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HEMOLYSIS AND LIPID PEROXIDATION IN HUMAN ERYTHROCYTES INCUBATED WITH ROUNDUP

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The effects of exposure of human erythrocytes to different concentrations of Roundup were studied, with particular attention to hemolysis, lipid peroxidation, acetylocholinoesterase activity (AChE) and hemoglobin oxidation. Human erythrocytes were incubated with Roundup at concentrations from 100 to 1500 ppm (100 μg /ml erythrocytes at 5% hematocrite), for 1 to 24 hours. The results show that Roundup decreases AChE activity, increases the level of methemoglobin, products of lipid peroxidation and hemolysis. Taking into account the limited accumulation of Roundup in the organism as well as the fact that 500 ppm was the threshold dose which caused changes in erythrocytes, it is possible to draw a conclusion that this pesticide is safe to human erythrocytes.

INTRODUCTION

Glyphosate was first prepared at Monsanto Agricultural Products Company in May 1970, and preliminary greenhouse testing data were available by early June of that year. When formulated as its monoisopropylamine salt, glyphosate is commonly sold as Roundup herbicide (Franz, Mao & Sikorski, 1997). In 1994, these glyphosate based herbicides were sold under more than 150 different trademarks.

Glyphosate is virtually non-toxic to mammals, birds, fish, insects, and most bacteria. In addition, glyphosate does not bioaccumulate in the tissues of animals or agricultural crops. The Environmental Protection Agency (EPA) classifies herbicides for acute toxicity in four categories where „I" is the most toxic and „IV" is the least toxic. Based on oral rat tests, the EPA currently rates glyphosate as a Category IV herbicide. More extensive studies have shown no evidence of mutagenic, carcinogenic, teratogenic, or allergenic activity in a wide battery of assays and tests. Glyphosate was designated as a Category E herbicide because there is „evidence of noncarcinogenicity for humans". Glyphosate is now produced commercially not only in the U.S. but also in Europe, Asia, and South America.

In a teratology study with rabbits, the maternal NOEL was 175 mg/kg/day and no developmental toxicity was observed in the fetuses at the highest dose tested (350 mg/kg/day) (Environmental Protection Agency U. S. (1992). *Pesticide tolerance for glyphosate*. Federal Register **57**, 8739-8740).

Rats given doses up to 3,500 mg/kg on days 6 to 19 of pregnancy had offspring with no teratogenic effects, but other toxic effects were observed in both the mothers and the fetuses. No toxic effects to the fetuses occurred at 1,000 mg/kg/day (Franz *et al*., 1997).

The compound does not cause mutations in microbes. The tests on eight different kinds of bacterial strains and on yeast cells were all negative. The compound possess little mutagenic risk to humans (Stevens, James & Sumner, 1991).

Rats and dogs and mice fed glyphosate over a wide range of doses showed no cancer-related effects directly due to the compound (Forest Service (1984). *Pesticide background statements*, Vol. I *Herbicides*. United States Dept. of Agriculture, Agriculture Handbook No. 633).

EPA has stated that there is sufficient evidence to conclude that glyphosate is not carcinogenic in humans (Environmental Protection Agency U. S. (1987). *Health advisory*. Office of Drinking Water).

Glyphosate caused no changes in the rate of body weight gain, in blood, nor in kidneys or liver. The studies were conducted at doses up to 500 mg/kg (*The Agrochemicals handbook* (1991). The Royal Society of Chemistry: Cambridge, England.).

Glyphosate is poorly absorbed from the digestive tract and is largely excreted unchanged by mammals. Ten days after treatment there were only minute amounts in the tissues of rats fed glyphosate for three weeks (Monsanto Company (1985). *Toxicology of glyphossate and roundup*

herbicide. Department of Medicine and Environmental Health: St. Louis, MO).

Cows, chickens, and pigs fed small amounts had undetectable levels (less than 0.05 ppm) in muscle tissue and fat. Levels in milk and eggs were also undetectable (less than 0.025 ppm). Nearly all glyphosate residues were rapidly eliminated by fish that had been once exposed for 10 to 14 days once these fish were transferred to glyphosate-free water. Glyphosate has no significant potential to accumulate in animal tissue (Malik, Barry & Kishore, 1989).

