**Effects of Hemazin SC 500 (terbuthylazine) on antioxidative enzymes in human erythrocytes *in vitro***

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*Abstract:* The aim of this work was to investigate the effect of the herbicide, commercial formulation Hemazin SC 500 containing terbuthylazine as an active compound on isoenzyme patterns and activities of CuZn superoxide dismutase (SOD1) and catalase (CAT), as well as on glutathione-S-transferase (GST) activity in human erythrocytes *in vitro*. The human erythrocytes were treated with Hemazin SC 500 in broad terbuthylazine concentrations (37 nmol/L - 37 μmol/L) for 1 and 3 h at the temperature of 37ºC. Native electrophoresis in control and treated samples revealed two SOD1 and one CAT isoform. Treatment did not affect SOD1 and CAT isoenzyme profile, but induced a change in their activities. Terbuthylazine at lower concentration has induced a significant increase of total SOD1 activity and decreased GST activity in samples incubated for 1 and 3 h. On the other hand, the highest increase of CAT activity was observed in the sample treated for 1 h with higher concentration of terbuthylazine. Hemazin SC 500 containing terbuthylazine induces changes in the erythrocyte antioxidative system whereby the response of individual enzymatic antioxidants depends on the concentration of the pesticide and the incubation time.

*Key words:* herbicide, hemolysate, SOD1, catalase, glutathione-S-transferase

TERBUTHYLAZINE AND ANTIOXIDATIVE ENZYMES OF ERYTHROCYTES

INTRODUCTION

Application of pesticides in agriculture is bidirectional. Pesticides lead to higher crop yields and obtaining sufficient quantities of food and reduce the occurrence of life-threatening illnesses. On the other hand, pesticides can have toxic effects on animals and humans that are not the primary targets of pesticide application. Terbuthylazine (C9H16ClN5) (Fig. 1) as active component of Hemazin SC 500 is a selective herbicide from the class of chloro-triazine and it inhibits photosynthesis in broad leaf weeds. It is applied in the protection of different crops: maize, sugar cane, olives, pineapple, *etc*.

Fig. 1 corrected.tif

Fig. 1. Structural formula of terbuthylazine.

The prohibition of atrazine (European Union, 2006) resulted in a significant increase of terbuthylazine application. According to the U.S. EPA Office of Pesticide Programs (OPP), Carcinogen List terbuthylazine exhibits slightly acute toxicity (category III), does not exhibit genotoxicity (classified in group D) and is not considered a carcinogen in humans.1 Because the data on the terbuthylazine toxicity are incomplete or ambiguous, according to the International Agency for Research on Cancer (IARC), terbuthylazine is classified in group 3 of the Carcinogen List: unclassifiable. There are not many studies on the toxicity of terbuthylazine and its metabolism in human organism. However, in experiments on rats it has been shown that the major metabolic pathway of terbuthylazine is hydrolysis of chlorine and mono and didealkylation, as well as hydroxylation of one or both dialkylamino groups of amines. Also, studies on rats have shown that terbuthylazine rapidly excreted from the body, completely metabolized and not accumulated in the tissue.2

It has been shown in the literature that erythrocytes represent a good model for the study of toxic effects of pesticides on human organism.3 Due to self-oxidation of hemoglobin and the presence of high content of polyunsaturated fatty acids, erythrocytes are susceptible to ROS (reactive oxygen species - superoxide anion radical (O2**.**-), hydrogen peroxide (H2O2) and hydroxyl radical (OH**.**)) formation. There is evidence that pesticides cause increased production of ROS in erythrocytes.4 Moreover, triazine pesticides (atrazine) show the same effect in erythrocytes.5 On the other hand, to our knowledge, there are no data on terbuthylazine impact on human erythrocytes. It was shown that terbuthylazine in algal cells lead to ROS formation.6 Also, the major products of degradation of terbuthylazine: terbuthylazine-desethyl and terbuthylazine-2-hydroxy,7 cause the formation of ROS in carp8 and red swamp crayfish.9

The main antioxidants in erythrocytes are antioxidative enzymes: copper-zinc superoxide dismutase (SOD1, CuZnSOD, EC 1.11.16), catalase (CAT, EC 1.11.1.6) and glutathione-S-transferase (GST, EC 2.5.1.18). The response of antioxidative enzymes in pesticide-treated erythrocytes has been demonstrated.3,10 Up till now, there were no reports published for the treatment of human erythrocytes with terbuthylazine *in vitro*.

