CLINICAL STUDY

Erythrocyte superoxide dismutase, glutathione peroxidase and catalase activities in healthy male subjects in Republic of Macedonia

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Abstract

Objectives: **The study was aimed to establish the reference values for erythrocyte antioxidant enzymes, superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) in helthy male subjects, as well as to detect their relation to age, cigarette smoking, moderate alcohol consumption, antiinflammatory drugs and supplements use and the possible correlation between individual parameters.**

Background: **Superoxide dismutase, glutathione peroxidase and catalase are the three main enzymes that control the biological effects of the reactive oxygen species (free radicals).**

Methods: **The authors have examined 111 healthy male blood donors aged 18–59 years. All studied enzymes were assayed in erythrocytes by manual techniques. The results were statistically evaluated by means of the ANOVA programme.**

Results: **The group of healthy individuals had a large dispersion of interindividual activities of antioxidant enzymes. Age, cigarette smoking, antiinflammatory drugs and dietary supplements use had no significant effect on the antioxidant enzymes activity. The activity of the enzymes showed normal Gaussian distribution. We established mean reference values for SOD, GPX and CAT activity. SOD negatively correlated with CAT (r = -0.199).**

Conclusions: **We established the reference values for erythrocyte SOD, GPX and CAT activity in male subjects. The large dispersion of activities of the investigated enzymes in healthy individuals does not allow to make a standard use of these parameters in clinical practise without establishing "own reference values" for each laboratory.** ***(Tab. 2, Fig. 4, Ref. 31.)***

Key words: superoxide dismutase, glutathione peroxidase, catalase, reference values, male subjects.

In healthy individuals, the antioxidative system defends tissues against free radical (prooxidants) attack. Free radicals are highly reactive molecules generated by the biochemical redox reactions that occur as a part of normal cell metabolism. The main free radical species that occur in the human body include:

— superoxide radical ($.O_2^-$);
— hydroxyl radical (.OH);
— nitric oxide radical (NO.);
— peroxyl radical (ROO.) (1).

Free radicals and peroxides are clearly involved in physiological phenomena such as synthesis of prostaglandins and tromboxans, but they are also involved in pathogenesis of various diseases, including atherosclerosis, inflammatory diseases, and cancer, and are thought to participate in aging processes (1, 2, 3). The biological effects of these highly reactive compounds are controlled *in vivo* by a wide spectrum of antioxidative defense mechanisms that can be identified in three classes:

— primary antioxidants (e.g. superoxide dismutase, catalase, gluthatione, peroxidase, ceruloplasmin, transferrin, ferritin) prevent the formation of new free radical species;

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— secondary antioxidants (e.g. vitamin e, vitamin C, ß-carotene, uric acid, bilirubin, albumin) remove newly formed free radicals before they can initiate chain reactions;
— tertiary antioxidants (e.g. DNA repair enzymes, methionine sulphoxide reductase) repair cell structures damaged by free radical attack (1, 2, 3).

In some clinical studies, one or several of these antioxidant enzymes were measured in blood as possible biological indicators, especially concerning hyperlipidemia and atherosclerosis, alcoholism, diabetes, cancer, etc. (4–9). There is no standardization of the determination methods of erythrocyte antioxidant enzymes. Analytical differences between laboratories make it difficult to compare the results obtained in different studies. A lot of references report on the activity of one antioxidative enzyme. In our laboratory, reference values for superoxide dismutase (SOD) and glutathione peroxidase (GPX) activity in female subjects were obtained within the framework of a project (10). Therefore, the aim of our study was to introduce catalase as a part of the antioxidant enzyme status and to determine the reference values of the three main antioxidative enzymes (AOE): SOD, GPX and catalase (CAT) in erythrocytes of healthy male subjects, in order to use our own reference values, that will be comparable with the activities of AOE in groups of patients with certain diseases as part of another project (data not published yet).

