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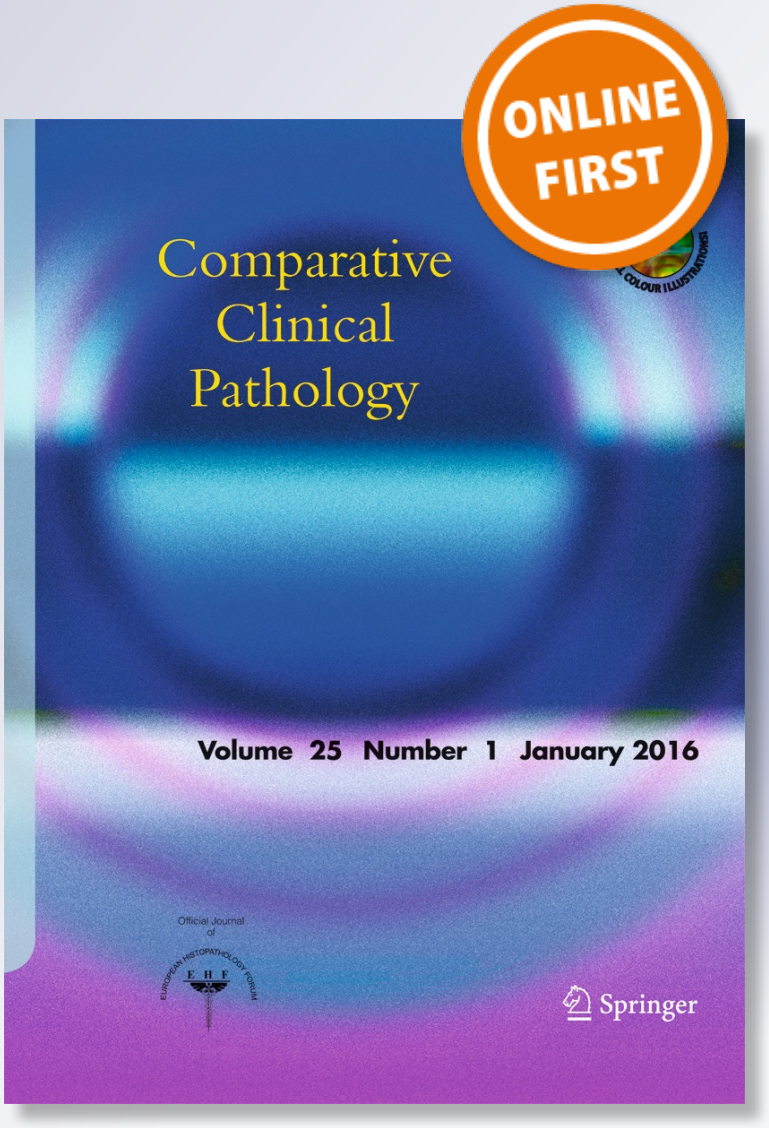

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ORIGINAL ARTICLE

Glyphosate-based herbicides with different adjuvants are more potent inhibitors of 3T3-L1 fibroblast proliferation and differentiation to adipocytes than glyphosate alone

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Abstract Glyphosate-based herbicides are extensively used for weed control all over the world. Thus, it is important to investigate the putative toxic effects of these commercial formulations which contain not only glyphosate but also different adjuvants. 3T3-L1 fibroblasts are a useful tool in the study of adipocyte differentiation. We have previously reported that a commercial glyphosate formulation inhibits proliferation and differentiation in these cells. In the present investigation, we further evaluated the effect of different commercial glyphosate-based herbicides on 3T3-L1 fibroblast proliferation and differentiation and compared their effect with glyphosate itself. After treatment of 3T3-L1 fibroblasts with different concentrations of three glyphosate formulations or glyphosate itself, the increase in cell number or cytosolic lipid accumulation was determined. To evaluate the presence of polyethoxylated adjuvants, matrix-assisted laser desorption/ionization (MALDI) analysis of the three commercial glyphosate-based herbicides was also performed. We found that the three commercial formulations tested were more potent inhibitors of both physiological processes: proliferation and differentiation to adipocytes of 3T3-L1 fibroblasts than glyphosate. We also found by MALDI analysis that the adjuvants were polyethoxylated in two of the formulations but not in the third one. It is well known the toxic effect of polyethoxylated adjuvants, but according to our results, non-polyethoxylated adjuvants may also contribute to the toxic effects. Our results further support the ability of glyphosate-based herbicides to disturb cellular physiology and highlight the importance of toxicological assessment of commercial formulations rather than glyphosate alone.

