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FORT and FORD: two simple and rapid assays in the evaluation of oxidative stress in patients with type 2 diabetes mellitus

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Abstract

The aim of the study was to evaluate the levels of free oxygen radicals and free oxygen radicals defense in patients with newly diagnosed type 2 diabetes mellitus (T2DM). The disease seems to be involved strongly in the production of reactive oxygen species. Forty-five patients with newly diagnosed T2DM and 20 apparently healthy individuals (control group) were included in the study. Reactive oxygen species were determined using the free oxygen radicals (FORT) test, which is based on the Fenton reaction. In this method, the hydroperoxides reacted with the transition metal ions liberated from the proteins and were converted to alkoxy and peroxy radicals. The radical species produced by the reaction, which are directly proportional to the quantity of lipid peroxides, interact with an additive that forms a radical molecule. Similarly, the free oxygen radicals defense (FORD) test uses preformed stable and colored radicals and determines the decrease in absorbance that is proportional to the blood antioxidant concentration. We found that (*a*) FORT levels were increased in diabetic patients (2.86 ± 0.56 mmol/L H_2O_2) compared with controls (1.87 ± 0.26 mmol/L H_2O_2) ($P < .0001$) and (*b*) FORD levels were lower in diabetic patients (1.23 ± 0.18 mmol/L Trolox) compared with controls (1.34 ± 0.14 mmol/L Trolox) ($P < .01$). The intraassay and interassay coefficients of variation were 3.7% and 6.2%, respectively, for FORT and 4.2% and 6.6%, respectively, for FORD. Determination of free oxygen radicals and free oxygen radicals defense seems to play an important role in the generation and evaluation of oxidative stress, an imbalance between oxidants and antioxidants that can lead to oxidative damage and is involved in the pathogenesis of several diseases, such as T2DM.

1. Introduction

Oxidative stress generally describes a condition in which cellular antioxidant defenses (superoxide dismutase, catalase, glutathione peroxidase, albumin, ceruloplasmin, vitamin C, vitamin E, β-carotene, reduced glutathione, and uric acid) are inadequate to completely detoxify free radicals that have been generated because of excessive production of reactive oxygen species (ROS), loss of antioxidant defenses, or, typically, both. Various pathologic processes such as atherosclerosis, cardiovascular diseases, neurologic diseases, cancer, diabetes, and obesity as well as the inflammatory processes of aging disrupt this balance between formation and neutralization of prooxidants by increasing the formation of free radicals in proportion to the available antioxidants, thus resulting in a state of oxidative stress [1-4].

Type 2 diabetes mellitus (T2DM) is a metabolic disorder characterized by hyperglycemia and insufficiency of secretion and/or action of endogenous insulin [5]. Increased oxidative stress is a widely accepted participant in the development and progression of diabetes and its complications [6-8]. Diabetes is usually accompanied by increased production of free radicals [7-10] or impaired antioxidant defenses [11-13]. High blood glucose level determines overproduction of ROS by the mitochondria electron transport chain. High reactivity of ROS determines chemical changes in virtually all the cellular components, leading to DNA and protein modification and lipid peroxidation [2]. Mechanisms by which increased oxidative stress is involved in the diabetic complications are partly known, including activation of transcription factors, advanced glycated end

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products, and protein kinase C [5]. Microvascular complications involve progressive degenerative process at the level of retina, renal glomerulus, and peripheral nerve. Macrovascular diseases involve accelerated arteriosclerosis of arteries that supply the heart, brain, and lower extremities [14].

The aim of this study was to evaluate the levels of free oxygen radicals and free oxygen radicals defense in newly diagnosed patients with T2DM because the disease is involved strongly in the increased production of ROS.

2. Materials and methods

2.1. Subjects

Patients with T2DM were recruited consecutively from the "Laiko" General Hospital in Athens. Forty-five patients with newly diagnosed T2DM (age, 55 ± 12 years; 23 men and 45 women) and 20 apparently healthy individuals (control group), matched for sex, age, and body mass index (BMI) with the patients with T2DM, were included in the study. Body weight and height were measured in light clothing without shoes, and BMI was calculated. Waist circumference was measured with a soft tape with the subject standing, midway between the lowest rib and the iliac crest. Patients were included provided that they did not have any current or past diagnosed micro- or macrovascular complication. Patients and controls were excluded if they had any chronic disease, such as atherosclerosis, coronary heart disease, obesity, neurologic disease, psychiatric disease, and autoimmune/inflammatory disease, or if they were current smokers, pregnant, or lactating. Patients were also excluded if they were currently using lipid-lowering drugs, angiotensin-converting enzyme inhibitors, angiotensin II receptor blockers, antiplatelets, anti-inflammatory agents, as well as antioxidants such as vitamin supplements or fish oil capsules. Blood samples were collected from the subjects in the morning, after an overnight fasting and before any medication was taken, at Laiko Hospital. Furthermore, patients were asked to refrain from exercise the day of the blood drawing and the day before. The FORT and FORD tests took place in "Aghia Sophia" Children's Hospital in Athens, and fresh whole blood was collected from the patients for a second time. The study was performed in accordance with the Helsinki Declaration of 1964 (as amended in 1983 and 1989) and was approved by the Institutional Ethics Committee of both hospitals.