Material and methods

Population samples

Our sample included 111 male healthy blood donors selected without known bias, aged between 18–59 years. The blood donors were recruited from the Republic Institute of Transfusiology, Skopje, Macedonia. Information concerning smoking, alcohol consumption, use of dietary supplements, and drug intake was obtained by questionnaires in the presence of a medical doctor. All of them gave written informed consent. The Ethic Committee of Doctor's Chamber of Republic of Macedonia approved all procedures that have been undertaken during the project.

Blood samples

Venous blood samples for measuring the enzyme activities were collected into heparinized tubes between 08.00 and 09.00 o'clock. Hemoglobin concentration was determined in K_3EDTA blood samples with a Coulter Microdif 18.

The blood was centrifuged at 2600 rpm for 15 minutes and plasma was removed. The erythrocytes were carefully sampled from the bottom of the tubes to minimize contamination with leukocytes, washed three times with isotonic saline solution, and lysed by addition (1/4, by vol.) of doubly distilled water containing 5 ml/L Triton x-100, followed by vigorous vortex-mixing and storage on ice for 10 minutes. Membrane-free hemolysate was obtained by centrifugation at 10 000x g for 5 minutes.

Antioxidant enzymes (AOE) were measured within the same day of collecting.

Enzyme assays

Assay of superoxide dismutase (SOD; EC.1.15.1.1) activity:

SOD activity was measured with RANSOD kits (cat. No. SD 125; Randox Labs., Crumlin, North Ireland). This method employs xanthine and xanthine oxidase to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. The superoxide dismutase activity is then measured by the degree of inhibition of this reaction. All diluted sample rates were converted into percentages of the sample diluent rate, and substracted from 100 % to give a percentage of inhibition. The activity was measured at 37 °C on a PU 8630 UV-VIS spectrophotometer, and absorbancy was monitored at 505 nm for 3 minutes. The unit of activity is defined as the amount of enzyme that inhibits the rate of the formazan dye formation by 50 %.

SOD units were obtained from standard curve using percentage inhibition of the samples (SOD units/ml of whole blood-absolute activity) and were converted to SOD units/g hemoglobin (specific activity). Standards were prepared by diluting a commercial SOD preparation (cat. No. SD 125) in order to obtain standard curve.

Assay of glutathione peroxidase (GPX; EC.1.11.1.9) activity:

GPX activity was determined with Ransel kits (Cat. No. RS 505; Randox Labs., Crumlin, North Ireland) at 37 °C on a PU 8630 UV-VIS spectrophotometer at 340 nm for 3 minutes. This assay, based on the method of Paglia and Valentine, requires cumene hydroperoxide as a substrate. Before analysis the samples were diluted 40 fold to a hemolysate by adding Drabkin's reagent (double strength) to inhibit the peroxidase activity of hemoglobin (11). The final concentrations of reagents in the assay were those recommended by the manufacturer. The activity of GPX was expressed in µmol/L/min for absolute activity and µmol/gHb/min for specific activity.

Assay of catalase (CAT; EC.1.11.1.6) activity:

Catalase activity was measured in hemolysates at 25 °C with PU 8630 UV VIS spectrophotometer by the method of Aebi (12). The decomposition of the substrate H_2O_2 was monitored spectrophotometrically at 240 nm for 3 minutes. Activity was expressed as k (rate constant of the first order reaction as defined by Aebi) (absolute activity) and k/g hemoglobin (specific activity).

Enzyme assays of each sample were performed in duplicate on 8630 PU UV-VIS spectrophotometer.

Data analyses

Statistical analyses were performed by using the commercial statistical package, Statistica for Windows, Version 5.0. Standard analyses to study interrelationships between enzyme activities and their potential determinants were used. Ability to fit to normal Gaussian distributions was investigated by calculation of standard skewness and kurtosis coefficients. Multiple regression analyses was used to investigate the influence of different variables on the enzyme activities by the ANOVA program. Spearman's rank correlation coefficient was used to investigate associations between enzyme activities. We studied partial correlation of the various enzyme activities after adjustment for the

