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Vitamins C and E reverse effect of herbicide-induced toxicity on human epidermal cells HaCaT: a biochemometric approach

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Abstract

The purpose of this study was to investigate and compare the cytotoxicity of glyphosate alone or included in Roundup 3 plus® modulated by the cytoprotective effects of additional antioxidants such as Vitamin C and Vitamin E on the human keratinocytes cell line HaCaT. An experimental design which allows to minimize the number of experiments was carried out to determine the optimal conditions for cytoprotection against herbicide-induced toxicity. It was shown that HaCaT cell line provides a useful model to study components with toxicity or antioxidant activity. Our results indicated that (i) glyphosate-based formulations can be responsible for oxidative damage to human epidermal cells, (ii) antioxidant compounds should be associated to herbicide formulations to decrease their deleterious effects on human skin. The use of an experimental design connected with the simplex method can be consider to be a fast technique to classify, with a limited number of experiments, the respective role of five parameters in the in vitro cytoprotection by antioxidants of herbicide-induced toxicity.

Keywords: Keratinocytes HaCaT; Experimental design; Cytotoxicity; Herbicide; Antioxidant activity

1. Introduction

Herbicides deserve particular attention since the general population is potentially exposed to such chemicals through many routes. Despite the obvious benefits of herbicide, their extensive use has posed a health risk to human (Delescluse et al., 1998; Jouan et al., 1998). Herbicides are often applied in combination with other active ingredients and/or surfactant. As well, glyphosate (*N*-phosphonomethylglycine) and Roundup 3 plus®, which is an aqueous solution of isopropylamine salt of glyphosate with a polyethoxylated tallow amine surfactant (POEA) added to enhance the efficacy of the herbicide, are nonselective herbicides

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that inhibit plant growth through interference with the production of essential aromatic amino acid. The adverse effects of glyphosate-based herbicides are periodically re-evaluated and the herbicide toxicity has been described for severe exposure conditions (Delescluse et al., 1998; Williams et al., 2000; Tsui and Chu, 2003). In that context, skin is one of the main potential portals of entry for herbicides (in case of dermal contact) and keratinocytes are certainly a good biological epidermal system for the evaluation of their cytotoxicity. The material obtained from primary keratinocyte culture is limited and may change with increasing numbers of passage. The use of the non-tumorigenic, spontaneously immortalized keratinocyte cell line HaCaT has the good advantage of providing an almost unlimited supply of identical cells, assuring high reproducibility; this cellular model was also selected because it possessed the enzyme equipment to bioactivate or detoxify xenobiotics (Delescluse et al., 1998), and it has been previously used to predict the coetaneous irritation of anionic surfactants (Wilhelm et al., 1994).

Numerous studies showed that antioxidant substances protect coetaneous cells against deleterious effects of environmental agents (as radiations or chemical products). Vitamin E (vit E) and Vitamin C (vit C) have been identified as nutrients. Trolox® is the commercial name for a water-soluble vit E analog which will be localized in aqueous cellular compartments exhibiting high antioxidant properties (Stewart et al., 1996). Vit C protects the skin through two separate mechanisms (i) it recycles α-tocopherol (vit E), the major antioxidant of biological membranes, from the tocopheroxyl free radical and, (ii) it acts directly as a scavenger of reactive oxygen and radical species. Vit C is the major water-soluble antioxidant found in the aqueous compartments of cells and extra-cellular fluids (Savini et al., 1999).

The purpose of this study was to investigate and compare the cytotoxicity of glyphosate alone or included in Roundup 3 plus® modulated by the cytoprotective effects of additional antioxidants such as vits C and E on the human keratinocyte cell line HaCaT. Using this approach, an experimental design which allows to minimize the number of experiments was carried out to determine the optimal conditions for cytoprotection against herbicide toxicity.

