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Environment permissible concentrations of glyphosate in drinking water can influence the fate of neural stem cells from the subventricular zone of the postnatal mouse☆

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ABSTRACT

The developing nervous system is highly vulnerable to environmental toxicants especially pesticides. Glyphosate pesticide induces neurotoxicity both in humans and rodents, but so far only when exposed to higher concentrations. A few studies, however, have also reported the risk of general toxicity of glyphosate at concentrations comparable to allowable limits set up by environmental protection authorities. *In vitro* data regarding glyphosate neurotoxicity at concentrations comparable to maximum permissible concentrations in drinking water is lacking. In the present study, we established an *in vitro* assay based upon neural stem cells (NSCs) from the subventricular zone of the postnatal mouse to decipher the effects of two maximum permissible concentrations of glyphosate in drinking water on the basic neurogenesis processes. Our results demonstrated that maximum permissible concentrations of glyphosate recognized by environmental protection authorities significantly reduced the cell migration and differentiation of NSCs as demonstrated by the downregulation of the expression levels of the neuronal *ß-tubulin III* and the astrocytic *S100B* genes. The expression of the cytoprotective gene *CYP1A1* was downregulated whilst the expression of oxidative stresses indicator gene *SOD1* was upregulated. The concentration comparable to non-toxic human plasma concentration significantly induced cytotoxicity and activated Ca^{2+} signalling in the differentiated culture. Our findings demonstrated that the permissible concentrations of glyphosate in drinking water recognized by environmental protection authorities are capable of inducing neurotoxicity in the developing nervous system.

1. Introduction

The nervous system develops with a very complex pattern of tightly regulated events including apoptosis, proliferation, differentiation of NSCs into neurons and astrocytes as well as cell migration, gliogenesis, synaptogenesis, and myelination. These events take place in a very precise and controlled fashion to ensure appropriate and undisturbed development. Any perturbation in these processes by a chemical entity adversely affects the physiology of the nervous system (Coullery et al., 2016; Tohyama, 2016). The developing nervous systems (fetus and infantile) are highly sensitive towards chemicals such as pesticides because of the poorly developed blood-brain barrier and enzyme systems (Costa et al., 2008; Pamies et al., 2018). Several studies have reported

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the association between early-life exposure to pesticides and neurological disorders in children (Parrón et al., 2011; Tohyama, 2016) and raised serious concerns to explore the possible neurotoxic effect of commonly employed pesticides (Pamies et al., 2018). Glyphosate is a herbicide which is widely exploited to improve the growth and productivity of genetically modified crops (Jasper et al., 2012). Chemically glyphosate is *N*-[Phosphonomethyl]glycine, a glycine analogue which exerts its herbicide actions by blocking the synthesis of essential aromatic amino acids through the Shikimate pathway, a pathway unique to plants, protozoa, bacteria, and fungi but lacking in mammalians and human beings. Thus glyphosate is generally considered as a safe pesticide and unlikely to cause acute toxicity in animals and human beings in normal doses (Song et al., 2012; Sribanditmongkol et al., 2012; Szepanowski et al., 2019). The European Commission for environment setup a Drinking Water Directive which considered 0.1 μg/L of glyphosate as a maximum allowable concentration (MAC) in drinking water (Dolan et al., 2013) while the United State Environment Protection Agency (USEPA) defined 700 μg/L of glyphosate as a maximum contamination level (MCL) (Larsen et al., 2012). Both MAC and MCL represent the maximum permissible concentrations in drinking water and are considered as non-observable adverse effect levels of glyphosate. Kinetic studies conducted in a rat model revealed that glyphosate achieved peak plasma concentration of 4500 μg/L within 5.16 h upon administration of a single oral dose of 400 mg/kg and followed the two-compartment model with an elimination half-life of 14.38 h (Anadón et al., 2009). Glyphosate is capable of crossing the blood-brain barrier and accumulates in the striatum, hypothalamus, and midbrain as reported in the rat model (Martínez et al., 2018). Glyphosate can also cross the placental barrier, being secreted in the urine and breast milk of the human beings (Honeycutt and Rowlands, 2014) indicating its potential for developmental toxicity. A strong link between glyphosate exposure and multiple health impairments (Clair et al., 2012; Gallegos et al., 2020; Ren et al., 2018; Sribanditmongkol et al., 2012; Tang et al., 2020) including mental disorders have also been reported in the literature (Bradberry et al., 2004). Several clinical studies have affirmed the association between the exposure to glyphosate formulations and the nervous system disorders in human beings (Barbosa et al., 2001), including Parkinson's (Caballero et al., 2018), meningitis (Sato et al., 2011) and loss of consciousness in adults (Zouaoui et al., 2013) while autism (von Ehrenstein et al., 2019), attention deficit and attention deficit hyperactivity disorders have been reported in children whose parents were exposed to glyphosate (Solomon et al., 2007). Additionally, glyphosate formulations induced behavioural disorders (Gallegos et al., 2016) and anxiety along with depression-like symptoms in rodents (Ait Bali et al., 2017). In all of these studies humans and rodents were exposed to glyphosate formulations rather than pure glyphosate and therefore the contribution of formulation additives in the neurotoxicity must not be overlooked (Neto da Silva et al., 2020).

A variety of mechanisms are involved in glyphosate induced neurotoxicities. Glyphosate and its formulation, for instance, tend to reduce neural cell migration due to their hypothyroid activity (Beecham and Seneff, 2016), disrupt neuronal plasticity (Dechartres et al., 2019), induce glutamate excitotoxicity, induce oxidative stress, decrease the expression of astrocyte marker (Cattani et al., 2017), increase the Ca^{2+} influx in hippocampus tissues of rodents (Cattani et al., 2014), reduce differentiation of neurons and synaptogenesis in the neocortex area by modulating the expression of regulatory genes controlling Wnt/B-catenin/Notch Pathway (Ji et al., 2018), and reduce the expression of 5HT-cells in the basolateral amygdala and medial prefrontal cortex (Ait Bali et al., 2017) of rodents. Glyphosate associated apoptosis in differentiated PC12 cell lines (Gui et al., 2012) and inhibitory effects on neurite development and growth (Coullery et al., 2016) at higher concentrations have also been reported in the literature. Glyphosate decreased the levels of 5-HT, dopamine, and nor-adrenaline in striatum, hypothalamus, and mid-brain regions of the rat brain when exposed for 6 days at doses several times higher than the NOAEL dose determined in preliminary experiments of that study (Martínez et al., 2018). Glyphosate induced oxidative stress and upregulated the proinflammatory genes IL6 and TNFα in human neuroblastoma SH-SY5Y cell line at 5 mM concentration (Martínez et al., 2020). Unfortunately, all of these studies were performed employing extremely high concentrations (*i.e*. several hundred to thousands times) of glyphosate rather than exploiting concentrations defined by environmental protection authorities of different countries and were more relevant to occupational exposure than the normal exposure, *i.e.* through nutrition.

Developmental neurotoxicity (DNT) is defined as the neurotoxic effects produced by a noxious substance in an organism during embryonic or postnatal life (Coecke et al., 2007; Giordano and Costa, 2012). The Organization of Economic Co-operation and Development (OECD) set developmental neurotoxicity guidelines TG426 for animal-based *in vivo* testing conducted during pregnancy and lactation. These testing systems are based upon functional endpoints such as memory, learning, auditory startle, motor activity, and brain morphometry (Tohyama, 2016). Among animal models, the mouse model is highly attractive for predicting human toxicities of the environmental toxicants because of a range of similarities between mice and human beings concerning genome sequence, metabolic pathways (Harper, 2010) and mechanism of neurodegenerative disorders such as Parkinson's and Alzheimer's (Blesa and Przedborski, 2014; Harper, 2010). Additionally, mice are easy to maintain, have shorter generation time and a high rate of reproduction. Mouse embryos help to predict developmental human neurotoxicity associated with food or environmental pollutants (Hafezparast et al., 2002; Uhl and Warner, 2015). Despite all these advantages, traditional animal-based *in vivo* cytotoxicity assays are associated with several limitations including low sensitivity, laborious work (Ait Bali et al., 2017; Wang et al., 2007), lengthy processes, high experimental costs (Coady et al., 2017) and animal ethical concerns, pushing the regulatory authorities to replace animal-based studies with robust cell-based *in vitro* assays for screening toxicities of chemicals including pesticides (Jang et al., 2014). Both the REACH regulations in European Union countries and Lautenberg amendment to toxic substances control act TSCA in the USA recommended the implementation of alternative testing methods, such as computational toxicology and bioinformatics, high-throughput screening methods or *in vitro* studies (Hartung and Sabbioni, 2011; Lilienblum et al., 2008).

