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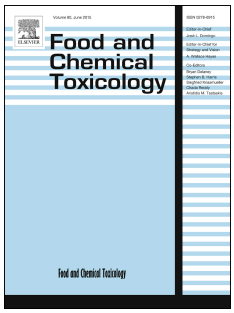

Postnatal exposure to a glyphosate-based herbicide modifies mammary gland growth and development in Wistar male rats

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PII: S0278-6915(18)30300-4

DOI: 10.1016/j.fct.2018.05.011

Reference: FCT 9765

To appear in: *Food and Chemical Toxicology*

Received Date: 13 March 2018

Revised Date: 4 May 2018

Accepted Date: 6 May 2018

Please cite this article as: Altamirano, G.A., Delconte, M.B., Gomez, A.L., Ingaramo, P., Bosquiazzo, Veró.L., Luque, E.H., Muñoz-de-Toro, Mó., Kass, L., Postnatal exposure to a glyphosate-based herbicide modifies mammary gland growth and development in Wistar male rats, *Food and Chemical Toxicology* (2018), doi: 10.1016/j.fct.2018.05.011.

FINAL VERSION

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ABSTRACT

Our aim was to evaluate whether postnatal exposure to a glyphosate-based herbicide (GBH) modifies mammary gland development in pre- and post-pubertal male rats. From postnatal day 1 (PND1) to PND7, male rats were injected subcutaneously every 48 h with either saline solution (vehicle) or 2 mg GBH/kg·bw. On PND21 and PND60, mammary gland and blood samples were collected. Estradiol (E2) and testosterone (T) serum levels, mammary gland histology, collagen fiber organization, mast cell infiltration, proliferation index, and estrogen (ESR1) and androgen receptor (AR) expression levels were evaluated. At PND21, GBH-exposed male rats exhibited greater development of the mammary gland with increased stromal collagen organization and terminal end buds (TEBs) compared to control rats. At PND60, the number of TEBs remained high and was accompanied by an increase in mast cell infiltration, proliferation and ESR1 expression in GBH-exposed male rats. In contrast, no effects were observed in E2 and T serum levels and AR expression in both days studied. Our results showed that a postnatal subacute treatment with GBH induces endocrine-disrupting effects in the male mammary gland *in vivo*, altering its normal development.

KEY WORDS:

Male rats; mammary gland; mast cells; glyphosate-based herbicide, estrogen receptor

Abbreviations: AR: androgen receptor; GBH: glyphosate-based herbicide; ESR1: estrogen receptor alpha; E2: estradiol; IOD: integrated optical density; LN: lymph node; P&H: picrosirius-hematoxylin; PND: postnatal day; TEBs: terminal end buds; T: testosterone

1. Introduction

Glyphosate (N-phosphonomethyl glycine) is an active ingredient in many commercially available broad-spectrum herbicides. Over the past decades, glyphosate-based herbicide (GBH) use has diversified and expanded significantly. Benbrook (2016) reported that the application of these herbicides increased ~ 100-fold worldwide from 1974 to 2014. In Argentina, increasing use of these chemicals has been associated with agricultural expansion due to the ongoing adoption of glyphosate tolerant genetically modified soybeans (CASAFE, 2012). In recent years, glyphosate and its major metabolite, aminomethylphosphonic acid (AMPA), have been detected in surface water, sediments and soil that surround horticultural production areas of different regions of Argentina (Aparicio et al., 2013; Bonansea et al., 2017; Lupi et al., 2015; Mac Loughlin et al., 2017; Primost et al., 2017; Ronco et al., 2016). These pesticide residues have also been detected in foodstuff (EFSA, 2017) and human urine (Connolly et al., 2017; Goen et al., 2017; Mills et al., 2017; Parvez et al., 2018) and serum samples (Kongtip et al., 2017). In addition, it has been demonstrated in rats that exposure to a mixture containing glyphosate is able to induce hepatoxicity (Docea et al., 2018) and that low doses of GBH provokes kidney and especially liver oxidative damage and non-alcoholic fatty liver disease (Mesnage et al., 2015a; Mesnage et al., 2017b). These findings suggest that there is a risk of environmental exposure to GBH and raise concern of its possible effects on the environment and human health.

