

# Chronic cypermethrin exposure alters mouse embryonic stem cell growth kinetics, induces Phase II detoxification response and affects pluripotency and differentiation gene expression

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## ABSTRACT

Worldwide uncontrolled use of synthetic pyrethroids contaminates water and soil leading to health hazards. Cypermethrin (CYP), the most used pyrethroid, induces detrimental effects on adults and embryos at different stages of development of several vertebrate species. In Mammals, CYP-induced alterations have been previously described in adult somatic cells and in post-implantation embryos. It remains unknown whether CYP has effects during pre-implantation development. Studies to assess pre-implantation embryo toxicity are complicated by the restricted number of blastocysts that may be obtained, either *in vivo* or *in vitro*. Embryonic stem cells (ESCs) are an *in vitro* model study that overcomes these limitations, as millions of pluripotent cells are available to the analysis. Also, ESCs maintain the same pluripotency characteristics and differentiation capacity of the inner cell mass (ICM) present in the blastocyst, from which they derive. In this work, using mouse R1 ESCs, we studied CYP-induced cell death, ROS production, the activation of oxidative stress-related and detoxification responses and the population growth kinetics following 72 h exposure at the 0.3 mM LD<sub>50</sub> dose. Also, the expression levels of pluripotency genes in exposed ESCs and of markers of the three germ layers after their differentiation into embryoid bodies (EBs) were determined. Two apoptotic waves were observed at 12–24 h and at 72 h. The increase of ROS production, at 24 h until the end of the culture period, was accompanied by the induction, at 48 h, of redox-related *Cat*, *Sod1*, *Sod2*, *Gpx1* and *Gpx4* genes. Up-regulation of *Cyp1b1*, but not of *Cyp1a1*, phase I gene was detected at 72 h and induction of *Nqo1*, *Gsta1* and *Ugt1a6* phase II genes began at 24 h exposure. The results show that exposed R1 ESCs activate oxidative stress-related and detoxification responses, although not sufficient, during the culture period tested, to warrant recovery of the growth rate observed in untreated cells. Also, CYP exposure altered the expression of *Oct-4* and *Nanog* pluripotency genes in ESCs and, when differentiated into EBs, the expression of *Fgf5*, *Brachyury* and *Foxa2*, early markers of the ectoderm, mesoderm and endoderm germ layers, respectively. NIH/3T3 cells, a differentiated cell line of embryonic origin, were used for comparison.

**Key words:** Cypermethrin; embryonic stem cells; cell growth; apoptosis; reactive oxygen species; detoxification response.

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## Introduction

Synthetic pyrethroids are the most worldwide diffused pesticides, largely used since the '80s in households and agriculture<sup>1</sup> because of their effectiveness and low toxicity compared to other insecticides, such as organophosphorus and carbamic ester compounds.<sup>2,3</sup> As a result of their diffusion and uncontrolled use, pyrethroids, by contaminating water and soil, have entered the food chain<sup>3,4,7</sup> becoming a serious human health hazard.<sup>8</sup> Cypermethrin (CYP), a type II pyrethroid insecticide, classified by the World Health Organization as moderately hazardous (class II), is the most widely used against a broad range of insects.<sup>9-11</sup>

CYP detrimental effects have been described on adults and during embryonic development of several vertebrate species. Specifically, earlier studies demonstrated that CYP generates oxidative stress and apoptosis in fish liver,<sup>12-14</sup> retina,<sup>15</sup> kidney<sup>12</sup> and gill,<sup>16</sup> rat brain and liver<sup>17</sup> cells and also in mouse macrophages.<sup>18</sup> In the mouse, by impairing endometrial receptivity, CYP severely reduces embryo implantation.<sup>19</sup>

It remains however unknown whether CYP has effects during pre-implantation, a developmental window with three main critical steps. The first occurs at the time of embryonic genome activation (2-cell or 4-cell stage in mouse and human, respectively)<sup>20</sup>, the second when the embryo reaches the morula stage,<sup>20</sup> followed by the most critical at the blastocyst stage, when, in our species, the great majority of embryos is lost.<sup>21</sup> Pre-implantation embryonic loss is either a consequence of a functional damage to the endometrium or to the cell lines that make up the blastocyst, *i.e.*, the trophectoderm or the inner cell mass (ICM), which will contribute, respectively, to the extra-embryonic tissue (*e.g.*, placenta) or to the foetus. A damage to the ICM cells, when not selective against the embryo, could, however, cause serious damage to the foetus or appear much later in development, childhood or adulthood.

Despite the blastocyst being one of the most vulnerable developmental stage in the life of a new individual, studies to access the embryo-toxicity of test substances, performed on model animals, are complicated by the required experimental setting and the restricted number of blastocysts that may be obtained, either *in vivo* or *in vitro*, for each single experiment. To this regard, embryonic stem cells (ESCs) are an *in vitro* model study that overcomes these limitations, as for each experiment millions of pluripotent cells are available to the analysis of the test substance. ESCs maintain the same pluripotency characteristics and differentiation capacity of the ICM from which they derive.<sup>22-24</sup>

In this work, using mouse ESCs (mESCs) as an *in vitro* model of the blastocyst's ICM, we studied CYP-induced alterations that, in Mammals, have been previously described in adult somatic cells and in post-implantation embryos. Specifically, cell growth, cell death, ROS production and the activation of a detoxification response were analysed in mESCs and NIH/3T3 cells, the latter, a differentiated cell line of embryonic origin, used for comparison. Also, the differentiation potential of exposed mESCs was determined through their development into embryoid bodies (EBs).