The studies performed hitherto on glyphosate show it to be toxic to a very small extent, but its very broad application call for further experiments. The broad activity spectrum and the growing general use of glyphosate cause an increase in exposure of a major part of society to its influence. Therefore, scientists ask themselves whether the result of experiments conducted in the past reflect in full the "true results of action of this herbicide".

Erroneous estimations of pesticide toxicity have happened in the past. This situation took place in the case of toxicity of the Agent Orange blend which included 2,4-D and 2,4,5-T. This blend was used by Americans for defoliation of the jungle in Vietnam. Both constituent compounds were considered to have low toxicity. After many years of research it was discovered that the toxicity of 2,4-D and 2,4,5-T is much higher than supposed before and that their metabolites 2,4-dichlorophenol and 2,4,5-trichlorophenol are more toxic than the parent compounds (Bukowska, Chajdys, Duda & Duchnowicz, 2000). Furthermore, in the process of synthesis of these compounds, 2% of dioxins is formed. Since tons of 2,4-D and 2,4,5-T were produced and used, high amount of the dioxins were formed with huge toxicity (Bukowska & Duda, 2001).

The toxic action of glyphosate, as well as other herbicides, is linked to the dose and time of action. Because of the widespread use and occurrence of this compound in many commercial preparations and the low toxicity which has been hitherto reported, new research is being carried out in order to avoid potential unknown toxic activities towards living organisms.

MATERIALS AND METHODS

The following biological material was used: human erythrocytes and hemoglobin were obtained from whole blood, taken from donors at the Blood Bank of Lodz.

Erythrocytes were separated from blood plasma and leukocytes by centrifugation (600 g, 10 min) at 4°C and washed three times with phosphate-buffered saline (PBS; 150 mmol l^{-1} NaCl, 1.9 mmol l^{-1} NaH_2PO_4, 8.1 $mmol^{-1}$ Na_2HPO_4, pH 7.4). In work, herbicyde commercialy named Roundup Ultra 360 Sl was used, its biological active component was Glyphosate [360 gram of isoprpropyloamino salt in one litre of preparate]. Chemicaly, Glyphosate is N-phosphonomethylglycine. The producer of herbicyde is Monsanto Europe S.A., Belgium.

Isolated erythrocytes at a hematocrit of 5% were treated at temperature 25°C for one hour with 100 - 1500 ppm Roundup. After this treatment various erythrocyte parameters were examined.

Hemolysis

The ratio of hemolysis was calculated from the equation:

$$H = (A_{pb} - A_0) / (A_{water} - A_0) \cdot 100\%$$

where: H — hemolysis of erythrocytes incubated with Roundup, A_{pb} — absorbance of sample, A_{water} — absorbance after complete hemolysis, A_0 — absorbance of the sample with 155 mM of NaCl

Lipid peroxidation

The Roundup-induced peroxidation of human erythrocytes and of their membrane lipids was investigated. Malonyldialdehyde (MDA), the final product of fatty acid peroxidation, reacts with TBA to form a colored complex. The level of TBARS was measured on the basis of the absorbance at the wavelength of 532 nm (Stock & Dormandy, 1971).

Hemoglobin

The concentration of hemoglobin was measured by the Drabkin method (Drabkin, 1946). Absorption spectra of hemoglobin were obtained in the wavelength range 440 nm to 700 nm using an automatic spectrophotometer (Specord M40) connected to a computer. Samples consisting of 1% aqueous solutions of oxyhemoglobin were incubated at 37°C in air with continuous mixing with doses from 10-300 ppm of Roundup. The percentage of met-Hb in the total Hb content was calculated from absorbance at 630 and 700 nm both for sample (100% met) and samples with control and glyphosat (pb):

$$\%\ met\text{-Hb} = 100\ (A_{pb630} - A_{pb700}) / (A_{100\%met\ 630} - A_{100\%\ met\ 700}).$$

Table 1. Percentage of hemolysis in control erythrocytes and in erythrocytes incubated with Roundup (100 -1500 ppm).

