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A step further toward glyphosate-induced epidermal cell death: Involvement of mitochondrial and oxidative mechanisms

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ABSTRACT

A deregulation of programmed cell death mechanisms in human epidermis leads to skin pathologies. We previously showed that glyphosate, an extensively used herbicide, provoked cytotoxic effects on cultured human keratinocytes, affecting their antioxidant capacities and impairing morphological and functional cell characteristics. The aim of the present study, carried out on the human epidermal cell line HaCaT, was to examine the part of apoptosis plays in the cytotoxic effects of glyphosate and the intracellular mechanisms involved in the apoptotic events. We have conducted different incubation periods to reveal the specific events in glyphosate-induced cell death. We observed an increase in the number of early apoptotic cells at a low cytotoxicity level (15%), and then, a decrease, in favor of late apoptotic and necrotic cell rates for more severe cytotoxicity conditions. At the same time, we showed that the glyphosate-induced mitochondrial membrane potential disruption could be a cause of apoptosis in keratinocyte cultures.

1. Introduction

The skin is the largest organ of the human body and its outermost layer, the epidermis, is made up of stratified epithelium. The epidermis is divided into four layers representing the differentiated stages of the main cells of epithelium, the keratinocytes that undergo a constant process of cell generation. Among other layers, the epidermis functions as a physical barrier that provides the first protection from physical and chemical traumas (Proksch et al., 2008). As it covers the entire surface of the body, the skin can undergo extensive exposure to environmental pollutants, including herbicides.

There are epidemiological lines of evidence that chronic exposure to glyphosate [N-(phosphonomethyl)glycine], an extensively used herbicide, induces genotoxic damage, especially in immune circulating cells (Bolognesi et al., 2009) and is associated with increasing frequency of cancers as

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non-Hodgkin's lymphoma (De Roos et al., 2003). More recently, it has been shown that glyphosate-based formulations had teratogenic effects on *Xenopus laevis* embryos (Paganelli et al., 2010) and that glyphosate alone induced clastogenic effects (Prasad et al., 2009) and carcinogenicity (George et al., 2010) respectively in mouse bone marrow and skin.

Experimentally, glyphosate is a chemical component studied alone or included in formulations for its deleterious effect on different types of cells (Marc et al., 2002, 2005; Benachour et al., 2007). Some epidemiological studies with glyphosate or its formulations reported cases of cutaneous toxicity from chemical burns to allergic contact dermatitis (Amerio et al., 2004; Penagos et al., 2004; Nagami et al., 2005). Nevertheless, little is known about the molecular mechanisms of topical glyphosate toxicity.

In a previous in vitro study we showed that glyphosate is an epidermal cytotoxic agent, affecting the intracellular antioxidant defence system, glutathione redox balance and increasing lipid peroxidation related to membrane disorders of cultured human keratinocytes (Gehin et al., 2005, 2006).

More recently, we identified some glyphosate-induced morphological and functional modifications by a combined confocal and atomic force microscopy approach (Elie-Caille et al., 2010; Heu et al., 2012). Indeed, glyphosate exposure of the human epithelial HaCaT cell line modified the cell morphology: stressed cells became shrunk and elongated and showed a significantly affected cell adhesion potential. Combined with hydrogen peroxide production, cytoskeleton disorganization and chromatin condensation, the data as a whole converged to a glyphosate-induced apoptotic process, a physiological pathway toward self-destruction when human keratinocytes were subjected to an in vitro oxidative stress (Lockshin and Zakeri, 2004). Our research has been underscored by other reports which show a glyphosate-induced apoptosis phenomenon in other types of cell (Benachour and Séralini, 2008; Clair et al., 2012).

Two studies (Mukherjee et al., 2010; Wang et al., 2010) have characterized apoptosis mechanisms in a HaCaT cell model involving several flow cytometry labeling techniques widely employed on other cell types for the same aim (Kumar et al., 2009; Li et al., 2009; Zakaria et al., 2009). Our oxidative stress model has been thus applied to the assessment of the apoptotic nature of glyphosate-induced epidermal cell death.

Overproduction of reactive oxygen species (ROS) has been shown to activate apoptosis (Simon et al., 2000). Svobodová et al. (2008) previously used DCFH-DA to determine the protective effect of a plant extract phenolic fraction against UVA-induced damages to HaCaT human keratinocyte cell line. Indeed, apoptosis may serve as a 'failsafe' device to prevent cells from proliferating in the face of a persistent oxidative stress (Kannan and Jain, 2000). The annexin V/propidium iodide labeling method led us to distinguish intact cells, early and late apoptotic cells, and necrotic or dead cells. Some publications reported studies on a HaCaT cell model with an annexin V/propidium iodide apoptosis characterization (Haberland et al., 2006; He et al., 2006).

An excess of ROS can lead to oxidative stress which precedes mitochondrial membrane permeabilization during apoptosis events (Fleury et al., 2002). Malatesta et al. (2008) used JC-1 labeling to characterize glyphosate's effects on hepatoma tissue culture cells. HaCaT cells were employed as a model for assessing of JC-1 efficiency by Zuliani et al. (2003).