## Materials and Methods

### Cells and cell cultures

The R1 mESC line (kindly provided by Dr. Nagy from Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada) was cultivated as reported in Rebuzzini *et al.*<sup>25</sup> Briefly, cells were grown in Knockout DMEM supplemented with 15% ESC Qualified FBS (Thermo Fisher Scientific, Waltham,

MA, USA), 2 mM L-glutamine, 1x non-essential amino acids, 0.5% penicillin/streptomycin (all from Life Technology, Carlsbad, CA, USA), 0.1 mM  $\beta$ -mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA) and 500 U/ml ESGRO-LIF (Millipore, Burlington, MA, USA). The STO cell line (American Type Culture Collection CRL-2225), used as feeder-layer, was maintained in DMEM (Sigma-Aldrich) supplemented with 10% FBS, 4 mM L-glutamine, 1x non-essential amino acids, 0.5% penicillin/streptomycin (all from Life Technology), 0.1 mM  $\beta$ -mercaptoethanol (Sigma-Aldrich) and 0.2 mg/mL geneticin (Sigma-Aldrich).

Cells were routinely passaged with 0.25% trypsin every 2-3 days, alternating a passage on STO feeder cells with two passages on gelatin-coated 100×20 mm Petri dishes.

The NIH/3T3 cell line, hereafter 3T3 (a generous gift by Prof. Giulotto, Department of Biology and Biotechnology, University of Pavia), was cultured in DMEM (Sigma-Aldrich) supplemented with 10% FBS, 4 mM L-glutamine, 1x non-essential amino acids, 0.5% penicillin/streptomycin (all from Life Technology) and 0.1 mM  $\beta$ -mercaptoethanol (Sigma-Aldrich). Cells were routinely passaged with 0.25% trypsin every 2-3 days.

### Cypermethrin preparation

$\alpha$ -cypermethrin [ $\alpha$ -Cyano-(3-phenoxyphenyl)-methyl 3-(2,2-dichlorovinyl)-2,2-dimethyl cyclopropane carboxylate] (Abcam, Cambridge, UK) was dissolved to a final concentration of 100 mM in dimethyl sulfoxide (DMSO). The solution was maintained at -20°C and renewed every 7 days, to ensure the stability of the molecule, according to manufacturer instructions.

### Determination of CYP LD<sub>50</sub> dose

CYP toxicity was carried out by measuring the alteration of growth of a massive cell culture, as described by Rebuzzini *et al.*<sup>26</sup> Briefly,  $3 \times 10^4$  R1 and 3T3 cells were seeded in 6 cm Petri dish (Corning) and exposed to increasing concentrations of CYP (0.01, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 or 1 mM) or to 0.01% DMSO (Control, CTR). After 72 h, the cells were collected in PBS 1x, pelleted at 1200 rpm for 8 min, lysed in 0.1 M NaOH and incubated at 50°C in water bath for 30 min. The number of cells was estimated by measuring the absorbance of the lysate at 260 nm.

### Cell growth curve determination

R1 and 3T3 cells were seeded at a density of  $5 \times 10^4$  and  $8 \times 10^4$ , respectively, and incubated with the determined CYP LD<sub>50</sub> concentration for 72 h. Cells were harvested after 8, 24, 48 and 72 h, and counted using a Bürker chamber.

### Apoptosis Annexin V assay

The ApopNexin™ FITC and propidium iodide (PI) kit assay (Merck Millipore) was used to detect apoptosis after CYP treatment. Briefly, 3T3 ( $1.5 \times 10^4$  cells) and R1 ( $10^5$  cells) were seeded in 6 cm Petri dishes and exposed to 0.6 mM and 0.3 mM CYP, respectively, for 8 (only 3T3), 12 (only R1), 24, 48 and 72 h. Cells were harvested according to the manufacturer instructions and analysed, after staining, by bivariate flow cytometry [FITC band in FL1 and PI band in the FL3 channel, flow cytometer FACS Lyric BD (BD Biosciences, Franklin Lakes, NJ, USA)]. A minimum of 50,000 events was acquired for each sample. Data were processed, plotted and analysed using the BD FACSuite™ flow cytometer software.

### Reactive oxygen species quantification

The cell-permeant 2',7'-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA) fluorescent probe (Thermo Fisher Scientific) was used to evaluate the intracellular levels of reactive oxygen species. R1 ( $10^5$  cells) and fibroblast 3T3 ( $2 \times 10^5$  cells) were seeded in 10

cm Petri dishes and exposed to 0.3 mM or 0.6 mM CYP, respectively, for 8 (3T3 only), 12 (R1 only), 24, 48 and 72 h (both cell lines). Then, cells were collected, centrifuged at 500 rpm for 5 min and resuspended in 1x PBS solution containing 10  $\mu$ M H<sub>2</sub>DCFDA. The mixture was incubated at 37°C for 30 min under dark conditions. The analyses of the samples were performed using a flow cytometer FACS Lyric BD (BD Biosciences). A minimum of 50,000 events were acquired for each sample. Data were processed, plotted and analysed using the BD FACSuite™ flow cytometer software.