Recent research showed that terbuthylazine lead to DNA damage11 and DNA instability in the culture of leukocytes and inhibition of SOD1 activity in erythrocytes after treatmen of whoole blood.12 Increases in the use of terbuthylazine and the lack of data on its toxicity point to the importance of examining its impact not only on humans but also on other living organisms. Also, the problem with the use of terbutilazine can be its stability and the stability of products of its degradation in soil and water,7 which are extremely toxic to aquatic organisms.8

Therefore, the aim of this study was to examine the changes in antioxidant activity enzymes in erythrocytes (SOD1 and CAT as enzymes of the first line of defense against ROS and GST as an enzyme of biotransformation) after acute exposure to commercial herbicide Hemazin SC 500 with a terbuthylazine as an active compound.

EXPERIMENTAL

*Reagents*

Commercial product Hemazine SC 500 with 500 g/L of terbuthylazine (N2-tert-butyl-6-chloro-N4-ethyl-1,3,5-triazine-2,4-diamine) as active substance was purchased by Agromarket (Serbia). Vacutainers for blood sampling with 3.2% sodium citrate solution were purchased from Greiner Bio-One (Austria). Nitro Blue Tetrazolium (NBT) and tetramethylethylenediamine (TEMED) were purchased from Fisher Bioreagents (USA), riboflavin and glycerin from Semikem (B&H), toluene, ethylenediaminetetraacetic acid (EDTA), potassium hexacyanoferrate(III) and iron(III)chloride hexahydratefrom Lach-Ner (Czech Republic). Hydrogen peroxide (30%) was purchased from Carlo Erba (France). Glutathione (98% purity) and 1-chloro-2,4-dinitrobenzene (CDNB, 99% purity) were purchased from Acros Organics (USA). All the other reagents and chemicals used were of analytical grade.

*Sample preparation*

Human blood was obtained from healthy volunteers. All procedures were approved by the Ethical Committee of the Medical Faculty of the University of Banja Luka, B&H number 18/4.14/18. The average age of volunteers (6 men and 12 women) was 23 years (from 21 to 25). All volunteers were healthy, did not take any therapies, were not smokers and had not come in contact with pesticides. On average, 6 mL of blood was taken from each person into a vacutainer containing citrate as an anticoagulant. The experiments were performed on the day of the collection. The blood was centrifuged at 900 g for 10 min (Centric 200R, TEHTNICA) and the plasma was separated. The precipitate of erythrocytes was washed three times with a cold physiological solution and used for treatment with Hemazin SC 500.

*Preparation of pesticides solution*

In the experiments, we used a commercial herbicide Hemazin SC 500 with a known concentration of terbuthylazine, therefore we amount of herbicides for treatment of erythrocytes calculated based on terbuthylazine concentration (37 nmol/L - 37 μmol/L). The lowest selected concentration of terbuthylazine (37 nmol/L) in our experiment was based on the data showing that the concentration of terbuthylazine in waterways on day on which terbuthylazine was applied on crops was 42 nmol/L.13 On the other hand, the solubility of terbuthylazine in water is 39 μmol/L14 and therefore we chose the 37 μmol/L as highest concentration for the treated erythrocytes. Terbuthylazine was dissolved in a saline solution.

