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Glyphosate-based herbicide induces hyperplastic ducts in the mammary gland of aging Wistar rats

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A B S T R A C T

Glyphosate-based herbicide (GBH) exposure is known to have adverse effects on endocrine-related tissues. Here, we aimed to determine whether early postnatal exposure to a GBH induces long-term effects on the rat mammary gland. Thus, female Wistar pups were injected with saline solution (Control) or GBH (2 mg glyphosate/kg/day) on postnatal days (PND) 1, 3, 5 and 7. At 20 months of age, mammary gland samples were collected to determine histomorphological features, proliferation index and the expression of steroid hormone receptors expression, by immunohistochemistry, and serum samples were collected to assess 17β-estradiol (E2) and progesterone (P4) levels. GBH exposure induced morphological changes evidenced by a higher percentage of hyperplastic ducts and a fibroblastic-like stroma in the mammary gland. GBH-treated rats also showed a high expression of steroid hormone receptors in hyperplastic ducts. The results indicate that early postnatal exposure to GBH induces long-term alterations in the mammary gland morphology of aging female rats.

1. Introduction

Glyphosate (N-phosphonomethyl glycine) is an active ingredient of broad-spectrum herbicide formulations. Although commercial formulations of glyphosate include other chemical compounds, which are classified as inert compounds (Székács and Darvas, 2018), glyphosate formulations have been proved to be more toxic than the compound in its technical grade (Benachour and Seralini, 2009; Defarge et al., 2016; Mesnage et al., 2014; Tsui and Chu, 2003). The use of glyphosate on crops to control weeds has increased all over the world as a result of the use of genetically modified crops and the occurrence of glyphosate-resistant weeds (Benbrook, 2016), among others. This expanded use of glyphosate-based herbicides (GBHs) has led to the presence of glyphosate and its primary metabolite, aminomethylphosphonic acid (AMPA), in several environmental matrices, such as water, soil, and air (Van Bruggen et al., 2018), as well as in food (for humans or livestock) (Bai and Ogbourne, 2016; Rendón-von Osten and Dzul-Caamal, 2017; Rodrigues and de Souza, 2018; Zoller et al., 2018). Consequently, residues of glyphosate have also been detected in human urine and serum samples (Gillezeau et al., 2019).

Some controversies have arisen regarding the carcinogenic and the endocrine-disrupting effects of glyphosate and GBHs. The International Agency for Research on Cancer (IARC; World Health Organization) concluded that “glyphosate is probably carcinogenic to humans (IARC Group 2A)” (IARC, 2015), whereas the European Food Safety Authority (EFSA) determined that “glyphosate is unlikely to pose a carcinogenic hazard to humans” (EFSA, 2015). It is important to highlight that the IARC examined studies of GBH and glyphosate, whereas the EFSA evaluated studies that used technical-grade glyphosate (Portier et al., 2016; Tarazona et al., 2017). This indicates that safety evaluations focused on glyphosate alone can underestimate toxicity and are insufficient to assess the relevance of human and environmental exposures to glyphosate and GBHs (Vandenberg et al., 2017).

It is known that many endocrine-disrupting chemicals (EDCs) mimic the endogenous estrogen functions or interfere with estrogen signaling pathways (Shanle and Xu, 2011). Regarding glyphosate and GBH, the Endocrine Disruptor Screening Program (EDSP) conducted by the US Environmental Protection Agency (EPA) concluded that the results so far obtained are not sufficient to classify glyphosate as an EDC (US EPA, 2015). However, several studies have shown that glyphosate may lead

Abbreviations: E_2, 17β-estradiol; ERα, estrogen receptor α; GBH, glyphosate-based herbicide; P_4, progesterone; PCNA, proliferating cell nuclear antigen; PND, postnatal day; PR, progesterone receptor

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to a disruption of endocrine-signaling systems. *In vitro* experiments have demonstrated that the exposure to glyphosate induces human breast cancer cell proliferation by activating estrogen receptor α (ERα), either directly (Thongprakaisang et al., 2013) or through a ligand-independent mechanism (Mesnage et al., 2017). Other *in vitro* studies using breast cancer cell lines have also demonstrated that glyphosate and GBH may induce cell damage, independently of estrogenic pathways (De Almeida et al., 2018; Stur et al., 2019). *in vitro* By using an *in vivo* model to evaluate estrogenic activity, we have previously found that GBH is able to modulate the expression of estrogen-sensitive genes in the rat uterus (Varayoud et al., 2017). In addition, we have found that early postnatal exposure to GBH affects the normal development of the uterus (Guerrero Schimpf et al., 2017) and the mammary gland of male rats (Altamirano et al., 2018), suggesting endocrine-disrupting effects. Interestingly, several studies have also demonstrated that exposure to GBH may have long-term consequences such as female reproductive failures (Ingaramo et al., 2016, 2017; Lorenz et al., 2019) and second-generation adverse effects (Milesi et al., 2018).

The rodent female mammary gland represents a potentially sensitive endpoint to study the toxicological effects of several environmental agents (Fenton, 2006). Although this gland begins its development during gestation, most of its growth occurs after birth (Filgo et al., 2016). In the rat, the mammary gland evolves from a primary main lactiferous duct. Then, by the 14th day of postnatal life, the ducts have branched in new ducts ended in terminal end buds (Russo and Russo, 1996). Due to this branching, any interference (such as that caused by chemicals) could alter the development of the mammary gland, causing lasting effects on the gland (Fenton, 2006).

