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Serum resistance to singlet oxygen in patients with diabetes mellitus in comparison to healthy donors

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ABSTRACT

Diabetes mellitus causes endothelial injury through oxidative stress involving reactive oxygen species and peroxides as well as inflammation, both of which consume antioxidant defenses. Singlet oxygen ($^1O_2$) is produced by leukocytes during inflammatory and biochemical reactions and deactivated by producing reactive oxygen species and peroxides. To determine whether serum was capable of deactivating $^1O_2$, we triggered a photo reaction in sera from 53 healthy donors and 52 diabetic patients. Immediately after light delivery, dichlorofluorescein was added and then its fluorescence was recorded. The mean capacity of $^1O_2$ or secondary oxidant deactivation was reduced in patients with diabetes mellitus. Hemolysis reduced deactivation of $^1O_2$-induced secondary oxidants in both healthy and diabetic patients. Body mass index, age, platelet counts, and blood cell numbers exerted a nonlinear influence. High levels of glycated hemoglobin were associated with an increased deactivation of oxidative species, whereas high-density lipoprotein cholesterol, total cholesterol, and the total cholesterol to high-density lipoprotein cholesterol ratio decreased the serum deactivation capacity. Oral antidiabetics bore no influence on deactivation, which was restored by insulin in women. Deactivation capacity was lower in women, who had half the complications found in men, suggesting that, with more severe diabetes mellitus, protection was maintained against complications. Resistance to $^1O_2$ should be considered during the monitoring of diabetes mellitus.

1. Introduction

Diabetes mellitus (DM) is the leading cause of blindness, noninjury amputation, end-stage kidney disease, and atherosclerosis through micro and macro blood vessel damage. Although the biochemical mechanisms that induce complications are complex, the role of reactive oxidative species (ROS) is underlined at all stages of diabetic development [1,2]. Infections are more frequent and severe in diabetic patients when singlet oxygen ($^1O_2$) is produced in vivo by activated neutrophils [3,4] or eosinophils [5], and during various biochemical reactions [6] including energy production and photo reactions [6,7]. Singlet oxygen is an excited form of molecular oxygen that is strongly oxidant for many biological

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targets, either directly or through the successive formation of various oxidative species. Singlet oxygen may react with various targets and/or generate ROS or peroxides with a much longer half-life [8-12]. However, the dichlorofluorescein (oxidized, fluorescent) (DCF) fluorescence obtained after the oxidation of dichlorofluorescein (reduced) (DCFH) is routinely used to reveal oxidizing species varied largely from one serum to the next [13], whereas the overall amount of detectable fluorescence was highly reproducible for a given serum. This indicated that not all sera could equally deactivate secondary ROS arising after the production of a standardized amount of $^1O_2$ resulting from a short photodynamic reaction. The aim of the present article was to determine whether sera obtained from diabetic patients had the same capacity for $^1O_2$ neutralization as sera obtained from a cohort of healthy blood donors. We also sought to identify any correlations between $^1O_2$ or ROS neutralization and patient characteristics.

2. Materials and methods

The study was conducted according to the protocol (NTS 2006-02) established between the Etablissement Français du Sang and the Nantes University Hospital, in accordance with the Helsinki declaration (1964/2000). The protocol was approved by the Nantes University Hospital ethics committee. Blood samples (Table 1) obtained from 53 healthy blood donors were analyzed on a per-donor basis or after having pooled their sera. None of the healthy donors had ever been previously diagnosed with a severe disease.

Sixty patients were consecutively recruited for this pilot study, but only 52 were included (25 women; mean age, 57.6 years; 27 men; mean age, 59.1 years) because some blood samples showed an excessive rate of hemolysis. Patients were diagnosed with type 2 (n = 45) or type 1 (n = 7) DM, and circumstances of diagnosis are detailed in Table 1. Following the initial diagnosis, complications were noted in 14 women and 20 men. These were divided into ocular, vascular, kidney, infectious, and neurological categories. To consider the severity of the disease, amputation, myocardial infarction, and kidney failures were counted as additive complications and reached a number of 27 in women and 63 in men. High blood pressure and body overweight were not considered as diabetic complications. Diabetes mellitus of either type 1 or 2 was considered as nonequilibrated in the case of symptomatic glycemia fluctuations, a recently discovered symptomatic and progressing complication. Glycated hemoglobin (HbA_{1c}) was considered separately, as certain patients were clinically in good health and stable but had only a level higher or equal to 6% of HbA_{1c} as a pejorative criterion.

Blood sampling was carried out between 9:00 AM and 4:00 PM for healthy donors and between 7:00 AM and 9:00 AM for patients with DM. No patients had eaten for 2 hours before sampling, and smoking was forbidden 2 hours before blood sampling and was in fact a study exclusion criterion. Ten milliliters of blood were drawn on sterile clot act dry tubes (Venosafe VF-054SP, Terumo Europe, Leuven, Belgium) and hemolysis was avoided as much as possible. Serum was collected avoiding hemoglobin aspiration under sterile conditions, separated into 2 aliquots, and frozen at –20°C until processed. The period between blood sampling and freezing did not exceed 40 minutes.

Donors and patients were assayed for viral status (HIV and hepatitis B), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), total cholesterol, triglycerides, HbA_{1c}, Na, K, Ca, total proteins and albumin, glucose, urea, creatinine, uric acid, total and conjugated bilirubin, and C-reactive protein. Blood counts, including platelets, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin concentration, and mean corpuscular hemoglobin, as well as blood typing, were also performed.

Assays for $^1O_2$ resistance of sera have been already published [13]. Briefly, absorption spectra were obtained with a Techcomp 8500 absorption spectrophotometer (Techcomp, Honk Kong, China) for each patient sample and each control, after dilution in water for injections (5% serum). The level of hemolysis was evaluated from the 413-nm peak absorption and the baseline by the 650-nm absorption where there is normally a minimal absorption. Value was subtracted to ratio, using a formula determined from preliminary studies [13].

