Effects of Roundup® Pesticide on the Stability of Human Erythrocyte Membranes and Micronuclei Frequency in Bone Marrow Cells of Swiss Mice

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Abstract: Pesticides can affect the health of living organisms through different mechanisms such as membrane denaturation. The evaluation of the deleterious effects of chemical agents on biological membranes can be performed through the analysis of the stability of erythrocytes against a concentration gradient of certain chemical agent in physiologic saline solution. This work analyzed the effect of the herbicide Roundup® on the membrane of human erythrocytes in blood samples collected with EDTA or heparin as anticoagulant agent. The results were analyzed through spectrophotometry at 540 nm and light microscopy. There was an agreement between spectrophometric and morphologic analyses. At the concentration limit recommended for agricultural purposes, Roundup® promoted 100% of hemolysis. The D_{50}Roundup® values obtained for human blood samples collected with EDTA were not significantly different from those obtained for samples collected with heparin. However, the lysis curves presented lower absorbance values at 540 nm in the presence of blood collected with EDTA in relation to that collected with heparin, probably due to hemoglobin precipitation with EDTA. This work also analyzed the effects of three different Roundup® doses (0.148, 0.754 and 1.28 mg/kg) on the micronuclei frequency in bone marrow cells of Swiss mice in relation to a positive control of cyclophosphamide (250 mg/kg). The two highest Roundup® doses showed the same genotoxicity level as the positive control.

Keywords: Roundup®, glyphosate, membrane stability, toxicity.

INTRODUCTION

Agriculture has been the largest source of environmental contamination by pesticides, although they have been used in other sectors. Some pesticides and their metabolites have been considered as pollutants of the bottom and surface of waters, soil [1-3] and atmosphere [4]. They are likely responsible for the loss of biodiversity and deterioration of natural habitats [5]. Increased awareness on the risks related to the intensive use of pesticides has led to a more critical attitude by the agricultural society to prevent future environmental damage [6].

Among herbicides, Roundup® is the most common [7] and most widely used in Brazil. Its active principle is glyphosate (N-phosphonomethylglycine), a derivative of the amino acid glycine. Glyphosate was patented in 1970 and its use in agriculture started in 1974 for the selective control of weeds in rice, maize and soybean crops [8]. Roundup® has around 48% (w/v) glyphosate, being applied in agriculture at concentrations of 5L/hectare [9].

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The USA environmental protection agency rates glyphosate as a less toxic category IV herbicide [10].

This non-selective herbicide inhibits the growth of plants by interfering in the production of essential aromatic amino acids, specifically by inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which is responsible for the biosynthesis of chorismate, which is an intermediate in the biosynthesis of phenylalanine, tyrosine and tryptophan. The inhibition of the synthesis of aromatic amino acids affects not only the synthesis of protein, but also the production of other metabolites such as folate, ubichinones and naphtochines, which create an impact on the general metabolism and on the processing of genetic information, depressing the generation of energy and the synthesis of chlorophyll and protein [11-13].

A low-cost and high-efficiency test to obtain information about the composition and structure of erythrocyte membranes and to investigate the effects of substances and their integrity is the erythrocyte osmotic fragility test (EOF), which assesses the amount of hemolysis obtained in the presence of a salt gradient [14,15].

The EOF test is frequently used in the diagnosis of hemoglobinopathies, mainly spherocytosis, by assessing the effect of drugs on the haematopoiesis [16], in the identification of

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membrane changes in individuals with cervical cancer and obstructive sleep apnea [17].

EOF expresses the ability of membranes in maintaining their structural integrity when exposed to osmotic stress [14]. This type of test includes monitoring the lysis of erythrocytes through the hemoglobin absorbance reading in a spectrophotometer with wavelength set at 540 nm [15].

EOF can be also used in evaluating the toxicity of pesticides [18-21].

A test, which is a variation of EOF, is the measure of the stability of erythrocyte membranes in physiological saline solution under a concentration gradient of the chemical agent to be evaluated [19,22,23].

Among the genotoxicity evaluation tests recommended by international agencies and government institutions, the *in vivo* micronucleus test in the bone marrow of rodents has been widely accepted and recommended for the evaluation and registration of new chemicals and pharmaceuticals that are yearly released in the world market [24].

