

C-Reactive Protein Is a Predictor for Developing Proteinuria in a Screened Cohort

Kunitoshi Iseki^{a, b} Chiho Iseki^{a, b} Kozen Kinjo^{a, b}

^aDialysis Unit, University Hospital of the Ryukyus, and ^bOkinawa General Health Maintenance Association, Nishihara, Japan

Key Words

C-reactive protein · Proteinuria · Estimated glomerular filtration rate · Chronic kidney disease

Abstract

Background: The relationship between C-reactive protein (CRP) and proteinuria is not known. **Methods:** We examined 20,077 screenees (men: 52.4%) of the Okinawa General Health Maintenance Association (OGHMA) registry who were examined between 2004 and 2006. Cross-sectional and longitudinal relationships between CRP and dipstick proteinuria were examined. The OGHMA central laboratory measured creatinine and CRP levels using an autoanalyzer (normal: <0.30 mg/dl). The glomerular filtration rate was estimated using the Japanese formula. **Results:** The prevalence of dipstick-positive proteinuria increased from 5.2% in screenees with a low CRP level of <0.10 mg/dl to 12.3% in those with high CRP levels (0.30–0.90 mg/dl). The CRP values did not affect the mean (SD) estimated glomerular filtration rate: 76.9 (13.7) with low CRP and 76.4 (15.1) with high CRP levels. We examined the relationship between baseline CRP and the development of proteinuria among subjects screened in 2004. Of 8,315 subjects without proteinuria examined again by 2006, 370 (4.4%) had developed proteinuria. The odds ratio (95% CI) for high CRP levels (0.30–0.90 mg/dl; reference CRP: <0.10 mg/dl) was 1.433 (1.013–2.028; $p = 0.0422$) after adjusting for multivariate variables, suggesting that CRP is closely associated with the prevalence

and incidence of proteinuria. **Conclusion:** A prospective study on the development of proteinuria among those with high CRP levels is warranted. Screenees with high CRP levels may need to be followed up carefully despite the absence of traditional risk factors for proteinuria.

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Introduction

C-reactive protein (CRP) is a nonspecific acute-phase reactant associated with inflammation. Individuals with chronic kidney disease (CKD) are exposed to several potential inflammatory stimuli as they often have traditional risk factors for atherosclerosis. In both the general population and dialysis patients, CRP is independently associated with cardiovascular outcome [1, 2]. Inflammation may be the unifying concept between kidney disease and the enhanced risk for cardiovascular disease in patients with CKD.

The prevalence of end-stage renal disease (ESRD) is high in Japan [3, 4], and may be due to the high incidence of CKD or the more pronounced progression of CKD in Japanese. In asymptomatic subjects of the general population, the prevalence of microalbuminuria and proteinuria is higher in the Japanese than in other populations [5–9]. A cross-sectional study revealed that microalbuminuria is associated with CRP. However, microalbuminuria is not usually measured in a mass screening setting. The inter-

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Kunitoshi Iseki, MD
Dialysis Unit
University Hospital of the Ryukyus
207 Uehara, Nishihara, Okinawa 903-0215 (Japan)
Tel. +81 98 895 1341, Fax +81 98 895 1416, E-Mail chihokun@med.u-ryukyu.ac.jp

Table 1. Demographics of the screened subjects (n = 20,077)

Variables	All (n = 20,077)	Low CRP level (n = 12,336)	High CRP level (n = 1,596)
Age at screening, years	49.4 (11.3)	48.7 (11.2)	50.4 (11.8)**
Men, n	10,523 (52.4%)	5,968 (48.4%)	940 (58.9%)**
Body mass index	24.1 (3.5)	23.2 (3.1)	26.0 (4.4)**
Systolic blood pressure, mm Hg	122.1 (16.2)	120.4 (16.0)	125.6 (16.7)**
Diastolic blood pressure, mm Hg	75.2 (10.6)	74.1 (10.4)	77.6 (11.0)**
Blood urea nitrogen, mg/dl	13.6 (3.6)	13.6 (3.6)	13.5 (3.7)
Serum creatinine, mg/dl	0.77 (0.19)	0.76 (0.18)	0.79 (0.18)**
eGFR, ml/min/1.73 m ²	76.5 (13.9)	76.9 (13.7)	76.4 (15.1)**
Total protein, g/dl	7.2 (0.4)	7.1 (0.4)	7.2 (0.4)**
Serum albumin, g/dl	4.3 (0.2)	4.4 (0.2)	4.3 (0.2)**
Total cholesterol, mg/dl	201.9 (33.8)	199.3 (32.7)	204.0 (35.8)*
Triglyceride, mg/dl	129.6 (109.7)	115.3 (86.4)	150.1 (113.5)**
Uric acid, mg/dl	5.7 (1.5)	5.4 (1.4)	6.1 (1.5)**
Fasting blood glucose, mg/dl	101.6 (21.4)	99.2 (17.7)	107.6 (28.6)**
Hemoglobin A1c, %	5.2 (0.7)	5.1 (0.6)	5.5 (1.0)**
Hematocrit, %	42.7 (4.1)	42.3 (4.1)	43.3 (4.0)**
Proteinuria, %	6.8	5.2	12.3**
CRP, mg/dl	0.11 (0.13)	0.05 (0.03)	0.47 (0.16)**

Data are means with SD in parentheses unless otherwise specified. Low CRP level: CRP <0.10 mg/dl. High CRP level: 0.30–0.90 mg/dl. Abnormally high levels of CRP (>0.90 mg/dl) were excluded from the study (n = 413; 2.0% of the total of 20,490). Proteinuria: dipstick 1+ or over. Screening was performed during 2004 to 2006 in Okinawa, Japan. * p < 0.05, ** p < 0.01.

action between CRP and CKD has not been investigated in a general population study. We have been studying the relationship between dipstick proteinuria and renal outcomes in a large community-based screening cohort. In the present study, we examined the effect of CRP on the development of dipstick-positive proteinuria.

Materials and Methods

Study Design

This study was an observational study in a community-based, longitudinal, limited-access data set designed to examine the relationship between CRP and CKD, and also the effect of CRP on the development of dipstick-positive proteinuria.

Between 2004 and 2006, the study population, a total of 20,610 participants aged 20–89 years, was examined at the Okinawa General Health Maintenance Association (OGHMA) central clinic. The OGHMA, a nonprofit organization founded in 1972 and currently under the direction of Drs. Ikemiya and Kinjo, conducts a large annual community-based health examination [8, 9]. Once each year, the staff, doctors and nurses visit residences and workplaces throughout the prefecture to perform health examinations or to examine those who visited the central clinic. All examinations were performed at the OGHMA laboratory, and participants visited voluntarily. The OGHMA personnel provided mass screen-

ing, informed the participants of their results and, when necessary, recommended further evaluation or treatment. This process included an interview concerning health status, a physical examination, and urine and blood tests. A nurse or doctor measured blood pressure using a standard mercury sphygmomanometer with the subject in the sitting position. Dipstick testing for proteinuria, hematuria and glucosuria (Ames Dipstick, Tokyo, Japan) was performed in spontaneously voided fresh urine. Proteinuria was defined as a dipstick urinalysis score of 1+ or more. The body mass index was calculated as weight (kilograms) divided by the square of the height (meters). Hypertension was defined as systolic blood pressure of ≥ 140 mm Hg and/or diastolic blood pressure of ≥ 90 mm Hg. Diabetes mellitus was defined as fasting blood glucose of ≥ 126 mg/dl or hemoglobin A1c of $\geq 6.0\%$.

Participants studied again during 2005 to 2006 were identified by their ID number. The annual numbers of participants were 11,887 in 2004, 12,815 in 2005 and 13,627 in 2006. The ethics committee of the OGHMA approved the study protocol. Only coded data were used for the study.

Laboratory Tests

Serum creatinine levels were measured using the automated enzymatic method. The glomerular filtration rate (GFR) was estimated using the equation developed by the Japanese Society of Nephrology: estimated GFR (eGFR) = $194 \times \text{serum creatinine}^{-1.094} \times \text{age}^{-0.287}$ ($\times 0.739$, if female). We defined CKD as an eGFR of less than 60 ml/min/1.73 m² [10]. High-sensitivity serum

Table 2. Mean (SD) levels of CRP based on eGFR and/or proteinuria (n = 20,077)

	CRP, mg/dl	
	proteinuria (-)	proteinuria (+) ¹
eGFR		
<60 ml/min/1.73 m ²	0.12 (0.13) n = 1,714	0.18 (0.18) n = 252
60–89 ml/min/1.73 m ²	0.11 (0.13) n = 14,137	0.15 (0.16) n = 908
≥90 ml/min/1.73 m ²	0.11 (0.14) n = 2,856	0.17 (0.17) n = 210
Dipstick for proteinuria		
(-) (n = 16,428)	0.11 (0.12)	
(+/-) (n = 2,279)	0.13 (0.14)	
1+ (n = 1,016)	0.15 (0.16)	
2+ (n = 304)	0.16 (0.16)	
3+ and over (n = 50)	0.18 (0.18)	

Data are means with SD in parentheses. Abnormally high levels of CRP (>0.90 mg/dl) were excluded from the study (n = 413; 2.0% of the total of 20,490). Screening was performed during 2004 to 2006 in Okinawa, Japan.

¹ Dipstick 1+ or over.

CRP levels were measured using the latex-enhanced immunonephelometric method (Daiichi-Chemical, Tokyo, Japan). Both within-variance and the coefficient of variance of the method were less than 5% of the normal range of less than 0.30 mg/dl.

Statistics

Data were analyzed by SAS/STAT software (version 6.03; SAS Institute, Tokyo, Japan). Student's t test and the χ^2 test were performed to compare the significance of discrete variables. The odds ratio (95% CI) was calculated by a logistic regression procedure using multivariate models. $p < 0.05$ was considered significant.

Results

A total of 20,610 subjects visited the OGHMA and were examined during 2004 to 2006. CRP and dipstick proteinuria data were available for the majority of the subjects (20,490; 99.4%). Abnormally high levels of CRP (>0.90 mg/dl) were excluded from the study (n = 413; 2.0% of the total), and therefore we studied a total of 20,077 subjects (97.4%). The demographics of the subjects are summarized in table 1. The prevalence of dipstick-positive proteinuria increased from 5.2% in screenees with a CRP level of <0.10 mg/dl to 12.3% in those with

Table 3. Demographics of screened subjects examined in 2004 (baseline) and followed up by 2006 (reexamined) versus those not reexamined in Okinawa, Japan (n = 11,626)

	Reexamined (n = 8,926)	Not reexamined (n = 2,700)
Age at screening, years	49.6 (10.1)	49.3 (12.7)
Men, n	5,216 (58.4%)	1,353 (50.1%)**
Body mass index	24.2 (3.4)	24.0 (3.5)**
Systolic blood pressure, mm Hg	122.4 (15.8)	122.5 (16.4)
Diastolic blood pressure, mm Hg	75.0 (10.5)	74.3 (10.7)**
Blood urea nitrogen, mg/dl	13.9 (3.6)	14.1 (3.8)*
Serum creatinine, mg/dl	0.79 (0.18)	0.77 (0.24)**
eGFR, ml/min/1.73 m ²	75.6 (13.5)	76.9 (14.5)**
Total protein, g/dl	7.1 (0.4)	7.2 (0.4)**
Serum albumin, g/dl	4.3 (0.2)	4.3 (0.2)
Total cholesterol, mg/dl	201.7 (32.9)	201.2 (34.9)
Triglyceride, mg/dl	132.4 (114.5)	128.6 (102.9)
Uric acid, mg/dl	5.8 (1.5)	5.6 (1.5)**
Fasting blood glucose, mg/dl	102.1 (20.9)	102.5 (23.3)
Hemoglobin A1c, %	5.2 (0.7)	5.2 (0.8)
Hematocrit, %	42.9 (4.0)	42.5 (4.1)**
Proteinuria, %	6.9	7.5
CRP, mg/dl	0.12 (0.13)	0.12 (0.13)

Data are means with SD in parentheses unless otherwise specified. Abnormally high levels of CRP (>0.90 mg/dl) at baseline were excluded from the study. Proteinuria: dipstick 1+ or over.

