Glyphosate-based herbicide induces long-lasting impairment in neuronal and glial differentiation

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Abstract
Glyphosate-based herbicides (GBH) are among the most sold pesticides in the world. There are several formulations based on the active ingredient glyphosate (GLY) used along with other chemicals to improve the absorption and penetration in plants. The final composition of commercial GBH may modify GLY toxicological profile, potentially enhancing its neurotoxic properties. The developing nervous system is particularly susceptible to insults occurring during the early phases of development, and exposure to chemicals in this period may lead to persistent impairments on neurogenesis and differentiation. The aim of this study was to evaluate the long-lasting effects of a sub-cytotoxic concentration, 2.5 parts per million of GBH and GLY, on the differentiation of human neuroepithelial stem cells (NES) derived from induced pluripotent stem cells (iPSC). We treated NES cells with each compound and evaluated the effects on key cellular processes, such as proliferation and differentiation in daughter cells never directly exposed to the toxicants. We found that GBH induced a more immature neuronal profile associated to increased PAX6, NESTIN and DCX expression, and a shift in the differentiation process toward glial cell fate at the expense of mature neurons, as shown by an increase in the glial markers GFAP, GLT1, GLAST and a decrease in MAP2. Such alterations were associated to dysregulation of key genes critically involved in neurogenesis, including PAX6, HES1, HES5, and DDK1. Altogether, the data indicate that subtoxic concentrations of GBH, but not of GLY, induce long-lasting impairments on the differentiation potential of NES cells.

Keywords
glyphosate-based herbicides, neural stem cell, neurodevelopment, neuronal differentiation, neurotoxicity
INTRODUCTION

Glyphosate-based herbicides (GBH) are systemic, non-selective, and post-emergent herbicides. GBH reached the market in 1974 and have become the most widely used worldwide pesticide.1–3 Frequently, the term GLY is used as a synonym of a wide range of herbicides formulations that only have the active ingredient in common. These formulations contain GLY in different salt forms mixed with different chemicals, as co-formulants, intended to facilitate permeability and absorption by plants. GLY is claimed non-toxic to humans and other animals, due to its action blocking a key enzyme in the signaling pathway for production of aromatic amino acids and secondary metabolites, which is not found in mammals.4–6 Intriguingly, despite its claimed low toxicity, GBH formulations have been shown to produce toxic effects at lower concentrations as compare to pure GLY.7–12

The detection of GLY in urine samples from farmers and non-farmer workers suggests that GBH exposure may occur also indirectly via food and contaminated water,13 but epidemiological data on its effects at relevant environmental levels is limited.14–20

Environmental chronic exposure to pesticides poses a potential risk for long-lasting neurotoxic effects, particularly when occurs during the development.21,22 The potential neurodevelopmental toxicity following GBH exposure may be linked to its capability to cross the placental and the blood–brain barrier, as supported by the presence of GLY in the brain and cerebrospinal fluid of subjects under acute intoxication.23–27

Considering the increasing evidence pointing to the possible occurrence of development exposure (prenatal and postnatal) to GBH, the aim of our study was to investigate the long-lasting effects of sub-cytotoxic concentrations of GLY or GBH (Roundup® Transorb) on human neural stem cells differentiation. We evaluated the effects of these compounds on human neuroepithelial stem cells (NES) derived from induced pluripotent stem cells (iPSC), never directly exposed to the chemicals, according to a protocol previously described.28 We found that subtoxic concentrations of GBH, but not GLY, induce a more undifferentiated state of NES cells. In addition, the differentiation pattern was skewed toward astroglia at the expense of mature neuronal cells.

MATERIALS AND METHODS

2.1 | Ethics statement

The neuroepithelial stem cells (NES) used in the study were granted from fibroblasts donors as written consent concerning the sampling and the line derived from an iPSC. In order to reprogram the cells, ethical permission was granted (Reprogrammering av mänskliga celler) Dnr 2012/208-31/3 with addendum 2012/856-32 and 2015/1097-31/1 approved by the Ethical review board (Regionala etikprövningsnämnden i Stockholm).

