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Brief communication

Glyphosate based- herbicide exposure affects gut microbiota, anxiety and depression-like behaviors in mice

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ABSTRACT

Recently, a number of studies have demonstrated the profound relationship between gut microbiota (GM) alterations and behavioral changes. Glyphosate-based herbicides (GBH) have been shown to induce behavioral impairments, and it is possible that they mediate the effects through an altered GM. In this study, we investigated the toxic effects of GBH on GM and its subsequent effects on the neurobehavioral functions in mice following acute, subchronic and chronic exposure to 250 or 500 mg/kg/day.

The effect of these acute and repeated treatments was assessed at the behavioral level using the open field, the elevated plus maze, the tail suspension and splash tests. Then, mice were sacrificed and the intestinal samples were collected for GM analysis.

Subchronic and chronic exposure to GBH induced an increase of anxiety and depression-like behaviors. In addition, GBH significantly altered the GM composition in terms of relative abundance and phylogenic diversity of the key microbes. Indeed, it decreased more specifically, *Corynebacterium*, *Firmicutes*, *Bacteroidetes* and *Lactobacillus* in treated mice.

These data reinforce the essential link between GM and GBH toxicity in mice and suggest that observed intestinal dysbiosis could increase the prevalence of neurobehavioral alterations.

1. Introduction

Emerging behavioral and molecular evidence from Germ Free mice reveals a relationship between the gut microbiota and brain disorders, and provides support for the idea that normal healthy gut bacteria may influence the development of the central nervous system and thereby its function (Wiley et al., 2017). In this regard, GF mice exhibited pronounced cognitive deficits and expressed higher anxiety and depression-like behaviors, paralleled with lower turnover rates of monoamines in several brain regions (Nishino et al., 2013).

Pesticide residues are a persistent and serious environmental problem; they are ubiquitous in food materials, water and soil. Because of their antimicrobial activity, pesticides have the potential to change the gut microbiota leading to disorders of energy metabolism, immune system function and psychoaffective functions (Jin et al., 2015). Glyphosate-based herbicide (GBH), the active ingredient present in Roundup® (Monsanto Company, St. Louis, MO), is the most heavily used organophosphate herbicide worldwide (Powles et al., 1996). It has been found that glyphosate's mechanism of action in plants is related to the disruption of the shikimate pathway, which is involved in the synthesis of the essential aromatic amino acids (Herrmann and Weaver, 1999). The currently accepted dogma is that glyphosate (Gly) is not harmful to humans or to any mammals because of the absence of the shikimate pathway in mammals (Samsel and Seneff, 2013). However, this pathway is present in gut bacteria, which plays an important and heretofore largely overlooked role in human physiology (Moco et al., 2012).

Because GBH-exposure has been shown to impact the neurobehavioral functions, and that altered gut mirobiota (GM) profiles have been associated with anxiety and depressive-like behavior (Bercik et al., 2011a, 2011b), it can be hypothesized that GBH-induced GM alterations may contribute in mediating behavioral changes. Thus, the purpose of the present study was to evaluate the impact of GBH on a healthy microbiota–gut–brain axis by investigating the gastrointestinal microbiota alterations and their subsequent effects on the neurobehavioral functions in mice.

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2. Material and methods

2.1. Pesticide

Roundup herbicide (glyphosate concentration 360 g/l in the form of glyphosate isopropylamine salt 486 g/l) with molecular formula $C_6H_{17}N_2O_5P$, molecular weight of 228.183 g/Mol, melting point 200 °C and density 1.218 g/cm^3 was used in the liquid commercial form supplied by Monsanto Company (St. Louis, MO, USA).

2.2. Animals and treatment

Male Swiss mice (1-month-old) were obtained from the animal husbandry of the Faculty of Sciences, Cadi Ayyad University, Marrakech, Morocco. The animals were housed in Plexiglas cages (30 cm × 15 cm × 12 cm) under standard conditions of temperature (22 ± 2 °C) and photoperiod 12 h/12 h (lights on at 08:00 h). Food and water were available *ad libitum*. All procedures were conducted in accordance with approved institutional protocols, and with the provisions for animal care and use prescribed in the scientific procedures on living animals, European Council Directive: EU2010/63. All efforts were made to minimize any animal suffering. The study was approved by the Council Committee of Research Laboratories of the Faculty of Sciences, Cadi Ayyad University, Marrakech.

Mice were subdivided to three experimental groups (acute, subchronic and chronic: $n = 18$/group), and each group was subjected either to orally gavages by NaCl 0.9% (control $n = 6$), by 250 mg/kg/day (n = 6) or 500 mg/kg/day (n = 6) of GBH. These doses were selected on the basis of Gly no-observed adverse effect level (NOAEL) of 500 mg/kg/day for subchronic toxicity (EPA, 1993). The mice assigned to the acute group received one administration of 0.3 ml of GBH, while the subchronic and chronic groups were treated daily by this volume for 6 and 12 weeks, respectively. Then, they were submitted to behavioral testing. On the last day of the experiment, the treated animals were sacrificed for gut microbiota analysis.

