# Oxidative stress and antioxidative potency are closely associated with diabetic retinopathy and nephropathy in patients with type 2 diabetes

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## ABSTRACT

**الأهداف:** دراسة علاقة الإجهاد التأكسدي في أمراض السكري والمضاعفات الوعائية.

**الطريقة:** أجريت دراسة مقطعية في قسم المختبرات، جامعة دوكيو الطبية، كوشجيا، اليابان وذلك خلال الفترة من 2010م حتى 2011م. تم قياس كلاً من تركيز الدم على الريق، والهيموجلوبين في السكري، والمصل الشحمي، وفقدان الالبومين البولي، وقاع العين، ومنعكس العرقوب، ومسبب عصد الكاحل، وسرعة موجة النبض لدى 51 شخص مصاب بمرض السكري من الدرجة الثانية و20 شخص سليم (مجموعة الشاهد)، كما تم فحص قاع العين ومنعكس العرقوب لدى المرضى. تم قياس الاجهاد التأكسدي باختبار ايضات المؤشرات الحيوية وفعالية اختبار مضادات الأكسده الحيوية في الجهاز التحليلي للجذر كما تم قياس نشاط انزيم سوبر أكسيد ديسميوتاز باستخدام رنين تدويم الالكترون.

**النتائج:** أظهرت النتائج أن المرضى الذين يعانون من مرض السكري لديهم ارتفاع ايضات المؤشرات الحيوية مقارنة بالأشخاص السليمين والذي يؤدي إلى ارتفاع ملحوظ في تطور اعتلال الشبكية. كان هنالك انخفاظ ملحوظ في فعالية اختبار مضادات الأكسده الحيوية لدى المرضى المدخنين، وكان هنالك علاقة عكسية مهمة إحصائياً مع مقدار الألبومين البولي($p$=0.029)، كما انخفض نشاط مصل انزيم سوبر أكسيد ديسميوتاز بشكل أحصائي مع أمراض اعتلال الشبكية لدى مرض السكري ($p$=0.017).

**خاتمة:** أظهرت مقاييس الجهاز التحليلي للجذر أن ارتفاع الإجهاد التأكسدي وانخفاض فعالية مضادات الأكسدة مرتبطة بجلوكوز الدم التالف، والتدخين، وتطور اعتلال أمراض الشبكية، وأمراض الكلى في المرضى المصابين بداء السكري.

**Objectives:** To examine involvement of oxidative stress in the pathogenesis and vascular complications of diabetes.

**Methods:** This cross sectional study was conducted at the Joint Laboratory Office (JLO), Dokkyo Medical University Koshigaya Hospital, Koshigaya, Japan from April 2010 to December 2011. Fasting glucose, glycosylated hemoglobin (HbA1c), serum lipids, urinary albumin excretion (UAE), ankle brachial index and pulse wave velocity were measured in 51 patients with type 2 diabetes and 20 healthy controls. The fundus oculi and Achilles' tendon reflex were also examined in the patients. Oxidative stress was measured by a reactive oxygen metabolites (ROM) test and antioxidant potency was evaluated by a biological antioxidant potential (BAP) test in the Free Radical Analytical System (FRAS)-4. Superoxide dismutase (SOD) activity was assayed using electron spin resonance (ESR).

**Results:** Diabetic patients tended to have increased ROM compared with healthy subjects, and ROM showed a marked increase with progression of diabetic retinopathy. A significant reduction of BAP was found in patients who were smokers, and BAP was significantly negatively correlated with UAE ($p$=0.029). Serum SOD activity significantly decreased with progression of diabetic retinopathy ($p$=0.017).

**Conclusion:** The FRAS-4 measurements showed that increased oxidative stress and decreased antioxidative potency are linked to deteriorated blood glucose control, heavy smoking, and progression of retinopathy and nephropathy in patients with type 2 diabetes.