Keywords Glyphosate-based herbicides · Glyphosate · 3T3-L1 fibroblasts · Differentiation · Proliferation · Adjuvants

Introduction

Glyphosate is the active ingredient of the most commonly used herbicide worldwide, mainly in countries such as USA, Argentina, Brazil, and Canada which have the largest plantings of glyphosate-resistant crops. Glyphosate acts by targeting the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which is involved in the synthesis of several essential amino acids in plants, but it is not found in animals (Gianessi 2005; Dill et al. 2008). Thus, glyphosate is considered to be safe to people and other vertebrates when used according to the manufacturer's instructions.

In Argentina, there has been an important increase of the area planted with glyphosate-resistant crops, mainly soybean. Accordingly, glyphosate was found in water and soil of an area of Buenos Aires planted with glyphosate-resistant crops (Peruzzo et al. 2008). Taking this into account, it is possible for this herbicide to spread in the ecosystem and reach plants, animals, and also the food chain. In addition, humans may be exposed to herbicide residues by agriculture practices (Acquavella et al. 2004).

A commercial formulation of glyphosate, Roundup®, has been reported to be genotoxic (Poletta et al. 2009) and to have teratogenic effects in *Xenopus laevis* and chicken embryos (Paganelli et al. 2010). It has also been shown that formulated glyphosate inhibits cell cycle progression from analysis of the

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first cell division of sea urchin embryos, a recognized model for cell cycle studies (Marc et al. 2004).

Glyphosate-based herbicides also contain adjuvants, such as polyethoxylated alkylamines (POEA), which facilitate the absorption of the herbicide and increase its effectiveness. These adjuvants are considered as "inert" but may also be toxic; in keeping with this, commercial formulations of glyphosate have been shown to be more toxic than the active ingredient itself (Richard et al. 2005; Benachour et al. 2007; Mesnage et al. 2013; Gasnier et al. 2009).

3T3-L1 fibroblasts are a useful tool in the study of adipocyte differentiation. After the addition of a differentiation mixture containing insulin, dexamethasone, and 3-isobutyl-1-methylxanthine (MIX), postconfluent 3T3-L1 fibroblasts reenter the cell cycle. This proliferation step is called mitotic clonal expansion (MCE). MCE precedes the adipogenic gene expression program leading to adipocyte differentiation (Qiu et al. 2001; Martini et al. 2009). Therefore, 3T3-L1 fibroblasts are an interesting mammalian cellular model that allows to evaluate the effect of environmental contaminants on two different physiological processes that take place in these cells: proliferation and differentiation. Accordingly, 3T3-L1 fibroblasts were previously used to evaluate the role of arsenic trioxide, as well as of a commercial formulation of the herbicide glyphosate and hexavalent chromium, on cell proliferation, survival, and differentiation (Wang et al. 2005; Martini et al. 2012, 2014)

We have previously shown that a commercial glyphosate formulation inhibits proliferation in 3T3-L1 fibroblasts and induces apoptosis, which is indicative of cellular damage and also inhibits the ability of this cell line to differentiate to adipocytes (Martini et al. 2012).

In the present investigation, we compared the effects on the proliferation and differentiation of 3T3-L1 fibroblasts of glyphosate itself and three different glyphosate-based herbicides, including the one previously reported, to analyze the contribution of glyphosate to the toxicity of commercial glyphosate formulations, and we also performed a comparative analysis by matrix-assisted laser desorption/ionization (MALDI) of the adjuvants present in these commercial formulated herbicides.

