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Could cholesterol bound to haemoglobin be a missing link for the occasional inverse relationship between superoxide dismutase and glutathione peroxidase activities?

Milan Nikolic [a], Danijela Vranic [b], Aurelija Spiric [b], Valentina Batas [b], Aleksandra Nikolic-Kokic [c], Petar Radetic [b], Lazar Turubatovic [b], Dusko P. Blagojevic [c], David R. Jones [a], Vesna Niketic [a], Mihajlo B. Spasic [a,c,*]

[a] *Department of Chemistry, University of Belgrade, Studentski trg 16, 11001 Belgrade, Serbia and Montenegro*
[b] *Institute of Meat Hygiene and Technology, Kacunskog 12, 11001 Belgrade, Serbia and Montenegro*
[c] *Department of Physiology, Institute of Biological Research, Bulevar Despota Stefana 142, 11060 Belgrade, Serbia and Montenegro*

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Abstract

The concept of an anti-oxidant defence system as a means to prevent oxidative cell damage implies balanced activities of anti-oxidant defence enzymes. As well as positive correlations between anti-oxidant enzyme activities in human erythrocytes, it has been observed that sometimes when glutathione peroxidase activity is increased, CuZn-superoxide dismutase activity is decreased. In our current study we have examined the plasma lipid profile and the anti-oxidant defence enzymes in erythrocytes from humans, pigs, and bulls. We found that a negative correlation existed between CuZn-superoxide dismutase and glutathione peroxidase activities in human erythrocytes when the concentrations of both plasma triglycerides and total cholesterol were high. This correlation was also found in pig erythrocytes, but not in bull erythrocytes. We propose that cholesterol could affect membrane lipid peroxidation and superoxide generation in erythrocytes *via* the recently found fraction of cholesterol bound to haemoglobin, termed haemoglobin-cholesterol.

Keywords: Anti-oxidant defence; Erythrocytes; Cholesterol; Haemoglobin; Humans; Pigs; Bulls; Plasma lipids

Erythrocytes are particularly vulnerable to oxidative stress because they are exposed to oxygen radicals that are continuously generated primarily due to the auto-oxidation of haemoglobin (Hb). There is a defence system against oxidative stress in erythrocytes. CuZn-superoxide dismutase (SOD; EC 1.15.1.1) catalyses the dismutation of superoxide $O_2^{\cdot-}$ to hydrogen peroxide (H_2O_2), which is then independently converted to water by catalase (CAT; EC 1.11.1.6) or by selenium-dependent glutathione peroxidase (GSH-Px; EC 1.11.1.9) [1]. Glutathione reductase (GR; EC 1.6.4.2) catalyses the reduction of oxidised GSH back into GSH, the latter being the co-substrate of GSH-Px [2]. The concept of an anti-oxidant defence system as a means to prevent oxidative cell damage implies the coordinated expression of anti-oxidant defence enzymes. In a group of 220 healthy Danish subjects erythrocyte SOD activity was positively correlated to CAT. In addition, CAT was positively correlated to GR. No significant correlations were found between GSH-Px and any of the other enzymes measured [3]. Any increase in SOD activity produces an excess of H_2O_2 that must be efficiently neutralised by either GSH-Px or CAT. Thus, the enzymatic activity of the first step (SOD) and second step (GSH-Px and/or CAT) must be balanced to prevent cell damage. Indeed,

* Corresponding author. Fax: +381 11 2761433.
E-mail address: spasa@ibiss.bg.ac.yu (M.B. Spasic).

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this relationship has been shown in cases of trisomy 21 (Down's syndrome) where an increase in SOD activity was accompanied by increases in GSH-Px and CAT activities [4–6].

