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Parameters of Oxidative Stress Status in Healthy Subjects: Their Correlations and Stability After Sample Collection

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It has been proposed that sample storage may have some influence on the parameters of oxidative stress status (OSS) in biological fluids. We measured four important OSS parameters in plasma of 23 healthy subjects and repeated the measurements in the same samples kept at –70°C after different time intervals. Hydroperoxides and total antioxidant capacity (TAC) were determined by ferrous ion oxidation in presence of xylenol orange (FOX) and ferric reducing antioxidant power (FRAP) assays, respectively. Sulfhydryls and carbonyls were measured spectrophotometrically. In fresh samples, OSS seemed to increase with age and relatively good correlations were found among different parameters. The mean values of hydroperoxides (6.08 μM), TAC (0.334 mM Trolox equivalent), and sulfhydryls (0.562 mM) in fresh samples did not show any significant change after 1, 7, and 30 days of storage. Mean carbonyl concentration determined after 1 day storage (2.0 nmol/mg protein) did not change after 30 days. However, extents of changes in hydroperoxide concentrations varied considerably from one individual to another, even after 1 day. A similar phenomenon was observed in TAC, but after 7 days. We suggest measuring hydroperoxides in fresh samples and TAC maximally after 1 week. Sulfhydryls and carbonyls showed more stability and can be measured at least 1 month after sample collection. J. Clin. Lab. Anal. 20:139–148, 2006.

Key words: oxidative stress; storage; hydroperoxide(s); total antioxidant capacity; sulfhydryl(s); carbonyl(s)

INTRODUCTION

Oxidative stress has been implicated to have an important pathophysiological role in several diseases like atherosclerosis (1), Alzheimer's disease (2), Parkinson's disease (3), and several other pathologies (4–6). To evaluate the degree of oxidative stress that a human is undergoing, i.e., the oxidative stress status (OSS), several methodologies have been proposed that evaluate the oxidation of different macromolecules, including lipids, proteins, and DNA (7) or measure the antioxidant capability in biological fluids and tissues (8).

It has been proposed that some markers of oxidative stress are not stable and sample storage may have some influence on analyses results (9–11). This can be due to ex vivo generation of oxidation products (12) or degradation of antioxidants. These effects can be decreased by analyzing samples within a few hours after sample collection, but it may not be possible in large

Abbreviations: DNPH, 2,4-dinitrophenylhydrazine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); FOX, ferrous ion oxidation in presence of xylenol orange; FRAP, ferric reducing antioxidant power; OSS, oxidative stress status; ROOH, hydroperoxide; SH, sulfhydryl; TAC, total antioxidant capacity.

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studies. Therefore, understanding the stability of parameters of OSS seems to be an important issue for the precise evaluation of OSS in individuals. However, a few authors have only sporadically addressed this.

We measured four common biomarkers of OSS in plasma of healthy individuals and evaluated the effect of sample storage on them. These parameters were:

- Lipid hydroperoxides (ROOHs) measured by the ferrous ion oxidation in presence of xylenol orange (FOX) assay (9).
- Total antioxidant capacity (TAC) determined by the ferric reducing antioxidant power (FRAP) assay (13,14).
- Sulfhydryl (SH) groups measured by using the reagent 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (15).
- Carbonyl content of proteins determined by using 2,4-dinitrophenylhydrazine (DNPH) (16,17).

These methods evaluate different aspects of OSS; FOX assay measures the degree of lipid peroxidation, FRAP assay evaluates the capacity of plasma at competing with free radicals, SH and carbonyls methods give an estimation of the extent of protein oxidation.

Furthermore, we evaluated the effect of the age of the individuals on the parameters of OSS in fresh samples and also correlations among different parameters.