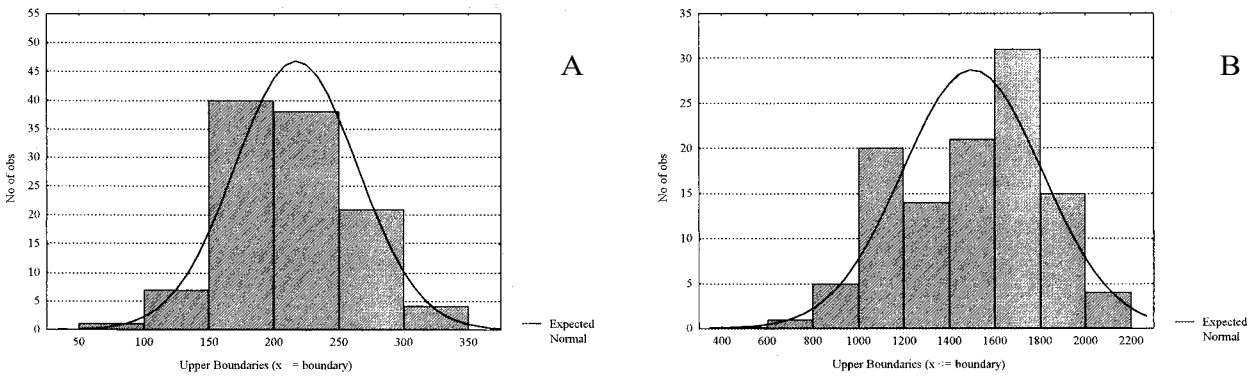

Fig. 1. Frequency distribution for erythrocyte superoxide dismutase activity (A — absolute activity expressed as U/ml; B — specific activity expressed as U/gHb).

effects of the confounding factors. The level of significance was $p<0.05$.

We established the reference intervals by using symmetrical empirical percentiles (2.5 and 97.5) for the erythrocyte SOD, GPX and CAT activity (absolute and specific activity) (13, 14).

Results

The covariantes included in the regression model were: age (years), smoking (0 = no, 1 = yes), tobacco consumption (smoker = 1, non-smoker = 0), moderate alcohol consumption (100 ml wine daily) (0 = no, 1 = yes), non-steroid anti-inflammatory drugs (0 = no, 1 = yes) and dietary supplements (0 = no, 1 = yes). Our subjects were from 18–59 years old with mean value 32.55±11.88 years. 54 % of them were cigarette smokers and 55.8 % declared moderate alcohol consumption. 35.1 % of them were using nonsteroid anti-inflammatory drugs, and only 25 % dietary supplements. Multiple-regression analysis has shown that age, cigarette smoking, moderate alcohol consumption and use of anti-inflamatory drugs as well as dietary supplements have no statistically significant effect on all antioxidant enzyme activities.

To establish reference intervals we tested the hypothesis of symmetrical Gaussian distribution by testing the skewness and kurtosis coefficient for each antioxidative enzyme.

Hypothesis for Gaussian distribution for SOD activity was obtained by the results from the testing of skewness and kurtosis coefficients that showed the following values:

— for absolute activity of SOD, coefficient of skewness was -0.598 with k_s = -0.12 and s_s = -0.23. Kurtosis coefficient was +1.17 with k_k = -0.36 and s_k = 0.45,

— for specific activity of SOD, coefficient of skewness was +0.598 with k_s = -0.23 and s_s = 0.23. Kurtosis coefficient was +1.17 with k_k = -0.36 with s_k = 0.45.

SOD showed symmetrical Gaussian distribution with 95% confidence interval (Figs 1A and 1B). The activity values of SOD were normally distributed around the arithmetical mean value with mean values $\bar{x}$±SD = 217.1±47.4 SODU/ml for absolute activity and $\bar{x}$±SD= 1501.6±308.7 SODU/gHb for specific activity (Tab. 1 and Figs 1A and 1B).

The hypothesis for Gaussian distribution of erythrocyte GPX activity was accepted by the results obtained from testing the skewness and kurtosis coefficients, that have shown these values:

— for absolute activity of GPX expressed in U/L, coefficient of skewness was +0.594 with k_s=0.61 and s_s=0.23. Coefficient of kurtosis was +1.17 with k_k=-0.24 and s_k=0.45;

— for specific activity of GPX expressed in U/gHb, coefficient of skewness was +0.595 with k_s=0.59 and s_s=0.229. Coefficient of kurtosis was +1.17 with k_k=-0.397 and s_k=0.45.