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Active oxygen and free radicals play important roles in immunological function and adenosine 5'-triphosphate production in intracellular mitochondria, and are stimulators of metabolic syndrome,[1] aging, inflammation, cancer and atherosclerosis.[2] Oxidative stress is accelerated in diabetes through activation of the polyol pathway, or through protein kinase C-dependent activation of reduced nicotinamide adenosine dinucleotide (phosphate) oxidase in hyperglycemia.[3] Reduced superoxide due to direct superoxide scavenging potentiated by nitrogenic or vasculo-myogenic relaxation may also cause oxidative stress.[4] Simple and accurate measurement of the oxidative state of a biological system could be of fundamental importance for clinical diagnosis, but there are few methods available for this purpose. Several clinical markers are known to reflect oxidative stress, including urinary 8-hydroxy-2'-deoxyguanosine, serum the advanced glycation end-product, creatol, malondialdehyde low-dense lipoprotein (MDA-LDL), carbonyl-modified protein, serum or urinary 8-isoprostane,[5] and proteolytic products of proteins.[6] Markers for antioxidant status include serum superoxide dismutase (SOD) activity, copper-, zinc- and manganese-SOD concentration, autoantibody to MDA-LDL, paraoxonase activity, PAF-acetylhydrolase, glutathione, and catechins. However, the sensitivities and specificities of these most markers have shown some limitations in terms of reliability. The d-reactive oxygen metabolites (ROM) test was developed as a simple, inexpensive and practical method to identify patients with a high level of oxidative stress and to follow the effect of treatment.[7] The Free Radical Analytical System (version 4: FRAS-4) expanded on this approach to permit more accurate measurement of oxidative stress and antioxidant potency.[8] The FRAS-4 measurements have been used in rheumatoid arthritis, obstructive sleep apnea,[9] renal failure,[10] and hypertension,[11] with findings that show that the system is useful for evaluating oxidative stress. The aim of the current study was to examine the involvement of oxidative stress and antioxidant potency in the pathogenesis of type 2 diabetes and associated vascular complications using the FRAS-4 method.

**Disclosure**. The authors have no conflict of interests, and the work was not supported or funded by any drug company.

**Methods.** This cross sectional study was conducted at the Joint Laboratory Office (JLO), Dokkyo Medical University Koshigaya Hospital, Koshigaya, Japan from April 2010 to December 2011. This study was approved by our institutional ethical review board and informed consent was provided by each patient. The subjects were 51 patients with type 2 diabetes (27 males, 24 females; mean age, 65.1 ± 9.8 years old) and 20 healthy subjects (10 males, 10 females, mean age; 52.3 ± 12.3 years old). We excluded patients with type 1 diabetes because of differences in the pathogenesis compared with type 2 diabetes. The patients under treatment with diet therapy alone (N=4), oral hypoglycemic therapy (N=26), and insulin therapy (N=21). Regarding smoking status, the patients were classified as non-smokers (N=35), mild smokers (N=3), and heavy smokers (N=13). Diabetic retinopathy was classified as no retinopathy (NDR, N=29), simple retinopathy (SDR, N=11), and proliferative retinopathy (PDR, N=11). Body weight, waist size, blood pressure, fasting plasma glucose, glycosylated hemoglobin (HbA1c; Japan Diabetes Society [JDS] value), serum and urinary C-peptide, and serum lipids including total cholesterol, triglyceride, high-density lipoprotein (HDL) cholesterol, and LDL cholesterol were measured in all subjects. Diabetic complications were evaluated by examination of the fundus oculi, urinary albumin excretion (UAE) in spot urination, Achilles' tendon reflex, and ankle brachial index (ABI). A ROM test for oxidative stress was performed using FRAS-4 (Wismerll, Tokyo, Japan).[7] The ROM values were expressed as Carratelli units (U-Carr). The method is to measure serum hydroperoxide concentration using some reagents, as a volume oxidative metabolite, utilizing photometry. We assessed the measurement values as a ROM volume from sera. A blood biological antioxidant potential (BAP) test for antioxidant potency was also performed using FRAS-4.[7] Ten µl of serum samples was dissolved in colored solution, previously prepared by mixing $FeCl_3$ with a thiocyanate derivative. After 5 minutes incubation at $37^0$C, such a solution loses color and the intensity of this chromatic change is directly proportional to the ability of serum to reduce, during the incubation, ferric ions to ferrous ions. Photometric reading was employed to assess the intensity of decoloration. A lyophilized human control serum with known antioxidant activity (µmol/l) was used to periodically calibrate the FRAS-4 system. Superoxide dismutase activity in blood was measured by electron spin resonance (ESR). Fifty µl of 2 mM hypoxanthine, 35 µl of 5.5 mM diethyl-enteramine-pentaacetic acid, and 50 µl of xanthine