In some human pathological samples it has been observed that often when GSH-Px is increased, SOD is reduced and *vice versa* [7–9]. Very small inter-species variations in terms of the amount of enzyme per gramme of Hb were found in the case of SOD activity, but large variations were found in the case of CAT and GSH-Px activities (up to a 150-fold difference between minimal and maximal values) [10]. Erythrocytes are also exposed to oxidative pressure from plasma (particularly H_2O_2 and nitric oxide). An imbalance between oxidative stress and the cell's anti-oxidant defence system may have adverse effects on cell membranes through the indiscriminate oxidation of susceptible molecules such as polyunsaturated fatty acids (PUFAs), the main substrates for lipid peroxidation [11]. Some investigators [12,13] have suggested that the alteration in the SOD/GSH-Px + CAT ratio rather than the absolute concentrations of anti-oxidant enzymes is an important indicator of cellular damage. This is because changes in this quotient correlate well with increases in lipid damage. This means that the sensitivity of a cell to free radicals apparently depends on the relationship between SOD and (CAT + GPx) rather than on absolute amounts of individual anti-oxidants [14]. We have recently detected cholesterol (Ch), associated with phospholipids, bound to Hb in human erythrocytes (termed Hb-Ch) [15]. In that report we showed that Hb-Ch was formed in a young healthy population as a consequence of a seasonally related elevated plasma cholesterol level (due to changes in plasma lipoprotein metabolism) [15]. Generally unsaturated fatty acids and phospholipids promote human Hb oxidation and reactive oxygen species (ROS) formation [16,17]. Cholesterol, on the other hand, has a protective effect [18]. The amount of Hb-Ch did not influence erythrocyte anti-oxidant enzyme activities, however, a higher amount of Hb-Ch changes correlations in the part of the anti-oxidant defence system relating to GSH, suggesting increased peroxidative pressure from plasma lipids (Nikolic et al., unpublished data).

As the influence of plasma lipids on anti-oxidant defence in erythrocytes was not examined in detail [19], we have now studied the levels of plasma lipids and the activity of the erythrocyte anti-oxidant enzymes in humans and compared the findings with animals (pigs and bulls) having different lipid metabolism, different plasma lipid profiles, and different erythrocyte anti-oxidant defence compositions. We also examined the content of Hb-Ch in human, pig, and bull erythrocytes.

Materials and methods

Chemicals. All the chemicals used were purchased from Sigma (USA) or Merck (Germany), unless indicated otherwise.

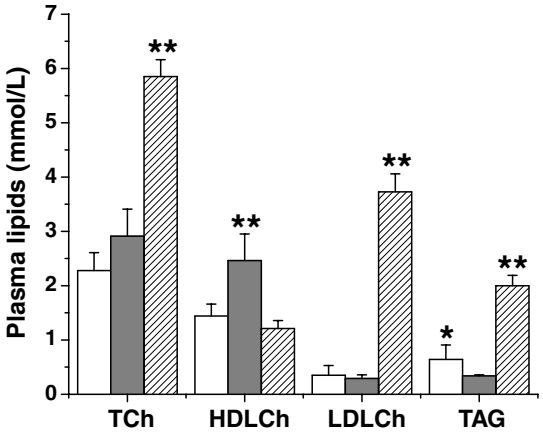

Fig. 1. The concentration of plasma lipids in pigs (white bars), bulls (grey bars), and humans (shaded bars). The error bars indicate the standard deviations. *$p < 0.01$ and **$p < 0.001$ significantly different from the other two species.

Subjects and blood sampling. Human blood samples were obtained by venapuncture following an overnight fast. Citrate (3.8%) was used as an anticoagulant. After an initial screening, 15 human subjects (9 males and 6 females from our research laboratories and institutions aged 40–57 years old) were selected for the study. They all had slightly elevated levels of plasma total cholesterol (T-Ch) and plasma triglycerides (TGs) (Fig. 1) according to the third report of the National Cholesterol Education Program [20]. Otherwise, normal haematological indices were present and the history of all participants did not reveal any relevant diseases, medical conditions or the taking of prescribed medication that could have influenced anti-oxidant enzyme activities. No unusual physical or drinking habits were observed. Informed consent was obtained from all the human volunteers prior to initialising the study. Blood samples from 12 pigs and 12 young bulls were obtained from the Institute of Meat Hygiene and Technology. The pigs were Swedish landrace (approximately 100 kg live weight, fattened in about 160 days). The young bulls were from crosses between Charloase and a domestic spotted breed (aged 480 days and about 650 kg in weight). Citrate (3.8%) was used as an anticoagulant for both pig and bull blood sampling. All measurements described below were carried out in duplicate using fresh plasma and erythrocyte lysates.