2.2 | Cell culture procedures and treatments

The human control NES cell line was generated from human iPSC as described.29 NES cells were kept proliferating in a 75 cm² tissue flask coated with poly-l-Ornithine (0.1 mg/ml Sigma-Aldrich) and laminin (5 mg/ml Sigma-Aldrich). The proliferative state was maintained by culturing the cells in serum-free media containing DMEM/F12 (1:1), L-Glutamine, and penicillin/streptomycin (1:100) (Life Technologies™) supplemented with B27 (1:500, Life Technologies™), N2 (1:100, Life Technologies™), EGF (10 ng/ml Invitrogen) and FGF (10 ng/ml, R&D Systems) and replacing the medium every day until cells reach confluence.

After reaching confluence, the cells were split using 1 ml of TrypLE TM Express (1x + Phenol red) by incubation for 3 min, at 37°C in 5% CO₂, followed by addition of 1 ml defined Trypsin Inhibitor (1x) (Gibco). These cells were then centrifugated at 1000 RPM for 4 min, then the supernatant was discarded and the cells were resuspended in a new fresh medium, counted, and plated at a density of 4 x 10⁵ cells/cm². These cells were defined as parent cells. After 24 h, parent cells, were exposed to 2.5 parts per million (ppm) of GBH (Roundup® Transorb, Monsanto do Brasil LTDA, São Paulo Brazil. 480 g/L of glyphosate) or equivalent water solution of glyphosate (GLY Sigma-Aldrich 45 521) diluted in the medium for 24 h. Control cells were grown in the same medium without the chemicals.

The subtoxic concentrations of GBH and GLY used in this study were selected based on dose response tests with cell viability, evaluated by MTT, trypan blue, and apoptosis, assessed by nuclear condensation identified by Hoechst 33342 (see Figure S2). While none of the GLY concentrations tested induced toxic effects, the highest concentration of GBH that did not exert cytotoxicity was 2.5 ppm. Therefore, it was selected for both compounds to evaluate possible long-lasting effects on neural differentiation, as described in Figures S1 and S2. After exposure, P-cells were split and seeded in fresh medium, (without GBH or GLY), at a density of 2 x 10⁴ cells/cm² for protein extraction or 3 x 10⁵ cells/cm² for RNA extraction and immunocytochemistry. This new generation of cells, not directly exposed to GBH or GLY, were termed daughter cells (D-cells) and were kept in a proliferating state by changing the medium every day until reaching 90% confluence (see Figure S1). After reaching confluence, proliferating D-cells were harvested for RNA and protein extraction, as well as for immunocytochemistry.

To evaluate the differentiation process, proliferating D-cells were deprived of FGF and EGF in the medium and maintained in DMEM/F12 (1:1) supplemented with N2 (1:100) and B27 (1:500) for 28 days of differentiation (dD28). The medium was replaced every other day for the first 14 days, and every 3 days until day 28. Differentiating D-cells were collected for analysis at dD3, dD6, dD12 and the final dD28. To count the total number of cells at dD28, cells were washed once with DPBS, and 0.4 ml of Accutase (Gibco) were added to each well that was then incubated at 37°C for 20 min. Afterward, 2 ml of medium were added, and cells were centrifuged at 1000 rpm for 3 min, resuspended in fresh medium, and counted with automated cell counter TC20 Bio-rad. Differentiated cells were either fixed for...
immunocytochemistry or harvested for RNA and protein extraction. The experimental design is depicted in Figure S1.

2.3 | EdU assay

The proliferation of D-cells was evaluated 24 h after plating the cells at $3 \times 10^4$ cells/cm². This method is based on the incorporation of thymidine analog nucleoside during the phase of DNA synthesis in the cell cycle. Cells were incubated with EdU (5-ethyl-2'-deoxyuridine) Click-iT® EdU Alexa Fluor® 488 at 10 $\mu$M final concentration, and incubated at 37°C, 5% CO₂, for 1.5 h. Following incubation, cells were fixed in 4% paraformaldehyde for 20 min, washed twice in PBS/BSA 3% and incubated for 30 min with 0.5 ml of Click-iT® reaction cocktail protected from the light. Cells were then washed once with PBS and counterstained with Hoechst 33342 (final concentration 5 mg/ml) for 15 min. After that, slides were mounted in DAKO mounting medium and analyzed in Zeiss Axioskop 2 mot plus fluorescence microscope.

