Urinary Hydrogen Peroxide and Renal Function Parameter of Type 2 Diabetes Mellitus Patients Consuming Metformin and Metformin-Sulfonylurea

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ABSTRACT
Objective: Diabetes is one of the primary causes of kidney failure. About 40% people with diabetes will get Chronic Kidney Disease (CKD). The previous study reported that oxidative stress played an important role in diabetic patients with kidney damage. However, the study did not take consideration for therapeutic treatment of the patients. This study purpose to determine urinary hydrogen peroxide concentration as a biomarker of oxidative stress in type 2 diabetes patients consuming metformin and metformin-sulfonylurea and to know its correlation with estimated Glomerular Filtration Rate (eGFR) and Urine Albumin to Creatinine Ratio (UACR) as a parameter of the renal function.
Methods: Blood and urine were collected from 114 type 2 diabetes outpatients in Pasar Minggu Community Health Center. The concentration of urinary hydrogen peroxide ($H_2O_2$) was measured using Ferrous Ion Oxidation Xylenol Orange 1 (FOX-1) method and was normalized with urine creatinine measured with kinetic Jaffe method. The value of eGFR was calculated based on serum creatinine using Cockcroft-gault, MDRD, dan CKD-EPI equation. Results: There were no significant difference in concentration of urinary $H_2O_2$ ($p = 0.228$), eGFR (Cockcroft-gault $p = 0.936$; MDRD $p = 0.779$; dan CKD-EPI $p = 0.671$), and UACR ($p = 0.938$) between the two groups of treatment. There was no correlation between urinary $H_2O_2$ with eGFR in all equations and between urinary $H_2O_2$ and UACR. In the other hand, moderate positive correlation showed in analysis between urinary $H_2O_2$ and UACR in patients with albuminuria ($r=0.457$; $p=0.001$). Results of linear regression analysis showed that $H_2O_2$ was the most and the only significant factor for increased UACR, even after controlled by age, gender, IMT, systolic blood pressure, HbA1c, hypertension status, smoking habit, exercise habit, and medicine. Conclusion: There was no significant difference in concentration of urinary $H_2O_2$ in type 2 diabetes patients consuming metformin and metformin-sulfonylurea. Urinary presumably will increase significantly together with the present of albuminuria, so it can not be used in early detection of renal function in patients without albuminuria.
Key words: Metformin, Sulfonylurea, Diabetes Mellitus, Estimated Glomerular Filtration Rate, Urine Albumin to Creatinine Ratio, Urinary Hydrogen Peroxide.
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INTRODUCTION
Indonesia has around 10 million people with diabetes, in which about 90% -95% of all diabetes patients are people with type 2 diabetes mellitus. It is predicted to reach the number of 16.3 million in 2040. Diabetes is one of the primary causes of kidney failure. About 40% people with diabetes will get chronic kidney disease (CKD). Oxidative stress is known to play an important role in the molecular mechanism of kidney damage in diabetes, as shown by identification of isoprostane, advanced oxidation protein products (AOPP) as a marker of oxidative stress lead to renal ischemia reperfusion injury. It develops when a number of reactive oxygen species (ROS) and antioxidants are imbalance and enzyme system cannot change ROS to unreactive species. It is known nephropathy diabetic could be induced at least in 5 pathways: abnormal electron transport in mitochondria, polyol pathway, production of advanced glycation end products (AGEs), Protein kinase C (PKC) pathway, and hexosamine pathway. Continuous hyperglycemia induce overproduction of ROS and it can result in kidney cell apoptosis. $H_2O_2$ is one type of ROS that can be measured in urine. An in vitro study reported that $H_2O_2$ exposure significantly increased apoptosis and reduced kidney cell survival. Urinary $H_2O_2$ has been used as a marker of oxidative stress in cancer patients. It also can be used as a marker of oxidative stress in healthy people who have a variety of lifestyles such as smoking, alcohol drinking, and exercise. However, the role of $H_2O_2$ as oxidative stress marker induces kidney damage in diabetes is still unclear. First line treatment of type 2 diabetes mellitus in Indonesia is a single/monotherapy of oral antidiabetic. The widely used medicine in first line therapy is metformin. If the treatment fails to reach the target in 3 months, patients will be received a combination of two oral antidiabetic drugs. Tight glycemic control can reduce the development and progression of diabetic nephropathy. However, it remains unknown whether oral anti-diabetic consumed by type 2 diabetes patients in Indonesia can reduce the risk of oxidative stress and diabetic complication in the kidney. Diabetic nephropathy is usually detected when patients have been entered the final stage. Therefore, it is necessary to do research to discover the early marker of diabetic nephropathy. This study aims to determine the difference of urinary $H_2O_2$ concentration in type 2 diabetic patients consuming metformin and metformin-sulfonylurea and to know its correlation with estimated Glomerular Filtration Rate (eGFR) and Urine Albumin to Creatinine Ratio (UACR) as a parameter of the renal function.
METHODS