Plasma lipids. Blood samples were centrifuged at 2000*g* for 15 min at 4 °C to determine the plasma lipids. Plasma T–Ch and TGs were determined based on enzymatic methods with Reanal kits (Hungary). High-density lipoprotein cholesterol (HDL–Ch) was measured in the supernatant following the precipitation of apoB-containing lipoproteins with dextran sulphate (Serva, Germany) and $MgCl_2$. Low-density lipoprotein cholesterol (LDL–Ch) was calculated using the Friedewald formula.

Erythrocyte anti-oxidant enzyme activities. The preparation of the erythrocyte lysates was as follows. After the citrate-treated blood samples were centrifuged at 2000*g* for 15 min at 4 °C, the plasma and buffy coat were discarded. The separated erythrocytes were washed three times with 0.9% NaCl. Washed cells (0.5 ml) were lysed by adding 3 ml of ice-cold distilled water followed by thorough mixing. The Hb concentration was then estimated in an aliquot of this haemolysate using the Drabkin method. To remove the Hb [21], 1.0 ml of an ethanol/chloroform (1:1, v/v) mixture was added to an aliquot (0.5 ml) of the haemolysate cooled on ice. This mixture was stirred constantly for 15 min before being diluted with 0.5 ml of distilled water. After centrifugation for 10 min at 1600*g*, the pale yellow supernatant was separated from the protein precipitate and was used to assay SOD enzyme activity.

The activities were determined using a Shimatzu UV-160 spectrophotometer. All enzyme measurements were carried out in duplicate and average data of GSH-Px activities were normalised using the Hb content (5 g %). SOD activity was measured according to the method described by McCord and Fridovich [22]. One unit of activity is defined as the amount

of enzyme necessary to decrease the rate of cytochrome *c* reduction to 50% of maximum at 25 °C and pH 7.8, using xanthine and xanthine-oxidase as the superoxide source. The activity of CAT was determined by the rate of H_2O_2 disappearance measured at 240 nm, according to Claiborne [23]. One unit of CAT activity is defined as the amount of enzyme that decomposes 1 mmol H_2O_2 per minute at 25 °C and pH 7.0. The activity of GSH-Px was determined by the GSH-dependent reduction of *t*-butyl hydroperoxide, using a modification of the assay described by Paglia and Valentine [24]. One unit of GSH-Px activity is defined as the amount needed to oxidise 1 nmol NADPH per minute at 25 °C and pH 7.0. GR activity was determined using the method of Glatze and co-workers [25]. This assay is based on NADPH oxidation concomitant with GSH reduction. One unit of GR activity is defined as the oxidation of 1 nmol NADPH per minute at 25 °C and pH 7.6.

Polyacrylamide gel electrophoresis. Twelve percent running and 4% stacking gels were employed. SOD from erythrocytes was diluted to 2 U/ml in a solution containing 12% glycerol, 0.5 mM Tris–HCl (pH 6.8), and 0.2 M EDTA before loading 50 µl well. Cow SOD was Perixinorm (Gruneutal Gmbh, Germany). Pig SOD was purified to homogeneity from mixed pig blood [26]. Proteins were identified by Coomassie staining. SOD activity in the gel was revealed by staining with 4-nitroblue tetrazolium (NBT). Protein bands were visualised using the reduction of NBT (0.25 mg/ml) with $O_2^{\cdot-}$ produced by photochemical reduction of riboflavin (0.1 mg/ml) with TEMED (1%).

Determination of Hb-Ch. The content of Hb-Ch was determined as previously described by Nikolic et al. [15]. Briefly, aliquots of the haemolysates, from which membranes were carefully removed, were extracted according to Reed and co-workers [27]. Cholesterol was enzymatically determined in the lipid extracts. The results are expressed as the percentage of total Hb. The total Hb content was measured as cyanmethemoglobin using the Drabkin method.