As the epidermis is continuously renewed, the keratinocytes undergo a specialized form of programmed cell death which needs to be well orchestrated to maintain skin homeostasis. Consequently, a deregulation of cell death mechanisms in the skin or an improper removal of damaged cells can lead to diseases or cancerous lesions (Lippens et al., 2009). Thus, the aim of the present study was to examine firstly the part of apoptosis in the cytotoxic effects of glyphosate in the human epidermal cell line, HaCaT and, secondly, the intracellular mechanisms involved in the apoptosis phenomenon as mitochondrial membrane potential disruption and ROS production increasing.

2. Materials and methods

2.1. Cell cultures

The immortalized human HaCaT cell line (ATCC, Teddington, UK) (Boukamp et al., 1988) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS; D. Dutscher, Brumath, France) and 80 mg/L of gentamicin in a humidified 5% CO_2 atmosphere at 37 °C. The keratinocytes were grown to confluence in 75 cm^2 culture flasks (D. Dutscher). The medium was removed every 48 h, and cells were subcultured every 7 days at a ratio of 1:6.

2.2. Cytotoxicity assays

HaCaT cells were seeded at a density of 1×10^4 cells per well in 100 μL FCS-supplemented DMEM on 96-multiwell culture plates (Nunc, D. Dutscher) and incubated overnight for adherence. The following day, the medium was removed and cells were incubated in FCS-free medium containing increasing concentrations of glyphosate (0, 5, 10, 20, 30, 40, 50, 60, and 70 mM) for nine incubation times (0.5, 1, 2, 4, 6, 9, 12, 15 and 18 h) in 37 °C–5% CO_2 atmosphere (glyphosate: powder purity ≥ 95%, Sigma–Aldrich, St. Louis, MO, USA). After the exposure periods, the reaction medium was removed, and the residual cell viability was measured in each well using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide procedure as previously described (Mosmann, 1983; Gehin et al., 2005). Results were expressed as percentage of controls (100% viability) according to the glyphosate concentrations, and the 50% inhibition concentrations (IC_{50}), i.e. the concentrations of glyphosate killing 50% of keratinocytes, were compared according to the four exposure times. Each experiment was done three times, and each determination was carried out in triplicate.

2.3. Flow cytometry

For all of the flow cytometry study, HaCaT cells were seeded in 25 cm^2 culture flasks (D. Dutscher,) at a density of 5×10^5 cells/DMEM supplemented with 10% (v/v) FCS and 80 mg/L of gentamicin in a humidified 5% CO_2 atmosphere at 37 °C for 24 h for cell attachment.

Flow cytometry analyses were performed on a FC500 cytometer (Beckman-Coulter, France). Analyses were performed on no less than 10,000 cells, using the CXP™ software (Beckman-Coulter).

2.4. Intracellular ROS (H_2O_2)

2′-7′-Dichlorodihydrofluorescein diacetate (DCFDA) dye was used to study the intracellular hydrogen peroxide generation in HaCaT cells. After 24 h of cell attachment, the cells were washed twice with 2 mL PBS and DCFDA dye was added at a concentration of 20 μM in 2 mL per flask. The flasks were placed in a humidified 5% CO_2 atmosphere at 37 °C for 30 min. Following incubation, the dye solution was removed, the cells were washed with 2 mL PBS and were treated with several concentrations of glyphosate in FCS-free media depending on different incubation periods: 30, 40, 53 and 70 mM for 0.5 h; 15, 20, 30 and 45 mM for 6 and 18 h. To ensure the dye specificity, a positive control is carried out, a treatment with tert-butyl hydroperoxide (tBHP) 15 μM for 18 h. Following exposure, and after supernatant recovery, the cells were washed with pre-warmed PBS and were collected by trypsinization; whereafter, the trypsin was removed by centrifugation. Cells were resuspended in PBS before flow cytometry.

The H_2O_2 positive cells are cells which have fluorescence intensity higher than the majority of control cells.

2.5. Apoptosis

After 24 h of cell attachment, cultured cells were exposed to several concentrations of glyphosate in FCS-free media depending on different incubation periods: 30, 40, 53 and 70 mM for 0.5 h; 15, 20, 30 and 45 mM for 6 and 18 h. Following exposure, and after supernatant recovery, the cells were washed with pre-warmed PBS and were collected by trypsinization; whereafter, the trypsin was removed by centrifugation. The cells were then stained with the FITC annexin V Apoptosis Detection Kit II (BD Biosciences, Franklin Lakes, USA) as recommended in the procedure. To ensure the dye specificity, a positive control with camptothecin and a negative control after a pretreatment with recombinant annexin V were carried out.

In the early stages of apoptosis, changes occur at the cell surface. One of these plasma membrane alterations is the translocation of phosphatidylserine (PS) from the inner side of the plasma membrane to the outer layer, by which PS becomes exposed at the external surface of the cell. Annexin V is a Ca^{2+} dependent phospholipid-binding protein with high affinity for PS. Hence this protein can be used as a sensitive probe for PS exposure upon the cell membrane. Translocation of PS to the external cell surface is not unique to apoptosis, but occurs also during cell necrosis. The difference between these two forms of cell death, during the initial stages of apoptosis, is that the cell membrane remains intact, while at the moment where necrosis occurs, the cell membrane loses its integrity and becomes leaky. Therefore, the measurement of annexin V, binding to the cell surface as indicative for apoptosis, has to be performed in conjunction with a dye exclusion test with propidium iodide to establish integrity of the cell membrane (Vermes et al., 1999). An increase in green fluorescence intensity shows an annexin V positive profile, therefore an early apoptosis state. An increase in the two dye intensities shows an annexin V and propidium iodide positive profile, i.e. a late apoptosis state. If only the blue fluorescence intensity increases, the cells are in necrosis or dead.

