Neural stem cells as a model for screening environmental toxicants and a pharmacologically active molecule

Dissertation

zur Erlangung des Grades

des Doktors der Naturwissenschaften

der Naturewissenschaftlich-Technischen Fakultät

der Universität des Saarlandes

Von

Muhammad Irfan Masood

Saarbrücken

2021

Tag des Kolloquiums:	**03. November 2021**
Dekan:	**Prof. Dr. Jörn Erik Walter**
Berichterstatter:	**Prof. Dr. Claus Jacob**
	Prof. Dr. Alexandra K. Kiemer
Vorsitz:	**Prof. Dr. Marc Schneider**
Akad. Mitarbeiter:	**Dr. Agnes-Valencia Weiß**

Die vorliegende Arbeit wurde von April 2016 bis Juli 2020 unter Anleitung von Herrn Prof. Dr. Claus Jacob in der Fachrichtung Pharmazie (Bioorganische Chemie) der Naturwissenschaftlisch-Technischen Fakultät der Universität des Saarlandes angerfertigt.

Dedicated to my Loving Family

Table of contents

Table of contents

Acknowledgements

Firstly, I would like to express my sincere gratitude to my supervisor Prof. Dr. Claus Jacob for accepting me as his PhD student and for his continuous support to accomplish my PhD project under his kind supervision. Kindness, devotion, dedication, vision and guidance, and motivation are some of the unique features of his personality which helped me to complete my thesis effectively.

I am extremely grateful to Prof. Dr. Alexandra K. Kiemer as my co-supervisor. She was always kind and provided me guidance concerning my project.

I am highly grateful to Prof. Dr. Karl Herbert Schäfer, University of Applied Sciences Kaiserslautern, Zweibruecken campus, Zweibruecken, who provided me the opportunity to complete the major part of research in his laboratory for almost three years. I always found him a humble, kind and devoted person. He guided me at every stage of my project in spite of his extremely busy routine.

My sincere thanks go to my colleagues with whom I have worked during my PhD. My deepest thanks go to Dr. Muhammad Jawad Nasim for his persistent support and motivation during my PhD.

I am extremely thankful to my colleagues at Saarland University, Saarbruecken. They are Dr. Muhammad Sarfraz, Dr. Abdul Baseer, Dr. Sharoon Griffin and Yannick Ney for their valuable suggestions and encouragement.

I would like to express my heartiest thanks to Dr. David Gründmann, Dr. Salam A Warda, Maximilian Weyland, Anne Christmann and other members of the Working Group Enteric Nervous system, University of Applied Sciences Kaiserslautern, Zweibruecken campus, Zweibruecken.

I would like to acknowledge my wife, Dr. Mahrukh Naseem for her constant support and encouragement throughout my work. Finally, my deepest thanks to my parents for their love, encouragement and prayers for my success.

List of Abbreviations

Abbreviations	Definitions
Aß	Amyloid beta
APP	Amyloid precursor peptide
AD	Alzheimer's disease
BAX	BCL2-associated X protein
Bcl2	B-cell lymphoma 2
CSF	Cerebrospinal fluid
CaMKII	Calmodulin-dependent protein kinase II
CYP	Cytochrome P450
cRBP	Cellular retinoid-binding proteins
DCX	Doublecortin-X
DEET	*N,N*-Diethyl-*meta*-toluamide
DW	Drinking water
ENDF1	Enhancement of neuronal differentiation factor 1
EPC	Environment permissible concentration
Erk	Extracellular signal-regulated kinases
FPR2	N-formyl peptide receptor 2
GFAP	Glial fibrillary acidic protein
GW	Ground water
GAP43	Growth associated Protein 43
MAPK	mitogen-activated protein kinases
HEK293	Human embryonic kidney 293 cells,
IL-1ß	Interleukin-1 beta

List of Abbreviations

MAC	Maximum acceptable concentration
MCL	Maximum contamination level
NECs	Neuroepithelial cells
NGF	Nerve growth factor
NO	Nitric oxide
NSCs	Neural stem cells
NSPCs	Neural stem progenitor cells
Nrf2	Nuclear factor erythroid 2–related factor 2
PC12	Pheochromocytoma12
PD	Parkinson's Disease
PS1/PS2	Presenilin ½
ROS	Reactive Oxygen Species
PI3K	Phosphoinositide 3-kinases
RWW	Raw wastewater
SDF 1	Stem cell derived factor-1
SVZ	Subventricular zone
SOD	Superoxide Dismutase
TNF-α	Tumor necrosis factor alpha
TrkA	Tropomyosin receptor kinase A
TSH	Thyroid stimulating hormone
TSW	Treated sewage water
USEPA	United States Environmental Protection Agency
TRX	Troxerutin
Wnt	Wingless and Int

List of Abbreviations

WWTP	Wastewater treatment plant

Summary

Primary neural stem cells (NSCs) from postnatal mice are a valuable, economical, ethically acceptable and sensitive *in vitro* model for screening environmental pollutants and plant molecules with neuromodulating properties.

An *in vitro* assay system based upon NSCs from the subventricular zone of postnatal mice was established to screen the neurotoxicities of pollutants occur in treated and untreated water samples collected from a local area of Baden-Wuerttemberg, Germany. The assay was successfully employed to explore the neurotoxic impact of Glyphosate at concentrations presumed to be safe in potable water. The same assay was also used to assess the neurogenerative and neuroprotective properties of Troxerutin flavonoid.

The NSCs model uncovered the deleterious effects of waterborne pollutants on the basic neurogenesis processes and the toxic potential of the Glyphosate molecule on neural cell differentiation, migration and cytoprotective genes. The model also revealed the neurogenerative activities of Troxerutin and its role in neuroprotection against the amyloid-ß42 induced inhibition of neuronal cell differentiation.

The NSCs based bioassay should be included in the existing battery of bioassays available for screening waterborne pollutants. The present study may be helpful for regulatory authorities to revise the permissible levels of pesticides in drinking water. The study established a new screening method for a flavonoid with neurogenerative properties.

Zusammenfassung

Primäre neurale Stammzellen (NSCs) aus postnatalen Mäusen sind ein wertvolles, kostengünstiges, ethisch akzeptables und empfindliches *invitro* Screening-Modell für Umweltschadstoffe und Pflanzenmoleküle mit neuromodulierenden Eigenschaften.

Ein auf diese NSCs basierter*in vitro* Assay, wurde etabliert, um die Neurotoxizität von Schadstoffen zu screenen, die in unbehandelten und behandelten Wasserproben aus einem lokalen Gebiet in Baden-Württemberg, Deutschland, enthalten sind. Dabei wurden die neurotoxischen Auswirkungen des Pestizids Glyphosat bei Konzentrationen untersucht, die in tragbarem Wasser als sicher gelten. Der gleiche Assay wurde auch verwendet, um die neurogenerativen und neuroprotektiven Eigenschaften vom Troxerutin-Flavonoid zu bewerten.

Die schädlichen Auswirkungen von wassergebundenen Schadstoffen auf die grundlegenden Prozesse der Neurogenese, das toxische Potenzial des Glyphosat-Moleküls sowie die neurogenerativen und neuroprotektiven Eigenschaften von Troxerutin gegen die Amyloid-ß42-induzierte Hemmung der Differenzierung neuronaler Zellen wurden mit diesem NSC Assay gezeigt.

Der auf NSCs basierende Bioassay sollte in die bestehende Batterie von Bioassays aufgenommen werden, die für das Screening von wassergebundenen Schadstoffen zur Verfügung stehen. Die Studie kann darüber hinaus für Regulierungsbehörden hilfreich sein, um die zulässigen Pestizidwerte im Trinkwasser zu überarbeiten. Im Rahmen der vorliegenden Studie wurde somit eine neue Screening-Methode für das Troxerutin Flavonoid mit neurogenerativen Eigenschaften etabliert.

Publications included in this thesis

Publication 1

Neural Stem Cell-Based *In vitro* Bioassay for the Assessment of Neurotoxic Potential of Water Samples.

Muhammad Irfan Masood, Natalie Tamara Hauke, Muhammad Jawad Nasim, Muhammad Sarfraz, Mahrukh Naseem, Karl Herbert Schäfer.

Journal of Environmental Sciences, 2021,101, 72–86.

Publication 2

Environment Permissible Concentrations of Glyphosate in Drinking Water can Influence the Fate of Neural Stem Cells from the Subventricular Zone of the Postnatal Mouse.

Muhammad Irfan Masood, Mahrukh Naseem, Salam A. Warda, María Angeles Tapia-Laliena, Habib ur Rehman, Muhammad Jawad Nasim , Karl Herbert Schafer.

Environmental Pollution, 2021, 270, 116179.

Publication 3

Troxerutin Flavonoid has Neuroprotective Properties and Increases Neurite Outgrowth and Migration of Neural Stem Cells from the Subventricular Zone.

Muhammad Irfan Masood, Karl Herbert Schäfer, Mahrukh Naseem, Maximilian Weyland, Peter Meiser.

PLoS ONE, 2020, 15(8), 0237025.

1. Introduction

The developing nervous system of mammals is immensely sensitive towards chemical stimuli due to the poorly developed enzyme systems and an immature blood-brain barrier [1, 2]. Any intrusion in the basic neurogenesis by an exogenous noxious substance inimically alters the physiology of the nervous system [3, 4]. Exposure to noxious chemical entities results in permanent mental defects at the later stages of the life of an organism [5]. Most of these noxious chemical entities are environmental pollutants. Human exposure to such pollutants occurs mainly through food and drinking water. Several of these environmental toxicants deposit in tissues, traverse the placenta and are secreted in the milk of feeding mothers, thus posing the risk of toxicity to mothers and their feeding neonates [6-8].

Numerous reports in the literature have delineated the interrelation between exposure to environmental pollutants and neurodegenerative diseases in neonates [4, 9]. Parkinson's disease (PD), dementia, behavioural disorders, loss of cognitive functions and Alzheimer's disease (AD) are neurological disorders associated with exposure to environmental neurotoxicants [10, 11]. In contrast, small molecules of edible plants such as flavonoids favourably modulate neurogenesis and hence have earned the interest of researchers who are in the quest to find natural remedies for treating neurodegenerative disorders [12-16].

1.1. Environmental toxicants and neurotoxicities

Neat and clean drinking water is vital for healthy human life. Water for human consumption is obtained both from ground and surface water reservoirs. Excessive anthropogenic activities, such as industrialization, intensive agricultural activities, animal farming and effluents from the wastewater treatment plants (WWTPs) are the most prominent factors contributing towards the contamination of water reservoirs. Synthetic inorganic substances, heavy metals, hydrocarbons, fungicides, detergents, pharmaceuticals and pesticides represent the major water pollutants [17-21].

1.1.1 Metal-based contaminants

Metals ions and their compounds are frequently reported as water pollutants. The ability to cross the placental barrier, blood-brain barrier and subsequent secretion into breast milk indicates the potential of metals to cause developmental neurotoxicity [22-28]. The metal ions exert neurotoxic effects by forming complexes with neurotransmitters, hormones, enzymes and other important macromolecules of the nervous system [29]. There is a great diversity in the mechanisms of neurotoxicity induced by metals contaminants. Cadmium, for instance, causes neurotoxicity by lipid peroxidation in the brain resulting in the decreased proliferation of NSCs and activation of the apoptosis pathway in the subventricular zone (SVZ) [28, 30]. Mercury induces memory defects, behavioural abnormalities and locomotive dysfunctioning in rodents by modulating the cholinergic receptors of the cells of the brain [31]. In cultured cells, exposure to mercuric compounds inhibits the neurite outgrowth and activates the pro-inflammatory markers [32]. Aluminium has been shown to reduce the proliferation, differentiation and migration of NSCs by exerting oxidative stress (OS) [33, 34]. Additionally, aluminium also increases the aggregation of amyloid-ß (Aß), thus increasing the risk of AD [23].

1.1.2. Nitrogenous and organic pollutants

Phthalates and bisphenol-A are organic neurotoxicants. Higher levels of phthalates have been reported in municipality and surface water of some European countries, including Germany and France [35, 36]. Exposure to phthalates can increase the risk of autism in children since phthalates have been shown to inhibit neurite growth in neurons [37]. Bisphenol-A reduces the proliferation, neuronal differentiation of cultured NSCs, and prevents cell migration in rodent's brain [38-42]. The higher concentrations of nitrogenous pollutants in groundwater reservoirs results from excessive decaying of plants, intense utilization of fertilizers, animal-manure for agricultural purpose, and industrial activities.

It is generally assumed that drinking water obtained from shallow wells of fewer than 30.4 meters depth provides a high risk of nitrogenous contaminants [43]. The increased concentrations of nitrogenous substances in treated water from the WWTPs, including those of some developed European countries, indicate the incapability of water clarification techniques in these countries [44]. Exposure of pregnant mothers to high nitrogenous contents through potable water has been found to induce neural tube defects in the embryos and anencephaly in newborns [45]. Nitrogenous substances adversely affect neural differentiation [46, 47].

1.1.3. Pesticides

Excessive and unjustifiable exploitation of pesticides to improve the production of crops has resulted in the contamination of water bodies of several countries across the globe. Contamination of water reservoirs by pesticides is common in agricultural countries [48]. Human exposure to pesticides occurs both directly and indirectly. Direct or occupational exposure occurs when pesticides are sprayed by agricultural workers for their intended purpose. Indirect exposure occurs through contaminated drinking water. Exposure to the lower levels of pesticides for a longer period could result in serious neurological disorders. The developing brain is particularly vulnerable to the neurotoxic effects of pesticides, especially organophosphates. Common neurological disorders associated with exposure to organophosphate pesticides include mood disturbances, impaired cognitive functions, psychomotor dysfunctioning, behavioural disorders, sensory disorders and defects in autonomic nervous system [49]. The prime reason for such higher concentrations of pesticides in freshwater bodies is the inefficient regulatory control in developing countries [50]. Even, developed European countries cannot claim to be free of this risk. A relatively high concentrations of pesticides in major water bodies is also linked to the effluents released from WWTPs into lakes and river systems [51]. With the growing population and reduced degree of precipitation, authorities in many developed countries are considering treated water from WWTPs as an alternative source of water for human consumption [52].

Both the treated and untreated water must be thoroughly evaluated before making them available for human consumption since higher levels of pollutants including pesticides, are reported in the treated water of many European countries [51, 53].

Basic physicochemical characterization of pollutants in water samples, as per current recommendations of environment protection authorities, is insufficient to assure the safety of the water [54]. Such analysis becomes more meaningful when physicochemical evaluations are combined with a complex battery of bioassays providing information about the cytotoxicity of waterborne pollutants [55]. Furthermore, the proposed bioassay battery must include several bioassays since water samples contain a cocktail of various groups of toxicants, including heavy metals, nitrogenous substances and pesticides. Each class of toxicants follows different biological targets and has diverse mechanisms of toxicities [56]. It is, therefore, necessary to include neurotoxicity assays in the bioassays battery for evaluating toxicants in water samples.

1.1.4. Glyphosate and neurotoxicities

A plethora of studies divulges the strong link between exposure to pesticides and neurological disorders in humans [4, 9]. These reports have encouraged researchers to unveil the neurotoxic potential of widely employed pesticides [2]. Glyphosate is an extensively exploited pesticides worldwide to revamp the productivity of genetically manipulated crops [57]. Glyphosate, chemically a glycine analogue, shows its herbicidal action by hindering the biosynthesis of amino acids in the weeds through the Shikimate pathway, a metabolic pathway distinct to fungi, protozoa, plants and bacteria but missing in mammals. Due to the lack of the metabolic target in mammals, acute toxicity of glyphosate with normal usage in agriculture is rare [58-60]. Nonetheless, several studies have reported the multiple systemic toxicities of glyphosate and glyphosate-based herbicides in humans. These toxicities have been associated with exposure to higher concentrations of glyphosate herbicides [58, 61-65].

Introduction

Glyphosate can cross the placental barrier and is also secreted into breast milk. These facts reflect the potential of glyphosate to cause developmental toxicities [66].

Besides general systemic toxicities, glyphosate-based herbicides also induce neurotoxicities including AD, PD, multiple sclerosis, meningitis, and loss of consciousness in adult humans. Autism, attention defects, behaviour disorders and seizures have been reported in neonates whose mothers were gotten exposure of glyphosate herbicides during pregnancy [64, 65, 67-72]. In addition, researchers have also reported anxiety and depression-like disorders in rodents after exposure to glyphosate [73].

Glyphosate and glyphosate formulations have been reported to induce neurotoxicities in cultured cells derived from the mammalian nervous system, through diverse mechanisms. Glyphosate induces apoptosis in differentiated PC12 cells by up-regulating apoptosis marker BAX and down-regulating the anti-apoptotic protein *Bcl2* [74, 75]. Neurotoxicity of glyphosate in the SH-SY5Y human neuroblastoma cell line has been associated with the activation of inflammatory markers TNF-α and IL6 [76]. Exposure of developing embryo of a mouse to glyphosate herbicide results in neurodevelopment abnormalities such as reduced neuronal differentiation and synaptogenesis in the neocortex due to the disruption in the regulation of genes related to Wnt/ß-Catenin/Notch signalling pathways [77].

During the peripartum period, glyphosate and its herbicide reduce neuronal plasticity of mothers in rodents [78]. Glyphosate herbicide has been shown to induce Ca^{2+} influx, glutamate excitotoxicity and OS in the hippocampus of rats [79, 80]. An aberrant electrical signalling pattern has been reported in the neocortex of rat pups after exposure to glyphosate [81]. The researchers have also reported the disruption in the enzymatic profile of neural cells upon long term exposure to glyphosate [69, 82]. Glyphosate concentration a few folds higher than the maximum contamination level in drinking water disrupted the blood-brain barrier without affecting the neuronal cell morphology [82].

Introduction

Higher concentrations of glyphosate inhibit the neurite growth of differentiated neurons from the hippocampus of rat embryos by modulating the CaMKII activities and the expression of Wnt5a [3]. Demyelination of differentiated neurons from dorsal root ganglia of mouse embryo after exposure to glyphosate has also been confirmed by researchers [83]. Glyphosate herbicides induce anxiety, depression and locomotive abnormalities in rats by reducing the expression of dopaminergic and serotoninergic neurons in the brain [73]. The decrease in sensitivity of dopaminergic receptor D1 in the brain of rats has been observed after exposure to glyphosate [84]. Prolonged exposure to glyphosate herbicide for a week or more has resulted in anomalous neurotransmission in the midbrain, striatum and hypothalamus regions of the brain of rats [85]. Researchers have postulated that glyphosate could reduce neural cell migration because of its inhibitory effects on thyroid activity [69]. Moreover, the decrease in the expression of the astrocytic marker in the brain of rats after acute exposure to glyphosate has also been reported [80].

The chemical structure of glyphosate is represented in Figure 1. Being a glycine analogue, glyphosate gets substituted at specific domains in important neural proteins and causes misfolding and aberrant configuration. Such substitutions may lead to neurodegenerative disorders such as AD, PD and amyotrophic lateral sclerosis in mammals. One of such substitutions of glyphosate is reported in the Aß peptide. Within the Aß peptide molecule, the substitution by glyphosate molecule results in interference in the activity of γ-secretase to strip off residues 41 and 42, leaving behind the soluble neurotoxic oligomeric Aß-42 which triggers AD [86].

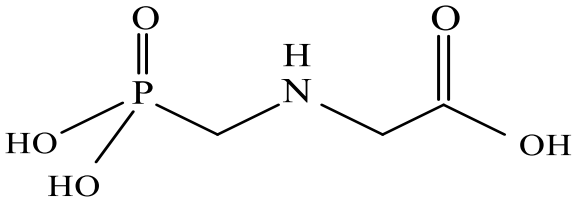


Figure 1.The chemical structure of glyphosate

Glyphosate-associated neurotoxicities reported by most researchers are generally associated with very high concentrations of glyphosate or glyphosate herbicide preparations [3, 78-80, 83]. These levels of glyphosate are more relevant to occupational exposure rather than normal exposure. Regulatory authorities have prescribed the environment permissible concentrations (EPC) of glyphosate in potable water. European Commission for Environment permitted 0.1 μgL^{-1} of glyphosate as a Maximum Allowable Concentration (MAC) in drinking water [87]. Similarly, the USA Environmental Protection Agency (USEPA) has permitted 700 $\mu gL^{-1}$of glyphosate as the Maximum Contamination Level (MCL) in drinking water [88].

It has been assumed that exposure to EPC of glyphosate is considered safe and rarely cause toxicity in mammals. Few reports have divulged the toxic effects of glyphosate at concentrations comparable to and few folds higher than the EPC of glyphosate in drinking water. Glyphosate at a concentration of 1 μgL^{-1}, for instance, significantly reduces the testosterone level in rats [89]. Exposure to glyphosate at a concentration of 1000 μgL^{-1} induces cytotoxicity in testicular Sertoli cells, reduces motility of human sperms, disrupts progesterone in the human-derived cell line [89-91]. Although few, but such studies have highlighted an alarming situation regarding the so-called EPC of glyphosate. Hence, these reports provide a solid foundation and compelling reasons for further evaluation of the neurotoxic impact of glyphosate at EPC. A relevant, sensitive neural cell model is a prerequisite to explore this fact.

1.2. Amyloid-ß and neurotoxicity

Amyloid-ß(Aß) is a class of bioactive peptides that consists of 28-43 amino acids. It is produced by enzymatic hydrolysis of transmembrane proteins amyloid precursor peptide (APP). Aß is produced by neurons and astrocytes. It usually exists as a soluble monomer that is secreted in the extracellular spaces from where it is cleared by cerebrospinal fluid (CSF) and the vascular system.

Introduction

Aß plays very useful roles in the nervous system, such as antimicrobial action, tumour suppression, repairing of the blood-brain barrier, repairing of brain tissues, regulation of synaptic functions and neuronal protection when produced in low concentrations. Exposure of the nervous system to environmental toxicants triggers the production of Aß, but the triggering response diminishes upon recovery. The CSF of a normal person contains several isoforms of Aß but the Aß40 and Aß42 are relatively in abundance. The imbalance between the production and clearance of Aß leads to the deposition of Aß peptides in the extracellular spaces where soluble monomers bind together to form oligomers or Aß plaques. The oligomeric and plaque forms of Aß are difficult to be digested and cleared by the enzymes of the nervous system, so such forms of Aß ultimately leads to neurotoxicity upon accumulation [92, 93]. The oligomeric Aß triggers a chain of pathological events in the nervous system such as activation of inflammatory pathways, OS, dysregulation of Ca^{2+} metabolism, hyperphosphorylation of the cytoskeleton protein tau, mitochondrial damage and inhibition of cell survival.

The pathological features of Aß neurotoxicity include neuritic plaque formation, neurofibrillary tangle formation, deformation of neurites and astrocytes, loss of synapsis and neuronal death. These pathologies, altogether are the hallmark of a neurodegenerative disorder such as AD. It is worth mentioning that Aß42 is more prone to aggregation and fibril formation than Aß40 [94, 95]. There are two possible mechanisms which may result in excessive production and the aggregation of Aß. The first mechanism involves the mutation in APP and peptide processing proteins Presenilin-1 and 2 whilst the second mechanism involves the triggering of Aß aggregation by exogenous substances such as heavy metals and pesticides [96, 97].

Pesticides directly interact with the oxidation chain of mitochondria of brain cells and increase the intracellular concentration of ROS, which in turn shifts the α-helix peptide structure to ß-sheet resulting in the aggregation of Aß a prominent feature of AD [98].

Introduction

The presence of an oligomeric form of Aß42, albeit at a very low concentration in the lateral ventricular part of the brain is sufficient to severely impair the learning and memory of the rodents. Researchers have postulated that Aß42 binds with cholinergic receptors in the hippocampus, cerebral cortex and forebrain of the brain of mammals and decreases the release of cholinergic neurotransmitters, which ultimately results in the impairment of memory [99]. Aß42-plaque deposition in the hippocampus induces cognitive defects of a Tg-mice expressing human APP, PS1 and PS2 genes [100]. In another study, researchers have delineated that the monomeric Aß inhibits apoptosis in the differentiated neuroblastoma cell line. On the other hand, the oligomeric form favours apoptosis by facilitating BCL2-BCN1 apoptosis complex formation [101].

Both oligomeric and protofibrils of Aß42 are capable of disrupting membrane integrity of differentiated neurons from human neuroblastoma cell line SH-SY5Y by inducing lipid peroxidation. Additionally, Aß42 also dysregulates the Ca^{2+} homeostasis and membrane depolarization [102]. The oligomeric Aß42 suppresses the differentiation and proliferation of the cultured NSCs by triggering the senescence pathway in NSPCs [103]. Interestingly, some researchers have reported that oligomeric Aß42 neither inhibits the neuronal differentiation of NSCs nor does induce apoptosis. Instead, the lower doses of Aß42 provide neurogenerative and neuroprotective effects. These findings contradicted the general hypothesis that Aß inhibits neurogenesis in the brain [104]. But a plethora of studies has repeatedly demonstrated deleterious effects of Aß42 deposition on neurogenerative processes. For instance, a study shows that four-day exposure of cultured neurons from the hippocampus to Aß42 has resulted in a strong reduction of synapse formation, neurite growth and arborization. These morphological changes under the influence of Aß42 are very similar to those reported in AD patients [105].

1.3. Flavonoids and neurogenerative properties

Natural molecules such as flavonoids modulate the fate of NSCs favourably. Several researchers have reported the augmenting impact of flavonoids on neural cell proliferation, differentiation, migration, neurite growth and mechanisms of neuroprotection [13, 16, 106-110].

Vegetables and fruit including apples, blueberries, blackberries, strawberries, white onions, red onions, sweet peppers, tomatoes, oranges, grapes, wines and have been studied extensively for their flavonoid contents [108].

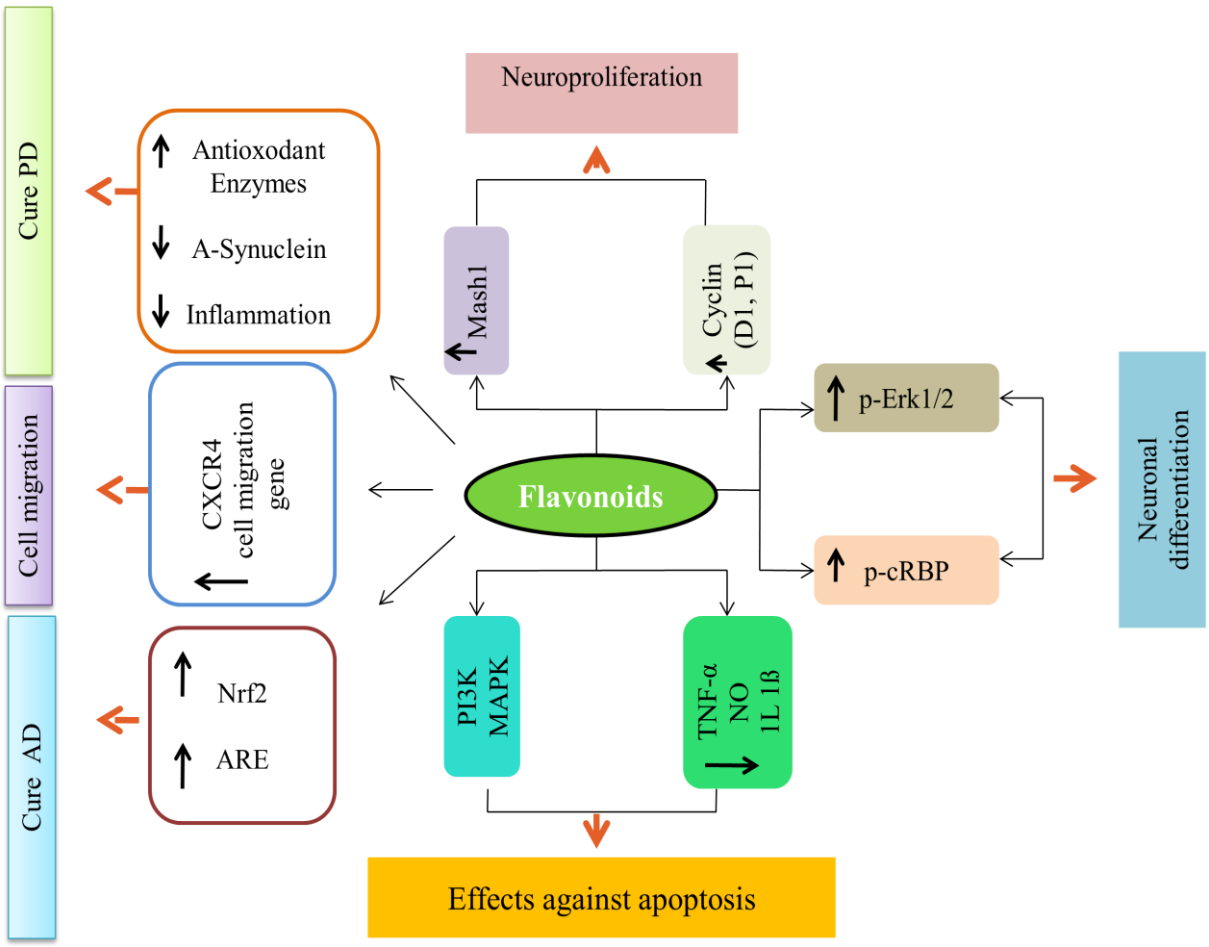


Figure 2. Diagram is summarizing the neuroprotective and neurogenerative properties of flavonoids. ↑: increase; ↓: decrease; α: alpha; ß: beta. The figure has been as adapted and redrawn from [13, 109, 112-118].

Neurogenerative and neuroprotective activities of flavonoids have been reported extensively in the literature [111]. Figure 2 summarizes the neuroprotective and neurogenerative properties of flavonoids. The poor aqueous solubility is a major shortcoming of many flavonoids. Which normally diminishes uptake of flavonoids by tissues of an organism.

1.4. Troxerutin and neurogenerative activities

Troxerutin (TRX), 3',4',7-*tris*[O-(2-hydroxyethyl)]rutin is a water-soluble derivate of the bioflavonoid rutin and is found abundantly in tea, coffee, vegetables and fruit. The chemical structure of TRX is represented in Figure 3. Literature has reported the cytoprotective activities of TRX for the liver and kidney and has also demonstrated the antidepressant, memory augmentation, anxiolytic and anti-neuroinflammatory properties of TRX in animal models [12]. Numerous studies have reported the cytoprotective and neuroprotective effects of TRX in rodents [99, 119-129].

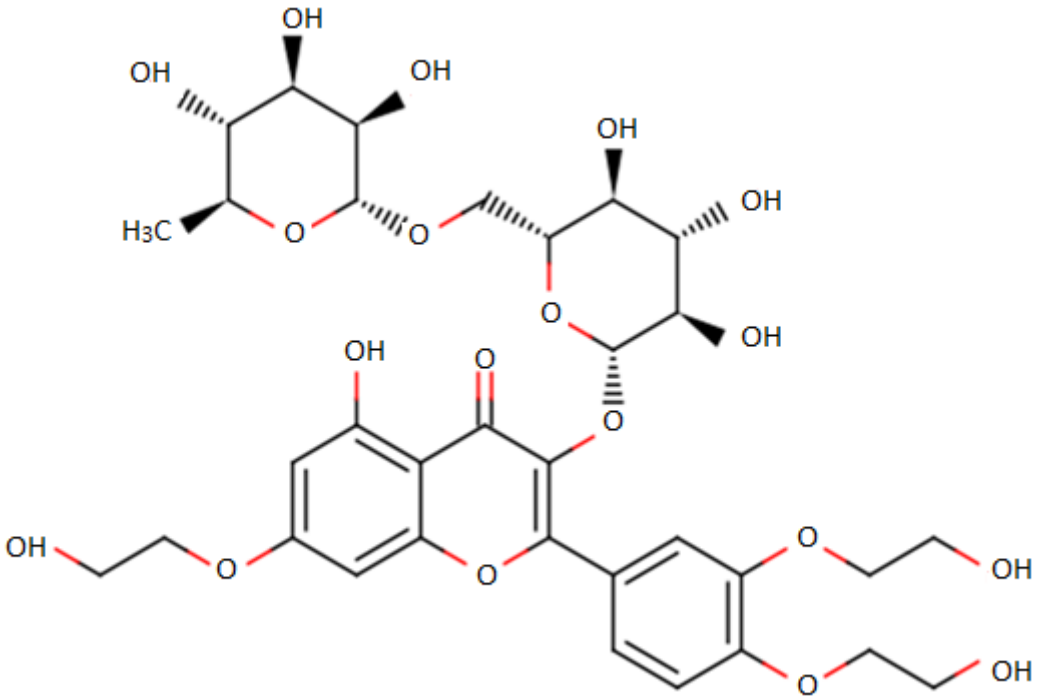


Figure 3. Chemical structure of TRX. The figure has been adopted and redrawn from [12].

Introduction

The neurogenerative and neuroprotective activities of TRX have been summarized in Table 1.

Table 1. Summary of neuroprotective and neurogenerative properties of TRX

Neuromodulating potentials	Biological activities	Ref.
Improves Aß(1-42) induced defects of memory in rats	• Ameliorates the performance of cholinergic system	[99, 119]
Improves cognitive functions, memory defects in diabetic rats.	• ↓OS, ↓Endoplasmic reticulum stress • Restores glucose cholesterol and fatty acid levels	[120]
Improves learning performance in diabetic rats	• ↓OS, • ↑Expression of glutamate-cysteine ligase catalyst in the hippocampus	[121]
Improves cognitive functions in rat	• Activates Nrf2/ARE pathways in hippocampus	[122, 123]
Neutralizes domoic acid-induced memory defects in a mouse	• ↓OS • ↓Neuroinflammation	[124]
Improves behavioral performance in D-galactose treated mice	• ↓OS • Anti-cholinesterase activity • Activates NGF/TrKA pathway in hippocampus	[125, 126]
Shows anxiolytic effects in rats	• Anti-inflammatory • ↑Expression of neurotrophic factors • Balance in serum glucose level	[127, 128]
Augments sensory motor functions in mice	• ↓Stroke volume • ↓Cerebral edema	[129]

Concerning impacts of TRX In cell culture, TRX has been found to ameliorate UVB induced cell migration inhibition in a HaCa T keratinocyte cell line [130]. A combination of TRX and cerebral-protein hydrolysate has been shown to induce cell migration in cultured human umbilical vein endothelial cell culture [131]. Most of the studies on neurogenerative properties of TRX are effect-based studies which have been conducted by exploiting *in vivo* models without a description of the individual process of neurogenesis. It will be worthwhile to screen the biological activities of TRX on the individual process of neurogenesis through a primary NSCs model. The information from the proposed NSCs assay will be highly valuable to optimize TRX as a future therapeutic candidate to deal with neurodegenerative disorders in humans.

1.5. Limitations of *in vivo* assays for screening toxicants and drug molecules

With the progress in research and drug development processes, the use of animals as a research tool has been increasing worldwide. Mouse, rats, hamsters, guinea pigs, dogs, chicken, fish and frogs are the most extensively employed models for screening newly developed drug molecules and toxicants. Millions of animals are sacrificed worldwide annually for research purpose, alone. In animal-based procedures, either a whole animal or its specific viscera are utilized. Upon completion of the experiment, the animal itself dies or is euthanized. Pain, distress and death experienced by an animal during the experiment is a matter of serious concern among animal welfare organizations [132]. Other limitations in using intact animals as experimental models are high total experimental cost, long duration of experiments, laborious work and need for a skilful person in animal handling [73, 133, 134].

These concerns have forced the regulatory authorities to opt for the 3 R concept “reduce, refine and replace” and to partially or entirely replace animal-based studies with robust *in vitro* cell culture techniques for screening of drug molecules and toxic chemicals [132, 135, 136].

In vitro screening techniques are either based upon microbial cells or cells isolated from the skin, liver, kidney and nervous system of animals which can be further propagated and maintained in a growth medium for months to years. Cell-based *in vitro* screening techniques drastically reduce the total number of animals exploited during a study [132].

1.6. NSCs for screening toxicants and pharmacologically active compounds

The nervous system is, to some extent, able to respond to injurious or toxic stimuli by the recruitment of a special kind of multipotent cells termed as NSCs [137]. The ability of NSCs to proliferate and differentiate into neurons, glia and oligodendrocytes enables them to repair the injury throughout the nervous system and to eliminate the toxic stimuli [138]. NSCs exist both in the developing and adult nervous system of mammals [139]. During earlier development of the brain, neuroepithelial cells (NECs) derived from the primary ectoderm fold-in to give rise to the neuroplate and finally the neural tube. NECs in neural tube undergo such a transition that their basal side is oriented outward, contacting the outer surface of the brain and the apical surface is oriented inward to form the ventricular zone. Within the ventricular zone of the brain, NECs are converted into multipotent radial glial cells, which later become NSCs (Figure 4) [140, 141].

The subventricular zone (SVZ), hippocampus, and the entire length of the gut are niches of NSCs [139, 142]. Within the nervous system, the symmetric cell division of NSCs results in cell proliferation while asymmetric cell division produces functional cells of the nervous system. NSCs undergo extensive proliferation in SVZ. During postnatal life, the size of SVZ gradually increases and reaches its maximum size limit at the end of the first week of the life of an organism and then decreases gradually with ageing. During adult life, NSCs mainly pool into the SVZ [140, 141, 143].

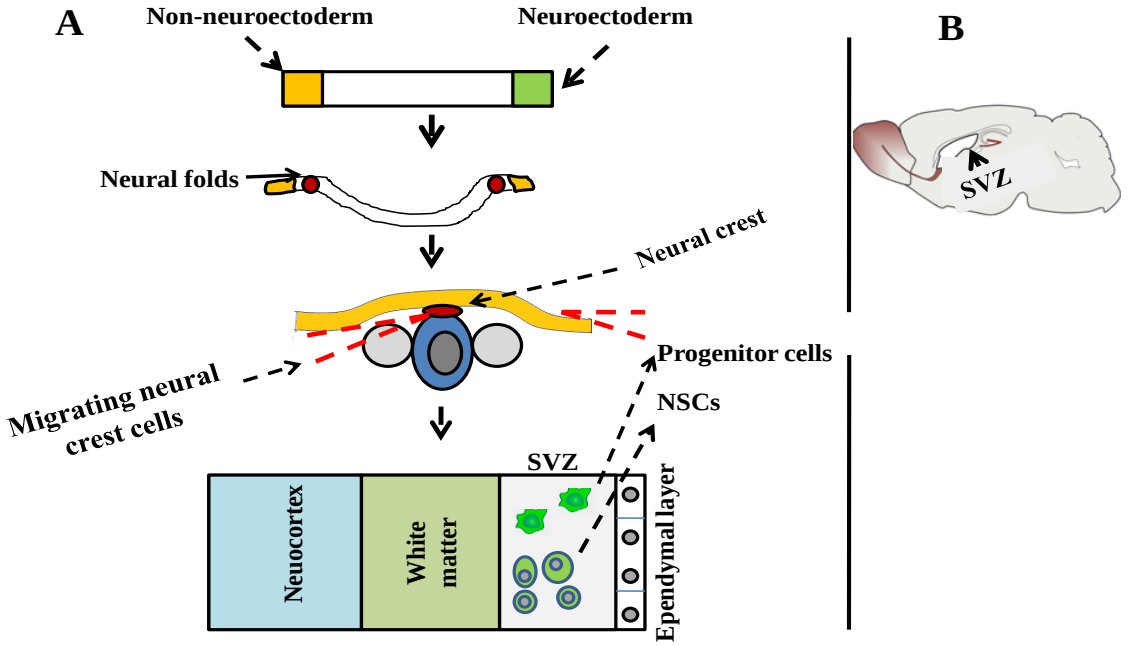


Figure 4. Panel A: The evolutionary stages in the development of the mouse brain. Panel B: The location of SVZ in the mouse brain. The figure has been adapted and redrawn from [144, 145].

The NSCs are more sensitive than the other fully developed cells of the body. NSCs are generally quiescent in the absence of exogenous chemical stimuli. These are the external stimuli trigger the changes in proliferation, migration and differentiation events of NSCs. Some of these exogenous chemical substances, including environmental pollutants, exert deleterious effects on the fate of NSCs. On the other hand, chemical substances such as plant flavonoids, provide augmenting effects on NSCs. NSCs from the developing nervous system are even more sensitive to external stimuli than their adult counterpart. Due to their higher sensitivity, NSCs provide a physiologically relevant, robust and easily quantifiable response at very low concentrations of the chemical entities [146-148].

The self-renewal property and multipotency are the unique properties of NSCs which differentiate them from the other cells of the nervous system. The response of NSCs to an external stimulus varies with the age and specific niche of the donor animal. Collectively these properties make NSCs an adaptable screening tool for both environmental toxicants and small plant molecules of pharmacological significance [149-151]. Easy extraction and handling and swift *in vitro* multiplication provide NSCs of SVZ an edge over NSCs from other niches [152, 153].

1.7. *In vitro* screening assays based upon NSCs

Earlier cell-based *in vitro* screening assays employed either genetically modified cells or cells originated from human and animal cancers. These cells, however, display less physiological relevancy. In comparison, primary NSCs have strong physiological relevance and have been shown to provide a broad spectrum of chemical effects on the nervous system with detailed molecular mechanisms [146, 148]. The *in vitro* assay based upon NSCs is considered a valuable, sensitive, robust and economical screening tool to replace traditional *in vivo* assays for screening neurotoxicities of water-borne pollutants at subtle concentrations. These *in vitro* assays have also proven their effectiveness in the discovery and optimization of new drug candidates for treating neurological disorders. In NSCs-based *in vitro* assay, the impact of a chemical compound or toxicant is evaluated on proliferation, viability, migration, differentiation and neurite growth of the cultured cells. [146, 148, 154-159]. The schematic illustration of NSCs based *in vitro* assay followed in the present study is depicted in Figure 5.

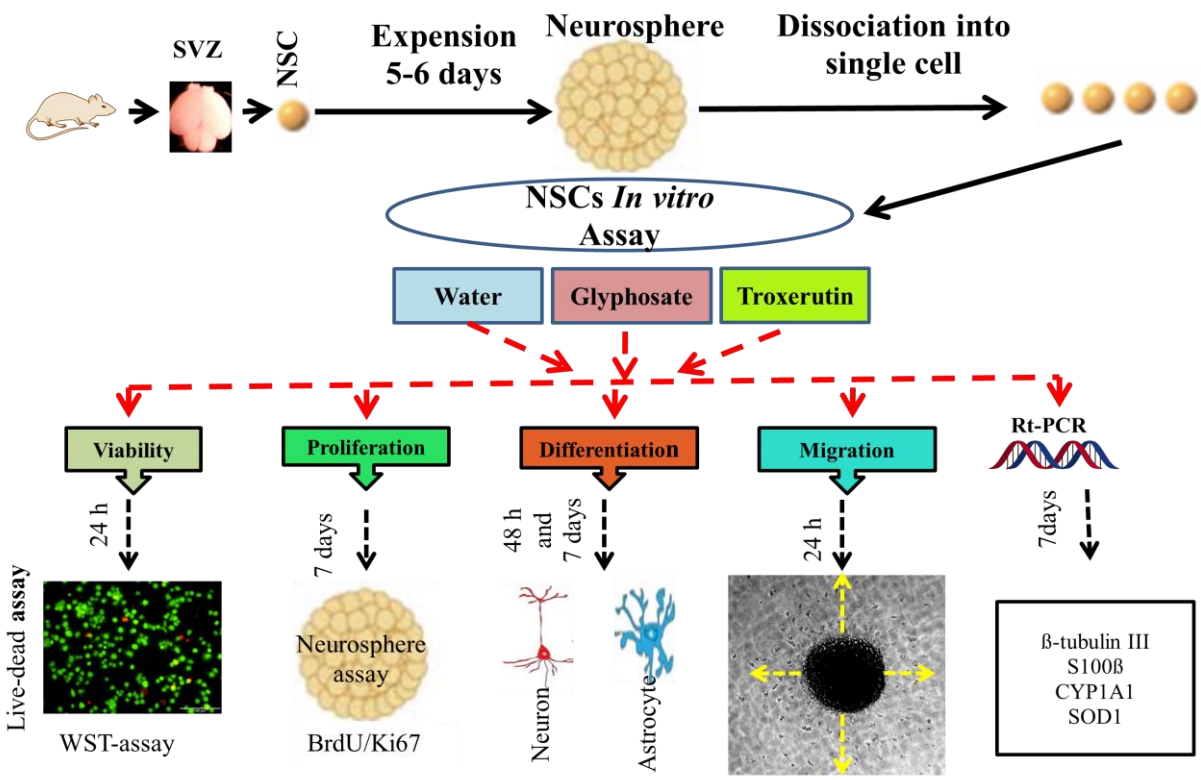


Figure 5. A layout and schematic illustration of the NSCs-based *in vitro* followed in the present study. NSCs were extracted from SVZ of the postnatal mice.

2. Aims of the thesis

Primary NSCs from the brain of the postnatal mouse offers a valuable, cost-effective and robust screening model for environmental toxicants. This NSCs-based *in vitro* model can also be employed to evaluate the neurogenerative potentials of small molecules obtained from various plants. The high sensitivity of NSCs enables them to respond to the neurotoxic potential of subtle levels of neurotoxicants. The main focus of the present thesis is to establish and utilize a robust, cost-effective and sensitive *in vitro* assay system based upon NSCs derived from the SVZ of the postnatal mice to screen the neurotoxicity of waterborne environmental toxicants and to explore the neurogenerative properties of the TRX flavonoid.

The first part of the thesis comprises the development and application of an *in vitro* assay system to screen the neurotoxicity of a complex assortment of pollutants present in both treated and untreated water samples collected from a WWTP and water supply facility near Weschnitz River in the German state of Baden-Wuerttemberg. The study supports the notion that NSCs-based *in vitro* assay may provide a robust, inexpensive and sensitive bio-tool to evaluate the quality of water samples.

The second objective of the thesis includes the application of the NSCs *in vitro* assay system to screen the neurotoxicity of the environment permissible non-toxic concentrations of glyphosate in potable water on the basic neurogenesis processes. The study supports the presumption that NSCs from the SVZ of the developing brain of the mouse may possess high sensitivity to uncover the neurotoxic potential of glyphosate at concentrations presumed to be safe in potable water.

The third objective of the thesis comprises the application of the NSCs *in vitro* assay system to explore the neurogenerative potential of TRX flavonoids. Additionally, the assay was also employed to explore the neuroprotective potential of TRX against the Aß42-induced neurotoxicity. The findings of this part of study may provide a basis for future research to elucidate the detailed molecular mechanisms governing the neuroaugmenting effects of TRX.

3. Results

3.1. Publication 1

Neural Stem Cell-based *In vitro* Bioassay for the Assessment of Neurotoxic Potential of Water Samples

Muhammad Irfan Masood, Natalie Tamara Hauke, Muhammad Jawad Nasim, Muhammad Sarfraz, Mahrukh Naseem, Karl Herbert Schäfer.

Journal of Environmental Sciences, 2021, (101), 72–86.

Available online at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/jes

JES JOURNAL OF ENVIRONMENTAL SCIENCES

www.jesc.ac.cn

Neural stem cell-based *in vitro* bioassay for the assessment of neurotoxic potential of water samples

Muhammad Irfan Masood[1,3,5,*], *Natalie Tamara Hauke*[2,4], *Muhammad Jawad Nasim*[1], *Muhammad Sarfraz*[1], *Mahrukh Naseem*[6], *Karl Herbert Schäfer*[3,*]

[1] *Division of Bioorganic Chemistry, School of Pharmacy, Saarland University, D-66123 Saarbrücken, Germany*
[2] *Applied Life Sciences, University of Applied Sciences Kaiserslautern, 66482 Zweibrücken, Germany*
[3] *ENS Group, University of Applied Sciences Kaiserslautern, 66482 Zweibrücken, Germany*
[4] *Abwasserverband Bergstrasse, Altau 10, 69469 Weinheim, Germany*
[5] *Institute of Pharmaceutical Sciences, University of Veterinary and Animal Sciences, 54000 Lahore, Pakistan*
[6] *Department of Zoology, University of Balochistan, Quetta 87550, Pakistan*

ARTICLE INFO

Article history:
Received 24 January 2020
Revised 26 July 2020
Accepted 31 July 2020

Keywords:
Neural stem cells
Subventricular zone
Neurotoxicity
Bioassay
Treated sewage effluents
Pesticides

ABSTRACT

Intensive agriculture activities, industrialization and growing numbers of wastewater treatment plants along river banks collectively contribute to the elevated levels of neurotoxic pollutants in natural water reservoirs across Europe. We established an *in vitro* bioassay based upon neural stem cells isolated from the subventricular zone of the postnatal mouse to evaluate the neurotoxic potential of raw wastewater, treated sewage effluent, groundwater and drinking water. The toxic potential of water samples was evaluated employing viability, proliferation, differentiation and migration assays. We found that raw wastewater could reduce the viability and proliferation of neural stem cells, and decreased the neuronal and astrocyte differentiation, neuronal neurite growth, astrocyte growth and cell migration. Treated sewage water also showed inhibitory effects on cell proliferation and migration. Our results indicated that relatively high concentrations of nitrogenous substances, pesticides, mercuric compounds, bisphenol-A, and phthalates, along with some other pollutants in raw wastewater and treated sewage water, might be the reason for the neuroinhibitory effects of these water samples. Our model successfully predicted the neurotoxicity of water samples collected from different sources and also revealed that the incomplete removal of contaminants from wastewater can be problematic for the developing nervous system. The presented data also provides strong evidence that more effective treatments should be used to minimize the contamination of water before release into major water bodies which may be considered as water reservoirs for human usage in the future.

* Corresponding authors.
E-mails: Irfan_masood_79@yahoo.com (M.I. Masood), karlherbert.schaefer@hs-kl.de (K.H. Schäfer).

https://doi.org/10.1016/j.jes.2020.07.028
1001-0742/

Introduction

The freshwater ecosystem is a highly valuable asset for human beings. Freshwater resources such as lakes and rivers have become polluted by a large variety of chemical substances predominantly derived from anthropogenic activities. These pollutants include complex synthetic inorganic substances, heavy metals and organic substances such as pesticides, insecticides, household cleaning agents (Héritier et al., 2017; Trintinaglia et al., 2015) and pharmaceuticals (Miege et al., 2009). With a rapidly growing world population, the available freshwater resources have become scarce, so water administrative authorities are forced to manage alternative sources of water for drinking (Jia et al., 2015). Treated wastewater is now increasingly considered as a part of the common water supply (Jaeger et al., 2015). Wastewater treatment plants are generally based upon conventional activated sludge and membrane bioreactor treatment techniques, which are inefficient in completely removing contaminants from wastewater (Celiz et al., 2009). The presence of hundreds of pharmaceuticals residues has been reported in effluents of wastewater treatment plants of various European and American countries (Miege et al., 2009).

Drinking water quality in Germany is coordinated through both local and international regulations, such as the Federal water act, Agenda 21 of UN (chapter-18), EU water framework directive 2000/60/EC (WRRL) and drinking water ordinances. Overall, the water supply in Germany is satisfactory. Anthropogenic factors and the decrease in summer precipitation due to climate change, however, suggest that the current water supply in Germany will reach its limits in the long term. In Germany, therefore, there is a need to utilize additional sources of water, such as the recycling of wastewater from sewage treatment plants. New advanced wastewater treatment technologies may assist in producing high-quality water that can even be employed for drinking purposes (Schmid and Bogner, 2018). The presence of high levels of pharmaceuticals and pesticides in water obtained from wastewater treatment plants in Germany reflects their inefficiency in completely removing water contaminants (Münze et al., 2017; Ternes, 1998). The Weschnitz River, which is one of the four tributaries to the Rhine River, is known to receive a huge volume of treated water from several wastewater plants in the area. High concentrations of pesticides have been reported in the water of the Weschnitz River, which is linked to sewage treatment plant effluents. Pesticide concentrations increase during the summer season because of intense agriculture activities. The Rhine River, in turn, has several catchment points for obtaining water for drinking purposes after further filtration (Quednow and Püttmann, 2007; Storck et al., 2015). To assess the quality of treated water as well as the efficacy of wastewater treatment plants, it is necessary to analyze both influents and effluents (Jaeger et al., 2015).

Most of the environmental monitoring programs rely on the physicochemical characterization of water samples (Niss et al., 2018), which becomes more powerful when combined with biological testing since the latter provides deep insight into the real toxic threat of water pollutants (Žegura et al., 2009). A single kind of bioassay is insufficient to explore all kinds of toxicities associated with a complex mixture of water contaminants, so common practice employs multiple bioassays for evaluating multiple types of toxicities (Jaeger et al., 2015). High total cost and ethical concerns voiced by animal welfare organizations have forced regulatory authorities to replace the traditional whole-animal-based toxicity assay systems with *in vitro* cytotoxicity testing (Burden et al., 2016). Major advantages of *in vitro* cell culture approaches include high efficacy, high reproducibility, high sensitivity, easy handling (Bianchi et al., 2015), and the ability to explore the molecular mechanism of toxicity (Poteser, 2017).

During the development of the nervous system, cell proliferation, migration, differentiation, synaptogenesis and myelination are processes that are highly sensitive to chemical exposure (Costa et al., 2008; Coullery et al., 2016). *In vitro* bioassay systems established for evaluation of the neurotoxicity of chemicals and water samples include zebrafish embryos, brain slices, synaptic systems, sensory systems, stem cells, primary cell models and cell lines (Hendricks and Pool, 2012; Legradi et al., 2018). Primary neural stem cells (NSCs) are basic building blocks of the nervous system. Their ease of isolation from the nervous system, ability to grow in an *in vitro* culture, self-renewal properties, differentiation into multiple lineages such as neurons and glial cells and capability of migrating from one location to another make NSCs a useful research tool (Bergström and Forsberg-Nilsson, 2012) for screening environmental toxicants (Tamm et al., 2006). The subventricular zone (SVZ) harbors the NSCs and represents the main site (Bollmann et al., 2014) of primary and secondary neurogenesis (Inta et al., 2008; Saha et al., 2012; Wang, 2015). Rapid proliferation and much easier handling make NSCs of SVZ a useful model for screening substances with neuromodulating properties (Liu et al., 2009).

We established an *in vitro* neurotoxicity bioassay based upon NSCs from SVZ of postnatal mice, to screen the neurotoxic potential of water samples on basic neurogenesis processes (viability, proliferation, differentiation and migration). Water samples were collected from a wastewater treatment plant and a drinking water distribution facility near the Weschnitz River located at two towns of the German state Baden-Wuerttemberg. Findings of this study will be highly valuable for environmental protection agencies and regulatory authorities to establish new standards for wastewater treatment plants to improve water quality.

1. Materials and methods

1.1. *Water sampling*

Water samples were collected from a wastewater treatment plant and drinking water distribution facilities near the Weschnitz River located in two towns of the German state Baden-Wuerttemberg. All samples were collected only once and on the same day. Sampling was performed according to the guidelines of DIN 38402–30:1998–07 (pre-treatment, homogenization and division of heterogeneous water samples) and following sampling standards: DIN 38402–11:2009–02 and DIN 38402–13:2016–09 for raw, treated and groundwater (Beuth, 2009, 2016). The sample of raw wastewater was collected from the inlet shaft before the primary clarifier,

while the sample of treated sewage water was collected from the drainage shaft after the secondary clarifier of a sewage treatment plant, Abwasserverband Bergstraße (AVB), Altau, Weinheim, Germany. The samples of groundwater and drinking water were collected from drinking water distribution facilities, Wasserzweckverband Badische Bergstraße, Wasserwerk, Hemsbach, Germany. The sampling cup was thoroughly washed first with 20% citric acid (NeolabMigge GmbH Heidelberg, Germany) and subsequently with distilled water before sampling. After sample collection, samples were transported in Duran glass transportation vessels which were tightly sealed. Samples were homogenized for 72 hr and then stored at 2–5°C until used for further analysis. Each sample volume was adjusted to 1 L. AVB is the biggest wastewater treatment plant in the area and receives the total sewage of seven communities in its vicinity. The AVB treats an annual wastewater volume of 12,000,000 to 18,500,000 m^3/year depending on rain events and groundwater levels in the associated area. The plant processes between 20,000 and 130,000 m^3 of wastewater each day depending upon the amount of precipitation (MWM, 2014). AVB plant treats wastewater by the following multiple steps: primary sedimentation, denitrification, nitrification, bio-P and sim-P.

1.2. *Chemical analysis of water samples*

Water samples were homogenized before chemical analysis. pH, electrical conductivity and O_2 concentration were measured using a multiparameter device (SensION156, Hach company, USA). Ammonium nitrogen and nitrate nitrogen were analyzed by Dr HACH-Lang Cuvette Kit systems with LCK304 and LCK339 kits using a VIS-spectrophotometer (D3000, Hach company, USA) following DIN photometry protocols (DIN ISO 15923-1:2014–07, EN ISO 6978: 2004 (DEV D11).

Pesticides *N,N*-Diethyl-*meta*-toluamide (DEET), mecoprop and terbutryn in water samples were characterized by LC-MS/MS (liquid chromatography) and dieldrin was characterized by GC–MS/MS (gas chromatography). All chromatographic analyses were carried out by an analytical laboratory (Limbach Analytics GmbH, District Court: HRB. Mannheim, Germany) with analysis reference number 17–10360 date Jul. 13, 2017. For LC-MS/MS analysis of DEET, mecoprop and terbutryn pesticides, the chromatographic separation was carried out by using a SunShell C18 CoreShell column (2.6 µm, 2.1 i.d. × 50 mm) through a Shimadzu Nexera2-HPLC system (Shimadzu, Japan) under a gradient program. Eluent A was composed of 0.1% formic acid in water and Eluent B of (0.5% formic acid and 20 mmol/L ammonium formate in methanol). The analytes were quantified by an ABSciex Triple Quad 5500-MS mass spectrometer (AB Sciex corporation, USA). For GC–MS/MS analysis, the chromatographic separation was carried out by GC column DB-35MS UI (30 *m* × 0.18 mm × 0.18 µm film) through an Agilent 7890 GC system and analytes were quantified by an Agilent 7010C Triple Quad mass spectrometer (Agilent, USA). Hydrogen gas was used as the mobile phase.

1.3. *Animals*

Wild-type Balb/c mice of both sexes of age 3–5 days were utilized. Animal preparations were conducted according to the guidelines of the local ethical committee and according to animal protection law in Rhineland-Palatinate, Germany.