* $p < 0.05$, ** $p < 0.01$.

CRP levels of 0.30–0.90 mg/dl. The CRP values did not affect the mean (SD) eGFR: 76.9 (13.7) for CRP levels of <0.10 mg/dl and 76.4 (15.1) for CRP levels of 0.30–0.90 mg/dl. Several variables were significantly different between subjects with low CRP (<0.10 mg/dl) and high CRP levels (0.30–0.90 mg/dl), such as body mass index, proteinuria, serum albumin, uric acid, blood pressure, total cholesterol, triglyceride and diabetes mellitus at baseline.

The relationship between baseline CRP, eGFR and dipstick proteinuria is shown in table 2. CRP levels were associated with degree of proteinuria, but not with GFR.

In 2004, a total of 11,626 screenees participated, 8,926 (76.8%) of whom had data on urine dipstick tests for proteinuria and CRP and were examined again by 2006. The baseline demographics were slightly different between those who were examined again and those who were not reexamined by 2006 (table 3). Nevertheless, the baseline levels of CRP were similar.

The incidence of proteinuria was related to the baseline serum CRP levels (fig. 1, 2). Among screenees without proteinuria (n = 8,315), the incidence was 4.4% of the

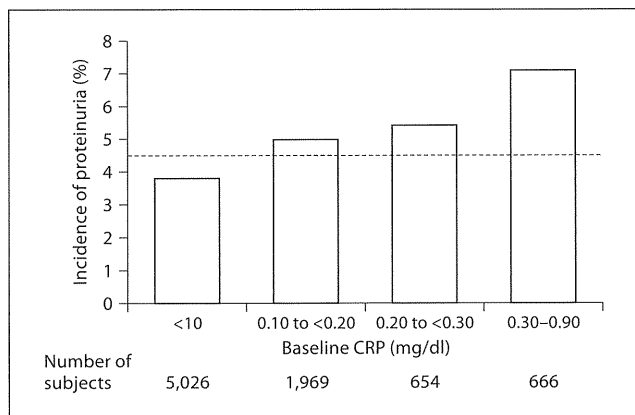


Fig. 1. Incidence of proteinuria by baseline CRP. Baseline screening was performed in 2004, and the follow-up study was performed by 2006. All screenees were negative for dipstick proteinuria in 2004 (n = 8,315).

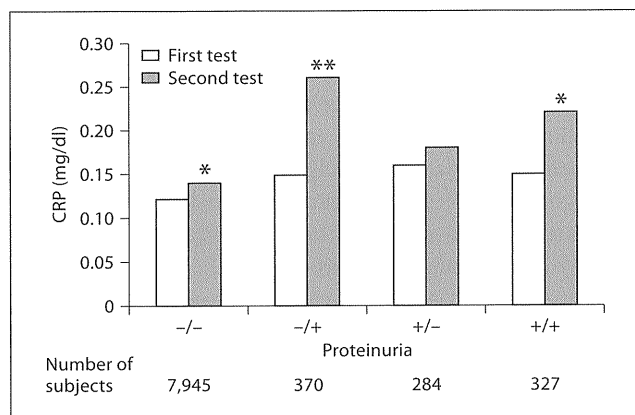


Fig. 2. Relationship between CRP and proteinuria. Mean levels of CRP by combining the levels of proteinuria, either absence or presence, at the first and second test. Baseline screening was performed in 2004, and the follow-up study was performed by 2006 (n = 8,926). * p < 0.05, ** p < 0.01, paired t test.

total: 3.8% in subjects with a CRP level of <0.10 mg/dl, 5.0% in those with CRP levels of 0.10 to <0.20 mg/dl, 5.4% in those with CRP levels of 0.20 to <0.30 mg/dl, and 7.1% in those with CRP levels of 0.30–0.90 mg/dl. The GFR-adjusted odds ratio (95% CI) of CRP was 1.433 (1.013–2.028; p = 0.0422) for developing proteinuria (table 4). Mean CRP levels are shown by the combination of the presence or absence of proteinuria at baseline and follow-

Table 4. Risk of developing proteinuria by baseline levels of CRP

CRP levels	Odds ratio	95% CI	p
Model 1			
<0.10 mg/dl	1.000		
<0.20 mg/dl	1.333	1.039–1.711	0.0239
<0.30 mg/dl	1.439	0.994–2.084	NS
0.30–0.90 mg/dl	1.933	1.389–2.688	<0.0001
Model 2			
<0.10 mg/dl	1.000		
<0.20 mg/dl	1.299	1.011–1.668	0.0408
<0.30 mg/dl	1.391	0.960–2.016	NS
0.30–0.90 mg/dl	1.888	1.356–2.628	0.0002
Model 3			
<0.10 mg/dl	1.000		
<0.20 mg/dl	1.081	0.835–1.401	NS
<0.30 mg/dl	1.112	0.760–1.627	NS
0.30–0.90 mg/dl	1.433	1.013–2.028	0.0422

Baseline CRP level was categorized into 4 groups: <0.10, <0.20, <0.30, and 0.30–0.90 mg/dl. Multivariate analysis was performed using the variables presented in the table. Subjects were those who were screened in 2004 and visited again by 2006 in Okinawa, Japan (n = 8,315). Variables used for analysis: none for model 1; age and sex for model 2; age, sex, body mass index, systolic blood pressure, diastolic blood pressure, serum creatinine, hematocrit, total cholesterol, triglyceride, serum albumin, uric acid and fasting blood glucose for model 3. NS = Not significant.

up (fig. 2). Those who developed proteinuria had significantly increased levels of CRP at the follow-up visit (paired t test: p = 0.0008).

Discussion

Dipstick proteinuria is the strongest predictor of ESRD among the commonly measured variables in a mass screening setting [11, 12]. We further investigated the role of CRP in the prevalence and incidence of proteinuria. CRP is not usually measured for screening purposes unless otherwise indicated. It is now included among the standard laboratory tests among our screening tools, however, because CRP is a known marker of atherosclerosis and a predictor of cardiovascular disease and microalbuminuria. CRP is a useful laboratory test for predicting the risk of developing proteinuria independently of the baseline GFR level. Therefore, CRP might be another marker for those at high risk of developing CKD and eventually ESRD, in addition to other traditional risk factors for proteinuria.

The higher the levels of proteinuria, the more rapidly CKD progresses. Renin-angiotensin system (RAS) inhibitors are beneficial in the treatment of proteinuric renal disease [13]. Except for the hypotensive action of RAS inhibitors, their effects are not well described. RAS inhibitors also effectively suppress chronic inflammation and therefore lower CRP [14]. The findings of the present study support the notion that chronic inflammation has a causative role in the development of proteinuria and cardiovascular disease.

Several studies have evaluated inflammatory markers in CKD and cardiovascular disease risk. CKD has both traditional and nontraditional risk factors [15, 16]. The relationship between GFR and proteinuria, however, may not be simple. CRP is often associated with an increased risk for cardiovascular disease even without CKD. The effects of proteinuria may be independent of the GFR level. Although Weiner et al. [17] recently reported that both CKD and inflammation are associated with an increased risk for cardiovascular disease and mortality, their study did not consider proteinuria. Proteinuria per se is a cause of inflammation. Higher proteinuria levels are associated with increased tubulointerstitial damage [18]. Lowering proteinuria levels effectively prevents the progression of GFR decline independently of lowering blood pressure [13].

The prevalence of stage 3 CKD is relatively high and is expected to increase with the growing elderly population, particularly in Japan [19, 20]. The incidence and prevalence of ESRD are also quite high in Japan and are generally expected to increase in Asian countries [7]. Therefore, it is important to define those at risk of developing ESRD among subjects with stage 3 CKD. Metabolic syndrome is a predictor of CKD and proteinuria [21, 22]. Obesity causes glomerulopathy [23], as confirmed by meta-analysis [24]. The results of the present study demonstrate a significant association between CRP and body mass index. Several mechanisms may underlie this entity; visceral adipocytes are often associated with hypercytokinemia, leading to inflammation [25].

The strength of this paper is that the registry used in this study is a well-described, community-based screening registry in Okinawa, Japan [11, 12], and that the number of subjects is sufficiently large. Renal outcomes were investigated using several variables including dipstick proteinuria. The subjects participated voluntarily and most were ambulatory. Both cross-sectional and follow-up studies supported the role of CRP in the development of proteinuria. The present study confirms that atherosclerotic processes are related to chronic inflammation.

This study has several limitations. First, it is a retrospective observational study, and pertinent information such as lifestyle-related factors, comorbid conditions and treatments was not available. Compared to the effects of body mass index and blood pressure, the effects of smoking and alcohol intake were small on the development of proteinuria [26]. Low intake of alcohol was rather inhibitory of proteinuria development. Subjects with proteinuria and high levels of CRP might have been treated with RAS inhibitors, which influence CRP levels and proteinuria. Since the participants were largely normotensive, the effect of RAS inhibitors would have been very small. Second, the causes of high CRP levels, 0.30–0.90 mg/dl and higher, are not clear. In the majority of subjects who were examined twice or more, CRP levels remained in the range of 0.15 mg/dl and proteinuria was not detected (fig. 2). Subjects with symptomatic urinary tract infection, other signs of infection, or a history of cardiovascular disease or malignancies were not completely excluded from the screening. We have excluded those with abnormally high levels of CRP (>0.9 mg/dl), therefore, such a possibility would be very small, if existent. Third, dipstick proteinuria is a semi-quantitative method and is not so accurate. But a positive result indicates that the proteinuria (or albuminuria) level is higher than that of the range of microalbuminuria. Microalbuminuria is relatively common in screened Japanese subjects [5, 6]. A significant relationship between CRP and microalbuminuria has been reported in apparently healthy individuals in a cross-sectional cohort [6]. Quantification of microalbuminuria is more sensitive than that of albuminuria, but it would require a longer follow-up to track the development of ESRD. The predictive power of proteinuria for the development of ESRD is significant.

In conclusion, CRP is a useful predictor of dipstick-positive proteinuria in a screening setting. In the present study, the risk of developing proteinuria was associated with increased CRP levels, even those with an increase within the normal range. Subjects with high CRP levels (0.30–0.90 mg/dl) may require further follow-up, despite the absence of traditional risk factors for CKD. The benefits of a comeasurement of CRP remains to be shown among the screened subjects with dipstick-negative proteinuria.

