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Acute uterine effects and long-term reproductive alterations in postnatally exposed female rats to a mixture of commercial formulations of endosulfan and glyphosate

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ABSTRACT

Endosulfan and glyphosate are widely used pesticides and have been associated to reproductive disorders. We examine the acute and long-term effects of postnatal exposure to commercial formulations of endosulfan (EF), glyphosate (glyphosate-based herbicide, GBH) and a mixture of both pesticides (MIX). After birth, female pups of Wistar rats received saline solution (CONTROL), EF (600 μg/kg of b.w/day), GBH (2 mg/kg of b.w/day) or a mixture (at the same doses) from postnatal day (PND) 1 to PND7. The uterine histology and expression of Hoxa10, estrogen (ERα) and progesterone (PR) receptors were evaluated on PND8. Reproductive performance was evaluated on gestational day 19. GBH and MIX rats showed an increment of 1) the incidence of luminal epithelial hyperplasia, 2) PR and Hoxa10 expression. EF modified ERα and Hoxa10 expression. During adulthood, MIX and GBH rats showed higher post-implantation losses while EF alone produced an increase of pre-implantation losses. We showed that the co-administration of both pesticides produced acute uterine effects and long–term deleterious reproductive effects that were similar to those induced by GBH alone. We consider important to highlight the necessity to evaluate the commercial pesticide mixture as a more representative model of human exposure to a high number of pesticides.

1. Introduction

Pesticides are a large and heterogeneous group of chemicals widely used in agriculture and in public health for prevention and control of pests; however it is assumed they may have adverse health effects (Hernandez et al., 2017; Mostafalou and Abdollahi, 2017). Even to very low levels of exposure, a number of pesticides is suspected may act as endocrine disruptor compounds (EDCs) and may be harmful to exposed people (Kim et al., 2017; Combarnous, 2017). The prenatal and postnatal periods of development until puberty are particularly sensitive to EDCs exposure, which might interfere with the physiology of normal endocrine-regulated events, leading to adverse effects later in life (Crain et al., 2008; Varayoud et al., 2014).

Endosulfan is an organochlorine pesticide belonging to class cyclodiene (Menezes et al., 2017). Despite its life-threatening toxic effects, endosulfan continues to be one of the most widely used agricultural pesticides, largely in the developing countries, due to its high efficacy, low cost and environmental stability. However, is a controversial agrochemical due to its potential for bioaccumulation, high persistence, low volatility, acute toxicity, and role as EDC (Ahmad et al., 2018; Milesi et al., 2015; Ingaramo et al., 2016a). Other polemic pesticide is glyphosate and its commercial formulation, glyphosate-based herbicides (GBHs). Glyphosate is the most widely used herbicide in the world for non-selective control of annual and perennial weeds, grasses and broadleaf plants (Siroski et al., 2016). Previous in vivo and in vitro studies showed that glyphosate and endosulfan could act as

Abbreviations: CL, corpora lutea; DAB, diaminobenzidine; DES, diethylstilbestrol; EDCs, endocrine disruptor compounds; EF, formulations of endosulfan; ERα, estrogen receptor alpha; GBH, glyphosate-based herbicide; GD, gestational day; GE, glandular epithelium; Hoxa10, Homeobox A10; IHC, immunohistochemistry; IOD, integral optical density; IS, implantation sites; LE, luminal epithelium; LEH, Luminal epithelial hyperplasia; PND, postnatal day; PR, progesterone receptor; RS, resorption sites; SEM, standard error of the mean; SS, subepithelial stroma

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EDCs affecting endocrine events in different organs/cells (Li et al., 2013; Senthilkumaran, 2015; Myers et al., 2016; Varayoud et al., 2008a, 2017).