The principle of the previously described measurement [13] is to analyze the speed of neutralization of ROS and/or peroxides induced by photodynamically induced $^1O_2$ by means of the DCFH/DCF system, activated DCFH being added to each sample assayed immediately *after* the end of light delivery and in a standardized manner. Rose bengal (RB) used as a source of $^1O_2$ under light exposure [14] may also produce small amounts of other oxidants, among them superoxide ions.

The area under the curve (AUC) for the change in DCF fluorescence over time was measured for each donor serum.

Table 1 – Healthy donors and patients description and circumstances of diagnosis

			Type 2 DM							Type 1 DM						
Patients	n	Age (y)	n	Age	IFD	EBW	Cv	Infect	OpC	n	Age	IFD	EBW	Cv	Infect	OpC
Women	25	57.6	22	58.7	18.3	7	7	1	2	3	45	8.3	0	2	1	1
Men	27	59.1	23	62.6	15.7	6	2	1	1	4	43	17	1	1	0	1
Whole	52	58.4	45	60.7	16.9	13	9	2	3	7	44	13.3	1	3	1	2
Donors																
Women	22	36.2	Range, 21-65													
Men	31	42.8	Range, 22-65													
Whole	53	40.1														

Complications leading to diagnosis are mentioned. IFD indicates interval from diagnosis; EBW, excess body weight; Cv, cardiovascular; Infect, infectious; OpC, ophthalmic complication.

The corrected value for each diabetic patient was divided by the DCF fluorescence AUC value for normal pooled sera samples prepared from the pool of healthy donors used as a reference. Reference ratio was equal to 1; a value higher than 1 indicated a greater AUC fluorescence than the reference pool used and thus a lower capacity for the given serum to neutralize $^1O_2$ and related ROS or peroxides as a function of time, whereas a lower value indicated a higher capacity.

Kolmogorov-Smirnov test for normality distributions, χ^2 test for group comparisons, Mann-Whitney *U* test for data comparisons, and Pearson test for correlations had been used when needed for a significance at $P < .05$. Cohorts equal to or larger than 8 had been considered for statistics.

3. Results

3.1. $^1O_2$ deactivation by sera from healthy donors

The typical evolution of DCF fluorescence with phototreated human serum firstly displayed a steep and almost linear increase during the initial 10 minutes then increased at a lower speed for up to 60 minutes. The shapes of the curve of DCF evolution of fluorescence and AUC values were similar to data already obtained [13]. Variations existed within the AUC recorded for each healthy donor (Fig. 1A) but were not linked to differences in the extinction coefficient, as they had been corrected for absorption differences. When sera had been pooled, the AUC value was 0.86 (the reference was in this particular case a previous human serum pool named *EFS 1* constituted from 75 healthy donors) with an optical density of 0.1 at 413 nm. The means calculated from the whole cohort (0.85; range, 0.44-1.51) for a mean optical density of 0.096 at 413 nm did not significantly differ when they were compared with the range of variations measured on a per-donor basis (AUC) for men (mean, 0.87; SD, 0.23; range, 0.44-1.51) or women (mean, 0.83; SD, 0.2; range, 0.54-1.38). Age bore a slight influence on the capacity of sera to deactivate secondary oxidants after exposure to $^1O_2$ and ROS within the range of ages assayed, although this capacity slightly decreased between 40 and 60 years of age (Fig. 1B). The donor's viral status, the biochemical parameters of each donor (that were all within the reference range), electrolytes, LDL-C, total bilirubin, total cholesterol, triglycerides, proteins including albumin and uric acid, glucose, and urea did not correlate with individual AUC measured DCF fluorescence. The hematological parameters were normal for each donor, and no correlation was found between DCF fluorescence ratio and any of these parameters.

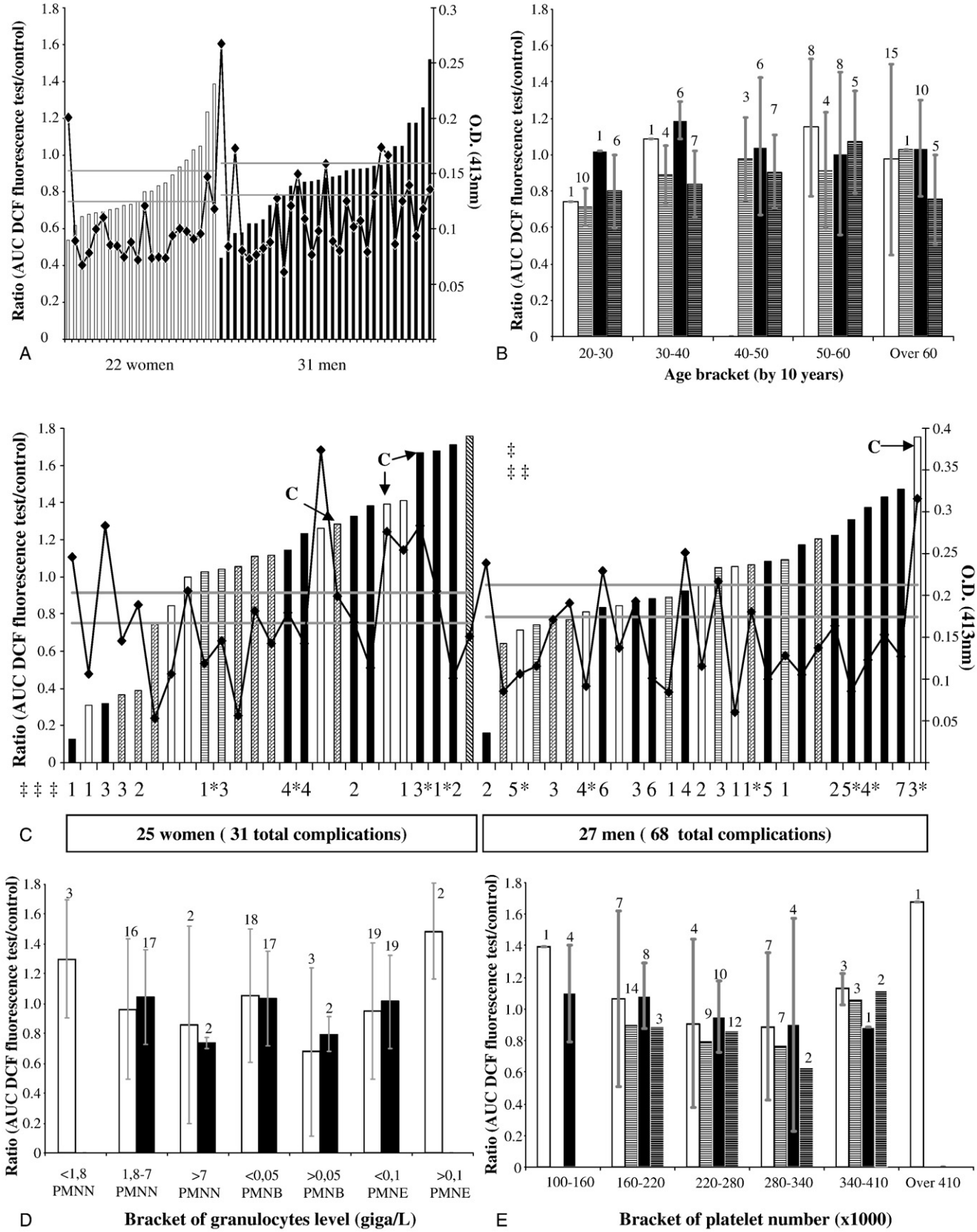
From the above data, the mean ratios of (presumably) healthy donors was 0.85 and it was arbitrarily decided, given the absence of other published data, that the reference range was + or −10%, that is, a 20% range.

