

Oxidative Damage and Antioxidant Status in Diabetes Mellitus and Rheumatoid Arthritis: A Comparative Study

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Abstract: The present work compares and correlates antioxidant defenses and oxidative stress markers in patients with Diabetes Mellitus (DM) and in those with Rheumatoid Arthritis (RA) in order to find out if new evidences for a possible relationship between these pathologies can be found. Stress markers and antioxidant defenses were tested in blood samples from RA, Type I and Type II DM patients. Erythrocytes (RBC) showed increased susceptibility to lipid peroxidation in both DM and RA, whereas basal oxidation was higher only in RA. Both pathologies showed elevated oxidized LDL concentration and decreased RBC membrane fluidity. In DM, plasma TRAP was decreased and showed an inverse correlation RBC oxidative damage. On the other hand, TRAP was normal in RA. In both pathologies, TRAP was not correlated with oxidized LDL. We can conclude that the peroxidative damage would occur through a complex mechanism, with different factors involved in both illnesses. In this way, an association between these diseases could not be established.

1. INTRODUCTION

In recent years, a great number of studies have investigated the possible role of Reactive Oxygen Species (ROS) in the etiology and pathogenesis of several diseases [1]. In healthy subjects, significant oxidative damage is prevented by a very complex and efficient antioxidant system, constituted by a number of interrelated antioxidant compounds and enzymes. The mechanisms and effects of lipid peroxidation in biological systems have been described in the development of Rheumatoid Arthritis (RA) [1-5] and Diabetes Mellitus (DM) [6-11].

In DM patients, it has been suggested that low-density lipoprotein (LDL) oxidation contributes to LDL accumulation and, therefore, to an accelerated development of arteriosclerosis [12]. Moreover, the overproduction of ROS due to persistent hyperglycemia produces oxidative protein damage, which would be related to the pathogenesis of diabetic's complications [13].

It has been reported that ROS play a role in perpetuating local inflammatory reactions in the joint in RA, where oxidative modification of proteins, lipids, and lipoproteins contribute to bone cell activation. Synovial fluid from rheumatoid patients contains products of lipid peroxidation that correlate with disease severity as was measured clinically and in experimental systems [8, 10, 12-14].

Abnormal erythrocyte membrane functions and properties related to oxidative stress have also been reported in RA

[12-15] and in both, insulin-dependent diabetes mellitus (Type I DM) and non insulin-dependent diabetes mellitus (Type II DM) [16-18]. Increase in malondialdehyde (MDA) concentration and decrease in one or more enzymatic and enzymatic antioxidant defenses such as vitamin E, beta-carotene, glutathione peroxidase, superoxide dismutase, and catalase activity have been reported in these diseases [2, 5, 11, 16]. Decreased values of total reactive antioxidant potential (TRAP) were found in RA and DM [19, 20].

Furthermore, there are many evidences that chronic systemic inflammation predispose to development of DM. Higher levels of insulin resistance in patients with inflammatory arthritis and an association between high CRP (C Reactive Protein) concentrations and insulin resistance, was reported by Dessein *et al.* [21]. Besides, increased concentrations of TNF-alpha (tumor necrotic factor) were found in acute and chronic inflammatory conditions (e.g., trauma, sepsis, infection, rheumatoid arthritis), in which a shift toward a pro-atherogenic lipid profile and impaired glucose tolerance occurs. TNF-alpha, produced by cells of the immune system and by adipocytes, may provide the link between inflammation and insulin sensitivity [22].

It is known that soluble interleukin-2 receptor (sIL2r), a marker of T-cell activation, is elevated in inflammatory processes, such as RA. Wadwa *et al.* have reported that elevated plasma sIL2r is associated with coronary artery calcification progression in Type 1 diabetics [23]. This finding supports the hypothesis that processes that lead to the development of the pathological cardiovascular arteriosclerosis associated with both diseases are closely related.

As we described above there are many evidences that high ROS concentrations and decreased antioxidant defenses

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are present in AR and DM. Also some stress or inflammatory signaling was found in both diseases, but an association between these pathologies has not yet been ruled out.

We designed the present study with the aim to determine whether antioxidant defenses and oxidative stress markers are similarly affected in patients with DM and in those with RA in order to point new evidences of a possible relationship between these pathologies.