Materials and methods

Chemicals

Dulbecco's modified Eagle's medium (DMEM), trypsin, insulin, 3-isobutyl-1-methylxanthine (MIX) dexamethasone, and glyphosate (GLY) were purchased from Sigma Chemical Co. (St. Louis, MO). 3T3-L1 fibroblasts were obtained from Asociación Banco Argentino de Células (origin: ATCC). Different glyphosate formulations were used: Glifosato Atanor (isopropylamine salt, 35.6 % *w*/*v* acid equivalent (ae), GLY-A) was from Atanor, Argentina; Roundup FG (monoammonium salt, soluble granules, 72 % *w*/*w* ae; GLY-FG) was from Monsanto Argentina, and Glifogran (monoammonium salt, soluble granules, 68.7 % *w*/*w* ae; GLY-Gl) was from Gleba, Argentina. According to the suppliers, these herbicides contain surfactants and water, but no specification is provided.

Cell cultures and treatments

3T3-L1 fibroblasts were cultured in DMEM + 10 % fetal bovine serum (FBS) with 100 μg/ml streptomycin, 100 U/ml penicillin, and 250 ng/ml Fungizone (DMEM + 10 % FBS). When indicated, GLY, GLY-A, GLY-FG, or GLY-Gl was added in DMEM + 10 % FBS as vehicle. Prior to addition of GLY or formulated herbicides to the cell plate, the appropriate solutions were prepared and neutralized with a small amount of NaOH, when it was necessary to keep the pH of the medium.

Cell counting in exponentially growing cells

3T3-L1 fibroblasts were cultured in 24-well plates until they reached 30 % confluence. At that moment, some wells were treated with different concentrations of GLY or GLY-based herbicides for 24 h, as indicated in each case, and others were treated with DMEM + 10 % FBS alone (control). At the end of these treatments, cells were trypsinized and resuspended in phosphate-buffered saline (PBS) and an aliquot was counted using a Neubauer chamber.

Induction of differentiation

To induce differentiation, 2-day postconfluent cells were treated with a differentiation mixture containing 10 μg/ml insulin, 0.5 mM 3-isobutyl-1methylxanthine (MIX), and 100 nM dexamethasone in DMEM + 10 % FBS (DM). Three days after the induction of differentiation, medium was replaced with DMEM + 10 % FBS supplemented with 10 μg/ml insulin. Then, the medium was changed every 2 days with DMEM + 10 % FBS. When indicated, GLY or glyphosate-based herbicides were added with the differentiation mixture to obtain the appropriate final concentration indicated in each experiment and were kept in the medium until the end of the differentiation.

Oil Red O staining

On day 7, adipocyte monolayers were washed three times with PBS and then fixed for 30 min with 4 % formaldehyde in PBS. Oil Red O (0.35 %) in isopropanol was diluted with water (3:2), filtered, and added to the fixed cell monolayers for

30 min at room temperature. Cells were then washed with water, and the stained triglyceride droplets in the cells were visualized and photographed.

MALDI analysis of glyphosate-based herbicides

Mass spectrometry (MS) experiments were carried out on a Bruker Daltonics Ultraflex II equipped with TOF/TOF ion optics and an Nd-YAG laser. MALDI analysis was performed by CEQUIBIEM (Faculty of Sciences, University of Buenos Aires). The system was calibrated immediately before analysis with a mixture of angiotensin, substance P, bombesin, ACTH, and somatostatin peptides within a range of 1000–3100 Da with a mass precision better than 50 ppm. One microliter of each glyphosate-based herbicide was diluted 10, 100, and 1000 times in water and mixed with 1 μl of a solution of α-cyano-4-hydroxycinnamic acid matrix prepared in 50 % ACN with 0.1 % TFA. The mixture was spotted on an Anchor Chip target; the droplet was allowed to evaporate before introducing the target into the mass spectrometer. Acquisitions were taken in manual modes. MS spectra were acquired in the positive reflector mode by summarizing 1000 single spectra (5 × 200) in the mass range from 0 to 5000 Da.

Statistical analysis

The experiments were carried out three times unless otherwise stated. All data is expressed as mean ± SE. Statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc test, and p values below 0.05 were considered significant.

Results

Effect of glyphosate-based herbicides and glyphosate on the proliferation of 3T3-L1 fibroblasts

In a previous work (Martini et al. 2012), we found that a glyphosate-based herbicide (GLY-A) is able to inhibit the proliferation of 3T3-L1 fibroblasts, as well as the accumulation of cytosolic lipids that takes place when these cells are induced to differentiate to adipocytes.