The development of the mammary gland is tightly regulated by fluctuations in the levels of endogenous hormones (Javed and Lteif, 2013). The ovarian steroid hormones, estrogen and progesterone (P_4), are major controllers of the lobuloalveolar development of the mammary gland both by direct receptor-mediated interactions and by stimulating growth factors (Davis and Fenton, 2013), which subsequently stimulate the proliferation of mammary epithelial cells (Hvid et al., 2012). In the rat mammary gland, both ERα and progesterone receptor (PR) have been found to be expressed in the epithelial compartment, which gives support to the fact that the hormonal regulation of mammary gland development involves mainly a receptor-mediated mechanism (Russo and Russo, 1998, 1999).

Taking into account all the mentioned antecedents, in the present study we aimed to evaluate whether neonatal exposure of rats to a GBH induces long-term effects on the female mammary gland. To this end, we evaluated: 1) the body weight (bw) of the animals along the experiment, and 2) the ovarian steroid hormone levels, the mammary gland morphology, and the proliferation and epithelial expression of classical endocrine-related proteins at 20 months of age.

2. Materials and methods

2.1. Animals

All the experimental protocols used in this study were approved by the Institutional Ethics Committee of the School of Biochemistry and Biological Sciences (Universidad Nacional del Litoral -UNL-, Santa Fe, Argentina) and performed in accordance with the principles and procedures outlined in the Guide for the Care and Use of Laboratory Animals issued by the US National Academy of Sciences. Animals were treated humanely and with regard for alleviation of suffering.

Rats of a Wistar-derived strain bred at the Department of Human Physiology of the School of Biochemistry and Biological Sciences of the UNL were used. The animals were maintained in a controlled environment (22 ± 2 °C; 14 h of light from 06:00 h to 20:00 h) in stainless steel cages with sterile pine wood shavings as bedding and had free access to pellet laboratory chow (16–014007 Rat-Mouse diet, Nutrición Animal, Santa Fe, Argentina) and tap water in glass bottles with rubber stoppers. The food was composed mainly of proteins (23%), raw fiber (6%) and minerals (10%), with a relative humidity of 12% (see Kass et al., 2012 and Andreoli et al., 2015 for more details). In a previous work, we checked for the presence of glyphosate in the diet (pellet chow and water) by using Ultra performance liquid chromatography–tandem mass spectrometer (UHPLC–MS/MS), and found no detectable levels (Milesi et al., 2018).

2.2. Experimental procedures

Pups were obtained from eight timed-pregnant rats housed singly. After delivery (postnatal day -PND- 0), pups were sexed according to the anogenital distance and litters of eight pups (preferably four males and four females) were left with each mother (Guerrero Schimpf et al., 2017). Female pups were cross-fostered among the mothers to minimize the use of siblings. This schedule allows including no more than two siblings in each group. Female pups from each foster mother were randomly assigned to one of the following postnatal treatment group: 1) Control group, receiving saline solution (n = 8), and 2) GBH group, receiving a commercial formulation of glyphosate diluted with saline solution (2 mg glyphosate/kg bw/day, n = 10). The remaining pups (females and males) were assigned to other experiments performed at our lab.

The glyphosate formulation used was Roundup FULL II®, a liquid water-soluble formulation containing 66.2% of glyphosate potassium salt as its active ingredient, coadjuvants and inert ingredients. As mentioned before, during the neonatal period, the mammary gland is highly susceptible to chemical compounds that could alter the normal development of the gland. The treatments were administered by subcutaneous injections in the nape of the neck every 48 h from PND 1 to PND 7. On each treatment day, a glyphosate solution was prepared according to the average bw of the pups, so asto administer 2 mg glyphosate/kg bw in a fixed volume of 40 μL. The dose of GBH was calculated based on the concentration of glyphosate acid (54 g of glyphosate per 100 mL of GBH). This dose is representative of the glyphosate residues found in soybean grains (Arregui et al., 2004; Test Biotech, 2013), and is in the order of magnitude of the environmental levels detected in our country (Bonansea et al., 2017; Peruzzo et al., 2008; Primost et al., 2017). As previously reported (Guerrero Schimpf et al., 2017), the postnatal treatment with GBH (with 2 mg/kg bw/day in pups every 48 h from PND 1 to PND 7) did not alter the maternal care or nursing of the experimental groups. In addition, the treatment led to no signs of local reaction or acute or chronic toxicity.

At weaning (PND 21), the offspring were kept under standard laboratory animal husbandry conditions. Along the experiment, animals were weighed on each treatment day (PND 1, 3, 5 and 7) as well as 24 h after the end of the experiment (PND 8), and then weekly (from PND 9 up to PND 30) or monthly (from PND 31 up to 20 months of age). To sacrifice all animals at the same stage of the estrous cycle, vaginal smears were performed every morning (Montes and Luque, 1988) starting on PND 570. Briefly, vaginal secretion was collected with a plastic pipette filled with 500 μL of saline solution (NaCl 0.9%) by inserting the tip of the pipette into the vaginal canal. The pipette bulb was firmly but gently depressed to expel the saline into the vagina and the saline was drawn back into the dropping pipette which was removed from the vaginal canal. Vaginal fluid was placed on glass slides and observed under an optical microscope to determine the stage of the estrous cycle, according to the predominant cells present (Montes and Luque, 1988; Manservisi et al., 2018). All animals were sacrificed by decapitation in the morning at the estrus closest to 20 months of age. Trunk blood was collected, samples were centrifuged and serum was stored at −20 °C until hormone assays were performed.

Traditionally and according to guidelines in toxico-pathological studies, the abdominal-inguinal mammary gland chain is examined (Ruehl-Fehlert et al., 2003). Thus, at sacrifice, one abdominal mammary gland was obtained, fixed in 10% (v/v) buffered formalin for

6 h at room temperature, and embedded in paraffin for histological studies (morphometric and immunohistochemical analysis).

