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ORIGINAL ARTICLE

Moderate levels of glyphosate and its formulations vary in their cytotoxicity and genotoxicity in a whole blood model and in human cell lines with different estrogen receptor status

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Abstract

In vitro studies were conducted to determine the short-term cytotoxic and genotoxic effects of pure glyphosate and two glyphosate formulations (Roundup® and Wipeout®) at concentrations relevant to human exposure using whole blood (cytotoxicity) and various cancer cell lines (cytotoxicity and genotoxicity). Pure glyphosate (pure glyph) and Roundup® (Ro) showed similar non-monotonic toxicological profiles at low dose exposure (from 10 µg/ml), whereas Wipeout® (Wo) demonstrated a monotonic reduction in cell viability from a threshold concentration of 50 µg/ml, when tested in whole blood. We evaluated whether using various cancer cells (the estrogen-E2-responsive HEC1A, MCF7 and the estrogen-insensitive MDA-MB-231) exposed to moderate doses (75–500 µg/ml) would indicate varied toxicity and results indicated significant effects in the HEC1A cancer cells. A non-monotonic reduction in cell viability was observed in HEC1A exposed to pure glyph (75–500 µg/ml) and proliferative effects were observed after exposure to Wo (75, 125 and 250 µg/ml). Genotoxicity assessment (test concentration 500 µg/ml) demonstrated DNA damage in the HEC1A and MDA-MB-231 cells. Adjuvants and/or glyphosate impurities were potential contributing factors of toxicity based on the differential toxicities displayed by Ro and Wo in human whole blood and the HEC1A cells. This study contributes to the existing knowledge about in vitro exposure to moderate concentrations of glyphosate or glyphosate formulations at cytotoxic and genotoxic levels. In addition, a suggestion on the relevance of the estrogen receptor status of the cell lines used is provided, leading to the need to further investigate a potential endocrine disruptive role.

Keywords Glyphosate · Roundup® · Wipeout® · Cytotoxicity · Genotoxicity

Abbreviations

EPSP	5-Enolpyruvylshikimate-3-phosphate synthase
EPA	Environmental Protection Agency
ER	Estrogen receptor
E-responsive	Estrogen responsive
Pure glyph	Pure glyphosate (99.5% purity)
Ro	Roundup®
Wo	Wipeout®
DI H_2O	Deionised water
FITC	Fluorescein isothiocyanate
SEM	Standard error of the mean
TM	Olive tail moment
TL	Tail length (µm)
TDNA	Tail DNA (%)
WHO	World Health Organization

Introduction

Glyphosate, *N*-(phosphonomethyl)glycine, is a low-molecular weight, organophosphate compound and the active ingredient found in several herbicide formulations, e.g. Roundup®. The addition of adjuvants to the active compound aids the penetration and stabilization of glyphosate in plants (Anadon et al. 2009; Amoros et al. 2007; Cartigny et al. 2004; Clair et al. 2012). Glyphosate-containing herbicides are defined as broad spectrum, non-selective, post-emergence herbicides (Abdullah et al. 1995; Steinmann

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et al. 2012). The herbicidal action of glyphosate involves the competitive inhibition of the shikimate pathway (a major biosynthetic pathway in plants required for the synthesis of amino acids), specifically an enzyme of this pathway known as 5-enolpyruvylshikimate-3-phosphate synthase (EPSP) (Gehin et al. 2005; Marchiosi et al. 2009). Glyphosate is claimed to be a relatively safe herbicide because its mode of action is selective (the shikimate pathway is only found in plants and microorganisms) (Vera-Candioti et al. 2013).

The versatility glyphosate displays in controlling a large spectrum of weeds has enabled it to become the largest selling herbicide worldwide (Baylis 2000; Benamu et al. 2010; Beuret et al. 2005; Woodburn 2000). Although glyphosate and glyphosate formulations were previously deemed to be safe, their toxicity has become a major focal point of research over recent years and in 2015, The World Health Organization's (WHO) International Agency for Research on Cancer (IARC) classified glyphosate as probably carcinogenic to humans (Acuri 2017; Paul and Pandey 2017). Following the IARC report and due to health concerns surrounding glyphosate-based herbicides, several countries including Holland, Denmark, Canada and Sweden placed restrictions, bans or issued statements of intent for banning/restricting these herbicides (Acuri 2017; Wylie 2015). The contentious nature of glyphosate is further highlighted in the landmark lawsuit, which resulted in Monsanto being held liable for Dewayne Johnson's (a former groundskeeper) terminal cancer, due to exposure to Monsanto's glyphosate formulation, Roundup (Associated Press 2018). There is an ever increasing need to understand the effects of glyphosate and glyphosate-based herbicides at low/moderate doses, as these levels are often within the range likely to be encountered by humans and animals in most circumstances (Vom Saal and Welshon 2006). The concentrations selected for this study included (at, below or within the range) the regulatory standards in the United States of America (EPA), Brazil (Agência Nacional de Vigilácia Sanitária) and health-based values set by the WHO for glyphosate in drinking water (0.7, 0.5 and 0.9 µg/ml, respectively) (Coutinho et al. 2008; WHO 2004). For moderate-exposure studies, concentrations selected were below and/or within the range of occupational and residential exposure reported and tested by Mladinic et al. (2009), and the maximum concentrations assessed were 92.8 and 580 µg/ml. This study concluded that concentrations relevant to human exposure did not pose a risk to human health. More recently, however, studies conducted by Luo et al. (2017) demonstrated the cytotoxic effects of Roundup in L-02 hepatocytes, at concentrations relevant to occupational exposure.

Studies have indicated the toxic potential of low doses of glyphosate in its pure form and in formulation in animal and human models. Literature has reported varying effects caused by short-term or long-term low-dose exposure to this herbicide. Intracellular biochemical alterations due to glyphosate (Larsen et al. 2012) and glyphosate formulations (Benedetti et al. 2004) have been documented in rat models. Alterations in immune function have been observed in fish (Kreutz et al. 2011). Similar results were observed in mussels exposed to technical-grade glyphosate (Lummato et al. 2013). The cytotoxicity, endocrine disruptive and genotoxic properties of glyphosate and glyphosate-based herbicides were confirmed in the HepG2 human liver cell line (Gasnier et al. 2009; Manas et al. 2009; Mesange et al. 2012), and HEK293 and JEG-3 human placental cell lines (Mesange et al. 2012). Reproductive alterations were also observed in studies conducted in rat testis, sertoli cells (De Liz Cavalli et al. 2013) and in Siluridae fish (Soso et al. 2007) when exposed to low levels of glyphosate/glyphosate-based herbicides.

Previous studies using human whole blood as a model showed greater sensitivity to the effects of pure glyphosate and its formulations and, therefore, it was chosen as one of the models for this study, which could be directly compared to previous findings. Duramad et al. (2006) described the advantages of using a whole blood system as opposed to isolated lymphocytes and advantages included the fact that whole blood more closely represents an in vivo system, which is characterized by a larger variety of immune cells which are able to contribute to a response. Co-stimulation and "cross-talk" between various immune cells and other cell types present in whole blood may lead to an amplified response to a toxin (Klinger and Jelkmann 2002; Krakauer 2002).

The cancer cell lines examined in this study were the MDA-MB-231 estrogen receptor (ER)-negative breast cancer cell line, the MCF7 (ER positive) breast cancer cell line and the HEC1A (ER positive) endometrial cancer cell line.

For genotoxicity studies, the comet assay (single-cell gel electrophoresis) was employed as an initial screening tool for DNA damage. This method is advantageous because it provides a simple, rapid and sensitive assessment of DNA strand breaks at a single-cell level (Fairbairn et al. 1995). The aim of this study was to assess the cytotoxic and genotoxic potential of pure glyphosate and two glyphosate-based herbicides, Roundup® and Wipeout®, at concentrations relevant to human exposure over short-term periods. Overall, this study contributes to the existing reports of glyphosate toxicity in humans as knowledge in this area still remains relatively limited.