In order to obtain these objectives, we evaluated the interrelationships among basal RBC oxidative damage, RBC susceptibility to oxidative stress, oxidized LDL concentration, RBC membrane fluidity, plasma antioxidant potential and routine biochemical parameters in blood samples from RA, Type I and Type II DM patients.

2. MATERIALS AND METHODOLOGY

2.1. Patients

DM - Studies were performed on 20 patients (10 females, 10 males) with Type I DM and 20 patients (10 females, 10 males) with Type II DM, with mean \pm SD age ranges of 58 ± 3 years and 60 ± 3 years, and treated with insulin and sulphonylureas in doses according to the needs of metabolic compensation, respectively. Their treatments followed the standards for clinical management of DM. At the time of the study none of the patients were taking glucocorticoids. Diabetes had been diagnosed between 8 and 20 years previously to the present study. All the patients were following a nutritionally controlled diet. None of the patients present any clinical symptoms of neuropathy, nephropathy or retinopathy.

RA - The study was performed on 20 female patients with mean age range of 58 ± 4 years. All the patients satisfied the 1987 American College of Rheumatology criteria for the diagnosis of RA and had rheumatoid factor positive. Those patients who had been receiving corticosteroid agents, and/or penicillamine for at least 3 months before the consultation were excluded from this study. However, patients who had been receiving ordinary dosages of non-steroidal anti-inflammatory drugs (NSAID) were not excluded. Patients presented common characteristics of long evolution of the disease (varying from 10 to 16 years) and they were not in reactive phase. None of RA patients were following any dietary advice.

Controls - Thirty healthy donors, whose ages and sex (proportion) matched with those of the patients, served as controls (20 females, 10 males; average age range 57 ± 5 years).

None of the patients or controls was a smoker or consumed alcohol or had any other chronic disease.

All subjects gave their informed consent for the study. The procedures were in accordance with the ethical standards of the Helsinki Declaration of 1975.

2.2. Chemicals

Tert-butyl hydroperoxide (*t*-BOOH), sodium azide, sodium dodecyl sulfate, and *o*-phenanthroline were purchased from Sigma, thiobarbituric acid (TBA) from Fluka, and Potassium iodide from Merck. All other reagents were of ana-

lytical grade. Routine biochemical analyses were performed using high-performance standardized kits from Boehringer-Mannheim Argentina.

2.3. Samples

Blood samples with and without heparin as anticoagulant were withdrawn from both the patients and the controls after an overnight fast. Blood serum and plasma were immediately separated by centrifugation at 3000 rpm for 5 min. After plasma was removed, the RBC were washed thrice with phosphate buffer saline (PBS), pH 7.4. Packed RBC, plasma, and serum were immediately used.

2.4. Oxidative Treatment of the RBC and Quantification of Lipid Peroxidation

This technique was performed to evaluate basal oxidative stress and susceptibility to an external oxidation of RBC from patients and healthy blood donors. Packed RBC were suspended in PBS 50% (v/v) and these dilutions were controlled by the determination of the respective haematocrits. Samples were incubated at 37°C with a final concentration of 3 mM of *t*-BOOH in the presence of 2.5 mM sodium azide. Aliquots, taken at 0, 15, 30, 45 and 60 minutes, were deproteinized with 20% trichloroacetic acid (TCA) and centrifuged at 2000 rpm for 10 min. Supernatants were separated and added to a solution containing 10% sodium dodecylsulfate, 13.4 mM butyl hydroxy toluene in ethanol, and 0.7% TBA and incubated for 15 minutes in a boiling water bath. After cooling, absorbance at 532 nm was recorded using a Shimadzu UV 210A double-beam spectrophotometer. The results were obtained using a calibration curve with 1,1,3,3-tetraethoxypropane as the standard and were expressed as nmol TBARS/ml packed RBC [24, 25].

2.5. LDL Precipitation

The LDL was precipitated from an aliquot of serum by aggregation with sodium citrate buffer 64 mM, heparin 50,000 UI/l, final pH 5.11, and subsequent centrifugation at 4,000 rpm for 10 minutes. The lipids of the precipitate were extracted using a mixture of chloroform-methanol 2:1 containing 0.002% butyl hydroxy toluene [26]. Lipid hydroperoxide and phospholipid concentrations in this fraction were determined.