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References

- 1 Iseki K, Tozawa M, Yoshi S, et al: Serum C-reactive protein and risk of death in chronic dialysis patients. *Nephrol Dial Transplant* 1999;14:1956–1960.
- 2 Zimmermann J, Herrlinger S, Pruy A, et al: Inflammation enhances cardiovascular risk and mortality in hemodialysis patients. *Kidney Int* 1999;55:648–658.
- 3 Nakai S, Wada A, Kitaoka T, et al: An overview of regular dialysis treatment in Japan (as of 31 December 2004). *Therap Apher Dial* 2006;10:476–497.
- 4 Iseki K: Chronic kidney disease in Japan. *Int Med* 2008;26:13–17.
- 5 Konta T, Hao Z, Abiko H, et al: Prevalence and risk factor analysis of microalbuminuria in Japanese general population: the Takahata study. *Kidney Int* 2006;70:751–756.
- 6 Nakamura M, Onoda T, Itai K, et al: Association between serum C-reactive protein levels and microalbuminuria: a population-based cross-sectional study in northern Iwate, Japan. *Int Med* 2004;43:919–925.
- 7 Iseki K: Chronic kidney disease in Japan: from early predictions to current facts. *Nephron Clin Pract* 2008;110:268–272.
- 8 Iseki K: The Okinawa screening program. *J Am Soc Nephrol* 2003;14(suppl 2):S127–S130.
- 9 Iseki K: Screening for renal disease: what can be learned from the Okinawa experience. *Nephrol Dial Transplant* 2006;21:839–843.
- 10 Matsuo S, Imai E, Horio M, et al: Revised equations for estimating glomerular filtration rate from serum creatinine in Japan. *Am J Kidney Dis* 2009;53:982–992.
- 11 Iseki K, Iseki C, Ikemiya Y, et al: Risk of developing end-stage renal disease in a cohort of mass screening. *Kidney Int* 1996;49:800–805.
- 12 Iseki K, Ikemiya Y, Iseki C, et al: Proteinuria and the risk of developing end-stage renal disease. *Kidney Int* 2003;63:1468–1474.
- 13 Ruggenti P, Perticucci E, Cravedi P, et al: Role of remission clinics in the longitudinal treatment of CKD. *J Am Soc Nephrol* 2008;19:1213–1224.
- 14 Fliser D, Wagner KK, Loos A, et al: Chronic angiotensin II receptor blockade reduces (intra)renal vascular resistance in patients with type 2 diabetes. *J Am Soc Nephrol* 2005;4:1135–1140.
- 15 Sarnak MJ, Levey AS, Schoolwerth AC, et al: Kidney disease as a risk factor for development of cardiovascular disease: a statement from the American Heart Association Councils on Kidney in Cardiovascular Disease, High Blood Pressure Research, Clinical Cardiology, and Epidemiology and Prevention. *Circulation* 2003;108:2154–2169.
- 16 Go AS, Chertow GM, Fan D, et al: Chronic kidney disease and the risks of death, cardiovascular events, and hospitalization. *N Engl J Med* 2004;351:1296–1305.
- 17 Weiner DE, Tighiouart H, Elsayed EF, et al: Inflammation and cardiovascular events in individuals with and without chronic kidney disease. *Kidney Int* 2008;73:1406–1412.
- 18 Remuzzi G, Bertani T: Is glomerulosclerosis a consequence of altered glomerular permeability to macromolecules? *Kidney Int* 1990;38:384–394.
- 19 Imai E, Horio M, Yamagata K, et al: GFR decline rate in Japanese general population: a longitudinal 10-year follow-up study. *Hypertens Res* 2008;31:433–442.
- 20 Imai E, Horio M, Iseki K, et al: Prevalence of chronic kidney disease (CKD) in the Japanese general population predicted by the MDRD equation modified by a Japanese coefficient. *Clin Exp Nephrol* 2007;11:156–163.
- 21 Tozawa M, Iseki C, Tokashiki K, et al: Metabolic syndrome and risk of developing chronic kidney disease in Japanese adults. *Hypertens Res* 2007;30:937–943.
- 22 Ninomiya T, Kiyohara Y, Kubo M, et al: Metabolic syndrome and CKD in a general Japanese population: the Hisayama study. *Am J Kidney Dis* 2006;48:383–391.
- 23 Kambham N, Markowitz GS, Valeri AM, et al: Obesity-related glomerulopathy: an emerging epidemic. *Kidney Int* 2001;59:1498–1509.
- 24 Wang Y, Chen X, Song Y, et al: Association between obesity and kidney disease: a systematic review and meta-analysis. *Kidney Int* 2008;73:19–33.
- 25 Krikken JA, Lely AT, Bakker SJ, et al: The effect of a shift in sodium intake on renal hemodynamics is determined by body mass index in healthy young men. *Kidney Int* 2007;71:260–265.
- 26 Yamagata K, Ishida K, Sairenchi T, et al: Risk factors for chronic kidney diseases in a community-based population: a 10-year follow-up study. *Kidney Int* 2007;71:159–166.

Methylglyoxal augments intracellular oxidative stress in human aortic endothelial cells

NORIKO MIYAZAWA¹, MICHIAKI ABE¹, TOMOKAZU SOUMA¹, MASAYUKI TANEMOTO¹, TAKAAKI ABE^{2,3}, MASAOKI NAKAYAMA⁴ & SADAYOSHI ITO^{1,4}

¹Tohoku University Hospital, Division of Nephrology, Endocrinology and Vascular Medicine, Sendai, Miyagi, 980-8574, Japan, ²Division of Medical Science, ³Department of Clinical Biology and Hormonal Regulation, Tohoku University Graduate School of Medicine, Sendai 980-8574, Japan, and ⁴Research Division of Dialysis and Chronic Kidney Disease, Tohoku University, Sendai, Miyagi, 980-8574, Japan

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Abstract

Methylglyoxal (MGO) is a non-enzymatic metabolite in the glycolytic pathway and its concentration in blood and tissues is elevated in diabetes and renal failure. MGO induces tissue injuries via ROS; however, the mechanism remains to be clarified. The present study examined the harmful actions of MGO. Human aortic endothelial cells were assessed under real-time fluorescent microscopy with continuous superfusion. Increases in intracellular ROS were measured with fluorescent indicator, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (DCFH-DA). The addition of MGO rapidly increased the ROS in a dose-dependent manner. The increment of DCF was entirely abolished by pre-treatment with superoxide anion scavenger and membrane-permeable catalase, indicating that MGO induces superoxide production. The increment was completely inhibited by 2-thenoyltrifluoroacetone or carbonyl cyanide 3-chlorophenylhydrazone and partially inhibited by N-methyl-L-arginine. These data suggest that MGO stimulates superoxide production from mitochondria and partially stimulates nitric oxide synthase in human endothelial cells.

Keywords: *Methylglyoxal, oxidative stress, endothelial cell, carbonyl stress*

Introduction

Endothelial dysfunction is the initial step in the development of atherosclerosis. Diabetes mellitus and end-stage renal disease (ESRD) cause atherosclerosis and arteriosclerosis through endothelial damage [1].

Methylglyoxal (MGO) is a highly reactive dicarbonyl compound produced mainly by the degradation of glucose, via fructose in the glycolytic pathway. MGO reacts with the peptide forming MGO-derived lysine dimer, one of the advanced glycation end-products (AGEs) [2]. MGO is also known as a protein-bound uremic toxin [3] and the free form of MGO also exists stably in plasma. The plasma level of MGO is elevated in diabetic patients [4], patients with end-stage renal disease [5] and the corresponding animal models [6–8]. Several studies have suggested that MGO

induces peritoneal tissue injury via oxidative stress [9]. MGO stimulates the transcription of angiotensin-2 expression, which causes endothelial cell death and acellular capillary formation in the retina [10] and kidney [11]. In addition, a substantial portion of glucose-induced tissue injuries are caused by MGO [12]. Based on such findings, increased attention has been focused on the role of MGO in various pathological conditions such as diabetes mellitus, renal failure and hypertension [12–14]. However, the biological actions of MGO have not been well studied.

Reactive oxygen species (ROS), such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) are crucial risk factors in the progression of endothelial damage [15–17]. We have recently reported that MGO itself is not an ROS, as

Correspondence: Michiaki Abe, Assistant Professor, Division of Nephrology, Endocrinology and Vascular Medicine, Tohoku University Hospital, 1-1 Seryo-cho, Aoba-ku, Sendai, 980-8574, Japan. Tel: 81-22-717-7163. Fax: 81-22-717-7168. Email: michiabe@mail.tains.tohoku.ac.jp

determined by a chemiluminescence method; however, we detected free radicals by an electron spin resonance spectrum method when MGO was present with H_2O_2 [18]. MGO induces ROS production in neutrophils [19], platelets [20] and vascular smooth muscle cells [21]. However, the pathway of ROS production induced by MGO has not been examined enough in aortic endothelial cells, especially in the acute period after stimulation.

In the present study, we determined if MGO at concentrations detected in physiological or pathological conditions induced ROS in human aortic endothelial cells (HAECs) *in vitro*. We also examined the pathways of MGO-induced ROS production.

Materials and methods

Cell culture and reagents

Human aortic endothelial cells (HAECs) were obtained from Cambrex (Charles City, IA) and cultured according to the supplier's instructions. Methylglyoxal, elastase, polyethylene-glycol binding catalase (PEG-Cat), 2-thenoyltrifluoroacetone (TTFA), carbonyl cyanide 3-chlorophenylhydrazine (CCCP), apocynin and N-methyl-L-arginine (L-NAME) were purchased from Sigma-Aldrich (St. Louis, MO) and 5- (and 6-) chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (DCFH-DA) was purchased from Invitrogen (Carlsbad, CA). Tiron and H_2O_2 were purchased from Dojindo (Kumamoto, Japan).

Assessment of direct reaction between DCFH-DA and MGO

To confirm that DCFH-DA does not chemically react with MGO in a cell-free condition, 1, 10 and 100 $\mu\text{mol/L}$ MGO or various concentrations of H_2O_2 were added to DCFH-DA in Falcon 96-well plates. Elastase was added to the well for conversion of DCFH-DA to DCFH 3 min before the addition of H_2O_2 or MGO. DCFH reacted with H_2O_2 or $ONOO^-$, but not O_2^- , and was converted to the fluorescent form of the probe oxidation product (2',7'-dichlorofluorescein, DCF). The fluorescent signals of DCF were captured with excitation at 480 nm and emission at 535 nm by using a Fluoroscan Ascent plate reader (Thermo Lab-systems, Waltham, MA). Data were analysed with Ascent software (Thermo Lab-systems, Waltham, MA).

Measurement of DCF under real-time fluorescent microscopy with a continuous superfusion system

HAECs at the 5–7th passage were cultured on 15 mm cover slips and grown to 80–90% confluence under 95% O_2 and 5% CO_2 . The cover slip was then placed on the stage of a real-time fluorescent microscope

IX71 (Olympus, Tokyo, Japan) and the chamber was set for continuous superfusion.

The temperature of the chamber was maintained at 37°C by warming the superfusate with a TC-344 and TA-29 thermo-warmer (Warner Instruments, Hamden, CT). The superfusion rate of the buffer was maintained at 1 ml/min with a PHD2000 syringe pump (Harvard Apparatus, Holliston, MA) and the chamber volume was kept at 5 mL by continuous aspiration. Signals of DCF were detected through an Ixon cool CCD video camera (Andor Co., Tokyo, Japan) equipped with an emission filter of 535 nm after excitation at 480 nm by a DG-4 and 175-W Xenon-Arc lamp (Sutter Instruments, Navato, CA). The signals were captured every 10 s and quantified with MetaFluor imaging software (Universal Imaging Co., Ypsilanti, MI).

Protocol

Cells were loaded with a membrane-permeable, non-fluorescent probe, DCFH-DA (5 $\mu\text{mol/L}$) for 15 min at 37°C in Hanks' Balanced Salt Solution (HBSS) under the light-shielding condition [22]. After washing the dye with superfusion buffer for 5 min, the vehicle buffer was exchanged with buffer that contained 0, 1, 10 or 100 $\mu\text{mol/L}$ MGO and cells were observed for 20 min. Then the fluorescence of DCF was recorded. For the inhibition study, cells were pre-treated with inhibitors such as Tiron, TTFA, CCCP, apocynin and L-NAME for 6 h prior to the study. In the case of PEG-catalase, the cells were treated for 15 min before loading DCFH-DA. To compare ROS productivity after each treatment, mean rates of oxidation (units/s) were calculated for 800 s immediately after starting MGO superfusion during every experiment. These values are rough but illustrative estimates of the rate of oxidation of the probe which is not constant in time (especially for higher rates).

Statistical analysis

All data from at least five independent experiments were expressed as means \pm SEM. The unpaired Student's *t*-test was used to analyse the data of mean oxidation rates in each independent experimental group.

