**Response to the Reviewer**

Dear dr. Nedić:

We want to express our thanks to the reviewers for the thorough and extensive review of the paper. We have carefully reviewed the comments and have revised the paper accordingly. We have tried to be thorough in writing this paper and perhaps that’s why we have gone out of the permitted page limit. The changes in the manuscript as well as added text are given in red (Reviewer A) and blue color (Reviewer B).

Reviewer A:

***Abstract***

According to the reviewer's suggestion, in the Abstract, we changed effect of terbuthylazine to effect of Hemazine SC 500.

Page 1, lane 26.

The name of the commercial preparation used for erythrocyte treatment is added to the keywords*.*

Page 1, lane 30.

***Introduction****This section is too long and has to be reduced by deleting all well-known facts and general knowledge on pesticides. Although the Table 1 is informative, it is better suited for a review
article. The aim of study has to be better defined. It is not clear why this study is important and which gaps in the existing knowledge it intends to fill.*

General parts related to pesticides have been excluded from the Introduction as well as Table I. We added a section that highlights what is known about the effect of terbuthylazine on human cells and on which basis we have defined the goal of our work by trying to investigate what is unknown. We emphasized that the treatment was made with a commercial preparation having the active component terbuthylazine.

Recent research showed that terbuthylazine lead to DNA damage17 and DNA instability in the culture of leukocytes and inhibition of SOD1 activity in erythrocytes after treatmen of whoole blood.18  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,9 which are extremely toxic to aquatic organisms.10

Therefore, the aim of this study was to examine the changes in antioxidant activity enzymes in erythrocytes (SOD1 and CAT as an 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 s as an active compound.

Page 3, lanes 77-87.

***Experimental design***

*There is a discrepancy between the number of blood donors mentioned in the description of experimental conditions (18, as stated in the line 1, Page 5) and those reported in the section Results and Discussion (6). The authors have to clearly state how many blood donors were actually used. It is also not clear why male and female donors were used for the study,
considering that the results were not focused on reporting findings in male or female subjects. Since donors of both sexes were engaged, it has to be clearly stated how
many male and female there were.*

Because the experiment was done in vitro conditions (we used isolated and purified erythrocytes) we did not take into account the differences between male and female donors. We assumed there was no hormone influence on the results in this case.

In MM we added that the blood was used by 18 volunteers (6 male and 12 female).

In the part of the Material and methods we tried to clarify the design of the experiment and the number of samples adding the following text:

The erythrocytes of 18 healthy volunteers was 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.

Page 4, lanes 126-128

In the MM is added that explains why the concentration of the herbicide calculated on the basis of terbuthylazine. 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 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.

Page 4, lane 128-130.

*The reason behind selection of 1 and 3 h of treatment has to be clearly
stated*

Consulting available literature for *in vitro* experiments in which erythrocytes were in conditions of acute exposure to pesticides, we have chosen incubation 1 and 3 hours.

In MM we add:

In preliminary studies, we have shown that erythrocytes do not lysis during incubation for 1 and 3 hours with given Hemazine concentrations based on Hb concentration measured in the supernatant.

Page 4, lane 130-132.

*More details on blood centrifugation have to be provided (type of the instrument, conversion of rpm into g, etc.).*

We mentioned the type of centrifuge used in the experiment and we have converted the rpm to g.

Centrifuge Centric 200R by Tehtnica was used at 900 g.

Page 4, lanes 111, 133. Page 5, lane 137.

*2.* ***Statistical analysis***

*Selected statistical analysis was not optimal for the study design.*

*Since there were more than two experimental groups, the Student’s t-test
seems not appropriate tool for the analysis*.

According to the reviewer's advice, statistical analysis of results has been improved and is being made One way ANOVA Tukey test for data comparison between controls and treated groups and treated grups to each other.

We changed:

*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 grups 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.*

Page 6, lanes 172-176.

*Furthermore, providing a median value and the range of the measured values
is also advisable.*

Mean values for the enzymes activities of control and treated groups we have mentioned in the text in the results and discussion section when we mentioned them.

Page 7, lanes 206-210; page 9, lanes 265-269; page 11, lanes 297-299.