3.2. $^1O_2$ deactivation by sera from diabetic patients

Shape of the curve of DCF evolution of fluorescence was similar to that recorded with healthy donors (unpublished data). The mean capacity of sera to deactivate secondary oxidants after exposure to $^1O_2$ and ROS was significantly decreased ($P < .02$ for women and $P < .04$ for men, ratio greater than the EFS pool) for diabetic patients as compared with healthy individuals in both the male and female cohorts. The influence of healthy patient sex was minimal, with the capacity of $^1O_2$ and ROS deactivation somewhat greater for women as compared with men. Women with DM had a corrected ratio 20% higher than healthy women and for men, this was 17% higher. Abnormal values were significantly worse for women than men ($P < .001$). Five of 7 patients with type 1 DM and 25 of 45 patients with type 2 DM had a reduced capacity to deactivate $^1O_2$ and ROS as compared with the reference range, with respective corrected ratios of 1.19 (1.084 for the whole type 1 DM cohort) and 1.31 (1.026 for the whole type 2 DM cohort). This did not correlate with serum absorption, particularly at the absorption wavelength at 413 nm for hemoglobin released from hemolysis following blood sampling (Fig. 1C). Correcting ratios for hemolysis increased the values without changing the overall number of patients with abnormal values. The highest observed fluorescence values in DM patients were similar to the highest values measured in presumed healthy individuals, but the number of individuals with values greater than the reference range was significantly greater (9/53 vs 32/52, $P < .05$) (Fig. 1A, B). The change in resistance to $^1O_2$ according to age in DM patients

Fig. 1 – Area under the curve of DCF fluorescence observed after addition of DCFH to phototreated (514 nm, 20 J/cm^2) sera in water (5%) containing RB (5 μg/mL). The AUC ratio represents values of test over control (pooled samples of all healthy sera). A, Individual variations of the serum resistance to $^1O_2$ in 53 healthy donors. White bars for healthy women and black bars for healthy men. Absorbance of 413 nm (black rhombus, secondary y-axis) does not correlate to the AUC ratio. B, The AUC ratio of diabetic serum/healthy pooled sera after correction for hemolysis according to age in diabetic women (w, white bars) or men (m, black bars) in comparison to healthy women (white bars with black lines) and men (black bars with white lines). Numbers above bars represent cohort sizes. C, Influence of diabetes, diabetes-related complications, or diabetes clinical stability on AUC ratio in women or men. Black bars for nonequilibrated type 2 DM, white bars for equilibrated type 2 DM, white bars with up diagonal black lines for equilibrated type 2 DM but with a nonequilibrated HbA_{1c}, white bars with horizontal black lines for type 1 DM, black bars with down diagonal white lines for nonequilibrated type 1 DM. The number of complications is noted on the x-axis, * when at least one complication is of infectious origin. Number of complications significantly differed between women and men ($P < .008$). ‡Healthy cohort from panel A vs diabetic patients: women, $P < .02$; men, $P < .04$. ‡‡Abnormal values in women vs men ($P < .001$). D, Influence of polymorphonuclear (N, neutrophils; B, basophils; E, eosinophils) number on AUC ratio of diabetic sera divided by healthy pooled sera after correction for hemolysis in diabetic women (white bars) or men (black bars). Numbers above bars represent cohort sizes. E, The AUC ratio after correction for hemolysis according to platelet number in diabetic women (white bars) or men (black bars) as compared with healthy women (white bars with black lines) or men (black bars with white lines). Cohort sizes are noted on the x-axis.

varied in a manner close to healthy patients (Fig. 1B). The resistance decreased for the first age groups and then increased at older than 60 years for women but was stable for men. An elevated body mass index (BMI) was associated with an increased capacity of $^1O_2$ and ROS deactivation in women but to a decreased capacity for women with morbid obesity (BMI >35) and for men. The date of DM discovery did not correlate with $^1O_2$ or ROS deactivation capacity.