Materials and methods

Chemicals

OxiSelect™ Comet Assay Kit (STA-351) and camptothecin were purchased from Cell biolabs Inc., dimethyl

sulphoxide (DMSO) was purchased from Merck, Dulbeco's Modified Eagle´s medium (DMEM) (+4.5 g/L glucose, + L-glutamine, containing phenol red) was supplied by Sigma, pure glyphosate (99.5% purity) was purchased from Supelco Analytical (USA), [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) was purchased from Duchefa Biochemie, Roundup® (360 g/L glyphosate potassium salt) and Wipeout® (500 g/L glyphosate potassium salt) were commercially available in local stores, and penicillin–streptomycin was supplied by Gibco, Invitrogen Corporation. Fetal calf serum (ThermoScientific), sodium chloride (NaCl), potassium chloride (KCl), disodium hydrogen phosphate ($Na_2HPO_4{\cdot}12H_2O$), potassium hydrogen phosphate (KH_2PO_4), Tris(hydroxymethyl) aminomethane and ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA) were all purchased from Merck, and 10% Trypsin in HBSS and Roswell Park Memorial Institute Medium (RPMI) (+25 mM HEPES, + L-glutamine) were purchased from Thermoscientific. Lipopolysaccharide (LPS) (from *Escherichia coli* 0111) was purchased from Sigma-Aldrich.

Whole blood cell culture and exposure

The effect of varying concentrations of pure glyphosate (pure glyph) and its formulations Roundup® (Ro) and Wipeout® (Wo) (0–500 µg/ml) on whole blood was assessed. Blood was collected by venipuncture (ethical clearance was obtained for the use of human blood samples) from healthy volunteers ($n=5$) in heparin-containing tubes (vacutainers). Blood samples were then diluted 1:10 in RPMI 1640 media (within 5 h of collection) supplemented with 50 µg/ml streptomycin and 50 U/ml penicillin. The whole blood (1 ml) samples were exposed to pure glyph, Ro and Wo (0–500 µg/ml) for 18 h at 37 °C. This study included the endotoxin, lipopolysaccharide (LPS, 5 µg/ml), found in Gram-negative bacteria (Ertel et al. 1995; Stoddard et al. 2010) as a positive control and pyrogen-free water was utilized for all the untreated controls in this study.

Breast cancer (MCF7 and MDA-MB-231) and endometrial cancer cell line culture (HEC1A) and exposure

The MDA-MB-231 and MCF7 breast cancer cell lines were used in this study, as these cell lines have proven to be suitable models for assessing the potential endocrine disruptive activity of xenobiotics in previous studies (Buteau-Lozano et al. 2008; Markaverich et al. 2002). The HEC1A cells are linked to fertility and, therefore, may in future provide additional insight into possible deleterious effects to human reproduction. Deleterious effects of glyphosate and Roundup to human reproduction have been demonstrated by Benachour et al. (2007) in human embryonic 293 and placental-derived JEG3 cells.

The two breast cancer cell lines, MCF7 (hormone responsive), MDA-MB-231 (hormone independent), were obtained from the American Type Culture Collection (ATCC), USA. HEC1A cells, an endometrial carcinoma cell line, were kindly donated by Dr. H. Davis (Nelson Mandela Metropolitan University, Eastern Cape, South Africa). All cell lines were grown in Dulbeco's Modified Eagle´s medium (DMEM) (+4.5 g/L glucose, + L-glutamine, phenol red) supplemented with 5% heat inactivated fetal calf serum, 50 U/ml penicillin and 50 µg/ml streptomycin. Cell cultures had been previously cultured in DMEM media containing phenol red, therefore, to maintain a stable estrogen-sensitive phenotype, as they may still present steroid memory effects (Glover et al. 1988) for this study cell lines were cultured in phenol-free medium after the removal of phenol red. All data obtained were, however, normalized against untreated controls (pyrogen-free water) to ensure that the effects observed were due to the different treatments in the cancer cell lines. Cell lines were routinely maintained under standard cell culture conditions, at 37 °C, 5% CO_2 and 90% humidity. Once cell cultures reached confluency (70–80%), cells were split and seeded into 24-well plates (100,000 cells/well) and allowed to attach overnight at 37 °C for use in subsequent experiments. The different cell lines were exposed to pure glyph and two glyphosate formulations Ro and Wo at varying concentrations (0–500 µg/ml) for a 24-h period (standardized lab protocol) at 37 °C. Camptothecin (100 µM), a DNA topoisomerase inhibitor (Hsiang et al. 1985), was used as a positive control in this study.

Cytotoxicity assay (MTT assay)

The MTT assay is a well-known method for the determination of cell viability. It is based on the cleavage of MTT (a tetrazolium salt) by metabolically active cells (via the enzyme, succinate dehydrogenase) to a purple formazan derivative (Denizot and Lang 1986). The MTT assay was conducted according to standardized lab protocols. Following herbicide exposure, samples were incubated in 0.5 mg/ml MTT reagent (in media) for 3 h (cancer cell lines) or 30 min (whole blood) at 37 °C based on the different levels of saturation of the dye in the different cell types. After incubation, the MTT reagent was aspirated and 1 ml of DMSO was added to solubilize the formazan product. Purple color formation was determined spectrophotometrically at 560 nm using a Biotek Powerwave XS microplate reader.

Single-cell gel electrophoresis (comet assay)

The comet assay (single-cell electrophoresis assay) is a very sensitive method used to determine DNA damage

(single-stranded DNA breaks) at a cellular level. This method is an electrophoretic and fluorescence microscopy assay based on the principle that fragmented DNA strands (due to strand breaks) migrate from cells that have been lysed to form a migration pattern that assumes the shape of a comet. The amount of DNA in the tail in comparison with the head and the length of the tail are related to the amount of DNA strand breaks (Anderson et al. 1998; Hovhannisyan 2010; Moller et al. 2000; Tice et al. 2000; Yared et al. 2002). The comet assay has been commonly used in environmental biomonitoring and toxicological studies in various organisms (Blasiak and Trzeciak 1998; Chuang and Hu 2004; Clements et al. 1997; Enciso et al. 2009; Jagetia and Rao 2011; Kumaravel and Jha 2006).

The exposure concentrations used for this study were chosen based on results obtained in the cell viability assay and a positive control reference concentration was chosen based on cytotoxicity results reported by Gasnier et al. (2009) in the HepG2 human liver cell line. The MCF7, MDA-MB-231 and HEC1A cancer cell lines (100,000 cells/well) were incubated in 24-well plates for 4 h at 37 °C, in the presence of pure glyph (500 and 1000 µg/ml), Wo (500 and 800 µg/ml) and Ro (500 and 800 µg/ml). Camptothecin was used as a positive control in this study (100 µM). After exposure, media was aspirated and the cells were trypsinized (2.5% trypsin) for 5 min at 37 °C in preparation for the comet assay.

The preparation of samples and the method used for the comet assay were conducted according to the instructions described in the OxiSelect™ Comet Assay Kit (Cell Biolabs, Inc).

Preparation of cell samples for the comet assay

Trypsinized cells were pooled (three sample wells) and centrifuged at 700×*g* for 2 min in a Merck Minispin Eppendorf AG centrifuge. The supernatant was discarded and the pelleted cells were washed with ice cold PBS (phosphate buffered saline, without Mg^{2+} and Ca^{2+}) and centrifuged at 700×*g* (Merck Minispin Eppendorf AG centrifuge). The cells (1×10^5 cells/ml) were then resuspended in ice cold PBS before the assay was conducted.

