

In vitro effect of the herbicide glyphosate on human blood platelet aggregation and coagulation

Efeito in vitro do herbicida glifosato na agregação plaquetária e coagulação sanguínea em humanos

Teresinha de Jesus C. Neiva¹

Ana Carolina R. Moraes¹

Rafaella Schwyzer¹

Cidônia de Lourdes Vituri²

Tania Rubia F. Rocha³

Diana M. Fries³

Márcio A. Silva⁷

Aloisio Luiz Benedetti⁸

Glyphosate [N-(phosphonomethyl)-glycine] is a broad-spectrum, non-selective, post-emergence herbicide that is extensively used in agriculture. Published data referring to the effects of this product on human health are contradictory. We showed previously that long-term treatment of rats with low doses of Glyphosate-Biocarb® may induce hepatic histological changes and bleeding without decreasing platelet counts. The aim of the current study was to investigate, in vitro, the effect of glyphosate on human blood platelet aggregation and coagulation. Materials and methods: Platelet aggregation was determined in the platelet-rich plasma using the agents: 6µM-adenosine diphosphate, 6µM-epinephrine and 4µg/mL-collagen. Pretreatment with 500µg/mL glyphosate showed significant hypofunction of the three aggregating agents. The inhibitory effect was dose-dependent at concentrations from 50 to 500 µg/mL. The release of ATP was lower for glyphosate-treated platelets after stimulation by collagen. On the other hand, glyphosate did not promote any inhibitory effects on prothrombin time, thromboplastin time and thrombin time. In conclusion, the results demonstrate that glyphosate promotes changes in the platelet metabolism with an inhibitory effect on primary hemostasis Rev. Bras. Hematol. Hemoter.

Key words: *Glyphosate; platelet aggregation; platelet secretion; blood coagulation; herbicide resistance.*

Introduction

Glyphosate (GP) is the active ingredient of Biocarb® or Roundup® (Brazil), marketed as a non-selective broad-spectrum, post-emergence herbicide, used against grasses, broad-leaf weeds and weeds in rice, corn and soybean plantations.^{1,2} The adverse effects of glyphosate and other components of its commercial formulation are periodically re-evaluated and toxicity of the pesticide has been described after severe exposure.^{3,4,5} It has been suggested that a particular toxicity of glyphosate-containing products is related to the surfactant components in its composition. With regular

usage, several regulatory agencies and scientific institutions worldwide have concluded that there is no indication of any human health concern with glyphosate and Roundup®.⁵ However, recent studies suggest that long-term exposition to glyphosate can cause toxicity in pregnant rats with deficient ossification of fetuses⁶, modifications in cell cycle activity,^{7,8} skin injuries,⁹ and increased rates of non-Hodgkin's lymphoma.¹⁰ Furthermore, we showed previously that long-term treatment of rats with low doses of Glyphosate-Biocarb® may induce hepatic histological changes, the leakage of hepatic intracellular enzymes such as alanine aminotransferase and aspartate aminotransferase as well as nasal

¹Hematologia Clínica. Departamento de Análises Clínicas-CCS – Universidade Federal de Santa Catarina – Florianópolis-SC.

²Citologia Clínica. Departamento de Análises Clínicas-CCS – Universidade Federal de Santa Catarina – Florianópolis-SC.

³Hemostasia. Laboratório de Hemostasia do Hospital das Clínicas, Faculdade de Medicina da USP – São Paulo-SP.

⁴Biologia Celular. Laboratório de Neurobiologia e Hematologia Celular e Molecular-BEG-CCB – Universidade Federal de Santa Catarina-SC.

Correspondence: Teresinha de Jesus Carvalho Neiva
Department of Clinical Analysis, CCS-UFSC
88040-900 – Florianópolis-SC – Brazil
Phone number: (55 48) 3319712
E-mail address: neiva@ccs.ufsc.br

bleeding but without decreasing platelet counts.¹¹

The haemostatic system depends on an interaction between the blood vessel wall and platelet circulating factors. The activity developed by platelets is considered an initial response in the regulation of the hemostatic mechanism and among their most important functions are adhesion, aggregation and secretion. Furthermore, pro-coagulant proteins participate in the consolidation of the hemostatic process. On considering bleeding events previously observed in rats, we investigated possible in vitro effects of glyphosate on platelets and blood clotting.

Material and Methods

Chemicals: Adenosine diphosphate (ADP), epinephrine, collagen and luciferin/luciferase were purchased from Chronolog Corporation, USA. Thromboplastin and ellagic acid phospholipid and thrombin reagents were purchased from Sigma Diagnostics, USA.

Subjects: This study involved 10 volunteer blood donors, who signed informed consent before participating in agreement with the Ethics Committee of the University Hospital Hemotherapy Clinic -HU-UFSC.

