EFFECT OF PESTICIDES ON RAT (*Rattus norvegicus*) ERYTHROCYTES ANTIOXIDANT ENZYMES *IN VITRO*

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Abstract: In the last century, maximum in herbicide production was achieved. Growing use of herbicides initiated the need for continuous evaluation of damaging effects of herbicides on human health and environment. Paraquat is the trade name for N,N′-dimethyl-4,4′-bipyridinium dichloride and one of the most widely used herbicides in the world. Although mechanism of paraquat toxicity remains undefined, a great portion of toxicity is attributed to the process of redox cycling. In this research, rat erythrocytes were exposed to various paraquat concentrations (0, 0.25, 0.5, 0.75, 1.25 mM). Changes in antioxidant enzymes activity, catalase and superoxide dismutase were determined, and also the activity of erythrocyte acetylcholinesterase. Obtained results show damaging effects of paraquat on erythrocytes due to oxidative stress.

INTRODUCTION

For thousands of years, man is investing enormous efforts and available resources aiming to eliminate weeds from plant crops. The use of pesticides and other agrochemicals caused a major increase in crop yield. During 1960s it has become obvious that herbicides have a great impact on the environment. One very frequently used herbicide in the world is paraquat. Paraquat is the trade name for N,N′-dimethyl-4,4′-bipyridinium dichloride. It is highly toxic for animals and humans. Acute exposure results in high mortality rate between 60 and 89% (Vale et al, 1987; Lin, 1990). Erythrocytes are efficient ROS scavengers due to antioxidative systems located in their cytoplasm. There are many studies about toxic effect of paraquat and other herbicides on erythrocytes (Ray et al, 2007; Bainy et al, 1994; Santi et al, 2011). Although the precise mechanism of paraquat toxicity has not been determined yet, it is known that redox cycling plays a great role in it. Redox cycling is a process in which paraquat is being reduced, and then re-oxidized in the presence of oxygen. It has two characteristics which are relevant for the development of paraquat toxicity: ROS formation and dissipation of cellular reducing equivalents (NADPH). Redox cycling induces oxidative stress in cells, and prolonged exposure to oxidative stress may lead even to cell death (Sies, 1985). In many studies performed on animals, paraquat induced oxidative stress, DNA damage and alterations of DNA repair systems (Ross et al, 1979; Dusinka et al, 1999). Even though several different antioxidants are located in erythrocytes, the most important elements of their antioxidative system are catalase and superoxide dismutase. Activities of these two enzymes are often used as bioindicators of ROS formation (Zelikoff et al, 1996). In this paper, activities of SOD and CAT in erythrocytes were observed after *in vitro* treatment with several different paraquat concentrations. Activity of erythrocytes acetylholinesterase was also studied. Many *in vitro* and *in vivo* studies showed inhibition of AChE by paraquat (Seto and Shinohara, 1987; Di Marzio et al, 1998; Ahmed et al, 2007; Santi et al, 2011). In this paper, the toxic effect of paraquat on rat erythrocytes was observed. We also wanted to show if rat erythrocytes could serve as appropriate models for *in vitro* studies of oxidative stress induced by pesticides.

MATERIALS AND METHODS

Blood was obtained from male Wistar rats by cardiac puncture in EDTA vials. Specimens were centrifuged at 3000 rpm for 10 minutes, and plasma and buffy coat were removed. Erythrocytes were washed three times with isotonic saline and centrifuged afterwards. After that, packed red blood cells were resuspended in isotonic saline and treated with several paraquat (Methyl viologen dichloride 98%, Sigma Aldrich) concentrations (0; 0.25; 0.5; 0.75 and 1.25 mM). Specimens were exposed to paraquat for 3 h with continuous mixing at 37°C. Hemolysis was performed on ice for 30 min, by adding equal volume of cold distilled water. Hemoglobin concentration was determined by the method of Drabkin and Austin, (1932). Protein content in samples was measured by the method of Lowry (1950).