### Cell cycle analysis

R1 (10<sup>5</sup> cells) and fibroblast 3T3 (2×10<sup>5</sup> cells) were seeded in 10 cm Petri dishes and exposed to 0.3 mM and 0.6 mM CYP, respectively. After 8 (3T3), 12 (R1), 24, 48 and 72 h, the cells were harvested, washed in 1x PBS and stained with 1.5 ml of 50  $\mu$ g/ml propidium iodide (PI), containing 100 U/ml of RNase and 0.05% Igepal (Sigma-Aldrich).

The analyses of the samples were performed using a flow cytometer FACS Lyric BD (BD Biosciences). A minimum of 50,000 events were acquired for each sample. Data were processed, plotted and analysed using the BD FACSuite™ flow cytometer software.

### Embryoid bodies formation

CTR and CYP-exposed ESCs were differentiated into EBs, following culture in the absence of LIF, using the hanging drop protocol.<sup>27</sup> After 3 days of culture, EBs were transferred into a 100 mm 0.1% agarose-coated Petri dish (Corning); after 2 more days, RNA was collected from about 100 EBs.

### RNA extraction, reverse transcription and real-time PCR

Total RNA was extracted from R1 (10<sup>5</sup> cells) and 3T3 (2×10<sup>5</sup> cells) cells after 8 (3T3), 12 (R1), 24, 48 and 72 h of CYP exposure and from EBs at the end of the differentiation period (5 days), using GenElute Mammalian Total RNA kit (Sigma-Aldrich) according to the manufacturer's instructions. Reverse transcription was performed in a final volume of 20  $\mu$ l reaction mixture containing 1  $\mu$ g of RNA, 1x PCR buffer, 5 mM MgCl<sub>2</sub>, 4 mM of each dNTP, 0.625  $\mu$ M oligo d(T)<sub>16</sub>, 1.875  $\mu$ M Random Hexamers, 20 U RNase inhibitor and 50 U MuLV reverse transcriptase (Thermo Fisher Scientific). The amplification programme was as follows: 25°C for 10 s, 42°C for 15 s, 99°C for 5 s.

One twentieth of the resulting cDNA was amplified in dupli-

cate by real-time PCR in 20  $\mu$ L reaction mixture with 200 nM of each primer (designed with Primer 3 software; Supplementary Table 1) and MESA GREEN qPCR MasterMix Plus for SYBR assay no ROX sample (Eurogentec, Liege, Belgium) at 1x as final concentration. The amplification reaction, performed in a Rotorgene 6000 (Corbett Life Science, Sydney, Australia), for the analysis of catalase (*Cat*), superoxide dismutase 1 and 2 (*Sod1*, *Sod2*), glutathione peroxidase 1 and 4 (*Gpx1*, *Gpx4*), cytochrome 1a1 (*Cyp1a1*), octamer-binding transcription factor 4 (*Oct-4*), homeobox protein NANOG (*Nanog*), T-Box Transcription Factor T (*Brachyury*), fibroblast growth factor 5 (*Fgf5*) and forkhead box protein A2 (*Foxa2*) transcripts expression was as follows: 95°C for 5 min, followed by 40 cycles at 95°C for 10 s, 60°C for 15 s, 72°C for 20 s. The amplification reaction for the analysis of cytochrome 1b1 (*Cyp1b1*), NAD(P)H quinone dehydrogenase (*Nqo1*), glutathione S-transferase alpha 1 (*Gsta1*) and UDP-glucuronosyltransferase 1 a 6 (*Ugt1a6*) transcripts expression was as follows: 95°C for 5 min, followed by 40 cycles at 95°C for 15 s, 60°C for 20 s, 72°C for 60 s.

The Rotorgene 6000 Series Software 1.7 was used for the comparative concentration analysis.  $\beta$ -2-microglobulin ( $\beta$ 2m) gene expression was used for sample normalization.<sup>28</sup>

### Statistical analysis

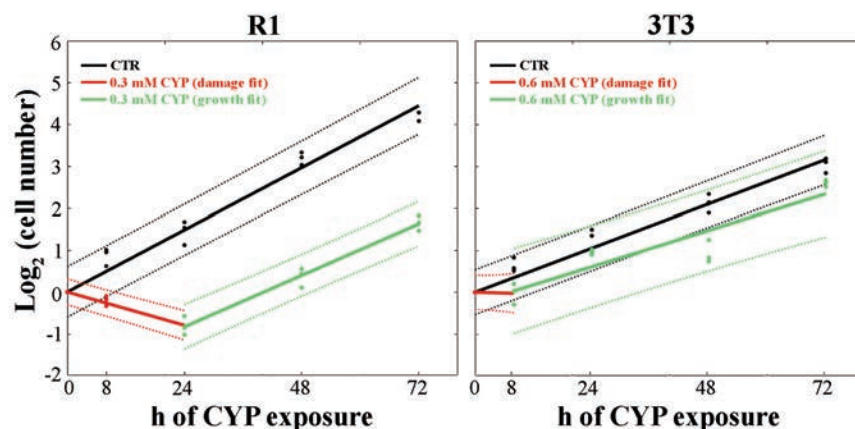
For each assay, three independent experiments were performed. The results were analysed using SigmaStat software. Data, expressed as mean  $\pm$  SD, were analysed by the one-way ANOVA and by the *post-hoc* LSD test.

