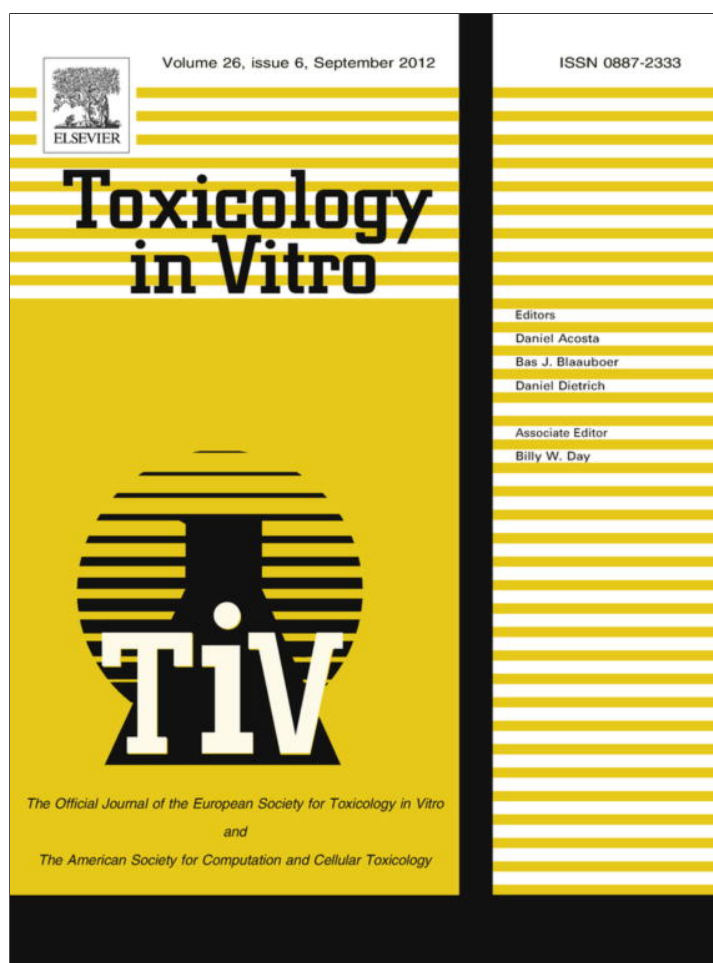




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A commercial formulation of glyphosate inhibits proliferation and differentiation to adipocytes and induces apoptosis in 3T3-L1 fibroblasts

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ABSTRACT

Glyphosate-based herbicides are extensively used for weed control all over the world. Therefore, it is important to investigate the putative toxic effects of these formulations which include not only glyphosate itself but also surfactants that may also be toxic. 3T3-L1 fibroblasts are a useful tool to study adipocyte differentiation, this cell line can be induced to differentiate by addition of a differentiation mixture containing insulin, dexamethasone and 3-isobutyl-1-methylxanthine. We used this cell line to investigate the effect of a commercial formulation of glyphosate (GF) on proliferation, survival and differentiation. It was found that treatment of exponentially growing cells with GF for 48 h inhibited proliferation in a dose-dependent manner. In addition, treatment with GF dilution 1:2000 during 24 or 48 h inhibited proliferation and increased cell death, as evaluated by trypan blue-exclusion, in a time-dependent manner. We showed that treatment of 3T3-L1 fibroblasts with GF increased caspase-3 like activity and annexin-V positive cells as evaluated by flow cytometric analysis, which are both indicative of induction of apoptosis. It was also found that after the removal of GF, remaining cells were able to restore proliferation. On the other hand, GF treatment severely inhibited the differentiation of 3T3-L1 fibroblasts to adipocytes. According to our results, a glyphosate-based herbicide inhibits proliferation and differentiation in this mammalian cell line and induces apoptosis suggesting GF-mediated cellular damage. Thus, GF is a potential risk factor for human health and the environment.

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1. Introduction

Glyphosate is the active ingredient of a broad-spectrum herbicide extensively used in agriculture and acts through inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which is essential in plants because it is required for the synthesis of aromatic aminoacids. Since this enzyme is absent in animals, this herbicide is supposed to be relatively non-toxic to them.