Transgenic crops are associated with different pesticide formulations, the most used worldwide are GBHs, followed by different insecticides including endosulfan, among others (Burella et al., 2018). Several studies have demonstrated presence of glyphosate and endosulfan residues, in soil, water or sediments in Argentina (Regaldo et al., 2018; Primost et al., 2017; Grondona et al., 2019; Aparicio et al., 2013). In agricultural topsoil samples from across the European Union, glyphosate was one of the most frequently compound found in soil samples and at the highest concentration (Silva et al., 2019).

In the agricultural practices the application of more than one pesticide at the same time is common so the human exposure to environmental contaminants is not limited to an individual compounds (Perobelli et al., 2010; dos Santos and Martinez, 2014). Therefore, separate toxicity assessment of single chemicals may not estimate adequately combined adverse effects of pesticides (Hernandez et al., 2017). A more realistic study of pesticide effects may be through evaluation of exposure effects to mixture of pesticides simultaneously. However, the large number of different combinations of chemicals, exposure patterns, and complex interactions makes it impossible to test all possible combinations, and experimental data sets for toxicity of mixtures are lacking (Hernandez et al., 2017). It has been demonstrated that the exposure to mixtures of pesticides increase the risk of spontaneous abortion and birth defects (Bhatt, 2000). In addition, adult rats exposed to a mixture of EDCs during perinatal period shows signs of early reproductive senescence (Johansson et al., 2016). The impact of exposure of pesticides on health in addition is conditionate by chemical formulation of these pesticides. Some commercial formulations of different pesticides contain surfactants and other compounds to increase spreading and penetrating power but the identity of adjuvants is generally not disclosed (Huston and Pignatello, 1997). A previous work determined the effects of glyphosate, endosulfan and mixtures of them on a South American caiman, *Caiman latirostris*, after in ovo exposure (Poletta et al., 2011). All parameters analyzed indicated a higher toxicity for the mixture of pesticides than for glyphosate formulation alone (Poletta et al., 2011; Hayes et al., 2006).

In the present study we evaluated the acute and long-term effects of a brief postnatal exposure to EF, GBH, and a mixture of them. We assessed: 1) the acute effects evaluating uterine differentiation on PND8, and 2) the long-term effects determining the reproductive performance of female adult rats.

2. Material and methods

2.1. Animals

All the procedures used in this study were approved by the Institutional Ethics Committee of the School of Biochemistry and Biological Sciences (Universidad Nacional del Litoral, 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 U.S. National Academy of Sciences. Rats of an inbred Wistar-derived strain from the Department of Human Physiology (Universidad Nacional del Litoral, Santa Fe, Argentina) were used. The animals were 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. The concentration of phytoestrogens in the diet was not evaluated; however, because food intake of control and GBH-treated rats was equivalent, we assumed that all animals were exposed to the same levels of phytoestrogens. Additional information regarding food composition was provided by Kass et al. (2012). To minimize additional exposures to EDCs, rats were housed in stainless steel cages with wood bedding, and tap water was supplied in glass bottles with rubber stoppers surrounded by a steel ring.