Comet assay sample slide preparation and cell lysis

Resuspended cells (10 µl) were combined with 100 µl molten comet agarose (comet agarose was heated to 95 °C for 20 min and maintained at 37 °C until use). The mixture (75 µl/well) was immediately placed onto an OxiSelect™ comet slide. Slides containing the cell agarose mixture were incubated at 4 °C (in the dark) for 15 min to allow the agarose to set. Slides were treated in pre-chilled lysis buffer (pH 10) for 60 min at 4 °C (in the dark) followed by treatment in alkaline solution (pH 13) for 30 min at 4 °C (in the dark).

Alkaline electrophoresis

Slides were subjected to alkaline electrophoresis for 18 min at 300 mA, neutralized in pre-chilled deionised water (DI H_2O) (twice for 2 min intervals) and washed in 70% ethanol (cold) for 5 min. The slides were then air dried. Once dried, the slides were incubated with 100 µl of 1 × Vista Green dye prepared in TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA) for 15 min at room temperature (in the dark).

Comet assay sample visualization and data analysis

Slides were visualized with the Zeiss Axio Vert. A1 fluorescence microscope (5×) using a FITC filter (7%). Images were obtained using an AxioCam MR3 Camera and stored in the Axio vision Rel.4.8 program. Image J Macro was used to calculate the tail length, % tail moment and % tail DNA. Data analysis was conducted according to the Comet assay guidelines outlined by Tice et al. (2000) for in vitro genotoxicity testing. Briefly, 50 cells were analyzed per slide (replicates of 3) from pooled cell cultures, per experimental treatment ($n = 3$).

Statistical analysis

All data were presented as mean ± standard error of the mean (SEM). ANOVA single-factor analysis (Microsoft Excel) was used to determine significant differences ($P \leq 0.05$, $P \leq 0.01$).

Non-linear least square regression models were applied to cytotoxicity data in human whole blood exposed to varying concentrations of pure glyph, Ro and Wo, using the Graphpad Prism 6 software package (San Diego, CA). Goodness of fit was assessed using R^2, R^2_{adjusted} values and by the assessment of upper and lower 95% confidence limits associated with the model fit. The Wald–Wolfowitz (runs) test was used to determine the deviation of the non-linear regression model from the experimental data. The models were used to estimate the concentration of glyphosate (in its pure form or within formulation) required to illicit a half maximal response (half maximal effective concentration, EC50).

Based on the observed biphasic nature of the curves obtained for pure glyph and Ro, a model describing a seven-parameter bell-shaped dose (combines two sigmoidal responses)–response curve was selected. This model was previously described by Antachopoulos et al. (2007) and is based on the following equation:

$$Y\min + (Y\max - Y\min)/1 + 10^{(\log EC50_1 - \log\ [x])*n_{H^1}} + Y\max_2 - Y\min/1 + 10^{(\log [x] - \log EC50_2)*n_H{}^2}, \quad (1)$$

where Y corresponds to the percentage cell viability. The logEC50 values (1 and 2) describe the half maximal responses from the two different phases of the regression curve. $n_H{}^1$ and $n_H{}^2$ are the slope parameters for the different phases of the curve and log [x] corresponds to the glyphosate concentrations.

Due to the complexity of the bell-shaped dose–response model (seven parameters) and the small number of data points ($n=6$), the choice of initial parameter estimates was crucial (Motulsky and Christopoulos 2003) and constraints were placed on the following parameters: Ymin, Ymax, $Y\max_2$, $n_H{}^1$, $n_H{}^2$ and x to allow the model to successfully converge.

The effects of Wo on whole blood showed a dose-dependent relationship; therefore, non-linear least square regression was used to model linear data. Fitting linear data with non-linear regression utilize a standard linear equation (refer to Eq. 2), however, fitting linear data with non-linear regression in Graphpad Prism 6 is advantageous as it allows greater options with respect to data analysis, e.g. the ability to apply weighting to the data and the ability automatically excludes outliers (Motulsky and Christopoulos 2003).

$$Y = m \times \log(x) + c, \quad (2)$$

where Y corresponds to the percentage cell viability, m is the slope of the curve, c is the intercept and log(x) corresponds to the glyphosate concentrations.

Results

Cell viability in human whole blood

The induction of cell toxicity by pure glyph, Ro and Wo in human whole blood exposure at low to moderate dose exposure (0.1–500 µg/ml) was determined using the MTT reduction assay (Fig. 1a1–c1). Non-linear regression models (Fig. 1a2, c2) were applied to the data to determine half maximal responses (EC50) (Table 1).

Pure glyph and the formulation Ro displayed non-monotonic effects on the cell viability in whole blood (Fig. 1a1 and b1). A minimal effect (when compared to the untreated control) on cell viability was observed at the lowest concentrations tested (0.1 and 0.7 µg/ml) in both treatments followed by a significant reduction in cell viability ($P \leq 0.01$) at pure glyph and Ro concentrations of 10 and 50 µg/ml compared to the untreated control. At a glyphosate concentration of 250 µg/ml, pure glyph showed a significant decrease ($P \leq 0.01$) in cell viability (51% ± 7.2), however, a non-significant increase ($P > 0.05$) was observed for the Ro treatment. The final phase of the both curves was characterized by a non-significant increase (levels near 100%) in cell viability at 500 µg/ml, for pure glyph (82% ± 18.9) and Ro (91% ± 16.4).

Pure glyph and Ro showed similar dose–response profiles characterized by an inverted bell-shaped curve. The bell-shaped curve model equation described the data accurately with R^2 and $R^2_{adjusted}$ values of 0.83, 0.79 for pure glyph and 0.98 for Ro (Fig. 1a2 and b2). The goodness of fit was further validated by relatively narrow 95% confidence intervals (upper and lower) around the fit. The Wald–Wolfowitz test indicated no significant deviation of the model for pure glyph ($P=0.9$) and Ro ($P=1.0$).

The effect of Wo on cell toxicity in whole blood showed a significant ($P \leq 0.01$) dose-dependent (monotonic) relationship (Fig. 1c1) from a threshold concentration of 50 µg/ml. The output of the linear regression model (Fig. 1c2) indicated a very good fit, with narrow upper and lower 95% confidence intervals being observed around the fit and R^2 and $R^2_{adjusted}$ values of 0.95 and 0.94, respectively. The results of the Wald–Wolfowitz test showed no significant deviation ($P=0.1$) of the model from the experimental data tested in this study.

Based on the half maximal inhibition (reduction) response values ($EC50_1$) shown in Table 1, Ro incurred a similar inhibitory effect (1.1 µg/ml) compared to pure glyphosate (1.3 µg/ml), with only a slight difference (15%) in EC50 values being observed. Wo (7.1 µg/ml) demonstrated the lowest inhibition effect in human whole blood.

Similar results were obtained for half maximal stimulation (induction) response values ($EC50_2$) in Ro (269.8 µg/ml) and Pure glyph (317.7 µg/ml). Ro produced a slightly greater (15%) half maximal stimulation than pure glyph.

LPS was a suitable positive control for this study, with significant reductions ($P \leq 0.01$) in human whole bloods cells being observed for pure glyph (63.28% ± 6.8), Ro (63.29% ± 3.4) and Wo (62.33% ± 0.1) at the concentration (5 µg/ml) tested.