In the last decade, genetically modified crops seeds which have a transgene of EPSPS resistant to glyphosate have been increasingly used in agriculture (Gianessi, 2005; Dill et al., 2008). According to a recent report (Dill et al., 2008), the global areas occupied by glyphosate-resistant crops are: soybean (54.2 million ha), maize (13.2 million ha), cotton (5.1 million ha), canola (2.3 million ha), alfalfa (0.1 million ha). USA, Argentina, Brazil and Canada have the largest plantings of glyphosate-resistant crops. In Argentina, there has been an important increase of the area planted with glyphosate-resistant crops, mainly soybean. Accordingly, in a recent study, 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). The widespread use of glyphosate-based herbicides to control weeds throughout the world requires the investigation of potential toxic effects.

Interestingly, the formulation of glyphosate used as an herbicide contains not only glyphosate but also adjuvants, such as POEA (polyoxyethyleneamine), which facilitate the absorption of the herbicide and increase its effectiveness. These compounds may also be toxic and it has been proposed that the commercial formulation of glyphosate is even more toxic than glyphosate itself (Benachour et al., 2007; Richard et al., 2005).

There is conflictive information about the safety of this herbicide. Some reports have not found any significant risk to human health by the use of glyphosate (De Roos et al., 2005; Solomon et al., 2007). However it has also been shown that a commercial formulation of glyphosate, Roundup®, is genotoxic, which was determined by the Comet assay and the Micronucleus test, in erythrocytes of broad-snouted caiman (*Caiman latirostris*) after *in ovo* exposure, at early embryonic stage, to different sub-lethal concentrations (Poletta et al., 2009). Thus, this caiman has been proposed as a sentinel species to evaluate genetic damage, which has significant consequences for short- and long-term survival of

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the natural species. In addition, teratogenic effects of glyphosate-based herbicides in vertebrates (*Xenopus laevis* and chicken embryos) through alterations in retinoic acid signaling has recently been reported (Paganelli et al., 2010).

It has also been shown that formulated glyphosate inhibits cell cycle progression from analysis of the first cell division of sea urchin embryos, a recognized model for cell cycle studies (Marc et al., 2004). On the other hand, glyphosate was proposed as an endocrine disruptor and it was found that Roundup® was always more toxic than its active ingredient. (Benachour et al., 2007; Richard et al., 2005; Benachour and Seralini, 2009). It was also reported that exposure of Wistar rats during pregnancy and lactation to Roundup® induced adverse reproductive effects on male offspring rats (Dallegrave et al., 2007).

3T3-L1 fibroblasts are a useful tool in the study of adipocyte differentiation (Rubin et al., 1978). After the addition of a differentiation mixture containing insulin, dexamethasone and 3-isobutyl-1-methylxanthine (MIX), post-confluent 3T3-L1 fibroblasts re-enter the cell cycle (Student et al., 1980). 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).

In the present investigation, we evaluated the effect of a commercial formulation of glyphosate used in agriculture on proliferation, survival and differentiation of 3T3-L1 fibroblasts.

2. Materials and methods

2.1. Chemicals

DMEM, trypsin and insulin were obtained from Invitrogen (Carlsbad, CA), 3-isobutyl-1-methylxanthine (MIX), dexamethasone and *N*-Acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA) were purchased from Sigma Chemical Co. (St. Louis, MO) and FITC Annexin V apoptosis detection kit from BD Pharmingen (San Diego, CA). 3T3-L1 fibroblasts were obtained from Asociación Banco Argentino de Células (origin: ATCC). Glyphosate formulation (48% w/v, isopropylamine salt) was from Atanor, Argentina. According to the supplier, this herbicide contains surfactants and water but no specification is provided.

2.2. Cell culture and treatment of 3T3-L1 fibroblasts

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, GF was added in DMEM + 10% FBS as vehicle. Prior to addition of GF to the cell plate, the appropriate dilution was prepared and neutralized with a small amount of NaOH, when it was necessary to keep the pH of the medium, since GF is acid.

2.3. Cell counting in exponentially growing cells

3T3-L1 fibroblasts were cultured in 24-well plates until they reached 30–40% confluence. At that moment, some wells were treated for 24 or 48 h with different doses of GF, as indicated in each case and others were treated with DMEM + 10% FBS alone (control). At the end of these treatments, cells were either trypsinized, resuspended in PBS and an aliquot was counted using a Neubauer chamber or incubated for 1 h at 37 °C with filtered MTT solution (5 mg/ml), which was added to each well to reach a final concentration of 1 mg/ml MTT. Afterwards, the supernatants were carefully aspirated and 200 µl of ice-cold ethanol was added to each well to dissolve the crystal product. Absorbance was measured at 570 nm with a plate-reader.