Herein, we first compared the dose-dependent effect of three different commercial glyphosate formulations: GLY-A, GLY-FG, and GLY-Gl and glyphosate itself (GLY) on proliferation of 3T3-L1 preadipocytes. The effect of equal amounts of glyphosate obtained from each of these three commercial formulations and from the pure chemical was analyzed. As it is shown in Fig. 1a, GLY-A significantly inhibited proliferation at concentrations of 178 and 89 ppm which correspond to dilutions of 1:2000 and 1:4000 of this commercial herbicide but not at 36 ppm (1:10,000 dilution) which is in agreement with our previous report (Martini et al. 2012). Similarly, the second herbicide tested, GLY-Gl, significantly inhibited proliferation at the two highest concentrations but not at 36 ppm (Fig. 1b). However, with the highest concentration of GLY-A (178 ppm), a decrease in cell number with respect to the number of cells that were present in the plates at the beginning of the experiment (zero time control, C0) was detected, since some cells had detached from the plate and were lost after 24 h.

In the case of GLY-FG, the number of cells had decreased with respect to C0 after 24-h treatment with concentrations of 178 and 89 ppm and many cells were lost. In addition, GLY-FG significantly inhibited proliferation at a concentration of 36 ppm, which was ineffective with the other two commercial herbicides analyzed (Fig. 1c).

We also tested the effect of glyphosate on proliferation of exponentially growing 3T3-L1 fibroblasts and found that it was not inhibitory at the concentrations assayed with the three glyphosate-based herbicides; the concentration of glyphosate had to be increased to 3600 ppm to have a significant inhibition of proliferation (Fig. 1d).

These results indicate that compounds different from glyphosate itself present in the commercial formulations importantly contribute to inhibit 3T3-L1 fibroblast proliferation. To further analyze this contribution, we estimated the concentration of each of these formulations of glyphosate necessary to obtain 50 % of the increase in cell number reached in untreated control cells after 24 h which is set at 100 %. As it is shown in Fig. 1e, where data from Fig. 1 a–d were summarized, this concentration is approximately 30 ppm for GLY-FG and 100 ppm for GLY-A and GLY-Gl. Negative values in Fig. 1e indicate a decrease in cell number with respect to the beginning of the experiment (C0) due to cells lost by detachment from plates. On the other hand, glyphosate is much less toxic than any of the commercial formulations tested.

Effect of glyphosate-based herbicides and glyphosate on the differentiation of 3T3-L1 fibroblasts

To further evaluate potential cytotoxic effects of the three glyphosate-based herbicides and glyphosate in 3T3-L1 fibroblasts, we investigated their effects on the differentiation to adipocytes that takes place when 2-day postconfluent 3T3-L1 fibroblasts are treated with a differentiation mixture. We found that all of them inhibited differentiation in a dose-dependent manner at similar doses as those found for inhibition of proliferation. Thus, we found that both GLY-A and GLY-Gl inhibited differentiation at 178 and 89 ppm but not at 36 ppm. Instead of that, GLY-FG inhibited differentiation at 89 and 36 ppm and cells did not survive at the higher dose of 178 ppm, as it is also shown in Fig. 1c. In addition, we found that GLY did not inhibit differentiation even at 178 ppm; inhibition was found with GLY 3600 ppm (Fig. 2).