MATERIALS AND METHODS

Reagents

Ammonium ferrous sulfate hexahydrate (cat. No. 09719), bovine serum albumin (BSA) (cat. No. A-3294), Bradford reagent (cat. No. B-6916), butylated hydroxytoluene (cat. No. B-1378), cumene hydroperoxide 88% (cat. No. 51329), 2,4-dinitrophenylhydrazine (DNPH, cat. No. 42210), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB, cat. No. D-8130), ethylenediaminetetraacetic acid (EDTA, cat. No. 25.404-5), ferric chloride hexahydrate (cat. No. 44944), glutathione (cat. No. G-4251), guanidine hydrochloride (cat. No. 50940), hydrogen peroxide (cat. No. H-0904), sodium phosphate dibasic (cat. No. S-7907), sodium phosphate monobasic (cat. No. S-9638), sulfuric acid 25% (cat. No. 84736), trichloroacetic acid solution 6.1 N (cat. No. T-0699), trifluoracetic acid (cat. No. 30.203-1), triphenylphosphine (TPP, cat. No. T8,440-9), tris[hydroxymethyl]aminomethane (trizma base) (cat. No. T-6066), 2,4,6-tris(2-pyridil)-s-triazine (cat. No. T 1253); Trolox ((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) (cat. No. 238813); xylenol orange (cat. No. 33825) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA; http://www.sigma-aldrich.com). Ethanol 99.8% (cat. No. 414608), Ethyl acetate (cat. No. 448256), hydrochloric acid 37% (cat. No. 403871) and methanol (HPLC-grade, cat. No. 412383) were purchased from Carlo Erba (Rodano Milano, Italy; http://www.carloerbareagenti.com). Acetic acid glacial (cat. No. A9014), potassium dihydrogen phosphate (cat. No. 4873) and sodium acetate trihydrate (cat. No. 6267) were obtained from Merck (Darmstadt, Germany; http://www.merck.de). Deionized water was prepared with MilliQ4 (Millipore, Molsheim, France; cat. No. ZD2023074).

Subjects

Twenty-three healthy volunteers (eight males and 15 females) aged 21–46 years (mean 31.5) were enrolled in the study according to the principles of the Declaration of Helsinki. All participants were in good health and were receiving no medications or dietary supplementation of vitamins or antioxidants. Five of them were smokers.

Blood Samplings and Study Design

Venous blood was collected into EDTA (for carbonyls and sulfhydryls [SH] measurements) or sodium citrate-coated (for hydroperoxides [ROOHs] and total antioxidant capacity [TAC] measurements) tubes after overnight fasting between 9 and 10 AM. Plasma was separated within 30 min by centrifugation at 1500*g* for 10 min at 4°C. Fresh samples were analyzed within 3 hr of sample collection. The rest of plasma samples were divided into aliquots and stored at –70°C for measurements performed later. Each aliquot was used only once for one measurement. ROOHs, TAC, and SH groups were measured in fresh plasma samples (day 0) and repeated on days 1, 7, and 30. The first measurements of carbonyls were performed on day 1 and repeated on day 30.

Determination of Hydroperoxides in Plasma by FOX Assay

ROOHs were determined by the FOX assay, as described by other authors (9,18) with minor modifications. The glassware was cleaned with warm concentrated nitric acid before use. FOX solution was made mixing two solutions A and B. Solution A was prepared dissolving butylated hydroxytoluene in pure methanol at 4.4 mM. Solution B was consisted of xylenol orange 1 mM and ammonium ferrous sulfate 2.5 mM dissolved in sulfuric acid 250 mM. Working solution was prepared by mixing A and B solutions at a proportion of 9:1, respectively. Working solution was kept at 4°C for a maximum of 2 weeks. The molar extinction coefficient for each working solution was determined by means of

a calibration curve with different concentrations of hydrogen peroxide in the range 0.1–25 μM. The concentration of hydrogen peroxide was measured spectrophotometrically (ε_{240}: 43.6) (19).

For measuring ROOHs in plasma, 90 μL of plasma was mixed with 10 μL triphenylphosphine (TPP) 20 mM in methanol (in quadruplicate) or with 10 μL of methanol (in quadruplicate) in 1.5-mL microcentrifuge vials. The vials were vortex-mixed every 10 min and incubated at room temperature in the dark for 30 min before adding 900 μL of FOX solution. The samples then were again incubated for 1 hr at room temperature in the dark, being vortex-mixed every 10 min, and centrifuged at 16,000 *g* for 7 minutes. Absorbance of the supernatants was determined at 560 nm by an spectrophotometer (Hewlett-Packard 8452AX; Hewlett-Packard, Palo Alto, CA, USA; http://www.hp.com). The absorbance of the samples treated with TPP was subtracted from non-treated samples to calculate the concentration of ROOHs.

The intraassay coefficient of variation ($CV_{intraassay}$) was calculated by measurements performed on a pool of plasma (n = 10). Cumene hydroperoxide diluted in methanol at the concentration of 6 μM was measured on each day of experiment to calculate the inter-assay coefficient of variation ($CV_{interassay}$).

Determination of the Total Antioxidant Capacity of Plasma by FRAP Assay

The FRAP assay was performed according to Benzie and Strain (13) with minor modifications. To prepare the FRAP solution, 10 mL of acetate buffer 300 mM, adjusted to pH 3.6 by the addition of acetic acid, were mixed with 1 mL of ferric chloride hexahydrate 20 mM dissolved in distilled water and 1 mL of 2,4,6-tris (2-pyridyl)-s-triazine (TPTZ) 10 mM dissolved in HCl 40 mM.