2.2. The FORT assay

Reactive oxygen species were determined using the FORT test (Callegari, Parma, Italy), a colorimetric assay based on the ability of transition metals, such as iron, to catalyze the breakdown of hydroperoxides (ROOH) into derivative radicals, according to the Fenton reaction. When 20 μL of blood sample was dissolved in an acidic buffer, the hydroperoxides reacted with the transition metal ions liberated from the proteins in the acidic medium and were converted to alkoxy ($RO^{\cdot}$) and peroxy ($ROO^{\cdot}$) radicals.

The radical species produced by the reaction interact with an additive (phenylenediamine derivative [$2CrNH_2$]) that forms a colored, fairly long-lived radical cation evaluable by spectrophotometer at 505 nm (linear kinetic-based reaction, 37°C). The intensity of the color correlates directly to the quantity of radical compounds and to the hydroperoxides concentration and, consequently, to the oxidative status of the sample according to the Lambert-Beer law (Form CR 2000, Callegari).

$$R\text{-}OOH + Fe^{2+} \rightarrow RO^{\cdot} + OH^{-} + Fe^{3+}$$
$$R\text{-}OOH + Fe^{3+} \rightarrow ROO^{\cdot} + H^{+} + Fe^{2+}$$
$$RO^{\cdot} + ROO^{\cdot} + 2CrNH_2 \rightarrow ROO^{-} + RO^{-} + [CrNH_2^{+\cdot}]$$

All FORT reagents were stored at room temperature and ready to use without additional preparations. Results are expressed as FORT U (FORT units), whereby 1 FORT U corresponds to 0.26 mg/L H_2O_2. The intraassay and interassay coefficients of variation were 3.7% and 6.2%, respectively, for FORT, whereas the linearity ranged from 1.22 to 4.56 mmol/L H_2O_2.

The assay is completed in 6 minutes. Data are available to suggest that the FORT test can satisfactorily assess the level of oxidative radicals in whole blood [15,16,24,36,37].

2.3. The FORD assay

The FORD test uses preformed stable and colored radicals and determines the decrease in absorbance that is proportional to the blood antioxidant concentration of the sample according to the Lambert-Beer law. In the presence of an acidic buffer (pH = 5.2) and a suitable oxidant ($FeCl_3$), the chromogen that contains 4-amino-*N,N*-diethylaniline sulfate forms a stable and colored radical cation photometrically detectable at 505 nm.

Antioxidant compounds in the sample reduce the radical cation of the chromogen, quenching the color and producing a decoloration of the solution, which is proportional to their concentration. The absorbance values obtained for the samples are compared with a standard curve obtained using Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a permeable cell derivative of vitamin E commonly used as an antioxidant.

$$Chromogen_{(no\ color)} + Fe^{2+} + H^{+} \rightarrow chromogen^{\cdot+}{}_{(purple)}$$
$$Chromogen^{\cdot+}{}_{(purple)} + AOH \rightarrow chromogen_{(no\ color)} + AO$$

All FORD reagents were stored at room temperature and ready to use without additional preparations. The intraassay and interassay coefficients of variation were 4.2% and 6.6%, respectively, for FORD, whereas the linearity ranged from 0.25 to 3.0 mmol/L Trolox.

The assay is completed in 6 minutes. There are no data available for the FORD test.