2.4. Determination of cell viability by trypan blue exclusion

3T3-L1 fibroblasts were cultured in 24-well plates until they reached 30–40% confluence. At that moment, some of the wells were treated with 1:2000 dilution of GF for 24 or 48 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 after addition of one volume of trypan blue 0.4% w/v, cells were counted in Neubauer chamber, as indicated above.

2.5. Determination of caspase-3 activity

3T3-L1 fibroblasts were cultured in 6 cm-plates until they reached 70–80% confluence. At that moment cells were treated with GF (1:2000 dilution) for 24 h. After treatments cells were washed twice with ice-cold PBS. Then lysis buffer (50 mM Tris-HCl pH 7.4 containing 1 mM EDTA, 0.01 mM digitonin, 0.2 mM PMSF and 7.5 µg/ml pepstatin) was added. Cells were scraped off the plate, transferred to an eppendorf tube and incubated for 30 min at 37 °C. At the end of the incubation, cells were disrupted using a syringe. The lysates were then centrifuged, and the amount of protein in the supernatant was measured by the method of Bradford (1976) with bovine serum albumin as protein standard. Samples containing 200 µg of total protein were assayed with Ac-DEVD-pNA (SIGMA) as a caspase-3 specific substrate. Absorbance was measured at 405 nm in a microplate-reader.

2.6. Annexin-V and PI staining

3T3-L1 fibroblasts were cultured in 6-well plates until they reached 30–40% confluence. The cells were then treated with GF for 24 h. Flow cytometric analysis with Annexin V-FITC was done according to the manufacturer's instructions (BD Pharmingen, San Diego, CA). Briefly, cells were trypsinized, centrifuged and washed twice with PBS. Then, cells were resuspended in binding buffer (BD Biosciences) and stained with 5 µl of annexin V-FITC at room temperature for 5 min in the dark, followed by the addition of 5 µl of PI for another 10 min. AnnexinV-FITC and PI emissions were detected by a BD FACS Aria II flow cytometer. The percentages of distribution of normal cells, cells in early apoptosis, and cells in late apoptosis or necrosis were calculated using WinMDI 2.9 software.

2.7. Differentiation induction of 3T3-L1 fibroblasts and Oil-Red-O staining

To induce differentiation, two-days postconfluent 3T3-L1 fibroblasts were treated with a differentiation mixture containing 10 µg/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine (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 insulin. Then medium was changed every 2 days with DMEM + 10% FBS. When indicated, GF was added, as described in each experiment, to obtain the appropriate final concentration.

3T3-L1 adipocyte monolayers (usually on day eight) were washed three times with phosphate-buffered saline (PBS) and then fixed for 30 min with 10% formaldehyde in PBS. Oil-Red-O (0.4%) in isopropanol was diluted with two volumes of water, filtered, and added to the fixed cell monolayers for 30 min at room temperature.

2.8. Cell counting after MCE

Three days after the addition of DMEM + 10% FBS alone (C) or the differentiation mixture without (DM) or with glyphosate formulation (DM + GF), 3T3-L1 cells from 24-well plates were washed

with PBS, trypsinized, resuspended in PBS and an aliquot was counted using a Neubauer chamber.

2.9. Statistical analysis

The experiments were carried out three times unless otherwise stated. All data were expressed as mean $\pm$ S.E. Statistical analysis was performed using Student t-test and p values below 0.05 were considered significant.

3. Results

3.1. Effect of glyphosate formulation on exponentially growing 3T3-L1 fibroblasts

To evaluate the effect of a commercial formulation of glyphosate (GF) used in agriculture on the proliferation of 3T3-L1 fibroblasts, we first investigated the effect of the addition of this formulation on exponentially growing cells. We found that treatment of exponentially growing 3T3-L1 fibroblasts with the commercial herbicide for 48 h provoked a dose-dependent inhibition of cell proliferation evaluated by two different approaches: by counting cells in Neubauer chamber or by using MTT colorimetric assay. As seen in Fig. 1A and B, GF (dilutions 1:5000 or lower) inhibited the proliferation of exponentially growing cells. The number of cells in plates treated with GF (1:2000 dilution) remained similar to the number of cells found in control plates at the beginning of

the experiment (zero time control, C0). When concentration of GF was increased (1:1000 dilution) several cells were detached from the plate and floated, resulting in a decrease in the number of cells with respect to zero time control, as seen in Fig. 1A.