## Results

We first determined the CYP dose that reduced growth of 50% (LD<sub>50</sub>); the LD<sub>50</sub> was 0.3 mM and 0.6 mM, for mouse R1 ESCs (R1) and 3T3, respectively (Supplementary Figure 1); all the experiments described below were performed at these specific doses. The presence of the DMSO vehicle did not significantly alter cell growth of both cell lines ( $P > 0.05$ ) (*data not shown*).

### Cell growth kinetics

R1 and 3T3 cell lines displayed different growth dynamics. A significant ( $P < 0.003$ ) decrease of the R1 cell number was observed up to 24 h following CYP exposure (Figure 1). Then, exposed cells



**Figure 1.** Fitted growth curves of CTR and CYP-exposed cells of R1 and 3T3 lines. Circles represent the experimental data; dotted lines are the upper and lower percentage bounds (95%).

grew, although at lower rate compared to CTR, as evidenced by the significant reduction ( $P=0.008$ ) of the regression slope (Figure 1); the population doubling (PD) time was higher in exposed (19.52 h) compared to that of CTR (16.21 h, as also shown earlier by Tamm *et al.*<sup>29</sup>) cells. In 3T3, the number of exposed cells significantly decreased ( $P=0.024$ ), compared to CTR, within 8 h of culture (Figure 1). Then, CTR and treated 3T3 cells grew at the same rate ( $P=0.133$ , comparison of the regression slopes, Figure 1) with similar PD time (22.78 h and 27.70 h, respectively). This PD time agrees with previous published data (<http://www.nih3t3.com/>) for untreated cells.

To understand whether the decrease in cell number observed was due to apoptosis and/or to alterations in the progression through the cell cycle stages, the Annexin V assay and the nuclear DNA content evaluation were used, respectively.

### Apoptosis

Figure 2 reports the fraction of R1 and 3T3 cells positive to Annexin V (AnV<sup>+</sup> early apoptotic marker), Annexin V and propidium iodide (AnV<sup>+</sup>/PI<sup>+</sup>, late apoptotic marker), or propidium iodide (PI<sup>+</sup>, cell death marker) after CYP exposure, expressed as fold change relative to CTR set at 1.

AnV<sup>+</sup> and AnV<sup>+</sup>/PI<sup>+</sup> R1 cells significantly increased after both 12 h (3.2- and 2.4-fold, respectively;  $P<0.05$ ) and 24 h (1.6- and 2.2-fold, respectively;  $P<0.05$ ) exposure, when compared to the CTR population (Figure 2A). At 48 h, only apoptotic PI<sup>+</sup> cells were detected, with a 1.4 fold-increase ( $P<0.05$ ). A second relevant wave of apoptosis was recorded after 72 h exposure with a 7.7-fold and a 5.3-fold increase of AnV<sup>+</sup> and AnV<sup>+</sup>/PI<sup>+</sup> cells, respectively (Figure 2A).

After 8 h exposure, AnV<sup>+</sup> and AnV<sup>+</sup>/PI<sup>+</sup> 3T3 cells increased (1.5 and 1.7-fold, respectively,  $P<0.001$ ), while PI<sup>+</sup> 3T3 cells decreased (0.6-fold,  $P<0.05$ ) compared to CTR (Figure 2B). After 24 h, a further increase ( $P<0.05$ ) up to 2.2- and 2.1-fold for AnV<sup>+</sup>

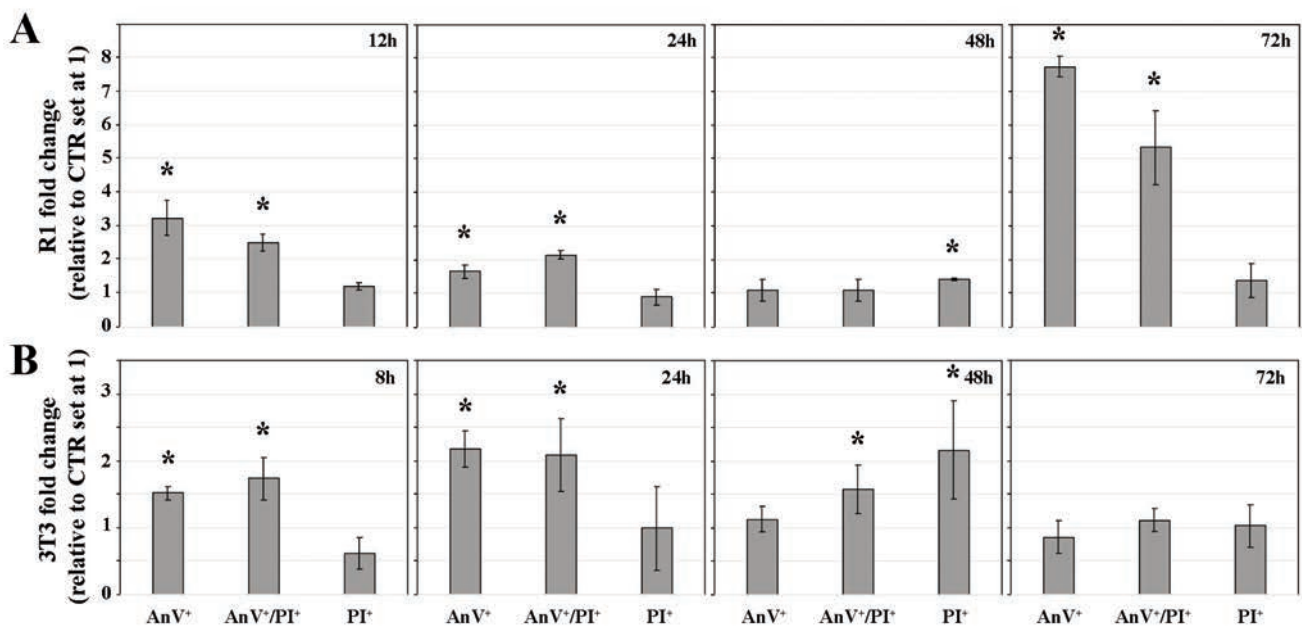
and AnV<sup>+</sup>/PI<sup>+</sup> cells, respectively, was observed, whereas PI<sup>+</sup> cells were not significantly different ( $P>0.05$ ) when compared to CTR. A peak (2.2-fold increase,  $P<0.05$ ) of cell death was observed after 48 h, whereas at 72 h exposed cells completely recovered, showing the same degree of early or late apoptosis as that of CTR ( $P>0.05$ ) (Figure 2B).