2. Materials and methods

2.1. Materials

Dubelcco's Modified Eagle's Minimum Essential Medium (DMEM), foetal calf serum (FCS), trypsin (0.25%) were from D. Dutscher (Brumath, France). *N*-(Phosphonomethyl)-glycine (glyphosate), Trolox®(6- hydroxy-2, 5, 7,8- tetramethylchroman- 2-carboxylic acid) (Vitamin E), ascorbic acid (Vitamin C), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dimethylsulfoxide (DMSO), *N* - (2- hydroxyethyl)piperazine- *N*′-(2- ethanesulfonic acid) (HEPES), were purchased from Sigma–Aldrich (Saint Quentin Fallavier, France). The herbicide Roundup 3 plus® (containing 21% (p/p) isopropylamine glyphosate salt (170 g/l), 8% (p/p) POEA and 71% (p/p) water and others minor ingredients) was from a commercial source, Monsanto (Paris, France) (Marc et al., 2002). Phosphate-buffered saline (PBS) without calcium and magnesium was purchased from VWR International (Cergy-Pontoise, France). The chemical structures of glyphosate, Vitamin C and Vitamin E were given in Fig. 1.

2.2. Cell culture

HaCaT, an immortalized human keratinocyte line was a generous gift from Nathalie Gault (Commissariat

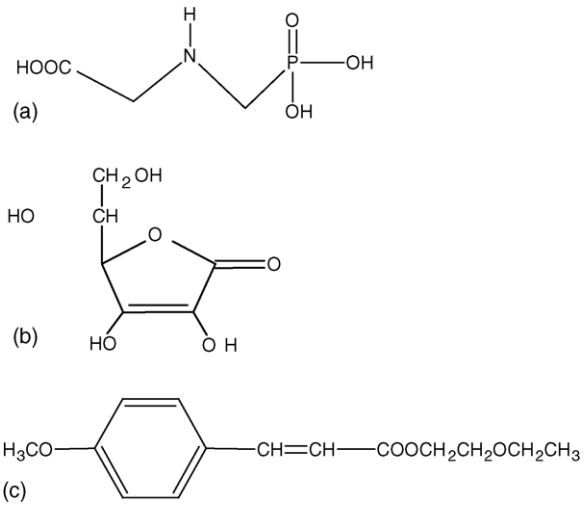

Fig. 1. The structure of glyphosate (a), Vitamin C (b) and Vitamin E (c).

à l'Energie Atomique, Bruyères Le Châtel, France) (Boukamp et al., 1988; Gault et al., 2002). Cells were cultured in DMEM medium supplemented with 10% (v/v) FCS, 1 M of HEPES in a humidified 5% CO_2 atmosphere at 37 °C. Cells were grown to confluence (from 0.7 to 1×10^6 cells/ml) in 75 cm^2 culture flasks (D. Dutscher, Brumath, France). The medium was removed every 48 h and cells were sub-cultured every 7 days. The cells were used at passages 3, 4 or 5 after thawing.

2.3. Treatment of cultures

HaCaT cells were seeded at a density from 4×10^4 to 6×10^4 cells per well in 100 μl culture medium containing 10% FCS on 96 multiwell culture plates and incubated overnight for adherence. The following day, the medium was removed and the cells were incubated in FCS-free medium containing concentrations of vit E and/or vit C (up to 200 μM). After the incubation period with antioxidant (up to 48 h), the medium was removed and the cells were incubated overnight in FCS-free medium containing increasing concentrations of glyphosate alone or included in Roundup 3 plus® (0–10–12.5–15–17.5–20–22 and 25 mM) and/or vit E and/or vit C (up to 200 μM). Each experiment was done twice, and each determination was done in triplicate.

2.4. Cytotoxicity assay

After the exposure period, the reaction medium was removed and the adhering cells were washed with PBS. 100 μl MTT solution (0.5 g/l in medium) was added to the culture wells. After incubation for 4 h at 37 °C, the MTT reaction medium was removed and formazan-blue was solubilised by 100 μl of DMSO. This assay was based on the reduction of the yellow tetrazolium salt MTT by the mitochondrial succinate dehydrogenase to form an insoluble formazan-blue product. Only viable cells with active mitochondria reduce significant amounts of MTT (Mosmann, 1983) and formazan-blue formation was quantified with a spectrophotometer at 570 nm. Values of absorbance were converted into percentage of residual viability. A curve of percentage of viability was represented according to the herbicide concentration. Two measurements (Y_m) have been selected: the inhibition concentration 50% (IC_{50}) which is the toxic concentration resulting in 50% cell death, and the slope of the part of the decreasing curve which directly depends to the kinetic of cell death.