Ten microliters of plasma were added to 1.8 mL of a freshly prepared FRAP solution in borosilicate test-tubes in quadruplicate and the absorbance was measured at 593 nm after 6 min of incubation at room temperature against a blank of acetate buffer. Trolox at two different concentrations of 0.2 and 0.4 mM was used to obtain a calibration curve on each day of experiment. The total antioxidant capacity (TAC), expressed as the "Trolox equivalent concentration," was then calculated by dividing the absorbance change (A_6–A_0) by the slope of the calibration curve. The sum of the absorbance of plasma diluted 180 times and the absorbance of FRAP solution was considered as A_0.

$CV_{intraassay}$ was calculated by measurements performed on a pool of plasma (n = 9). Slopes of the calibration curves were used to calculate $CV_{interassay}$.

Determination of Sulfhydryls in Plasma

SH groups in plasma were measured by a spectrophotometric method using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB or Ellman's reagent) (15) with minor changes. Sixty microliters of plasma were mixed with 200 μL of Tris-EDTA buffer (Tris base 0.25 M, EDTA 20 mM, pH 8.2) in 1.5-mL microcentrifuge tubes in quadruplicate. Subsequently, 15 μL of DTNB 10 mM in methanol were added to the samples except one blank to which the same quantity of methanol was added. After 15 min of incubation at room temperature in the dark, 1 mL of methanol was added to all samples and they were centrifuged at 3,000*g* for 10 min after another 5 min of incubation. The absorbance of the supernatant was subsequently measured at 412 nm by a spectrophotometer and the concentrations of SH groups were calculated (ε: 13,600 $Cm^{-1} M^{-1}$).

A freshly prepared plasma pool was used to calculate the $CV_{intraassay}$ (n = 10). A plasma pool divided in aliquots and kept at −20°C was used to calculate the $CV_{interassay}$. It was thawed shortly before analysis each day. Furthermore, a fresh solution of glutathione at a concentration of 0.5 mM dissolved in phosphate buffer 50 mM pH 7 was also used to calculate $CV_{interassay}$.

Determination of Carbonyl Groups in Plasma

Carbonyl groups in plasma were determined using the reagent 2,4-dinitrophenyl hydrazine (DNPH) as described by other authors (16,17) with minor changes. Fifteen microliters of plasma were mixed with 400 μL of DNPH 15 mM in HCl 2 N in 1.5-mL microcentrifuge tubes in quadruplicate. Four hundred microliters of HCl 2 N was added to 15 μL of plasma in duplicate as blanks. The samples were each vortex-mixed for 10 min and after 1 hr of incubation at room temperature in the dark, 1 mL of trichloroacetic acid (TCA) 14% (w/v) was added. After another 10 min of incubation all the samples were centrifuged at 11,000*g* for 5 min. Then the supernatant was discarded and precipitates were washed three times with 1 mL of a mixture of ethanol:ethyl acetate, 1:1 (v/v) and centrifuged after 10 min. Finally, the protein precipitates were dissolved in 900 μL of guanidine hydrochloride 6 M in KH_2PO_4 20 mM adjusted to pH 2.3 with diluted trifluoroacetic acid. Subsequently, the samples were placed in a water bath at 37°C for 1 hr and then incubated for another 2 hr in the dark at room temperature. The absorbance was measured at 360 nm with a spectrophotometer against guanidine solution as blank. The concentration of carbonyls was calculated using a molar extinction coefficient (ε) of 22,000 (16). Protein concentrations in samples were determined by the Bradford assay (20) modified by Macart and Gerbaut (21). Measurements

were performed by a microplate reader (Bio-Rad 3550; Bio-Rad, Hercules, CA, USA; http://www.bio-rad.com) at 595 nm and calibration curves were prepared with bovine serum albumin as standard.

A freshly prepared pool of plasma was used to calculate the $CV_{intraassay}$ (n = 10). Another pool of plasma divided in aliquots and kept at –20°C was used to calculate the $CV_{interassay}$. It was thawed shortly before analysis each day.

Statistics

Comparisons between day 0 and days 1, 7, and 30 were performed by paired Student's *t*-test using the program SigmaStat version 3.00 for Windows (SPSS Inc., Chicago, IL, USA). Regression analyses were performed using the same software.

RESULTS

Hydroperoxides in Plasma

The molar extinction coefficients of FOX solutions were in the range 38,000–42,000.