2.4 | RNA extraction, cDNA synthesis and qRT-PCR

RNA was extracted by peqGOLD Total RNA Kit (PEQLAB Biotechnologie GmbH, VWR) and on-column DNase digestion (RNase-free DNase set; Qiagen VWR). To determine RNA concentration and to assess the purity, a NanoDrop 1000 spectrophotometer (Thermo Scientific) was used. cDNA was prepared using at least 1 $\mu$g of mRNA and maxima first strand cDNA synthesis Kit, according to manufacturer instructions (Thermo Fischer Scientific). qPCR reactions were performed by adding 5 $\mu$l of cDNA, 7.5 $\mu$l of SYBR Green PCR Master Mix (Applied Biosystems), and 0.2 $\mu$M of primers mix. The reactions were performed using a QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific) and results were analyzed by Applied Biosystems QuantStudio™ 3 & 5 Real-Time PCR System software. Amplification conditions were proceeded as hold stage at 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 seconds at 95°C and 1 min at the annealing temperature. The specificity of the qRT-PCR reactions was confirmed by melting curve analyses performed at the final dissociation stage. The primers used are described in Table 1.

The expression values were normalized against the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) and the relative change in gene expression was analyzed and calculated according to the $2^{\Delta\Delta Ct}$ method.

2.5 | Immunocytochemistry, immunoblotting and image analysis

2.5.1 | Immunocytochemistry

Cells were fixed for 30 min in 4% paraformaldehyde at room temperature following three times wash with PBS. Cells on a coverslip were incubated with PBS 0.1% Triton X-100 for 3 min and then washed with PBS three times. After that, cells were blocked for 1 h with 10% Goat serum in PBS, and subsequently incubated overnight at 4°C in primary antibodies. Primary antibodies were then removed, cells washed with PBS three times, and incubated with secondary antibodies for 2 h at room temperature. Next, samples were washed

**TABLE 1** Primer set used for qPCR

| Gene  | Forward | Reverse | Ta (°C) |
|-------|---------|---------|---------|
| TRKB  | 5′-TGTGCCTCCACTTGTCAG-3′ | 5′-ATCCATACCCACCCACTCAA-3′ | 56 |
| PAX6  | 5′-AGGAGGGGGAGAGATAACAC-3′ | 5′-GCCCTCCCTGATTAGAACAC-3′ | 60 |
| MAP2  | 5′-GATGAGGAGTCCACGATCAACG-3′ | 5′-ACCAGGCTTACTTGTCTCTC-3′ | 60 |
| DCX   | 5′-GGCAAATTTTCCAGGACCAC-3′ | 5′-CAGAAGGCGCTCAAACTGG-3′ | 60 |
| GFA   | 5′-GATCAACTCAGCGCCAACG-3′ | 5′-CTCCTCCTCCAGGGGATGC-3′ | 60 |
| HPRT  | 5′-ACCCACCAGAAGTGGAGTA-3′ | 5′-AAGCAGATGGCAGAAGACT-3′ | 60 |
| DKK1  | 5′-TCCAGGAGAAAAATTAGAGA-3′ | 5′-CTGTGGACGAGTCTGATA-3′ | 56 |
| CDDN1 | 5′-TCTCTCCAAAATGCGAGAG-3′ | 5′-TGAGGCCGTAGTAGGACAG-3′ | 56 |
| P16   | 5′-GTTGACCTGGGCTAGG-3′ | 5′-CTTCATCGGGGATGTCTG-3′ | 60 |
| HES1  | 5′-TCAACAGCAACGGGAATATA-3′ | 5′-CCCGAGCTATCTTTCTTCA-3′ | 56 |
| HES5  | 5′-ACATCGTGGATGGCCTGTC-3′ | 5′-AGCAGCTCATCTGCGTC-3′ | 58 |
| TUBB3 | 5′-CTCAGGGGGCTTGGGACAT-3′ | 5′-CAGGAGTGGCAGTTCAC-3′ | 56 |
| P21   | 5′-ACACGCTCCCCAGGTAGTG-3′ | 5′-AGCAGATGGCAGACGTC-3′ | 60 |
| NESTIN| 5′-TCAGCTTCCAGGACCGCAAGC-3′ | 5′-GAGCAAGATCCAGAAGGCG-3′ | 60 |
| GLAST | 5′-TCACAGGATTCGTCCTCC-3′ | 5′-CGAGGGCATCCTTGATTG-3′ | 60 |
| GLT1  | 5′-TGGCTGCTGAGAGGATGAGA-3′ | 5′-ACTCGATGTGGGAGTCAATGG-3′ | 60 |
three times with PBS and counterstained with 4',6-diamidino-2-phenylindole (DAPI), 1:100 (Sigma-Aldrich).