Neural stem cells (NSCs) are a unique bio-tool to understand brain physiology and allow *in vitro* screening of compounds for neuromodulating properties. NSCs based assay bridges a gap between the preclinical data and the clinical practices (Wang, 2015). NSCs occur both in developing and adult mammalian and human brain. The subventricular zone (SVZ), the dentate gyrus of the hippocampus (Guo et al., 2012), and the entire length of the gut (Grundmann et al., 2016; Rauch et al., 2006; Schäfer et al., 2003) are the major niches of NSCs.

SVZ represents the largest pool of NSCs (Inta et al., 2008) and is the main site of neurogenesis (Inta et al., 2008; Saha et al., 2012; Wang, 2015). Multipotency, self-renewal properties (Shoemaker and Kornblum, 2016), variable response to an external stimulus with the age of the donor animal, and the specific region of extraction (Bixby et al., 2002; Urbán and Guillemot, 2014) make NSCs a versatile *in vitro* screening model. Easy isolation and

handling, and a fast *in vitro* growth rate provides NSCs from the SVZ an edge over their counterpart (Liu et al., 2009).

Several *in vitro* NSCs assays for DNT have been used to investigate the effects of chemicals including pesticides on proliferation, migration, differentiation, neurite growth, viability, neurotransmission, and mRNA gene expression of neural cell lineages (Coecke et al., 2007; Lein et al., 2007; Salama et al., 2015). Furthermore, these approaches increase the speed and reliability of neurotoxic screening of chemicals (Lein et al., 2007). A successful DNT *in vitro* method should include more than one endpoints which correspond with human neurodevelopment process. These methods should be capable of quantifying the extent of changes in each specific endpoint with the changing concentrations of the test compound (Crofton et al., 2011). The majority of neurotoxicity studies of glyphosate were conducted at concentrations several thousand times higher than those permissible for drinking water, while a few studies reported glyphosate induced toxicities in the liver, kidney (Mesnage et al., 2015), and the Sertoli cells of rat (Clair et al., 2012) at concentrations closer to MCL. What happens when maximum permissible concentrations of glyphosate in drinking water (MAC and MCL) for neurotoxicity studies are applied is mainly unknown. So in the present study, we established an *in vitro* neurotoxicity assay system based upon NSCs from SVZ of the postnatal mouse to assess the effects of permissible concentrations of glyphosate in drinking water on the viability, proliferation, differentiation, migration and gene expression of NSCs for a maximum of seven days. Additionally, 7000 μg/L of glyphosate which represents the concentration comparable to non-toxic human plasma (Aris and Leblanc, 2011; Kwiatkowska et al., 2016) was also evaluated for its effects on NSCs viability.

2. Materials and methods

2.1. Test compound

In the given study the effects of glyphosate [*N*-(Phosphonomethyl)glycine, ≤100%] compound with Mol. Wt. 169.07 (1071-83-6, Sigma-Aldrich, Taufkirchen, Germany) were evaluated on viability, proliferation, differentiation and migration of NSCs from SVZ of the postnatal mouse.

2.2. Animals

Male Balb/c wild-type mice at the age of 3 days were employed in the present study to obtain NSCs. For each biological replicate, tissues were obtained from a set of 3 animals and a total of 24 animals were exploited in this study. Animals were kept in the pathogen-free environment with a standard constant temperature (23 ± 2 °C) and under 12 h light and 12 h dark cycle. Animals were sacrificed via decapitation by an authorized person without employing anaesthesia. The animal handling and sacrifice were performed following the guidelines and recommendations of animal protection legislation in Rhineland-Palatinate, Germany. Since experiments were not performed directly on living animals and NSCs were isolated from the sacrificed animals, no special approval was required for this study. Animal sacrifice was, however, reported to the local ethics committee on animal experiments at the University of Applied Sciences Kaiserslautern, Germany.

2.3. Extraction and culture of neural stem cells from the subventricular zone of the postnatal mice

The extraction and culture of NSCs from the SVZ of the postnatal mice were performed following the protocols described in the literature (Bender et al., 2017). Tissues from three mice were employed for each experiment. Instantly after decapitation, the mouse brain was removed and immediately stored in the chilled MEM-medium (Life Technology, Eugene, OR. USA) supplemented with 1% streptomycin/penicillin (Applichem, Darmstadt, Germany). The SVZ was dissected from both hemispheres exploiting a stereomicroscope and then transferred in 1 mL of accutase enzyme (HyClone-GE, Utah, USA) and incubated for 20 min at 37 °C. The SVZ tissues were triturated gently with 23 and 27 gauge needles 4 times each. The enzyme was removed by centrifugation of cell suspension for 5 min at 100× *g*. The cell pellet was resuspended in 5 mL of the proliferation medium [DMEM/F12-glutamax, (Life technology, Eugene, USA), β-mercaptoethanol, 2% B-27 without vitamin A (Gibco, Paisley, UK), 1% Penicillin/streptomycin, EGF 10 ng/mL and FGF 20 ng/mL (Immunotool, Friesoythe, Germany)] with initial NSCs seeding number adjusted at 500,000. Neurospheres appeared within 3 days. NSC's culture was further continued for 5 days to obtain the required cell number. About one half of the medium was replaced by fresh proliferation medium every 3 days. Cell number was precisely counted by the trypan blue method before each experiment.

2.4. Viability and cytotoxicity assays

The effects of glyphosate on NSCs viability were determined by a live-dead assay using Calcein-AM and Propidium iodide. The live-dead assay was performed for proliferation culture incubated in a 24-well plate with glyphosate for 24 h. The assay was performed as described in the literature (Sadeh et al., 2016). Cytotoxicity was assessed by WST-1 cytotoxicity assay following kit methods. The detail of the Live-dead assay and WST-1 assay have been given in the supplementary materials.

2.5. Calcium imaging

The effects of glyphosate on Ca^{2+} signal changes were evaluated in an adherent neuron/astrocyte co-culture differentiated from NSCs. Around 50,000 NSCs were attached to a 15 mm glass coverslips pre-coated with PDL and incubated in a colorless differentiation medium (composition closely resembled with proliferation medium except growth factors were omitted and B-27 with Vitamin A was used) into each well of a 6 well plate for 48 h without glyphosate. Changes in Ca^{2+} homeostasis were recorded after acute exposure to glyphosate for 2 min. The details of the method have been provided in the supplementary materials.

2.6. Proliferation assay

Effects of 0.1 μg/L and 700 μg/L of glyphosate on NSCs proliferation were explored through clonogenic assay and immunostaining of proliferation markers BrdU/Ki67. The clonogenic assay was performed for seven days in 96-well plates and the readouts such as neurosphere number and diameter were recorded on 3rd, 5th, and 7th day of the incubation. For BrdU/Ki67 proliferation assay, cells were initially proliferated in 24 well plates for seven days followed by fixation of cell culture and immunostaining for BrdU and Ki67 proliferation markers. The details of proliferation methods are provided in the supplementary material.

2.7. In vitro differentiation assay

NSCs were differentiated for a period of 7 days to evaluate the impact of 0.1 μg/L and 700 μg/L of glyphosate upon neuronal and glial cells differentiation (Zhang et al., 2015). Cells were allowed to attach on to 12 mm glass coverslips coated with ECM-gel (E1270, Sigma-Aldrich, Taufkirchen, Germany) in a 24-well plate containing

differentiation medium with and without glyphosate. After incubation, cells were fixed and immunostained for *ß-tubulin III* (Mouse-anti β-tubulin III, MAB1637, Merk, Darmstadt, Germany) as a neuronal marker and *GFAP* (Rabbit anti-GFAP, Z0334, Dako, Glostrup, Denmark) as an astrocytic marker. The method has been described in detail in the supplementary materials.

2.8. Immunostaining

Fixed cultures were immunostained for neuronal, astrocytic, and cell proliferation markers by following a protocol previously reported (Bernas et al., 2017). The details of the immunostaining method have been provided in the supplementary materials.

2.9. Neurosphere migration assay

Neurosphere migration assay was performed to evaluate the effect of 0.1 μg/L and 700 μg/L of glyphosate on cell migration for 24 h. The assay was performed by following a previously reported procedure (Masood et al., 2021).