The mean concentrations of ROOHs on day 0 ($6.08 \pm 2.68\,\mu M$), day 1 ($5.65 \pm 2.31\,\mu M$), day 7 ($5.33 \pm 2.70\,\mu M$), and day 30 ($5.15 \pm 1.99\,\mu M$) showed a slight decrease in time, although these changes were not statistically significant (Fig. 1A). However, large differences were observed between individual changes. There was a week correlation between fresh samples and the samples stored for 1 day ($r = 0.668$, $P = 0.025$). This correlation became even weaker after 7 and 30 days (Table 1). Similarly, the range of individual changes and the mean of absolute values of individual changes showed a variability, which increased considerably in time (Table 1).

The intra- and interassay coefficients of variation ($CV_{intraassay}$ and $CV_{interassay}$) were calculated as described in Materials and Methods and were 12.8% (n = 10) and 16.8% (n = 20), respectively.

Total Antioxidant Capacity of Plasma

The mean concentration of TAC remained constant and no significant change was observed among day 0 (0.334 ± 0.083 mM), day 1 (0.314 ± 0.070 mM), day 7 (0.344 ± 0.087 mM), and day 30 (0.332 ± 0.065 mM) (Fig. 1B).

Individual sample changes did not show considerable variability after 1 and 7 days and the correlation between fresh samples and days 1 and 7 measurements were very good ($r = 0.923$, $P < 0.001$ and $r = 0.896$, $P < 0.001$, respectively). The measurements after 30 days showed a slightly higher variability and the correlation with fresh samples was weaker (Table 1).

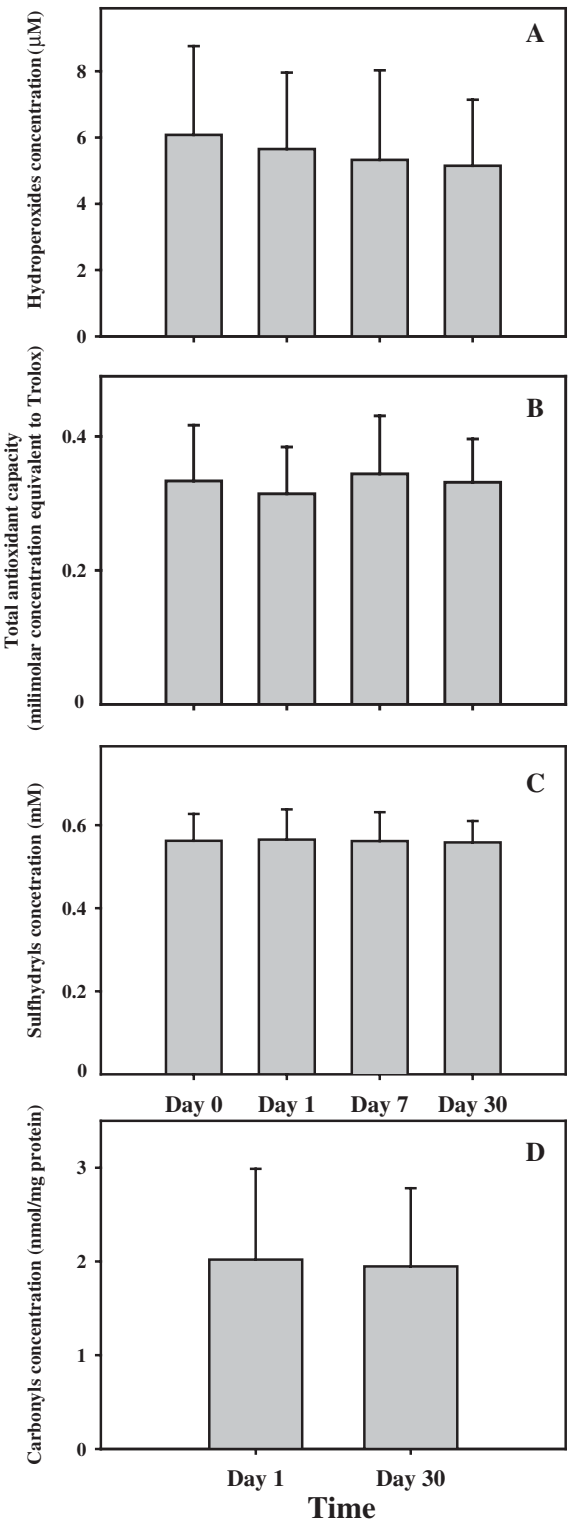

Fig. 1. Determination of hydroperoxides (**A**), total antioxidant capacity (**B**), sulfhydryls (**C**), and carbonyls (**D**) in plasma. Measurements were performed on fresh plasma samples of healthy individuals (day 0) and repeated on aliquots of the same samples stored at –70°C after 1, 7, and 30 days. Differences between day 0 and days 1, 7, and 30 were not statistically significant in any of the four figures. Values represent means and error bars show standard deviation; n = 11 in A and n = 23 in B–D.