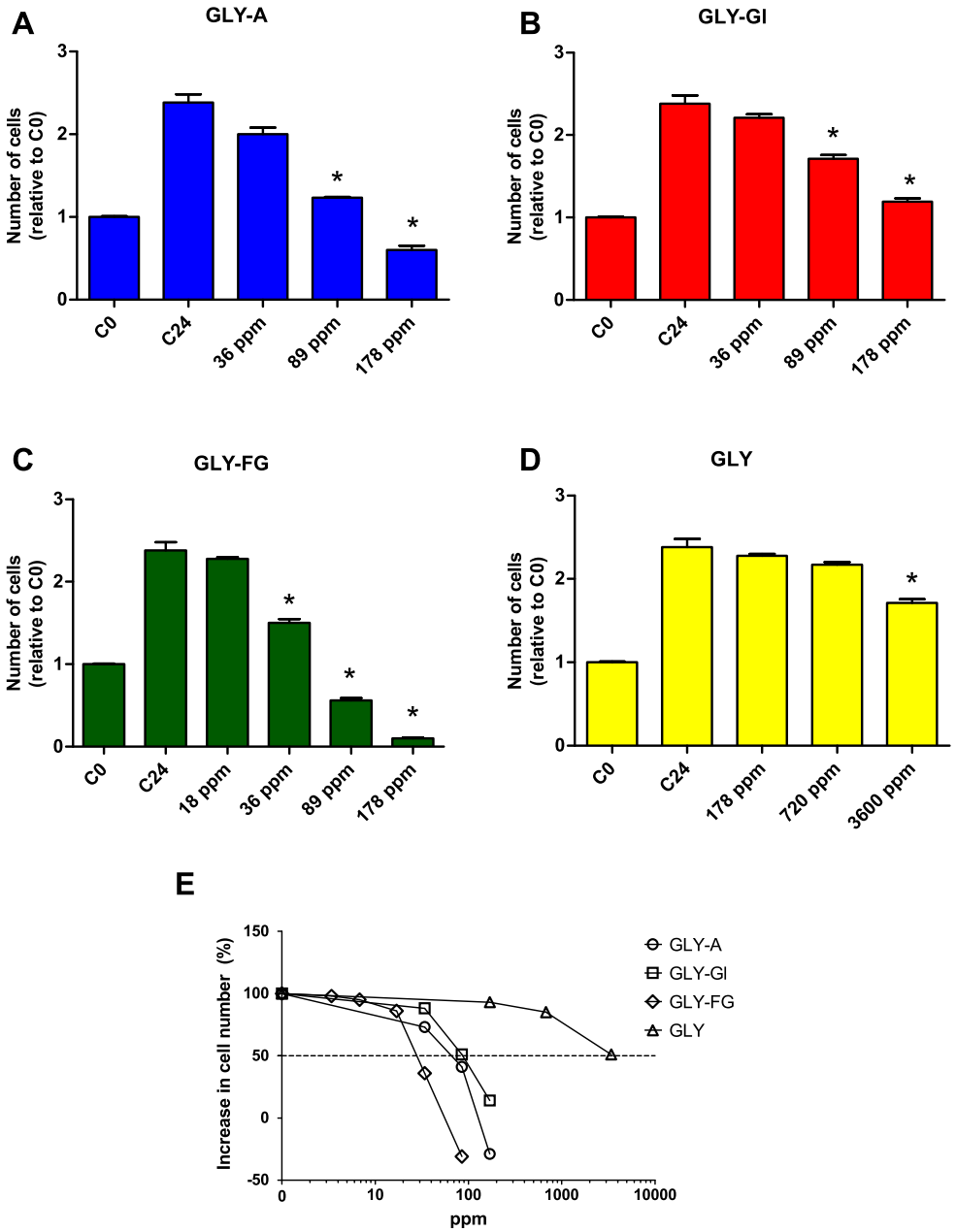

Fig. 1 Dose-dependent effect of three glyphosate-based herbicides and glyphosate alone on exponentially growing 3T3-L1 fibroblasts. Cells were cultured in 24-well plates until they reached 30–40 % confluence. At that time, two plates were counted as zero time control (C0). Others were treated for 24 h with medium (C24) or different concentrations in parts per million of GLY-A (**a**), GLY-Gl (**b**), GLY-FG (**c**), or GLY (**d**) as indicated in each case. At the end of these treatments, cells were trypsinized and counted in Neubauer chamber as indicated in "Materials and methods" section. Results are expressed relative to C0 which is set to 1 and represent mean ± SE of three independent experiments. *Significantly different from C24, $p < 0.05$ (ANOVA). These results are summarized in the graph shown in **e**

MALDI analysis of the adjuvants in the three glyphosate-based herbicides tested

We compared the adjuvants present in these three formulated herbicides using MALDI analysis (Fig. 3). Both Gly-FG and Gly-Gl showed the presence of several peaks with a difference in mass of 44 kDa which is indicative of the presence of polyethoxylated adjuvants that vary in the length of the ethoxylated chain since the repetitive unit is –CH2-CH2-O–. In addition, GLY-FG spectrum was centered on 1018 *m*/*z* and GLY-Gl on 770 *m*/*z* (Fig. 3). Thus, polyethoxylated adjuvant in GLY-FG has higher mass than in GLY-Gl.