2.2. Experimental design

Female rats (90 days old) were housed with males of proven fertility. The day on which sperm was found in the vaginal smear was designated as Gestational day 1 (GD1). Pregnant rats were housed singly, and, at delivery, pups were sexed according to the anogenital distance. To minimize the use of siblings and avoid potential litter effects, offspring of the same litter were distributed between different mothers. Cross-fostered litters were adjusted to eight pups, prioritizing a maximum of the female pups per litter when possible. When fewer than eight females were available, an appropriate number of males were retained. Female pups were assigned to one of four neonatal treatment groups: 1) control group treated with saline solution (Control, n = 34); 2) GBH group treated with a commercial formulation of glyphosate diluted with saline solution at a dose of 2 mg of glyphosate/Kg of body weight/day (n = 38); 3) EF group treated with a commercial formulation of endosulfan diluted with saline solution at a dose of 600 μg of endosulfan/kg of body weight/day (n = 38); 4) MIX group treated with a mixture of both pesticides at the same doses of GBH and EF groups (n = 38). The GBH was a liquid water-soluble formulation containing 66.2% of glyphosate potassium salt, as its active ingredient, coadjuvants and inert ingredients. EF and GBH doses were selected based on previous evidence of adverse effect on uterine development and functionality using pure endosulfan and GBH (Ingaramo et al., 2016a, 2017; Milesi et al., 2012, 2015). The selected dose of GBH is in the order of magnitude of 1) the environmental levels detected in our country (Aparicio et al., 2013; Arregui et al., 2004; Peruzzo et al., 2008) and 2) the oral reference dose defined by EPA (EPA, 1993). The EF used was Thionex EC (Makhteshim Chemical Works Ltd.) a formulation containing 400 g of ensosulfan/L. The dose of EF is according to the no observed effect level (NOEL) defined by EPA, 600 μg/kg/d (EPA, 2007) for endosulfan. As we mentioned before, the MIX group received the mixture of pesticides administrated simultaneously (at above-described doses). The different treatments were given on postnatal days (PND) 1, 3, 5 and 7 by sc injections in the nape of the neck. The MIX group received two injections daily with individual doses of formulation of each pesticide. No signs of acute toxicity were observed by exposure to the pesticides, and no significant differences in weight between exposed and control pups were recorded during the experiment (data no shown). After the end of the treatment, one group of female pups were sacrificed by decapitation on PND8 (to evaluate the acute effects during neonatal period, 8 pups/group) and uterine tissues were collected. Another group of female rats were weaned on PND21, housed at four per cage, and held without further treatment until adulthood. On PND90, female rats neonatally exposed to each one of the treatments, were housed for two consecutive weeks with sexually mature untreated males of proven fertility to allow several possible mating. Every morning, vaginal smears were obtained to check for the presence of spermatozoa (Montes and Luque, 1988). The first day on which a sperm-positive smear was detected, was considered GD1. Pregnant female rats were assigned to a fertility test to evaluate the reproductive performance on GD19 (n = 26–30/experimental group). The experimental design is shown in Fig. 1.

2.3. Sample collection

Eight rats from each neonatal treatment group were weighted and sacrificed by decapitation on PND8 to evaluate acute effects of pesticides on uterine differentiation. Uterine horns were removed, fixed by immersion in 4% paraformaldehyde buffer for 6 h at 4 °C and processed for histology and immunohistochemistry (IHC).