*Treatment of Erythrocytes with Commercial Herbicide Hemazin SC 500 and Lysis*

The erythrocytes of 18 healthy volunteers were used in experiments. The experiments were repeated 3 times with 6 volunteers (samples) per experiment. Each of the 6 samples was divided into 4 aliquots (1 control and 3 treated). We've presented results for one experiment with 6 volunteers. The erythrocytes were treated with Hemazine SC 500 calculated on terbuthylazine (control (0), 37 μmol/L, 3.7 μmol/L and 37 nmol/L) for 1 and 3 h at the temperature of 37ºC with consistent steering. In preliminary experiments we have shown that there is no lysis of erythrocytes for the selected concentrations of terbuthylazine under these conditions. After incubation erythrocytes were centrifuged at 900 g for 10 min and the supernatant was discarded. Treated erythrocytes (washed 2 times with saline after treatment) were lysed with cold distilled water 1:3 (v/v) (in order to prevent protein denaturation) and toluene 1:1 (v/v) (to remove lipids). Then they being swirled in the vortex and standing in the refrigerator for 1 h, the lysed erythrocytes were centrifuged at 900 g for 25 min. The lysed erythrocytes were used for further analysis, while membranes were removed. Hemoglobin (Hb) concentration was determined by using the Drabkin and Austin methodat 545 nm.15

*Preparation of SOD1 fractions*

In one part of the lysate, hemoglobin was removed by using the Tsuchihashi method16 and the remaining solution was used to analyze the SOD1 isoenzyme pattern and activity. Prior to removal of hemoglobin in the samples, its content was determined. The volume of each of the samples applied to the gel for electrophoresis corresponded to an equal amount of hemoglobin.

*Native polyacrylamide gel electrophoresis*

For the separation of SOD1 isoforms we used 10% polyacrylamide gels and 8% polyacrylamide gels for the separation of CAT isoforms. The buffer for electrophoresis (pH 8.3) contained 24.8 mmol/L Tris and 192 mmol/L glycine. Before being loaded on the gel, samples were mixed with the loading buffer (50 mmol/L Tris pH 6.8, 10% glycerol and 0.1% bromophenol blue) in the 1:3 ratio. After electrophoresis, SOD1 isoforms were determined by specific staining. The gels were incubated in the staining solution consisting of 0.25 mmol/L NBT, 0.13 mmol/L riboflavin, 4 mmol/L Tris buffer pH 7.8, 1 mmol/L EDTA and 2.72 mmol/L TEMED.17 CAT activity was determined in hemolysate after native polyacrylamide gel electrophoresis and specific staining. Prior to staining, the gels were incubated in 0.003% H2O2 for 5 min. The staining solution used for CAT activity contained 1% FeCl3 and 1% KFe(CN)6.18 Quantification of SOD1 and CAT activities on the native gel were performed using the Image Master Total Lab TL 120 software (Nonlinear Dynamics Ltd., Durham, USA). Total SOD1 activity represents the sum of individual isoforms (SOD1 A and SOD1 B) activities for each sample.

*Spectrophotometric Determination of GST Activity*

According to the method of Habig *et al.*19 GST activity was determined in hemolysate. This method was based on binding substrate (1-chloro-2,4-dinitrobenzene (CDNB)) to sulfhydryl group of reduced glutathione (GSH) in reaction catalyzed by GST. The formation of the conjugate CDNB-GSH, with a maximum absorption at 340 nm, was measured during 150 s (every 30 s) at 37 °C. GST activity was expressed in U/g Hb (μM GSH/min x g Hb).

*Statistical Analysis*

Data are given as mean ± SE for 6 healthy volunteers. For statistical analysis we used the One way ANOVA Tukey test for data comparison between controls and treated groups and treated groups to each other. In each experiment, control blood samples and samples treated with terbuthylazine were taken from the same person. The experiments were repeated three times.

RESULTS AND DISCUSSION

The obtained results indicate that changes in SOD1, CAT and GST activities occur in the human erythrocytes treated with commercial herbicide Hemazine SC 500 *in vitro*. Even though, Hemazine's active compound is terbuthylazine with concentration 500 g/L, Hemazin also contains other components which are not listed in the product specification. In our paper we focused discussion on the influence of the active component, terbuthylazine on antioxidative metabolism, although there is a possibility of contribution of terbuthylazine's accompanying components to changes in antioxidative metabolism of erythrocyte.