Cell viability (MTT assay) and genotoxicity assessment (comet assay) in human cancer cell lines

The main focus of this study was to determine the toxic (cytotoxic and genotoxic) effects of moderate dose, short-term exposure of pure glyph and two glyphosate formulations, Ro and Wo on three cancer cell lines, MCF7, HEC1A and MDA-MB-231. In the cell viability study, the three cell

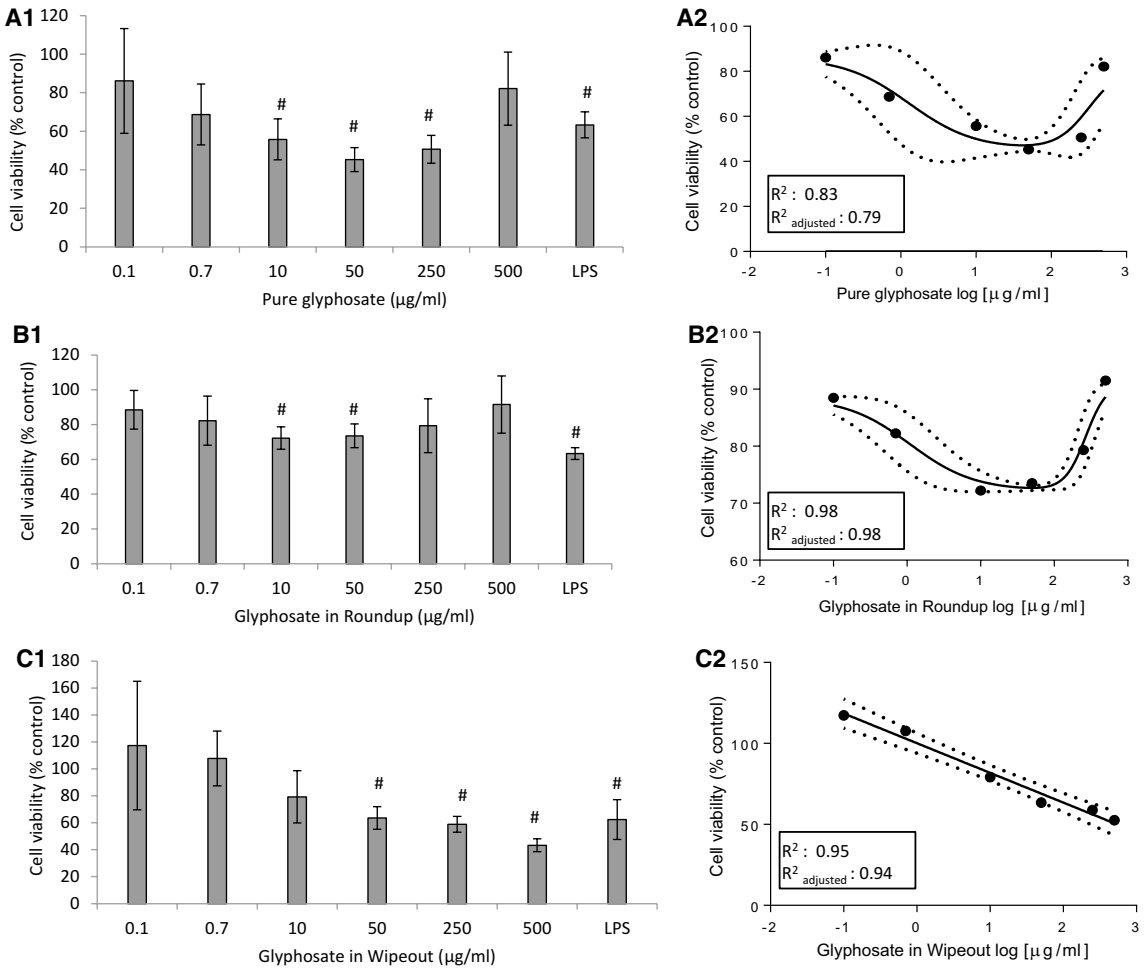

Fig. 1 The effect of **a1** pure glyph (99.5%) and its formulations, Ro (**b1**) and Wo (**c1**) on the cell viability in human whole blood. The cells were exposed over an 18-h period at varying herbicide concentrations (0–500 µg/ml). Lipolysaccharide (LPS, 5 µg/ml) was used as a positive control in this study. Data points represent the means of five replicates (±SEM). ANOVA single-factor analysis was used to determine significant differences from the untreated control (not shown) $^{*}P \leq 0.05$, $^{\#}P \leq 0.01$. Non-linear regression analysis using Graphpad Prism 6 software was used to describe the effect of pure glyph (**a2**), Ro (**b2**) and Wo (**c2**) in human whole blood. **a2** Bell-shaped curve (biphasic curve): $Y\text{min} + (Y\text{max} - Y\text{min})/1 + 10^{(\log EC50_1 - \log[x]) * nH1} + Y\text{max}_2 - Y\text{min}/1 + 10^{(\log[x] - \log EC50_2) * nH2}$, **b2** bell-shaped curve (biphasic curve): $Y\text{min} + (Y\text{max} - Y\text{min})/1 + 10^{(\log EC50_1 - \log[x] * nH1} + Y\text{max}_2 - Y\text{min}/1 + 10^{(\log[x] - \log EC50_2) * nH2}$, **c2** linear curve: $Y = m * \log(x) + c$. Goodness of fit was assessed by R^2, R^2_{adjusted} values and upper and lower 95% confidence intervals around the fit (dash lines)

Table 1 EC50 values of pure glyph and two formulations (Ro and Wo) in human whole blood and the 95% confidence intervals of the modeled parameters

Test substance	Model	$EC50_1$ (µg/ml)	$EC50_2$ (µg/ml)	Slope	Intercept
Pure glyph	BSC	1.3 (0.2–10.1)[a]	317.7 (124.2–814.7)	−1.0, −2.0[b]	N/A
Ro	BSC	1.1 (0.3–3.9)	269.8 (190.5–382.8)	−1.0, −3.0[b]	N/A
Wo	LC	7.1[c]	N/A	−18.34 (−22–(−) 14.7)	100.1 (94.0–106.4)

BSC bell-shaped curve, *LC* linear curve, *N/A* not applicable

[a]Values in parenthesis indicate upper and lower 95% confidence limits for the corresponding parameter

[b]Constrained values chosen to represent the slope factors $n_H{}^1$ and $n_H{}^2$, respectively

[c]The EC50 value was derived by the following equation: $EC50 = 10^{(\log[x]_{max} + \log[x]_{min})/2}$

lines were exposed to varying concentrations of glyphosate (75–500 µg/ml) and equivalent glyphosate concentrations in Ro and Wo over a 24-h period. The cytotoxic effects of pure glyphosate and its formulations (Ro and Wo), on the MCF-7, MDA-MB-231 and the HEC1A cell lines, were determined using the MTT assay (Fig. 2). Results obtained were expressed relative to untreated controls.

Increasing concentrations of pure glyph (75–500 µg/ml) indicated no significant effect on the MCF-7 and MDA-MB-231 cell lines when compared to the untreated control (Fig. 2a). A significant ($P \leq 0.05$, $P \leq 0.01$) dose-independent decrease in cell viability was observed for the HEC1A cell line when exposed to varying concentrations of pure glyphosate with an overall average inhibition of 26.5% ± 1.8. Glyphosate in the Ro formulation did not induce significant cytotoxic effects on the three cancer cell lines at the concentrations tested (Fig. 2b). The Wo formulation showed no significant effects on the cell viability of the MCF-7 and MDA-MB-231 cell lines (Fig. 2c); however, a significant ($P \leq 0.05$, $P \leq 0.01$) dose-independent increase in cell viability was observed in the HEC1A cell line at 75 (59% increase in cell viability), 125 (54% increase in cell viability) and 250 µg/ml (54% increase in cell viability) (Fig. 2c). A non-significant decrease in cell viability (8% decrease in cell viability) occurred in the HEC1A cell line exposed to a Wo concentration of 500 µg/ml (Fig. 2c). Camptothecin (100 µM) was a suitable positive control in this study and induced a significant ($P \leq 0.05$, $P \leq 0.01$) decrease in cell viability (below 80%) in all three cell lines when compared to the untreated control.

