Title: The effect of glyphosate-based herbicide Roundup and its co-formulant, POEA, on the motoric activity of rat intestine – *In vitro* study

Author: Magdalena Chłopecka Marta Mendel Natalia Dziekan Wojciech Karlik

PII: S1382-6689(16)30327-1
DOI: http://dx.doi.org/doi:10.1016/j.etap.2016.12.010
Reference: ENVTOX 2680

To appear in: *Environmental Toxicology and Pharmacology*

Received date: 2-6-2016
Revised date: 22-12-2016
Accepted date: 23-12-2016

Please cite this article as: Chłopecka, Magdalena, Mendel, Marta, Dziekan, Natalia, Karlik, Wojciech, The effect of glyphosate-based herbicide Roundup and its co-formulant, POEA, on the motoric activity of rat intestine – In vitro study.Environmental Toxicology and Pharmacology http://dx.doi.org/10.1016/j.etap.2016.12.010

The effect of glyphosate-based herbicide Roundup and its co-formulant, POEA, on the motoric activity of rat intestine - *in vitro* study

Magdalena Chłopecka[a], Marta Mendel[a], Natalia Dziekan[a] and Wojciech Karlik[a]

[a]Warsaw University of Life Sciences, Faculty of Veterinary Medicine, Department of Preclinical Sciences, Division of Pharmacology and Toxicology, 8, Ciszewskiego St., 02-786 Warsaw, Poland

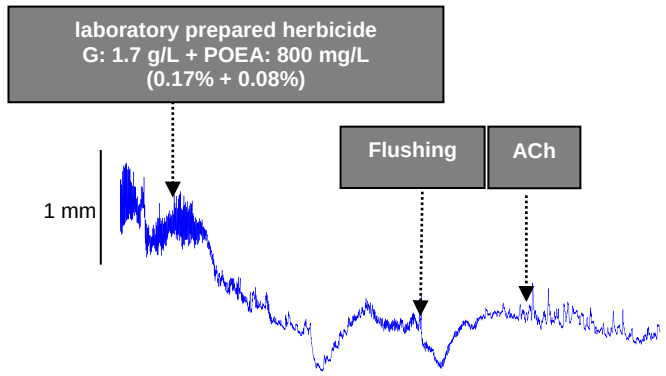
corresponding author: marta_mendel@sggw.pl, Division of Pharmacology and Toxicology, Department of Preclinical Sciences, Faculty of Veterinary Medicine, Warsaw University of Life Sciences, 8 Ciszewskiego St., 02-786 Warsaw, Poland;

phone: +48 225936037, fax: +48 225936065

Graphical abstract

$R\text{-}N\text{-}(CH_2CH_2O)_m\text{-}H(CH_2CH_2O)_n\text{-}H$

polyoxyethylene tallow amine (POEA)



The effect to the application of laboratory prepared herbicide on the jejunum strips motoric activity: a sample recording

Highlights

- both, POEA and Roundup affect significantly the motoric activity of rat jejunum
- POEA exhibits high toxicity towards jejunum smooth muscle
- the interaction between glyphosate and POEA is of antagonistic type

Abstract

The study was aimed at evaluating the effect of Roundup, polyoxyethylene tallow amine (POEA) and mixture of glyphosate and POEA in different levels on the motoric activity of jejunum strips. The incubation in the Roundup solutions caused a significant, mostly miorelaxant, reversible reaction of smooth muscle; only in the highest tested dose which is

equivalent to the agricultural concentration (1% corresponding to 1.7 g glyphosate/L) there was an irreversible disturbance of the spontaneous contractility and reactivity. The incubation in POEA solutions in the range of low doses (0.256; 1.28; 6.4 mg/L) resulted in a biphasic muscle reaction (relaxation and contraction); whereas in the range of high doses, i.e. 32; 160 and 800 mg/L (agricultural spray concentrations) induced only a miorelaxant, irreversible response. The results indicate very high toxicity of POEA which exceeds the toxicity of the commercial formulations. Besides, it is postulated that glyphosate and POEA may display antagonistic interaction towards the motoric activity of gastrointestinal tract.

Keywords: Roundup, POEA, Glyphosate, Motoric activity, Jejunum strips, Interaction

ACh – acetylcholine, POEA – polyethyloxylated tallow amine, Isop – isoproterenol, MK-HS – modifies Krebs-Henseleit solution, PPP – plant protection product

1. Introduction

Pesticides containing glyphosate (*N*-phosphonomethyl-glycine) are nowadays the most used herbicides worldwide. They gained tremendous popularity thanks to their high effectiveness in crop plants protection against weeds and low toxicity of the glyphosate towards mammals, including humans (Dill et al., 2010; Williams et al., 2000). However, during the last decades there has been a rising number of data revealing the possible toxicity of glyphosate and its commercial products on animals. Interestingly, the results of various experiments indicate biological activities of glyphosate containing pesticides and glyphosate itself that are not always possible to observe while conducting toxicological studies required for the registration of plant protection products (PPP) (Mesnage et al., 2015).

It is generally accepted that the toxicity of commercial glyphosate herbicides exceeds significantly the toxicity of glyphosate. This was confirmed in numerous *in vivo* and *in vitro*

studies (Contardo-Jara et al., 2009; El-Shenawy, 2009; Howe et al, 2004; Mesnage et al., 2015; Richard et al., 2005). The exposure to high doses of Roundup-type products causes serious poisonings in human (Chang et al., 1999; Roberts et al., 2010; Stella and Ryan, 2004) although the toxicity of glyphosate alone towards mammals is very low. It is worth emphasizing that though glyphosate is approved as an active substance for the use in plant protection products (Commission Directive 2001/99/EC) and as such is widely considered, its activity towards the target organisms (weeds) is also questionable. This is due to the fact that the recommended agricultural dilutions of the herbicide formulations contain the active ingredient in a concentration exhibiting probably no herbicidal activity (Séralini, 2015) what suggests that glyphosate should be considered as a declared active ingredient. It is speculated that the higher toxicity of commercial products might result from the presence of so called inert ingredients (Brausch et al., 2007; Mann et al., 2009; Mesnage et al., 2015, 2014, 2013; Moore et al., 2012). Nowadays, one of the most commonly used herbicides is Roundup. It is an aquatic solution of glyphosate, used in the form of isopropylamine salt and other co-formulants which are confidential for regulatory purposes but certainly include polyethyloxylated tallow amine (POEA). The data obtained in numerous studies point out very high toxicity of POEA towards animals which exceeds clearly the toxicity of glyphosate and its commercial products (Folmar et al., 1979; Howe et al., 2004; Mesnage et al., 2015, 2013; Moore et al., 2012; Seok et al., 2011; Servizi et al., 1987; Tsui and Chu, 2003). The results of experiments on cell models and animals as well as the clinical signs observed in case of intoxication might indicate that POEA is the ingredient that is responsible for most of the toxic effects of glyphosate containing herbicides. Besides, this data points out the possible interactions between the declared ingredient and the co-formulant (Benachour and Seralini, 2009; Frontera et al., 2011; Guilherme et al., 2012; Kim at al., 2013; Mesnage et al., 2015; Song et al., 2012a).