Complications (Fig. 1C) were statistically more frequent in men than in women ($P < .008$), but there was no relationship between the number of complications and the capacity to deactivate ROS after $^1O_2$ production. Thirty-one diabetes-related complications had been diagnosed in 14 of 25 women (9/14 had a decreased capacity to deactivate $^1O_2$ or ROS) and 68 complications in 20 of 27 men (11/20 had a decreased capacity to deactivate $^1O_2$ or ROS). A reduced $^1O_2$ and ROS deactivation capability was observed for complications diagnosed either before or after blood sampling for the present study in 3 patients with a cataract (mean ratio, 0.987) and 13 diabetic retinopathies (mean ratio, 1.106; 1.089 for 5 women, 1.117 for 8 men) or nephropathy (mean ratio, 1.027 for 13 patients; 3 women) whether the DM was equilibrated or not. Four cancers (affecting 3 women and 1 man) had been diagnosed, giving high ratios of AUC fluorescence (thus, the lowest capacity to deactivate $^1O_2$). Three of these tumors had been diagnosed before DM, the DM being the consequence of cancer treatments in 2 cases (1 hypothalamic germinoma and 1 kidney carcinoma with operated pancreatic metastases). The fourth case was a uterine cancer discovered in a patient with a type 2 DM 18 years after the DM diagnosis. In this patient, the DM had never been stabilized either before or after cancer treatment. The lowest $^1O_2$ and ROS deactivation was observed in a nonstabilized type 1 DM patient (1.757), whereas nonstabilized type 2 DM patients and/or patients with clinically active complications had a score of 1.082. Nonstabilized diabetes was found to impair the capacity of $^1O_2$ and ROS deactivation more than the number of active complications, given that 14 patients with values greater than the reference range (5 women) out of 20 had been found to be unstable. Ten patients (4 women) had active infectious complications. The mean capacity to deactivate $^1O_2$ or ROS was decreased because the ratio was 1.254 (1.171 for men, 1.379 for women). The type 2 DM patients clinically stabilized, with the exception of a high HbA_{1c} level (>6%) or with nonclinically progressing complications, demonstrated a normal deactivation capacity (0.876). Although the patient cohorts were very small in some cases, a propensity toward decreasing AUC of DCF fluorescence after $^1O_2$ production proportional to the number of neutrophils basophils and eosinophils was noticeable in both men and women (Fig. 1D). Similarly to the results already observed in healthy donors (normal or elevated number of platelets), the sera of patients with diabetes were found to have a lower capacity of $^1O_2$ deactivation when their blood had a platelet count less than or greater than the upper limit of the reference range (Fig. 1E). Monocyte or lymphocyte counts did not influence $^1O_2$ deactivation.

Glucose exerted no influence on the value of the AUC ratio of DCF fluorescence (Fig. 2A). On the contrary, the capacity to deactivate $^1O_2$ decreased according to the percentage of HbA_{1c} within the female cohort ($P < .05$) and to a lesser extent within the male cohort (Fig. 2B). An elevated total cholesterol (≥2.08 g/L) or HDL-C level (0.6 g/L) reduced the capacity to deactivate reactive species after $^1O_2$ production in women, whereas LDL-C (low or normal in any case) had little influence in women but increased the AUC ratio in men (Fig. 2C). There was an inverse relationship, observed mainly in the female group ($P < .03$), between the ratio of total cholesterol to HDL-C and the capacity to deactivate $^1O_2$ in diabetic patients (Fig. 2D, E). This was not observed in healthy patients. There was no correlation or trend toward a correlation with C-reactive protein, albumin, free or conjugated bilirubin, urea, uric acid, creatinine, or the presence of microalbuminuria.

Treatments also influenced the capacity of deactivating ROS or peroxides after $^1O_2$ production. The DCF fluorescence ratio decreased only when insulin was associated with oral hypoglycemics or statin, whereas neither of these classes of medications used alone had an effect (Table 2). A short-acting insulin had been used in 23 patients, as a combination of 2 in 16 patients and as a combination of short, intermediate, and long acting in 4 patients. Although frequently associated to other types of insulin, long-acting insulin seemed to increase the serum $^1O_2$ deactivating capacity. Despite a more severe DM, justifying the use of several antidiabetic medications including several types of insulin, it remained possible to bring back $^1O_2$ deactivating capacity to normal values (Table 2). Statins or vitamin supplementation (11 patients) did not seem to have any effect on the DCF fluorescence ratio after $^1O_2$ production.

4. Discussion

Diabetes mellitus and glucose excursions occurring in non- or poorly stabilized DM result in oxidative stress that has been postulated to promote the development of complications [2,15] of great personal social and economic significance. Among complications, sensitivity to infections and the gravity of these infections are more significant in diabetic patients for a plethora of reasons. Photo produced $^1O_2$ could represent a model accounting, to a certain extent, for what happens during inflammatory reactions involving polymorphonuclear leukocytes. One cannot completely exclude however that polymorphonuclear leukocyte oxidative activity in physiological conditions would induce different effects, as they produce other oxidizing species although of a lower redox potential.