The overall findings of the cell viability study suggest that glyphosate and the two formulations do not display

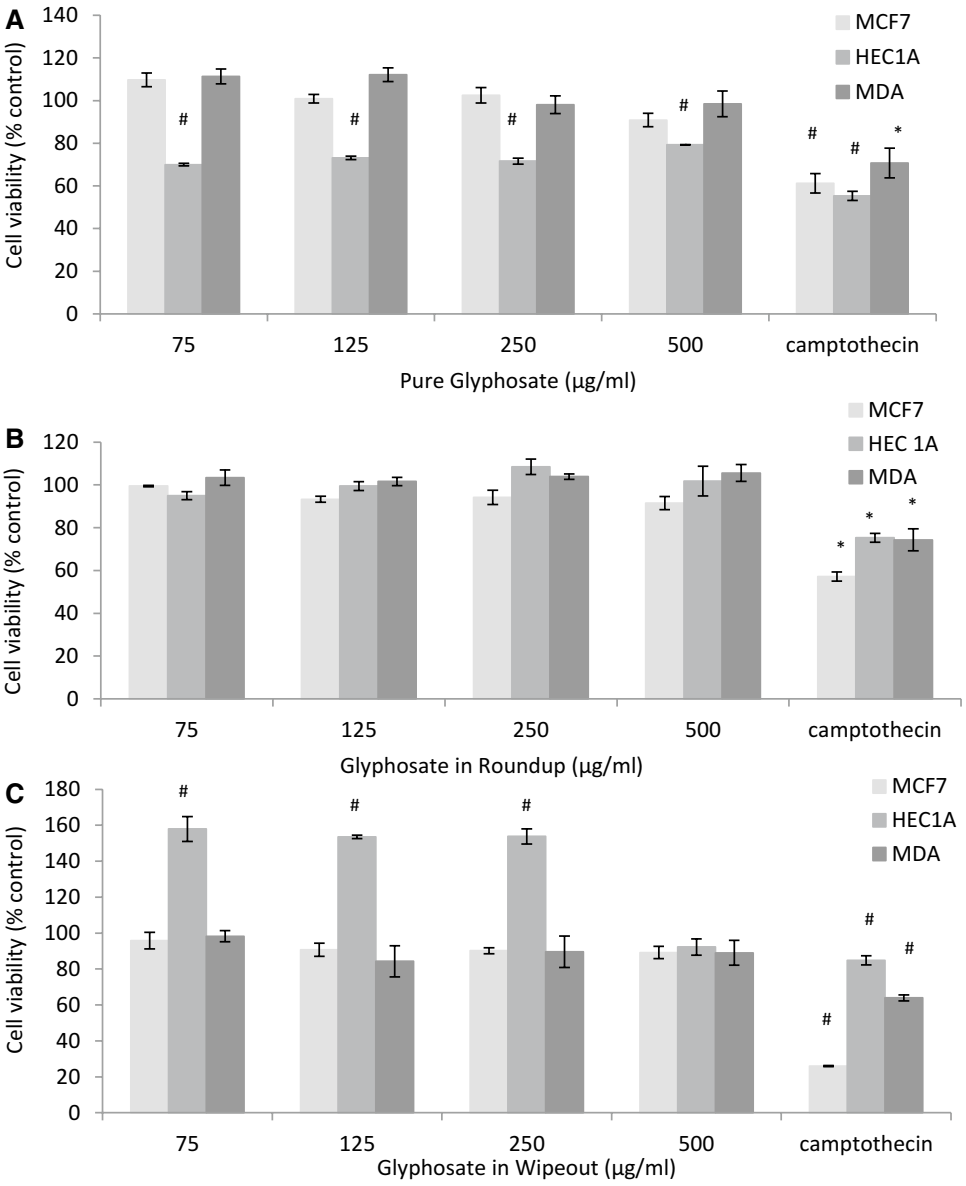

Fig. 2 The effect of **a** pure glyph (99.5%) and its formulations, Ro (**b**) Wo (**c**) on the cell viability of the cancer cell lines (MCF-7, HEC1A and MDA-MB-231). The cancer cell lines were exposed over a 24-h period at varying herbicide concentrations (0–500 µg/ml). Camptothecin (100 µM) was used as a positive control in this study. Data points represent the means of three replicates (± SEM). ANOVA single-factor analysis was used to determine significant differences from the untreated control (not shown) $^{*}P \leq 0.05$, $^{\#}P \leq 0.01$

a cytotoxic effect in the MCF-7 (ER positive) and MDA-MB-231 (ER negative) cell lines under the experimental conditions tested. Pure glyph and Wo induced inhibitory and proliferative effects, respectively, in the HEC1A cell line (ER positive), although the toxic effects observed were not dose dependent. Ro was found to be less cytotoxic (not significant) in the HEC1A cell line compared to the Wo formulation and pure glyphosate.

Test concentrations selected for genotoxicity studies were based on the results of the cell viability study and reference concentrations were chosen based on glyphosate concentrations reported in literature to incur genotoxic damage in human cell lines. In the cell viability, study concentrations of glyphosate and its formulations as high as 500 µg/ml showed no significant responses in the MCF-7 (glyph: 90%, Ro: 91% and Wo: 89% cell viability) and MDA-MB-231 (glyph: 98%, Ro: 105% and Wo: 89% cell viability) breast cancer cell lines. A Wo concentration of 500 µg/ml indicated non-significant alteration in the cellular growth of the HEC1A (92% cell viability) endometrial cancer cell line; therefore, this concentration was selected for genotoxicity analysis to determine whether any damage was present at a genomic level. Gasnier et al. (2009) reported initial toxicity of pure glyphosate at 1000 µg/ml and the highest reported concentration for initial toxicity for the Roundup® (Roundup 7,2®) formulation was 800 µg/ml in cell viability tests (MTT assay) conducted on the HepG2 liver cell line. These concentrations were, therefore, chosen as reference concentrations.

Potential DNA damage due to glyphosate and glyphosate formulation exposure in the HEC1A, MCF-7 and MDA-MB-231 cell lines was determined using the comet assay. After exposure to different concentrations of pure glyph (500 and 1000 µg/ml), Ro (500 and 800 µg/ml) and Wo (500 and 800 µg/ml) over a 4-h period, images developed in the comet assay (Fig. 3) from each experimental treatment and were analyzed using Image J, recorded and presented as TL (µm) and TM (arbitrary units). Discrepancies were observed in the calculated TDNA (%) (data not shown) when compared to TL and TM; this has been documented in previous literature (Ündeğer and Başaran 2005). A correlation study between TL and TM (data not shown) indicated a positive correlation (R^2 values close to 1) and, therefore, these two parameters were better suited to describe DNA damage observed in the cancer cell lines.

Results of DNA damage induced by exposure to pure glyphosate in the three cancer cell lines are shown in Fig. 4.