### Distribution of cells in the cell cycle phases

Next, the nuclear DNA content during the different cell cycle phases was evaluated using a one-parametric analysis of PI-stained nuclei.

In R1 CTR cells, the recorded distribution in G<sub>0</sub>/G<sub>1</sub> (32%), in S (54%) and in G<sub>2</sub>/M (14%) phases confirms previous findings of our and other groups.<sup>30,31</sup> When exposed to CYP, the frequency of R1 cells distributed within the three phases of the cell cycle remained unaltered up to 24 h (Supplementary Table 2). At 48 and 72 h, the frequency of cells in G<sub>0</sub>/G<sub>1</sub> significantly decreased ( $P<0.001$ ), in association with a significant increase of cells in G<sub>2</sub>/M phase (Supplementary Table 2), suggesting an activation of a G<sub>2</sub>/M cell cycle block at both time points.

About 67% of unexposed 3T3 cells were in G<sub>0</sub>/G<sub>1</sub> phase, 14% in S phase and 18% in G<sub>2</sub>/M phase (Supplementary Table 2 and Tai *et al.*<sup>32</sup>). After 8 and 24 h of CYP exposure the distribution of cells in the different phases of the cell cycle remained unaltered compared to CTR (Supplementary Table 2), instead, at 48 h, the frequency of cells in G<sub>0</sub>/G<sub>1</sub> increased ( $P<0.001$ ) while the frequency of cells in S and in G<sub>2</sub>-phase significantly decreased ( $P<0.001$ ) (Supplementary Table 2), suggesting an activation of a G<sub>0</sub>/G<sub>1</sub> block of the cell cycle. The distribution of cells in the three phases returns not significantly different compared to CTR after 72 h exposure. Then, we determined whether reactive oxygen species (ROS) are induced by CYP. Also, we evaluated whether an antioxidant cellular response is triggered, by analysing the expression of *Cat*, *Sod1*, *Sod2*, *Gpx1* and *Gpx4* genes, which code for major



**Figure 2.** Annexin V positive (AnV<sup>+</sup>), Annexin V and propidium iodide positive (AnV<sup>+</sup>/PI<sup>+</sup>) and propidium iodide positive (PI<sup>+</sup>) cells in R1 (A) and 3T3 (B) cell lines, detected 8 (3T3), 12 (R1), 24, 48 or 72 h after CYP exposure. \* $P<0.001$ .

antioxidant proteins against ROS.

### ROS production and redox-related gene expression response

Exposed R1 cells showed 1.2-fold increase ( $P < 0.05$ ) in ROS production beginning at 24 h culture. ROS production continues to increase after both 48 and 72 h (1.3 and 1.6 fold, respectively,  $P < 0.05$ ) (Figure 3A). In exposed R1 cells, ROS production was paralleled by the induction ( $P < 0.001$ ) of *Cat*, *Sod1*, *Sod2*, and *Gpx4* gene expression, at 48 h culture, and also of *Gpx1* ( $P < 0.05$ ) at 72 h (Figure 3B and Supplementary Table 3).

Compared to CTR, exposed 3T3 cells displayed higher levels of ROS at 24 h and 48 h (1.3- and 1.2-fold, respectively;  $P < 0.05$ ) (Figure 3A), but not at 8 h and 72 h. Of the five gene transcripts (*Cat*, *Sod1*, *Sod2*, *Gpx1* and *Gpx4*) analysed, no difference between CTR and exposed cells was detected within 8 h, whereas, after 24 h, cells displayed significant increase ( $P < 0.05$ ) of *Cat* expression and slight, but significant, decrease ( $P < 0.05$ ) of *Gpx1* and *Sod2* transcripts. *Sod2* transcripts remained low in exposed cells after 48 h culture. At 72 h, slight ( $P < 0.05$ ) *Cat* and *Gpx4* induction was detected (Figure 3B and Supplementary Table 4).

To determine if the detoxification mechanisms, known to be involved in the processes of elimination of environmental toxicants,<sup>33</sup> were activated in response to CYP exposure, the expression profile of cytochromes P450 *Cyp1a1* and *Cyp1b1* (AHR-regulated of phase I) and *Nqo1*, *Gst1a* and *Ugt1a6* (AHR-regulated of phase II) enzymes was evaluated.