2.5.2 | Image analysis

For analysis, random images with the same exposure time were captured by fluorescent microscope (Zeiss Axioskop mot plus) from three independent experiments. Cellular protein quantification was performed by measuring the fluorescence intensity of each marker normalized by the area of the region of interest. The perimeter and area of each cluster were measured using the freehand selection tool, calibrated by the microscope scale bar using FIJI software (NHI). The measurements are represented as percentage from controls. For representative images, pictures were acquired in confocal microscope Zeiss LSM 800.

2.5.3 | Western blot (WB)

Proteins were either extracted from six multi-well, in lysis buffer with protease and phosphatase inhibitor, followed by mechanical lysis in tissue lyzer for 1 min at 30 Hz and protein quantification by Pierce BCA Protein assay kit (ThermoFisher). For 100 mm dishes, cells or tissue lyzer for 1 min at 30 Hz and protein quantification by Pierce BCA Protein assay kit (ThermoFisher). For 100 mm dishes, cells or tissue lyzer for 1 min at 30 Hz and protein quantification by Pierce BCA Protein assay kit (ThermoFisher).

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The antibodies used are described as following (Table 2):

| Antibody                     | Concentration |
|------------------------------|---------------|
| ICQ TUBB3-TUJ1 (Biolegend 802001) | 1:1000        |
| ICQ GFAP (Sigma G3893)       | 1:500         |
| ICQ Ki-67 (Cell signaling #12202) | 1:500       |
| ICQ NESTIN (Millipore MAB5326) | 1:500         |
| WB                            | 1:1000       |
| ICQ GLAST (Abcam ab416)      | 1:500         |
| ICQ GLT-1 (Millipore AB1783) | 1:500         |
| ICQ NICD (Abcam, ab8925)     | 1:500         |
| ICQ MAP2 (Sigma-Aldrich M1406) | 1:1000     |
| ICQ Anti-mouse 594 (Thermo Fisher A-11005) | 1:500 |
| ICQ Anti-rabbit 488 (Thermo Fisher A-11008) | 1:500 |
| ICQ Anti-Guinea Pig 546 (Thermo Fisher A-11074) | 1:500 |
| WB Cleaved Caspase 3 (Asp175) (Cell signaling #9664) | 1:1000 |
| WB P21 (sc-471-G)            | 1:1000        |
| WB Actin (Sigma-A3853)       | 1:5000        |
| WB IRDye 800CW Goat anti-Mouse (926–32 210) | 1:5000 |
| WB IRDye 680RD Goat anti-Rabbit (926–68 071) | 1:5000 |

2.6 | Spontaneous Ca²⁺ activity

To determine the spontaneous calcium (Ca²⁺) activity in the differentiated cells, cultures were incubated at 37° C, 5% CO₂ during 30 min in a medium containing 5 µM Fluo-3/AM with Pluronic F-127 at 0.1%. Following rinsing the cell culture, a KREBS-Ringer’s solution was added (NaCl 119 mM, KCl 2.5 mM, NaH₂PO₄ 1 mM, CaCl₂ 2.5 mM, MgCl₂ 1.3 mM, 4-[hydroxyethyl]-1-piperazineethanesulfonic acid 20 mM and D-glucose 11 mM). Serial images, Imagens were acquired at 0.2 Hz with an upright fluorescence microscope and a ×20/1.0 water dipping objective (Carl Zeiss) equipped with a high-speed wavelength switcher (DG-4, Sutter Instrument) and a cooled EMCCD camera (Cascade II:512, Photometrics). MetaFluor (Molecular Devices) was used to control all devices and to analyze acquired images. Spontaneous activity in cells was determined with a minimum of two well-defined spontaneous Ca²⁺ peaks exceeding 15% of baseline over a 10-min period.