oxidase (0.272 unit/ml) were mixed in a test tube. The solution was then placed in a flat cell for spectrometer and 5.5-dimethyl-l-pyrrorine-l-oxide-$O_2$-, the spin adduct was measured by ESR spectrometry as follows: serum were added together with phosphate buffered saline and measured by ESR. The SOD activity could reflect scavenging power of superoxide by added serum antioxidants.

Data are presented as the mean ± standard deviation. Statistical analyses were performed using the Statistical Package for Social Sciences (Japan IBM, Tokyo, Japan). The significance of correlations between the 2 variables was determined by simple regression analysis. Parametric comparisons were made by analysis of variance (ANOVA). Comparisons among multiple groups were evaluated by Bonferroni test if the ANOVA was significant. A $p<0.05$ was considered statistically significant.

**Results.** The clinical characteristics of the patients and the healthy control subjects are summarized in **Table 1**. The diabetic patients were significantly older than the healthy controls ($p<0.001$) (**Table 1**). The mean body mass index (BMI) was 24.7 ± 4.3 kg/m$^2$ and the duration of diabetes was 12.8 ± 9.2 years (**Table 1**). The mean levels of fasting plasma glucose was 177.8 ± 79.0 mg/dl, and HbA1c (JDS value) was 9.2 ± 1.8% mg/dl. Smoking status and BMI did not differ significantly between the 2 groups. Diabetic patients showed a tendency for an increase in ROM compared with healthy subjects (**Table 1**), but there was no significant difference in the BAP or SOD activity between the 2 groups. The relationships of ROM, BAP, and SOD measurements with clinical markers were investigated in the patients (**Table 2**). The ROM had a significantly positive correlation with female gender (r=0.355, $p=0.011$) and HbA1c values (r=0.281, $p=0.045$), and a marked increase in ROM was observed with progression of diabetic retinopathy. The BAP was significantly augmented with a shift of therapy from diet and oral hypoglycemic agents to insulin (r=0.286, $p=0.042$). The BAP was significantly lower in smokers and showed a tendency to be lower with progression of diabetic retinopathy. The BAP also had a significant negative correlation with log UAE (r=-0.333, $p=0.029$). Serum SOD activity in diabetic patients showed a tendency to be lower in smokers (r=-0.272, $p=0.056$) and significantly decreased with progression of diabetic retinopathy (r=-0.337, $p=0.017$). The differences in BAP and SOD measurements based on smoking status in diabetic patients are summarized in **Figure 1**. The BAP in heavy smokers was significantly lower than in non-smokers ($p=0.005$) and mild smokers ($p=0.023$). The SOD activity also exhibited a marked reduction in heavy smokers compared to patients in the other smoking categories. The BAP and SOD activity based on the degree of diabetic retinopathy are shown in **Figure 2**. Patients with PDR showed a tendency for a decrease in BAP, and a significant reduction in SOD activity ($p=0.021$) compared to patients with SDR. Correlations between oxidative stress markers and clinical markers in the diabetic patients are shown in **Figure 3**. There was a significant positive correlation between ROM and serum HbA1c (JDS values) (r=0.281, $p=0.045$), and a significant negative correlation between BAP and log UAE (r=-0.333, $p=0.029$).