The micronucleus test emerged as one of the most recommended methods to assess chromosome damages, since this method allows the reliable evaluation of both chromosome loss and rupture [25]. This test is able to reveal the action of clastogenic agents (which break chromosomes) and aneugenic agents (which induce aneuploidy or abnormal chromosome segregation) [26].

The effect of Roundup®, not individual glyphosate, on the stability of human erythrocyte membranes was evaluated by spectrophotometry and light microscopy, using blood samples collected with EDTA or heparin as anticoagulant agent, as well as its genotoxicity on the bone marrow of Swiss mice.

MATERIAL AND METHODS

Ethics

This work was previously approved by the institutional Ethics Committee. The experiments on animals followed the Ethical Principles in Animal Research adopted by the Brazilian College for Animal Experimentation (COBEA).

Collection of Blood Samples

Blood samples (3 mL) were collected from 8 human volunteers (with mean age of 24 ± 3 years, healthy, non-smokers, non-users of drugs or medications and, especially, not consumers of alcoholic beverages) through intravenous puncture after an 8-12 hours fasting period. The blood collections were performed in evacuated tubes (BD, Juiz de Fora, Brazil) containing 50 µL of 25 mmol/L K_2EDTA or 50 µL of heparin as anticoagulant.

Reagents and Equipment

The NaCl used was of brand Synth and had purity degree of 99.5%, which was properly corrected in the preparation of solutions. The volume measures were performed in refractory glass burette and automatic pipettes (Labsystems, model Finnpipette Digital). The mass measures were performed on a digital scale label AND, model 870. The incubations were performed in a Marconi thermostat-controlled water bath, MA model 184 (Piracicaba, SP, Brazil). The absorbance readings were performed in Micronal spectrophotometer model B-442 (São Paulo, SP, Brazil). The microscopic analyses were performed in an Olympus microscope, model CX-41, coupled to a digital camera.

Determination of the Stability of Human Erythrocytes against Roundup® in Physiologic Saline Solution

Duplicate sets of test tubes (Eppendorf®) with physiologic saline solutions (NaCl 0.9%) from 0 to 40 µL/100 mL Roundup® (40 µL/dL), which concentration range is within the acceptable limit for use of the herbicide in agriculture, according to the manufacturer, were pre-incubated at 37 °C during 5 minutes. After the addition of 10 µL of blood samples collected with EDTA or heparine, homogenization and incubation at 37 °C for 30 minutes, the flasks were centrifuged for 10 minutes at 1300 *g* and the supernatant was analyzed by spectrophotometry at 540 nm. The supernatant and the precipitate were stained with Leishman's stain and analyzed by light microscopy.

Determination of the Stability of Human Erythrocytes against Roundup® in Aqueous Solutions

Duplicate sets of test tubes (Eppendorf®) with aqueous solutions from 0 to 40 µL/100 mL Roundup® were submitted to the same procedure described in the previous section to evaluate the stability of human erythrocytes against Roundup® in the absence of saline solution.

Determination of the Erythrocytes Lysis Curves

The dependence of the $\mathbf{A_{540}}$ values on the Roundup® concentrations were adjusted by sigmoidal regression lines, given by the Boltzmann equation,

$$A_{540} = \frac{A_1 - A_2}{1 + e^{(D - D_{50})/dD}} + A_2 \quad (1),$$

where $\mathbf{A_1}$ and $\mathbf{A_2}$ are the A_{540} values that represent the minimum and maximum hemolysis plateaus, **D** is the Roundup® concentration, $\mathbf{D_{50}}$ represents the Roundup® concentration that causes 50% of hemolysis, and **dD** is the amplitude of the sigmoidal transition between $\mathbf{A_1}$and $\mathbf{A_2}$.