2.7 | Statistical analyses

Independent experiments were performed in triplicate and subjected to the Shapiro–Wilk and Brown–Forsythe tests for normality and homogeneity of variance, respectively. Normally distributed data were analyzed by two-tailed unpaired T-test (T) or one-way ANOVA (F) with Dunnett’s multiple comparison post hoc test. Welch’s ANOVA (W) test and Dunnett’s T3 post hoc test were used when the variance was heterogeneous. When the data were not normally distributed, two-tailed Mann–Whitney (U) or Kruskal–Wallis (H) with Dunn’s post hoc test were used. The data are reported in the text with their...
respective statistical tests and \( p \) values. Graphs are shown as mean, ± SD, post-hoc or \( p \) values test. Software Prism 8 (GraphPad Software, Inc., La Jolla, CA, USA) was used and differences were considered significant at \( p < .05 \).

3 | RESULTS

3.1 | Exposure to GBH inhibits proliferation, and alters the expression of key neural stem cell markers and neurogenesis regulators in proliferating D-cells

Initially, to investigate whether exposure to 2.5 ppm of GBH or GLY has long-lasting effects on NES cells, we evaluated proliferation rate in daughter cells (D-cells), never directly exposed to the chemicals. We found a reduction of proliferating cells in GBH-treated cultures, as demonstrated by a decreased EdU uptake, compared to controls \( [F (2, 38) = 12.09; p < .001] \), while the same concentration of GLY did not exert an effect on it (Figure 1A–D). To elucidate the molecular mechanisms underlying the observed alterations, we analyzed the expression of key genes regulating proliferation and cell cycle, including cyclin dependent kinase inhibitor 2A (CDKN2A, also known as \( P16 \)) and cyclin dependent kinase inhibitor 1A (CDKN1A, \( P21 \)). We found no change in \( P16 \) expression \( [F (2, 28) = 0.1424; p = .8679] \), but up-regulated \( P21 \) expression \( [F (2, 28) = 7.105; p = .0032] \), also indicated by WB analysis (Figure 1E,F).

To evaluate whether GBH or GLY could lead to long-lasting effects, we analyzed the expression of key neural stem cell genes: NESTIN (an intermediate filament in neuroepithelial cells), paired box 6 (PAX6, a transcription factor involved in neuronal commitment), and doublecortin (DCX, a microtubule-associated protein marker for progenitors committed toward neuronal fate). The expression of NESTIN, was increased in GBH-treated cells as compared to control cultures \( [F (2, 28) = 3.672; p = .0383] \), while the expression of PAX6 and DCX, was decreased \( [F (2, 28) = 4.671; p = .0178] \) and \( [F (2, 28) = 7.208; p = .0030] \), respectively (Figure 2A).

Due to its crucial role in neurogenesis and gliogenesis, we also studied the activity of the Notch pathway. Although we did not find altered expression for the NOTCH receptor \( [F (2, 26) = 2.211; p = .1298] \), neither in GBH nor GLY exposed D-cells, the Notch intracellular domain (NICD), a receptor domain released in the cytoplasm following Notch activation, was increased upon GBH treatment, as showed by immunofluorescence analysis \( [F (2, 47) = 12.87; p < .0001] \) (Figure 2B–D,F). In addition, the expression of Hairy/Enhancer of Split 1 (HES1), one of the main Notch effectors, was upregulated compared with controls \( [F (2, 28) = 5.266; p = .0114] \), while the Hairy/Enhancer of Split 5 (HES5), another important Notch effector, did not show a statistical difference in GBH-treated proliferating D-cells, as compared to control \( [F (2, 27) = 0.2962; p = .7460] \) (Figure 2G). The Wnt signaling is another relevant pathway involved in the regulation of neural stem cells proliferation, thus we checked the expression of its well-established inhibitor, Dickkopf Inhibitor