Results

MGO did not chemically produce DCF in the cell-free assay

DCFH-DA is a fluorescent indicator used extensively to detect intracellular H_2O_2 , but it may have potential artifacts. We assessed the direct interaction between MGO and DCFH-DA by using microplate readers to confirm that DCFH-DA is useful as a fluorescent

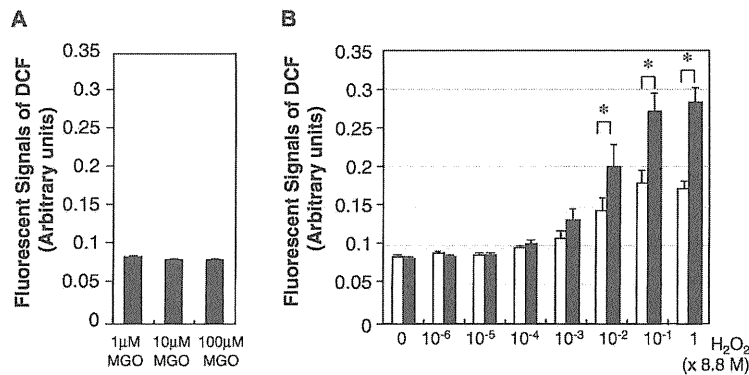


Figure 1. DCFH-DA did not react directly with MGO. (A) MGO did not form DCF from DCFH. (B) DCFH-DA formed DCF from DCFH in the presence of H₂O₂ at concentrations higher than 8.8 mmol/L. Elastase was added to form DCFH from DCFH-DA. Open bars, absence of elastase; closed bars, presence of elastase. * $p < 0.05$ absence of elastase vs the presence of elastase; # $p < 0.05$ vs H₂O₂ 8.8*10⁻⁴ mol/L.

marker to measure intracellular H₂O₂ and ONOO⁻. The amount of DCF in the presence of elastase was increased in a H₂O₂-concentration dependent manner and significantly greater than the amount in the absence of elastase at concentrations of H₂O₂ greater than 8.8 mM (Figure 1B). However, MGO did not directly convert DCFH to DCF (Figure 1A). These results suggested that MGO is not a ROS molecule that chemically reacts with DCFH, so DCFH-DA is available in the next protocol with HAECs.

MGO induced DCF formation dose-dependently

MGO increased the amount of intracellular DCF in HAECs. In order to evaluate the rate of increase, the intensities of the DCF were plotted against the elapsed time (Figure 2) and the mean rates of oxidation were calculated (Figure 3).

MGO increased the DCF signals in a dose- and time-dependent manner. Superfusion of MGO at the concentration of 10 and 100 μmol/L significantly increased the production of ROS. The rate for 0

(control), 1, 10 and 100 μmol/L MGO was 0.51 ± 0.18, 0.42 ± 0.09, 1.90 ± 0.12* and 2.64 ± 0.23 units/s*, respectively (* $p = 0.0029$, 10 μmol/L MGO vs control, $p = 0.0014$, 100 μmol/L MGO vs control).

Inhibition study of MGO-induced ROS formation

To determine the ROS production by MGO in HAECs (Figure 3), the cells were pre-treated with a membrane-permeable type of catalase (50 U/mL of PEG-Cat) or O₂⁻ scavenger [23] (1 mmol/L of Tiron). Pre-treatment with either PEG-Cat (0.26 ± 0.25 units/s, $p = 0.00071$ vs 100 μmol/L MGO) or Tiron (0.29 ± 0.07 units/s, $p = 0.00005$ vs 100 μmol/L MGO) completely abolished the MGO-induced DCF to the vehicle level. These data suggest that MGO promotes the production of O₂⁻ and that the O₂⁻ is converted to H₂O₂, which increases the intracellular levels of DCF.

Next, we investigated which pathway was involved in the MGO-induced O₂⁻ production. Cells were pre-treated with an uncoupler of oxidative phosphorylation

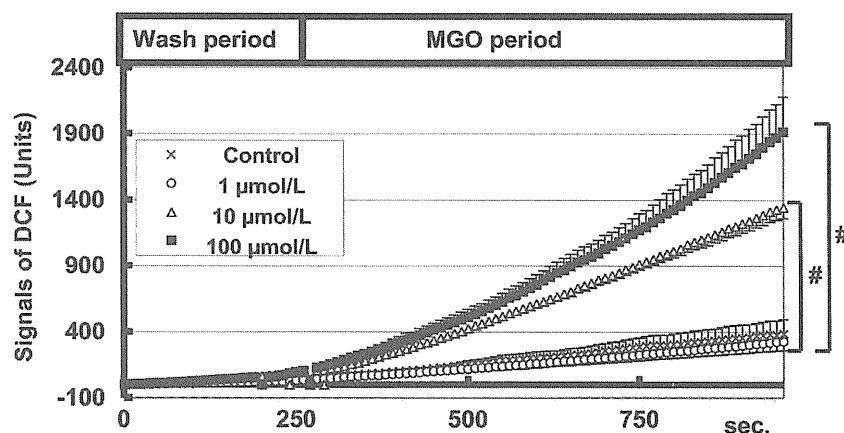


Figure 2. Intracellular ROS production by MGO. Increases in the amounts of DCF indicate the production of MGO-induced ROS. Acute infusion of MGO induced the production of intracellular ROS in a dose- and time-dependent manner in HAECs. The wash period indicates the vehicle in the each experiment. # $p < 0.05$, control vs 10 μmol/L or 100 μmol/L.

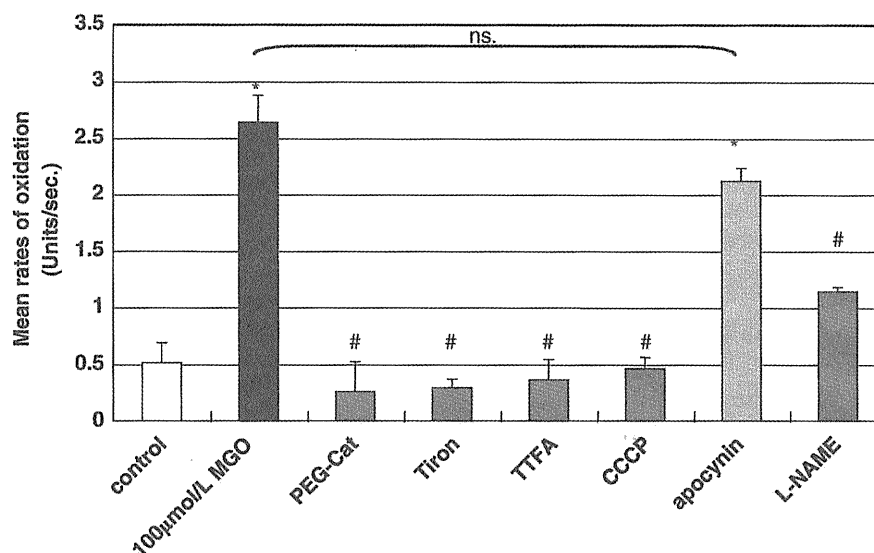


Figure 3. Effects of inhibitors on the MGO-induced ROS production. MGO (100 µmol/L) significantly increased the intracellular ROS production (* $p < 0.05$). Pre-treatment with PEG-Cat, Tiron, TTFA or CCCP completely inhibited the 100 µmol/L MGO-induced ROS (# $p < 0.05$). Pre-treatment with apocynin did not significantly inhibit the ROS formation vs 100 µmol/L MGO (ns), but significantly increased the formation of ROS vs control (* $p < 0.05$). Pre-treatment with L-NAME partially and significantly inhibited the formation of ROS (# $p < 0.05$).

that abolishes the mitochondrial membrane proton gradient (5 µmol/L of CCCP), an inhibitor of the mitochondrial electron transport chain complex II (10 µmol/L of TTFA), nitric oxide synthase (NOS) inhibitor (100 µmol/L of L-NAME) or an inhibitor of NADPH oxidase (NOX) (1 mmol/L of apocynin) for 6 h. Increases in the level of DCF were completely inhibited to the level of vehicle by pre-treatment with CCCP (0.46 ± 0.09 units/s, $p = 0.0001$ vs 100 µmol/L MGO) or TTFA (0.36 ± 0.18 units/s, $p = 0.0002$ vs 100 µmol/L MGO). Pre-treatment with L-NAME (1.14 ± 0.04 units/s, $p = 0.0012$ vs 100 µmol/L MGO) significantly suppressed the response to 54% of the response induced by 100 µmol/L MGO. These results suggest that pathological concentrations of MGO increase the intracellular production of $O_2^{\cdot-}$ in HAECs and that the production is mainly mediated by the mitochondrial electron-transport pathway and partially mediated by the NOS pathway. However, pre-treatment with apocynin also partially suppressed the production of DCF, although not significantly (2.12 ± 0.11 units/s, $p = 0.09$ vs 100 µmol/L MGO, $p = 0.0016$ vs control).

Discussion

MGO is one of the uremic toxins found in patients with diabetic nephropathy and ESRD, but its contribution to tissue toxicity has remained controversial. In the present study, we demonstrate that MGO increased the production of ROS in HAECs by using real-time fluorescent microscopy. For the first time, we demonstrated that the increase occurred immediately

after exchanging the vehicle buffer with MGO in the chamber. DCFH is useful as an indicator of $ONOO^-$ as well as H_2O_2 [24]. Superoxide anion is converted into H_2O_2 or $ONOO^-$, so DCF is able to indirectly measure the total amounts of superoxide. Tiron inhibited DCF after MGO stimulation; thus, probably MGO primarily stimulates the production of $O_2^{\cdot-}$. Tiron has been reported to be a Ca^{2+} chelator in addition to a scavenger of $O_2^{\cdot-}$ like a SOD mimetic [25]. Therefore, it is possible that tiron diminishes ROS production via another unknown pathway.

Certain levels of MGO exist in normal tissues throughout the whole body and the plasma level of MGO is increased in hyperglycaemic conditions (1–4 µmol/L) [26], in end-stage renal disease (9.7–11.5 µmol/L) [5] or by ageing [27,28]. In the present study we used 1, 10 or 100 µmol/L of MGO, which are the concentrations found in the plasma of healthy persons, diabetic patients and ESRD patients, respectively. MGO is a key component in pathological conditions such as DM and ESRD. We measured the plasma concentration of MGO in healthy individuals and found that it was less than 1 µmol/L. Even after healthy individuals consumed beverages, MGO concentrations were not greater than 1 µmol/L (unpublished data). Thus, the daily intake of food does not appear to contribute to endothelial toxicity in healthy individuals, because our results show that a low level of MGO does not increase the production of intracellular ROS. A previous study showed that cigarette smoke contains 0.19–0.83 µmol of MGO per cigarette [29]. Chronic oral administration of MGO at 0.69 mmol per kg of body weight led to

kidney collagen accumulation in an animal study [30]. Thus, the toxicity of MGO may be involved not only in some disease conditions, but also in activates such as smoking and the large intake of foods containing high levels of MGO.

The present study demonstrated that pathological concentrations of MGO rapidly elicit ROS production in human endothelial cells. Previous studies have used rather high concentrations of MGO to demonstrate its biological actions [19–21]. The higher sensitivity of our system may be due to our superfusion system and real time measurements of ROS. When cells are treated with MGO for a prolonged period under culture conditions, secondary changes may occur, and these changes may obscure the detection of subtle changes. Since our system allowed real-time measurements in the absence of the influences of secondary changes, we were able to detect significant effects of MGO at low concentrations.

Davidson and Duchon [31] reported that mitochondria play an important role in production of $O_2^{\cdot-}$ in the endothelial cells. Superoxide is generated from two main sites in the inner mitochondrial membrane, NADH dehydrogenase at complex I and at the interface between ubiquinone and complex III. High glucose loads stimulate mitochondrial $O_2^{\cdot-}$ production from the latter site in aortic endothelial cells [32] and retinal endothelial cells [33]. Rosca et al. [34] showed that the MGO-induced modification of mitochondrial complex III was associated with the excessive formation of $O_2^{\cdot-}$ in streptozotocin-derived diabetic rats. Long-standing hyperglycaemia alters the mitochondrial function and increases the formation of $O_2^{\cdot-}$ due to alterations in mitochondrial metabolism [35]. Shangari et al. [36] reported that a monocarbonyl compound metabolized from glucose, glyoxal markedly increased ROS-induced cytotoxicity in rat hepatocytes. In the present study, two types of mitochondrial inhibitors (TTFA and CCCP) completely suppressed the MGO-induced ROS. TTFA inhibits mitochondrial electron transport chain complex II and CCCP uncouples oxidative phosphorylation, which abolishes the mitochondrial membrane proton gradient. Our results suggest that the mitochondrial pathway is crucial for MGO-induced ROS production, which probably originates from $O_2^{\cdot-}$.