2.5. Preliminary assays

Preliminary assays were necessary to determine the levels of the quantitative parameters such as herbicide and antioxidant concentrations. Herbicides and antioxidants were first tested on viability of HaCaT plated in 96-multiwell culture plates (6×10^4 cells per well). Twenty-four hours after plating, the medium was discarded and fresh medium containing the herbicides (0–10–12.5–15–17.5–20–22–25–30 and 35 mM), or the antioxidants (up to 1 mM) were added to cell cultures. After 24 h, cellular viability was determined by MTT test.

2.6. Chemometric methodology

As shown in Table 1, five parameters were finally evaluated: two qualitative parameters, herbicide formulation (x_1) and antioxidant substance (x_2), with respectively two levels (glyphosate alone and glyphosate included in Roundup 3 plus®) and three levels (vit C, vit E and vit C + vit E), and three quantitative parameters with three levels: antioxidant concentration (x_3)

Table 1
The five parameters (from x_1 to x_5) and the three levels (from 1 to 3) for each parameter

Parameter	Level		
	1	2	3
Herbicide formulation (x_1)	Glyphosate	Roundup 3 plus®	
Antioxidant substance (x_2)	Vit C	Vit E	Vit C + vit E
Antioxidant concentration (x_3) (μM)	0	100	200
Incubation period of antioxidant (x_4) (h)	0	24	48
Cell density (x_5)	4×10^4	5×10^4	6×10^4

Table 2
Second-order experimental design for five parameters (from x_1 to x_5), where each level was coded from 1 to 3: 18 experiments

Experiment number	x_1	x_2	x_3	x_4	x_5
1	1	1	1	1	1
2	1	1	2	2	2
3	1	1	3	3	3
4	1	2	1	1	2
5	1	2	2	2	3
6	1	2	3	3	1
7	1	3	1	2	1
8	1	3	2	3	2
9	1	3	3	1	3
10	2	1	1	3	3
11	2	1	2	1	1
12	2	1	3	2	2
13	2	2	1	2	3
14	2	2	2	3	1
15	2	2	3	1	2
16	2	3	1	3	2
17	2	3	2	1	3
18	2	3	3	2	1

x_1: herbicide formulation, x_2: antioxidant substance, x_3: antioxidant concentration (μM), x_4: incubation period of antioxidant (h), x_5: cell density.

at 0, 100 and 200 μM, incubation period of antioxidant (x_4) at 0, 24 and 48 h and seeded cell density (x_5) at 4, 5 and 6×10^4/100 μl. The range of parameters was chosen in relation with the preliminary assays. As opposed to the traditional method which would study separately each parameter which influences the cell viability, the chemometric approach was based on the use of a matrix of experiments (experimental design) by which the simultaneous variations of all parameters can be studied (Box and Wilson, 1955; Cochran and Cox, 1957; Fell et al., 1988). In Table 2, each level was coded to have a variation from 1 to 3. This significantly reduces the number of experiments, as compared with the traditional method. A two-order mathematical model was used which linked the calculated response Y_c, representing the IC_{50} value or the slope value, and the influencing factors (x_i):

$$\begin{aligned} Y_c = {} & a_0 + a_1x_1 + a_2x_2 + a_3x_3 + a_4x_4 + a_5x_5 \\ & + a_{12}x_1x_2 + a_{13}x_1x_3 + a_{14}x_1x_4 + a_{15}x_1x_5 \\ & + a_{23}x_2x_3 + a_{24}x_2x_4 + a_{25}x_2x_5 + a_{34}x_3x_4 \\ & + a_{35}x_3x_5 + a_{45}x_4x_5 + a_{11}x_1^2 + a_{22}x_2^2 \\ & + a_{33}x_3^2 + a_{44}x_4^2 + a_{55}x_5^2 \end{aligned} \quad (1)$$

The a_i, a_{ii} and a_{ij} are constants, which represent the effects of the parameters. The total number of experiments was 18.