On the contrary, the spectrum of GLY-A did not show peaks with this difference of 44 kDa in mass suggesting the presence of non-polyethoxylated adjuvants in this formulation in spite of its ability to inhibit proliferation and differentiation in 3T3-L1 fibroblasts similarly to GLY-Gl.

Discussion

In keeping with our previous report of cytotoxic effects of a glyphosate formulation in 3T3-L1 fibroblasts, herein, we found that different commercial glyphosate-based herbicides were able to inhibit proliferation and differentiation to

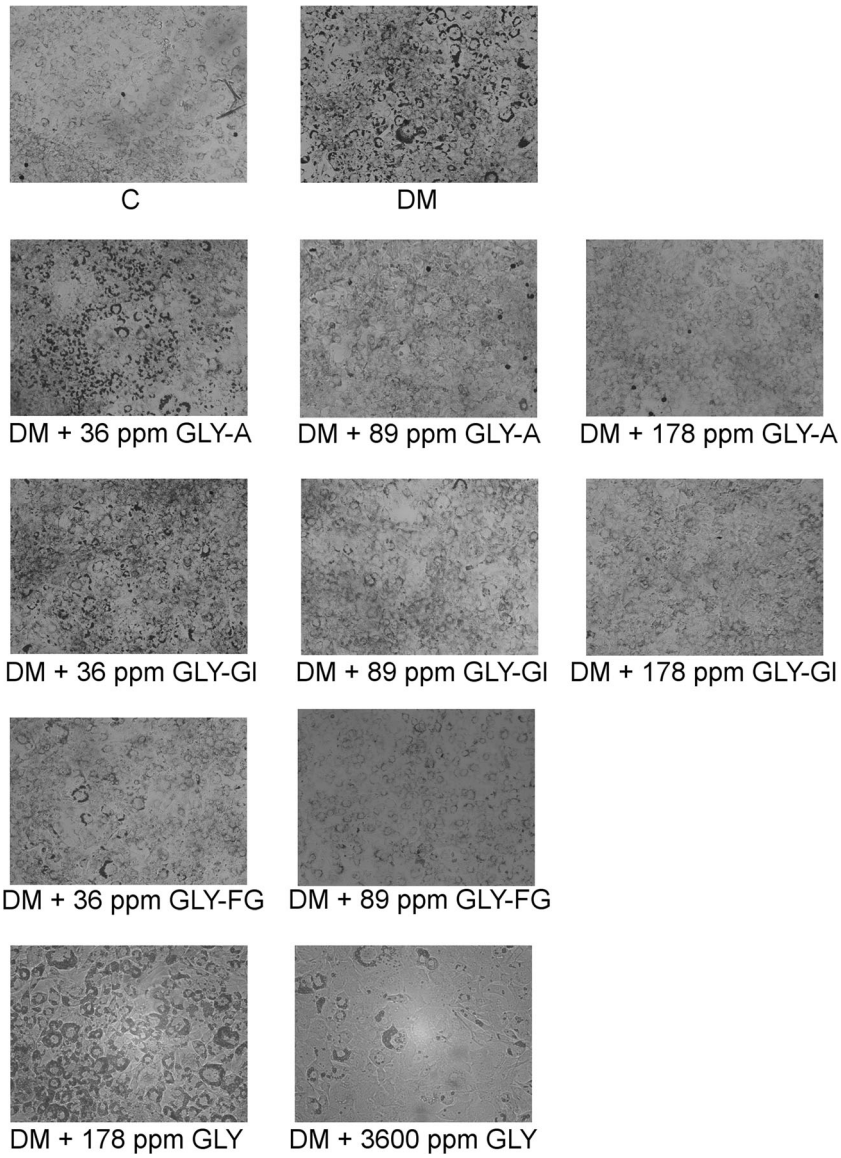

Fig. 2 Dose-dependent effect of three glyphosate-based herbicides and glyphosate on differentiation of 3T3-L1 fibroblasts to adipocytes. Two-day postconfluent 3T3-L1 fibroblasts were treated with the following: DMEM + 10 % FBS alone (C) or with the addition of the following: differentiation mixture (DM) or DM + each of the glyphosate-based herbicides (GLY-A, GLY-GL, GLY-FG) or DM + GLY, at the concentrations indicated in each case. Glyphosate-based herbicides or GLY was also added in the fresh medium supplemented with insulin that was added 3 days after induction of differentiation. Eight days after induction of differentiation, adipocytes were stained with Oil Red O. Stained triglyceride droplets in the cells were visualized and photographed as indicated in "Materials and methods" section. Results shown are from a representative experiment repeated three times with similar results

adipocytes of 3T3-L1 fibroblasts in a dose-dependent manner and all of them are more potent inhibitors than glyphosate itself.