The results of our previous studies revealed a significant effect of glyphosate on the motoric activity of the gastrointestinal tract (Chłopecka et al., 2014). The alterations of jejunal smooth muscle activity were observed, if glyphosate was used in doses reflecting its concentrations measured in the blood of human with only slight or no clinical symptoms of glyphosate poisoning (Aris and Leblanc, 2011; Roberts et al., 2010). Our interest in understanding the effect of glyphosate on gastrointestinal motoric activity results from a pharmacokinetic study which revealed that shortly after oral exposure over 30% of administered dose of glyphosate was found in the wall of small intestine. Consequently, the amount of glyphosate found there clearly exceeds the amounts found in other tissues (Brewster et al., 1991). Therefore, the next object of our studies included the verification of the effect of a commercial herbicide containing glyphosate and POEA on the motoric activity of gastrointestinal preparations, as well as the evaluation of the interaction between the declared active ingredient and the POEA towards the motoric activity of intestine smooth muscle.

Materials and methods

2.1.Chemicals and media

Acetylcholine chloride (ACh), isoproterenol hemisulfate (Isop), glyphosate (*N*-phosphonomethyl-glycine) (Sigma Chemicals Co, St. Louis, USA, CAS: 1071-83-6), Roundup ULTRA 170 SL (170 g of glyphosate in the form of isopropylamine salt/L and 80 g/L of POEA corresponding to 17% and 8%, respectively) (Monsanto Europe S.A./N.V., Antwerpia, Belgium), polyethyloxylated tallow amine containing oxide/tallow amine ratio: 15:1 (POEA) (Dr. Ehrenstorfer GmbH, Augsburg, Germany, CAS: 61791-26-2), $CaCl_2$ (Merck, Darmstadt, Germany), NaH_2PO_4 (Fluka Chemie, AG, Buchs, Switzerland), NaCl, KCl, $MgSO_4$, $NaHCO_3$ and glucose (Avantor, Gliwice, Poland) were used for preparing and conducting the experiments. Modified Krebs-Henseleit solution (MK-HS) containing NaCl

(123.76 mM), KCl (5 mM), $CaCl_2$ (2.5 mM), $MgSO_4$ (1.156 mM), $NaHCO_3$ (14.5 mM), KH_2PO_4 (2.75 mM) and glucose (12.5 mM) was used as the incubation medium. MK-HS maintained the pH value of 7.35 (7.30 – 7.40) throughout long-term experiments while heated up to 37°C and bubbled continuously with carbogen (95% O_2 + 5% CO_2). All substance used to the experiments were dissolved in MK-HS.

2.2. Animals

The experiments were carried out on intestinal strips isolated from male Wistar rats (weighting approx. 250 g). The use of animals, all procedures involving animals and their tissues were approved by the Local Ethics Committee (approval number 8/2011). The rats had free access to feed and water. The animals were euthanized in chambers filled with carbon dioxide (CO_2) (Everitt and Gross, 2006).

2.3. Preparation of intestine strips and registration of their motoric activity

Immediately after rat's euthanasia the abdominal cavity was opened and fragments of jejunum were incised. Intestine strips were placed in warm (37°C) MK-HS and prepared as described in former experiments (Chłopecka et al., 2007). The preparations were incubated in MK-HS in the chambers of Schuler Organ Bath set (Hugo-Sachs Elektronik, Harvard Apparatus, USA). The experiments were carried out under isotonic conditions, under a load of 0.5 g. The registration of the data was performed through a bridge amplifier (DBA, type 660, Hugo-Sachs Elektronik, Harvard Apparatus, USA) and PowerLab (ADInstruments, Australia). Subsequently, the graphical records were analyzed by Chart v7.0 program and Excel (MS Office XP Professional).

2.4. Design of experiments

Each experiment started with 60-minute preincubation supplemented with three exchanges of the incubation medium every 20 minutes. Subsequently, the strips were exposed to two

reference substances, ACh and Isop, used in the reference concentrations of 1 μM and 0.1 μM, respectively (Chłopecka et al., 2007). The response of strips to the reference substances was registered and MK-HS was exchanged. Once the spontaneous motoric activity of jejunum strips stabilized after flushing with MK-HS, the incubation chambers were filled with tested solutions. The intestine strips were incubated for 3 – 15 minutes in selected solutions of tested compounds and then flushed with fresh MK-HS. At the end of each experiment all preparations were re-exposed to ACh in the reference concentration in order to verify their viability. If it was necessary, tested solutions were buffered with 0.1 M sodium hydroxide to obtain a pH value of 7.35. In experiments aimed at evaluating the effect of Roundup non-buffered solution were used as well.

2.4.1. Experiments with Roundup

The incubation chambers were filled with Roundup solution at concentrations amounting to 0.003, 0.014, 0.068, 0.34, and 1.7 g of glyphosate, i.e. declared active ingredient (d.a.i.)/L. The range of tested doses corresponds to the highest environmental levels, concentrations found in human blood and finally, concentrations used for agricultural dilution. The highest used concentration of Roundup referred to the amount of glyphosate in the field dilution of the herbicide used at the low application rate (i.e. 1% of Roundup containing 170g of glyphosate/L and 800 mg/L of POEA). The intestine strips were incubated in the presence of Roundup and then flushed with fresh MK-HS (pH 7.35). The dilutions of Roundup and concentrations of main formulants (glyphosate and POEA) used in particular treatments are presented in Table 1.

2.4.2. Experiments with polyethyloxylated tallow amine (POEA)

The incubation chambers were filled with POEA solutions at concentrations amounting to 1.28, 6.4, 32, 160, and 800 mg/L. The concentrations of the co-formulant corresponded to its concentrations in Roundup solutions diluted in MK-HS (Table 1). Next, POEA was

additionally used in two other concentrations, i.e. 0.256 and 0.051 mg/L, in order to reach the "non-effective" concentration of the test substance. The intestine strips were incubated in the presence of different POEA solutions and then flushed with fresh MK-HS (pH 7.35).

2.4.2. Experiments with mixture of glyphosate and POEA

The incubation chambers were filled with a mixture of glyphosate and POEA dissolved in MK-HS at the following concentrations: (i) 1.7 g/L and 800 mg/L (laboratory prepared herbicide), (ii) 1.7 g/L + 0.051 mg/L of the declared active ingredient and the co-formulant, respectively. The intestine strips were incubated in the presence of the described mixtures and then flushed with fresh MK-HS (pH 7.35).

