International Journal of BioMedicine 3(1) (2013) 15-19

INTERNATIONAL JOURNAL OF BIOMEDICINE

CLINICAL RESEARCH

Correlation between Antioxidant Enzymes Activity and Intraerythrocyte Concentration of Fe, Mg, Zn, Cu in Pulmonary Arterial Hypertension and Cor Pulmonale in Children with Congenital Lung Disease and Cystic Fibrosis

Elena M. Vasilyeva, PhD,ScD[1], Michael I. Bakanov, PhD, ScD[1], Kirill A. Zykov, PhD, ScD[2] , Julia V. Solovjeva[1], Anna O. Bogatyreva, PhD[2], Anna V. Rvacheva, PhD[2], Olga I. Simonova, PhD, ScD[1],Victoria B. Beilina, PhD[2], Elena I. Kaznacheeva, PhD[2], Tatyana N. Kuznetzova, PhD[2], Natalia N. Mazanova[1]

[1]*Scientific Centre for Children's Health of Russian Academy of Medical Sciences,*
[2]*Moscow State University of Medicine and Dentistry,*
Moscow, Russian Federation

Abstract

Significant changes in the levels of the potential prooxidant Cu (increase) and the antioxidant Zn (decrease) in plasma were revealed in children having bronchopulmonary dysplasia (BPD) complicated by pulmonary arterial hypertension (PAH) and chronic cor pulmonale (CCP) when compared with the control. The Zn / Cu ratio in the blood plasma of patients with BPD, especially in CCP, was found to be lower than in the control group ($p<0.001$). This could indicate the activation of the prooxidant processes; simultaneously, the total antioxidant status (AOS) decreased. No significant increase in the intracellular free ("ionized" (i)) form of magnesium (iMg) was found; in fact, the concentration of iFe in all the patient groups was higher than in the control. An increase in the iCu and iZn levels (nonprotein-bound) was observed in the blood cells of the affected children. A significant increase in the glutathione peroxidase activity in the CCP patients may indicate an accumulation of organic peroxides, and partially compensate for the lesser activity of superoxide dismutase (SOD) and other antioxidants. The Zn / Cu and iZn/ iCu ratios were reduced in patients with CCP when compared with patients with PD without CCP.

Key words: *free ions, glutathione peroxidase, superoxide dismutase, chronic cor pulmonale, pulmonary arterial hypertension*

Introduction

According to modern concepts, pulmonary arterial hypertension (PAH) is a multifactorial pathology where the pathogenesis of PAH involves the participation of various biochemical processes and cell types. It was P. White who proposed the term "cor pulmonale" (CP) in 1931. Forty years ago, an expert committee of the World Health Organization defined cor pulmonale as "hypertrophy of the right ventricle resulting from diseases affecting the function and/ or structure of the lungs . . ." It is known that one of the conditions necessary for the long-term adaptation to extreme environmental conditions is a modification of the lipid composition of the membranes, directed towards altering the information and energy flows as well as the functional activity of cells. For an optimum flow of the metabolic processes, a human being requires at least 10 trace elements: Fe, Cu, Mn, I, Zn, Cr, Se, Mo, Co, and F. Concentrations of the macro-and micronutrients in the blood and

***Corresponding author**: Elena M. Vasilyeva PhD, ScD, Laboratory of Clinical Biochemistry, Scientific Center for Children's Health of Russian Academy of Medical Sciences, 2/62, Lomonosov ave., Moscow, 117963, Russia.*
Tel: 7-499-1254954. E-mail: Elena-vasilieva-44@yandex.ru

the cells are very strictly regulated by the biological mechanisms that provide homeostasis in healthy subjects and those in the pre-disease state [1]. Any change in the concentration of even one of the elements usually results in changes in the levels of the others. An adverse impact of different endogenous and exogenous factors can disrupt the balance between the levels of the divalent ions (pro- and antioxidants), which promotes the development of oxidative stress (OS). Violations against the background of OS may be due to enzyme activity alterations, including the activity of the antioxidant enzymes (their immediate inactivation or the oxidative violation of the nucleic acids encoding them and regulating the activity of the transcription factors) [2]. In the current literature, very little information is available regarding the copper, zinc, magnesium and iron levels in the erythrocytes, especially in children.