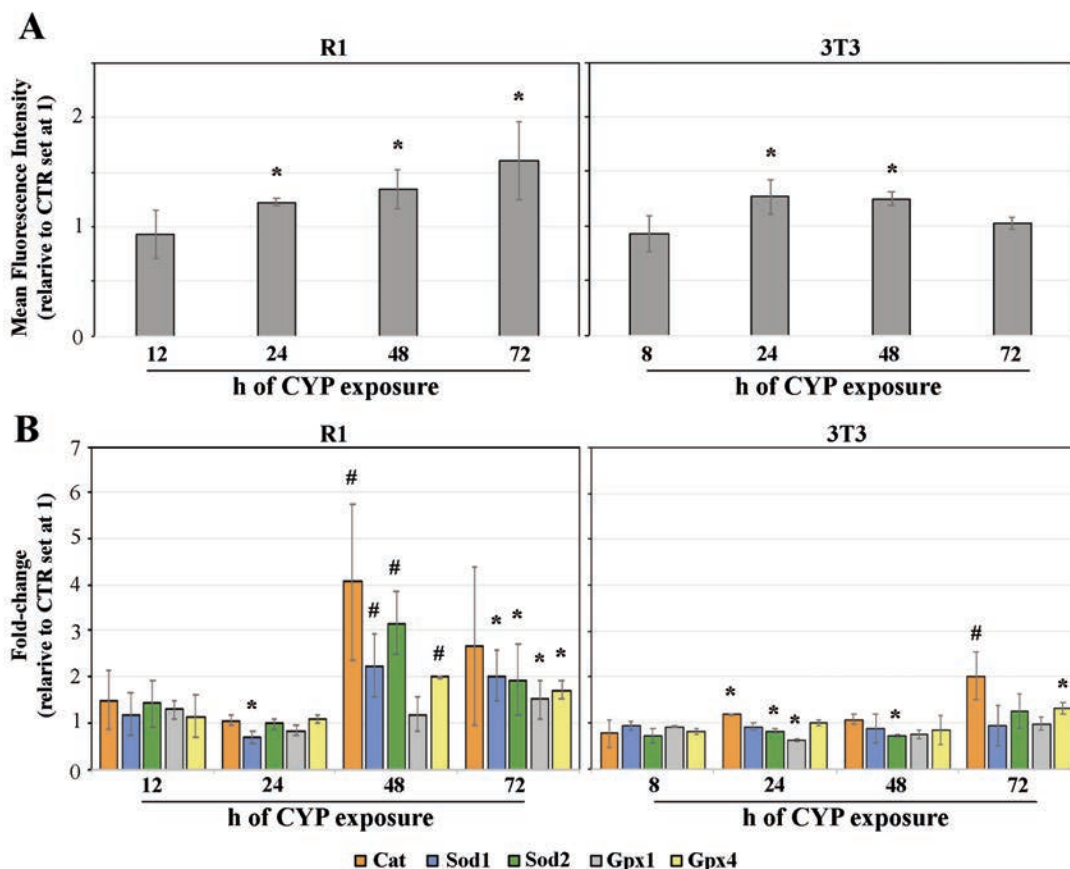
### Phase I and phase II gene expression response

In exposed R1 mESCs, of the two Phase I genes, the level of *Cyp1a1* transcripts was similar to CTR at 12 and 72 h whereas it was lower ( $P < 0.05$ ) at both 24 and 48 h; *Cyp1b1* was down regulated ( $P < 0.05$ ) at 24 and 48 h but up regulated ( $P < 0.001$ ) at 72 h culture. *Ugt1a6* Phase II gene was initially down regulated ( $P < 0.05$ ), but then it was up regulated ( $P < 0.001$ ) from 24 h until the end of the culture period. Also up regulated ( $P < 0.001$ ) were *Gst1a* (from 48 h) and *Nqo1* (at 72 h) (Figure 4 and Supplementary Table 3).

Compared to CTR, of the five genes analysed, three were upregulated in 3T3 exposed cells: *Cyp1a1* ( $P < 0.05$ ) at 24 h; *Ugt1a6* ( $P < 0.001$ ) from 24 h, *Gst1a* ( $P < 0.001$ ) from 48 h. The expression of the other two genes (*Cyp1b1* and *Nqo1*) remained unaltered ( $P > 0.01$ ) (Figure 4 and Supplementary Table 4).

### Pluripotency and germ cell layers gene expression

When R1 mESCs were exposed to CYP for 12 and 24 h, they displayed a significant reduction ( $P < 0.001$ ) of *Oct-4* and *Nanog* expression compared to CTR. To the contrary, after 72 h exposure, for *Oct-4*, or 48 h and 72 h exposure, for *Nanog*, the expression significantly increases ( $P < 0.001$ ) (Figure 5A and Supplementary Table 5). The altered expression of two genes of the pluripotency core might influence the differentiation capacity of exposed R1 ESCs. To test whether CYP-exposed mESCs maintain their ability to form the three germ layers, they were differentiated into EBs for



**Figure 3.** A) ROS content in R1 and 3T3 cell populations. B) Expression profile of *Cat*, *Sod1* and 2 and of *Gpx1* and 4 ROS redox-response genes, at different time points in the presence of CYP. The expression values of CTR samples were set at 1 for the calculation of the n-fold change. Values are expressed as mean  $\pm$  SD. \* $P < 0.05$ ; # $P < 0.001$ .

