

Original Research

Heavy Metal-Induced Oxidative Stress and Changes in Physiological Process of Free Radicals in the Blood of White Stork (*Ciconia ciconia*) Chicks in Polluted Areas

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Abstract

The aim of this study was to examine the impact of Ca, Mg, Fe, Na, K, Zn, Cd, and Pb upon enzymatic activity of superoxide dismutase (SOD), catalase (CAT), and ceruloplasmine (CP) diurnal and nocturnal activity, and the content of thiobarbituric acid-reactive substances (TBARS), and their interaction with free radicals, in the blood of the White Stork (*Ciconia ciconia*), an altricial bird, during postnatal development, in polluted areas (copper manufacture) and in control environments.

The age of chicks examined from an output from an egg was increased from 19 to 54 days. Samples of investigated wing venous blood were taken for AAS analyses of element concentration. We collected blood samples via veni-puncture of the brachial vein of chicks.

We have stated significant interactions between Cd, Ca, and Mg, and TBARS, SOD, CAT, and CP activity. Interaction with Fe, Na, K, Zn, and Pb were not significant. We observed regularities in the course of relationships in the case of Cd; interactions of Cd-enzyme activity were negative in the control environment, both during the day time and at night. The prevalence of Cd participation in element-enzymes interaction takes place. Ca- and Mg-relationships were more differential; Ca-enzymes interactions were significant only during the day in polluted environments and all of them were positive. Relations with Mg were positive during the day and negative at night, but significant in polluted areas only.

We conclude that physiological activity of antioxidant systems SOD, CAT, and CP, and content of TBARS-active products are determined by concentrations of physiological elements and toxic heavy metals. These groups of elements influenced enzymatic activity both through excess and deficiency of their concentration in the environment. Simultaneously, we have not stated significant interactions with other microelements, thus we can conclude about their lack of important interactions on enzymatic activity.

Keywords: *Ciconia ciconia*, free radicals, toxic heavy metals, interactions, environmental stress, antioxidant enzyme activity

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Introduction

In the last few years researchers have seen declines in the populations of White Stork in Poland and West Europe particularly, and increases in the mortality rate amongst chicks, which have been linked to the pollution of their environment by heavy metals [1-3]. Research by these authors has linked concentrations of toxic metals in the organs of birds with higher mortality amongst chicks, and with a fall in fecundity. This indicates a necessity to determine the stages and mechanisms by which pollutants enter birds tissues during their development in the nest.

Metals' toxicity increases mortality rates of nestlings and adult birds and reduces productivity of their populations in various types of environmental pollution. In addition, they may give rise to anaemia, and to improper functioning of immunological matches [1-6]. Metals exert toxic effects on animals if they interfere with important biochemical reactions and metabolism. The threshold concentration at which such deleterious effects occur is usually higher for essential elements than for non-essential ones, although the "window for essentiality" for some elements is quite narrow. Unlike many organic chemicals, metals cannot be metabolized into less toxic components. When released into the environment by human activities, metals have long residence times in soils and may continue to exert harmful effects on the environment long after the source of pollution has ceased to operate [7, 8, 3, 5].

Interactions between chemical elements and antioxidant enzymatic activity plays an important role in the ecotoxicological response of an organism in its environment. Macro- and microelements, and toxic heavy metals have their differential ecotoxicological impact upon the course of the level of pro- and antioxidant activity of enzymes and on the development of lipoperoxidation processes. Thus studies on these relationships are necessary and they are very advisable for explanation of these coherences. We can find some attempts for explanation of the effects of these interactions, but they are rather concerned with laboratory conditions and ways of raising animals, e.g. Sanchez et al. [9] have studied copper-induced oxidative stress in the three-spined stickleback (*Gasterosteus aculeatus*), a raised fish, and its relationship with hepatic metal levels. Similar studies were undertaken by Zhikic et al. [10], who studied activities of superoxide dismutase and catalase in erythrocytes and plasma transaminases of goldfish (*Carassius auratus gibelio*) exposed to cadmium. Stajn et al. [11] examined the effects of exogenous cadmium on antioxidant defense systems in the kidney, and the possible protective role of Se against Cd toxicity, in male Wistar albino rats, under laboratory conditions.