Several studies have reported the adverse effects of GBH exposure on both female and male reproductive systems at low and environmentally relevant doses (Cai et al., 2017; Guerrero Schimpf et al., 2017; Ingaramo et al., 2017; Ingaramo et al., 2016; Nardi et al., 2017; Varayoud et al., 2017). In male rats, glyphosate exposure modifies testicular

function and morphology, decreases testosterone serum levels, increases aromatase expression level, and alters sperm production, suggesting that GBH could act as an endocrine disruptor *in vivo* (Cassault-Meyer et al., 2014; Dallegrave et al., 2007; Owagboriaye et al., 2017; Romano et al., 2010). In addition, glyphosate was shown to reduce aromatase enzyme activity in human placental cells (Richard et al., 2005) and induce human breast cancer cell proliferation by directly activating the estrogen receptor alpha (ESR1) *in vitro* (Thongprakaisang et al., 2013). In contrast, Mesnage et al. (2017a) showed that glyphosate activates ESR1 through a ligand-independent mechanism in hormone-dependent human cancer cells. Therefore, the estrogenic potential effect of glyphosate remains under investigation. In general, new epidemiological and toxicological studies as well as human biomonitoring are urgently needed to determinate whether GBH could be considered an endocrine disruptor and to improve safety standard (Myers et al., 2016; Vandenberg et al., 2017).

Given the effects on male reproductive organs and the suggested estrogenic properties of this compound, male mammary gland development could also be affected. The male mammary gland of rats has been used by several researchers as a useful model to study the effects of potential endocrine disruptors that may affect the risk of breast cancer in humans (Filgo et al., 2016; Mandrup et al., 2016; Mandrup et al., 2015). Recently, we showed that prenatal exposure to the endocrine disruptor bisphenol A induces a growth delay and decreases the expression of androgen receptor (AR) in the pre-pubertal male rat mammary gland (Kass et al., 2015) and that early postnatal exposure to endosulfan induces premalignant lesions in the mammary gland of post-pubertal male rats (Altamirano et al., 2017). There is evidence that chronic exposure to GBH increases mammary tumor incidence in adults rats (Seralini et al., 2014). However, to date, the

effects of postnatal GBH exposure on the male mammary gland *in vivo* remain mostly unknown. Therefore, the aim of the present study was to evaluate whether early postnatal exposure to GBH affects mammary gland growth and development in pre- and post-pubertal male rats.

2 Materials and methods

2.1 Animals

Sexually mature female rats (90 days old) of a Wistar-derived strain bred at the Department of Human Physiology (Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral (UNL), Santa Fe, Argentina) were used. The animals were maintained in a controlled environment (22 ± 2°C; 14 h of light) and had free access to pellet laboratory chow (16-014007 Rat-Mouse diet, Nutrición Animal, Santa Fe, Argentina). All the experimental protocols were approved by the Ethical Committee of the Facultad de Bioquímica y Ciencias Biológicas, UNL. Animals were treated humanely and with regard for alleviation of suffering.

2.2 Experimental procedures

Females in proestrus were caged overnight with males of proven fertility. The day on which the sperm was found in the vagina was designated day 1 of gestation. The mating pairs were then separated, and the pregnant female rats were housed individually in stainless steel cages. After delivery (PND0), the pups were weighed and sexed according to the anogenital distance, and the litter size was adjusted to eight pups (four males and four females) per mother. Male pups from each mother were assigned to two neonatal treatment groups (8-10 pups/group/postnatal day): a) the control group that received a saline solution and b) the GBH group that received a commercial formulation

of glyphosate dissolved in saline solution at 2 mg/kg·bw. The composition of the glyphosate commercial formulation used (Roundup FULL II®) is a liquid water-soluble preparation containing glyphosate potassium salt, as its active ingredient, adjuvants and inert ingredients. According to the manufacture, the amount of equivalent glyphosate acid was 54 %. The dose of 2 mg/kg was calculated based on this data (54 g of glyphosate per 100 ml of GBH) and the average body weight of the pups on each treatment day. Therefore, according to the weight (i.e.: 6–7 g per pup on PND1), male offspring were subcutaneously injected every 48 h from PND1 to PND7 with an herbicide solution containing approximately 0.013 mg of glyphosate per injection (Ingaramo et al., 2017). For this experiment, the dose of GBH was selected based on previous reports (Guerrero Schimpf et al., 2017; Ingaramo et al., 2017) that used the reference dose (RfD) for glyphosate proposed by the U.S. Environmental Protection Agency (U.S. EPA, 1993). Although the RfD for glyphosate was 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 (Guerrero Schimpf et al., 2017).

Male rats were sacrificed at pre-puberty (PND21) and post-puberty (PND60), and both blood and mammary gland samples were obtained. From the blood collected by decapitation, serum samples were obtained and stored at -80°C until estradiol (E2) and testosterone (T) levels were quantified by chemiluminescence on both days studied. One abdominal-inguinal mammary gland chain was randomly chosen to be processed for whole-mount evaluation, and the contralateral gland was fixed in 10% (v/v) buffered formalin for 6 h at room temperature and embedded in paraffin for histological examination and immunohistochemistry assays (Kass et al., 2015).