The aim of the study was to explore the changes in the concentrations of the anti-oxidants, pro-oxidants and cations of copper, zinc, iron and magnesium in the blood plasma and the concentrations of these free ions in the erythrocytes in children with bronchopulmonary dysplasia complicated by pulmonary arterial hypertension and chronic cor pulmonale.

Material and Methods

Between 2007 and 2011 we examined more than 300 children with various forms of BPD. In general, these were patients with congenital bronchopulmonary malformations and cystic fibrosis (CF). Patients were divided into 3 groups: the 1st group (n=110) included children with bronchopulmonary dysplasia (BPD) without CP and PAH; the 2nd group (n=80) had children with BPD and PAH; the 3rd group (n=52) included children with BPD and chronic cor pulmonale (CCP). As a control (reference the 4th group) data obtained from apparently healthy children without BPD was used (n=30). All the children were surveyed in the Scientific Center for Children's Health of RAMS. All children were about the same age, although patients with CCP were slightly older than those patients without PAH and CCP (Table 1).

Blood was collected into tubes with heparin-lithium; whole blood 50 µl was collected for determination of glutathione peroxidase (GP) activity using Randox kits on a DU 530 spectrophotometer (Beckmann Coulter, USA). Next, the blood was centrifuged, plasma was separated and its antioxidant status (AOS) was analyzed using Randox kits and magnesium, iron, and phosphorus ionic content was determined using an automatic Beckmann Coulter Synchron CX-5Δ analyzer (USA). The amount of zinc and copper was determined spectrophotometrically using Sentinel kits (Italy), ionized calcium (Ca2 +) in the ion-selective Microlyte analyzer ("Konelab").

The erythrocytes were then washed thrice with cold physiological solution, following by cell sedimentation. After the last wash, 0.5 mL of the mixed erythrocyte sediment was thoroughly but carefully added to 2.0 mL of deionized water and frozen for complete cell hemolysis. Then, 0.005 mL of cells was drawn from the mixed pellet for erythrocyte counting in the probe. After hemolysis superoxide dismutase activity (SOD) in the cells was determined using the Randox standard kits and the level of free (nonprotein-bound) Cu ion (iCu), Zn ion (iZn), Fe ion (iFe), Mg ion (iMg) was determined using an automatic Beckmann Coulter Synchron CX-5Δ analyzer, and malone dialdehyde (MDA) content was determined by a reaction with TBA. To determine the free intracellular ions after lysate defrosting, first the hemoglobin and proteins were removed using trichloroacetic acid (TCA) by slowly adding it to 5% final concentration.

Statistical data analysis was carried out using Statistical software. The differences between groups were verified statistically, significant at probability forecasting ($p<0.05$). The correlation coefficient was computed by the Pearson method.

Results

The phosphorus level in the blood plasma of patients with BPD was higher than in the control group ($p_{1/4}<0.001$, $p_{2/4}<0.01$), while the level of Ca^2 was significantly lower in patients with CCP than in the control group ($p_{1/4}<0.01$). The Mg and Fe levels in the blood plasma of patients did not differ from those of