2.7. Simplex optimization process

To optimize the mathematical model (Y_c) representing the combination with the highest value of IC_{50} (low toxicity) and the lowest value of slope (high kinetic of cell death) given by the experimental design, a Simplex method was used (Nelder and Mead, 1965; Baker, 1985; Carlin and Louis, 1996; Culioli, 1994). This way, the Y_c value was calculated for m sets of starting conditions where m represented the number of parameters to be optimized, plus one. The point corresponding to the optimal value of Y_c was then reflected in relation to the surface that was defined by the other points. This gave a new set of starting conditions. Once again, the point with the optimal value of Y_c was reflected and the process was repeated until the same conditions were obtained.

3. Results

3.1. Measurement of IC_{50} and slope of Roundup 3 plus® and glyphosate alone

The IC_{50} values of glyphosate alone and glyphosate included in Roundup 3 plus® on HaCaT cells were 22 mM and 19.5 mM respectively and their slope values were respectively −2.8 and −7.2 (Fig. 2). Therefore, these preliminary results suggested that Roundup 3 plus® was more toxic on epidermal cells than pure glyphosate. In the experimental design, seven concentrations of herbicides were finally assayed (10–12.5–15–17.5–20–22 and 25 mM) to determine the IC_{50} and slope variations.

3.2. Measurements of effects of vit C and vit E on HaCaT cell viability

Both vits C and E have been identified as antioxidant nutrients. In normal human keratinocyte cultures, supplementation of vits C and E did not affect HaCaT cell viability up to 1 mM (data not shown). This is in accordance with Shen et al. (2001) who reported that vits C and E did not induce cytotoxicity nor DNA

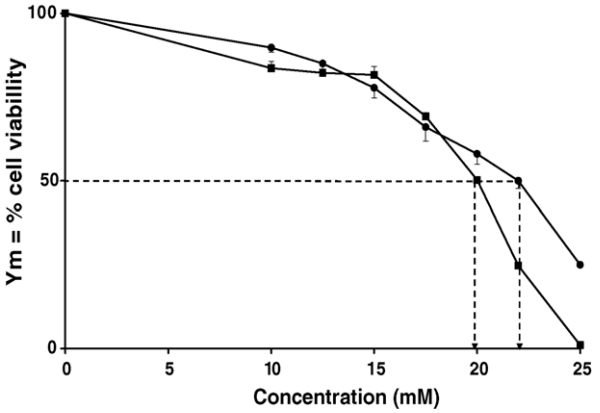

Fig. 2. Cytotoxicity profiles of glyphosate (●) and Roundup 3 plus® (■) in cultured HaCat cells after 24 h of treatment. The results expressed as percentage of controls, are given as means ± standard deviation of at least three independent experiments, each being performed in triplicate.

oxidative damage of primary human keratinocytes. For these reasons, the protective effects of these antioxidants at concentrations 100 and 200 μM were evaluated.

3.3. Experimental design

Using the chemometric methodology, the effects of the herbicide formulation (parameter x_1), the antioxidant substance (parameter x_2), the antioxidant concentration (parameter x_3), the incubation period of antioxidant (parameter x_4) and the cell density (parameter x_5) were studied on HaCaT cells, and the experimental Y_c values representing the IC_{50} or the slope values were determined. All experiments were repeated twice. The variance coefficients of the Y_c values were less than 4% in most cases, indicating high reproducibility and good stability for the system. The results (i.e., experimental Y_c values) were processed by computer and the parameters of Eq. (1) were obtained. The fitting of the model to the results was respectively 0.987 and 0.988 for the IC_{50} and the slope values. With two replicates, the Student's *t*-test, associated with the pooling statistical method (Cochran and Cox, 1957), was used to provide the basis for precision to determine the significance of the model coefficients: all variables were significant for both measurements (data not shown). The predicted (Y_c) and measured (Y_m) values for all experiments are given in Table 3 for IC_{50} and in Table 4 for