2.3 E2 and T levels serum levels

The E2 and T levels were determined by chemiluminescence assays using an Immulite® 2000 system (Siemens Healthcare SA, Argentina), following the manufacturer's specifications. The assays had a detection limit of 15 pg/ml for E2 and of 0.15 ng/ml for T. The intra-assay coefficient of variation for E2 was 6.7%, whereas that for T was 10.1% (Altamirano et al., 2017).

2.4 Mammary gland whole-mount evaluation

As previously described (Altamirano et al., 2017), images of mammary gland whole-mounts from PND21 were recorded using a Spot Insight V3.5 color video camera attached to a Stemi 305 stereomicroscope (ZEISS, Argentina), whereas those from PND60 were recorded using a Sony Alpha a58 DSLR Camera (Sony Latin America, Inc.). All images were analyzed with ImageJ software (NIH, USA; https://imagej.nih.gov/ij). The unequal illumination (shading correction) was corrected, and the measurement system was calibrated with a reference slide. All evaluations were carried out blinded to the treatment group on mammary gland N°4. Different mammary gland parameters, including total area, perimeter, distance between mammary glands N°4 and 5, longitudinal growth and number of terminal end buds (TEBs), were analyzed on mammary gland whole-mount images (Altamirano et al., 2017; Kass et al., 2015).

2.5 Histological analysis and collagen organization

For histological examination, paraffin sections (5 µm) of mammary gland samples collected on PND21 and PND60 were cut and stained with Sirius Red (Direct Red 80, Sigma-Aldrich, Argentina) in picric acid solution (picrosirius) counterstained with

Harris hematoxylin (Kass et al., 2001). The development of ducts and the lobuloalveolar structure as well as collagen organization were evaluated using Olympus BH2 light microscopy (Olympus Optical Co., Ltd., Japan).

For analysis of the organization of the collagen fibers in the mammary gland stroma, the collagen birefringence of picrosirius-stained samples was quantified by polarization microscopy (Montes, 1996). For each animal, 10 images were captured, digitized and analyzed. The stromal area occupied by organized collagen was measured as integrated optical density (IOD) by using the Image-Pro-Plus 4.1.0.1® system (Media Cybernetics, Silver Spring, USA). The digitized images were converted to gray scale, and the IOD was calculated as a linear combination of the average gray intensity and the relative area occupied by the stromal area. The results are expressed in arbitrary units (Altamirano et al., 2017; Kass et al., 2001).

2.6 Cell proliferation, steroid receptor expression and mast cell infiltration

Consecutive 5 µm sections were immunoassayed to evaluate the proliferative index (Ki67) and the expression of AR and ESR1 on PND21 and PND60. Sections from two different depths were used to evaluate the expression of each protein. Immunoperoxidase staining was performed as previously described (Kass et al., 2015). In addition, for identification of mast cells, rat mast cell proteinase-I (RMCP-I) was detected in mammary gland sections on PND60. Immunostaining was performed with the immunoperoxidase technique after periodic acid and sodium borohydrate incubation to block endogenous peroxidase activity (Varayoud et al., 2004). Sections were incubated overnight at 4°C with primary antibodies against Ki67 (Dako, Glostrup, Denmark), AR (N-20, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), ESR1

(clone 6-F11, Novocastra Laboratories Ltd., UK) and RMCP-I (Moredun Scientific Ltd., Edinburgh, Scotland). Anti-rabbit (Zymed Laboratories Inc., USA) or anti-mouse (Instituto de Salud y Ambiente del Litoral, Santa Fe, Argentina) secondary antibodies (biotin-conjugated) were used, and reactions were developed using an avidin-biotin peroxidase method with diaminobenzidine (Sigma-Aldrich) as a chromogen substrate. Each run included negative controls in which the primary antibody was replaced with non-immune mouse or rabbit serum (Sigma-Aldrich).

The percentages of Ki67, AR and ESR1 expression in the mammary epithelial cells were quantified in two different tissue sections per animal, and at least 2000 cells per tissue section were analyzed. The volume fraction (Vv) of mast cells in mammary tissue was determined using the point counting procedure with an Olympus BH2 microscope (Olympus Optical Co., Ltd., Tokyo, Japan) and a Dplan 40X objective (Olympus) (Luque et al., 1996). Briefly, a square grid inserted in a focusing eyepiece was used, and the volume fraction ratio was defined as the number of incident points in the studied cell (mast cell) divided by the total number of incident points in the volume unit (whole mammary gland section) (Durando et al., 2007).