**Table 1** - Clinical characteristics of the patients and healthy controls included in a study conducted at the Joint Laboratory Office (JLO), Dokkyo Medical University Koshigaya Hospital, Koshigaya, Japan.

| Variables | Control (N=20) | Diabetes mellitus patients (N=51) | *P*-value |
|---|---|---|---|
| ***Gender*** | | | |
| Male | 10 | 27 | |
| Female | 10 | 24 | |
| Age, years | 52.3 ± 12.3 | 65.1 ± 9.8 | <0.0001 |
| DM duration, years | - | 12.8 ± 9.2 | |
| ***Therapy*** | - | | |
| Diet | | 4 | |
| OHA | | 26 | |
| Insulin | | 21 | |
| ***Smoking*** | | | |
| None | 15 | 35 | |
| Mild | 0 | 3 | |
| Heavy | 13 | 13 | |
| FPG, mg/dl | - | 177.8 ± 79.0 | |
| HbA1c, % | - | 9.2 ± 1.8 | |
| Body mass index | 24.3 ± 3.5 | 24.7 ± 4.3 | 0.7465 |
| ROM (U Carr) | 237.1 ± 39.4 | 260.3 ± 54.8 | 0.0894 |
| BAP (μEq/L) | 2504.8 ± 191.2 | 2560.4 ± 425.8 | 0.4508 |
| SOD (U/ml) | 12.6 ± 6.0 | 12.3 ± 4.1 | 0.8272 |
| ***Retinopathy*** | - | | |
| NDR | | 29 | |
| SDR | | 11 | |
| PDR | | 11 | |

DM - diabetes mellitus, OHA - oral hypoglycemic agent, FPG - fasting plasma glucose, HbA1c - glycosylated hemoglobin, ROM - reactive oxygen metabolites, U Carr - Carratelli units, BAP - biological antioxidant potential, SOD - superoxide dismutase, NDR - no retinopathy, SDR - simple retinopathy, PDR - proliferative retinopathy

**Table 2 -** Relationships of reactive oxygen metabolites (ROM), biological antioxidant potential (BAP), and superoxide dismutase (SOD) measurements with clinical markers in patients with type 2 diabetes.