2.6. Peroxide Determination

Chloroform-methanol of the lipid extracts were evaporated under vacuum at a temperature below 25°C and the residues were dissolved with 50 μ l of absolute ethanol. The lipid hydroperoxide concentrations were measured as described in Cimato *et al.* [27]. Peroxidation index was expressed as nmol of hydroperoxides per mg of phospholipid.

2.7. Phospholipid Determination

Total phospholipid content was determined by the Stewart method [28]. The lipid extract in chloroform-methanol was dried in a bath at 37°C under a current of warm air, redissolved in chloroform, and added to a solution of ammonium ferrothiocyanate (27.03 g $\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$ + 30.4 g NH_4SCN in 1 liter of H_2O). The biphasic system was vigorously mixed for 1 minute and centrifuged for 10 minutes at

3000 rpm to facilitate phase separation. The chloroform phase was removed and its optical density was recorded at 472 nm in a Shimadzu UV 210 double-beam spectrophotometer.

2.8. Membrane Fluidity

RBC membrane fluidity was studied using electron spin resonance (ESR) spectroscopy through the determination of the order parameter (S). S gives a measure of the degree of structural order in the membrane, with values of S = 1 for a rapidly spin-label motion about only one axis and S = 0 for a rapid isotropic motion, an increase in S value denoting the effect of a decrease in the membrane fluidity. RBC were labeled according to the procedure described in Facorro *et al.* [29]. ESR spectra were recorded at 20°C using an X-band ESR Spectrometer Bruker ECS 106 (Berlin, Germany). The spectrometer settings were: center field 3445.00 G, sweep width 100.00 G, microwave power 20.0 mW, modulation frequency 50 KHz, modulation amplitude 0.2 G, conversion time 40.96 ms, receiver gain 5.00.10⁴, and resolution 1024 points.

2.9. TRAP of Plasma Samples

Plasma antioxidant capacity was evaluated as the protection provided to RBC by an aliquot of the plasma sample when it was added to a pool of normal RBC that was subjected to oxidative stress by *t*-BOOH, with reference to a control without plasma. The oxidative stress was then evaluated by TBARS production [29].

Washed packed RBC isolated from the blood pool of healthy donors were suspended in PBS 50% (v/v). Plasma samples were added so that a volume ratio of 3:1 (RBC 50%/plasma) was obtained. In samples used as controls that exhibit 100% peroxidation, PBS replaced plasma. Stress by *t*-BOOH and evaluation of TBARS production were performed in a manner similar to that described previously.

The TBARS production in the plateau of the oxidation curve (45 minutes) was used to estimate the Total Reactive Antioxidant Potential (TRAP), which was calculated using the following expression:

$$TRAP(\%) = 100 \left[1 - \frac{A_p(t) - A_p(t_0)}{A(t) - A(t_0)} \right]$$

where:

$A_p(t)$ = absorbance at 532 nm after 45 minutes of peroxidation in the presence of plasma.

$A_p(t_0)$ = absorbance at 532 nm without peroxidation ($t = 0$) in the presence of plasma.

$A(t)$ = absorbance at 532 nm after 45 minutes of peroxidation in the absence of plasma.

$A(t_0)$ = absorbance at 532 nm without peroxidation ($t = 0$) in the absence of plasma.

2.10. Statistical Analysis

Statistical analyses were performed using analysis of variance (ANOVA following post hoc Bonferoni's test) and Pearson correlation coefficient. All data were reported as mean \pm SD, and $p < 0.05$ was considered to be significant.

RESULTS

The biochemical parameters: glycemia, fructosamine, uric acid, triglycerides, creatinine, total cholesterol, HDL-cholesterol, LDL-cholesterol, and total lipids in diabetic patients, rheumatoid arthritic patients, and in the respective healthy controls are shown in Table 1. Statistically significant increase in the value of glucose ($p < 0.005$) and fructosamine ($p < 0.01$) was found in fasting diabetic patients (both Type I and Type II) with reference to the controls. No significant differences were found in the other biochemical parameters. In RA patients, all biochemical evaluations showed normal values. The hematological control routines showed normal values in both groups, DM and RA, so the data are not shown.

