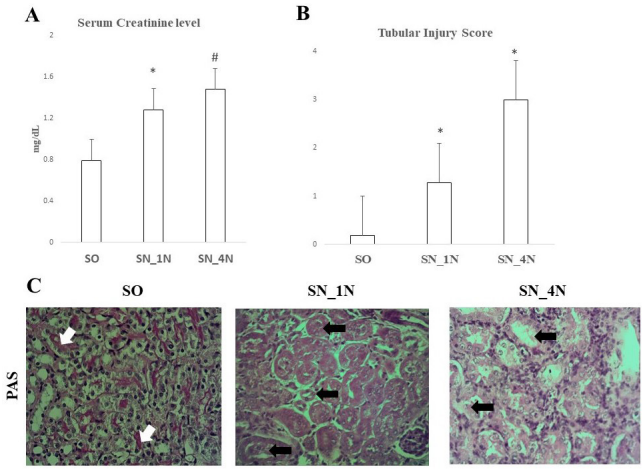

Figure 1: Functional and histological disruption occurred after SN. (A) Serum Creatinine Levels. Increased serum creatinine levels were tested statistically using one way ANOVA and post-hoc Fisher's LSD test. (B) Tubular Injury Score. (C) Histological appearance of kidney specimens with PAS staining. 400x magnification. SO group demonstrated normal histological appearance with brush border and intact epithelial cells (white arrows). SN group demonstrated tubular injury with cash formation, brush border loss, inflammatory cells infiltration and effacement of epithelial cells (black arrows). *: p<0.05 vs SO group; #: p<0.05 vs. SN_1N group.

Correlation of pre pro-ET-1 and eNOS expression with tubular injury

Prepro-ET-1 and eNOS expressions can be seen from the RT-PCR results as shown in Fig. 2.

Further, we calculated the correlation between prepro-ET-1 and eNOS expression with tubular injury. Prepro-ET-1 shows a positive correlation with tubular injury (p value: 0.004, r: 0.768), while eNOS shows negative correlation with tubular injury, but is not statistically significant (p value: 0.236, r: -.0354).

DISCUSSION

Chronic renal failure can be indicated by a progressive

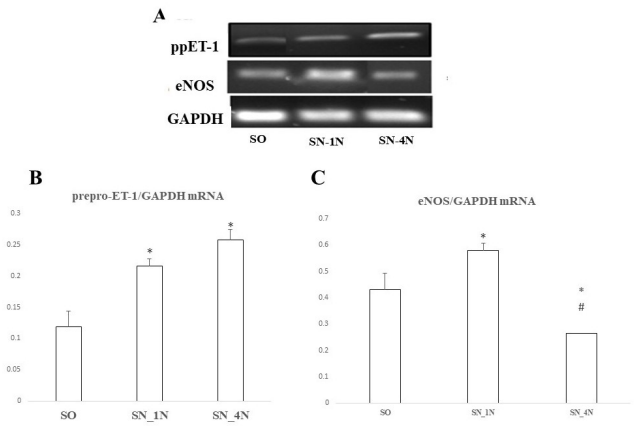

Figure 2: Upregulation of ppET-1 mRNA and downregulation of eNOS mRNA in SN group. (A) Representative image of pre-pro-ET-1 and eNOS mRNA expressions. (B) The mean pre pro-ET-1 expression has an increasing tendency with increasing experiment time. (C) The mean difference in eNOS expression in each group shows a non-linear picture; increased from SO vs SN_1N, then decreased from SN_1N vs SN_4N. *: p <0.05 vs SO group, #: p<0.05 vs SN_1N group.

decrease in glomerular filtration rate (7), while the glomerular filtration rate can be determined by changes in serum creatinine, BUN, and serum cystatin-C levels (8). Based on the results of data analysis, there has been an increase in serum creatinine levels that were statistically significant in the SN_1N and SN_4N groups against the SO group (controls). These results indicate that there has been a decline in kidney function in the model mice used, so it is expected to represent chronic kidney failure in humans.

A decrease in the glomerular filtration rate can be caused by damage to the nephrons in the kidneys. It is well known that tubular and interstitial injuries are the biggest contributors to the loss of kidney function (9,10). In this study, the results obtained were a significant increase in the severity of tubular injury from group data. The highest average was obtained in the SN_4N treatment group which underwent the longest trial time.

A decrease in the number of nephrons progressively causes an increase in the glomerular filtration rate in each normal nephron. This adaptive mechanism is used to maintain the total glomerular filtration rate, but can subsequently cause tubular and glomerular injury. This injury results in a further glomerular barrier dysfunction (11). One of the many peptides released in this condition is ET-1. It has been widely investigated that ET-1 has a large role in the progression of chronic kidney failure. In this study, the results of ET-1 expression analysis in the SO, SN_1N, and SN_4N groups showed a trend of increasing expression as time added and had a significant difference in value.

Endothelin-1 (ET-1) has a protective effect through

activation of kidney tubules Endothelin B Receptor (ETBR). ETBR activation causes vasodilation because NO is produced by the endothelium via the eNOS pathway and prostascyclin6. In this study, there were statistically significant differences in the mean eNOS expression between all treatment groups. The increase and decrease in eNOS expression in this study is also stated in a reasearch by Manotham et al. (2004) that stated the earliest response in the subtotal nephrectomy model is intrarenal vasodilation with relatively weak dilatation of the efferent arteriole, which then causes glomerular hyperfiltration and hypertension (12). While in the chronic phase, about 2 weeks, there is a reduction in the number of peritubular capillaries which coincides with the development of severe tubulointerstitial injury (4).

The results of the correlation analysis between tubular injury and ET-1 expression in this study were statistically significant with values. While the Spearman correlation coefficient (r) was obtained at 0.768 (sig. 2-tailed). This value indicates that ET-1 expression has a relationship that is directly proportional to the increase in mean tubular injury score and has a moderate relationship strength. These results reinforce the theory that previously widely adopted, namely ET-1 will cause damage to podocytes, a formation of scar tissue, and tubular cell death due to toxic effects on the condition of proteinuria (13).

Another mechanism that can cause tubular injury by ET-1 is the constriction of peritubular capillaries which is followed by surrounding hypoxic tubule injuries (14). The occurrence of vasoconstriction is certainly caused by a decrease in vasodilator eNOS expression. The eNOS expression correlation with a tubular injury in this study has a value of p = 0.236 (sig. 2-tailed) which means that the correlation is not statistically significant. In addition, the value of the Spearman correlation results (r): -0,354, which means the correlation of eNOS expression and tubular injury is inversely proportional and has a very weak correlation strength. These results are not in accordance with the assumptions made based on the mechanism that has been studied previously, where the expression of eNOS and tubular injury scores will influence each other significantly and have a strong relationship.

CONCLUSION

Imbalance between vasoconstriction and vasodilatation might contribute to deterioration of renal tubular injury in kidney fibrosis model.

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