The values of GPX activity were normally distributed around the arithmetical mean value (Figs 2A and 2B). The mean values

Tab. 1. Antioxidant enzymes activity in male human erythrocytes (mean±SD).

	SOD		GPX		CAT	
	U/ml	U/gHb	U/L	U/gHb	k	k/gHb
N (111)						
Range	217.1±47.4	1501.6±308.7	7369±3246	51.5±23.4	29.5±5.83	205.4±43.3
CV %	21.83	20.56	44	45.4	19.76	21.1

SOD — superoxide dismutase, SOD U/ml — superoxide dismutase expressed as absolute activity, SOD U/gHb — superoxide dismutase expressed as specific activity, GPX — glutathione peroxidase, GPX U/L — glutathione peroxidase expressed as absolute activity, GPX U/gHb, glutathione peroxidase expressed as specific activity, CATk — absolute activity of catalase - a rate constant of the first order reaction as defined by Aebi, CAT k/gHb — specific activity of CAT, N — number of subjects, CV— coefficient of variation.

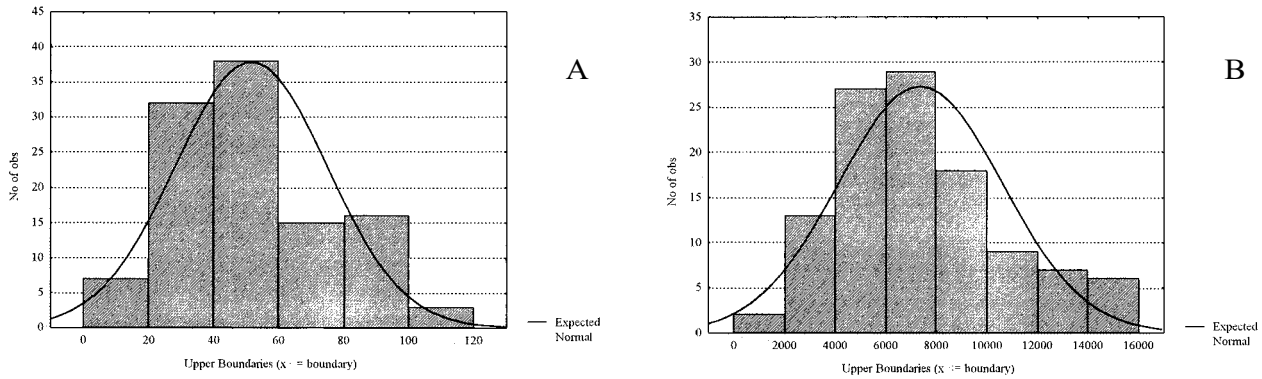

Fig. 2. Frequency distribution for erythrocyte glutathione peroxidase activity (A — absolute activity expressed as U/L; B — specific activity expressed as U/gHb).

of GPX were $\bar{x}$±SD=7369.3±3246 U/L and 51.5±23.4 U/gHb (Tab. 1).

The hypothesis for Gaussian distribution of catalase activity was accepted by the results obtained from testing the coefficients of skewness and kurtosis that have shown these values:

— for absolute activity of CAT, coefficient of skewness was +0.594 with k_s=0.457 and s_s=0.229. Coefficient of kurtosis was 1.17 with k_k=0.028 with s_k=0.45;

— for specific activity of CAT expressed as k/gHb, coefficient of skewness was +0.594 with k_s=0.382 and s_s=0.229. Coefficient of kurtosis was +1.183 with k_k=-0.467 with s_k=0.455.

The catalase activity was normally distributed around the arithmetical mean value (Figs 3A and 3B). The mean values of CAT were $\bar{x}$±SD=29.5±5.8 k, for absolute activity and 205.4±43.3 k/gHb for specific activity (Tab. 1).

