Toxicology 376 (2017) 2–14

Contents lists available at ScienceDirect

Toxicology

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

Neonatal exposure to a glyphosate based herbicide alters the development of the rat uterus

Marlise Guerrero Schimpf, María M. Milesi, Paola I. Ingaramo, Enrique H. Luque, Jorgelina Varayoud*

Instituto de Salud y Ambiente del Litoral, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral – Consejo Nacional de Investigaciones Científicas y Técnicas, Santa Fe, Argentina

ARTICLE INFO

Article history:
Received 22 December 2015
Received in revised form 26 May 2016
Accepted 6 June 2016
Available online 7 June 2016

Keywords:
Glyphosate based herbicide
Uterus
Luminal epithelial hyperplasia
Progesterone receptor
Hoxa10
Estrogen receptor alpha

ABSTRACT

Glyphosate-based herbicides (GBHs) are extensively used to control weeds on both cropland and non-cropland areas. No reports are available regarding the effects of GBHs exposure on uterine development. We evaluated if neonatal exposure to a GBH affects uterine morphology, proliferation and expression of proteins that regulate uterine organogenetic differentiation in rats. Female Wistar pups received saline solution (control, C) or a commercial formulation of glyphosate (GBH, 2 mg/kg) by sc injection every 48 h from postnatal day (PND) 1 to PND7. Rats were sacrificed on PND8 (neonatal period) and PND21 (prepubertal period) to evaluate acute and short-term effects, respectively. The uterine morphology was evaluated in hematoxylin and eosin stained sections. The epithelial and stromal immunophenotypes were established by assessing the expression of luminal epithelial protein (cytokeratin 8; CK8), basal epithelial proteins (p63 and pan cytokeratin CK1, 5, 10 and 14); and vimentin by immunohistochemistry (IHC). To investigate changes on proteins that regulate uterine organogenetic differentiation we evaluated the expression of estrogen receptor alpha (ERα), progesterone receptor (PR), Hoxa10 and Wnt7a by IHC. The GBH-exposed uteri showed morphological changes, characterized by an increase in the incidence of luminal epithelial hyperplasia (LEH) and an increase in the stromal and myometrial thickness. The epithelial cells showed a positive immunostaining for CK8, while the stromal cells for vimentin. GBH treatment increased cell proliferation in the luminal and stromal compartment on PND8, without changes on PND21. GBH treatment also altered the expression of proteins involved in uterine organogenetic differentiation. PR and Hoxa10 were deregulated both immediately and two weeks after the exposure. ERα was induced in the stromal compartment on PND8, and was downregulated in the luminal epithelial cells of gyphosate-exposed animals on PND21. GBH treatment also increased the expression of Wnt7a in the stromal and glandular epithelial cells on PND21. Neonatal exposure to GBH disrupts the postnatal uterine development at the neonatal and prepubertal period. All these changes may alter the functional differentiation of the uterus, affecting the female fertility and/or promoting the development of neoplasias.

1. Introduction

Glyphosate (*N*-phosphonomethyl glycine) is the active ingredient of a number of broad-spectrum herbicide formulations, widely used all over the world to control weeds on both cropland and non-cropland areas (Baylis, 2000; Woodburn, 2000; Cerdeira et al., 2007; Duke and Powles, 2008). Commercial formulations of glyphosate include other chemical compounds that act as solvents, adjuvants, preservatives or surfactants. Although these substances are classified as inert compounds, it has been demonstrated that the formulations of glyphosate

Abbreviations: CK, cytokeratin; DES, diethylstilbestrol; EDCs, endocrine-disrupting chemicals; ERα, estrogen receptor alpha; GBHs, glyphosate-based herbicides; IARC, International Agency for Research on Cancer; IHC, immuhistochemistry; IOD, integral optical density; LEH, luminal epithelial hyperplasia; PND, postnatal day; PR, progesterone receptor; RfD, reference dose; U.S. EPA, United StatesEnvironmental Protection Agency; Vv, Volume fraction.

* Corresponding author at: Instituto de Salud y Ambiente del Litoral (ISAL), Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Casilla de Correo 242, Santa Fe 3000, Argentina.
E-mail address: varayoud@fbcb.unl.edu.ar (J. Varayoud).

http://dx.doi.org/10.1016/j.tox.2016.06.004
0300-483X/

are more toxic than the compound in its technical grade (Richard et al., 2005; Benachour and Seralini, 2009; Mesnage et al., 2014). In Argentina, the areas of lands in transgenic glyphosate-resistant soybean production have extensively increased, and that has been accompanied by an increase in the herbicide use (Cerdeira et al., 2011). To date, more than 200 million litters of GBHs are applied every year in our country (Aparicio et al., 2013).

Although glyphosate has been considered to have low persistency, the magnitude of environmental impact depends on the rate and frequency of glyphosate application (Mamy et al., 2010). In Argentina, a monitoring study carried out within the main area of soybean production, revealed levels of glyphosate range from 0.1 to 0.7 mg/l in surface waters and 0.5–5 mg/kg in sediments and soil (Peruzzo et al., 2008; Aparicio et al., 2013). Other studies reported the presence of glyphosate residues in pre-harvest soybean (Arregui et al., 2004; Test Biotech, 2013) and in crops at harvest (Agricultural Marketing Service – U.S. Department of Agriculture, 2013). In addition, Curwin et al. (2007a,b) reported glyphosate detection in the urine of families living in farms and nonfarm households, although the estimated exposure levels to glyphosate were several orders of magnitude below thereference dose (RfD) proposed by the U.S. Environmental Protection Agency (U.S. EPA, 1993).