Statistical analysis. The data are presented as means ± standard deviation (SD). Differences among parameters were assessed by analysis of variance (ANOVA) followed by Tukey's post hoc comparison test. Statistical significance was established by protocols as described in Hinkle and colleagues [28]. A p value <0.05 was considered statistically significant.

Results

The results obtained in our study are illustrated in Figs. 1–4 and Table 1. The human, pig, and bull plasma lipid parameters are shown in Fig. 1. The concentrations of human T-Ch, LDL-cholesterol, and TGs were all significantly higher than those of pigs and bulls. However, the concentration of human HDL-Ch was significantly lower than that measured in bulls. When the parameters for pigs and bulls were compared, we found that the concentrations of T-Ch and HDL-Ch were significantly higher in bulls, whereas the concentration of TGs was higher in pigs. No difference in the level of LDL–Ch was apparent between pigs and bulls.

The activities of the erythrocyte anti-oxidant enzymes are illustrated in Fig. 2. There were no differences in the activities of both GSH-Px and GR between the three species. In bull erythrocytes SOD activity was significantly higher when compared to both human and pig erythrocytes. CAT activity was found to be significantly higher in pig erythrocytes when compared to human and bull erythrocytes.

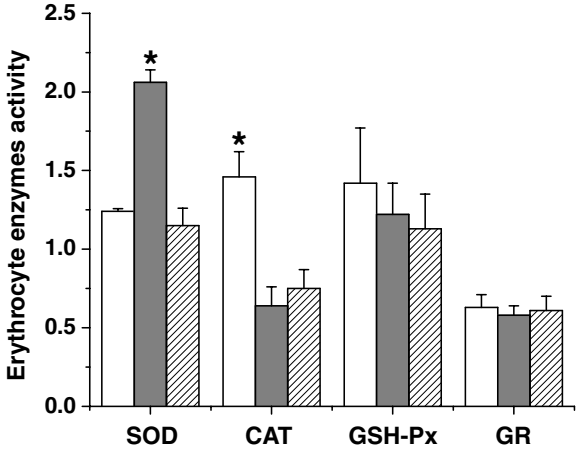

Fig. 2. The activity of anti-oxidant enzymes in erythrocytes from pigs (white bars), bulls (grey bars), and humans (shaded bars). The units of activity are described in Materials and methods. The values for CAT, GSH-Px, and GR activities shown are reduced by a factor of 10 to allow their visualisation in the figure. The error bars indicate the standard deviations. *$p < 0.001$ significantly different from the other two species.

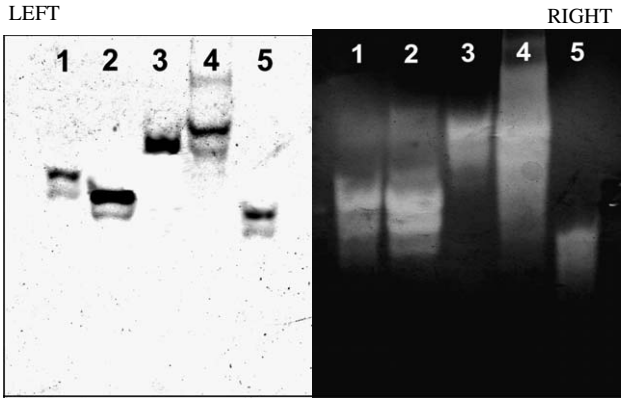

Fig. 3. Non-reducing polyacrylamide gel electrophoresis of SOD. Preparations containing 2 U of protein activity were loaded onto a 12% gel. The picture is separated into two panels. The left panel represents proteins stained with Coomassie blue and the right panel represents the activity of SOD (stained with NBT). The lanes are: 1, SOD from pig erythrocytes (method of Tsuchihashi); 2, SOD purified from pig erythrocytes; 3, SOD from bull erythrocytes (method of Tsuchihashi); 4, SOD purified from bull erythrocytes; 5, SOD from human erythrocytes (method of Tsuchihashi).