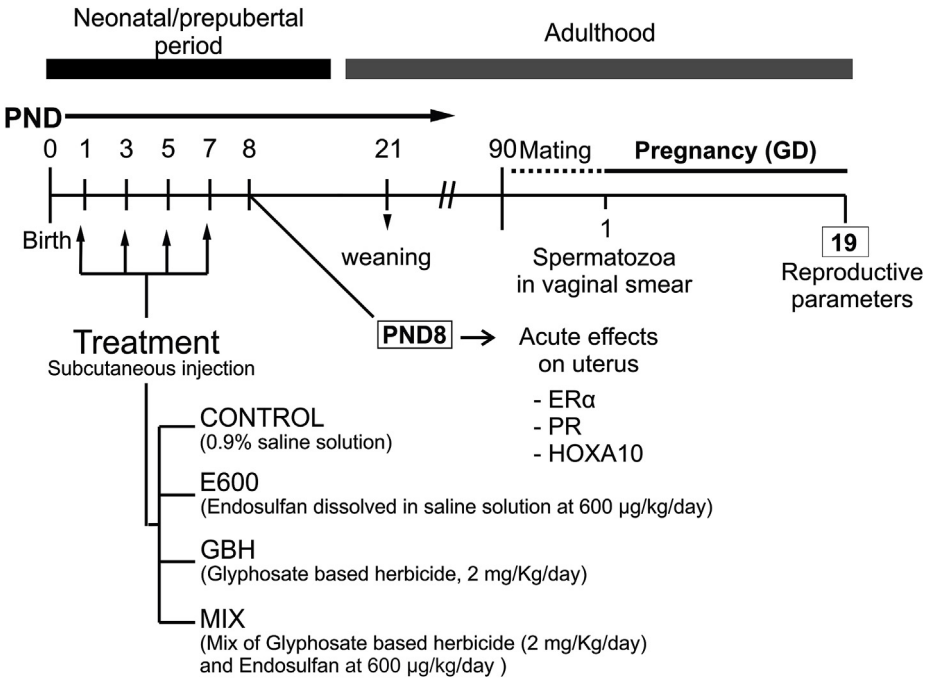

Fig. 1. Schematic representation of the experimental protocol used to investigate the effects of subcutaneous exposure to a glyphosate-based herbicide (GBH), Commercial formulation of endosulfan (EF) and a mixture of these compounds (MIX) on the uterus of rats on postnatal day (PND) 8 and gestational day (GD) 19.

2.4. Histological analysis

Uterine longitudinal sections (5 mm 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 40X objective (numerical aperture = 0.65; Olympus). 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 as was previously described by us (Guerrero Schimpf et al., 2017). The thickness of the subepithelial stroma and myometrium layers was analyzed by Image Pro-Plus 5.0.2.9 system (Media Cybernetics, Silver Spring, MD), as previously described (Guerrero Schimpf et al., 2017). Briefly, the images were recorded with a Spot Insight V3.5 color video camera, attached to a microscope (Olympus). To spatially calibrate the Image Pro-Plus analyzer, square grids from Neubauer's chamber images were captured. At least 10 fields were recorded in each section using a Dplan 40x objective (numerical aperture = 0.65; Olympus).

2.5. Immunohistochemistry

2.5.1. Antibodies

The following primary antibodies were used: mouse monoclonal antibody to estrogen receptor alpha (ERα) (clone 6F-11, 1:800 dilution; Novocastra, Newcastle upon Tyne, UK), rabbit polyclonal anti-progesterone receptor (PR) (A/B isoforms, A0098, 1:50 dilution; Dako Corp., Carpinteria, CA), and goat polyclonal antibody to Homeobox A10 (Hoxa10) (sc-17159, 1:200 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA). Anti-rabbit and anti-mouse secondary antibodies (biotin conjugate, B8895/B8774) were purchased from Sigma. An anti-goat secondary antibody (biotin conjugate, sc-2042) was purchased from Santa Cruz Biotechnology Inc.

2.5.2. Assay

Immunohistochemistry was performed to evaluate protein expression of ERα, PR and Hoxa10 in uteri of rats on PND8. Briefly, uterine sections 5 µm thick were de-paraffinized and rehydrated in a series of xylene and ethanol washes and then subjected to a microwave pretreatment for antigen retrieval. After blocking endogenous peroxidase activity and non-specific binding sites, samples were incubated in a humid chamber first with the specific primary antibody (14–16 h at 4 °C) and then with the corresponding biotin-conjugated secondary antibody (30 min at room temperature): anti-rabbit (1:200 dilution) and anti-mouse (1:100 dilution). Each immunohistochemical run included positive controls (sections from tissues known to express the proteins of interest) and negative controls (in which the primary antibody was replaced by non-immune serum of the species used to generate the primary antibody). The reaction was developed using the avidin-biotin-peroxidase method and diaminobenzidine (DAB) (Sigma) as a chromogen substrate. Samples were mounted with permanent mounting medium (Eukitt, Sigma-Aldrich).