Table 1.
The ionic homeostasis and antioxidant status in patients with BPD

Groups / Parameters	BPD (1)	BPD +PAH (2)	BPD +CCP (3)	Control (4)
Age	9.2±0.4	10.8±0.7	13.3±0.5	11.0±0.6
Plasma				
Ca^{2+} mmol/L	1.167±0.006	1.180±0.009	1.129±0.019	1.189±0.006
Pi mmol/L	1.45±0.02	1.39±0.03	1.33±0.04	1.23±0.05
Cu µmol/L	10.2±0.13	11.7±0.9	11.0±0.3	9.38±0.26
Zn µmol/L	21.2±0.1	20.9±0.5	20.4±0.5	22.5±0.3
Fe µmol/L	15.4±0.5	16.9±1.2	14.5±1.4	15.0±1.3
Mg mmol/L	0.94±0.01	0.94±0.02	0.9±0.02	0.9±0.02
Zn/Cu	1.85±0.05	2.16±0.14	1.73±0.08	2.44±0.07
AOC mmol/L	1.10±0.03	1.18±0,07	1.05±0.04	1.60±0.13
Erythrocytes / 10^{12} Erythrocytes				
iFe µmol/Er	55.3±1.1	53.3±2.0	51.2±2.4	43.2±0.3
iCu µmol/Er	4.6±0.07	4.61±0.3	4.43±0.15	4.02±0.03
iZn µmol/Er	25.9±0.3	26.5±1.0	24.3±0.5	22.0±0.2
iMg mmol/Er	0.36±0.01	0.37±0.01	0.36±0.01	0.34±0.01
iZn/iCu	6.04±0.02	6.52±0.28	5.48±0.2	5.47±0.4
GP UE /L	44.9±2.3	34.8±2.9	64.7±9.0	39.6±3.8
SOD UE /Er	120.6±5.4	135.5±14.0	109.9±7.6	130.2±15.0
МДА µmol/Er	36.4±1.7	35.0±2.9	33.2±2.6	19.1±2.7

Note*: See article text.*

the control group. The increase in the level of the prooxidant Cu ($p_{1/4}<0.01$, $p_{3/4}<0.01$, $p_{2/4}<0.05$) and the decrease in the level of the antioxidant Zn ($p_{1/4}<0.01$, $p_{2/4}<0.05$) in the blood plasma of patients were significant.

It is known that Cu and Zn are part of the active site of the Cu-Zn-SOD, and they are actively involved in regulating the activity of other enzymes. We found that the Zn/Cu ratio in the blood plasma of patients with BPD was less than in the controls ($p_{1/4, 3/4}<0.001$). This may indicate the activation of the prooxidant processes, which are in turn associated with a lower AOS ($p_{1/4}<0.01$, $p_{2/4}<0.05$, $p_{3/4}<0.001$). The reduction in the Zn/Cu ratio and AOS was especially pronounced in patients with CCP.

The level of the free intracellular iMg showed no significant changes; however, the iFe level was high in all the patient groups when compared with the controls ($p_{1/4, 2/4}<0.001$, $p_{3/4}<0.01$). An increase in the iCu ($p_{1/4}<0.001$, $p_{2/4}=0.05$, $p_{3/4}<0.05$) and iZn ($p_{1/4}<0.001$, $p_{2/4, 3/4}<0.01$) levels in the blood cells of the children with the BPD was observed when compared with controls. Simultaneously, the iZn/iCu ratio was found to increase, although it was significant only in the 2nd group ($p_{2/4}<0.05$).

A marked decrease in the SOD activity was seen in the erythrocytes accompanied by a sharp increase in the glutathione peroxidase (GP) activity, especially in patients with CCP ($p<0.05$); only this group of patients showed direct correlation between the SOD and the level of pulmonary pressure (r=+0.5). The MDA level was high in all the patients with BPD when compared with the control group ($p<0.01$); however, as the disease progressed, a tendency for the MDA level to decrease was observed.

Thus, PAH is associated with a slight increase in Ca^{2+}, Cu and AOS levels in the blood plasma. However, the addition of CCP reduces these parameters. The GP activity is reduced ($p<0.05$), while the SOD activity is increased in patients with PAH when compared with the patients of the 1st group. The addition of CCP leads to a sharp rise in the GP activity ($p_{2,3}<0.01$) and a subsequent inhibition of the SOD activity.