2.7 Statistical analysis

All data are expressed as the mean ± SEM. An exploratory analysis was first conducted to confirm the normal distribution of the data (Shapiro–Wilk test) and variance homogeneity (Levene's test). For determination of whether a change in body weight affected the whole-mount parameters, multiple lineal regression (whole-mount parameter ~ treatment + body weight) analyses were performed with each of the parameters evaluated as a dependent variable and the treatment and body weight as

independent variables. After discarding the effect of body weight, the evaluation of mammary whole-mount parameters was analyzed using the *t* test to compare the experimental and control groups. The variables that did not show a normal distribution were analyzed by the Mann-Whitney U test. All analyses were carried out using R software (The R Foundation for Statistical Computing http://www.r-project.org/). Values with $p<0.05$ were considered significant.

3. Results

No signs of acute or chronic toxicity were observed in the litters, and no differences were found in the weight gain between treated and control pups during the treatment (PND1-PND7). At PND21, the pup body weight was similar between the experimental groups (Control: 34.39±0.96 g *vs* GBH: 35.85±0.78 g), whereas in post-pubertal (PND60) male rats exposed to GBH, a significant increase in the body weight was observed (Control: 218.10±3.82 g *vs* GBH: 230.70±2.98 g; $p<0.05$).

3.1 Mammary gland growth of pre-pubertal male rats.

To assess whether mammary growth was affected by postnatal exposure to GBH in pre-pubertal male rats, we analyzed mammary whole-mount parameters and performed a histological examination at PND21. Representative images are shown in Fig. 1. Postnatal subacute exposure to GBH increased the total area, perimeter and number of TEBs in pre-pubertal GBH-exposed rats compared to control rats (Table 1, $p<0.05$). In contrast, the distance between mammary gland N°4 and 5 was similar between the groups.

Although postnatal subacute exposure to GBH affected the mammary growth, no differences were found in the histological examination of mammary epithelial structures in pre-pubertal male rats. In both experimental groups, the ducts were characterized by a single or double layer of epithelial cells surrounded by connective tissue stroma and embedded in a developed adipose tissue with low intracellular lipid content (Fig. 1). Nevertheless, pre-pubertal GBH-exposed rats showed a higher collagen birefringence level than control rats (Control: 0.015±0.002 IOD *vs* GBH: 0.021±0.003 IOD; $p<0.05$), indicating a more organized collagen fiber.

3.2 Mammary gland development in post-pubertal male rats.

Given the mammary histoarchitecture alterations observed in pre-pubertal male rats after postnatal GBH exposure, the mammary gland development was also analyzed in post-pubertal male rats. Representative images of mammary whole-mount samples and histological characterization are shown in Fig. 2. First, considering the small statistically significant increase in body weight in the GBH-exposed post-pubertal rats, multiple linear regression analysis was performed to evaluate the effect of this change on each of the whole-mount parameters. Based on this analysis, this increase in the body weight had no effect on the whole-mount parameters analyzed at PND60 (data not shown). Mammary glands of GBH-exposed male rats exhibited augmented longitudinal ductal growth and an increased number of TEBs compared with those of control rats at PND60 (Table 1; $p<0.05$). However, mammary total area and perimeter did not show significant differences between the experimental groups. Additionally, the mammary histological analysis showed that the lumen of both ducts and lobuloalveolar structures was less dilated in post-pubertal GBH-exposed rats than control ones (Fig. 2). Furthermore,

collagen birefringence was similar between the experimental groups (Control: 0.17±0.03 IOD *vs* GBH: 0.17±0.02 IOD).

3.3 Endocrine environment and mammary gland cellular markers in pre- and post-pubertal male rats

To evaluate whether postnatal subacute exposure to GBH induced changes in proliferation and the mammary gland endocrine environment that could be associated with the previously observed morphological alterations in pre- and post-pubertal male mammary glands, we assessed T and E2 serum levels as well as Ki67, ESR1 and AR protein expression. No differences among treatments were observed in either T or E2 serum levels at both PNDs analyzed (Table 2). As expected, post-pubertal male rats showed higher serum levels of T and E2 than pre-pubertal rats.

The percentages of epithelial cells that expressed Ki67, ESR1 and AR in the male mammary gland are shown in Fig. 3. At PND21, the proliferation index as well as ESR1 and AR expression did not differ significantly between GBH-exposed and control rats. In contrast, at PND60, GBH almost doubled the proliferation index and ESR1 expression compared to those of the control rats ($p<0.05$), whereas AR expression was similar between the groups.

3.4 Evaluation of mast cells in the mammary gland of post-pubertal male rats.

Considering that postnatal subacute exposure to GBH increased the number of TEBs and the proliferation index in the mammary gland at PND60, mast cell infiltration was quantified in the mammary gland of post-pubertal male rats. Postnatal subacute exposure to GBH also induced a higher number of mast cells in the stroma that

surrounded both ducts and lobuloalveolar structures compared with those of control rats at this age (Fig. 4; $p<0.05$).