2.6. Mitochondrial transmembrane potential

After 24 h of cell attachment, keratinocytes were treated as for the apoptosis study. The cells were then stained with the mitochondria staining kit (Sigma CS0390) as recommended by the procedure. To ensure the dye specificity, a negative positive control with valinomycin was carried out.

In the data representation, a point represents any cell with its red fluorescence intensity in abscissa and its green fluorescence intensity in ordinate.

The changes in the mitochondrial membrane potential were monitored using JC-1 (5,5V,6,6V-tetrachloro-1,1V,3,3V-tetraethylbenzimidazolcarbocyanine iodide). When administered to living cells, JC-1 accumulates in the mitochondria where it emits either red or green fluorescence, depending on the mitochondrial membrane potential (Reers et al., 1991); therefore, the shift from red to green fluorescence is considered as a reliable indication of a drop in the mitochondrial membrane potential.

Indeed, the decrease of the red/green fluorescence ratio represents the loss of mitochondrial red fluorescent aggregates in favor of the accumulation of cytoplasmic green fluorescent monomers.

It has to be mentioned that the successive assay steps induced, in the case of an 18 h-incubation period, the lost of a majority of cells, preventing us to collect enough data in extreme conditions.

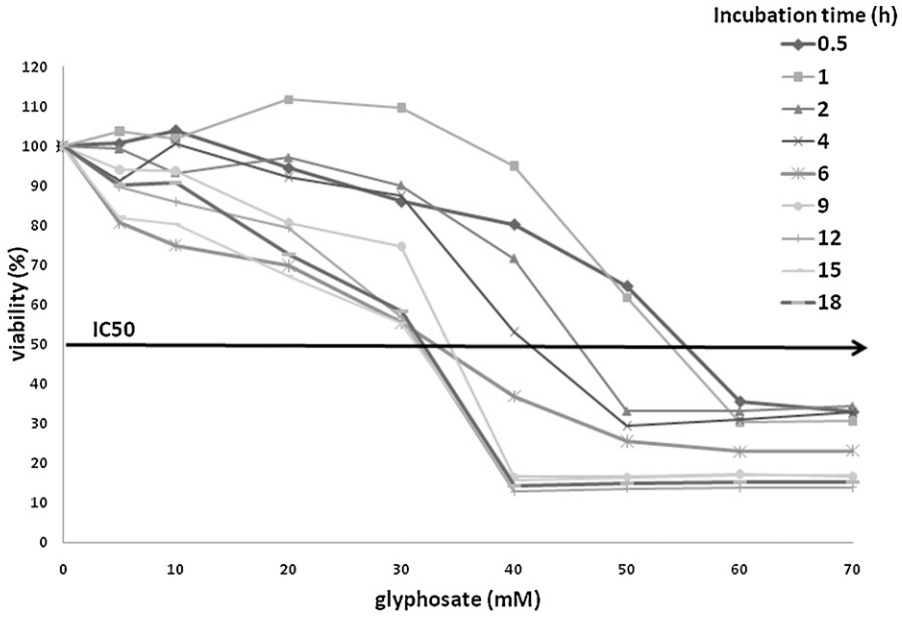
3. Results

3.1. Glyphosate impact on HaCaT cell viability

The detailed study of cell viability allowed us to choose the best conditions to examine the glyphosate-induced apoptosis phenomenon. The percentages of HaCaT cell viability and the IC_{50} values recorded after glyphosate treatment were investigated at different concentrations of glyphosate (5–70 mM) and several incubation periods (0.5–18 h). Looking at IC_{50} values, cytotoxic profiles shared into three groups (Fig. 1(a)). From 6 to 18 h incubation periods, cytotoxic profiles were superimposed and presented an IC_{50} value, approximately 30 mM. For shorter incubation periods, 0.5 and 1 h, the IC_{50} increased to 53 mM. For intermediate incubation periods, 2 and 4 h, cytotoxic profiles progressively shifted from an IC_{50} value of 45–41 mM of glyphosate. Based on that, 3 incubation periods were selected: 0.5 and 18 h as border times, and 6 h, since it corresponds to the first relatively short incubation time which shows a “18 h-like” profile.

The table in Fig. 1(b) gathers cytotoxicity values obtained at different experimental conditions (incubation times and glyphosate concentrations) chosen for the following investigations.

a



b

Cytotoxicity (%)		Glyphosate (mM)						
		15	20	30	40	45	53	70
Incubation time (h)	0.5			15	20		45	67
	6	28	30	45		69		
	18	18	27	42		85		

Fig. 1 – Glyphosate-induced cytotoxicity on HaCaT cells. Cytotoxicity profiles (a) expressed as percentages of residual cell viability compared with controls, after 0.5, 1, 2, 4, 6, 9, 12, 15 and 18 h of treatment of confluent HaCaT cell cultures. Results are given as means of three independent experiments, performed in triplicate. Table (b) reports the cytotoxicity percentages for each three incubation times and four glyphosate concentrations chosen for the study (IC_{50} values are highlighted in bold).