Other investigations conducted in laboratory conditions were concerned with the role of toxic and heavy metals on antioxidant activity [12-16]. Some of these papers deal with mechanisms involved in metal-induced oxidative damage and characterized heavy metal toxicity in connection with oxidative stress, and antioxidant nutrients [17-22]. Only very few papers were

concerned to study these effects under field conditions [23-25].

We can thus state the lack of studies about these relationships in natural conditions. Only Uchida et al. [26] investigated the reduction of erythrocyte catalase and superoxide dismutase activities in human male inhabitants of Cd-polluted areas in the Jinzu River basin (Japan). We can also find several general research studies that are more widespread, i.e. biogeochemical and element-enzyme interactions. However, they concern laboratory conditions, as a rule. Thus some papers have analyzed biogeochemical interactions affecting hepatic trace elements in aquatic birds [27]. Among others, Irato et al. [28] studied metal-metal interactions in rat liver and kidney and their relations with thioneins activity. Remaining papers that assumed the effects of laboratory and field investigations concerning toxic metals intoxication during particular physiological periods in birds and raising mammals, e.g. Benito et al. [29], studied ecological determinations of trace elements in blood collected from birds feeding in an area affected by a toxic spill.

The aim of this study was to examine the impact of macroelements and heavy metals of various chemical groups (Ca, Mg, Fe, Na, K, Zn, Cd, and Pb) upon enzymatic activity of most important antioxidant enzymes of the blood of White Stork (*Ciconia ciconia*), during post-natal development. We have taken under consideration the correlation between the level of Ca and heavy metals and the content of thiobarbituric acid-reactive substances (TBARS), activity of superoxide dismutase (SOD), catalase (CAT), and ceruloplasmine (CP) in diurnal and nocturnal phase, in polluted environments and control areas.

Experimental Procedures

Blood samples for analyses were collected in 2005 from young White Storks developing in relatively pure environment and treated as a control (Kłopot village; 52°07'56.3"N, 14°42'10.4"E with no industry in a radius of 150 km; SW Poland). (For more description of this area see also Tryjanowski et al. [30]). Samples were also collected in a small distance from Głogów (51°39'32.6"N, 16°04'49.9"E; SW Poland), where copper manufacture is localized. Głogów Copper Manufacture produces copper and lead from mining. Głogów plant copper leads to an active pro ecological activity towards the direction of wide creation of zones of safety. Green fields consists of about 50% of protective areas of this manufacturing complex. Forests are present in about 32% of this area. Acid soils are subjected by calcification technology.

The age of Stork chicks studied from output from an egg was changed from 19 up to 54 days. For elimination of diurnal rhythm changes all examinations were started at 10 a.m. and ended at 12 a.m.

Samples of investigated wing venous blood were taken for further analyses of Ca, Mg, Fe, Na, K, Zn, Cd, and Pb concentrations. The content of elements were then de-

terminated using a Perkin-Elmer atomic absorption spectrophotometer. Standard curves were prepared using standardized samples from Merck. Concentrations of elements were given in terms of $\mu\text{g}\cdot\text{g}^{-1}$ of dry weight (ppm dw).

We collected blood samples via veni-puncture of the brachial vein of Stork chicks. They were retrieved from the nest and placed into individual ventilated cotton sacks. Blood (5 ml) was collected using a 5 ml syringe washed up with EDTA. Samples were kept in a chilled cooler before transportation to the laboratory. After centrifugation, plasma samples were frozen at -20°C and stored until analysis. Our behavioral observations as well as physical examinations of the birds suggested that all of them were physically healthy.