Table 3
Measured Y_m and calculated Y_c IC_{50} (inhibition concentration 50%) in 18 assayed conditions of the experimental design

Experiment number	x_1	x_2	x_3	x_4	x_5	IC_{50}		e (%)
						Y_m	Y_c	
1	Glyphosate	Vit C	0	0	4×10^4	22.5	23	2.17
2	Glyphosate	Vit C	100	24	5×10^4	20.6	21	1.90
3	Glyphosate	Vit C	200	48	6×10^4	23.9	24.2	1.23
4	Glyphosate	Vit E	0	0	5×10^4	23.7	23.9	0.83
5	Glyphosate	Vit E	100	24	6×10^4	23.6	23.9	1.25
6	Glyphosate	Vit E	200	48	4×10^4	21.6	21.8	0.91
7	Glyphosate	Vit C + vit E	0	24	4×10^4	21.4	20.9	2.39
8	Glyphosate	Vit C + vit E	100	48	5×10^4	21.3	21	1.42
9	Glyphosate	Vit C + vit E	200	0	6×10^4	19.0	19.7	3.55
10	Roundup	Vit C	0	48	6×10^4	17.6	18.2	3.29
11	Roundup	Vit C	100	0	4×10^4	18.1	18.9	4.23
12	Roundup	Vit C	200	24	5×10^4	16.9	17.3	2.31
13	Roundup	Vit E	0	24	6×10^4	16.7	17.1	2.33
14	Roundup	Vit E	100	48	4×10^4	16	16.8	4.76
15	Roundup	Vit E	200	0	5×10^4	17.6	17.9	1.67
16	Roundup	Vit C + vit E	0	48	5×10^4	16.9	17.3	2.31
17	Roundup	Vit C + vit E	100	0	6×10^4	21.8	21.2	2.83
18	Roundup	Vit C + vit E	200	24	4×10^4	16.7	17	1.76

x_1: herbicide formulation, x_2: antioxidant substance, x_3: antioxidant concentration (μM), x_4: incubation period of antioxidant (h), x_5: cell density.

Table 4
Measured Y_m and calculated Y_c slope in 18 assayed conditions of the experimental design

Experiment number	x_1	x_2	x_3	x_4	x_5	Slope		e (%)
						Y_m	Y_c	
1	Glyphosate	Vit C	0	0	4×10^4	−3.8	−3.7	2.63
2	Glyphosate	Vit C	100	24	5×10^4	−11.4	−11.2	1.75
3	Glyphosate	Vit C	200	48	6×10^4	−23.2	−23.8	2.58
4	Glyphosate	Vit E	0	0	5×10^4	−6.4	−6.3	1.56
5	Glyphosate	Vit E	100	24	6×10^4	−23.6	−24	1.66
6	Glyphosate	Vit E	200	48	4×10^4	−19.4	−19.8	2.06
7	Glyphosate	Vit C + vit E	0	24	4×10^4	−2.4	−2.5	4.0
8	Glyphosate	Vit C + vit E	100	48	5×10^4	−14.2	−13.9	2.11
9	Glyphosate	Vit C + vit E	200	0	6×10^4	−18.7	−18.8	0.53
10	Roundup	Vit C	0	48	6×10^4	−25.6	−25.4	0.78
11	Roundup	Vit C	100	0	4×10^4	−18.4	−18.3	0.54
12	Roundup	Vit C	200	24	5×10^4	−26.6	−26.7	0.37
13	Roundup	Vit E	0	24	6×10^4	−20	−19.7	1.5
14	Roundup	Vit E	100	48	4×10^4	−29.6	−30.2	2.02
15	Roundup	Vit E	200	0	5×10^4	−19.9	−20.2	1.48
16	Roundup	Vit C + vit E	0	48	5×10^4	−34.4	−33.9	1.45
17	Roundup	Vit C + vit E	100	0	6×10^4	−17	−16.8	1.17
18	Roundup	Vit C + vit E	200	24	4×10^4	−33	−32.7	0.90

x_1: herbicide formulation, x_2: antioxidant substance, x_3: antioxidant concentration (μM), x_4: incubation period of antioxidant (h), x_5: cell density.