2.10. RT-PCR experiments

RT-PCR experiments were performed for NSCs differentiated with and without exposure to 0.1 μg/L and 700 μg/L concentrations of glyphosate. Around 500,000 cells were differentiated into each well of a 6 well plate coated with ECM gel for 7 days. The concentration of RNA was determined by NanoDrop™ spectrophotometer (Thermo fischer Scientifics, USA) whilst the purity was assessed by following the instructions reported in the literature (Wilfinger et al., 1997). The starting concentration of RNA extracted from the cell culture was 303−356 ng/μL and the final concentration of RNA for cDNA synthesis in RT-PCR reaction was adjusted at 100 ng/μL in 20 μL of the reaction mixture. Extraction of RNA, reverse transcription and final qPCR analysis were performed as described in the supplementary materials. Genes which were amplified as a part of this study were provided in Table 1.

2.11. Statistical analysis

Data were analyzed statistically using descriptive statistics and non-parametric Kruskell-Wallis test with post hoc Dunn's test. For two treatments experiments, Mann Whitney equation was employed. Differences between values were considered statistically significant with a probability value of $*p < 0.05$.

3. Results

3.1. Effect of glyphosate on NSCs viability and cytotoxicity

The results of the live-dead assay revealed non-significant effects of 0.1 μg/L and 700 μg/L concentrations of glyphosate on the viability of NSCs after 24 h of incubation (Fig. 1A). However, non-toxic human plasma concentration *i.e.* 7000 μg/L and the 36,000 μg/L of glyphosate (a toxic concentration reported in the previous literature) significantly reduced the viability of NSCs (Fig. 1B). Since higher concentrations of glyphosate significantly reduced cell viability, WST-1 cytotoxicity was only performed for higher concentrations. Both higher concentrations of glyphosate significantly enhanced the cytotoxicity (Fig. 1C) upon 24 h of incubation when compared to the vehicle control.

3.2. Glyphosate enhanced the Ca^{2+} uptake

It has been reported in the literature that glyphosate herbicide alters Ca^{2+} homeostasis in the brain tissues of rodents upon acute exposure (Cattani et al., 2014, 2017). To investigate the impact of glyphosate on Ca^{2+} signalling in the differentiated culture of NSCs from SVZ of the postnatal mouse calcium imaging experiments were performed. Our preliminary experiments revealed that 0.1 μg/L and 700 μg/L did not exhibit noteworthy response (data not presented) so we exploited a rather higher concentration of 7000 μg/L and a reported toxic concentration of 36,000 μg/L (Cattani et al., 2014). Our results demonstrated that upon acute exposure for 2 min, 7000 μg/L of glyphosate activated the Ca^{2+} signalling (excitation ratio at 340/380 nm) by 16% (Fig. 1D) whilst 36,000 μg/L of glyphosate activated Ca^{2+} signalling by 32% when compared to the C1 buffer control (Fig. 1E).

3.3. Effect of glyphosate on NSCs proliferation

Glyphosate interrupts the normal proliferation of cells by acting as an analogue of the amino acid glycine, a non-essential amino acid essentially required by proliferating cells. Effects of glyphosate on cultured cells preliminary depend upon the concentration of glyphosate and the type of target cells (Ji et al., 2018; Li et al., 2013; Thongprakaisang et al., 2013). We evaluated the effect of 0.1 μg/L and 700 μg/L of glyphosate on NSCs proliferation. The clonogenic assay did not reveal any significant difference between control and two concentrations of glyphosate concerning neurosphere number and mean diameter (Supplementary materials Fig. S1A and B) at all observation time points. We further confirmed our results by BrdU/Ki67 double staining experiment and found non-significant effects on the total percentage of BrdU + ve cells, Ki67+ve cells, and the ratio BrdU/Ki67 (Supplementary material Fig. S1C).

3.4. Effect of glyphosate on the differentiation of NSCs

Glyphosate and glyphosate formulations modulate functions of the blood-brain barrier, neurons (Martinez and Al-Ahmad, 2019), alter neurite growth (Coullery et al., 2016), and growth of astrocytes (Cattani et al., 2014; Ramírez-Duarte et al., 2008). We performed differentiation experiments for 7 days to explore whether 0.1 μg/L and 700 μg/L concentrations of glyphosate affect neuronal/astrocyte differentiation and their morphological features. Although the effect of both tested concentrations of glyphosate on the percentage of differentiated neurons was non-significant, yet both concentrations *i.e.* 0.1 μg/L and 700 μg/L significantly reduced the percentage of astrocytes. Interestingly, 0.1 μg/L of glyphosate exhibited a 20% reduction in the percentage of astrocytes when

Table 1
Primer sets of neural cell lineages and cytoprotection genes used in qRT-PCR.

Genes	Gene reference	Forward primer	Reverse primer
ß-tubulin III	NM_023,279	CGAGACCTACTGCATCGACA	CATTGAGCTGACCAGGGAAT
S100B	NM_009115.3	GCTGACCACCATGCCCCTGTAG	CTGGCCATTCCCCTCCTCTGTC
CYP1A1	31981814	CTCTTCCCTGGATGCCTTCAA	GGATGTGGCCCTTCTCAAATG
SOD1	NM_011434.2	CCAGTGCAGGACCTCATTTT	CACCTTTGCCCAAGTCATCT
GAPDH	NM_008084	GACCCCTTCATTGACCTCAACTACAT	TGATGGCATGGACTGTGGTCATGA