Pure glyphosate induced significant DNA damage ($P \leq 0.01$) in the HEC1A cell line at 500 µg/ml (TL: 0.36, TM: 26,282) and at the reference concentration of 1000 µg/ml (TL: 0.24, TM: 14,519) indicated by a significant increase ($P \leq 0.01$) in the TL and TM when compared to the untreated control (TL: 0.13, TM: 4720). The results of the comet assay for the MCF-7 cell line after pure glyph treatment showed extremely low levels of DNA damage at both 500 µg/ml (TL: 0.13, TM: 7324) and 1000 µg/ml (TL: 0.141, TM: 3063) as no significant increases in comet formation were observed. A significant level ($P \leq 0.01$) of DNA

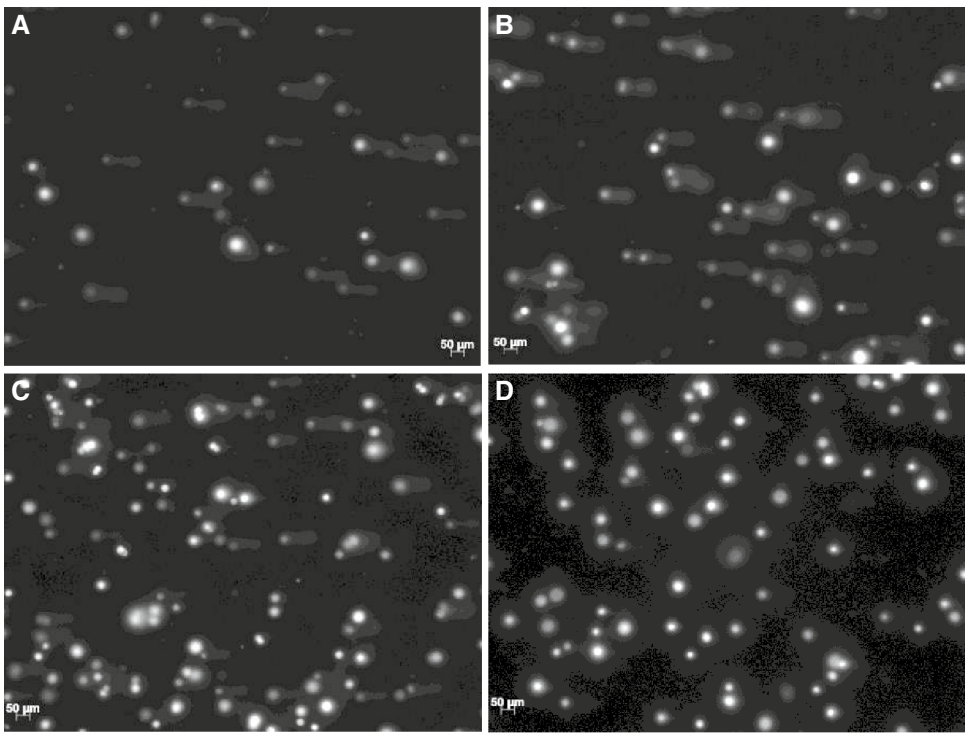

Fig. 3 A representation of (HEC1A endometrial carcinoma cancer cell line) images developed in the comet assay (fluorescence microscope), **a** exposure to 500 µg/ml pure glyph **b** exposure to 500 µg/ml Ro **c** exposure to 500 µg/ml Wo **d** untreated control. Cells were exposed for a period of 4 h at 37 °C. FITC filter, magnification bar = 50 µm

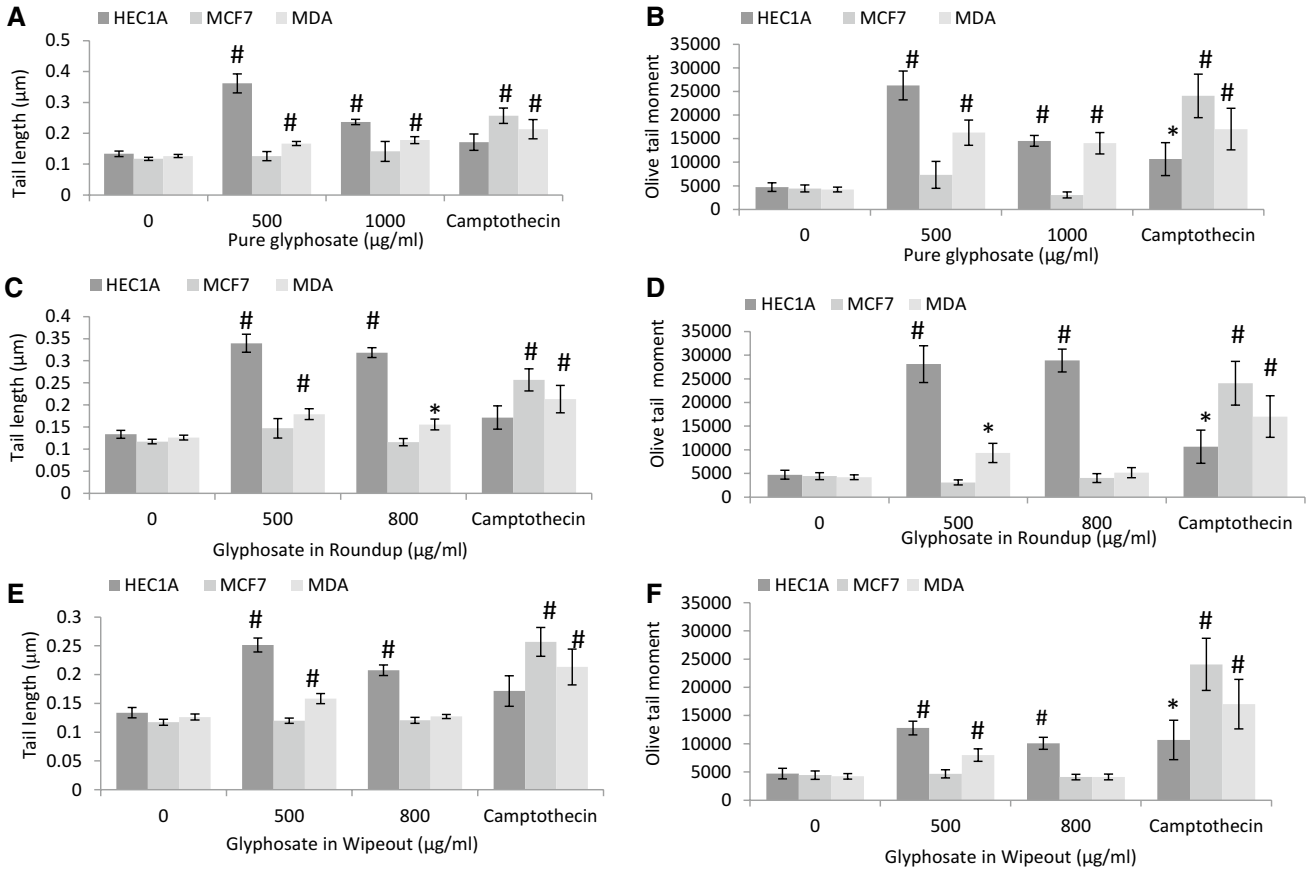

Fig. 4 DNA damage in HEC1A, MCF7 and MDA-MB-231 cancer cell lines presented as TL (µm): **a** pure glyph **c** Ro and **e** Wo and TM, **b** pure glyph, **d** Ro and **f** Wo. Cells were exposed for 4 h at 37 °C at test and reference herbicidal concentrations. Camptothecin (35 µg/ml) was the positive control in this study and data points represent the means of 50 cells analyzed per experimental treatment (±SEM). ANOVA single-factor analysis was used to determine significant differences from the untreated control. $^{*}P \leq 0.05$, $^{\#}P \leq 0.01$

damage was observed in the MDA-MB-231 cell line when compared to the untreated cells with an increase in TL and TM after exposure to 500 µg/ml (TL: 0.17, TM: 16,267) and 1000 µg/ml (TL: 0.18, TM: 14,027) of pure glyph. The positive control (100 µM camptothecin) showed genotoxic activity in all three cell lines. The observed effects of the pure glyphosate treatments in the three cell lines show evidence of the genotoxicity in the HEC1A and MDA-MB-231 cancer cell lines. No conclusive evidence of genotoxic effects was observed in the MCF-7 breast cancer cell line.