4. Discussion

In recent years, the effects of endocrine-disrupting chemicals on the development of the male mammary gland of rodents has received increased attention due to its sensitive response to estrogenic and/or androgenic compounds (Altamirano et al., 2017; Filgo et al., 2016; Kass et al., 2015; Kolla et al., 2017; Mandrup et al., 2015; Vandenberg et al., 2013). In the current study, postnatal subacute exposure of male rats to GBH during a critical period of development resulted in alterations in the growth and development of the mammary gland in pre- and post-pubertal rats without systemic toxicity. At PND21, GBH-exposed rats exhibited greater development of the male mammary gland and an increased number of TEBs compared to control rats. After puberty, the number of TEBs remained high and was accompanied by changes in the proliferation index and ESR1 expression in GBH-exposed male rats. Interestingly, high infiltration of mast cells was induced by postnatal exposure to GBH at PND60.

Notwithstanding the sexual dimorphism that exists in terms of mammary growth, there is evidence that mammary gland development is similar between female and male rats before puberty (Cardy, 1991; Filgo et al., 2016). Mammary gland development is characterized principally by a tubular epithelial pattern, and the presence of TEBs is observed in both male and female rats until puberty (Filgo et al., 2016). Exposure to endocrine disruptors during certain stages of development has been shown to cause abnormal mammary development in pre-pubertal male rats (Altamirano et al., 2017; Filgo et al., 2016; Kass et al., 2015). Our results showed that early postnatal exposure to

GBH could accelerate the mammary growth of pre-pubertal male rats. This was shown by an increment of the mammary total area and perimeter in GBH-exposed rats at PND21. Considering that TEBs are highly proliferative terminal ductal structures and primarily responsible for epithelial extension through the fat pad (Filgo et al., 2016; Paine and Lewis, 2017), the increased number of TEBs found in GBH-exposed rats could explain the greater development of the male mammary gland at this stage. Postnatal exposure to E2 increases the number of TEBs together with an increment in cell proliferation and alterations of T levels and ESR1 expression in male rats at puberty (Miousse et al., 2013). In our study, despite the high number of TEBs observed, the proliferation index and steroid hormone receptor expression as well as T and E2 serum levels were not affected by GBH exposure in pre-pubertal male rats.

Several researchers have demonstrated that a stiffer extracellular matrix due to an increase in collagen density influences epithelial cell proliferation and promotes initiation and progression of breast cancer in animal studies (Levental et al., 2009; Provenzano et al., 2008). In this regard, we showed that the exposure to endosulfan during the first week of age induces an increment of stromal collagen deposition that is associated with a higher incidence of hyperplastic mammary lesions (Altamirano et al., 2017). In the present study, the observed increment in collagen birefringence could be allowing the deregulation of proliferative structures in the male mammary gland, increasing the number of TEBs in pre-pubertal GBH-exposed animals.

As puberty progresses in the rat, the mammary ductal growth occurs exponentially at the same time that the TEBs begin to differentiate into terminal ends and the alveolar buds within mammary gland become mature structures, demonstrating dense branching

density (Filgo et al., 2016; Masso-Welch et al., 2000). In addition, the sexually mature control male mammary gland presents a permanent lobuloalveolar structure with an indistinct alveolar lumen that sometimes contains small amounts of secretory material, and the ducts have a pseudostratified or striated epithelium (Cardy, 1991; Filgo et al., 2016; Rudmann et al., 2005). Our results showed that although similar epithelial structures were found in both experimental groups, male rats exposed to GBH presented less dilated alveoli and ducts than control rats at PND60. The implication of this finding is unknown but could be the consequence of a more proliferative structure in the post-pubertal stage.

In vitro, the exposure to glyphosate induces an increment in mammary cell proliferation and ESR1 activation and/or ESR1 expression (Mesnage et al., 2017a; Thongprakaisang et al., 2013). In this *in vivo* experiment, we analyzed different markers of reproductive effect in the mammary gland and showed that the proliferation index and ESR1 expression were augmented by postnatal subacute exposure to GBH in post-pubertal male rats, whereas AR expression as well as T and E2 serum levels were similar between the groups. Given that estrogens through their receptors promote mammary gland proliferation and ductal elongation (Fendrick et al., 1998), these findings could explain the increase in the longitudinal ductal growth and the high number of TEBs that remained in GBH-exposed male rats at PND60. Despite similar E2 and T serum levels between groups, the increased proliferation index observed in GBH-exposed rat could be the result of the activation of ESR1 through a ligand independent manner as was described by Mesnage et al. (2017a). In addition, it has been reported that glyphosate alone may induce DNA methylation in human cells (Kwiatkowska et al., 2017). In our study, epigenetic modifications of *ESR1*, induced by GBH exposure, could be

responsible for the increased expression of this receptor without changes in E2 serum levels, like is shown for endosulfan in the uterus (Milesi et al., 2015; Milesi et al., 2017).