Samples of investigated venous blood taken for analyses were representative of a level of oxidative stress processes by measuring lipid peroxidation through malondialdehyde (MDA), the last product of lipid breakdown caused by oxidative stress. The lipid peroxidation process was measured by TBARS method [31]. Intact blood was used for measurement of SOD activity (estimated from the extent of inhibition of superoxide (O_2^-) dependent quercetine transformation, according to the method of Kostyuk et al. [32]. CAT activity was determined in the plasma blood (after centrifugation $3000 \times \text{min}^{-1}$; 5 min) by the amount of H_2O_2 consumed $\times \text{min}^{-1} \times \text{l}^{-1}$ with molibdate ammonium [33]. CP activity was estimated using *p*-phenilenodiamine [34].

The results are expressed as mean \pm S.D. for animals in day and night time separately. Significant differences among the means were measured using a multiple range test at min. $P < 0.05$. Data not having a normal distribution were log transformed. Student *t*-test with 95% confidence intervals ($\alpha = 0.05$) was applied to determine significance of differences between element concentrations in types of environment day and night. Correlations were calculated between element concentrations in the blood of chicks and enzymatic activity of SOD, CAT, and CP (linear regression). Significance of these correlations (regression coefficients) was examined using ANOVA for correlation test. The approximation method by multinomial of 5-degree (least second power method) was also applied. Arithmetic mean concentrations of elements in blood were estimated by using two-way ANOVA.

Results

We have ascertained statistically significant interactions between concentrations of toxic heavy metals (Cd) and physiological elements (Ca, Mg), and the content of TBARS products and enzymatic activity of SOD, CAT, and CP in the blood of White Stork (*Ciconia ciconia*) chicks during their nesting development in SW Poland; interactions of Cd-enzyme activity were negative for reference of control environment, both during the day and at night (e.g. for TBARS products giv-

en in Fig. 1). It is worthy for underlining the prevalence of cadmium participation in element-enzymes interaction (Table 1).

The relationships with macroelements were more differential; Ca-enzyme interactions were significant only during the day in polluted environments and all of them were positive (Table 1). However, relationships between Mg and all investigated enzymes were positive during the day, and negative at night. Simultaneously, they were significant in polluted areas only (Table 1).