Micronuclei Frequency in the Bone Marrow of Swiss Rats Treated with Roundup® Solutions

In order to carry out micronuclei frequency experiments, Swiss mice aged from 7 to 12 weeks, provided by Iquego (Goiânia, Goiás, Brazil), kept in plastic cages at 26 ± 2.0 °C, with light-dark cycles of 12 h, and *ad libitum* access to food and water were divided into the following groups: group I (negative control, which received saline solution); group II (0.148 mg/kg Roundup®); group III (0.754 mg/kg Roundup®); group IV (1.28 mg/kg Roundup®); group V (positive control, which received 250 mg/kg cyclophosphamide). Drugs were prepared in 0.2 mL of sterile saline solution and intraperitoneally administrated. Twenty-four hours after treatment, the animals were sacrificed by cervical dislocation, and then the bone marrow of both femurs was

collected for the preparation of slides for the micronuclei research. The bone marrow was collected with the aid of a 1 ml syringe filled with saline. The femur was washed with saline and cell suspension was collected in a test tube containing saline. The cell suspension was centrifuged for 5 minutes at 1300 *g*, discarding the supernatant and retaining a volume of 0.5 ml in the tube for later re-suspension and homogenization of the cell precipitate. From the resulting suspension, a small drop was removed and placed in one extremity of the blade for the performance of smears in duplicate. The smears were stained with Leishman's stain after drying and the slides were dried at room temperature.

Editions of Graphics and Statistical Analyses

The editing of graphics and statistical analyses were performed with the aid of the Origin 7.5 Professional software (Microcal Inc., Northampton, Massachusetts, USA). The regression lines were only considered significant when p was lower than 0.05. The comparisons of means between groups were performed by analysis of variance (ANOVA), with $p < 0.05$ indicating statistically significant differences.

RESULTS

The Fig. (**1A**) shows the dependence of the absorbance at 540 nm, which reflects the hemolysis intensity against Roundup® concentrations using heparin as anticoagulant [not difference was verified using EDTA]; the Fig. (**1B**) shows the hemolysis intensity on the salt concentrations. The Fig. (**2**) shows Roundup® concentration in physiological saline solution using human blood samples collected with heparin (Fig. **2A**) and EDTA (Fig. **2B**). The D_{50} values (N = 8), which represent the Roundup® concentration that causes 50% of hemolysis, were not significantly different ($p = 0.91$) when determined with blood samples collected with heparin (D_{50} = 31.91 ± 3.86µL/dL) compared to samples collected with EDTA (D_{50} = 31.725 ± 3.03µL/dL), which means were compared through the analysis of variance (ANOVA).

Some solutions considered in Fig. (**2A**), which shows human blood samples collected in heparin, were also examined through light microscopy. The results are shown in Fig. (**3**). In a concentration prior to the lysis transition (5.32 µL/dL) of the sigmoidal curve (Fig. **2A**), the erythrocytes appear with aspect closest to normal, some of them with oval

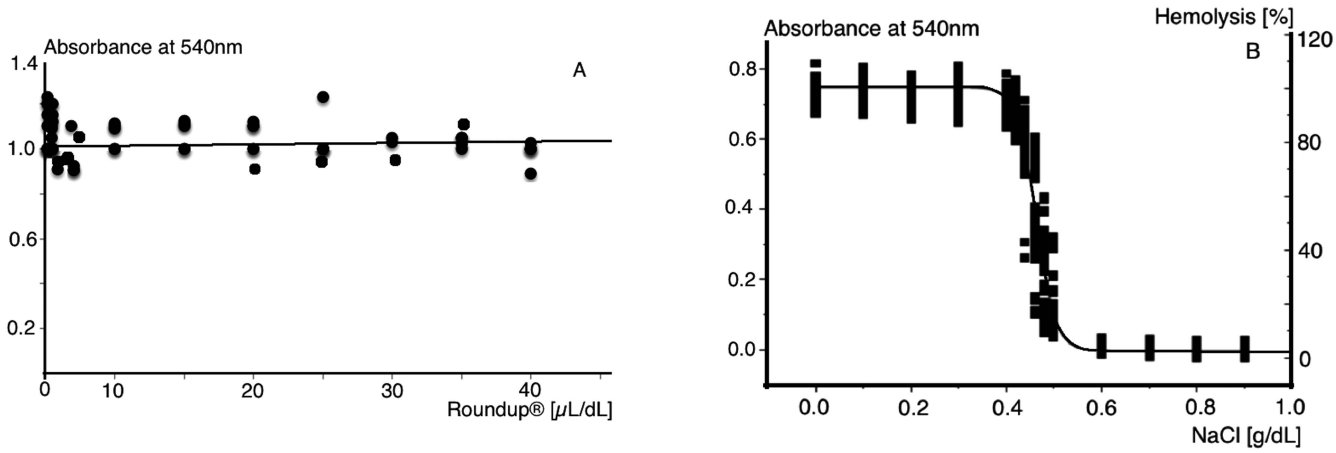

Fig. (1). A. Stability of human erythrocytes in function of the Roundup® concentration in aqueous solution, without salt. Blood samples were collected using heparin as anticoagulant agent. **B.** Hemolysis against crescent salt concentration.