2.5.3. Quantification of protein expression

The expression of ERα, PR and Hoxa10 in the uterine samples of PND8 was evaluated by image analysis using the Image Pro-Plus 5.0.2.9 system (Media Cybernetics, Silver Spring, MD, USA), as previously described (Milesi et al., 2012). Briefly, images were recorded with a Spot Insight V3.5 color video camera attached to an Olympus BH2 microscope (illumination: 12 V halogen lamp, 100 W, equipped with a stabilized light source; Olympus, Tokyo, Japan) using an objective A-Plan 20x/0.45 (Carl Zeiss Microscopy, LLC, USA) and converted to gray scale. The integrated optical density (IOD) was measured as a linear combination of the average gray intensity and the relative area occupied by positive cells. Because the IOD is a dimensionless parameter, the results are expressed as arbitrary units. Protein expression was quantified in the subepithelial stroma (a 45-µm-wide area adjacent to the epithelium, from the basement membrane toward the outer layers) and luminal epithelia of PND8 samples. At least 10 randomly selected fields per section and two sections per rat (separated by 50 µm from each other) were assessed.

2.6. Evaluation of reproductive performance

Control (n = 26 rats) and pesticide-exposed female rats (n = 30 rats per group) with a sperm-positive smear were housed separately, and their reproductive performance was evaluated on GD19. Sperm-positive females were killed on GD19. The ovaries were dissected, and the numbers of profusely irrigated corpora lutea (CL) were counted by direct visualization with the aid of a stereo microscope (Leica). The two-horned uteri were removed and visually inspected to identify resorption sites (RS) and implantation sites (IS), following criteria previously described in (Varayoud et al., 2011). The RS were defined as endometrial sites with an appended amorphous mass without a fetus, and the number of IS as the result of the total number of placentae with live fetuses plus the total number of RS (Ingaramo et al., 2016b). The fetuses per dam were counted. Fertility parameters were calculated as follows: the pregnancy rate was calculated as the number of pregnant females/number of females housed with a male × 100; fertility potential: number of IS/number of CL × 100; the rate of pre-implantation loss: [(number of CL − number of IS)/number of CL] × 100, whereas the rate of post-implantation loss was calculated as [(number of IS – number of fetuses)/number of IS] × 100 (Perobelli et al., 2012).

2.7. Statistic

All results were expressed as the mean ± standard error of 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). The variables were analyzed by the Kruskal–Wallis analysis to obtain the overall significance (testing the hypothesis that the response was not homogeneous across treatments), and differences between groups were determined using the Dunn post-hoc test. The data of incidence of LEH on PND8 were analyzed using Fischer's exact Test. All the analyses were carried out using R software (The R Foundation for Statistical Computing http://www.r-project.org/). Differences were considered significant at $P < 0.05$.

3. Results

3.1. Acute effects of pesticide exposure on uterine differentiation

The acute effects of pesticides were determined evaluating morphological parameters such as, thickness of stromal and myometrial compartments and the incidence of LEH. In addition, the expression of key proteins associated with uterine differentiation were evaluated on PND8. The selected proteins were ERα, PR and Hoxa10.

3.1.1. Morphological parameters

Table 1 shows the morphological changes induced by exposure to pesticides. After GBH exposure we detected an increment in the thickness of stromal and myometrial compartments (circular and longitudinal). In addition, an increase of incidence of LEH was observed in GBH group. On the contrary, EF did not affect the morphological parameters of the uterus. As we showed in Fig. 2, when animals were exposed to the mixture of pesticides, the morphological changes were similar to GBH alone.

3.1.2. Expression of proteins associated with uterine differentiation

3.1.2.1. ERα. The luminal epithelial expression of ERα did not show differences between all experimental groups (Fig. 3). When we compared the expression of ERα in stromal and myometrial compartments, GBH and EF showed an increased expression of ERα. The up regulation of ERα elicited by GBH and EF exposure was not detected in the MIX group. Representative photomicrographs of uterine immunodetection of ERα are shown (Fig. 6).

3.1.2.2. PR. The PR expression was detected only in the luminal epithelial cells and stromal cells. A clear induction of PR was detected in the GBH group, in both cellular compartments (luminal epithelium and stroma) (Fig. 4). EF exposure did not affect the PR expression. The MIX exposure had a similar effect to GBH in both cellular compartment (luminal epithelium and stroma). Representative photomicrographs of uterine immunodetection of PR are shown (Fig. 6).