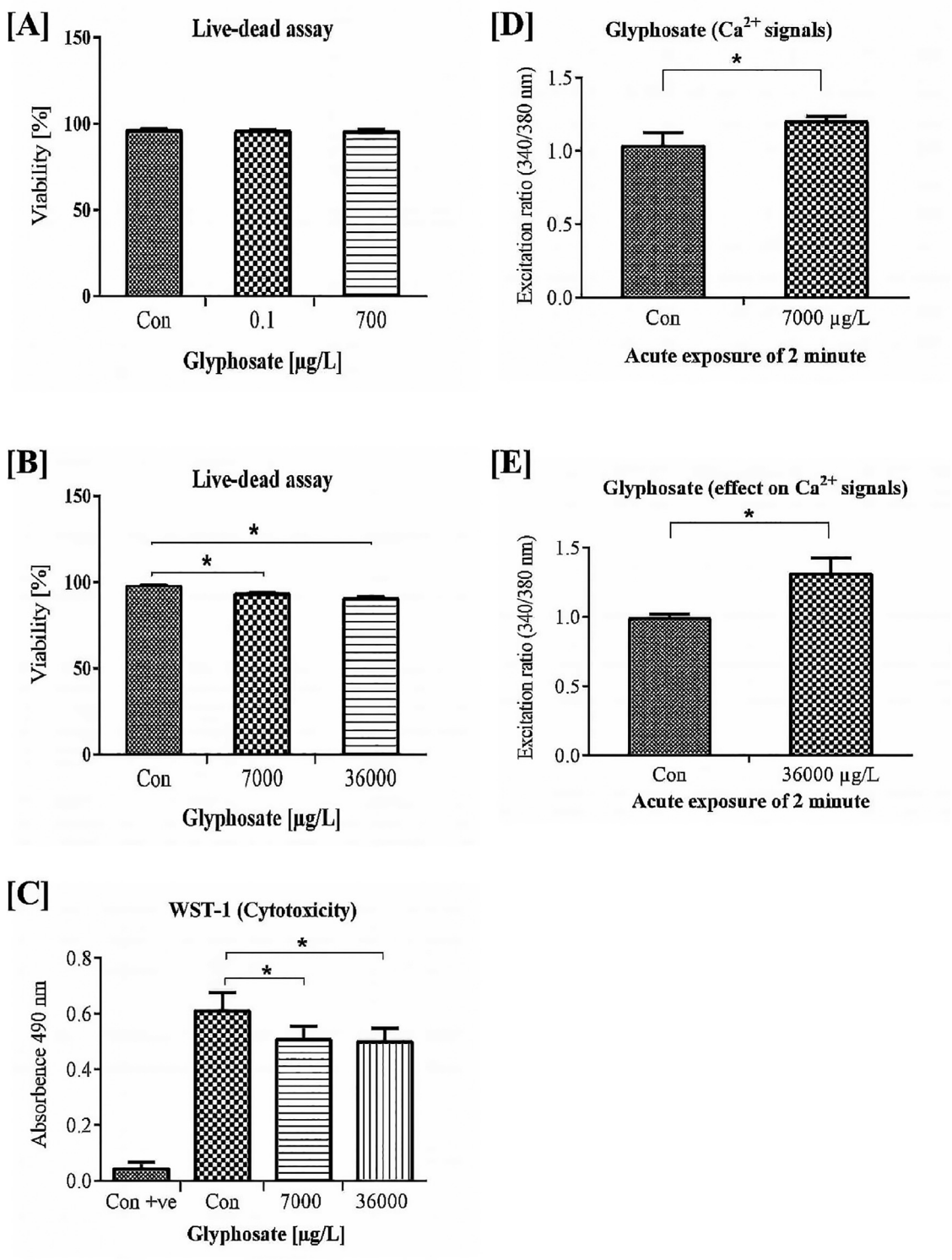

Fig. 1. Glyphosate reduced viability, induced acute cytotoxicity, and Ca^{2+} influx in NSCs culture. [A-B] Represent the viability in terms of percentage of NSCs following 24 h exposure to maximum permissible and higher concentrations of glyphosate. The percentage of viable cells was calculated from the total cell count in the phase-contrast pictures. [C] Represents the WST-1 cytotoxicity. The lower spectrometric absorbence values of glyphosate treatments at 490 nm as compared to control reflect poor viability due to general cytotoxicity. Con + ve (Control + ve was 0.2% Triton x 100). [D-E] Represent the increase in fluorescent excitation ratio in differentiated NSCs after acute exposure to 7000 µg/L and 36,000 µg/L of glyphosate respectively. Experiments were performed as five independent replicates *(n = 5)* with 3 technical replicates for each treatment condition. 0.1 µg/L: MAC; 700 µg/L: MCL; Con: vehicle control. Data are mean ± SD. *p < 0.05.

compared to the control whilst 700 μg/L presented only a 15% reduction (Fig. 2). Although the effect on total and mean neurite length was non-significant (Fig. 3B), 700 μg/L of glyphosate significantly increased the percentage of non-neurite neurons (Fig. 3A). Surprisingly, only 0.1 μg/L of glyphosate significantly reduced the astrocyte soma area (Fig. 4).

3.5. Glyphosate reduced cell migration

In a recent study, it was postulated that glyphosate may affect the migration of the cells of the nervous system due to its inhibitory effects on thyroid-stimulating hormone, a hormone which regulates the cell migration process (Beecham and Seneff, 2016). The neurosphere migration assay is a useful *in vitro* bio-tool that effectively reveals the neural cell migration process. Results of our neurosphere migration assay demonstrated that 700 μg/L of glyphosate significantly reduced the cell migration in NSCs culture upon 24 h of incubation (Fig. 5).

3.6. RT-PCR analysis

Real-time RT-PCR was performed to comprehend the effects of 0.1 μg/L and 700 μg/L of glyphosate on the expression of neural cell lineage-specific genes *i.e. ß-tubulin III, S100β* (Wang and Bordey, 2008) as well as those which play important roles in neuroprotection and respond to toxic stimuli *i.e. CYP1A1* and *SOD1* (Milani et al., 2011; Wójtowicz et al., 2019) in differentiated cultures incubated for 7 days. Our results demonstrated that exposure of differentiating NSCs to 700 μg/L of glyphosate significantly reduced the expression of *ß-tubulin III* mRNA (67.7% of the control) whilst exposure to 0.1 μg/L of glyphosate significantly reduced the expression of *S100B* (63% of the control) (Fig. 6A). Concerning the *CYP1A1* gene, 0.1 μg/L of glyphosate significantly reduced the expression (71% of the control). The 700 μg/L of glyphosate strongly increased the *SOD1* mRNA expression by 190% as compared to control (Fig. 6B).

4. Discussions

The WHO recognized glyphosate as one of the safest pesticides which is unlikely to cause acute toxicity in normal utilisation with an oral LD_{50} value in rodents being several thousand times higher than MAC and MCL, the maximum permissible concentrations in drinking water recommended by Environmental Protection authorities of EU and USA (Dolan et al., 2013; Larsen et al., 2012; Song et al., 2012). Neurotoxicity of glyphosate has been reported in human beings (Ait Bali et al., 2017; Shaw, 2017; Zheng et al., 2018), rodents (Cattani et al., 2014, 2017; Gallegos et al., 2016; Hernández-Plata et al., 2015; Joaquim et al., 2014; Roy et al., 2016) and NSCs cultures (Coullery et al., 2016). Most of the published neurotoxicity studies were performed *in vivo* in rodent models exploiting glyphosate formulations at doses several thousand times higher than permissible concentrations in drinking water. Only a few studies reported glyphosate-induced toxicities in the liver, kidney (Mesnage et al., 2015), and Sertoli cells (Clair et al., 2012) at doses closer to MCL. These reports inspired us to employ an *in vitro* NSCs model from the developing brain of the mouse to explore whether concentrations generally considered as permissible in drinking water by regulatory authorities lead to neurotoxic effects or if they open "toxic" windows, minor molecular changes, which can end up in severe damages when several microenvironmental factors, each harmless, act synergistically. The exploitation of cells from the mouse for predicting human neurotoxicity of xenobiotics including pesticides has been justified because of the greater similarities between mice and human beings concerning genome sequences, metabolic pathways (Harper, 2010), mechanism of neurological disorders such as Alzheimer' and Parkinson's (Blesa and Przedborski, 2014). Additionally, the susceptibility of a mouse to environment toxicants is comparable to that of the human beings (Hafezparast et al., 2002). NSCs from the SVZ of the postnatal mice have been utilized by researchers to unveil the effects of potentially toxic compounds, such as pesticide and enzyme inhibitors on NSCs proliferation and differentiation into neurons and astrocytes (Bender et al., 2017; Park and K, 2018). In the present study, we established an *in vitro* model based on NSCs from SVZ of the postnatal mouse to screen the neurotoxic effects of MAC (0.1 μg/L) and MCL (700 μg/L) of glyphosate. Our results demonstrated the significant inhibitory effects of permissible concentrations of glyphosate on NSCs differentiation and migration with modulation in the expression levels of cell lineage-specific and cytoprotective genes. Additionally, significant neurotoxicity in NSCs culture was also observed at a concentration comparable to non-toxic human plasma glyphosate concentration.

4.1. Glyphosate reduced the viability and induced cytotoxicity in the cultured NSCs

Our result demonstrated that glyphosate concentrations several times higher than MAC and MCL reduced the NSCs viability and induced cytotoxicity after 24 h of incubation. Our findings were consistent with the previous studies which reported that glyphosate concentrations several thousand times higher than so-called permissible concentrations for drinking water reduced the viability of neural stem cells from rat embryonic hippocampus (Coullery et al., 2016) and induced cytotoxicity at dosages equivalent to 36,000 μg/L of glyphosate (Cattani et al., 2014, 2017), in human buccal epithelial cells (Koller et al., 2012), in rat testes Sertoli cells (Cavalli et al., 2013), and in SH-SY5Y neuroblastoma cell line (Martínez et al., 2020). Interestingly, our data not only confirmed the cytotoxicity at the concentration of 36,000 μg/L but also at the non-toxic human plasma concentration (7000 μg/L) in NSCs cultures. The toxicity of so-called non-toxic human plasma concentration (Aris and Leblanc, 2011; Kwiatkowska et al., 2016) was probably observed due to the relatively longer glyphosate acute exposure time in our approach and also the intrinsically higher sensitivity of mouse culture towards glyphosate than those of rat and cell lines employed by researchers in their studies (Cattani et al., 2014, 2017; Cavalli et al., 2013; Martínez et al., 2020; Popova et al., 2017). Reduced survival of the cells of the nervous system due to environmental toxicants exposition in early development results in neurological disorders such as week memory, defective locomotive performance (Gorini et al., 2014), and poor IQ scores in children (Tseng et al., 2014).

4.2. Glyphosate disrupted Ca^{2+} homeostasis upon acute exposure

Concerning effects on Ca^{2+} signal activation, we found that not only higher concentration of glyphosate (36,000 μg/L) but also 7000 μg/L of glyphosate, a concentration comparable to non-toxic for human (Aris and Leblanc, 2011; Kwiatkowska et al., 2016), stimulated the Ca^{2+} signalling in the differentiated culture of NSCs from the mouse SVZ upon acute exposure. Our findings are in accordance with those reported in previous studies that acute exposure of hippocampus cells from postnatal rat to glyphosate formulation resulted in increased Ca^{2+}up take. Activation of NMDA receptors, voltage-dependent Ca^{2+} channels and activation of CaMKII played a key role in Ca^{2+} influx (Cattani et al., 2014, 2017). Enhanced Ca^{2+} influx was also reported in Sertoli cells of rat testis after acute exposure to 7200 μg/L and 36,000 μg/L of glyphosate.