At a concentration of glyphosate of 36 ppm, only GLY-FG was able to inhibit proliferation and differentiation of 3T3-L1 fibroblasts. A higher concentration (89 ppm) was necessary with both GLY-A and GLY-Gl. On the other hand, a concentration of glyphosate of 3600 ppm was needed to have inhibitory effects similar to those obtained with the commercial formulated herbicides. Therefore, the adjuvants present in all these formulations seem to contribute to the toxic effects in agreement with previous reports (Adam et al. 1997; Benachour et al. 2007; Mesnage et al. 2013; Piola et al. 2013).

We used acute treatments with subagriculture concentrations of glyphosate-based herbicide in our assays; lower concentrations might be required for toxic effects in chronic or repetitive exposure to the commercial herbicides as it takes place in the areas planted with glyphosate-resistant crops.

It is known that polyethoxylated adjuvants can be present in formulations of glyphosate. The toxicity of these compounds, such as POEA-15, as well as its important contribution to glyphosate-based herbicides toxic effects was previously reported (Adam et al. 1997; Mesnage et al. 2013). More recently, because of the increasing number of reports where toxic effects of these adjuvants are shown, some formulations of glyphosate have replaced polyethoxylated adjuvants. Taking this into account, we analyzed three glyphosate-based herbicides by MALDI and searched for peaks with difference in mass of 44 kDa in their spectra, which corresponds to different chain length of the polyethoxylated adjuvants. It was found that GLY-FG and GLY-Gl but not GLY-A showed