FIGURE 1 Effects of GBH and GLY exposure on proliferation of D-cells. (A–D) Percentage of Click-iT® EdU thymidine analog incorporation in proliferating D-cells derived from control, GBH and GLY, nuclei of EdU positive cells are seen in green. (E) \( P16 \), and \( P21 \) gene expression after exposure to GBH or GLY. (F) Measurement of \( P21 \) protein by western blot in cells exposed to GBH (arbitrary units relative to control). Post hoc test \( p \) values are shown in statistically significant different groups.
FIGURE 2 Effects of GBH and GLY exposure on key neural stem cell markers and neurogenesis regulators in proliferating D-cells. (A) NESTIN, PAX6 and DCX gene expression after exposure to GBH or GLY. (B–D) Immunocytochemistry for NESTIN and Notch intracellular domain (NICD) in D-cells exposed to GBH and GLY. Fluorescence measurement of NESTIN (E) and NICD (F) in cells exposed to GBH and GLY. G NOTCH, HES1, HES5, DKK1 gene expression in proliferating D-cells after exposure to GBH or GLY. Post hoc test p values are shown in statistically significant different groups.

1 (DKK1), which was upregulated in GBH exposed cells compared with control [W (2.000, 15.26) = 11.30; p < .0010] (Figure 2G). No alterations were observed after GLY treatment.

3.2 Exposure to GBH induces morphological alteration and long-lasting changes of neuronal and glial markers in differentiated D-cells

Next, we investigated the long-lasting effects of GBH and GLY on spontaneous differentiation. Analyses of the major genes related to neuroepithelial, neuronal, and glial cells, including NESTIN, DCX, TUBB3, MAP2, GLT1, GLAST and GFAP were conducted in differentiated D-cells at the day of differentiation 28 (dD28).

GBH induced an increased gene expression of NESTIN [F (2, 25) = 19.38; p < .0001] (Figure 3A), confirmed by immunocytochemistry [F (2, 40) = 24.30; p < .0001] (Figure 3B–E), and DCX [H (2, 25) = 14.00; p = .0009] (Figure 3A), a marker for immature neurons. The expression of TUBB3, an early-born neuron marker, was increased [W (2.000, 15.73) = 5.508; p = .0154] as confirmed by immunocytochemistry [W (2.000, 20.02) = 14.39; p < .0001] (Figure 3A,F–I). Conversely, we observed a decreased expression of
FIGURE 3  Legend on next page.
Considering the extensive use of GBH, it was worthy of note that in several studies the biological effects of whole GBH formulations are not taken into account, when GLY concentrations are reported in food or biological fluids. In herbicides, active ingredients such as GLY are responsible for the intended effects, although commercialized formulations contain different co-formulants, as ethoxylated compounds, to improve solubilization and penetration in the plants. In the case of GBH, the presence of these co-formulants significantly enhances the toxicity of pure glyphosate. Several studies have indicated that GLY can reach the developing embryo, inducing toxic effects, since is able to diffuse through the placenta. In agreement, GLY exposure in rats, during perinatal phases, results in neurobehavioral, estrogenic, and thyroid hormone alterations in offspring. Considering the extensive use of GBH, it is relevant to evaluate the effect of formulations, particularly when the exposure occurs during development. Therefore, our study aimed at evaluating the long-lasting effects of GBH or GLY on neurodevelopmental critical processes, such as proliferation and differentiation. The concentration used in our experiments (2.5 ppm) is in the range of residues found in several food types (0.001–18.53 ppm), and cases of mild-acute intoxication (11.48–61 ppm), but higher than the one measured in umbilical cord serum (0.2–94.9 ppb) and the estimated for chronic ingestion. It is worthy of note that in several studies the biological effects of whole GBH formulations are not investigated in detail, when GLY concentrations are reported in food or biological fluids.

With the present study we wanted to investigate the neurotoxic potential of both GLY and GBH using the human iPSC-derived neuroepithelial-like stem cells (NES cells), which have proven to be a powerful in vitro model to investigate developmental neurotoxicity. Our results showed that daughter cells (D-cells) NES cells never directly exposed to GBH, display alterations in proliferation and neuronal differentiation, pointing to the occurrence of long-lasting effects induced by exposing parent cells.