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

The isolation and culture of NSCs from mice SVZ for our experiments was done according to the protocol published in the literature (Bender et al., 2017). Three animals were used for each experiment. Immediately after decapitation, mice brains were removed and stored in ice-chilled MEM-medium (Life Technology, Eugene, USA) with 1% penicillin/streptomycin (ThermoFisher, Waltham, MA, USA). SVZ were dissected from both hemispheres under an inverted microscope (SZX7, Olympus, Japan) and transferred to 1 mL of HyQtase enzyme solution (HyClone-GE, USA) followed by incubation for 20 min at 37°C. Brain tissues were triturated gently using 23 gauges and 27 gage needles 3–4 times each. HyQtase was immediately removed by centrifugation of cell suspension at 100 ×*g* for 5 min. The cell pellet was then seeded in T25 culture containing 5 mL of the proliferation medium (DMEM/F12 GlutaMax™, Life Technology, USA), 2% B-27 without vitamin A, 1% penicillin/streptomycin, ß-mercaptoethanol (Gibco, Paisley, UK), 10 ng/mL EGF and 20 ng/mL FGF (Immunotool, Germany) with the initial cell number of 500,000 to expand the cells for further experiments. Neurospheres generally appear within 3–4 days. To achieve maximum yield and cell numbers, the culture was incubated for 5 days. About half of the medium was changed every 3 days. Before starting each individual experiment, the numbers of cells were counted using the Trypan Blue method. Viability, proliferation, differentiation and migration assays were carried out by reconstituting water samples into lyophilized DMEM-F12 medium. For proliferation and viability assays, reconstituted samples were supplemented by the proliferation culture as mentioned above. For differentiation and migration assays, growth factors were omitted and the B-27 without Vitamin A was replaced by B-27 with Vitamin A (Gibco, UK). All experiments were performed in 5 replicates ($n = 5$).

1.5. *Preparation of water samples for bioassay*

A very important aspect of treating the cell-based assay with the water samples involves the adequate utilization of the water samples for medium preparation. We achieved a double-concentrated DMEM-F12 culture medium through lyophilization, which could be appropriately diluted with the water samples to be tested. The desired reconstituted medium comprising the original concentration of the ingredients of the cell culture medium was obtained with a twofold dilution of the lyophilized medium with water samples. Thus, the complete cytotoxic profile of water contaminants was achieved (Niss et al., 2018). Each water sample was initially filtered with a coarse filter followed by centrifugation (Centrifuge5–804, Eppendorf, Germany) at 20,000 r/min for 10 min to remove small visible particles. For each water sample including control, 10 mL of DMEM F-12 medium was lyophilized by freeze-drying with an Alpha 1–2 L Duplus (Fisher Bioblock Scientific, France) in a 50-mL falcon tube for about 15 hr. to reduce its volume to half. The lyophilized DMEM F-12 was re-

constituted with each of the water samples so that the final volume of water sample in DMEM medium constituted 50% of the total volume. A control sample was prepared by reconstituting with double-distilled water. Other reconstituted wastewater samples were named Con for control, RWW for wastewater, TSW for treated sewage water, GW for ground water and DW for drinking water. Immediately after reconstitution of lyophilized DMEM medium with water samples, the pH of all the samples was adjusted to 7.4. Samples were filtered through a membrane filter (0.22 µm) inside the sterile bench to remove microbial contamination.

1.6. *Calcein and propidium iodide live-dead assay*

A Calcein/Propidium iodide live-dead assay was performed to evaluate the effect of water samples on the viability of NSCs. Calcein-AM (3100MP, Life Technology, USA) is a non-fluorescent compound which is readily taken up by live cells and enzymatically converted into a green fluorescent marker. Propidium iodide (Sigma-Aldrich, Germany) provides red fluorescence and serves as a marker for dead cells. Around 25,000 NSCs were expanded in reconstituted water samples supplemented by growth factor rmFGF (20 ng/mL) and rmEGF (10 ng/mL) into a 24-well plate for a period of 24 hr. At the end of the incubation period, neurospheres were formed, which were collected and dissociated enzymatically and mechanically into a single-cell suspension, followed by washing the cells three times with PBS. The cells were subsequently incubated with live and dead assay reagent containing calcein 3 µmol/L and propidium iodide 2.5 µmol/L at 37°C for 15 min. At least five independent microscopic fields were taken with the 20× lens of a fluorescent microscope (Olympus CKX41SF, Olympus Corporation, Japan) for each water sample in each replicate. Green cells were counted as live cells while red cells were counted as dead cells.

1.7. *NSC proliferation assay*

Effects of water samples on NSC proliferation were determined through the neurosphere clonogenic assay. A neurosphere clonogenic assay is especially useful in those cases where the effect on proliferation is robust. The neurosphere number count indicates the self-renewal property of NSCs, and the neurosphere diameter indicates cell proliferation within the neurosphere architecture (He et al., 2013). Clonogenic parameters, such as the number and diameter of neurospheres, are measured periodically as defined by previous researchers (Xiong et al., 2011). Around 1000 cells were seeded in 200 µL of each water sample reconstituted in DMEM F-12 proliferation medium into each well of a 96-well plate. The plate was incubated at 37°C for a total of 7 days. The medium was changed every 3 days. For clonogenic assays, the numbers and mean diameters of neurospheres for each treatment condition were counted on days 3, 5 and 7 of the culture. On each specific time point, the whole well of a 96-well plate was scanned by using the 4× objective of a microscope with phase-contrast mode. Neurosphere parameters were recorded by CELL-SENS version 1.17.

1.8. *Differentiation assay*

In vitro differentiation was performed for 7 days to quantify the neurons and astrocytes generated from NSCs (Zhang et al., 2015). NSCs were first expanded in standard DMEM F-12 proliferation medium for 5–6 days as described above. Neurospheres were dissociated into a single cell suspension followed by seeding 20,000 cells on a 12 mm glass coverslip coated with extracellular matrix (ECM) for 1 hr. After cell adherence, cells were exposed to reconstituted water samples for the following 7 days. At the end of the incubation period, cells were fixed in a solution of 4% paraformaldehyde at room temperature for 20 min followed by washing with PBS three times. The fixed cultures were processed further for immunostaining.

1.9. *Migration assay*

NSCs were first proliferated in standard DMEM F-12 proliferation medium supplemented with growth factors, for 5–6 days. Then 15–20 neurospheres were allowed to attach at the surface of a 12 mm glass coverslip coated with Poly-D-lysine (10 µg/mL) and incubated in a 24 well plate for 1hr. placed in an incubator at 37°C. Attached neurospheres were exposed to reconstituted water samples for the next 24 hr. Phase-contrast photographs of each well were taken by a microscope using a 4× objective. Measurements were performed according to the protocol described by previous researchers (Baumann et al., 2014). Briefly, four radii of each differentiated neurosphere were calculated by measuring the distance travelled by migrated cells at a right angle to the edge of a neurosphere core from all four directions **(Fig. 5)**. At least 15 neurospheres were included for each replicate per each condition.

1.10. *Immunostaining*

Differentiated fixed cell cultures were immunostained for the neuronal and astrocyte markers. Staining was performed by following a published protocol with slight changes (Bernas et al., 2017). Immediately after differentiation, cells were fixed with 4% paraformaldehyde solution (Sigma-Aldrich, Germany) for 30 min. Cell permeability was enhanced by incubating cells with a solution of Triton 100× (0.3%) for 10 min at room temperature followed by washing with PBS-tween once and with PBS twice. Cell surfaces were blocked by incubating them with 10% Normal donkey serum (Merck, Germany) for 1 hr at room temperature. Cultures were subsequently incubated at room temperature for 1 hr. with the following primary antibody solutions in PBS with 5% blocking agent: (mouse-anti ß-tubulin III, 1:500) (MAB1637, Merck, Germany) and (rabbit anti-GFAP, 1:500) (Z0334, Dako, Denmark). The cultures were subsequently washed thrice with PBS. Cultures were then further incubated with Alexafluor 488 & Alexafluor 594 conjugated donkey antibodies (Life technology, USA) for 1 hr, and finally washed thrice with PBS. Finally, the nuclei of cells in culture were counterstained by DAPI (1:500) (Sigma-Aldrich, Germany) for 10 min then finally washed with PBS. The fluorescent mounting medium was used to fix coverslips on glass sides (Ostenfeld and Svendsen, 2004).

Table 1 – General quality parameters of water samples.

Parameter	RWW	TSW	GW	DW	Safety limit
Color pH	Black gray 7.8	Colorless 6.2	Colorless 6.7	Colorless 7.2	Colorless 6.5–8.5
Conductivity (μS/cm)	261	716	822	1013	2500
O_2 concentration (mg/L)	0.78	6.74	2.86	5.13	5.0
NH_4^+ (mg/L)	124.6	0.688	0.409	0.004	0,5
NO_3^- (mg/L)	1.13	8.600	0.111	0.145	50

RWW: raw wastewater; TSW: treated sewage water; GW: groundwater; DW: drinking water; N/A: not available.
Safety limit for drinking water (Organization, 2017).

Table 2 – Pesticide traces in water samples.

Pesticide	RWW	TSW	GW	DW	Safety limit
Dieldrin (μg/L)	<0.01	<0.01	<0.01	Undetectable	0.03
Diethyltoluamid (μg/L)	0.75	0.13	<0.01	Undetectable	0.10
Mecoprop (μg/L)	0.03	0.14	<0.01	Undetectable	0.10
Terbutryn (μg/L)	0.18	0.19	0.02	Undetectable	0.03

Safety limit for drinking water (Directive, 2006; Organization, 2017) .

1.11. *Statistical analysis*

Data were analyzed statistically using descriptive statistics and the Kruskal Wallis test with the post hoc Dunn's test. Data were accepted as statistically significant under a probability range of 5%.

2. Results

2.1. *Physicochemical characterization of water samples*

Table 1 Represents the general quality parameters of the water samples. Both RWW and TSW contained higher concentrations of ammonium-and nitrate-nitrogen when compared to the GW and DW, while electrical conductivity values were lower in the case of RWW and TSW. The quantitative analysis of the pesticides revealed that dieldrin was present under the safe limit in all water samples. DEET and terbutryn were not detected, whilst mecoprop was found within the safe limit in DW. The concentrations of both DEET and terbutryn were found to be elevated in RWW and TSW. All four pesticides were detected in GW, but none of them was above the safe limit, see Table 2. Chromatograms of dieldrin, DEET, terbutryn and mecoprop pesticides are given in **Appendix A Figs. S1-S12.**

2.2. *NSC-based bioassay*

We initially performed baseline studies to perceive if the lyophilization and subsequent reconstitution of DMEM/F-12 medium with water samples adversely affect the medium quality. In baseline studies we performed viability, proliferation, and differentiation assays for normal DMEM/F-12 and lyophilized DMEM/F-12 reconstituted with double-distilled water. Our baseline study showed insignificant difference between normal DMEM/F-12 and reconstituted DME/F-12 in terms of viability, proliferation, differentiation and migration of NSC culture (**Appendix A Figs. S13-S14**).

2.2.1. *Viability and proliferation assay*

The Calcein-AM/PI live-dead assay is widely used to determine the toxic effects of chemical compounds in mammalian cells, and a previous researcher employed this assay to determine the effects of tap water contaminants on the viability of water flea, a bio-indicator for water toxicity (Teplova et al., 2010). We performed Calcein-AM/propidium iodide live-dead assays to evaluate the acute toxicity of the water samples on NSCs when exposed for 24 hr. The results demonstrated that RWW significantly reduced the percentage of living cells when compared to the Con (**Fig. 1a**). The neurosphere clonogenic assay was performed to evaluate the effect of water samples on NSC proliferation. RWW significantly reduced the count and mean diameter of neurospheres (**Fig. 1b, c and Fig. 2**) when compared to the Con and all other treatment conditions at all observation time points. A significant difference between RWW and TSW was, however, only observed on day 3. We also observed a significant difference between the number and mean diameter of TSW *vs.* Con and TSW *vs.* GW only on day 7.

2.2.2. *Differentiation assay*

We carried out a differentiation assay to evaluate the effect of water samples on the multipotency of NSCs. Differentiated cells were identified by immunostaining of ß-tubulin III as a neuronal marker, and GFAP as an astrocyte marker. In the differentiation experiment, the reason for focusing only on differentiated neurons and astrocytes was their pivotal role in the development and functions of a nervous system and high vulnerability to toxic insult. Additionally, the neuronal/astrocyte co-culture system is a proven *in vitro*

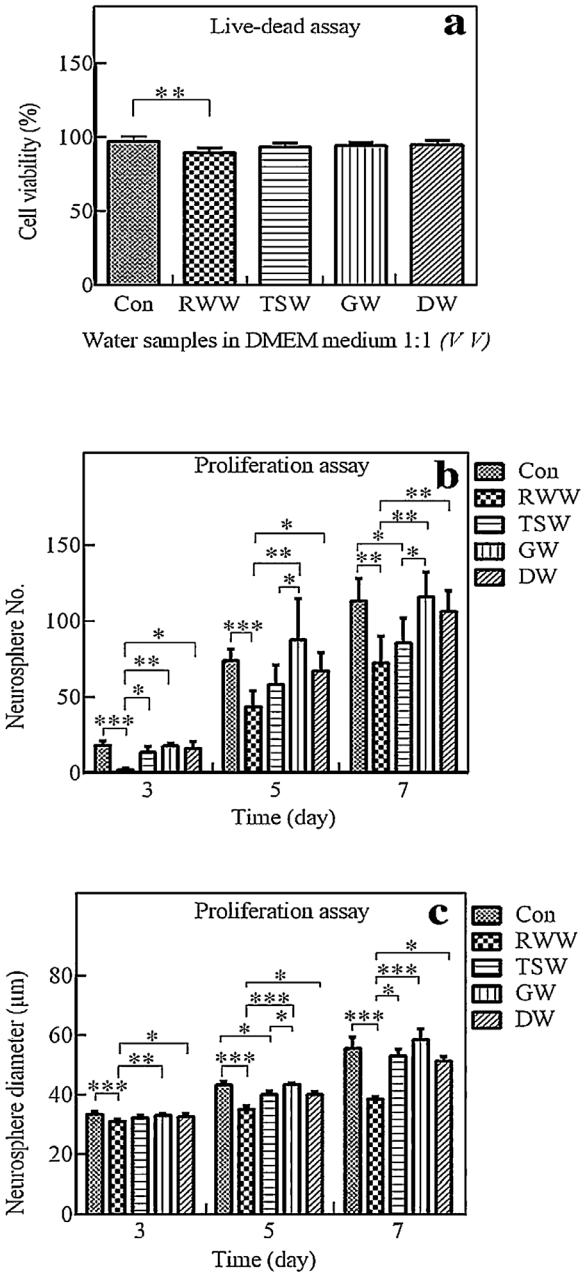


Fig. 1 – Calcein/Propidium iodide viability and clonogenic proliferation assay of NSCS upon treatment with water samples. For viability, NSCs were exposed to water treatments and control for 24 hr in a 96-well plate. (a) Represents the percentage of viable cells (stained with Calcein-AM) which were calculated from the total cell count obtained through phase-contrast pictures. Proliferation assay was performed on 96-well plate for 7 days. The whole well was scanned by a phase-contrast microscope using a 4× objective. (b) Represents the mean neurosphere number for each treatment condition at different observation time points. (c) Represents the mean diameter. Con: control; RWW: raw wastewater; TSW: treated sewage water; GW: groundwater and DW: drinking water. Data are mean ± SEM of 5 independent experiments ($n = 5$). *$p \leq 0.05$, **$p < 0.01$ and ***$p < 0.001$.

model and has been used extensively in toxicology studies (Anderl et al., 2009; De Simone et al., 2017; Deng and Poretz, 2003; Jiang et al., 2015). Percentages of differentiated cells and neurite outgrowth and the percentage of each glass coverslip area covered by differentiated cells were calculated using CELL-SENS and image J software. RWW significantly reduced the percentage of neurons and astrocytes and significantly increased the percentage of double-negatives (cells non-reactive to both neuronal and astrocyte markers) when compared to the Con and other water treatments (**Fig. 3a**). The strongest difference in the percentage of neurons was observed between RWW and DW (37%), whilst the percentage of astrocytes differed significantly between RWW and GW (26%). Similar trends with respect to the percentage of area covered by differentiated neurons and astrocytes cells in each microscopic field were observed for RWW and all other water treatments (**Fig. 3b**). RWW strongly inhibited the earlier neurite growth and hence gave rise to the highest percentage of neuriteless neurons (67% of total neurons) when compared to all other water treatments. Concerning the percentage of neuriteless neurons, the highest difference of around 95% was calculated between RWW *vs.* Con and RWW *vs.* DW (**Fig. 3c**). The morphological features of differentiated neurons and astrocytes under different water treatments are shown in the Fig. 4. Neurons were well-grown with extended neurites and multiple branches when exposed to Con, GW, and DW. In contrast, the cells exposed to RWW were observed to be detached at the end of observation period, and the ones that survived demonstrated highly depressed growth, with intermingled deformed neurites without a well-developed branching pattern. Similarly, cells exposed to TSW also demonstrated depressed neuronal growth, with less dense neurites when compared to other water treatments. A leaf-like morphology of astrocytes was observed for all samples except RWW, which demonstrated depressed growth with elongated thread-like morphology

2.2.3. *Migration assay*

The neurosphere migration assay is a very strong and relevant tool for screening the effect of environmental pollutants on neural cell migration in developmental neurotoxicity studies. The assay effectively mimics the *in vivo* cell migration process (Fritsche et al., 2011). The neurosphere migration assay was performed to measure cell migration under the influence of water samples for 24 hr, and calculations were done using CELL-SENS software. RWW significantly reduced the mean migration distance travelled by the differentiated cells when compared to the Con, GW and DW. A significant difference concerning the mean distance travelled by differentiated cells was also calculated between Con and TSW. The strongest difference was calculated between Con *vs.* RWW (34%) and RWW *vs.* DW 33% (**Fig. 5**).

3. Discussion

Several ecological studies identified industrial discharges and inefficiency in wastewater treatment techniques as two main sources for high concentrations of cytotoxic and neurotoxic

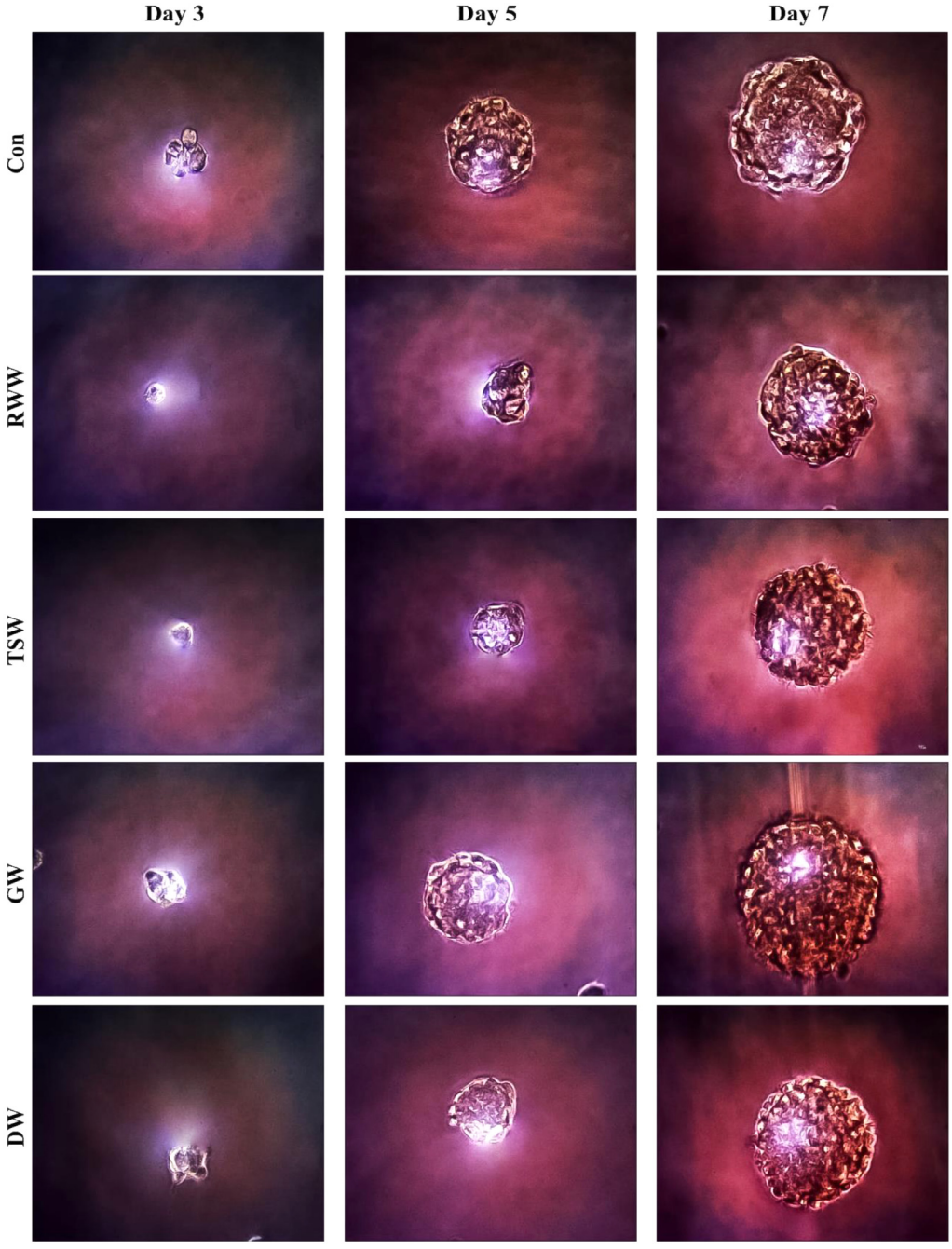


Fig. 2 – Growing sizes of neurosphere on days 3, 5 and 7 of incubation with water samples. NSCs were cultured in a 96-well plate. On day 3 of incubation, neurospheres were not completely rounded. On days 5 and 7, neurospheres acquired rounded shape. Pictures were taken with a phase-contrast microscope using a 20× objective.

pollutants present both in wastewater and treated wastewater (Fricke et al., 2015; Fritsche et al., 2018; Gerhardt, 2019; Héritier et al., 2017; Karlsson et al., 2020; Le et al., 2017; Miege et al., 2009; Poteser, 2017; Trintinaglia et al., 2015). NSC is an attractive and robust cell-based *in vitro* model to investigate the neurotoxicity of toxic substances. An *in vitro* assay based upon NSC was successfully employed to explore the neurotoxicity of tap water samples (with nickel pollutant) on neuroproliferation and neuronal differentiation (Zhou et al., 2019). In the present study, we developed an *in vitro* assay employing NSCs isolated from SVZ of postnatal Balb/c mice to assess the neurotoxicity of wastewater, effluent from the wastewater treatment plant, groundwater and drinking water. We selected lyophilized DMEN/F-12 medium reconstituted by double-distilled water as a control for the rest of our assays. Our baseline study showed that the lyophilization and reconstitution processes did not adversely affect the DEME/F-12 media quality for NSC culture.

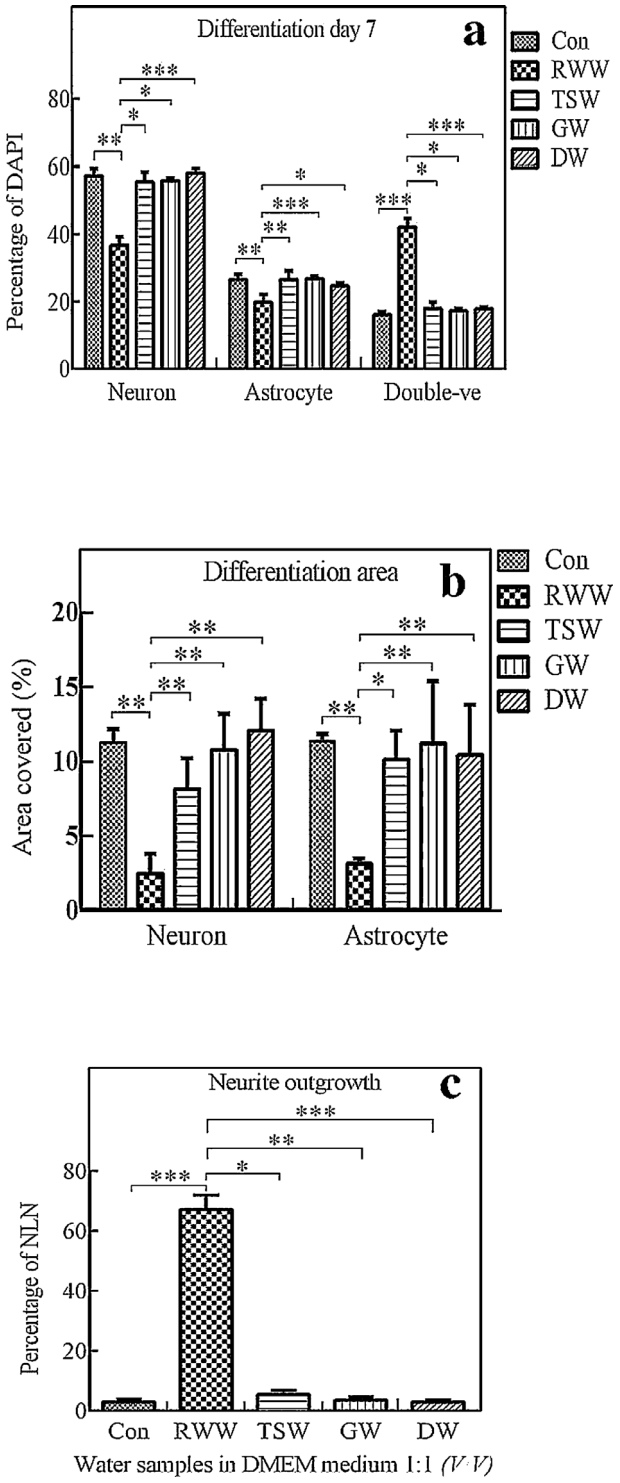


Fig. 3 – NSC differentiation after 7 days of incubation with water samples. Percentage of neurons and astrocytes was calculated from total DAPI +ve nuclei count. Area of 12 mm glass coverslip covered by differentiated cells was calculated from 4 × 4 mosaic pictures taken with 4× objective of a fluorescent microscope. (a) Represents the percentage of neurons, astrocytes and the cell which were non-immunoreactive for both neuronal and astrocyte cell markers. (b) Represents the percentage of area covered by differentiated cells on each coverslip. (c) Indicates the percentage of neuriteless neurons (NLN) calculated from the total neuronal count. Values are mean ± SEM of 5 independent replicates ($n = 5$).*$p \leq 0.05$, **$p < 0.01$ and ***$p < 0.001$.

3.1. *Physicochemical characterization of water samples*

Our results demonstrated that the levels of nitrogenous contaminants and pesticides were below the safe limit in GW and DW. RWW and TSW contained relatively higher concentrations of ammonium compounds, nitrates, pesticides DEET, terbutryn and mecoprop. Wastewater treatment plants working in some EU countries are unable to completely remove pollutants from raw wastewater. A study conducted in Spain reported that effluents of three different wastewater treatment plants contained higher concentrations of pesticides as compared to the untreated raw wastewater. The presence of relatively higher concentrations of pollutants in the effluents of wastewater treatment plants was due to the release of pollutants from the plants themselves (Köck-Schulmeyer et al., 2013). The presence of high nitrate contents in water is a product of highly decayed plant contents, usage of animal manure and leakage of septic tank effluents, and excessive use of fertilizers (Huang et al., 2018; Manassaram et al., 2005). High ammonium levels in wastewater (Seruga et al., 2019) and wastewater treatment plant effluents have been recently reported (Huang et al., 2018). An excessive amount of nitrogenous contaminants in treated wastewater reflects the inefficiency of the wastewater treatment process (Santos et al., 2008). Periconceptional exposure of human mothers to a high concentration of nitrate through drinking water resulted in neural tube defects in the fetus and anencephaly in newborns (Croen et al., 2001) and neural tube defects in zebrafish embryo by disrupting estrogen receptors (Jannat et al., 2014).

In Germany, a high concentration of DEET was reported in water samples collected from different sources. The Danube river in German territory, for instance, which receives a huge amount of water from widely dispersed wastewater treatment plants in the area (Loos et al., 2017), was reported to contain a high concentration of DEET in untreated wastewater and effluents of wastewater treatment plants (Launay et al., 2013), in surface water and wastewater influents (Aronson et al., 2012). DEET is an environmental toxicant whose toxic concentration has been detected in marine water near nuclear power plants. DEET is considered toxic and neurotoxic (Abou-Donia et al., 1996; de Assis Martini et al., 2017). Terbutryn is a commonly employed herbicide and an environmental toxicant, with high concentrations detected in drinking water and processed food in several countries (Villarini et al., 2000). A high concentration of terbutryn was reported in surface water of the Llobregat River near Catalonia, Spain (Rubirola et al., 2019). In another study conducted in Germany, toxic levels of terbutryn were not only detected in untreated raw water but also in effluents of wastewater treatment plants (Le et al., 2017). A European study revealed the presence of DEET and terbutryn at high concentrations in most of the effluents tested (Loos et al., 2013). In Sweden and Denmark, high concentrations of mecoprop pesticide were detected both in untreated water and wastewater treatment plant effluents (Bollmann et al., 2014). Mecoprop was also detected in stream water in the German countryside. However, the concentration of mecoprop was below the safe limit (Schulte-Oehlmann et al., 2011). A high concentration of mecoprop was reported in groundwater collected from Weaver and Gowy catchments in the UK. The concentration

detected was higher than EU drinking water quality standards (Idowu et al., 2014). The presence of dieldrin in wastewater treatment plant effluents (Kenny et al., 2017), marine surface water and in potable water was also reported by researchers (Díaz-Barriga Arceo et al., 2015; Kenny et al., 2017).

3.2. *Effects on viability*

Only RWW significantly reduced the percentage of viable cells when compared to the Con. A study conducted in Slovenia reported on the cytotoxicity of untreated wastewater and wastewater treatment plant effluents in different dilutions toward a human hepatoma cell line (Žegura et al., 2009). Severe neurotoxicity in fish upon exposure to diluted effluent from a wastewater treatment plant was reported in Noksan (South–Korean) due to high concentrations of pesticides and industrial compounds that were not removed effectively by the wastewater treatment plant (Park et al., 2009). The possible reason for the inhibitory effects of RWW on NSC viability is the complex combination of pollutants derived from anthropogenic activities whose release was not controlled. Polycyclic aromatic hydrocarbons (Tang et al., 2003), phthalates (Lim et al., 2009), methyl mercury (Farina et al., 2011) and pesticides (Lin et al., 2018) are notorious for their neurotoxicity. High concentrations of polycyclic aromatic hydrocarbons were reported in the Neckar River in Southern Germany (Vincze et al., 2015). Moreover, elevated levels of phthalate in municipal wastewater (Fromme et al., 2002), methyl mercury in different lakes and rivers (Euractive, 2018) and pesticides such as atrazine, terbuthylazine, metazachlor in different water beds in Germany (Karlsson et al., 2020) were also observed. A high concentration of ammonium compounds in RWW may contribute to neurotoxicity since an excess of ammonium compounds induces cytotoxicity in murine myeloma cells (Martinelle and Häggström, 1993). The literature also reported the neurotoxicity of DEET (Swale et al., 2014) and cytotoxicity of terbutryn (Villarini et al., 2000). The role of these pesticides in the neurotoxicity of RWW is not clearly understood, since the levels employed in published studies were several times higher than those detected in our RWW and TSW samples. Poor neural cell viability in children due to neurocytoxicity induced by environmental pollutants during the early stage of life results in neurodevelopmental disorders such as defective locomotive functions, weak memory (Tseng et al., 2013) and poor IQ scores (Gorini et al., 2014). Our results indicated that wastewater treatment plants sufficiently reduced the toxic potential of pollutants in the studied water samples.

3.3. *Effect on cell proliferation*

Counting the number and diameter of neurospheres at specific time intervals provides an estimation of NSC proliferation capability (Lu and Wong, 2005). Our results demonstrated that both RWW and TSW inhibited NSC proliferation. RWW inhibited proliferation at all observation time points, whilst TSW inhibited proliferation only at later stages of incubation when compared to the Con and GW. Our findings are consistent with a published study showing that both the raw wastewater influent and treated effluents significantly reduced the proliferation of a human embryonic kidney cell line by altering the expression of cell cycle regulatory proteins, due to the presence of multiple contaminants in the water samples (Ren et al., 2017). Untreated sewage effluent contained pharmaceutical contaminants including atenolol, caffeine, hypnotics and antihypertensive drugs, and was reported to strongly inhibit cell proliferation in the brain of *Prochilodus lineatus* (Pérez et al., 2018). The decreased NSC proliferation with RWW and TSW could be attributed to the presence of one or more environmental pollutants with potential cell proliferation inhibition properties. Inhibitory effects of organic mercury compounds (Bose et al., 2012), bisphenol-A (Tiwari et al., 2015), the pesticide DEET (Parihar et al., 2013), and nitrates (Solari et al., 2009) on the proliferation of NSCs and effects of pharmaceutical contaminants on fish brain cells (Pérez et al., 2018) at ultra-low doses were reported by researchers. Moreover, the literature reveals that a high amount of methyl mercury has been found in a large number of lakes and surface water deposits across Europe, including Germany (Euractive, 2018). DEET pesticide was found in elevated concentrations in the Danube river (Loos et al., 2017),treated and untreated wastewater (Launay et al., 2013) and in surface water (Aronson et al., 2012). A very high concentration of bisphenol-A was also observed both in treated and untreated wastewater in different cities of Germany (Fromme et al., 2002). Decreased proliferation of NSCs in the brain is linked to poor memory, learning dysfunction (Parihar et al., 2013) and schizophrenia at early ages in children (Reif et al., 2006). Future studies need to conduct dose-response assays for pure DEET and nitrates since both pollutants were found in high concentrations in RWW and TSW, especially when these pollutants pose inhibitory effects on NSC proliferation. Furthermore, our findings demonstrated that wastewater treatment plants did not remove the pollutants completely, resulting in the inhibitory effects of TSW on cell proliferation.

3.4. *Effect on differentiation*

RWW significantly decreased the percentage of neurons, astrocytes, neuronal growth area, and astrocyte growth area, and increased the percentage of neuriteless neurons when compared to the control. Although no significant difference was observed between Con *vs*.TSW, yet TSW depressed the neurite growth and elongated the astrocytes when compared to Con, GW, and DW (Fig. 3). TSW also exhibited neutralizing effects since areas covered by differentiated neurons and astrocytes were significantly higher than those with RWW treatment. Many environmental pollutants frequently detected in treated and untreated water bodies of European countries were studied for their effect on neuronal and astrocytes differentiation of NSCs. Ammonium compounds (Braissant et al., 2002), nitrates (Solari et al., 2009), methyl mercury (Tamm et al., 2006), and bisphenol-A (Fujiwara et al., 2018; Tiwari et al., 2015) were reported to have inhibitory effects on neuronal differentiation, whilst artificial sweeteners (Cong et al., 2013), the pesticide DEET (Christen et al., 2017), Dieldrin (Richardson et al., 2006) and phthalates (You et al., 2018) were reported to exhibit inhibitory effects on neurite growth. Pizzurro et al. (2014) in their study reported the inhibitory effects of diazinon pesticide on astrocyte differentiation and development. The inhibitory ef-

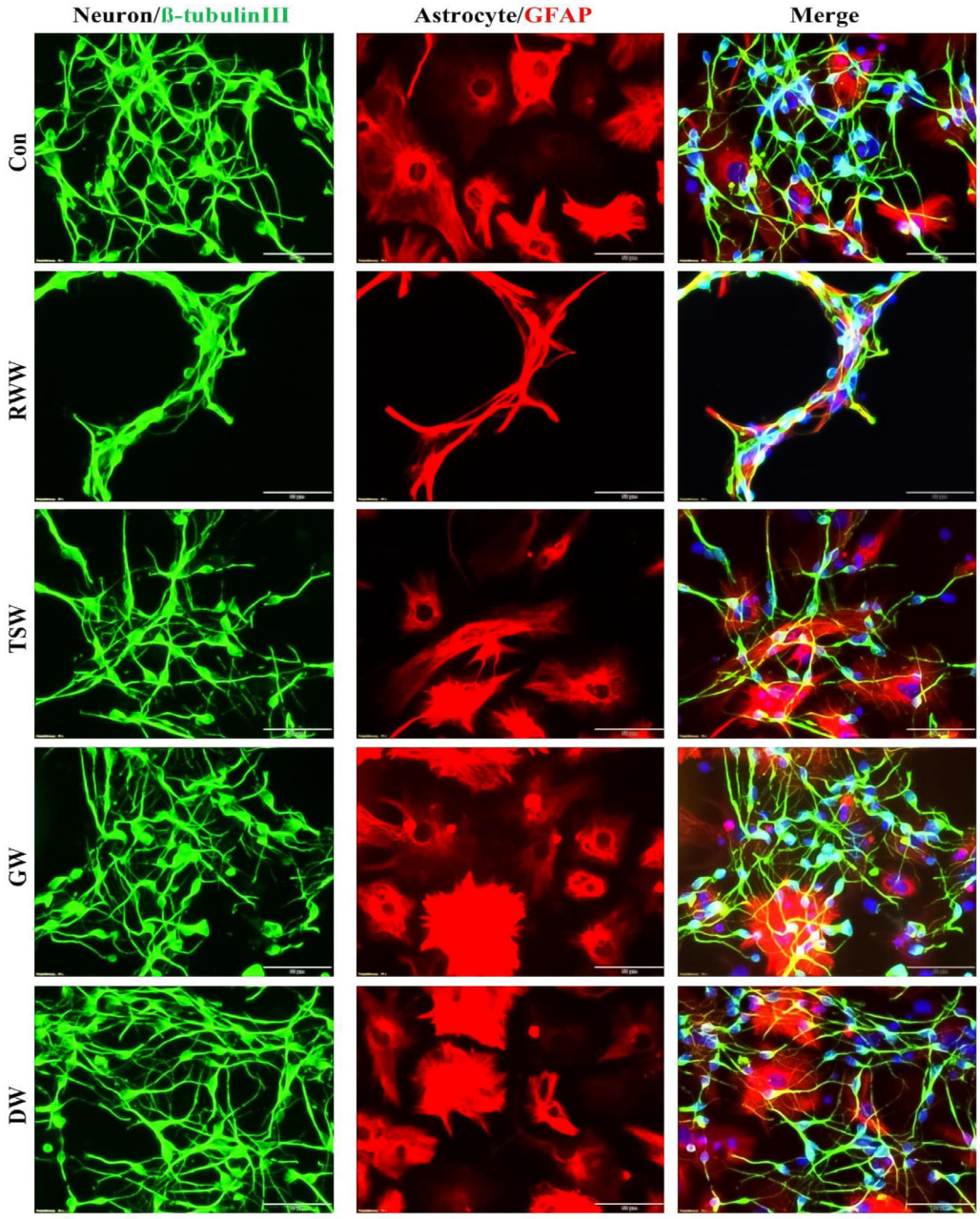
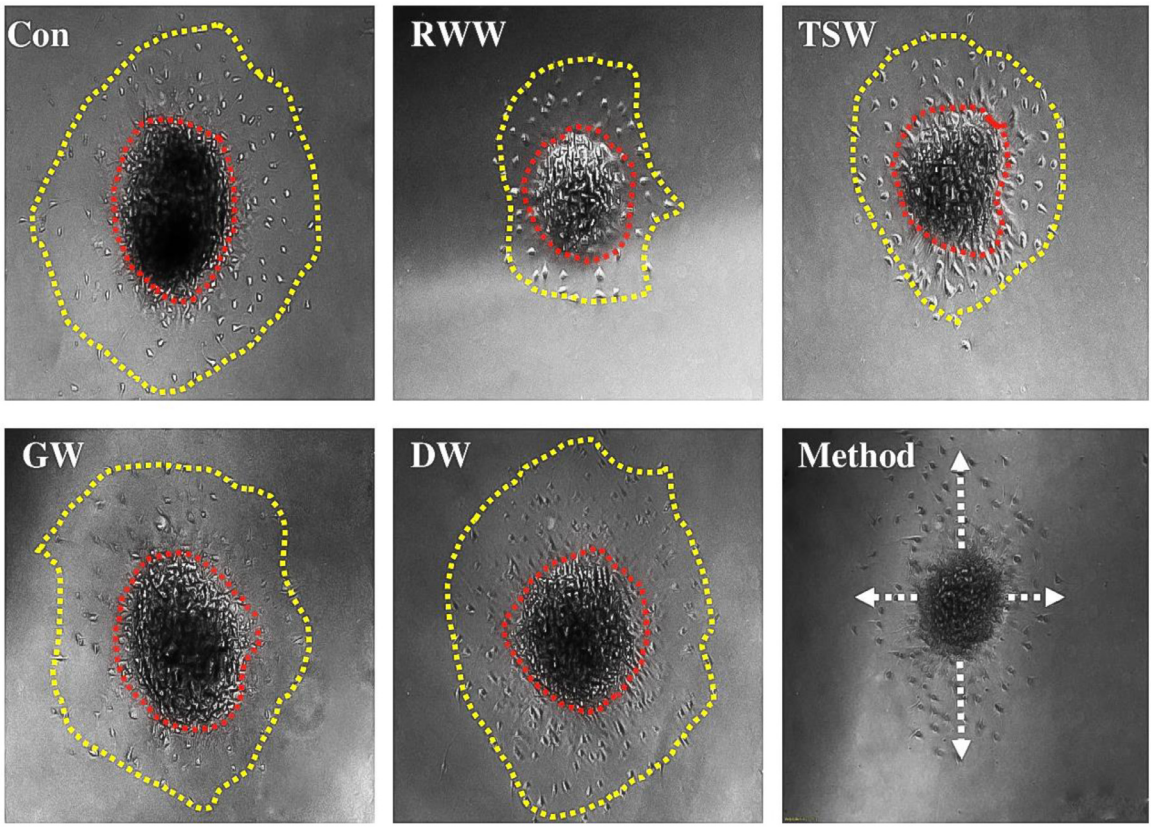


Fig. 4 – NSC differentiation on ECM-coated glass coverslip after 7 days of incubation. Green cells are ß-tubulin III+ve (ß-tub) neurons with fibrous neurites. Red cells with leaf-like morphology are GFAP+ve astrocytes. RWW strongly inhibited neurite outgrowth and branching and also caused neurite deformations. Astrocytes under RWW have elongated morphology unlike with other treatments, which have leaf-like morphology. TSW treatment showed less dense neurite growth as compared to Con, GW, and DW. Pictures were taken with 40× objective. .

fects of these pollutants on neuronal and glial differentiation were evaluated at very low dose levels. Studies reported the presence of organic mercury compounds (Euractive, 2018; Fricke et al., 2015), bisphenol-A (Gerhardt, 2019), artificial sweeteners (Scheurer et al., 2009), the pesticide DEET (Loos et al., 2017) and phthalates (Fromme et al., 2002) at high levels both in untreated raw water and treated water samples collected from different locations in Germany. We speculate that besides other contaminants in RWW, DEET also contributed to the inhibitory effects on neurodifferentiation. Poor neurite growth is implicated in autism (Gilbert and Man, 2017), and poor astrocyte development is linked to mood disorders (Koyama, 2015) in human beings. In future studies, it is very important to investigate the neuro-inhibitory effects of DEET and dieldrin at concentrations we detected in RWW and TSW. Dieldrin was found at very low concentration, yet its synergis-



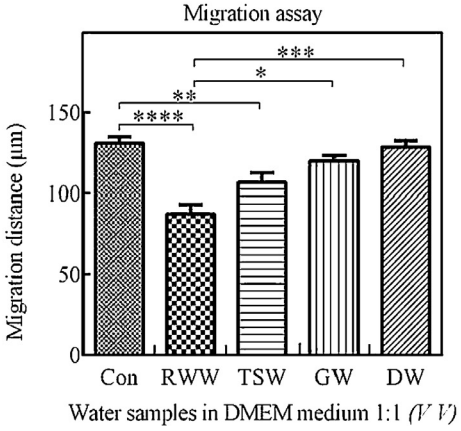


Fig. 5 – Neurosphere migration assay for determination of the effect of water samples on cell migration. Migration assay was performed in a 24-well plate. Phase-contrast pictures of neurospheres were taken 24 hr after incubation with 10× objective of a phase-contrast microscope. The yellow dotted line in images encircles the area occupied by migrated cells and the red dotted line encloses the neurosphere core. White arrows in the method image indicate the distance travelled by migrating cells away from the edge of the neurosphere core. Bar graph represents the mean distance travelled by migrating cells in four directions for each treatment condition. Values are presented as mean ± SEM of 5 independent replicates ($n = 5$).*$p \leq 0.05$,$p < 0.01$ and ***$p < 0.001$, ****$p < 0.0001$.**

tic effects with other complex mixture of pollutants must not be ignored, since chemical interaction between environmental pollutants can result in an increase or decrease in the toxicity of an individual compound (Krishnan and Brodeur, 1994). One such type of interaction was reported in the literature in which dieldrin synergistically interacted with H_2O_2 to cause severe toxicity in rat thymocytes (Chimeddorj et al., 2013).

3.5. *Effect on cell migration*

Cell migration is an important subset of the neurogenesis process. In the event of brain trauma or injury, SVZ is the first area where NSC stem cell proliferation and migration take place to repair the injured tissues (Galindo et al., 2018). Both RWW and TSW inhibited cell migration when compared to Con and DW. Many environmental toxicants, such as organic mercury compounds, steroidal drugs, the environmental toxicant bisphenol-A and pesticides, inhibit the migration of cells in the nervous system (Fahrion et al., 2012; Ishido and Suzuki, 2010a, 2010b). The occurrence of these environmental toxicants was reported both in wastewater and wastewater treatment plant effluents of different countries of the world (Gbondo-Tugbawa et al., 2010; Mohapatra et al., 2011; Pauwels et al., 2008), including Germany (Aronson et al., 2012; Euractive, 2018; Gerhardt, 2019). Although we could not find any published study to directly support our findings, we assume that the inhibitory effects of RWW and TSW on cell migration might be due to the presence of many environmental toxicants in our samples that were not completely removed by the water treatment plant. A decrease in neural cell migration increases the risk of epilepsy in human beings (Stouffer et al., 2016).

Although neurotoxicity of DEET and mecoprop in human beings (Petrucci and Sardini, 2000; Wiles et al., 2014), and teratogenic effects of terbutryn in an animal model (Meulenbelt et al., 1988; Velisek et al., 2012) at very low dose levels were reported in the literature, data regarding the neurotoxic concentrations of these pesticides in living body fluids is lacking. Prospective researchers are suggested to conduct biotransformation studies parallel to dose-response toxicity studies of DEET, terbutryn and mecoprop pesticides.

4. Conclusions

Our results demonstrated that groundwater and drinking water presented no sign of inhibitory effects on any of the neurogenerative processes (viability, proliferation, differentiation and migration) in an *in vitro* neurotoxicity assay based upon NSCs from SVZ of postnatal mice. Raw wastewater inhibited all neurogenesis processes, while treated sewage water showed inhibitory effects only on proliferation and migration. Through this study, we confirmed that an *in vitro* NSC-based assay provides a highly sensitive and robust system for neurotoxicity screening of diluted water samples from different sources with different levels of contamination. The assay also unveiled the tendency of treated sewage water to inhibit the differentiation of NSCs. Finally, it can be concluded that NSC-based *in vitro* assays offer a very good platform for screening water pollutants with neurotoxic potential, and should be considered as an integral part of other bioassays for evaluating the quality of water samples containing a mixture of chemical pollutants with different modes of action before these water samples are released into the environment or considered for human consumption. These findings also reflect the inefficiency of wastewater treatment plants in the studied area in completely removing the toxic pollutants and direct the attention of water management authorities to review their protocols regarding wastewater treatment practices.

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.

Acknowledgments

We are thankful to the Higher Education Commission (HEC), Pakistan (Ref. PD/OS-II/Batch-VI/Germany/2015/77159/17755) and the University of Applied Sciences Kaiserslautern, Germany for providing financial support to execute the present project.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jes.2020.07.028.

REFERENCES

Abou-Donia, M.B., Wilmarth, K.R., Abdel-Rahman, A.A., Jensen, K.F., Oehme, F.W., Kurt, T.L., 1996. Increased neurotoxicity following concurrent exposure to pyridostigmine bromide, DEET, and chlorpyrifos. Fundam. Appl. Toxicol 34, 201–222.

Anderl, J.L., Redpath, S., Ball, A.J., 2009. A neuronal and astrocyte co-culture assay for high content analysis of neurotoxicity. J. Vis. Exp. 27, 1173.

Aronson, D., Weeks, J., Meylan, B., Guiney, P.D., Howard, P.H., 2012. Environmental release, environmental concentrations, and ecological risk of N, N-Diethyl-m-toluamide (DEET). Integr. Environ. Assess. Manag. 8, 135–166.

Baumann, J., Barenys, M., Gassmann, K., Fritsche, E., 2014. Comparative human and rat "neurosphere assay" for developmental neurotoxicity testing. Curr. Protoc. Toxicol. 59, 24 12.21. 11-12.21.

Bender, M., Schwind, L., Grundmann, D., Martin, M., Klotz, M., Götz, C., et al., 2017. Impact of protein kinase CK2 inhibitors on proliferation and differentiation of neural stem cells. Heliyon 3, e00318.

Bergström, T., Forsberg-Nilsson, K., 2012. Neural stem cells: brain building blocks and beyond. Ups. J. Med. Sci. 117, 132–142.

Bernas, S.N., Leiter, O., Walker, T.L., Kempermann, G., 2017. Isolation, culture and differentiation of adult hippocampal precursor cells. Bio. Protoc. 7, e2603.

Beuth. 2009. German standard methods for the examination of water, waste water and sludge - General information (group A) - Part 11: sampling of waste water (A 11). Available:https://www.beuth.de/en/standard/din-38402-11/108855969 . Accessed date: July 07, 2020.

Beuth. 2016. German standard methods for the examination of water, waste water and sludge - General information (group A) - Part 13: sampling from aquifers (A 13). Available:https://www.beuth.de/en/draft-standard/din-38402-13/258660287 . Accessed: July 02, 2020.

Bianchi, E., Goldoni, A., Trintinaglia, L., Lessing, G., Silva, C., Nascimento, C., et al., 2015. Evaluation of genotoxicity and cytotoxicity of water samples from the Sinos River Basin, southern Brazil. Braz. J. Biol. 75, 68–74.

Bollmann, U.E., Tang, C., Eriksson, E., Jönsson, K., Vollertsen, J., Bester, K., 2014. Biocides in urban wastewater treatment plant influent at dry and wet weather: concentrations, mass flows and possible sources. Water Res. 60, 64–74.

Bose, R., Onishchenko, N., Edoff, K., Janson Lang, A.M., Ceccatelli, S., 2012. Inherited effects of low-dose exposure to methylmercury in neural stem cells. Toxicol. Sci. 130, 383–390.

Braissant, O., Henry, H., Villard, A.M., Zurich, M.G., Loup, M., Eilers, B., et al., 2002. Ammonium-induced impairment of axonal growth is prevented through glial creatine. J. Neurosci. 22, 9810–9820.

Burden, N., Benstead, R., Clook, M., Doyle, I., Edwards, P., Maynard, S.K., et al., 2016. Advancing the 3Rs in regulatory ecotoxicology: a pragmatic cross-sector approach. Integr. Environ. Assess. Manag. 12, 417–421.

Celiz, M.D., Pérez, S., Barceló, D., Aga, D.S., 2009. Trace analysis of polar pharmaceuticals in wastewater by LC-MS-MS: comparison of membrane bioreactor and activated sludge systems. J. Chromatogr. Sci. 47, 19–25.

Chimeddorj, T., Suzuki, T., Murakane, K., Inai, M., Satoh, M., Oyama, Y., 2013. Synergistic increase in cell lethality by dieldrin and H_2O2 in rat thymocytes: effect of dieldrin on the cells exposed to oxidative stress. Chemosphere 93, 353–358.

Christen, V., Rusconi, M., Crettaz, P., Fent, K., 2017. Developmental neurotoxicity of different pesticides in PC-12 cells *in vitro*. Toxicol. Appl. Pharmacol. 325, 25–36.

Cong, W.N., Wang, R., Cai, H., Daimon, C.M., Scheibye-Knudsen, M., Bohr, V.A., et al., 2013. Long-term artificial sweetener acesulfame potassium treatment alters neurometabolic functions in C57BL/6 J mice. PLoS ONE 8, e70257.

Costa, L.G., Giordano, G., Guizzetti, M., Vitalone, A., 2008. Neurotoxicity of pesticides: a brief review. Front. Biosci. 13, 1240–1249.

Coullery, R.P., Ferrari, M.E., Rosso, S.B., 2016. Neuronal development and axon growth are altered by glyphosate through a WNT non-canonical signaling pathway. Neurotoxicology 52, 150–161.

Croen, L.A., Todoroff, K., Shaw, G.M., 2001. Maternal exposure to nitrate from drinking water and diet and risk for neural tube defects. Am. J. Epidemiol. 153, 325–331.

de Assis Martini, G., Rogero, S.O., Rogero, J.R., 2017. Cytotoxic effects caused by *N, N* -diethyl-meta-toluamide and radiation in *Perna perna* mussels. Ecotoxicol. Environ. Contam. 12, 33–37.

De Simone, U., Caloni, F., Gribaldo, L., Coccini, T., 2017. Human co-culture model of neurons and astrocytes to test acute cytotoxicity of neurotoxic compounds. Int. J. Toxicol. 36, 463–477.

Deng, W., Poretz, R.D., 2003. Oligodendroglia in developmental neurotoxicity. Neurotoxicology 24, 161–178.

Díaz-Barriga Arceo, S., Martínez-Tabche, L., Álvarez-González, I., López López, E., Madrigal-Bujaidar, E., 2015. Toxicity induced by dieldrin and chlorpyrifos in the freshwater crayfish *Cambarellus montezumae* (Cambaridae). Rev. Biol. Trop. 63, 83–96.

Directive, G., 2006. Directive 2006/118/EC of the European Parliament and of the Council of 12 December 2006 on the protection of groundwater against pollution and deterioration. Off. J. Euro. Union L372, 19–31.

Euractive, 2018. Mercury in the water: 40% of Europe's lakes and rivers contaminated. Available: https://www.euractiv.com/section/health-consumers/news/les-eaux-europeennes-restent-largement-polluees-par-le-mercure/. Accessed April 10, 2020.

Fahrion, J.K., Komuro, Y., Li, Y., Ohno, N., Littner, Y., Raoult, E., et al., 2012. Rescue of neuronal migration deficits in a mouse model of fetal Minamata disease by increasing neuronal Ca^{2+} spike frequency. Proc. Natl. Acad. Sci. U.S.A. 109, 5057–5062.

Farina, M., Rocha, J.B., Aschner, M., 2011. Mechanisms of methylmercury-induced neurotoxicity: evidence from experimental studies. Life Sci. 89, 555–563.

Fricke, I., Götz, R., Schleyer, R., Püttmann, W., 2015. Analysis of sources and sinks of mercury in the urban water cycle of Frankfurt am Main, Germany. Water (Basel) 7, 6097–6116.

Fritsche, E., Barenys, M., Klose, J., Masjosthusmann, S., Nimtz, L., Schmuck, M., et al., 2018. Current availability of stem cell-based *in vitro* methods for Developmental Neurotoxicity (DNT) testing. Toxicol. Sci. 165, 21–30.

Fritsche, E., Gassmann, K., Schreiber, T., 2011. Neurospheres as a model for developmental neurotoxicity testing. Methods Mol. Biol. 758, 99–114.

Fromme, H., Küchler, T., Otto, T., Pilz, K., Müller, J., Wenzel, A., 2002. Occurrence of phthalates and bisphenol A and F in the environment. Water Res. 36, 1429–1438.

Fujiwara, Y., Miyazaki, W., Koibuchi, N., Katoh, T., 2018. The effects of low-dose bisphenolA and bisphenol F on neural differentiation of a fetal brain-derived neural progenitor cell line. Front. Endocrinol. 9, 1–9.

Galindo, L.T., Mundim, M.T., Pinto, A.S., Chiarantin, G.M., Almeida, M.E., Lamers, M.L., et al., 2018. Chondroitin sulfate impairs neural stem cell migration through ROCK activation. Mol. Neurobiol. 55, 3185–3195.

Gbondo-Tugbawa, S.S., McAlear, J.A., Driscoll, C.T., Sharpe, C.W., 2010. Total and methyl mercury transformations and mass loadings within a wastewater treatment plant and the impact of the effluent discharge to an alkaline hypereutrophic lake. Water Res. 44, 2863–2875.

Gerhardt, A., 2019. Plastic additive bisphenol A: toxicity in surface-and groundwater crustaceans. J. Toxicol. Risk Assess. 5, 1–10.

Gilbert, J., Man, H.Y., 2017. Fundamental elements in autism: from neurogenesis and neurite growth to synaptic plasticity. Front. Cell. Neurosci. 11, 1–29.

Gorini, F., Muratori, F., Morales, M.A., 2014. The role of heavy metal pollution in neurobehavioral disorders: a focus on autism. Rev. J. Autism Dev. Disord. 1, 354–372.

He, N., Jin, W., Lok, K., Wang, Y., Yin, M., Wang, Z., 2013. Amyloid-β 1–42 oligomer accelerates senescence in adult hippocampal neural stem/progenitor cells via formylpeptide receptor 2. Cell Death Dis. 4, e924.

Hendricks, R., Pool, E.J., 2012. Rapid *in vitro* tests to determine the toxicity of raw wastewater and treated sewage effluents. Water SA 38, 807–812.

Héritier, L., Duval, D., Galinier, R., Meistertzheim, A.L., Verneau, O., 2017. Oxidative stress induced by glyphosate-based herbicide on freshwater turtles. Environ. Toxicol. Chem. 36, 3343–3350.

Huang, J., Kankanamge, N.R., Chow, C., Welsh, D.T., Li, T., Teasdale, P.R., 2018. Removing ammonium from water and wastewater using cost-effective adsorbents: a review. J. Environ. Sci. 63, 174–197.