2.3. Hormone assays

Serum samples stored at −20 °C were thawed and 17β-estradiol (E_2) and P_4 serum levels were assessed using the Ultra-Sensitive Estradiol Radioimmunoassay Kit DSL4800 (Immunotech, Beckman Coulter, Czech Republic) and Kit LI4043F1 (EIAgen, Adaltis Srl., Italy), respectively, according to the manufacture's guidelines. All the samples were run in duplicate. The assay sensitivity was 2.2 pg/mL for E_2 and 0.105 ng/mL for P_4. The intra- and interassay coefficients of variation were ≤8.9 and 12.2% for E_2 and ≤9.1 and 13.9% for P_4.

2.4. Immunohistochemistry

A standard immunohistochemical technique was performed, following protocols previously described by our laboratory (Muñoz-de-Toro et al., 1998). Briefly, mammary longitudinal sections (5 μm thick) were deparaffinized and rehydrated in graded ethanol solutions.

After microwave pretreatment for antigen retrieval, the endogenous peroxidase activity and non-specific binding sites were blocked. Primary antibodies were used at the dilutions mentioned in Table 1 and incubated overnight at 4 °C. After incubation with biotin-conjugated secondary antibodies (Table 1) for 30 min, reactions were developed using a streptavidin-biotin peroxidase method and diaminobenzidine (Sigma–Aldrich, Buenos Aires, Argentina) as a chromogen substrate.

Each immunohistochemical run included negative controls in which the primary antibody was replaced by non-immune rabbit or mouse serum (Sigma-Aldrich). For immunodetection, the samples were counter-stained with hematoxylin (Biopur, Rosario, Argentina). Samples were dehydrated and mounted with permanent mounting medium (Eukitt, Sigma-Aldrich).

2.5. Histological analysis and morphometry

Mammary gland sections (5-μm thick) were stained using hematoxylin and eosin for histological examination, which was performed by a trained pathologist (AA, see Acknowledgments) blinded to the experimental group.

Images of stained mammary gland sections were recorded using a Spot Insight V3.5 color video camera attached to an Olympus BH2 microscope (Olympus Optical Co., Ltd., Tokyo, Japan). All images were analyzed with ImageJ software (NIH, USA; https://imagej.nih.gov/ij), and all the evaluations were performed in at least 10 randomly selected fields per section. Two or three sections, at least 30 μm apart from each other, were examined in a blinded fashion.

2.5.1. Morphometric analysis of the parenchyma

The proportion of mammary gland parenchyma (namely ducts or alveoli) was measured using a Dplan 4X objective (numerical aperture = 0.10; Olympus BH2) by applying an orthogonal line grid mask on the whole image. The volume fraction (Vv) was calculated by applying the formula given by Weibel (1969): Vv = Pi/P, where Pi is the number of incident points over ducts, alveoli or adipose tissue, and P is the number of incident points over the whole image (Durando et al., 2007). The results are expressed as percentage of ducts and percentage of alveoli, respectively. We also calculated the ratio between ducts and alveoli (i.e.: percentage of ducts/percentage of alveoli) as a measurement of mammary gland development.

Additionally, the presence of cysts containing white fluid ("milk cysts"), as described previously by Masso-Welch et al. (2000), was quantified and expressed as incidence and multiplicity. Moreover, the diameter of cysts was measured to compare between groups.

Hyperplastic ducts were quantified and classified as mild, moderate or florid (four, five or more than five layers of epithelial cells lining the ducts, respectively), as has been previously described (Durando et al., 2011; Singh et al., 2000), using a Dplan 40 X objective (numerical aperture = 0.65; Olympus BH2). To obtain the proportion of hyperplastic ducts, we evaluated three sections per mammary gland that were at least 30 μm apart from each other, and 75 ducts per section were analyzed.

2.5.2. Morphometric analysis of the stromal compartment

As mentioned above, the proportion of adipose tissue was calculated by applying the formula given by Weibel (1969). The results are expressed as percentage of adipose tissue.

The thickness of the stroma surrounding the moderate and florid hyperplastic ducts was analyzed with a Dplan 40 X objective (numerical aperture = 0.65; Olympus BH2) by measuring the average length from the basement membrane of each duct towards the stroma.

The density of stromal nuclei was calculated using a Dplan 20 X objective (numerical aperture = 0.40; Olympus BH2) as the ratio between the area occupied by stromal nuclei*an* and the total area occupied by adipose tissue*at*, covering an area of 0.25 mm^2 (Durando et al., 2007).

2.5.1.1. Quantification of mast cells. Mammary gland sections (5-μm thick) were stained with toluidine blue to detect mast cells (Varayoud et al., 2004). The number of mast cell within the stroma surrounding mammary ducts was calculated using a Dplan 20 X objective (numerical aperture = 0.40; Olympus BH2) as follows: (MC/TA) x 100, where MC is the number of mast cells associated with each duct and TA is the total area occupied by the connective stroma of the duct (measured from the basement membrane towards the adipose tissue).

2.5.1.2. Quantification of protein expression. We next evaluated the expression of ERα, PR and the proliferating cell nuclear antigen (PCNA) as a proliferation marker. Two mammary tissue sections per animal were evaluated and at least 2000 epithelial cells per tissue section were analyzed, using a Dplan 40 X objective (numerical aperture = 0.65; Olympus BH2). The percentages of ERα, PR and PCNA were calculated in normal ducts and moderate plus florid hyperplastic ducts.

2.5.1.3. Statistical analysis. All data are expressed as the mean ± SEM and were analyzed using a Mann-Whitney *U* test. All analyses were carried out using the R software (The R Foundation for Statistical Computing http://www.r-project.org/). Values with $p < 0.05$ were considered significant.

Table 1
Antibodies used for immunohistochemistry.

