Circular RNA expression profiles in hippocampus from mice with perinatal glyphosate exposure

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A B S T R A C T

Glyphosate is the active ingredient in numerous herbicide formulations. The roles of glyphosate in embryo-toxicity and neurotoxicity have been reported in human and animal models. Recently, several studies have reported evidence linking neurodevelopmental disorders (NDDs) with gestational glyphosate exposure. However, the role of glyphosate in neuronal development is still not fully understood. Our previous study found that perinatal glyphosate exposure resulted in differential microRNA expression in the prefrontal cortex of mouse offspring. However, the mechanism of glyphosate-induced neurotoxicity in the developing brain is still not fully understood. Considering the pivotal role of Circular RNAs (circRNAs) in the regulation of gene expression, a circRNA microarray method was used in this study to investigate circRNA expression changes in the hippocampus of mice with perinatal glyphosate exposure. The circRNA microarrays revealed that 663 circRNAs were significantly altered in the perinatal glyphosate exposure group compared with the control group. Among them, 330 were significantly upregulated, and the other 333 were downregulated. Furthermore, the relative expression levels of mmu-circRNA-014015, mmu-circRNA-28128 and mmu-circRNA-29837 were verified using quantitative real-time polymerase chain reaction (qRT-PCR). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses demonstrated that stress-associated steroid metabolism pathways, such as aldosterone synthesis and secretion pathways, may be involved in the neurotoxicity of glyphosate. These results showed that circRNAs are aberrantly expressed in the hippocampus of mice with perinatal glyphosate exposure and play potential roles in glyphosate-induced neurotoxicity.

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1. Introduction

Glyphosate, a phosphonomethyl amino acid derivative, is commonly used as a broad-spectrum, non-selective, post-emergence herbicide and is widely used in both agricultural and non-agricultural activities, such as gardening [1]. Glyphosate-based herbicides (GBH) are the most utilized agrochemicals in the world [2]. Approximately two-thirds of the total volume of GBH used has been delivered to the environment [3], and it induces soil erosion [4]. It has been thought that the use of GBH does not cause adverse effects to mammals [5]. However, recently attention has been drawn to the overuse of glyphosate and its harm to human health. Animal studies suggest that glyphosate may be neurotoxic to the developing brain [6,7]. In vitro studies have revealed that glyphosate can pass through the blood brain barrier and placental barrier [8]. Results from the Childhood Autism Risks from Genetics and Environment study provide further evidence for an association between neurodevelopmental disorders (NDDs) and gestational organophosphate exposure, particularly glyphosate [9]. However, the mechanism of glyphosate-induced neurotoxicity in the developing brain is still not fully understood.

Novel studies have provided insight into the possible roles of environmental and epigenetic factors in NDDs [10,11]. Our previous study showed that alterations of the miRNAs and the target genes associated with neurogenesis and neuron differentiation may play

a role in the neurotoxicity of glyphosate [12]. However, it is likely that other factors are also involved, and these mechanisms are yet to be fully elucidated.

Circular RNAs (circRNAs) are a special class of non-coding RNAs (ncRNAs) which may interact with miRNAs [13]. CircRNAs may act as miRNA sponges and could play an important role in regulating gene expression through a circRNA-miRNA gene pathway. There is increasing evidence that circRNA-mediated gene expression regulation is widespread and critical in mammals. Recent studies reveal a striking regulation of circRNAs during neuronal development [14]. Altered expression of circRNAs may contribute to the post-transcriptional modulation of genes involved in central nervous system diseases [15].

Little is known about the functions of circRNAs in developing brain with perinatal glyphosate exposure. Specifically, there are no reports on the expression profiles of circRNAs in developing brain with perinatal glyphosate exposure. Herein, we established a perinatal glyphosate exposure model and performed microarray detection to profile the expression of circRNAs in the hippocampus from exposed mice. Then, we investigated circRNAs with differential expression after perinatal glyphosate exposure.

2. Materials and methods

2.1. Reagents

The pesticide used in this study was a commercial formulation marketed in China as Roundup® (Monsanto Company, St. Louis, MO, USA), containing 48 g glyphosate isopropylamine salt per 100 cm^3 of product (equivalent to 35.6% w/v of glyphosate acid).

2.2. Animals

Qualified specific pathogen-free pregnant ICR mice (aged 9—11 weeks), weighing 40—50 g, obtained from Shanghai Laboratory Animal Center at the Chinese Academy of Sciences (Shanghai, China). The mice were kept for 1 week under controlled temperature and humidity with a day/night cycle and access to standard laboratory food and water *ad libitum*. The study was approved by the Institutional Animal Care and Use Committee of Hangzhou Medical College (Hangzhou, China) and based on the NIH Guide for the Care and Use of Laboratory Animals.