Idowu, I., Alkhaddar, R., Atherton, W., 2014. Possible source term of high concentrations of mecoprop-p in leachate and water quality: impact of climate change, public use and disposal. Environ. Technol. 35, 2055–2067.

Inta, D., Alfonso, J., von Engelhardt, J., Kreuzberg, M.M., Meyer, A.H., van Hooft, J.A., et al., 2008. Neurogenesis and widespread forebrain migration of distinct GABAergic neurons from the postnatal subventricular zone. Proc. Natl. Acad. Sci. U.S.A. 105, 20994–20999.

Ishido, M., Suzuki, J., 2010a. Inhibition by rotenone of mesencephalic neural stem-cell migration in a neurosphere assay *in vitro*. Toxicol. In Vitro 24, 552–557.
Ishido, M., Suzuki, J., 2010b. Quantitative analyses of inhibitory effects of bisphenol A on neural stem-cell migration using a neurosphere assay *in vitro*. J. Health. Sci. 56, 175–181.
Jaeger, N., Moraes, J., Klauck, C., Gehlen, G., Rodrigues, M., Ziulkoski, A., 2015. Cytotoxicity assays to evaluate tannery effluents treated by photoelectrooxidation. Braz. J. Biol. 75, 53–61.
Jannat, M., Fatimah, R., Kishida, M., 2014. Nitrate (NO_3 (-)) and nitrite (NO_2 (-)) are endocrine disruptors to downregulate expression of tyrosine hydroxylase and motor behavior through conversion to nitric oxide in early development of zebrafish. Biochem. Biophys. Res. Commun. 452, 608–613.
Jia, A., Escher, B.I., Leusch, F.D., Tang, J.Y., Prochazka, E., Dong, B., et al., 2015. *In vitro* bioassays to evaluate complex chemical mixtures in recycled water. Water Res. 80, 1–11.
Jiang, D., Lim, S., Kwak, M., Ryu, Y.K., Mintz, C.D., 2015. The changes of oligodendrocytes induced by anesthesia during brain development. Neural Regen. Res. 10, 1386–1387.
Karlsson, A.S., Lesch, M., Weihermüller, L., Thiele, B., Disko, U., Hofmann, D., et al., 2020. Pesticide contamination of the upper Elbe River and an adjacent floodplain area. J. Soils Sediments 20, 2067–2081.
Kenny, J., Webber, B., Howe, E., Holden, R., 2017. Pesticide removal through wastewater and advanced treatment: full-scale sampling and bench-scale testing. Water Sci. Technolo. 77, 739–747.
Köck-Schulmeyer, M., Villagrasa, M., de Alda, M.L., Céspedes-Sánchez, R., Ventura, F., Barceló, D., 2013. Occurrence and behavior of pesticides in wastewater treatment plants and their environmental impact. Sci. Total Environ. 458, 466–476.
Koyama, Y., 2015. Functional alterations of astrocytes in mental disorders: pharmacological significance as a drug target. Front. Cell. Neurosci. 9, 261.
Krishnan, K., Brodeur, J., 1994. Toxic interactions among environmental pollutants: corroborating laboratory observations with human experience. Environ. Health Perspect. 102, 11–17.
Launay, M., Kuch, B., Dittmer, U., Steinmetz, H., 2013. Occurrence of organic pollutants in treated wastewater effluent and surface waters under the influence of urban catchment areas.. Istanb. Int. Solid Waste. Water Wastewater Congr. Avaiable: http://www.emwis.org/thematicdirs/events/2013/05/istanbul-international-solid-waste-water-and-wastewater-congress. Accessed: April 05, 2020.
Le, T.D.H., Scharmüller, A., Kattwinkel, M., Kühne, R., Schüürmann, G., Schäfer, R.B., 2017. Contribution of waste water treatment plants to pesticide toxicity in agriculture catchments. Ecotoxicol. Environ. Saf. 145, 135–141.
Legradi, J., Di Paolo, C., Kraak, M., Van Der Geest, H., Schymanski, E., Williams, A., et al., 2018. An ecotoxicological view on neurotoxicity assessment. Environ. Sci. Eur. 30, 46.
Lim, C.K., Kim, S.K., Ko, D.S., Cho, J.W., Jun, J.H., An, S.Y., et al., 2009. Differential cytotoxic effects of mono-(2-ethylhexyl) phthalate on blastomere-derived embryonic stem cells and differentiating neurons. Toxicology 264, 145–154.
Lin, J., Zhao, H.-.S., Qin, L., Li, X.N., Zhang, C., Xia, J., et al., 2018. Atrazine triggers mitochondrial dysfunction and oxidative stress in quail (*Coturnix C. coturnix*) cerebrum via activating xenobiotic-sensing nuclear receptors and modulating cytochrome P450 systems. J. Agric. Food Chem. 66, 6402–6413.
Liu, Y., Lacson, R., Cassaday, J., Ross, D.A., Kreamer, A., Hudak, E., et al., 2009. Identification of small-molecule modulators of mouse SVZ progenitor cell proliferation and differentiation through high-throughput screening. J. Biomol. Screen. 14, 319–329.
Loos, R., Carvalho, R., António, D.C., Comero, S., Locoro, G., Tavazzi, S., et al., 2013. EU-wide monitoring survey on emerging polar organic contaminants in wastewater treatment plant effluents. Water Res. 47, 6475–6487.
Loos, R., Tavazzi, S., Mariani, G., Suurkuusk, G., Paracchini, B., Umlauf, G., 2017. Analysis of emerging organic contaminants in water, fish and suspended particulate matter (SPM) in the Joint Danube Survey using solid-phase extraction followed by UHPLC-MS-MS and GC–MS analysis. Sci. Total Environ. 607, 1201–1212.
Lu, F., Wong, C.S., 2005. A clonogenic survival assay of neural stem cells in rat spinal cord after exposure to ionizing radiation. Radiat. Res. 163, 63–71.
Manassaram, D.M., Backer, L.C., Moll, D.M., 2005. A review of nitrates in drinking water: maternal exposure and adverse reproductive and developmental outcomes. Environ. Health Perspect. 114, 320–327.
Martinelle, K., Häggström, L., 1993. Mechanisms of ammonia and ammonium ion toxicity in animal cells: transport across cell membranes. J. Biotechnol. 30, 339–350.
Meulenbelt, J., Zwaveling, J., Van Zoonen, P., Notermans, N., 1988. Acute MCPP intoxication: report of two cases. Hum. Toxicol. 7, 289–292.
Miege, C., Choubert, J., Ribeiro, L., Eusèbe, M., Coquery, M., 2009. Fate of pharmaceuticals and personal care products in wastewater treatment plants–conception of a database and first results. Environ. Pollut. 157, 1721–1726.
Mohapatra, D.P., Brar, S.K., Tyagi, R.D., Surampalli, R.Y., 2011. Occurrence of bisphenol A in wastewater and wastewater sludge of CUQ treatment plant. J. Xenobiot. 1, 9–16.
Münze, R., Hannemann, C., Orlinskiy, P., Gunold, R., Paschke, A., Foit, K., et al., 2017. Pesticides from wastewater treatment plant effluents affect invertebrate communities. Sci. Total Environ. 599, 387–399.
MWM, 2014. Weinheim sewage treatment plant, Weinheim wastewater treatment plant generates around 150% of its own electricity requirements itself. Available: https://www.mwm.net/mwm-kwk-bhkw/newsletter-mwm-newsflash/newsflash-07-dezember-2014/klaeranlage-weinheim/. Accessed: July 02, 2020.
Niss, F., Rosenmai, A.K., Mandava, G., Örn, S., Oskarsson, A., Lundqvist, J., 2018. Toxicity bioassays with concentrated cell culture media—A methodology to overcome the chemical loss by conventional preparation of water samples. Environ. Sci. Pollut. Res. Int. 25, 12183–12188.
Organization, W.H., 2017. Guidelines for drinking-water quality: fourth edition incorporating first addendum, 4th ed + 1st add. Available: https://www.who.int/water_sanitation_health/publications/gdwq4-1st-addendum/en/. Accessed: January 19, 2020.
Ostenfeld, T., Svendsen, C.N., 2004. Requirement for neurogenesis to proceed through the division of neuronal progenitors following differentiation of epidermal growth factor and fibroblast growth factor-2-responsive human neural stem cells. Stem Cells 22, 798–811.
Parihar, V.K., Hattiangady, B., Shuai, B., Shetty, A.K., 2013. Mood and memory deficits in a model of Gulf War illness are linked with reduced neurogenesis, partial neuron loss, and mild inflammation in the hippocampus. Neuropsychopharmacology 38, 2348–2362.
Park, S.Y., Kim, S.J., Rhee, Y., Yum, S.-.S., Kwon, T.D., Lee, T.K., 2009. Genotoxic and neurotoxic potential in marine fishes exposed to sewage effluent from a wastewater treatment plant. Mol. Cell. Toxicol. 5, 265–271.
Pauwels, B., Noppe, H., De Brabander, H., Verstraete, W., 2008. Comparison of steroid hormone concentrations in domestic and hospital wastewater treatment plants. J. Environ. Eng. 134, 933–936.

Pérez, M.R., Rossi, A.S., Bacchetta, C., Elorriaga, Y., Carriquiriborde, P., Cazenave, J., 2018. In situ evaluation of the toxicological impact of a wastewater effluent on the fish Prochilodus lineatus: biochemical and histological assessment. Ecol. Indic. 84, 345–353.
Petrucci, N., Sardini, S., 2000. Severe neurotoxic reaction associated with oral ingestion of low-dose diethyltoluamide-containing insect repellent in a child. Pediatr. Emerg. Care 16, 341–342.
Pizzurro, D.M., Dao, K., Costa, L.G., 2014. Diazinon and diazoxon impair the ability of astrocytes to foster neurite outgrowth in primary hippocampal neurons. Toxicol. Appl. Pharmacol. 274, 372–382.
Poteser, M., 2017. Cell-based *in vitro* models in environmental toxicology: a review. Biomonitoring 4, 11–26.
Quednow, K., Püttmann, W., 2007. Monitoring terbutryn pollution in small rivers of Hesse, Germany. J. Environ. Monit. 9, 1337–1343.
Reif, A., Fritzen, S., Finger, M., Strobel, A., Lauer, M., Schmitt, A., et al., 2006. Neural stem cell proliferation is decreased in schizophrenia, but not in depression. Mol. Psychiatry 11, 514–522.
Ren, X., Kou, Y.Y., Kim, T., Chae, K.J., Ng, H.Y., 2017. Toxicity study of reclaimed water on human embryonic kidney cells. Chemosphere189 390–398.
Richardson, J.R., Caudle, W.M., Wang, M., Dean, E.D., Pennell, K.D., Miller, G.W., et al., 2006. Developmental exposure to the pesticide dieldrin alters the dopamine system and increases neurotoxicity in an animal model of Parkinson's disease. FASEB J. 20, 1695–1697.
Rubirola, A., Quintana, J., Boleda, M.R., Galceran, M.T., 2019. Analysis of 32 priority substances from EU water framework directive in wastewaters, surface and drinking waters with a fast sample treatment methodology. Int. J. Environ. Anal. Chem. 99, 16–32.
Saha, B., Ypsilanti, A.R., Boutin, C., Cremer, H., Chédotal, A., 2012. Plexin-B2 regulates the proliferation and migration of neuroblasts in the postnatal and adult subventricular zone. J. Neurosci. 32, 16892–16905.
Santos, I.R., Costa, R.C., Freitas, U., Fillmann, G., 2008. Influence of effluents from a wastewater treatment plant on nutrient distribution in a coastal creek from southern Brazil. Braz. Arch. Biol. Technol. 51, 153–162.
Scheurer, M., Brauch, H.J., Lange, F.T., 2009. Analysis and occurrence of seven artificial sweeteners in German waste water and surface water and in soil aquifer treatment (SAT). Anal. Bioanal. Chem. 394, 1585–1594.
Schmid, S., Bogner, F., 2018. What Germany's university beginners think about water reuse. Water (Basel) 10, 731.
Schulte-Oehlmann, U., Oehlmann, J., Keil, F., 2011. Before the curtain falls: endocrine-active pesticides–A German contamination legacy. Rev. Environ. Contam. Toxicol. 213, 137–159.
Seruga, P., Krzywonos, M., Pyżanowska, J., Urbanowska, A., Pawlak-Kruczek, H., Niedźwiecki, Ł, 2019. Removal of ammonia from the municipal waste treatment effluents using natural minerals. Molecules 24, 3633.
Solari, M., Paquin, J., Ducharme, P., Boily, M., 2009. P19 neuronal differentiation and retinoic acid metabolism as criteria to investigate atrazine, nitrite, and nitrate developmental toxicity. Toxicol. Sci. 113, 116–126.
Storck, F.R., Sacher, F., Brauch, H.J., 2015. Hazardous and emerging substances in drinking water resources in the Danube River Basin. In: Liska, I. (Ed.), The Danube River Basin. Springer, Berlin, pp. 251–270.
Stouffer, M.A., Golden, J.A., Francis, F., 2016. Neuronal migration disorders: focus on the cytoskeleton and epilepsy. Neurobiol. Dis. 92, 18–45.
Swale, D.R., Sun, B., Tong, F., Bloomquist, J.R., 2014. Neurotoxicity and mode of action of N, N-diethyl-meta-toluamide (DEET). PLoS ONE 9, e103713.
Tamm, C., Duckworth, J., Hermanson, O., Ceccatelli, S., 2006. High susceptibility of neural stem cells to methylmercury toxicity: effects on cell survival and neuronal differentiation. J. Neurochem. 97, 69–78.
Tang, Y., Donnelly, K., Tiffany-Castiglioni, E., Mumtaz, M., 2003. Neurotoxicity of polycyclic aromatic hydrocarbons and simple chemical mixtures. J. Toxicol. Environ. Health A. 66, 919–940.
Teplova, V.V., Andreeva-Kovalevskaya, Z.I., Sineva, E.V., Solonin, A.S., 2010. Quick assessment of cytotoxins effect on *Daphnia magna* using *in vivo* fluorescence microscopy. Environ. Toxicol. Chem. 29, 1345–1348.
Ternes, T.A., 1998. Occurrence of drugs in German sewage treatment plants and rivers. Water Res. 32, 3245–3260.
Tiwari, S.K., Agarwal, S., Seth, B., Yadav, A., Ray, R.S., Mishra, V.N., et al., 2015. Inhibitory effects of bisphenol-A on neural stem cells proliferation and differentiation in the rat brain are dependent on Wnt/β-catenin pathway. Mol. Neurobiol. 52, 1735–1757.
Trintinaglia, L., Bianchi, E., Silva, L., Nascimento, C., Spilki, F., Ziulkoski, A., 2015. Cytotoxicity assays as tools to assess water quality in the Sinos River basin. Braz. J. Biol. 75, 75–80.
Tseng, I.L., Yang, Y.F., Yu, C.W., Li, W.H., Liao, V.H.C., 2013. Phthalates induce neurotoxicity affecting locomotor and thermotactic behaviors and AFD neurons through oxidative stress in *Caenorhabditis elegans*. PLoS ONE 8, e82657.
Velisek, J., Stara, A., Machova, J., Dvorak, P., Zuskova, E., Prokes, M., et al., 2012. Effect of terbutryn at environmental concentrations on early life stages of common carp (*Cyprinus carpio* L.). Pestic. Biochem. Physiol. 102, 102–108.
Villarini, M., Scassellati-Sforzolini, G., Moretti, M., Pasquini, R., 2000. *In vitro* genotoxicity of terbutryn evaluated by the alkaline single-cell microgel-electrophoresis"comet" assay. Cell Biol. Toxicol. 16, 285–292.
Vincze, K., Scheil, V., Kuch, B., Köhler, H.R., Triebskorn, R., 2015. Impact of wastewater on fish health: a case study at the Neckar River (Southern Germany) using biomarkers in caged brown trout as assessment tools. Environ. Sci. Pollut. Res. Int. 22, 11822–11839.
Wang, C., 2015. Application of *in vitro* models in developmental neurotoxicity and pharmaceutics research. J. Mol. Pharm. Org. Process Res. 3, 122–128.
Wiles, D., Yee, J., Castillo, U., Russell, J., Spiller, H., Casavant, M., 2014. A lethal case of DEET toxicity due to intentional ingestion. J. Anal. Toxicol. 38, 696–698.
Xiong, F., Gao, H., Zhen, Y., Chen, X., Lin, W., Shen, J., et al., 2011. Optimal time for passaging neurospheres based on primary neural stem cell cultures. Cytotechnology 63, 621–631.
You, M., Dong, J., Fu, Y., Cong, Z., Fu, H., Wei, L., et al., 2018. Exposure to Di-(2-ethylhexyl) phthalate during perinatal period gender-specifically impairs the dendritic growth of pyramidal neurons in rat offspring. Front. Neurosci. 12, 444.
Žegura, B., Heath, E., Černoša, A., Filipič, M., 2009. Combination of *in vitro* bioassays for the determination of cytotoxic and genotoxic potential of wastewater, surface water and drinking water samples. Chemosphere 75, 1453–1460.
Zhang, T., Li, W., Wang, F., Zhang, Y., Xu, A., Shen, Y., 2015. Differentiation and proliferation potential of neural stem cells in subventricular zone of mice *in vitro*. Chin. J. Repar. Reconstr. Surg. 29, 766–771.
Zhou, Y., Fu, Y., Bai, Z., Li, P., Zhao, B., Han, Y., et al., 2019. Neural differentiation of mouse neural stem cells as a tool to assess developmental neurotoxicity of drinking water in Taihu Lake. Biol. Trace Elem. Res. 190, 172–186.

3.2. Publication 2

Environment Permissible Concentrations of Glyphosate in Drinking water can Influence the fate of Neural Stem Cells from the Subventricular Zone of the Postnatal Mouse.

Muhammad Irfan Masood, Mahrukh Naseem, Salam A. Warda, María Angeles Tapia-Laliena, Habib ur Rehman, Muhammad Jawad Nasim , Karl Herbert Schafer.

Environmental Pollution, 2021, 270, 116179.

Environmental Pollution 270 (2021) 116179

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

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

Muhammad Irfan Masood [a, b, c, *], Mahrukh Naseem [d], Salam A. Warda [b], María Ángeles Tapia-Laliena [f], Habib ur Rehman [g], Muhammad Jawad Nasim [a], Karl Herbert Schäfer [b, e, **]

[a] Division of Bioorganic Chemistry, School of Pharmacy, Saarland University, Saarbrücken, D-66123, Germany
[b] Working Group Enteric Nervous System, University of Applied Sciences Kaiserslautern, Campus Zweibrücken, 66482, Germany
[c] Institute of Pharmaceutical Sciences, University of Veterinary and Animal Sciences, Lahore, 54000, Pakistan
[d] Department of Zoology, University of Balochistan, Quetta, 87550, Pakistan
[e] Department of Pediatric Surgery Mannheim, University Medicine Mannheim, University of Heidelberg, Mannheim, 68167, Germany
[f] Medical Faculty Mannheim, University of Heidelberg, Mannheim, D- 68167, Germany
[g] Department of Physiology, University of Veterinary and Animal Sciences, Lahore, 54000, Pakistan

ARTICLE INFO

Article history:
Received 26 June 2020
Received in revised form
3 November 2020
Accepted 26 November 2020
Available online 5 December 2020

Keywords:
Glyphosate
Neurotoxicity
Neural stem cells
Environment
Cytoprotective

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

☆ This paper has been recommended for acceptance by Wen Chen.

* Corresponding author. Division of Bioorganic Chemistry, School of Pharmacy, Saarland University, Saarbrücken, D-66123, Germany.
** Corresponding author. Working Group Enteric Nervous System, University of Applied Sciences Kaiserslautern, Campus Zweibrücken, 66482, Germany.
E-mail addresses: irfan@uvas.edu.pk, irfan_masood_79@yahoo.com (M.I. Masood), karlherbert.schaefer@hs-kl.de (K.H. Schäfer).

https://doi.org/10.1016/j.envpol.2020.116179
0269-7491/

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

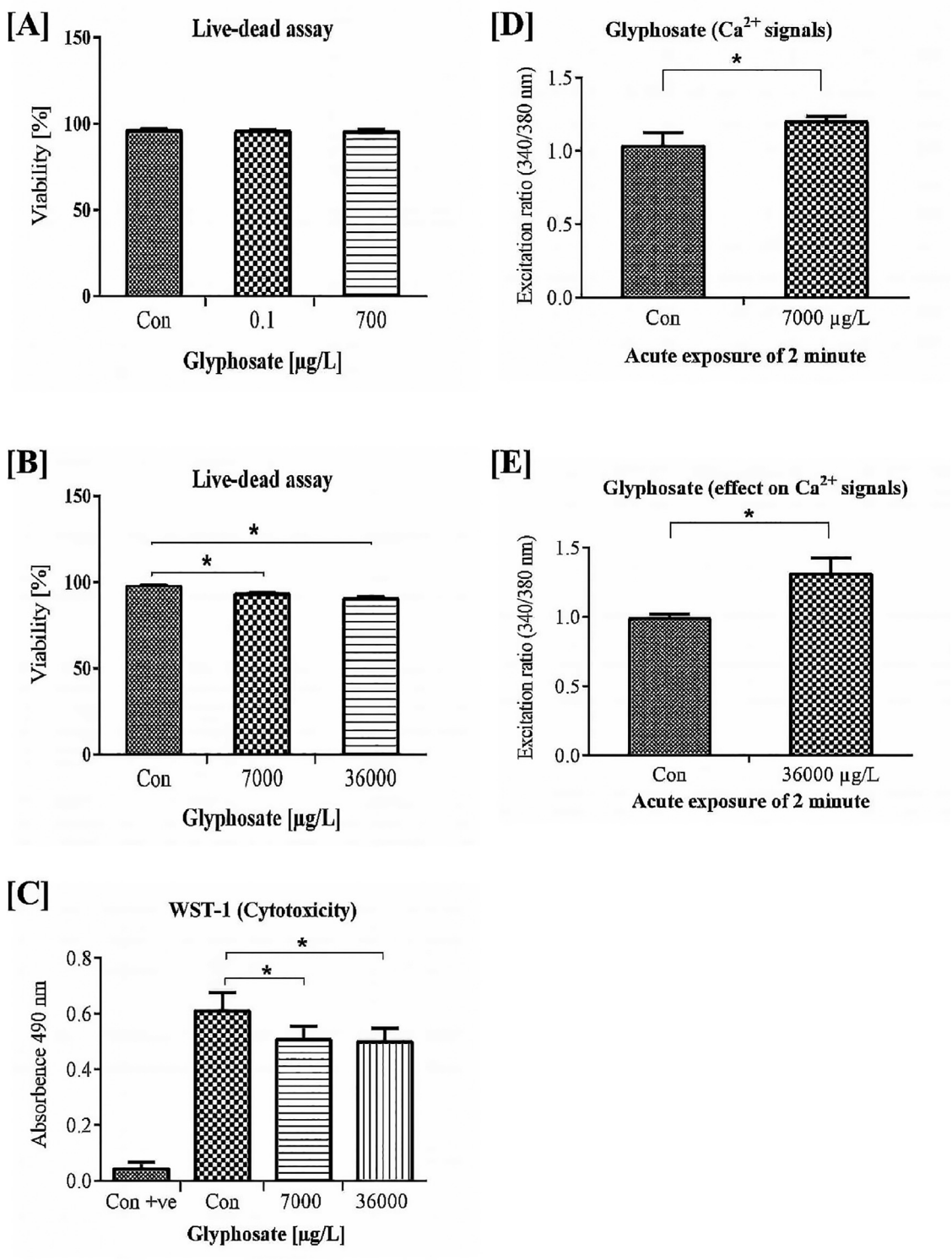


Fig. 1. Glyphosate reduced viability, induced acute cytotoxicity, and Ca^{2+} influx in NSCs culture. [A-B] Represent the viability in terms of percentage of NSCs following 24 h exposure to maximum permissible and higher concentrations of glyphosate. The percentage of viable cells was calculated from 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.

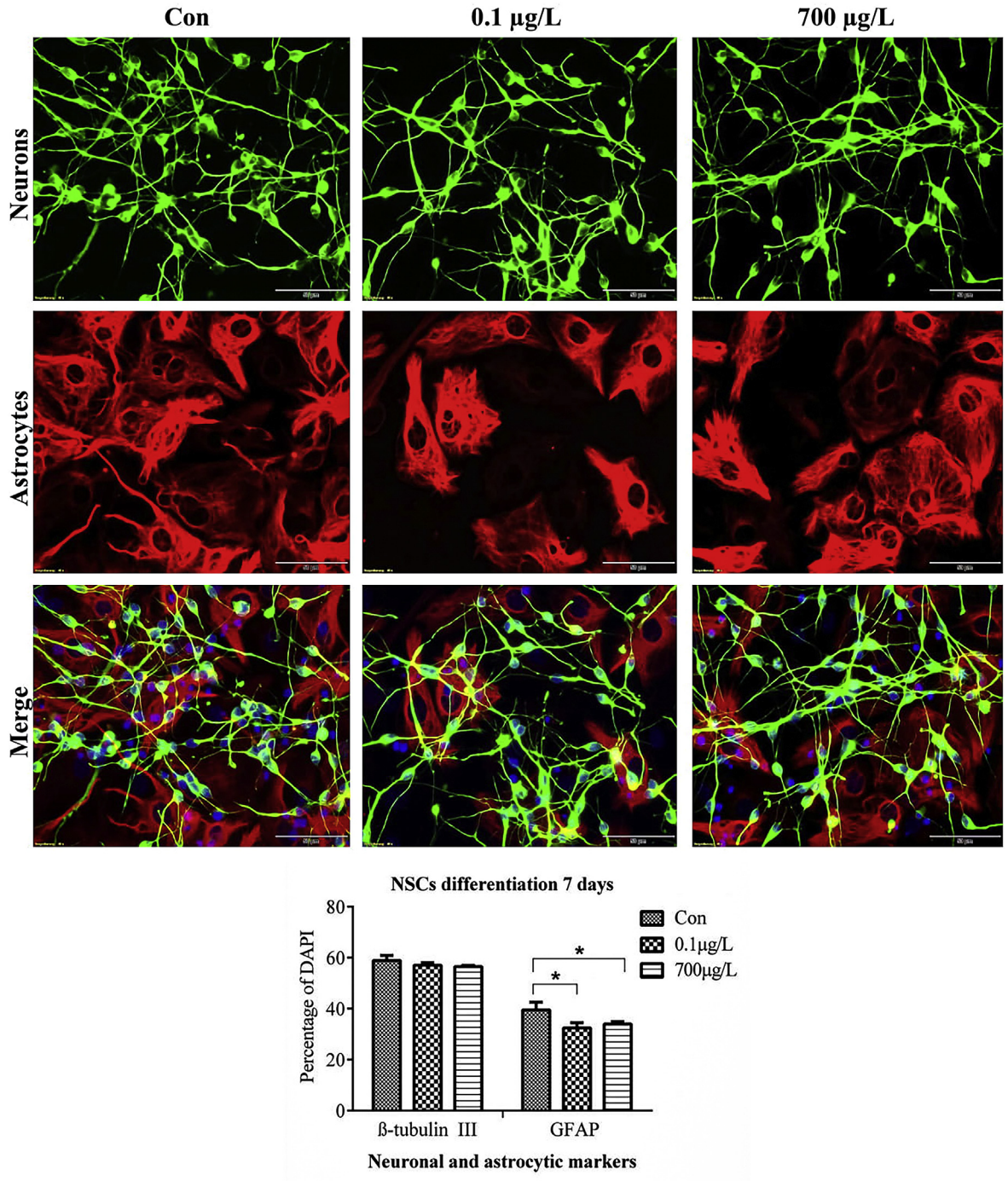


Fig. 2. Effects of glyphosate on neuronal and astrocytic differentiation of NSCs after 7 days of incubation. Green cells are β-tubulin III + ve neurons and red cells are GFAP + ve astrocytes. Percentages of neurons and astrocytes represented in the graph were calculated from the total DAPI nuclei count. 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.)

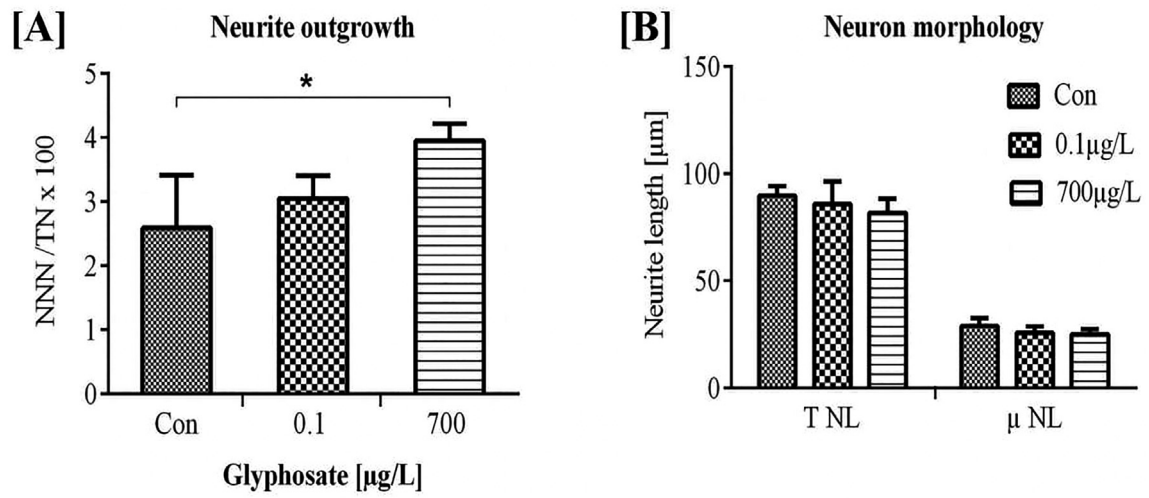


Fig. 3. Glyphosate effects on neurite outgrowth of differentiated neurons. NSCs were differentiated for 7 days. [A] Represents the percentage of non-neurite neurons which was calculated from the total neuronal count in each microscopic field. [B] Represents the neurite length which was measured from 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.

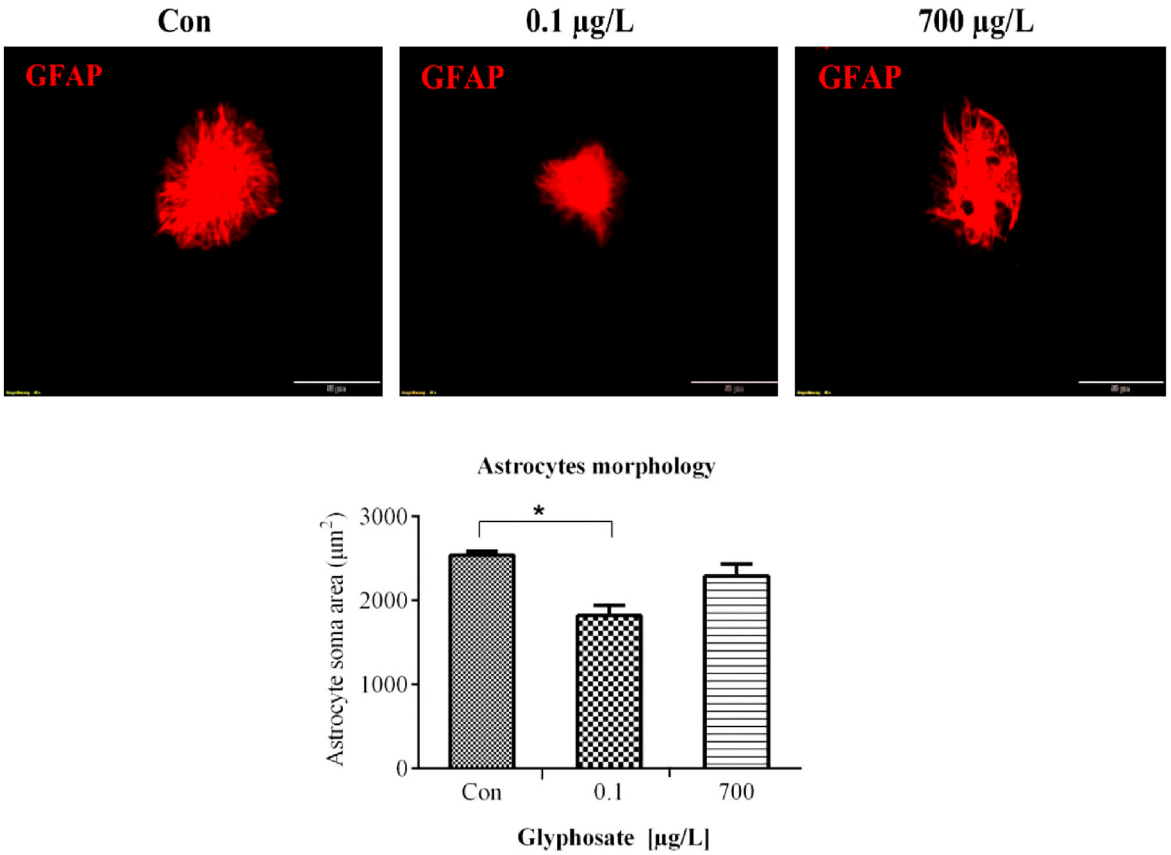


Fig. 4. Effects of glyphosate on astrocyte soma area after 7 days of differentiation. Differentiated astrocytes were stained for GFAP. Most of the astrocytes showed a leaf-like morphology under all treatment conditions. Around 100 cells were included in the measurement from each condition in each replicate. 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,

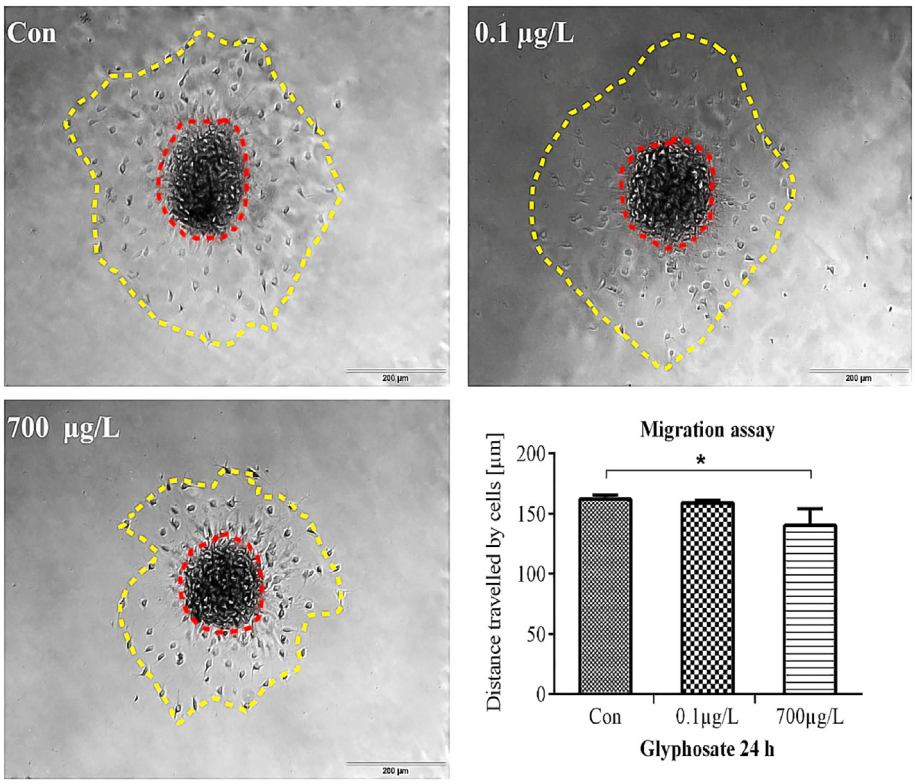


Fig. 5. Glyphosate reduced cell migration after 24 h of incubation. Untreated neurospheres were attached on to PDL coated glass coverslips and differentiated in differentiation medium into each well of a 24-well plate with and without glyphosate for 24 h. Pictures of neurospheres were captured with a 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.)

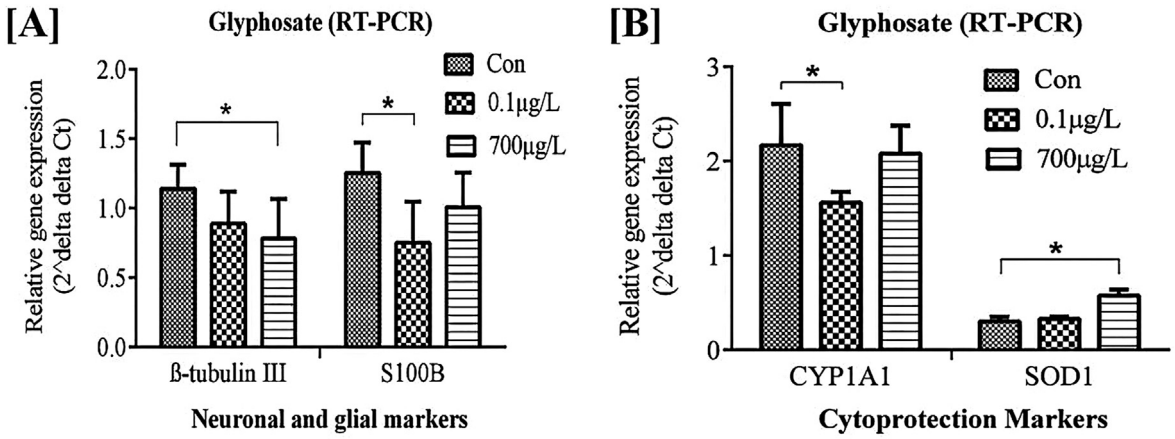


Fig. 6. qRT-PCR of genes expression in differentiated NSCs exposed to glyphosate for 7 days. qRT-PCR was used to determine mRNA expression of cell lineage-specific genes and those involved in the cellular response to the toxic stimuli. [A] Represents the relative gene expression of neuronal and astrocyte 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.

Acknowledgements

The present project was funded by the University of Applied Sciences, Kaiserslautern, Germany and the Higher Education Commission Government of Pakistan (Ref. PD. OS-B6 Germany-2015/77159/17755). The funding agencies provided stipend for authors and money to cover expenses associated with consumables but did not take part in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.116179.

References

Abranches, E., O'Neill, A., Robertson, M.J., Schaffer, D.V., Cabral, J.M., 2006. Development of quantitative PCR methods to analyse neural progenitor cell culture state. Biotechnol. Appl. Biochem. 44, 1–8.
Ait Bali, Y., Ba-Mhamed, S., Bennis, M., 2017. Behavioral and immunohistochemical study of the effects of subchronic and chronic exposure to glyphosate in mice. Front. Behav. Neurosci. 11, 146.
Anadón, A., Martinez-Larranaga, M., Martinez, M., Castellano, V., Martínez, M., Martin, M., Nozal, M., Bernal, J., 2009. Toxicokinetics of glyphosate and its metabolite aminomethyl phosphonic acid in rats. Toxicol. Lett. 190, 91–95.
Aris, A., Leblanc, S., 2011. Maternal and fetal exposure to pesticides associated to genetically modified foods in Eastern Townships of Quebec. Canada. Reprod. Toxicol. 31, 528–533.
Bal-Price, A.K., Hogberg, H.T., 2011. Effect of pesticides on neuronal and glial cell differentiation and maturation in primary cultures. In: Smiljanic, T. (Ed.), Pesticides-The Impacts of Pesticides Exposure. IntechOpen, Rijeka, Croatia, pp. 341–354.
Barbosa, E.R., Leiros da Costa, M.D., Bacheschi, L.A., Scaff, M., Leite, C.C., 2001. Parkinsonism after glycine-derivate exposure. Mov. Disord. 16, 565–568.
Baumann, J., Barenys, M., Gassmann, K., Fritsche, E., 2014. Comparative human and rat "neurosphere assay" for developmental neurotoxicity testing. Curr. Protoc. Toxicolo. 59, 12.21.11-24.
Beecham, J.E., Seneff, S., 2016. Is there a link between autism and glyphosate-formulated herbicides? J. Autism 3, 1.
Benachour, N., Séralini, G.-E., 2009. Glyphosate formulations induce apoptosis and necrosis in human umbilical, embryonic, and placental cells. Chem. Res. Toxicol. 22, 97–105.
Bender, M., Schwind, L., Grundmann, D., Martin, M., Klotz, M., Götz, C., Montenarh, M., Schäfer, K.-H., 2017. Impact of protein kinase CK2 inhibitors on proliferation and differentiation of neural stem cells. Heliyon 3, e00318.
Bernas, S.N., Leiter, O., Walker, T.L., Kempermann, G., 2017. Isolation, culture and differentiation of adult hippocampal precursor cells. Bio. Protoc. 7, e2603.
Betancourt, A.M., Burgess, S.C., Carr, R.L., 2006. Effect of developmental exposure to chlorpyrifos on the expression of neurotrophin growth factors and cell-specific markers in neonatal rat brain. Toxicol. Sci. 92, 500–506.
Bixby, S., Kruger, G.M., Mosher, J.T., Joseph, N.M., Morrison, S.J., 2002. Cell-intrinsic differences between stem cells from different regions of the peripheral nervous system regulate the generation of neural diversity. Neuron 35, 643–656.
Blesa, J., Przedborski, S., 2014. Parkinson's disease: animal models and dopaminergic cell vulnerability. Front. Neuroanat. 8, 155.
Bradberry, S.M., Proudfoot, A.T., Vale, J.A., 2004. Glyphosate poisoning. Toxicol. Rev. 23, 159–167.
Caballero, M., Amiri, S., Denney, J.T., Monsivais, P., Hystad, P., Amram, O., 2018. Estimated residential exposure to agricultural chemicals and premature mortality by Parkinson's disease in Washington state. Int. J. Environ. Res. Publ. Health 15, 2885.
Cai, W., Zhang, F., Zhong, L., Chen, D., Guo, H., Zhang, H., Zhu, B., Liu, X., 2020. Correlation between CYP1A1 polymorphisms and susceptibility to glyphosate-induced reduction of serum cholinesterase: a case-control study of a Chinese population. Pestic. Biochem. Physiol. 162, 23–28.
Cattani, D., Cavalli, V.L.d.L.O., Rieg, C.E.H., Domingues, J.T., Dal-Cim, T., Tasca, C.I., Silva, F.R.M.B., Zamoner, A., 2014. Mechanisms underlying the neurotoxicity induced by glyphosate-based herbicide in immature rat hippocampus: involvement of glutamate excitotoxicity. Toxicology 320, 34–45.
Cattani, D., Cesconetto, P.A., Tavares, M.K., Parisotto, E.B., De Oliveira, P.A., Rieg, C.E.H., Leite, M.C., Prediger, R.D.S., Wendt, N.C., Razzera, G., 2017. Developmental exposure to glyphosate-based herbicide and depressive-like behavior in adult offspring: implication of glutamate excitotoxicity and oxidative stress. Toxicology 387, 67–80.
Cavalli, V.L.d.L.O., Cattani, D., Rieg, C.E.H., Pierozan, P., Zanatta, L., Parisotto, E.B., Wilhelm Filho, D., Silva, F.R.M.B., Pessoa-Pureur, R., Zamoner, A., 2013. Roundup disrupts male reproductive functions by triggering calcium-mediated cell death in rat testis and Sertoli cells. Free Radic. Biol. Med. 65, 335–346.
Clair, É., Mesnage, R., Travert, C., Séralini, G.-É., 2012. A glyphosate-based herbicide induces necrosis and apoptosis in mature rat testicular cells in vitro, and testosterone decrease at lower levels. Toxicol. Vitro 26, 269–279.
Coady, K.K., Biever, R.C., Denslow, N.D., Gross, M., Guiney, P.D., Holbech, H., Karouna-Renier, N.K., Katsiadaki, I., Krueger, H., Levine, S.L., 2017. Current limitations and recommendations to improve testing for the environmental assessment of endocrine active substances. Integrated Environ. Assess. Manag. 13, 302–316.
Coecke, S., Goldberg, A.M., Allen, S., Buzanska, L., Calamandrei, G., Crofton, K., Hareng, L., Hartung, T., Knaut, H., Honegger, P., 2007. Workgroup report: incorporating in vitro alternative methods for developmental neurotoxicity into international hazard and risk assessment strategies. Environ. Health Perspect.

115, 924–931.
Costa, L.G., Giordano, G., Guizzetti, M., Vitalone, A., 2008. Neurotoxicity of pesticides: a brief review. Front. Biosci. 13, 1240–1249.
Coullery, R.P., Ferrari, M.E., Rosso, S.B., 2016. Neuronal development and axon growth are altered by glyphosate through a WNT non-canonical signaling pathway. Neurotoxicology 52, 150–161.
Crofton, K.M., Mundy, W.R., Lein, P.J., Bal-Price, A., Coecke, S., Seiler, A.E., Knaut, H., Buzanska, L., Goldberg, A., 2011. Developmental neurotoxicity testing: recommendations for developing alternative methods for the screening and prioritization of chemicals. ALTEX 28, 9–15.
Culbreth, M.E., Harrill, J.A., Freudenrich, T.M., Mundy, W.R., Shafer, T.J., 2012. Comparison of chemical-induced changes in proliferation and apoptosis in human and mouse neuroprogenitor cells. Neurotoxicology 33, 1499–1510.
Dechartres, J., Pawluski, J.L., Gueguen, M.M., Jablaoui, A., Maguin, E., Rhimi, M., Charlier, T.D., 2019. Glyphosate and glyphosate-based herbicide exposure during the peripartum period affects maternal brain plasticity, maternal behaviour and microbiome. J. Neuroendocrinol. 31, e12731.
Degl'Innocenti, D., Ramazzotti, M., Sarchielli, E., Monti, D., Chevanne, M., Vannelli, G.B., Barletta, E., 2019. Oxadiazon affects the expression and activity of aldehyde dehydrogenase and acylphosphatase in human striatal precursor cells: a possible role in neurotoxicity. Toxicology 411, 110–121.
Dolan, T., Howsam, P., Parsons, D.J., Whelan, M.J., 2013. Is the EU drinking water directive standard for pesticides in drinking water consistent with the precautionary principle? Environ. Sci. Technol. 47, 4999–5006.
Estácio, S.G., Leal, S.S., Cristóvão, J.S., Faísca, P.F., Gomes, C.M., 2015. Calcium binding to gatekeeper residues flanking aggregation-prone segments underlies non-fibrillar amyloid traits in superoxide dismutase 1 (SOD1). Biochim. Biophys. Acta 1854, 118–126.
Fathi, M.A., Han, G., Kang, R., Shen, D., Shen, J., Li, C., 2020. Disruption of cytochrome P450 enzymes in the liver and small intestine in chicken embryos in ovo exposed to glyphosate. Environ. Sci. Pollut. Res. Int. 27, 16865–16875.
Gallegos, C.E., Bartos, M., Bras, C., Gumilar, F., Antonelli, M.C., Minetti, A., 2016. Exposure to a glyphosate-based herbicide during pregnancy and lactation induces neurobehavioral alterations in rat offspring. Neurotoxicology 53, 20–28.
Gallegos, C.E., Bartos, M., Gumilar, F., Raisman-Vozari, R., Minetti, A., Baier, C.J., 2020. Intranasal glyphosate-based herbicide administration alters the redox balance and the cholinergic system in the mouse brain. Neurotoxicology 77, 205–215.
Ghosh, C., Hossain, M., Solanki, J., Dadas, A., Marchi, N., Janigro, D., 2016. Pathophysiological implications of neurovascular P450 in brain disorders. Drug Discov. Today 21, 1609–1619.
Gilbert, J., Man, H.-Y., 2017. Fundamental elements in autism: from neurogenesis and neurite growth to synaptic plasticity. Front. Cell. Neurosci. 11, 359.
Giordano, G., Costa, L.G., 2012. Developmental neurotoxicity: some old and new issues. ISRN Toxicol 814795, 2012.
Gorini, F., Muratori, F., Morales, M.A., 2014. The role of heavy metal pollution in neurobehavioral disorders: a focus on autism. Rev. J.Autism Dev. Disord. 1, 354–372.
Grundmann, D., Markwart, F., Scheller, A., Kirchhoff, F., Schäfer, K.-H., 2016. Phenotype and distribution pattern of nestin-GFP-expressing cells in murine myenteric plexus. Cell Tissue Res. 366, 573–586.
Gui, Y.-x., Fan, X.-n., Wang, H.-m., Wang, G., 2012. Glyphosate induced cell death through apoptotic and autophagic mechanisms. Neurotoxicol. Teratol. 34, 344–349.
Guo, W., Patzlaff, N.E., Jobe, E.M., Zhao, X., 2012. Isolation of multipotent neural stem or progenitor cells from both the dentate gyrus and subventricular zone of a single adult mouse. Nat. Protoc. 7, 2005–2012.
Hafezparast, M., Ahmad-Annuar, A., Wood, N.W., Tabrizi, S.J., Fisher, E.M., 2002. Mouse models for neurological disease. Lancet Neurol. 1, 215–224.
Ham, J., Lim, W., Whang, K.-Y., Song, G., 2020. Butylated hydroxytoluene induces dysregulation of calcium homeostasis and endoplasmic reticulum stress resulting in mouse Leydig cell death. Environ. Pollut. 256, 113421.
Harper, A., 2010. Mouse models of neurological disorders—a comparison of heritable and acquired traits. Biochim. Biophys. Acta 785–795, 1802.
Harrill, J.A., Freudenrich, T., Wallace, K., Ball, K., Shafer, T.J., Mundy, W.R., 2018. Testing for developmental neurotoxicity using a battery of in vitro assays for key cellular events in neurodevelopment. Toxicol. Appl. Pharmacol. 354, 24–39.
Hartung, T., Sabbioni, E., 2011. Alternative in vitro assays in nanomaterial toxicology. Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol. 3, 545–573.
Héritier, L., Duval, D., Galinier, R., Meistertzheim, A.L., Verneau, O., 2017. Oxidative stress induced by glyphosate-based herbicide on freshwater turtles. Environ. Toxicol. Chem. 36, 3343–3350.
Hernández-Plata, I., Giordano, M., Díaz-Muñoz, M., Rodríguez, V.M., 2015. The herbicide glyphosate causes behavioral changes and alterations in dopaminergic markers in male Sprague-Dawley rat. Neurotoxicology 46, 79–91.
Herzine, A., Laugeray, A., Feat, J., Menuet, A., Quesniaux, V., Richard, O., Pichon, J., Montécot-Dubourg, C., Perche, O., Mortaud, S., 2016. Perinatal exposure to glufosinate ammonium herbicide impairs neurogenesis and neuroblast migration through cytoskeleton destabilization. Front. Cell. Neurosci. 10, 191.
Hogberg, H.T., Kinsner-Ovaskainen, A., Coecke, S., Hartung, T., Bal-Price, A.K., 2010. mRNA expression is a relevant tool to identify developmental neurotoxicants using an in vitro approach. Toxicol. Sci. 113, 95–115.
Honeycutt, Z., Rowlands, H., 2014. Glyphosate Testing Report: Findings in American Mothers' Breast Milk, Urine and Water. Moms Across America. https://www.gentechvrij.nl/wp-content/uploads/2018/09/Gly-testing-rapport-MOMS-18.pdf. (Accessed 20 October 2020).
Inta, D., Alfonso, J., von Engelhardt, J., Kreuzberg, M.M., Meyer, A.H., van Hooft, J.A., Monyer, H., 2008. Neurogenesis and widespread forebrain migration of distinct GABAergic neurons from the postnatal subventricular zone. Proc. Natl. Acad. Sci. U. S. A. 105, 20994–20999.
Ishido, M., Suzuki, J., 2010. Inhibition by rotenone of mesencephalic neural stem-cell migration in a neurosphere assay in vitro. Toxicol. Vitro 24, 552–557.
Jang, Y., Kim, J.-E., Jeong, S.-H., Cho, M.-H., 2014. Towards a strategic approaches in alternative tests for pesticide safety. Toxicol. Res. 30, 159.
Jasper, R., Locatelli, G.O., Pilati, C., Locatelli, C., 2012. Evaluation of biochemical, hematological and oxidative parameters in mice exposed to the herbicide glyphosate-Roundup®. Interdiscipl. Toxicol. 5, 133–140.
Jeon, S., Lee, S.-H., Roh, J., Kim, J.-E., Bunch, H., 2020. Glyphosate influences cell proliferation in vitro. Life 13, 54–65.
Ji, H., Xu, L., Wang, Z., Fan, X., Wu, L., 2018. Differential microRNA expression in the prefrontal cortex of mouse offspring induced by glyphosate exposure during pregnancy and lactation. Exp. Ther. Med. 15, 2457–2467.
Joaquim, A.d.O., Macrini, D.J., Ricci, E.L., Rodrigues, P.A., Spinosa, H.d.S., Suffredini, I.B., Bernardi, M.M., 2014. Effects of exposure to glyphosate in male and female mice behavior in pubertal period. Braz. J. Vet. Res. Anim. Sci. 51, 194–203.
Kim, H.Y., Wegner, S.H., Van Ness, K.P., Park, J.J., Pacheco, S.E., Workman, T., Hong, S., Griffith, W., Faustman, E.M., 2016. Differential epigenetic effects of chlorpyrifos and arsenic in proliferating and differentiating human neural progenitor cells. Reprod. Toxicol. 65, 212–223.
Koller, V.J., Fürhacker, M., Nersesyan, A., Mišík, M., Eisenbauer, M., Knasmueller, S., 2012. Cytotoxic and DNA-damaging properties of glyphosate and Roundup in human-derived buccal epithelial cells. Arch. Toxicol. 86, 805–813.
Koyama, Y., 2015. Functional alterations of astrocytes in mental disorders: pharmacological significance as a drug target. Front. Cell. Neurosci. 9, 261.
Kwiatkowska, M., Jarosiewicz, P., Michałowicz, J., Koter-Michalak, M., Huras, B., Bukowska, B., 2016. The impact of glyphosate, its metabolites and impurities on viability, ATP level and morphological changes in human peripheral blood mononuclear cells. PloS One 11, e0156946.
Larsen, K., Najle, R., Lifschitz, A., Virkel, G., 2012. Effects of sub-lethal exposure of rats to the herbicide glyphosate in drinking water: glutathione transferase enzyme activities, levels of reduced glutathione and lipid peroxidation in liver, kidneys and small intestine. Environ. Toxicol. Pharmacol. 34, 811–818.
Lein, P., Locke, P., Goldberg, A., 2007. Meeting report: alternatives for developmental neurotoxicity testing. Environ. Health Perspect. 115, 764–768.
Li, Q., Lambrechts, M.J., Zhang, Q., Liu, S., Ge, D., Yin, R., Xi, M., You, Z., 2013. Glyphosate and AMPA inhibit cancer cell growth through inhibiting intracellular glycine synthesis. Drug Des. Dev. Ther. 7, 635.
Lilienblum, W., Dekant, W., Foth, H., Gebel, T., Hengstler, J., Kahl, R., Kramer, P.-J., Schweinfurth, H., Wollin, K.-M., 2008. Alternative methods to safety studies in experimental animals: role in the risk assessment of chemicals under the new European Chemicals Legislation (REACH). Arch. Toxicol. 82, 211–236.
Liu, J., Sridhar, J., Foroozesh, M., 2013. Cytochrome P450 family 1 inhibitors and structure-activity relationships. Molecules 18, 14470–14495.
Liu, Y., Lacson, R., Cassaday, J., Ross, D.A., Kreamer, A., Hudak, E., Peltier, R., Mclaren, D., Muñoz-Sanjuan, I., Santini, F., 2009. Identification of small-molecule modulators of mouse SVZ progenitor cell proliferation and differentiation through high-throughput screening. J. Biomol. Screen 14, 319–329.
Martinez, A., Al-Ahmad, A.J., 2019. Effects of glyphosate and aminomethylphosphonic acid on an isogeneic model of the human blood-brain barrier. Toxicol. Lett. 304, 39–49.
Martínez, M.-A., Ares, I., Rodríguez, J.-L., Martínez, M., Martínez-Larrañaga, M.-R., Anadón, A., 2018. Neurotransmitter changes in rat brain regions following glyphosate exposure. Environ. Res. 161, 212–219.
Martínez, M.-A., Rodríguez, J.-L., Lopez-Torres, B., Martínez, M., Martínez-Larrañaga, M.-R., Maximiliano, J.-E., Anadón, A., Ares, I., 2020. Use of human neuroblastoma SH-SY5Y cells to evaluate glyphosate-induced effects on oxidative stress, neuronal development and cell death signaling pathways. Environ. Int. 135, 105414.
Masood, M.I., Hauke, N.T., Nasim, M.J., Sarfraz, M., Naseem, M., Schäfer, K.H., 2021. Neural stem cell-based in vitro bioassay for the assessment of neurotoxic potential of water samples. J. Environ. Sci. 101, 72–86.
Mesnage, R., Arno, M., Costanzo, M., Malatesta, M., Séralini, G.-E., Antoniou, M.N., 2015. Transcriptome profile analysis reflects rat liver and kidney damage following chronic ultra-low dose Roundup exposure. Environ. Health 14, 70.
Milani, P., Gagliardi, S., Cova, E., Cereda, C., 2011. SOD1 transcriptional and post-transcriptional regulation and its potential implications in ALS. Neurol. Res. Int. 2011, 458427. https://doi.org/10.1155/2011/458427.
Neto da Silva, K., Garbin Cappellaro, L., Ueda, C.N., Rodrigues, L., Pertile Remor, A., Martins, R.d.P., Latini, A., Glaser, V., 2020. Glyphosate-based herbicide impairs energy metabolism and increases autophagy in C6 astroglioma cell line. J. Toxicol. Environ. Health 83, 153–167.
Ohtaki, N., Kamitani, W., Watanabe, Y., Hayashi, Y., Yanai, H., Ikuta, K., Tomonaga, K., 2007. Downregulation of an astrocyte-derived inflammatory protein, S100B, reduces vascular inflammatory responses in brains persistently infected with Borna disease virus. J. Virol. 81, 5940–5948.
Pamies, D., Block, K., Lau, P., Gribaldo, L., Pardo, C.A., Barreras, P., Smirnova, L., Wiersma, D., Zhao, L., Harris, G., 2018. Rotenone exerts developmental neurotoxicity in a human brain spheroid model. Toxicol. Appl. Pharmacol. 354, 101–114.
Park, K.-Y., M S, K., 2018. Inhibition of proliferation and neurogenesis of mouse