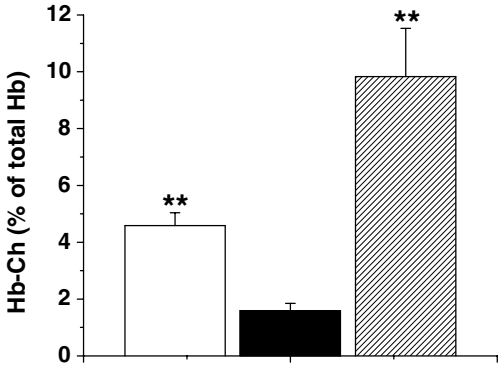

Fig. 4. The level of Hb-Ch from pig (white bar), bulls (grey bar), and human (shaded bar) haemolysates. The error bars indicate the standard deviations. **$p < 0.001$ significantly different from the other two species.

Table 1
Correlation analysis of the activities of erythrocyte anti-oxidant defence enzymes in humans (a), pigs (b), and bulls (c)

	SOD	CAT	GSH-Px	GR
Humans (a)				
SOD		−0.029	**−0.728***	−0.228
CAT			0.490	−0.430
GSH-Px				0.034
GR				
Pigs (b)				
SOD		−0.051	**−0.789***	0.071
CAT			0.440	−0.580
GSH-Px				−0.191
GR				
Bulls (c)				
SOD		**−0.759***	0.118	0.165
CAT			−0.324	−0.241
GSH-Px				−0.467
GR				

Significant correlations are indicated in bold text. Statistical significance was analysed by the ANOVA test.
* $p < 0.01$.

The correlation coefficients between erythrocyte anti-oxidant enzyme activities within each species are shown in Table 1. There was a negative relationship between SOD and GSH-Px activities both in human and pig erythrocytes (Table 1, a and b). A negative correlation between the activities of SOD and CAT was found in bull erythrocytes (Table 1, c).

Differences in the electrophoretic pattern of erythrocyte SOD from humans, pigs, and bulls are shown in Fig. 3. The isoelectric points of pig, human, and bull SOD were found to be 5.8, 5.6, and 4.8, respectively. According to these values the net protein charge on SOD varied more significantly for pigs (lane 1) and humans (lane 5) than for bulls (lane 3). SOD purified to homogeneity from mixed pig blood (lane 2) revealed heterogeneity (70% Leu; 30% Val on position 29) [26]. The same result was obtained for pigs (mixed blood from five pigs, lane 1).

Fig. 4 illustrates the level of Hb-Ch in erythrocytes from pigs, bulls, and humans. A higher level (nearly threefold more) of Hb-Ch was found in pigs when compared with bulls. Humans had at least twofold more Hb-Ch when compared with pigs.

Discussion

The main source of ROS in erythrocytes is the production of $O_2^{\cdot -}$, *via* the auto-oxidation of oxyhaemoglobin to methaemoglobin [29,30]. Excessive production of ROS creates a situation known as oxidative stress when reactions of ROS with macromolecules can cause a wide spectrum of cell damage including lipid peroxidation, inactivation of enzymes, alteration of intracellular oxidative-reduction stage, and DNA mutations. Under normal conditions erythrocytes contain sufficient scavenger enzymes such as SOD, CAT, and GSH-Px to protect against free radical injury. In addition, the specific activities of these enzymes in erythrocytes are higher than in most other tissues in the body [31]. It is known that some of the $O_2^{\cdot -}$ scavenging enzymes can respond to conditions of increased oxidative stress with compensatory increases in their activities [32,33]. Among SOD activities cellular distribution of enzymes is also important and determined mostly by p*I* values and according to our results human erythrocytes SOD is similar to that pigs but both differ from bulls.