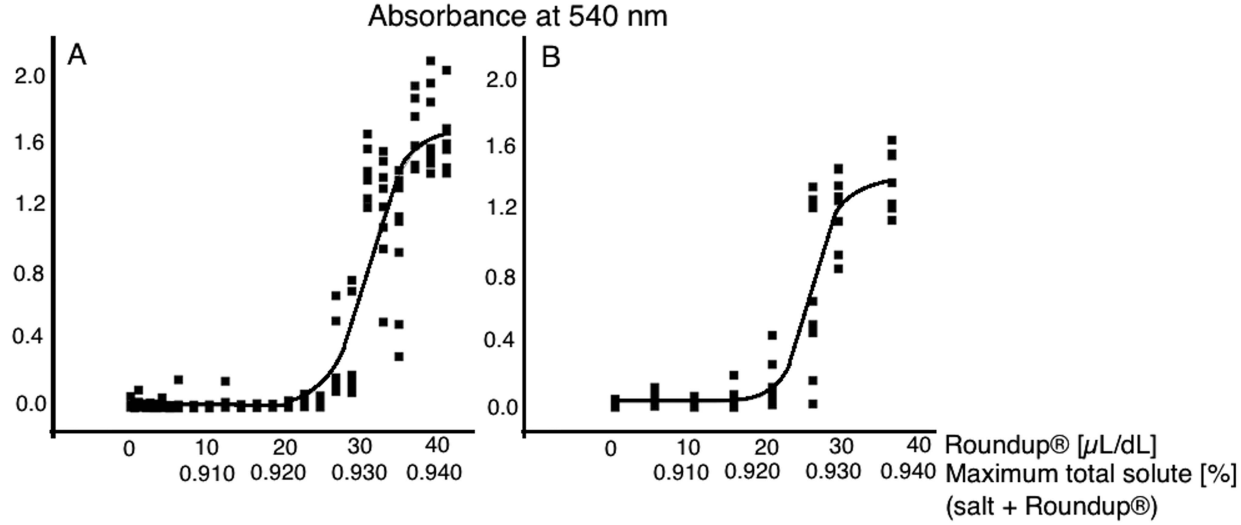

Fig. (2). Stability of human erythrocytes in function of the Roundup® concentration in physiological saline solution. Blood samples were collected using heparin (**A**) or EDTA (**B**) as anticoagulant agent.