3.2. *Glyphosate-dependent production of ROS*

A DCFH-DA labeling was employed to evaluate the intracellular oxidative status by a cytometric assay. Fig. 2 describes the percentage of H_2O_2 positive cells function of the percentage of cytotoxicity for the 3 incubation times. While the H_2O_2 positive cell percentage globally grew with the glyphosate cytotoxicity for the 3 incubation periods, subtle differences in this general increase appeared. Indeed, for a 0.5 h-incubation, the increase of H_2O_2 positive cells began for a 20% cytotoxicity treatment whereas it was measured for 6 and 18 h respectively at 45 and 42% of cytotoxicity. At similar cytotoxic values closed to IC_{50}, there were more (two fold) H_2O_2 positive cells at 18 h than at 6 h.

3.3. *Apoptosis study*

Apoptotic glyphosate-treated HaCaT cells were detected by double staining with annexin V (green) and propidium iodide (blue).

In Fig. 3(a), the cloud of points takes the shape of an arrowhead that demonstrated the natural HaCaT cells autofluorescence and prevented us defining the usual 4 square areas characterizing the death cell state. A negative control allowed us to distinguish autofluorescence from annexin V and propidium iodide labeling and so consider that this autofluorescence as too low to interfere with the dye signal.

HaCaT cells were exposed to different glyphosate cytotoxic conditions (0, 15 and 45%) for a 0.5 h-incubation. At 15% cytotoxicity, the cloud of point initiated a shift to early apoptotic area. Then, at 45% cytotoxicity, lot of cells reached not only early but also late apoptotic state and even a necrotic state.

Fig. 3(b) reports quantitative data of cell percentage in every cell state. For a short incubation time (0.5 h), we noticed a weaker healthy cell percentage than for the two longer incubation times (6 and 18 h); in the same way, the early apoptotic cell percentages evolved as a "bell shape" with the glyphosate cytotoxicity reaching a maxima at 15% cytotoxicity.

For the 3 incubation times, the early apoptotic cell percentages fall sharply at drastic conditions: 67, 69 and 70% cytotoxicity for respectively 0.5, 6 and 18 h-incubation times. In parallel, the late apoptotic percentages began to increase at IC_{50} conditions until reaching high percentages up to 5 fold for 0.5 h, and 10 fold for 6 and 18 h. In drastic conditions ($\gg IC_{50}$), the percentage of necrotic cells declined while incubation times increased and corresponded to 30, 24 and 9% of total cells respectively for 0.5, 6 and 18 h.

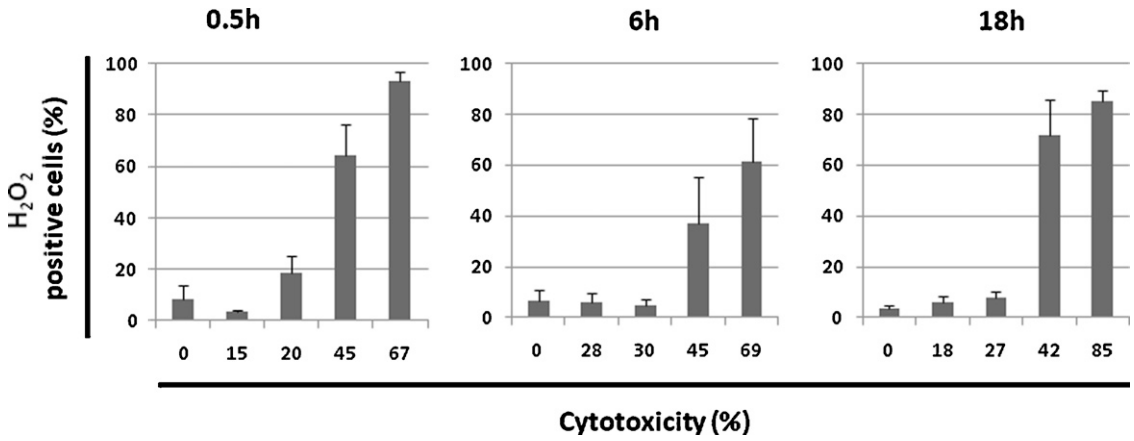


Fig. 2 – Glyphosate dependent production of ROS in HaCaT cells. Histograms show the percentages of H_2O_2 productive cell function of glyphosate-induced cytotoxicity. The quantitative data of flow cytometry analysis are given as mean of three independent experiments; the standard deviation is evaluated for each H_2O_2 positive cell percentage on histograms.

To free ourselves from even weak healthy cell number variabilities, Fig. 3(c) points out, under piled histograms, the redistribution of the 3 death states: the necrosis evolution is stackable to Fig. 3(b), while it underlines more clearly the early-to-late apoptosis transition.

3.4. *Mitochondrial membrane potential study*

In Fig. 4(a), for a 0.5 h incubation time, the cell cloud moved from the top left to the bottom right of the graph when the glyphosate cytotoxicity increased. Indeed, in untreated samples, one population could be observed. It exhibited high red and relatively low green signals. Consequently, this population is containing the most energized mitochondria.