GSH-dependent enzymes in the anti-oxidant system are separately regulated, probably *via* the concentration of (reduced) GSH and the cellular redox status [34]. The higher GSH-Px activity observed in pig erythrocytes (Fig. 2) may be explained by the increased formation of peroxides. GSH-Px is an enzyme capable of acting not only on H_2O_2 but also on lipid peroxides. GSH-Px reduces lipid hydroperoxides thereby indirectly protecting hydrophobic membrane compartments. SOD and CAT, on the other hand, primarily operate in a hydrophilic environment. Therefore, a comprehensive understanding of the anti-oxidant system should be based on the knowledge of activities and mutual interactions of enzymes involved in free radical detoxication [14]. To act as an anti-oxidant GSH-Px requires reduced GSH that is then oxidised. The regeneration of GSH is catalysed by GR. The activity of GR is related to the concentration of GSH, which therefore acts as more than a substrate for GSH-Px. The function of GSH is not only for scavenging ROS, but also for the detoxification of xenobiotics and carcinogens and the regulation of immune functions and maintenance of protein structure, function, and turnover [35]. GR activity maintains a high reduced/oxidised glutathione (GSH/GSSG) ratio in normal erythrocytes. Unchanged GR activity in erythrocytes from humans, pigs, and bulls suggested no significant difference in GSH turnover. GSH, together with its related enzyme GSH-Px, mainly interacts with H_2O_2 and lipid peroxides and functions as the major scavenger of ROS in erythrocytes [36].

In the present study a significantly higher CAT activity was observed in pig erythrocytes. CAT has a dual function as it catalyses the decomposition of H_2O_2 into oxygen and water (catalase activity). It also oxidises electron donors such as ethanol, methanol, and phenols (peroxidative activity). This higher CAT activity suggests an increase in H_2O_2 generation in pig erythrocytes. An increased concentration of H_2O_2, which inhibits some protective enzymes including SOD, allows the production of hydroxyl radicals, especially in the presence of catalytically active metals [37,38]. Only at higher concentrations of H_2O_2 the role of CAT becomes increasingly prominent [39].

It is not uncommon that anti-oxidant defence systems are altered in response to various diseases [40,41]. Together our results indicate that the anti-oxidant defence system in human erythrocytes with preserved homeostasis finely retunes its composition according to plasma oxidative demands. An increase in some plasma lipid components

may potentiate membrane lipid peroxidation in erythrocytes and decrease intra-erythrocyte production of $O_2^{\cdot -}$, which could result in a negative correlation between SOD and GSH-Px activities found in our experiments. The discovery of the new Hb-Ch modification could explain how cholesterol may influence the organisation of the anti-oxidant defence system in erythrocytes. As the preferred method of lowering plasma lipid peroxidation (measured by quality biomarkers) is suggested to lose weight and reduce cholesterol intake, rather than taking large amounts of vitamin E supplements [42], our results are in agreement with these suggestions. Further studies should be performed to clarify the relationship among lipids, lipid peroxidation, and anti-oxidant enzymes. These studies are currently in progress in our laboratories.