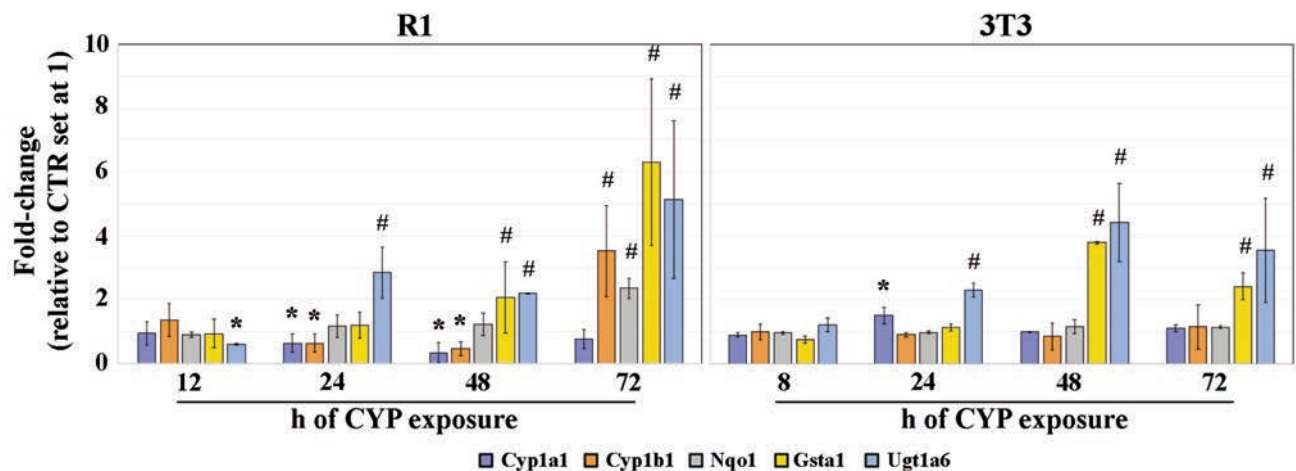
5 days. As expected, *Fgf5*, *Brachyury*, and *Foxa2* (markers of the ectoderm, mesoderm and endoderm, respectively) were highly induced in CTR EBs when compared to undifferentiated mESCs (2.3, 60.0 and 5.9-fold change, respectively; *data not shown*).

CYP-exposed ESCs maintain the ability to form EBs, but displayed altered expression of the markers of three germ layers. Specifically, the expression of *Fgf5* in EBs obtained from ESCs exposed to CYP for 12 h and 72 h is similar to CTR EBs (Figure 5B; Table 6S), whereas it is reduced ( $P<0.001$ ) in EBs obtained from ESCs exposed to CYP for 24 and 48 h. The number of *Brachyury* gene transcripts is significant altered ( $P<0.001$ ), either lower or higher (Figure 5B; Supplementary Table 6), in EBs derived from mESCs exposed to CYP when compared to CTR EBs. *Foxa2* gene transcripts are significantly ( $P<0.001$ ) overexpressed in EBs obtained from CYP-exposed ESCs for 12, 48 and 72 h (Figure 5B and Table 6S).

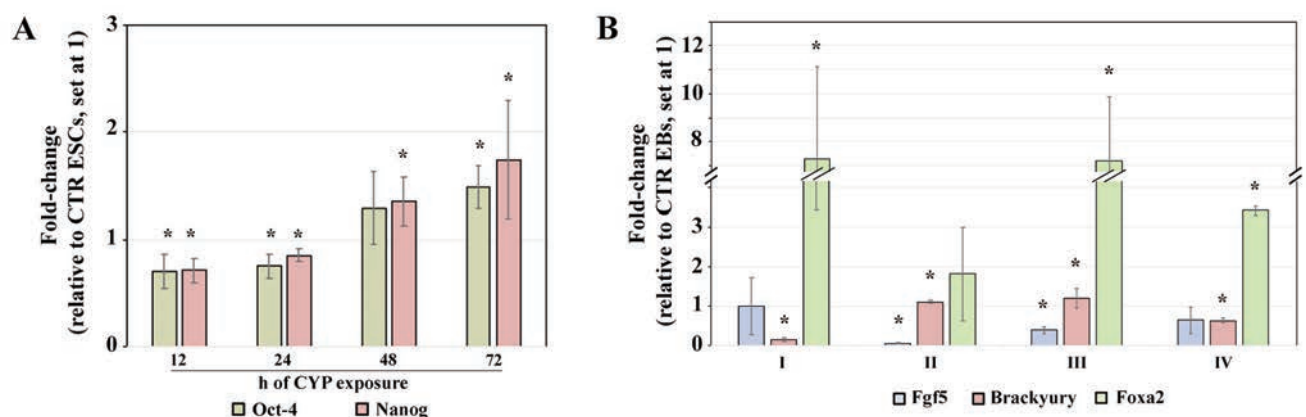
## Discussion

The results of this study highlight three main responses of ESCs following exposure to the pyrethroid insecticide cypermethrin: alterations of the i) cell growth kinetics; ii) phase II detoxification genes induction; and iii) expression of the pluripotency and the three germ layers gene markers.