Table 1. Biochemical Parameters in Rheumatoid Arthritis, Diabetes and Control Groups

	Control (n=30)	DM Type I (n=20)	DM Type II (n=20)	RA (n=20)
Glucose (mg/dl)	82 \pm 9	146 \pm 9 *	240 \pm 19 *	88 \pm 11
Fructosamine (umol/dl)	180 \pm 17	378 \pm 20**	356 \pm 21**	190 \pm 17
Haematocrit (%)	40 \pm 5	44 \pm 3	45 \pm 2	38 \pm 2
Total Cholesterol (mg/dl)	195 \pm 11	210 \pm 13	232 \pm 40	195 \pm 13
LDL -Choles. (mg/dl)	120 \pm 13	141 \pm 11	130 \pm 23	138 \pm 12
HDL -Choles. (mg/dl)	49 \pm 4	47 \pm 5	56 \pm 9	51 \pm 4
Triglycerides (mg/dl)	127 \pm 36	142 \pm 15	161 \pm 46	118 \pm 25
Total Lipids (mg/dl)	680 \pm 200	710 \pm 200	795 \pm 115	630 \pm 52
Creatinin (mg/dl)	0.92 \pm 0.28	0.94 \pm 0.32	0.88 \pm 0.41	0.83 \pm 0.35
Uric Acid (mg/dl)	4.8 \pm 0.9	5.3 \pm 0.6	5.2 \pm 0.6	3.8 \pm 0.3

Results are expressed as mean \pm SD. Significant differences: * $p < 0.005$; ** $p < 0.01$.

Kinetics studies of TBARS production in RBC after oxidative treatment with *t*-BOOH were performed to evaluate the RBC susceptibility to an oxidative stress (Fig. 1). The TBARS concentration at time zero is an indicator of the basal oxidation of RBC. The erythrocytes of RA patients showed greater amount of basal oxidation products than those of the controls ($p < 0.01$), whereas no statistically significant differences were observed in DM patients (Type I and Type II) neither regard to the control nor between them. Both DM and RA erythrocytes showed an increased susceptibility to *t*-BOOH-induced oxidation, which was evidenced by the higher levels of TBARS after 30 minutes of incuba-

tion, with reference to the controls. In RA-RBC the increase in the lipid peroxidation products was higher compared with DM-RBC ($p < 0.01$). No significant differences were found between both types of DM.

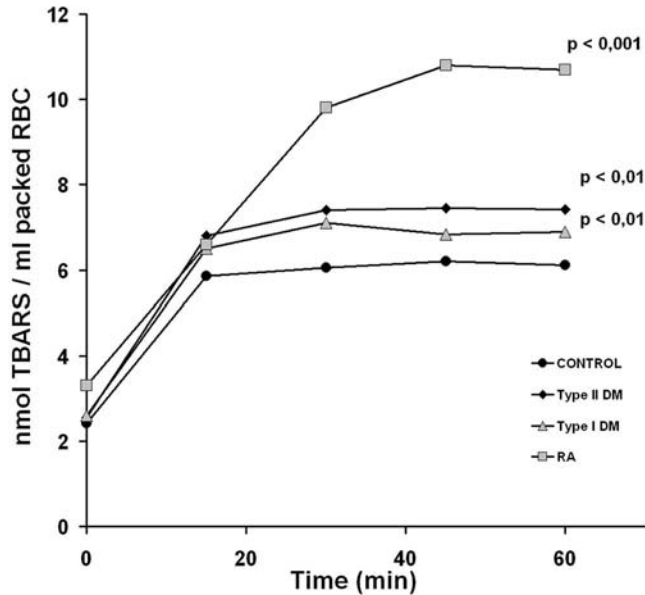


Fig. (1). Kinetics profile of oxidative stress in RBC treated with t-BOOH at 37°C. Oxidative products were measured in RBC of Type I DM (Δ) (n=20), Type II DM (\blacklozenge) (n=20), RA (\square) (n=20), patients and healthy controls (\bullet) (n=30), by the thiobarbituric acid assay. Results are expressed as nmol TBARS/ml packed RBC.