Discussion

The decrease in ionized calcium observed in BPD is probably due to the development of osteopenia and osteoporosis in the majority of the patients. The violation of bone mineral density was diagnosed almost in 30% patients with CCP. A negative correlation was found between the iP and the SOD activity in the patients of the 1st group (r=-0.36). Evidence indicates [3] that hyperphosphatemia may be a result of cytolysis; besides, the liberation of phosphate and potassium from the cells, and magnesium to a lesser degree, takes place during hemolysis, and the application of cytostatics, which is consistent with our data. A slight increase in iMg was observed in all the subgroups of patients. There is evidence to believe that ischemia reduces the ATP level and increases the levels of free intracellular magnesium in the heart and other organs because magnesium is associated with the ATP and RNA in the cells. It is believed that the increase in the Mg in ischemia is very important for further organ reperfusion [4].

It is known that Zn has an antioxidant effect, being a co-factor in the stabilization of the cytoplasmic membranes that are damaged by the products of lipid peroxidation. Also, Zn blocks the absorption of the prooxidant micronutrients. The effect of copper, however, similar to the iron ions, is dose-dependent. An adverse impact of various endogenous and exogenous factors can disturb the equilibrium, which ultimately contributes to the development of OS. The increase in the Zn/Cu ratio indicates the stability of the antioxidant protection. Rakitskiy VN and Yudina TV (2005) TV observed a clear correlation between the magnitude of the Zn/Cu ratio and the intensity of radical formation [5].

In this study, we found correlations between the pulmonary pressure level and the Zn level in the blood plasma of patients of the 2nd group (r=-0.46). A correlation was also noted between the Zn level in the blood plasma and AOS in patients with CCP (r=+0.45). Several recent papers have described the correlation between the level of trace elements and asthma which is the most studied among the bronchopulmonary disorders. Data on the zinc level in the serum of patients with bronchial asthma (BA) are contradictory; however, a correlation between this ion and asthma is undeniable [6]. Many experiments show a decrease in the SOD activity with an increase in the serum Zn concentration [7]. We, however, found no correlation between the SOD level activity and Zn in the plasma and the cells.

During hypoxia, most patients with BPD exhibited erythrocytes that were different from those formed under normal conditions. These had a shorter lifetime, showed changes in their physical and chemical properties, as well as expressed some changes in their metabolic features [8]. There is evidence that the lipid peroxidation within the organism is significantly activated under stress conditions. Tissue hypoxia leads to an increase in the iFe which is closely correlated with the accumulation of the products of lipid peroxidation.

Singlet oxygen and H_2O_2 can interact in the presence of free iron, liberating the highly reactive hydroxyl radical (·OH). It is found that high • OH levels promote an increase in the accumulation of free iron and copper ions in the storage places, which in turn leads to a fresh increase in the production of free radicals [9]. This is consistent with our data on the simultaneous increase in the iFe level and the accumulation of MDA in the erythrocytes of patients with BPD. We found a negative correlation between the level of GP activity and iFe in patients with CCP (r=-0.35). The iFe level showed a correlation with the MDA content in patients with PAH (r=+0.41). According Kocyigit A., et al. (2004), in children with asthma, the elevated MDA level did not return to the normal level even after treatment. The authors suggested that such a permanently high MDA level indicates "a chronic inflammatory process" [10].

Oxidative stress leads to the oxidation of arachidonic acid and the formation of a new series of prostanoid mediators called isoprostanes, which increase bronchoconstriction and exudation. In the neutrophils, in the presence of the chloride ions, myeloperoxidase metabolizes H_2O_2 to produce hypochlorous acid, which is a strong oxidant [11]. During hypoxia, the ATP synthesis gets terminated, phospholipases are activated by the Ca^{2+} ions, permeability of the intracellular membranes becomes increased, Fe^{2+} ions migrate from the membrane compartments in the cytoplasm and activate the lipid peroxidation. Metabolic acidosis is a potential factor for the accumulation of Fe^{2+}, and an "alkalization" of the plasma with sodium bicarbonate (a well-known method in clinical practice) is pathogenetically justified, even under the conditions of a slight deficiency of bases [12].