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References

[1] B. Chance, H. Sies, A. Boveris, Hydroperoxide metabolism in mammalian organs, Physiol. Rev. 59 (1979) 527–605.
[2] M. Gul, F.Z. Kutay, S. Temocin, O. Hanninen, Cellular and clinical implications of glutathione, Indian J. Exp. Biol. 38 (2000) 625–634.
[3] H.R. Andersen, J.B. Nielsen, F. Nielsen, P. Grandjean, Antioxidative enzyme activity in human erythrocytes, Clin. Chem. 43 (1997) 562–568.
[4] P.M. Sinet, F. Lavelle, A.M. Michelson, H. Jerome, Superoxide dismutase activities of blood platelets in Trisomy 21, Biochem. Biophys. Res. Commun. 67 (1975) 904–909.
[5] P.M. Sinet, A.M. Michelson, A. Bazin, J. Lejeune, H. Jerome, Increase in glutathione peroxidase activity in erythrocytes from Trisomy 21 subjects, Biochem. Biophys. Res. Commun. 67 (1975) 910–915.
[6] M.-C. Pastor, C. Sierra, M. Dolade, E. Navarro, N. Brandi, E. Cabre, A. Mira, A. Seres, Antioxidant enzymes and fatty acid status in erythrocytes of Down syndrome patients, Clin. Chem. 44 (1998) 924–929.
[7] B. Golse, Q. Debray, K. Ruget, A.M. Michelson, Dosages errythrocytaires de la superoxyde dismutase 1 et de la glutathion peroxidase dans les schizophrenies de l'adulte, La Nouvelle Press Medicale 7 (1978) 2070–2071.
[8] B. Golse, P. Debray-Ritzen, P. Durosay, K. Ruget, A.M. Michelson, Pertubation de deux enzymes: la superoxyde dismutase 1 et de la glutathion peroxidase dans la psychose infantiles de developpement (autisme infantile), Revue de Neurologie (Paris) 134 (1978) 699–705.
[9] B. Golse, P. Debray-Ritzen, K. Ruget, A.M. Michelson, Dosages errythrocytaires et plaquettaires des superoxyde dismutases (1 et 2) et de la glutathion peroxidase dans les psychoses infantiles de developpement, La Nouvelle Press Medicale 7 (1978) 1952–1954.
[10] J. Maral, K. Puget, A.M. Michelson, Comparative study of superoxide dismutase, catalase and glutathione peroxidase levels in erythrocytes of different animals, Biochem. Biophys. Res. Commun. 77 (1977) 1525–1535.
[11] A. Crastes de Paulet, Les lipides membranaires: un cible priviligiee des radicaux lebres, Cah. Nutr. Diet 22 (1987) 23–33.
[12] J.B. de Haan, F. Cristiano, R.C. Iannello, I. Kola, Cu/Zn-superoxide dismutase and glutathione peroxidase during aging, Biochem. Mol. Biol. Int. 35 (1995) 1281–1297.
[13] M.E. Percy, A.J. Dalton, V.D. Markovic, D.R. McLachlan, J.T. Hummel, A.C. Rusk, D.F. Andrews, Red cell superoxide dismutase, glutathione peroxidase and catalase in Down syndrome patients with and without manifestations of Alzheimer disease, Am. J. Med. Genet. 35 (1990) 459–467.
[14] C. Michiels, M. Raes, O. Toussaint, J. Remacle, Importance of Se-glutathione peroxidase, catalase and Cu/Zn-SOD for cell survival against oxidative stress, Free Radic. Biol. Med. 17 (1994) 235–248.
[15] M. Nikolic, D. Stanic, N. Antonijevic, V. Niketic, Cholesterol bound to hemoglobin in normal human erythrocytes: a new form of cholesterol in circulation? Clin. Biochem. 37 (2004) 22–26.
[16] C.C. LaBrake, L.W. Fung, Phospholipid vesicles promote human hemoglobin oxidation, J. Biol. Chem. 267 (1992) 16703–16711.
[17] A.A. Akhrem, G.M. Andreyuk, M.A. Kisel, P.A. Kiselev, Hemoglobin conversion to hemichrome under the influence of fatty acids, Biochem. Biophys. Acta 992 (1989) 191–194.
[18] J. Szebeni, K. Toth, Lipid peroxidation in hemoglobin-containing liposomes. Effects of membrane phospholipid composition and cholesterol content, Biochim. Biophys. Acta 857 (1986) 139–145.
[19] M. Spasic, P. Durosay, K. Puget, A.M. Michelson, Comparison of pharmacokinetic and cell binding properties of turkey Cu-SOD and *E. coli* Mn-SOD, Free Rad. Res. Commun. 1 (1986) 189–199.
[20] Executive Summary of the Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III), JAMA 285 (2001) 2486–2497.
[21] M. Tsuchihashi, Zur Kernntnis der Blutkatalase, Biochem. Z. 140 (1923) 65–74.
[22] J.M. McCord, I. Fridovich, The reduction of cytochrome *c* by milk xantine oxidase, J. Biol. Chem. 243 (1968) 5753–5760.
[23] A. Claiborne, in: R.A. Greenwald (Ed.), Handbook of Methods for Oxygen Radical Research, CRC Press Inc., Boca Raton, 1984, pp. 283–284.
[24] D.E. Paglia, N.W. Valentine, Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase, J. Lab. Clin. Med. 70 (1967) 74–77.
[25] D. Glatzle, J.P. Vuilleumier, F. Weber, K. Decker, Glutathione reductase test with whole blood: a convenient procedure for the assessment of the riboflavin status in humans, Experientia 30 (1974) 665–668.
[26] K. Hering, S.M. Kim, A.M. Michelson, F. Otting, K. Puget, G.J. Steffens, L. Flohe, The primary structure of porcine Cu–Zn superoxide dismutase. Evidence for allotypes of superoxide dismutase in pigs, Biol. Chem. Hoppe Seyler. 366 (1985) 435–445.
[27] C.F. Reed, G. Swisher, V. Marinetti, E.G. Eden, Studies of the lipid of the erythrocyte. I. Quantitative analysis of the lipids of normal human red blood cell, J. Lab. Clin. Med. 56 (1960) 281–289.
[28] E.D. Hinkle, W. Wiersma, G.S. Jurs, in: Applied Statistics for Behavioral Sciences, Houghton Mifflin Company, Boston, 1994, p. 574.
[29] J.M. Rifkind, E. Nagababu, S. Ramasamy, L.B. Ravi, Hemoglobin redox reactions and oxidative stress, Redox Rep. 8 (2003) 234–237.
[30] C.C. Winterbourn, Haemoglobin oxidation and free radical production in the red cell, Biomed. Biochim. Acta 42 (1983) S134–S138.
[31] L. Guemouri, Y. Artur, B. Herbeth, Biological variability of superoxide dismutase, glutathione peroxidase, and catalase in blood, Clin. Chem. 37 (1991) 1932–1937.
[32] D.V. Godin, S.A. Wohaieb, M.E. Gernett, A.D. Goumeniouk, Antioxidant enzyme alterations in experimental and clinical diabetes, Mol. Cell. Biochem. 84 (1998) 223–231.
[33] J. Yam, L. Frank, R.J. Roberts, Oxygen-toxicity: comparision of lung biochemical responses in neonatal and adult rats, Pediat. Res. 12 (1978) 115–119.
[34] A. Jovanovic-Galovic, D.P. Blagojevic, G. Grubor-Lajsic, R. Worland, M.B. Spasic, Role of antioxidant defense during different stages