*SOD1 activity*

In all samples (control and treated erythrocytes) incubated for 1 and 3 h at 37°C with terbuthylazine, two superoxide dismutase isoforms (labeled with SOD1 A and SOD1 B) were detected with *R*f values (*Rf*= ): *R*fSOD1 A = 0.340 ± 0.066 and *Rf*SOD1 B = 0.510 ± 0.047 (Fig. 2A and B).

gelovi SOD1 1.tif

Fig. 2. The representative native electrophoresis gel with separated SOD1 isoforms. In SOD fraction of the control sample (0) and treated groups were detected SOD1 A and SOD1 B isoforms. Erythrocytes were treated with 37 μmol/L, 3.7 μmol/L and 37 nmol/L terbuthylazine and incubated for 1 h (A) and 3 h (B) at 37°C.

Available literature data shown that change of antioxidative metabolism of erythrocytes depends of type of pesticide (structure), treatment conditions and concentrations of pesticides.3,20,21 Although, terbuthylazine treatment did not lead to any changes in the isoenzyme profiles, it did induce changes in SOD1 activity (Fig. 3). Terbuthylazine in all concentrations (except 37 μmol/L, incubation 1 h and 37 nmol/L, incubation for 3 h) significantly changed SOD1 activity in the samples incubated for 1 and 3 h compared to the controls (420694±18995 U/g Hb) for 1h and 3h (420197±28530 U/g Hb). After 1 h of incubation, SOD1 activity increased in the samples treated with concentrations: 37 nmol/L (p<0.01) (527926±29308 U/g Hb) and 3.7 μmol/L (p<0.005) (667220±27949 U/g Hb), as well as after 3 h incubation with concentration of 3.7 μmol/L (p<0.005) (597906±25600 U/g Hb) (Fig. 3). A significant decrease in SOD1 activity (p<0.01) (270598±23388 U/g Hb) was measured only at the highest concentrations of terbuthylazine after incubation for 3 h. The statistically significant difference between treatments for both incubation periods was between 3.7 μM and lower and higher terbuthylazine concentrations (Fig. 3).

Fig. 3 corrected.tif

Fig. 3. Total SOD1 (sum of activities of SOD1 A and SOD1 B) relative activity in samples incubated for 1 and 3 h at 37°C. Quantification of SOD1 relative activity on the native gel was performed using Image Master Total Lab TL 120 software. Results are presented as Mean ± SE. \*\* p<0.01; \*\*\* p<0.005.

Erythrocytes are exposed to oxygen radicals that are continuously generated primarily due to auto-oxidation of oxy hemoglobin to methemoglobin.22 The earlier examination of antioxidative defense enzyme activities *in vitro* has shown that SOD1 possesses a constant specific activity and may be inhibited irreversibly by the cyano group (CN-), reversibly by H2O2 or by copper chelators such as DDC (diethyldithiocarbamate). Later it has been shown that HS- enhances O2**.**- scavenging activity of the bovine erythrocyte SOD1 by about twofold.23 Our results show that at certain concentrations, terbuthylazine as chloro-triazine increases the SOD1 activity in erythrocytes (Fig. 3). In the work of Gultekin and coauthors it was shown that the decrease in SOD1 and CAT activities is statistically significant with the increase in pesticide concentrations and is more pronounced for longer incubation periods.24 However, the authors have also shown that at lower pesticide concentrations SOD1 activity increased (by 2% after incubation of 1 h and 13% for a 4 h incubation period for the concentration of 0.01 g/L chlorpyrifos-ethyl). The treatment of Commercial SOD1 with a low concentration of the same pesticides (0.01 g/L) induced an increase in SOD1 activity by 1.34%. As sulfur-containing compounds can activate SOD1 at certain concentrations and inhibit SOD1 at higher concentrations as a result of SOD1 damage,25 according to our results it seems that it may also be true for certain chlorine-containing compounds (terbuthylazine). In other papers it has also been shown that low concentrations of pyrethroid insecticide20 and herbicide10 lead to an increase in SOD1 activity in erythrocytes, whereas high concentrations decrease SOD1 activity when compared to control. Results of this paper related to the highest terbuthylazine concentration of 37 μmol/L show that 3 h incubation leads to a decrease in SOD1 activity (36%) (p<0.01). Statistically significant decrease of SOD1 activity was demonstrated in the treatment of rat erythrocyte for 3 h with organochlorine insecticide endosulfan and organophosphorus insecticide chlorpyrifos.4 Reduction of activity may be due to the oxidative protein changes that can result in increased susceptibility to proteolysis and protein denaturation.26