Dose/hours	1 h	5 h	24 h
control	0.70 ± 0.49	1.05 ± 0.53	3.41 ± 0.90
100 ppm	0.59 ± 0.39	1.13 ± 0.76	5.57 ± 2.16
500 ppm	0.62 ± 0.36	1.25 ± 1.01	11.12 ± 6.93*
1000 ppm	0.90 ± 0.32	2.89 ± 1.19*	18.85 ± 7.84*
1500 ppm	1.28 ± 0.49*	5.72 ± 3.01*	23.62 ± 7.18*

*Significantly different from the control ($P < 0.05$). Mean ± SD of 12 experiments.

Acetylocholinoesterase activity assay

Acetylocholinoesterase activity was assayed by the method of Ellman Ciurtney, Andress and Featherstone (1961). Briefly, to 2 ml of erythrocyte suspensions, diluted with a buffered solution of NaCl (145 mM NaCl, 5 mM Na - phosphate buffer, pH 8.0) to a hematocrit value of 0.05%, 20µl of Ellman reagent was added (final concentration 10^{-4} M).

The kinetics of acetylthiocholine iodide hydrolysis (80µl) was recorded spectrophotometrically (SPECORD M40) at 22°C and the rate of the reaction was calculated from the equation:

$$V = \frac{\Delta A^{413} F}{13600 \times 1000} \cdot \left[\frac{\text{mol}_{\text{acetylthiocholine}}}{\text{min} \times \text{ml of packed cells}} \right]$$

where ΔA^{413} is the increase of absorbance at 413 nm during 1 min and F is the dilution in respect to a hematocrit of 100% (in our experiments equal to 2000).

Statistics

The data were given as arithmetic means ± SEM. Statistical evaluation of the data was performed by the Student's t-test. The level of significance was chosen as $P < 0.05$ (Gondko, Zgirski & Adamska, 1994).

RESULTS

In the present work we have measured changes in some essential biochemical parameters crucial to the correct function of erythrocytes. The effects of Roundup on hemolysis, lipid peroxidation, autoxidation of hemoglobin and activity of ACHE in erythrocytes were investigated.

It was found that the investigated compound causes slight hemolysis of human erythrocytes, but the differences were statistically significant starting at the dose of 1500 ppm after 1 hour of incubation and 500 ppm after 24 hours of incubation (Table 1).

Roundup significantly increases the concentration of thiobarbituric acid-reactive substances (TBARS) in erythrocytes (Table 2).

Incubation of hemoglobin with specified doses (100, 500, 1000, 1500 ppm) of Roundup decreased oxyhemoglobin concentration and increased the level of met-Hb. A significant decrease of α and β peaks was observed with a simultaneous increase of the 630 nm peak which is typical for methemoglobin - Table 2.

Roundup is used as a herbicide, but in exceptional cases may serve as an insecticide. It is known that thionophosphate insecticides block the activity of acetylcholinesterase. Roundup does not have the typical structure of thionophosphate insecticides, since it is a thione phosphonate. It is, however, a phosphoric compound and potentially it could be presumed to influence the activity of AChE.

In this work, the influence of Roundup on AChE activity and kinetics was studied. The following doses were used: 100 ppm, 500 ppm, 1000 ppm, 1500 ppm. The incubation was conducted for 1 hour with erythrocytes at 5% hematocrite. The activity kinetics was determined according to the method of Ellman (1961).

A decrease of AChE activity was found depend-

Table 2. Lipid peroxidation in control erythrocytes and erythrocytes incubated with Roundup.

Dose	Absorbance λ=532nm
control	0.041 ± 0.019
100 ppm	0.047 ± 0.015
500 ppm	0.050 ± 0.019*
1000 ppm	0.055 ± 0.029*
1500 ppm	0.066 ± 0.027*

*Significantly different from the control ($P < 0.05$). Mean ± SD of 10 experiments.

Table 3. Percentage of met-Hb in erythrocytes incubated with Roundup.

	1 h	5 h	24 h
Control	3.21 ± 1.39	5.19 ± 1.44	14.60 ± 4.51
100 ppm	4.01 ± 1.51	5.70 ± 1.15	15.09 ± 3.47
500 ppm	4.31 ± 1.55	6.03 ± 1.13	19.57 ± 4.70*
1000 ppm	4.68 ± 1.29*	6.65 ± 1.80*	24.57 ± 4.76*
1500 ppm	4.98 ± 1.92*	7.90 ± 1.77*	29.76 ± 7.64*

*Significantly different from the control ($P < 0.05$). Mean ± SD of 12 experiments.