In a recent report, a consensus statement analyzeddifferent results related to GBHs (Myers et al., 2016).Some studies indicate that GBHs disrupt endocrine-signalling systems *in vitro* (Richard et al., 2005; Gasnier et al., 2009; Thongprakaisang et al., 2013; Defarge et al., 2016). Few *in vivo* studies have dealt with the effects of GBHs, and no reports are available regarding the consequence of GBHs exposure during critical periods of developmentonthe female reproductive tract.

The female reproductive tract and particularly the uterus are highly sensitive to developmentally disruptive effects of hormonal steroids and natural or synthetic endocrine-disrupting chemicals (EDCs) (Spencer et al., 2012; Varayoud et al., 2014). Transient disruption of the normal developmental program has long-term adverse consequence for uterine function and reproductive health (Varayoud et al., 2008, 2011; Milesi et al., 2012; Milesi et al., 2015). In the present work we hypothesized that early postnatal exposure to a GBH might interfere with normal uterine development and differentiation. We evaluated the effects of neonatal exposure to a low dose of a GBH on the uterine morphology, the cell proliferation and the expression of proteins involved in uterine organogenetic differentiation, such as, ERα, PR, Hoxa10 (a member of the Hox gene family) and Wnt7a (a member of the Wnt gene family). The effects were determined at two time points: i) shortly after the end of the exposure period (PND8, neonatal period) to evaluate the acute response to GBH exposure, and ii) two weeks after the end of the exposure period (PND21, prepubertal period), to investigate whether the effects persisted and/or were manifested in a stage distant from the GBH exposure. The selection of proteins to be evaluated was based on their role in uterine organogenetic differentiation. Hoxa10 and Wnt7a, regulate several developmental pathways that guide uterine growth and differentiation during embryogenesis and postnatal development (Benson et al., 1996; Miller and Sassoon, 1998; Spencer et al., 2012). These molecules are also dynamically expressed in adult endometrium, where they play a pivotal role on embryo implantation (Bagot et al., 2000; Dunlap et al., 2011). Because of many EDCs exert their actions through the interaction with sex steroid hormone receptors (Roy et al., 2009), we postulate that uterine ERα and PR proteins could be affected by a GBH developmental exposure.

2. Materials and methods

2.1. Animals

All procedures used in this study were approved by the Institutional Ethic Committee of the School of Biochemistry and Biological Sciences (Universidad Nacional del Litoral, Santa Fe, Argentina), and were performed in accordance with the principles and procedures outlined in the Guide for the Care and Use of Laboratory Animals issued by the U.S. National Academy of Sciences. Inbred Wistar strain rats were bred at the Department of Human Physiology (Santa Fe, Argentina) and housed under a controlled environment (22 °C ± 2 °C; lights on from 06:00 to 20:00 h) with free access to pellet laboratory chow (16–014007 Rat-Mouse Diet, Nutrición Animal, Santa Fe, Argentina) and tap water. For more information regarding the food composition, see Kass et al. (2012) and Andreoli et al. (2015). To minimize additional exposure to EDCs, rats were housed in stainless steel cages with sterile pine wood shavings as bedding, and tap water was supplied in glass bottles with rubber stoppers surrounded by a steel ring.

2.2. Experimental design

Pups were obtained from 8 to 10 timed-pregnant Wistar rats per group housed singly. After parturition (PND0), pups were sexed according to anogenital distance and litters of eight pups (preferably four males and four females) were left per mother. Female pups from each mother were randomly assigned to the following neonatal treatment groups: 1) control group receiving saline solution, and 2) GBH group receiving a commercial formulation of glyphosate dissolved in saline solution (2 mg/kg b.w). The glyphosate formulation used was Roundup FULL II®, a liquid water-soluble formulation containing 66.2% of glyphosate potassium salt, as its active ingredient, coadjunvants and inert ingredients. Substances (40 μl) were administered by s.c. injection in the nape of the neck every 48 h from PND1 to PND7. Each treatment day, the dose was calculated based on the average body weight of the pups. The dose of GBH was selected based on the reference dose (RfD)for glyphosate proposed by the U.S. Environmental Protection Agency (U.S. EPA, 1993). Although the RfD for glyphosate is based on oral exposure, the subcutaneous via is the unique administration route that warrants the whole incorporation of a chemical compound when an early postnatal exposure model is used.Eight rats from each neonatal treatment group were weighted and sacrificed by decapitation on PND8 and PND21 to evaluate acute and short-term effects, respectively. Uterine horns were removed, fixed by immersion in 4% paraformaldehyde buffer for 6 h at 4 °C and processed for histology and IHC.

2.3. Histological analysis

Uterine longitudinal sections (5 μm thick) were stained with hematoxylin and eosin and examined by light microscope (Olympus BH2 microscope; Olympus, Tokyo, Japan) to analyze the uterine morphology. Three sections per animal separated 25 μm from each other were evaluated. First, we quantified the number of luminal epithelial layers using a Dplan 40× objective (numerical aperture = 0.65; Olympus) on PND8 and 20× objective (numerical aperture = 0.40; Olympus) on PND21. Luminal epithelial hyperplasia (LEH) was established as a luminal epithelium with more than four cellular layers. A total of 10 fields were evaluated/section and the results were expressed as% of incidence of LEH. The number of uterine glands was determined on 10 randomly selected fields using a Dplan 20× objective. Finally, the thickness of the subephitelial stroma and myometrium layers was analyzed by