Superoxide reacts with nitric oxide (NO) very quickly and forms $ONOO^-$ [37]. Zou et al. [38] demonstrated that increases in the level of $ONOO^-$ reduced the amount of BH_4 and increased the uncoupling form of eNOS. Cai [39] suggested that H_2O_2 originating from vascular NAD(P)H oxidases propagates its own production via the enhancement of several pathways of ROS production including uncoupling eNOS. The uncoupling eNOS increases the production of $O_2^{\cdot-}$. However, our protocols of measuring DCF could not distinguish the production of H_2O_2 from $ONOO^-$. Thus, we could not distinguish uncoupling

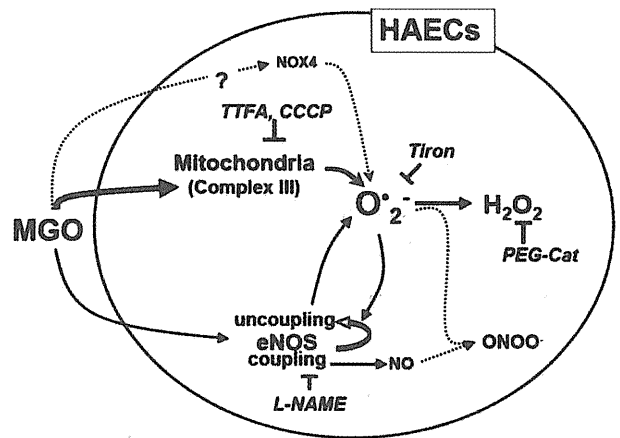


Figure 4. Putative pathways by which MGO induces ROS production in HAECs. To increase $O_2^{\cdot-}$ production, MGO primarily affects the mitochondria and partially affects eNOS. MGO might also potentially affect Nox. The increment of intracellular $O_2^{\cdot-}$ increases the uncoupling of eNOS and probably trigs a vicious cycle on the oxidative stress.

eNOS from coupling eNOS to consider their individual contributions to the ROS production. $ONOO^-$ is also a harmful ROS molecule and L-NAME inhibits uncoupling eNOS as well as coupling eNOS. Based on our results, eNOS is associated with the MGO-mediated ROS production. These findings suggest that MGO triggers a vicious cycle involved in formation of uncoupling eNOS (Figure 4). This pathway may augment the MGO toxicity in the cells.

In vascular endothelial cells, the Nox family is an important source of $O_2^{\cdot-}$. Nox2 and Nox4 are abundantly expressed in human endothelial cells [40]. A previous study suggested that Nox was responsible for the MGO-induced $O_2^{\cdot-}$ formation in mesangial cells [41]. However, we did not find a significant contribution of Nox to the acute ROS production induced by MGO in HAECs when we used apocynin to inhibit Nox. Apocynin is activated in the presence of H_2O_2 and myeloperoxidase (MPO) [42] and it inhibits the activation of Nox2 by inhibition of the translocation of p47phox. A recent report found that apocynin is an antioxidant and is not used as an NADPH oxidase inhibitor [43]. HAEC has a rare expression pattern of MPO that is different from neutrophils. Furthermore, the pre-treated apocynin was washed out before the experimental period, so apocynin could not exert antioxidant effects in our protocol. Nox4 requires p22phox for its activity but does not require other regulatory sub-units [44]. It is possible that Nox4 produced superoxide in our study, but we could not address this possibility in the present study.

Recently we reported that MGO reacted with H_2O_2 , forming a novel carboxy-methyl radical [18]. The radical was formed higher under MGO with H_2O_2 than under glyoxal (GO) with H_2O_2 . This observation is consistent with our supplemental data (online version only) showing that the ROS production rate by MGO

(4.11 ± 0.79 units/s) is greater than that by GO (0.82 ± 0.05 units/s, $p = 0.02$). As MGO reacts with glutathione and forms S-D-lactoylglutathione, it is possible that MGO decreases the intracellular capacity of antioxidants [45], but GO does not [46]. On the other hand, H_2O_2 is known as an endothelial-derived hyperpolarization factor [47]. Thus, MGO may reduce H_2O_2 localized around endothelial cells and damage the artery.

MGO is a radical modulator that forms advanced glycation end-products (AGEs). Aminoguanidine (AG) inhibits AGE formation from glucose and methylglyoxal [48]. AGE is also an inducer of $O_2^{\cdot-}$. We examined the ability of AG and arginine (L-Arg) to inhibit AGE formation from MGO. As shown in the supplemental figure (online version only), both L-Arg and AG entirely inhibited the ROS production (mean oxidation rates (units/s): MGO alone, 4.11 ± 0.79 ; MGO with L-Arg, 0.014 ± 0.01 ; MGO with AG, -0.09 ± 0.06 ; control, 0.31 ± 0.12). MGO reacts with guanidine residue [49] and produces superoxide, which is inhibited by AG [50]. Arginine is a kind of guanidino compound. It is possible that the extracellular $O_2^{\cdot-}$ generated from L-Arg and MGO was continuously drained from the chamber because of our continuous superfusion system. As DCF is an accumulative indicator, it is probable that AG almost completely inhibited ROS by the basal production and that we observed a photo-bleaching phenomenon of DCF. Because the guanidine residue is a constituent of cyclic GMP, which is an intracellular signal of NO to dilate vessels, MGO may interfere with NO functions. It has been reported that MGO activated caspase-3 and induced the cell death of endothelial cells [51]. The expression and activity of caspase-3 was stimulated by superoxide [51]. This study supports our finding that MGO stimulates the production of superoxide in endothelial cells. Here, we show the mechanism by which MGO produces ROS in endothelial cells. Further studies are needed to conclusively determine if the produced ROS is derived from $O_2^{\cdot-}$.

In conclusion, we found that high concentrations of MGO rapidly induce the production of ROS in HAECs. The putative source of the ROS is the mitochondrial pathway and also partially the eNOS pathway (Figure 4). The increased level of MGO in patients with renal dysfunction and/or diabetes probably plays an important role in the endothelial dysfunction and is a putative risk for developing atherosclerosis.

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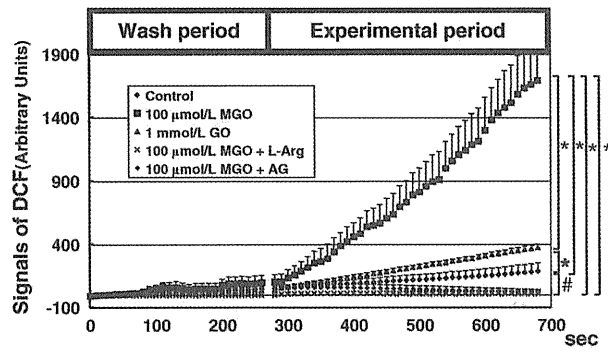
Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

- [1] Davignon J, Ganz P. Role of endothelial dysfunction in atherosclerosis. *Circulation* 2004;109:III27–III32.
- [2] Shinohara M, Thornalley PJ, Giardino I, Beisswenger P, Thorpe SR, Onorato J, Brownlee M. Overexpression of glyoxalase-I in bovine endothelial cells inhibits intracellular advanced glycation endproduct formation and prevents hyperglycemia-induced increases in macromolecular endocytosis. *J Clin Invest* 1998;101:1142–1147.
- [3] Yavuz A, Tetta C, Ersoy FF, D'intini V, Ratanarat R, De Cal M, Bonello M, Bordoni V, Salvatori G, Andrikos E, Yakupoglu G, Levin NW, Ronco C. Uremic toxins: a new focus on an old subject. *Semin Dial* 2005;18:203–211.
- [4] Han Y, Randell E, Vasdev S, Gill V, Gadag V, Newhook LA, Grant M, Hagerty D. Plasma methylglyoxal and glyoxal are elevated and related to early membrane alteration in young, complication-free patients with type I diabetes. *Mol Cell Biochem* 2007;305:123–131.
- [5] Miyata T, Horie K, Ueda Y, Fujita Y, Izuhara Y, Hirano H, Uchida K, Saito A, van Ypersele de Strihou C, Kurokawa K. Advanced glycation and lipidoxidation of the peritoneal membrane: respective roles of serum and peritoneal fluid reactive carbonyl compounds. *Kidney Int* 2000;58:425–435.
- [6] Beisswenger PJ, Drummond KS, Nelson RG, Howell SK, Szewgold BS, Mauer M. Susceptibility to diabetic nephropathy is related to dicarbonyl and oxidative stress. *Diabetes* 2005;54:3274–3281.
- [7] Rabbani N, Sebekova K, Sebekova K Jr, Heidland A, Thornalley PJ. Accumulation of free adduct glycation, oxidation, and nitration products follows acute loss of renal function. *Kidney Int* 2007;72:1113–1121.
- [8] Staniszewska MM, Nagaraj RH. Upregulation of glyoxalase I fails to normalize methylglyoxal levels: a possible mechanism for biochemical changes in diabetic mouse lenses. *Mol Cell Biochem* 2006;288:29–36.
- [9] Nakayama M, Sakai A, Numata M, Hosoya T. Hyper-vascular change and formation of advanced glycation endproducts in the peritoneum caused by methylglyoxal and the effect of an anti-oxidant, sodium sulfite. *Am J Nephrol* 2003;23:390–394.
- [10] Yao D, Taguchi T, Matsumura T, Pestell R, Edelstein D, Giardino I, Suske G, Ahmed N, Thornalley PJ, Sarthy VP, Hammes HP, Brownlee M. Methylglyoxal modification of mSin3A links glycolysis to angiopoietin-2 transcription. *Cell* 2006;124:275–286.
- [11] Yao D, Taguchi T, Matsumura T, Pestell R, Edelstein D, Giardino I, Suske G, Rabbani N, Thornalley PJ, Sarthy VP, Hammes HP, Brownlee M. High glucose increases angiopoietin-2 transcription in microvascular endothelial cells through methylglyoxal modification of mSin3A. *J Biol Chem* 2007;282:31038–31045.
- [12] Cantero AV, Portero-Otín M, Ayala V, Auge N, Sanson M, Elbaz M, Thiers JC, Pamplona R, Salvayre R, Nègre-Salvayre A. Methylglyoxal induces advanced glycation end product (AGEs) formation and dysfunction of PDGF receptor-beta: implications for diabetic atherosclerosis. *FASEB J* 2007;21:3096–3106.
- [13] Wang X, Desai K, Chang T, Wu L. Vascular methylglyoxal metabolism and the development of hypertension. *J Hypertension* 2005;23:1565–1573.