Table 2 shows the reference intervals of AOE activity in male subjects calculated by using mean ±SD interval. It should be noted that these calculated ranges are only valid for the enzymatic assays used.

The reference interval for erythrocyte SOD ($\bar{x}$±2SD) was from 122.4 to 331.9 SODU/ml for absolute activity and from 884.2 to 2119.0 SODU/gHb for specific activity (Tab. 2).

The reference interval ($\bar{x}$±SD) for erythrocyte GPX activity expressed as absolute activity was from 4123 U/L to 10 615 U/L and from 28.1 U/gHb to 74.9 U/gHb for specific activity (Tab. 2). The reference interval ($\bar{x}$±2SD) for erythrocyte catalase activity was from 17.9 to 41.1 k for absolute activity, and from 118.8 to 292.0 k/gHb for specific activity (Tab. 2).

The interindividual coefficients of variation for each specific activity of AOE studied were 20.5 % for SOD, 45.4 % for GPX and 21.1 % for CAT (Tab. 1).

Correlation between antioxidant enzyme activities: We found statistically significant negative correlation between erythrocyte catalase and superoxide dismutase activity with r=-0.24 and $p<0.05$ (for the activities expressed as absolute activities) (Fig. 4A) and r=-0.199 with $p<0.05$ (for the activities expressed as specific activities) (Fig. 4B). We found no statistically significant correlation between GPX and catalase or SOD activity.

Discussion

Superoxide dismutase, glutathione peroxidase and catalase are three main enzymatic defense systems of the organism against free radicals and peroxides. Since literature data have shown no standardization of the determination methods, we attempted to identify the factors affecting activities of SOD, GPX and CAT as well as to establish our "own reference values" for male individuals.

We found no significant changes in the acivities of erythrocyte antioxidant enzymes associated with ageing. Guemouri et al (15) described statistically significant changes of all antioxidant enzyme activities in correlation with age, but their sample included individuals aged 4–65 years. They found out that activities appeared rather stable in adults <65 years old but decreased for most enzymes in the elderly. Our population group was under the age less of 60 years. In the study of Ceballos-Picot (16), based on the results from 167 individuals between 1 month and 67 years of age, there was a negative correlation between

Tab. 2. Reference values for antioxidant enzyme activities in male human erythrocytes (N=111).

	SOD		GPX		CAT	
	U/ml	U/gHb	U/L	U/gHb	k	k/gHb
Age (18-59)	122.4-333.9[a]	884.2-2119[a]	4123-10615[b]	28.1-74.9[b]	17.9-41.1[a]	118.8-292.0[a]

N — number of cases, SOD U/ml — superoxide dismutase expressed as absolute activity, SOD U/gHb — superoxide dismutase expressed as specific activity, GPX U/L — glutathione peroxidase expressed as absolute activity, GPX U/gHb, glutathione peroxidase expressed as specific activity, CATk — absolute activity of catalase - a rate constant of the first order reaction as defined by Aebi, CAT k/gHb — specific activity of catalase, [a] — mean±2SD, [b] — mean±SD.