The lipohydroperoxides/phospholipids (nmol/mg) ratio in the LDL-fraction of the plasma lipoproteins was increased in DM patients with reference to the controls ($p < 0.05$ for both groups), with no statistically significant difference between Type I and Type II DM. RA patients showed a higher level ($p < 0.001$) of oxidized LDL compared with DM patients (Fig. 2).

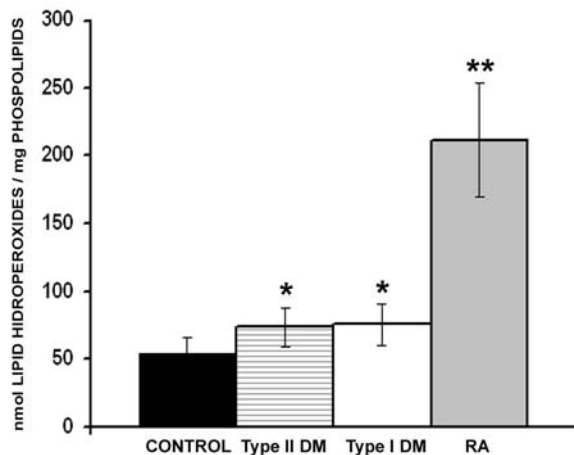


Fig. (2). Oxidized LDL concentration in plasma of Type I DM (n=20) (\square), Type II DM (n=20) (\equiv), RA (n=20) (\blacksquare) patients and healthy controls (n=30) (\blacksquare). Lipid hydroperoxides were measured in the LDL fraction and results were expressed as nmol lipid hydroperoxides / mg of phospholipids. Data are represented as the mean \pm SD. Statistically significant increases compare to controls: * $p < 0.05$ and ** $p < 0.001$

To evaluate changes in physical membrane during the oxidative process, membrane fluidity was estimated by the calculation of the order parameter (S). As shown in Fig. (3), this parameter increased in both DM and RA groups in a similar proportion ($p < 0.01$). As is known, an increase in the S value (higher membrane order) is the consequence of a decrease in the RBC membrane fluidity.

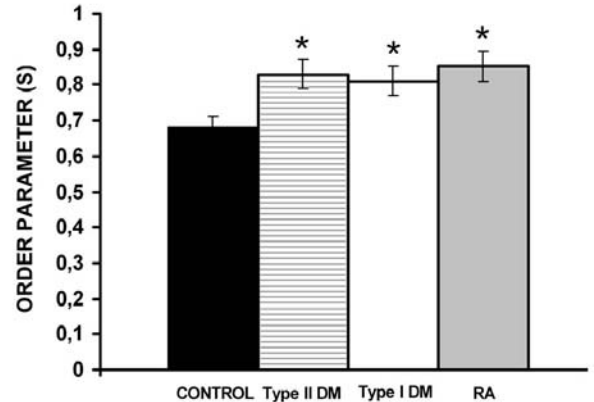


Fig. (3). Order parameter of RBC in Type I DM (n=20) (\square), Type II DM (n=20) (\equiv), RA (n=20) (\blacksquare) patients and healthy controls (n=30) (\blacksquare). RBC were labeled with 5-DSA and the order parameter S was calculated from its ESR spectrum. An increase in the value of S indicates a decrease in membrane fluidity. Data are represented as the mean \pm SD. Statistically significant increases compare to controls: * $p < 0.01$

As Fig. (4) shows, plasma TRAP of both Type I and Type II DM patients showed lower values compared with controls ($p < 0.001$), but no significant difference was found for TRAP between RA patients and health controls.