subventricular zone neural stem cells by a mitochondrial inhibitor rotenone. J. Life Sci. 28, 1397–1405.
Parrón, T., Requena, M., Hernández, A.F., Alarcón, R., 2011. Association between environmental exposure to pesticides and neurodegenerative diseases. Toxicol. Appl. Pharmacol. 256, 379–385.
Pizzurro, D.M., Dao, K., Costa, L.G., 2014. Diazinon and diazoxon impair the ability of astrocytes to foster neurite outgrowth in primary hippocampal neurons. Toxicol. Appl. Pharmacol. 274, 372–382.
Popova, D., Karlsson, J., Jacobsson, S.O., 2017. Comparison of neurons derived from mouse P19, rat PC12 and human SH-SY5Y cells in the assessment of chemical- and toxin-induced neurotoxicity. BMC Pharmacol. Toxicol. 18, 42.
Ramírez-Duarte, W.F., Rondón-Barragán, I.S., Eslava-Mocha, P.R., 2008. Acute toxicity and histopathological alterations of Roundup® herbicide on" cachama blanca"(Piaractus brachypomus). Braz. J. Vet. Res. 28, 547–554.
Rauch, U., Hänsgen, A., Hagl, C., Holland-Cunz, S., Schäfer, K.-H., 2006. Isolation and cultivation of neuronal precursor cells from the developing human enteric nervous system as a tool for cell therapy in dysganglionosis. Int. J. Colorectal Dis. 21, 554–559.
Ren, X., Li, R., Liu, J., Huang, K., Wu, S., Li, Y., Li, C., 2018. Effects of glyphosate on the ovarian function of pregnant mice, the secretion of hormones and the sex ratio of their fetuses. Environ. Pollut. 243, 833–841.
Richard, S., Moslemi, S., Sipahutar, H., Benachour, N., Seralini, G.-E., 2005. Differential effects of glyphosate and roundup on human placental cells and aromatase. Environ. Health Perspect. 113, 716–720.
Rosen, D.R., Siddique, T., Patterson, D., Figlewicz, D.A., Sapp, P., Hentati, A., Donaldson, D., Goto, J., O'Regan, J.P., Deng, H.-X., 1993. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. Nature 362, 59–62.
Roy, N.M., Carneiro, B., Ochs, J., 2016. Glyphosate induces neurotoxicity in zebrafish. Environ. Toxicol. Pharmacol. 42, 45–54.
Sadeh, N., Oni-Biton, E., Segal, M., 2016. Acute live/dead assay for the analysis of toxic effects of drugs on cultured neurons. Bio-protocol 6, e1889.
Saha, B., Ypsilanti, A.R., Boutin, C., Cremer, H., Chédotal, A., 2012. Plexin-B2 regulates the proliferation and migration of neuroblasts in the postnatal and adult subventricular zone. J. Neurosci. 32, 16892–16905.
Salama, M., Lotfy, A., Fathy, K., Makar, M., El-emam, M., El-gamal, A., El-gamal, M., Badawy, A., Mohamed, W.M., Sobh, M., 2015. Developmental neurotoxic effects of Malathion on 3D neurosphere system. Appl. Transl. Genom. 7, 13–18.
Samsel, A., Seneff, S., 2013. Glyphosate's suppression of cytochrome P450 enzymes and amino acid biosynthesis by the gut microbiome: pathways to modern diseases. Entropy 15, 1416–1463.
Santes-Palacios, R., Ornelas-Ayala, D., Cabañas, N., Marroquín-Pérez, A., Hernández-Magaña, A., del Rosario Olguín-Reyes, S., Camacho-Carranza, R., Espinosa-Aguirre, J.J., 2016. Regulation of human cytochrome P4501A1 (hCYP1A1): a plausible target for chemoprevention? BioMed Res. Int. 5341081, 2016.
Sato, C., Kamijo, Y., Yoshimura, K., Ide, T., 2011. Aseptic meningitis in association with glyphosate-surfactant herbicide poisoning. Clin. Toxicol. 49, 118–120.
Schäfer, K.-H., Hagl, C.I., Rauch, U., 2003. Differentiation of neurospheres from the enteric nervous system. Pediatr. Surg. Int. 19, 340–344.
Sea, K., Sohn, S.H., Durazo, A., Sheng, Y., Shaw, B.F., Cao, X., Taylor, A.B., Whitson, L.J., Holloway, S.P., Hart, P.J., 2015. Insights into the role of the unusual disulfide bond in copper-zinc superoxide dismutase. J. Biol. Chem. 290, 2405–2418.
Seth, B., Yadav, A., Agarwal, S., Tiwari, S.K., Chaturvedi, R.K., 2017. Inhibition of the transforming growth factor-β/SMAD cascade mitigates the anti-neurogenic effects of the carbamate pesticide carbofuran. J. Biol. Chem. 292, 19423–19440.
Shaw, W., 2017. Elevated urinary glyphosate and clostridia metabolites with altered dopamine metabolism in triplets with autistic spectrum disorder or suspected seizure disorder: a case study. Integr. Med. 16, 50–57.
Shoemaker, L.D., Kornblum, H.I., 2016. Neural stem cells (NSCs) and proteomics. Mol. Cell. Proteomics 15, 344–354.
Solomon, K.R., Anadon, A., Carrasquilla, G., Cerdeira, A.L., Marshall, E., Sanin, L.-H., 2007. Coca and poppy eradication in Colombia: environmental and human health assessment of aerially applied glyphosate. Rev. Environ. Contam. Toxicol. 190, 43–125.
Song, H.-Y., Kim, Y.-H., Seok, S.-J., Gil, H.-W., Hong, S.-Y., 2012. In vitro cytotoxic effect of glyphosate mixture containing surfactants. J. Kor. Med. Sci. 27, 711–715.
Sribanditmongkol, P., Jutavijittum, P., Pongraveevongsa, P., Wunnapuk, K., Durongkadech, P., 2012. Pathological and toxicological findings in glyphosate-surfactant herbicide fatality: a case report. Am. J. Forensic Med. Pathol 33, 234–237.
Szepanowski, F., Kleinschnitz, C., Stettner, M., 2019. Glyphosate-based herbicide: a risk factor for demyelinating conditions of the peripheral nervous system? Neural Regen. Res. 14, 2079–2080.
Tang, J., Hu, P., Li, Y., Win-Shwe, T.-T., Li, C., 2017. Ion imbalance is involved in the mechanisms of liver oxidative damage in rats exposed to glyphosate. Front. Physiol. 8, 1083.
Tang, Q., Tang, J., Ren, X., Li, C., 2020. Glyphosate exposure induces inflammatory responses in the small intestine and alters gut microbial composition in rats. Environ. Pollut. 261, 114129.
Thongprakaisang, S., Thiantanawat, A., Rangkadilok, N., Suriyo, T., Satayavivad, J., 2013. Glyphosate induces human breast cancer cells growth via estrogen receptors. Food Chem. Toxicol. 59, 129–136.
Tohyama, C., 2016. Developmental neurotoxicity test guidelines: problems and perspectives. J. Toxicol. Sci. 41, SP69–SP79.
Tseng, I.-L., Yang, Y.-F., Yu, C.-W., Li, W.-H., Liao, V.H.-C., 2014. Correction: phthalates induce neurotoxicity affecting locomotor and thermotactic behaviors and AFD neurons through oxidative stress in Caenorhabditis elegans. PloS One 9, e99945.
Uhl, E.W., Warner, N.J., 2015. Mouse models as predictors of human responses: evolutionary medicine. Curr. Pathobiol. Rep. 3, 219–223.
Urbán, N., Guillemot, F., 2014. Neurogenesis in the embryonic and adult brain: same regulators, different roles. Front. Cell. Neurosci. 8, 396.
von Ehrenstein, O.S., Ling, C., Cui, X., Cockburn, M., Park, A.S., Yu, F., Wu, J., Ritz, B., 2019. Prenatal and infant exposure to ambient pesticides and autism spectrum disorder in children: population based case-control study. BMJ 364, l962.
Wang, C., 2015. Application of in vitro models in developmental neurotoxicity and pharmaceutics research. J.Mol. Pharm. Org. Process Res. 3, 122–128.
Wang, D.D., Bordey, A., 2008. The astrocyte odyssey. Prog. Neurobiol. 86, 342–367.
Wang, M.-W., Hao, X., Chen, K., 2007. Biological screening of natural products and drug innovation in China. Phil. Trans. Biol. Sci. 362, 1093–1105.
Wang, Y., Branicky, R., Noë, A., Hekimi, S., 2018. Superoxide dismutases: dual roles in controlling ROS damage and regulating ROS signaling. J. Cell Biol. 217, 1915–1928.
Wilfinger, W.W., Mackey, K., Chomczynski, P., 1997. Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. Biotechniques 22, 474–481.
Wójtowicz, A.K., Sitarz-Głownia, A.M., Szczęsna, M., Szychowski, K.A., 2019. The action of di-(2-ethylhexyl) phthalate (DEHP) in mouse cerebral cells involves an impairment in aryl hydrocarbon receptor (AhR) signaling. Neurotox. Res. 35, 183–195.
Xia, Y., Yang, X., Lu, J., Xie, Q., Ye, A., Sun, W., 2020. The endoplasmic reticulum stress and related signal pathway mediated the glyphosate-induced testosterone synthesis inhibition in TM3 cells. Environ. Pollut. 260, 113949.
Zhang, T., Li, W., Wang, F., Zhang, Y., Xu, A., Shen, Y., 2015. Differentiation and proliferation potential of neural stem cells in subventricular zone of mice in vitro. Chin. J. Reparative Reconstr. Surg. 29, 766–771.
Zheng, Q., Yin, J., Zhu, L., Jiao, L., Xu, Z., 2018. Reversible Parkinsonism induced by acute exposure glyphosate. Park. Relat. Disord. 50, 121.
Zouaoui, K., Dulaurent, S., Gaulier, J., Moesch, C., Lachâtre, G., 2013. Determination of glyphosate and AMPA in blood and urine from humans: about 13 cases of acute intoxication. Forensic Sci. Int. 226, e20–e25.

3.3. Publication 3

Troxerutin Flavonoid has Neuroprotective Properties and Increases Neurite Outgrowth and Migration of Neural Stem Cells from the Subventricular Zone.

Muhammad Irfan Masood, Karl Herbert Schäfer, Mahrukh Naseem, Maximilian Weyland, Peter Meiser.

PLoS ONE, 2020, 15(8), 0237025.

PLOS ONE

RESEARCH ARTICLE

Troxerutin flavonoid has neuroprotective properties and increases neurite outgrowth and migration of neural stem cells from the subventricular zone

Muhammad Irfan Masood[1,2,3]*, Karl Herbert Schäfer[2]*, Mahrukh Naseem[4], Maximilian Weyland[2], Peter Meiser[5]

1 Division of Bioorganic Chemistry, School of Pharmacy, Saarland University, Saarbrücken, Germany, **2** ENS Group, University of Applied Sciences Kaiserslautern, Zweibrücken, Germany, **3** Institute of Pharmaceutical Sciences, University of Veterinary and Animal Sciences, Lahore, Pakistan, **4** Department of Zoology, University of Balochistan, Quetta, Pakistan, **5** Medical Scientific Department GM, URSAPHARM Arzneimittel GmbH, Saarbrücken, Germany

* karlherbert.schaefer@hs-kl.de (KHS); Irfan_masood_79@yahoo.com, mohammed.irfan.masood@gmail.com (MIM)

OPEN ACCESS

Citation: Masood MI, Schäfer KH, Naseem M, Weyland M, Meiser P (2020) Troxerutin flavonoid has neuroprotective properties and increases neurite outgrowth and migration of neural stem cells from the subventricular zone. PLoS ONE 15(8): e0237025. https://doi.org/10.1371/journal.pone.0237025

Editor: Michal Hetman, University of Louisville, UNITED STATES

Received: March 18, 2020

Accepted: July 17, 2020

Published: August 14, 2020

Peer Review History: PLOS recognizes the benefits of transparency in the peer review process; therefore, we enable the publication of all of the content of peer review and author responses alongside final, published articles. The editorial history of this article is available here: https://doi.org/10.1371/journal.pone.0237025

Data Availability Statement: All relevant data are within the manuscript and its Supporting Information files.

Abstract

Troxerutin (TRX) is a water-soluble flavonoid which occurs commonly in the edible plants. Recent studies state that TRX improves the functionality of the nervous system and neutralizes Amyloid-ß induced neuronal toxicity. In this study, an *in vitro* assay based upon Neural stem cell (NSCs) isolated from the subventricular zone of the postnatal balb/c mice was established to explore the impact of TRX on individual neurogenesis processes in general and neuroprotective effect against ß-amyloid 1–42 (Aß42) induced inhibition in differentiation in particular. NSCs were identified exploiting immunostaining of the NSCs markers. Neurosphere clonogenic assay and BrdU/Ki67 immunostaining were employed to unravel the impact of TRX on proliferation. Differentiation experiments were carried out for a time span lasting from 48 h to 7 days utilizing ß-tubulin III and GFAP as neuronal and astrocyte marker respectively. Protective effects of TRX on Aß42 induced depression of NSCs differentiation were determined after 48 h of application. A neurosphere migration assay was carried out for 24 h in the presence and absence of TRX. Interestingly, TRX enhanced neuronal differentiation of NSCs in a dose-dependent manner after 48 h and 7 days of incubation and significantly enhanced neurite growth. A higher concentration of TRX also neutralized the inhibitory effects of Aß42 on neurite outgrowth and length after 48 h of incubation. TRX significantly stimulated cell migration. Overall, TRX not only promoted NSCs differentiation and migration but also neutralized the inhibitory effects of Aß42 on NSCs. TRX, therefore, offers an interesting lead structure from the perspective of drug design especially to promote neurogenesis in neurological disorders i.e. Alzheimer's disease.

Funding: We are very thankful to The University of Applied Sciences, Kaiserslautern, Germany and The Higher Education Commission Government of Pakistan (Ref. PD/OS-II/Batch-VI/Germany/2015/77159/17755) for providing financial support in the execution of the present project. These funding agencies provided support in term of stipend for authors and expenses concerning chemicals but did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. Additionally, Ursapharm Arzneimittel GmbH provided support in the form of salary for author PM, but did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The specific roles of these authors are articulated in the 'author contributions' section.

Competing interests: The authors have read the journal's policy and have the following potential competing interests: PM is a paid employee of Ursapharm Arzneimittel GmbH. This does not alter our adherence to PLOS ONE policies on sharing data and materials. There are no patents, products in development or marketed products associated with this research to declare.

Introduction

Neural stem cell (NSC) is a structural and functional unit of the nervous system [1] which deals with the traumatic events or neuronal losses with ageing. Being multipotent (capable of differentiating into glial cells, oligodendrocytes and neurons) in nature and having self-renewal properties [2], NSCs recapitulate the nervous system development processes such as proliferation, differentiation, migration, synaptogenesis and myelination [3, 4]. NSCs occur both in developing and adult nervous system of all mammalians including human [5]. Within the brain, NSCs are located mainly in the Subventricular zone (SVZ) and the dentate gyrus [6]. NSCs response to the external stimuli varies with the age of the donor [7], site in the nervous system and due to the diversity in their local environment [8, 9]. The SVZ presents the major niche of NSCs where primary and secondary neurogenesis occurs primarily [10–12].

The biggest hurdle in curing neurodegenerative disorders involves the irreversible damage to the neuronal cells which could no longer be replaced or repaired. High self-renewal potential, multipotency and multidirectional fate are a few rather unique characteristic features associated with NSCs which highlight the significance of these cells to serve as a promising tool to decipher the biochemical mechanisms underlying neurodegenerative disorders [13]. Screening small molecules which induce desired types of neurons from NSCs is highly valuable not only for regenerative medicine but also for the development of new drug candidates [14]. Interestingly, plant-based molecules, especially flavonoids, modulate the fate of NSCs favourably as confirmed by several *in vitro* culture systems. Enhanced proliferation of NSCs from multiple niches was observed when exposed to epimedium flavonoids [15], icariin [16, 17] and morin hydrate [18] whilst baicalin [19, 20], apigenin [21] and quercetin have proven their efficacy in inducing neuronal differentiation of NSCs [22]. Quercetin-3-o-glucuronide was reported to promote the cell migration of NSCs of mouse hippocampus [23]. It is important to note, however, that the bioavailability of flavonoids is generally low due to its inherent physicochemical properties.

Troxerutin (TRX) (3',4',7-tris[O-(2-hydroxyethyl)]rutin) is a water-soluble derivative of the bioflavonoid rutin extracted from the Japanese pagoda tree, which is also found abundantly in tea, coffee, vegetables and fruits. TRX exhibits several biological activities and cytoprotective effects against apoptosis, mitotic and necrotic cell death of liver, kidney and brain. TRX is also considered as an interesting drug candidate for multiple neurological disorders since it demonstrates antidepressant activity (because of its anti-inflammatory action), augments memory in animal models and provides anxiolytic actions (by reducing serum cortisol level) [24] and ameliorated the impairments of spatial learning and memory in a rat Alzheimer's model [25]. Moreover, TRX alleviates UV-B induced apoptosis, cell growth arrest, migration restriction, proliferation inhibition and DNA damaged in cultured HaCaT human immortal keratinocytes [26]. Intriguingly, TRX was reported to exhibit neuroprotective effects against the cholesterol-induced oxidative stress through its antioxidant properties and by enhancing phosphor inositide3 kinase/Akt activation in mouse hippocampus models. TRX exerts a neuroprotective role under endoplasmic reticulum induced stress by inhibiting the activities of caspase-3 and caspase-12. Overall, TRX is an excellent candidate which could be exploited to improve neuronal survival *i.e.* in Alzheimer's disease [27, 28].

Amyloid-ß (Aß) is a brain peptide with the size of approximately 4kDa derived from the amyloid precursor protein (APP) by enzymatic cleavage. Aß42 is the most hydrophobic form of Aß which is more fibrillogenic and forms the plaques in the brain [29]. Polymerization of monomeric Aß into soluble oligomer and insoluble fibril mass triggers Alzheimer's disease [30]. Predominantly, Aß deposition occurs in the hippocampus region of the brain which ultimately leads to the neuronal death due to oxidative stress. The production and aggregation of

Aß protein increase with aging [31]. Literature reveals that Aß42 inhibits proliferation and differentiation of NSCs from mouse hippocampus [32]. Flavonoids have proven their efficacy in improving synaptic functions against the Aß42 induced neurotoxicity [33]. TRX is capable of restoring memory loss and learning incapability induced by Aß42 in a rat model. These effects of TRX were associated with antioxidative action, anti-inflammatory effects and the capability to up-regulate cholinergic receptors in the animal brain [25]. Since neuroprotective and neuroaugmentation properties of TRX have already been reported in the literature, it would be interesting to decipher the effect of TRX on neurogenesis processes. So the aim of the present study is to establish an *in vitro* NSCs model from SVZ of the postnatal mice to investigate the effects of TRX on proliferation, migration and differentiation of NSCs and its neuroprotective effects against oligomeric Aß42 on the differentiation of NSCs.

Materials and methods

Animal dissection and cell culture

In the present study, NSCs were isolated from SVZ of postnatal Balb/c wild-type mice of 3–5 days old. Animals were housed under specific pathogen-free conditions on a 12 h light/12h dark cycle according to German regulations in the animal house facilities of the medical faculty Homburg, next to the Zweibrücken Campus. Animals were transported in warmed boxes and killed immediately after arrival by decapitation. Around three animals were employed used for each set of experiment. The total number of animals used for this study was 21. An authorized and well-trained researcher sacrificed the animals by decapitation without anaesthesia. Animal preparations in this study were carried out in strict accordance with the recommendation in the Guide for the care and use of laboratory animals according to animal protection law in Rhineland-Palatinate State, Germany. Since no experiment was directly performed on the living animals and only tissues were taken from the dead animals, no separate approval was necessary and the animal killing only has to be reported to the local Committee on the Ethics of Animal Experiments, University of Applied Sciences Kaiserslautern. NSCs were isolated and subsequently cultured according to the procedure reported in the literature with few necessary modifications [34]. Immediately after decapitation, mice brains were collected and stored in ice-chilled MEM-medium (Life technology, Eugene, USA) containing1% penicillin/streptomycin (Thermofischer, Waltham, USA). Under an inverted microscope (Olympus, Tokyo, Japan) SVZ was separated from both hemispheres followed by mechanical and enzymatic digestion with HyQtase enzymes (HyClone-GE, Utah, USA) and dissociated into single-cell suspensions. Approximately 100,000 cells were seeded in a proliferation medium DMEM/F12 (Life technology, Eugene, USA)containing 2% B-27 without antioxidants (Gibco, Paisley UK), 1% Penicillin/streptomycin, ß-mercaptoethanol, EGF 10 ng/mL and FGF 20 ng/mL (Immunotool, Friesoythe, Germany) in a T25 culture flask (Greiner, Frickenhausen, Germany). After 6 days NSCs proliferated to generate neurospheres. About half of the medium was replaced every alternative day. Before starting every individual experiment, neurospheres were dissociated and cell numbers were counted employing trypan blue (Gibco, Paisley, UK). All experiments were performed in five replicates ($n = 5$).

Proliferation assay

The neurosphere clonogenic assay is a simple but robust assay which provides information about the effect of compounds on NSCs proliferation. Neurosphere's diameter and number were two readouts recorded at different time points of incubation [35]. Neurosphere diameter indicates NSCs multiplication within a neurosphere whilst neurosphere number is an indicator of self-renewal properties of NSCs [32]. At least 1000 cells were seeded in 200 μL of

proliferation medium into each well of a 96-well plate and medium was change on every alternative day. The cells were exposed to different concentrations of TRX (Y000497, Sigma-Aldrich, Taufkirchen, Germany) (25 μM, 50 μM and 100 μM). Neurosphere diameter and number were recorded on 3rd, 5th and 7th day of the culture. The effect of TRX was further confirmed by BrdU/Ki67 double staining. The ratio of BrdU-negative to the Ki67positivecells provides an accurate estimation of the amount of proliferating cells [36, 37]. NSCs cells were proliferated in proliferation medium supplemented by growth factors with and without TRX for 7 days. 4 h before the completion of incubation, proliferating cells were exposed to 10 μM BrdU (Sigma-Aldrich, Taufkirchen, Germany) followed by dissociation of neurospheres into a single-cell suspension. Around 20,000 cells were seeded on each of a 12 mm glass coverslips coated with PDL (Sigma-Aldrich, Taufkirchen, Germany) for 2 h. Cells were fixed with 4% Paraformaldehyde at 25˚C for 20 min. BrdU/Ki67double staining was then performed as described in the immunostaining section. To confirm the stem cell nature of NSCs, NSCs after 7 days of proliferation were dissociated into a single cell suspension and seeded on ECM gel (Sigma-Aldrich, Taufkirchen, Germany) coated glass coverslips followed by fixation and stained for basic stem cell marker Nestin and a secondary NSCs marker GFAP. For calculating cell percentages, 30 microscopic fields at a magnification of 200 x were included in the observation.

Differentiation assay

Effect of TRX on the differentiation of NSCs into neuronal and astrocyte cells was determined through a differentiation assay performed for 7 days and 48 h by exploiting all three test concentrations of TRX. Approximately 20,000 cells were attached on each of 12 mm glass coverslips coated with ECM gel. Cells were differentiated in the DMEM F12 medium comprising of a similar composition as employed for cell proliferation but excluding growth factors. NSCs in the differentiation medium were exposed to TRX for a given period followed by cell fixation and immunostaining for neuronal and astrocyte markers. Percentages of neurons and astrocytes were calculated and morphological parameters such as total neurite length, mean neurite length, %age of non-neurite neurons and soma area of astrocytes were quantified. For calculating cell percentages, 30 microscopic fields with a 20 x objective were captured and included in the observation. For morphological analysis, 100 cells were included in the analysis for each condition in each replicate. Morphological measurements were carried out for 7 days as well as for 48 h of incubation. Additionally, concentration produced optimum effect was used for counter toxicity testing against Amyloid-ß42 aggreSure AS72216 (AnaSpec EGT Group, California, USA) induced toxicity.

Immunostaining

Adhered cells were fixed by exposure to 4% paraformaldehyde for 20 min at room temperature. After two times washing with PBS, cells were stored at 4˚C until staining. Staining was performed following a recently published protocol [38]. Briefly, fixed cells were incubated with Triton 100 x 0.3% for 10 min at room temperature to enhance permeability followed by washing with PBS-tween one time and PBS 2 times. Non-specific binding was blocked with 10% normal donkey serum (Merck, Darmstadt, Germany) for 1 h at room temperature. Cultures were then incubated with primary antibodies mouse-anti-ß tubulin-IIIMAB1637 (1:500; Merck, Darmstadt, Germany), Nestin MAP353 (1:300; Merck, Darmstadt, Germany), rabbit anti-GFAP Z0334 (1:500, DakoGlostrup, Denmark), rat anti-BrdU (1:250; AbDserotec, Kidlington, UK) and rabbit-anti Ki67 (1:250; Abcam Cambridge, UK) at room temperature for 1hfollowed by washing 3 times with PBS. The samples were visualized using Alexafluor 488

(1:1000) and Alexafluor 594 (1:1000) conjugated donkey antibodies (Life Technology, Eugene USA) for another one hour and finally washing 3 times with PBS. DAPI was used for nuclear staining and incubated at room temperature for 10 min followed by final washing in PBS. Fluorescence supporting mounting medium was used to fix coverslips on glass slides. For BrdU, predenaturation of nucleic acid was achieved with 2N HCl prior to blocking and the acid was neutralized by borate buffer 0.1 M with pH 8.5 [39]. The rest of the steps were identical to those used previously.

Neurosphere migration assay

NSCs were proliferated in proliferation medium supplemented by growth factor for 5–6 days in the absence of TRX. Around 12–15 neurospheres were allowed to attach on each of the 12 mm glass coverslips coated with PDL (Sigma-Aldrich, Taufkirchen, Germany) then incubated and differentiated in a differentiation medium excluding growth factor for a period of 24 hat 37˚C with 5% CO_2. The incubation was performed with and without TRX At the end of incubation, phase-contrast pictures were capture with a 4 x objective. Cell migration was evaluated by calculating the mean distance travelled by migrating cells away from the edge of the neurosphere in four directions measured at a right angle to the edge of the neurosphere core to the furthest migrated cells [40].

Statistics

All results were presented as mean ± SEM which were calculated by exploiting descriptive statistics and non-parametric Kruskal-Wallis test with post hoc Dunn's test. Mann-Whitney pair wise test was employed in the case of two treatments.

Results

Identification of NSCs in vitro and the effects of TRX

We rated the stemness of NSCs culture using general stem cell markers Nestin and glial fibrillary acidic protein (GFAP), a reactive glial and astrocyte marker [41]. After 7 days of proliferation with and without TRX treatments, cells were fixed and immunostained. Our results indicated that each cell from every treatment condition was immunoreactive to a general NSCs marker Nestin. However, we also found a fraction of cells co-expressing Nestin and secondary NSCs marker GFAP in every treatment condition including control. TRX at 100 μM concentration significantly reduced the percentage of cells only expressing single Nestin marker when compared to all other treatments (Control vs 100 μM: $p = 0.046$., 25 μM vs 100 μM: $p = 0.0018$., 50 μM vs 100 μM: $p = 0.0043$) but increased the percentage of cells co-expressing Nestin and GFAP when compared with 25 μM ($p = 0.0013$) and 50 μM ($p = 0.0043$) concentrations (Figs 1 and 2D). Difference between Control and two lower concentrations of TRX was statistically non-significant concerning all calculated cell percentages.

TRX effects on proliferation

We performed proliferation assay as previously reported that flavonoids enhance the proliferation of NSCs in an *in vitro* cultures [15, 16]. The results of clonogenic assay revealed that in the absence of growth factors, NSCs failed to proliferate and died on the 7^{th} day in all tested concentrations except in Control+ve supplemented with growth factors where cells rapidly proliferated to form neurospheres at all observation time points (Fig 3). To investigate the augmenting effects of TRX on NSCs proliferation in the presence of growth factors, a set of a clonogenic assay where proliferation medium supplemented with growth factors for all

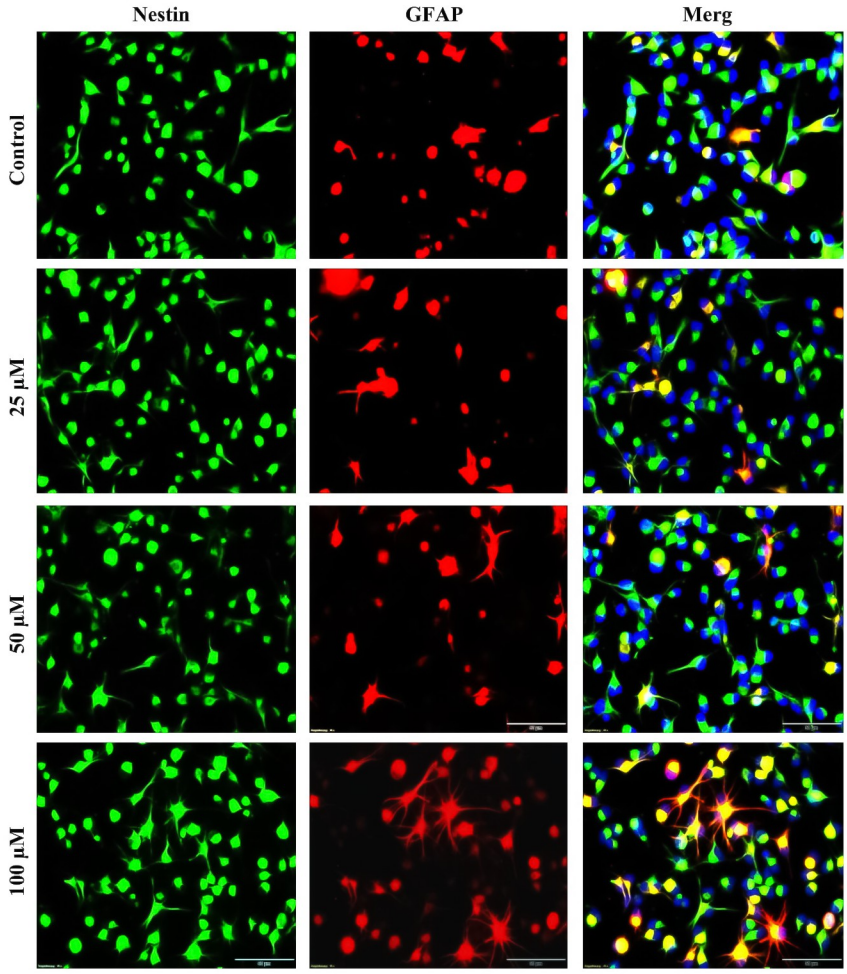


Fig 1. Immunostaining of NSCs for stem cell markers after seven days of proliferation. Green and red represent Nestin+ve and GFAP+ve cells, respectively. Yellow to orange cells in the merge images represent NSCs co-expressing both Nestin and GFAP. The highest concentration of TRX (100 μM) exhibited more GFAP and more doubled stained cells when compared to all other treatments. Pictures were captured with a 40 x objective of a fluorescent microscope. TRX; Troxerutin. Scale bar measures 50 μm.

https://doi.org/10.1371/journal.pone.0237025.g001

treatment conditions was performed. The results clearly indicated that none of the tested TRX concentrations exhibited significant effect on NSCs proliferation parameters (neurosphere number and neurosphere mean diameter) during all observation points when compared to the Control (Fig 2A and 2B). The results of the study were further confirmed with more sensitive proliferation assay BrdU/Ki67 immunostaining. After seven days of proliferation, the effect of all tested concentrations of TRX on percentage of BrdU cells, Ki67 cells the ratio of BrdU/Ki67 were non-significant when compared to the Control (Fig 2C). Overall, the results demonstrate that TRX did not promote cell proliferation during any of the observation time points in the presence and absence of mitogenic growth factors.

TRX enhanced neurite growth in neurons differentiated from NSCs

Being multipotent in nature, NSCs give rise to neurons, oligodendrocytes and glial cells on differentiation. NSCs differentiation mainly depends upon the environmental signals [42]. Plant

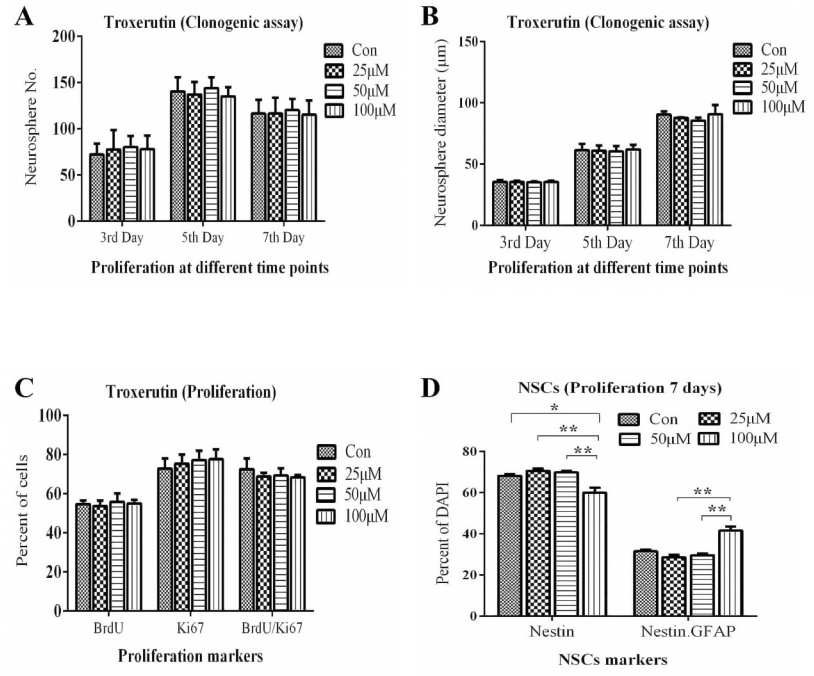


Fig 2. TRX effect on neural stem cell markers and proliferation of NSCs. TRX effect on proliferation was determined through neurosphere clonogenic and by BrdU/Ki67 double staining. **A**: Represents neurosphere number. **B**: Represents neurosphere mean diameter. **C**: represents the percentage of S-phase marker BrdU, Ki67 and the ratio of both markers. Percentage of BrdU+ve and Ki67 +ve cells were calculated from the total DAPI nuclei count. **D**: Represents the percentage of NSCs which were exclusively Nestin+ve and the fraction of NSCs co-expressing Nestin and GFAP. Every cell from each treatment condition was immunoreactive to general NSCs Nestin. Percentages of Nestin+ve and GFAP+ve cells were calculated from the total DAPI stained nuclei count. The experiments were performed as five replicates *(n = 5)*. Con; Vehicle control. Data were calculated as the mean ± SEM.*p<0.05,** p<0.01.

https://doi.org/10.1371/journal.pone.0237025.g002

flavonoids are known to induce NSCs differentiation by interacting with genes regulating cell fate [20, 43–46]. In addition to neuronal differentiation, flavonoids also improve the neurite growth of differentiated neuronal cells [19]. Single-cell suspension of NSCs was differentiated for 7 days and 48 h in the presence and absence of TRX in 25 μM, 50 μM and 100 μM concentrations. Short term incubation was performed to evaluate the effect of TRX on early neuronal and astrocytes differentiation and also on their morphological characteristics. TRX increased the neuronal expression and decreased the GFAP astrocyte cells expression in a dose-dependent manner both on 7 days and 48 h of incubation. It is, however, important to mention that the results are statistically non-significant. Nevertheless, TRX in high concentration (*i.e.* 100 μM) exhibited a 15.9% increase in neuronal expression and an 11% decrease in astrocyte cells expression when compared to the vehicle Control. A similar trend was observed on 48 h incubation where the higher concentration increased neuronal expression by 19% with minimum effects on astrocyte expression (Fig 4A and 4B). TRX significantly decreased the percentage of non-neurite neurons (*i.e.* 2.2 folds compared to Control ($p = 0.0027$)) on 7th day at 100 μM (Figs 4C and 5). In the case of 48 h incubation, both 50 μM and 100 μM of TRX significantly reduced the percentage of non-neurite neurons *i.e.* 1.7 fold ($p = 0.05$) and 2.5 fold ($p = 0.0053$), respectively when compared to the Control. Additionally, 6–12% cells were double-stained for both neuronal and astrocyte markers during 48 h incubation (Figs 4D and 6). TRX reduced the percentage of double-stained cells in a dose-dependent manner and a

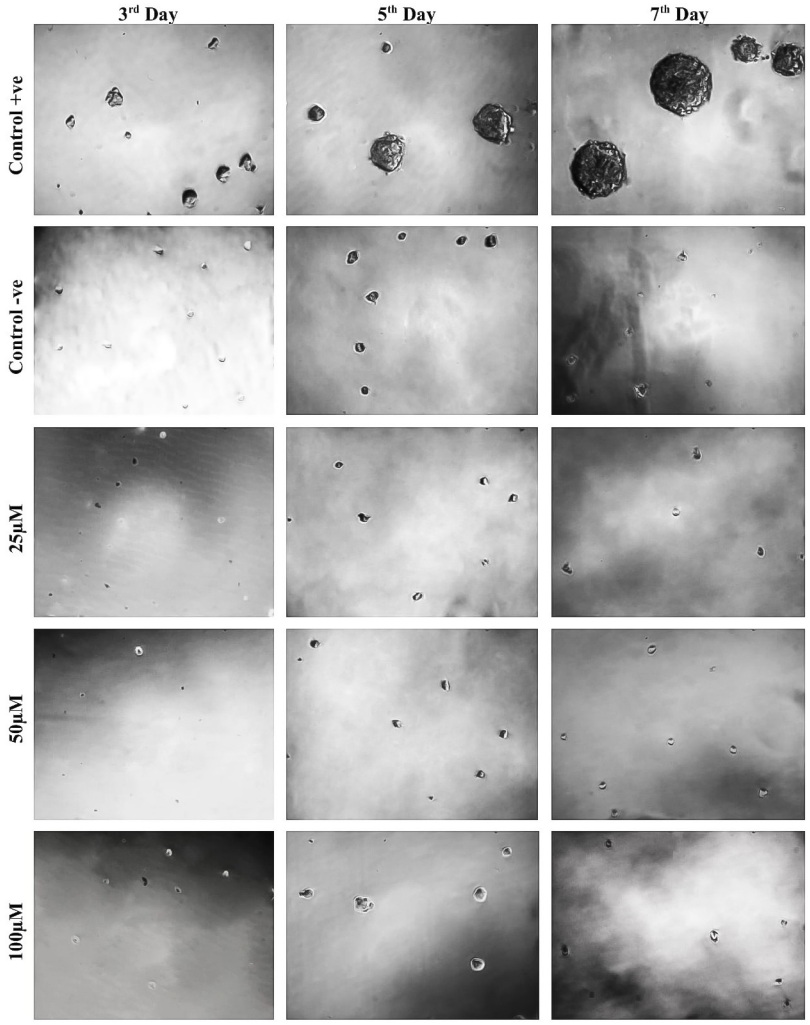


Fig 3. Neurosphere clonogenic assay for NSCs proliferation. The medium of Control+ve was supplemented with EGF and FGF growth factors. Growth factors were completely omitted from the Control-ve and all TRX treatments. Cells proliferated rapidly in positive control and formed neurospheres whose size increased with the increase in the incubation period. Cells in all other treatment conditions failed to proliferate and completely died on 7th day of observation. Pictures were captured with a 10 x objective of a phase-contrast microscope.

https://doi.org/10.1371/journal.pone.0237025.g003

significant difference was observed for control vs 50 μM ($p = 0.044$) and Control vs 100 μM ($p = 0.01$) concentrations.

Total neurite length and mean neurite length per neuron were calculated as morphological parameters for neurons, while the cell body area was calculated for GFAP+ astrocytes. Morphological analysis was performed after 7 days employing an *in vitro* differentiation culture for all tested concentrations of TRX. The results revealed that TRX increased the total and mean neurite length in a dose-dependent manner after 7 days of incubation. Concerning total neurite length, a significant difference was observed between Control vs 50 μM ($p = 0.014$) and Control vs 100 μM ($p = 0.0009$) TRX stimulation (Figs 7A and 8). Neurite arborisation was assessed by calculating arborisation index as reported in the literature [47]. None of the tested concentrations of TRX affected the arborisation index after 7 days of differentiation. However,

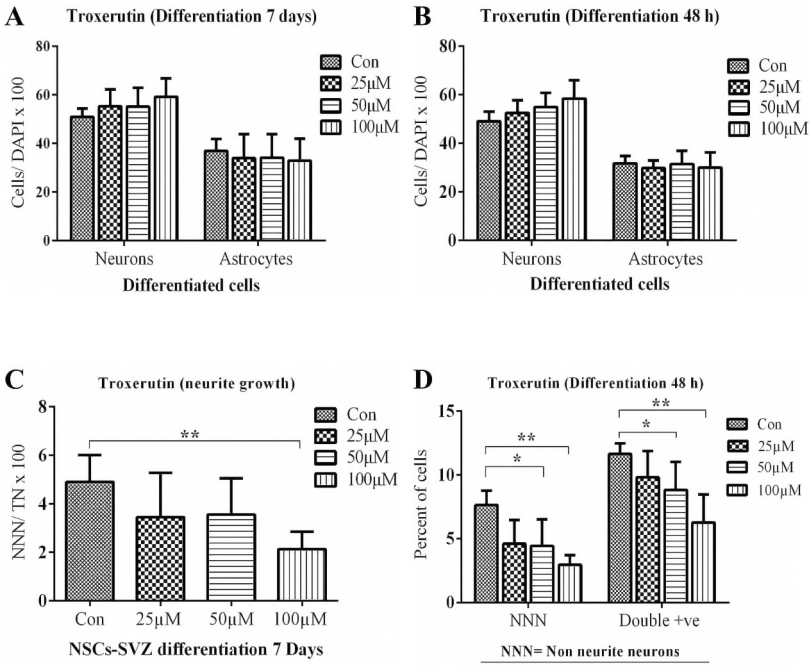


Fig 4. TRX effect on NSCs differentiation after 7 days and 48 h of incubation. ß-tubulin-III was employed as a neuronal marker and GFAP was exploited as an astrocyte marker. **A**: Represents the percentage of neurons and astrocytes after 7 days of differentiation exposed to different concentrations of TRX. **B**: represents the percentage of cells after 48 h of incubation. **C**: Represents the decrease in the percentage of non-neurite neurons; (NNN) with increasing concentration of TRX after 7 days. **D**: Represents the decrease in the percentage of non-neurite neurons and the double positive cells (Co-expressing ß-tubulin III and GFAP) with increasing concentrations of TRX after 48 h. NNN percentage was calculated from the total neuronal count. Only after 48 h of incubation time, cells immunoreactive to both neuronal and astrocyte markers (double+ve) could be detected. Data are presented as mean ± SEM. $^{*}p<0.05$, $^{**}p<0.01$.

https://doi.org/10.1371/journal.pone.0237025.g004

TRX significantly enhanced the number of neurite branching tips when compared to the Control (Fig 9) at both 50 μM *(p = 0.017)* and 100 μM *(p = 0.002)* concentrations. TRX increased the astrocyte soma area at 25 μM (*p = 0.05*) when compared to the Control and all other concentrations of TRX after 7 days of incubation (Figs 7C and 8). Morphological parameters of neurons and astrocytes for short term incubation of 48 h were only determined for high concentration of 100 μM because of optimum effects of this higher concentration on NSCs differentiation upon longer incubation. Interestingly TRX significantly increased total and mean neurite length and decreased the astrocyte's soma area at 100 μM when compared to the control (*p = 0.05*) (Figs 7B–7D and 10). Moreover, a non-significant difference concerning NSCs viability was also observed between the Control and 100 μM TRX Fig S A in S1 File).

Neuroprotective effect of TRX against Aß42induced depression in NSCs differentiation

Oligomeric form of Aß42 is implicated in Alzheimer's disease, a leading neurological disorder characterized by progressive loss of memory and cognitive functions [48, 49]. Oligomeric Aß42 decreases the neuronal and astrocytes differentiation of NSCs culture [32] whilst natural products, such as flavonoids including TRX, have proven their efficacy against the Aß42

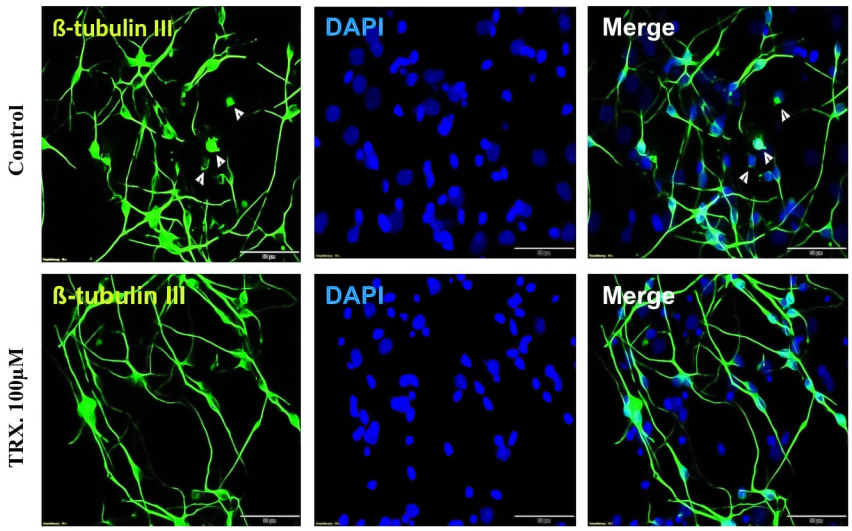


Fig 5. Effects of TRX on NNN after 7 days of differentiation. NSCs were differentiated for 7 days in the presence of TRX. Cells were fixed and immunostained for ß-tubulin III neuronal markers. Green cells represent neurons whilst blue rounded bodies indicate nuclei stained by nuclear dye DAPI. Control condition presented more neurons without neuritis (NNN) than the TRX as indicated by white arrows in the top left and top right images. Fluorescent images were captured with a 40 x objective of a microscope with scale bar measures 50 μm.

https://doi.org/10.1371/journal.pone.0237025.g005

induced neurotoxicity both in *in vitro* and *in vivo* models [25, 49–51]. In this set of experiment, an *in vitro* model based upon NSCs isolated from SVZ of the developing mouse was established to unveil the neutralizing effect of TRX against the oligomeric Aß42 induced inhibition in the differentiation of NSCs and neurite growth. A concentration of 100 μM of TRX was selected as an optimal concentration to evaluate its neuroprotective effects against Aß42 induced inhibition in neuronal differentiation. Aß42 was employed in 10 μM concentration since this concentration has been reported to exhibit strong inhibitory effects on the neuronal differentiation [32] and the neurite growth in the differentiated neurons [52]. Moreover, the literature reveals that lower concentrations of Aß42 exhibit neurogenerative effects rather than neurotoxicity through a compensatory mechanism of brain repair [32, 53]. Finally, WST-1 cytotoxicity assay was performed to investigate the impact of Aß42 on the cell viability in differentiated culture for 48 h and a non-significant difference between the Control and the Aß42 (10 μM) was observed (Fig S B in S1 File). Aß42 significantly reduced the amount of neurons when compared to the Control ($p = 0.034$) and the Aß42+TRX combination ($P = 0.009$) after 48 h incubation (Fig 11A). The amount of non-neurite neurons significantly increased as compared to the Control ($p = 0.003$) and the combination Aß42+TRX ($p = 0.016$) in the remaining neurons. Aß42 treatment significantly increased the amount of cells co-expressing the neuronal and astrocytes markers when compared to the Control ($p = 0.005$) and Aß42+TRX combination ($P = 0.036$) (Figs 11B and 12). Aß42 reduced the total neurite length and mean neurite length of each neuron when compared with combination Aß42+TRX treatment ($p = 0.004$) (Figs 11C and 13) and also reduced total neurite length by 27% and mean neurite length by 19% when compared with the Control. Concerning the astrocyte soma area, both Aß42 ($p = 0.008$) and combination Aß42+TRX ($p = 0.007$) significantly reduced the soma area when compared to the Control (Figs 11D and 13). However, a non-significant difference was observed between Aß42 and Aß+TRX for this parameter.

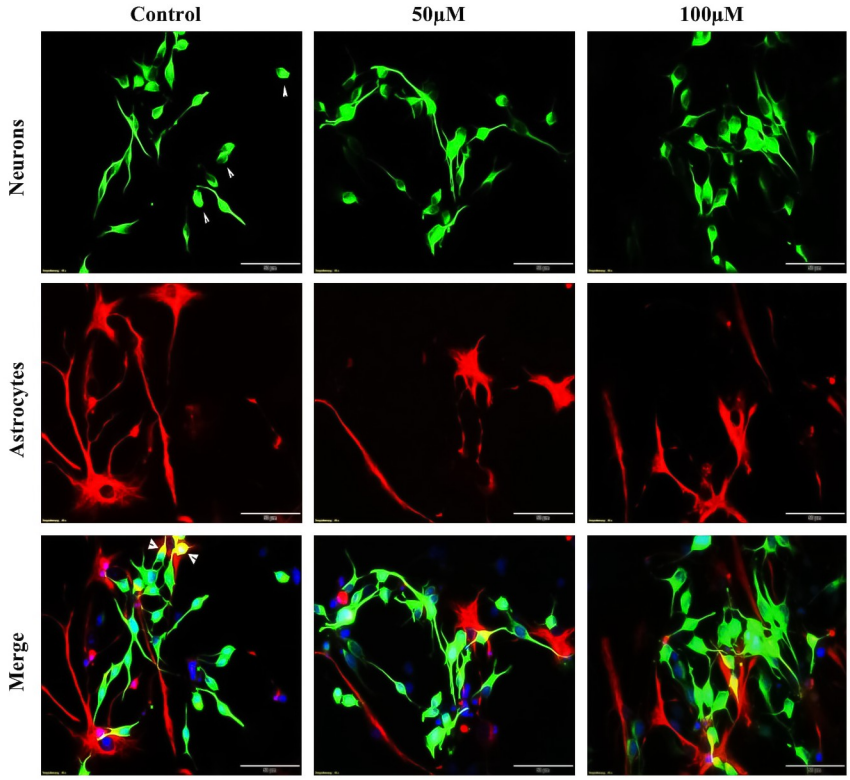


Fig 6. Effects of TRX on NNN after 48 h of differentiation. NSCs were differentiated for 48 h in the presence of TRX. Cells were fixed and immunostained for ß-tubulin III neuronal markers and GFAP astrocyte marker. Green cells represent neurons, red cells indicate astrocytes and blue rounded bodies present nuclei stained by nuclear dye DAPI. Control condition exhibited more neurons without neuritis (NNN) than TRX as indicated by white arrows in the top left image and more cells co-stained for neuronal and astrocyte (yellow cells) indicated by a white arrow in the bottom left images. Fluorescent images were captured with a 40 x objective of a microscope with scale bar 50 μm.

https://doi.org/10.1371/journal.pone.0237025.g006

TRX stimulates cell migration in NSCs culture

Stimulatory effects of TRX on cell migration have already been reported for different kinds of cells [26, 54]. TRX induces cell migration of human umbilical vein endothelial cells in combination with cerebroprotein hydrolysate [54]. To evaluate whether TRX influences migration in NSCs culture, neurosphere migration assays were performed. TRX significantly enhanced the cell migration at 50 μM ($p = 0.012$) and 100 μM ($p = 0.006$), while 25 μM of TRX did not exhibit any significant increase after 24 h incubation (Fig 14).

Discussion

The self-renewal capacity and the differentiation into multiple cell types of the nervous system are characteristic features which make NSCs a very useful bio-tool for the screening of molecules which exert supporting effects on neural cell proliferation [15, 16, 55], differentiation [19, 20, 22], migration [23] and synaptogenesis [45]. Flavonoids serve as effective agents which provide antioxidative protection and neuroprotection. TRX is a derivate of rutin which serves as neuroprotective and neurogenerative agent [25, 28, 33, 56]. Additionally, TRX proved its effectiveness in avoiding Aß42 induced memory defects in a mouse model [27]. In the present study, an *in vitro* NSC based assay from SVZ of the postnatal mouse was established to evaluate

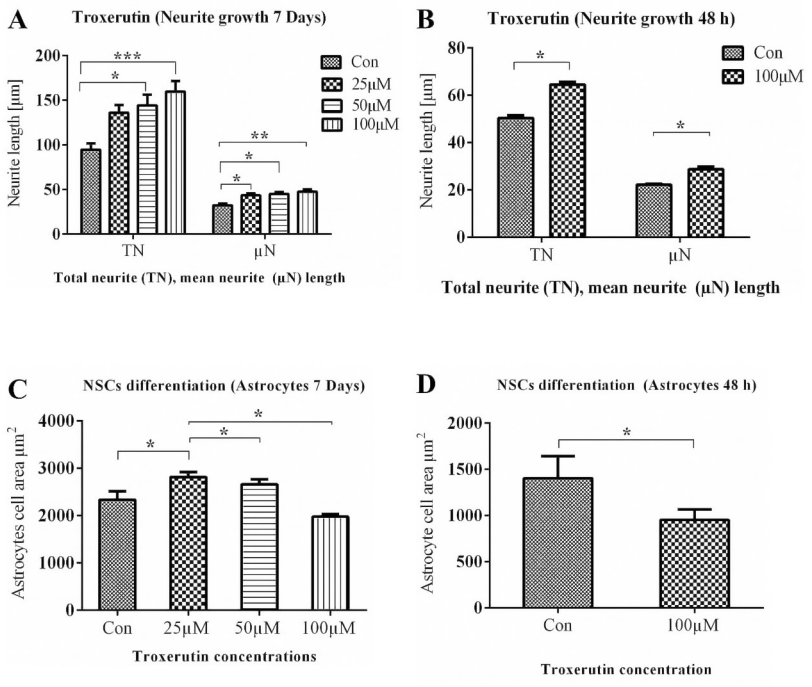


Fig 7. Effects of TRX on morphological parameters of neurons and astrocytes differentiated from NSCs after 7 days and 48 h of incubation. Approximately100 cells were employed for morphological analysis in each replicate for each condition. **A**: Represents TN and µN after 7 days. **B**: represents TN and µN after 48 h. **C**: Represents the astrocytes soma area after 7 days. **D**: represents astrocytes soma area after 48 h of incubation. The study was performed in 5 replicates *(n = 5)*. Data are presented as mean ± SEM. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. TN; total neurite length, µN; mean neurite length.

https://doi.org/10.1371/journal.pone.0237025.g007

the effect of TRX on basic neurogenesis processes and to quantify neuroprotective effects in Aß42 challenges, where neuronal differentiation and neurite outgrowth are compromised.

Routinely, NSCs are identified in an *in vitro* culture by the immunostaining of NSCs/ neural progenitor cells (NPCs) markers namely DCX, Atoh1, SOX2, Nestin and GFAP. DCX is a marker for NPCs and not pure NSCs. Atoh1 is the marker for NSCs of the Cochlar nucleus. SOX2 is the marker of NSCs in the developing brain during early embryonic phase. Nestin is the widely employed marker for both NSCs and NPCs in the developing and adult nervous system [57–59]. Nestin is an intermediate filamentous protein widely expressed by NSCs from the mammalian nervous system [15]. There exists a class of NSCs which co-express nestin and the reactive glial cell marker GFAP. So both Nestin and GFAP are considered as NSCs markers [60]. NSCs which co-express Nestin and GFAP comprise of radial glial cells which present neural stem cell properties [61, 62]. These cells predominantly exist in the SVZ of both developing and adult brain and are capable of differentiation into neurons, oligodendrocytes and astrocytes *in vitro* [63, 64]. In this study, both nestin and GFAP immunostainings were performed to identify NSCs isolated from SVZ culture proliferated for 7 days in the presence of different concentrations of TRX. The immunostaining results indicated that cells treated with TRX (100 µM) presented a tendency to increase the percentage of cells co-expressing both stem cell markers Nestin and GFAP when compared to all other treatments. It is already reported in the literature that around 30% of the total NSCs isolated from mammalian brain stained for stem cell marker nestin, also co-stained for astrocyte marker GFAP [41]. These

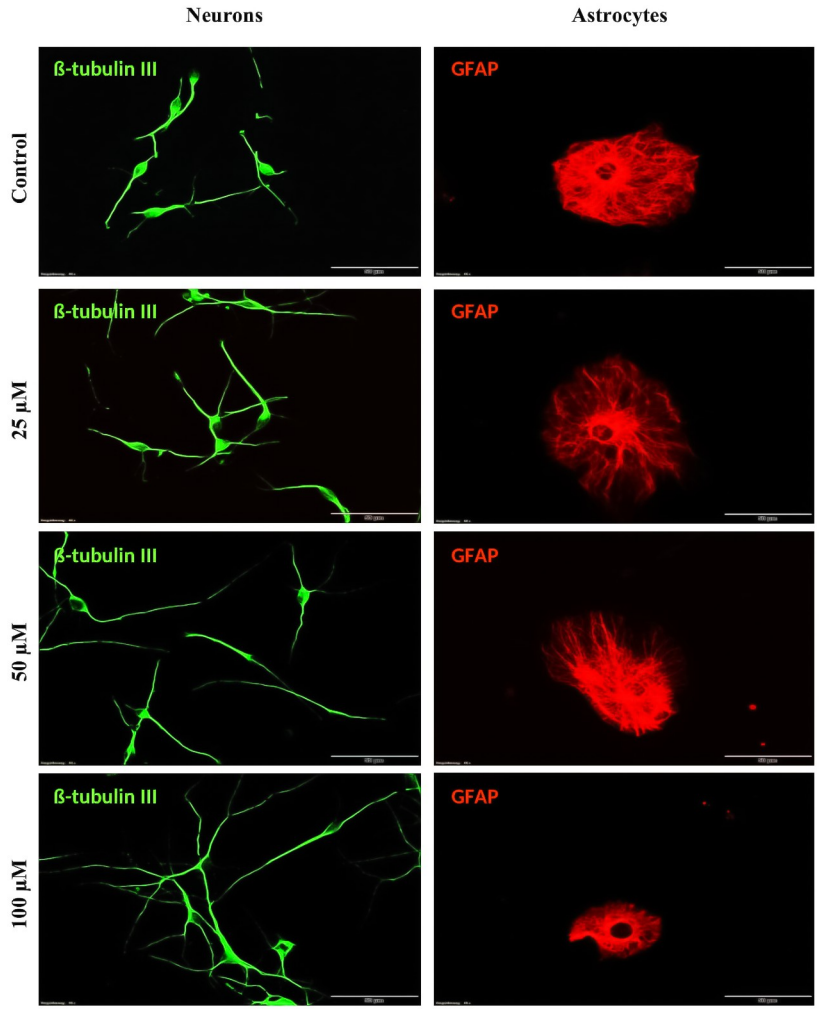


Fig 8. Effect of TRX on neurite growth of neurons and soma area of astrocyte after 7 days of incubation. NSCs were differentiated on ECM coated glass coverslips fixed and then stained for a neuronal marker (ß-tubulin-III) and astrocyte marker (GFAP). Green cells represent are neurons. Neurons under TRX treatments exhibited elongated neurites with more branching points when compared to control. Red cells indicate astrocytes. Most of the astrocytes cells presented leaf-like morphology. Astrocytes treated with TRX in less than 25 μM concentration demonstrated expended soma area when compared with all other treatment conditions. Cells presented a smooth surface with no deformation. All measurements were performed exploiting Cell SENE software. Pictures were captured with 40 x objective of a fluorescent microscope with scale bar 50 μm.

https://doi.org/10.1371/journal.pone.0237025.g008

doubled stained NSCs give rise to neurons and glial cells at the time of birth and give rise to adult NSCs in the SVZ thus allowing a continuous supply of NSCs for regenerative processes throughout the life [65]. Additionally GFAP expressing NSCs from SVZ quickly turn into functional neurons in response to brain injury [66]. Another similar property of NSCs is their ability to facilitate neuronal cell migration [67]. Taken together, our immunostaining results suggested that all cells cultured from mouse SVZ were NSCs as they expressed stem cell markers in proliferation culture and then differentiated into neurons and astrocytes on subsequent differentiation experiments.