TABLE 1. Comparison among measurements performed on fresh samples (day 0) and those carried out after 1, 7, and 30 days*

	Day 0 vs. day 1			Day 0 vs. day 7			Day 0 vs. day 30[d]		
	Correlation (r (p))[a]	Range of changes (%)[b]	Mean of absolute values (%)[c]	Correlation (r (p))[a]	Range of changes (%)[b]	Mean of absolute values (%)[c]	Correlation (r (p))[a]	Range of changes (%)[b]	Mean of absolute values (%)[c]
Hydroperoxides	0.668 (0.025)	−54.8 to +79.2	34.2	0.158 (0.685)	−57.2 to +155.5	58.6	0.083 (0.844)	−59.3 to +181.8	58.1
Total antioxidant capacity	0.923 (<0.001)	−18.0 to +14.5	8.7	0.896 (<0.001)	−10.6 to +22.2	10.5	0.729 (<0.001)	−22.7 to +31.3	15.8
Sulfhydryls	0.758 (<0.001)	−13.6 to +13.8	6.9	0.753 (<0.001)	−10.0 to +19.6	7.6	0.705 (<0.001)	−18.2 to +12.8	6.9
Carbonyls	–	–	–	–	–	–	0.714 (<0.001)	−37.9 to +50.6	22.8

*Four parameters of oxidative stress were measured in fresh samples (except for carbonyl, which were measured after one day) and repeated on aliquots of the same samples stored at −70°C at different time intervals.
[a]Regression analyses were performed between results of day_x and fresh samples (day_0).
[b]Range of individual sample changes ((day x − day 0)/day 0 × 100).
[c]Mean of absolute values of individual changes.
[d]Data of carbonyls represent comparison between day 1 and day 30.

$CV_{intraassay}$ and $CV_{interassay}$ were calculated as described in Materials and Methods and were 6.7% (n = 9) and 6.8% (n = 40), respectively.

Sulfhydryls in Plasma

The mean concentration of SH groups on day 0 (0.562 ± 0.065 mM) did not show any significant change after 1 (0.565 ± 0.072 mM), 7 (0.561 ± 0.069 mM), and 30 (0.558 ± 0.051 mM) days (Fig. 1C).

Changes in individual samples showed the lowest variabilities. Correlations between different days were acceptable and remained almost constant even after 30 days (Table 1).

$CV_{intraassay}$ was 1.3% (n = 10). $CV_{interassay}$ was calculated as described in Materials and Methods and was 5.8% (n = 10) for pool of plasma and 6.0% (n = 10) for glutathione at 0.5 mM concentration.

Carbonyls in Plasma

The mean concentration of carbonyls on the day 1 (2.0 ± 1.0 nmol/mg protein) did not show any significant change after 29 days (1.9 ± 0.8 nmol/mg protein) (Fig. 1D). There was a fairly good correlation between the two measurements. Individual changes after 29 days showed some variability, but the mean of absolute values of changes (22.8%) was not so high.

$CV_{intraassay}$ and $CV_{interassay}$ were calculated as described in Materials and Methods and were 14.1% (n = 10) and 19.3% (n = 11), respectively.

Correlations

Positive and strong associations were observed between the age of the subjects and their ROOHs and carbonyls concentrations, while TAC and SHs showed inverse correlations with age (Table 2).

Regression analyses between different assays are presented in Fig. 2. Positive associations were observed between TAC and SHs and between ROOHs and carbonyls, while inverse correlations were found in all other combinations. TAC and carbonyls did not seem to have a good correlation.