2.3. Perinatal glyphosate exposure model

Sixteen pregnant ICR mice were randomly divided into two groups of eight mice. In the control group, pregnant mice were provided with purified water. In the glyphosate-treated group, pregnant mice were provided with drinking water containing 0.38% glyphosate (1% Roundup®) from embryonic day (E) 14 to postnatal day (PND) 7 (the day of birth considered as PND 0), then provided with normal drinking water. The dose administered was equivalent to 50 mg of glyphosate/kg/day, which was 1/20th of the glyphosate no-observed-adverse-effect level for glyphosate described previously [5]. The offspring received glyphosate indirectly via pregnancy and lactation. Weaning occurred on PND 21, then the offspring were provided with normal drinking water. A total of 8 male offspring from each group were sacrificed on PND 28, the brains were quickly removed, and the hippocampi were isolated on an ice pad.

2.4. RNA isolation

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. The concentrations of the RNA samples were determined by the OD260/OD280 ratio using a NanoDrop ND-1000 instrument (Agilent, Santa Clara, CA, USA). The integrity of the RNA was assessed by electrophoresis on a denaturing agarose gel.

2.5. CircRNA microarray analysis

The sample preparation and microarray hybridization were performed according to Arraystar's standard protocols. The total isolated RNA was taken from pooled samples of each group. Briefly, total RNA was digested with Rnase R (Epicentre, Inc.) to remove linear RNAs and enrich for circular RNA. Then, the enriched circular RNA was amplified and transcribed into fluorescent cRNA utilizing a random priming method (Arraystar Super RNA Labeling Kit; Arraystar). The labeled cRNA was hybridized onto the Arraystar Mouse circRNA Array v2 (8 × 15 K, Arraystar), and the arrays were scanned by the Agilent Scanner G2505C. Agilent Feature Extraction software (version 11.0.1.1, USA) was used to analyze acquired array images. Quantile normalization and subsequent data processing were performed using the limma package in R software. Differentially expressed circRNAs between the two groups were identified through Fold Change filtering. Hierarchical Clustering was performed to show the differential circRNAs expression pattern among samples. circRNAs with an absolute value of fold change ≥2.0 and a p-value ≤ 0.05 were selected as significantly differentially expressed.

2.6. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

After determining the optimal annealing temperatures, qRT-PCR was performed in the ABI PRISM® 7500 Sequence Detection System (Applied Biosystem, Foster City, CA, USA) using SYBR Green qPCR SuperMix (Invitrogen, Carlsbad, CA, USA). The primers used for the qRT-PCR analysis were as follows: 5′-GAGAAGGCTCCGAAAGAATA-3' (sense) and 5' -CACTTGGAAGGCTGTAAACAT-3' (anti-sense) for mmu-circRNA-014015; 5′-AGGAATTGCCCATTGTCAGC-3' (sense), 5′-CCCAGACCATCCCACATCAC-3' (anti-sense) for mmu-circRNA-28128; 5′-AACGGGAAAATCCAAAGCAC-3' (sense), 5′-CAGGA-CAGGAAGACAGAACTCG-3' (anti-sense) for mmu-circRNA-29837; and 5′-CACTGAGCAAGAGAGGCCCTAT-3' (sense), 5′- GCAGC-GAACTTTATTGATGGTATT-3' (anti-sense) for GAPDH. Both the perinatal glyphosate exposure group and the control group had three independent samples and all samples were conducted in triplicate. The PCR conditions were a 95 °C denaturation for 10 min, followed by 40 cycles at 95 °C for 10 s and 60 °C for 60 s. Melt curve analysis was carried out after the PCR to confirm primer specificity. The relative expression of circRNAs was calculated using the $2^{-\Delta\Delta Ct}$ method [16].

2.7. Computational bioinformatics analysis

CircRNA/miRNA interactions were predicted with Arraystar's homemade miRNA target prediction software based on TargetScan (http://www.targetscan.org/vert_71/) and miRanda (www.microrna.org/). Then, a circRNA-miRNA gene network was generated to visualize the interactions using Cytoscape. GO (Gene Ontology) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were used to annotate the functions of the targets genes.

2.8. Statistical analysis

All data are presented as the mean ± standard deviation (SD). Statistical significance was tested by Student's *t*-test, and a P-value < 0.05 was considered to be statistically significant.