Toxicology 387 (2017) 67–80

Contents lists available at ScienceDirect

Toxicology

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

Developmental exposure to glyphosate-based herbicide and depressive-like behavior in adult offspring: Implication of glutamate excitotoxicity and oxidative stress

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ARTICLE INFO

Keywords:
Glyphosate-based herbicide
Glutamate excitotoxicity
Oxidative stress
Depressive-like behavior
NMDA glutamate receptor
Molecular docking

ABSTRACT

We have previously demonstrated that maternal exposure to glyphosate-based herbicide (GBH) leads to glutamate excitotoxicity in 15-day-old rat hippocampus. The present study was conducted in order to investigate the effects of subchronic exposure to GBH on some neurochemical and behavioral parameters in immature and adult offspring. Rats were exposed to 1% GBH in drinking water (corresponding to 0.36% of glyphosate) from gestational day 5 until postnatal day (PND)-15 or PND60. Results showed that GBH exposure during both prenatal and postnatal periods causes oxidative stress, affects cholinergic and glutamatergic neurotransmission in offspring hippocampus from immature and adult rats. The subchronic exposure to the pesticide decreased L-[^{14}C]-glutamate uptake and increased $^{45}Ca^{2+}$ influx in 60-day-old rat hippocampus, suggesting a persistent glutamate excitotoxicity from developmental period (PND15) to adulthood (PND60). Moreover, GBH exposure alters the serum levels of the astrocytic protein S100B. The effects of GBH exposure were associated with oxidative stress and depressive-like behavior in offspring on PND60, as demonstrated by the prolonged immobility time and decreased time of climbing observed in forced swimming test. The mechanisms underlying the GBH-induced neurotoxicity involve the NMDA receptor activation, impairment of cholinergic transmission, astrocyte dysfunction, ERK1/2 overactivation, decreased p65 NF-κB phosphorylation, which are associated with oxidative stress and glutamate excitotoxicity. These neurochemical events may contribute, at least in part, to the depressive-like behavior observed in adult offspring.

1. Introduction

Glyphosate (*N*-phosphonomethyl-glycine) is an organophosphorus compound, widely used as a broad-spectrum, post-emergent, non-selective herbicide, as well as a crop desiccant. It has been used in both agricultural and non-agricultural activities (e.g. gardening). Commercial formulations containing glyphosate account for approximately 60% of the world market of non-selective herbicides (IBAMA, 2014; IARC, 2015). In Brazil, the planting of glyphosate-resistant soybeans (GRS) has greatly enhanced the consumption of glyphosate formulations in crops.

Glyphosate-based herbicides (GBH) are frequently formulated as isopropylamine salts and surfactants may be added to formulations (FAO, 2000/2001; IPCS, 1994). The use of polyethyloxylated tallowamine (POEA) as a surfactant in GBH formulations facilitate the herbicide uptake by plants (Székács and Darvas, 2012). It has been suggested that formulations containing surfactants may be even more toxic than the glyphosate alone to mammal cells (de Liz Oliveira Cavalli et al., 2013; Mesnage et al., 2013; Sribanditmongkol et al., 2012).

In 2000, William and coworkers reviewed the data concerning the safety of GBH to human and animal health. They suggested that the use of these herbicides did not cause adverse effects to mammals (Williams

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http://dx.doi.org/10.1016/j.tox.2017.06.001
Received 2 February 2017; Received in revised form 21 May 2017; Accepted 10 June 2017
Available online 13 June 2017
0300-483X/

et al., 2000). However, in a recently statement of concerns over the use of GBH and risks associated with exposures, Myers et al. (2016) reported a considerable number of animal and epidemiology studies published in the last decade demonstrating GBH toxicity to animals and humans. Based on these data, the authors concluded that human exposures to glyphosate are rising and the current safety standards for GBH are outdated and may fail to protect public health or the environment.

The presence of glyphosate and its main metabolite AMPA (aminomethylphosphonic acid) are generally detected together and more frequently in soil and sediments, ditches and sewers, rivers and streams (Contardo-Jara et al., 2009; Aparicio et al., 2013; Battaglin et al., 2017). Thus, non-target organisms may be exposed to glyphosate through drinking water (Larsen et al., 2012; Mbanaso et al., 2014). Indeed, evidences of human exposure to this herbicide has been shown through the detection of glyphosate in urine samples of people living in farm and non farm household (Acquavella et al., 2004; Conrad et al., 2017).

A recent study also demonstrated the presence of glyphosate in maternal and umbilical cord serum in pregnant women who gave birth in three provinces of Thailand (Kongtip et al., 2017). The authors showed that pregnant women who work in agriculture or live with families that work in agriculture had higher exposures to the herbicide glyphosate when compared to pregnant women who were not agriculturists/farmers by occupation. In this context, studies involving the transport kinetics of glyphosate across the BeWo cell monolayer (used as model of human trophoblast) and across the placental barrier in the *ex vivo* perfusion system showed that the transport of glyphosate through placenta is possible, even though is considered low. After 2.5 h of perfusion, the percentage of compound transferred to the fetal compartment was about 15% for glyphosate (Mose et al., 2008) and after 24 h of BeWo cell experiments, the percentage of glyphosate transported was near 24% (Poulsen et al., 2009).