TABLE 2. Correlations of different parameters of oxidative stress in fresh plasma of healthy subjects with their age

	Correlation with age (r)
Hydroperoxides	0.765 ($p = 0.006$, n = 11)
Total antioxidant capacity	−0.342 ($p = 0.129$, n = 23)
Sulfhydryls	−0.580 ($p = 0.007$, n = 23)
Carbonyls	0.661 ($p = 0.0015$, n = 23)

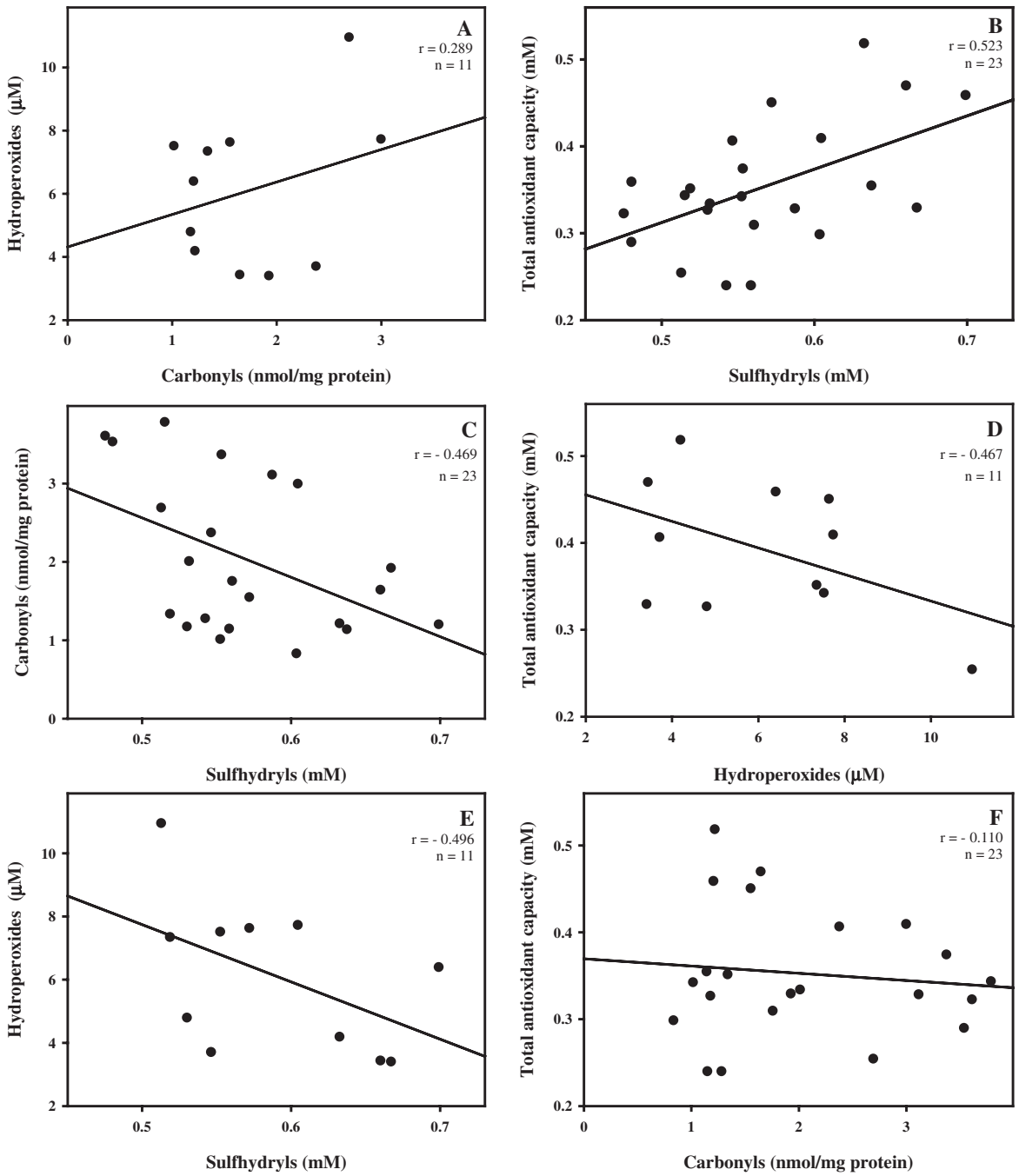

Fig. 2. Correlations among different parameters of oxidative stress status in plasma. Hydroperoxides (μM), total antioxidant capacity (millimolar concentration equivalent to Trolox), sulfhydryls (mM), and carbonyls (nmol/mg protein) were measured in fresh plasma samples of healthy individuals. Values represent the mean of quadruplicate measurements of individual plasma samples.

DISCUSSION

Effects of sample storage on concentrations of four important biomarkers of oxidative stress status were evaluated in plasma samples of 23 healthy adult subjects (Fig. 1; Table 1).