Subject
Fifty subject consuming metformin and 64 subjects consuming metformin-sulfonylurea have been selected in this study. All of the study subjects consume the medicine for at least 4 months. The local ethical committee, Faculty of Medicine, Universitas Indonesia, Dr. Cipto Mangunkusumo Hospital (44/UN2.F1/ETIK/1/2016) approved this study and all patients had given informed consent before blood and urine sampling.

Urine and Blood Collections
Patients included in this study were in a state of fasting when blood and urine were collected. Exclusion criteria were patient with hematuria and severe anemia. We interviewed the patients who come to Pasar Minggu Community Health Center to identified patients fulfilling the inclusion criteria. Then, we made an appointment to collect blood and urine sample. Urine collected from every participant as much as 30 ml in a plastic pot. Urine samples were allowed to stand 2 hours until separate into two parts. The clear part was taken and put into several microtubes. Urine in the microtube stored at -80°C until analyzed. Blood was taken by certified phlebotomist from an accredited laboratory as much as 10 ml. Then, the samples were analyzed in the laboratory to measure creatinine and HbA1c.

Measurement of eGFR

eGFR defined based on three formulation, Cockcroft-Gault, The Modification of Diet in Renal Disease (MDRD) and The Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) as shown in Table 1.

Measurement of urine creatinine
Urine creatinine was measured using the Creatinine Colorimetric Detection kit (Catalog No.ADI-907-030A, Enzo Life Science, USA). The principle was based on Jaffe reaction. Creatinine in urine will react with the picric acid in alkali condition resulting orange color and measured at 490 nm. A urine sample was diluted in 1:20 by deionized water. The diluted sample, standard, and blank (50 μl) were pipetted to appropriate well. Then, 100 μl of creatinine detection reagent (the saturated picric acid and NaOH 1 N) was added to the well. Sample and reagent were incubated for 30 minutes at room temperature. Then, absorbance was measured at 490 nm. The calibration curve for measurement urine creatinine is prepared from creatinine standard at concentration of 20, 10, 5, 2.5, 1.25, 0.625, 0.3125 mg/dL.

Measurement of urine albumin
Urine albumin was measured using the BCG Albumin Kit (Catalog No. MA24-1KT, SIGMA). Standard albumin solution was made to 5.0, 4.0, 3.0, 2.0, 1.5, 1.0, and 0.5 g/dL. The assay performed in duplicate. Five μl blank, standard, and sample solution were put into each well. Bromocresol Green (BCG) was then added into the well. The plate was incubated for 5 minutes, and the absorbance was measured at λ 620 nm.