Interestingly, mast cell infiltration was also increased in the stroma that surrounded the epithelial structures in post-pubertal GBH-exposed rats. Lilla and Werb (2010) have shown that mast cells are present throughout normal postnatal mammary development and regulate TEBs and duct cell proliferation during puberty, mostly through mast cell-released factors. Therefore, the increase in mast cells could contribute to TEB maintenance and duct elongation in the male mammary gland of GBH-exposed rats. The TEB structure is highly susceptible to an oncogenic insult (Paine and Lewis, 2017), and increased mast cell infiltration could heighten the risk of malignant transformation of these structures, considering that they have been linked to intraductal proliferation that could progress to carcinoma (Russo and Russo, 1996). The release of histamine after mast cell degranulation could be the link between mast cell infiltration and intraductal proliferation because it has been shown that histamine promotes tumor cell proliferation and mammary carcinoma growth (Aponte-Lopez et al., 2018). In GBH-exposed males, the number of mast cells in the post-pubertal mammary gland was doubled as compared to non-exposed animals. Similar results were observed after *in utero* exposure to the xenoestrogen bisphenol A; however, in this case the increase in mast cell infiltration occurs in the female rat mammary gland stroma that were spatially associated with hyperplastic ducts (Durando et al., 2007).

Despite having no effect on mammary gland growth, another interesting result obtained in our study was the increase in body weight in post-pubertal male rats following GBH

treatment. Such an increase in body weight was not observed in pre- and post-pubertal female GBH-exposed rats in experiments using the same dose and window of exposure as the one in our experiment (Guerrero Schimpf et al., 2017; Ingaramo et al., 2016), suggesting that this effect could be gender-specific. Exposure to some endocrine-disrupting chemicals, termed “obesogens”, during pregnancy and/or lactation can cause weight gain later in life by altering lipid homeostasis to promote adipogenesis and lipid accumulation (Darbre, 2017). Further studies are needed to determinate whether early postnatal GBH exposure affects lipid homeostasis in post-pubertal male rats.

Taken together, our results show that exposure to GBH during the first week of life has an endocrine disruption effect in the male mammary gland *in vivo*, inducing the accelerated development of the mammary gland tree in pre-pubertal rats and the persistence of highly proliferative structures in post-pubertal animals together with high ESR1 expression. It should be noted that all these observed effects were due to the exposure to the complex mixture of the active principle (glyphosate) accompanied by several adjuvants. It is known that GBH-commercial formulations are more potent, or toxic, than glyphosate alone to non-target organism including mammals (Mesnage and Antoniou, 2018; Mesnage et al., 2013; Mesnage et al., 2015b; Myers et al., 2016). Additional studies are needed to demonstrate whether these effects on male mammary gland development are caused by glyphosate alone or its combination with adjuvants.

Declaration of interest

The authors declare that there are no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by grants from the Universidad Nacional del Litoral (CAI+D program) and the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT). These funding sources were not involved in the study design, the collection, analysis or interpretation of the data, the writing of the report, or the decision to submit the article for publication.

Acknowledgments

We would like to thank Dr. Pablo M. Beldomenico (Laboratorio de Ecología de Enfermedades, Instituto de Ciencias Veterinarias del Litoral (ICiVet-Litoral), UNL-CONICET) for advice on statistics and Juan Grant and Laura Bergero (ISAL; UNL-CONICET) for technical assistance and animal care. G.A.A. and A.L.G. are fellows and L.K., P.I., V.L.B. and E.H.L. are Career Investigators of CONICET.

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Figure legends

Figure 1. Whole mounts and picrosirius-hematoxylin (P&H) images of male mammary glands of GBH-exposed pre-pubertal rats. At PND21, GBH-exposed rats showed larger mammary glands than control rats and visible terminal end buds **(arrows).** In addition, GBH-exposed rats exhibited a greater connective tissue area surrounding the epithelial structures than control rats **(arrow heads)**. LN: lymph nodes.

Figure 2. Mammary gland development of post-pubertal male rats exposed to GBH. Representative images of whole mounts and picrosirius-hematoxylin (P&H) are shown. At PND60, GBH-exposed rats showed an increased longitudinal ductal growth and number of terminal end buds compared to those of control rats. Also, GBH-exposed rats presented ducts and lobuloalveolar structures less dilated than control rats. LN: lymph nodes.

Figure 3. Proliferation index and steroid hormone receptors expression in pre- and post-pubertal male rats postnatally exposed to GBH. Percentage of **(A)** proliferation index (Ki67), **(B)** ERS1 and **(C)** AR-positive cells are shown. Bars represent the mean values ± SEM of 6-9 male rats/group (*$p<0.05$; Mann-Whitney U test). **(D)** At PND60, representative images of Ki67, ESR1 and AR expression in mammary ducts. Arrows indicate positive cells. Scale bars = 50 μm.