The proliferation of NSCs in an *in vitro* culture condition can only be maintained when cells are supported by essential growth factors. Among these, brain-derived neurotrophic

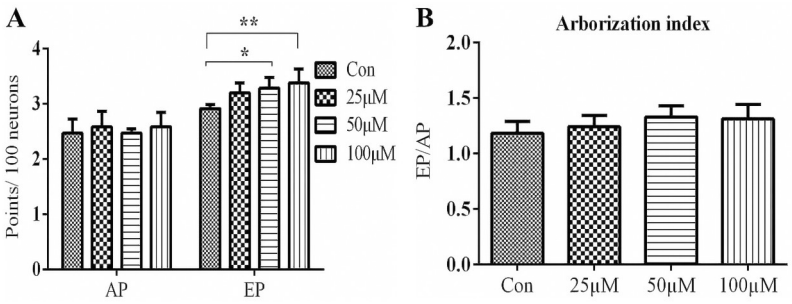


Fig 9. Effect of TRX on neurite arborisation after 7 days of differentiation. Neurite arborisation measurements were carried out for NSCs culture differentiated for 7 days with different concentrations of TRX. Around 100 neurons were included in the observation for each condition in each replicate. **A**: Represents the effects of TRX on neurite attachment point; AP and neurite ending points; EP. AP is an indicator of primary neurite number and EP is an indicator of neurite branching. **B**: Represents the neurite arborisation index which is described as the ratio of EP to that of AP. Data are presented as mean ± SEM. $^{*}p \leq 0.05$.

https://doi.org/10.1371/journal.pone.0237025.g009

factors (BDNF), fibroblast growth factor (FGF) [68] and epidermal growth factor (EGF) are very important [69]. In the absence of growth factors, such as EGF and FGF, NSCs failed to proliferate and died within a few days of culture. The literature reveals that various natural flavonoids facilitate the maintenance of the *in vitro* proliferation of NSCs independent of the

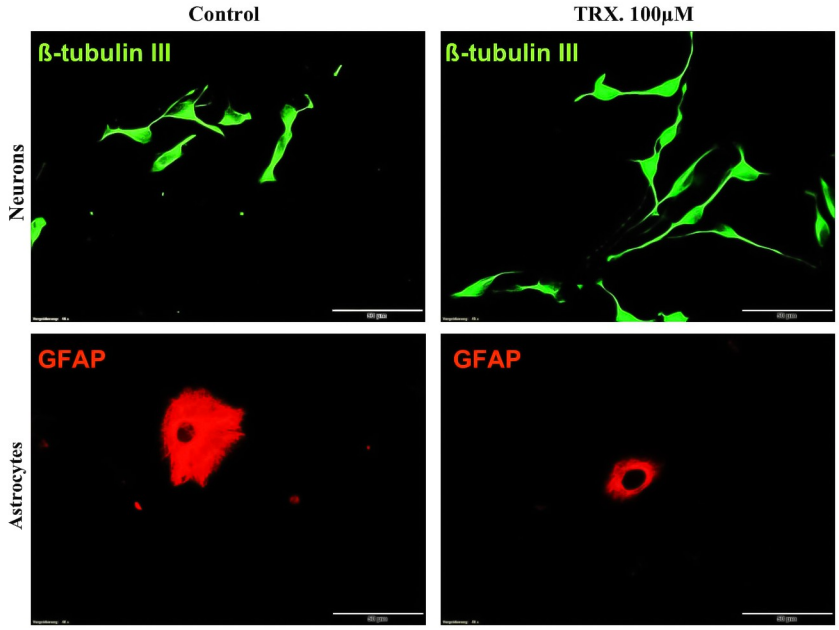


Fig 10. Effect of TRX on neurite growth of neurons and soma area of astrocyte after 48 h of incubation. NSCs differentiated on ECM coated glass coverslip for 48 h of incubation. Cells were immunostained for a neuronal marker (ß-tubulin III) and an astrocyte marker (GFAP). Green cells represent neurons. Neurons treated with TRX present elongated neurites with more branching points when compared to control. Red cells represent astrocyte cells stained for GFAP. Astrocytes presented both leaf-like and star-like morphology but leaf-like morphology was dominant over star shaped cells. Astrocyte cells treated with The Control exhibited larger soma area when compared to cell treated with 100 μM of TRX. Cells presented a smooth surface with no deformation. All measurements were performed exploiting Cell-SENE software. Pictures were captured with a 40 x objective with 50 μm scale bar.

https://doi.org/10.1371/journal.pone.0237025.g010

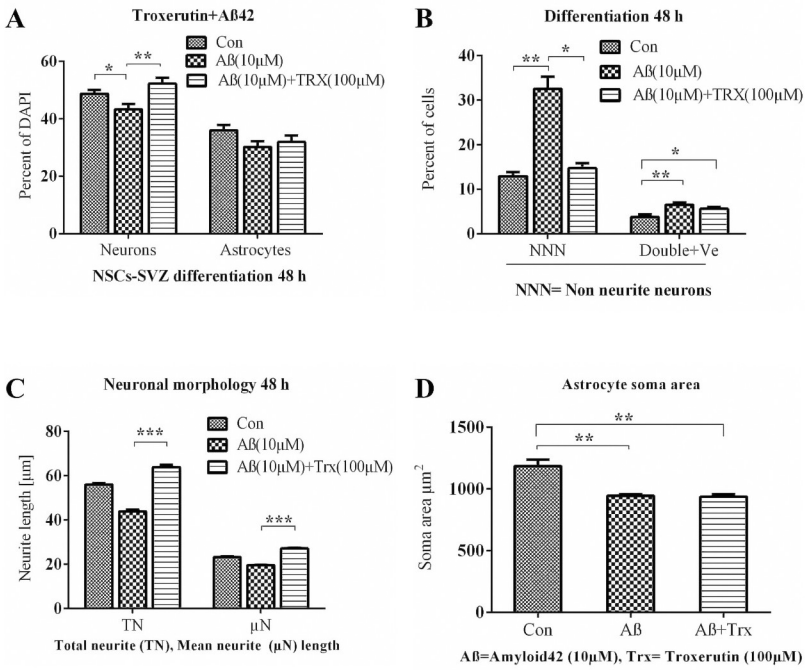


Fig 11. Neuroprotective effects of TRX flavonoid against Aß42 induced depression of differentiation after 48 h of differentiation on ECM coated glass coverslips. A: Represents the percentage of cells differentiated from NSCs. **B**: represents the percentage of non neurite neurons differentiated from total neurons and the percentage of cells double +ve for both neuronal marker ß-tubulin III and astrocyte marker (GFAP). **C** and **D** represent the effects on neurite outgrowth per neuron and astrocyte soma area, respectively. The data were conceived exploiting Cell SENE software. Percentage of differentiated neurons and astrocytes were calculated from the total DAPI nuclei count. At least 100 cells were included in observation for morphological analysis for each condition in each replicate. Data are presented as mean ± SEM. Experiments were performed as 5 replicates (n = 5).$^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

https://doi.org/10.1371/journal.pone.0237025.g011

growth factors. Epimedium flavonoids, for instance, promote the proliferation of cultured NSCs from postnatal rat hippocampus [15]. Icariin has been reported to promote the proliferation of NSCs by up-regulating cell cycle gene D1 and protein p21 [16]. Results of our clonogenic and BrdU/KI67 immunostaining assays clearly indicated that none of the tested TRX concentrations exhibited stimulatory or inhibitory effects on the proliferation of NSCs irrespective of the presence or absence of essential neurotrophic factors. Previous studies reported a stimulatory effect of TRX on cell proliferation other than NSCs. 10 µM concentration of TRX, for instance, alleviated UV induced arrest in the proliferation of cultured human keratinocytes cell lines (HaCaT) by upregulating miRNA -181a-5p [26]. Moreover, TRX in combination with cerebroprotein lysate enhanced the proliferation of human umbilical vein endothelial cells HUVECs [54].

Being multipotent in nature, NSCs give rise to functional cells of the nervous system on differentiation in response to environmental stimuli [15]. In this study NSCs were differentiated in the presence of different concentrations of TRX for 48 h and for a relatively long period of 7 days. Purpose of short term differentiation was to evaluate the effect of TRX on early neuronal differentiation and neurite outgrowth. One week differentiation of NSCs provided neurons with well-developed neurite and branching pattern. Higher concentrations of TRX stimulated the neurite outgrowth and decreased the percentage of non-neurite neurons when compared to the vehicle control during both incubation periods. Effect of TRX on neurite arborisation

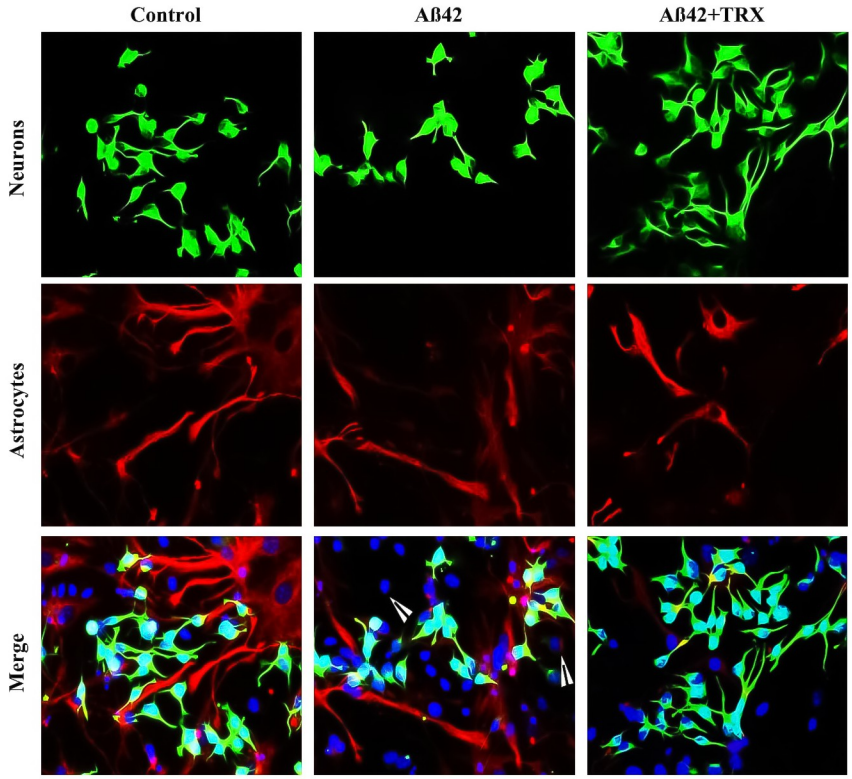


Fig 12. Neuroprotective effect of TRX against Aß42 induced inhibition in the amount of neurons and astrocytes after 48 h of differentiation. Green cells present ß-tubulin III neurons which were found denser in control and Aß42 +TRX. Red cells represent GFAP astrocytes which were observed in both leaf-like and star-like morphology. Astrocytes were relatively denser in Control as compared to other treatment conditions. Pictures were captured with a 40 x objective of a fluorescent microscope. Scale bar is 50 μm.

https://doi.org/10.1371/journal.pone.0237025.g012

index was non-significant at lower concentration but the higher concentrations of 50 μM and 100 μM significantly enhanced the number of neurite branching tips. Although there are no data available to represent the effect of TRX on the differentiation of NSCs, several studies describe the neurogenerative effects of flavonoids. Baicalin, for instance, enhanced the neuronal fate of cultured NSCs isolated from rat hippocampus [46] and also enhanced the neurite outgrowth by upregulating phosphorylation of Erk1/2 [19]. Baicalin also stimulated the neuronal differentiation and inhibited glial differentiation of rat embryonic NSCs by modulating the function of transcription factor stat3 and basic helix-loop-helix gene family [20]. Quercetin is another flavonoid which enhanced neurogenesis and synaptogenesis by stimulating brain-derived neurotrophic factors and phosphorylating cyclic AMP response binding protein (pCRBP) [22]. In another study quercetin enhanced neurite outgrowth and affected the percentage of neuronal cells by upregulating Gap-43 and cAMP in a cultured N1E115 cell line [44]. Prenylated flavonoid ENDF1 enhanced the axonal length and branching density in the cultured neurons from the dorsal ganglion by upregulating the expression of microtubule binding-protein gene DCX and maintaining the Ca^{2+} haemostasis in neurons [70]. Oral administration of flavonoids rich dried root extracts of Chinese herb *Scutellaria baicalensis* Georgi stimulated the axonal growth against experimentally induced spinal injury in a rat model by upregulating the expression of NF-H expression in neurons [71]. Isoquercitrin

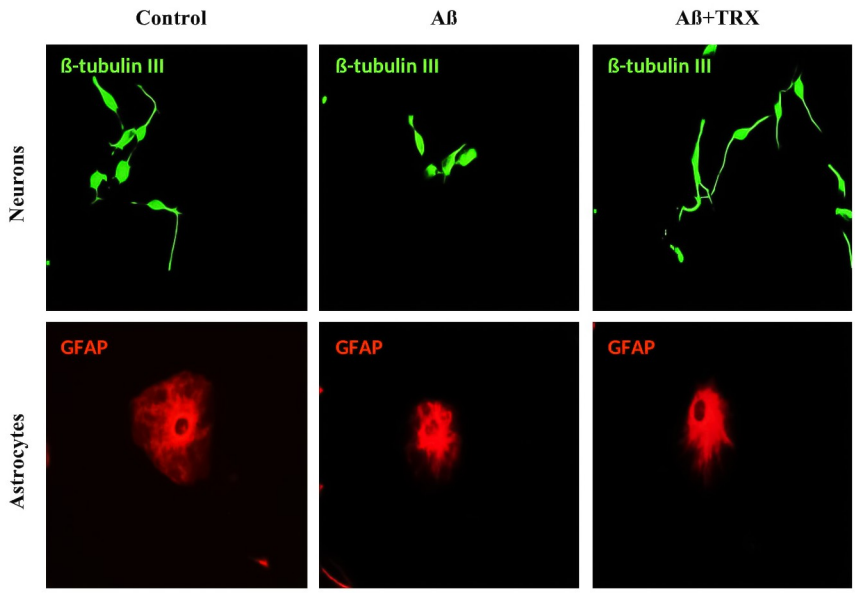


Fig 13. Neuroprotective effect of TRX against Aß42 induced neurite growth inhibition after 48 h incubation. Green cells represent ß-tubulin III neurons. Neurons treated with TRX+Aß42 exhibited elongated neurites with more branching when compared with control. Red cells represent GFAP astrocyte which under control condition exhibited larger soma area when compared with Aß and combination TRX+Aß42 treatment. Cells demonstrated a smooth surface with no deformation. All measurements were performed by employing Cell SENE software. Pictures were captured with a 40 x objective with 50 μm scale bar.

https://doi.org/10.1371/journal.pone.0237025.g013

flavonoids promoted the axonal elongation in of the cultured NG108-15 cells by reducing the activity of RhoA kinase [45]. A high concentration of TRX also decreased the percentage of double positive cells (immunoreactive to both neuronal and astrocyte markers) which was only observed during 48 h of incubation. The occurrence of cells co-expressing neuronal markers ß-tubulin III and glial markers GFAP in SVZ of the developing brain is supported by the previously reported literature. These cells are neural progenitors and on long term differentiation, give rise to either neurons or glial cells [72, 73]. In this study, 100μM TRX presented a tendency to reduce the percentage of ß-tubulin/GFAP double-positive cells and, at the same time, increased the percentage of neurons.

TRX in high concentration (100 μM) significantly reduced astrocyte soma area as compared to control and two other lower concentrations. The literature reveals that TRX exhibits a mitigating effect in Parkinson's 6-OHDA rat model not only *via* antioxidation activity but also through inhibition of astroglial GFAP expression partially by modulating the function of PI3K/ERβsignalling pathway [56]. In another study, baicalin inhibited astrocyte differentiation of rat embryonic NSCs by interacting with basic helix-loop-helix genes and transcription factor stat3 [20]. Reduced oxidative stress stimulates the neuronal differentiation of NSCs [74]. TRX exhibits antioxidant activities by interacting with reactive oxygen species [75]. Since TRX exerts neuroprotective and neurogenerative effects through its anti-oxidative actions in the brain tissues of rat [54], it was assumed that anti-oxidation activity might also be associated with enhanced neuronal differentiation and neurite growth. Detailed anti-oxidation and molecular studies are required to prove this assumption.

Aß42 decreased the percentage of neurons, decreased the neurite outgrowth and neurite length after 48 h of incubation. Literature reveals that Aß42 decreased the percentage of

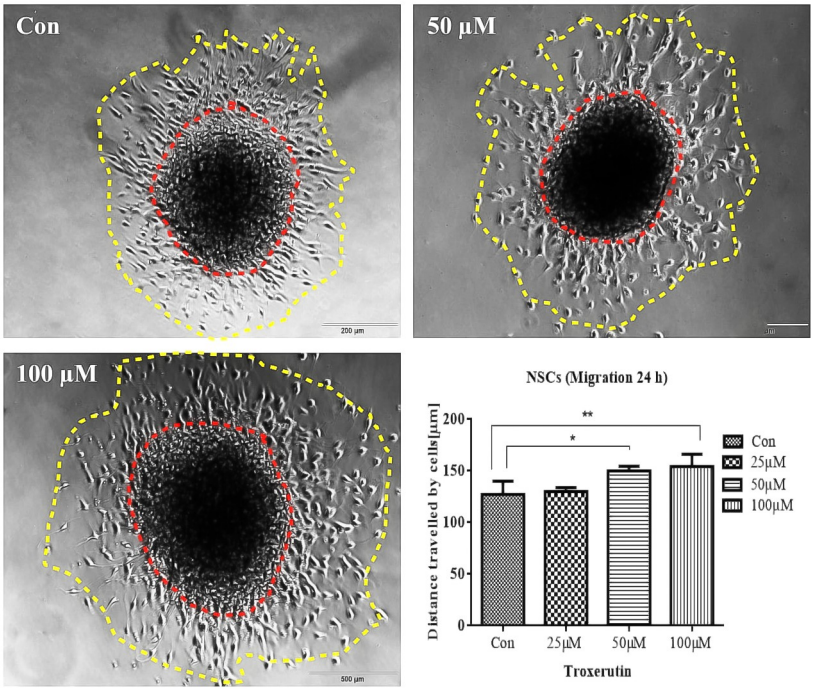


Fig 14. TRX enhances the migration of differentiated cells from NSCs cultured for 24 h. NSCs were proliferated to develop neurospheres which were adhered to PDL coated glass coverslips and incubated in differentiation mediumwith and without the presence of TRX for 24 h. The central dark area encircled by The red dotted ring is the neurospheres core consists of the heterogeneous population of cells. Results representmean distance travelled by cells from the core in all four directions (**Graph**). The migrated cells are enclosed in yellow dotted ring. For each condition, 10-15neurospheres were included in the observation. The study was performed in 5 replicates *(n = 5)*. Pictures were captured with 10 x objective of a phase-contrast microscope. Data are presented as the mean ± SEM.

https://doi.org/10.1371/journal.pone.0237025.g014

neurons differentiated from the cultured mouse NSCs isolated from the hippocampus [32]. Another *in vitro* study reported that Aß42, on short term incubation, significantly inhibited the axonal growth and synapsis formation in cultured cortical and hippocampus cells. These inhibitory effects of Aß42 are similar to those defined in transgenic mouse and Alzheimer pathology [76]. Moreover, short term exposure to Aß42 inhbits neurite growth of cultured PC12 cells due to an oxidative stress and mitochondrial dysfunction as described by researchers [52]. TRX, in high concentration (*i.e.*100 μM), neutralized the inhibitory effects of Aß42 (10 μM) on neuronal differentiation, neurite outgrowth, neurite extension after 48 h incubation. TRX protects hippocampus neurons from the neurotoxic effects of Aß42 by ameliorating antioxidant enzymes and attenuating elevated acetylcholinesterase enzyme levels. Additionally TRX also reduced the apoptosis on chronic treatment of 14 days [33]. Apigenin is an aglycone flavone which improves the memory defect induced by Aß25-35 in mouse by several mechanisms including antioxidant actions, reduction of acetylcholinesterase activity and modulationof phosphor-CREB, BNDF and TrkB [77]. Quercetin has been reported to exhibita protective effect against Aß42 induced lipid peroxidation in cultured hippocampus cell culture from postnatal rat [78].

Countertoxicity effects of a flavonoid against Aß 42 depend upon the ability of a flavonoid to prevent the fibrillization of the later [49]. Two major structural requirements for the anti-fibrilization effect of a flavonoid molecule involve the number of aromatic rings and the

number of hydroxyl groups present in the molecule. Aromatic rings bind with the hydrophobic amino acid residues through covalent bonds and hydroxyl groups of a flavonoid interact with hydrophilic amino acid residues of the peptide backbone of Aß 42 leading to the disaggregation effects or prevention of fibrillization. The number of these functional groups are, therefore, directly proportional to the antifibrils activity against Aß 42. Gallocatechin gallate and theaflavin exhibited 100% efficacy in preventing fibrils formation of Aß 42 in an extracellular chemical reaction since these molecules provide a higher number of aromatic rings and hydroxyl groups in their structures as compared to other compounds tested (Phan 2019). A number of these aromatic rings and reactive hydroxyl groups in TRX molecule [79] is comparable to that of theaflavin [80], rutin [81] and more than that of gallocatechin gallate [82] so it was assumed that the neutralizing activity of TRX against Aß 42 induced inhibition in neuronal differentiation and neurite growth in our experiments might be due to the disaggregating or antifibrillization effects of TRX. Future detailed studies are needed to prove if TRX prevents fibrillization of Aß42 in a reaction mixture.

The findings of this study indicated that TRX can stimulate cell migration in NSCs culture in the absence of any stress. Ma et al., reported in their study that TRX in combination with cerebroprotein hydrolysate, induced cell migration of human umbilical vein endothelial cells [54]. In another study, TRX induced HaCa cells migration by ameliorating UV induced migration restriction [26]. Stimulatory effects of TRX on the migration of cell lines are mediated through modulation of regulatory gene miR-181a-5p and transcription factor integrin ß3 mRNA [26, 54]. Quercetin has been reported to induce murine NSCs migration under differentiation conditions with concomitant up-regulation of CXCR4 gene in an *in vitro* experiment [23]. CXCR4 is a receptor protein for chemokine SDF1. CXCR4/SDF1 signalling pathway which is implicated in the development of various tissues including the nervous system. One of the major roles of SDF1/CXCR4 involves the regulation of neuronal cell migration of various kinds in cortex and cerebellum areas of the brain. Knock out of either CXCR4 or SDF1 genes in mouse resulted in severe defects in granular cell migration [83].

Conclusions

The present study revealed that TRX not only promoted the neuronal differentiation of cultured NSCs of the postnatal mouse but also stimulated neurite outgrowth and neurite extension as well as cell migration in the absence of any inhibitory stimuli. Moreover, TRX also neutralized the inhibitory effects of Aß42 oligomer on neuronal differentiation and neurite outgrowth. These findings provide clues about the role of TRX in neurogenesis and curing Aß42 dependent neurological disorders. TRX, in stark contrast to unmodified rutin and other flavonoids, is easily water soluble, which makes this unique molecule a suitable candidate for oral applications. Indeed, detailed investigations are required to explore molecular pathways governing stimulatory effects of TRX in neurite growth, cell migration and neuroprotective actions against neurotoxic peptides implicated in neurodegenerative disorders.

Supporting information

S1 File.
(DOCX)

Acknowledgments

We are grateful to Anne Braun, Anna Baumann and Steven Schulte for technical support in laboratory-based work and Dr. Abdul Baseer for administrative work.

Author Contributions

Conceptualization: Muhammad Irfan Masood, Karl Herbert Schäfer.

Data curation: Muhammad Irfan Masood, Mahrukh Naseem, Maximilian Weyland, Peter Meiser.

Formal analysis: Karl Herbert Schäfer.

Funding acquisition: Karl Herbert Schäfer.

Investigation: Muhammad Irfan Masood, Karl Herbert Schäfer.

Methodology: Muhammad Irfan Masood.

Project administration: Karl Herbert Schäfer.

Resources: Karl Herbert Schäfer, Peter Meiser.

Software: Muhammad Irfan Masood, Mahrukh Naseem, Maximilian Weyland.

Supervision: Karl Herbert Schäfer.

Visualization: Karl Herbert Schäfer.

Writing – original draft: Muhammad Irfan Masood, Mahrukh Naseem, Maximilian Weyland, Peter Meiser.

Writing – review & editing: Muhammad Irfan Masood, Mahrukh Naseem, Maximilian Weyland, Peter Meiser.

References

1. Bergström T, Forsberg-Nilsson K. Neural stem cells: brain building blocks and beyond. Ups J Med Sci. 2012; 117(2):132–42. https://doi.org/10.3109/03009734.2012.665096 PMID: 22512245.
2. Shoemaker LD, Kornblum HI. Neural stem cells (NSCs) and proteomics. Mol Cell Proteomics. 2016; 15 (2):344–54. https://doi.org/10.1074/mcp.O115.052704 PMID: 26494823
3. Coullery RP, Ferrari ME, Rosso SB. Neuronal development and axon growth are altered by glyphosate through a WNT non-canonical signaling pathway. Neuro Toxicol. 2016; 52:150–61. https://doi.org/10.1016/j.neuro.2015.12.004 PMID: 26688330.
4. Okano H. Neural stem cells and strategies for the regeneration of the central nervous system. Proc Jpn Acad Ser B Physiol Sci. 2010; 86(4):438–50. https://doi.org/10.2183/pjab.86.438 PMID: 20431266
5. Gage FH. Mammalian neural stem cells. Science. 2000; 287(5457):1433–8. https://doi.org/10.1126/science.287.5457.1433 PMID: 10688783.
6. Guo W, Patzlaff NE, Jobe EM, Zhao X. Isolation of multipotent neural stem or progenitor cells from both the dentate gyrus and subventricular zone of a single adult mouse. Nat Protoc. 2012; 7(11):2005–12. https://doi.org/10.1038/nprot.2012.123 PMID: 23080272
7. Urbán N, Guillemot F. Neurogenesis in the embryonic and adult brain: same regulators, different roles. Front Cell Neurosci. 2014; 8. https://doi.org/10.3389/fncel.2014.00396 PMID: 25505873
8. Bixby S, Kruger GM, Mosher JT, Joseph NM, Morrison SJ. Cell-intrinsic differences between stem cells from different regions of the peripheral nervous system regulate the generation of neural diversity. Neuron. 2002; 35(4):643–56. https://doi.org/10.1016/s0896-6273(02)00825-5 PMID: 12194865.
9. Kim H-T, Kim I-S, Lee I-S, Lee J-P, Snyder EY, Park KI. Human neurospheres derived from the fetal central nervous system are regionally and temporally specified but are not committed. Exp Neurol. 2006; 199(1):222–35. https://doi.org/10.1016/j.expneurol.2006.03.015 PMID: 16714017.
10. Wang C. Application of in vitro models in developmental neurotoxicity and pharmaceutics research. J Mol Pharm Org Process Res. 2015; 3:122–8. https://doi.org/10.4172/2329-9053.1000e122
11. Saha B, Ypsilanti AR, Boutin C, Cremer H, Chédotal A. Plexin-B2 regulates the proliferation and migration of neuroblasts in the postnatal and adult subventricular zone. J Neurosci. 2012; 32(47):16892–905. https://doi.org/10.1523/JNEUROSCI.0344-12.2012 PMID: 23175841
12. Inta D, Alfonso J, Von Engelhardt J, Kreuzberg MM, Meyer AH, Van Hooft JA, et al. Neurogenesis and widespread forebrain migration of distinct GABAergic neurons from the postnatal subventricular zone.

Proc Natl Acad Sci USA. 2008; 105(52):20994–9. https://doi.org/10.1073/pnas.0807059105 PMID: 19095802

13. Zhang B, Yan W, Zhu Y, Yang W, Le W, Chen B, et al. Nanomaterials in Neural-Stem-Cell-Mediated Regenerative Medicine: Imaging and Treatment of Neurological Diseases. Advanced Materials. 2018; 30(17):1705694. https://doi.org/10.1002/adma.201705694 PMID: 29543350.
14. Kong S-Y, Park M-H, Lee M, Kim J-O, Lee H-R, Han BW, et al. Kuwanon V inhibits proliferation, promotes cell survival and increases neurogenesis of neural stem cells. PLoS One. 2015; 10(2):e0118188. https://doi.org/10.1371/journal.pone.0118188 PMID: 25706719
15. Yao R, Zhang L, Li X, Li L. Effects of Epimedium flavonoids on proliferation and differentiation of neural stem cells in vitro. Neurol Res. 2010; 32(7):736–42. https://doi.org/10.1179/174313209X459183 PMID: 19703337.
16. Fu X, Li S, Zhou S, Wu Q, Jin F, Shi J. Stimulatory effect of icariin on the proliferation of neural stem cells from rat hippocampus. BMC Complement Altern Med. 2018; 18(1):34. https://doi.org/10.1186/s12906-018-2095-y PMID: 29378551
17. Yang P, Guan YQ, Li YL, Zhang L, Zhang L, Li L. Icariin promotes cell proliferation and regulates gene expression in human neural stem cells in vitro. Mol Med Report. 2016; 14(2):1316–22. https://doi.org/10.3892/mmr.2016.5377 PMID: 27278906.
18. He Q, Jia Z, Zhang Y, Ren X. Morin hydrate promotes inner ear neural stem cell survival and differentiation and protects cochlea against neuronal hearing loss. J Cell Mol Med. 2017; 21(3):600–8. https://doi.org/10.1111/jcmm.13005 PMID: 27709784
19. Li M, Tsang KS, Choi ST, Li K, Shaw PC, Lau KF. Neuronal differentiation of C17. 2 neural stem cells induced by a natural flavonoid, baicalin. Chembiochem. 2011; 12(3):449–56. https://doi.org/10.1002/cbic.201000570 PMID: 21290546.
20. Li Y, Zhuang P, Shen B, Zhang Y, Shen J. Baicalin promotes neuronal differentiation of neural stem/progenitor cells through modulating p-stat3 and bHLH family protein expression. Brain Res. 2012; 1429:36–42. https://doi.org/10.1016/j.brainres.2011.10.030 PMID: 22088824.
21. Taupin P. Apigenin and related compounds stimulate adult neurogenesis: Mars, Inc., the Salk Institute for Biological Studies: WO2008147483. Expert Opin Ther Pat. 2009; 19(4):523–7. https://doi.org/10.1517/13543770902721279 PMID: 19441930.
22. Tchantchou F, Lacor PN, Cao Z, Lao L, Hou Y, Cui C, et al. Stimulation of neurogenesis and synaptogenesis by bilobalide and quercetin via common final pathway in hippocampal neurons. J Alzheimers Dis. 2009; 18(4):787–98. https://doi.org/10.3233/JAD-2009-1189 PMID: 19661619.
23. Baral S, Pariyar R, Kim J, Lee H-S, Seo J. Quercetin-3-O-glucuronide promotes the proliferation and migration of neural stem cells. Neurobiol Aging. 2017; 52:39–52. https://doi.org/10.1016/j.neurobiolaging.2016.12.024 PMID: 28110104.
24. Azarfarin M, Farajdokht F, Babri S, Salehpour F, Taghizadeh M, Mohaddes G. Effects of troxerutin on anxiety-and depressive-like behaviors induced by chronic mild stress in adult male rats. Iran J Basic Med Sci. 2018; 21(8):781. https://doi.org/10.22038/IJBMS.2018.26915.6582 PMID: 30186563
25. Babri S, Amani M, Mohaddes G, Alihemmati A, Ebrahimi H. Protective effects of troxerutin on β-amyloid (1–42)-induced impairments of spatial learning and memory in rats. Neurophysiology. 2012; 44(5):387–93. https://doi.org/10.1007/s11062-012-9309-6
26. Lee KS, Cha HJ, Lee GT, Lee KK, Hong JT, Ahn KJ, et al. Troxerutin induces protective effects against ultraviolet B radiation through the alteration of microRNA expression in human HaCaT keratinocyte cells. Int J Mol Med. 2014; 33(4):934–42. https://doi.org/10.3892/ijmm.2014.1641 PMID: 24503859.
27. Lu J, Wu D-m, Zheng Y-l, Hu B, Cheng W, Zhang Z-f, et al. Troxerutin counteracts domoic acid–induced memory deficits in mice by inhibiting CCAAT/enhancer binding protein β–mediated inflammatory response and oxidative stress. The Journal of Immunology. 2013; 190(7):3466–79. https://doi.org/10.4049/jimmunol.1202862 PMID: 23420885.
28. Lu J, Wu D-m, Zheng Z-h, Zheng Y-l, Hu B, Zhang Z-f. Troxerutin protects against high cholesterol-induced cognitive deficits in mice. Brain. 2011; 134(3):783–97. https://doi.org/10.1093/brain/awq376 PMID: 21252113.
29. Selkoe DJ. Alzheimer's disease: genes, proteins, and therapy. Physiol Rev. 2001; 81(2):741–66. https://doi.org/10.1152/physrev.2001.81.2.741 PMID: 11274343.
30. Thapa A, Woo E-R, Chi EY, Sharoar MG, Jin H-G, Shin SY, et al. Biflavonoids are superior to monoflavonoids in inhibiting amyloid-β toxicity and fibrillogenesis via accumulation of nontoxic oligomer-like structures. Biochemistry. 2011; 50(13):2445–55. https://doi.org/10.1021/bi101731d PMID: 21322641.
31. Cutler RG, Kelly J, Storie K, Pedersen WA, Tammara A, Hatanpaa K, et al. Involvement of oxidative stress-induced abnormalities in ceramide and cholesterol metabolism in brain aging and Alzheimer's

disease. Proc Natl Acad Sci USA. 2004; 101(7):2070–5. https://doi.org/10.1073/pnas.0305799101 PMID: 14970312

32. He N, Jin W, Lok K, Wang Y, Yin M, Wang Z. Amyloid-β 1–42 oligomer accelerates senescence in adult hippocampal neural stem/progenitor cells via formylpeptide receptor 2. Cell Death Dis. 2013; 4(11): e924. https://doi.org/10.1038/cddis.2013.437 PMID: 24263098

33. Farajdokht F, Amani M, Bavil FM, Alihemmati A, Mohaddes G, Babri S. Troxerutin protects hippocampal neurons against amyloid beta-induced oxidative stress and apoptosis. EXCLI journal. 2017; 16:1081. https://doi.org/10.17179/excli2017-526 PMID: 29285004

34. Bender M, Schwind L, Grundmann D, Martin M, Klotz M, Götz C, et al. Impact of protein kinase CK2 inhibitors on proliferation and differentiation of neural stem cells. Heliyon. 2017; 3(6):e00318. https://doi.org/10.1016/j.heliyon.2017.e00318 PMID: 28649667

35. Xiong F, Gao H, Zhen Y, Chen X, Lin W, Shen J, et al. Optimal time for passaging neurospheres based on primary neural stem cell cultures. Cytotechnology. 2011; 63(6):621–31. https://doi.org/10.1007/s10616-011-9379-0 PMID: 21858692

36. Tanaka R, Tainaka M, Ota T, Mizuguchi N, Kato H, Urabe S, et al. Accurate determination of S-phase fraction in proliferative cells by dual fluorescence and peroxidase immunohistochemistry with 5-bromo-2′-deoxyuridine (BrdU) and Ki67 antibodies. J Histochem Cytochem. 2011; 59(8):791–8. https://doi.org/10.1369/0022155411411090 PMID: 21551319

37. Kee N, Sivalingam S, Boonstra R, Wojtowicz J. The utility of Ki-67 and BrdU as proliferative markers of adult neurogenesis. J Neurosci Methods. 2002; 115(1):97–105. https://doi.org/10.1016/s0165-0270(02)00007-9 PMID: 11897369.

38. Bernas S, Leiter O, Walker T, Kempermann G. Isolation, culture and differentiation of adult hippocampal precursor cells. Bio Protoc. 2017; 7(21):e2603. https://doi.org/10.21769/BioProtoc.2603

39. Ostenfeld T, Svendsen CN. Requirement for Neurogenesis to Proceed through the Division of Neuronal Progenitors following Differentiation of Epidermal Growth Factor and Fibroblast Growth Factor-2–Responsive Human Neural Stem Cells. Stem Cells. 2004; 22(5):798–811. https://doi.org/10.1634/stemcells.22-5-798 PMID: 15342944.

40. Baumann J, Barenys M, Gassmann K, Fritsche E. Comparative human and rat "neurosphere assay" for developmental neurotoxicity testing. Curr Protoc Toxicol. 2014; 59(1):12.21. 1–12.21. 4. https://doi.org/10.1002/0471140856.tx1221s59 PMID: 24898107.

41. Messam CA, Hou J, Major EO. Coexpression of nestin in neural and glial cells in the developing human CNS defined by a human-specific anti-nestin antibody. Exp Neurol. 2000; 161(2):585–96. https://doi.org/10.1006/exnr.1999.7319 PMID: 10686078.

42. Mahabadi VP, Movahedin M, Semnanian S, Mirnajafi-zadeh J, Faizi M. In vitro differentiation of neural stem cells into noradrenergic-like cells. Int J Mol Cell Med. 2015; 4(1):22–31. PMID: 25815279;

43. Frandsen J, Narayanasamy P. Flavonoid enhances the glyoxalase pathway in cerebellar neurons to retain cellular functions. Sci Rep. 2017; 7(1):5126. https://doi.org/10.1038/s41598-017-05287-z PMID: 28698611

44. Ming-Ming C, Zhi-Qi Y, ZHANG L-Y, Hong L. Quercetin promotes neurite growth through enhancing intracellular cAMP level and GAP-43 expression. Chinese journal of natural medicines. 2015; 13(9):667–72. https://doi.org/10.1016/S1875-5364(15)30064-9 PMID: 26412426.

45. Palazzolo G, Horvath P, Zenobi-Wong M. The flavonoid isoquercitrin promotes neurite elongation by reducing RhoA activity. PLoS One. 2012; 7(11):e49979. https://doi.org/10.1371/journal.pone.0049979 PMID: 23209630

46. Zhuang PW, Cui GZ, Zhang YJ, Zhang MX, Guo H, Zhang JB, et al. Baicalin regulates neuronal fate decision in neural stem/progenitor cells and stimulates hippocampal neurogenesis in adult rats. CNS Neurosci Ther. 2013; 19(3):154–62. https://doi.org/10.1111/cns.12050 PMID: 23302221

47. Ho S-Y, Chao C-Y, Huang H-L, Chiu T-W, Charoenkwan P, Hwang E. NeurphologyJ: an automatic neuronal morphology quantification method and its application in pharmacological discovery. BMC Bioinformatics. 2011; 12(1):230. https://doi.org/10.1186/1471-2105-12-230 PMID: 21651810

48. Irwin K, Sexton C, Daniel T, Lawlor B, Naci L. Healthy Aging and Dementia: Two Roads Diverging in Midlife? Front Aging Neurosci. 2018; 10. https://doi.org/10.3389/fnagi.2018.00275 PMID: 30283329

49. Zuo L, Hemmelgarn BT, Chuang C-C, Best TM. The role of oxidative stress-induced epigenetic alterations in amyloid-β production in Alzheimer's disease. Oxid Med Cell Longev. 2015; 2015. https://doi.org/10.1155/2015/604658 PMID: 26543520

50. Sato M, Murakami K, Uno M, Nakagawa Y, Katayama S, Akagi K-i, et al. Site-specific inhibitory mechanism for amyloid β42 aggregation by catechol-type flavonoids targeting the Lys residues. J Biol Chem. 2013; 288(32):23212–24. https://doi.org/10.1074/jbc.M113.464222 PMID: 23792961

51. Phan HT, Samarat K, Takamura Y, Azo-Oussou AF, Nakazono Y, Vestergaard MdC. Polyphenols Modulate Alzheimer's Amyloid Beta Aggregation in a Structure-Dependent Manner. Nutrients. 2019; 11 (4):756. https://doi.org/10.3390/nu11040756 PMID: 30935135

52. Kurz C, Ungerer I, Lipka U, Kirr S, Schütt T, Eckert A, et al. The metabolic enhancer piracetam ameliorates the impairment of mitochondrial function and neurite outgrowth induced by ß-amyloid peptide. Br J Pharmacol. 2010; 160(2):246–57. https://doi.org/10.1111/j.1476-5381.2010.00656.x PMID: 20218980

53. Brothers HM, Gosztyla ML, Robinson SR. The physiological roles of amyloid-β peptide hint at new ways to treat Alzheimer's disease. Front Aging Neurosci. 2018; 10:118. https://doi.org/10.3389/fnagi.2018.00118 PMID: 29922148

54. Ma W, Wang S, Liu X, Tang F, Zhao P, Cheng K, et al. Protective effect of troxerutin and cerebroprotein hydrolysate injection on cerebral ischemia through inhibition of oxidative stress and promotion of angiogenesis in rats. Mol Med Report. 2019; 19(4):3148–58. https://doi.org/10.3892/mmr.2019.9960 PMID: 30816516

55. Xiong L-L, Chen Z-W, Wang T-H. Nerve growth factor promotes in vitro proliferation of neural stem cells from tree shrews. Neural Regen Res. 2016; 11(4):591. https://doi.org/10.4103/1673-5374.180743 PMID: 27212919

56. Baluchnejadmojarad T, Jamali-Raeufy N, Zabihnejad S, Rabiee N, Roghani M. Troxerutin exerts neuroprotection in 6-hydroxydopamine lesion rat model of Parkinson's disease: Possible involvement of PI3K/ERβ signaling. Eur J Pharmacol. 2017; 801:72–8. https://doi.org/10.1016/j.ejphar.2017.03.002 PMID: 28284752.

57. Bazan E, Alonso F, Redondo C, Lopez-Toledano M, Alfaro JM, Reimers D, et al. In vitro and in vivo characterization of neural stem cells. Histol Histopathol. 2004. https://doi.org/10.14670/HH-19.1261 PMID: 15375770

58. Zhang J, Jiao J. Molecular biomarkers for embryonic and adult neural stem cell and neurogenesis. Biomed Res Int. 2015; 2015. https://doi.org/10.1155/2015/727542 PMID: 26421301

59. Völker J, Engert J, Völker C, Bieniussa L, Schendzielorz P, Hagen R, et al. Isolation and characterization of neural stem cells from the rat inferior colliculus. Stem cells international. 2019; 2019. https://doi.org/10.1155/2019/5831240 PMID: 31781242

60. Zalfa C, Grasselli C, Santilli G, Ferrari D, Lamorte G. GFAP Delta as Divergent Marker of human glial progenitors. Stem Cell Res Ther. 2018; 8(437):2. https://doi.org/10.4172/2157-7633.1000437

61. Berg DA, Bond AM, Ming G-l, Song H. Radial glial cells in the adult dentate gyrus: what are they and where do they come from?. F1000 Research. 2018; 7. https://doi.org/10.12688/f1000research.12684.1 PMID: 29568500

62. Kriegstein A, Alvarez-Buylla A. The glial nature of embryonic and adult neural stem cells. Annu Rev Neurosci. 2009; 32:149–84. https://doi.org/10.1146/annurev.neuro.051508.135600 PMID: 19555289.

63. Shimada IS, LeComte MD, Granger JC, Quinlan NJ, Spees JL. Self-renewal and differentiation of reactive astrocyte-derived neural stem/progenitor cells isolated from the cortical peri-infarct area after stroke. J Neurosci. 2012; 32(23):7926–40. https://doi.org/10.1523/JNEUROSCI.4303-11.2012 PMID: 22674268

64. Obernier K, Alvarez-Buylla A. Neural stem cells: origin, heterogeneity and regulation in the adult mammalian brain. Development. 2019; 146(4):dev156059. https://doi.org/10.1242/dev.156059 PMID: 30777863

65. Merkle FT, Tramontin AD, García-Verdugo JM, Alvarez-Buylla A. Radial glia give rise to adult neural stem cells in the subventricular zone. Proc Natl Acad Sci USA. 2004; 101(50):17528–32. https://doi.org/10.1073/pnas.0407893101 PMID: 15574494

66. Duan CL, Liu CW, Shen SW, Yu Z, Mo JL, Chen XH, et al. Striatal astrocytes transdifferentiate into functional mature neurons following ischemic brain injury. Glia. 2015; 63(9):1660–70. https://doi.org/10.1002/glia.22837 PMID: 26031629.

67. Malatesta P, Appolloni I, Calzolari F. Radial glia and neural stem cells. Cell Tissue Res. 2008; 331 (1):165–78. https://doi.org/10.1007/s00441-007-0481-8 PMID: 17846796.

68. Huang L, Wang G. The effects of different factors on the behavior of neural stem cells. Stem Cells Int. 2017; 2017. https://doi.org/10.1155/2017/9497325 PMID: 29358957;.

69. Arsenijevic Y, Weiss S, Schneider B, Aebischer P. Insulin-like growth factor-I is necessary for neural stem cell proliferation and demonstrates distinct actions of epidermal growth factor and fibroblast growth factor-2. J Neurosci. 2001; 21(18):7194–202. https://doi.org/10.1523/JNEUROSCI.21-18-07194.2001 PMID: 11549730

70. Bieler L, Vogl M, Kirchinger M, Urmann C, Riepl H, Bandtlow CE, et al. The prenylflavonoid ENDF1 overrules central nervous system growth inhibitors and facilitates regeneration of DRG neurons. Front Cell Neurosci. 2019; 13:332. https://doi.org/10.3389/fncel.2019.00332 PMID: 31396054

71. Zhang Q, Zhang L-X, An J, Yan L, Liu C-C, Zhao J-J, et al. Huangqin flavonoid extraction for spinal cord injury in a rat model. Neural Regen Res. 2018; 13(12):2200. https://doi.org/10.4103/1673-5374.241472 PMID: 30323153

72. Dráberová E, Del Valle L, Gordon J, Marková V, Šmejkalová B, Bertrand L, et al. Class III β-tubulin is constitutively coexpressed with glial fibrillary acidic protein and nestin in midgestational human fetal astrocytes: implications for phenotypic identity. J Neuropathol Exp Neurol. 2008; 67(4):341–54. https://doi.org/10.1097/NEN.0b013e31816a686d PMID: 18379434.

73. Rieske P, Azizi SA, Augelli B, Gaughan J, Krynska B. A population of human brain parenchymal cells express markers of glial, neuronal and early neural cells and differentiate into cells of neuronal and glial lineages. Eur J Neurosci. 2007; 25(1):31–7. https://doi.org/10.1111/j.1460-9568.2006.05254.x PMID: 17241264.

74. Hegewald C, Alt R, Hetz S, Cross M, Acikgoez A, Till H, et al. Reduced oxygen stress promotes propagation of murine postnatal enteric neural progenitors in vitro. Neurogastroenterol Motil. 2011; 23(10): e412–e24. https://doi.org/10.1111/j.1365-2982.2011.01761.x PMID: 21815967

75. Shan Q, Zhuang J, Zheng G, Zhang Z, Zhang Y, Lu J, et al. Troxerutin reduces kidney damage against BDE-47-induced apoptosis via inhibiting NOX2 activity and increasing Nrf2 activity. Oxid Med Cell Longev. 2017; 2017. doi.org/10.1155/2017/6034692.

76. Evans NA, Facci L, Owen DE, Soden PE, Burbidge SA, Prinjha RK, et al. Aβ1–42 reduces synapse number and inhibits neurite outgrowth in primary cortical and hippocampal neurons: A quantitative analysis. J Neurosci Methods. 2008; 175(1):96–103. https://doi.org/10.1016/j.jneumeth.2008.08.001 PMID: 18761375.

77. Liu R, Zhang T, Yang H, Lan X, Ying J, Du G. The flavonoid apigenin protects brain neurovascular coupling against amyloid-β 25-35-induced toxicity in mice. J Alzheimers Dis. 2011; 24(1):85–100. https://doi.org/10.3233/JAD-2010-101593 PMID: 21297270.

78. Ansari MA, Abdul HM, Joshi G, Opii WO, Butterfield DA. Protective effect of quercetin in primary neurons against Aβ (1–42): relevance to Alzheimer's disease. J Nutr Biochem. 2009; 20(4):269–75. https://doi.org/10.1016/j.jnutbio.2008.03.002 PMID: 18602817

79. Xin X, Zhang M, Li X, Lai F, Zhao G. Biocatalytic synthesis of acylated derivatives of troxerutin: their bioavailability and antioxidant properties in vitro. Microb Cell Fact. 2018; 17(1):130. https://doi.org/10.1186/s12934-018-0976-x PMID: 30134913

80. Leung LK, Su Y, Chen R, Zhang Z, Huang Y, Chen Z-Y. Theaflavins in black tea and catechins in green tea are equally effective antioxidants. The Journal of nutrition. 2001; 131(9):2248–51. https://doi.org/10.1093/jn/131.9.2248 PMID: 11533262

81. Enogieru AB, Haylett W, Hiss DC, Bardien S, Ekpo OE. Rutin as a potent antioxidant: implications for neurodegenerative disorders. Oxid Med Cell Longev. 2018; 2018. https://doi.org/10.1155/2018/6241017 PMID: 30050657

82. Wilkins C, De Bruijn J, Korver O, Frost D, Weinges K. Isolation and identification of (—)-gallocatechin gallate and circular dichroism of green tea flavanols. J Sci Food Agric. 1971; 22(9):480–4. https://doi.org/10.1002/jsfa.2740220913

83. Mithal DS, Banisadr G, Miller RJ. CXCL12 signaling in the development of the nervous system. J Neuroimmune Pharmacol. 2012; 7(4):820–34. https://doi.org/10.1007/s11481-011-9336-x PMID: 22270883

4. Discussion

The *in vitro* assay based upon NSCs offers a versatile, sensitive, robust, cost effective and physiologically relevant screening platform for diverse materials having neuromodulating potential including environmental toxicants and compounds of pharmacological significance [3,141, 153]. The NSCs from the developing SVZ provide several advantages over their adult equivalents such as, easy availability, isolation, culture, rapid growth rate and very high sensitivity to chemical stimuli [148]. Within the scope of the present thesis, an *in vitro* assay system based upon the NSCs from the SVZ of postnatal mouse has been established to explore the inhibitory effects of neurotoxicants occur in water samples (raw wastewater: RWW, treated sewage water: TSW, groundwater: GW and drinking water: DW) and very low concentrations of glyphosate pesticide molecule.

The same assay has also found its utility to demonstrate the neurogenerative and neuroprotective properties of pharmacologically active flavonoid TRX. The NSCs based *in vitro* assay has been able to confirm the presumption regarding potential inhibitory effects of both treated and untreated water samples on the basic neurogenic processes. The findings of the present study have confirmed the high sensitivity of NSCs model to reveal the inhibitory impacts of extremely low concentrations of glyphosate on neural cell differentiation and migration. The model has been successfully employed to reveal the genes involved in regulating neural cell lineages and cytoprotective responses. In addition, the model has also been able to demonstrate the neurogenerative and neuroprotective properties of TRX flavonoid. The major findings of these investigations will now be discussed in detail.

4.1. Impact of treated and untreated polluted water on the fate of NSCs.

The natural water reservoirs available for human consumption are being depleted worldwide majorly due to the rapid growth of human population and gradual decrease in precipitation. The water management authorities of several countries including European ones, are now considering the recycling of high-quality water from wastewater to make it suitable for human consumption [52].

Unfortunately, WWTPs operating in various countries have been found inefficient in completely removing or reducing water-borne pollutants to safe levels [160]. Water produced by WWTPs along with water clarification facilities, must be subjected to the series of analyses before treated water is available for human consumption.[56]. These analyses should include both the physicochemical characterization and biological testing of water [54, 55]. *In vitro* bioassays currently available for evaluating the quality of water are based upon cells derived from mammals and cancer cell lines. Since water samples contain a heterogeneous mixture of pollutants with diverse mechanisms of toxicity, it is therefore recommended to employ multiple biological assays by utilizing multiple cell types derived from different physiological systems of an organism [56]. Several researchers have evaluated the quality of water samples collected from different sources by employing non-neural cells derived from animals. Such assays could only provide information about the general and genotoxicity of water-borne pollutants [55, 56, 161-163]. The data concerning neurotoxicity of the water-borne pollutants is still lacking, perhaps due to the lack of a suitable model. Primary NSCs are the building block of the nervous system respond quickly to the exogenous stimuli in highly sensitive manner. Therefore, these are considered as a useful bio-tool to reveal neurotoxicity associated with pollutants present in water samples collected from different sources [164].

In the present work, NSCs based *in vitro* assay was employed to explore the toxic impact of water-borne pollutants on the viability, proliferation, differentiation and migration of neural cells. Both treated and untreated water samples were tested in the given study [165].

4.1.1. Impact on the viability of NSCs

The Live-dead *in vitro* assay demonstrated the significant reduction in the viability of NSCs upon exposure to RWW for 24 h. The TSW, GW and DW exhibited insignificant impact on the viability of NSCs. The inhibitory effect of RWW on cell viability was probably due to the presence of a blend of environmental pollutants at relatively higher concentrations.

The presence of high concentrations of environmental pollutants in raw water bodies has been reported in various European countries, including Germany [36, 166, 167]. The findings of this study positively indicate that treatment of wastewater by a WWTP has been found efficient in reducing the concentration of pollutants in TSW to the levels which did not show direct effects on the viability of NSCs. The results also indicate that GW and DW collected from the water distribution facility near the Weschnitz River located at the German state Baden-Wuerttemberg display no direct risk of acute cytotoxicity in the cells of nervous system. The findings of viability assay did not show the signs of acute neural cytotoxicity of TSW. Poor viability of neural cells during early childhood as a result of exposure to environmental pollutants have been correlated with locomotive abnormalities, memory defects and cognitive dysfunctions [168, 169].

4.1.2. Impact on proliferation and differentiation of NSCs

The neurosphere clonogenic assay demonstrated that RWW significantly inhibits the proliferation of NSCs during each of the observation time points. In comparison, TSW exhibited the inhibitory effect only on day 7 of incubation. Water pollutants including pharmaceuticals (antihypertensive drugs, atenolol, caffeine), nitrates, organic mercury, bisphenol-A and pesticide such as *N*,*N*-Diethyl-*meta*-toluamide (DEET) are strong inhibitors of neural cell proliferation even at very low concentrations [38,47, 170-172]. The inhibition of the proliferation of NSCs by TSW on day 7 of incubation reflects that WWTP has not been able to completely eliminate the neural cell proliferation inhibitors. Findings of this study are in agreement with the published studies which affirmed the occurrence of relatively high concentrations of NSCs-proliferation inhibitors both in raw water and treated sewage water collected from different cities of European countries including Germany [35, 173, 174]. Reduced proliferation of NSCs during early age of life enhances the risk of Schizophrenia [175].

The NSCs differentiation assay is an important assay in assessment of the toxic impact of environmental pollutants on the functional cells of the nervous system.

In differentiation assay, the NSCs are differentiated into neurons and astrocytes with and without the presence of pollutants. The impact of pollutants is recorded by calculating the percentages of differentiated cells and measuring the growth of neurites and soma as final outcomes. Neurons are the basic functional cells of the nervous system. The astrocytes are second most important cells of the nervous system which are also called as supporting cells of the nervous system. Both of these cell types display high sensitivity to the chemical stimuli including, environmental pollutants [176, 177]. Results of the differentiation assay demonstrated that after a week of differentiation, RWW could reduce the percentage of neurons and astrocytes differentiated from NSCs. A strong inhibition in the growth of neurites and soma of astrocytes has also been observed after an exposure to RWW. All other tested water samples including TSW exhibited non-significant impacts on the differentiation parameters when compared to the control.

Environmental pollutants such as nitrogenous waste methyl mercury, bisphenol-A have been reported to reduce the neuronal differentiation of NSCs [39, 46, 47, 171, 178]. Artificial sweeteners pesticides DEET, dieldrin and phthalates reduce the neurite growth of neurons [179-182]. Moreover, another potential pollutant diazinon (insecticide) inhibits the growth and development of astrocytes. The presence of high concentrations of such pollutants have been reported both in natural water reservoirs and treated sewage water in European countries including Germany [35, 166, 183-185]. Consequently, depressed neurite growth could result in autism in children [37]. Similarly, mood disorders are associated with poor astrocyte development [186]. The findings of the differentiation assay indicate that treatment by WWTP was successful in removing or reducing the concentrations of environmental pollutants which could disrupt the neuronal and astrocytes differentiation of NSCs since TSW reveals comparable impact to that of control.

