Thiol-producing microbiota of the intestine modulate oxidative stress and inflammation in Chronic Kidney Disease

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Ika Nindya Kadariswantiningsih[1*], Mochammad Thaha[2,3], Cahyo Wibisono Nugroho[2,3], Maulana A. Empitu[4]

ABSTRACT

Background: Inflammation and oxidative stress are among the key contributing risk factors for cardiovascular disease (CVD) in chronic kidney disease (CKD). The human gut is the home for microbiota, of which some of the gram-negative gut bacteria are known to produce low-molecular-weight thiols including glutathione (GSH). GSH is used by mammals and eukaryotic cells as a substrate of glutathione peroxidase to eliminate reactive oxygen species. This study aims to investigate whether the presence of thiol-producing gut microbiota in CKD affects the level of oxidative stress and inflammation markers.

Methods: This study examined the stool sample of 41 CKD patients at three hospitals in Surabaya, Indonesia to identify the gram-negative bacteria producing thiols. Based on Ellman's assay result, the study participants were grouped into patients with thiol-producing and non-thiol-producing gram-negative gut microbiota.

The markers of inflammation, high sensitivity C-reactive protein (hs-CRP), total antioxidant capacity (TAC), and urinary oxidative stress marker 8-hydroxy-2'-deoxyguanosine (8-OHdG) were compared between the two groups.

Results: The group with thiol-producing gut microbiota exhibited lower levels of plasma hs-CRP and urinary 8-OHdG compared to the group with non-thiol-producing gram-negative gut microbiota. In contrast, the group with thiol-producing gut microbiota has higher TAC compared to the group with non-thiol-producing gram-negative gut microbiota.

Conclusion: This study emphasized that the phenotypical diversity regarding the ability to produce low-molecular-weight thiols might modulate inflammation and oxidative stress as CVD risk factors in CKD.

[1] Department of Medical Microbiology, Faculty of Medicine, Universitas Airlangga, Indonesia
[2] Department of Internal Medicine, Faculty of Medicine, Universitas Airlangga, Indonesia
[3] Department of Internal Medicine, Universitas Airlangga Hospital, Indonesia
[4] Department of Pharmacology and Therapeutics, Faculty of Medicine, Universitas Airlangga, Indonesia

*Correspondence to:
Ika Nindya Kadariswantiningsih;
Department of Medical Microbiology, Faculty of Medicine, Universitas Airlangga, Indonesia;
ika.nindya@fk.unair.ac.id

INTRODUCTION

Chronic Kidney Disease (CKD) affects one-tenth of the general population worldwide with a mortality rate of 20-50% in the United States.[1] The number of deaths due to CKD has almost doubled compared to two decades ago,[2] and the incidence and prevalence of CKD are expected to increase given the ongoing obesity epidemic, autoimmune disease in a younger demographics,[3] and the growing elderly population.[4] The spectrum of CKD ranges from subtle kidney damage marked with micro-albuminuria to end-stage renal disease (ESRD), which require dialysis or kidney replacement therapy.[5]

Cardiovascular disease (CVD) is the primary cause of morbidity and mortality among CKD patients.[6] More than 20% of patients starting dialysis are dead within the first year[9], making the prognosis of ESRD worse than most cancers.[7] The CVD risk factors contributing to CKD include chronic inflammation, oxidative stress, protein-energy wasting, disordered mineral metabolism, and deficiency of endogenous calcification inhibitors.[8]

Among these factors, chronic inflammation and oxidative stress are regarded as key contributing factors to CVD in CKD.

The human digestive tract contains > 10^{14} microbes that have significant physiological roles on metabolic functions, such as vitamin synthesis, digestion of polysaccharides from plants, and other critical tasks such as balancing local and systemic immunity.[9] Recent research results showed that microbiota imbalance also occurs in diseases seemingly not correlated with the gastrointestinal tract such as cardiovascular and its progression.[10] Gut bacterial DNA fragments have been detected in the blood of CKD patients and correlated with increased levels of plasma C-reactive protein, interleukin-6, and D-lactate.[10,11] However, the current understanding of the crosstalk between microorganisms and the host in the context of CKD is still in its infancy, and the role of particular gut bacterial species is still not completely understood.