It is known that 60% of the Cu in the blood is found in the erythrocytes and leukocytes, mostly determined in the combined

form, specifically as the copper-carrying protein ceruloplasmin. The stability of erythrocytes to hemolysis is caused by the bond of the ceruloplasmin with a specific receptor protein on the membrane of the erythrocyte. The tripeptide bond with Cu ensures the supply of Cu and Fe into the cell in a non-toxic form. Copper has anti-inflammatory properties, and reduces the expression of autoimmune diseases. Copper also enhances the action of prostacyclin, helping it to bind to the receptors; however, zinc weakens this bonding [1].

As copper deficiency leads to a twofold increase in the peroxide processes, an increase in the Cu level in patients with BPD can be qualified as a compensatory response. However, no difference in the Cu levels in the patient subgroups has been revealed. It is known that Zn inhibits the oxidation of the free radicals catalyzed by Fe at the cellular level. We identified an increase in the iZn level; on the one hand, this increase can be positive, as it occurs against the background of a significant increase in the iFe level. On the other hand, this increase in the level of free ions in the cell indicates a lower degree of bonding of these ions with proteins, and this therefore, indicates their non-participation in the function of many enzymes [1].

Rukgauer M., et al. (2001), found that the decrease in the Zn level is associated with the violation of the structure and functional activity of the SOD and its ability to remove the reactive oxygen [13]. There is evidence to support that the release of H_2O_2 from the alveolar macrophages leads to the development of pulmonary fibrosis. Cu-Zn-SOD-deficient (SOD -/-) mice have found to generate lesser numbers of H_2O_2 and have less oxidative stress; they do not have pulmonary fibrosis and the redistribution of type-I collagen in the lung fibroblasts. The formation of H_2O_2 mediated by the Cu Zn-SOD is inhibited in the absence of iron-sulfur proteins [14]. This is consistent with our data regarding the increase in the free iFe in the erythrocytes of patients in all the subgroups. The decrease in the SOD activity can be partly attributed to the compensatory mechanisms in these children.

The overexpression of SOD has been shown to lead to the formation of a stable pathological phenotype in animal tissues; approximately 20% of the radicals do not have the time to react with the SOD; it is assumed that they migrate through the cell membrane and stimulate lipid peroxidation and the oxidative degradation of proteins. The tendency of the oxidized proteins to aggregate slows their enzymatic hydrolysis [2]. There is evidence that adults with asthma are exposed to intense OS, and the GP activity in the erythrocytes is reduced [4].

In our study, only a small number of patients have gotten the asthma; perhaps the age factor may have also influenced the GP activity. It is well known that children adapt more easily even to very adverse conditions, much better than adults. Besides, the glutathione system is the main antioxidant mechanism in the airways.

Many antioxidants in high doses are toxic and can even become prooxidants. In pathology, the ability of the mitochondria to generate energy could be impaired. Therefore, the oxidative modification of proteins (OMP) is often regarded as one of the earliest and most reliable markers of OS. The oxidation products of the proteins in the oxidative tissue damage appear earlier and are more stable when compared with the products of lipid peroxidation. During OMP, including lipoproteins, a dysfunction of the proteolytic system is noted. Protein aggregates contain large quantities of SOD, accompanied by a reduction in its activity.