4.1.3. Impact on cell migration

During any injury either physical or induced by environmental toxicants, SVZ is the major niche in the brain of mammals which provides spontaneous response by activating the proliferation of NSCs and

their subsequent migration towards the injured area in order to start the repairing process [187]. Neurosphere migration assay offers a valuable tool to quantify the changes in cell migration process as a result of exposure to environmental toxicants [156]. Migration assay demonstrated a significant reduction in cell migration after an exposure to both the RWW and TSW. Impact of RWW on neural cell migration was stronger than TSW. Steroidal drugs, pesticides, bisphenol-A and organic mercury have been known to inhibit neural cell migration [40-42]. Presence of such neurotoxic pollutants above the safety limit has been reported both in wastewater and the treated wastewater of several countries across the globe including Germany [166, 183, 188-191]. Poor neural cell migration during the early age of childhood escalates the risk of epilepsy in later stages of human life [192].

Taken together, the findings of this study reflect that NSCs might be a simple but useful model for roughly ranking the neurotoxicity of water samples. Such water samples can be classified on basis of levels of pollutants and their source of origin. The study also indicates that WWTP operating in the German state of Baden-Wuerttemberg is not fully successful in eliminating or reducing the concentrations of pollutants which have toxic impact on the neural cell proliferation and migration processes. On the basis current findings, it can be argued that the TSW may not be suitable for human consumption if used without additional treatments. The study has also indicated that GW and DW collected from the water distribution facility near the Weschnitz River in the German state of Baden-Wuerttemberg demonstrated no risk of toxicity in the assays performed on NSCs. WWTPs in various countries, including European countries, operate on the basis of traditional membrane bioreactor treatment and activated sludge [160].Such water treatment techniques are capable of removing biodegradable organic macropollutants, microorganisms and large suspended particles. The efficacy of WWTPs to remove micropollutants such as pharmaceuticals, pesticides and nanosized particulate materials has been shown to be poor [162]. In the present study the neuroinhibitory impactof of TSW might stem from such inefficacy.

4.2. Impact of subtle concentrations of glyphosate on the fate of NSCs

The NSCs from the developing brains of mammals are more sensitive to the exogenous stimuli than those of adult brains. Due to high sensitivity, these cells provide prominent, robust and easily quantifiable responses to the subtle concentrations of environmental toxicants [146-148]. Keeping in view these inspiring qualities of NSCs from the developing brain, an *in vitro* assay based upon NSCs from the SVZ of the postnatal mouse was exploited to evaluate the impact of EPC of glyphosate in potable water *i.e.* MAC and MCL on the basic processes of neurogenesis. In addition, modifications in gene expression of neural cell lineages and cytoprotective responses have also been evaluated. In parallel, 7000 μgL^{-1} of glyphosate has been evaluated for its acute toxicity on NSCs culture [193]. Researchers have reported the concentrations of glyphosate equivalent to 7000 μgL^{-1} in the plasma of human subjects who have been without toxicity [194, 195].

4.2.1. Impact of glyphosate on the viability of NSCs

The findings of live-dead assay indicate the non-significant impact of both, MAC and MCL of glyphosate, on the viability of NSCs after an exposure of 24 h. Interestingly, 7000 μgL^{-1} of glyphosate has been previously reported by researchers as a concentration in human plasma without toxicity [194, 195]. However, this concentration has significantly decreased the viability of NSCs due to the induction of cytotoxicity in the present study. The reduced viability of NSCs induced by7000 μgL^{-1} of glyphosate, indicates the high sensitivity of the NSCs model to this concentration of glyphosate. In contrast to previously published studies that have reported the cytotoxicities of glyphosate in the cells of the nervous system at 100s of folds higher concentrations. The current study is reporting cytoxicity at much lower concentration [3, 76, 79, 80].

In an attempt to elucidate the potential role of Ca^{2+} homeostasis behind cytotoxicity, the 7000 μgL^{-1} and higher concentration of glyphosate induced Ca^{2+} influx in NSCs culture upon acute exposure which has not been observed after exposure to MAC and MCL of glyphosate during preliminary experiments. These findings are in agreement with the previous reports showing the induction of Ca^{2+} influx in the Sertoli cells at a concentration comparable to 7000 $\mu gL^{-1}$of glyphosate [196]. Sensitivity to the changes in Ca^{2+} homeostasis in the present study indicates the higher sensitivity of the employed NSCs model, since previous models have reported the sensitivity at 36000 μgL^{-1} of glyphosate or higher [79]. The increase in Ca^{2+} influx is related to the excessive intracellular production of ROS and increase in the release of glutamate which collectively leads to the death of cells [80]. Alternatively, disturbance in Ca^{2+} homeostasis may result in the disturbance of metabolic pathways implicated in cell fate determination [197]. Poor survival of neural cells due to exposure to environmental toxicants causes poor memory and defects in cognitive functions in children [169, 198]. Since MAC and MCL represent the important concentrations of glyphosate from the perspective of public safety and it initially presented no acute cytotoxic response, therefore, these have been evaluated for further neural effects, if any.

4.2.2. Impact of glyphosate on proliferation and differentiation of NSCs

Being analogue to the amino acid glycine, glyphosate obstructs the normal proliferation pathways of the cells [77, 199]. Results of the present study demonstrated that the impact of MAC and MCL of glyphosate on the proliferation of NSCs is comparable to that of control. In the previous studies, it has been reported that the impacts of glyphosate on cell proliferation vary according to the nature of target cells and the concentrations of glyphosate employed [200]. In their study, Li and co-workers demonstrated the inhibition of the proliferation of prostate and ovarian cancer cell lines at higher concentrations of glyphosate.

Conversely, stimulatory effects have been observed when the concentrations are reduced to one half [199]. In another study, glyphosate has been found to enhance the proliferation of human kidney HEK293 cells when utilized at concentrations ranging from 0.6-18 µM. Interestingly, concentrations outside this range did not affect cell proliferation [201]. Although MCL of glyphosate employed in the present study fell within the range of 0.6-18 µM, yet, MCL has not been able to alter the proliferation of NSCs. The divergence between the present study and the published reports could be attributed to the difference in types of cells utilized since glyphosate response varies with the type of target cells [200, 201].

No published data has been found regarding the effects of very low concentrations of glyphosate on the proliferation of NSCs to compare directly to the present study. Uniquely, the present study is novel and may provide a solid basis for in-depth investigation in future using the NSCs model optimized in the present study. Such investigations may be more meaningful if these could include molecular studies to explore the impact of EPC of glyphosate (MAC and MCL) on the expression of genes responsible for regulating the proliferation of NSCs.

During differentiation from NSCs, neurons and astrocytes are highly vulnerable to the impact of exogenous stimuli, including pesticides [159]. Immunostaining of differentiated cultured NSCs is a simple and useful way to quantify the alterations in differentiated cells induced by pesticides. In routine practices, ß-*tubulin* III is utilized as a general neuronal marker and *GFAP* as an astrocytic marker [202-204]. In the present study, the differentiation assay demonstrated that MCL of glyphosate tends to depress the neurite outgrowth whilst MAC significantly reduces the percentage of astrocytes and their growth. Glyphosate has been found to reduce the neurite outgrowth in the cultured hippocampus neurons by reducing the expression of Wnt5a protein and down-regulating CaMKII. It is noteworthy to mention that the concentrations of glyphosate employed in the published study were several 1000s fold higher (2-4 mgL^{-1}) than MAC and MCL employed in the present study [3].

These findings confirm the notion once again that the NSCs from SVZ of the postnatal mouse are highly sensitive towards glyphosate neurotoxicity compared to the experimental models exploited by other researchers.

Concerning the impact of glyphosate on astrocytes, Cattani and co-workers have reported the inhibitory effects in a rat model. On the contrary, some other researchers reported the stimulatory effects of glyphosate on the proliferation and growth of astrocytes in the telencephalon area of the brain of fish [79, 80, 205]. The discrepancy among the published reports regarding astrocyte growth and development might be attributed to the variation in the models and concentrations of glyphosate employed since glyphosate response varies significantly with concentrations and animal models exploited [200]. Reduced astrocyte development under the influence of the MAC of glyphosate in the present study is probably related to the interaction between the lower concentration of glyphosate and the molecular pathways regulating the energy metabolism in astrocytes. This notion is supported by a published study which has demonstrated a decrease in proliferation and development of astrocytes upon exposure to lower micromolar concentrations of glyphosate [206]. Poor neurite growth results in autism whilst poor astrocytes development has been implicated in mood disorders in children [37, 186].

4.2.3. Impact of glyphosate on the migration of neural cells

After completion of proliferation NSCs along with progenitor cells, differentiated neurons and astrocytes tend to migrate from a neurogenic niche to their final destination in the nervous system. The process of cell migration is highly sensitive to foreign stimuli since any perturbation by a noxious substance significantly increases the risk of epilepsy, heterotopias and schizophrenia in humans [207]. Researchers have established a link between thyroid-stimulating hormone (TSH) deficiency and defects in neural cell migration in mammals after developmental exposure to glyphosate herbicide [208].

The MCL of glyphosate in the present study significantly reduced cell migration. Although there is no published study to directly support these findings, but sufficient studies are available to support the notion that herbicides inhibit or reduce the migration of neural cell. The Oxadiazon, for instance, reduces the neuronal cell migration in the primary culture of the striatal cella at a non-toxic concentration by over-expressing acylphosphatase implicated in ion transport, apoptosis and cell differentiation [209].

Another herbicide glufosinate inhibits the cell migration from the SVZ of the mouse at concentrations several folds lower than the concentration considered safe by environment regulatory authorities [210]. Prospective researchers are encouraged to investigate further the possible link between the inhibition of TSH and reduction in neural cell migration at EPC of glyphosate as employed in the present study.

4.2.4. Impact of glyphosate on gene expression

Researchers have proposed that the determination of changes in the expression of genes (genes involved in the key neurogenesis processes) under the influence of chemical entities provides a sensitive and valuable tool for screening the potential neuromodulators. Such analysis is capable of unveiling the neurotoxicity of very low concentrations of toxicants which could not be explored through the traditional immunostaining assays, otherwise [202, 211]. *ß-tubulin III* is a protein marker expressed in the soma and axons of the general population of neurons. It is a widely used neuronal marker employed in developmental studies and plays an important role in neuronal development. *ß-tubulin III* has also been implicated in the neurite outgrowth processes [76, 212].

The results of the present study demonstrated that exposure to glyphosate at MCL downregulated the expression of *ß-tubulin III*. These findings are in agreement with those of Martinez and co-workers who have reported the decrease in expression of *ß-tubulin III* in SH-SY5Y cell culture upon exposure to glyphosate [76]. *S100B* is a mature astrocytic marker which regulates the proliferation and growth of astrocytes, coordinates axonal and neurite growth and plays a neuroprotective role [213].

Discussion

In the present study, the MAC of glyphosate downregulated the expression of *S100B* in differentiated NSCs culture which is consistent with a published study that has reported the decrease in expression of *S100B* in rat pups after developmental exposure to glyphosate herbicide [79, 80]. The downregulation of the *S100B* in the neural cells during the postnatal period may lead to defects in brain development during future life [214]. The findings of the present study indicate that the determination of gene expression of neuronal and astrocytic markers is a valuable endpoint to discriminate between neurotoxicity and glialtoxicity and should be included in the *in vitro* bioassay battery employed for the evaluation of developmental neurotoxicity in future.

Enzymes of the Cytochrome 450 (CYP) family are involved in the generation of essential metabolites and detoxification of xenobiotics. The dysregulation in the expression of CYP genes results in an enhanced vulnerability of an organism to pollutants and the depletion of important bio-molecules [69]. *CYP1A1* is the only member of the CYP family that is expressed in the brain of both humans and rodents [215, 216]. Researchers have reported that glyphosate and its herbicide formulations inhibit the expression of CYP family of enzymes in mammals [69].

In the present study, the MAC of glyphosate significantly downregulated the expression of *CYP1A1* in differentiated NSCs and this outcome is in agreement with published studies which state that glyphosate herbicide exerts an inhibitory effect on the expression of *CYP1A1* in non-neural tissues of rodents, fish, cow and in TM3 cell line [217-220]. The inhibitory impacts of glyphosate on the expression of *CYP1A1*in neural tissues have not been reported previously. Uniquely, the present study is the first study which has demonstrated the inhibitory impacts of glyphosate on the expression of *CYP1A1* gene in cultured NSCs isolated from the brain of developing mouse.

Another cytoprotective response deals with OS. Excessive production of ROS takes place when a cell is exposed to environmental pollutants. These ROS exert a deleterious impact on macromolecules of cells [19].

In general, cells respond to the overproduction of ROS by up-regulating superoxide dismutase (SOD) to neutralize OS [221]. SOD exists in three isoforms. The most important isoform, SOD1, interacts with superoxide radicals and converts them into less toxic H_2O_2 [222-225]. The MCL of glyphosate significantly up-regulated the expression of *SOD1* which is consistent with the published study which has reported the up-regulation of *SOD1* in hepatic tissues of rat after exposure to glyohosate [226].

The increase in the expression of *SOD1* in the differentiated culture of NSCs after exposure to MCL of glyphosate indicates the possible risk of OS for the cells of the nervous system at EPC of glyphosate.

Taken together, findings of the present study indicate that NSCs from the SVZ of the developing mouse are highly sensitive to the toxic potentials of subtle concentrations of glyphosate. The observed changes in the expression of neuronal *ß-tubulin III*, astrocytic *S100ß* and the cytoprotective responses *(CYP1A1 and SOD1)* markers in the differentiated culture of NSCs upon exposure to EPC of glyphosate proposed that quantification of gene expression could be a potential readout for the determination of developmental neurotoxicity of the subtle concentrations of glyphosate.

4.3. Impact of TRX flavonoid on the fate of NSCs

Plants have been considered as a potential source of natural molecules with attractive biological activities to treat a variety of ailments for mankind [227]. Amongst plant-based molecules, flavonoids have specifically earned the interest of neuroscientists for a couple of decades due to their neurogenerative and neuroprotective properties against neurological disorders [13, 16, 107, 110, 111]. TRX is a water-soluble flavonoid that has already proven its neurogenerative and neuroprotective activities against several neurological disorders in rodents [12, 99, 122, 123, 127, 129, 130]. Most of the studies concerning TRX, however, are effect-based *in vivo* studies lacking a precise mechanism of action.

It is worthful to uncover the augmenting effects of TRX on the basic processes of neurogenesis. Such information could be highly valuable to optimize the TRX as a lead compound in dealing with neurodegenerative disorders in future. To screen plant-based molecules such as TRX, NSCs based *in vitro* assay pose an ideal choice due to its ability to recapitulate processes involved in neurogenesis [3, 149, 154, 228].

In this part of the thesis, NSCs *in vitro* assay was exploited to explore the augmenting effects of TRX on the major neurogenesis processes and its neuroprotective properties against the inhibitory impacts of oligomeric Aß42 on neural cell differentiation [229].

4.3.1. Neurogenerative effects of TRX flavonoid

The NSCs are best recognized by employing the general NSCs marker Nestin along with GFAP which has been used as a secondary marker. Around 30% of mammalian NSCs are double-stained for Nestin and GFAP [16, 230, 231]. The doubly stained NSCs respond quickly to the injury of the brain by differentiating into neurons and supporting cells [232]. Additionally, doubly stained NSCs also facilitate neuronal cell migration [233]. Results of the present study demonstrated that the higher concentration of TRX employed in the present study significantly increased the percentage of doubly stained NSCs and therefore exhibited strong activating effects in producing quick response to the injury of the brain.

The proliferation of NSCs relies on multiple growth factors [147, 234]. Flavonoids have been shown to stimulate the proliferation of NSCs by up-regulation of genes and proteins involved in the cell cycle [16, 112]. Researchers have demonstrated the stimulatory effects of TRX on the proliferation of non-neural cells [131]. Intriguingly, TRX did not exhibit modulating impact on the proliferation of NSCs in the present study. In differentiation experiments, however, TRX has been found to stimulate the branching tips and length of neurites in differentiated neurons from NSCs. Various studies in the literature have also revealed the augmenting effects of flavonoids on the differentiation and growth of neuronal cells.

Baicalin flavonoid, for instance, enhances the ratio of neurons differentiated from NSCs and their neurite outgrowth by up-regulating the phosphorylation of the Erk1/2 pathway [106]. Quercetin improves neurite outgrowth by up-regulating Gap43 and cAMP in a cultured neuronal cell line [235]. The Phenylated flavonoid ENDF1 enhances the neurite branching density by up-regulating the expression of the DCX gene associated with microtubule-binding protein [14].

The stimulatory impacts of TRX on neurite outgrowth and extension were most probably associated with the antioxidant activity of TRX since reduced OS has been shown consistently to enhance neuronal differentiation of NSCs [236].

The results of the neurosphere migration assay demonstrated that TRX significantly increased the cellular migration of the differentiated culture of NSCs. The flavonoids such as quercetin induce neural cell migration in a mouse by increasing the expression of the Chemokine SDF1 receptor gene [13]. Several studies have reported the inducing impacts of TRX on the migration of umbilical vein endothelial cells when employed in combination with hydrolysates of cerebroprotein [131]. Researchers have reported that TRX induces migration of cell lines by modulating regulatory genes of transcription factors [130, 131]. The present study is the first *in vitro* study which has demonstrated the augmenting impact of TRX on neurite growth and neural cell migration.

On the other hand, the study demonstrated that TRX has not been unable to exert stimulatory impact on the proliferation of neural cells and no concerning published reports have been found. It makes the present study the first to report the impact of TRX on neural cell proliferation which has been found to be non-significant. The exact molecular mechanism of stimulatory actions of TRX on neurite growth and neural cell migration needs to be determined in future studies.

4.3.2. Neuroprotective effects of TRX flavonoid against Aß42

In another set of differentiation experiments, TRX has been found to neutralize the inhibitory effects of Aß42 on neuronal differentiation, neurite outgrowth and neurite extension. Oligomeric Aß42 is implicated in neurological disorders such as AD [237, 238]. Aß42 exhibits its neurotoxicity by reducing the differentiation of neurons, synaptogenesis and neurite outgrowth principally by inducing OS [103, 105, 239]. Several *in vivo* studies have reported the neuroprotective activities of TRX against Aß42 induced memory loss, learning defects and locomotive abnormalities in animal models. Researchers have reported that TRX neutralizes Aß42-induced neurotoxicity by ameliorating dysregulated processes including OS, neuroinflammation, synaptic deformation, elevated acetylcholine esterase level and disrupted action potential [99, 119, 240]. Researchers have also reported the anti-apoptotic action as one of the mechanisms of neuroprotective activity of TRX against the Aß42-induced toxicity [119, 120, 241, 242].

In the present assay, the neuroprotective property of TRX against the Aß42-induced toxicity was probably associated with the anti-oxidant activity of TRX as many other flavonoids evaluated against the neurotoxicity of Aß42 display anti-oxidant as a principal mechanism of neuroprotection [119, 120, 241]. The neurotoxicity of Aß42 is mainly associated with oligomeric and fibrillar forms of Aß42. Many flavonoids possess the natural ability to prevent the transformation of a non-toxic monomeric form of Aß42 to a toxic fibrilar or oligomeric form [243]. Two structural features of a flavonoid molecule prerequisite for its ant-fibrillization activity against Aß42 are the presence of i) aromatic rings ii) hydroxyl groups in the molecules of flavonoid. The aromatic ring of a flavonoid interacts with hydrophobic amino acid residues whilst the hydroxyl group of a flavonoid interacts with the hydrophilic amino acid residue of Aß42. Such chemical interactions result in the disaggregation or the prevention of fibrllization of Aß42 [243]. The TRX possesses all the structural features prerequisite for prevention of fibrilization of Aß42 so it is can be assumed that TRX prevents oligomerirzation of Aß42 (see Figure 3).

This assumption is supported by the findings of Wang and co-workers which have demonstrated that a flavonoid, rutin, attenuates the toxicity of Aß in neuroblastoma cells by preventing the oligomerization of Aß [244]. The TRX has a great structural similarity to that of rutin [245]. Further studies are required to explore whether TRX is capable of preventing oligomerization of Aß42 through chemical interactions.

5. Conclusions

The present study involves the development of cost-effective and physiologically relevant *in vitro* assay system based upon the primary NSCs derived from the SVZ of the developing brain of the mouse. The NSCs-based model provided a robust, sensitive and economical bio-tool to differentiate neurotoxic impacts of a complex assortment of pollutants found in treated and untreated water samples of various origins. The highly sensitive nature of the assay was able to reveal the neurotoxic impacts of subtle concentrations of glyphosate, an environmental pollutant. The utility of the assay was further reflected by its potential to assess the neurogenerative potential of flavonoid Troxerutin.

The *in vitro* assay has revealed that GW and DW samples collected from a water facility located at Hemsbach town in the German state of Baden-Wuerttemberg present no threat to the basic neurogenic processes. The absence of neurotoxicity in the present study confirms the suitability of GW and DW for human consumption. The RWW adversely affected all of the studied processes of neurogenesis reinforcing its toxic impacts on animals. In the same manner, TSW has also not been found safe since it adversely affected the proliferation and migration of neural cells. The findings of this part of the study indicate the incapability of WWTP operating in the study area to completely eliminate or reduce the levels of neurotoxicants which could adversely impact the processes of proliferation and migration of neural cells. The study suggests that TSW is not fit for human consumption unless it is subjected to additional extensive purification processes and treatments. The detected neurotoxic responses indicate that NSCs-*in* vitro may be a very useful screening tool for the assessment of the quality of water sampled from different sources having different levels of contaminants.

Glyphosate is the most extensively employed pesticide and is included in the list of leading environmental pollutants. It is assumed to be non-toxic at or below EPC (MAC and MCL) in potable water as per recommendations of environment regulatory authorities. Conversely, the assay based on the NSCs model in the present study highlighted the neurotoxic potential of glyphosate at presumably safe EPC ranges.

Conclusion

While establishing neurotoxic potentials, these were the gene expression experiments which conclusively indicated that EPC of glyphosate are capable of inducing oxidative stress and increasing the susceptibility of neural cells to toxic compounds. It can be safely concluded that gene-expression experiments as a part of *in vitro* assay system are vital to uncover the neurotoxicities associated with sublethal or non-toxic concentrations of glyphosate or other neurotoxicants. Moreover, the subtle concentration of glyphosate has been found to induce acute toxicity and activate Ca^{2+} signalling in the cultured NSCs. Prospective researchers need to verify the findings and conduct detailed molecular studies to uncover molecular pathways involved in the neurotoxic effects of EPC of glyphosate present in potable water. If confirmed, such findings should encourage the regulatory authorities to review the safety standards concerning EPC of glyphosate in potable water.

The assay developed in the present study using the NSCs has also been found to be successful in screening natural compounds with neurogenerative properties. Through this assay, flavonoid TRX has been shown to possess a neuroaugmenting impact on the basic neurogenic processes. In parallel, the TRX could successfully reverse the inhibitory impacts of oligomeric Aß42 on neuronal differentiation and neurite growth. In conclusion, TRX is a powerful neuroprotective flavonoid exerting a positive impact on the basic neurogenic processes and reversing the deleterious impacts induced by amyloid deposition. Theoretically, such model should be capable of evaluating similar molecules for their neuroprotective properties.

The findings of the present study imply that TRX is a useful pharmacological lead compound. To move forward, these findings necessitate the implementation of detailed molecular studies to explore the molecular pathways governing neurogenerative/neuroprotective properties of TRX. To elucidate further details, prospective researchers may choose to evaluate the possible role of TRX in the disaggregation of the toxic oligomeric form of Aß42 into non-toxic monomeric form. Such studies may lead to optimization of the TRX as a potential therapeutic candidate for treating neurodegenerative disorders.

6. References

1. Costa, L. G., Giordano, G., Guizzetti, M. & Vitalone, A. (2008) Neurotoxicity of pesticides: a brief review, *Front Biosci.* **13**, 1240-1249.

2. Pamies, D., Block, K., Lau, P., Gribaldo, L., Pardo, C. A., Barreras, P., Smirnova, L., Wiersma, D., Zhao, L. & Harris, G. (2018) Rotenone exerts developmental neurotoxicity in a human brain spheroid model, *Toxicol Appl Pharmacol.* **354**, 101-114.

3. Coullery, R. P., Ferrari, M. E. & Rosso, S. B. (2016) Neuronal development and axon growth are altered by glyphosate through a WNT non-canonical signaling pathway, *Neurotoxicol.* **52**, 150-161.

4. Tohyama, C. (2016) Developmental neurotoxicity test guidelines: problems and perspectives, *J Toxicol Sci.* **41**, SP69-SP79.

5. Grandjean, P. & Landrigan, P. J. (2006) Developmental neurotoxicity of industrial chemicals, *Lancet.* **368**, 2167-2178.

6. Jurewicz, J., Polańska, K. & Hanke, W. (2013) Exposure to widespread environmental toxicants and children's cognitive development and behavioral problems, *Int J Occup Med Environ Health.* **26**, 185-204.

7. Perlroth, N. H. & Branco, C. W. C. (2017) Current knowledge of environmental exposure in children during the sensitive developmental periods, *J Pediatr (Rio J).* **93**, 17-27.

8. Tran, N. Q. V. & Miyake, K. (2017) Neurodevelopmental disorders and environmental toxicants: epigenetics as an underlying mechanism, *Int J Genomics.* **2017**, 7526592.

9. Parrón, T., Requena, M., Hernández, A. F. & Alarcón, R. (2011) Association between environmental exposure to pesticides and neurodegenerative diseases, *Toxicol Appl Pharmacol.* **256**, 379-385.

10. Landrigan, P. J., Graham, D. G. & Thomas, R. D. (1994) Environmental neurotoxic illness: research for prevention, *Environ Health Perspect.* **102**, 117-120.

11. Medehouenou, T. C. M., Ayotte, P., Carmichael, P.-H., Kröger, E., Verreault, R., Lindsay, J., Dewailly, É., Tyas, S. L., Bureau, A. & Laurin, D. (2019) Exposure to polychlorinated biphenyls and organochlorine pesticides and risk of dementia, Alzheimer's disease and cognitive decline in an older population: a prospective analysis from the Canadian Study of Health and Aging, *Environ Health.* **18**, 1-11.

12. Azarfarin, M., Farajdokht, F., Babri, S., Salehpour, F., Taghizadeh, M. & Mohaddes, G. (2018) Effects of troxerutin on anxiety-and depressive-like behaviors induced by chronic mild stress in adult male rats, *Iran J Basic Med Sci.* **21**, 781-786.

References

13. Baral, S., Pariyar, R., Kim, J., Lee, H.-S. & Seo, J. (2017) Quercetin-3-O-glucuronide promotes the proliferation and migration of neural stem cells, *Neurobiol Aging.* **52**, 39-52.

14. Bieler, L., Vogl, M., Kirchinger, M., Urmann, C., Riepl, H., Bandtlow, C. E., Klimaschewski, L., Aigner, L. & Couillard-Despres, S. (2019) The prenylflavonoid ENDF1 overrules central nervous system growth inhibitors and facilitates regeneration of DRG neurons, *Front Cell Neurosci.* **13**, 332.

15. Li, F., Dong, H., Gong, Q., Wu, Q., Jin, F. & Shi, J. (2015) Icariin decreases both APP and Aβ levels and increases neurogenesis in the brain of Tg2576 mice, *Neuroscience.* **304**, 29-35.

16. Yao, R., Zhang, L., Li, X. & Li, L. (2010) Effects of Epimedium flavonoids on proliferation and differentiation of neural stem cells in vitro, *Neurological research.* **32**, 736-42.

17. Bondy, S. & Campbell, A. (2017) Water Quality and Brain Function, *Int J Environ Res Public Health.* **15**, 2.

18. Fawell, J. & Nieuwenhuijsen, M. J. (2003) Contaminants in drinking water, *Br Med Bull.* **68**, 199-208.

19. Héritier, L., Duval, D., Galinier, R., Meistertzheim, A. L. & Verneau, O. (2017) Oxidative stress induced by glyphosate-based herbicide on freshwater turtles, *Environ Toxicol Chem.* **36**, 3343-3350.

20. Miege, C., Choubert, J., Ribeiro, L., Eusèbe, M. & Coquery, M. (2009) Fate of pharmaceuticals and personal care products in wastewater treatment plants–conception of a database and first results, *Environ Pollut.* **157**, 1721-1726.

21. Trintinaglia, L., Bianchi, E., Silva, L., Nascimento, C., Spilki, F. & Ziulkoski, A. (2015) Cytotoxicity assays as tools to assess water quality in the Sinos River basin, *Braz J Biol.* **75**, 75-80.

22. Alexandrov, P. N., Pogue, A. I. & Lukiw, W. J. (2018) Synergism in aluminum and mercury neurotoxicity, *Integr Food Nutr Metab.* **5**.

23. Bondy, S. C. (2010) The neurotoxicity of environmental aluminum is still an issue, *Neurotoxicol.* **31**, 575-581.

24. Björnberg, K. A., Vahter, M., Berglund, B., Niklasson, B., Blennow, M. & Sandborgh-Englund, G. (2005) Transport of methylmercury and inorganic mercury to the fetus and breast-fed infant, *Environ Health Perspect.* **113**, 1381-1385.

25. Chandio, T. A., Khan, M. N., Muhammad, M. T., Yalcinkaya, O. & Kayis, A. F. (2020) Co-exposure of neurotoxic contaminants (Pb and Mn) in drinking water of Zhob District, Baluchistan Pakistan, *Environ Nanotechnol Monit Manag.* **14**, 100328.

26. Crinnion, W. J. (2000) Environmental medicine, part three: long-term effects of chronic low-dose mercury exposure, *Altern Med Rev.* **5**, 209-223.

References

27. Oteiza, P. I., Keen, C. L., Han, B. & Golub, M. S. (1993) Aluminum accumulation and neurotoxicity in Swiss-Webster mice after long-term dietary exposure to aluminum and citrate, *Metabolism.* **42**, 1296-1300.

28. Wang, H., Engstrom, A. K. & Xia, Z. (2017) Cadmium impairs the survival and proliferation of cultured adult subventricular neural stem cells through activation of the JNK and p38 MAP kinases, *Toxicology.* **380**, 30-37.

29. Gupta, V. K., Singh, S., Agrawal, A., Siddiqi, N. J. & Sharma, B. (2015) Phytochemicals mediated remediation of neurotoxicity induced by heavy metals, *Biochem Res Int.* **2015**.

30. Rehman, K., Fatima, F., Waheed, I. & Akash, M. S. H. (2018) Prevalence of exposure of heavy metals and their impact on health consequences, *J Cell Biochem.* **119**, 157-184.

31. Fischer, C., Fredriksson, A. & Eriksson, P. (2007) Coexposure of neonatal mice to a flame retardant PBDE 99 (2, 2′, 4, 4′, 5-pentabromodiphenyl ether) and methyl mercury enhances developmental neurotoxic defects, *Toxicol Sci.* **101**, 275-285.

32. Parran, D., Mundy, W. & Barone Jr, S. (2001) Effects of methylmercury and mercuric chloride on differentiation and cell viability in PC12 cells, *Toxicol Sci.* **59**, 278-290.

33. Nam, S. M., Kim, J. W., Yoo, D. Y., Kim, W., Jung, H. Y., Choi, J. H., Hwang, I. K., Seong, J. K. & Yoon, Y. S. (2016) Effects of aluminum on the reduction of neural stem cells, proliferating cells, and differentiating neuroblasts in the dentate gyrus of D-galactose-treated mice via increasing oxidative stress, *J Vet Sci.* **17**, 127-136.

34. Reichert, K. P., Schetinger, M. R. C., Pillat, M. M., Bottari, N. B., Palma, T. V., Gutierres, J. M., Ulrich, H., Andrade, C. M., Exley, C. & Morsch, V. M. (2019) Aluminum affects neural phenotype determination of embryonic neural progenitor cells, *Arch Toxicol.* **93**, 2515-2524.

35. Fromme, H., Küchler, T., Otto, T., Pilz, K., Müller, J. & Wenzel, A. (2002) Occurrence of phthalates and bisphenol A and F in the environment, *Water Res.* **36**, 1429-1438.

36. Vincze, K., Scheil, V., Kuch, B., Köhler, H. R. & Triebskorn, R. (2015) Impact of wastewater on fish health: a case study at the Neckar River (Southern Germany) using biomarkers in caged brown trout as assessment tools, *Environ Sci Pollut Res Int.* **22**, 11822-11839.

37. Gilbert, J. & Man, H.-Y. (2017) Fundamental elements in autism: from neurogenesis and neurite growth to synaptic plasticity, *Front Cell Neurosci.* **11**, 359.

38. Pérez, M. R., Rossi, A. S., Bacchetta, C., Elorriaga, Y., Carriquiriborde, P. & Cazenave, J. (2018) In situ evaluation of the toxicological impact of a wastewater effluent on the fish Prochilodus lineatus: biochemical and histological assessment, *Ecological indicators.* **84**, 345-353.

39. Fujiwara, Y., Miyazaki, W., Koibuchi, N. & Katoh, T. (2018) The effects of low-Dose Bisphenol a and Bisphenol F on neural Differentiation of a Fetal Brain-Derived neural Progenitor cell line, *Front Endocrinol (Lausanne).* **9**, 24.

References

40. Fahrion, J. K., Komuro, Y., Li, Y., Ohno, N., Littner, Y., Raoult, E., Galas, L., Vaudry, D. & Komuro, H. (2012) Rescue of neuronal migration deficits in a mouse model of fetal Minamata disease by increasing neuronal Ca2+ spike frequency, *Proc Natl Acad Sci U S A.* **109**, 5057-5062.

41. Ishido, M. & Suzuki, J. (2010) Inhibition by rotenone of mesencephalic neural stem-cell migration in a neurosphere assay in vitro, *Toxicol In Vitro.* **24**, 552-557.

42. Ishido, M. & Suzuki, J. (2010) Quantitative analyses of inhibitory effects of Bisphenol A on neural stem-cell migration using a neurosphere assay in vitro, *J Heal Sci.* **56**, 175-181.

43. Manassaram, D. M., Backer, L. C. & Moll, D. M. (2006) A review of nitrates in drinking water: maternal exposure and adverse reproductive and developmental outcomes, *Environ Health Perspect.* **114**, 320-327.

44. Santos, I. R., Costa, R. C., Freitas, U. & Fillmann, G. (2008) Influence of effluents from a wastewater treatment plant on nutrient distribution in a coastal creek from southern Brazil, *Braz Arch Biol Technol.* **51**, 153-162.

45. Croen, L. A., Todoroff, K. & Shaw, G. M. (2001) Maternal exposure to nitrate from drinking water and diet and risk for neural tube defects, *Am J Epidemiol.* **153**, 325-331.

46. Braissant, O., Henry, H., Villard, A.-M., Zurich, M.-G., Loup, M., Eilers, B., Parlascino, G., Matter, E., Boulat, O. & Honegger, P. (2002) Ammonium-induced impairment of axonal growth is prevented through glial creatine, *J Neurosci.* **22**, 9810-9820.

47. Solari, M., Paquin, J., Ducharme, P. & Boily, M. (2009) P19 neuronal differentiation and retinoic acid metabolism as criteria to investigate atrazine, nitrite, and nitrate developmental toxicity, *Toxicol Sci.* **113**, 116-126.

48. Sankhla, M. S., Kumari, M., Sharma, K., Kushwah, R. S. & Kumar, R. (2018) Water contamination through pesticide & their toxic effect on human health, *Int J Res Appl Sci Eng Technol.* **6**, 967-970.

49. Alavanja, M. C., Hoppin, J. A. & Kamel, F. (2004) Health effects of chronic pesticide exposure: cancer and neurotoxicity, *Annu Rev Public Health.* **25**, 155-197.

50. Sandoval-Herrera, N., Mena, F., Espinoza, M. & Romero, A. (2019) Neurotoxicity of organophosphate pesticides could reduce the ability of fish to escape predation under low doses of exposure, *Sci Rep.* **9**, 10530.

51. Münze, R., Hannemann, C., Orlinskiy, P., Gunold, R., Paschke, A., Foit, K., Becker, J., Kaske, O., Paulsson, E. & Peterson, M. (2017) Pesticides from wastewater treatment plant effluents affect invertebrate communities, *Sci Total Environ.* **599**, 387-399.

52. Schmid, S. & Bogner, F. X. (2018) What Germany's university beginners think about water reuse, *Water.* **10**, 731.

References

53. Ternes, T. A. (1998) Occurrence of drugs in German sewage treatment plants and rivers, *Water Re.* **32**, 3245-3260.

54. Niss, F., Rosenmai, A. K., Mandava, G., Örn, S., Oskarsson, A. & Lundqvist, J. (2018) Toxicity bioassays with concentrated cell culture media—a methodology to overcome the chemical loss by conventional preparation of water samples, *Environ Sci Pollut Res Int.* **25**, 12183-12188.

55. Žegura, B., Heath, E., Černoša, A. & Filipič, M. (2009) Combination of in vitro bioassays for the determination of cytotoxic and genotoxic potential of wastewater, surface water and drinking water samples, *Chemosphere.* **75**, 1453-1460.

56. Jaeger, N., Moraes, J., Klauck, C., Gehlen, G., Rodrigues, M. & Ziulkoski, A. (2015) Cytotoxicity assays to evaluate tannery effluents treated by photoelectrooxidation, *Braz J Biol.* **75**, 53-61.

57. Jasper, R., Locatelli, G. O., Pilati, C. & Locatelli, C. (2012) Evaluation of biochemical, hematological and oxidative parameters in mice exposed to the herbicide glyphosate-Roundup®, *Interdiscip Toxicol.* **5**, 133-140.

58. Sribanditmongkol, P., Jutavijittum, P., Pongraveevongsa, P., Wunnapuk, K. & Durongkadech, P. (2012) Pathological and toxicological findings in glyphosate-surfactant herbicide fatality: a case report, *Am J Forensic Med Pathol.* **33**, 234-237.

59. Song, H.-Y., Kim, Y.-H., Seok, S.-J., Gil, H.-W. & Hong, S.-Y. (2012) In vitro cytotoxic effect of glyphosate mixture containing surfactants, *J Korean Med Sci.* **27**, 711-715.

60. Szepanowski, F., Kleinschnitz, C. & Stettner, M. (2019) Glyphosate-based herbicide: a risk factor for demyelinating conditions of the peripheral nervous system?, *Neural Regen Res.* **14**, 2079-2080.

61. Lelan, F., Boyer, C., Thinard, R., Remy, S., Usal, C., Tesson, L., Anegon, I., Neveu, I., Damier, P. & Naveilhan, P. (2011) Effects of human alpha-synuclein A53T-A30P mutations on SVZ and local olfactory bulb cell proliferation in a transgenic rat model of Parkinson disease, *Parkinsons Dis.* **2011**, 987084.

62. Amerio, P., Motta, A., Toto, P., Pour, S. M., Pajand, R., Feliciani, C. & Tulli, A. (2004) Skin Toxicity from Glyphosate-Surfactant Formulation, *J Toxicol Clin Toxicol.* **42**, 317-319.

63. Bradberry, S. M., Proudfoot, A. T. & Vale, J. A. (2004) Glyphosate poisoning, *Toxicol Rev.* **23**, 159-167.

64. Chen, H.-H., Lin, J.-L., Huang, W.-H., Weng, C.-H., Lee, S.-Y., Hsu, C.-W., Chen, K.-H., Wang, I.-K., Liang, C.-C. & Chang, C.-T. (2013) Spectrum of corrosive esophageal injury after intentional paraquat or glyphosate-surfactant herbicide ingestion, *Int J Gen Med.* **6**, 677-83.

References

65. Dallegrave, E., Mantese, F. D., Oliveira, R. T., Andrade, A. J., Dalsenter, P. R. & Langeloh, A. (2007) Pre-and postnatal toxicity of the commercial glyphosate formulation in Wistar rats, *Arch Toxicol.* **81**, 665-673.

66. Sato, C., Kamijo, Y., Yoshimura, K. & Ide, T. (2011) Aseptic meningitis in association with glyphosate-surfactant herbicide poisoning, *Clin Toxicol (Phila).* **49**, 118-120.

67. Barbosa, E. R., Leiros da Costa, M. D., Bacheschi, L. A., Scaff, M. & Leite, C. C. (2001) Parkinsonism after glycine-derivate exposure, *Mov Disord.* **16**, 565-568.

68. Caballero, M., Amiri, S., Denney, J. T., Monsivais, P., Hystad, P. & Amram, O. (2018) Estimated Residential Exposure to Agricultural Chemicals and Premature Mortality by Parkinson's Disease in Washington State, *Int J Environ Res Public Health.* **15**, 2885.

69. Samsel, A. & Seneff, S. (2013) Glyphosate's suppression of cytochrome P450 enzymes and amino acid biosynthesis by the gut microbiome: pathways to modern diseases, *Entropy.* **15**, 1416-1463.

70. Zouaoui, K., Dulaurent, S., Gaulier, J., Moesch, C. & Lachâtre, G. (2013) Determination of glyphosate and AMPA in blood and urine from humans: About 13 cases of acute intoxication, *Forensic Sci Int.* **226**, e20-e25.

71. Shaw, W. (2017) Elevated urinary glyphosate and clostridia metabolites with altered dopamine metabolism in triplets with autistic spectrum disorder or suspected seizure disorder: A case study, *Integr Med (Encinitas).* **16**, 50-57.

72. Solomon, K. R., Anadon, A., Carrasquilla, G., Cerdeira, A. L., Marshall, E. & Sanin, L.-H. (2007) Coca and poppy eradication in Colombia: Environmental and human health assessment of aerially applied glyphosate, *Rev Environ Contam Toxicol.* **190**, 43-125.

73. Ait Bali, Y., Ba-Mhamed, S. & Bennis, M. (2017) Behavioral and Immunohistochemical Study of the Effects of Subchronic and Chronic Exposure to Glyphosate in Mice, *Front Behav Neurosci.* **11**, 146.

74. Gui, Y.-x., Fan, X.-n., Wang, H.-m. & Wang, G. (2012) Glyphosate induced cell death through apoptotic and autophagic mechanisms, *Neurotoxicol Teratol.* **34**, 344-349.

75. Zheng, Q., Yin, J., Zhu, L., Jiao, L. & Xu, Z. (2018) Reversible Parkinsonism induced by acute exposure glyphosate, *Parkinsonism Relat Disord.* **50**, 121.

76. Martínez, M.-A., Rodríguez, J.-L., Lopez-Torres, B., Martínez, M., Martínez-Larrañaga, M.-R., Maximiliano, J.-E., Anadón, A. & Ares, I. (2020) Use of human neuroblastoma SH-SY5Y cells to evaluate glyphosate-induced effects on oxidative stress, neuronal development and cell death signaling pathways, *Environ Int.* **135**, 105414.

77. Ji, H., Xu, L., Wang, Z., Fan, X. & Wu, L. (2018) Differential microRNA expression in the prefrontal cortex of mouse offspring induced by glyphosate exposure during pregnancy and lactation, *Exp Ther Med.* **15**, 2457-2467.

References

78. Dechartres, J., Pawluski, J. L., Gueguen, M. M., Jablaoui, A., Maguin, E., Rhimi, M. & Charlier, T. D. (2019) Glyphosate and glyphosate-based herbicide exposure during the peripartum period affects maternal brain plasticity, maternal behaviour and microbiome, *J Neuroendocrinol.* **31**, e12731.

79. Cattani, D., Cavalli, V. L. d. L. O., Rieg, C. E. H., Domingues, J. T., Dal-Cim, T., Tasca, C. I., Silva, F. R. M. B. & Zamoner, A. (2014) Mechanisms underlying the neurotoxicity induced by glyphosate-based herbicide in immature rat hippocampus: involvement of glutamate excitotoxicity, *Toxicology.* **320**, 34-45.

80. Cattani, D., Cesconetto, P. A., Tavares, M. K., Parisotto, E. B., De Oliveira, P. A., Rieg, C. E. H., Leite, M. C., Prediger, R. D. S., Wendt, N. C. & Razzera, G. (2017) Developmental exposure to glyphosate-based herbicide and depressive-like behavior in adult offspring: implication of glutamate excitotoxicity and oxidative stress, *Toxicology.* **387**, 67-80.

81. McConnell, E. R., McClain, M. A., Ross, J., LeFew, W. R. & Shafer, T. J. (2012) Evaluation of multi-well microelectrode arrays for neurotoxicity screening using a chemical training set, *Neurotoxicol.* **33**, 1048-1057.

82. Martinez, A. & Al-Ahmad, A. J. (2019) Effects of glyphosate and aminomethylphosphonic acid on an isogeneic model of the human blood-brain barrier, *Toxicol Lett.* **304**, 39-49.

83. Szepanowski, F., Szepanowski, L.-P., Mausberg, A. K., Albrecht, P., Kleinschnitz, C., Kieseier, B. C. & Stettner, M. (2018) Differential impact of pure glyphosate and glyphosate-based herbicide in a model of peripheral nervous system myelination, *Acta Neuropathol.* **136**, 979-982.

84. Hernández-Plata, I., Giordano, M., Díaz-Muñoz, M. & Rodríguez, V. M. (2015) The herbicide glyphosate causes behavioral changes and alterations in dopaminergic markers in male Sprague-Dawley rat, *Neurotoxicology.* **46**, 79-91.

85. Martínez, M.-A., Ares, I., Rodríguez, J.-L., Martínez, M., Martínez-Larrañaga, M.-R. & Anadón, A. (2018) Neurotransmitter changes in rat brain regions following glyphosate exposure, *Environ Res.* **161**, 212-219.

86. Samsel, A. & Seneff, S. (2016) Glyphosate pathways to modern diseases V: amino acid analogue of glycine in diverse proteins, *J Biol Phys Chem.* **16**, 9-46.

87. Dolan, T., Howsam, P., Parsons, D. J. & Whelan, M. J. (2013) Is the EU drinking water directive standard for pesticides in drinking water consistent with the precautionary principle?, *Environ Sci Technol.* **47**, 4999-5006.

88. Larsen, K., Najle, R., Lifschitz, A. & Virkel, G. (2012) Effects of sub-lethal exposure of rats to the herbicide glyphosate in drinking water: glutathione transferase enzyme activities, levels of reduced glutathione and lipid peroxidation in liver, kidneys and small intestine, *Environ Toxicol Pharmacol* **34**, 811-818.

References

89. Clair, É., Mesnage, R., Travert, C. & Séralini, G.-É. (2012) A glyphosate-based herbicide induces necrosis and apoptosis in mature rat testicular cells in vitro, and testosterone decrease at lower levels, *Toxicol In Vitro.* **26**, 269-279.

90. Anifandis, G., Amiridis, G., Dafopoulos, K., Daponte, A., Dovolou, E., Gavriil, E., Gorgogietas, V., Kachpani, E., Mamuris, Z. & Messini, C. I. (2017) The In Vitro Impact of the Herbicide Roundup on Human Sperm Motility and Sperm Mitochondria, *Toxics.* **6**, 2.

91. Young, F., Ho, D., Glynn, D. & Edwards, V. (2015) Endocrine disruption and cytotoxicity of glyphosate and roundup in human JAr cells in vitro, *Integr Pharm Toxicol Gentocicol.* **1**, 12-19.

92. Brothers, H. M., Gosztyla, M. L. & Robinson, S. R. (2018) The physiological roles of amyloid-β peptide hint at new ways to treat Alzheimer's disease, *Front Aging Neurosci.* **10**, 118.

93. Pearson, H. A. & Peers, C. (2006) Physiological roles for amyloid β peptides, *J Physiol.* **575**, 5-10.

94. Choi, H. & Koh, S. (2016) Interaction between Amyloid Beta Toxicity and the PI3K Pathway in Alzheimer's Disease, *J Alzheimers Dis Parkinsonism.* **6**, 2161-0460.1000269.

95. Paula, V. d. J. R. d., Guimarães, F. M., Diniz, B. S. & Forlenza, O. V. (2009) Neurobiological pathways to Alzheimer's disease: Amyloid-beta, TAU protein or both?, *Dement Neuropsychol.* **3**, 188-194.

96. Maltsev, A., Bystryak, S. & Galzitskaya, O. (2011) The role of β-amyloid peptide in neurodegenerative diseases, *Ageing Res Rev.* **10**, 440-452.

97. Tang, B. L. (2020) Neuropathological Mechanisms Associated with Pesticides in Alzheimer's Disease, *Toxics.* **8**, 21.

98. Heys, K. A., Shore, R. F., Pereira, M. G. r. & Martin, F. L. (2017) Levels of organochlorine pesticides are associated with amyloid aggregation in apex avian brains, *Environ Sci Technol.* **51**, 8672-8681.

99. Babri, S., Amani, M., Mohaddes, G., Alihemmati, A. & Ebrahimi, H. (2012) Effect of troxerutin on synaptic plasticity of hippocampal dentate gyrus neurons in a β-amyloid model of Alzheimer′ s disease: An electrophysiological study, *Neurophysiology.* **44**, 387-393.

100. Park, H., Kang, S., Nam, E., Suh, Y.-H. & Chang, K.-A. (2019) The protective effects of PSM-04 against beta amyloid-induced neurotoxicity in primary cortical neurons and an animal model of Alzheimer's disease, *Front Pharmacol.* **10**, 2.

101. Guglielmotto, M., Monteleone, D., Piras, A., Valsecchi, V., Tropiano, M., Ariano, S., Fornaro, M., Vercelli, A., Puyal, J. & Arancio, O. (2014) Aβ1-42 monomers or oligomers have different effects on autophagy and apoptosis, *Autophagy.* **10**, 1827-1843.

References

102. Yasumoto, T., Takamura, Y., Tsuji, M., Watanabe-Nakayama, T., Imamura, K., Inoue, H., Nakamura, S., Inoue, T., Kimura, A. & Yano, S. (2019) High molecular weight amyloid β1-42 oligomers induce neurotoxicity via plasma membrane damage, *FASEB J.* **33**, 9220-9234.

103. He, N., Jin, W., Lok, K., Wang, Y., Yin, M. & Wang, Z. (2013) Amyloid-β 1–42 oligomer accelerates senescence in adult hippocampal neural stem/progenitor cells via formylpeptide receptor 2, *Cell Death Dis.* **4**, e924.

104. López-Toledano, M. A. & Shelanski, M. L. (2004) Neurogenic effect of β-amyloid peptide in the development of neural stem cells, *J Neurosci.* **24**, 5439-5444.

105. Evans, N. A., Facci, L., Owen, D. E., Soden, P. E., Burbidge, S. A., Prinjha, R. K., Richardson, J. C. & Skaper, S. D. (2008) Aβ1–42 reduces synapse number and inhibits neurite outgrowth in primary cortical and hippocampal neurons: A quantitative analysis, *J Neurosci Methods.* **175**, 96-103.

106. Li, M., Tsang, K. S., Choi, S. T., Li, K., Shaw, P. C. & Lau, K. F. (2011) Neuronal differentiation of C17. 2 neural stem cells induced by a natural flavonoid, baicalin, *Chembiochem.* **12**, 449-456.

107. Oberbauer, E., Urmann, C., Steffenhagen, C., Bieler, L., Brunner, D., Furtner, T., Humpel, C., Bäumer, B., Bandtlow, C. & Couillard-Despres, S. (2013) Chroman-like cyclic prenylflavonoids promote neuronal differentiation and neurite outgrowth and are neuroprotective, *J Nutr Biochem.* **24**, 1953-1962.

108. Spencer, J. P. (2010) The impact of fruit flavonoids on memory and cognition, *Br J Nutr.* **104**, S40-S47.

109. Spencer, J. P. (2010) Beyond antioxidants: the cellular and molecular interactions of flavonoids and how these underpin their actions on the brain, *Proc Nutr Soc.* **69**, 244-260.

110. Wang, T.-y., Li, Q. & Bi, K.-s. (2018) Bioactive flavonoids in medicinal plants: Structure, activity and biological fate, *Asian J Pharm Sci.* **13**, 12-23.

111. Vauzour, D., Vafeiadou, K., Rodriguez-Mateos, A., Rendeiro, C. & Spencer, J. P. (2008) The neuroprotective potential of flavonoids: a multiplicity of effects, *Genes Nutr.* **3**, 115-126.

112. Fu, X., Li, S., Zhou, S., Wu, Q., Jin, F. & Shi, J. (2018) Stimulatory effect of icariin on the proliferation of neural stem cells from rat hippocampus, *BMC Complement Altern Med.* **18**, 34.

113. Jing, X., Shi, H., Zhu, X., Wei, X., Ren, M., Han, M., Ren, D. & Lou, H. (2015) Eriodictyol Attenuates β-Amyloid 25–35 Peptide-Induced Oxidative Cell Death in Primary Cultured Neurons by Activation of Nrf2, *Neurochem Res.* **40**, 1463-1471.

114. Li, Y., Zhuang, P., Shen, B., Zhang, Y. & Shen, J. (2012) Baicalin promotes neuronal differentiation of neural stem/progenitor cells through modulating p-stat3 and bHLH family protein expression, *Brain Res.* **1429**, 36-42.

References

115. Mani, S., Sekar, S., Barathidasan, R., Manivasagam, T., Thenmozhi, A. J., Sevanan, M., Chidambaram, S. B., Essa, M. M., Guillemin, G. J. & Sakharkar, M. K. (2018) Naringenin decreases α-synuclein expression and neuroinflammation in MPTP-induced Parkinson's disease model in mice, *Neurotox Res.* **33**, 656-670.

116. Tchantchou, F., Lacor, P. N., Cao, Z., Lao, L., Hou, Y., Cui, C., Klein, W. L. & Luo, Y. (2009) Stimulation of neurogenesis and synaptogenesis by bilobalide and quercetin via common final pathway in hippocampal neurons, *J Alzheimers Dis.* **18**, 787-798.

117. Zheng, L. T., Ock, J., Kwon, B.-M. & Suk, K. (2008) Suppressive effects of flavonoid fisetin on lipopolysaccharide-induced microglial activation and neurotoxicity, *Int Immunopharmacol.* **8**, 484-494.

118. Zhuang, P. W., Cui, G. Z., Zhang, Y. J., Zhang, M. X., Guo, H., Zhang, J. B., Lu, Z. Q., Isaiah, A. O. & Lin, Y. X. (2013) Baicalin regulates neuronal fate decision in neural stem/progenitor cells and stimulates hippocampal neurogenesis in adult rats, *CNS Neurosci Ther.* **19**, 154-162.

119. Farajdokht, F., Amani, M., Bavil, F. M., Alihemmati, A., Mohaddes, G. & Babri, S. (2017) Troxerutin protects hippocampal neurons against amyloid beta-induced oxidative stress and apoptosis, *EXCLI journal.* **16**, 1081.

120. Lu, J., Wu, D.-m., Zheng, Z.-h., Zheng, Y.-l., Hu, B. & Zhang, Z.-f. (2011) Troxerutin protects against high cholesterol-induced cognitive deficits in mice, *Brain.* **134**, 783-797.

121. Zhang, S., Li, H., Zhang, L., Li, J., Wang, R. & Wang, M. (2017) Effects of troxerutin on cognitive deficits and glutamate cysteine ligase subunits in the hippocampus of streptozotocin-induced type 1 diabetes mellitus rats, *Brain Res.* **1657**, 355-360.

122. Gao, M., Kang, Y., Zhang, L., Li, H., Qu, C., Luan, X., Liu, L. & Zhang, S. (2020) Troxerutin attenuates cognitive decline in the hippocampus of male diabetic rats by inhibiting NADPH oxidase and activating the Nrf2/ARE signaling pathway, *Int J Mol Med.* **46**, 1239-1248.

123. Zhang, S., Yuan, L., Zhang, L., Li, C. & Li, J. (2018) Prophylactic use of troxerutin can delay the development of diabetic cognitive dysfunction and improve the expression of Nrf2 in the hippocampus on STZ diabetic rats, *Behav Neurol.* **2018**, 8678539.

124. Liu, F., Huang, Y. & Fu, P. (2013) Troxerutin Counteracts domoic acidinduced memory deficits in mice by inhibiting CCAAT/Enhancer binding protein ß-mediated inflammatory response and oxidative stress, *J Immunol.* **190**, 3466-3479.

125. Lu, J., Wu, D.-m., Hu, B., Cheng, W., Zheng, Y.-l., Zhang, Z.-f., Ye, Q., Fan, S.-h., Shan, Q. & Wang, Y.-j. (2010) Chronic administration of troxerutin protects mouse brain against D-galactose-induced impairment of cholinergic system, *Neurobiol Learn Mem.* **93**, 157-164.

References

126. Lu, J., Wu, D. m., Hu, B., Zheng, Y. l., Zhang, Z. f. & Wang, Y. j. (2010) NGF-Dependent activation of TrkA pathway: A mechanism for the neuroprotective effect of troxerutin in D-galactose-treated mice, *Brain Pathol.* **20**, 952-965.

127. Bayandor, P., Farajdokht, F., Mohaddes, G., Diba, R., Hosseindoost, M., Mehri, K., Zavvari Oskuye, Z. & Babri, S. (2019) The effect of troxerutin on anxiety-and depressive-like behaviours in the offspring of high-fat diet fed dams, *Arch Physiol Biochem.* **125**, 156-162.

128. Hoseindoost, M., Alipour, M. R., Farajdokht, F., Diba, R., Bayandor, P., Mehri, K., Rad, S. N. & Babri, S. (2019) Effects of troxerutin on inflammatory cytokines and BDNF levels in male offspring of high-fat diet fed rats, *Avicenna J Phytomed.* **9**, 597-605.

129. Mohammadi, M., Rahmani, M., Kaeidi, A., Bazmandegan, G., Hakimizadeh, E. & Allahtavakoli, M. (2020) Effect of Troxerotin on permanent cerebral ischemia in male mice, *J Jiroft Uni Med Sci.* **7**, 374-383.

130. Lee, K. S., Cha, H. J., Lee, G. T., Lee, K. K., Hong, J. T., Ahn, K. J., An, I.-S., An, S. & Bae, S. (2014) Troxerutin induces protective effects against ultraviolet B radiation through the alteration of microRNA expression in human HaCaT keratinocyte cells, *Int J Mol Med.* **33**, 934-942.

131. Ma, W., Wang, S., Liu, X., Tang, F., Zhao, P., Cheng, K., Zheng, Q., Zhuo, Y., Zhao, X. & Li, X. (2019) Protective effect of troxerutin and cerebroprotein hydrolysate injection on cerebral ischemia through inhibition of oxidative stress and promotion of angiogenesis in rats, *Mol Med Rep.* **19**, 3148-3158.