Given the fact that the majority of gut microbiota are gram-negative bacteria, of which some can

produce thiols including glutathione,[9,12] they may be involved in modifying the balance between oxidant and pro-oxidant state in CKD. This study aims to investigate whether gut microbiota diversities regarding their ability to produce thiols, may modulate oxidative stress and inflammation in CKD.

METHODS

Participants in this cross-sectional study were recruited consecutively from outpatient clinics and hemodialysis units of a government hospital and two private hospitals in Surabaya, Indonesia from March 2017 to August 2017. The inclusion criteria were patients aged 21 years or older who suffered from CKD at any stage and agreed to be involved in this study. The diagnosis of CKD was confirmed by the National Kidney Foundation Kidney Disease Outcomes Quality Initiative (NKF K-DOQI) criteria based on the CKD-EPI equation (eGFR£60 ml/min/1,73m²; urine albumin, albumin to creatinine ratio). Exclusion criteria include signs of infection, overt infection, fever in the last three days, acute inflammatory disease, and malignancy. The study protocol was approved by the Ethics Committee of Universitas Airlangga Hospital Surabaya, Indonesia with the reference number of 093/IGH/2017. Study participants underwent a detailed review of their disease history, physical examinations, and laboratory measurements at the time of enrollment. Subjects were classified into two groups based on the ability of their gut microbiota to produce thiols and the level of inflammation, and oxidative stress markers were compared between the two groups.

Participants underwent blood and urine sampling early in the morning. Serum creatinine, serum cystatin-C, and HbA1c were measured based on established laboratory methods previously explained.[13] High sensitivity C-reactive protein (hs-CRP) was used as an inflammatory biomarker. hs-CRP was measured in the blood using particle enhanced turbidimetry (Roche Diagnostic, CA, USA). Malondialdehyde (MDA) was used as a biomarker of oxidative stress. MDA and 8-OHdG were measured in the blood serum process by high-performance liquid chromatography (HPLC) method using Agilent 1100. Total antioxidant capacity was measured using enzyme-linked immunosorbent assay.

Stool samples were collected within one week of inflammation and oxidative stress markers measurements. The stool was cultured in Oxoid MacConkey Agar No. 3 agar plate (Thermo-Fisher Scientific, MA, USA) to selectively grow the gram-negative bacteria. The isolated colony, which were previously grown, were randomly selected and sub-cultured in Oxoid MacConkey Agar No. 3 agar plate (Thermo-Fisher Scientific, MA, USA) to confirm the gram negativity of the bacteria. The determination of thiol producing status of the confirmed gram-negative colony was done using Ellman's assay, as previously described.[14]

The normality of the quantitative data was analyzed using the Saphiro-Wilk normality test. For normally distributed variables, data were shown as mean ± SD. For skewed variables, data were displayed as median and interquartile range (1st quartile - 3rd quartile). To investigate the difference between the two groups, normally distributed data were analyzed using the Student's *t*-test, while data that were not normally distributed were analyzed using the Mann-Whitney U-test. The Chi-square test was used to evaluate the difference in categorical data between the two groups. All tests were two-tailed with a significance level set at 0.05. The statistical analyses were performed using XLSTAT software version 2016.02.28451.

RESULTS

In this study, a total of 71 patients aged 21 years or older with a diagnosis of CKD were recruited. Among them, 41 participants gave their consent for stool sample examination and microbiota identification. As many as 31 participants were identified to have non-thiol-producing gram-negative bacteria, while the remaining ten were found to have thiol-producing gram-negative bacteria. The 41 participants consisted of patients with CKD at stages 1-5. The proportion of CKD patients with type 2 diabetes, hypertension, and congestive heart disease between the thiol-producing and non-thiol-producing groups were not significantly different (Table 1).

The plasma hs-CRP level was measured to determine whether there was a difference in inflammation status between the thiol-producing and non-thiol producing groups. The hs-CRP level in CKD patients with thiol-producing gut microbiota (0.9 IQR 0.8 – 1.7 mg/L) was lower compared to patients with non-thiol producing bacteria (1.8 IQR 1.2 – 4.8 mg/L, P-value = 0.026, Fig. 1).