Figure 4. Mast cells quantification in post-pubertal male rats. **(A)** Volume density (Vv x 100) of mast cells. Bars represent the mean values ± SEM of 6-9 male rats/group (*$p<0.05$; Mann-Whitney U test). **(B)** Representative images of RMCP-I protein expression at PND60. Arrow heads indicate positive mast cells. Scale bars = 50 μm. Inset: high magnification view of mast cells in the mammary gland stroma; scale bars = 25 μm.

Table 1. Mammary gland whole mount analysis of male rats exposed to GBH during the first week of life.

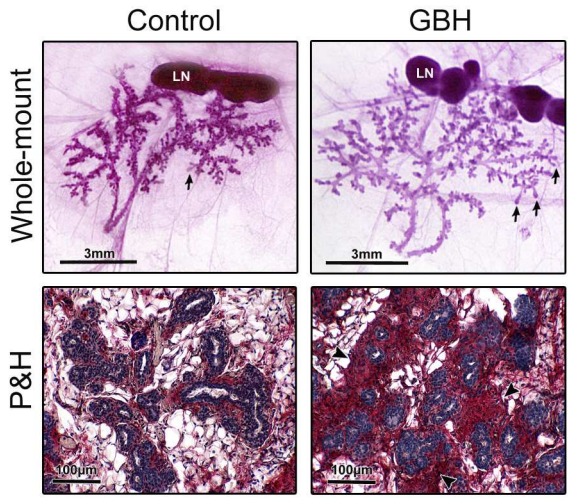
	Control	GBH
PND21		
Total area (mm^2)	15.5±3.0	24.2±2.7*
Perimeter (mm)	13.1±3.5	22.0±1.3*
Distance between MG No. 4 and No. 5 (mm)	2.3±0.4	2.5±0.4
TEBs (No.)	4.3±1.4	11.0±2.4*
PND60		
Total area (mm^2)	471.3±19.5	490.8±22.0
Perimeter (mm)	95.2±4.1	97.3±5.8
Longitudinal growth (mm)	17.1±0.5	18.7±0.6*
TEBs (No.)	2.8±0.6	5.1±0.8*

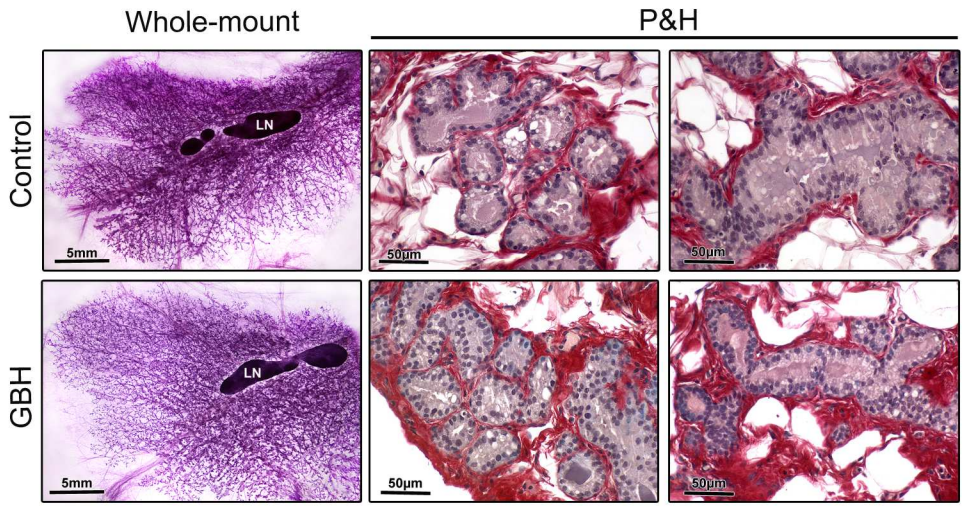
Values are expressed as mean ± SEM of 8-10 male rats/group/day.
MG: mammary gland; TEBs: terminal end buds.
*$p<0.05$ (Unpaired t test)