Measurement of urinary H2O2
H2O2 is a marker of oxidative stress and contained in urine that can be measured. H2O2 measurement in the urine was conducted by FOX assay. The principle of this method is the oxidation of Fe3+ to Fe2+ by oxidizing agents in the samples, binding with Xylenol Orange (XO) and made a complex color that can be measured at 560 nm. The complex color of Fe3+-XO is very sensitive to pH and optimum pH for performing this method is 1.7 to 1.8. FOX is not a specific method for the measure of H2O2. Other oxidizing agents also can oxidize Fe3+ into Fe2+. Therefore, this method is modified with the presence of catalase enzyme. Urinary hydrogen peroxide was measured by calculating the absorbance difference in the sample with catalase enzyme and on samples without catalase, in which catalase will selectively destroy H2O2. The calibration curve for measurement hydrogen peroxide is prepared from 30% H2O2 standard at a concentration of 30, 25, 12.5, 6.25, 3.125, and 1.5625 mg/dL.

Statistical Analyses
All data are expressed as sum (percentages) or mean ± standard deviation or standard error of the mean. Independent t-test was applied in parametric data to compare the two treatments. Meanwhile, Mann-Whitney test was used for non-parametric data. Differences were considered significant at p < 0.05. Correlation between two variables was analyzed using bivariate test (person or spearman test). Linear regression analysis with stepwise method also conducted to know the most predictive factor for UACR. The results were significantly correlated if p < 0.05.

RESULTS AND DISCUSSION

Calibration and linear regression analysis of urinary creatinine
Linear regression analysis showed a very good and significant coefficient of determination in both groups. The coefficient of determination for metformin group was 0.9998 with the equation of y=0.0879x+0.012. Meanwhile, the coefficient of determination for combination group was 0.9997 with an equation of y= 0.0859x+0.014.

Calibration and linear regression analysis of urinary hydrogen peroxide
Linear regression analysis showed a very good and significant coefficient of determination (r= 0.9992) with linear equation y=0.005x+0.0097).

Calibration and linear regression analysis of albumin
Linear regression analysis showed a very good and significant coefficient of determination in both groups. The coefficient of determination for metformin group was 0.9995 with an equation of y=0.1972x+0.0087. Meanwhile, a coefficient of determination for combination group was 0.9991 with an equation of y=0.1958x+0.0083.

Subject characteristics
Complete data were obtained from 114 patients consist of 50 people (9 men, 41 women) consuming metformin and 64 people (10 men, 54 women) consuming a combination of metformin-sulfonylurea. Table 2 shows the basic characteristic of a study subject. Most of the study subjects in both groups were elderly. However blood pressure of most participants still under control. The urinary creatinine and serum creatinine in the two treatment groups were still in normal range, which indicating that most of the participants in this study have an apparently normal renal function. Effective antihypertensive treatment, such as angiotensin I-converting enzyme inhibitors or angiotensin receptor blocker in patients can postpone or prevent diabetic nephropathy.

Subject who consuming metformin-sulfonylurea has higher HbA1c than metformin group, however, no significant difference between the two groups (p=0.066) statistically (Table 3). The mean of systolic and diastolic blood pressure of two groups was still in the normal ranges (124.80 ± 17.46; 126.56 ± 16.26 and 78.00 ± 8.08; 78.44 ± 7.39). The mean UACR in the combination group was higher than in metformin group, even not statistically significant due to the nature of wide variation of individual concentration.

Mean of urinary H2O2 in the whole subjects is 80.80 ± 11.02 μmol/g creatinine, whereas mean H2O2 level in healthy people as reported was 5.66 ± 8.27 μmol/g creatinine with the same analytical methods. Thus, the
average concentration of urinary H$_2$O$_2$ in this study subjects was much higher than normal subjects. The level of urinary H$_2$O$_2$ between the two groups also does not have a significant difference (p=0.228). Urinary H$_2$O$_2$, eGFR, and UACR were non-parametric, so we used Spearman correlation to determine the correlation between them. Analysis of correlation between urinary H$_2$O$_2$ and eGFR showed that there was no correlation between urinary H$_2$O$_2$ with eGFR and UACR. However, there was a moderate correlation between urinary H$_2$O$_2$ and UACR in albuminuria patients (r=0.457; p=0.001) (Figure 1).

The linear regression analysis results indicate that hydrogen peroxide was the most predictive factor for UACR, even after controlled by age, gender, IMT, systolic blood pressure, HbA1c, hypertension status, smoking habit, exercise habit and medicine (Table 4).