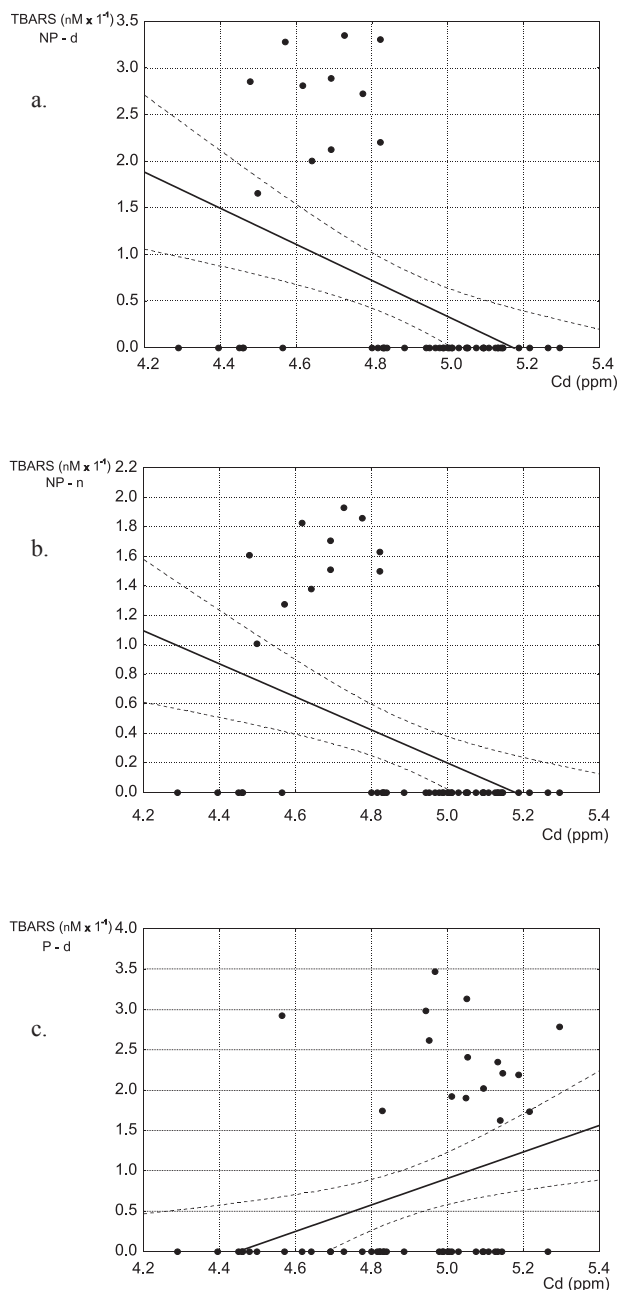


Fig. 1. Interactions between cadmium concentration and TBARS product contents in the blood of White Stork (*Ciconia ciconia*) chicks during their nesting development in SW Poland; in non-polluted environments (NP) during the day (a) and at night (b), and in polluted environments (P) during the day (c).

Table 1. Interactions element-enzyme in the blood of White Stork (*Ciconia ciconia*) chicks during their nesting development in SW Poland (superoxide dismutase SOD, catalase CAT, ceruloplasmine CP diurnal and nocturnal activity, the content of thiobarbituric acid-reactive substances TBARS, NP – not polluted area, P – polluted area, d – day, n – night).

Interaction	Regression equation	p	r
Cd – TBARS NP d	$y = -1.9350x + 10.0090$	0.0010	-0.4333
Cd – TBARS NP n	$y = -1.1200x + 5.7991$	0.0000	-0.4290
Cd – TBARS P d	$y = 1.6430x - 7.3110$	0.0080	0.3595
Cd – SOD NP d	$y = -282.4000x + 1467.1000$	0.0060	-0.3672
Cd – SOD NP n	$y = -195.0000x + 1015.2000$	0.0150	-0.3291
Cd – SOD P d	$y = 152.0700x - 683.2000$	0.0090	0.3502
Cd – CAT NP d	$y = -9.1350x + 47.4230$	0.0080	-0.3590
Cd – CAT NP n	$y = -12.9600x + 66.6490$	0.0030	-0.4020
Cd – CAT P d	$y = 8.7238x - 39.3200$	0.0000	0.4104
Cd – CP NP d	$y = -4.2140x + 21.8330$	0.0000	-0.4101
Cd – CP NP n	$y = -3.1600x + 16.3230$	0.0000	-0.4243
Cd – CP P d	$y = 5.9479x - 27.1300$	0.0000	0.4395
Ca – TBARS P d	$y = 146.6548 - 715.5306$	0.0010	0.3965
Ca – SOD P d	$y = 354.5674 - 1076.2140$	0.0000	0.3253
Ca – CAT P d	$y = 247.3974 - 864.9002$	0.0000	0.4051
Ca – CP P d	$y = 6.8964 - 51.0945$	0.0020	0.4070
Mg – TBARS P d	$y = 175.3547 - 789.5000$	0.0100	0.3960
Mg – TBARS P n	$y = -8.4566 + 39.7053$	0.0021	-0.2001
Mg – SOD P d	$y = 13.4657 - 67.0007$	0.0010	0.3178
Mg – SOD P n	$y = -186.7308 + 1240.0064$	0.0011	-0.4022
Mg – CAT P d	$y = 9.4653 - 53.0400$	0.0030	0.3440
Mg – CAT P n	$y = -6.3846 + 61.2400$	0.0001	-0.3946
Mg – CP P d	$y = 1.3990 - 8.2350$	0.0110	0.4380
Mg – CP P n	$y = -116.7330 + 780.4550$	0.0070	-0.4108

Physiological activity of most important enzymes investigated (SOD, CAT, CP) and TBARS products are determined by concentration of physiological elements and toxic heavy metals. Macroelements, however, influenced enzymatic activity both through excess and deficiency of their concentration in the blood and in the environment. Simultaneously, we have not stated significant interactions with microelements, that is to say about their lack of important interactions on enzymatic activity.