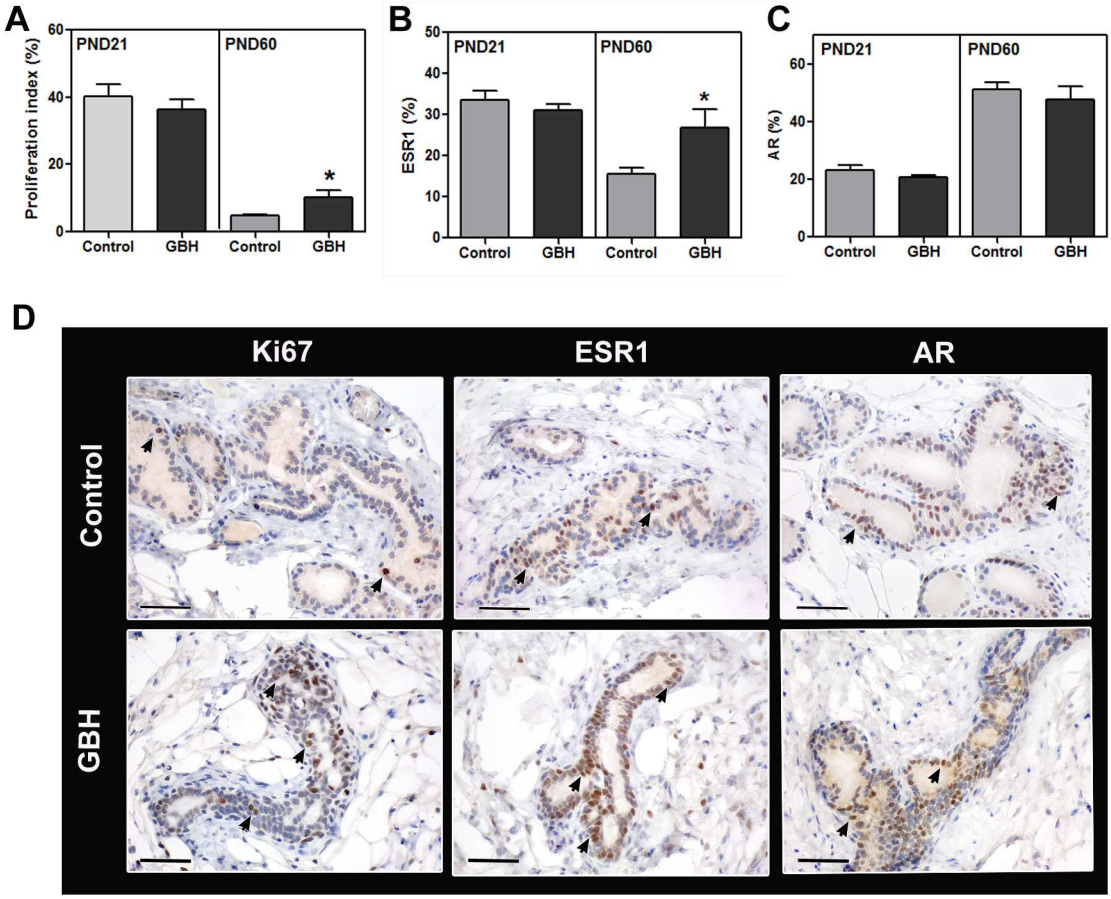
Table 2. Serum concentration of E2 and T in GBH-postnatally exposed pre- and post-pubertal male rat.

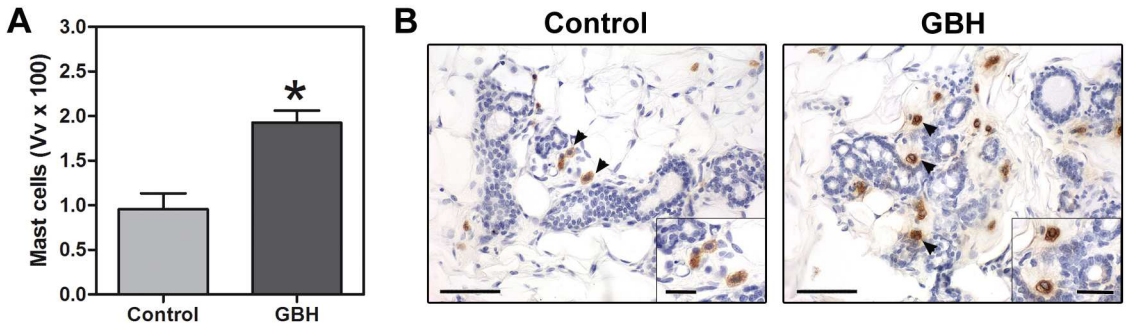
	Control	GBH
PND21		
E2 (pg/ml)	27.40±0.98	27.24±2.58
T (ng/ml)	0.29±0.04	0.26±0.02
PND60		
E2 (pg/ml)	42.75±0.09	36.32±3.48
T (ng/ml)	2.16±0.35	3.11±0.38

Values are expressed as mean ± SEM of 8-10 male rats/group/day.









Highlights

- Postnatal subacute exposure to GBH accelerates mammary gland development in male rats
- GBH augments mast cell infiltration in the mammary stroma of post-pubertal males
- GBH increases mammary gland proliferation and estrogen receptor alpha expression in post-pubertal males