of preadult life cycle in European corn borer (*Ostrinia nubilalis*, Hubn.): diapause and metamorphosis, Arch. Insect Biochem. Physiol. 55 (2004) 79–89.
[35] A. Meister, M.E. Anderson, Glutathione, Annu. Rev. Biochem. 52 (1983) 711–760.
[36] S.K. Aggarwal, A.S. Saini, Forms and detoxification of oxygen free radicals and peroxide in erythrocyte, Indian J. Hematol. 2 (1984) 39–44.
[37] M.B. Yim, P.B. Chock, E.R. Stadtman, Enzyme function of copper, zinc superoxide dismutase as a free radical generator, J. Biol. Chem. 268 (1993) 4099–4107.
[38] M. Pelad-Kamar, J. Lotem, I. Wirguin, L. Weiner, A. Hermalin, Y. Groner, Oxidative stress mediates impairment of muscle function in transgenic mice with elevated level of wild-type Cu/Zn superoxide dismutase, Proc. Natl. Acad. Sci. USA 94 (1997) 3883–3890.
[39] M. Kurata, M. Suzuki, N.S. Agar, Antioxidant systems and erythrocyte life-span in mammals, Comp. Biochem. Physiol. B 106 (1993) 477–487.
[40] L.W. Oberley, T.D. Oberlay, Role of antioxidant enzymes in cell immortalization and transformation, Mol. Cell. Biochem. 84 (1988) 147–153.
[41] J.K. Gambhir, P. Lali, A.K. Jain, Correlation between blood antioxidant levels and lipid peroxidation in rheumatoid arthritis, Clin. Biochem. 30 (1997) 351–355.
[42] B. Halliwell, How i became a biochemist, IUBMB Life 56 (2004) 569–570.