To determine the state of oxidative stress, we measured the plasma TAC and urinary level of 8-OHdG. The cystatin-c-adjusted TAC value in CKD patients with thiol-producing gut microbiota (1.3 IQR 0.8 – 1.9 mmol/L) was higher compared to patients with non-thiol producing bacteria (0.4 IQR 0.30 – 0.8, P-value = 0.0009, Fig. 2). In contrast, the urinary 8-OHdG level of CKD patients with thiol-producing gut microbiota (0.25 IQR 0.02 –

Table 1. Characteristics of Study Participants

Characteristic	CKD patients with non-thiol producing GN microbiota (n= 31)	CKD patients with thiol producing GN microbiota (n= 10)	p-value
Age, years	60 (52-64)	58 (56-62)	NS
Sex ratio, M/F	17/14	6/4	NS
Kidney Parameters			
eGFR, ml/minute/1.73 m2	6 (5-31)	53 (34-82)	0.002
Serum Creatinine, mg/dL	9.3 (2.3-14)	1.5 (0.8-2.3)	0.003
Serum Cystatin-C, mg/L	6.6 (2.0-7.7)	1.4 (0.9-1.8)	0.003
Cholesterol Profile			
Total Cholesterol, mg/dL	204 (162-227)	217 (159-253)	NS
LDL Cholesterol, mg/dL	117 (92-145)	142 (83-160)	NS
HDL Cholesterol, mg/dL	38 (35-50)	57 (47-60)	NS
Cardiovascular Disease and Risk Factors			
Type 2 Diabetes, n (%)	21 (67)	9 (90)	NS
Hypertension, n (%)	30 (96)	9 (90)	NS
CHF, n (%)	11 (35)	2 (20)	NS

Data are expressed in Median (Interquartile range)

CHF: congestive heart failure; eGFR: estimated glomerular filtration rate; GN: gram-negative; NS: no significance. $p < 0.05$ was considered significant*.
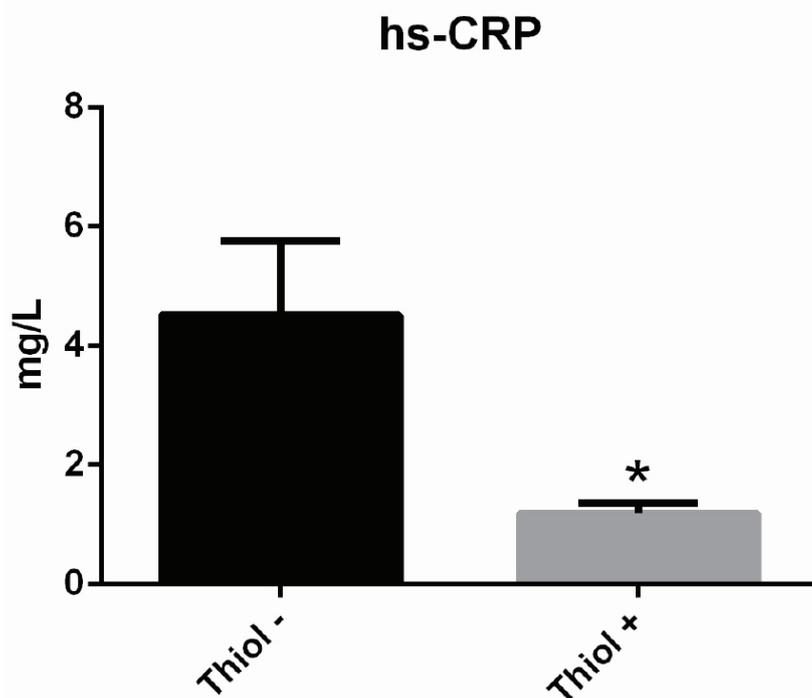

Figure 1   Plasma hs-CRP level in CKD patients with thiol-producing gut microbiota versus non-thiol-producing gut microbiota. * P-value=0.026

0.58 mmol/L) was significantly lower compared to patients with non-thiol producing bacteria (0.54 IQR 0.20 – 1.0 mmol/L, P-value=0.041, Fig. 3).