Comet formation in the HEC1A, MCF-7 and MDA-MB-231 cell line after exposure to the glyphosate formulations Ro and Wo are shown in Fig. 4 (c–f). Results suggest that both Ro and Wo cause significant DNA damage ($P \leq 0.01$) in the HEC 1A cell line at a concentration of 500 µg/ml and a reference concentration of 800 µg/ml. Ro (500 µg/ml and 800 µg/ml) and Wo (500 µg/ml and 800 µg/ml) did not stimulate significant comet formation in the MCF-7 cell line compared to the untreated control. The tail length results obtained for the MDA-MB-231 cell line indicates significant ($P \leq 0.05$, $P \leq 0.01$) DNA damage by the Ro formulation at 500 µg/ml (TL: 0.18) and 800 µg/ml (TL: 0.16). With respect to the Wo formulation, results indicated a significant ($P \leq 0.05$, $P \leq 0.01$) increase in tail length at 500 µg/ml (TL: 0.16). Results for the reference concentration (800 µg/ml), however, did not indicate a significant change in the TL and TM (for both Ro and Wo formulations).

Genotoxicity was observed in both the HEC1A and MDA-MB-231 cancer cell lines when exposed to pure glyph, Ro and Wo. Comparatively pure glyphosate and the two formulations showed a significantly higher ($P \leq 0.001$) genotoxic effect in the HEC1A (ER positive) endometrial cancer cell line when compared to the MDA-MB-231 (ER negative) breast cancer cell line. When examining the results of test concentration (500 µg/ml) there was no significant difference in the genotoxic effect displayed by pure glyphosate when compared to Ro and Wo in the MDA-MB-231 cancer cell line. Pure glyph was observed to be significantly ($P \leq 0.001$) more genotoxic than the Wo formulation and exhibited a statistically similar genotoxic effect to the Ro formulation in the HEC1A cancer cell line.

Discussion

The current study presents findings for the cytotoxic and genotoxic potential of pure glyph and glyphosate formulations (Ro and Wo) in humans. The potential relevance of the estrogen receptor status of the cell lines investigated is also discussed. The parameters chosen for this study equate to a low/moderate-dose short-term exposure (the maximum exposure time was 24 h).

Cytotoxic effects in human whole blood exposed to glyphosate

Cell viability results of human whole blood exposed to pure glyph, Ro and Wo confirmed that all three treatments (pure glyph, Ro and Wo) showed significant toxicity (Fig. 1a1–c1) to the cells at varying concentrations. Evidence for two different dose responses was demonstrated in this study. Pure glyph and Ro showed similar toxicological profiles characterized by an inverted bell-shaped dose–response curve (Fig. 1a2, b2). Low dose exposure toxicity studies have demonstrated non-monotonic dose–response curves which are often characterized by an inverted U, U, β or J shape (Conolly and Lutz 2004; Vandenberg et al. 2012). The link between non-monotonic dose–response relationships and overcompensations in cell systems (known as hormesis) due to disruptions in homeostasis has been proposed (Calabrese and Baldwin 2001); however, the relevance and significance of this theory to the results presented in this study requires further investigations. The results in this study do, however, present scope for future investigations into possible hormetic effects at low-dose glyphosate exposure in humans.

EC50 values describing the inhibitory ($EC50_1$) and the stimulation ($EC50_2$) phase of the dose–response curves for pure glyph and Ro suggested that both treatments exhibit similar inhibitory and stimulatory effects with a minor difference (15%) in EC50 values being reported. The formulation Wo showed a linear dose-dependent inhibition of whole blood cells from a threshold concentration of 50 µg/ml (Fig. 1c2); however, the calculated EC50 value (7.1 µg/ml) was greater than that obtained for pure glyph and Ro, which strongly suggests that Wo showed a lower inhibitory effect in human whole blood.

Similar to the findings in the present study a report by Pieniążek et al. 2004 demonstrated the toxic capacity of glyphosate and a glyphosate formulation Roundup ULTRA 360 SL in human erythrocytes, with the biochemical disruption of crucial biological parameters, e.g. the oxidation of hemoglobin to methemoglobin and lipid peroxidation being observed in blood cells exposed to glyphosate alone and in formulation at concentrations of 500 and 1000 µg/ml respectively. However, in contrast to the present work, Roundup indicated a higher toxicity than glyphosate alone. The majority of studies in literature have demonstrated evidence that glyphosate formulations display greater toxicity than the active ingredient in a variety of different models such as human peripheral blood mononuclear cells after a 96-h exposure period Martinez et al. (2007), human-derived buccal epithelial cells exposed to glyphosate and Roundup concentration ranges of 10–20 µg/ml (Koller et al. 2012) and human placental cells exposed to glyphosate and Roundup concentration ranges of 0.2–2% (Richard et al. 2005). Some studies have proposed that the amplification of toxicity observed in formulation may be due to synergistic actions between glyphosate and the adjuvants present in formulation. This theory was highlighted in mitochondrial bioenergetics studies in Wistar rat mitochondria conducted by Peixoto (2005) and for the common adjuvant in glyphosate-based herbicides, namely TN-20 by Kim et al. (2013) in studies conducted in the rat heart cell line H9C2. More recently, studies conducted by Kwiatkowska et al. (2014) have evidenced the toxic effects of impurities commonly found in glyphosate formulations (*N*-(phosphonomethyl) iminodiacetic acid, *N*-methylglyphosate, hydroxymethylphosphonic acid and bis-(phosphonomethyl)amine), at low to high dose exposure (~ 2–1000 µg/ml), in human erythrocytes. Results from the study suggested that slightly greater toxicity was induced by these compounds when compared to pure glyphosate.

Findings by Bolognesi et al. (1997) are in agreement with the work presented in this study, when comparing the DNA-damaging activity (DNA breaks and alkali labile sites) of glyphosate and Roundup (300 µg/ml) in rat liver and kidney, with no significant differences observed in the relative toxicities after exposure of the two herbicidal treatments. Although pure glyph did not demonstrate a lower toxicity when compared to the formulations in this work, the varying toxicities displayed by the two different formulations led this study to infer that additives in formulation played a role. This study focused on the short-term effects and long-term studies may reveal different toxicity profiles than the initial profiles obtained under the current experimental conditions; however, future work would be required to conclusively state this. Human serum albumin, an important carrier of various substances, e.g. drugs in human blood has been shown to bind to glyphosate (Nowak and Shaw 1995; Yue et al. 2008); however, to the best of our knowledge, this has not been demonstrated for adjuvants reported to be present in glyphosate formulations, particularly POEA, the most commonly used non-ionic surfactant in glyphosate formulations; therefore, future studies should incorporate HSA-binding studies which would allow further insight into the bioavailability of different compounds to biological targets and the comparative toxicities of glyphosate and its formulations in human blood.

Cytotoxic and genotoxic effects of glyphosate in human cancer cell lines

The HEC1A (estrogen-E2 responsive) endometrial cancer cell line and two breast cancer cell lines MCF-7 (estrogen-E2 responsive) and MDA-MB-231 (estrogen insensitive) were exposed to varying concentrations of pure glyph (75–500 µg/ml) and equivalent glyphosate concentrations in the Ro and Wo formulation. The three herbicidal treatments displayed different toxicities (as observed in cell viability results in Fig. 2) on the cancer cell lines suggesting that the herbicidal cytotoxicity was cell type dependent. In our experiments, no cytotoxic effects were observed in any of the cell lines when exposed to the Ro formulation (Fig. 2b). Pure glyph and Wo showed no effect on the cell viability of the MCF-7 and MDA-MB-231 breast cancer cell lines (Fig. 2a, c). The most significant results were observed in the HEC1A cell line (Fig. 2a).