***Results and Discussion***

*This section is not presented well. The limitations of study approach are
not stated or discussed. Considering that the study was performed in vitro, the whole part of
discussion focused on metabolism (Pages 12-13) is superfluous. The authors should find a way to better discuss their most important/interesting findings, and provide a better literature review of relevant papers. At least an elementary discussion on potential toxic effects of other
ingredients of the tested formulation is mandatory.*

Due to better visibility and presentation of results, gels and activities for SOD 1 and CAT were separated. In the previous version of the Manuscript, Fig.1 consisted of parts A, B and C, and in the new version there are two figures: Fig. 1 (A and B gels with separated SOD1 isoforms) and Fig. 2. (total relative activity of SOD1 isoforms). Also Fig. 2 in the previous version was separated in the same way: Fig. 3 (A and B contains native gels with separated CAT isoforms) and Fig. 4 contains the relative activity of CAT.

At the beginning of the Results and discussion, we indicated the possible influence of the accompanying components in the Hemazin commercial preparation. Since the accompanying components not listed in the product specification, we focused our discussion on the influence of the active component, terbuthylazine on antioxidant metabolism.

We added a part:

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.

Page 6, lanes 180-186.

Through the Results and discussion section we tried to emphasize our results and compare them with the relevant literature. Modified parts are marked red. As the second Reviewer requested that the discussion on the metabolism of terbuthylazine remain, we tried to shorten it and make it more readable.

Please see in MS:

Page 7, lanes 200-203; 211-213.

Page 8, lanes 229-230; 242-243.

Page 9, lanes 264-270.

Page 10, lanes 278-285.

Page 11, lanes 307-317.

Page 12, lanes 329-335.

***Conclusion***

Please see:

Page 12, lanes 345-347.

Reviewer B:

*1. I think it is necessary to add the chemical structure (formula) of the used herbicide to the text!*

We've added the chemical formula and structure of the herbicide terbuthylazine in the Introduction section.

Page 2, lanes 43-48.

*2. Please add the used concentrations (or just the range) of the herbicide to the abstract text.*

We have added a range of concentrations of herbicides that we used in the Abstract.

Page 1, lane 19-20.

*3. It is better to specify the used xenobiotic in the abbreviated title of the paper (Page 2, line 7): terbuthylazine instead of just pesticide.*

The short title of manuscript now is:

TERBUTHYLAZINE AND ANTIOXIDATIVE ENZYMES OF ERYTHROCYTES

Page 1, lane 33.

*4. The authors discussed (justifiably!) the metabolism/biotransformation of terbuthylazine in the Results and discussion part of the text. Accordingly, it would be useful to give in the introductory section of the manuscript basic information about (route paths) exposure/absorption, disposition/distribution and elimination of this xenobiotic in the human body!*

According to the reviewer's advice in the introduction section we added a part about the metabolism of terbutilazine in rats. In our available literature we have not found data on the metabolism of terbutilazine in the human body.

There are not many studies on the toxicity of terbuthylazine and its metabolism in human organisms. However, in experiments on rats it has been shown that the major metabolic pathways 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.

Page 2, lanes 55-60.

*5. I would not say that incubation for 1-3 hours is “a prolonged exposure” to something… just exposure or acute exposure (Page 2, line 8).*

We have changed the term exposure to acute exposure or just exposure (treatment) in text.

*6. Blood of total 18 apparently healthy volunteers was used for experiments. It is not clear: from that “pool” of samples, (just) six were used for each individual parameter (3 x 6 = 18), and every single sample (of that 6) was divided to 4 aliquots (1 x control + 3 x for treatment with 3 different concentrations of herbicide) (Page 5 vs. Page 6, line 7 etc.)!?*

In MM we added the clarification:

The erythrocytes of 18 healthy volunteers (6 man and 12 women) was used in experiments. The experiments were repeated 3 times with 6 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.

Page 4, lanes 126-128

*7. Please add in which ratio (v/v) the treated erythrocytes are lysed (including toluene): Page 6, lines 3-4).*

We added:

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).

Page 5, lanes 135-136.

*8. Please add how GST activity is expressed (units of activity; Page 7, line 14).*

We added:

GST activity was expressed in U/g proteins (μM GSH/min/g Hb).

Page 6, lane 169.

***Technical issues:***

We corrected all technical issues. Please see MS.