2.5. Data analysis and statistics

All data are expressed as percent of the reaction caused by the reference substances. The contraction and relaxation provoked by ACh and Isop, respectively, in the reference doses are defined as 100% (positive control). The negative control is expressed as a contractile and relaxant response of jejunum strips to the filling of incubation chambers with pure MK-HS (pH 7.35).

Results are expressed as mean values (±SD) from 5-7 separate experiments. The reaction of rat isolated jejunum strip to the tested compound is considered as significant if its strength differs statistically from the force of the reaction to the negative control treatment. If the response to a single treatment included both, contraction and relaxation, and both were statistically significant, such reaction was described as significant biphasic reaction. In the statistical analysis a one-way analysis of variance (ANOVA) with post hoc LSD Fisher test was used. Values of $p \leq 0.05$ are considered to be significant. Data were analyzed using StatSoft.Inc. (2014), STATISTICA (data analysis software system), version 12. (www.statsoft.com).

1. Results

3.1 The effect of Roundup on the motoric activity of rat isolated jejunum strips

The incubation of rat jejunum strips in MK-HS containing buffered solutions of Roundup (pH 7.35) resulted always in significant disturbations of the spontaneous motoric activity, irrespective of Roundup concentration. The observed reaction was almost always transient, only the use of Roundup solution containing 1.7 g d.a.i./L, resulted in a constant, irreversible reaction. The dominant character of the registered response was miorelaxant and the reaction was dose-dependent in the range of doses 0.003 – 0.034 g d.a.i./L (Fig.1A). The miorelaxation was accompanied by a significant contraction only when the strips were incubated in Roundup solution containing 0.003 g d.a.i./L. The force of the miorelaxant response of jejunum strips to Roundup containing 0.003 and 0.034 g d.a.i./L amounted to 49.7±26 and 110.5±41.7% of the reaction induced by Isop, respectively (Fig. 1A). The incubation of jejunum preparations in the presence of the highest concentration of Roundup (1.7 g d.a.i./L) provoked a permanent (remaining after flushing) inhibition of smooth muscle reactivity to ACh and disturbed spontaneous motoric activity (Tab. 2). The use of Roundup solution containing 0.34 g d.a.i./L resulted also in a constant but only partial attenuation of ACh-induced reactivity (weaker about 20 %).

The incubation of intestine preparations in a non-buffered Roundup solution containing 1.7 g d.a.i./L (pH 6.0) resulted in a very clear and constant miorelaxation which was 1.5 times stronger than the reaction caused by buffered solutions of Roundup containing the same amount of glyphosate. In contrary, the use of non-buffered Roundup solution containing 0.34 g d.a.i./L (pH 6.7) caused a miorelaxant reaction which force amounted to approx. 50 % of the reaction caused by buffered solutions of Roundup containing the same amount of glyphosate (Fig. 2).

3.2 The effect of POEA on the motoric activity of rat isolated jejunum strips

The application of POEA solutions caused considerable disturbances of the spontaneous motoric activity of rat isolated jejunum segments. However, the character of the observed reactions was dependent on POEA concentration. The use of POEA in higher concentrations (32, 160, and 800 mg/L) resulted in smooth muscle relaxation (Fig. 1B), whereas the use of POEA solutions of lower concentrations (0.256, 1.28 and 6.4 mg/L) induced a biphasic reaction, the preparations underwent both miorelaxation and contraction. The first statistically significant biphasic reaction (contraction and relaxation) was noticed after application of POEA solution in a concentration of 0.256 mg/L. Its force amounted then to 38.5 ±13.7 and 42.9±25.5% of the contraction and relaxation evoked by Isop and ACh, respectively. The maximum relaxant and contractile response was registered if POEA was used at a concentration of 6.4 mg/L. It amounted to 129.5±43.7 % of the reaction caused by Isop and 91.6±39.6 % of the reaction induced by ACh (Fig. 1B). The incubation of jejunum strips in the presence of the solutions containing high concentrations of POEA (32, 160 and 800 mg/L) over 3-5 minutes resulted in more advanced disturbances of the spontaneous contractility and reactivity loss (no response to ACh application), even after POEA was removed from the incubation chambers and replaced by fresh M K-HS (Tab. 2).

3.3. The effect of mixture of glyphosate and POEA on the motoric activity of rat isolated jejunum strips

The incubation of jejunum preparations in the solution of laboratory prepared herbicide containing 1.7g/L and 800 mg/L of glyphosate and POEA respectively, generated only a myorelaxant response which strength came to 100.4 ± 44.3 % of the control treatment with Isop. The incubation of jejunal smooth muscle in laboratory prepared herbicide solution over five minutes produced an irreversible reaction which continued even after exchanging M K-HS. The exchange of the incubation medium caused neither the return of the spontaneous contractility which remained disturbed nor the comeback of the proper reactivity to ACh (Fig.

3, Tab. 2). The use of a mixture containing glyphosate (1.7 g/L) and POEA in the "non-effective" concentration of 0.051 mg/L resulted in strips' myorelaxation. The observed reaction was completely reversible (Fig. 3, Tab. 2) and did not contain a contractile component. The force of the myorelaxant reaction of the strips incubated in the presence of the mixture was significantly lower and amounted to only half of the reaction induced by glyphosate applied alone in the corresponding concentration.

2. Discussion

The results presented herein clearly indicate that Roundup, a commercial herbicide, and POEA, the most used co-formulant in glyphosate-based pesticides, affect significantly the motoric activity of gastrointestinal smooth muscle when used in a wide range of doses. The significant reaction induced by Roundup was observed when the herbicide was used in doses which correspond to highest environmental concentrations of Roundup found in some water sediments bins (Aparicio et al., 2013; Frontera et al., 2011; Giesy et al., 2000). Besides, the result which indicate that toxic doses of Roundup refer to glyphosate concentrations measured in the blood of glyphosate-herbicide poisoned people who exhibited only slight poisoning symptoms seems to be the major finding of this study. The mean plasma concentration values were 0.04, 0.072 and 1.37g/L for minor, moderate and fatal poisonings, respectively (Roberts et al., 2010). Considering the fact that the presented results were obtained in *in vitro* experiments it must be emphasized that the concentrations of glyphosate reported in the general population (dietary exposure) are alarmingly close to the lowest effective of Roundup concentrations used in this experiment (Aris and Leblanc, 2011). The results of our study are consistent with data gained on rat ventricular strips model where demonstrable disorders of cardiac electrophysiological actions were observed if Roundup was used at doses corresponding to the range of concentrations used in our study (Gress et al., 2015). Most of experiments on various *in vitro* (Clair et al., 2012; Gasnier et al., 2009; Gress et al., 2015,