The FOX assay proposed by Nourooz-Zadeh et al. (9) is a simple spectrophotometric method that represents an alternative to more sophisticated and cumbersome methodologies (22) for measurement of hydroperoxides (ROOHs) in biological fluids. This assay is based on the reaction of lipid ROOHs with Fe^{2+} and its oxidation to

Fe^{3+} under acidic conditions, which is in turn determined spectrophotometrically by measuring the absorbance of its colored complex with xylenol orange at 560 nm. The measured signal is authenticated by prior reduction of plasma ROOHs with triphenylphosphine (TPP), a selective ROOH reductant. This method has been applied to measure ROOHs in different pathologies like diabetes (23,24), systemic lupus erythematosus (25), and familial hypercholesterolemia (19).

Our results showed that there was a slight decrease in the mean concentration of ROOHs in time (Fig. 1A). Although this change was not statistically significant, there were large interindividual differences in the loss of ROOHs after storage. For example, after 30 days of storage at –70°C the concentration changes varied from –59.3% to +181.8% compared to the first analyses (Table 1). There was a weak correlation between ROOHs concentrations of days 0 and 1 ($r = 0.668$, $P = 0.025$), while there was no correlation between the measurements of fresh samples and after 7 ($r = 0.158$, $P = 0.685$) and 30 ($r = 0.0834$, $P = 0.844$) days. Therefore, our results indicate that samples stored at –70°C undergo considerable changes compared to fresh samples in their ROOHs content evaluated by FOX method.

In one study, it was shown that plasma samples with or without butylated hydroxytoluene had large interindividual differences in ROOHs content changes after 6 and 60 weeks of storage at –70°C (26). However, the samples were not measured earlier than 6 weeks in this study. Our results indicate that there is a time-dependent variation of samples beginning from the first day of storage, evidenced by the weak correlation between measurements of day 0 and day 1 ($r = 0.668$, $P = 0.025$). However, determinations on the first day seemed to be much closer to fresh samples as compared to measurements performed later (Table 1). In another study it was observed that an exogenous ROOH (15(S)-HPETE, which is an eicosatetraenoic acid hydroperoxide) added to plasma was stable only for 2 weeks at –70°C (27). The situation may be different for biological samples, because different ROOHs may have different stabilities (28).

Mean plasma ROOH concentrations in our subjects ($6.08 \pm 2.68\ \mu M$) appeared to be slightly higher than studies that reported 3.76 μM (24) and 4.1 μM (23) in healthy subjects, but lower than another article in which a concentration of 8.47 μM was reported (26).

FRAP is a simple and reliable colorimetric method commonly used for measuring the TAC of biological fluids (13). This assay evaluates the capacity of a sample to reduce ferric ions inside a complex with 2,4,6-tripiridyl-s-triazine (TPTZ) to ferrous ion. During this process a blue color is developed and subsequently measured at 593 nm (13). This assay has been widely used for nutritional studies (29–31) and the evaluation of oxidative stress status in various pathologies (32,33). However, the effects of sample storage on FRAP values have not been addressed by most authors.

Our results showed that the mean levels of TAC did not have any significant changes after different time intervals (Fig. 1B). Individual changes in FRAP values showed larger differences after 30 days compared to those after 1 and 7 days and varied from −22.7 to +31.3% compared to fresh determinations. When correlations between fresh sample measurements and days 1, 7, and 30 were evaluated, a gradual decrease in correlation coefficient was observed (Table 1). This fact indicates that although there is an acceptable correlation still after 30 days, the precision decreases moderately after 30 days compared to earlier measurements. This point emphasizes the importance that samples should be analyzed after a maximum of 1 week to have a higher assay precision.

The mean FRAP value of fresh plasma was 0.334 ($\pm$ 0.083) mmol Trolox equivalent concentration. Each mole of Trolox in our assay had an equal effect to 2.1 moles of Fe^{2+}. Thus this value is equivalent to 0.69 mmol of Fe^{2+} per liter. This value is very similar to the findings of Cao et al. (30), who reported a mean of 0.388 ($\pm$ 0.027) mmol Trolox equivalent concentration in plasma samples. A higher value of 1.01 ($\pm$ 0.13) mmol of Fe^{2+} per liter has been reported by other authors (31), which may be due to differences among studied populations.

SH groups were measured by a spectrophotometric assay using the reagent DTNB (15). This simple assay has long been used by various authors to determine the extent of oxidation of proteins in chronic renal failure (34), rheumatoid arthritis (35,36) and cystic fibrosis (37). It has been proposed that samples stored at 4°C upto 2 days or frozen at –70°C are acceptable for SH determination (15). However, no experimental data has been described supporting this notion.