Antibodies	Dilution	Supplier
Primary		
Anti- ERα (clone 6F-11)	1/50	Novocastra (Newcastle upon Tyne, UK)
Anti-PR (A/B isoforms)	1/400	Dako Corporation (Carpinteria, CA, USA)
Anti-PCNA (clone PC -10)	1/1000	Novocastra
Anti-Vimentin (clone V9)	1/100	Novocastra
Secondary		
Anti-mouse (B8774)	1/100	Sigma-Aldrich (St. Louis, MO, USA)
Anti-rabbit (B8895)	1/200	Sigma-Aldrich

ERα, estrogen receptor α; PCNA, proliferating cell nuclear antigen; PR, progesterone receptor.

3. Results

3.1. Effects of GBH exposure on body weight

Taking into account that our experimental model aimed to evaluate the long-term consequences of GBH exposure, we first determined

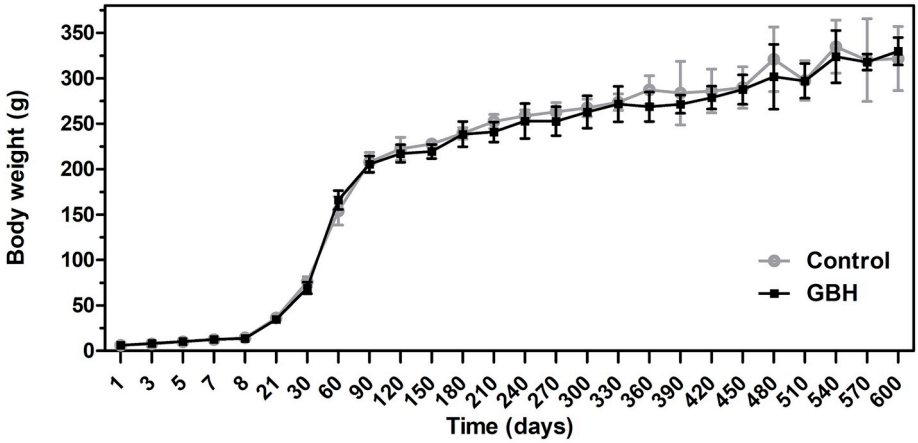

Fig. 1. Body weight of rats exposed to saline solution (Control) or glyphosate-based herbicide (GBH) from postnatal day (PND) 1–20 months of age.

whether GBH exposure altered the bw of the animals. We found no significant differences in bw gain between Control and GBH-treated females from birth to adulthood (Fig. 1).

3.2. Long-term effects of postnatal GBH exposure

3.2.1. Ovarian steroid levels

The serum levels of E_2 and P_4 in the Control and GBH-exposed rats showed no statistical differences (E_2: Control: 38.97 ± 2.06 pg/mL vs GBH: 33.26 ± 4.10 pg/mL, p = 0.244; P_4: Control: 10.09 ± 2.49 ng/mL vs GBH: 10.61 ± 3.61 ng/mL, p = 0.888).

3.2.2. GBH exposure induced hyperplastic ducts accompanied by modifications in the stromal compartment in the mammary gland

Regarding the morphological structure of the mammary gland, we detected that Control and GBH-exposed rats showed a similar percentage of the total area occupied by adipose tissue (89.9% Control and 90.4% GBH group; p = 0.762), as well as no differences in the percentage of ducts (Control: 6.92 ± 0.85%; GBH: 8.53 ± 1.38%) and alveoli (Control: 3.15 ± 1.05%; GBH: 1.09 ± 0.34%) (p = 0.76 and p = 0.17, for ducts and alveoli, respectively). However, the ratio between ducts and alveoli was higher in GBH rats (Control: 3.80 ± 1.28 and GBH: 13.82 ± 3.99; p = 0.036), suggesting different development degrees between groups.

An important observation was the difference related to the ductal epithelial layering of the mammary gland (Fig. 2A). Considering the age of the rats, both experimental groups presented hyperplastic ducts, but GBH-exposed rats showed a higher percentage (Control: 6.56 ± 0.81% vs GBH: 11.20 ± 1.25%, p = 0.006, Fig. 2B). These animals also presented a higher number of moderate and florid hyperplastic ducts (moderate hyperplastic ducts: Control: 0.79 ± 0.20% vs GBH: 2.23 ± 0.44%, p = 0.014; florid hyperplastic ducts: Control: 0.73 ± 0.18% vs GBH: 2.42 ± 0.45%, p = 0.009, Fig. 2B).

Again, the presence of cysts (Fig. 2C) was associated with the age of the animals and no differences were detected regarding incidence (7/8 and 8/10 for Control and GBH, respectively) or multiplicity (1.94 ± 0.61 and 2.05 ± 0.81, for Control and GBH, respectively). However, the average diameter of cysts was higher in GBH-exposed rats (Control: 395.6 ± 104.6 μm vs GBH: 855.0 ± 106.9 μm; p = 0.009, Fig. 2D).

We next evaluated the stroma surrounding hyperplastic ducts and found that the thickness of the stroma layer was not modified due to the treatment with GBH (Control: 28.21 ± 3.80 μm vs GBH: 26.73 ± 2.80 μm, p = 0.958). However, the stroma of GBH-treated rats showed a high cellularity evidenced by a high number of nuclei in a fixed area, related to that of Control animals (Control: 285.4 ± 19.74 cells/mm^2 vs GBH: 391.6 ± 36.67 cells/mm^2, p = 0.043, Fig. 3A and D). Strikingly, in the GBH group, the normal adipose tissue was replaced by a fibroblastic-like stroma, with a strong presence of vimentin-positive cells (Fig. 3B).

We quantified the number of mast cells in the stroma surrounding normal and hyperplastic ducts and found that mast cells were more abundant within the stroma of GBH-treated rats than within that of controls (Control: 45.31 ± 11.22 cells/mm^2 vs GBH: 79.03 ± 12.93 cells/mm^2, p = 0.043, Fig. 3C and E).