*Catalase activity*

A large amount of CAT was present in the erythrocytes, and only the liver contained more of this enzyme, which participates in the defense against free radicals together with other enzymes for antioxidant protection in the cell. Catalase is effective at relatively high H2O2 content, while low concentrations are removed by glutathione peroxidase. We detected one CAT isoform with *R*f values *R*fCAT = 0.429 ± 0.106 in the control sample and the treated sample incubated for 1 and 3 h at 37°C (Fig. 4).

CAT GELOVI 1.tif

Fig. 4. The representative native electrophoresis gel with one separated CAT (catalase) isoform in erythrocyte lysate: control (0) and groups treated with 37 μmol/L, 3.7 μmol/L and 37 nmol/L terbuthylazine incubated for 1 h (A) and 3 h (B) at 37°C.

The statistically significant increase in CAT activity (24%) was observed in the samples treated for 1 h with 37 μmol/L of terbuthylazine (289699±7990 U/g Hb) (p<0.01) (Fig. 5). In other treatments compared to control (233419±3719 U/g Hb) for 1 h and 3 h (229566±4950 U/g Hb), CAT activity did not change significantly for either of incubation periods. Between treatments, statistical significance (p<0.005) was detected among 37 nmol/L (223793±13887 U/g Hb) and 3.7 μmol/L concentration of terbuthylazine after 1 h incubation (Fig. 5).

Fig. 5 corrected.tif

Fig. 5. Relative CAT activity in samples control (0) and groups treated with 37 μmol/L, 3.7 μmol/L and 37 nmol/L terbuthylazine incubated for 1 and 3 h. Quantification of CAT relative activity on the native gel was performed using Image Master Total Lab TL 120 software. Results are presented as Mean ± SE. \*\* p<0.01; \*\*\* p<0.005.

Santi *et al*. in the treatment of human erythrocyte 1 h with isoxazolidinone herbicide clomazone shown that all concentrations of herbicide led to a decrease in CAT activity.10 Increase of CAT activity has been demonstrated in human erythrocyte treated 1 h with organophosphorus insecticide trichlorfon,27 while CAT activity remained unchanged for all concentrations of organophosphate insecticide diazinon treated 1 and 3 h.21 Obtained results show that CAT activity is the highest in conditions of exposure to the highest concentration of terbuthylazine (Fig. 5) for which we can assume that are the most prooxidative as other authors have shown.28 Increased H2O2 concentration, resulting from the inhibition of CAT activity29 with superoxide anion, can inhibit SOD1 activity.30 It has been shown that the anti-oxidant system, at the level of coordinated expression, functions in the domain of positive correlation between SOD1 and CAT.

*GST activity*

In contrast to the SOD1 and CAT, glutathione-dependent enzymes in the anti-oxidant system are separately regulated, probably via the concentration of (reduced) glutathione and the redox status of the cell. GST is an antioxidant enzyme, and, in addition, it belongs to the enzymes of Phase II biotransformation. GST possesses the ability to catalyze the conjugation of a reduced form of glutathione with xenobiotics for the purpose of detoxification.

A statistically significant reduction in GST activity for the lowest concentration of terbuthylazine after incubation with 37 nmol/L for 1h (5.604±0.646 U/g Hb) and 3 h (5.024±0.815 U/g Hb) was observed compared to control (7.910±0.796 U/g Hb) for 1 h and 3 h (9.374±0.648 U/g Hb) (Fig. 6).

Fig. 6 corrected.tif

Fig. 6. GST (glutathione-S-transferase) activity in samples control (0) and groups treated with 37 μmol/L, 3.7 μmol/L and 37 nmol/L terbuthylazine incubated for 1 and 3 h. Results are presented as Mean ± SE. \* p<0.05: \*\* p<0.01.

Erythrocytes treatment with higher concentrations of terbuthylazine induced a statistically significant increase in GST activity (p<0.01) compared to the lowest concentration, which was especially pronounced after 3 h of incubation (Fig. 6).