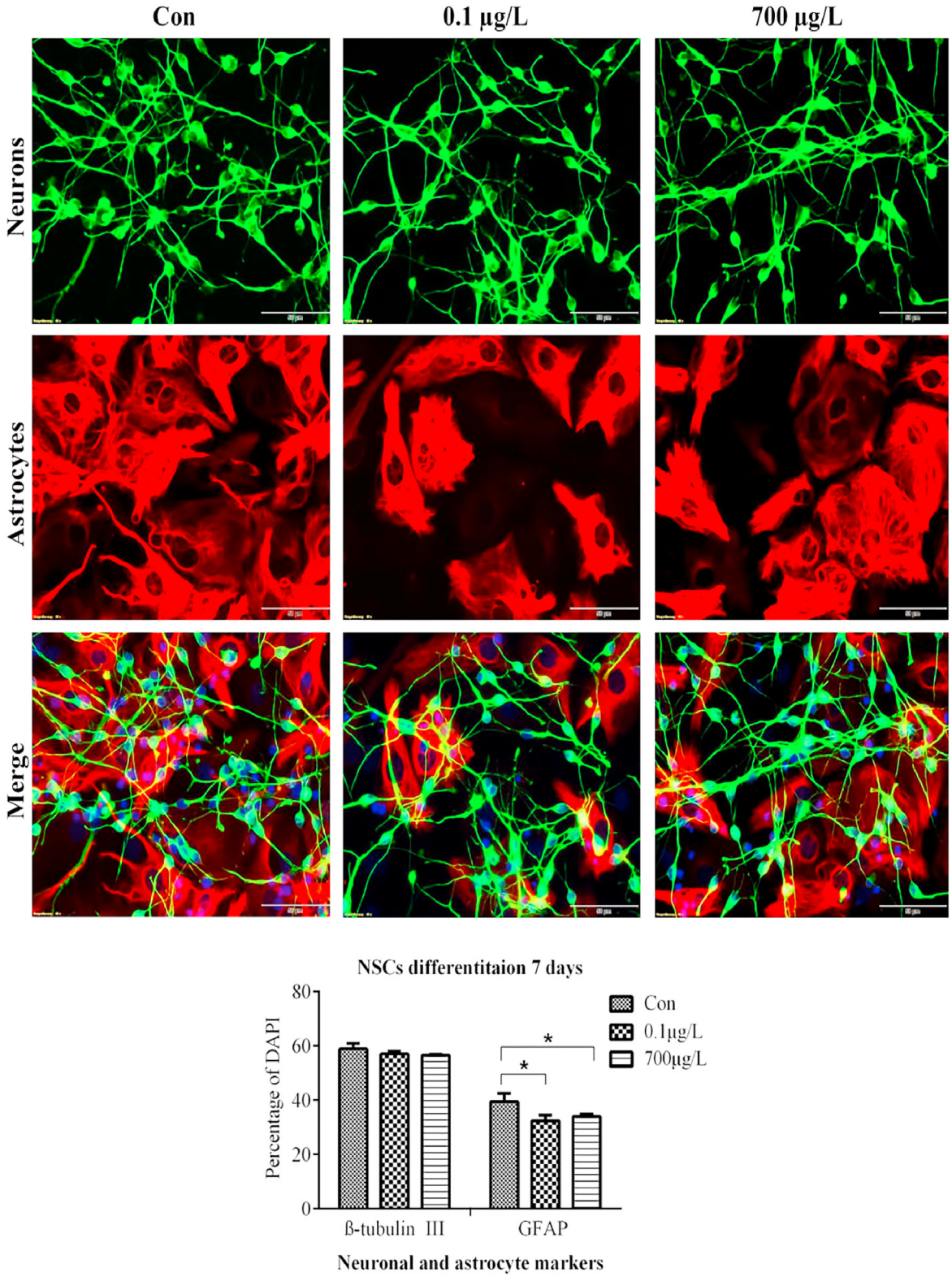

Fig. 2. Effects of glyphosate on neuronal and astrocytic differentiation of NSCs after 7 days of incubation. Green cells are β-tubulin III + ve neurons and red cells are GFAP + ve astrocytes. Percentages of neurons and astrocytes represented in the graph were calculated from the total DAPI nuclei count. Neurons were well developed with healthy neurites in all conditions. Astrocytes were mostly having leaf-like morphology. Pictures were taken with a 40 x lens of a fluorescent microscope. The experiment was performed as 5 independent replicates with 3 technical replicates for each treatment condition. Data are mean ± SD. Scale bars: 50 µm *$p < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

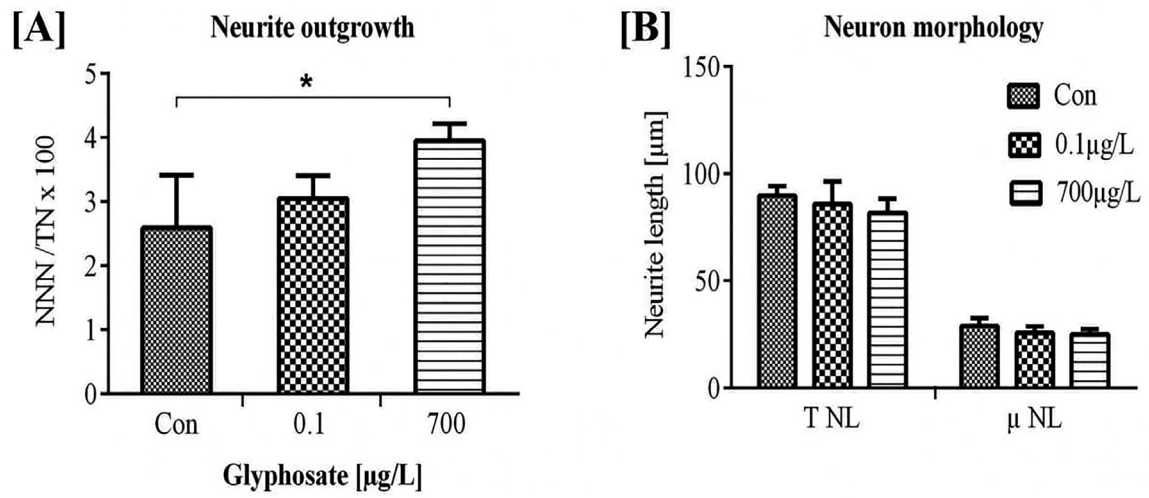

Fig. 3. Glyphosate effects on neurite outgrowth of differentiated neurons. NSCs were differentiated for 7 days. [A] Represents the percentage of non-neurite neurons which was calculated from the total neuronal count in each microscopic field. [B] Represents the neurite length which was measured from at least 100 neurons for each condition in each replicate. Neurite length was measured using Cell-SENS software. NNN: non-neurite neurons; TN: total number of neurons; TNL: total neurite length. µ NL; mean neurite length. The assay was performed as five independent experiments (n = 5) with 3 technical replicates for each treatment condition. Data are mean ± SD. *p < 0.05.

