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Original Article

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 the 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 Cockroft-gault, MDRD, dan CKD-EPI equation. **Results:** There were no significant difference in concentration of urinary H_2O_2 (p = 0.228), eGFR (Cockroft-Gault p = 0.936; MDRD p = 0.779; dan CKD-EPI p= 0.671), and UACR (p = 0.838) 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.[1] It is predicted to reach the number of 16.3 million in 2040.[1,2] 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[3], advanced oxidation protein products (AOPP)[4] as a marker of oxidative stress lead to renal ischemia reperfusion injury.[5] 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.[6] Continuous hyperglycemia induce overproduction of ROS and it can result in kidney cell apoptosis.[7]

H_2O_2 is one type of ROS that can be measured in urine.[8] An in vitro study reported that H_2O_2 exposure significantly increased apoptosis and reduced kidney cell survival.[9] Urinary H_2O_2 has been used as a marker of oxidative stress in cancer patients.[6] 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.[10] 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.[11] Tight glycemic control can reduce the development and progression of diabetic nephropathy.[12] 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 stated 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 serum and HbA1c.

Measurement of eGFR

eGFR defined based on three formulation, Cockroft-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.[13] 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. MAK124-1KT, 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 H_2O_2

H_2O_2 is a marker of oxidative stress and contained in urine that can be measured. H_2O_2 measurement in the urine was conducted by FOX assay. The principle of this method is the oxidation of Fe^{2}+ to Fe^{3}+ 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 Fe^{3}+-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 H_2O_2. Other oxidizing agents also can oxidize Fe^{2+} into Fe^{3+}. 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 H_2O_2.[7] The calibration curve for measurement hydrogen peroxide is prepared from 30% H_2O_2 standard at a concentration of 50, 25, 12.5, 6.25, 3.125, and 1.5625 mg/dL.

Statistical Analyses

All data are expressed as sum (percentages) or mean ± standar 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.[14]

Subject who consuming metformin-sulfonylurea has higher $HbA1_C$ 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 H_2O_2 in the whole subjects is 80.80 ± 11.02 µmol/g creatinine, whereas mean H_2O_2 level in healthy people as reported was 5.66 ± 8.27 µmol/g creatinine with the same analytical methods.[10] Thus, the

average concentration of urinary H_2O_2 in this study subjects was much higher than normal subjects. The level of urinary H2O2 between the two groups also does not have a significant difference (p=0.228). Urinary H_2O_2, eGFR, and UACR were non-parametric, so we used Spearman correlation to determine the correlation between them. Analysis of correlation between urinary H_2O_2 and eGFR showed that there was no correlation between urinary H_2O_2 with eGFR and UACR. However, there was a moderate correlation between urinary H_2O_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_2O_2 level was influenced by coffee drinking.[15] 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_2O_2 in both groups were not a significant difference. It's showed that there was 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.[16]

Mean of urinary H_2O_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.[10] 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).[10] Bivariate analysis between urinary H_2O_2 and eGFR showed that there was no correlation between them. This result is presumably caused by wide variation of intra-individual urinary H_2O_2. Yuen & Benzie (2003) investigated urinary H_2O_2 as a potential biomarker of oxidative stress. They found that large biological variation can limit the usefulness of urine H_2O_2 even when concentration corrected by creatinine.[17]

High concentration of urinary H_2O_2 in diabetic patients causes by the interaction between RAS and ROS which related to renal dysfunction. Angiotensin-II-induced increases in H_2O_2 production in the renal medulla. Renal medullary NF-κB activation also has positively correlated with local H_2O_2 production in the kidney.[18] Patina et al (2014) also reported that H_2O_2 is oxidative stress biomarker which increased in diabetic rats renal medulla.[19]

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.[14] This study showed that urinary H_2O_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_2O_2 and UACR in patients with albuminuria (Figure. 1). Urinary hydrogen peroxide presumably will increase significantly together

Table 1: Formula of eGFR

Equation	eGFR (mL/minutes/1,73 m2)
Cockroft-Gault	((140 – age (years)) (Body Weight) (0.85 for female))/(SCr x 72)
Modification of Diet in Renal Disease (MDRD)	$186 \times (sCr\ (mg/dL))^{-1.154} \times age\ (years)^{-0.203}$
	×0.742 (female)
CKD-EPI	Female with SCr ≤ 0.7
	$(SCr/0.7)^{-0.329} \times 0.993^{age} \times 144$
	Female with Scr >0.7
	$(SCr/0.7)^{-1.209} \times 0.993^{age} \times 144$
	Male with Scr ≤ 0.9
	$(SCr/0.7)^{-1.209} \times 0.993^{usia} \times 144$
	Male with Scr > 0.9
	$(SCr/0.9)^{-1.209} \times 0.993^{age} \times 141$