At a 15% cytotoxicity condition, the cell population exhibited an intermediate fluorescence profile and represented a cell subpopulation with an intermediate mitochondrial membrane potential.

Close to the IC_{50} condition (45% cytotoxicity), the cell population presented a low red signal and high green fluorescence profile that is typical of the lowest energized mitochondria.

Then, after a glyphosate treatment at IC_{50} conditions, the shift to higher green fluorescence signals of cells indicates the massive collapse of the mitochondrial potential.

We globally observed the same trend for 6 and 18 h (data not shown) which indicates the mitochondrial membrane potential disruption.

In Fig. 4(b), quantitative data, representing the red to green fluorescence ratio, are reported to the control condition value (100%). We can observe, for the three incubation times, a glyphosate concentration dependent decrease of the red/green ratio.

The ratio clearly dropped more quickly for 0.5 h than for long incubation times (6 and 18 h), for which a more progressive diminution was performed. Indeed, at conditions close to IC_{50} for each incubation time, the red/green ratio is lower (15.4%) at 0.5 h than at 6 h (55.3%) or 18 h (49.4%).

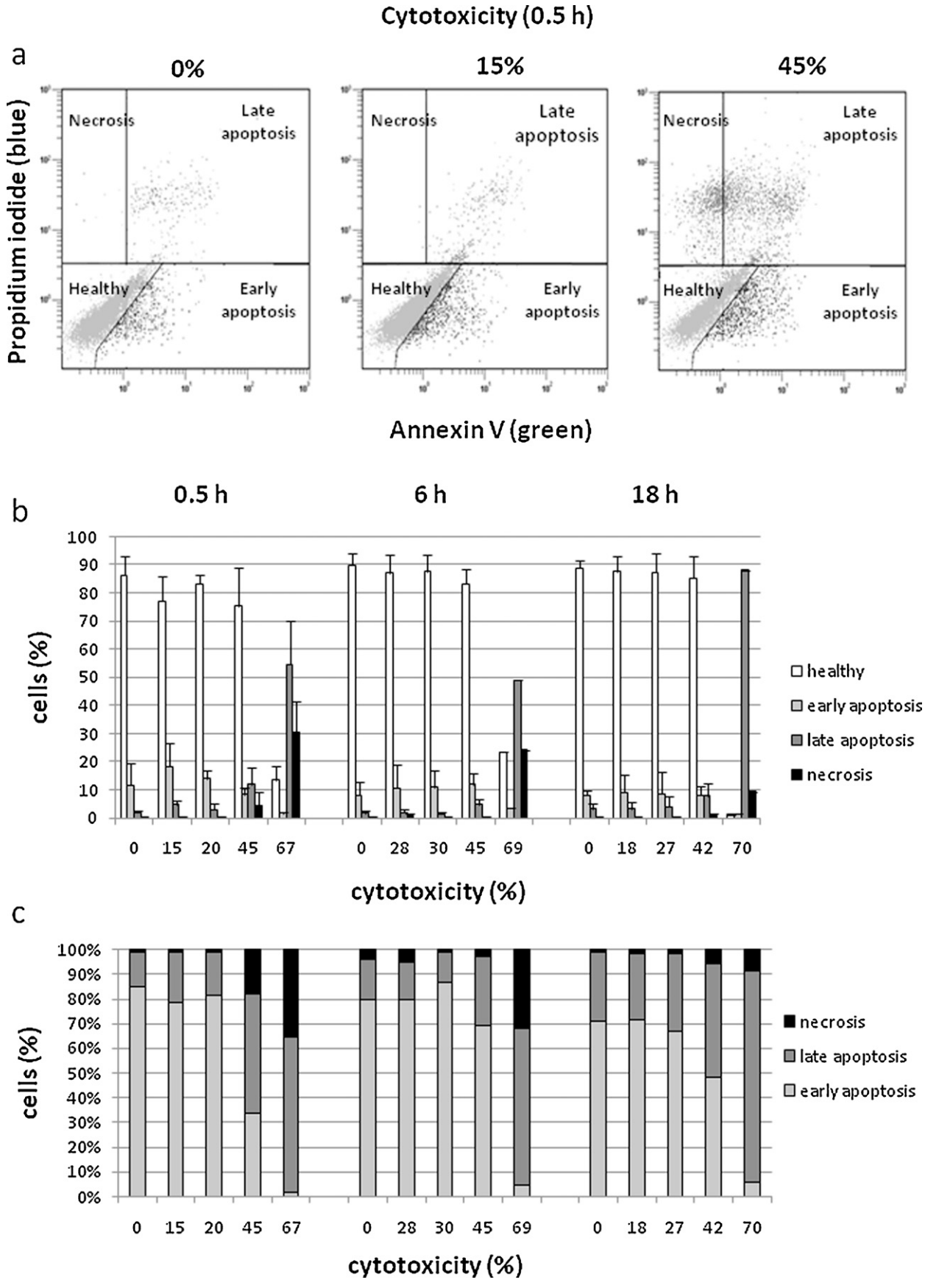
4. Discussion

The skin has the potential for high exposure to environmental pollutants including herbicides. Glyphosate was for a long time considered to be essentially harmless in normal use or during chronic exposure (Williams et al., 2000). However, glyphosate penetrates the epidermis of even slightly compromised skin even more effectively than healthy skin (Nielsen et al., 2007). Using human cells, it was shown that glyphosate-based formulations act as endocrine disruptors, decreasing the mRNA levels of the CYP aromatase and inhibiting its activity (Richard et al., 2005; Gasnier et al., 2009).