3.2.3. GBH exposure modified the epithelial expression of classical endocrine-related proteins

Finally, we evaluated whether postnatal exposure to GBH induced changes in proliferation and endocrine-related proteins that could be associated with the induction of hyperplastic ducts and a fibroblastic-like stroma. In the normal ducts, the expression of all the proteins evaluated was similar between groups. However, the expression of steroid hormone receptors (ERα and PR) in the moderate plus florid hyperplastic ducts of GBH-treated animals was statistically higher than in Control animals (ERα: Control: 12.07 ± 1.56% vs GBH: 16.61 ± 1.26%, p = 0.033, Fig. 4A and D; PR: Control: 15.53 ± 2.79% vs GBH: 21.07 ± 1.16%, p = 0.037, Fig. 4B and E). The epithelial proliferation index was similar in both groups when normal (Control: 25.38 ± 3.75% vs GBH: 31.41 ± 3.64%, p = 0.274) and moderate plus florid hyperplastic ducts (Control: 40.20 ± 7.79% vs GBH: 51.10 ± 4.73%, p = 0.203, Fig. 4C and F) were analyzed.

4. Discussion

To our knowledge, this is the first study to determine the long-term effects of postnatal exposure to a low dose of a GBH on the mammary gland morphology of aging female rats. Although the development of the mammary gland has windows of increased susceptibility to external influences, these windows are lost when exposure starts during adulthood (Davis and Fenton, 2013; Eighmy et al., 2018), and this event highlights the relevance of exposing animals as early as possible. In the present study, we selected a model of exposure during the first postnatal week of life, a period which has already been proved to be highly susceptibility to hormonal and chemical challenge, not only for the mammary gland (Altamirano et al., 2017, 2018) but also for other organs (Guerrero Schimpf et al., 2017; Milesi et al., 2015, 2017; Monje et al., 2009; Rodríguez et al., 2010). Our present study provides compelling evidence that developmental exposure to GBH induces hyperplastic ducts and, in association with fibroblastic-like stroma, higher number of mast cells and alterations in the expression of steroid receptors.

Hyperplastic ducts are considered premalignant structures and precursors of neoplastic lesions (Singh et al., 2000). This kind of