D-cells, following exposure to GBH, exhibited a reduced cell proliferation rate that likely reduced the pool of differentiating neurons resulting in a decrease of postmitotic neurons. Changes in cell cycle phases and proliferation of neural stem cells have significant effects on neural fate commitment and possibly be linked to the onset of neurodevelopmental disorders. NES cells after GBH exposure showed a reduced proliferation rate associated with...
upregulation of two key genes, namely P21 and DKK1. The well-known inhibitor of cyclin dependent kinase (CDK), P21, reduces DNA synthesis by disrupting DNA polymerase activation, while DKK1 inhibits the canonical Wnt signaling pathway, which plays a critical role in promoting proliferation and self-renewal of neural stem cells. GBH did not affect the expression of NOTCH, a transmembrane receptor involved in neurogenesis and neural stem cell maintenance, but increased the release of NICD, the receptor’s intracellular domain needed for downstream activation of the signaling pathway. One of the main Notch signaling effectors is HES1, a transcription factor involved in the self-renewal and maintenance of

![Figure 4](image-url)
stemness in neural stem cells.\textsuperscript{58-59} The expression of HES1 was upregulated in proliferating D-cells, however, the expected effect on proliferation was possibly counteracted by P21 upregulation, resulting in an inhibition of cell cycle progression.

Proliferating D-cells exposed to GBH showed alterations of major neural stem cell and neural progenitor cell markers, such as NESTIN, PAX6 and DCX. The intermediate filament NESTIN, a cytoskeletal protein buffering mechanical stress, is expressed in embryonic and adult proliferating regions of the central nervous system and is a marker for neuroepithelial stem cells.\textsuperscript{60-61} The increased NESTIN expression at both mRNA and protein level suggests that GBH, prevents differentiation of NES cells forcing them into a stemness state.\textsuperscript{62-63}

During neurodevelopment, NES cells give rise to radial glia that further divide to maintain the stem cell population, while generating neurons, and later glial cells.\textsuperscript{64-69} In our NES cell model, a preferential expression of neuroepithelial and neural rosette markers (e.g., PAX6, SOX3, PLZF) is described, indicating an earlier stage of CNS development.\textsuperscript{70}

Among them, PAX6 is an essential transcription factor promoting neuronal commitment, but its continued expression in neuronal progenitors leads to failed neuronal differentiation.\textsuperscript{70,71} In our study, PAX6 was downregulated in proliferating D-cells GBH-exposed, providing an explanation for the impaired neuronal commitment resulting in a reduction of the young neuron marker (DCX),\textsuperscript{72,73} in the proliferating D-cells.

Impaired expression of HES1 leads to upregulation of proneuronal genes, premature neurogenesis, and defects on neurulation. On the other hand, its continuous expression antagonizes proneuronal genes preventing neuronal commitment and maturation.\textsuperscript{74-80} Since glial differentiation is inhibited by proneuronal genes, an upregulation of HES1 drives cells toward gliogenesis by blocking neurogenesis promoting genes.\textsuperscript{81-85} Therefore, GBH-induced increased expression of HES1 is likely a molecular pathway involved not only in the maintenance of D-cells in the neural stem state, but also in preventing neurogenesis and shifting cell fate toward glial cells.

The canonical \(\beta\)-catenin-dependent Wnt signaling pathway, activated based on glycoproteins binding to Frizzled and LRP6 receptor, is also involved in embryonic development, cell fate determination and neuronal differentiation.\textsuperscript{86} Activation of Wnt signaling promotes the expression of proneuronal markers, while its disruption inhibits neuronal differentiation.\textsuperscript{87-89} As Wnt acts promoting neurogenesis, its downregulation by inhibitors, such as DKK1, induces a decreased neuronal production and increased gliogenesis.\textsuperscript{90-92} Accordingly, the DKK1 upregulation in proliferating D-cells, following GBH exposure, further explain the altered neurons-glial cells proportions.

Therefore, PAX6 downregulation, HES1 and DKK1 upregulation in proliferating D-cells support the impairment in neuronal differentiation, as shown by a decrease of young neurons (DCX). Moreover, the alteration of these signaling pathways, during proliferating phases, further explain the decrease of post-mitotic neurons (MAP2) and increase of glial marker observed later in differentiated D-cells.

In differentiated D-cells, GBH exposure induced an increase in NESTIN, suggesting a larger cell population retained in an undifferentiated state, and prevented to differentiate.\textsuperscript{93-94} Our results also suggest that GBH prevent young neurons maturation, as shown by increased DCX and TUBB3 expression and downregulation of the post-mitotic neuronal marker MAP2.