Conclusion

We noted the adverse effect of the high levels of intraerythrocytic free iron in patients with BPD. Not very marked differences in the iFe levels were observed in patients of the 2nd group compared with the 1st group, which indicates that the therapy subcompensates for the relevant biological processes. It is known that the use of antioxidants alone offers little promise, as these drugs cannot neutralize the Fe^{2+}, which are the main link for the free radical reactions. However, the increase in the iFe level indicates the progression of the oxidative stress and the involvement of a growing number of free iron ions in this process. Probably, children with BPD, especially at elevated pulmonary pressure levels, will benefit if iron chelators are included in the treatment. A significant increase in the GP activity in CCP patients may indicate an accumulation of organic peroxides and partially compensate for the low activity of the SOD and other antioxidants. The Zn/Cu and iZn/iCu ratios are reduced in patients with CCP when compared with the patients not affected with CCP. Probably, patients with CCP require drugs containing Zn, in addition to the primary therapy for the prevention of intensification of the OS, which is fundamental to the development of multiple organ failure [12,15]. The accumulation of toxic radicals, as it is known, is not instantaneous, and occurs over a certain time period.

References

1. Avtsyn AP, Zhavoronkov AA, Rim MA, Strochkova LS. Human microelementoses. M.: Medicine, 1991. [in Russian].
2. Dubinina EE, Pustygina AB. Free radical processes in aging, neurodegenerative diseases, and other pathological conditions. Biomedical chemistry 2007: 53(4):351-372. [in Russian].
3. Arkhipova N. Exchange of phosphate in health and disease. Bulletin of St Peter State Medical Acad 2004; 4 (5):71-74. [in Russian].
4. Murphy E, Freudenrich CC, Lieberman M. Cellular magnesium and Na/Mg exchange in heart cells. Ann Rev Physiol 1991; 53:273-287.
5. Rakitskiy VN. Yudina TV. Antioxidant and microelement status of the organism: present-day diagnostic problems. Vestn Ross Akad Med Nauk 2005; (3):33-6. [in Russian].
6. Urushidate S, Matsuzaka M, Okubo N. Association between concentration of trace elements in serum and bronchial asthma among Japanese general population. J Trace Elem Med Biol 2010; 24:236-24.
7. Hughes S, Samman S. The effect of zinc supplementation in humans on plasma lipids, antioxidant status and thrombogenesis. J Am Coll Nutr 2006; 25(4):285-91.
8. Kudryashov AM, Titova NM, Savchenko AA, Kudryashov EV. The content of ascorbic acid and its oxidized forms at the aging red blood cells produced in the conditions of normal and intense erythropoiesis. Biomedical Chemistry 2005; 51(1):53-59. [in Russian].
9. Kocyigit A, Armutcu F, Gurel A, Ermis B. Alteration in plasma essential trace elements selenium, manganese, zinc, copper and iron concentrations and the possible role of these elements on oxidative status in patients with childhood asthma. Biol Trace Elem Res 2004; 91(1): 31-41.
10. Kocyigit A, Armutcu F, Gurel A, Ermis B. Alterations in plasma essential trace elements selenium, manganese, zinc,

copper and iron concentrations and possible role of these elements on oxidative status in patients with childhood asthma. Biol Trace Elem Res 2004: 97:31-41.
11. Barnes PJ, Shapiro SD, Pauwels RA. Chronic obstructive pulmonary disease: molecular and cellular mechanisms. Eur Respir J 2003; 22:672-688.
12. Orlov YuP, Dolgich VT. Iron metabolism in biological systems (biochemical, pathophysiological and clinical perspectives). Biomedical chemistry 2007; 53(1): 25-38.
13. Rukgauer M, Neugebauer RJ, Plecko T. The relation between selenium, zinc and cooper concentration and the trace element dependent antioxidative status. J Trace Elem Med Biol 2001; 15:73-78.
14. He C, Murthy S, McCormic ML. Mitochondrial Cu,Zn-Superoxide dismutase mediates pulmonary fibrosis by augmenting H_2O_2 generation. J Biol Chem 2011; 286(17):15597-15607.
15. Inoue K, Branigan D, Xiong Z-G. Zink-induced neurotoxicity mediated by transient receptor potential Melastatin 7 channels. J Biol Chem 2010; 285(10): 7430-7439.