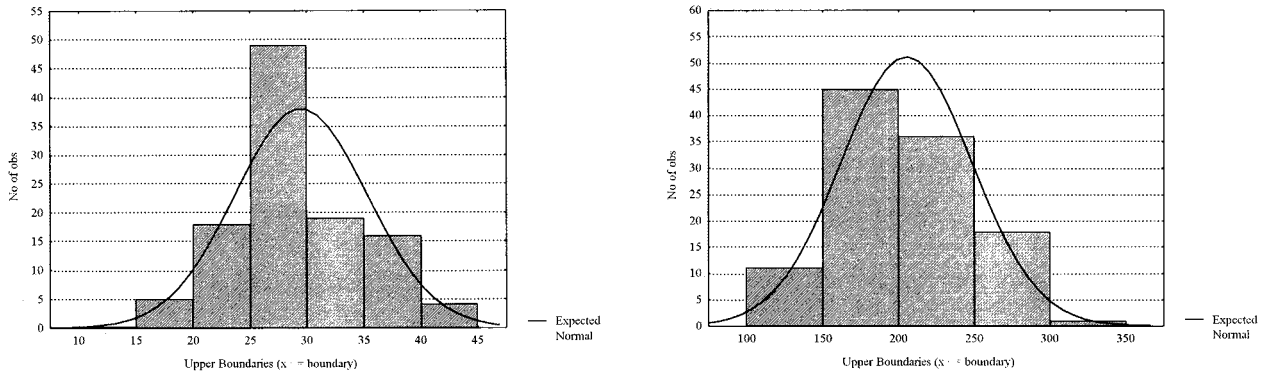

Fig. 3. Frequency distribution for erythrocyte catalase activity (A — absolute activity expressed as k units of Aebi; B — specific activity expressed as k/Hb).

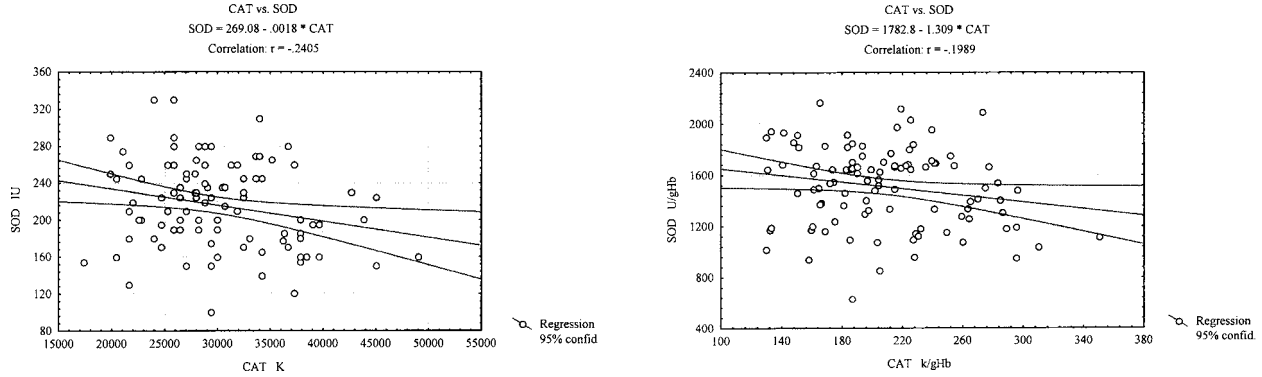

Fig. 4. Correlation between activities of catalase (CAT) and superoxide dismutase (SOD) (A — correlation between absolute activities of CAT and SOD; B — correlation between specific activities of CAT and SOD) after adjustment for the units of enzyme activities.

age and activities of SOD and positive correlation with GPX activity. In the same report there was no significant change in the enzyme activites associated with ageing in the group aged 25–40 years and in the group aged 40–63 years (that were similar to our group) (16). Andersen et al (17) reported about age-related decrease in erythrocyte SOD. They found no age-related changes for GPX and CAT activity (17). These results are partially in accordance with our results. Bolzan et al (18) reported no significant changes in the catalase activity associated with ageing. In the same study they reported about significant negative correlation of SOD activity with age and positive correlation between GPX and age (18). Conversely, Volkovova K et al (19) reported about significant negative correlation of catalase activity with age, but no significant correlation of SOD and GPX activity with age. In accordance with these findings and with our results are the results of Habif (20), who reported about no significant change in GPX activity between different age groups. Winterbourn (21) also reported about no significant change of the activity of SOD due to age that is in accordance with our results.

Important decrease in the enzyme activities in elderly subjects aged 65–95 years were reported by Guemouri L et al (15). Schafer and Thorling (22) also described a reduction of erythrocyte GPX in the elderly, as did Jozwiak and Jasnowska for erythrocyte SOD in such individuals (23). In the same study the later authors found that erythrocyte GPX and CAT were higher in subjects aged 65–80 years than in those aged 20–50 years (23).

Some differences between the studies may be due to factors other than age, since random population samples were not used, and selection bias may have affected the results.