Our data showed that the mean concentrations of SHs on different days of analysis did not show any significant change (Fig. 1C). The interindividual differences seemed to be small even after 30 days (mean of absolute values of individual changes, 6.9%). Correlations between measurements performed on fresh samples and on different days showed similar correlation coefficients from the first to the 30th day (Table 1). These annotations confirm that storage of samples may not change the stability of SH groups at least for 1 month. Some researchers investigated glutathione stability in induced sputum (38) and in biological tissues (10) but these can be hardly compared with blood.

Different values have been reported for SH concentrations in healthy subjects in literature. Himmelfarb et al. (34) reported a low value of 0.279 (±0.012) mM for SH concentration in plasma, while other authors observed higher values of 0.421 (±0.075) mM (37), 0.567 (±0.071) mM (36), and 0.591 (±0.085) mM (39), which are very similar to our results (0.562±0.065 mM).

Recently, there has been considerable attention focused on increased concentrations of carbonyls in various diseases and carbonyl content has been by far the most commonly used marker of protein oxidation (40). For determination of the carbonyl groups in plasmatic proteins, a spectrophotometric method was applied, which is based on measuring the absorbance of dinitrophenylhydrazone derivatives at 360 nm after treating the plasma samples with DNPH (16,17). This method has been used for measuring carbonyl content in Alzheimer's disease (41), diabetes (39), rheumatoid arthritis (42), chronic renal failure (32,34), cystic fibrosis (37), and several other diseases.

There was no significant difference between two measurements performed after 1 and 30 days (Fig. 1D). Although some individual samples showed moderate changes after 30 days compared to their first day determinations (the range of changes was –37.9 to +50.6%), the mean of absolute values was not so high (22.8%). Furthermore, a good correlation was found between the measurements on the first and 30th days ($r = 0.714$, $P < 0.001$) (Table 1). Considering the relatively low precision of this method compared to the others in our hands (intra- and interassay coefficients of variation were 14.1% and 19.3%, respectively), it may be suggested that carbonyl contents in plasma kept at –70°C at least after 1 month are stable and sample storage does not considerably change analysis results. It has also been proposed by other authors that carbonyls may be stored for a long time without considerable changes (43). Carbonyls could not be measured in fresh samples due to the long and laborious nature of the assay, thus no comment can be made about the fresh samples. However, considering that one month storage at –70°C did not significantly change the carbonyl concentrations, it can be assumed that carbonyls are relatively stable over time.

Different values like 0.53 (±0.14) (37), 0.80 (±0.30) (32), and 0.97 nmol/mg protein (44) have been reported as the mean concentration of carbonyls in plasma of healthy subjects. In one study, a positive correlation was shown between age and carbonyl concentrations, and values of 1.33 and 1.58 nmol/mg protein were reported for the concentration of carbonyls in young and elderly subjects, respectively (45). Our results (2.0 nmol/mg protein) were close to the findings of these authors but higher than the results of other authors. These disagreements in the literature may be explained by differences in the technical details of measurements. For example, we noticed that the 15 min of incubation at 37°C proposed by Levine et al. (16) were not enough to dissolve the proteins in guanidine 6 M and may lead to an underestimation of the carbonyl content.

The strongest association with age was observed for ROOHs ($r = 0.765$), followed by carbonyls ($r = 0.661$) and sulfhydryls ($r = -0.580$). TAC showed only a weak inverse correlation with age (Table 2). It has been also reported by other authors that OSS may be influenced by age of the individual subjects (45).

ROOHs and carbonyls are both products of oxidation and evaluate the extents of lipid peroxidation and protein oxidation, respectively. A correlation was observed between these two parameters (Fig. 2A), which indicates an association between these two aspects of oxidative stress. Similarly, a correlation was observed between TAC and SHs (Fig. 2B). Both of these parameters assess the defense mechanisms of plasma against free radicals, which may be the reason of this association. As expected, an inverse correlation was found between carbonyls and SHs (Fig. 2C), because both of them are protein oxidation markers. ROOHs showed relatively well inverse correlations with TAC and SHs (Fig. 2D and E), while a poor correlation was observed between TAC and carbonyls (Fig. 2F), which indicates a poor association between these parameters.

CONCLUSIONS

Four parameters of oxidative stress determined in plasma of healthy subjects showed a correlation with the age of the individuals. There were also relatively good correlations among most of the parameters measured in this study. Furthermore, our results confirmed the importance of early sample analysis for ROOHs determinations with FOX assay and showed that SHs and carbonyls have reasonable stabilities after storage at –70°C up to 30 days. For TAC measurements by FRAP assay, it may be suggested that an earlier determination (before 1 week) gives more precise results.

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