On the other hand, it was also found that treatment of exponentially growing 3T3-L1 fibroblasts with 1:2000 dilution of GF for 24 or 48 h blocked cell proliferation while the total number of untreated control cells continuously increased within this time (Fig. 1C). When we analyzed by trypan blue exclusion the percentage of dead cells within the total number of cells shown in Fig. 1C we found that there was only 2–3% of dead cells in control samples while this percentage increased to 17% and 35% after treatment with GF for 24 or 48 h, respectively (Fig. 1D). Total cell number (both trypan blue-positive and negative) in GF-treated-cells remained as it was at the beginning of the experiment (with no loss of cells by detachment), suggesting a complete arrest of cell division under these experimental conditions.

3.2. Effect of glyphosate formulation removal on exponentially growing 3T3-L1 fibroblasts

On the other hand, we wanted to evaluate if cells were able to recover their ability to proliferate after removing the herbicide. Thus, we treated 3T3-L1 fibroblasts with GF for 24 h, changed the medium to a medium free of GF and analyzed cell number at different time points: 0, 24 and 72 h after removal of the herbicide. As seen in Fig. 2, while the number of cells increased with time in un-

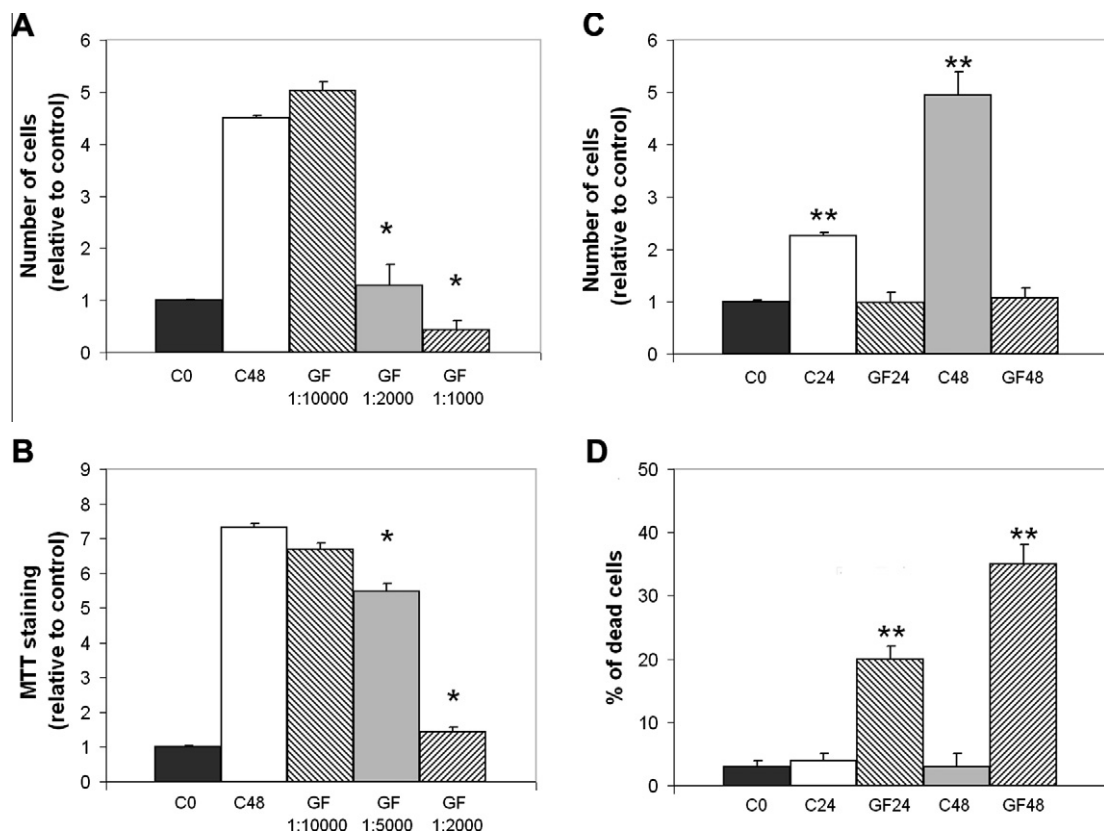


Fig. 1. Dose and time-dependent effect of glyphosate formulation on exponentially growing 3T3-L1 fibroblasts. Cells were cultured in 24-well plates until they reached 30–40% confluence. At that time, two plates were counted as zero time control (C0). Others were treated for 48 h with medium (C48) or different dilutions of glyphosate formulation (GF) as indicated in each case. At the end of these treatments, cells were either trypsinized and counted in Neubauer chamber (A) or incubated with filtered MTT solution (B) as indicated in methods. Both results are expressed relative to C0 which is set to 1. Other plates were treated for 24 or 48 h with medium (C24 and C48, respectively) or glyphosate formulation final dil 1:2000 (GF24 and GF48, respectively) as indicated in each case. At the end of these treatments, cells were trypsinized and counted after trypan blue addition in Neubauer chamber as indicated in methods. (C) Total cells relative to C0 which is set to 1 or (D) percentage of dead cells in each preparation. Results represent mean $\pm$ S.E. of three independent experiments. *Significantly different from C48, $p < 0.05$ (Student t-test). **Significantly different from C0, $p < 0.05$ (Student t-test).