132. Doke, S. K. & Dhawale, S. C. (2015) Alternatives to animal testing: A review, *Saudi Pharm J.* **23**, 223-229.

133. Coady, K. K., Biever, R. C., Denslow, N. D., Gross, M., Guiney, P. D., Holbech, H., Karouna-Renier, N. K., Katsiadaki, I., Krueger, H. & Levine, S. L. (2017) Current limitations and recommendations to improve testing for the environmental assessment of endocrine active substances, *Integr Environ Assess Manag.* **13**, 302-316.

134. Wang, M.-W., Hao, X. & Chen, K. (2007) Biological screening of natural products and drug innovation in China, *Philos Trans R Soc Lond B Biol Sci.* **362**, 1093-1105.

135. Hartung, T. & Sabbioni, E. (2011) Alternative in vitro assays in nanomaterial toxicology, *Wiley Interdiscip Rev Nanomed Nanobiotechnol* **3**, 545-573.

136. Jang, Y., Kim, J.-E., Jeong, S.-H. & Cho, M.-H. (2014) Towards a strategic approaches in alternative tests for pesticide safety, *Toxicol Res.* **30**, 159-68.

137. Wang, C. (2015) Application of in vitro models in developmental neurotoxicity and pharmaceutics research, *J Mol Pharm Org Process Res.* **3**, 122-128.

138. Xu, W., Lakshman, N. & Morshead, C. M. (2017) Building a central nervous system: The neural stem cell lineage revealed, *Neurogenesis.* **4**, e1300037.

References

139. Guo, W., Patzlaff, N. E., Jobe, E. M. & Zhao, X. (2012) Isolation of multipotent neural stem or progenitor cells from both the dentate gyrus and subventricular zone of a single adult mouse, *Nat Protoc.* **7**, 2005-2012.

140. Rushing, G. & Ihrie, R. A. (2016) Neural stem cell heterogeneity through time and space in the ventricular-subventricular zone, *Front Biol (Beijing).* **11**, 261-284.

141. Zhao, X. & Moore, D. L. (2018) Neural stem cells: developmental mechanisms and disease modeling in pp. 1-6, Cell Tissue Res,

142. Dettmann, H. M., Zhang, Y., Wronna, N., Kraushaar, U., Guenther, E., Mohr, R., Neckel, P. H., Mack, A., Fuchs, J. & Just, L. (2014) Isolation, expansion and transplantation of postnatal murine progenitor cells of the enteric nervous system, *PloS one.* **9**, e97792.

143. Inta, D., Alfonso, J., von Engelhardt, J., Kreuzberg, M. M., Meyer, A. H., van Hooft, J. A. & Monyer, H. (2008) Neurogenesis and widespread forebrain migration of distinct GABAergic neurons from the postnatal subventricular zone, *Proc Natl Acad Sci U S A.* **105**, 20994-20999.

144. Ma, D. K., Bonaguidi, M. A., Ming, G.-l. & Song, H. (2009) Adult neural stem cells in the mammalian central nervous system, *Cell Res.* **19**, 672-682.

145. Preston, M. & Sherman, L. S. (2012) Neural stem cell niches: critical roles for the hyaluronan-based extracellular matrix in neural stem cell proliferation and differentiation, *Front Biosci (Schol Ed).* **3**, 1165-79.

146. Gorba, T. & Conti, L. (2013) Neural stem cells as tools for drug discovery: novel platforms and approaches, *Expert Opin Drug Discov.* **8**, 1083-1094.

147. Huang, L. & Wang, G. (2017) The effects of different factors on the behavior of neural stem cells, *Stem Cells Int.* **2017**, 9497325.

148. Tasneem, S., Farrell, K., Lee, M.-Y. & Kothapalli, C. R. (2016) Sensitivity of neural stem cell survival, differentiation and neurite outgrowth within 3D hydrogels to environmental heavy metals, *Toxicol Lett.* **242**, 9-22.

149. Shoemaker, L. D. & Kornblum, H. I. (2016) Neural stem cells (NSCs) and proteomics, *Mol Cell Proteomics.* **15**, 344-354.

150. Bixby, S., Kruger, G. M., Mosher, J. T., Joseph, N. M. & Morrison, S. J. (2002) Cell-intrinsic differences between stem cells from different regions of the peripheral nervous system regulate the generation of neural diversity, *Neuron.* **35**, 643-656.

151. Urbán, N. & Guillemot, F. (2014) Neurogenesis in the embryonic and adult brain: same regulators, different roles, *Front Cell Neurosci.* **8**, 396.

152. Kim, H.-J. (2019) Regulation of neural stem cell fate by natural products, *Biomolecules & therapeutics.* **27**, 15-24.

References

153. Liu, Y., Lacson, R., Cassaday, J., Ross, D. A., Kreamer, A., Hudak, E., Peltier, R., Mclaren, D., Muñoz-Sanjuan, I. & Santini, F. (2009) Identification of small-molecule modulators of mouse SVZ progenitor cell proliferation and differentiation through high-throughput screening, *J Biomol Screen.* **14**, 319-329.

154. Salama, M., Lotfy, A., Fathy, K., Makar, M., El-emam, M., El-gamal, A., El-gamal, M., Badawy, A., Mohamed, W. M. & Sobh, M. (2015) Developmental neurotoxic effects of Malathion on 3D neurosphere system, *Appl Transl Genom.* **7**, 13-18.

155. Saeidnia, S., Manayi, A. & Abdollahi, M. (2015) From in vitro experiments to in vivo and clinical studies; pros and cons, *Curr Drug Discov Technol.* **12**, 218-224.

156. Fritsche, E., Barenys, M., Klose, J., Masjosthusmann, S., Nimtz, L., Schmuck, M., Wuttke, S. & Tigges, J. (2018) Current availability of stem cell-based in vitro methods for Developmental Neurotoxicity (DNT) testing, *Toxicol Sci.* **165**, 21-30.

157. Poteser, M. (2017) Cell-based in vitro models in environmental toxicology: a review, *Biomonitoring.* **4**, 11-26.

158. Jannat, M., Fatimah, R. & Kishida, M. (2014) Nitrate (NO3(-)) and nitrite (NO2(-)) are endocrine disruptors to downregulate expression of tyrosine hydroxylase and motor behavior through conversion to nitric oxide in early development of zebrafish, *Biochemical and biophysical research communications.* **452**, 608-13.

159. Bal-Price, A. K., Hogberg, H. T., Buzanska, L., Lenas, P., van Vliet, E. & Hartung, T. (2010) In vitro developmental neurotoxicity (DNT) testing: relevant models and endpoints, *Neurotoxicol.* **31**, 545-554.

160. Celiz, M. D., Pérez, S., Barceló, D. & Aga, D. S. (2009) Trace analysis of polar pharmaceuticals in wastewater by LC-MS-MS: Comparison of membrane bioreactor and activated sludge systems, *J Chromatogr Sci.* **47**, 19-25.

161. Yu, Y., Wu, B., Jiang, L., Zhang, X.-X., Ren, H.-Q. & Li, M. (2019) Comparative analysis of toxicity reduction of wastewater in twelve industrial park wastewater treatment plants based on battery of toxicity assays, *Sci Rep.* **9**, 3751.

162. Etteieb, S., Kawachi, A., Han, J., Tarhouni, J. & Isoda, H. (2015) Bioanalytical tests for assessing cytotoxicity and estrogenicity effects of treated wastewater on mammalian cell lines, *Energy Procedia.* **74**, 878-885.

163. Makene, V. W., Tijani, J. O., Petrik, L. F. & Pool, E. J. (2016) Evaluation of cytotoxicity and inflammatory activity of wastewater collected from a textile factory before and after treatment by coagulation-flocculation methods, *Environ Monit Assess.* **188**, 471.

164. Bergström, T. & Forsberg-Nilsson, K. (2012) Neural stem cells: brain building blocks and beyond, *Ups J Med Sci.* **117**, 132-142.

References

165. Masood, M. I., Hauke, N. T., Nasim, M. J., Sarfraz, M., Naseem, M. & Schäfer, K. H. (2021) Neural stem cell-based in vitro bioassay for the assessment of neurotoxic potential of water samples, *J Environ Sci.* **101**, 72-86.

166. Euractive (2018, September 24) Mercury in the water: 40% of Europe's lakes and rivers contaminated in

167. Karlsson, A. S., Lesch, M., Weihermüller, L., Thiele, B., Disko, U., Hofmann, D., Vereecken, H. & Spielvogel, S. (2020) Pesticide contamination of the upper Elbe River and an adjacent floodplain area, *J Soils Sediments*, 2067-2081.

168. Tseng, I.-L., Yang, Y.-F., Yu, C.-W., Li, W.-H. & Liao, V. H.-C. (2013) Phthalates induce neurotoxicity affecting locomotor and thermotactic behaviors and AFD neurons through oxidative stress in Caenorhabditis elegans, *PLoS one.* **8**, e82657.

169. Gorini, F., Muratori, F. & Morales, M. A. (2014) The role of heavy metal pollution in neurobehavioral disorders: a focus on autism, *Rev JAutism Dev Disord.* **1**, 354-372.

170. Bose, R., Onishchenko, N., Edoff, K., Janson Lang, A. M. & Ceccatelli, S. (2012) Inherited effects of low-dose exposure to methylmercury in neural stem cells, *Toxicol Sci.* **130**, 383-390.

171. Tiwari, S. K., Agarwal, S., Seth, B., Yadav, A., Ray, R. S., Mishra, V. N. & Chaturvedi, R. K. (2015) Inhibitory effects of bisphenol-A on neural stem cells proliferation and differentiation in the rat brain are dependent on Wnt/β-catenin pathway, *Mol Neurobiol.* **52**, 1735-1757.

172. Parihar, V. K., Hattiangady, B., Shuai, B. & Shetty, A. K. (2013) Mood and memory deficits in a model of Gulf War illness are linked with reduced neurogenesis, partial neuron loss, and mild inflammation in the hippocampus, *Neuropsychopharmacology.* **38**, 2348-2362.

173. Launay, M., Kuch, B., Dittmer, U. & Steinmetz, H. (2013) Occurrence of organic pollutants in treated wastewater effluent and surface waters under the influence of urban catchment areas, *ISTANBUL3WCONGRESS 2013*, 152.

174. Loos, R., Carvalho, R., António, D. C., Comero, S., Locoro, G., Tavazzi, S., Paracchini, B., Ghiani, M., Lettieri, T. & Blaha, L. (2013) EU-wide monitoring survey on emerging polar organic contaminants in wastewater treatment plant effluents, *Water Res* **47**, 6475-6487.

175. Reif, A., Fritzen, S., Finger, M., Strobel, A., Lauer, M., Schmitt, A. & Lesch, K. (2006) Neural stem cell proliferation is decreased in schizophrenia, but not in depression, *Mol Psychiatry.* **11**, 514-522.

176. Anderl, J. L., Redpath, S. & Ball, A. J. (2009) A neuronal and astrocyte co-culture assay for high content analysis of neurotoxicity, *JoVE (Journal of Visualized Experiments)*, e1173.

177. Jiang, D., Lim, S., Kwak, M., Ryu, Y. K. & Mintz, C. D. (2015) The changes of oligodendrocytes induced by anesthesia during brain development, *Neural regeneration research.* **10**, 1386.

References

178. Tamm, C., Duckworth, J., Hermanson, O. & Ceccatelli, S. (2006) High susceptibility of neural stem cells to methylmercury toxicity: effects on cell survival and neuronal differentiation, *J Neurochem.* **97**, 69-78.

179. Cong, W.-n., Wang, R., Cai, H., Daimon, C. M., Scheibye-Knudsen, M., Bohr, V. A., Turkin, R., Wood III, W. H., Becker, K. G. & Moaddel, R. (2013) Long-term artificial sweetener acesulfame potassium treatment alters neurometabolic functions in C57BL/6J mice, *PLoS One.* **8**, e70257.

180. Christen, V., Rusconi, M., Crettaz, P. & Fent, K. (2017) Developmental neurotoxicity of different pesticides in PC-12 cells in vitro, *Toxicol Appl Pharmacol.* **325**, 25-36.

181. Richardson, J. R., Caudle, W. M., Wang, M., Dean, E. D., Pennell, K. D., Miller, G. W., Richardson, J. R., Caudle, W. M., Wang, M. & Dean, E. D. (2006) Developmental exposure to the pesticide dieldrin alters the dopamine system and increases neurotoxicity in an animal model of Parkinson's disease, *FASEB J.* **20**, 1695-1697.

182. You, M., Dong, J., Fu, Y., Cong, Z., Fu, H., Wei, L., Wang, Y., Wang, Y. & Chen, J. (2018) Exposure to Di-(2-ethylhexyl) phthalate during perinatal period gender-specifically impairs the dendritic growth of pyramidal neurons in rat offspring, *Front Neurosci.* **12**, 444.

183. Gerhardt, A. (2019) Plastic Additive Bisphenol A: Toxicity in Surface-and Groundwater Crustaceans, *J Toxicol Risk Assess.* **5**, 017.

184. Loos, R., Tavazzi, S., Mariani, G., Suurkuusk, G., Paracchini, B. & Umlauf, G. (2017) Analysis of emerging organic contaminants in water, fish and suspended particulate matter (SPM) in the Joint Danube Survey using solid-phase extraction followed by UHPLC-MS-MS and GC–MS analysis, *Sci Total Environ.* **607**, 1201-1212.

185. Scheurer, M., Brauch, H.-J. & Lange, F. T. (2009) Analysis and occurrence of seven artificial sweeteners in German waste water and surface water and in soil aquifer treatment (SAT), *Anal Bioanal Chem.* **394**, 1585-1594.

186. Koyama, Y. (2015) Functional alterations of astrocytes in mental disorders: pharmacological significance as a drug target, *Front Cell Neurosci.* **9**, 261.

187. Galindo, L. T., Mundim, M. T., Pinto, A. S., Chiarantin, G. M., Almeida, M. E., Lamers, M. L., Horwitz, A. R., Santos, M. F. & Porcionatto, M. (2018) Chondroitin sulfate impairs neural stem cell migration through ROCK activation, *Mol Neurobiol.* **55**, 3185-3195.

188. Gbondo-Tugbawa, S. S., McAlear, J. A., Driscoll, C. T. & Sharpe, C. W. (2010) Total and methyl mercury transformations and mass loadings within a wastewater treatment plant and the impact of the effluent discharge to an alkaline hypereutrophic lake, *Water Res.* **44**, 2863-2875.

References

189. Mohapatra, D. P., Brar, S. K., Tyagi, R. D. & Surampalli, R. Y. (2011) Occurrence of bisphenol A in wastewater and wastewater sludge of CUQ treatment plant, *J Xenobiotic.* **1**, 9-16.

190. Pauwels, B., Noppe, H., De Brabander, H. & Verstraete, W. (2008) Comparison of steroid hormone concentrations in domestic and hospital wastewater treatment plants, *J Environ Eng.* **134**, 933-936.

191. Aronson, D., Weeks, J., Meylan, B., Guiney, P. D. & Howard, P. H. (2012) Environmental release, environmental concentrations, and ecological risk of N, N-diethyl-m-toluamide (DEET), *Integr Environ Assess Manag.* **8**, 135-166.

192. Stouffer, M. A., Golden, J. A. & Francis, F. (2016) Neuronal migration disorders: focus on the cytoskeleton and epilepsy, *Neurobiol Dis.* **92**, 18-45.

193. Masood, M. I., Naseem, M., Warda, S. A., Tapia-Laliena, M. A., Rehman, H., Nasim, M. J. & Schäfer, K. H. (2021) Environment permissible concentrations of glyphosate in drinking water can influence the fate of neural stem cells from the subventricular zone of the postnatal mouse, *Environ Pollut.* **270**, 116179.

194. Aris, A. & Leblanc, S. (2011) Maternal and fetal exposure to pesticides associated to genetically modified foods in Eastern Townships of Quebec, Canada, *Reprod Toxicol.* **31**, 528-533.

195. Kwiatkowska, M., Jarosiewicz, P., Michałowicz, J., Koter-Michalak, M., Huras, B. & Bukowska, B. (2016) The impact of glyphosate, its metabolites and impurities on viability, ATP level and morphological changes in human peripheral blood mononuclear cells, *PLoS One.* **11**, e0156946.

196. Cavalli, V. L. d. L. O., Cattani, D., Rieg, C. E. H., Pierozan, P., Zanatta, L., Parisotto, E. B., Wilhelm Filho, D., Silva, F. R. M. B., Pessoa-Pureur, R. & Zamoner, A. (2013) Roundup disrupts male reproductive functions by triggering calcium-mediated cell death in rat testis and Sertoli cells, *Free Radic Biol Med.* **65**, 335-346.

197. Ham, J., Lim, W., Whang, K.-Y. & Song, G. (2020) Butylated hydroxytoluene induces dysregulation of calcium homeostasis and endoplasmic reticulum stress resulting in mouse Leydig cell death, *Environ Pollut* **256**, 113421.

198. Tseng, I.-L., Yang, Y.-F., Yu, C.-W., Li, W.-H. & Liao, V. H.-C. (2014) Correction: Phthalates Induce Neurotoxicity Affecting Locomotor and Thermotactic Behaviors and AFD Neurons through Oxidative Stress in Caenorhabditis elegans, *PloS one.* **9**, e99945.

199. Li, Q., Lambrechts, M. J., Zhang, Q., Liu, S., Ge, D., Yin, R., Xi, M. & You, Z. (2013) Glyphosate and AMPA inhibit cancer cell growth through inhibiting intracellular glycine synthesis, *Drug Des Devel Ther.* **7**, 635-43.

References

200. Thongprakaisang, S., Thiantanawat, A., Rangkadilok, N., Suriyo, T. & Satayavivad, J. (2013) Glyphosate induces human breast cancer cells growth via estrogen receptors, *Food Chem Toxicol.* **59**, 129-136.

201. Jeon, S., Lee, S.-H., Roh, J., Kim, J.-E. & Bunch, H. (2020) Glyphosate influences cell proliferation in vitro, *All Life.* **13**, 54-65.

202. Abranches, E., O'Neill, A., Robertson, M. J., Schaffer, D. V. & Cabral, J. M. (2006) Development of quantitative PCR methods to analyse neural progenitor cell culture state, *Biotechnol Appl Biochem.* **44**, 1-8.

203. Park, K.-Y. & Man, S. K. (2018) Inhibition of Proliferation and Neurogenesis of Mouse Subventricular Zone Neural Stem Cells by a Mitochondrial Inhibitor Rotenone, *J Life Sci.* **28**, 1397-1405.

204. Seth, B., Yadav, A., Agarwal, S., Tiwari, S. K. & Chaturvedi, R. K. (2017) Inhibition of the transforming growth factor-β/SMAD cascade mitigates the anti-neurogenic effects of the carbamate pesticide carbofuran, *J Biol Chem.* **292**, 19423-19440.

205. Ramírez-Duarte, W. F., Rondón-Barragán, I. S. & Eslava-Mocha, P. R. (2008) Acute toxicity and histopathological alterations of Roundup® herbicide on" cachama blanca"(Piaractus brachypomus), *Braz J Vet Res.* **28**, 547-554.

206. Neto da Silva, K., Garbin Cappellaro, L., Ueda, C. N., Rodrigues, L., Pertile Remor, A., Martins, R. d. P., Latini, A. & Glaser, V. (2020) Glyphosate-based herbicide impairs energy metabolism and increases autophagy in C6 astroglioma cell line, *J Toxicol Environ Health A.* **83**, 153-167.

207. Baumann, J., Barenys, M., Gassmann, K. & Fritsche, E. (2014) Comparative human and rat "neurosphere assay" for developmental neurotoxicity testing, *Curr Protoc Toxicolo.* **59**, 12.21.1-24.

208. Beecham, J. E. & Seneff, S. (2016) Is there a link between autism and glyphosate-formulated herbicides?, *J Autism.* **3**, 1.

209. Degl'Innocenti, D., Ramazzotti, M., Sarchielli, E., Monti, D., Chevanne, M., Vannelli, G. B. & Barletta, E. (2019) Oxadiazon affects the expression and activity of aldehyde dehydrogenase and acylphosphatase in human striatal precursor cells: A possible role in neurotoxicity, *Toxicology.* **411**, 110-121.

210. Herzine, A., Laugeray, A., Feat, J., Menuet, A., Quesniaux, V., Richard, O., Pichon, J., Montécot-Dubourg, C., Perche, O. & Mortaud, S. (2016) Perinatal exposure to glufosinate ammonium herbicide impairs neurogenesis and neuroblast migration through cytoskeleton destabilization, *Front Cell Neurosci* **10**, 191.

References

211. Hogberg, H. T., Kinsner-Ovaskainen, A., Coecke, S., Hartung, T. & Bal-Price, A. K. (2010) mRNA expression is a relevant tool to identify developmental neurotoxicants using an in vitro approach, *Toxicol Sci.* **113**, 95-115.

212. Betancourt, A. M., Burgess, S. C. & Carr, R. L. (2006) Effect of developmental exposure to chlorpyrifos on the expression of neurotrophin growth factors and cell-specific markers in neonatal rat brain, *Toxicol Sci.* **92**, 500-506.

213. Wang, D. D. & Bordey, A. (2008) The astrocyte odyssey, *Prog Neurobiol.* **86**, 342-367.

214. Ohtaki, N., Kamitani, W., Watanabe, Y., Hayashi, Y., Yanai, H., Ikuta, K. & Tomonaga, K. (2007) Downregulation of an astrocyte-derived inflammatory protein, S100B, reduces vascular inflammatory responses in brains persistently infected with Borna disease virus, *J Virol.* **81**, 5940-5948.

215. Ghosh, C., Hossain, M., Solanki, J., Dadas, A., Marchi, N. & Janigro, D. (2016) Pathophysiological implications of neurovascular P450 in brain disorders, *Drug Discov Today.* **21**, 1609-1619.

216. Liu, J., Sridhar, J. & Foroozesh, M. (2013) Cytochrome P450 family 1 inhibitors and structure-activity relationships, *Molecules.* **18**, 14470-14495.

217. Cai, W., Zhang, F., Zhong, L., Chen, D., Guo, H., Zhang, H., Zhu, B. & Liu, X. (2020) Correlation between CYP1A1 polymorphisms and susceptibility to glyphosate-induced reduction of serum cholinesterase: A case-control study of a Chinese population, *Pestic Biochem Physiol.* **162**, 23-28.

218. Heymann, A.-K., Schnabel, K., Billenkamp, F., Bühler, S., Frahm, J., Kersten, S., Hüther, L., Meyer, U., von Soosten, D. & Trakooljul, N. (2021) Effects of glyphosate residues and different concentrate feed proportions in dairy cow rations on hepatic gene expression, liver histology and biochemical blood parameters, *Plos one.* **16**, e0246679.

219. Larsen, K., Najle, R., Lifschitz, A., Maté, M. L., Lanusse, C. & Virkel, G. L. (2014) Effects of sublethal exposure to a glyphosate-based herbicide formulation on metabolic activities of different xenobiotic-metabolizing enzymes in rats, *Int J Toxicol.* **33**, 307-318.

220. Xia, Y., Yang, X., Lu, J., Xie, Q., Ye, A. & Sun, W. (2020) The endoplasmic reticulum stress and related signal pathway mediated the glyphosate-induced testosterone synthesis inhibition in TM3 cells, *Environ Poll.* **260**, 113949.

221. Wang, Y., Branicky, R., Noë, A. & Hekimi, S. (2018) Superoxide dismutases: Dual roles in controlling ROS damage and regulating ROS signaling, *J Cell Biol.* **217**, 1915-1928.

222. Estácio, S. G., Leal, S. S., Cristóvão, J. S., Faísca, P. F. & Gomes, C. M. (2015) Calcium binding to gatekeeper residues flanking aggregation-prone segments underlies non-fibrillar amyloid traits in superoxide dismutase 1 (SOD1), *Biochim Biophys Acta.* **1854**, 118-126.

References

223. Milani, P., Gagliardi, S., Cova, E. & Cereda, C. (2011) SOD1 transcriptional and posttranscriptional regulation and its potential implications in ALS, *Neurol Res Int.* **2011**, 458427.

224. Rosen, D. R., Siddique, T., Patterson, D., Figlewicz, D. A., Sapp, P., Hentati, A., Donaldson, D., Goto, J., O'Regan, J. P. & Deng, H.-X. (1993) Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis, *Nature.* **362**, 59-62.

225. Sea, K., Sohn, S. H., Durazo, A., Sheng, Y., Shaw, B. F., Cao, X., Taylor, A. B., Whitson, L. J., Holloway, S. P. & Hart, P. J. (2015) Insights into the role of the unusual disulfide bond in copper-zinc superoxide dismutase, *J Biol Chem.* **290**, 2405-2418.

226. Tang, J., Hu, P., Li, Y., Win-Shwe, T.-T. & Li, C. (2017) Ion Imbalance Is Involved in the Mechanisms of Liver Oxidative Damage in Rats Exposed to Glyphosate, *Front Physiol.* **8**, 1083.

227. Chandran, R. & Abrahamse, H. (2020) Identifying plant-based natural medicine against oxidative stress and neurodegenerative disorders, *Oxid Med Cell Longev.* **2020**.

228. Okano, H. (2010) Neural stem cells and strategies for the regeneration of the central nervous system, *Proc Jpn Acad Ser B Phys Biol Sci.* **86**, 438-450.

229. Masood, M. I., Schäfer, K. H., Naseem, M., Weyland, M. & Meiser, P. (2020) Troxerutin flavonoid has neuroprotective properties and increases neurite outgrowth and migration of neural stem cells from the subventricular zone, *PloS one.* **15**, e0237025.

230. Messam, C. A., Hou, J. & Major, E. O. (2000) Coexpression of nestin in neural and glial cells in the developing human CNS defined by a human-specific anti-nestin antibody, *Exp Neurol.* **161**, 585-596.

231. Zalfa, C., Grasselli, C., Santilli, G., Ferrari, D. & Lamorte, G. (2018) GFAP Delta as Divergent Marker of Human Glial Progenitors, *Stem Cell Res Ther.* **8**, 2.

232. Duan, C. L., Liu, C. W., Shen, S. W., Yu, Z., Mo, J. L., Chen, X. H. & Sun, F. Y. (2015) Striatal astrocytes transdifferentiate into functional mature neurons following ischemic brain injury, *Glia.* **63**, 1660-1670.

233. Malatesta, P., Appolloni, I. & Calzolari, F. (2008) Radial glia and neural stem cells, *Cell Tissue Res.* **331**, 165-178.

234. Arsenijevic, Y., Weiss, S., Schneider, B. & Aebischer, P. (2001) Insulin-like growth factor-I is necessary for neural stem cell proliferation and demonstrates distinct actions of epidermal growth factor and fibroblast growth factor-2, *J Neurosci.* **21**, 7194-7202.

235. Ming-Ming, C., Zhi-Qi, Y., ZHANG, L.-Y. & Hong, L. (2015) Quercetin promotes neurite growth through enhancing intracellular cAMP level and GAP-43 expression, *Chin J Nat Med.* **13**, 667-672.

References

236. Shan, Q., Zhuang, J., Zheng, G., Zhang, Z., Zhang, Y., Lu, J. & Zheng, Y. (2017) Troxerutin reduces kidney damage against BDE-47-induced apoptosis via inhibiting NOX2 activity and increasing Nrf2 activity, *Oxid Med Cell Longev.* **2017**, 6034692.

237. Irwin, K., Sexton, C., Daniel, T., Lawlor, B. & Naci, L. (2018) Healthy Aging and Dementia: Two Roads Diverging in Midlife?, *Front Aging Neurosci.* **10**.

238. Zuo, L., Hemmelgarn, B. T., Chuang, C.-C. & Best, T. M. (2015) The role of oxidative stress-induced epigenetic alterations in amyloid-β production in Alzheimer's disease, *Oxid Med Cell Longev.* **2015**, 604658.

239. Kurz, C., Ungerer, I., Lipka, U., Kirr, S., Schütt, T., Eckert, A., Leuner, K. & Müller, W. (2010) The metabolic enhancer piracetam ameliorates the impairment of mitochondrial function and neurite outgrowth induced by ß-amyloid peptide, *Br J Pharmacol.* **160**, 246-257.

240. Babri, S., Mohaddes, G., Feizi, I., Mohammadnia, A., Niapour, A., Alihemmati, A. & Amani, M. (2014) Effect of troxerutin on synaptic plasticity of hippocampal dentate gyrus neurons in a β-amyloid model of Alzheimer′ s disease: an electrophysiological study, *Eur J Pharmacol.* **732**, 19-25.

241. Ansari, M. A., Abdul, H. M., Joshi, G., Opii, W. O. & Butterfield, D. A. (2009) Protective effect of quercetin in primary neurons against Aβ (1–42): relevance to Alzheimer's disease, *J Nutr Biochem.* **20**, 269-275.

242. Lu, J., Wu, D.-m., Zheng, Y.-l., Hu, B., Cheng, W., Zhang, Z.-f. & Li, M.-q. (2013) Troxerutin counteracts domoic acid–induced memory deficits in mice by inhibiting CCAAT/enhancer binding protein β–mediated inflammatory response and oxidative stress, *J Immunol.* **190**, 3466-3479.

243. Phan, H. T., Samarat, K., Takamura, Y., Azo-Oussou, A. F., Nakazono, Y. & Vestergaard, M. d. C. (2019) Polyphenols Modulate Alzheimer's Amyloid Beta Aggregation in a Structure-Dependent Manner, *Nutrients.* **11**, 756.

244. Wang, S.-w., Wang, Y.-J., Su, Y.-j., Zhou, W.-w., Yang, S.-g., Zhang, R., Zhao, M., Li, Y.-n., Zhang, Z.-p. & Zhan, D.-w. (2012) Rutin inhibits β-amyloid aggregation and cytotoxicity, attenuates oxidative stress, and decreases the production of nitric oxide and proinflammatory cytokines, *Neurotoxicology.* **33**, 482-490.

245. Xin, X., Zhang, M., Li, X., Lai, F. & Zhao, G. (2018) Biocatalytic synthesis of acylated derivatives of troxerutin: their bioavailability and antioxidant properties in vitro, *Microb Cell Fact.* **17**, 130.

7. Supplementary material:

7.1. Supplementary material for Publication 1: Neural stem cell-based *in vitro* bioassay for the assessment of neurotoxic potential of water samples.

Supplementary Data

Neural stem cell-based *in vitro* bioassay for the assessment of neurotoxic potential of water samples

Muhammad Irfan Masood[1,3,5,*], Natalie Tamara Hauke[2], Muhammad Jawad Nasim[1], Muhammad Sarfraz[1], Mahrukh Naseem[6], Karl Herbert Schäfer[3,*]

1. Division of Bioorganic Chemistry, School of Pharmacy, Saarland University, D-66123 Saarbrücken, Germany
2. Applied Life Sciences, University of Applied Sciences Kaiserslautern, 66482 Zweibrücken, Germany
3. ENS Group, University of Applied Sciences Kaiserslautern, 66482 Zweibrücken, Germany
4. Abwasserverband Bergstrasse, Altau 10, 69469 Weinheim, Germany
5. Institute of Pharmaceutical Sciences, University of Veterinary and Animal Sciences, 54000 Lahore, Pakistan
6. Department of Zoology, University of Balochistan, Quetta 87550, Pakistan

[*]Corresponding authors. E-mail:Irfan_masood_79@yahoo.com(Muhammad Irfan Masood); karlherbert.schaefer@hs-kl.de (Karl-Herbert Schäfer).

Supporting method

Characterization of pesticides impurities in water samples by LC/GC-MS/MS

Pesticides diethyltoluamide (DEET), mecoprop and terbutryn in water samples were characterized by LC-MS/MS (liquid chromatography) and dieldrin was characterized by a GC-MS/MS (gas chromatography). Water samples were sent to an analytical firm "Limbach Analytics GmbH, Mannheim laboratory, Edwin Reis Strasse.6-10, 68229, Mannheim, Company headquarters. Mannheim District court: HRB 720967, Managing director, Dr Gerold Appelt, Dr Jürgen Grochowski" with analysis reference number: 17-10360 date 13.07.2017. The complete analysis and interpretation of the data were done by the firm. For LC-MS/MS analysis, the chromatographic separation was carried out by using SunShell C18 CoreShell (2.6 µm, 2.1 i.d. x 50 mm) column through the Shimadzu Nexera2-HPLC system (Shimadzu, Japan) under gradient program. Eluent A was composed of 0.1% formic acid in water and Eluent B of (0.5% formic acid and 20mmol/L ammonium format in methanol).The analytes were quantified by ABSciex Triple Quad 5500-MS mass spectrometer (AB Sciex corporation, USA). For GC-MS/MS analysis, the chromatographic separation was carried out by GC column DB-35MS UI (30 m × 0.18 mm × 0.18 µm film) through the Agilent 7890 GC system and analytes were quantified by Agilent 7010C Triple Quad mass spectrometer (Agilent, USA). Hydrogen gas was used as a mobile phase. Standard solutions of dieldrin, DEET and terbutryn contained 100ng/L of the each pesticide while standard solution of mecoprop contained 50 ng/L.

Supporting figures

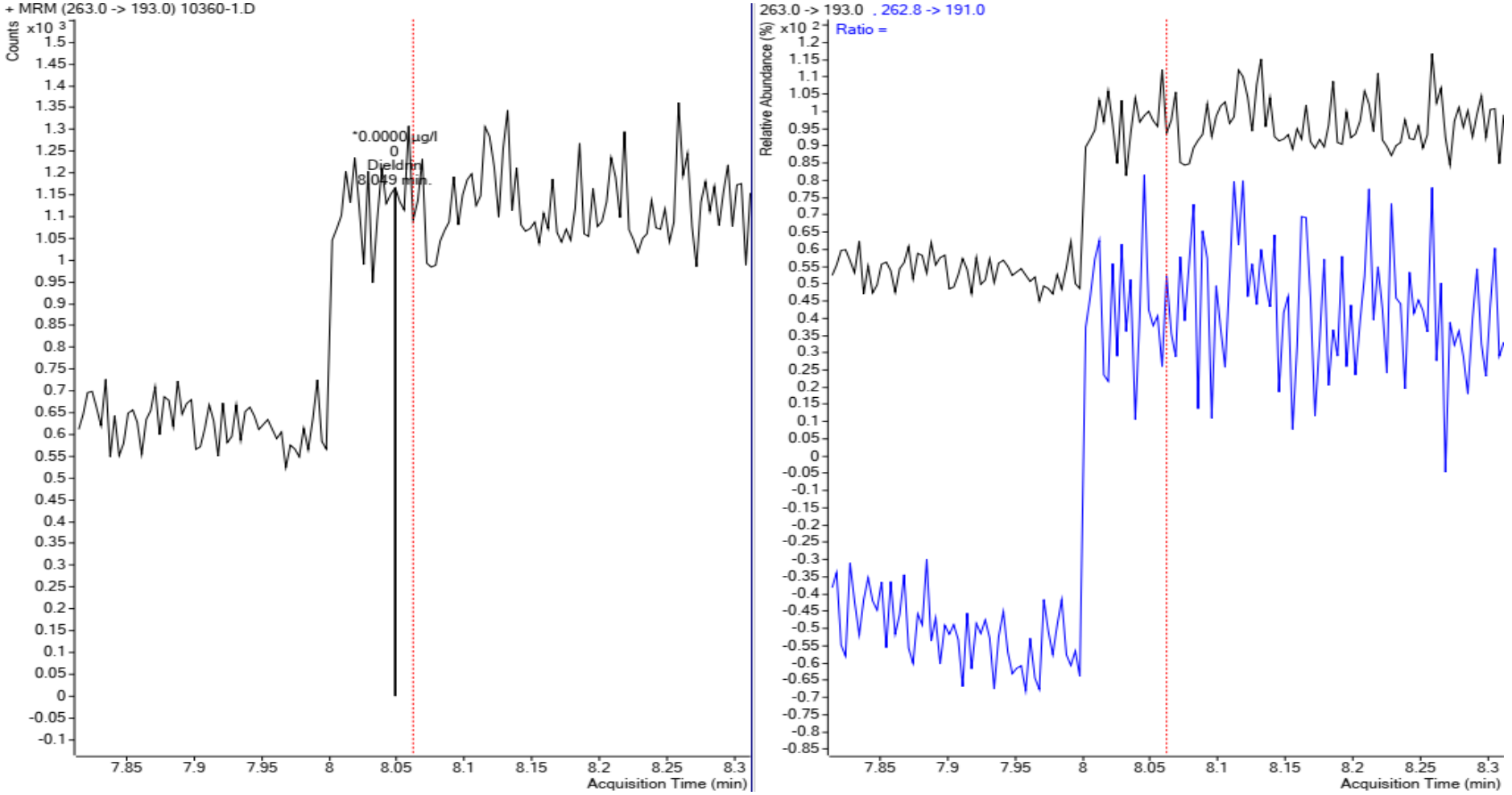


Fig. S1 Ion chromatogram of pesticide dieldrin in RWW. Signal intensity was measured as cps (count per second) along y-axis against the time in min along the x-axis. RWW: raw wastewater.

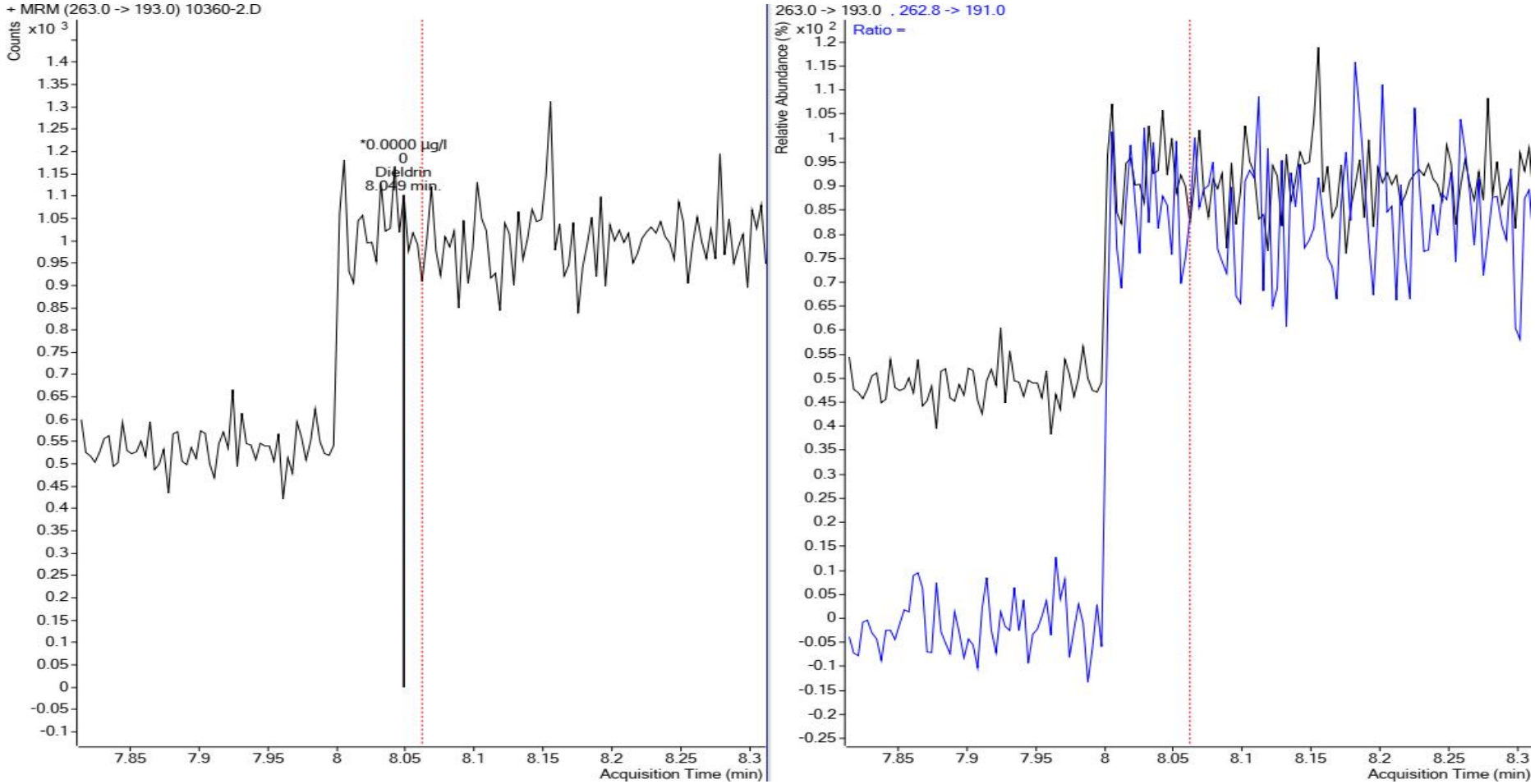


Fig. S2 Ion chromatogram of pesticide dieldrin in TSW. Signal intensity was measured as cps (count per second) along y-axis against the time in min along the x-axis. TSW: treated sewage water.

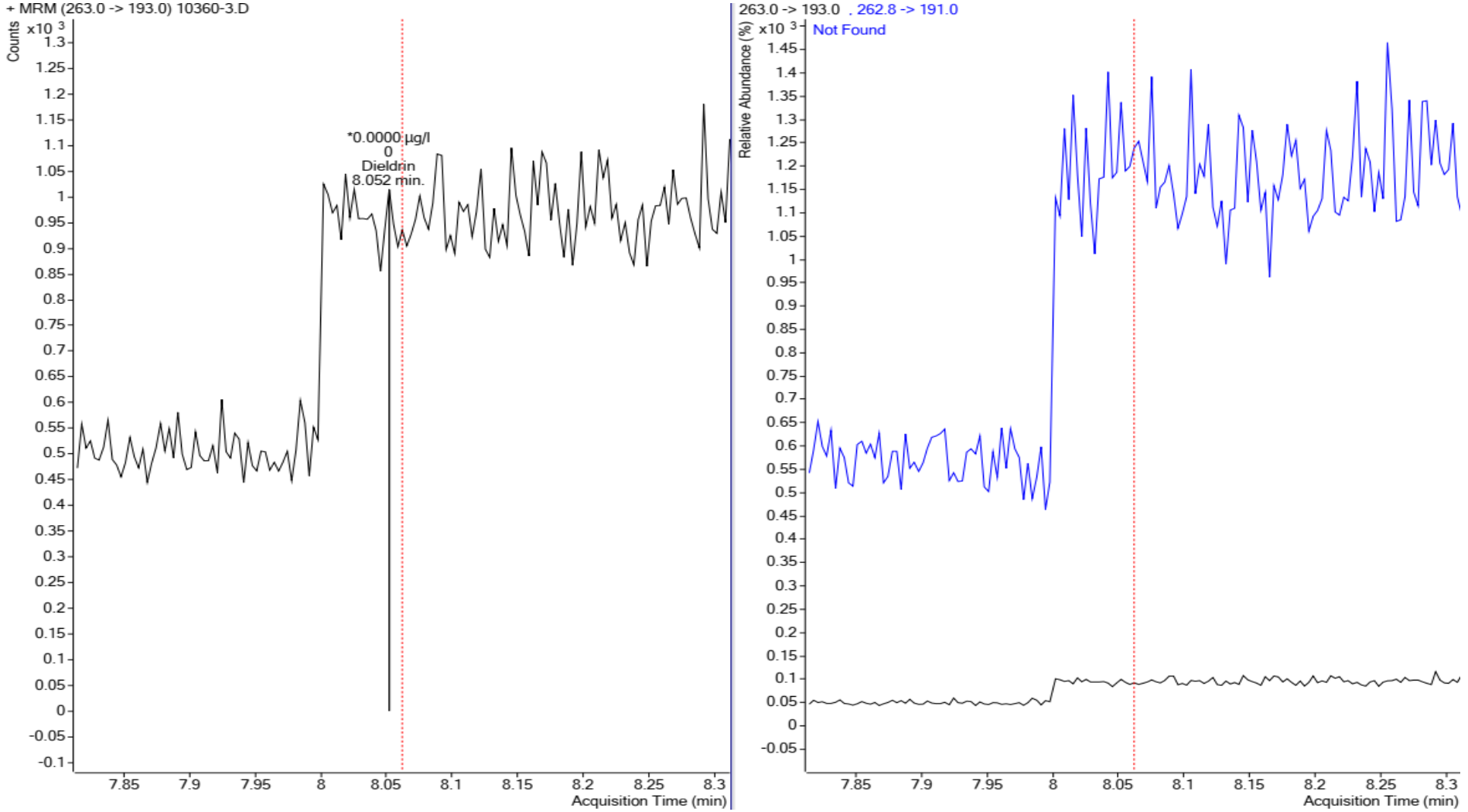


Fig. S3 Ion chromatogram of pesticide dieldrin in GW. Signal intensity was measured as cps (count per second) along y-axis against the time in min along the x-axis. GW: groundwater.

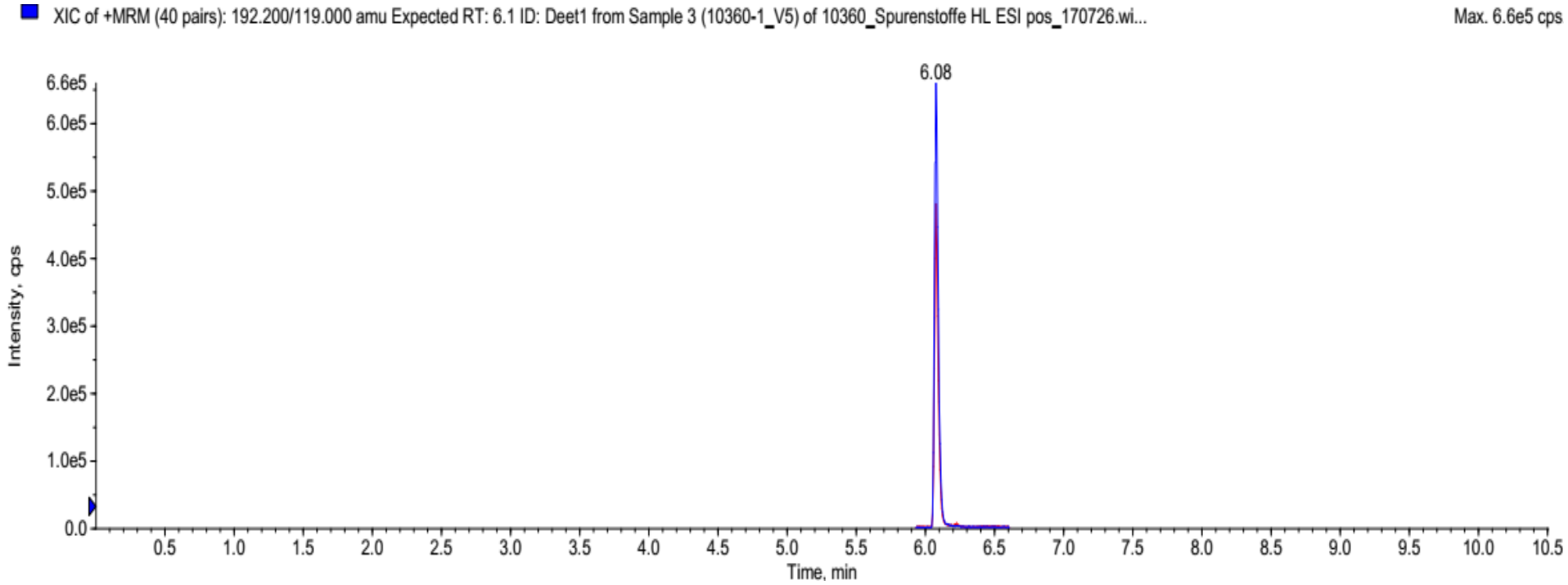


Fig. S4 Ion chromatogram of pesticide DEET in RWW. Signal intensity was measured as cps (count per second) along y-axis against the time in min along the x-axis.

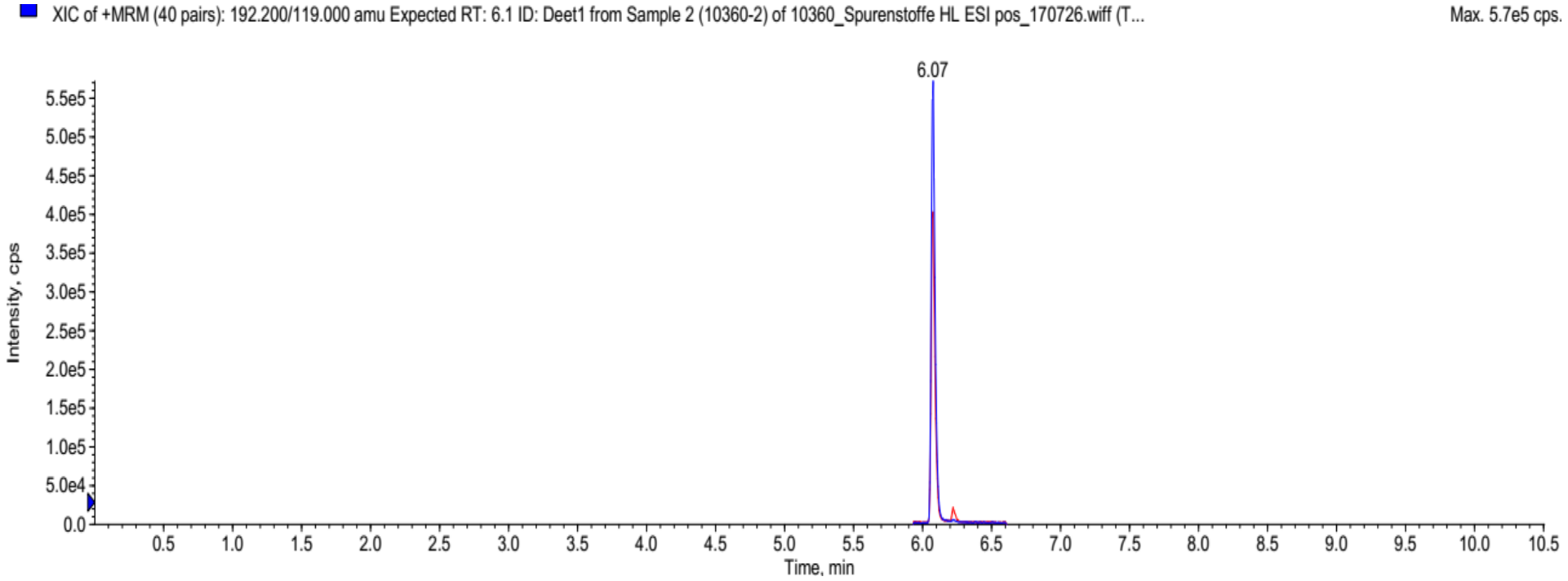


Fig. S5 Ion chromatogram of pesticide DEET in TSW. Signal intensity was measured as cps (count per second) along y-axis against the time in min along the x-axis.

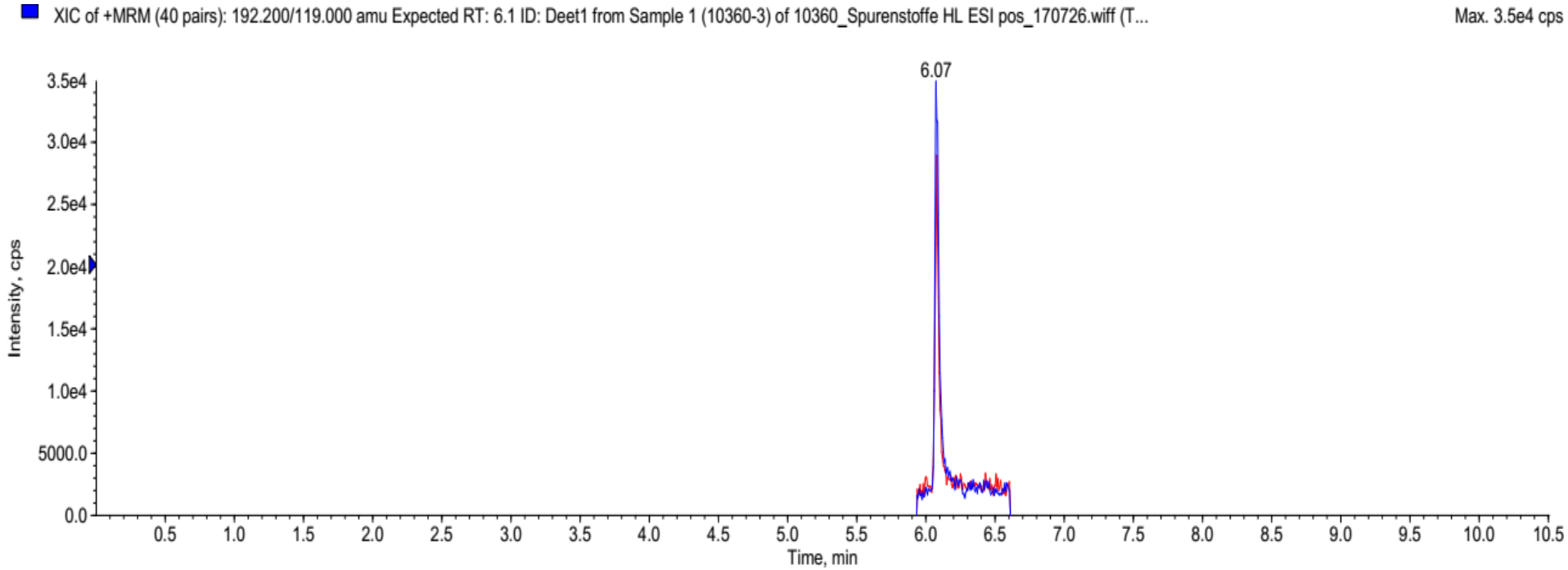


Fig. S6 Ion chromatogram of pesticide DEET in GW. Signal intensity was measured as cps (count per second) along y-axis against the time in min along the x-axis.

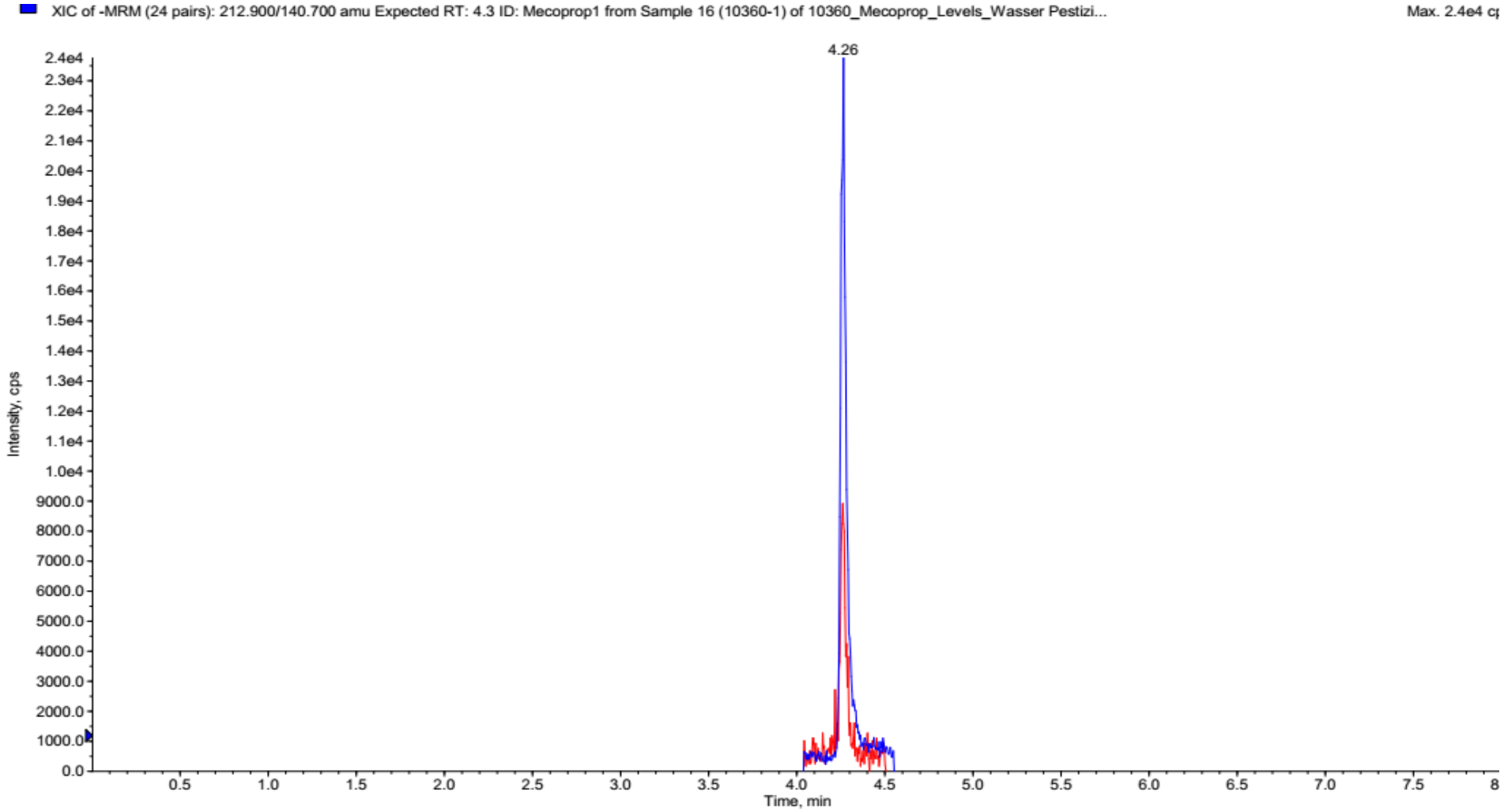


Fig. S7 Ion chromatogram of pesticide Mecoprop in RWW. Signal intensity was measured as cps (count per second) along y-axis against the time in min along the x-axis.