- [14] Wang X, Chang t, Jiang B, Desai K, Wu L. Attenuation of hypertension development by aminoguanidine in spontaneously hypertensive rats: role of methylglyoxal. *Am J Hypertens* 2007;20:629–636.
- [15] Brownlee M. Biochemistry and molecular cell biology of diabetic complications. *Nature* 2001;414:813–820.
- [16] Cai H, Harrison DG. Endothelial dysfunction in cardiovascular disease. The role of oxidant stress. *Circ Res* 2000;87:840–844.
- [17] Johansen JS, Harris AK, Rychly DJ, Erqul A. Oxidative stress and the use of antioxidants in diabetes. *Cardiovasc Diabetol* 2005;4:5–15.
- [18] Nakayama M, Saito K, Sato E, Nakayama K, Terawaki H, Ito S, Kohno M. Radical generation by the non-enzymatic reaction of methylglyoxal and hydrogen peroxide. *Redox Rep* 2007;12:125–133.
- [19] Ward RA, McLeish KR. Methylglyoxal: a stimulus to neutrophil oxygen radical production in chronic renal failure? *Nephrol Dial Transplant* 2004;19:1702–1707.
- [20] Leoncini G, Poggi M. Effects of methylglyoxal on platelet hydrogen peroxide accumulation, aggregation and release reaction. *Cell Biochem Funct* 1996;14:89–95.
- [21] Chang T, Wang R, Wu L. Methylglyoxal-induced nitric oxide and peroxynitrite production in vascular smooth muscle cells. *Free Radic Biol Med* 2005;38:286–293.
- [22] Ho HY, Cheng ML, Lu FJ, Chou YH, Stern A, Liang CM, Chiu DT. Enhanced oxidative stress and accelerated cellular senescence on glucose-6-phosphate dehydrogenase (G6PD)-deficient human fibroblasts. *Free Radic Biol Med* 2000;29:156–169.
- [23] Seki S, Flavahan NA, Smedira NG, Murray PA. Superoxide anion scavenger restore NO-mediated pulmonary vasodilation after lung transplantation. *Am J Physiol* 1999;276:H42–H46.
- [24] Wang H, Joseph JA. Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. *Free Radic Biol Med* 1999;27:612–616.
- [25] Ghosh M, Wang HD, McNeill JR. Tiron exerts effects unrelated to its role as a scavenger of superoxide anion: effects on calcium binding and vascular responses. *Can J Physiol Pharmacol* 2002;80:755–760.
- [26] Odani H, Shinzato T, Matsumoto Y, Usami J, Maeda K. Increase in three α,β -dicarbonyl compound levels in human uremic plasma: specific *in vivo* determination of intermediates in advanced Maillard reaction. *Biochem Biophys Res Commun* 1999;256:89–93.
- [27] Hipkiss AR. Dietary restriction, glycolysis, hormesis and ageing. *Biogerontology* 2007;8:221–224.
- [28] Schoneich C. Protein modification in aging: an update. *Exp Gerontol* 2006;41:807–812.
- [29] Fujioka K, Shibamoto T. Determination of toxic carbonyl compounds in cigarette smoke. *Environ Toxicol* 2006;21:47–54.
- [30] Golej J, Hoeger H, Radner W, Unfried G, Lubec G. Oral administration of methylglyoxal leads to kidney collagen accumulation in the mouse. *Life Sci* 1998;63:801–807.
- [31] Davidson SM, Duchon MR. Endothelial mitochondria: contributing to vascular function and disease. *Circ Res* 2007;100:1128–1141.
- [32] Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, Yorek MA, Beebe D, Oates PJ, Hammes HP, Giardino I, Brownlee M. Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature* 2000;404:787–790.
- [33] Du Y, Miller CM, Kern TS. Hyperglycemia increases mitochondrial superoxide in retina and retinal cells. *Free Radic Biol Med* 2003;35:1491–1499.
- [34] Rosca MG, Mustata TG, Kinter MT, Ozdemir AM, Kern TS, Szweda LI, Brownlee M, Monnier VM, Weiss MF. Glycation of mitochondrial proteins from diabetic rat kidney is associated with excess superoxide formation. *Am J Physiol Renal Physiol* 2005;289:420–430.
- [35] Hink U, Li H, Mollnau H, Oelze M, Matheis E, Hartmann M, Skatchkov M, Thaiss F, Stahl RA, Warnholtz A, Meinertz T, Griendling K, Harrison DG, Forstermann U, Munzel T. Mechanisms underlying endothelial dysfunction in diabetes mellitus. *Circ Res* 2001;88:e14–e22.
- [36] Shangari N, Chan TS, Popovic M, O'Brien PJ. Glyoxal markedly compromises hepatocyte resistance to hydrogen peroxide. *Biochem Pharmacol* 2006;71:1610–1618.
- [37] Huie RE, Padmaja S. The reaction of NO with superoxide. *Free Radic Res Commun* 1993;18:195–199.
- [38] Zou MH, Shi C, Cohen RA. Oxidation of the zinc-thiolate complex and uncoupling of endothelial nitric oxide synthase by peroxynitrite. *J Clin Invest* 2002;109:817–826.
- [39] Cai H. NAD(P)H oxidase-dependent self-propagation of hydrogen peroxide and vascular disease. *Circ Res* 2005;96:18–22.
- [40] Sorescu D, Weiss D, Lassègue B, Clempus RE, Szöcs K, Sorescu GP, Valppu L, Quinn MT, Lambeth JD, Vega JD, Taylor WR, Griendling KK. Superoxide production and expression of Nox family proteins in human atherosclerosis. *Circulation* 2002;105:1429–1435.
- [41] Ho C, Lee PH, Huang WJ, Hsu YC, Lin CL, Wang JY. Methylglyoxal-induced fibronectin gene expression through Ras-mediated NADPH oxidase activation in renal mesangial cells. *Nephrology* 2007;12:348–356.
- [42] Touyz RM. Apocynin, NADPH oxidase, and vascular cells. A complex matter. *Hypertension* 2008;51:172–174.
- [43] Heumüller S, Wind S, Barbosa-Sicard E, Schmidt HHHW, Busse R, Schröder K, Brandes RP. Apocynin is not an inhibitor of vascular NADPH oxidases but an antioxidant. *Hypertension* 2008;51:211–217.
- [44] Frederick LM, von Loehneysen K, Dinauer MC, Knaus UG. Functional analysis of Nox4 reveals unique characteristics compared to other NADPH oxidases. *Cell Signal* 2006;18:69–82.
- [45] Thornalley PJ. Modification of the glyoxalase system in human red blood cells by glucose *in vitro*. *Biochem J* 1988;254:751–755.
- [46] Akhand AA, Hossain K, Mitsui H, Kato M, Miyata T, Inagi R, Du J, Takeda K, Kawamoto Y, Suzuki H, Kurokawa K, Nakashima I. Glyoxal and methylglyoxal trigger distinct signals for MAP family kinases and caspase activation in human endothelial cells. *Free Radic Biol Med* 2001;31:20–30.
- [47] Shimokawa H, Morikawa K. Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in animals and humans. *J Mol Cell Cardiol* 2005;29:725–732.
- [48] Li W, Ota K, Nakamura J, Naruse K, Nakashima E, Oiso Y, Hamda Y. Antiglycation effect of glioclazide on *in vitro* AGE formation from glucose and methylglyoxal. *Exp Biol Med* 2008;233:176–179.
- [49] Li Y, Cohenford MA, Dutta U, Dain JA. The structural modification of DNA nucleosides by nonenzymatic glycation: an *in vitro* study based on the reactions of glyoxal and methylglyoxal with 2'-deoxyguanosine. *Anal Bioanal Chem* 2008;390:679–688.
- [50] Nohara Y, Usui T, Kinoshita T, Watanabe M. Generation of superoxide anions during the reaction of guanidino compounds with methylglyoxal. *Chem Pharm Bull* 2002;50:179–184.
- [51] Quagliaro L, Piconi L, Assaloni R, Da Ros R, Szabó C, Ceriello A. Primary role of superoxide anion generation in the cascade of events leading to endothelial dysfunction and damage in high glucose treated HUVEC. *Nutr Metab Cardiovasc Dis* 2007;17:257–267.

Supplementary Material



Supplementary Figure 1. Vehicle buffer was continuously superfused during the wash period and then switched to buffer that contained MGO (100 $\mu\text{mol/L}$) or glyoxal (GO, 1 mmol/L) during the experimental period. Signals of DCF were detected and captured by a 535-nm emission filter with 480-nm excitation wavelength every 10 s. The responses by MGO were measured in the presence or absence of 10 mmol/L Aminoguanidine (AG) or 100 $\mu\text{mol/L}$ L-arginine (L-Arg). * $p < 0.05$, # $p = 0.016$ control vs 100 μM MGO + AG.

Biological Effects of Electrolyzed Water in Hemodialysis

Masaaki Nakayama^a Shigeru Kabayama^c Hirofumi Nakano^d Wan-Jun Zhu^{a, c}
Hiroyuki Terawaki^a Keisuke Nakayama^a Kiyoshi Katoh^b Toshinobu Satoh^b
Sadayoshi Ito^{a, b}

^aResearch Division of Dialysis and Chronic Kidney Disease, Tohoku University Graduate School of Medicine,

^bDepartment of Blood Purification, Tohoku University Hospital, Sendai, ^cNihon Trim Co. Ltd., Osaka,

^dDialysis Center, Kashima Hospital, Iwaki, Japan

Key Words

Hemodialysis · Electrolyzed water · Oxidative stress · Methylglyoxal · Neutrophils

Abstract

Background/Aims: The application of electrolyzed water (EW) at the cathode side to manufacture reverse osmosis (RO) water and hemodialysis (HD) solution can actually lead to less oxidative capacity in chemical terms. The present study examined the biological actions of this water on human polymorphonuclear leukocytes (PMNs), and the clinical feasibility of applying this technology to HD treatment. **Methods:** RO water using EW (e-RO) exhibited less chemiluminescence in luminol-hydrogen peroxide and higher dissolved hydrogen levels (–99.0 ppb) compared with control RO water. The effects of e-RO on PMN viability were tested. HD using e-RO was performed for 12 consecutive sessions in 8 patients for the feasibility test. **Results:** Basal cellular viability and function to generate superoxide radicals of PMNs were better preserved by e-RO application. In the clinical trial, reductions of blood pressure were noted, but no adverse events were observed. There were no changes in the blood dialysis parameters, although methylguanidine levels were

significantly decreased at the end of study. **Conclusion:** The present study demonstrated the capacity of e-RO to preserve the viability of PMNs, and the clinical feasibility of applying this water for HD treatment. The clinical application of this technology may improve the bio-compatibility of HD treatment.

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Introduction

Evidence suggests that enhanced oxidative stress plays a crucial role in poor outcomes of patients on maintenance hemodialysis (HD) [1, 2]. In addition to a uremic milieu [1], several factors in the HD system have been found to be involved in the pathological mechanism of these poor outcomes, including bio-incompatibility of the dialysis membrane, contamination of the HD solution and loss of antioxidants during HD [3–7].

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Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

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Masaaki Nakayama, MD, Tohoku University Graduate School of Medicine
Research Division of Dialysis and Chronic Kidney Disease
1-1 Seiryomachi, Aoba-ku, Sendai 980-8574 (Japan)
Tel. +81 22 717 7399, Fax +81 22 717 7469
E-Mail mnakayama@mail.tains.tohoku.ac.jp

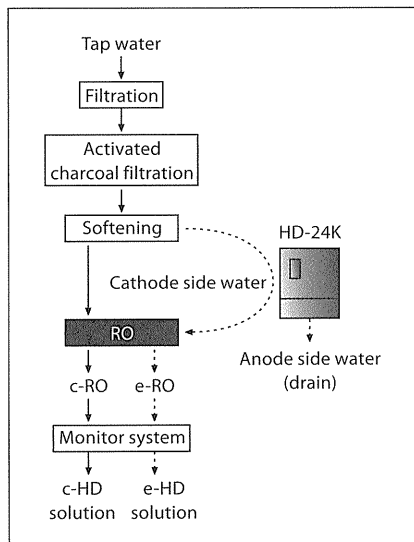


Fig. 1. Manufacturing process for HD solution using reverse osmosis water from the cathode-side of electrolyzed water. c-RO = control reverse osmosis water; e-RO = reverse osmosis water using electrolyzed water at the cathode side.

Water electrolysis renders 2 types of water: acidic water at the anode side and alkali water at the cathode side. It has been shown that alkali water (called electrolyzed water, EW) at the cathode side exhibits unique chemical properties, such as highly dissolved hydrogen with antioxidative capacity [8, 9]. An *in vitro* study showed that chemical reactions to generate superoxide anions and hydrogen peroxide (H_2O_2) were suppressed by EW treatment [8]. Based on these findings, therapeutic application of EW or water with highly dissolved hydrogen has been studied in animal models of diabetes [10, 11] and ischemic brain injury [12].