Our results showed no significant changes in AOE activities associated with ageing.

We observed no signifficant differences in AOE activities between smokers and non-smokers. This finding agrees with that of Andersen et al (17), Bolzan (18), Guemoury (15), and Durak (24).

Thoth et al (25) hypothesized that the antioxidant activities and protective abilities of erythrocytes from cigarette smokers might be greater than in erythrocytes from non-smokers. They found that erythrocytes from smokers contained more CAT than did those from nonsmokers, but contained the same activities of GPX (25). We did not confirm these results; our multiple-regression analysis showed no significant effect of cigarette consumption on the activity of all erythrocyte antioxidant enzymes. Conversely to the findings of Thoth et al (25), Bolzan et al (18)

observed significant decrease of catalase activity in female cigarette smokers in comparison with nonsmokers. Volkovova (19) found significant decrease of the GPX activity in smokers in comparision with non-smokers, but the other antioxidative enzymes were not significantly changed in the group of smokers. Leonard (26) in 40 healthy adults (40 years old or less) not specifying their gender, also found no significant differences of activity of SOD and GPX between smoking and non-smoking volunteers. Our results suggest that smoking does not lead to oxidant stress in the enzymatic antioxidant defense system.

In our study the reference interval for erythrocyte SOD activity in male subjects was 884.2 – 2119 U/gHb, for erythrocyte GPX activity 28.1 – 74.9 U/gHb and for CAT activity 118.8 – 292 k/gHb (specific activity). The literature survey revealed that there are many differences in the reference values and intervals of the antioxidant enzyme activities. It probably depends on the differences in the examination methods, number of studied subjects, their gender, age, and some other factors as cigarette smoking, dietary supplements, etc. That is why each laboratory should estimate their "own reference values". Our reference intervals are in agreement with the results reported by Guemouri et al (15) who investigated 1242 individuals, and Leonard who investigated 40 subjects (26). Hopkins and Tudhope in 62 cases have got similar reference values for the GPX activity (27). Our results about Gaussian distribution and the reference interval are in a good agreement with the results of the investigation of Witerbourn et al (21). Other authors report about the reference values of AOE activity, but they use different methods, and express the activity in U/mg Hb, or mU/mgHb (18), (16) or U/mmol Hb or U/μmol Hb (28). Durak used U/ml to express the AOE activity (24). Lux and Naidoo studied the biological variability of SOD and GPX activities in the erythrocytes of 12 healthy subjects and reported an interindividual coefficient of variation of 16 % for SOD and 27 % for GPX (29). The interindividual coefficients of variation for the same AOE reported here were 20.56 % for SOD, 45.4 % for GPX and 21 % for CAT, that were in agreement with the results of Lux and Naidoo, and with the results of Bolzan, who confirmed the highest variability of GPX (29, 18).

Finally, we have found a negative correlation between SOD and CAT in erythrocytes. Our results are in agreemant with the results of Volkovova et al (19). Conversely Guemouri et al found positive correlation between SOD and CAT erythrocyte activity (15). However, the results obtained by both groups of authors as well as our results seem to be logical. SOD is the first antioxidant defence system in erythrocytes that produces superoxide anion in the reaction of autooxidation of hemoglobin (30). CAT is thought to account for a notable part of the destruction of H_2O_2, which is partly generated by SOD (31). Because the production of H_2O_2 in the physiologycal conditions is relatively low, we suppose that there is a different affinity of CAT towards the substrate (H_2O_2) in the studied subjects.

In conclusion, by using three simple and sensitive assays we estimated the reference values for erythrocyte SOD, GPX and CAT activities in male subjects, that are not affected by age, cigarette smoking, moderate alcohol consumption, antiinflamatory drugs and dietary supplements use. We have confirmed that AOE activity in human blood exhibits a wide interindividual variability (e.g. the erythrocyte antioxidant enzymes are inconsistent), that is highest for GPX activity. Having this in mind and the fact of no standardisation of the methods for measuring the AOE activity support the assumption that each laboratory should use its "own reference values".

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