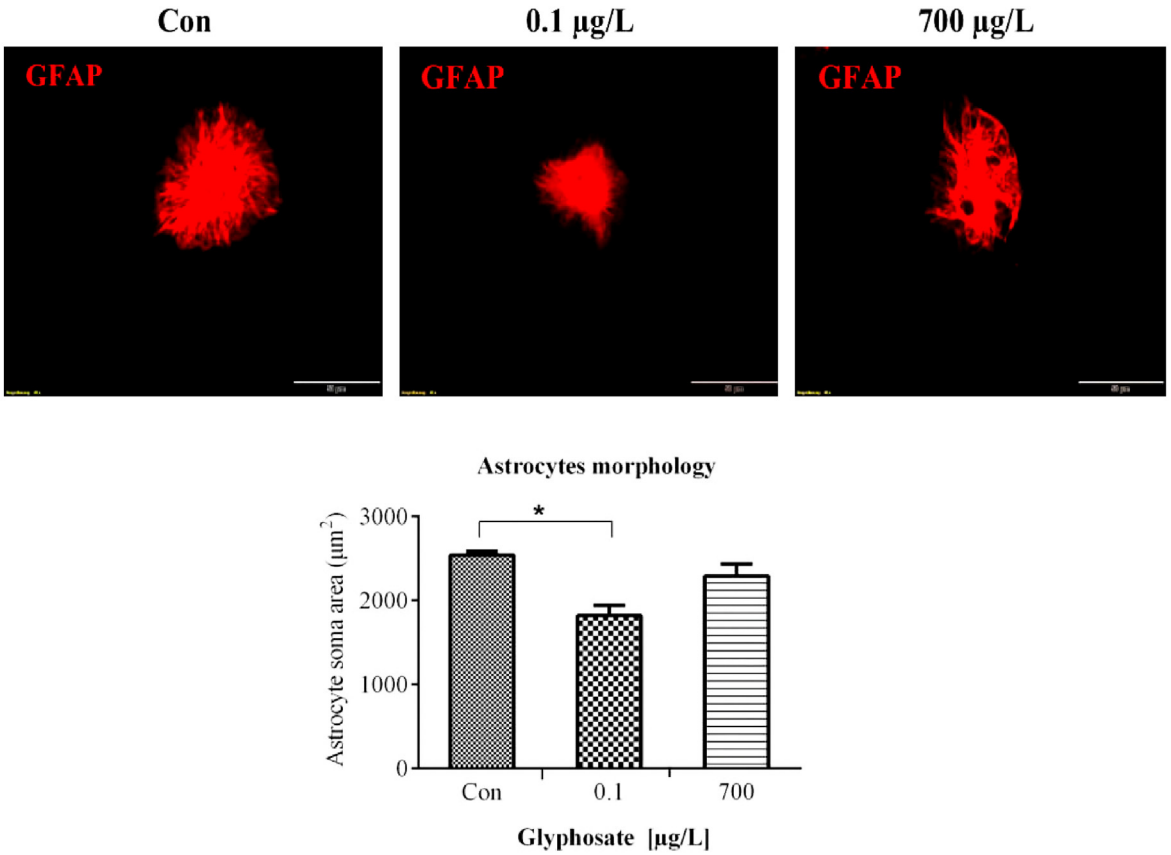

Fig. 4. Effects of glyphosate on astrocyte soma area after 7 days of differentiation. Differentiated astrocytes were stained for GFAP. Most of the astrocytes showed a leaf-like morphology under all treatment conditions. Around 100 cells were included in the measurement from each condition in each replicate. Measurements were performed using CELL-SENS software. Representative pictures were taken with a 40× objective of a fluorescent microscope. The assay was performed as five independent experiments *(n = 5)* with 3 technical replicates for each treatment condition. Data are mean ± SD. Scale bars: 50 µm *P < 0.05.

Activation of phosphatidylinositol 3 kinase, protein kinase c, mitogen-activated protein kinase p38 MAPK, and ERK played a key role in Ca^{2+} influx in Sertoli cells (Cavalli et al., 2013). Intriguingly, in our study Ca^{2+} signalling activation was observed not only at the reported neurotoxic concentration 36,000 µg/L (Cattani et al., 2014) but also at the lower concentration of glyphosate *i.e.*7000 µg/L,

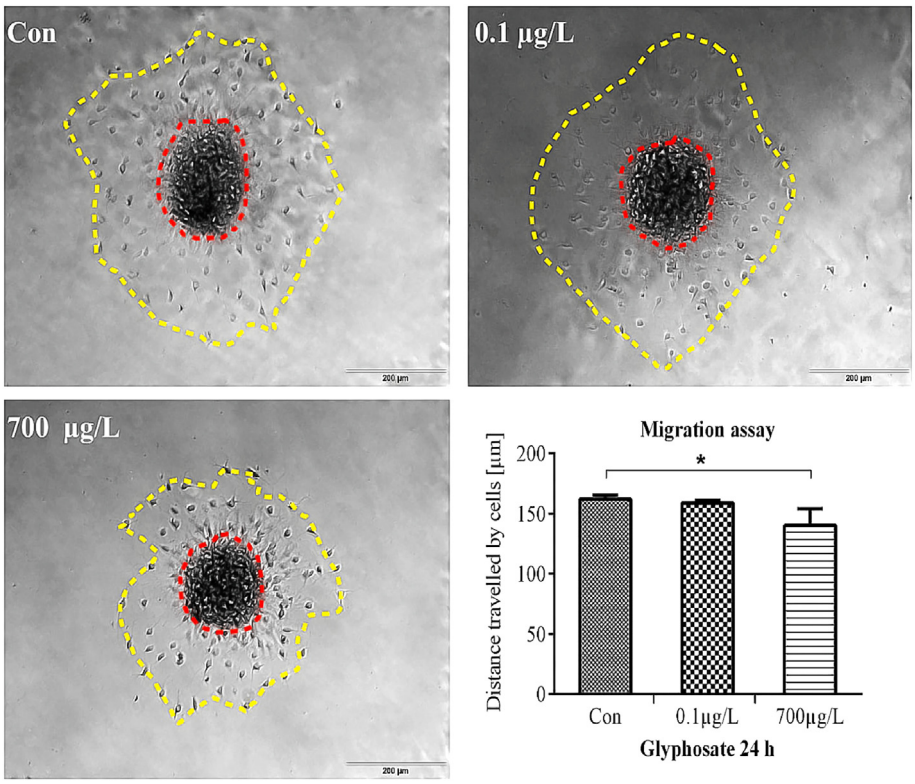

Fig. 5. Glyphosate reduced cell migration after 24 h of incubation. Untreated neurospheres were attached on to PDL coated glass coverslips and differentiated in differentiation medium into each well of a 24-well plate with and without glyphosate for 24 h. Pictures of neurospheres were captured with a phase-contrast microscope for each condition. The bar graph represents the mean distance travelled by cells from the edge of a neurosphere core to the widest destination in all four directions. Representative Images were taken with a 10× objective of a phase-contrast microscope. The outer yellow circles in the phase-contrast images indicate the area covered by the migrated cells and the inner red circles enclose the neurosphere core. The core of neurosphere consists of a mixed population of undifferentiated, partially differentiated and dead NSCs. The assay was performed as five independent experiments *(n = 5)* with 3 technical replicates for each treatment condition. Data are mean ± SD. *p < 0.05. Scale bar is 200 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

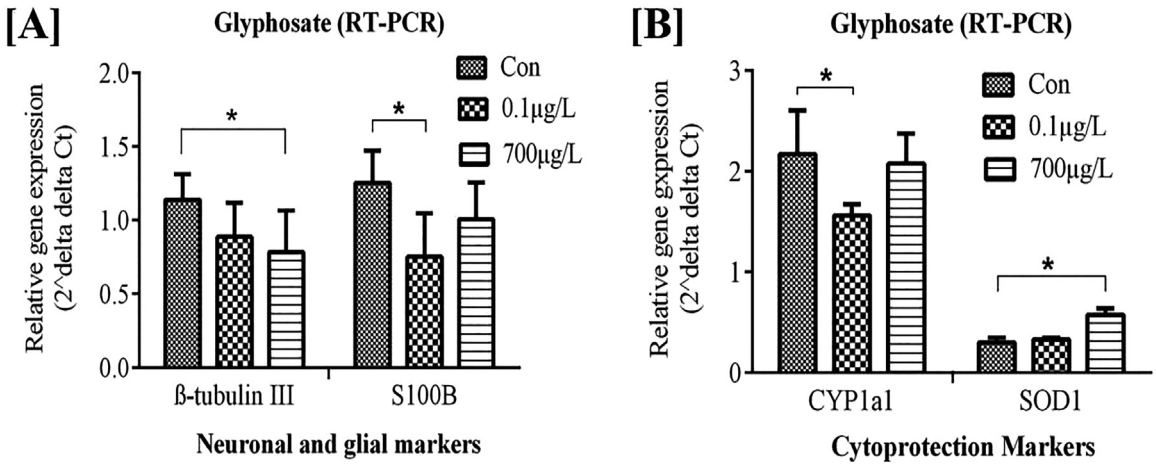

Fig. 6. qRT-PCR of genes expression in differentiated NSCs exposed to glyphosate for 7 days. qRT-PCR was used to determine mRNA expression of cell lineage-specific genes and those involved in the cellular response to the toxic stimuli. [A] Represents the relative gene expression of neuronal and astrocyte lineages specific genes. [B] Represents the relative gene expression of metabolic marker CYP1A1 and oxidative stress indicator SOD1. Data were normalized to the reference gene GAPDH and represented in bar graphs as mean ± SD. The qRT-PCR experiment was performed as a triplicate. *p < 0.05.

being much closer to the concentration which induced Ca^{2+} influx in cells other than those of the nervous system (Cavalli et al., 2013). These findings affirm the high sensitivity of our model towards the neurotoxic effects of glyphosate. Interestingly, associations between Ca^{2+} signalling activation and cytotoxicity was also observed regarding two glyphosate concentrations employed. The increased Ca^{2+} influx is related to the increase in the generation of intracellular reactive oxygen species and excessive release of glutamate which ultimately leads to cell death as reported in the literature (Cattani et al., 2014). Moreover, disruption in Ca^{2+} homeostasis

leads to disarray in cellular metabolism implicated in determining cell fate (Ham et al., 2020). Since none of the permissible concentrations of glyphosate inhibited the viability of NSCs or activated Ca^{2+} influx, we performed proliferation, differentiation, migration assays and gene expression analysis for MAC and MCL concentrations of glyphosate in the subsequent experiments.