Our present study was designed to cast light on the structural and functional correlations in epidermal cell behavior in relation to glyphosate-induced cytotoxicity through a deep investigation of molecular mechanisms impaired by oxidative stress. Toward this aim, we carried out experiments on the human epidermal cell line, HaCaT, to examine the cytotoxic and apoptotic effects of glyphosate, as well as the subcellular mechanisms involved in the apoptosis phenomenon as ROS production and mitochondrial membrane potential disruption.

Firstly, we determined the most relevant in vitro conditions to study the glyphosate-induced cell death phenomenon. Nine incubation periods, from 0.5 to 18 h, were tested: the cytotoxicity profiles evolved from 0.5 to 4 h, with decreasing IC_{50} values (glyphosate concentrations from 53 to 45 mM respectively), but remained unchanged from 6 to 18 h, with a similar IC_{50} value (around 30 mM). These superimposed cytotoxic profiles for such a wide range of times lead us to conclude that the threshold of toxicity depends not only on the dose but also on the time of contact, as recently observed by Gasnier et al. (2010), on another human cell line model.

Following a morphological study we published on HaCaT cells investigating 0.5 and 18 h of glyphosate contact (Elie-Caille et al., 2010), we conducted an interesting intermediate incubation period of 6 h to characterize events implied in glyphosate-induced cell death. Indeed, the 6 h-condition


Cytotoxicity (0.5 h)
a
0%
15%
45%
Necrosis
Late apoptosis
Healthy
Early apoptosis
Propidium iodide (blue)
Annexin V (green)
b
0.5 h
6 h
18 h
cells (%)
healthy
early apoptosis
late apoptosis
necrosis
0 15 20 45 67
0 28 30 45 69
0 18 27 42 70
cytotoxicity (%)
c
cells (%)
necrosis
late apoptosis
early apoptosis
cytotoxicity (%)