It is important to notice the predominance of cadmium participation in element-enzyme interactions (cadmium have significant impact upon biochemical activity of most important enzymes, especially superoxide dismutase and catalase). Simultaneously, we have stated negative element-enzyme interactions at night compared with those during the day. This can suggest prominently weakest

enzymatic activity during night in comparison with the daytime.

As we have ascertained in this paper, significant element-enzymes interactions are predominant in polluted areas, especially in the case of physiological elements (Ca, Mg). We can thus explain this tendency by intensive and prevailing metabolic reactions of toxic metals in redox reactions, which cause the priority of these metals to physiological elements, reflected by their influence upon enzymatic activity of antioxidant enzymes. The results of our studies show that concentrations of hardly toxic heavy metals gradually increased during nestling development, and in polluted areas were significantly higher as in control. This was probably due to a higher contamination of soils by them in polluted areas. Our investigations on White Stork chicks indicate the role of element-enzymes

interactions impact upon the definite image of the hemoglobin content and the values of red blood picture. The results of this study provide evidence that White Stork from control areas have better conditions for growth and development than in polluted ones. They also show the necessity to know the stages of growth to understand bioaccumulation processes of elements in chicks. Metal concentrations in the blood of chicks may be influenced by physiological responses of species to distinct metals, and by the greater or lesser bioavailability of these metals. In our studies on White Stork chicks we found correlations of element concentrations in the blood and the type of environment, particularly between element level and environmental stress. We concluded that the use of hematocrit and hemoglobin to assess the health and condition of birds is questionable, and may give a positive association with miscellaneous environmental loads.

Discussion

We can conclude from our results that physiological activity of most important enzymes, which were investigated, i.e. SOD, CAT, and CP, and TBARS products, is determined by their concentration, especially for physiological elements (Ca, Mg, Fe, Na, K) and toxic heavy metals (Cd, Pb). It seems that macroelements influenced enzymatic activity both through excess and deficiency of their concentration in the environment. Simultaneously, we have not observed significant interactions with microelements, which indicates their lack of important interactions on enzymatic activity. Previously we also analyzed pro oxidant and antioxidant balance processes in the blood of young storks and their age dependencies in these types of environments [35].

In our investigations we have observed negative element-enzyme interactions at night, compared with those during the day (Table 1). Results suggest the prominently weakest enzymatic activity during the night in comparison with the daytime. Simultaneously, we found a marked circadian variation in CP activity and in TBARS products in the blood of White Stork chicks. Our results testify to a significant decrease in the level of lipid peroxidation processes at night time as well as in the activity of CP [35]. However, we cannot compare our results with data from other authors on studying circadian rhythms for this group of animals because of their absence in the literature, as far as our knowledge.

Lipid peroxidation in cell membrane and subcellular organelles has been proposed as a primary mechanism for cellular membrane dysfunction and tissue injury associated with free-radical initiated processes, through redox reaction by metals. Elevated concentrations of lipid peroxides may disturb relations between protective and damaging factors in the tissues and at molecular level, testifying to changes in redox status. Although much is known about the chemistry of lipid peroxidation processes and cellular antioxidant defense mechanisms, chronobiological stud-

ies are needed to quantify the various cellular components involved in these processes and a better understanding of their role in physiological processes. Chronomes of putative anti- and prooxidants have recently been mapped to explore their damaging role as markers in redox status of tissues [36, 37].

Periodically changing environmental factors, such as food availability, temperature, or social stimuli, can synchronize avian circadian rhythms, but the most important synchronizer is the periodic alteration of light intensity with melatonin as the effector molecule [36]. Melatonin has been found to be an effective antioxidant and oxygen free radical scavenger (ROS). Reactive oxygen species, antioxidants and their induced redox alterations have been previously found to influence a number of gene expression and signal transduction pathways [37-40].