3.1.2.3. Hoxa10. Finally, we evaluated Hoxa10, a homeobox protein with a clear and high nuclear expression in the stromal and myometrial cells. The results showed that the GBH exposure increased the expression of Hoxa10 in stromal and myometrial compartments (Fig. 5). EF exposure increased the Hoxa10 expression only in myometrium (Fig. 5B). Again, the MIX exposure also increased the expression of Hoxa10 in stromal and myometrial compartments, similar to GBH alone. Representative photomicrographs of uterine immunodetection of Hoxa10 are shown in Fig. 6.

3.2. Long-term effects of neonatal exposure to commercial formulation of pesticides

Then we investigated the long-term effects on reproductive performance using a fertility test. The GBH group showed an increased percentage of post-implantation losses on GD19 (Fig. 7F). In contrast, when the animals were exposed to EF alone (EF group), we detected a reduction in the number of fetuses per dams (Fig. 7B), IS (Fig. 7D), and an increase in the percentage of pre-implantation losses (i.e., number of oocytes not fertilized or embryo loss before implantation) (Fig. 7E). Moreover, a decreased fertility potential on GD19 (Fig. 7G) was found in EF group. Finally, when the animals were exposed to both pesticides (MIX group) the effects were similar to GBH alone, with an increase of post-implantation losses.

4. Discussion

The current study was conducted to evaluate the effects of postnatal exposure to a mixture of controversial and widely used pesticides, evaluating the acute and long-term effects of exposure. We determined the effects of EF and GBH on uterine differentiation (on PND8) and reproductive performance of female adult rats.

Endosulfan has been banned in many countries but is still in use in India, China, Brazil, South Korea, and Israel being India one among the largest producer, consumer and exporter of endosulfan in the world (Ghosh et al., 2018). On the other hand, glyphosate is currently the most used pesticide in agriculture worldwide, mainly because of its

Table 1
Morphologic parameters in experimental groups.

Variable	Control	EF	GBH	MIX
Subepithelial stroma thickness (μm)	83.6 ± 3.1	77.2 ± 3.1	110.6 ± 2.1**	118.6 ± 7.8***
Circular myometrium thickness (μm)	33.4 ± 1.8	30.8 ± 1.5	42.2 ± 1.3*	44.9 ± 2.9**
Longitudinal myometrium thickness (μm)	13.2 ± 1.1	18.9 ± 1.2	21.4 ± 0.8**	24.8 ± 1.3***
Incidence of LEH (%)	1/8	1/8	6/8*	6/8*

The values are expressed as the mean ± SEM. LEH, Luminal epithelial hyperplasia. *, $p < 0.05$ vs. control; **, $p < 0.01$ vs. control; ***, $p < 0.001$ vs control.