Several studies have demonstrated that pre- and postnatal exposure to different types of pesticides may be associated with neurological and neuropsychiatric effects. Even at low concentrations, exposure to neurotoxins during central nervous system (CNS) development can promotes injuries to brain areas like prefrontal cortex and hippocampus, causing losses in cognitive functions (Ainge et al., 2007; Chen et al., 2012; Mogensen et al., 2007). This exposure to neurotoxicants can be very subtle and became more evident only in older ages (Bondy and Campbell, 2005; Rice and Barone, 2000).

Utilizing the zebrafish vertebrate model system to study early effects of glyphosate and GBH exposure, Roy et al. (2016)- found morphological abnormalities in the developing brain as well as a decrease in genes expressed in differents regions of the brain. Another study using zebrafish model demonstrated an impaired neuronal development caused by glyphosate exposure during the embryonic development period. The exposure led to an inhibition of carbonic anhydrase activity with production of ROS especially in branchial regions, triggered cellular apoptosis resulting in several types of malformations (Sulukan et al., 2017). It was recently demonstrated that initial axonal differentiation and growth of cultured neurons is affected by glyphosate. Biochemical approaches revealed that glyphosate led to a decrease in Wnt5a level, a key factor for the initial neurite development and maturation, as well as inducing a down-regulation of CaMKII activity. Additionally, these changes might be reflected in a subsequent neuronal dysfunction (Coullery et al., 2016).

In our previously work regarding maternal exposure to GBH we demonstrated that exposure during pre and postnatal periods leads to calcium overload and glutamate excitotoxicity in immature offspring hippocampus (Cattani et al., 2014). The mechanism underlying such effects involves the calcium influx by activating *N*-methyl-D-aspartate (NMDA) glutamate receptors and voltage-dependent calcium channels, which leads to oxidative stress and neural cell death in immature rat hippocampus (PND15). The mechanism involved in NMDA receptor overactivation by glyphosate is not elucidated. The glutamate excitotoxicity with subsequent intracellular calcium influx is one of the main factors for reactive oxygen species (ROS) generation in brain (Emerit et al., 2004; Gilgun-Sherki et al., 2001; Kaur and Ling, 2008; Zamoner et al., 2008), leading to oxidative damage.

The present study was conducted in order to evaluate if these effects of maternal exposure to GBH during gestational and suckling periods, previous observed in immature offspring hippocampus (PND15) (Cattani et al., 2014), persist until adulthood (postnatal day 60, PND60). In this context, several mechanistic/neurochemical endpoints were evaluated in this follow-up study, as well as the behavioral alterations induced by the subchronic herbicide exposure in adult animals, trying to provide benchmark data for a risk assessment for GBH to neural cells. Moreover, given the similarity of glyphosate structure to glycine and glutamate we investigated whether glyphosate might affect the glutamate neurotransmission system by using molecular docking of glyphosate to glycine and glutamate binding sites on the NMDA glutamate receptors.

2. Material and methods

2.1. Radiochemical and compounds

L-[^{14}C]-glutamate (specific activity 9.62 GBq/mmol) was purchased from Amersham (Oakville, Ontario, Canada). [$^{45}Ca^{2+}$]-$CaCl_2$ (specific activity of 321 kBq/mg of Ca^{2+}) and Optiphase Hisafe III biodegradable liquid scintillation were purchased from Perkin Elmer (Waltham, MA). Anti-p44/42 MAP Kinase (anti-ERK1/2), anti-phospho-p44/42 MAP kinase (anti-phospho ERK1/2), antibodies were from Cell Signaling Technology, Inc. (USA). The herbicide Roundup Original® (Homologation number 00898793) containing glyphosate 360 g/L is a commercial formulation registered in the Brazilian Ministry of Agriculture, Livestock and Supply (Ministério da Agricultura, Pecuária e Abastecimento – MAPA). The Immobilon™ Western chemiluminescent HRP substrate was obtained from Millipore. The NF-κB p65 (Total) InstantOne ELISA and InstantOnePhospho-NF-kB p65 (Ser536) ELISA kits were purchased from eBioscience® (San Diego, CA, USA). All other chemicals were of analytical grade.

2.2. Animals

Wistar rats were bred in animal facility and maintained in an air-conditioned room (21 ± 1 °C) with controlled lighting (12 h/12 h light/dark cycle). On the day of birth, the litter size was culled to eight pups (4 males and 4 females, whenever possible). Litters smaller than eight pups were not included. Pelleted food (Nuvital, Nuvilab CR1, Curitiba, PR, Brazil) and tap water were available *ad libitum*. All animals were observed for clinical signs of toxicity related to chemical treatment. All experimental procedures were conducted in accordance to the rules and laws of ethical recommendations of the local Ethical Committee for Animal Use at Federal University of Santa Catarina (CEUA/UFSC, Protocol #PP00820), which is in accordance with Brazilian Council for Control of Animal Experimentation (CONCEA).

2.3. Animal treatment

The experimental protocol of maternal exposure to GBH used herein was previously described by us (Cattani et al., 2014). Wistar rats were mated and the day of appearance of the vaginal plug was considered gestational day 0 (GD0). Pregnant females were housed individually and divided into 2 groups (4 dams in each group): control group provided with tap water and GBH group provided with 1% GBH in drinking water (corresponding to 0.36% of glyphosate). Maternal exposure to tap water or GBH-containing drinking water was started at gestational day 5 (GD5) and continually up to postnatal day 15 (PND15) or up to postnatal day 60 (PND60). Thus, the offspring were exposed *in utero*,