4.3. Effect of glyphosate on NSCs proliferation

Neither MAC nor MCL concentrations of glyphosate presented any modulating effect on NSCs proliferation. The impact of glyphosate on cell proliferation are versatile depending upon the dose and target cells employed. For instance, pico and micromolar concentration of glyphosate increased the proliferation in human breast cancer cell line T47D (Thongprakaisang et al., 2013) whilst 50 mM concentration of glyphosate inhibited proliferation of human ovarian and prostate cancer lines. Glyphosate concentration higher than 100 mM inhibited the proliferation of normal human immortal ovarian and prostate cell lines (Li et al., 2013). Another study reported that 0.6–18 μM of glyphosate increased the proliferation of human embryonic kidney cells (HEK293) by upregulating transcription factors, JUN, MYC, FOS, and ERG1. Interestingly, higher or lower concentrations of glyphosate did not affect the HEK293 proliferation (Jeon et al., 2020). Although the MCL of glyphosate used in our study lies within this concentration range (0.6–18 μM), yet MCL did not modulate the proliferation of NSCs. The discrepancy in our results and the published report (Jeon et al., 2020) was most probably due to the different type of target cells exploited in our study since glyphosate response varies significantly according to the target cells (Thongprakaisang et al., 2013). To the best of our knowledge, only a few researchers determined the effect of pure glyphosate on the proliferation of cell culture. Although these researchers employed cell lines instead of primary neural cell culture and used glyphosate concentrations several hundred times higher than the maximum concentration we employed in our proliferation experiment, still they did not report any effect on the cell proliferation (Culbreth et al., 2012; Harrill et al., 2018). Since there is no study to compare our results concerning the effect of glyphosate on the proliferation of the primary NSCs, we reported the effect of environmentally recognized permissible concentration of glyphosate on the proliferation of NSCs from the mammalian brain for the first time to establish the preliminary ground for future in-depth investigations.

4.4. Glyphosate affected the differentiation of NSCs

The nervous system contains different types of cells *i.e.* neurons, glial, microglial, and endothelial cells. Each of the given cell types maintains a specific role. These cells are highly sensitive towards chemical insults. Exposure to noxious substances during the cell differentiation phase results in serious consequences in the future life of an organism. Pesticides are known to disrupt neuronal and astrocytes differentiation (Bal-Price and Hogberg, 2011). Immunostaining of a differentiated culture of NSCs is an appropriate and very useful technique to identify multiple lineages of the cells of the nervous system (Abranches et al., 2006). β-tubulin III is a general neuronal marker, GFAP and S100β are astrocyte markers which have been successfully employed by researchers for unveiling neurotoxicities of pesticides in differentiated NSCs (Park and K, 2018; Seth et al., 2017). Our results demonstrated that both permissible concentrations of glyphosate did not affect the neuronal percentage and neurite length. Interestingly, MCL of glyphosate showed a tendency to increase the percentage of non-neurite-neurons when compared to the control, indicating the inhibitory effects of glyphosate on neurite outgrowth. Several pesticides inhibit neurite outgrowth indirectly by inhibiting the synthesis of fibronectin from astrocytes as observed in hippocampus cultures. Fibronectin is an important extracellular matrix protein which promotes neurite outgrowth (Pizzurro et al., 2014). Glyphosate decreased the neurite growth and maturation in the cultured hippocampus neurons of a rat by decreasing the expression of Wnt5a level and downregulating CaMKII (Coullery et al., 2016). It is worthful to mention that in our study the concentration of glyphosate was several thousand times lower than the one reported in the literature, confirming the notion that NSCs culture from mouse SVZ is highly sensitive towards glyphosate neurotoxicity as compared to the hippocampus culture from rat (Coullery et al., 2016). Although both the MAC and MCL of glyphosate decreased the percentage of astrocytes in differentiated culture, a relatively increased response was observed for MAC of glyphosate as compared to MCL. Additionally, MAC of glyphosate significantly reduced the astrocytes' soma area. Inhibitory effects of glyphosate formulation on expression levels of astrocytes of hippocampus cultures from rat pups have been previously reported (Cattani et al., 2014, 2017). Contrary to our findings, glyphosate herbicide formulation was reported to increase the astrocyte proliferation with concomitant loss of neurons in the telencephalon of the fish's brain (Ramírez-Duarte et al., 2008). The discrepancy in the published reports concerning the expression of astrocytes could be related to the different animal models exploited and different concentrations of glyphosate employed since glyphosate response significantly varies with the doses and target organisms and cells (Thongprakaisang et al., 2013). The intranasal administration of glyphosate in mice for four weeks, for instance, increased the percentage of astrocytes in the anterior olfactory but showed no effect on astrocytes in the hippocampus, substantia nigra, striatum, or prefrontal cortex (Gallegos et al., 2020). The decrease in astrocyte soma area after exposure to MAC of glyphosate could be related to the interaction of this low concentration of glyphosate to the molecular pathway governing the energy metabolism of the astrocyte. This notion is supported by a study which reported that low micromolar concentration of glyphosate-herbicide reduced the cell growth and proliferation of astrocytic cell line C6 by disrupting the energy metabolism (Neto da Silva et al., 2020). However, in this published study, the researchers exploited glyphosate formulation rather than pure glyphosate and a cell line rather than primary cell culture. So detailed molecular studies, therefore, are needed to unveil the inhibitory mechanism of MAC of glyphosate on astrocytes differentiated from our NSCs culture. Poor neurite outgrowth results in autism (Gilbert and Man, 2017), and abnormal astrocytic development is implicated in mood disorder in children (Koyama, 2015).

4.5. Glyphosate reduced cell migration

After mitosis, proliferating neural stem cells, progenitor cells, glial and differentiated neuronal cells tend to migrate from the neurogenic niches to their final destinations in the brain. Any abnormality in the cell migration process ultimately leads to serious brain development disorders in future life. Common neurological disorders associated with the cell migration defects include heterotopias, schizophrenia, epilepsy, and lissencephaly (Baumann et al., 2014). Several studies have reported the correlation between the deficiency of thyroid-stimulating hormone (TSH) and the neuronal cell migration disorders in rat and human offspring whose mothers were exposed to TSH inhibitors during pregnancy. Glyphosate can chelate manganese ions (Mn) leading to serum manganese deficiency which affects pituitary manganese-dependent Protein phosphatase-1 (PP1) enzyme function and ultimately leads to the strong reduction of TSH in serum (Beecham

and Seneff, 2016). In our study, MCL glyphosate significantly inhibited cell migration. Although we did not find any published *in vitro* study in which inhibitory effects of glyphosate were evaluated on cell migration yet there is a plethora of published studies reporting inhibitory effects of pesticides including herbicides on neural cell migration. The herbicide Oxadiazone, for instance, inhibited the migration of neuronal striatal cells in cultured primary neuronal precursor cells of the human striatal primordium at non-cytotoxic concentration. The inhibitory effects of Oxadiazone were mediated through overexpression of acylphosphatase (ACYP2), a marker associated with apoptosis, cell differentiation, and ion transportation and which is overly expressed on fibroblasts from Alzheimer's patient (Degl'Innocenti et al., 2019). An assay based on rat embryonic mesencephalic neural stem cells revealed the inhibitory effects of the pesticide Rotenone on cell migration in a dose-dependent manner (Ishido and Suzuki, 2010). Inhibition of neuroblast cell migration from SVZ in C57B1/6 mice in response to prenatal exposure to the herbicide Glufosinate ammonium at a dose several times less than the ones defined by Environmental protection authorities has also been reported. These effects were correlated with the impact of Glufosinate on the cytoskeleton (Herzine et al., 2016).