Mesnage et al., 2014; Richard et al., 2005) and *in vivo* (Tsui and Chu, 2003; Contardo-Jara et al., 2009; Mann et al., 2009) models point out higher toxicity of glyphosate-containing trade formulations in comparison to the toxicity of the glyphosate alone. The results presented by Gress et al. (2015) show that Roundup leads to a dose-dependent strong cardiotoxic effect under *in vitro* conditions. In contrary, the incubation of ventricular strips in the presence of corresponding concentrations of pure glyphosate caused only mild disturbances in cardiac action potential parameters. The results obtained herein indicate greater toxicity of the commercial formulation used in the range of higher doses, i.e. 0.068 up to 1.7 g d.a.i./L, towards the employed experimental model in comparison to glyphosate used alone. The force of relaxant reaction of Roundup in a dose of 0.068 and 0.34 g d.a.i./L was approx. twice as big as the reaction to pure glyphosate (Chłopecka et al., 2014) and in the highest concentration, i.e. containing 1.7 g d.a.i./L, Roundup provoked permanent disturbations of spontaneous contractility and reactivity to ACh (Tab. 2). On the other hand, the reduction of the contractile part of the biphasic reaction induced by Roundup administered at lower concentrations and the maintenance of biphasic effect in case of glyphosate use (Chłopecka et al., 2014). might suggest that in the range of low doses glyphosate toxicity exceeds the toxicity of the whole formulation. It is generally agreed that the presence of co-formulants, especially non-ionic surfactants including the most used one, i.e. polyethyloxylated tallow amine, is responsible for the increase of glyphosate-containing products' toxicity. According to the results gained in experiments evaluating the dose-response relationship for POEA, the co-formulant shows indeed very high toxicity that clearly exceeds the toxicity of the declared active ingredient and trade formulations. This observation is in agreement with data obtained in various biological models (Folmar et al., 1979, Howe et al., 2004, Servizi et al., 1987; Tsui and Chu, 2003). The first significant disturbations of the spontaneous motoric activity of jejunum strips observed as a biphasic response was noticed when POEA was applied in a very

low concentration of only 0.256 mg/L. Unfortunately, it is very difficult to refer this data to *in vivo* conditions since there is no toxicokinetic data for POEA in mammals. However, taking into account occasional data obtained from post-mortem analysis of herbicides' poisoning cases the measured concentration of POEA in blood was 1900 times higher (Song et al., 2012a) than the lowest effective concentration (0.256 mg/L) presented here. The high toxicity of POEA was confirmed additionally in *in vitro* studies aimed at cytotoxicity evaluation of different co-formulants. Mesnage et al. (2013) revealed that POEA was the most toxic ingredient of glyphosate-based herbicides in regards to human cellular models. The first signs of significant alteration of cellular respiration processes and disturbed membrane integrity were observed when POEA was used in concentrations of 1-3 ppm (1-3 mg/L) (Mesnage et. al., 2013). On the other hand, the earliest remarkable disturbances of the motoric activity was registered if POEA was administered in a concentration four times lower, i.e. 0.256 mg/L. This observation endorses the usefulness of organ models in toxicological studies. The strongest smooth muscle reaction, both contractile and relaxant, was induced by the co-formulant in the concentration of 6.4 mg/L (Fig. 1B), the applications of POEA at higher doses (starting from 32 mg/L) generated only myorelaxant response and progressive and irreversible disturbances of the spontaneous motoric activity and reactivity to ACh what confirms the high toxicity of POEA towards jejunal smooth muscle strips and indicates toxic damage of tissues. Sudden and rapid intensification of disturbances induced by increasing doses of POEA were found in mammalian cell lines models too (Song et al., 2012b). The analysis of dose-response curve reveals that the cytotoxic effect increases slowly and gradually in the range of low doses and then the inflection point occurs – the cytotoxicity of POEA at the concentration of 25 µM elevates very suddenly.

Our results indicate that pH value of solution affects the reaction of Roundup (Fig. 2). More advanced myorelaxation of jejunum strips incubated in non-buffered Roundup solution

containing 1.7 g d.a.i./L, pH 6.0 in comparison to the reaction evoked by the incubation in a buffered solution containing the same amount of Roundup might confirm the enhancement of glyphosate biological activity by acidic conditions (Fig. 2) (Chłopecka et al., 2014; Hedberg and Wallin, 2010). However, the increase of the pH value of the solution containing 0.34 g d.a.i./L up to 6.7 caused a disappearance of this dependence. Ethoxylated amines belong to the group of non-ionic surfactants because their hydrophilic part is uncharged. Nevertheless, they are defied as weak bases. Therefore, in the acidic environment ethoxylated amines are present in the form of cations whereas in the neutral and alkaline solutions they remain in the form of non-ionized particles (Cullum, 1994). The loss of the ionization of tallow amines enhances their toxicity due to the augmentation of their ability to cause non-specific damage of biological membranes and to penetrate into the cells. It explains significantly higher toxicity of the buffered Roundup solution (0.34 g d.a.i./L, pH 7.35) in the applied experimental model. The correlation between increased pH value and the enhanced toxicity of POEA and POEA-containing herbicides was pointed out previously by other authors in the biological and calculation models (Tsui and Chu, 2003; Schüürmann, 1990). Furthermore, the ability of POEA to affect cell membrane permeability has been confirmed in many studies (Benachour and Séralini, 2009; Clair et al., 2012; Hedberg and Wallin, 2010; Kim et al., 2013; Mesnage et al., 2013; Song et al., 2012b).

It is postulated that the toxicity of the whole herbicide formulation results from the very high toxicity of POEA and the final formulation toxicity is actually equal with the toxicity shown by the co-formulant (Howe et al., 2004; Moore et al., 2012; Seok et al., 2011). According to some *in vitro* and *in vivo* studies, there is a kind of synergy between the glyphosate and the POEA that provokes the intensification of the toxic effect induced by commercial formulations (Frontera et al., 2011; Kim et al., 2013; Song et al., 2012a). The results presented herein clearly indicate an interaction between individual components of Roundup