The expression pattern of HES1 and DKK1 fluctuated during the differentiation process, with both genes upregulated at the beginning (dD3) and the end (dD28) of the differentiation process, but no difference observed between these phases (dD6 and dD12). Their altered expression at the differentiation process may have further contributed to the decrease of mature neurons and increase in glial cells, since HES1 inhibits neurogenesis and promotes gliogenesis,\textsuperscript{78-80} and DKK1 leads to decreased neuronal production and increased gliogenesis.\textsuperscript{90-92}

On the other hand, although PAX6 was decreased in proliferating D-cells, explaining a reduction of neuronal progenitors fate commitment in early this phase, its upregulation in differentiated D-cells suggests that they were maintained as progenitors, since its sustained expression after neuronal commitment prevents differentiation.\textsuperscript{79}

As previously mentioned, following GBH exposure a higher proportion of cells were kept in the neural stem and neuronal progenitor stage, possibly due to HES1, PAX6 and DKK1 misexpression, leading to an increased stemness state. Therefore, these populations of NES could be guided by HES5 to differentiate toward glial cells, later in development. HES5 has been described to anticipate and promote gliogenesis, in addition to regulating the timing of transition between neurogenesis and the beginning of gliogenesis.\textsuperscript{82,95-97} Although its expression did not change in proliferating D-cells or at the beginning of the differentiation process, it steadily increased starting from dD12 being significant at dD28. Since the generation of astroglia in our cell model takes place from dD21,\textsuperscript{44} our data suggest that upregulation of HES5, during later phases of the differentiation process, is a key factor to the increased glial-neuronal proportion, as detected by the increase of glial markers GLT1, GLAST and GFAP. In addition, PAX6 higher expression during differentiation, may contribute to the increase in glia by promoting the expression of GLT-1 in astrocytes and influencing their maturation.\textsuperscript{98-99}

Overall, our findings demonstrate that, following GBH exposure, the maintenance of undifferentiated cells, decreased mature neuronal populations, as well as the switch in neurogenesis toward gliogenesis, is supported by the alterations observed in HES1, HES5, DKK1 and PAX6 not only in proliferating, but also in differentiated D-cells.

Moreover, the increase in undifferentiated cells and decrease in differentiated neurons is further supported by the functional analysis of (Ca\(^{2+}\)) activity. Ca\(^{2+}\) spontaneous activity in networks of neural progenitors has been shown to promote their proliferation.\textsuperscript{100-101} The increased activity correlates with inhibition of neuronal differentiation, as observed following exposure to environmental chemicals.\textsuperscript{102-103} Therefore, our data on higher spontaneous Ca\(^{2+}\) activity in differentiated D-cells further point to GBH interfering with neuronal differentiation and maturation processes.

In our cell model, during the differentiation process neural stem cells, neurons, and glia are progressively arranged in cluster structures connected to each other by neuronal processes. Following exposure
to GBH, cells formed fewer number of clusters, but with larger area and perimeter. These effects are supported by altered HES1 and PAX6 expression, as well as the increased Ca2+ spontaneous activity, leading to larger population of cells in self-renew process.

Accordingly, after GBH exposure, the clusters of differentiated D-cells presented a higher number of actively proliferating cells, as shown by Ki-67. Additionally, the decrease in apoptosis, shown by cleaved caspase 3 and nuclear condensation in the GBH group, may also contribute to the higher number of cells per clusters.

Neither gene expression alterations nor morphological changes were observed in proliferating or differentiated D-cells exposed to GLY. These results are in agreement with previous reports showing that commercial formulations are more toxic than the active ingredients alone.11,12,32,108 The presence of surfactants that alter the cell membrane permeability and increase the availability of GLY to the cells,30,109 is likely a critical factor accounting for the differences in GLY and GBH toxic effects that we observed in our study.

5 | CONCLUSIONS

In conclusion, our study demonstrates that GBH, but not GLY, induces long-lasting effects in human neural stem cells. GBH promotes an undifferentiated state, decreases neuronal differentiation, and shifts the differentiation toward a glial fate. The present data suggest that the altered expression of PAX6, HES1, HES5, and DKK1 contributes to the detrimental effects observed in both proliferating and differentiating cells. GBH are produced in different formulations and are largely distributed into the environment. Further studies are needed to evaluate the possible neurodevelopmental adverse effects associated with different commercial formulations.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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