Table 4. Activity of acetylcholinesterase in erythrocytes incubated with Roundup for 1 hour.

	AChE Activity [moles of acetylthiocholine hydrolysed/min./ml of packed cells]·10^{-6}
control	6.8 ± 2.0
100 ppm	6.4 ± 2.1
500 ppm	5.7 ± 1.9*
1000 ppm	5.4 ± 1.8*
1500 ppm	4.9 ± 1.7*

*Significantly different from the control ($P < 0.05$). Mean ± SD of 12 experiments.

ent on the dose of Roundup used. The differences were statistically significant already at the dose of 100 ppm of Roundup.

DISCUSION

Blood is a tissue which performs important roles in the organism: it transports oxygen and nutritive compounds. It is, however, worth remembering that it can also transport toxic compounds. They can reach various organs and damage them. They act in the first line on cells which take part in their transport, i.e. blood components. Therefore, all studies on toxicity of different compounds conducted on erythrocytes have a justification.

Short-term toxicological studies carried out using rats test species indicate LD_{50} values of 5000 mg/kg for glyphosate, glyphosate isopropylamine (IPA) salt, and Roundup herbicide. In a 21-day dermal study (Franz *et al,.* 1997), glyphosate was applied to the skin of New Zealand white rabbits using 10 rabbits of each sex per dose (5 with intact and 5 with abraded skin). The levels of glyphosate tested were 100, 1000, or 5000 mg/kg/day. Treatment-related effects were observed only in the high-dose groups. Based on the data obtained, the NOEL (no-observable-effect-level) for males and females is 1000 mg/kg/day, and the LOEL (Lowest-observable-effect-level) for males and females is 5000 mg/kg/day.

A 2-year chronic feeding and carcinogenicity study was carried out using male and female Sprague-Dawley rats fed diets containing 0, 2000, 8000, or 20000 ppm of glyphosate. These levels were equivalent to 0, 89, 362, or 940 mg/kg/day, respectively, for the females. Treatment related effects were observed only in the high-destose group (Franz *et al.*, 1997).

The studies of Sopinska, Grochala and Niezgoda (2000) showed that roundup is toxic to fish, handicapping the function of the defensive immunological system and liver and kidney function.

The research obtained in our work seems not to be alarming. We have obtained changes in erythrocyte function, but at a dose as high as 500 ppm per 5% hematocrite. The level of met-Hb increased, so did the amount of TBARS and hemolysis, while the activity of AChE decreased.

A significant fact is the lack of accumulation? Some compounds, such as e.g. phenols and dioxins accumulate and may be active after a long accumulation period, which seems to be especially dangerous. In the case of Roundup it is known that it undergoes fast biodegradation in the environment and that water is especially favorable to these processes (Dubbin, Sposito & Zavarin, 2000).

Numerous toxicological studies and extensive knowledge of the physical and chemical properties

of glyphosate indicate that it does not concentrate in the tissues of humans or animals. Studies have shown that glyphosate residues in crops fed to livestock are very low. In studies conducted with laboratory animals given high doses, the highest level of absorption of glyphosate following oral dosing was approximately 33 percent. In these studies, was stated that, 94 percent glyphasate was excreted unmetabolized in the urine within five days. Tissue retention was less than 0.1 ppm at ten days after dosing. These extremely low tissue retention rates should not be surprising, since glyphosate is a water-soluble compound. As a general rule, water-soluble compounds, unlike oil-soluble compounds, do not tend to bioaccumulate in tissue (Franz *et al.*, 1997).

Glyphosate is one of a few herbicides approved by the EPA (Environmental Protection Agency) for controlling weeds in delicate aquatic environments. That's why the EPA established an MCL (Maximum Contaminant Level) for glyphosate, which is 700 parts per billion. The potential for glyphosate presence in raw water does not itself represent a hazard to public health. During sampling of drinking water supplies, glyphosate has never even come remotely close to approaching the MCL. In addition, glyphosate is rated Category E by the EPA, or "evidence of non-carcinogenicity for humans," the most favorable category.

Based on the data on lack of accumulation of Roundup in the organism and the very high threshold dose it may be concluded that this is a compound with low toxicity towards human erythrocytes at doses which might potentially occur in the human body.

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