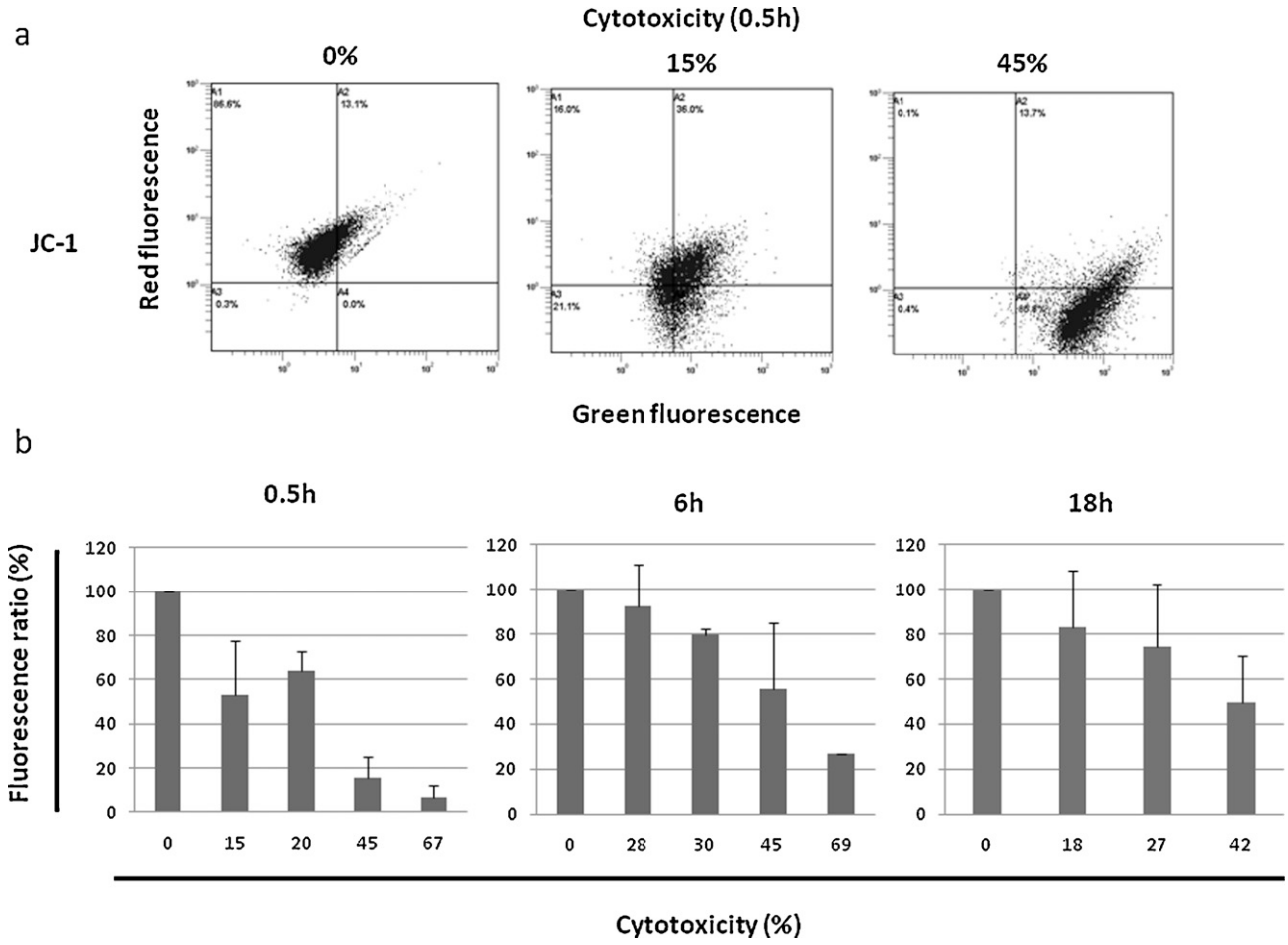


Fig. 4 – Mitochondrial membrane potential after glyphosate treatment. 0.5 h-flowcytometric profiles (a): fluorescence intensities in green (in abscissa) and in red (in ordinate). Histograms (b) showing the red/green fluorescence ratio function of glyphosate cytotoxicity for 0.5, 6 and 18 h incubation periods. Quantitative data of flow cytometric analysis are given as mean of three independent experiments, the standard deviation is evaluated for each fluorescence ratio on histograms.

presents the particular advantage to combine at once a relative short time and a "18 h-like" cytotoxic profile.

It has been shown that cell death was more likely to be provoked by glyphosate-based formulations than by the glyphosate alone (Benachour et al., 2007; Benachour and Séralini, 2008; Clair et al., 2012; Richard et al., 2005). These authors suggest that the adjuvants found in glyphosate preparations might also play a role in total cell death, through necrosis characterized by organelle alterations with mitochondrial and cell membranes swelling and ruptures. Indeed, adjuvants could provoke a glyphosate bioaccumulation or time-delayed effects. In our case, where pure glyphosate solutions were used, the results showed similar cytotoxic profiles between 6 and 18 h, demonstrating that there is no bioaccumulation or delayed effect of glyphosate in cultured HaCaT cells. Therefore, we proposed to study glyphosate effects in a concentration range (15–70 mM) previously validated in our laboratory (Elie-Caille et al., 2010; Gehin et al., 2005, 2006) and by others (Malatesta et al., 2008) without the presence of any adjuvants to interfere in the specific mechanisms of the glyphosate-induced cell death.

In order to complete our biochemical measurements of glyphosate-induced oxidative stress in HaCaT cells (Gehin et al., 2005, 2006), we presently explored ROS production with DCFH-DA labeling, which is frequently applied to the determination of the oxidative effect of cellular insults. This technique is easy to use, extremely sensitive toward the intracellular redox status, inexpensive and ideally suited to follow the kinetics of ROS production (Eruslanov and Kusmartsev, 2010). However, it is important to understand the limitations of using

Fig. 3 – Apoptosis study of HaCaT cells after glyphosate treatment. Cell populations after 0.5 h-glyphosate incubation (a), with their green fluorescence intensity in abscissa (annexin V) and their blue fluorescence intensity in ordinate (propidium iodide) were gated to distinguish healthy, early apoptotic, late apoptotic and necrotic or dead cells at 0, 15 and 45% cytotoxicity. Percentages of cells in each cellular (b) or death (c) state function of glyphosate cytotoxicity for 0.5, 6 and 18 h incubation are given as mean of three independent experiments, the standard deviation is evaluated for each cell percentage.

this probe: although DCFH-DA is proposed to measure the concentration of H_2O_2 in cells, it has to be noted that superoxide anion, nitric oxide (Rao et al., 1992) and singlet oxygen (Daghastanli et al., 2008) are also capable of oxidizing DCFH. In this assay, cells accumulate fluorescence proportionally to a total quantity of ROS products.

Taking into account of all these parameters, we applied this method to our epidermal oxidative stress in vitro model. We observed that glyphosate provoked ROS production in a dose dependent manner, which differs according to the 3 tested incubation times. Indeed, at similar cytotoxic levels, the short 0.5 h-treatment induced a more drastic oxidative phenomenon than a longer treatment (Fig. 2). We can therefore propose the hypothesis that cells need a minimal delay beyond 0.5 h to initiate antioxidant molecular responses through the numerous regulation pathways (Bickers and Athar, 2006).

Oxidative stress is one of the major biological causes of apoptosis. Annexin V/propidium iodide double staining is the most widely used methods to demonstrate apoptosis. A 0.5 h-incubation period allowed us to identify early cellular events. We observed firstly an increase in the number of early apoptotic cells for a low cytotoxicity level (15%), and then, secondly, a decrease, in favor of late apoptotic and necrotic cell rates for stronger cytotoxic conditions. Bearing this in mind, and in order to eventually initiate cell adaptation abilities toward glyphosate treatment, it is relevant to investigate apoptotic mechanisms under longer incubation time conditions. Apoptosis is known to be a dynamic, rapidly occurring event. Thus, longer incubation conditions should promote greater levels of late apoptotic and necrotic cells; Foldbjerg et al. (2009), who studied the mechanism of cell death caused by silver, showed that cells testing positive for apoptosis at an early time point (0.5 h) could be detected as necrotic cells after longer exposures. It has to be underlined that these authors amalgamated the two cell death processes, i.e. late apoptosis and necrosis. Contrary to those findings, our analysis of the flow cytometric apoptotic spectra distinguished three different cell death states: early apoptosis, late apoptosis and necrosis. As Foldbjerg et al. suggested it, in the context of glyphosate-induced stress, this dynamic is true only for apoptosis, with at first an early apoptotic cell rate that, afterwards, decreased in favor of late apoptotic cell emergence with time. But in contrast to Foldbjerg's report, we noticed a surprising simultaneous decrease of necrosis cell rate.