Oxidative stress, namely, the imbalance between ROS production and deactivation, can include autooxidation of glucose, shifts in redox balances, and reduced or impaired activities of antioxidant defense enzymes. The aim of our

Fig. 2 – Biochemical parameters influencing AUC of DCF fluorescence ratio (AUC ratio) in diabetic sera divided by healthy pooled sera in women (white bars) or men (black bars) after RB addition (5 μg/mL) and light delivery (514 nm, 20 J/cm^2) A, Glycaemia. B, Hemoglobin A_{1c} level. ‡Relationship between HbA_{1c} and ratio (female group) ($P < .05$). C, Total cholesterol, LDL-C, and HDL-C level. D, The AUC ratio (white bars) of the diabetic women cohort as a function of the total cholesterol to HDL-C ratio (curve with black square). Women reference range is between the 2 horizontal lines. ‡Inverse relationship between ratio and total cholesterol to HDL-C ratio ($P < .03$). E, The AUC ratio (black bars) of the diabetic men cohort as a function of the total cholesterol to HDL-C ratio (curve with white square). Men reference range is between the 2 horizontal lines.

study was to determine, by means of a standardized photo reaction produced within the sera, whether a long-term elevated production of ROS in patients with diabetes mellitus could lead to the consumption of antioxidant defenses and impair resistance to $^1O_2$ produced either physiologically when O_2 is involved as an oxidant or during inflammatory process-

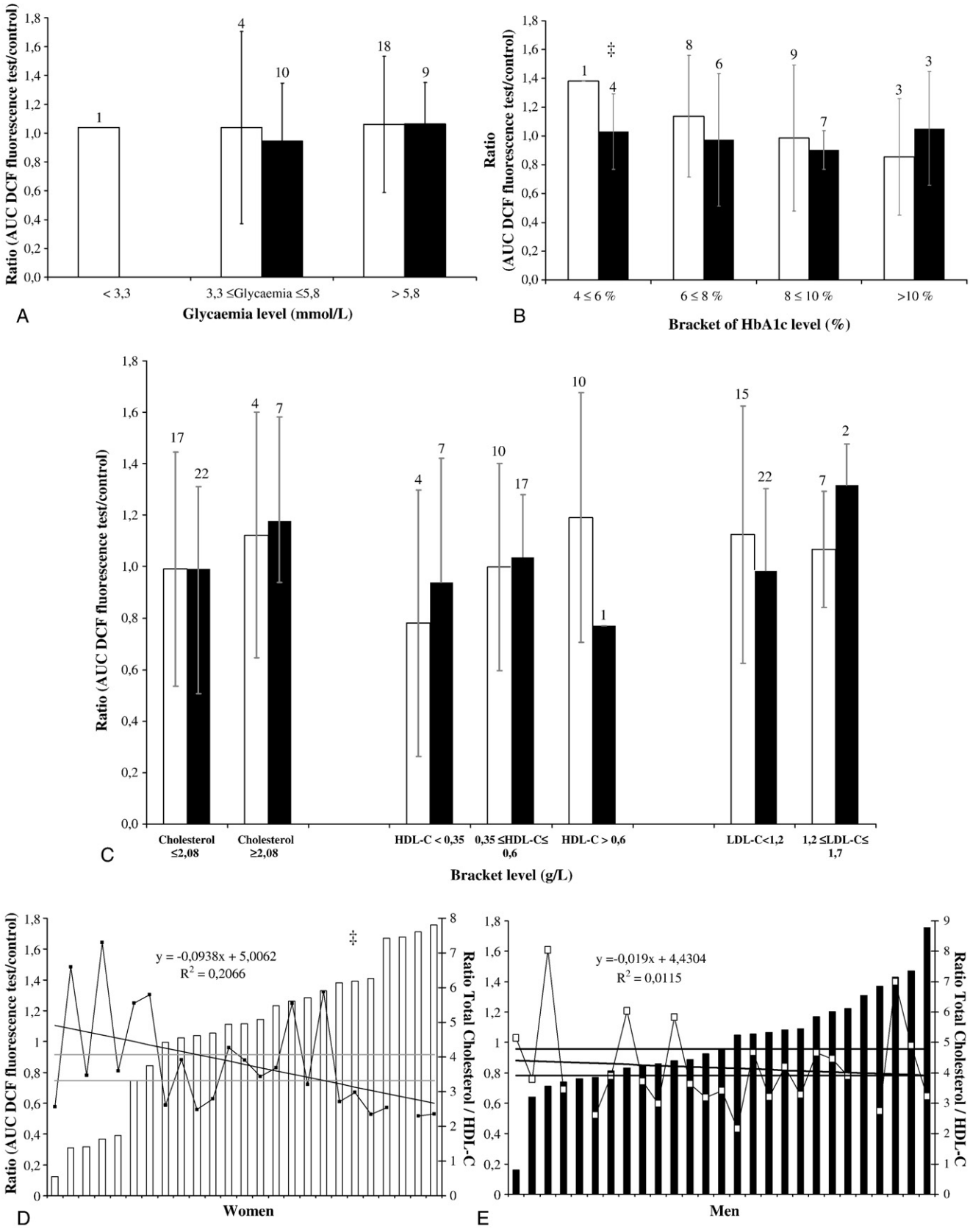


Table 2 – Influence of treatments given to patients with DM on $^1O_2$ deactivation capacity

	Control, 53 P, 22 W, (SD)	T1 + T2 DM, 52 P, 25 W, (SD)	O, 5 P, 1 W, (SD)	S, 2 P, 0 W, (SD)	O + S, 6 P, 5 W, (SD)	1 Insulin			2 Types of insulin			3 Types of insulin
						SAI, 3 P, 2 W, (SD)	IAI, 3 P, 2 W, (SD)	LAI, 13 P, 4 W, (SD)	SAI & IAI, 2 P, 1 W, (SD)	SAI & LAI, 14 P, 6 W, (SD)	IAI & LAI, 1 W, (SD)	SAI & IAI & LAI, 4 P, 3 W, (SD)
Whole cohort	0.85 (0.2)	1.034 (0.4)	1.152 (0.089)	1.39 (0.12)	1.17 (0.22)	1.08 (0.68)	1.457 (0.196)	0.89 (0.42)*	1.112 (0.396)	0.941 (0.428)*	1.056	0.972 (0.458)
Women	0.87 (0.20)	1.06 (0.35)†	1.262	/	1.13 (0.23)	1.074 (0.965)	1.53 (0.21)	0.688 (0.52)	1.392	1.12 (0.54)	1.056	0.888 (0.522)
Men	0.832 (0.220)	1.002 (0.310)	1.124 (0.074)	1.39 (0.12)	1.373	1.091	1.309	0.980 (0.367)	0.832	0.806 (0.290)	/	1.225

Mean ratios (group over control) of DCF fluorescence after $^1O_2$ production (after RB addition [5 μg/mL] and light delivery [514 nm, 20 J/cm^2]) in sera of diabetic patients or healthy donors measured as AUCs for 66 minutes. Control group consisted of samples of each serum pooled. P indicates persons; W, women; O, oral antidiabetics alone; S, statins alone; S + O, statins and oral antidiabetics; SAI, short-acting insulin; IAI, intermediate-acting insulin; LAI, long-acting insulin.