Table 1
Demographic, clinical, and biochemical parameters of the study subjects

	Controls	T2DM	*P*
Male/female (n)	10/10	23/22	.93
Age (y)	53.8 (11.5)	55.1 (11.8)	<.001
BMI (kg/m^2)	28.7 (4.2)	29.3 (5.7)	.62
Waist circumference (cm)	98.9 (6.1)	102.4 (4.9)	.34
Duration of diabetes (y)[a]	-	5.0 (4.0-9.5)	
Fasting glucose (mg/L)	897 (8.1)	1509 (60.5)	<.001
LDL-C (mg/L)	1006 (195)	1351 (354)	<.001
HDL-C (mg/L)	597 (108)	452 (117)	<.001
Triglycerides (mg/L)[a]	840 (637-1063)	1440 (845-1860)	<.001
Fasting insulin (μU/L)[a]	10.3 (6.8-22.4)	14.3 (8.9-23.8)	.03
HOMA-IR[a]	2.4 (1.9-6.4)	5.9 (2.7-9.8)	<.001
HbA_{1c} (%)	5.3 (4.5-6.2)	7.1 (4.7-12.0)	<.001
hs-CRP (mg/L)[a]	1.2 (0.9-1.8)	1.4 (0.6-3.2)	.35
Ferritin (μg/L)[a]	81.0 (36.8-154.6)	86.0 (48.5-160.0)	.52
WBC count (10^3/L)	7142.2 ± 1859.7	7236.5 ± 1942.7	.62
Lymphocyte count (10^3/L)	2186.2 ± 590.3	2228.2 ± 689.5	.72
Monocyte count (10^3/L)	420.6 ± 193.4	478.7 ± 217.7	.28

Data are shown as mean ± SD or as number (percentage).
[a] Median value (interquartile range).

2.4. Measurement of lipids, high-sensitivity C-reactive protein, ferritin, insulin, and hemoglobin A_{1c}

Blood chemistry including measurements of serum glucose, total cholesterol, triglycerides, high-density lipoprotein cholesterol (HDL-C), and high-sensitivity C-reactive protein ([hs-CRP] cardiophase-CRP) was performed using the Siemens Advia 1650 Clinical Chemistry System (Siemens Healthcare Diagnostics, Tarrytown, NY). Ferritin concentrations were determined by latex-particle–enhanced immunonephelometric assay on the BN ProSpec nephelometer (Dade Behring, Siemens Healthcare Diagnostics, Liederbach, Germany), whereas serum insulin levels were determined with the Siemens Advia Centaur Analyzer (Siemens Healthcare Diagnostics, Tarrytown, NY).

Hemoglobin A_{1c} (HbA_{1c})was measured using weak-cation–exchange high-performance liquid chromatography with the Bio-Rad Variant Hemoglobin Testing System (Bio-Rad Laboratories, Hercules, CA) with a nondiabetic range of 4.0% to 6.0%.

The homeostasis assessment model (HOMA) according to the formula HOMA index = fasting insulin (in micro–international units per milliliter) × fasting glucose (in millimoles per liter)/22.5 was used as a marker of insulin resistance (HOMA-IR).

2.5. Statistical analysis

Analyses were performed using the SPSS 12.0 (SPSS, Chicago, IL) statistical package. All variables were tested for normal distribution of the data. Differences between the controls and patients with T2DM were examined using the Student *t* test or the Mann-Whitney *U* test as appropriate, whereas a χ^2 test was used for categorical data. The Pearson and the Spearman correlation coefficients for parametric and nonparametric variables, respectively, were used to look for associations between the plasma concentrations of FORT and FORD with the study parameters. *P* values < .05 (2-sided) were considered statistically significant.

3. Results

The demographic, clinical, and biochemical parameters of the study population are shown in Table 1. Patients with T2DM had higher values of glucose ($P < .001$), low-density lipoprotein cholesterol (LDL-C) ($P < .001$), triglycerides ($P < .001$), and HOMA-IR ($P < .001$) and lower values of HDL-C ($P < .001$) than controls. Serum hs-CRP and ferritin concentrations as well as white blood cell (WBC), lymphocyte, and monocyte counts did not differ significantly between controls and patients with T2DM.

The main results of the study showed that the FORT levels in whole blood were significantly higher in the patients with T2DM compared with controls (2.86 ± 0.56 vs 1.87 ± 0.26 mmol/L H_2O_2, respectively; $P < .0001$) (Fig. 1). In addition, the levels of FORD in whole blood were significantly lower in the patients with T2DM in comparison with the controls (1.23 ± 0.18 vs 1.34 ± 0.14 mmol/L Trolox, respectively; $P = .01$) (Fig. 2.). In the diabetes group, the levels of FORT were higher in men in comparison with women (3.25 ± 0.50 vs 2.54 ± 0.36 mmol/L H_2O_2, respectively; $P < .001$), whereas the levels of FORD were not different between men and women (1.24 ± 0.17 vs 1.20 ± 0.19 mmol/L Trolox, respectively; $P = .58$). In the control group, no differences were found between men and women in levels of either FORT (1.94 ± 0.34 vs 1.83 ± 0.21 mmol/L H_2O_2, respectively; $P = .48$) or FORD (1.35 ± 0.13 vs 1.33 ± 0.15 mmol/L Trolox, respectively; $P = .82$).