Table 2: Basic subject characteristics

Variables	Metformin	Metformin-sulfonylurea
Male	9 (18)	10 (16)
Female	41 (82)	54 (84)
Age (years)	59.90 ± 7.24	58.81 ± 7.26
Body weight (kg)	61.12 ± 10.66	59.10 ± 8.49
Body height (cm)	152.06 ± 6.30	151.51 ± 6.48
Body Mass Index (BMI) (kg/m2)	26.36 ± 3.93	25.77 ± 3.63
Duration of diabetes mellitus (years)	5.56 ± 4.18	7.76 ± 5.93
Daily exercise	40 (80)	38 (59.38)

Table 3: Clinical characteristic of study subjects

Variables	Metformin (n=50)	Metformin-sulfonylurea (n=64)	p
HbA1C (%)	8.58 ± 1.77	10.73 ± 12.71	0.803
Systole (mmHg)	124.80 ± 17.29	126.56 ± 16.26	0.612
Diastole (mmHg)	78.00 ± 8.08	78.44 ± 7.39	0.749
Urine creatinine (mg/mL)	0.10 ± 0.06	0.09 ± 0.06	0.173
Serum creatinine (mg/dL)	0.85 ± 0.41	0.83 ± 0.49	0.648
H_2O_2 (μM)	19.35 ± 2.38	23.18 ± 1.83	0.065
H_2O_2 (μmol/g creatinine)	68.19 ± 14.14	90.96 ± 16.31	0.228
eGFR (ml/min/1.73 m2)			
Cockroft-Gault	93.14 ± 5.8	93.75 ± 4.92	0.936
MDRD	95.86 ± 5.94	97.77 ± 5.30	0.779
CKD-EPI	84.18 ± 3.78	86.14 ± 3.25	0.671
Albumin (mg/dL)	24.71 ± 6.22	90.21 ± 34.13	0.696
UACR (mg/g)	326.49 ± 112.19	1893.54 ± 536.96	0.838

Table 4: Linear Regression Analysis for UACR Using Stepwise Method

Variables	Beta (standardized coefficients)	p
H_2O_2 (μmol/g creatinine)	0.376	<0.001*

UACR, Urine Albumin to Creatinine Ratio *p < 0.05, significant; adjusted by age, gender, BMI, systolic blood pressure, HbA1c, hypertension status, smoking habit, exercise habit and medicine

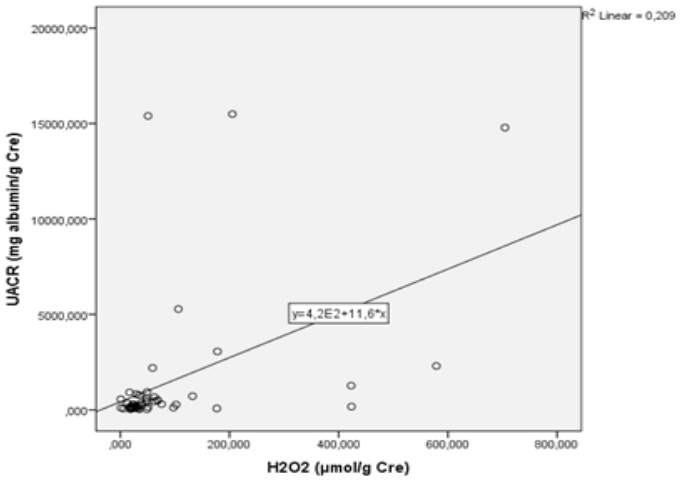

Figure 1: Correlation between urinary H_2O_2 and UACR on albuminuria patients

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.

Standard protocol on approvals, registration, & patient consents

This study has been registered in Ethics Committee, Faculty of Medicine, Universitas Indonesia–Dr. Cipto Mangunkusumo Hospital (No.44/UN2. F1/ETIK/I/2016). Clinical examinations were undertaken using questionnaire and informed consent was given to subjects before sampling

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 present 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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