Some study reported that urinary H$_2$O$_2$ level was influenced by coffee drinking. To avoid the bias effect of drink and foods, patients were fasting at least 8 hours before taking their urine and blood. The concentration of urinary H$_2$O$_2$ in both groups were not a significant difference. It's showed that there were no different effect to prevent oxidative stress between metformin and combination of metformin-sulfonylurea therapy. This result was similar to the previous study which found not significantly affect between metformin and sulfonylurea in all-cause mortality in type 2 diabetic patients.

Mean of urinary H$_2$O$_2$ in the metformin group (19.35 ± 2.38) and in the metformin-sulfonylurea group (23.18 ± 1.83) were higher than the concentration in healthy people (0.4 to 5.71 μM) as reported previously which analyzed with the same analytical methods. Urinary hydrogen peroxide levels standardized by urine creatinine value (53.62 ± 13.84 μmol/g creatinine) still higher when compared to healthy subjects (5.66 ± 8.27 μmol/g creatinine). Bivariate analysis between urinary H$_2$O$_2$ and eGFR showed that there was no correlation between them. This result is presumably caused by wide variation of intra-individual urinary H$_2$O$_2$.

High concentration of urinary H$_2$O$_2$ in diabetic patients causes by the interaction between RAS and ROS which related to renal dysfunction. Angiotensin-II-induced increases in H$_2$O$_2$ production in the renal medulla. Renal medullary NF-κB activation also has positively correlated with local H$_2$O$_2$ production in the kidney. Patina et al (2014) also reported that H$_2$O$_2$ is oxidative stress biomarker which increased in diabetic rats renal medulla.

Up to now, glomerular hyperfiltration leading to microalbuminuria is the earliest clinical marker of renal disease, which will progress into a renal damage, thus develop microalbuminuria and reduced glomerular filtration rate. This study showed that urinary H$_2$O$_2$ and UACR as the parameter of albuminuria has no correlation in total samples. In the other hand, moderate positive correlation showed in analysis between urinary H$_2$O$_2$ and UACR in patients with albuminuria (Figure 1). Urinary hydrogen peroxide presumably will increase significantly together.
with the present of albuminuria, so it can not be used in early detection of renal function in patients without albuminuria. This study has limitations such as the relatively small number of sample that can not represent the real condition. Nature unstable of $H_2O_2$ in urine may affect this study due to the sample trip from a place of sampling and analysis. However, we tried to minimize the degradation of $H_2O_2$ by maintaining the temperature with dry ice in a cool box, however, the advantages of this study are using a sample of patients with type 2 diabetes mellitus who fasting before blood and urine samples were taken.

**CONCLUSION**

In conclusion, there were no significant difference in concentration of urinary $H_2O_2$ in type 2 diabetes patients consuming metformin and metformin-sulfonylurea. Urinary $H_2O_2$ presumably will increase significant together with the presen of albuminuria, so it can not be used in early detection of renal function in patients without albuminuria.

**CONFLICT OF INTEREST**

The authors declare that they have no conflict of interest.

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**ABBREVIATIONS USED**

CKD: Chronic Kidney Disease; AOPP: Advanced Oxidation Protein Products; ROS: Reactive Oxygen Species; AGEs: Advanced Glycation End Products; PKC: Protein Kinase C; eGFR: estimated Glomerular Filtration Rate; MDRD: The Modification of Diet in Renal Disease; UACR: Urine Albumin to Creatinine Ratio; CKD-EPI: The Chronic Kidney Disease Epidemiology Collaboration; BCG: Bromocresol Green; XO: Xylenol Orange; RAS: The Renin–Angiotensin System.

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11. Ohira SI, Kirk AB, Dasgupta PK. Automated measurement of urinary creatinine concentration and analysis. However, we tried to minimize the degradation of $H_2O_2$ by maintaining the temperature with dry ice in a cool box, however, the advantages of this study are using a sample of patients with type 2 diabetes mellitus who fasting before blood and urine samples were taken.