Furthermore, in the diabetes group, we found significant positive correlations between FORT levels and hs-CRP (Fig. 3), BMI (Fig. 4), triglyceride concentrations, and waist

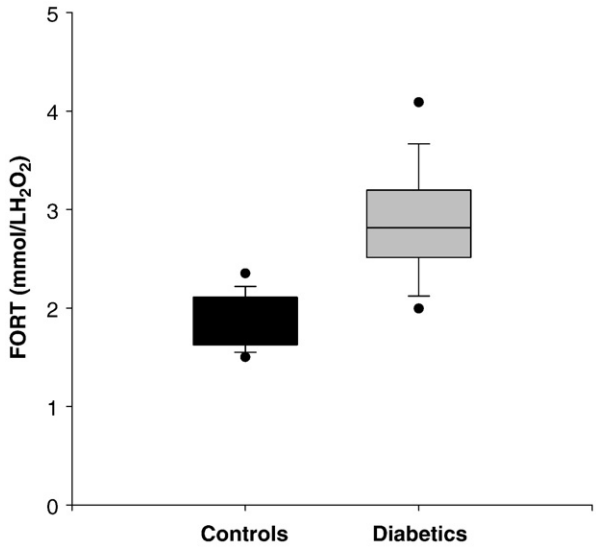

Fig. 1. Box plot (5th-95th percentiles) of FORT levels (in millimoles per liter H_2O_2) in whole blood in diabetic patients and controls.

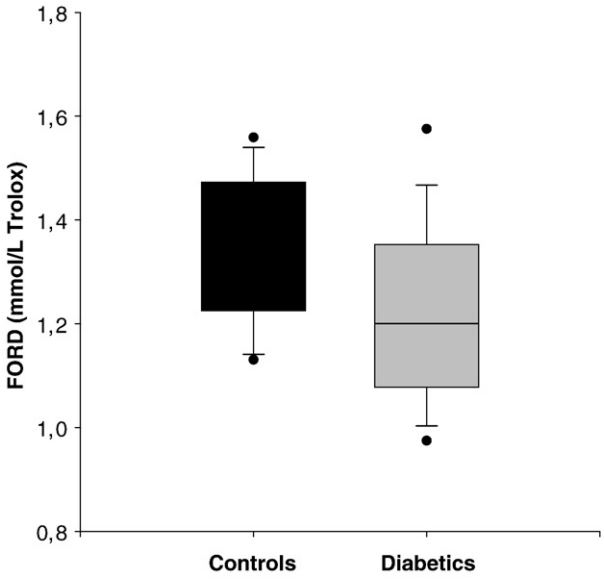

Fig. 2. Box plot (5th-95th percentiles) of FORD levels (in millimoles per liter Trolox) in whole blood in diabetic patients and controls.

circumference. The FORT levels were also associated with WBC count, lymphocyte count, insulin, and LDL-C. No significant associations were found with age, HOMA-IR, glucose, uric acid, HbA_{1c}, HDL-C, γ-glutamyltransferase, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) (data not shown), ferritin, neutrophil count, and monocyte count ($P > .05$) (Table 2). The levels of FORD were associated significantly only with HDL-C ($r = 0.32$, $P = .04$).

In the control group, no significant correlations were found between FORT or FORD levels and the demographic, clinical, and biochemical parameters.

4. Discussion

Excess production of free oxygen radicals or impaired oxygen defense results in a state of oxidative stress that has been linked to cell damage and apoptosis. Oxidative stress is associated with many pathologic processes in a variety of chronic diseases such as T2DM [17-23]. Because of the fact that active free oxygen radicals are rapidly metabolized in vivo, their evaluation represents an extremely difficult task.

The 2 assays, FORT and FORD, are based on a simple stand-alone device that is designed as a point-of-care test using freshly collected whole blood [24].

It is well known that in T2DM evidence of lipid peroxidation was observed with high plasma [25] and urine [26-28] isoprostane levels. In addition, MDA levels were higher than those in the normal subjects and correlated with the degree of glycemic control achieved [29]. Nitrotyrosine formation is increased in plasma of diabetic patients [30-31]. Even at the DNA level, it has been proven that hyperglycemia independently increases 8-hydroxy-2′ deoxyguanosine levels in urine and plasma of patients with T2DM [32-35]. In agreement with the above studies, in this study, we showed that the FORT levels in whole blood were significantly higher in the patients with T2DM compared with controls. We also observed that the levels of FORT were higher in men in comparison with women.