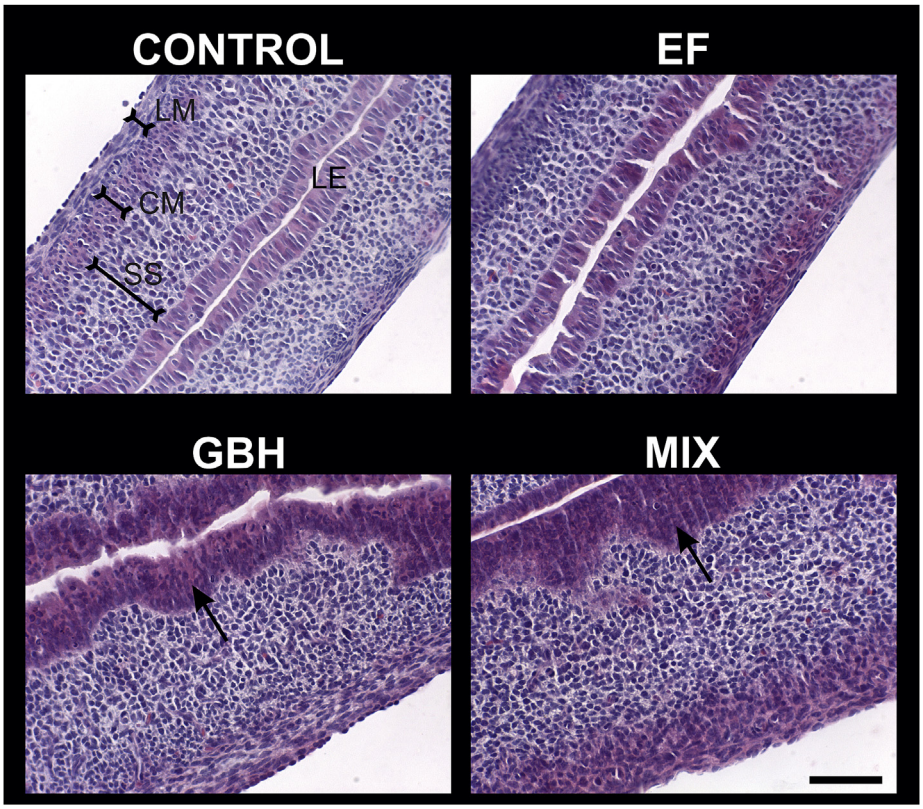

Fig. 2. Representative photomicrographs illustrating the morphological changes in hematoxilin and eosin stained uterine sections of experimental groups on PND8. An increase of subepithelial stroma, circular and longitudinal myometrium was observed in GBH and MIX group. At epithelial level, we detected an increment in the luminal epithelial hyperplasia (evidenced by an increase in the number of epithelial layers) in GBH and MIX group (arrow). LE, luminal epithelium; LM, longitudinal myometrium; CM, circular myometrium; SS, subepithelial stroma. Scale bar: 50 μm.

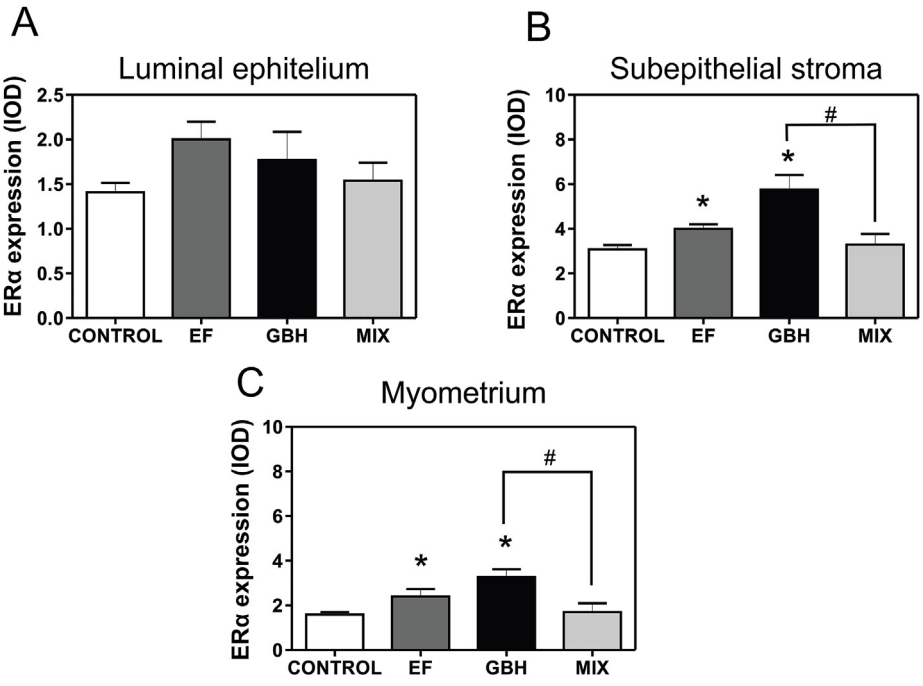

Fig. 3. Effects of neonatal GBH, EF and MIX exposure on ERα protein expression in uterus of rats on PND8. Quantification of ERα immunostaining in the Luminal epithelium, subepithelial stroma and myometrium (A–C) is expressed as integrated optical density (IOD). Note that ERα expression was increased in subepithelial stroma and myometrium in GBH and EF group. Each column represents the mean ± SEM (n = 8 rats per group). *, $P < 0.05$ vs. The control group; #, $P < 0.05$ vs. GBH group.