and unambiguously a synergistic reaction can be ruled out. In contrary, POEA masks the effect of other ingredients of Roundup if administered in the highest dose whereas the application of Roundup in one of the lower doses allows to observe the clear antagonistic interaction between individual components of the mixture. The following findings support this hypothesis (i) no summing of the effects of glyphosate and POEA and the loss of biphasic character of the reaction in comparison to the reaction to glyphosate alone (Chłopecka et al., 2014; Fig. 1), (ii) complete reversibility of the reactions induced by Roundup containing 32 and 160 mg/L of POEA in comparison to persistent reaction to POEA alone, (iii) significantly weaker reaction caused by Roundup than the reaction provoked by POEA solution used in corresponding doses. The phenomenon of antagonism between ingredients of the formulation expressed as diminished force of the reaction is the clearest when strips were incubated in Roundup containing 0.014g/L and 6.4 mg/L of glyphosate and POEA, respectively (Fig. 1). The produced reaction includes then only relaxation (no contractile response) of decreased strength that does not correspond to the sum of reactions caused by individual components, the magnitude of myorelaxation is twice times lower than the reaction registered when POEA was added in the same dose alone. The possible antagonistic interaction between glyphosate and POEA have been recently confirmed by other authors in *in vivo* and *in vitro* studies (Guilherme et al., 2012; Kim et al., 2013; Song et. al., 2012a). The results of tests conducted on fibroblast-like cells and alveolar cells line indicated a remarkable decrease of this co-formulant cytotoxicity measured in LDH assay when glyphosate was present in the solution at the same time (Song et al., 2012a). In the opinion of various authors the type of interaction between glyphosate and POEA (antagonism, synergism or no glyphosate-POEA interaction) and secondarily the eventual toxic effect of the mixture might result to a great extend from the ratio between both ingredients in the mixture (Kim et al., 2013; Song et al., 2012a). A confirmation of this opinion can be found in the results presented herein. The addition of

POEA in a very low, “non-effective”, dose of 0.051 mg/L reduces approx. twice the force of glyphosate-evoked (1.7 g/L) reaction on intestine contractility and the reaction remains reversible (Fig. 3, Tab. 2). The biological half-life of glyphosate in the environment oscillates between 2-14 and 7-70 days and in case of POEA between 13-18 hours and 21-42 days dependent on the environmental conditions (Frontera et al., 2011; Giesy et al., 2000; Wang et al., 2005; Mann et al., 2009). The discrepancies in the degradation time suggest that both chemicals might be present in water, food or feed as residues in changeable concentrations and in variable ratio and thus produce don’t fully predictable effect.

Conclusions

Summing up, the results presented herein justify the speculation that Roundup and its main co-formulant contribute to clinical manifestations of herbicide exposure observed in the course of the acute poisoning and might provoke a danger for the general population continuously exposed of these substances present in food and water. Possibly the consequences of glyphosate-based herbicides and POEA exposure to small but repeatable doses expressed as temporary disturbances of gut motility can be expected in human and animals who do not show clear clinical signs of herbicide poisoning. The obtained data point out the necessity of toxicological data verification in respect to co-formulants contained in glyphosate-based formulations and possibly highlights the need for the extension of toxicological test battery for trade formulations. It is shown that POEA affects very strongly gastrointestinal motoric activity and its toxicity exceeds the toxicity of glyphosate and POEA-containing trade formulation. There is a clear antagonistic interaction between the declared active ingredient and main Roundup co-formulant towards the activity of gastrointestinal smooth muscles. Nevertheless, the question regarding the possible effect of other non-declared ingredients of commercial herbicides on gastrointestinal motility remains unanswered.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgment

The study was supported by a research Grant from the National Science Centre Poland No. UMO-2011/01/B/NZ7/01497.

References

1. Aparicio, V.C., De Gerónimo, E., Marino, D., Primost, J., Carriquiriborde, P., Costa, J.L., 2013. Environmental fate of glyphosate and aminomethylphosphonic acid in surface waters and soil of agricultural basins. Chemosphere. 93(9), 1866-73. doi: 10.1016/j.chemosphere.2013.06.041
2. Aris, A., Leblanc, S., 2011. Maternal and fetal exposure to pesticides associated to genetically modified foods in Eastern Townships of Quebec. Can. Reprod. Toxicol. 31, 528–533, dx.doi.org/10.1016/j.reprotox.2011.02.004
3. Benachour, N., Séralini, G.E., 2009. Glyphosate formulations induce apoptosis and necrosis in human umbilical, embryonic, and placental cells. Chem. Res. Toxicol. 22(1), 97-105, doi: 10.1021/tx800218n
4. Brausch, J.M., Beall, B., Smith, P. N., 2007. Acute and Sub-Lethal Toxicity of Three POEA Surfactant Formulations to Daphnia magna. Bull. Environ. Contam. and Toxicol. 78(6), 510-4.
5. Brewster, D.W., Warren, J., Hopkins, W.E. 2nd, 1991. Metabolism of glyphosate in Sprague-Dawley rats: tissue distribution, identification, and quantitation of

glyphosate-derived materials following a single oral dose. Fundam. Appl. Toxicol. 17 43–51.

6. Chang, C.Y., Peng, Y.C., Hung, D.Z., Hu, W.H., Yang, D.Y., Lin, T.J., 1999. Clinical impact of upper gastrointestinal tract injuries in glyphosate-surfactant oral intoxication. Hum. Exp. Toxicol. 18(8), 475-8.
7. Chłopecka, M., Dziekan, N., Mendel, M., Bakała, A., Małdyk, J., Wiechetek, M., 2007. Evaluation of the time-stability of an alternative research model based on isolated rat gastrointestinal strips. J. Physiol. Pharmacol. 58 Suppl 3, 73-86.
8. Chłopecka, M., Mendel, M., Dziekan, N., Karlik, W., 2014. Glyphosate affects the spontaneous motoric activity of intestine at very low doses - in vitro study. Pestic. Biochem. Physiol. 113, 25-30. doi: 10.1016/j.pestbp.2014.06.005
9. Clair, E., Mesnage, R., Travert, C., Séralini, G.É., 2012. A glyphosate-based herbicide induces necrosis and apoptosis in mature rat testicular cells in vitro, and testosterone decrease at lower levels. Toxicol. In Vitro. 26(2), 269-79. doi: 10.1016/j.tiv.2011.12.009
10. Commission Directive 2001/99/EC amending Annex I to Council Directive 91/414/EEC concerning the placing of plant protection products on the market to include glyphosate and thifensulfuron-methyl as active substances, 2001. O. J. L 304, 14-16.
11. Contardo-Jara, V., Klingelmann, E., Wiegand, C., 2009. Bioaccumulation of glyphosate and its formulation Roundup ultra in Lumbriculus variegatusand its effects on biotransformation and antioxidant enzymes. Environ. Pollut. 157, 57–63, dx.doi.org/10.1016/j.envpol.2008.07.027