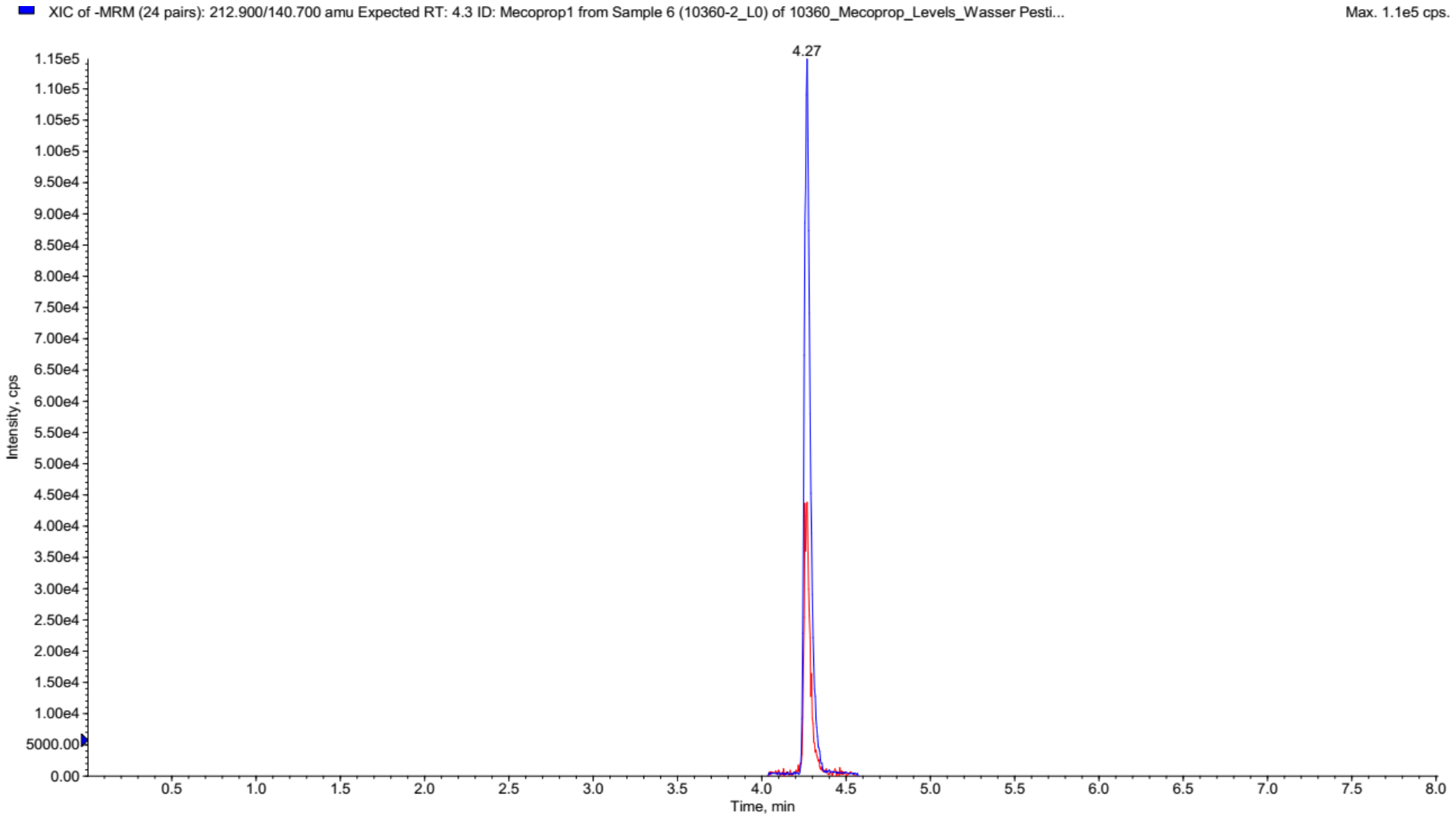


Fig. S8 Ion chromatogram of pesticide Mecoprop in TSW. Signal intensity was measured as cps (count per second) along y-axis against the time in min along the x-axis.

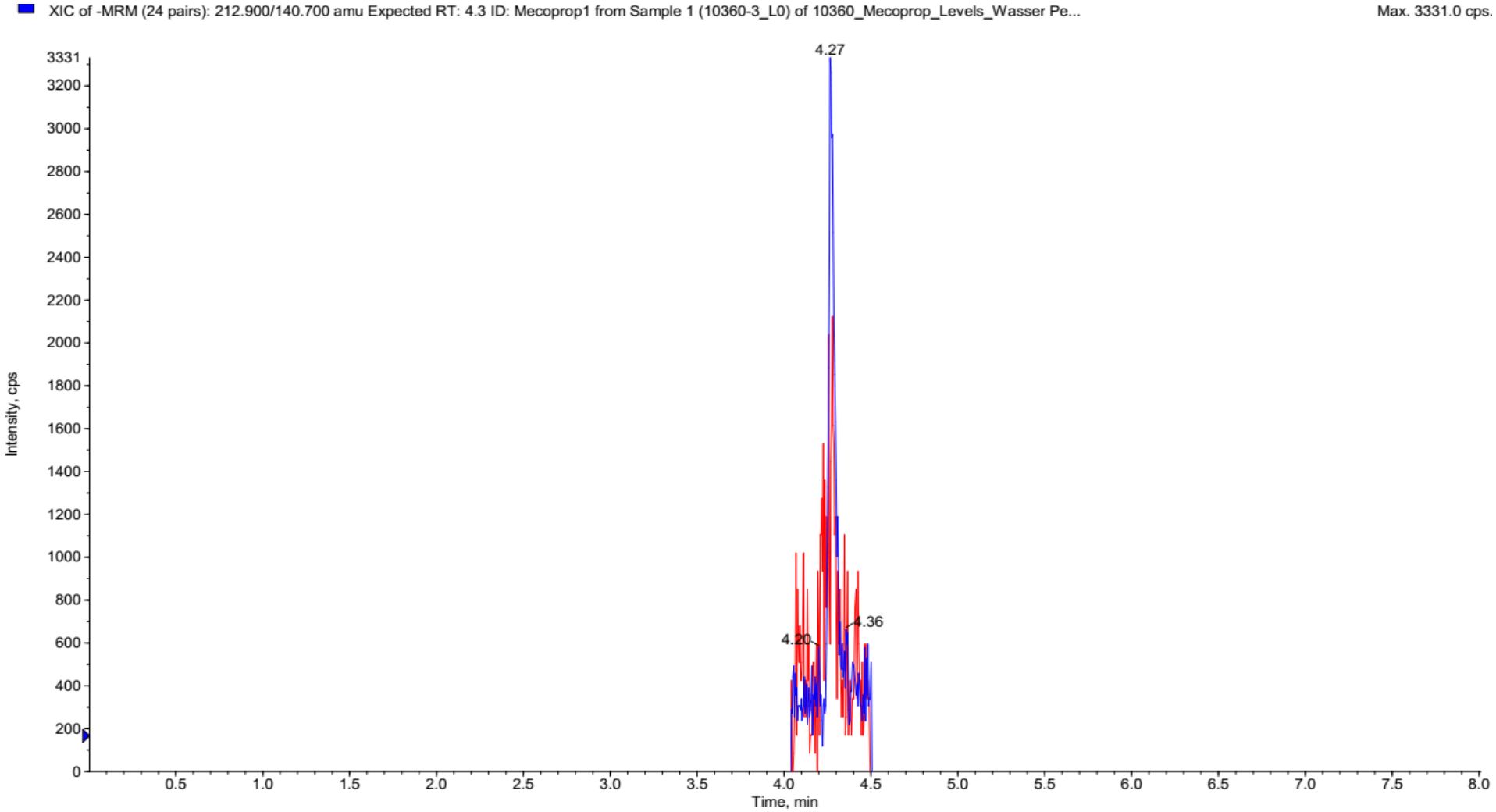


Fig. S9 Ion chromatogram of pesticide Mecoprop in GW. Signal intensity was measured as cps (count per second) along y-axis against the time in min along the x-axis.

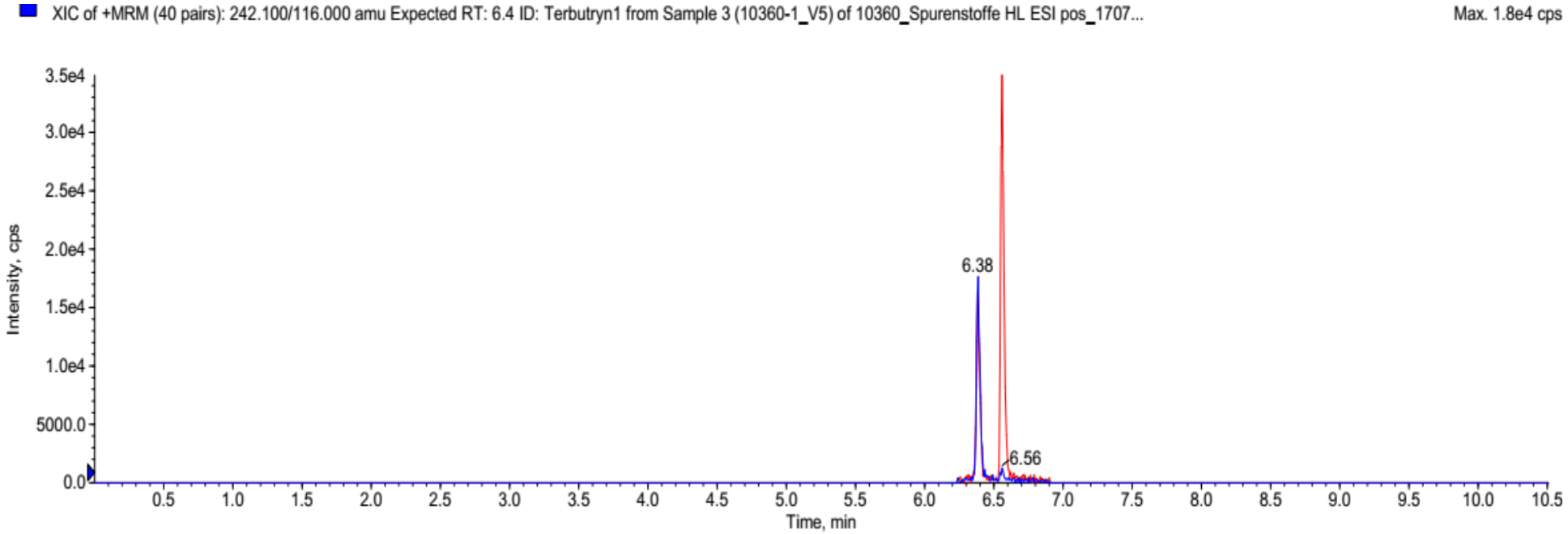


Fig. S10 Ion chromatogram of pesticide terbutryn in RWW. Signal intensity was measured as cps (count per second) along y-axis against the time in min along the x-axis.

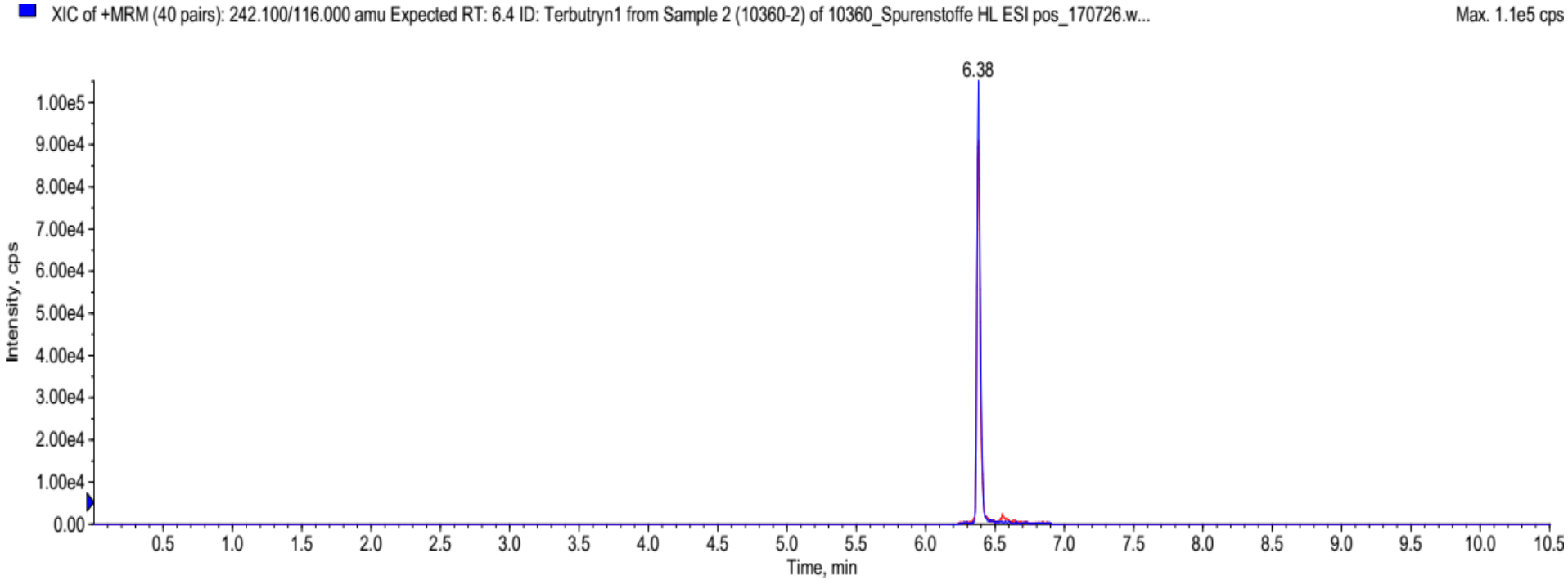


Fig. S11 Ion chromatogram of pesticide terbutryn in TSW. Signal intensity was measured as cps (count per second) along y-axis against the time in min along the x-axis.

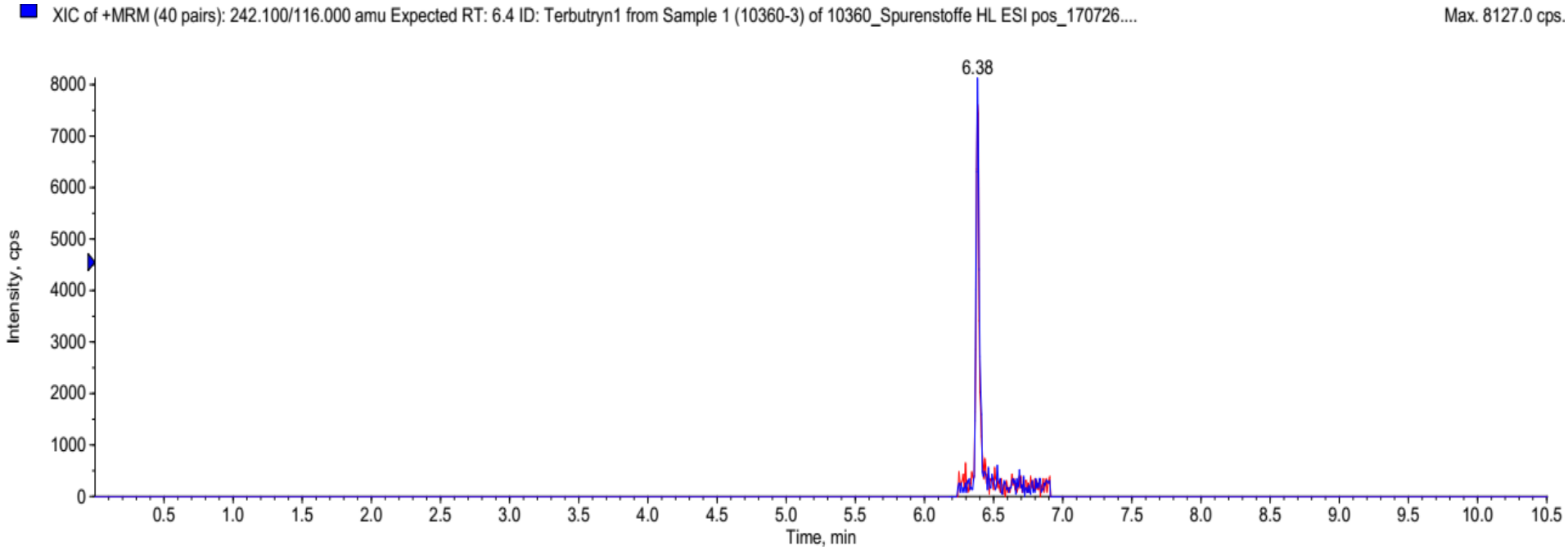


Fig. S12 Ion chromatogram of pesticide terbutryn in GW. Signal intensity was measured as cps (count per second) along y-axis against the time in min along the x-axis.

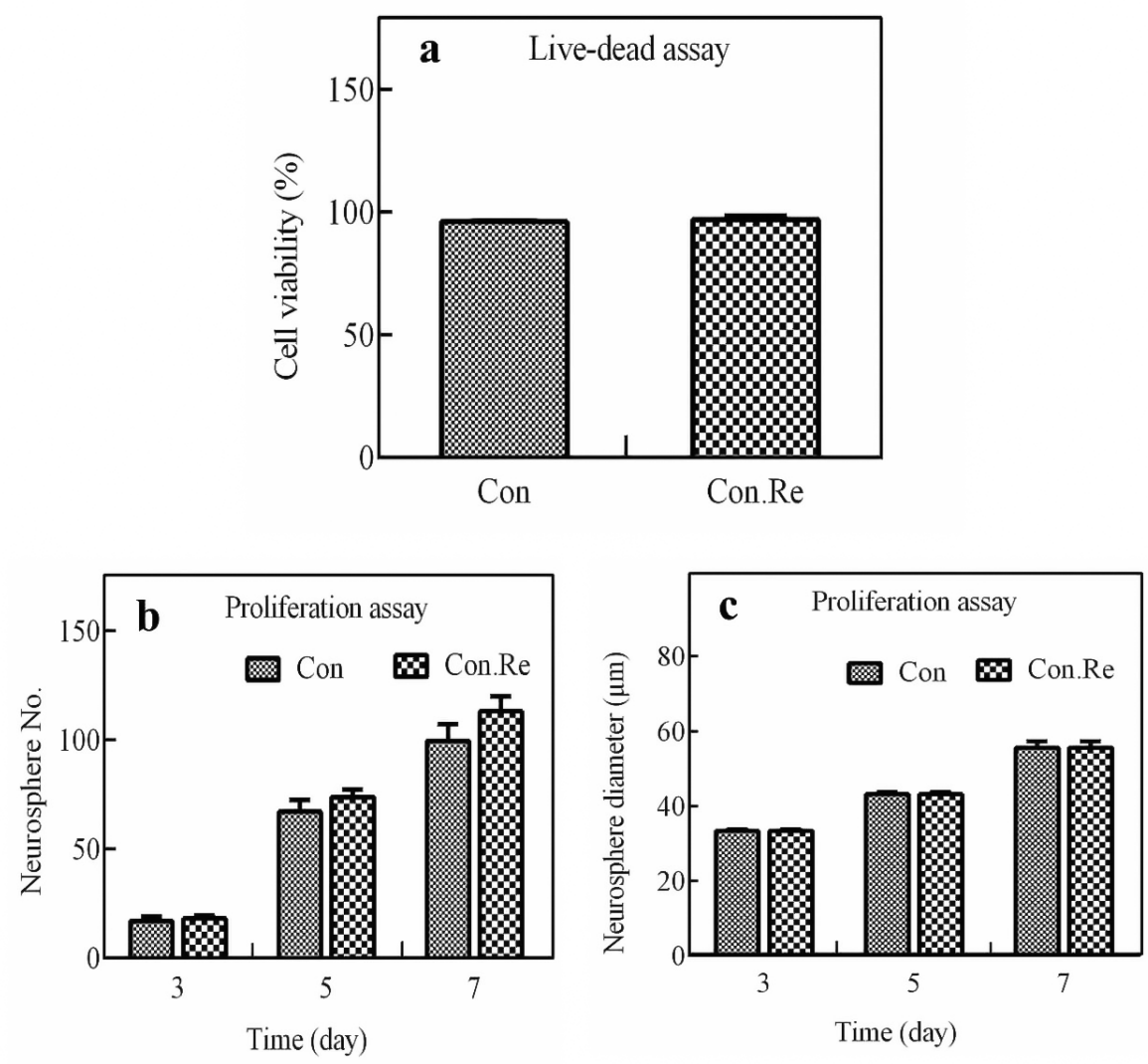


Fig. S13 Viability and proliferation assays for evaluating the effect of media processing parameters on neural stem cells. CON: normal control included the neural stem cell culture in normal DMEM-F.12 medium. CON.RE: reconstituted control included the neural stem cell culture in lyophilized DMEM-F.12 medium reconstituted with double distilled water (Normal control for water samples experiments). Neurosphere clonogenic proliferation assay was performed in a 96 well-plate. (a) Represents the mean neurosphere number for each condition. (b) Represents the neurosphere diameter for each condition. Viability was determined through Calcein/ Propidium Iodide Live and dead assay performed in 24-well plate. (c) Represents the percentage of Calcein stained live cells. Percentage viability was calculated from the total cell count in phase-contrast pictures. Experiments were performed in 5 independent replicates (n=5) with 3 technical replicates for each experimental condition.

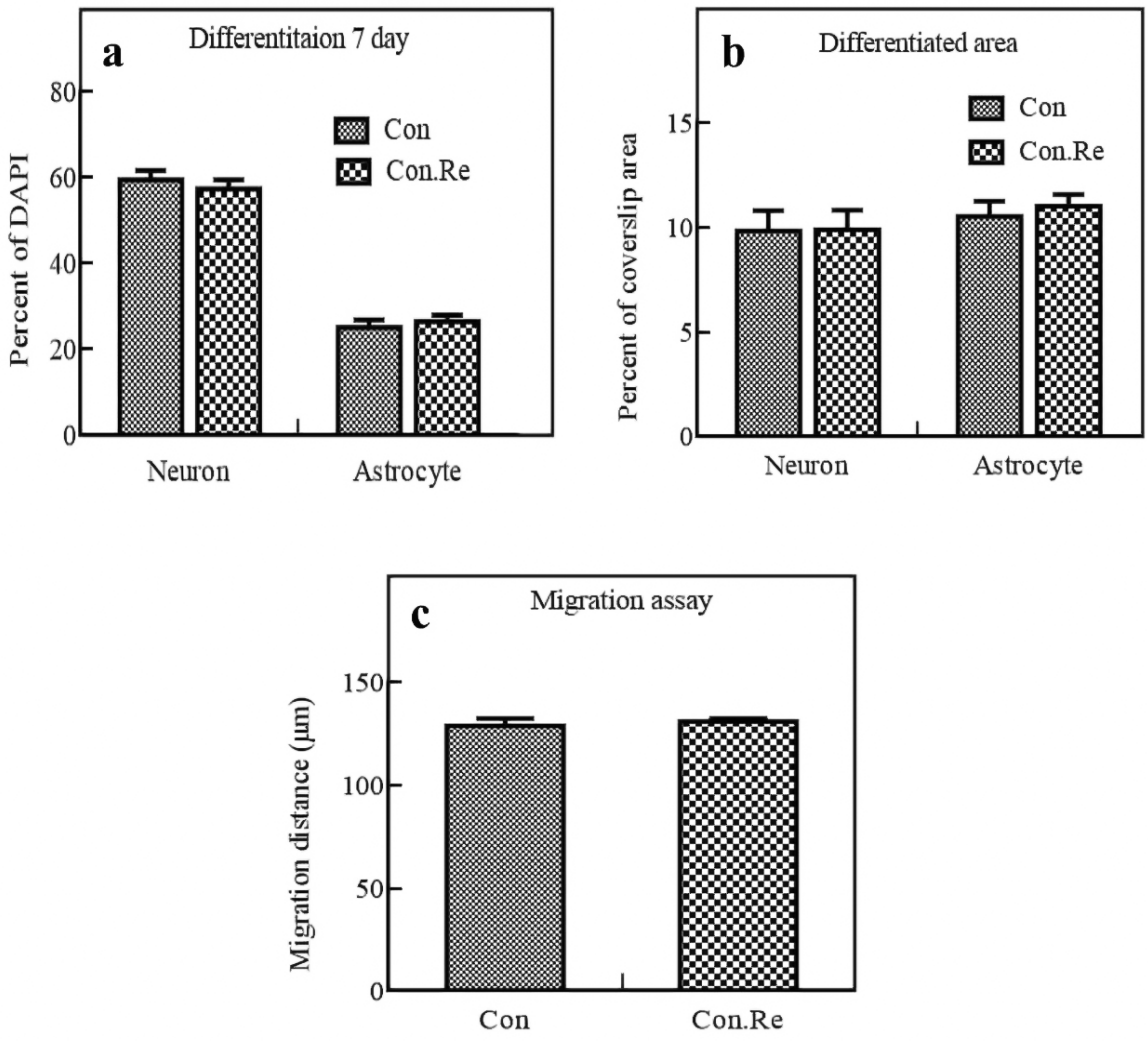


Fig. S14 Differentiation and migration assays for evaluating the effect of media processing parameters on neural stem cells. Differentiation experiments were carried out by seeding neural stem cells on ECM coated 12 mm glass coverslips in 24 well plates. ß-tubulin-III was used as a neuronal marker and GFAP was used as an astrocyte marker. Total of 20 microscopic fields was captured with a 20x lens of a fluorescent microscope in each technical replicate for each condition. (a) Represents the percentage of both neurons and astrocytes from total nuclei count stained with DAPI. (b) Represents the % of the area of a glass coverslip covered by differentiated neurons and astrocytes. A whole glass coverslip was scanned by a fluorescent microscope and then the area was calculated by using Image J. **(c)** Represents the mean distance travelled by migrating cells from a neurosphere core. Phase-contrast pictures of neurospheres attached on glass coverslips were taken with a phase-contrast microscope and the migration distance was calculated by using CELL-SENE software. For differentiation and migration assays, 5 independent experiments (n=5) were performed with 3 technical replicates for each condition.

7. Supplementary material:

7.2. Supplementary material for Publication 2: Environment permissible concentrations of glyphosate in drinking water can influence the fate of neural stem cells from the subventricular zone of the postnatal mouse.

Supplementary materials

Supportive Methods

Test compound

In the given study the effects of glyphosate [N-(Phosphonomethyl)glycine, ≤100%] compound with Mol. Wt. 169.07 (CAS# 1071-83-6, Sigma-Aldrich, Taufkirchen, Germany) were evaluated on the viability, proliferation, differentiation, and migration of NSCs from SVZ of the postnatal mouse. Glyphosate was dissolved in double-distilled water and vortexes thoroughly until completely dissolved. A stock solution of (7000 µg/L x 100). Working solutions were made from the stock solution by diluting in double-distilled water. Working solutions were filtered through a membrane filter under a sterile bench before use. The final volume of the glyphosate solution into the cell culture medium was 1% of the total volume. The control well contained sterile double-distilled water with volume 1% of the total volume of cell culture medium.

Viability and cytotoxicity assays

For the live-dead assay, approximately 10000 NSCs were seeded in proliferation medium with and without glyphosate test concentrations into each well of a 24-well plate for 24 h. Cells were washed with PBS and then incubated with the Live-dead reagent (containing 3µM Calcein-AM and 2.5µM propidium iodide in PBS) for 15 min at 37°C in dark. Approximately pictures of 10 microscopic fields were captured for both green and red fluorescence as well as for phase-contrast modes by a fluorescent microscope CKX41SF (Olympus Corporation, Tokyo, Japan) using 20 x objective for each condition in each replicate. Calcein-AM is a cell-permeant dye preferably taken up by living cells. Intracellularly, Calcein-AM is converted into a green fluorescent molecule thus indicating living cells. Propidium iodide enters the dead cells through the damaged membrane and is intercalated into the nucleus and gives red fluorescence (Sadeh et al., 2016). We employed WST-1 cytotoxicity tests only for those concentrations of glyphosate which produced significant effects on the viability of NSCs in the live-dead assay. Around 40000 cells were seeded into each well of a transparent flat-bottom 96-well plate and were kept in a proliferation medium for 24 h. Then cell culture was exposed to glyphosate for a further 24 h. WST-1 cytotoxicity assay was performed according to Kit instructions WST-1 (05015944001, Roche Diagnostics, Mannheim, Germany). The photometric analysis for WST-1 assay was performed by fluorescent multi-plate reader GENios (TECAN, Austria).

Calcium imaging

Experimental conditions for determination of changes in Ca^{2+} signal due to glyphosate exposure were set up by following the approach described by researchers in their study with necessary modification (Bufe et al., 2015; Bufe et al., 2012). Differentiation medium was removed after 48 h of incubation and cells were gently washed with C1 buffer (2.5 mM glucose,130 mM NaCl, 5 mM KCl, 10 mM HEPES, and 2 mM $CaCl_2$, pH 7.2). Cells were loaded with 2 µM Fluor-4AM (F14201, Life Technology, Eugene, USA) in C1 buffer and incubated for 45 min at room temperature followed by washing twice with C1 buffer. The glass coverslip was then transferred to a small plastic Petri dish equipped with a glass-bottom containing 2 mL of C1 buffer. Ca^{2+} dependent fluorescent changes were recorded by Carl Zeiss cell observer SD confocal microscope using 20 x objectives on green fluorescence mode by using AxioVision 4.8 Sp2 software (Carl Zeiss Micro-imaging GmbH, Jena, Germany). Cells were initially exposed to C1 buffer as vehicle control for 2 min then further 2 min for glyphosate and finally 2 min for 30 µM ATP +ve control. Ca^{2+} response was defined as an increase in excitation ratio at 340/380 nm that was 4 times higher than baseline noise (Bufe et al., 2015; Bufe et al., 2012). At least 100 cells were included in the observation for each condition in each replicate.

Proliferation assay

Neurosphere clonogenic assay was performed by following the approaches described by researchers in their studies. Neurosphere number and diameter were two readouts of the clonogenic assay (Baumann et al., 2014; Fritsche et al., 2018). Diameter implies the proliferation of NSCs within the neurosphere architecture and number indicates the self-renewal property of NSCs (He et al., 2013). Clonogenic assay was performed by seeding 1000 NSCs in 200µL of proliferation medium into each well of a 96-well plate with and without glyphosate. One half of the medium was replaced by a fresh proliferation medium on every 3^{rd} day. The whole well of a 96-well plate was scanned with a 4 x lens of a microscope (CKX41SF, Olympus Corporation, Tokyo, Japan) using phase-contrast mode at each observation time point (*i.e.* on 3^{rd}, 5^{th}, and 7^{th} day of the incubation) (Xiong et al., 2011), the neurosphere number was counted and neurosphere diameters were measured by a Cell-SENS software. Glyphosate effect on NSCs proliferation was further confirmed by a more sensitive assay based upon immunostaining for proliferation markers BrdU and Ki67. Ki67 is the nuclear protein marker expressed during every phase of the cell cycle except the resting phase. BrdU is expressed exclusively during the S-phase of the cell cycle. Calculating a ratio

of BrdU to the Ki67 positive cells gives an accurate estimation of actually proliferating cells (Tanaka et al., 2011). In our experiment, BrdU/Ki67 proliferation was performed in the same way as reported by researchers in their study (Masood et al., 2020).

In vitro differentiation assay

Around 20000 cells were allowed to adhere at the surface of each of 12 mm glass coverslips coated with ECM (E1270, Sigma-Aldrich, Taufkirchen, Germany) for 1h. The culture was differentiated in a differentiation medium with and without 0.1 µg/L and 700 µg/L of glyphosate in a 24-well plate. On completion of differentiation, cells were fixed with 4% paraformaldehyde and then stained for neuronal and astrocytes markers. The percentage of neurons and astrocytes was calculated. Total neurite length and mean neurite length were measured as neuronal morphological parameters while the soma area was recorded as a morphological readout of astrocytes. For each condition of each replicate, 30 microscopic fields with 200 x magnifications were taken with a fluorescent microscope (CKX41SF, Olympus, Tokyo, Japan). Quantification of morphological parameters of differentiated neurons and astrocytes was done using Cell-Sens software. Neurite outgrowth was quantified by calculating the percentage of non-neurite neurons from the total neuronal count. While total, and mean neurite length of neurons and the soma area were measured in fluorescent images by using the tracing tool of Cell-SENS software (Olympus, Tokyo, Japan). Around 100 cells were included in morphological observations for each treatment condition in each technical replicate.

Immunostaining

Fixed cultures were immunostained for the neuronal, astrocytes, and cell proliferation markers as previously reported (Bernas et al., 2017). Briefly, cells were exposed to 0.3% solution of triton x 100 for 10 min at 25°C to improve cell permeability followed by washing once with PBS-tween and twice with PBS. The culture was incubated with Normal donkey serum (S30, Merck, Darmstadt, Germany) 10% for 1 h to mask nonspecific binding of antibodies at 25°C followed by incubation for 1h with primary antibodies reconstituted in 5% blocking agent. Rat anti-BrdU (OBT0030G, AbDSerotec, Kidlington, UK), Rabbit anti-Ki67 (ab16667, Abcam, Cambridge, UK) were primary antibodies used as proliferation markers. Mouse-anti ß-tubulin III (MAB1637, Merk, Darmstadt, Germany) was a neuronal marker and Rabbit anti-GFAP (Z0334, Dako, Glostrup, Denmark) was the astrocytic marker used in our study. The culture was washed thrice with PBS and exposed to Alexafluor 594 and Alexafluor 488

conjugated secondary antibodies (Life Technology, Eugene, OR. USA) for 1 h at 25°C followed by washing thrice with PBS. The cell nuclei were counterstained by incubating the culture with DAPI (Sigma-Aldrich, Taufkirchen, Germany) for 10 min followed by final washing three times in PBS. Glass coverslips with stained culture were fixed on a glass slide by a fluorescent mounting medium. For BrdU staining, the predenaturation of nucleic acid was done by incubating the fixed proliferated cells on glass coverslips with 2N HCl solution for 15 min at 37°C. Acid was neutralized by 0.1 M Na-borate buffer of pH 8.5 followed by washing twice with PBS (Ostenfeld and Svendsen, 2004). The rest of the procedures were similar to those of other antibody stainings.

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 500000 cells were differentiated into each well of a 6 well plate coated with ECM gel for 7 days. Cells were then detached enzymatically and total RNA from the differentiation culture was extracted by TRIzol® (Thermofischer, Waltham, USA) following manufacturer instructions. Total RNA was quantified by Nanodrop spectrometer (Thermo Fisher Scientific, Wilmington, DE, USA) and then stocked at -80°C until used for PCR. Reverse transcription to complementary DND (cDNA) was done with 100 ng of RNA with a final volume of 20 µL by using a reverse transcription kit BioScript and following Kit method (Bioline London, UK). Initially priming premix (RNA, random hexamer, dNTP, and DEPC-treated water) was heated at 70°C for 5 min followed by cooling in ice. In the second step, reaction premix (Reverse transcriptase, RT-buffer, RNAse inhibitor, and DEPC-water) was mixed with priming premix, and the reaction was performed as (10 min 20°C, 60min 40°C and 10 min 70°C). Real-time PCR was performed using the SensiMix™SYBR®Low ROX Kit (Bioline London, UK) using QuantStudio™ 5 System272530370 (Waltham, USA). The following reaction condition was set on the instrument to amplify target mRNA in the differentiated NSCs culture: "95°C for 3 min, 40 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. After 40 cycles, the dissociation curve was generated for the determination of the melting point of the amplified cDNA and to authenticate the production of a single gene product (Tahir et al., 2019). Gene expression data were normalized to the reference gene GAPDH. Relative gene expression of all target genes was calculated using equation ($2^{-\Delta\Delta CT}$) described by researchers (Hegewald et al., 2011; Soleimani et al., 2011).

Supportive results

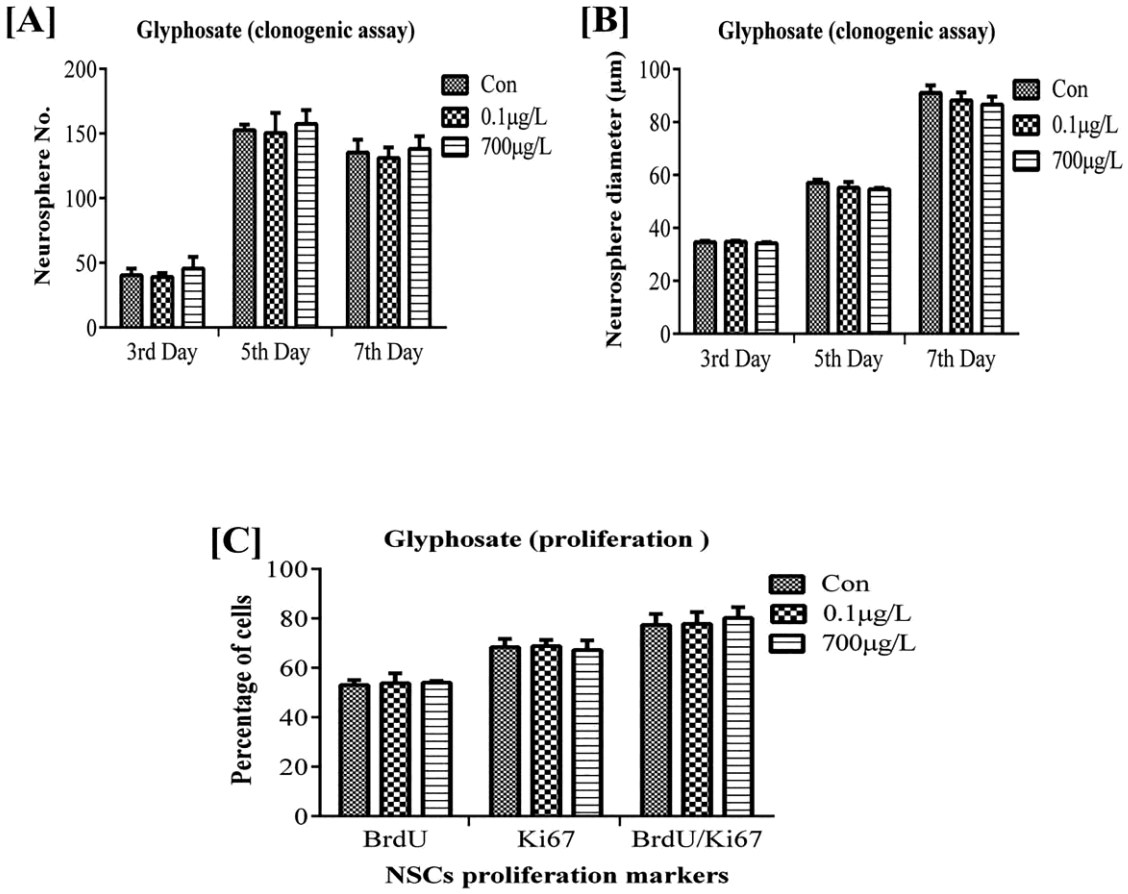


Fig. S1. Glyphosate effects on NSCs proliferation. Neurosphere clonogenic assay and BrdU/Ki67 immunostaining were performed to evaluate the effect of Environment permissible concentrations of glyphosate on the proliferation of NSCs in a 96-well plate. [A] Represents the neurosphere number. [B] Represents the mean diameter of neurospheres. Neurosphere number and diameter were measured by Cell-SENS software. [C] Represents the percentage of BrdU +ve and Ki67 +ve cells which were calculated from the total DAPI stained nuclei count. The assay was performed as five independent experiments *(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.

References

Baumann, J., Barenys, M., Gassmann, K., Fritsche, E., 2014. Comparative human and rat "neurosphere assay" for developmental neurotoxicity testing. Curr. Protoc. Toxicolo. 59, 12.21.11-24.

Bernas, S.N., Leiter, O., Walker, T.L., Kempermann, G., 2017. Isolation, Culture and Differentiation of Adult Hippocampal Precursor Cells. Bio. Protoc. 7, e2603.

Bufe, B., Schumann, T., Kappl, R., Bogeski, I., Kummerow, C., Podgórska, M., Smola, S., Hoth, M., Zufall, F., 2015. Recognition of Bacterial Signal Peptides by Mammalian Formyl Peptide Receptors A NEW MECHANISM FOR SENSING PATHOGENS. J. Biol. Chem. 290, 7369-7387.

Bufe, B., Schumann, T., Zufall, F., 2012. Formyl peptide receptors from immune and vomeronasal system exhibit distinct agonist properties. J. Biol. Chem. 287, 33644-33655.

Fritsche, E., Barenys, M., Klose, J., Masjosthusmann, S., Nimtz, L., Schmuck, M., Wuttke, S., Tigges, J., 2018. Current availability of stem cell-based in vitro methods for Developmental Neurotoxicity (DNT) testing. Toxicol. Sci. 165, 21-30.

He, N., Jin, W., Lok, K., Wang, Y., Yin, M., Wang, Z., 2013. Amyloid-β 1–42 oligomer accelerates senescence in adult hippocampal neural stem/progenitor cells via formylpeptide receptor 2. Cell Death Dis. 4, e924.

Hegewald, C., Alt, R., Hetz, S., Cross, M., Acikgoez, A., Till, H., Metzger, R., Metzger, M., 2011. Reduced oxygen stress promotes propagation of murine postnatal enteric neural progenitors in vitro. Neurogastroenterol. Motil. 23, e412-e424.

Masood, M.I., Schäfer, K.H., Naseem, M., Weyland, M., Meiser, P., 2020. Troxerutin flavonoid has neuroprotective properties and increases neurite outgrowth and migration of neural stem cells from the subventricular zone. PLoS One 15, e0237025.

Ostenfeld, T., Svendsen, C.N., 2004. Requirement for Neurogenesis to Proceed through the Division of Neuronal Progenitors following Differentiation of Epidermal Growth Factor and Fibroblast Growth Factor-2–Responsive Human Neural Stem Cells. Stem Cells 22, 798-811.

Sadeh, N., Oni-Biton, E., Segal, M., 2016. Acute Live/Dead Assay for the Analysis of Toxic Effects of Drugs on Cultured Neurons. Bio-protocol 6, e1889.

Soleimani, A., Zulkifli, I., Omar, A., Raha, A., 2011. Neonatal feed restriction modulates circulating levels of corticosterone and expression of glucocorticoid receptor and heat shock protein 70 in aged Japanese quail exposed to acute heat stress. Poult. Sci. 90, 1427-1434.

Tahir, S.K., Yousaf, M.S., Ahmad, S., Shahzad, M.K., Khan, A.F., Raza, M., Majeed, K.A., Khalid, A., Zaneb, H., Rabbani, I., 2019. Effects of Chromium-Loaded Chitosan Nanoparticles on the Intestinal Electrophysiological Indices and Glucose Transporters in Broilers. Animals 9, 819.

Tanaka, R., Tainaka, M., Ota, T., Mizuguchi, N., Kato, H., Urabe, S., Chen, Y., Fustin, J.-M., Yamaguchi, Y., Doi, M., 2011. Accurate determination of S-phase fraction in proliferative cells by dual fluorescence and peroxidase immunohistochemistry with 5-bromo-2'-deoxyuridine (BrdU) and Ki67 antibodies. J. Histochem. Cytochem. 59, 791-798.

Xiong, F., Gao, H., Zhen, Y., Chen, X., Lin, W., Shen, J., Yan, Y., Wang, X., Liu, M., Gao, Y., 2011. Optimal time for passaging neurospheres based on primary neural stem cell cultures. Cytotechnology 63, 621-631.

7. Supplementary material:

7.3. Supplementary material for Publication 3: Troxerutin flavonoid has neuroprotective properties and increases neurite outgrowth and migration of neural stem cells from the subventricular zone.

Supplementary material

Calcein/Propidium iodide Live-dead assay

Calcein/propidium live-dead assay was performed to evaluate if test concentrations of troxerutin flavonoid show cytotoxicity in NSCs culture. Around 10000 NSCs were proliferated in DMEM /F-12 differentiation medium into each well of a 96 well plate precoated With ECM gel for 48 h with and without exposure to 25 µM, 50 µM and 100 µM concentrations of troxerutin. The live-dead assay was performed in the same way as described by the researchers in their protocol (1). The live-dead reagent contains Calcein-AM (C3100MP, Life Technology, Eugene, USA) as a marker for viable cells and propidium iodide (P1304MP, Thermofischer, Waltham, USA). Calcein is a colourless compound and easily permitted through the cell membrane of viable cells. With in the living cell, calcein is converted into a fluorescent marker and gives green fluorescence. Propidium iodide enters only enter through the broken cell membrane of dead cells and is intercalated into the nucleic acid and gives red fluorescence. At least five images of independent microscopic fields were capture for each of phase contrast, green and red fluorescent modes by a 20 x objective of a fluorescent microscope (Olympus Corporation, Tokyo, Japan) for each condition in each replicate. Percentage of calcein +ve viable cells was obtained from the total cell count in the phase-contrast images. The experiment was performed as five replicates.

WST-1 cytotoxicity assay

Cytotoxicity of 10µM Aß42 was assessed through WST-1 cytotoxicity assay. Around 40000 NSCs were differentiated into each well of a 96-well plate pre-coated with PDL for 48 h in the presence of Aß42. Control was the vehicle used for making Aß42 working solution. WST-1 assay was performed by following the Kit method (WST-1, Roche, Mannheim, Germany).

Photometric absorbance was measured using a GENios fluorescent multi-plate reader (TECAN, Austria).

Supporting results and figure.

We found a non-significant difference between the control and all the tested concentrations of troxerutin concerning the viable percentage of NSCs after 7 days exposure (Fig SA). WST-1 assay revealed that 10µM Aß42 insignificantly affected the viability of neurons/astrocytes co-culture differentiated for 48 h (Fig SB).

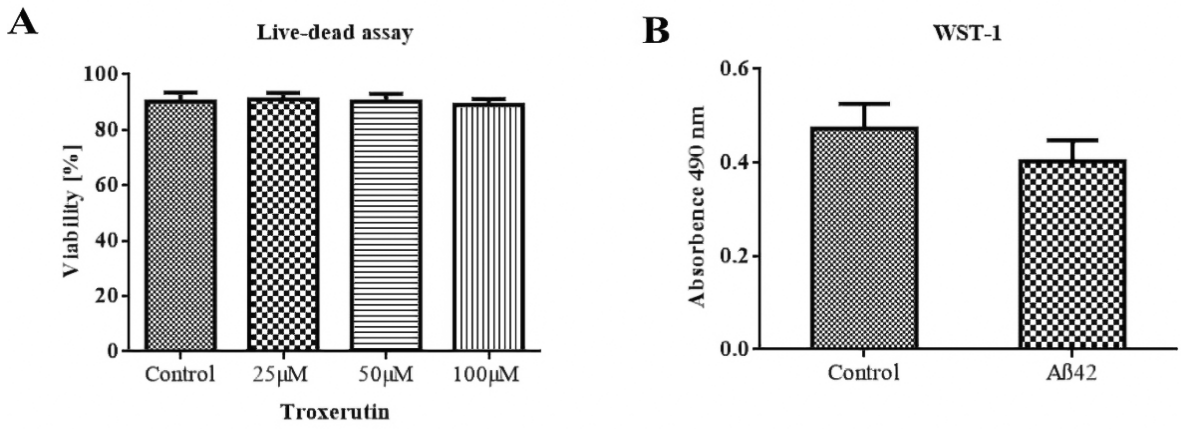


Fig S. Live-dead and WST-1 cytotoxicity assays. Calcein/propidium iodide live-dead assay was performed for three test concentrations of TRX. NSCs were exposed to TRX for 48 h followed by staining with Calcein AM as a live cell marker and propidium iodide as a dead cell marker. **A:** Represents the percentage of viable cells which was calculated from the total cell count. WST-1 was performed for differentiated culture of NSCs for 48 h exposed to 10µM Aß42. Test was performed in a 96 well plate. **B:** Represents the spectrophotometer absorbance values. Con; Control, TRX; troxerutin. Data are presented as mean±SEM. Experiments were performed as 5 replicates (n=5).

Moreover, a non-significant difference concerning NSCs viability was also observed betweenthe Control and 100µM TRX.

Supplementary raw data

Values in each table are from 5 independent experiments and are the values behind the bar graph.

Fig 2. TRX effect on neural stem cell markers and proliferation of NSCs.

Fig.2(A) Neurosphere Number

3rd day				5th day				7th day			
Con	25μM	50μM	100μM	Con	25μM	50μM	100μM	Con	25μM	50μM	100μM
59	60	69	62	160	135	149	142	114	116	115	104
88	112	98	96	148	157	162	146	142	146	141	136
62	64	71	64	136	142	134	121	106	111	117	116
77	70	86	83	119	121	140	137	113	108	109	97
75	83	78	85	139	130	135	129	108	102	119	123

Fig.2(B) Neurosphere Diameter

3rd day				5th day				7th day			
Con	25μM	50μM	100μM	Con	25μM	50μM	100μM	Con	25μM	50μM	100μM
34	35	36	35	55	56	54	56	90	87	85	104
38	37	36	37	57	57	58	61	87	88	84	89
35	35	34	35	65	62	64	63	93	89	84	88
36	35	35	36	66	66	62	64	89	87	90	88
35	36	35	35	64	64	64	66	93	88	85	85

Fig.2(C)

BrdU				Ki67				BrdU/Ki67			
Con	25μM	50μM	100μM	Con	25μM	50μM	100μM	Con	25μM	50μM	100μM
52	49,7	50	53	66	68	72	70	67	66	65	67
54	52	52,8	54	69	74,8	76	77	69	69	68	67,7
55,2	54,8	57,6	54	74	75	76	77	70,7	69	69	68
55,6	56	59	56	77	78,9	77	80	74,8	69	70	69
56,7	56	60	57,8	78	80	85	84	81	71	75	70

Fig.2(D)

Nestin				Nestin.GFAP			
Con	25μM	50μM	100μM	Con	25μM	50μM	100μM
68,8	72	72	64	31	27	27	36
71	74	70	62	28,9	25	31	38
68	71	69	65	32	28	32	43
68	68	67	53	32	32	29	47
65	68	71	56	34	31	29	44

Fig 4. TRX effect on NSCs differentiation after 7 days and 48 h of incubation**.**

Fig 4(A)

Neurons				Astrocytes			
Con	25µM	50µM	100µM	Con	25µM	50µM	100µM
41,6	47	45	52	51	46,8	47,8	45
46,5	51,9	52	54	41	37	36	36,9
60,8	64	62,9	67	28	25,9	27	25
48,9	52,8	53	55	40,7	38	37	35
56,9	61	63	68	24	22	23	23

Fig (B)

Neurons				Astrocytes			
Con	25µM	50µM	100µM	Con	25µM	50µM	100µM
56	59	61,7	65	30	27	29	25
36	47	50	51,2	41	33,6	38	40
43,6	48	48	49	36	32	36,6	27
52,9	53	59	62,8	27	30	26	32
56,9	56	56	64	25	26,5	28	26

Fig.4(C)

NNN/TN x 100			
Con	25µM	50µM	100µM
4,1	2,7	2,7	2,2
3,3	2,6	2,3	2,7
3,2	2,4	3,1	1
4,7	2,9	3,6	2
9,2	6,7	6,1	2,8

Fig.4(D)

NNN				Double +ve			
Con	25µM	50µM	100µM	Con	25µM	50µM	100µM
10	6,1	4,5	3	11,4	7,1	5,6	4,53
10,8	7,1	8	4	9,8	12,9	11,7	4
6,2	3,6	3,8	3	10	9,7	8,4	5,9
5,5	2,9	2,9	3	14	9,5	9,5	9,1
5,7	3,4	2,99	1,84	13	9,9	8,98	7,9

Fig 7. Effects of TRX on morphological parameters of neurons and astrocytes differentiated from NSCs after 7 days and 48 h of incubation.

Fig 7 (A)

TN				μN			
Con	**25μM**	**50μM**	**100μM**	**Con**	**25μM**	**50μM**	**100μM**
82	106	106	120	28	38	37	40
79	130	133	160	28	41	45	47
101	149	157	158	36	46	48	48
116	155	150	194	38	49	50	56
96	140	176	167	32	44	45	48

Fig 7 (C)

Astrocyte area			
Con	**25μM**	**50μM**	**100μM**
1870	2434	2255	2195
2700	3002	2715	1946
2747	3041	2835	1884
2361	2754	2780	1952
1996	2836	2719	1912

Fig 7 (B)

TN		μN	
Con	**100μM**	**Con**	**100μM**
53,44	63,33	22,44	26,8
48,84	64,5	21,6	27,4
47,6	66,6	21,8	31,5
52,9	67,6	24	30,8
49,5	60,5	21,4	27,7

Fig 7 (D)

Astrocyte area	
Con	**100μM**
1143	1042
1165	905
1467	778
1645	1055
1601	985

Fig 9. Effect of TRX on neurite arborisation after 7 days of differentiation.

Fig 9 (A)

AP				EP			
Con	25µM	50µM	100µM	Con	25µM	50µM	100µM
2,3	2,38	2,45	2,53	2,82	2,9	2,99	3
2,45	2,91	2,59	2,98	2,96	3,32	3,19	3,47
2,35	2,4	2,46	2,29	2,87	3,26	3,37	3,27
2,34	2,37	2,38	2,47	3	3,18	3,45	3,52
2,92	2,87	2,47	2,66	2,92	3,35	3,42	3,65

Fig 9 (B)

Arborization			
Con	25µM	50µM	100µM
1,22	1,21	1,22	1,18
1,2	1,14	1,23	1,16
1,22	1,36	1,37	1,43
1,28	1,34	1,45	1,42
1	1,16	1,38	1,37

Fig 11. Neuroprotective effects of TRX flavonoid against Aß42 induced depression of differentiation after 48 h of differentiation on ECM coated glass coverslips.

Fig 11(A)

Neurons			Astrocytes		
Con	Aß(10µM)	Aß+TRX	Con	Aß(10µM)	Aß+TRX
47,9	40	60	40	33,8	37,8
46	41,7	52	38	35	34
45,9	38,8	50	37	24	29
52,9	47	48,5	29	28	25
51	48,8	51	36	30	34

Fig 11(B)

NNN			Double]ve		
Con	Aß(10µM)	Aß+TRX	Con	Aß(10µM)	Aß+TRX
14,6	29	14,2	4,9	6,4	6,6
12,3	41	14,7	4,9	8,3	6,5
13	25	14,4	4,2	6,5	5
10	36	18,6	3	5,4	4,6
14,9	31,5	12	2,16	6,2	5,6

Fig 11(C)

TN			µN		
Con	Aß(10µM)	Aß+TRX	Con	Aß(10µM)	Aß+TRX
58,49	46,49	60,15	24,58	19,84	27,23
54,84	43,39	65,78	22,94	19,03	27,29
55,25	44,21	66,45	22,27	20,29	28,05
55,62	41,35	63,13	23,19	18,98	26,16
56,05	43,86	63,8775	23,245	19,535	27,1825

Fig 11(D)

Astrocyte area		
Con	Aß(10µM)	Aß+TRX
1076	922	946
1110	978	856
1182	969	977
1375	911	967
1185,75	945	936,5

Fig 14. TRX enhances the migration of differentiated cells from NSCs cultured for 24 h.

Distance travelled by cells			
Con	25μM	50μM	100μM
132	129	143	151
126	133	148	172
124	124	154	143
145	131	151	161
110	133	154	145

Fig S. Live-dead and WST-1 cytotoxicity assays.

Fig S(A)

Viability (%)			
Con	25μM	50μM	100μM
89	93	95	91
86	89	89	89
93	93	90	91
94	88	88	88
89	92	89	86

Fig S(B)

Absorbence	
Con	Aß42
0,3533	0,3678
0,6026	0,515
0,4907	0,3474
0,5695	0,499
0,347	0,2876

References

1. Sadeh N, Oni-Biton E, Segal M. Acute Live/Dead Assay for the Analysis of Toxic Effects of Drugs on Cultured Neurons. Bio-protocol. 2016; 6:15. doi: 10.21769/BioProtoc.1889.

8. List of publications

1) M. I. Masood, N. T. Hauke, M. J. Nasim, M. Sarfraz, M. Naseem, K.-H. Schäfer**.** Neural stem cell-based *in vitro* bioassay for the assessment of neurotoxic potential of water samples.

Journal of Environmental Sciences, 101, 72–86 (2021).

2) M. I. Masood, M. Naseem, S. A. Warda, M. A. Tapia-Laliena, H. Rehman, M. J. Nasim , K.-H. Schäfer. Environment permissible concentrations of glyphosate in drinking water can influence the fate of neural stem cells from the subventricular zone of the postnatal mouse.

Environmental Pollution, 270, 116179 (2021).

3) M. I. Masood, K.-H. Schäfer, M. Naseem, M. Weyland, P. Meiser. Troxerutin flavonoid has neuroprotective properties and increases neurite outgrowth and migration of neural stem cells from the subventricular zone.

PLoS ONE, 15(8), 0237025 (2020).

4) M. Vaso, W. Ali, **M. I. Masood**, Muhammad Jawad Nasim, Rainer Lilischkis, Karl-Herbert Schäfer, Marc Schneider, Vilma Toska Papajani and Claus Jacob. Nanosizing *Nigella*: A Cool Alternative to Liberate Biological Activity.

Current Nutraceuticals, 2020, *1*, 0-0.

5) M. Sarfraz, S. Griffin, T. G. Sad, R. Alhasan, M. J. Nasim, **M. I. Masood**, K. H. Schäfer, C. E. C. C. Ejike, C. M. Keck, C. Jacob, A. P. Ebokaiwe, Milling the Mistletoe: Nanotechnological Conversion of African Mistletoe (*Loranthus micranthus*) Into antimicrobial Materials.

Antioxidants, *7*(4), 60 (2018).

6) S. Griffin, **M. I. Masood**, M. J. Nasim, M. Sarfraz, A. P. Ebokaiwe, K-H. Schäfer, C. M. Keck, C. Jacob. Natural Nanoparticles: A Particular Matter Inspired by Nature.

Antioxidants, 7(1) (2018)

7) M. J. Nasim, P. Denezhkin, M. Sarfraz, R. Leontiev, Y. Ney, A. Kharma, S. Griffin, **M. I. Masood,** C. Jacob. The Small Matter of a Red Ox, a Particularly Sensitive Pink Cat, and the Quest for the Yellow Stone of Wisdom.

Current Pharmacology Reports, 4, 380–396 (2018).

List of publications

---

8) L. Faulstich, S. Griffin, M. J. Nasim, **M. I. Masood**, W. Ali, S. Alhamound, Y. Omran, H. Kim, A. Kharma, K.-H. Schäfer, R. Lilischkis, M. Montenarh, C. Keck, C. Jacob. Nature's Hat-trick: Can we use sulfur springs as ecological source for materials with agricultural and medical applications?. *International Biodeterioration & Biodegradation*, 119, 678-686 (2017).