| Variables | ROM (Carr) | | BAP (μEq/l) | | SOD (U/ml) | |
|---|---|---|---|---|---|---|
| | Correlation coefficient, *P*-value | | | | | |
| Gender (Male/Female) | 0.3551 | 0.0106 | -0.1198 | 0.4025 | 0.1899 | 0.1865 |
| Age (years) | -0.1282 | 0.3701 | 0.0921 | 0.5205 | -0.0414 | 0.7754 |
| Diabetes mellitus duration (years) | -0.2261 | 0.1105 | -0.0031 | 0.9828 | -0.0038 | 0.9789 |
| Therapy (Diet/OHA/Insulin) | -0.0162 | 0.9100 | 0.2864 | 0.0416 | -0.0212 | 0.8836 |
| Smoking (No/mild/heavy) | 0.2076 | 0.1438 | -0.3591 | 0.0097 | -0.2715 | 0.0564 |
| Body mass index | -0.1284 | 0.3694 | 0.1666 | 0.2427 | 0.0219 | 0.8800 |
| Waist girth | -0.0382 | 0.8277 | 0.2287 | 0.1864 | 0.1057 | 0.5520 |
| Systolic blood pressure (mm Hg) | 0.1141 | 0.4254 | -0.0899 | 0.5305 | -0.1438 | 0.3191 |
| Diastolic blood pressure (mmHg) | 0.0710 | 0.6205 | -0.1174 | 0.4119 | -0.1457 | 0.3126 |
| Fasting plasma glucose (mg/dl) | 0.0907 | 0.5268 | 0.0232 | 0.8716 | -0.1963 | 0.1719 |
| Glycosylated hemoglobin (%) | 0.2814 | 0.0454 | 0.0377 | 0.7926 | 0.1555 | 0.2807 |
| Insulin resistance index (μU/ml) | 0.0438 | 0.8088 | 0.3330 | 0.0579 | -0.0428 | 0.8131 |
| S-CPR (ng/ml) | 0.2591 | 0.0934 | 0.1846 | 0.2358 | -0.0977 | 0.5328 |
| U-CPR (μg/day) | 0.0054 | 0.9707 | 0.2124 | 0.1473 | 0.1629 | 0.2737 |
| Homeostasis model assessment for insulin resistance | 0.2886 | 0.1034 | 0.4171 | 0.0157 | -0.1293 | 0.4734 |
| Total cholesterol (mg/dl) | -0.0494 | 0.7619 | -0.2620 | 0.1024 | 0.2943 | 0.0689 |
| Triglycerides (mg/dl) | 0.0306 | 0.9328 | -0.1553 | 0.2817 | -0.0791 | 0.5889 |
| High-density lipoprotein cholesterol (mg/dl) | -0.1217 | 0.3995 | -0.1217 | 0.3998 | 0.2143 | 0.1393 |
| Low density lipoprotein cholesterol (mg/dl) | 0.095 | 0.4136 | -0.0836 | 0.5639 | 0.1718 | 0.2378 |
| Retinopathy (NDR/SDR/PDR) | 0.1949 | 0.1714 | -0.2430 | 0.0810 | -0.3373 | 0.0166 |
| Log 10 urinary albumin excretion (mg/g Cr) | -0.1879 | 0.2795 | -0.3329 | 0.0291 | 0.0619 | 0.6968 |
| ATR (+/±/-) | 0.0192 | 0.8933 | -0.0483 | 0.7359 | 0.0564 | 0.6970 |
| R-ABI | -0.1202 | 0.5123 | 0.0819 | 0.6559 | -0.0417 | 0.8208 |
| R-PWV (m/s) | -0.2236 | 0.9308 | -0.1797 | 0.3250 | -0.1302 | 0.4775 |
| Biological antioxidant potential (μEq/l) | -0.0555 | 0.6990 | ND | | ND | |
| Superoxide dismutase (U/ml) | 0.1419 | 0.3253 | 0.2235 | 0.1186 | ND | |

Statistical analysis was performed using linear regression analysis. OHA - oral hypoglycemic agent, S-CPR - serum C-peptide immunoreactivity, U-CPR - urine C-peptide immunoreactivity, NDR - no retinopathy, SDR - simple retinopathy, PDR - proliferative retinopathy, ATR - Achilles tendon reflex, + - positive, ± - weak positive, - - negative, R-ABI - right ankle-brachial index, R-PWV - right pulse wave velocity, ND - not determined

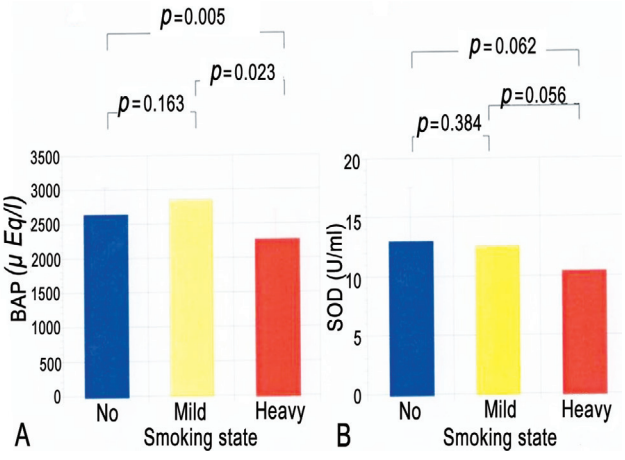


**Figure 1 -** Differences in (**A**) biological antioxidant potential (BAP) and (**B**) superoxide dismutase (SOD) activity in patients with type 2 diabetes classified by cigarette smoking status.