12. Cullum, D.C., 1994. Surfactant types; classification, identification, in : Cullum, D.C. (Ed), Separation, in Introduction to surfactant analysis. Springer Science+Business Media Dordrecht, pp. 17-41.
13. Dill, M.G., Sammons, R.D., Feng, P.C.C., Kohn, F., Kretzmer, K., Mehrsheikh, A., Bleeke, M., Honedder, J.L., Farmer, D., Wright, D., Haupfear, E.A., 2010. Glyphosate: discovery, development, applications, and properties, in: V.K. Nandula (Ed.), Glyphosate Resistance in Crops and Leeds: History, Development, and Management, John Wiley and Sons Inc, Hoboken, pp 1–33.
14. El-Shenawy, N.S., 2009. Oxidative stress responses of rats exposed to Roundup and its active ingredient glyphosate. Environ. Toxicol. Pharmacol. 28, 379-385.
15. Everitt, J.I., Gross, E.A., 2006. Euthanasia and necropsy, in:. Suckow, M.A, Weisbroth, S.H., Franklin, C.L. (Eds.), The Laboratory Rat. Elsevier Academic Press, London, pp. 665–678.
16. Folmar, L.C., Sanders, H.O., Julin, A.M., 1979. Toxicity of the herbicide glyphosphate and several of its formulations to fish and aquatic invertebrates. Arch. Environ. Contam. Toxicol. 8(3), 269-78.
17. Frontera, J.L., Vatnick, I., Chaulet, A., Rodríguez, E.M., 2011. Effects of glyphosate and polyoxyethylenamine on growth and energetic reserves in the freshwater crayfish Cherax quadricarinatus (Decapoda, Parastacidae). Arch. Environ. Contam. Toxicol. 61(4), 590-8., doi: 10.1007/s00244-011-9661-3
18. Gasnier, C., C. Dumont, N. Benachour, E. Clair, M.C. Chagnon, G.E. Séralini, 2009. Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines, Toxicology. 262, 184–191, dx.doi.org/10.1016/j.tox.2009.06.006
19. Giesy, J.P., Dobson, S., Solomon, K.R., 2000. Ecotoxicological Risk Assessment for Roundup Herbicide. Rev.. Environ. Contam. Toxicol. 167, 35-120.

20. Gress, S., Lemoine, S., Puddu, P.E., Séralini, G.E., Rouet, R., 2015. Cardiotoxic Electrophysiological Effects of the Herbicide Roundup(®) in Rat and Rabbit Ventricular Myocardium In Vitro. Cardiovasc Toxicol. 15(4), 324-35. doi: 10.1007/s12012-014-9299-2

21. Guilherme, S., Santos, M.A., Barroso, C., Gaivão, I., Pacheco, M., 2012. Differential genotoxicity of Roundup(®) formulation and its constituents in blood cells of fish (Anguilla anguilla): considerations on chemical interactions and DNA damaging mechanisms. Ecotoxicology. 21(5), 1381-90. doi: 10.1007/s10646-012-0892-5

22. Hedberg, D., Wallin, M., 2010. Effects of Roundup and glyphosate formulations on intracellular transport, microtubules and actin filaments in Xenopus laevis melanophores, Toxicol. In Vitro 24, 795–802, dx.doi.org/10.1016/j.tiv.2009.12.020

23. Howe, C.M., Berrill, M., Pauli, B.D., Helbing, C.C., Werry, K., Veldhoen, N., 2004. Toxicity of glyphosate-based pesticides to four North American frog species, Environ. Toxicol. Chem. 23, 1928–1938, dx.doi.org/10.1897/03-71

24. Kim, Y.H., Hong, J.R., Gil, H.W., Song, H.Y., Hong, S.Y., 2013. Mixtures of glyphosate and surfactant TN20 accelerate cell death via mitochondrial damage-induced apoptosis and necrosis. Toxicol. In Vitro. 27(1), 191-7. doi: 10.1016/j.tiv.2012.09.021

25. Mann R.M., Hyne R.V., Choung C.B., Wilson S.P., 2009. Amphibians and agricultural chemicals: review of the risks in a complex environment. Environ. Pollut. 157(11), 2903-27, doi: 10.1016/j.envpol.2009.05.015

26. Mesnage, R., Bernay, B., Séralini, G.E., 2013. Ethoxylated adjuvants of glyphosate-based herbicides are active principles of human cell toxicity. Toxicology. 313(2-3), 122-8, doi: 10.1016/j.tox.2012.09.006

27. Mesnage R., Defarge N., Spiroux de Vendômois J., Séralini G.E., 2014. Major pesticides are more toxic to human cells than their declared active principles. Biomed. Res. Int. 2014:179691., doi: 10.1155/2014/179691

28. Mesnage, R., Defarge, N., Spiroux de Vendômois, J., Séralini, G.E., 2015. Potential toxic effects of glyphosate and its commercial formulations below regulatory limits, Food Chem. Toxicol. 84, 133-53. doi: 10.1016/j.fct.2015.08.012

29. Moore, L.J., Fuentes, L., Rodgers, J.H. Jr, Bowerman, W.W., Yarrow, G.K., Chao, W.Y., Bridges, W.C. Jr., 2012. Relative toxicity of the components of the original formulation of Roundup to five North American anurans. Ecotoxicol. Environ. Saf. 78, 128-33, doi: 10.1016/j.ecoenv.2011.11.025

30. Richard, S., Moslemi, S., Sipahutar, H., Benachour, N., Seralini, G.E., 2005. Differential effects of glyphosate and Roundup on human placental cells and aromatase. Environ. Health Perspect. 113, 716–720, dx.doi.org/10.1289/ehp.7728

31. Roberts, D.M., Buckley, N.A., Mohamed, F., Eddleston, M., Goldstein, D.A., Mehrsheikh, A., Bleeke, M.S., Dawson, A.H., 2010. A prospective observational study of the clinical toxicology of glyphosate-containing herbicides in adults with acute self-poisoning. Clin. Toxicol. (Phila.) 48, 129–136, dx.doi.org/10.3109/15563650903476491

32. Schüürmann, G., 1990. Quantitative structure-property relationships for the polarizability, solvatochromic parameters and lipophilicity. Quant. Struct.-Act. Relat. 9, 326-333. doi: 10.1002/qsar.19900090406

33. Seok, S.J., Park, J.S., Hong, J.R., Gil, H.W., Yang, J.O., Lee, E.Y., Song, H.Y., Hong, S.Y., 2011 Surfactant volume is an essential element in human toxicity in acute glyphosate herbicide intoxication. Clin. Toxicol. (Phila). 49(10), 892-9. doi: 10.3109/15563650.2011.626422

34. Séralini G.E., 2015. Why glyphosate is not the issue with Roundup. J. Biol. Phys. Chem. 15(3), 111-119., doi: 10.4024/12SE15R.jbpc.15.03

35. Servizi, J.A., Gordon, R.W., Martens, D.W., 1987. Acute toxicity of Garlon 4 and Roundup herbicides to salmon, Daphnia, and trout. Bull. Environ. Contam. Toxicol. 39(1), 15-22.