Pure glyphosate caused a significant decrease (non-monotonic) in cellular growth in the HEC1A cell line at all the concentrations tested (Fig. 2a), indicating potential mitochondrial function disruption as measured by the MTT assay (via mitochondrial succinate dehydrogenase) at glyphosate concentrations as low as 75 µg/ml. Mitochondrial disruption by glyphosate and glyphosate formulations has been confirmed in literature with in vivo studies in rat mitochondria by Olorunsogo et al. (1979) indicating the potential of glyphosate (60–120 µg/ml) to uncouple mitochondrial oxidative phosphorylation and a more recent study by Gasnier et al. (2010) reported that the cell toxicity mechanism of different Roundup formulations (25 µg/ml) in human hepatic cell lines (HepG2 and Hep3B) was via the inhibition of mitochondrial succinate dehydrogenase.

Wo concentrations between 75 and 250 µg/ml induced a significant non-monotonic proliferative effect in this cell line (Fig. 3c). In vitro studies by Lin and Garry (2000) showed similar findings with proliferative effects in the MCF-7 breast cancer cell line being observed after exposure to glyphosate and Roundup at concentrations of 2.28 and 10 µg/ml, respectively. The contrasting results obtained for pure glyphosate and Wo suggest that adjuvants and/or glyphosate impurities found in the commercial formulation may have induced the proliferation of the HEC1A cells.

The comet assay was used to determine the DNA-damaging effects of glyphosate and its formulation at the highest test concentration (500 µg/ml) from the cell viability studies and reference concentrations for pure glyphosate (1000 µg/ml) and the two formulations (800 µg/ml). The reference concentrations were chosen according to initial toxicity results observed by Gasnier et al. (2009) in the HepG2 liver cell line. A reference concentration was utilized in this study to determine whether concentrations above 500 µg/ml would induce significant comet formation (positive control). The theory that cytotoxicity is not always directly associated with genotoxicity has been well documented, and the use of a sensitive tool such as the comet assay enables the determination of DNA damage at a single-cell level thus overcoming the limitations encountered when interpreting cytotoxicity data (Hartmann and Speit 1997; Manas et al. 2009; Vock et al. 1998).

We have demonstrated the ability of pure glyph and its formulations to induce DNA-damaging effects by the formation of comets in the HEC1A cells with a significant increase in the tail length being observed at the test concentration of 500 µg/ml (Fig. 4) when compared to the untreated control. Similar findings were observed in the MDA-MB-231 cell line and significant DNA damage was induced by pure glyph, Ro and Wo at the test concentration. Interestingly, a significantly ($P \leq 0.001$) greater genotoxic effect was observed in the HEC1A cell line compared to the MDA-MB-231 cell line. Based on this result, it can be assumed that the MDA-MB-231 cells show less sensitivity to the herbicide than the HEC1A cells. This may be due to the several factors including the presence or absence of ER receptors, the presence of different cofactors and co-regulators that may impact the binding potential of ligands (Vandenberg et al. 2012).

Significant comet formation was observed in the HEC1A cell line at the reference pure glyph (1000 µg/ml), Wo and Ro concentrations. In the MDA-MB-231 cell line, DNA damage was observed at the reference glyphosate and Ro concentration; however, no significant comet formation was observed in the Wo formulation. The exact reason for this is still unclear but there has been some evidence that confounding results in the comet assay due to high cytotoxicity are cell type specific (Hartmann et al. 2003). Findings by Frei et al. (2001) showed that reduced cell viability in primary rat hepatocytes did not induce differences in the tail length.

To the best of our knowledge, there are no reports documenting the effects of pure glyph and its formulations in the HEC1A endometrial cancer cell line. This cell line is estrogen responsive and although not confirmed in the current study, it is possible that glyphosate and the two formulations (Ro and Wo) potentially induce toxic effects in the HEC1A endometrial cancer cells via modulation of the ERα and ERβ receptors. Other studies involving the use of hormone-dependent cell lines have proven the ability of glyphosate and glyphosate-based herbicides to act as a xenoestrogens (George and Shukla 2011). Thongprakaisang et al. (2013) demonstrated proliferative effects induced by glyphosate after low dose exposure in hormone-dependent T47D breast cancer cells and Gasnier et al. (2009) showed the endocrine disruptive activity of glyphosate and four Roundup formulations in the MDA-MB-231-MB435-kb2 (androgen receptor positive) breast cancer cell line and the HepG2 liver cell line at low dose exposure, with endocrine effects reported within 24 h. Further studies involving the full characterization of

the interactions of pure glyphosate and glyphosate formulations with ERα and ERβ receptors in the HEC1A cell line are required to fully understand the endocrine modulating capacity of this herbicide in this cell line. This study has proven the suitability of the HEC1A cancer cell line model for assessing the cytotoxic and genotoxic effects of glyphosate.

Although most studies have reported no significant effects in the hormone-independent MDA-MB-231 cell line exposed to glyphosate and glyphosate formulations (Buteau-Lozano et al. 2008; Thongprakaisang et al. 2013), this study demonstrated the genotoxic activity displayed by pure glyphosate and its formulation in the MDA-MB-231 breast cancer cell line. MDA-MB-231 cancer cells are described as hormone-independent cells that express extremely low or no levels of ER α and ER β receptors, and are characterized by estrogen-independent proliferation (Adams et al. 2007; Wang and Kilgore 2002). To the best of our knowledge, this effect has not been shown in glyphosate-centralized studies, and suggests that glyphosate and glyphosate formulations may potentially induce cell damage via non-estrogenic mechanisms as well. Ventura et al. (2012) demonstrated the non-estrogenic effect (G2/M phase arrest) in the ER-negative MDA-MB-231 cell line exposed to the pesticide, chlorpyrifos, commonly defined as an endocrine disrupter. The study indicated that this mechanism was potentially based on the capacity of chlorpyrifos to bind to tubulin sites thus altering the formation of microtubules (Ventura et al. 2012). It must be taken into consideration that the primary focus of research using the estrogen-insensitive MDA-MB-231 breast cancer cell line was based on assessing the cytotoxic and genotoxic effects of glyphosate and glyphosate-based herbicides in cell lines with different estrogen receptor status and thus, experimental methods selected will generally not explore other mechanisms.

Pure glyph and Ro were found to be more genotoxic ($P \leq 0.001$) than Wo in the HEC1A cell line. The genotoxic effect of all three herbicide treatments was statistically similar in the MDA-MB-231 cell line. The results are relatively similar to the findings observed in human whole blood in this study.

No significant genotoxic effect was observed in the MCF-7 breast cancer cell line when exposed to pure glyph and the two formulations under the experimental conditions tested. Previous studies have, however, shown the endocrine disruptive activity of glyphosate and its formulations in this cell line (Hokanson et al. 2007; Kojima et al. 2004).

Conclusion

In summary, adjuvants and/or glyphosate impurities found in glyphosate formulations may potentially play a role in glyphosate toxicity as indicated by the preliminary evidence (differential toxicological profiles observed for Wo and Ro in human whole blood and the HEC1A cancer cell line) obtained. Cytotoxicity results at concentrations relevant to occupational and residential exposure to glyphosate and glyphosate formulations observed in the three cancer cell lines suggest that toxicity varies depending on cell type, with the most significant results observed in the HEC1A cancer cell line exposed to pure glyph and Wo. Moderate concentrations (500 µg/ml) of pure glyph, Ro and Wo induced genotoxic effects in the HEC1A and MDA-MB-231 cancer cell lines, respectively; this suggests that glyphosate may display various mechanisms of toxicity. This study has also provided preliminary insights into the significance of the estrogen receptor status of cell lines, as a foundation for future studies investigating the potential endocrine disruptive capacity of pure glyph and its formulations.

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Conflict of interest All authors declare that they have no conflict of interest in the publication.

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