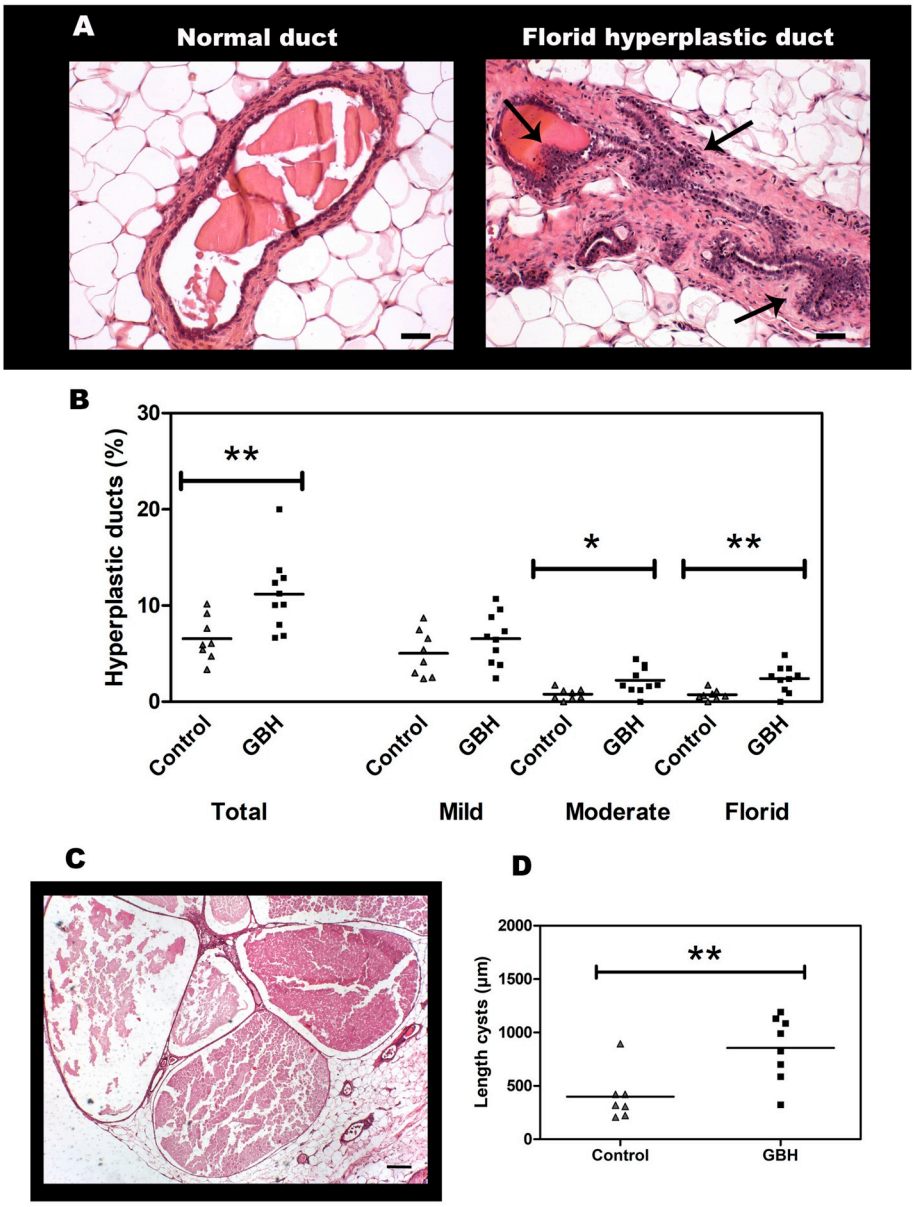

Fig. 2. (A) Representative images of a normal duct (Control group) and a florid hyperplastic duct (GBH group). Arrows indicate six or more layers of epithelial cells. Scale bar: 50 µm. (B) Quantification of normal and hyperplastic ducts. On the left, a higher incidence of total hyperplastic ducts in the GBH-treated rats is shown. On the right, a higher incidence of moderate and florid hyperplastic ducts in the GBH-treated animals is shown. The median value is represented by a straight line through each plot. *p < 0.05, **p < 0.01 (Mann-Whitney *U* test). (C) Representative image of a cyst is shown. Scale bar: 300 µm. (D) Neonatal exposure to GBH increased the average diameter of cysts. The median value is represented by a straight line through each plot. **p < 0.01 (Mann-Whitney *U* test).

degeneration of the ductal epithelium has been observed in rats perinatally exposed to estrogenic compounds, when terminal end buds are developing (Davis and Fenton, 2013), and may also occur spontaneously in aging rats (Eighmy et al., 2018). As expected, in the present study, we found hyperplastic ducts in both Control and GBH-exposed rats. However, we also found an increase in the percentage of these premalignant structures in the GBH group, mainly due to a higher number of moderate and florid hyperplastic ducts. According to these results, we could suggest that the exposure to a low dose of a GBH during early postnatal development might enhance the sensitivity of the mammary gland to develop preneoplastic lesions. These results are in agreement with those of other authors who observed an increase in these lesions during adulthood (Kolla et al., 2018; Murray et al., 2007; Tucker et al., 2018) and aging female rodents perinatally exposed to other EDCs, such as genistein, perfluorooctanoic acid and bisphenols (Padilla-Banks et al., 2006; White et al., 2009). The increase in the percentage of these aberrant structures may be explained by a higher incidence of florid (Ventura et al., 2016) or moderate hyperplastic ducts or both (Durando et al., 2011).

By using the same experimental protocol, we have previously detected uterine morphological changes known as luminal epithelial hyperplasia in pre-pubertal rats exposed to GBH (Guerrero Schimpf et al., 2017) and a high sensitivity to an exogenous estradiol treatment, suggesting that GBH-exposed rats have greater susceptibility to neoplastic uterine lesions (Guerrero Schimpf et al., 2018).

According to the literature, some but not all hyperplastic ducts turn into neoplastic lesions. This is due to the existence of two different types of hyperplastic ducts, called “initiated” and “initiated and promoted”. The difference between both is that the epithelial cells of the “initiated and promoted” hyperplastic ducts interact with stromal elements, attracting mast cells and stimulating local regulatory factors. These actions result in increased synthesis of proteoglycans, which, in turn, affect cell proliferation, desmoplasia, and angiogenesis (Russo and Russo, 1996). Our present results are consistent with the “initiated and promoted” type because GBH exposure induced not only an increased incidence of hyperplastic ducts, but also the presence of a fibroblastic-like stroma replacing the normal adipose tissue, and an increased number of mast cells in the stroma surrounding the ducts, as discussed later.

It is known that the stromal compartment plays an important role in inducing epithelial tumors, not only in the mammary gland (Maffini