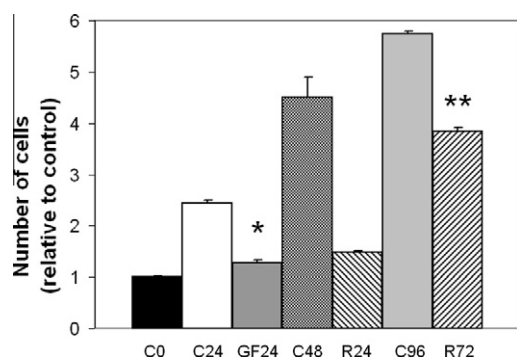


Fig. 2. Effect of removing glyphosate formulation from exponentially growing cells. Cells were cultured in 24-well plates until they reached 30–40% confluence. At that time, two plates were counted as zero time control (C0) and six plates were treated, in duplicates, for 24, 48 or 96 h with medium (C24, C48 and C96, respectively). Other six plates were treated with glyphosate formulation dil 1:2000 and after 24 h, two plates were counted (GF24) and in the others the medium was changed to a medium free of herbicide for 24 and 72 h (R24 and R72, respectively) as indicated in each case. At the end of these treatments, cells were trypsinized and counted in Neubauer chamber as indicated in methods. Results are expressed relative to control which is set to 1 and represent mean $\pm$ S.E. of three independent experiments. *Significantly different from C24, $p < 0.05$; **Significantly different from GF24, $p < 0.05$ (Student t-test).

treated cells, in cells treated with GF for 24 h, the number of cells was similar to that found in control plates at the beginning of the experiment and remained constant 24 h after removal of GF. However, 72 h after this removal, cell number increased, which indicates that proliferation was recovered after a lag period.

3.3. Glyphosate formulation-mediated induction of apoptosis in 3T3-L1 fibroblasts

To test if apoptosis was responsible for the death of 3T3-L1 fibroblasts observed after GF treatment, we investigated caspase-3-like activity in these cells using a synthetic substrate that contains the cleavage site recognized by caspase-3. A threefold increase in caspase-3-like activity was found after the treatment of cells with glyphosate-based herbicide for 24 h (Fig. 3A).

To further evaluate the induction of apoptosis by GF treatment, 3T3-L1 fibroblasts were stained with Annexin V-FITC and PI and analyzed by flow cytometry. It was found that living cells (Annexin V-FITC (–)/PI (–)) were decreased after treatment with the commercial herbicide in both doses tested, while the cells in early apoptosis (Annexin V-FITC (+)/PI (–)) and those in late apoptosis or necrosis (Annexin V-FITC (+)/PI (+)) were both increased in a dose dependent-manner (Fig. 3B). We found that treatment with GF (1:2000 dilution) increased the percentage of early apoptotic cells, from 1.6% in control cells to 11%, which is indicative of induction of apoptosis (Fig. 3C).

3.4. Effect of glyphosate formulation on differentiation of 3T3-L1 fibroblasts to adipocytes

Since 3T3-L1 fibroblasts is a useful tool to study differentiation to adipocytes, we also investigated the effect of GF on this process. To induce differentiation of 3T3-L1 fibroblasts, 2 days post confluent cells were treated with a differentiation mixture (containing insulin, dexamethasone and 3-isobutyl-1-methylxanthine) for 3 days and then this mixture was replaced with medium supplemented with insulin for the next 2 days. Finally, medium was changed every 2 days until differentiation occurred, usually on days 8–10. As it can be seen in Fig. 4, triglyceride accumulation in the cytosol can be visualized by Oil-Red-O staining in differentiated cells. On the contrary, when GF was present in the differenti-

ation mixture and also in the medium supplemented with insulin, differentiation was severely inhibited. However, if 3 days after the removal of GF, the undifferentiated-fibroblasts were treated with differentiation mixture, they were able to accumulate lipids droplets in the cytosol. Thus, differentiation was recovered after removing the herbicide.