DISCUSSION

This study showed that there were significant differences in plasma TAC and hs-CRP as well as urinary 8-OHdG levels between CKD patients with thiol-producing and those with non-thiol producing gut microbiota. This result indicates that the phenotypical diversity of gut microbiota, which in this context is the ability to produce thiols, might modulate inflammation and oxidative stress in CKD. This result strengthens previous findings that the diversity of gut microbiota affects diseases that occur outside of the intestinal tract, including CKD.[9,10]

The human intestinal tract is an environment housing a diverse and complex community of microbiota. The microbial community in the human gut is estimated to contain more than 1000 species of bacteria and 100-fold more genes compared to the human genome.[9] Therefore, the amount and variety of functional peptides and proteins produced by gut microbiota are thought to be a significant modulator in human health.[9,10] Recent evidence showed that dysbiosis in gut microbiota might have major consequences that are both beneficial and detrimental to the human physiology.[9]

Recent advances in highthrough put sequencing analysis have revealed that some clusters of bacterial species are essential for human health.[15] However, this study did not look for any significant bacterial species in CKD but rather employed a phenotypical identification without identifying the species. The hypothesis is that the secreted substance from the vast number of bacteria may have a systemic consequence for the host, whom in this context are CKD patients.

Among the vast majority of the gut microbial community are gram-negative bacteria, of which some can produce thiols.[16] The dominant thiol produced by eukaryotes and aerobic gram-negative bacteria is low-molecular-weight glutathione (GSH).[17] The GSH is a ubiquitous sulfhydryl-containing tripeptide used by most mammalian cells to neutralize reactive oxygen species (ROS).[17] The GSH is used as a substrate by glutathione peroxidase (GPx) to eliminate hydrogen peroxide, a potent source of ROS, by linking two GSH molecules together to form oxidized glutathione (GSSG).[17]

In CKD, oxidative stress is a result of dominant ROS production compared to anti-oxidant generation. The oxidant in hypertensive CKD may
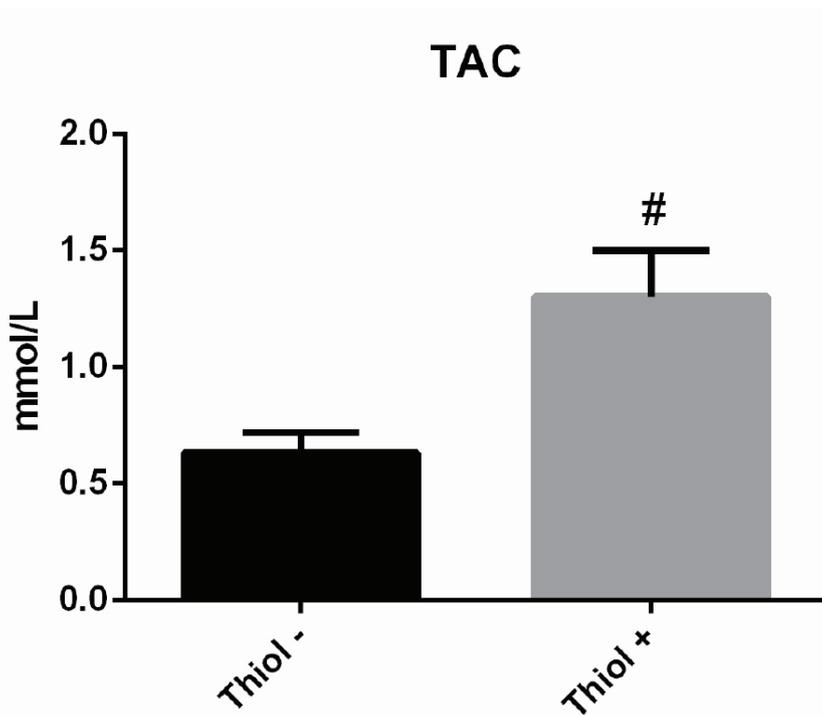
TAC