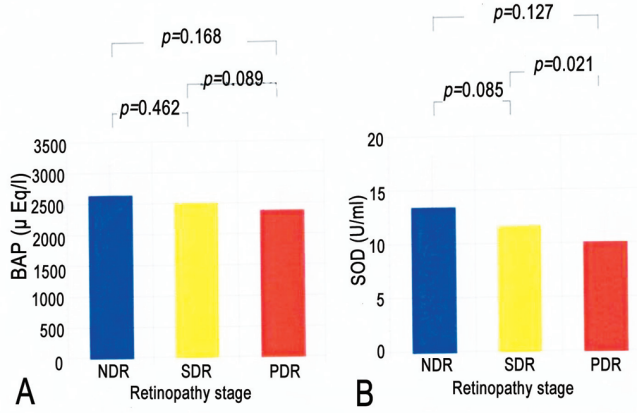


**Figure 2 -** Differences in (**A**) biological antioxidant potential (BAP) and (**B**) superoxide dismutase (SOD) activity in patients with type 2 diabetes classified by stage of diabetic retinopathy. NDR, no diabetic retinopathy, SDR - simple diabetic retinopathy, PDR - proliferative diabetic retinopathy

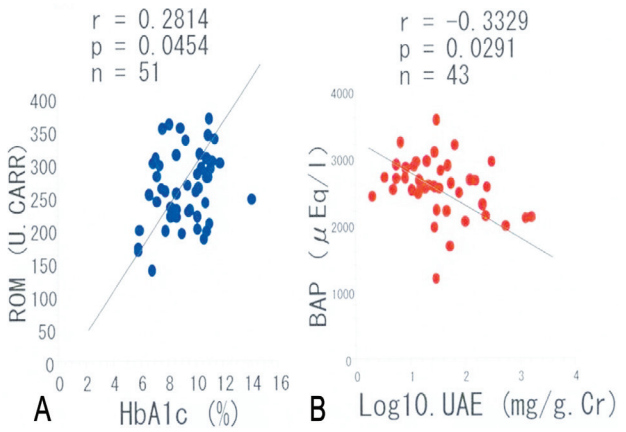


**Figure 3 -** Correlations of (**A**) reactive oxygen metabolites (ROM) with serum glycosylated hemoglobin (HbA1c) levels (Japan Diabetes Society value), and (**B**) biological antioxidant potential (BAP) with logarithmic values of urinary albumin excretion (UAE) in patients with type 2 diabetes.

**Discussion.** The purpose of this study was to examine the involvement of oxidative stress and antioxidant potency in the pathogenesis of type 2 diabetes and associated complications using FRAS-4 measurements. We found that deterioration of blood glucose control, heavy smoking, and development or progression of diabetic retinopathy and nephropathy was all associated with increased oxidative stress and a reduced antioxidant status. Such changes may cause atherosclerosis, aging, and cancer.