the slope. Using Eq. (1), the Y_c values were calculated for the different values of the parameters, where the coefficients a_i, a_{ii}, a_{ij} represent the effects. The five parameters (from x_1 to x_5) were classified for IC_{50} and slope measurements. Fig. 2, which represents the effects of the parameters on the IC_{50} values, shows that: $x_1 \gg x_4 \sim x_5 \sim x_3 > x_2$. Fig. 3, which represents the effects on the slope values, shows a nearly similar classification ($x_1 \sim x_4 > x_3 > x_5 > x_2$) (see Fig. 4).

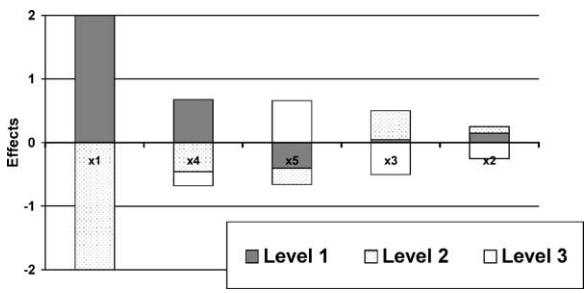

Fig. 3. Effects of the parameters on the IC_{50} (inhibition concentration 50%) values where x_1 represents the herbicide formulation (level 1: glyphosate; level 2: Roundup 3 plus®), and x_2 the antioxidant substance (level 1: Vitamin C; level 2: Vitamin E; level 3: Vitamin C + Vitamin E), x_3 the antioxidant concentration (μM) (level 1: 0; level 2: 100; level 3: 200), x_4 the incubation period of antioxidant (h) (level 1: 0; level 2: 24; level 3: 48), x_5 the cell density (level 1: 4×10^4; level 2: 5×10^4; level 3: 6×10^4).

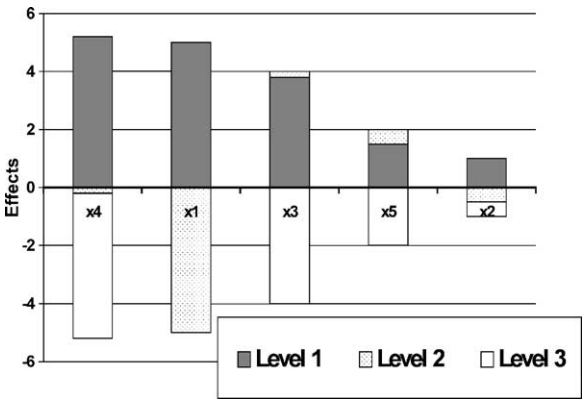

Fig. 4. Effects of the parameters on the slope values, where x_1 represents the herbicide formulation (level 1: glyphosate; level 2: Roundup 3 plus®), x_2 the antioxidant substance (level 1: Vitamin C; level 2: Vitamin E; level 3: Vitamin C + Vitamin E), x_3 the antioxidant concentration (μM) (level 1: 0; level 2: 100; level 3: 200), x_4 the incubation period of antioxidant (h) (level 1: 0; level 2: 24; level 3: 48), and x_5 the cell density (level 1: 4×10^4; level 2: 5×10^4; level 3: 6×10^4).