We also investigated the effect of the addition of GF on the proliferation process that takes place in the first 2–3 days after induction of differentiation of 3T3-L1 fibroblasts to adipocytes, which is called MCE. As shown in Fig. 5, 3 days after the addition of the differentiation mixture, there was a twofold increase in the number of cells. However, this increase was inhibited by GF in a dose-dependent manner.

4. Discussion

In this paper, we analyzed the effect of a formulation of glyphosate on the proliferation and differentiation to adipocytes of 3T3-L1 fibroblasts since this mixture, which exact composition is unknown, is the commercially available herbicide used for weed control and thus, is the way in which this herbicide is spread in the ecosystem.

Herein, we found that GF was able to inhibit the re-entry in the cell cycle that takes place when differentiation was induced in 2 days post-confluent 3T3-L1 fibroblasts. This is in agreement with a previous report that showed the ability of Roundup® to block cell cycle progression by inhibiting G2/M transition in sea urchin at a concentration 1–10 mM of glyphosate (Marc et al., 2004), which is similar to the ones we tested in the experiments shown in Fig. 5, where dilutions of GF from 1:1000 (2.1 mM glyphosate) to 1:5000 (0.42 mM glyphosate) were used.

Consistently, we also found that a dilution 1:2000 of GF inhibited the proliferation of exponentially growing cells as seen in Fig. 1, and increased the number of dead cells evaluated by trypan blue exclusion (Fig. 1D).

We investigated if the increase in cell death was due to apoptosis and found that treatment with GF increased caspase-3-like activity in these cells which suggests activation of apoptosis. This result was confirmed by the increase in annexin V-FITC (+)/PI (–) cells after treatment with GF. Accordingly, the induction of apoptosis by glyphosate and Roundup® in other cell lines has recently been reported (Benachour and Seralini, 2009).

In spite of the induction of apoptosis, we found that the removal of the herbicide allowed cells to restore proliferation suggesting that some cells were not affected or successfully recovered from the damage. In keeping with this, we found (Fig. 3), that with treatment of GF (1:2000 dilution) a percentage of the cells, but not all of them, were labeled by annexin V-FITC and/or PI, while the rest (annexin V-FITC (–) and PI (–), were not undergoing apoptosis although proliferation seemed to be inhibited with this GF treatment (Fig. 1), which suggests that these cells might be under the process of cell fate decision that takes place prior to commitment to apoptosis.

On the other hand, taking into account that it has been reported that glyphosate-based herbicides disturb the differentiation pattern involved in embryogenesis (Paganelli et al., 2010), we tested the ability of GF to interfere with the adipogenic program involved in the differentiation of 3T3-L1 fibroblasts. According to our data, if GF was present during the induction of differentiation of 3T3-L1 fibroblasts, adipogenesis was inhibited. As stated above, we also found inhibition of MCE by GF treatment.

There is conflictive information about the requirement of MCE for adipocyte differentiation. Some reports suggested that MCE is a pre-requisite for adipocyte differentiation (Yeh et al., 1995; Patel and Lane, 2000). It has also been reported that blockage of adipo-