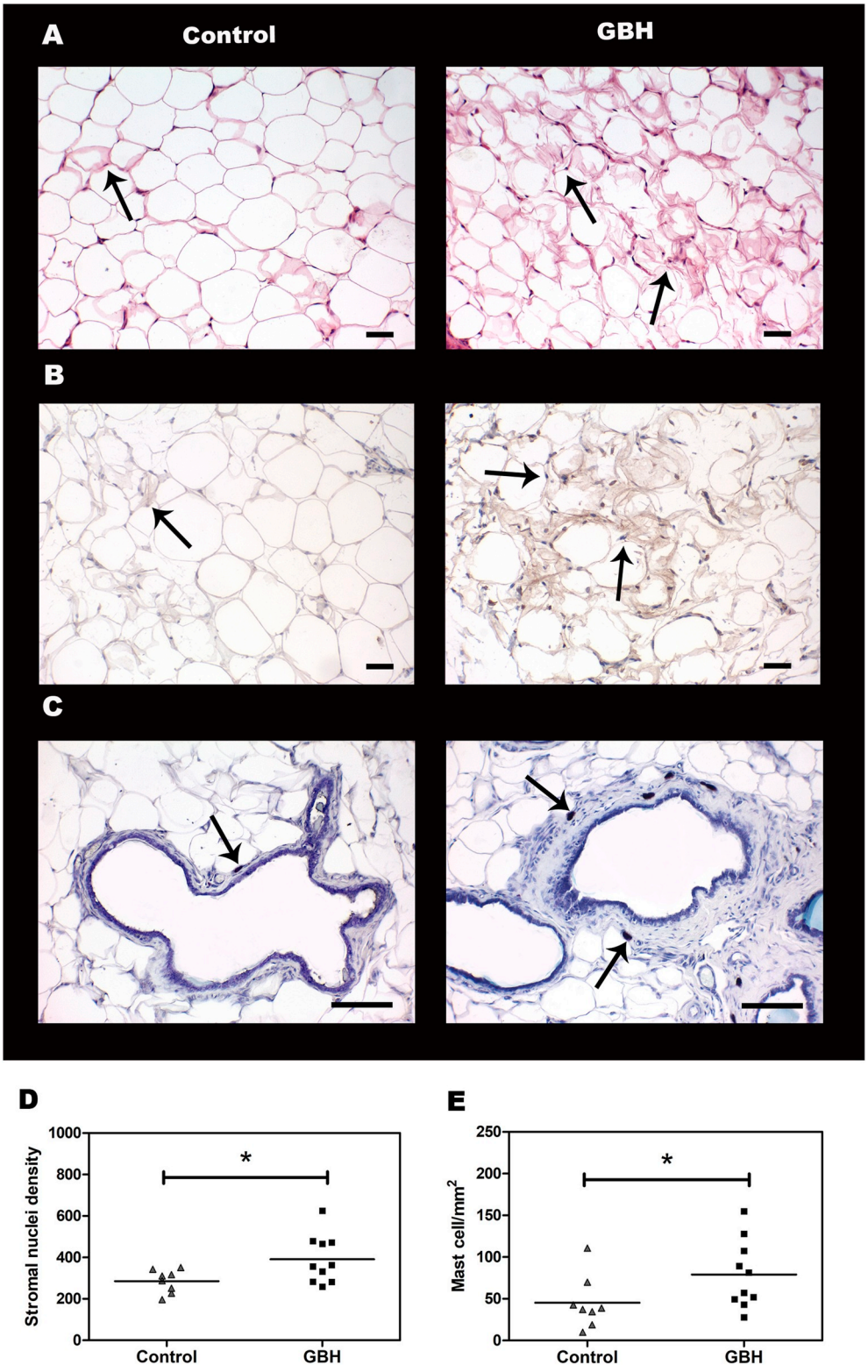

Fig. 3. Representative photomicrographs of mammary glands from aging female rats postnatally exposed to vehicle (Control) or glyphosate-based herbicide (GBH). Tissue sections were stained either with (A) hematoxylin and eosin to evaluate the stromal nuclei density in adipose tissue or with (C) toluidine blue to identify mast cells (arrows). (B) Representative photomicrographs show vimentin immunoreaction; positive cells are indicated by the arrows. Scale bar: 50 μm. Early postnatal exposure to GBH increased the density of stromal nuclei (D) and the number of mast cells surrounding ducts (E). The median value is represented by a straight line through each plot. *p < 0.05 (Mann-Whitney *U* test).

et al., 2004, 2005) but also in the prostatic gland (Barclay et al., 2005; Hayward et al., 2001). Specifically, in the mammary gland, the stromal compartment responds to epithelial alterations by starting a response that could be manifested as matrix stiffening due to an increase in the collagen density providing support for the tumor tissue (Burks et al., 2017; Kass et al., 2007; Meng et al., 2001). In the present study, the adipose tissue of GBH-animals was characterized by the presence of an increased number of nuclei and vimentin-positive cells. Therefore, it is reasonable to hypothesize that exposure to GBH during an early stage of development may alter the tissue organization, and that these alterations in turn generate abnormal tissue structures. By using the same experimental protocol, but administering endosulfan (another agrochemical compound), Altamirano et al. (2017) observed an increase in stromal collagen deposition associated with hyperplastic ducts in postpubertal male rats.