SOD fraction was obtained using Tsuchihashi method. SOD isoenzyme pattern and activity in SOD fraction were detected by native PAGE and specific staining (Beauchamp and Fridovich, 1971). After electrophoresis gels were soaked in staining solution (for 20 ml of staining solution: 4 µL TEMED, 4 mg NBT, 1 mg riboflavin, 0.5 M EDTA, 0.05 M Tris HCl pH 7.8). Catalase activity was measured in hemolysate samples using native PAGE and specific staining for catalase on gels by the method of Woodbury (1971). Before staining, gels were soaked in 0.0003% H_2O_2 for 5 min. Staining solution for catalase activity detection was comprised of 1% $FeCl_3$ and 1% $KFe(CN)_6$. Relative enzyme activity was

determined using TotalLab software. Acetylcholinesterase activity was determined in hemolysate (Ellman et al, 1961). According to this method, the substrate (acetylcholiniodide) is hydrolised by AChE to acid and thiocholine. Catalytic activity was measured by the formation of the yellow anion 5-thio-2 nitrobenzoate at 421 nm, which is formed in reaction of thiocholine with DTNB (5,5'-dithiobis-2-nitrobenzoic acid).
All results were expressed as mean ± SD. Statistical analysis was performed using ANOVA analysis of variances, and Tukey's test for analysis of interindividual differences between groups. Differences were significant at $^{**}p<0.01$.

RESULTS AND DISCUSSION

In SOD fraction only one SOD isoform was detected with Rf value ~ 0.65 (Fig.1). Incubation of erythrocytes in solution containing paraquat caused changes in SOD activity (Table 1). Lower concentrations of paraquat (0.25 and 0.5 mM) increased SOD activity by 32% and 55% respectively. In sample treated with 0.75 mM Pq, SOD activity was slightly decreased in comparison to control sample. The highest concentration (1.25 mM) caused minor inhibition of SOD activity by 13% (Fig. 3). In normal physiological conditions, SOD eliminates superoxide radicals formed by toxic effect of paraquat. Cytoplasmatic CuZnSOD is responsible for the dismutation of superoxide radical in erythrocytes (Fridovich, 1975). It has been demonstrated earlier that even exogenous application od SOD reduces toxic effect of paraquat (Autor, 1974). In another paper *in vitro* treatment of red blood cells showed that higher concentrations (1 and 1.5 mM) caused increase of SOD activity (Bainy et al, 1994). They explained this as a result of enhanced antioxidative capacity in erythrocytes. Their study also showed that concentration of 1.5 mM blocked the rise of activity, and the lowest concentration (0.5 mM) didn't cause changes.

Table 1. Values of SOD, CAT and AChE activity in samples.
Results are presented as Mean±SD.

Pq concentration mM	SOD U/mg protein	CAT U/mg protein	AChE mol/µg Hb
Control	6595,86 ± 355,02	1512,82±86,98	0,218±0,031
0,25	8684,92 ± 756,48	1948,88±139,70	0,203±0,038
0,5	10224,44 ± 708,22	1823,92±207,95	0,225±0,019
0,75	6501,35 ± 529,66	1493,48±257,57	0,19±0,009
1,25	5759,29 ± 406,54	1172,3±237,67	0,164±0,01

Differences between previously mentioned study and ours can be explained with different experiment conditions and incubation period. There are many papers which show that high levels of ROS may inactivate antioxidant enzimes. Production of superoxide radical can induce but also inhibit SOD activity (Kerr et al, 1988). In some studies long-term exposure of rats to Pq led to SOD and GPx inhibition (Ray et al, 2007). Many herbicides may suppress antioxidant enzymes in higher concentration (Wright et al, 1997; Bukowska, 2003). Paraquat can potentially inhibit processing of the human Mn-dependent SOD precursor hMnSOD in mitochondria which results with accumulation of the precursor and thereby decline of SOD activity (Wright et al, 1997). Inhibition of CuZn SOD in erythrocytes can be a result of CAT inhibition and hydrogen peroxide accumulation (Bukowska, 2003).