Furthermore, EW has been used to manufacture HD solution to reduce oxidative stress in patients [13–15]. To date, the limited clinical experience using this technology shows that EW suppresses oxidative/inflammatory markers in HD patients [13, 14]; however, most of the biological and clinical effects of EW remain unclear. The present study aimed to: (1) examine the biological action of EW in terms of whether it could ameliorate injury to human polymorphonuclear leukocytes, which may play a central role in excess inflammation or oxidative stress in HD patients [16], and (2) to test the clinical feasibility of applying this technology to HD treatment.

Materials and Methods

Manufacture of Test Solutions

Details of the manufacturing process were reported previously [15]. Briefly, test solutions were manufactured as follows (fig. 1): prefiltered water was processed by activated charcoal filtration and water softening to supply the water electrolysis system HD-24K (Nihon Trim, Osaka, Japan), where water was electrolyzed by direct current supply to the anode and cathode electrode plates. Water at the anode side was drained out, and EW was collected to supply the reverse osmosis equipment (MH500CX, Japan Water System Corp., Tokyo, Japan) at 500 ml/min. The intensity of the electrolysis was adjusted to maintain a $pH \leq 10.0$. The reverse osmosis water made by EW (e-RO) was supplied to a personal HD monitoring system (DBB-22B, Nikkiso, Tokyo, Japan) to make the HD solution by mixing with a liquid dialysis solution concentrate.

The pH of the e-RO ranged from 9.0 to 10.0, with mean dissolved hydrogen levels of 99.0 ppb. The dissolved hydrogen level was detected by a gas analyzer (DH-35A hydrogen gas analyzer, Mitsuwa Rikagaku, Osaka, Japan). The hemodialysis solution made by e-RO (e-HD) did not differ from the control HD (c-HD) solution in respect of the electrolyte composition or pH; however, the former solution had a higher level of dissolved hydrogen (80 vs. 0 ppb).

Biological Effects of EW on Polymorphonuclear Leukocytes Measurement of Cellular Viability

Human polymorphonuclear leukocytes (PMNs) were obtained from healthy volunteers or patients on chronic HD as indicated. Briefly, whole blood was withdrawn from healthy volunteers, and heparinized samples were placed onto the Mono-poly Resolving Medium (Dainippon Pharmaceutical, Osaka, Japan) to collect PMNs. After centrifugation at 1,800 rpm for 30 min at $18^\circ C$, the intermediate PMN-enriched layer was recovered, washed, and resuspended in RPMI 1640 culture medium supplemented with 10% fetal calf serum or phosphate buffered saline (PBS). Collected PMNs were adjusted to a concentration of 5×10^5 cells/ml. 50 μl of cell suspension was placed in a 96-well microplate and 50 μl of the test solution containing methylglyoxal (MGO), a toxic dicarbonyl compound elevated in uremic patients, and/or 4- β phorbol 12- β -myristate 13- α -acetate (PMA; a stimulator of the respiratory burst of PMNs) in PBS, was added. The viability of PMNs was examined using a commercially available kit (CellTiter-Glo[®] luminescent cell viability assay, Promega Corp., Wisc., USA) as previously reported [17]. After each treatment, 100 μl of the sample was placed in a microplate and 100 μl CellTiter-Glo[®] reagent (Promega), which contains beetle luciferin and luciferase was added immediately. This reacts with adenosine triphosphate (ATP), which was released from lysed cells, to produce oxyluciferin; it also generates chemiluminescence as a function of the increased ATP levels. After incubation, luminescence was measured using a chemiluminescence analyzer (GloMax[™] 20/20n luminometer, Promega). Each measurement was made 5 times, and the mean values for each sample were calculated for analysis after correction for cell-free levels.

Measurement of Radical Generation from PMN

The rate of superoxide release from human PMNs was determined by measuring the reduction of ferricytochrome C, as reported elsewhere [18]. PMNs obtained by the same procedure described above were adjusted to a concentration of 1×10^6 cells/ml

Fig. 2. Feasibility study protocol of HD applying e-RO based HD solution (e-HD). e-RO = reverse osmosis water using electrolyzed water at the cathode side; A = the start of the first e-HD session (first week); B = the start of the fourth e-HD session (second week); C = the start of c-HD after 12 e-HD sessions (post-e-HD).

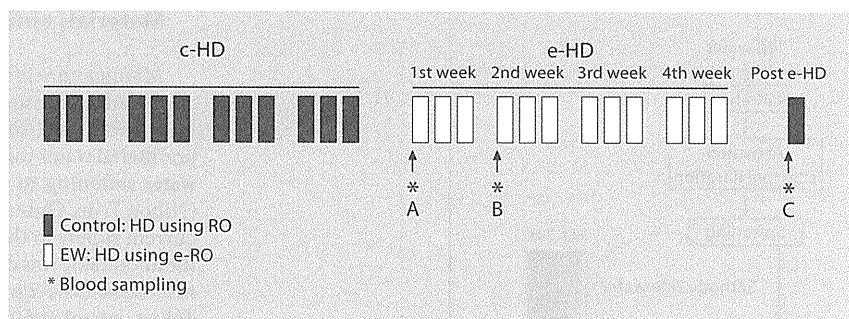


Table 1. Patient demographics

Case	Sex	Age years	HD vintage months	Underlying renal disease	Dialyser	Prescription	
						AHD	statin
1	female	65	58	NS	BG-1.6	ARB	-
2	male	35	111	CGN	APS-2.1	-	-
3	female	64	216	CGN	BG-1.8	-	-
4	male	70	70	DN	BG-1.8u	ARB	-
5	male	75	77	CGN	AMBC-1.5	ARB	+
6	male	67	335	CGN	APS-185	-	-
7	male	72	116	DN	BG-2.1	CCB+ARB	-
8	male	70	6	NS	PS-1.6	ARB	-

AHD = Anti-hypertensive drug; ARB = angiotensin receptor blockade; CCB = calcium channel blocker; CGN = chronic glomerulonephritis; DN = diabetic nephropathy; NS = nephrosclerosis.

in a solution of 80 μM cytochrome C diluted in PBS. Immediately after adding 10 μl of control or PMA (100 μg) to 700 μl of the sample, 250 μl of the sample was placed on the microplate. Absorbance was measured for 15 min at 550 nm, and was expressed as nanomoles of superoxide production per 0.25×10^6 cells/10 min.

In these 2 experiments, PBS was prepared either with control reverse osmosis water (c-RO) or e-RO. Manufactured e-RO was stocked in the closed flask immediately after being made and then used in the study.

Clinical Feasibility of a HD System Using EW Patients and Study Design

Eight patients on regular HD treatment at Kashima Hospital Dialysis Center (Iwaki, Japan) were enrolled in the trial (table 1). They consisted of 6 men and 2 women, with a mean age of 67 years (range 35–75 years) and a mean dialysis duration of 85 months (range 6–33.5 months). Their underlying renal diseases were chronic glomerulonephritis in 4 patients, nephrosclerosis in 2 and diabetic nephropathy in 2. All patients had been on regular HD treatment 3 times a week for 4 h (n = 6) or 5 h (n = 2) each session. All patients had been using high-flux membrane dialyzers. Among them, 4 were receiving an angiotensin receptor blocker, 1 was receiving an angiotensin receptor blocker and a calcium

channel blocker, and 1 was receiving a statin. Patients who were taking ascorbic acid or tocopherol were excluded.

Patients were treated by regular HD regimen using c-HD solution for 1 month, followed by the use of e-HD solution for another month. Both 1-month regimens comprised 3 sessions per week, for a total of 12 sessions (fig. 2). During the study, no changes in HD modes or concomitant medications were made.

Blood sampling was obtained just before each HD session. Blood was immediately centrifuged and serum was stored at -80°C until measurements were made by commercially available kits or high performance liquid chromatography (HPLC). Interleukin-6 was assessed by the CLEIA method (Human IL-6 CLEIA, Fujirebio C, Tokyo, Japan), highly sensitive C reactive protein by nephrometry (N High Sensitive CRP, Dade Behring, Marburg, Germany), creatol and methylguanidine by HPLC, 8-OHdG by an enzyme-linked immunosorbent assay (ELISA) kit (high sensitive 8-OHdG check, Nikken C, Shizuoka, Japan) and pentosidine by an ELISA kit (FSK pentosidine, Fushimi C, Maru-game, Japan).

All patients were monitored regarding subjective symptoms during the study periods. Blood pressure was measured using a sphygmomanometer at the upper arm with the patient in the supine position just before and after each HD session. The mean of the 12 measurements obtained was determined.

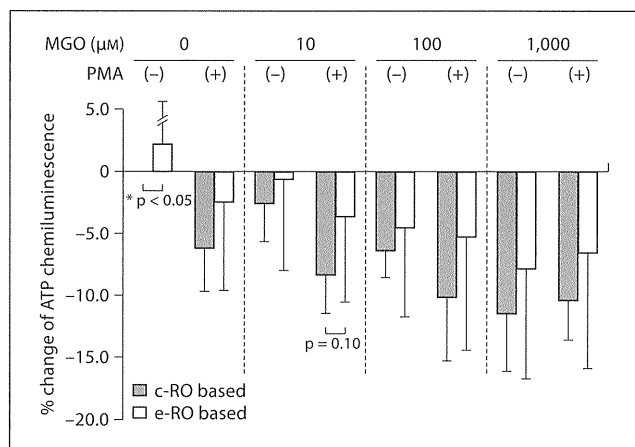


Fig. 3. Effect of e-RO: human neutrophil viability against MGO and/or PMA. Neutrophils from 7 healthy volunteers were suspended in PBS (n = 7). c-RO = treatment by control RO water; e-RO = treatment by RO water by electrolysis. Data are expressed as means \pm SD. * p < 0.05.

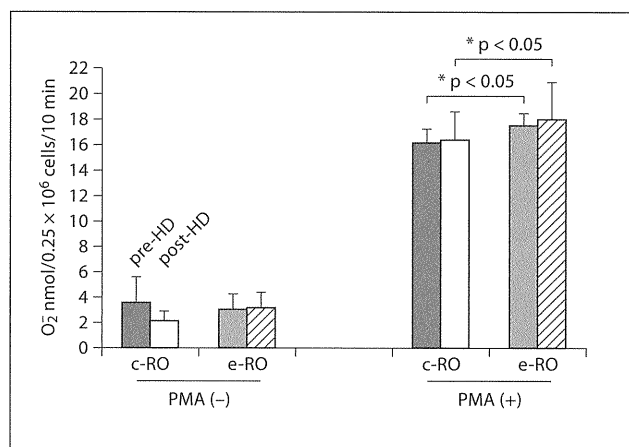


Fig. 4. Effect of e-RO: superoxide anion generation from human neutrophils by PMA. Neutrophils from patients on hemodialysis were suspended in PBS (n = 6). c-RO = treatment by control RO water; e-RO = treatment by RO water by electrolysis. Data are expressed as means \pm SD. * p < 0.05.

The present study was approved by the ethical committees of Kashima Hospital and Tohoku University (No. 2006-101, No. 2007-207), and informed consent was obtained from all patients. All values are expressed as mean \pm SD. Paired t test and 1-way repeated measure analysis of variance were used for statistical analysis. p < 0.05 was considered statistically significant.

Results

Changes in ATP-chemiluminescence of PMNs against MGO in the presence or absence of PMA are shown in figure 3. At basal levels (MGO 0 μ M) in the absence of PMA, ATP-chemiluminescence was significantly higher with e-RO than c-RO (p < 0.05). Significant decreases in ATP-chemiluminescence were associated with MGO levels in both the e-RO and c-RO groups (p < 0.001 for both groups). However, no differences were found between the e-RO and c-RO groups in MGO levels. For PMA load, further significant decreases in ATP-chemiluminescence were associated with MGO levels in the e-RO and c-RO groups (at MGO levels 0–100 μ M, p < 0.01 in both groups); however, no differences were found between c-RO and e-RO.

Figure 4 shows generated superoxide anions in both groups. Under basal levels, no differences were found between groups, whereas in stimulated conditions, levels of generated radicals were significantly higher for e-RO than for c-RO (p < 0.05 in pre-HD and post-HD).