4. Discussion

Firstly, our data show that the parameter x_1 (herbicide formulation) was the most significant parameter; it means that IC_{50} and slope measurements depended directly on the herbicide formulation. It should be pointed out that the IC_{50} ranging obtained in glyphosate included in Roundup 3 plus® experiments was lower than IC_{50} ranging in glyphosate alone experiments (Fig. 2, Table 3). The difference between commercial glyphosate formulation and glyphosate alone is the inclusion of POEA and other minor ingredients which enhance the efficacy of the herbicide but, in the same time, may influence the cytotoxicity of the chemical. Therefore, either the formulation products of Roundup 3 plus® were directly responsible for a part of cytotoxicity (Martinez et al., 1990; Martinez and Brown, 1991; Wilhelm et al., 1994), or glyphosate and formulation products acted in synergy. Indeed, it has been recently shown that glyphosate and surfactants present in Roundup 3 plus® exerted synergic effects in cell cycle dysfunction in vivo (Marc et al., 2002). This supports the idea that the formulation products might favour the penetration of active ingredient, glyphosate, in the epidermal cells. Although Williams et al. (2000) reported that glyphosate absorption through human skin was very low, less than 2%, our data confirm that the presence of surfactants might increase the kinetic of intracellular passage of glyphosate probably by a mechanism resulting from interactions between cell membrane and surfactant.

Secondly, it appeared that the parameter x_2 (antioxidant substance) was the least significant parameter. As we expected under others reports (Clement-Lacroix et al., 1996; Shen et al., 2001; Kim et al., 2002), vit C and vit E did not induce cytotoxicity in cultured human keratinocytes, but surprisingly, the cytoprotective effect of associated vit C + vit E was lower than vit C or vit E alone, and the activities of vit C or vit E were similar (both scavenge some of radicals generated by cellular metabolism). The transport mechanisms of vit C in HaCaT cells have not yet been characterized, as they have been in other cells (Rumsey and Levine, 1998). However, Savini et al. (1999) suggested the presence of an active transport system saturable at 1 mM vit C; but they also pointed out the rapid decomposition of vit C in cell culture media (half-life: 0.9 h). In our work, the assayed concentrations of vit C were lower (100 and 200 μM), and so the cell line probably showed the greatest ability to maintain gradient of vit C across the plasma membrane. Vit E is the most significant physiologic membrane-associated antioxidant, but there are limitations to testing its role in the aqueous medium of cell culture; it would be interesting to compare the relative efficiency of water-soluble Trolox® versus lipophilic vit E in a human reconstituted epidermis or after loading in colloidal vectors like nanospheres.

Finally, it can be noted that the three quantitative experimental parameters (antioxidant concentration: x_3, incubation period of antioxidant: x_4, and cell density: x_5) are in an intermediate place, between the qualitative parameters (x_1 and x_2). Concerning the antioxidant dependent parameters, the quantitative parameters x_3 and x_4 were more significant than the qualitative parameter x_2. The pre-incubation of antioxidant, during 24 or 48 h before herbicide contact, did not modify the cytoprotection compared to simultaneous herbicide-antioxidant co-incubation. This contrasts with the results of Clement-Lacroix et al. (1996) which showed that the overnight pre-incubation of epidermal cells with vitamin E before irradiation developed a better cytoprotection against UVA-induced oxidative processes.

Using the Simplex method, 30 iterative processes were performed by the computer to calculate the optimal parameters values, which provide simultaneously a maximum IC_{50} value and a minimum slope value. These optimal values are, 6×10^4 cells per well and 42 h of pre-incubation with 75% Vitamin C–25% Vitamin E at the concentration of 190 μM were needed to modulate the toxic effect of Roundup 3 plus®.

In conclusion, although herbicide concentrations in the human environment are very low, our results indicated that (i) glyphosate-based formulations can be responsible for oxidative damage to human epidermal cells, (ii) antioxidant compounds should be associated to herbicide formulations to decrease their deleterious effects on human skin. Other measurements as induction of cytochromes P450 or cellular antioxidant status appeared to be much sensitive than cytotoxicity test for determining the biological effects of herbicides. However, the use of an experimental design connected with the Simplex method can be considered to be a fast technique to classify, with a limited number of experiments, the respective role of five parameters in the in vitro cytoprotection by antioxidant of herbicide-induced toxicity.

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