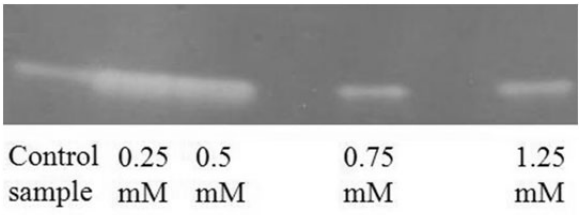

Figure 1. After native electrophoresis, one SOD isoform was detected on the gel. Lower paraquat concentrations induced SOD activity in erythrocytes while higher paraquat concentrations, 0.75 and 1.25 mM caused significant decrease.

One catalase isoform was detected on polyacrylamide gels with Rf value ~0.325 (Fig.2). Paraquat initiated changes in catalase activity also (Table 1). The highest increase in CAT activity (29%) has been observed in the sample containing the lowest Pq concentration (0.25 mM). Sample group treated with 0.5 mM Pq also showed significant increase (21%) in comparison to control samples. However, probes treated with higher paraquat concentration exhibited decline in enzymatic activity. Test samples incubated in 0.75 and 1.25 mM Pq expressed a reduction in activity by 1.2 % and 22 % respectively, in comparison to control samples (Table 1, Fig. 3). In previously mentioned study (Bainy et al, 1994) CAT activity also increased with the paraquat concentration, but until concentration of 1.5 mM was achieved. At this point, further increase was blocked. They explained activity increase as a response of antioxidant mechanisms on higher prooxidant level. In our study, the correlation between CAT activity and Pq concentrations can be observed. However, increase was altered at 0.75 mM Pq. Higher concentration causes decrease in CAT activity. What can be the reason of these differences between these two similar studies? Perhaps incubation period of 1 hour which has been used in the other reasearch is not enough for substantial increase in CAT ativity. On the other hand, long term exposure to Pq toxicity caused decline in CAT and SOD activity (Ray et al, 2007). Catalase is efficient in relatively high content of H_2O_2. Low concentrations are removed by glutathione peroxidase (Cohen et al, 1963). Superoxide radical can inhibit CAT activity also (Bagnyukova et al. 2006). It is possible that higher Pq concentrations led to inefficient removal of superoxide anion from the erythrocyte cytosol, which caused CAT activity reduction. Interference between antioxidative protection elements can cause their inhibition (Modesto et al, 2006).

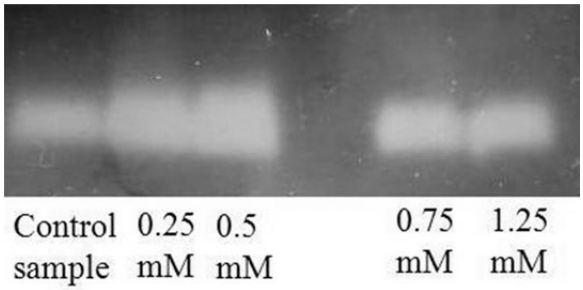

Figure 2. In comparison to control sample, lower concentrations of paraquat 0.25 and 0.5 mM caused significant increase in CAT activity, while treatment with higher concentrations, 0.75 and 1.25 mM significantly decreased the activity.