Isolation of platelets: Nine parts of blood drawn by venipuncture were mixed with one part of 3.8% trisodium citrate. Platelet rich plasma (PRP) was prepared by centrifuging the citrated blood at room temperature for 7 min at 180 g. The platelet concentration was adjusted to 3.0 x 10⁸ cells/mL with autologous plasma.

Measurement of platelet aggregation (PA): PA was determined by the turbidimetric method of Born¹² using a Chronolog. Copr. USA aggregometer. Aliquots of 400 µL of a platelet suspension were transferred into small cuvettes and stirred at a constant speed of 180g at 37°C. The platelets were pre-incubated with different concentrations of GP or vehicle (saline) for 5 min at 37°C, prior to the addition of 6µM ADP, 6µM epinephrine or 4µg/mL collagen. The degree of aggregation (%) was recorded continually for 5 min after the addition of the agonists.

Measurement of platelet ATP secretion induced by Collagen: platelet ATP secretion (%) was evaluated using the luciferin/luciferase system described by Ingerman-Wojenski & Silver.¹³ The platelets were pre-incubated with different concentrations of GP or vehicle (saline) for 5 min at 37°C, prior to the addition of 4µg/mL collagen.

Measurement of Blood Coagulation: nine parts of blood drawn by venipuncture were mixed with one part of 3.8% trisodium citrate. Blood was centrifuged for 10 min at 900 g and the plasma stored at -20°C until use. Activated prothrombin time (PT), activated partial thromboplastin time (APTT) and thrombin time (TT) were evaluated according to the method described by Triplett *et al.*¹⁴ Plasma was pre-incubated with GP or vehicle (saline) for 180 sec. at 37 °C and coagulation time was measured using a Net Lab Digital Coagulation System.

Results

The platelet aggregation profile was tested with three different aggregating agents (6µM ADP, 6µM epinephrine and 4µg/mL Collagen) in PRP pre-treated with increasing concentrations of GP and incubated during 5 min at 37°C. The effects of GP on platelet aggregation are shown in Table 1.

The aggregation effects of all agonists were diminished in the presence of GP. From 125 µg/mL of GP onwards, the aggregation was significantly inhibited when compared to the control. The most significant effect was observed at a concentration of 500 µg/mL of GP.

Table 1. Effects of glyphosate (GF) on platelet aggregation induced by 6µM-ADP, 6µM-Epinephrine and 4µg/mL-Collagen. Platelets were pre-incubated with GP for 5 min at 37 °C before stimulation

Treatment	ADP (%)	Epinephrine (%)	Collagen (%)
Control (saline)	86.3 ± 2.7	92.0 ± 6.4	75.2 ± 9.8
Glyphosate 50 µg/ml	75.6 ± 1.6	74.0 ± 7.4	62.8 ± 10.2
125 µg/ml	47.6 ± 4.0*	31.8 ± 0.8*	29.6 ± 6.1 *
500 µg/ml	20.3 ± 2.9*	15.6 ± 2.1*	7.6 ± 3.3 *

ADP - Adenosine diphosphate. P value is calculated using the Tukey Kramer test (n=10); *p < 0.05

Table 2. Effects of glyphosate (GF) on ATP platelet secretion stimulated by 4 µg/mL-Collagen

Treatment	ATP-secretion (%)
Control (saline)	100 ± 0.0
Glyphosate 50 µg/ml	74.5 ± 3.7 *
125 µg/ml	30.8 ± 5.6 *
500 µg/ml	1.2 ± 0.1 *

ATP - Adenosine triphosphate. P value is calculated using the Tukey Kramer test (n=10); *p < 0.05

Table 3. Effects of glyphosate (GF) (500µg/mL) on prothrombin time (PT), partial thromboplastin time (APTT) and thrombin time (TT). Each value represents the mean ± SD of 10 independent experiments

Test/ Treatment	Time(s)
PT/Control	13.16 ± 0.16
GF-500 µg/mL	12.9 ± 0.14
APTT/Control	31.34 ± 0.63
GF-500 µg/mL	31.25 ± 0.52
TT/Control	17.0 ± 0.13
GF-500 µg/mL	18.0 ± 0.07

GF - glyphosate; PT - prothrombin time; APTT - partial thromboplastin time; TT - thrombin time. P value is calculated using the Tukey Kramer test (n=10).

A decreased production of ATP was observed from the GP-treated platelets after stimulation with 4 µg/mL collagen as is demonstrated in Table 2. A significant decrease in ATP secretion was already observed at a concentration of 50 µg/mL of GP when compared to the control. The maximum inhibition was observed with 500 µg/mL of GP.

The blood coagulation profile was based on the results of prothrombin time (PT), thromboplastin time (APTT) and thrombin time (TT) (Table 3). The samples were incubated with a high concentration of GP (500 µg/mL) for 3 minutes at 37°C before stimulating clotting. None of the three parameters was affected by the glyphosate under experimental conditions even at such a high concentration.