Compared to control, CYP-exposed R1 ESCs display a massive 45% decrease in number during the first 24 h, followed by proliferation resumption during the remaining culture period. This resumption is characterized by significant longer PD time which contributes to explain the slower growth rate. The activation of a first apoptotic wave, observed soon after 12-24 h exposure, might represent a short-term response, perhaps needed for the elimination of highly damaged cells from the population. It is well known that mESCs undertake facilitated cell death<sup>34,35</sup> because of the lack of checkpoints controlling the G<sub>1</sub>-S and the intra-S<sup>30,34,36</sup> transition



**Figure 4.** Expression profile of *Cyp1a1*, *Cyp1b1* (Phase I), *Nqo1*, *Gst1* and *Ugt1a6* (Phase II) detoxification genes, at different time points in the presence of CYP. The expression values of CTR samples were set at 1 for the calculation of the n-fold change. Values are expressed as mean  $\pm$  SD. \* $P<0.05$ ; # $P<0.001$ .



**Figure 5.** A) Expression profile of *Oct-4* and *Nanog* pluripotency marker genes of ESCs exposed for 12, 24, 48 and 72 h to CYP; the expression values of CTR ESCs were set at 1 for the calculation of the n-fold change. B) Expression profile of *Fgf5*, *Brachyury* and *Foxa2*, early gene markers of the three germ layers, of EBs obtained from ESCs exposed to CYP for 12 (I), 24 (II), 48 (III) or 72 (IV) h. The expression values of CTR EBs were set at 1 for the calculation of the n-fold change.

phases. A second wave of CYP-induced apoptosis is observed at 72 h, when ROS production is further increased, contributing to the elimination of cells arrested in G<sub>2</sub>/M phase at 48 h and 72 h exposure, likely those with unreparable damages. The increase of the production of ROS is also accompanied by the induction, beginning at 48 h CYP treatment, of redox-related *Cat*, *Sod1*, *Sod2*, *Gpx1* and *Gpx4* genes.

In parallel, CYP exposure also triggers detoxification endogenous mechanisms,<sup>37,38</sup> with the up-regulation of only one, *Cyp1b*, of the two phase I genes studied and of phase II *Nqo1*, *Gst1* and *Ugt1a6* genes. Interestingly, phase II induction occurs earlier (beginning at 24 h) than that of phase I genes (at 72 h). These results highlight a major role, in pluripotent R1 ESCs, of phase II genes in the detoxification of pyrethroids, including  $\beta$ -cypermethrin, as shown earlier in human hepatocytes<sup>39</sup> and in zebrafish embryos.<sup>40</sup> In addition to *Cyp1b1*, we anticipated that the mechanism of detoxification of pyrethroids in undifferentiated stem cells might be mediated by members of the cytochrome P450 family other than *Cyp1a1*, such as *Cyp9a10*.<sup>41</sup>

Interestingly, CYP exposure altered the expression of *Oct-4* and *Nanog* pluripotency genes in ESCs and, when differentiated into EBs, the expression of *Fgf5*, *Brachyury*, and *Foxa2*, early markers of the ectoderm, mesoderm and endoderm germ layers, respectively. Our results highlight, for the first time, that pyrethroids might be able to perturb the inherent characteristics of pluripotency and differentiation capacity of ESCs.

Compared to R1 ESCs, the behaviour of 3T3 embryonic fibroblasts to CYP exposure evidences differences to both cell growth kinetics and detoxification response. In fact, these cells display a mild 10% decrease in cell number within 8 h exposure, followed by return to a PD time and growth rate comparable, throughout the remaining culture period, to those of control 3T3. The population growth rate appears to be unaffected by cell death, detected by the late apoptotic markers at 48 h culture, when the G<sub>0</sub>/G<sub>1</sub> checkpoint is activated. The growth rate recovery observed could also have been facilitated by the early (24 h) activation of the detoxification phase I (*Cyp1a1*) and phase II (*Ugt1a6*) response, the latter (*Ugt1a6* and *Gst1*) maintained until the end of the culture period. The increase of ROS production, initiated at 24 h, is no longer detected at the end of the culture period, when only *Cat* and *Gpx4*, out of six redox-related genes analysed, are up-regulated.

The results here reported on 3T3 cells confirmed previous observations that the metabolism of differentiated cells better tolerates the exposure to toxic molecules,<sup>42-44</sup> when compared to undifferentiated cells. For example, differentiated SH-SY5Y neuroblastoma cells displayed higher tolerance to all-trans-retinoic acid<sup>42</sup> or to inhibitors of the electron-transport chain mitochondrial complex I,<sup>44</sup> when compared to their undifferentiated counterpart. Also, differentiated human intestinal Caco-2 cells were more resistant to some inorganic compounds (*i.e.*, SiO<sub>2</sub>, ZnO), than the respective undifferentiated population.<sup>43</sup>

In conclusion, this is the first study to describe the adaptive response of pluripotent stem cells of embryonic origin to continuous exposure to CYP, one of the most common and widely used pyrethroid. The results show that R1 ESCs activate oxidative stress-related and detoxification responses, although not sufficient, during the culture period tested, to warrant recovery of the growth rate observed in untreated cells. Also, our study highlights potential detrimental effects of CYP exposure on the differentiation capacity of ESCs into the three germ layers, a pivotal step for the embryo proper formation. These results, obtained using the most robust *in vitro* model of the ICM, from which the foetus derives, anticipate possible effects exerted by CYP during pre-implantation development.

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