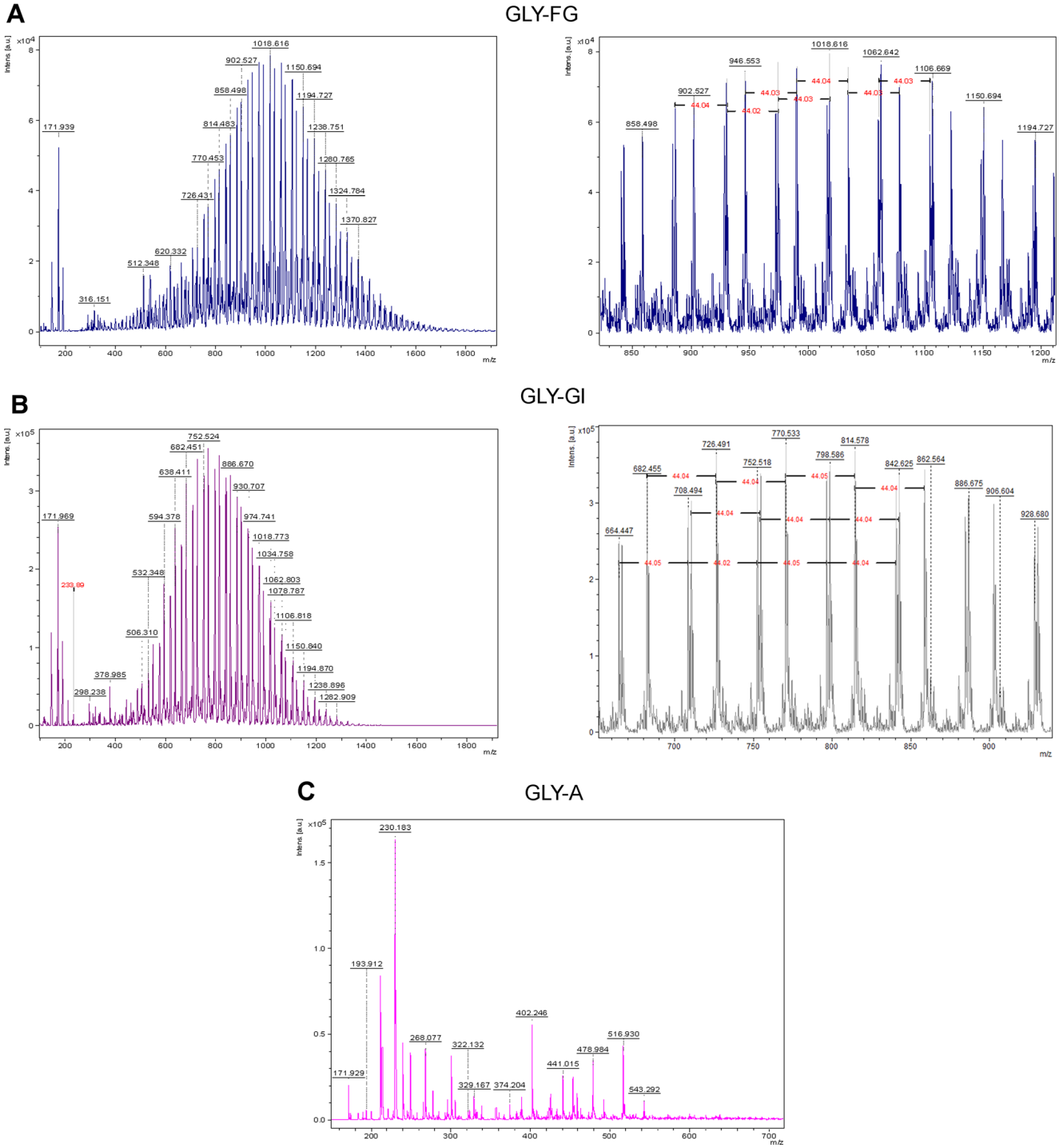

Fig. 3 MALDI analysis of the three glyphosate-based herbicides. MALDI analysis of the three glyphosate-based herbicides was performed as indicated in "Materials and methods" section. GLY-FG (**a**) and GLY-Gl (**b**) spectra, in both cases: on the *left*, complete spectrum is shown, and on the *right*, several peaks with difference in mass of 44 kDa are indicated. GLY-A (**c**) complete spectrum is shown

several peaks with this difference in mass (Fig. 3). These results suggest that GLY-A contains non-polyethoxylated adjuvants. However, it was able to inhibit proliferation and differentiation in a similar way as GLY-GL which contains polyethoxylated adjuvants according to the data obtained from MALDI analysis.

We confirmed that the inhibitory effects of commercial formulations were increased with respect to glyphosate alone when polyethoxylated adjuvants were present. In keeping with Mesnage et al. (2013), we also found polyethoxylated and non-polyethoxylated adjuvants in different commercial glyphosate-based herbicides by MALDI analysis. According to the

formulations tested in that previous report, the absence of polyethoxylated adjuvants implies a less toxic effect. In contrast, herein, we found that the replacement of polyethoxylated by non-polyethoxylated adjuvants not necessarily indicates a decrease in the toxic effect of the formulation. Thus, according to our results, non-polyethoxylated adjuvants may also contribute to the toxic effects.

These data further emphasize the importance of the evaluation of the cytotoxic effects of glyphosate formulations rather than the active ingredient, glyphosate, and it would be important to be taken into account by policy-makers for regulatory purposes.

In conclusion, glyphosate-based herbicides containing polyethoxylated and non-polyethoxylated adjuvants were more potent inhibitors than glyphosate alone of two physiological processes that take place in 3T3-L1 fibroblasts: proliferation and differentiation to adipocytes which contribute to highlight the importance of toxicological assessment of whole formulations used in agriculture practices as these are the mixtures actually spread in the environment.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Human and animal rights and informed consent This article does not contain any studies with human participants or animals performed by any of the authors.

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