It is important to notice the predominance of cadmium participation in element-enzymes interactions (Table 1). Moreover, when comparing our results it must take under consideration that most research emphasized significant impact of toxic heavy metals (particularly cadmium and lead) upon biochemical activity of most important enzymes, especially superoxide dismutase and catalase, e.g. Uchida et al. [26] have indicated that erythrocyte catalase and Cu/Zn-SOD activities are reduced as a result of long-term Cd exposure in Cd-polluted areas. They also suggested that erythrocyte catalase and Cu/Zn-SOD activities may be sensitive markers for predicting renal tubular damage due to chronic Cd exposure. Besides, since Cd generates ROS and inhibits antioxidant enzyme activities in erythrocytes (and kidneys), antioxidant enzyme activities in erythrocytes may become noninvasive biological markers for assessing and predicting renal tubular injury [26]. Similar results were obtained by Stajn et al. [11]. These authors suggested that Cd accumulation in the kidneys of rats, due to chronic dietary intake of Cd, is associated with marked alterations of antioxidant defense system (AOS). However, they do not indicate an obvious impairment of the kidney AOS. Furthermore, Cd-induced injury is not prevented by simultaneous intake of Se, which induces significant improvement of the kidney AOS. Therefore, the role of altered AOS in the development of Cd-induced nephrotoxicity, although possible, is not completely clear [11] and thus cannot be explained.

The importance of cadmium as a modulator of antioxidant enzymatic activity in erythrocytes and plasma transaminases is emphasized by Zhikic et al. [10]. They concluded that Cd induces appearance of anemia and alters the metabolism of proteins and carbohydrates. They observed the decreased activity of SOD in erythrocytes during Cd exposure, which indicates the presence of ROS-induced peroxidation, which leads to the destruction of red blood cell membranes. Moreover Congiu et al. [41] stated that chronic Cd administration in starlings yields a positively correlated increase in GSH (hepatic glutathione) levels (GSH is known to protect cells from oxidative damage through its oxidation as GSSG via Se-dependent GSH-Px (glutathione peroxidase). However, Nakahama

et al. [14] points that cadmium is slightly inhibitory to oxidant enzyme activities in animals.

Our results stated in this paper indicate significant element-enzyme interactions in the polluted areas, especially in the case of physiological elements (Ca, Mg). We can thus explain this tendency by intensive and prevailing access of toxic metals in redox reactions. This causes the priority of these metals to physiological elements, reflected by their influence upon enzymatic activity of antioxidant enzymes. These stages of reactions have been stated by Ercal et al. [13]. They found that transition metals act as catalysts in oxidative reactions of biological macromolecules, therefore toxicities associated with these metals might be due to oxidative tissue damage. Redox-active metals, such as iron, copper and zinc, undergo redox cycling, whereas redox-inactive metals, such as lead, cadmium, mercury, deplete cells' major antioxidants, particularly thiol-containing antioxidants and enzymes. Additionally, either redox-active or redox-inactive metals may cause an increase in the production of reactive oxygen species (ROS). Enhanced generation of ROS can overwhelm cells' intrinsic antioxidant defenses, and result in a condition known as "oxidative stress." Cells under oxidative stress display various dysfunctions due to lesions caused by ROS to lipids, proteins and DNA. So it can be suggested that metal-induced oxidative stress in cells can be partially responsible for toxic effects of heavy metals [13, 24, 25, 42]. In accordance with this we can consider that direct relationships exist among physiological elements (especially Ca, Mg, K, Fe) and toxic heavy metals, in the sphere of their participation and modification of peroxidation processes [27, 12, 24, 15]. Toxic heavy metals (Cd, Pb, Hg, Cr) exposure also affects the status of essential elements (Ca, Mg, K, Fe), which causes further decrease of antioxidant processes and detoxification processes. Thus, early detection and treatment of metals burden is important for successful detoxification. Optimization of nutritional status is also paramount to the prevention and treatment of metal toxicity [12, 22] in animals in polluted environments.