In accordance with the strong link between the "initiated and

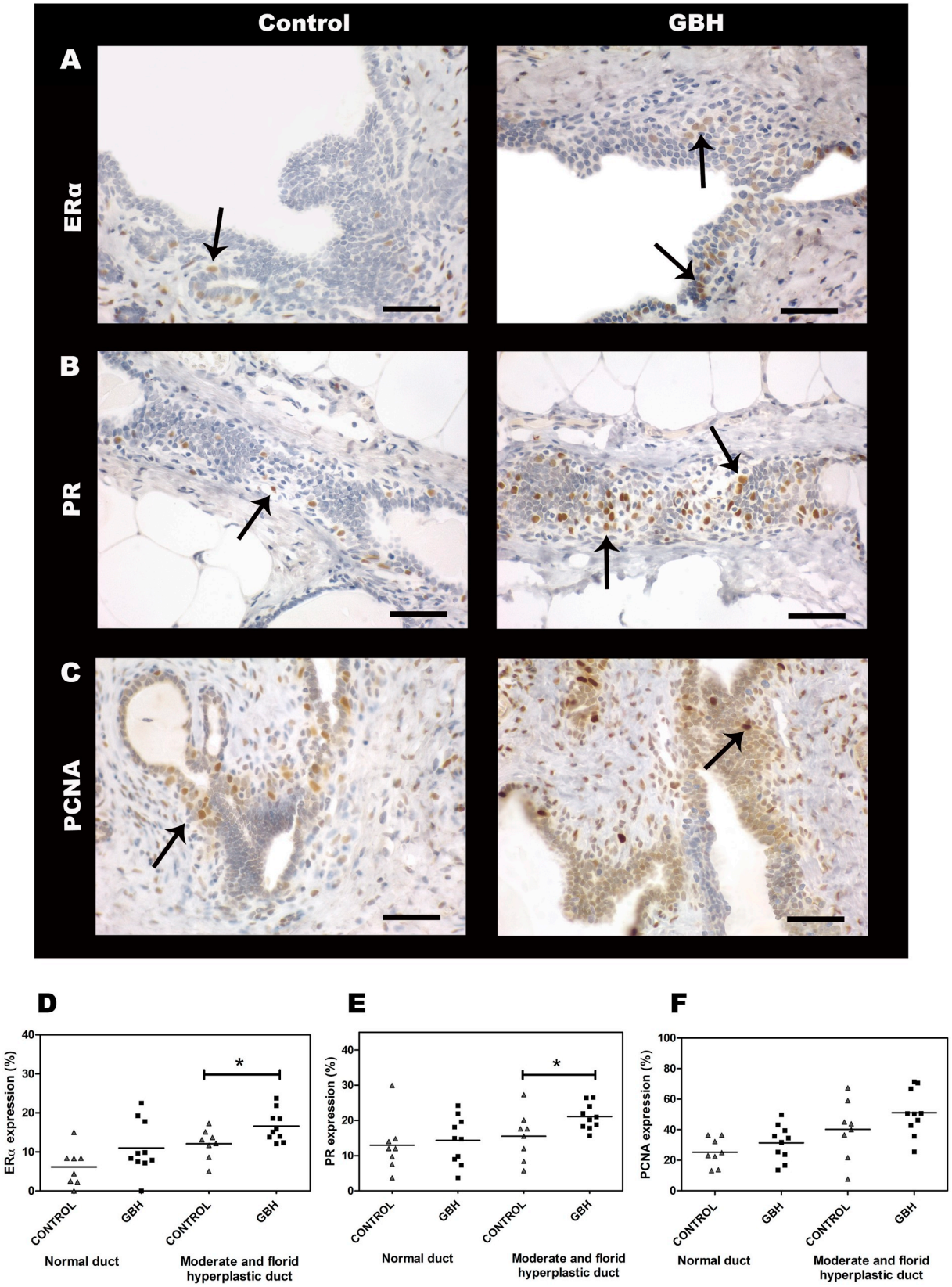

Fig. 4. Expression of steroid hormone receptors and proliferation index in aging female rats postnatally exposed to vehicle (Control) or glyphosate-based herbicide (GBH). Representative photomicrographs of (A) estrogen receptor alpha (ERα), (B) progesterone receptor (PR) and (C) proliferation index (PCNA) immunoreactions in epithelial cells of florid hyperplastic ducts. Positive cells are indicated by the arrows. Scale bar: 50 μm. GBH increased the expression of ERα (D) and PR (E), but not that of PCNA (F) in moderate plus florid hyperplastic ducts. The median value is represented by a straight line through each plot. *$p < 0.05$ (Mann-Whitney *U* test).

promoted" type of hyperplastic ducts and mast cells, we decided to quantify these cells in the stroma surrounding ducts and found an increase in the number of mast cells in animals treated with GBH. As mentioned earlier, mast cells surrounding the hyperplastic ducts could increase the risk of malignant transformation of the preneoplastic lesions to carcinoma (Eighmy et al., 2018; Russo and Russo, 1996). Indeed, it is known that these cells are involved in promoting angiogenesis in breast cancer (Aponte-López et al., 2018), reproductive tissue (Varayoud et al., 2004), and within tumors (Aoki et al., 2003). Although it is common to find inflammatory cells infiltrating within or adjacent to alveoli and ducts in the mammary gland of aging female rats (Eighmy et al., 2018), in our experiment, the GBH treatment induced an almost two-fold increase in the number of mast cells. Similarly, and supporting our results, Altamirano et al. (2018) have recently observed a higher presence of mast cells in the mammary gland of post-pubertal male rats postnatally exposed to GBH.

In the present study, GBH-exposed animals showed an increase in the ERα protein expression in moderate and florid hyperplastic ducts, suggesting a possible action of this receptor in the pre-neoplastic lesions. It is plausible to infer that epithelial cells are already primed and could thus respond more strongly to estradiol during adulthood. Murray et al. (2007) also observed an increase in the percentage of ERα expression and a higher proliferation of hyperplastic ducts in adult rats prenatally exposed to bisphenol AA. Taken together, these results contribute to a growing body of evidence supporting the potential interaction of GBHs with the estrogen signaling pathways (Gasnier et al., 2009; Mesnage et al., 2017; Richard et al., 2005; Thongprakaisang et al., 2013; Varayoud et al., 2017).

GBH also caused an increase in PR protein expression in moderate and florid hyperplastic ducts. Indeed, the alterations in the expression of PR and ERα proteins could occur previous to the development of hyperplasia. Similarly to our present results, in a previous study we found that early postnatal exposure to GBH modified the uterine PR expression during neonatal and prepubertal periods (Guerrero Schimpf et al., 2017). Therefore, the incidence of hyperplastic ducts promoted by GBH exposure seems to be a consequence of a mis-regulation of endocrine signaling pathways, although we found no differences in the serum hormone levels, at least in aging female rats. In accordance with our results, previous studies found no changes in the E_2 and P_4 serum levels of pregnant female rats (Ingaramo et al., 2016) and pre- and post-pubertal males (Altamirano et al., 2018; Gomez et al., 2019) perinatally exposed to GBH.

As it is broadly known, GBHs are commercialized in the form of mixtures consisting of glyphosate (active principle) and different co-formulants. These co-formulants are considered to be "inert" additives and are devoid of pesticide activity. Consequently, the information about substance content in pesticide formulation is usually not provided by pesticide companies (Defarge et al., 2016; Kniss, 2017). However, these formulations contain surfactants, used to increase the effectiveness of the herbicide by increasing the solubility of glyphosate and its absorption by the plants. Several research groups have revealed that GBHs are more toxic than the active ingredient, suggesting that co-formulants can also be a source of toxicity (Defarge et al., 2016; Mesnage and Antoniou, 2018; Mesnage et al., 2014). Taking these points into account, and under the conditions of our present study, we are not able to ascribe the morphological changes to glyphosate or to co-formulants or to both acting together. Thus, further research is needed to clarify whether glyphosate, the adjuvants or their combination are responsible for the effects observed in the mammary gland. We are conscious that our experimental design has some limitations, such as the small number of animals used, the administration of the compound in a single dose, and a single time point studied. However, we would like to highlight our commitment to continue our studies regarding the use of GBHs, and will thus attempt to address these limitations in future studies.

In conclusion, although aging induces morphological changes in the rat mammary gland, our results provide evidence that exposure to a low dose of a GBH during a critical period of development alters the morphology of the mammary gland, by perturbing its histoarchitecture, increasing the number of hyperplastic ducts, leading to higher ERα and PR protein expression in hyperplastic ducts, and leading to several stromal changes, including a higher number of cell nuclei and a higher number of mast cells.

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Declaration of competing interest

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

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