The ROM level was used as an indicator of oxidative stress. This marker has also been used in obstructive sleep apnea,[9] hemodialysis,[10] and essential hypertension.[11] Our results showed that ROM was significantly positively correlated with HbA1c. This is of interest, since chronic hyperglycemia is involved in development and progression of diabetic micro- and macro angiopathy. Among metabolic derangements, the advanced glycation end-product hypothesis is the most compatible with the theory of "hyperglycemic memory".[12] This pathogenesis induces oxidative stress that leads to endothelial damage, which is considered to be the initial change in the atherosclerotic process[13] and diabetic microangiopathy. Taken together, these findings suggest that oxidative stress induced by diabetes can result in diabetic neuropathy, retinopathy and nephropathy. The correlation of ROM with HbA1c, but not with FPG, suggests that oxidative stress is involved in chronic or postprandial hyperglycemia. Postprandial hyperglycemia is linked to cardiovascular disease through oxidative stress,[14] and repetitive postprandial fluctuation in the glucose concentration (rather than stable hyperglycemia) evokes monocyte adhesion to endothelial cells.[15] However, we failed to find a relationship of ROM with ABI or PWV, which may reflect limitations in the sensitivity and specificity of the methods. We did not examine the postprandial plasma glucose level, but it is possible that this may be correlated with ROM. Our results showed that BAP and SOD activity in diabetic patients who were heavy smokers were markedly reduced compared to other patients. The relationship between cigarette smoking and oxidative stress has been examined in several studies. For example, Fearon et al[16] reported that exogenous factors such as smoking contribute to oxidative stress in cardiovascular disease. Lin et al[17] showed that cigarette smoke is a mixture of chemicals that cause direct or indirect oxidative stress in different cell lines, including human acute monocytic leukemia cells. Smoking also increases the adverse effects of obesity on cardiovascular health.[18] These results and our findings indicate that excessive oxidative stress caused by smoking induces diminished antioxidant potency in diabetic patients. Hyperglycemia produces oxidative stress and leads to acute endothelial dysfunction in blood vessels.[19] We found a significant negative correlations of diabetic retinopathy and nephropathy with antioxidant potency markers, such as BAP and SOD. These data provide evidence that diminished antioxidant potency may induce acute endothelial dysfunction in blood vessels, in addition to the effect of excess oxidative stress. Sakane et al[20] demonstrated that oxidative stress promoted atherosclerosis in the retinal arteries in the Japanese population, which supports our data showing involvement of oxidative stress in diabetic retinopathy. Oxidative stress has also been suggested to play a role in diabetes-related neuronal damage.[21] However, we failed to find a significant relationship between oxidative stress markers and diabetic neuropathy, as measured by the Achilles' tendon reflex. This discrepancy may be due to limitations in the number of patients or the sensitivity of the reflex.

Mitiglinide[22] and gliclazide[23] among oral hypoglycemic agents have been reported as pharmacological drugs to improve oxidative stress. In the current study, only 3 of the 26 patients who received oral hypoglycemic agents were treated with gliclazide, and the drug was withdrawn on the day of the study. Therefore, a pharmacological effect on oxidative stress is unlikely. Angiotensin-converting enzyme inhibitors and angiotensin II type 1 receptor blockers enhance superoxide scavenging by polymorphonuclear leukocytes from diabetic patients.[24] Therefore, these

antihypertensive drugs were withdrawn one day before the study to eliminate this pharmacological effect on oxidative stress. We have also shown in vitro that amelioration of high blood glucose levels in diabetic patients played an important role in antioxidant efficacy of a thiazolidine derivative.[25] Based on this finding, we plan to investigate, whether thiazolidine derivatives, which are anti-diabetic drugs can ameliorate oxidative stress in vivo in diabetic patients. We also note that Nakamura et al[26] found that urinary protein excretion and 8-hydroxy-2'-deoxyguanosine, an oxidative stress marker, were significantly reduced in patients with early-stage chronic kidney disease treated with benidipine, compared to those treated with amlodipine. This is consistent with our finding of a significant negative correlation of BAP with log UAE.

The present study shows an important limitation. We initially designed to get an age-matching value between the diabetic patients and the healthy controls in this study, however unfortunately the result was that the diabetic patients were significantly older than the healthy controls, because of including some relatively younger healthy controls working in our hospital. Therefore, we need to keep in mind that we always should consider as a bias-marked differences in aging, when we compare clinical markers between diabetic patients and healthy controls in the present study.

In conclusion, FRAS-4 measurements showed that an increase in oxidative stress and a decrease in antioxidative potency are strongly associated with deteriorated blood glucose control and heavy smoking in patients with type 2 diabetes, and are linked to progression of diabetic retinopathy and nephropathy.

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