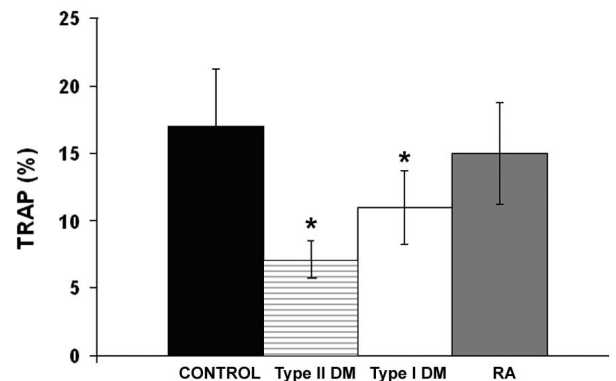


Fig. (4). TRAP of plasma of in Type I DM (n=20) (\square), Type II DM (n=20) (\equiv), RA (n=20) (\blacksquare) patients and healthy controls (n=30) (\blacksquare). RBC from a blood pool of healthy donors were incubated with t-BOOH in the presence and absence of plasma. TBARS were measured after 45 minutes of incubation. TRAP was calculated as the inhibition (%) of TBARS formation in the presence of plasma relative to the control without plasma. Data are represented as the mean \pm SD. Statistically significant decrease compare to controls: * $p < 0.001$

To find possible relationships between oxidative and biochemical parameters in both groups of patients, linear regression studies were carried out. In DM patients, the TRAP

and the RBC lipid peroxidation showed an inverse correlation ($r = -0.667, p < 0.05$), whereas in RA group, they did not show any correlation. Among biochemical parameters, TRAP showed an inverse correlation with fructosamine ($r = -0.735, p < 0.05$) in DM patients, directly with uric acid levels in both DM ($r = 0.435, p < 0.05$) and RA ($r = 0.548, p < 0.05$); and no correlation with oxidized LDL in both DM and RA.

DISCUSSION

During health, ROS production is low and lipid peroxidation is inhibited by the combined activities of the antioxidant systems present in the plasma. The loss of the normal oxidant-antioxidant equilibrium may either be due to a decreased antioxidant defense or to an increased generation of oxidants.

In DM, persistent hyperglycemia may lead to high production of oxygen-free radicals, which are generated in different processes: direct autoxidation of glucose [30], progressive non enzymatic glycosylation of proteins [8] and activation of enzymes that produces oxidant species and subsequent enhanced oxidative stress (NADPH oxidases, nitric oxide synthase, and xanthine oxidase) [31-33].

In RA, a chronic immune-inflammatory multisystem disease, the polymorphonuclear leukocytes are activated and ROS are generated in excessive amounts [34]. This enhanced oxidation plays a significant role in tissue damage and chronic inflammation process [4, 35]. In both pathologies, these increased ROS concentrations cause lipid peroxidation, leading to toxic damage of tissues. Previous papers reported, as an index of ROS production, high TBARS levels in plasma of patients with RA [2, 3, 5] and DM [9, 11]. In this paper we focus in the analysis of ROS effects on RBC. Our results showed that although patients with DM and RA have increased RBC susceptibility to induced oxidative stress, in RA this increase was higher. Besides, basal oxidative damage of RBC was only detected in RA patients. All these results indicated that the overproduction of ROS in RA was more important than that in DM.

The tissue damage caused by ROS must be controlled by several antioxidant mechanisms to avoid the cascade of oxidative chain reactions [36].

In both RA and DM patients, the decrease of the absolute values of the different antioxidant agents (vitamin E, vitamin C, superoxide dismutase, catalase, glutathione reductase, transferrin, ceruloplasmin, etc.) has been reported by many authors [2, 13]. The plasma levels of each antioxidant can be measured separately, but these measures do not truly reflect the susceptibility of all the system to oxidative injury. The measurement of TRAP gives an idea of the total potential of the plasma antioxidant system rather than the relative efficiency of each individual antioxidant [37]. In a previous article [19], we reported significant decrease of TRAP levels in Type II DM patients. In the present article, our results indicated that TRAP was also decreased in Type I DM, without significant difference between Type II and Type I DM. It is known that among the biochemical control parameters, the uric acid concentration plays an important role in TRAP value [38]. Thus, it could be considered that the decrease of TRAP could be related with a low uric acid level. The TRAP

values showed a direct correlation with the plasmatic levels of uric acid in DM patients, but there was no significant difference in the plasmatic concentration of the uric acid in DM groups with reference to healthy controls (Table 1). The results of this study suggest that despite being an important component of the plasmatic antioxidant defense system, uric acid is not responsible for the decrease in TRAP.