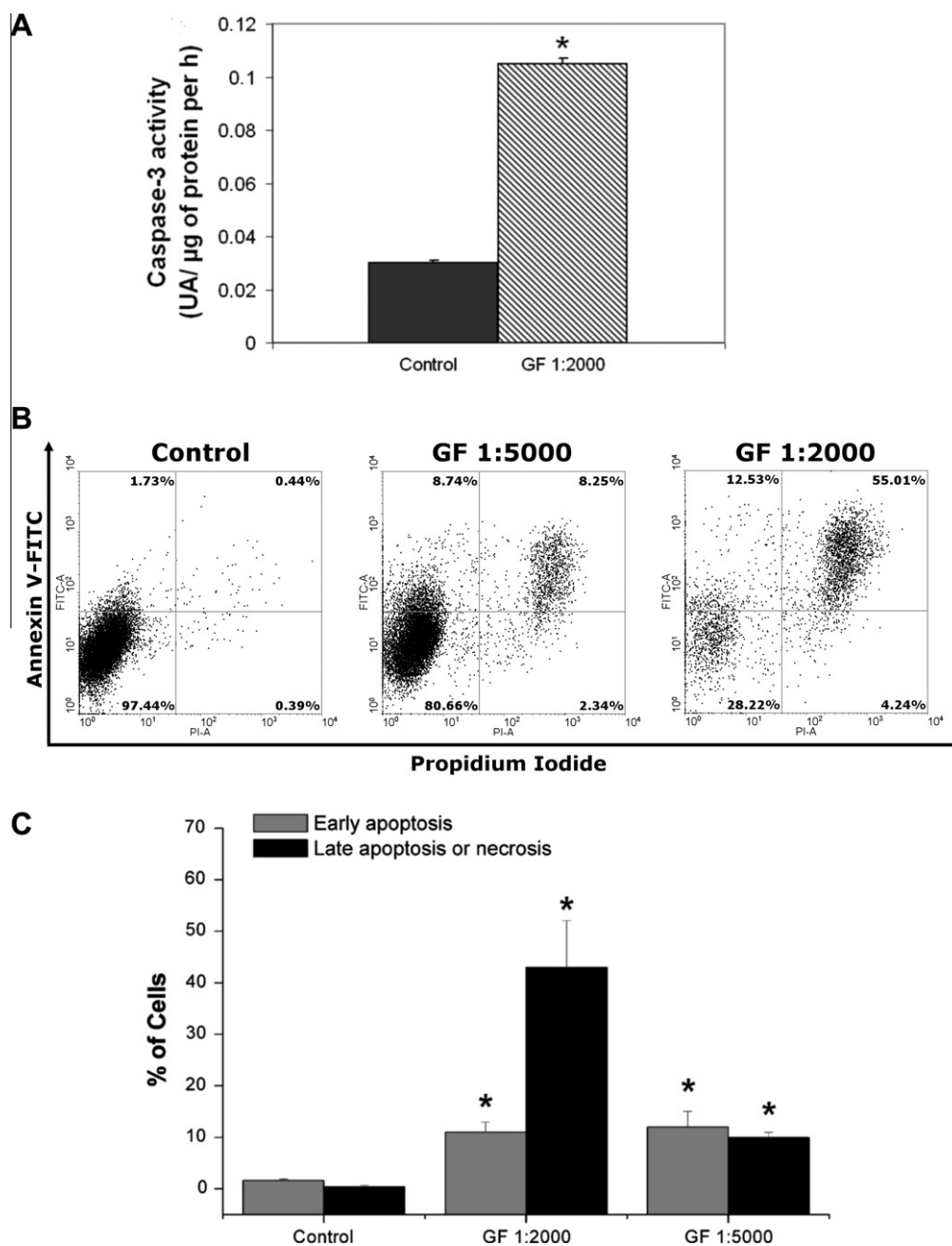


Fig. 3. Evaluation of apoptosis induction by glyphosate formulation in 3T3-L1 fibroblasts by caspase-3 activity and flow cytometry. 3T3-L1 fibroblasts were cultured in 6-cm plates until they reached 70–80% confluence. At that time, plates were treated with: DMEM + 10% FBS alone (Control), or with the addition of glyphosate formulation dil 1:2000 (GF 1:2000). After 24 h, cells were lysed and the amount of protein was measured by the method of Bradford, with bovine serum albumin as protein standard. An aliquot containing 200 μg of protein was assayed with Ac-DEVD-pNA, a caspase-3-substrate, as indicated in methods (A). Results represent mean ± S.E. of three independent experiments. *Significantly different from C, $p < 0.05$ (Student t-test). Other cells were cultured in 6-well plates until they reached 70–80% confluence. At that time, plates were treated with: DMEM + 10% FBS alone (Control), or with the addition of glyphosate formulation dil 1:5000 (GF 1:5000) or dil 1:2000 (GF 1:2000). After 24 h, cells were trypsinized, treated with Annexin V-FITC according to the manufacturer's instructions and analyzed in a BD FACS Aria II flow cytometer (B and C). Results shown in panel B are from a representative experiment repeated three times with similar results. The percentage of normal cells, cells in early apoptosis and cells in late apoptosis or necrosis, shown in the corresponding quadrant, was calculated using WinMDI 2.9 software. In the lower panel (C), the percentage of cells in early apoptosis and those in late apoptosis or necrosis in control cells and in cells treated with two dilutions of glyphosate formulation is shown. Results represent mean ± S.E. from three independent experiments. *Significantly different from Control, $p < 0.05$ (Student t-test).

cyte differentiation can occur with no alteration of MCE (Liao and Lane, 1995; Jin et al., 2000). Moreover, in agreement with Qiu et al. (2001), we have shown that differentiation of 3T3-L1 cells can occur in the absence of MCE (Martini et al., 2009). Taking this into account, in the present investigation, we proved that both MCE and differentiation are inhibited by GF treatment.