* Statistical significance vs the whole cohort of DM patients at $P < .05$.

† Vs healthy cohort; $P < .02$ for women and $P > .04$ for men.

es, diseases, or infections. Singlet oxygen is the common name used for the 2 metastable states of molecular oxygen with higher energy than the ground state triplet oxygen. Singlet oxygen is produced during biochemical or inflammatory reactions by neutrophils through myeloperoxidase-dependent mechanisms [3] and by eosinophils through a powerful peroxidase-catalyzed mechanism. Activated granulocytes (neutrophils and eosinophils) may in addition produce a variety of other oxidants including hypochlorous acid, hydrogen peroxide, and superoxide anion. All of these participate in the destruction of pathogenic agents directly through oxidative processes or through signaling pathways [16]. The effects of these reactions will thus depend to a certain extent on the capacity of each individual's serum to neutralize $^1O_2$ and the ROS/peroxides produced. Singlet oxygen may react with many targets producing ROS that will in turn deactivate, progressively producing peroxides, which themselves may produce ROS. The lifetime and the radius of action of $^1O_2$ are short, ranging from 3 microseconds in water to 30 microseconds in lipids [17,18]. Secondarily induced ROS or peroxides can have a much longer half-life [12]. In addition, $^1O_2$ can reactivate from superoxide anion via the Russel reaction [19].

The direct measurement of ROS, including $^1O_2$-induced ROS, is nearly impossible on a routine basis [20] or in vivo [18,21]. Total antioxidant status measurements may be more physiologically relevant because they take into account the potential synergistic or antagonistic [22] effects of antioxidants that appear to be important in vivo, but also nonspecific compounds with antioxidant properties such as uric acid, albumin, and bilirubin [23]. None of assays already developed take into account the influence of hemolysis, although it had been shown to be increased in diabetic patients [24]. The DCFH-DCF system is routinely used to detect ROS generated whatever the source with a reasonable accuracy, although it is nonspecific [25]. The total antioxidant capacity in plasma of type 1 DM patients was shown to be lower than that of healthy subjects [24] and comparable to values observed using other methods in type 2 DM patients [26,27] and to our own results involving $^1O_2$ deactivation. Techniques used consider mainly one given class of products (ie, lipid peroxides) or explore the capacity of deactivating oxidizing species produced by leukocytes after exogenous stimulation, and one cannot exclude that this capacity could be reduced [27-29]. Using photodynamic reactions, during which $^1O_2$ is massively produced in a very short time by the interaction of a chemical, which does not react with any component of the serum and an emission of light, measures the resistance to a physiological oxidative species, deactivating according to physiological pathways.

Hemolysis, which occurs physiologically but also during blood sampling, also causes the release of many molecules likely to play a role in antioxidative processes [30,31]. Heme had been found to be a potent inducer of inflammation in mice and is counteracted by heme oxygenase [32]. It is thus not completely surprising that hemolysis plays a role in determining the capacity of sera to deactivate $^1O_2$ and ROS. Regardless of the pathway used, hemolysis would have more consequences in diabetic patients who show an excessive production of ROS [33].

Hemoglobin A_{1c} accounts for 5% of the total hemoglobin in a healthy adult. Hemoglobin A_{1c} in the red blood cell is considered the criterion standard for assessing long-term plasma glucose control and is a key therapeutic target for the prevention of diabetes-related complications. We thus expected to see high HbA_{1c} levels associated with a low capacity for deactivation and not the contrary. Although the physiological effects of HbA_{1c} are unclear, except for an increased affinity for O_2 [34,35] and its inflammatory reactions [36], a correlation between HbA_{1c}, $^1O_2$, and ROS deactivation has never been demonstrated. Hemoglobin A_{1c} could represent not only a biomarker for the management of diabetes mellitus treatments but also a marker of a reduced oxygenation, given that O_2 has been shown to be able to decrease the glycosylation [37]. One could hypothesize that an increased level of deoxyhemoglobin [38] would correlate with a reduced production of $^1O_2$ and a reduced consumption of anti-$^1O_2$ defenses in diabetic patients. This is also in agreement with other findings, demonstrating no special direct toxicity [39] of advanced glycation ends products. Sex bore little influence on $^1O_2$ and ROS deactivation but the rate of complications in men was twice that of women, according to literature data. The influence of total cholesterol to HDL-C ratio was particularly marked in women who had less diabetes-related complications than men. As diabetes complications are said to be ROS related, this suggests that hormones play a determining and protective role toward the vasculature but have little impact on $^1O_2$ and ROS deactivation. One could hypothesize that, in women, the defenses are more efficient than in men and that these are mobilized to offset a higher rate of ROS production but possibly overcome at a later stage. Such a hypothesis could explain why a high BMI had a greater influence in men on $^1O_2$ and ROS deactivation (with the exception of morbid obesity in women) and also applies to the relationship between peripheral polymorphonuclear cells and capacity of $^1O_2$ and ROS deactivation as well as a higher ratio of AUC in women as compared with men during comparable infectious complications. The number of leukocytes was associated with a better capacity to deactivate $^1O_2$ and ROS, which is in agreement with the literature [28,29]. One can suggest that an incident inflammation can lead to a transient mobilization of antioxidative agents, increasing $^1O_2$ and ROS deactivation, if defenses are not chronically consumed. Inflammation is linked to the evolution of cardiovascular disease and acute coronary syndrome through the modification of adhesion molecule expression toward platelets and the secretion of chemokines by vascular endothelial cells [40]. From this point of view, it is worth considering that a low or elevated number of platelets was associated with a reduced capacity of $^1O_2$ and ROS deactivation, which is in agreement with other findings [41], although superoxide anion enhances platelet function [42]. This result could be related to the use of sera rather than plasma [43] or to an undetected disease with a high platelet number, which is known to provoke an oxidative stress during normal aggregation [44].