36. Song, H.Y., Kim, Y.H., Seok, S.J., Gil, H.W., Hong, S.Y., 2012a. In vitro cytotoxic effect of glyphosate mixture containing surfactants. J. Korean. Med. Sci. 27(7), 711-5, doi: 10.3346/jkms.2012.27.7.711

37. Song, H.Y., Kim, Y.H., Seok, S.J., Gil, H.W, Yang, J.O., Lee, E.Y., Hong, S.Y., 2012b. Cellular toxicity of surfactants used as herbicide additives. J. Korean. Med. Sci. 27(1), 3-9, doi: 10.3346/jkms.2012.27.1.3

38. Stella, J., Ryan, M. 2004. Glyphosate herbicide formulation: a potentially lethal ingestion. Emerg. Med. Australas. 16(3), 235-9. doi: 10.1111/j.1742-6723.2004.00593.x

39. Tsui, M.T., Chu, L.M., 2003. Aquatic toxicity of glyphosate-based formulations: comparison between different organisms and the effects of environmental factors. Chemosphere. 52(7), 1189-97.

40. Wang, N., Besser, J.M., Buckler, D.R., Honegger, J.L., Ingersoll, C.G., Johnson, B.T., Kurtzweil, M.L., Macgregor, J., McKee, M.J., 2005. Influence of sediment on the fate and toxicity of a polyethoxylated tallowamine surfactant system (MON 0818) in aquatic microcosms. Chemosphere. 59(4), 545-51.

41. Williams, G.M., Kroes, R., Munro, I.C., 2000. Safety evaluation and risk assessment of the herbicide Roundup and its active ingredient, glyphosate, for humans. Regul. Toxicol. Pharmacol. 31, 117–165.

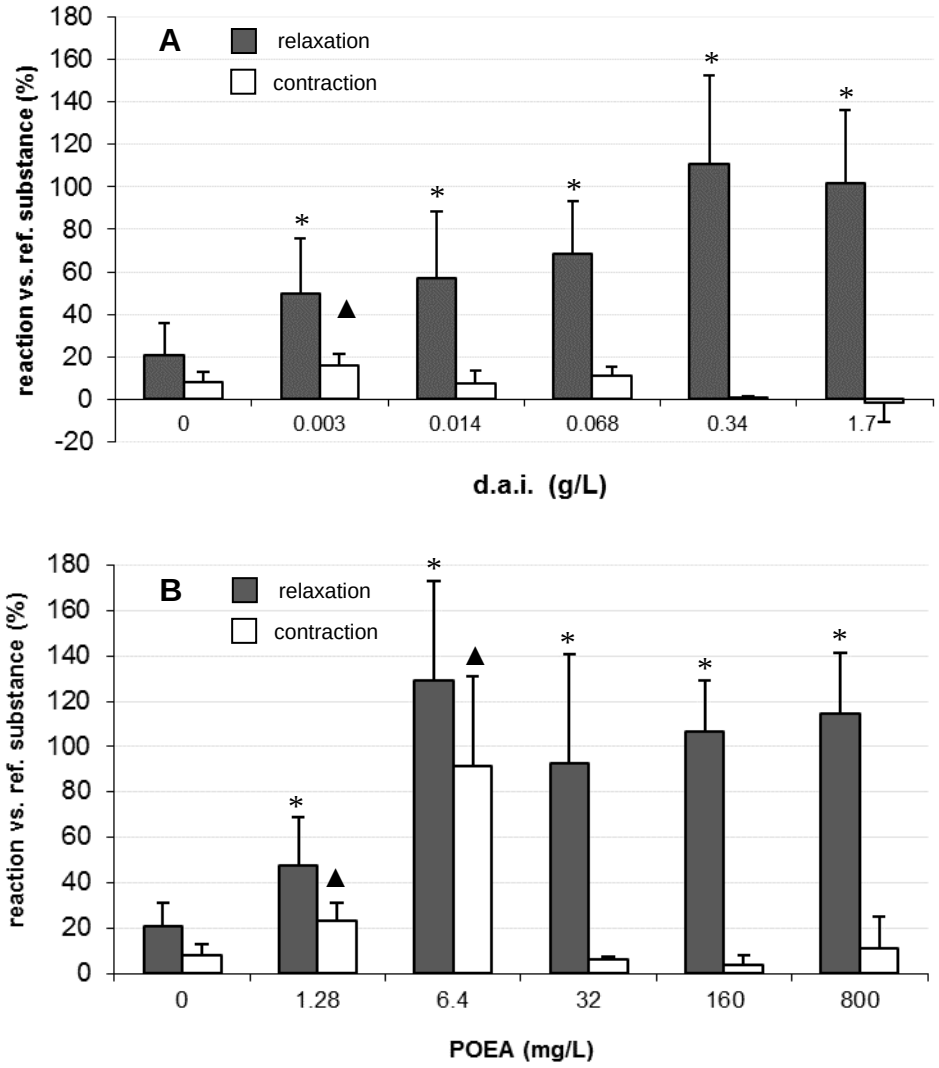


Fig. 1. Effect of Roundup (A) and POEA (B) solutions (pH 7.35) on the spontaneous motoric activity of isolated jejunum strips. The dilution of Roundup solutions are expressed as concentrations of declared active ingredient (d.a.i. = glyphosate). The concentrations of POEA correspond to the dilutions of Roundup formulation.

Data is expressed as a mean of independent experiments (n=6-7, ± SD, *$p \leq 0.05$ vs. relaxation control, ▲$p \leq 0.05$ vs. contraction control). The strength of the reaction is expressed as a percentage of muscle strips response to isoproterenol (muscle relaxation) or acetylcholine (muscle contraction) application in the reference concentration (0.1μM and 1μM, respectively). The response of muscle strips to the flushing with MK-HS without any tested substance was measured and expressed as control („0").

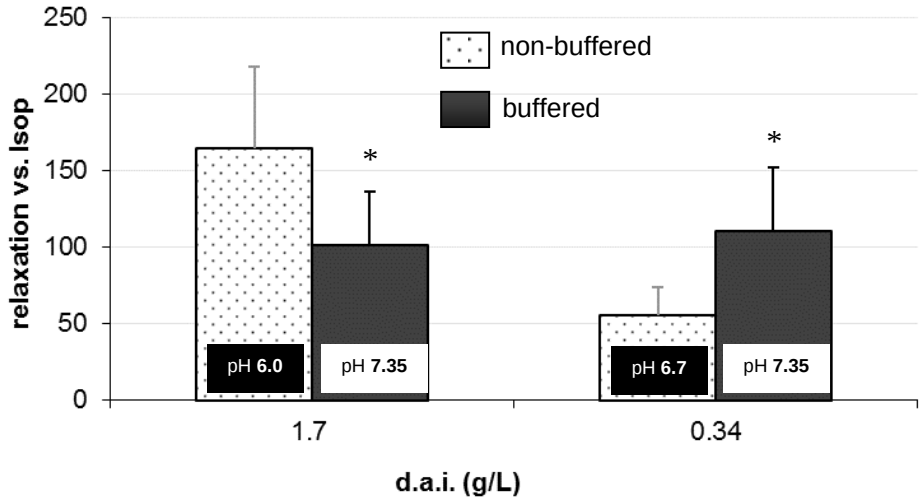


Fig. 2. Effect of Roundup: 1.7 and 0.34 g d.a.i./L in buffered and non-buffered solutions on the spontaneous motoric activity of isolated jejunum strips.