2.2.1. Behavioral assessments

2.2.1.1. Open field test. This test is the commonly used to assess locomotor activity and emotional reactivity in rodents placed into novel environments (Wilson et al., 1976). The apparatus was a square field (50 × 50 × 50 cm), within each mouse was placed, and allowed to move freely for 20 min. The time spent in the center area (15 × 15 cm) as an index of anxiety behavior was recorded using a video camera (JVC), and analyzed by Ethovision XT Noldus 8.5 video tracking program (Noldus Information Technology b.v., Wageningen, Netherlands).

2.2.1.2. Elevated-plus maze test. This test is used to assess anxiety-like behavior in rodents (Handley and Mithani, 1984). The apparatus elevated to a height of 45 cm above the floor, comprised two opposing open arms (OA) (50 × 5 cm) and two closed arms (CA) (50 × 5 × 15 cm), which joined at a central square area (5 × 5 cm) to form a plus sign. Animals were tested individually for 5 min, by placing them in the center of the maze platform, with the head facing an open arm. The time spent in the OA and CA as well as the number of entries to each arm were quantified using Ethovision XT Noldus 8.5 video tracking program, allowing us to evaluate the anxiety index, according to Cohen et al. (2013) method, expressed as: anxiety index = 1 − [([open arm time/total time] + [open arm entries/total number of entries])/2]).

2.2.1.3. The tail suspension test. This is one of the accepted behavioral tests used to evaluate potential antidepressant-like effects in rodents (Cryan and Holmes, 2005). The mice were individually suspended by the tail above the ground, with adhesive tape placed about 40 cm from the floor. A single 6 min session was recorded for each animal. The total time spent immobile during the last 4 min of a session was scored.

2.2.1.4. Splash test. This test assess grooming behavior, defined as cleaning of the fur by licking or scratching, after vaporization of 10% sucrose solution onto the mouse's dorsal coat (David et al., 2009). The latency to initiate a grooming behavior as well as the duration of grooming was recorded during 5 min after the vaporization of sucrose solution. Previous works in mice have shown that in the splash test, chronic stress decreases grooming behavior, a form of motivational behavior considered to parallel indifferent behavior as a symptom in depression (Isingrini et al., 2010).

2.2.2. Sample collection and abundances of intestinal microbiota determination

In mammals, the lower gastrointestinal tract (the small intestine, caecum and large intestine) contains a variety of distinct microbial habitats, in which physiological variations along its lengths include chemical and nutrient gradients, as well as compartmentalized host immune activity, known to influence bacterial community composition (for review see Gregory et al., 2016). Thus, to examine the effects of glyphosate-based herbicide on the composition of bacterial communities, intestinal samples were obtained from control and GBH-exposed mice at euthanasia, after behavioral tests completion. For each animal, sample of the intestinal tract starting from the duodenum to the end of the large intestine was collected directly into sterile tube, diluted ten times in sterile physiological water (NaCl 9 g/l), homogenized by vortexing for 10–15 min. Bacterial strains were counted using dilution / spreading method after the incubation at 37 °C for 72 h.

2.2.3. Phoenix system identification method

The Phoenix identification method uses modified conventional, fluorogenic, and chromogenic substrates. Combination panels for investigational use only (PMIC/ID-33, catalog no. 448587) for both identification and susceptibility testing were used for this comparison. Software V7. 00A/V5.91A was used for this study. The ID side contains 45 wells with dried biochemical substrates and 2 fluorescent control wells. The ID broth was inoculated with bacterial colonies adjusted to a 0.5 McFarland standard by using a Crystal Specnephelometer (BD Diagnostics), according to the manufacturer's recommendations. The specimen was logged and loaded into the instrument within the specified timeline of 30 min. Quality control and maintenance were performed according to the manufacturer's recommendations.

3. Statistical analysis

To compare data from behavioral tests and gut microbiota analysis between groups (control and treated), a statistical analysis of the different independent variables was performed by two-way ANOVA (treatment and treatment duration), using the Sigma Plot software 11.0. *Post hoc* analysis was performed using Holm-Sidak *post hoc* test. Results are presented as mean ± standard error of the mean (S.E.M). The significance threshold was set at $p < 0.05$.

4. Results

4.1. Behavioral changes after GBH exposure

Repeated exposure to GBH elicited evident emotional behavioral alterations in mice, as assessed by various behavioral tests: open field, elevated plus-maze, tail suspension and splash test.

Our results indicated that the control groups of each test showed no significant difference as a function of treatment duration, except for the controls of the open field test. In fact, we observed a significant increase in controls of subchronic (81.77 ± 5.15 s) and chronic treatment groups (76.63 ± 5,19 s) compared to the acute control group (34.68 ± 3.13 s) ($t = 8.47$ and $t = 7.56$ respectively, with 10 degrees