In this study, insulin was found to be the only treatment leading to the restoration of the $^1O_2$ and ROS deactivation capacity. This fits in well with the trend toward using insulin early during diabetes mellitus care [45]. Long-acting insulin seemed to have better capacity for restoring the $^1O_2$ and ROS deactivation. The insulin structure includes 6 cysteine residues for 51 amino acids when the antioxidant role of sulphur has been underlined [46,47]. These findings are borne out by other studies reporting an intensification of oxidative stress [26] and inflammation in type 2 DM despite antihyperglycemic treatment [48,49]. Oral antidiabetics in our series of patients were unable to restore a normal $^1O_2$ and ROS deactivation capacity, although some of them, such as glimepiride, have been shown to reduce glucose, mononuclear activation, and free radical–quenching properties [50].

The capacity of $^1O_2$ deactivation diminishes in diabetic patients as compared with healthy donors. Despite a weaker capacity of deactivation as compared with men, women experienced a much lower rate of complications. However, an abnormally low deactivation capacity could be considered as being associated with a poor prognosis given that, at least at the time of blood sampling, it indicates that defenses are overcome and the flood gates are opened to complications. In addition to HbA_{1c} or glycemia, resistance to oxidative species should be considered at time of diagnosis [51] and during the monitoring of DM.

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REFERENCES

[1] Giaccari A, Sorice G, Muscogiuri G. Glucose toxicity: the leading actor in the pathogenesis and clinical history of type 2 diabetes—mechanisms and potentials for treatment. Nutr Metab Cardiovasc Dis 2009;19:365-77.
[2] Ceriello A, Esposito K, Piconi L, et al. Oscillating glucose is more deleterious to endothelial function and oxidative stress than mean glucose in normal and type 2 diabetic patients. Diabetes 2008;57:1349-54.
[3] Hampton MB, Kettle AJ, Winterbourn CC. Inside the neutrophil phagosome: oxidants, myeloperoxidase and bacteria killing. Blood 1998;92:3007-17.
[4] Badwey JA, Karnovsky ML. Active oxygen species and the functions of phagocytic leukocytes. Annu Rev Biochem 1980; 49:695-726.
[5] Kanofsky JR, Hoogland H, Wever R, et al. Singlet oxygen production by human eosinophils. J Biol Chem 1988;263: 9692-6.
[6] Kanofsky JR. Singlet oxygen production by biological systems. Chem Biol Interact 1989;70:1–28.
[7] Weishaupt KR, Gomer CJ, Dougherty TJ. Identification of singlet oxygen as the cytotoxic agent in photoinactivation of a murine tumor. Cancer Res 1976;36:2326-9.
[8] Wright A, Hawkins CL, Davies MJ. Photo-oxidation of cells generates long-lived intracellular protein peroxides. Free Radic Biol Med 2003;34:637-47.
[9] Kon T, Tanigawa T, Hayamizu K, et al. Singlet oxygen quenching activity of human serum. Redox Rep 2004;9:9325-30.