In addition, the levels of FORD in whole blood were significantly lower in the patients with T2DM in comparison

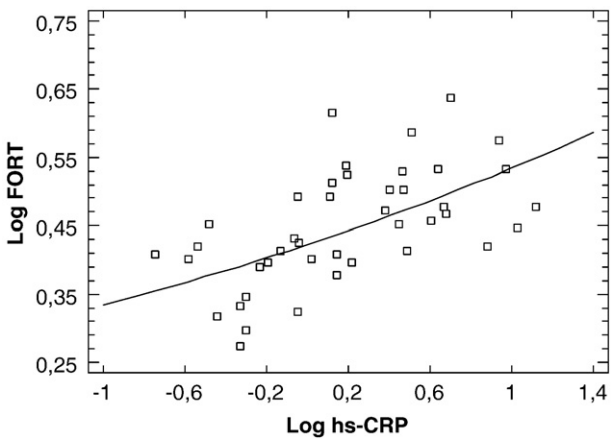

Fig. 3. Correlation of FORT values with hs-CRP values in diabetic patients.

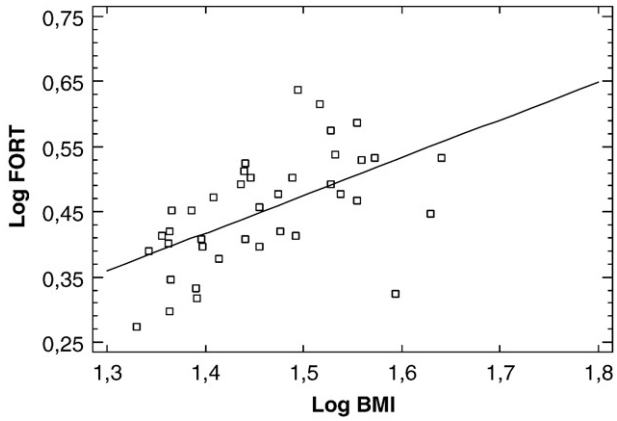

Fig. 4. Correlation of FORT values with BMI values in diabetic patients.

Table 2
The relationship (Pearson or Spearman correlation) between FORT and the studied variables in the patients with T2DM

	r	*P*
BMI	0.51	.001
Waist circumference	0.48	.005
LDL-C	0.30	.05
Triglycerides	0.47	.002
hs-CRP	0.62	<.0001
Insulin	0.31	.04
WBC count	0.40	.01
Lymphocyte count	0.32	.04

No significant correlations were found with age, HOMA-IR, glucose, uric acid, HbA_{1c}, HDL-C, γ-glutamyltransferase, AST, ALT, ALP, ferritin, neutrophil count, and monocyte count ($P > .05$).

with the controls. We did not observe any difference in FORD levels between men and women.

In the control group, no differences were found between men and women in levels of either FORT or FORD.

The increase of FORT levels and the decrease of FORD levels in whole blood in the patients with T2DM agree with the fact that hyperglycemia engenders free radicals and also impairs the endogenous antioxidant defense system in many ways during diabetes. These facts can lead to damage of cellular organelles and enzymes, increased lipid peroxidation, and development of insulin resistance.

We appreciate that it is very important to monitor the total oxidant and the total antioxidant status of a patient with T2DM with 2 simple, rapid, and reliable assays. The FORT test is completed in 6 minutes and the FORD test in the same time.

Furthermore, in the diabetes group, we found significant positive correlations between FORT levels and hs-CRP and triglyceride concentrations. The positive correlation between FORT levels and hs-CRP, a commonly used marker of low-grade inflammation, has also been described previously [1,24,36,37]. The levels of FORD were associated significantly only with HDL-C. These correlations may implicate a possible link among oxidative stress, inflammation, and lipid metabolism that can involve not only diabetes but other pathologic conditions as well.

In the same group, we did not find any significant associations with age, BMI, waist circumference, duration of diabetes, HbA_{1c}, glucose, lipids, uric acid, insulin, HOMA-IR, ferritin, AST, ALT, ALP, WBC count, or subtypes of WBC count.

In the control group, we did not find any significant correlations between FORT or FORD levels and the demographic, clinical, and biochemical parameters.

5. Conclusion

The 2 assays, FORT and FORD, are simple, rapid, reliable, and reproducible and can be used as a point-of-care test using freshly collected whole blood. They measure free oxygen radicals and free oxygen radicals defense, respectively. This information is important in the generation of oxidative stress, which can lead to oxidative damage and is involved in the pathogenesis of several diseases such as T2DM and its complications. As a result, the FORT and FORD assays consist a useful tool for the clinical physician in monitoring the oxidant and antioxidant status of a patient intending to improve his life.

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