Figure 2. Total antioxidant capacity in CKD patients with thiol-producing gut microbiota versus non-thiol-producing gut microbiota. TAC value was adjusted according to the plasma Cystatin-C level. # P-value=0.0009
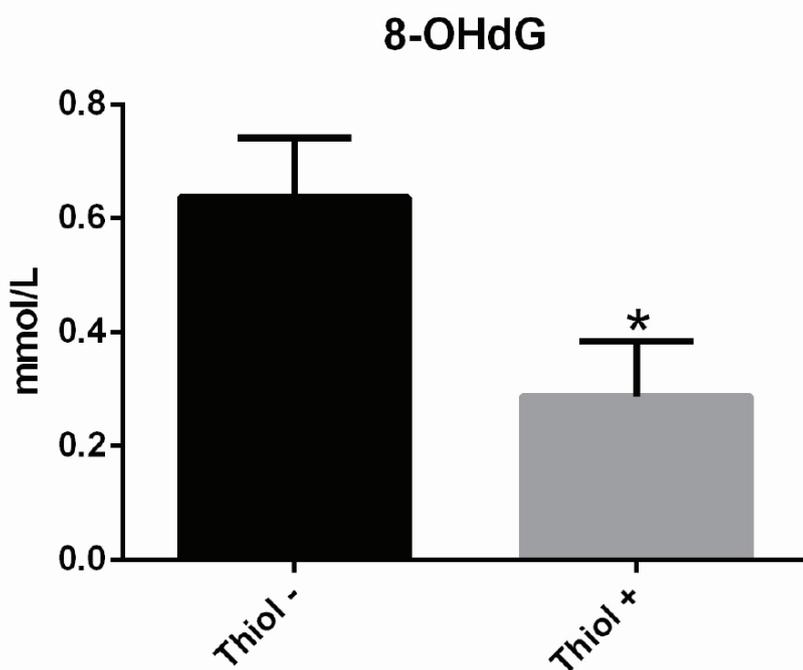
8-OHdG

Figure 3. Urine 8-OHdG level in CKD patients with thiol-producing gut microbiota versus non-thiol-producing gut microbiota. The 8-OHdG is adjusted according to the estimated glomerular filtration rate. * P-value=0.041

originate from the uncoupled nitric oxides (NO) due to the inhibition of nitric oxide synthase (NOS), activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and activation of xanthine oxidoreductase and mitochondrial respiratory enzymes.[18,19] ROS generation causes lipid peroxidation and cell injury in multiple organs.[18]

In this study, the abundance of thiol-producing bacteria might contribute to attenuate the oxidative stress in CKD patients. The antioxidant capacity in this group is higher, indicating the better ability of the system to neutralize ROS production than individuals with non-thiol-producing gut microbiota. The finding that urinary 8-OHdG was lower in individuals with thiol-producing gut microbiota supports this result. The urinary 8-OHdG is a dominant form of ROS, which is excreted by cells in the kidney and urinary tract. Consequently, it has been widely used as an oxidative stress marker in CKD.

The generation of ROS and cell injury in CKD ignite an inflammatory process via the induction of pro-cytokine release, which in turn increases the production of acute-phase protein hs-CRP.[20] The hs-CRP has been utilized as an inflammation biomarker and correlated well with the long-term outcome of CVD and CKD.[21] Although it has no diurnal variation, hs-CRP is affected by the level of cholesterol LDL, body fat percentage, and the level of oxidant.[22,23] In this study, the lower oxidative stress status might implicate a milder systemic inflammation; thereby we observed that CKD individuals with thiol-producing gut microbiota showed a lower hs-CRP level.

CONCLUSION

The result of this study may contribute as an initial indication that the phenotypical diversity of gut microbiota, regardless of their species, might modulate inflammation and oxidative stress in CKD. However, this study only employed non-parametrical comparison without any correlation insight, which becomes a limitation to its generalization. Thus, studies with larger sample sizes and more data points in a prospective design are required to further elucidate the clinical consequences of the phenotypical variation of gut microbiota.

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DISCLOSURE

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