In addition, we showed that differentiation could be restored after removal of the glyphosate-based herbicide. Thus, after a lag

period, cells were able to recover, which indicates that, at least some cells, were not irreversibly inhibited by GF treatment. As far as we know, this is a new finding and may be relevant from the toxicological and environmental point of view, because it suggests that interruption of exposure to the herbicide may be sufficient to recover cellular functions.

In this paper a subagricultural concentration of GF was used corresponding to 1:2000 dilution (240 mg/l or 0.05%, v/v), while dilu-

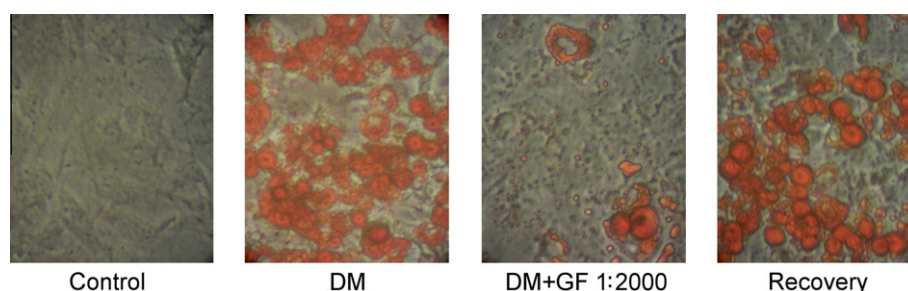


Fig. 4. Effect of glyphosate formulation on differentiation of 3T3-L1 fibroblasts to adipocytes. Two days post-confluent 3T3-L1 fibroblasts were treated with: DMEM + 10% FBS alone (C), or with the addition of: differentiation mixture (DM), or differentiation mixture + glyphosate formulation dil 1:2000 (DM + GF 1:2000). GF 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, but 3 of the dishes treated with DM + GF 1:2000, which had been without GF for the last 3 days, were induced to differentiate in the absence of GF (recovery). Stained triglyceride droplets in the cells were visualized and photographed as indicated in methods. Results shown are from a representative experiment repeated five times with similar results.

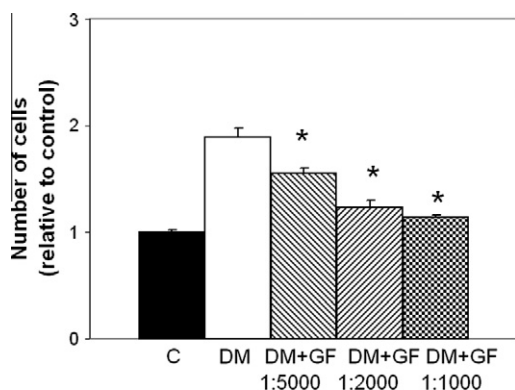


Fig. 5. Effect of the addition of different concentrations of glyphosate formulation on MCE. Two days post-confluent 3T3-L1 fibroblasts were treated with: DMEM + 10% FBS alone (C), or with the addition of: differentiation mixture (DM), or differentiation mixture + glyphosate formulation at different dilutions (DM + GF 1:1000 to 1:5000). After 3 days of induction of differentiation, cells were trypsinized and counted as indicated in methods. Results are expressed relative to control which is set to 1 and represent mean $\pm$ S.E. of three independent experiments. *Significantly different from DM, $p < 0.05$ (Student t-test).

tions from 1% to 10% (v/v) are usually suggested by suppliers. This subagriculture concentration of herbicide is high with respect to concentrations found in water or soil of areas planted with glyphosate-resistant crops (0.10–0.70 mg/l and 0.5–5 mg/kg, respectively) (Peruzzo et al., 2008). However, in our experiments, this concentration of GF was used in acute treatments. Lower doses might be required for the toxic effect in chronic or repetitive exposure to the herbicide as it takes place in the areas planted with resistant crops.

In addition, since apoptosis depends on the success or failure of the repairing process that usually takes place in vivo to counteract the insult that may trigger apoptosis and this is a probabilistic event, repetitive exposure may favor the appearance of this cytotoxic effect.

According to our results, GF 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 after addition of a differentiation mixture. Taking into account that the area planted with glyphosate-resistant crops is increasing and this requires repetitive application of GF, precaution must be taken since our results indicate that GF may provoke alterations of cellular functions which are indicative of cellular damage, and therefore, is a potential risk factor for human health and the environment.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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