Many articles [2, 3, 5, 34] have reported deficiencies in some of the individual plasma antioxidants (vitamin E, riboflavin, superoxide dismutase, and glutathione reductase) in RA patients, but only few have reported a decrease in the total plasmatic antioxidant capacity [18, 39]. On the contrary, in this study, no significant decrease was found in TRAP of plasma samples of RA patients. Although free radicals that were generated within the synovial fluid may escape to the external medium, this process appears to be insufficient to produce a severe loss of the total plasmatic antioxidant capacity but sufficient for producing oxidative damage.

Furthermore, it was found that the loss of antioxidant capacity is statistically associated with an increment of the oxidative damage of RBC and high levels of fructosamine in DM patients. It is supposed that the extended exposure to hyperglycemia and the consequent non enzymatic, post transitional modification of proteins [40] could be the reason for the development of most diabetic complications. If the glycosylation process modified proteins of the plasma antioxidant system, it could be a cause of the leak of antioxidant capacity found in the diabetic patients. Whereas, the results of this study in patients with RA suggest that erythrocyte oxidative damage could exist under normal plasma total antioxidant status. The decreased membrane fluidity of RBC and higher susceptibility to oxidative stress could be linked to a redox alteration associated with RA, which is not reflected by plasma TRAP. So, it could be considered that the increased susceptibility of erythrocytes to oxidative stress may be mediated through a TRAP-dependent mechanism in Type I and Type II DM but not in RA patients.

Diabetes is a disease accompanied by an increase in the risk of pathological cardiovascular arteriosclerosis. In recent years, many published reports showed that nonspecific oxidation of LDL is a factor involved in the development of atherogenic plaque [12]. The link between modified LDL and atherogenesis is based, in part, on studies showing that these lipoproteins, or their components, could promote the adhesion of monocytes to the endothelial cells, chemotaxis, cytotoxicity, modulation of the expression of growth factors, and the capability for the sequestration of macrophages. Furthermore, there are evidences suggesting that subjects with RA are characterized by acceleration of atherosclerotic process of arterial wall that could be the result of persistent endothelial dysfunction induced by chronic inflammation and immune deregulation [41, 42]. Oxidized LDL species appear to be present in rheumatoid synovial fluid [43]. Wada *et al.* found that autoantibodies against oxidized LDL appear to be associated with the degree of carotid atherosclerosis in patients with RA and are independent of traditional risk factors for atherosclerotic diseases [44].

This study showed an increase in LDL-lipohydroperoxides levels and a decrease in RBC membrane fluidity in both DM and RA patients. The results are in agreement with pre-

vious papers, which report that the nonspecific oxidation of LDL increases the rigidity of erythrocyte and plasma membranes of endothelial cells [18].

It could be assumed that the increase in the oxidation of LDL in DM patients would be related to the decrease of the antioxidant plasmatic capacity, but we have found by linear regression studies that these two parameters were directly related neither in DM nor in RA. Also, very high levels of oxidized LDL but normal TRAP were found in RA patients. This could indicate that the protective mechanism against oxidative damage of LDL would be independent of the antioxidant capacity of the plasma in both pathologies.

The results in both types of diabetic (Type I and Type II) showed no significant differences in the RBC basal oxidation, RBC susceptibility to oxidative stress, LDL peroxidation, and TRAP values. These findings suggest that the mechanisms that promote the oxidative changes and/or accelerate the secondary complications of diabetes are similar in Type I and Type II, regardless of the factors that lead to the illness.

Could the decrease in plasmatic defense be the primary reason of oxidative damage in diabetic patients? In this study, we found that TRAP and RBC peroxidation showed an inverse correlation. So, it would indicate that the decrease in the TRAP value would be responsible for the loss of the normal oxidative balance and of the increase in RBC membrane lipoperoxidation susceptibility. On the other hand no correlation was found between TRAP and lipoperoxidation of RBC in RA patients. This would indicate that the overproduction of ROS was responsible for the oxidative damage, without evident modifications of the plasmatic antioxidant system.

CONCLUSIONS

As we have discussed, the magnitude of this damage and its relationship with the antioxidant system were not the same in these pathologies. So taking into account the results obtained in this study we can conclude that although ROS would have an important role in the development of the oxidative damage of RBC and LDL in both DM and RA, this peroxidative damage would occur through a complex mechanism with different factors involved in both diseases. Further investigations will be required to find out if a real association between RA and DM could be established.

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