[10] Girotti AW. Translocation as a means of disseminating lipid hydroperoxide-induced oxidative damage and effector action. Free Radic Biol Med 2008;44:956-68.
[11] Tanielian C, Mechin R, Seghrouchni R, et al. Mechanistic and kinetic aspects of photosensitization in the presence of oxygen. Photochem Photobiol 2000;71:12-9.
[12] Olivier D, Douillard S, Lhommeau I, et al. PDT of culture media containing serum induces a delayed toxicity in vitro. Radiat Res 2009;172:451-62.
[13] Olivier D, Douillard S, Lhommeau I, et al. Secondary oxidants in human serum exposed to singlet oxygen: the influence of hemolysis. Photochem Photobiol Sci 2009;8:1476-86.
[14] Redmond RW, Gamlin JN. A compilation of singlet oxygen yields from biologically relevant molecules. Photochem Photobiol 1999;70:391-475.
[15] Fearon IM, Faux SP. Oxidative stress and cardiovascular disease: novel tools give (free) radical insight. J Mol Cell Cardiol 2009;47:372-81.
[16] Schäfer M, Werner S. Oxidative stress in normal and impaired wound repair. Pharmacol Res 2008;58:165-71.
[17] Benssasson RV, Land EJ, Truscott TG. Activated forms of oxygen. Excited states and free radicals in biology and medicine. Oxford University Press, USA 1993:102-41.
[18] Snyder JW, Skovsen E, Lambert JD, et al. Optical detection of singlet oxygen from single cell. Phys Chem Chem Phys 2006;8: 4280-93.
[19] Russel GA. Deuterium-isotope effects in the autoxidation of aralkyl hydrocarbons. Mechanism of the interaction of peroxy radicals. J Am Chem Soc 1957;79:3871-7.
[20] Waring WS, Mishra V, Maxwell SRJ. Comparison of spectrophotometric and enhanced chemiluminescent assays of serum antioxidant capacity. Clin Chim Acta 2003;338:67-71.
[21] Tsimikas S. Measures of oxidative stress. Clin Lab Med 2006; 26:571-90.
[22] Huang D, Ou B, Prior RL. The chemistry behind antioxidant capacity assays. J Agric Food Chem 2005;53:1841-56.
[23] Kikugawa K, Oikawa N, Miyazawa A, et al. Interaction of nitric oxide with glutathione or cysteine generates reactive oxygen species causing DNA single strand breaks. Biol Pharm Bull 2005;28:998–1003.
[24] Matteucci E, Giampietro O. Oxidative stress in families of type 1 diabetic patients. Diabetes Care 2000;23:1182-6.
[25] Hafer K, Iwamoto KS, Schiestl RH. Refinement of the dichlorofluorescein assay for flow cytometric measurement of reactive oxygen species in irradiated and bystander cell populations. Radiat Res 2008;169:460-8.
[26] Pavlatou MG, Papastamataki M, Apostolakou F, et al. FORT and FORD: two simple and rapid assays in the evaluation of oxidative stress in patients with type 2 diabetes mellitus. Metabolism 2009;58:1657-62.
[27] Medina LO, Veloso CA, de Abreu Borges E, et al. Determination of the antioxidant status of plasma from type 2 diabetic patients. Diabetes Res Clin Pract 2007;77:193-7.
[28] Noritake M, Katsura Y, Shinomiya N, et al. Intracellular hydrogen peroxide production by peripheral phagocytes from diabetic patients. Dissociation between polymorphonuclear leucocytes and monocytes. Clin Exp Immunol 1992;88:269-74.
[29] Sato N, Kashima K, Tanaka Y, et al. Effect of granulocyte-colony stimulating factor on generation of oxygen-derived free radicals and myeloperoxidase activity in neutrophils from poorly controlled NIDDM patients. Diabetes 1997;46: 133-7.
[30] Andersen HR, Nielsen JB, Nielsen F, et al. Anti oxidative enzyme activities in human erythrocytes. Clin Chem 1997;43: 562-8.
[31] Nagababu E, Rifkind JM. Heme degradation by reactive oxygen species. Antioxid Redox Signal 2004;6:967-78.
[32] Wagener FA, Eggert A, Boerman OC, et al. Heme is a potent inducer of inflammation in mice and is counteracted by heme oxygenase. Blood 2001;98:1802-11.
[33] Inouye M, Moi T, Sumino K. Glycated hemoglobin and lipid peroxidation in erythrocytes of diabetic patients. Metabolism 1999;48:205-9.
[34] Samaja M, Melotti D, Carenini A, et al. Glycosylated haemoglobins and the oxygen affinity of whole blood. Diabetologia 1982;23:399-402.
[35] Ditzel J, Nielsen NV, Kjaergaard JJ. Hemoglobin AIc and red cell oxygen release capacity in relation to early retinal changes in newly discovered overt and chemical diabetics. Metabolism 1979;28:440-7.
[36] Watanabe J, Grijalva V, Hama S, et al. Hemoglobin and its scavenger protein haptoglobin associate with apoA-1–containing particles and influence the inflammatory properties and function of high density lipoprotein. J Biol Chem 2009; 284:18292-301.
[37] Smith RJ, Koenig RJ, Binnerts A, et al. Regulation of hemoglobin AIc formation in human erythrocytes in vitro. Effects of physiologic factors other than glucose. J Clin Invest 1982;69:1164-8.
[38] Scano G, Filippelli M, Romagnoli I, et al. Hypoxic and hypercapnic breathlessness in patients with type I diabetes mellitus. Chest 2000;117:960-7.
[39] Chuyen NV, Arai H, Nakanishi T, et al. Are food advanced glycation end products toxic in biological systems? Ann N Y Acad Sci 2005;1043:467-73.
[40] Wauthier JL, Schmidt AM. Protein glycation, a firm link to endothelial cell dysfunction. Circ Res 2004;95:233-8.
[41] Watala C. Blood platelets reactivity and its pharmacological modulation in diabetes mellitus. Curr Pharm Des 2005;11: 2277-8.
[42] Handin RI, Karabin R, Boxer GJ. Enhancement of platelet function by superoxide anion. J Clin Invest 1977;59:959-65.
[43] Ghiselli A, Serafin IM, Natella F, et al. Total antioxidant capacity as a tool to assess redox status: critical view and experimental data. Free Radic Biol Med 2000;29:1106-14.
[44] Freedman JE. Oxidative stress and platelets. Arterioscler Thromb Vasc Biol 2008;28:11-6.
[45] Nyenwe EA, Jerkins TW, Umpierrez GE, et al. Management of type 2 diabetes: evolving strategies for the treatment of patients with type 2 diabetes. Metabolism 2011;60:1–23.
[46] Jain SK, Velusamy T, Croad JL, et al. L-cysteine supplementation lowers blood glucose, glycated hemoglobin, CRP, MCP-1, and oxidative stress and inhibits NF-kappaB activation in the livers of Zucker diabetic rats. Free Radic Biol Med 2009;46: 1633-8.
[47] Montes-Cortes DH, Hicks JJ, Ceballos-Reyes GM, et al. Chemical and functional changes of human insulin by in vitro incubation with blood from diabetic patients in oxidative stress. Metabolism 2010;59:935-42.
[48] Farah R, Shurtz-Swirski R, Lapin O. Intensification of oxidative stress and inflammation in type 2 diabetes despite antihyperglycemic treatment. Cardiovasc Diabetol 2008;7:20.
[49] Kelly AS, Thelen AM, Kaiser DR, et al. Rosiglitazone improves endothelial function and inflammation but not asymmetric dimethylarginine or oxidative stress in patients with type 2 diabetes mellitus. Vasc Med 2007;12:311-8.
[50] Schiekofer S, Rudofsky GJ, Andrassy M, Schneider J. Glimepiride reduces mononuclear activation of the redox-sensitive transcription factor nuclear factor–kappa B. Diabetes Obes Metab 2003;5:251-61.
[51] Fajans SS, Herman WH, Oral EA. Insufficient sensitivity of hemoglobin A(1C) determination in diagnosis or screening of early diabetic states. Metabolism 2011;60:86-91.