Data is expressed as a mean of independent experiments (n=5-6, ± SD, *$p \leq 0.05$ vs. reaction to non-buffered solution). The strength of the relaxation is expressed as a percentage of muscle strips response to isoproterenol applied in the reference concentration (0.1μM).

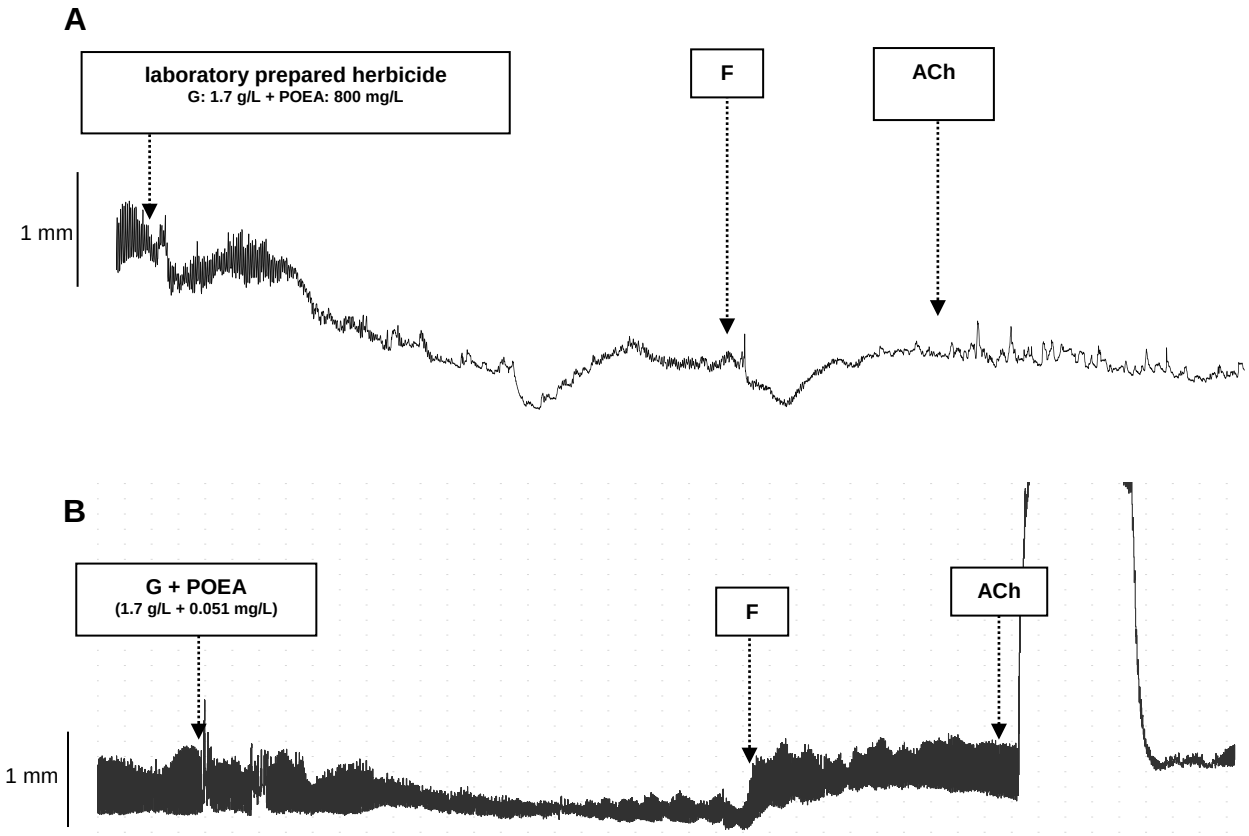


Fig. 3. A representative recording of isolated jejunum strip's activity: A. reaction to the application of laboratory prepared herbicide B. reaction to the application of a mixture of glyphosate and "non – effective" concentration of POEA.

G – glyphosate

POEA – polyoxyethylene tallowamine

F – flushing

ACh - acetylocholine

Table 1. The concentrations of formulants (glyphosate and POEA) at decreasing dilutions of Roundup ULTRA 170 SL.

FORMULATION/ FORMULANT/	UNITS	CONCENTRATIONS					
R-p	%	**100**[a]	1[b]	0.2	0.04	0.008	0.0016
G	g/L	**170**	1.7	0.34	0.068	0.014	0.03
POEA	mg/L	**$8x10^4$**	800	160	32	6.4	1.28

[a] concentrated formulation

[b] agricultural spray (in-use dilution)

Note that in experiments with POEA the concentration of 0.256 and 0.051 mg/L were additionally tested.

G – glyphosate

POEA – polyoxyethylene tallow amine

R-p – Roundup

Table 2. Effect of glyphosate, POEA, Roundup and mixtures of glyphosate and POEA on the spontaneous motoric activity of isolated jejunum strips.

	SOLUTIONS (pH 7.3)				
	G[1] (1.7 g/L)	**R-p** (**G**: 1.7 g/L + **POEA**: 800 mg/L)	**laboratory prepared herbicide** (**G**: 1.7 g/L + **POEA**: 800 mg/L)	**G + POEA** (1.7 g/L + 0.051 mg/L)	**POEA** (800 mg/L)
relaxation (%)	100.2 ± 33.7	101.6 ± 34.6	100.4 ± 44.3	49.9 ± 17.9*	114.7 ± 26.6
contraction (%)	14.9 ± 7.8	absent	absent	absent	absent
type of reaction	reversible	**irreversible**	**irreversible**	reversible	**irreversible**

[1]results obtained in the previous study (Chłopecka et al., 2014)

Data is expressed as a mean of 6-7 independent experiments (± SD). The strength of the reaction is expressed as a percentage of jejunum strips response to isoproterenol (0.1 μM) or acetylcholine (1 μM), *p≤0,05 vs. glyphosate.

G – glyphosate

POEA – polyoxyethylene tallow amine

R-p – Roundup