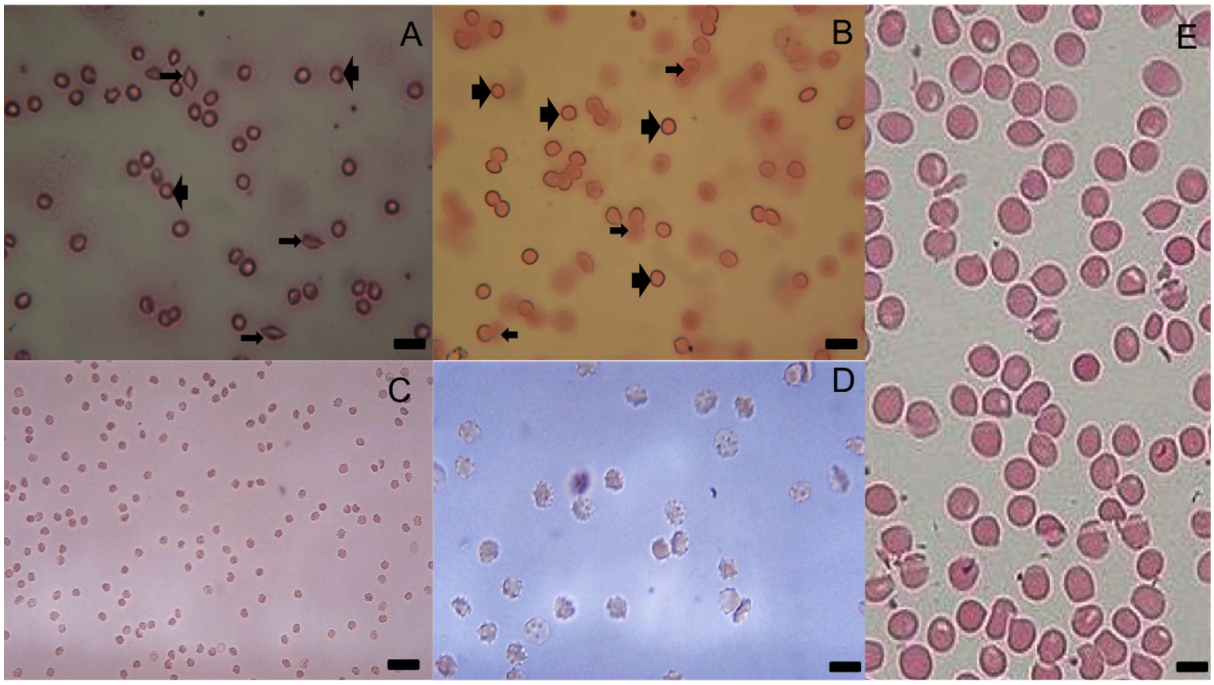

Fig. (3). Microphotographs of human erythrocytes in physiological saline Roundup® solution at 5.32 (**A**), 27.68 (**B**) and 31.91 µL/dL (**C** and **D**). Micrograph of erythrocytes in physiologic solution (**E**) was shown to indicate normal size and shape. Under Roundup® concentrations prior to the lysis sigmoidal transition (**A** and **B**), the most of the red blood cells are intact (large arrows), just any erythrocyte are disrupted (thin arrows). Roundup® at 0.851 mmol/L, and near the lysis line, erythrocytes also appear wilted (**C** and **D**), showing visible spicules at light microscopy, which indicates complete disruption (**D** is high magnification from **C** samples]. Bar=8µm.

shape, but with intact cell limit (Fig. **3A**), the similar is observed at 27.68 µL/dL of Roundup® concentration, which also precedes the lysis half-transition but closest $\mathbf{D_{50}}$ (Fig. **3B**). At 31.91 µL/dL, which represent concentrations around the $\mathbf{D_{50}}$ value, the erythrocytes are broken presenting spicules that indicates the disruption or lysis (Fig. **3C** and **3D**), these data are better observed in higher magnification in Fig. (**3D**).

Within the concentration range recommended for use in agriculture, Roundup® did not show genotoxicity (Fig. **4**) only at the lowest concentration tested (0.148 mg/kg of animal). At 0.754 and 1.28 mg/kg of animal, the micronuclei frequency was significantly higher in relation to the negative control, but not in relation to the positive control (cyclophosphamide), which means that at these concentrations, Roundup® showed the same genotoxicity level as cyclophosphamide.

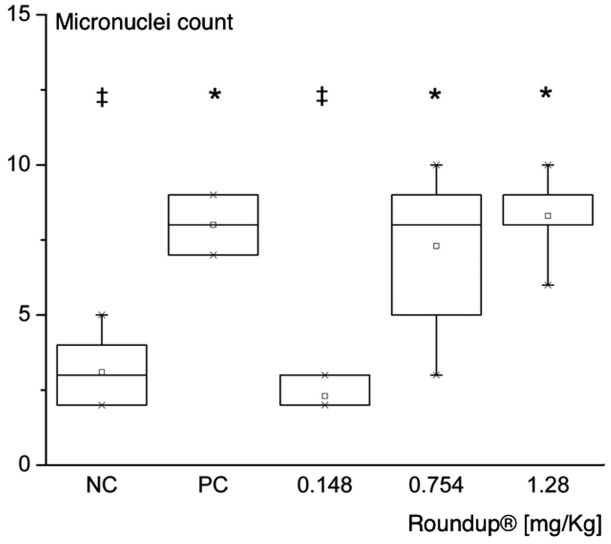

Fig. (4). Micronuclei frequency in the bone marrow of Swiss rats. NC and PC are negative and positive controls, respectively. * and ‡ indicate statistically significant difference (Tukey test) in relation to NC and PC, respectively.