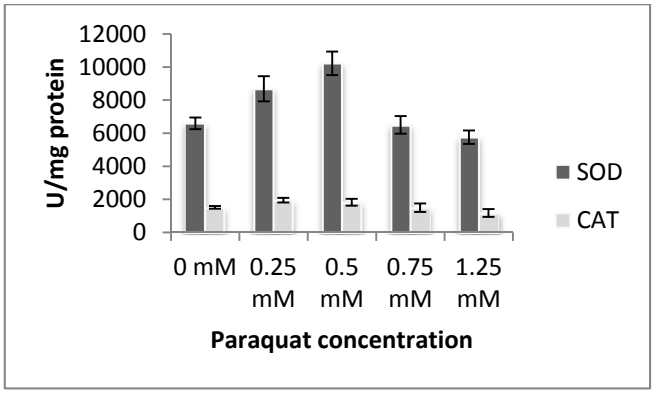

Figure 3. Enzyme activity (SOD and CAT) in rat erythrocytes treated with different concentrations of paraquat.

The lowest paraquat concentrations did not affect AChE activity in samples (Fig. 4). Still, minor inhibition (13%) has been observed in probe treated with 0.75 mM paraquat. The highest used Pq concentration caused significant reduction of AChE activity. In this sample, AChE activity has been inhibited by almost 25% compared to the control value (Table 1). Study of Pq effect on AChE activity showed ability of this herbicide to inhibit the activity *in vitro*. Many experiments showed numerous herbicides can inhibit AChE activity in erythrocytes (Seto i Shinohara, 1987; Thiermann et al, 2005; Bukowska et al, 2006).

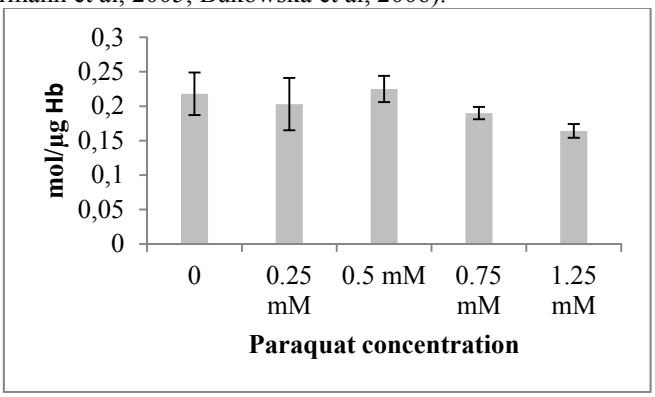

Figure 4. Activity of AChE in rat erythrocytes treated with different concentrations of paraquat.

It has been shown earlier that paraquat competitively binds to the anionic site and that is why his inhibitory effect is reversible (Belleau and Tani, 1966). Seto i Shinohara (1987) noticed ability of Pq to inhibit AChE in vitro in human and electric eel erythrocytes (*Electrophorus electricus*). Significant inhibition by Pq was observed also in carp erythrocytes (*Cyprinus caprio*) (Nemcsók et al, 1984; Szabó et al, 2002). Localization of the AChE on erythrocytes membrane predetermined enzyme's susceptibility to negative effect of ROS. Changes in AChE activity can be the consequence of direct negative impact of ROS, or herbicides which induce ROS generation (Bukowska, 2006). Under conditions of excessive oxidative stress, erythrocyte membrane damage may be a result of the decreased activity of membrane-bound enzymes (Lee et al, 2003). Peroxidation products can also influence AChE activity (Kale et al, 1999). There is

also evidence that shows high hydrogen peroxide concentrations (10^{-3}M) significantly decrease AChE activity by lowering V_{max} (Shallreuter et al, 2004). Inhibitory effect of herbicides and other xenobiotics can be considered as primary mechanism of acute toxicity, hence the control of AChE activity should be intergral part in the diagnosis and poisoning treatment.

CONCLUSIONS

Red blood cells are more susceptible to oxidative stress and ROS-derived changes than other cell types because of high hemoglobine and oxygen content. They are continuously exposed to high oxygen concentration and have limited ability to replace damaged biomolecules by *de novo* protein synthesys. On the other hand, they are very well protected against oxidative damage by their intracelular antioxidant enzymes. This research proves damaging effects of paraquat on erythrocytes due to oxidative stress. Towards a better understanding of mechanisms of paraquat toxicity on organisms, larger clinical studies are required.

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