Nel et al. (2006) suggested a hierarchical oxidative stress model, in which low levels of ROS activate cellular defense mechanisms whereas high levels activate cell death. We have already envisaged that a short incubation (0.5 h), even at low cytotoxic conditions (20%) could cause a sudden increase of ROS production concomitant with an unready operational antioxidant defense system; we can speculate that such conditions might generate an intracellular ROS level exceeding a certain threshold, thus tipping the cells toward necrosis. By the same token, a long glyphosate exposure could allow the cell antioxidant responses to be engaged to prevent the breach of the critical threshold. In their review, Orrenius et al. (2011) postulated that chemical toxicity might be associated with multiple modes of cell death depending on the dose of a given toxic agent. Here, we demonstrated that the toxic-induced death pathway depended also on the time exposure. Overall, over longer HaCaT treatment periods and when the global keratinocyte population is considered, the cell divisional state might also favor better adaptation phenomena toward glyphosate.

Apoptosis signals occur through multiple independent pathways, but several of them converge on mitochondria which constitutes a critical event in the apoptotic process. The MTT assay suggests that glyphosate affects mitochondrial activity in a dose-dependent manner. Flow cytometry with JC-1 staining showed the disruption of mitochondrial membrane potential in the glyphosate-treated cells, indicating that the mitochondrial apoptotic pathway played a pivotal role in glyphosate-induced apoptosis of HaCaT cells. Indeed, the mitochondrial-dependent apoptosis pathway is activated by various intracellular mechanisms that enhanced the permeability of the mitochondrial membrane.

For comparable cytotoxic levels, the glyphosate treatment effects were more drastic for the mitochondrial membrane potential at short, rather than for longer incubation periods. Indeed, the impact was revealed at low cytotoxic conditions until a value of 45% for cytotoxicity was achieved. For longer incubation periods, the less spectacular mitochondrial potential decrease could appear ambiguous, but, on the contrary, this suggested that the cell antioxidant responses had time to be engaged.

In the same way, numbers of ROS positive cells increased earlier in the case of a 0.5 h incubation time (from 20% cytotoxicity). Nevertheless, we have to underline the slight shift between the mitochondrial membrane potential impairment and the ROS overproduction. This interesting observation could be explained through two ways. We can firstly hypothesize a difference of sensitivity between the methods performed for ROS measurement and those for mitochondrial membrane potential determination. We may secondly envisage that the mitochondrial membrane potential disruption signaling pathway could occur precociously, and that an excess in ROS production would be a consequence of the massive collapse of the mitochondrial potential. Moreover, it is believed that mitochondria are the major source of intracellular ROS, which play a key role in the induction and execution of apoptosis following application of various stress agents (Kumar et al., 2009). In their review, Ly et al. (2003) described the two possibilities of the apoptosis chronology, with a mitochondrial potential disruption as an apoptosis cause or a consequence. In our glyphosate-induced oxidative stress model, the mitochondrial membrane potential disruption could well be a cause of apoptosis.

In conclusion, the role of mitochondria in the glyphosate-induced cytotoxic pathway was highlighted, leading to ROS overproduction and resulting in oxidative stress, apoptosis and, in particular conditions, in necrosis. It is now apparent that the oxidative imbalance is not only glyphosate dose-dependent, but also directly related to the exposure time when short and long exposure periods are considered; this conceptual advance could concern many other environmental toxic agents.

For long exposure periods, while we noticed a more important ROS positive cell population after 18 h than after 6 h, our data did not point out any other differences between these two conditions. Glyphosate-induced oxidative stress

consequences seem to be similar between these two conditions. A conceivable explanation in this lack of differences would be that the oxidative consequences remain stabilized from 6 h-exposure.

This time dependence would require a deep investigation at the molecular scales of various cellular ways of death, wherein caspases represent the main mediators. However, caspases might also be involved in non-apoptotic signaling pathways (Lippens et al., 2009), pointing out the complexity of the cellular effects exerted by toxic chemicals (Li et al., 2010). Besides, the better knowledge of the molecular mechanisms of the glyphosate cytotoxicity in the human skin is inescapable to envisage a possible reversion of the deleterious effects.

Glyphosate-based formulations are the most widely used herbicides across the world and this agent constitutes a major environmental pollutant, which may initially impact human skin in its role of biological barrier. We were able here to consistently demonstrate the impact of low glyphosate doses on cutaneous healthy cells. Knowing the increase of the penetration of this chemical agent in the epidermal layer of skin even after slight abrasion (Nielsen et al., 2007), our results confirm the potential public health risk of this agent.

Conflict of interest statement

Nothing declared.

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