DISCUSSION

The Roundup® concentrations used in this study were within the concentration range recommended by its manufacturer in Brazil (5 to 40 mL Roundup®/100 L of water). Within this limit (approximately 200 ppm of Roundup®), 100% of lysis of human erythrocytes was achieved, according to spectrophotometric analyses (Fig. **2A** and Fig. **2B**).

These analyses showed that human erythrocytes suffer 50% of lysis (D_{50}) at Roundup® concentrations of 31.91 ± 3.86µL/dL. Indeed, at light microscopy, erythrocytes are intact at Roundup® concentrations much smaller than the lysis half-transition region (Fig. **2A** and **2B**). The shape and diameter (around 8 µm) of erythrocytes are similar than that found in physiologic solution (Fig. **2E**), however, they also undergo volume contraction, deformation and lysis as the Roundup® concentration increases (Fig. **2C** and Fig. **2D**).

This effect is due to the Roundup® action and not a result of the nature of anticoagulants used in the blood collection, because no statistically significant difference was observed between D_{50} values obtained for the Roundup® action on human erythrocytes collected both with heparin and EDTA. However, a closer observation of Fig. (**2A**) in relation to Fig. (**2B**) shows that the absorbance values at 540 nm are

generally smaller in the presence of blood collected with EDTA than with heparin. The explanation for this difference would be the occurrence of EDTA-induced precipitation of part of the population of hemoglobin molecules. This explanation is in agreement with the decrease on the hemoglobin concentration in the blood of carps when blood samples are collected with EDTA [27].

The results obtained in this study are relevant for the care of the health of people who are in direct or indirect contact with Roundup®, which is considered one of the less toxic herbicides in market [EPA-1992], and also because the micronuclei frequency performed at minimum, intermediate and maximum *roundup* solution concentrations showed the occurrence of genotoxic effects in the last two (Fig. **4**). Other studies have shown the same genotoxic effect, but at higher concentrations [28], also showing that the micronuclei frequency increases when the glyphosate concentration increases [29], These studies, however, used a solution of gliphosate, not purified Roundup®. Therefore, other studies are necessary to verify the individual actions of particular substances present in Roundup® and their effects on organisms.

These data may justify at least in part the glyphosate poisoning symptoms, which include abdominal pain, vomiting, excess liquid in the lungs, headaches, loss of consciousness, heart palpitations, facial numbness and itchiness [30-32]. The destruction of erythrocytes decreases the ability to bring on oxygen to the blood, causing hypoxia in several tissues.

Erythrocyte lysis exacerbation in blood samples from blood banks had already been reported in literature [33], which also reports the occurrence of increased activity of acetyl-cholinesterase (AChE), lipoperoxidation of membranes and meta-hemoglobin levels due to the action of glyphosate. Those authors used glyphosate concentrations between 100 and 1500 ppm and verified that 500 ppm was the dose that caused changes in erythrocytes. This dose is slightly above the D_{50} value (equivalent to approximately 313 ppm), obtained in this study for human blood samples.

Concentrations such as those would hardly be reached in the blood of end consumer of foods grown under the action of Roundup®, considering the accumulation of glyphosate from Roundup® in food and consumer [33], but of course, these levels could be more easily achieved by people who directly handle the herbicide in agriculture.

CONCLUSIONS

At the concentration limit recommended by the manufacturer for use of Roundup® in agriculture, 100% of lysis of human erythrocytes is observed. The D_{50} values of Roundup® on human erythrocytes were not significantly different for blood samples collected with EDTA in relation to those collected with heparin. Moreover, the absorbance values at 540 nm were lower in the presence of blood collected with EDTA than in the presence of blood collected with heparin, probably due to the precipitation of a small fraction of hemoglobin molecules with EDTA. Besides the acute hemolytic action, Roundup® showed strong genotoxicity in the bone marrow cells of Swiss mice at doses of 0.754 and 1.28 mg/kg.

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