Table 2. Change in laboratory parameters

	Pre	Post	p value
WBC, n/ μ l	6,795 \pm 863	6,644 \pm 945	0.35
Hb, g/dl	11.3 \pm 1.2	11.2 \pm 1.2	0.19
Creatinine, mg/dl	11.0 \pm 3.2	10.6 \pm 3.0	0.07
BUN, mg/dl	64.6 \pm 6.2	60.8 \pm 9.0	0.1

Data are means \pm SD. Blood samples were obtained from the arteriovenous fistula before starting dialysis. Pre = e-HD day 1 (fig. 1); Post = c-HD day 13 (fig. 1); WBC = white blood cells; Hb = hemoglobin; BUN = blood urea nitrogen.

Changes in laboratory parameters during the clinical trial of e-HD are shown in table 2. No changes were observed in white blood cell count, hemoglobin, blood urea nitrogen and creatinine levels. Figure 5 shows changes in inflammatory or oxidative stress markers in the blood. There were significant decreases in plasma methylguanidine levels (p < 0.05; fig. 5c). There were also significant decreases in mean blood pressure during the study in the c-HD and e-HD groups between the pre- and post-HD sessions (p < 0.01 and p < 0.001, respectively; table 3). No adverse symptoms were observed in any patient.

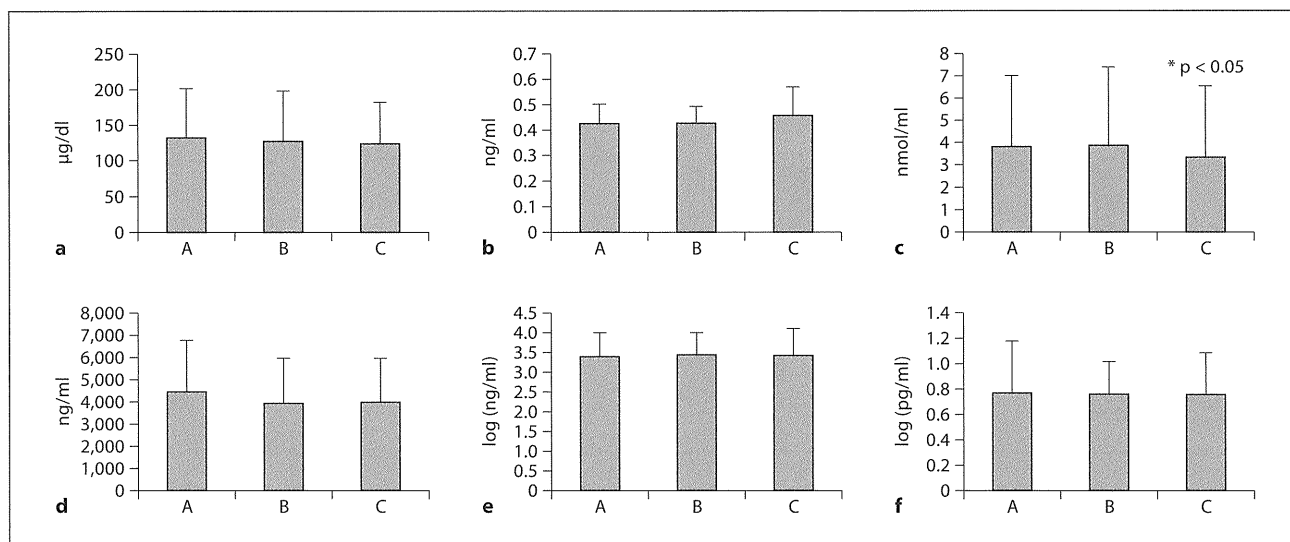


Fig. 5. Changes in laboratory variables after HD using the e-RO based HD solution. **a** Creatol. **b** 8-OHdG. **c** Methylguanidine. **d** Pentosidine. **e** Highly sensitive C reactive protein. **f** Interleukin-6. Data are expressed as means \pm SD. A = the start of the first e-HD session (first week); B = the start of the fourth e-HD session (second week); C = the start of c-HD after 12 e-HD sessions (post-e-HD). * $p < 0.05$.

Discussion

It has been demonstrated that e-RO exhibits antioxidative capacity [13–15]. The present study aimed to investigate the biological effect of e-RO on the viability of PMNs, and the clinical feasibility of applying this technology to HD treatment.

Plasma MGO levels are reportedly elevated in patients on dialysis [19, 20]. MGO is highly reactive and biologically toxic due to the formation of advanced glycation end products, protein and DNA modifications [21–23]. Recently, we demonstrated that MGO plays a role in the enhancement of injury to PMNs in combination with H_2O_2 [24], which may be involved with the pathological mechanism of microinflammation in dialysis patients.

In the c-RO group in the present study (fig. 3), decreased viabilities of PMNs were found to be associated with the MGO level, and the toxicity of MGO was further increased in the presence of PMA. Basal cellular viability was better preserved in the e-RO group than in c-RO group. Furthermore, MGO-related PMN injury in the e-RO group was relatively ameliorated compared with the c-RO group, although differences did not reach statistical significance. In addition, in PMNs of uremic patients (fig. 4), the cellular function to generate superoxide anions by a physiological respiratory burst was significantly higher with e-RO than with c-RO.

Table 3. Blood pressure and heart rate pre- and post-dialysis

	c-HD (n = 12)	e-HD (n = 12)	p value
Pre-dialysis			
SBP, mm Hg	165 \pm 25	157 \pm 21	<0.01
DBP, mm Hg	85 \pm 12	81 \pm 10	<0.05
MBP, mm Hg	112 \pm 15	106 \pm 12	<0.01
HR, beats/min	76 \pm 12	78 \pm 14	0.71
BW gain, %	3.8 \pm 0.85	4.0 \pm 0.93	0.9
Post-dialysis			
SBP, mm Hg	124 \pm 19	117 \pm 15	<0.01
DBP, mm Hg	69 \pm 11	64 \pm 9	<0.001
MBP, mm Hg	87 \pm 13	81 \pm 11	<0.001
HR, beats/min	80 \pm 12	81 \pm 14	0.63
Volume removed, kg	2.2 \pm 0.8	2.4 \pm 0.8	0.97

Data are the mean \pm SD levels of 12 HD sessions in each patient. BW gain = Inter-dialytic body weight gain over dry weight; c-HD = hemodialysis using control reverse osmosis water; DBP = diastolic blood pressure; e-HD = hemodialysis using reverse osmosis water by electrolyzed water; HR = heart rate; MBP = mean blood pressure; pre-dialysis = just before the hemodialysis session; post-dialysis = just after the hemodialysis session; SBP = systolic blood pressure; volume removed = intra-dialytic volume removal per HD session.

These findings indicate that the application of e-RO does not stimulate PMNs, but rather benefits PMNs in preserving cellular viability as compared with c-RO. However, suppression of MGO toxicity by e-RO application was not clearly demonstrated in this study (fig. 3). In terms of the lack of e-RO effect on MGO cytotoxicity in this study (fig. 3), we think that the change in chemical properties of e-RO, such as levels of dissolved hydrogen, may contribute to our findings, as indicated by recent studies that suggested a significant role for dissolved hydrogen in ameliorating oxidative stress [25, 26]. Further studies are needed in this area to address the context of the preservation of e-RO chemical properties.

Next, we examined the feasibility of the application of this technology to regular HD treatment. No symptomatic adverse events were observed during the study period, although there were nonsymptomatic reductions in mean blood pressure before and after the HD session. No changes were found in laboratory parameters or in interdialytic changes of body weight, which may indicate the feasibility of an e-HD system.

In previous reports, Huang et al. [13, 14] showed decreases in surrogate markers of inflammation and oxidative stress following the introduction of an e-HD system. In the present short trial, plasma methylguanidine was significantly decreased, but no other parameters, including C-reactive protein, interleukin-6, creatol and 8-OHdG, were changed (fig. 5). We propose the following

reasons for these contradictory results. First, methylguanidine is the nonspecific oxidative end product that could reflect the overall oxidative milieu, and thus could be changed significantly. Second, we only enrolled clinically stable patients, and thus those who presented with inflammation or malnutrition were not included in the study. This clinical background might influence the results of our study.

Interestingly, MBP was decreased by the introduction of e-HD. Taken together, the findings of decreased MBP and no changes in heart rate or inter-dialytic body weight gain lead us to speculate that e-HD may lead to vasodilation. Vasoconstrictive action by radical oxygen species such as peroxynitrite could play a role in increased blood pressure in HD patients, and dissolved hydrogen in EW may reduce superoxide anions, as previously reported [8], during the course of e-HD to suppress its pathological process. Further studies are needed in this area.

There are some unresolved issues in the present HD system. Data on the microbiological quality of the dialysis solution were not available, and water volume consumed per session was not clearly measured. These issues need to be clarified in follow-up studies.

In conclusion, the present study demonstrated the capacity of e-RO to preserve the viability of PMNs, and the clinical feasibility of using this water for HD treatment. The clinical application of this technology may improve bio-compatibility of HD treatment.

References

- Himmelfarb J, Stenvinkel P, Ikizler TA, Hakim RM: The elephant in uremia: oxidant stress as a unifying concept of cardiovascular disease in uremia. *Kidney Int* 2002;62:1524–1538.
- Becker BN, Himmelfarb J, Henrich WL, Hakim RM: Reassessing the cardiac risk profile in chronic hemodialysis patients: a hypothesis on the role of oxidant stress and other non-traditional cardiac risk factors. *J Am Soc Nephrol* 1997;8:475–486.
- Morena M, Delbosc S, Dupuy AM, Canaud B, Cristol JP: Overproduction of reactive oxygen species in end-stage renal disease patients: a potential component of hemodialysis-associated inflammation. *Hemodial Int* 2005;9:37–46.
- Wratten ML, Tetta C, Ursini F, Sevanian A: Oxidant stress in hemodialysis: prevention and treatment strategies. *Kidney Int Suppl* 2000;76:S126–S132.
- Himmelfarb J, Lazarus JM, Hakim R: Reactive oxygen species production by monocytes and polymorphonuclear leukocytes during dialysis. *Am J Kidney Dis* 1991;17:271–276.
- Nakayama K, Terawaki H, Nakayama M, Iwabuchi M, Sato T, Ito S: Reduction of serum antioxidative capacity during hemodialysis. *Clin Exp Nephrol* 2007;11:218–224.
- Morena M, Cristol JP, Bosc JY, Tetta C, Forret G, Leqer CL, et al: Convective and diffusive losses of vitamin C during haemodiafiltration session: a contributive factor to oxidative stress in haemodialysis patients. *Nephrol Dial Transplant* 2002;17:422–427.
- Shirahata S, Kabayama S, Nakano M, Miura T, Kusumoto K, Gotoh M, Hayashi H, Otsubo K, Morisawa S, Katakura Y: Electrolyzed-reduced water scavenges active oxygen species and protects DNA from oxidative damage. *Biochem Biophys Res Commun* 1997;234:269–274.
- Hiraoka A, Takemoto M, Suzuki T, Shinohara A, Chiba M, Shirao M, Yoshimura Y: Studies on the properties and real existence of aqueous solution systems that are assumed to have antioxidant activities by the action of 'active hydrogen'. *J Health Sci* 2004;50:456–465.
- Kim MJ, Kim HK: Anti-diabetic effects of electrolyzed reduced water in streptozotocin-induced and genetic diabetic mice. *Life Sci* 2006;79:2288–2292.
- Kim MJ, Jung KH, Uhm YK, Leem KH, Kim HK: Preservative effect of electrolyzed reduced water on pancreatic beta-cell mass in diabetic db/db mice. *Biol Pharm Bull* 2007;30:234–236.
- Ohsawa I, Ishikawa M, Takahashi K, Watanabe M, Nishimaki K, Yamagata K, Katsura K, Katayama Y, Asoh S, Ohta S: Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals. *Nat Med* 2007;13:688–694.