In recent research papers it was determined that the effect of antioxidant supplementation following heavy metals exposure. They suggest that antioxidants may play an important role in abating some health hazards of heavy metals in connection with interaction of physiological free radicals (health effects). So multiple mechanisms may be responsible for ROS production in toxic metal exposure. Among them, alterations in thiol status, increased lipid peroxidation, production of ROS, and damage to cell's antioxidant defense systems are well known for all redox-active and inactive elements. Chelators of various metals are given to increase excretion of metals, but unfortunately, there are many numerated [41, 13, 15].

On the basis of our research we can summarize that statistically significant interactions exist between toxic heavy metals (Cd) and physiological elements (Ca, Mg). Also, the enzymatic activity of SOD, CAT, and CP, and TBARS content occurring in the blood of White Stork

chicks. Interactions with Fe, Na, K, Zn, and Pb were not significant. We can observe regularities in the course of relationships investigated in the case of cadmium; interactions Cd-enzymes activity were negative for control environment, both during the day and at night time. Relationships with macroelements were differential; interactions Ca-enzymes were significant only during the day in polluted environments and all of them were positive. However, relations between Mg and investigated enzymes were positive during the day, and negative at night time, but significant in polluted areas only.

We can thus conclude that physiological activity of enzymes (SOD, CAT, CP) and TBARS products is determined by concentrations of physiological elements and toxic heavy metals. These groups of chemicals influenced enzymatic activity both through excess and deficiency of their concentration in the environment. Simultaneously, we have not found significant interactions with microelements, that is to say about their lack of evident interactions on enzymatic activity. On the other hand, it is important to notice the predominance of cadmium participation in element-enzyme interactions (Table 1). Moreover, it must take under consideration that most research emphasized significant impact of toxic heavy metals (particularly Cd and Pb) upon biochemical activity of most important enzymes, especially superoxide dismutase and catalase. We have also found negative element-enzymes interactions at night, compared with those during the day time. It can so be suggested that prominently weakest enzymatic activity occurs during the night compared with the day.

As we have registered in this paper, significant element-enzyme interactions are predominant in the polluted areas, especially in the case of physiological elements. We can thus explain this tendency by intensive and prevailing metabolic pathways of toxic metals in redox reactions, which cause the priority of these metals to physiological elements, reflected by their influence upon enzymatic activity of antioxidant enzymes.

Summary and Conclusions

In conclusion, our results showed that significant interactions between toxic heavy metals (Cd) and physiological elements (Ca, Mg), and enzymatic activity of enzymes: SOD, CAT, and CP and the content of TBARS occurs in blood of White Stork chicks. Relations among Fe, Na, K, Zn, and Pb, and enzyme activity were not significant. We can observe regularities in the course of relationships in the case of Cd; Cd-enzymes interactions activity were negative for control environment, both during the day and at night. We have stated that enzyme relations with macroelements were differential; interactions of Ca-enzymes were significant during the day in polluted environments and all of them were positive. However, relations with Mg were positive during the day, and negative at the night. Simultaneously, they were significant in polluted areas.

We can suggest that activity of SOD, CAT, and CP and the content of TBARS is determined by physiological elements and toxic metals. These groups of chemicals influenced enzymatic activity both through excess and deficiency of their concentration in the environment. Simultaneously, we have not stated significant interactions with microelements, that is to say about their lack of important interactions on enzymatic activity. It is important to notice Cd predominance participation in element-enzyme interactions. We have found negative element-enzyme interactions at night, compared with those during the day. This can suggest that prominently weakest enzymatic activity occurs during the night in comparison with the day. Moreover, significant element-enzyme interactions are predominant in polluted areas, especially in the case of physiological elements (Ca, Mg). We can thus explain this tendency by intensive and prevailing metabolic reactions of toxic metals in redox reactions. This causes the priority of these metals to physiological elements, reflected by their influence upon enzymatic activity of antioxidant enzymes.

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