4.6. Glyphosate modulated gene expression

Although immunostaining is an ideal technique for quantitative analysis of different phenotypes of the cells of the nervous system, it only reveals the proteins which are expressed within the cells. Gene expression at the mRNA level is a very useful tool to identify early and subtle effects of neurotoxins on various kinds of differentiated cells of the nervous system with detailed mechanisms (Abranches et al., 2006; Hogberg et al., 2010). *ß-tubulin III* represents the only tubulin protein of this class which is neuronal specific (Betancourt et al., 2006) expressed in differentiated neurons and axons (Kim et al., 2016) and plays an important role in their development (Martínez et al., 2020). Only MCL of glyphosate down-regulated the *ß-tubulin III* expression in our study. Our findings are in agreement with published literature which stated the down-regulation of *ß-tubulin III* gene expression in cultured neuroblastoma SH-SY5Y cells after glyphosate exposure (Martínez et al., 2020). *S100B* is a protein which occurs in the nucleus and cytoplasm of a wide variety of cells. *S100B* is located on chromosome 21q22.3. Within the nervous system, *S100β* is expressed by mature astrocytes and the ones which enclose the blood vessels. The basic role of S*100B* in the developing nervous system involves the coordination of neurite and axonal growth, augmentation of the astrocytes proliferation, and neuronal protection (Wang and Bordey, 2008). Reduced expression of astrocyte marker *S100B* upon developmental exposure of rat pups to glyphosate-based herbicide has already been reported in the literature (Cattani et al., 2014, 2017) which is in agreement with our finding. Here we found that already MAC reduced the expression of *S100B* mRNA. Down-regulation of *S100B* expression resulted in brain development problems during the postnatal period in rodents (Ohtaki et al., 2007). Cytochrome 450 (CYP) is a family of enzymes implicated in the detoxification of exogenous substances and the biosynthesis of important metabolites. Disruption in the expression of the CYP enzyme family by inhibitors not only increases the vulnerability of organisms to environmental toxicants but also inhibits the synthesis of various amino acids (Samsel and Seneff, 2013). Among other members, *CYP1A1* is the only enzyme expressed in extra-hepatic tissues (Liu et al., 2013). *CYP1A1* is mainly expressed in BBB and also in other brain tissues of both humans and rodents (Ghosh et al., 2016). Tight regulation of *CYP 1A1* is a prerequisite for normal physiological functions of the body since overexpression of *CYP1A1* results in the production of toxic metabolites. Pro-inflammatory cytokines TNF-α, IL-6, and IL1β down-regulate *CYP1A1* in hepatocytes in mice (Santes-Palacios et al., 2016). Glyphosate has been reported to inhibit the CYP enzyme family. Glyphosate, for instance, has been reported to down-regulate the mRNA expression of aromatase enzyme *CYP19A1* in human placental cell line on short term exposure to a concentration less than the ones found in agricultural workers (Richard et al., 2005). Deregulation in mRNA expression of *CYP1A1* disrupts the defence process in the mouse brain neocortical cells and increases the susceptibility towards the environmental toxicants (Wójtowicz et al., 2019). Glyphosate and its roundup formulation reduced the CYP enzyme activity and reduced the mRNA expression of *CYP19* in human placental JEG3 cell line when employed at non-toxic concentrations (Richard et al., 2005). In another study, roundup formulation of glyphosate down-regulated the mRNA expression of *CYP1A2* and *CYP1A4* in liver tissues of the chicken embryo (Fathi et al., 2020). Inhibitory effects of glyphosate herbicide formulations on *CYP1A1* in rodents and fish (Cai et al., 2020) and decreased mRNA expression of CYP1A1 gene in TM3 cell lines due to glyphosate exposure (Xia et al., 2020) have been reported in the literature which is in agreement with our study since MAC of glyphosate down-regulated the expression of *CYP1A1* gene.

Reactive oxygen species (ROS) such as superoxide radicals and H_2O_2 in low concentrations serve as signalling molecules and mediate the processes of cell proliferation, migration, and differentiation (Wang et al., 2018). Overproduction of ROS occurs when cells are exposed to environmental toxicants. An excessive amount of ROS exerts destructive effects on the important macromolecules of cells such as DNA, proteins and lipids (Héritier et al., 2017). Oxidative stress mildly upregulates the expression of superoxide dismutase enzymes gene and as a consequence, these enzymes interact with toxic superoxide radicals and convert them into relatively less toxic substances, such as oxygen and H_2O_2. In mammalians, there are three isotypes of *SOD i.e. SOD1, SOD2, and SOD3. SOD1* is the most abundant enzyme which exists in almost every mammalian cell (Wang et al., 2018). The *SOD1* gene is located on chromosome 21 of human beings. The *SOD1* gene encodes for the superoxide dismutase enzyme whose basic role involves the detoxification of highly toxic superoxide species and converting them into relatively less toxic hydrogen peroxide ions (Estácio et al., 2015; Milani et al., 2011; Rosen et al., 1993; Sea et al., 2015). In our study, MCL of glyphosate enhanced the expression of *SOD1* which is in agreement with the published studies which reported the up-regulation of *SOD* genes in liver tissues of the turtle after glyphosate-based herbicide exposure for 96 h (Héritier et al., 2017) and overexpression of *SOD1* gene in hepatic tissues of the rat on long term exposure (Tang et al., 2017). The increased expression of *SOD1* reflects the first signs of oxidative stress imposed by glyphosate.

A few studies also reported mild to moderate neurotoxicity in humans along with the concentration of glyphosate detected in the body fluids. The magnitude of these concentrations of glyphosate detected in the body fluids of the human was several hundred to thousands of times higher than MAC and MCL of glyphosate. The accidental intake of glyphosate herbicide by human beings, for instance, resulted in severe meningitis. The concentration of glyphosate detected in CSF was 122.5 μg/mL and the patient presented with a high level of the pro-inflammatory marker in CSF (Sato et al., 2011). Mild CNS symptoms along with CVS and respiratory disturbances were reported in suicidal cases with 61,000 μg/L as a mean serum concentration of glyphosate (Zouaoui et al., 2013). Furthermore, *in vitro* cell-based studies revealed that

glyphosate at concentration 10 times higher than MCL exhibited a mild inhibitory effect on the viability of cultured human kidney cell lines 293, placenta JEG3 cells, and Umbilical cord vein HUVE cell line (Benachour and Séralini, 2009) while 1000 μg/L of glyphosate significantly reduced the viability of Sertoli cells of rat (Clair et al., 2012). To the best of our knowledge, the present study is the first one to report *in vitro* neurotoxicity induced by very low concentrations of pure glyphosate which are permissible in drinking water by the regulatory authorities. These reports reflect the high sensitivity of our developmental neurotoxicity *in vitro* models.

5. Conclusions

The present *in vitro* study based upon SVZ of the postnatal mouse revealed that which types of neural cell and at what stage of the neurodevelopment process were affected by very low concentrations of pure glyphosate. Furthermore, we observed the neurotoxic effects of glyphosate at concentrations recognized by environment regulatory authorities as permissible concentrations in drinking water. Our study also revealed that gene expression endpoints may serve as very useful readouts for investigating the neurotoxicity of glyphosate. Combining immunostaining with gene expression endpoints in *in vitro* testing provides a highly valuable approach to speed up the neurotoxicity screening process for regulatory purposes leading to the restricted consumption and tight control on newborn exposure to glyphosate with developmental neurotoxicity. Moreover, a concentration that was only a few folds higher than MCL significantly exhibited cytotoxicity and Ca^{2+} signal activation in the differentiated NSCs cell. Our findings signify the need to review the safety standards established by environmental protection agencies concerning safe glyphosate concentrations in drinking water. Future studies, however, are required to unveil the detailed molecular mechanisms of neurotoxicity induced by maximum permissible concentrations of glyphosate in NSCs of the developing nervous system. It is also worthful for prospective researchers to include NSCs from a human embryo in the screening program.

Credit author statement

Muhammad Irfan Masood: Conceptualization, Methodology, Investigation, Software and Writing – original draft, Data curation. Mahrukh Naseem: Software, Staistical calculation, Writing – original draft. Salam A Warda: Software, Data curation, Statistical analysis. María Ángeles Tapia-Laliena: Investigation, Data curation. Habib ur Rehman: Editing and reviewing, Writing - original draft. Muhammad Jawad Nasim: Data curation, Editing and reviewing. Karl Herbert Schäfer: Supervision, Conceptualization, Resource, Reviewing article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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