Discussion

Glyphosate (GP) is the active ingredient in the most widely used herbicide in the world and has been chiefly studied by weed control and laboratory bioassays. We showed previously that long-term treatment of rats with low doses of Glyphosate-Biocarb® may induce hepatic histological changes, the leakage of hepatic intracellular enzymes as well as nasal bleeding without decreasing platelet counts.¹¹ The objective of the current study was to determine in vitro whether GP would affect platelet function and coagulation.

Human blood platelets were incubated in the presence of GP for 5 minutes at 37°C. The effect of GP on platelet aggregation is shown in Table 1. Treatment with 500 µg/mL of GP was enough to exhibit significant inhibition by three types of agonists [ADP (79.7%), epinephrine (84.4%) and collagen (89.8%)]. The inhibitory effect was dose dependent for concentrations of between 50 and 500 µg/mL. The results demonstrate that GP promoted changes in the platelet metabolism with an inhibitory effect on primary hemostasis. This can explain previous observations of hemorrhagic effects after exposure of rats to GP.

Platelet activation occurs in two stages following the addition of agents such as ADP, epinephrine and collagen. The primary phase of platelet activation by ADP is characterized by shape change and platelet aggregation. This is followed by a second phase which includes the release of dense granule contents, such as 5-HT, ADP and ATP, that potentiate platelet aggregation.^{15,16} In this study, we observed that shape change and the primary phase induced by the three agonists were not affected by the presence of GP. On the other hand, the second phase was significantly inhibited.

ATP production was used as an index of platelet secretion capacity. We observed decreased secretion of ATP by platelets pre-treated with GP (Table 2) after stimulation with collagen. The maximum inhibition was observed at 500

µg/mL of GP. These results suggest that inhibition of granule release may be the mechanism involved in the anti-aggregation effect caused by GP.

The influence of different pesticides on the platelet metabolism and blood coagulation has been studied.¹⁷⁻²⁰ Krug and Berndt¹⁷ demonstrated that carbamate-type pesticides inhibited platelet aggregation and the formation of thromboxane B₂. Lox¹⁸ demonstrated that the insecticide malathion affects blood coagulation in rats. Furthermore, pesticides produce both hypertension and cardiac arrhythmias.²¹

In the current study, screening tests were carried out in relation to the activated prothrombin time (PT), activated partial thromboplastin time (APTT) and thrombin time (TT) to evaluate the effects of GP on blood coagulation enzymes. A high concentration of GP (500 µg/mL) did not prolong the clotting time of three parameters evaluated under experimental conditions. It is worth noting that pre-incubation of plasma at the maximum dosage (500 µg/mL) did not promote inhibitory action on fibrinogen (TT); additionally, it did not have an inhibitory effect upon the activity of the other plasma proteins (as measured by PT and APTT) as shown in Table 3.

Conclusion

These effects suggest that GP activity in human blood is limited to primary hemostasis. Since reduced platelet function can play an important role as an adverse effect, further experiments will be needed to elucidate the biochemical platelet alterations which are associated to the capacity of GP to inhibit ATP secretion and platelet aggregation. In conclusion, the results demonstrate that GP caused changes in the platelet metabolism with an inhibitory effect on primary hemostasis.

Resumo

O glifosato [N-(fosfonometil)-glicina] é um herbicida pós-emergente não seletivo de amplo espectro muito utilizado na agricultura. Dados da literatura referentes aos efeitos desse produto na saúde humana são contraditórios. Em estudos prévios demonstramos que ratos previamente tratados com glifosato apresentavam lesões hepáticas e sangramento sem alterações quantitativas de plaquetas. O objetivo do presente estudo é investigar os efeitos in vitro do glifosato (GP) na agregação plaquetária e coagulação sanguínea em humanos. A agregação plaquetária foi determinada em plasma rico em plaquetas (PRP) usando os agentes adenosina difosfato (ADP) 6 µM, epinefrina 6 µM e colágeno 4 µg/mL. Pré-tratamento com GP 500 µg/mL demonstrou significativa hipofunção dos três agentes agregantes. O efeito inibitório foi dose dependente em concentrações de 50-500 µg/mL. Utilizando-se a quantificação de ATP como um índice da capacidade de secreção plaquetária, foi observado diminuição da liberação das plaquetas tratadas com GP. Por outro lado, o GP não promoveu efeito inibidor no tempo de protrombina (TP), tempo de tromboplastina parcial ativada (ATPP)

e tempo de trombina (TT). Em conclusão, os resultados demonstram que o GP promove mudanças no metabolismo plaquetário com efeito inibitório na hemostasia primária. Rev. Bras. Hematol. Hemoter.

Palavras-chave: Glifosato; agregação plaquetária; secreção plaquetária; coagulação sanguínea; resistência ao herbicida.

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