El-Demerdash in rabbit erythrocytes measured decreased in GST activity for all concentrations of synthetic pyrethroids insecticide lambda-cyhalothrin incubated for 4 h,3 while treatment for 3 h with organochlorine insecticide endosulfan and organophosphorus insecticide chlorpyrifos in rat erythrocytes induced increased in GST activity.4

As already mentioned in the Introduction section, there is no data on the metabolism of terbuthylazine in human cells only in rat cells. So far, the best studied mechanism of terbuthylazine detoxification is in plant cells which, among others, involves conjugation with glutathione catalysed with GST. In plants chloro-triazines are metabolized at the chloro- or 2-position of the triazine ring by hydrolytic dehalogenation via a nonenzymic constituent of plant sap to the corresponding hydroxytriazines.31 Another important reaction of the chloro group involves an enzyme-mediated conjugation with glutathione to form a series of S-bound amino-acid conjugates. These compounds can rearrange to form N-bound amino-acid conjugates. A third metabolic reaction involves oxidation of the alkyl-amino side chains located at the 4- and 6-positions of the triazine ring, prior to sugar conjugation or N-dealkylation. In the case where the alkyl amino group contains a CN-, hydrolysis leads to amide and carboxylic acid formation on the alkyl group. These three competing reactions can result in a complex mixture of Phase I metabolites (simple metabolites) and Phase II metabolites (conjugates of simple metabolites) that can occur either free or bound in various plant matrices.

Neefjes and coworkers showed that GST from erythrocyte is a marker of oxidative damage.32 A decrease in GST activity at the lowest concentration of terbuthylazine that we obtained may be due to an induction of antioxidative metabolism especially SOD1, which proved to be most sensitive in response to different concentrations of terbuthylazine during acute exposure (Fig. 3). On the other hand, at high concentrations of terbuthylazine during 3 h incubation, GST activity increases which highlight the importance of GST in defense of ROS in prooxidative conditions when SOD is inhibited.

CONCLUSIONS

The results obtained have shown the activation of SOD1 at lower concentrations of terbuthylazine and CAT at higher concentrations of terbuthylazine during acute incubation of 1 h. Meanwhile, GST, as an enzyme of the second stage of oxidative stress defense, is activated at higher terbuthylazine concentrations during 3 h of incubation. Such results may indicate the organism’s strategy in oxidative stress protection. As a consequence of chronic exposure to low concentrations of pesticides, changes can occur in antioxidant capacity at the systemic level. Because of the potential impact of the accompanying compounds of the commercial preparation Hemazin SC 500, on antioxidative metabolism of erythrocytes, future research will be focused on the study of influence of pure terbuthylazine.

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*Conflict of interest:* The authors report no conflicts of interest.

ИЗВОД

**Ефекат Хемазина SC 500 (тербутилазин) на антиоксидативне ензиме у хуманим еритроцитима *in vitro***

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Циљ нашег рада је био да се испита ефекат хербицида, комерцијалног назива Хемазин SC 500, са тербутилазином као активном компонентом на изоензимски профил и активност CuZn супероксид дисмутазе (SOD1) и каталазе (CAT) као и на активност глутатион-S-трансферазе (GST) у хуманим еритроцитима *in vitro*. Хумани еритроцити су третирани тербутилазином у широком опсегу концентрација (37 nmol/L - 37 μmol/L) 1 и 3 h на 37ºC. Нативном електрофорезом су у контролним и узорцима третираним са тербутилазином детектоване двије SOD1 изоформе и једна CAT изоформа. Третман са пестицидом није довео до промјена у изоензимским профилима SOD1 и CAT али је изазвао промјену њихове активности. Тербутилазин при ниским концентрацијама је индуковао значајно повећање укупне SOD1 активности и смањење GST активности у узорцима еритроцита инкубираним 1 и 3 h. С друге стране, највеће повећање CAT активности је измјерено у узорцима третираним 1 h са високим концентрацијама тербутилазина. Тербутилазин индукује промјене у антиоксидативном систему еритроцита при чему одговор појединачних ензимских антиоксиданата зависи од концентрације пестицида и времена инкубације.

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