high efficacy and low residual activity in soil and the introduction of glyphosate-resistant field crops (Okada et al., 2019). Glyphosate-resistant weeds have now been found in 18 countries worldwide, with significant impacts in Brazil, Australia, Argentina and Paraguay (Gilbert, 2013). The regular and extensive use of these compounds may affect both target and non-target organisms, and the long-term effects of low level exposure to one pesticide are greatly influenced by concomitant exposure to other pesticides (Aktar et al., 2009). Besides, due to endosulfan slow biodegradation property, it persists in the environment (air, soil sediments and water) increasing the chances of human exposure (Ghosh et al., 2018). Glyphosate, on the other hand, is considered to decay fast in soil, however, Glyphosate was also found in soil for longer periods of time when adverse soil/climate conditions are present (e.g. dry soil, low temperatures) or in soils with strong adsorption capacity (Bento et al., 2019). The worldwide use of these chemicals implies that humans are continuously exposed to single pesticides or to combination of various pesticides, often in low concentrations, that may elicit similar effects despite belonging to different

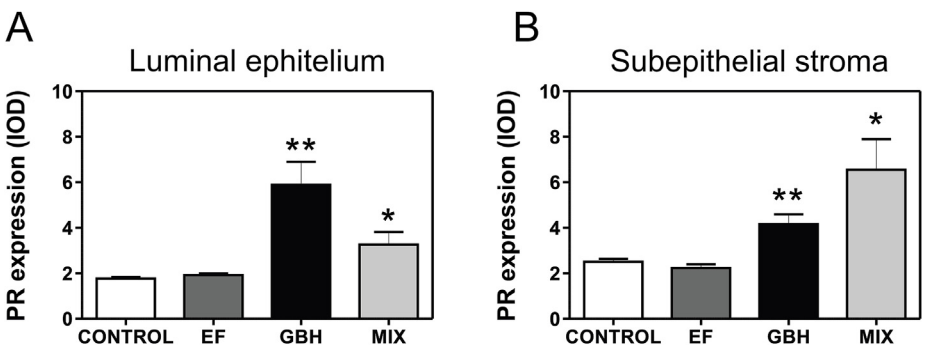

Fig. 4. Effects of neonatal GBH, EF and MIX exposure on PR protein in rats on PND8. Quantification of PR immunostaining in the luminal epithelium and subepithelial stroma (A–B) is expressed as integrated optical density (IOD). Note that the expression of PR in both zones was affected in GBH- and MIX-exposed rats. Each column represents the mean ± SEM (n = 8 per group). *, P < 0.05; **, P < 0.01.

chemical families (Hernandez et al., 2017). However, most of the mixtures to which we are exposed, and its effects are unknown. Several studies have shown association between a unique pollutant with some diseases, however, there are evidences that a mixture of diverse chemicals can elicit a different effect due to interactions of compounds (Hayes et al., 2006; Gomez-Gimenez et al., 2018; Soloneski et al., 2016). The study of chemical mixtures is limited because there is infinite number of combinations of possible contaminants, and often we do not know which is most important, or which dose ranges should be investigated, or which biologic end points should be studied (Carpenter et al., 2002).

In the present study, we compared the effects of commercial formulations of glyphosate and endosulfan, with the intention to define the effects of the combination of low doses of both pesticides. The selected doses are according to our previous published results (Guerrero Schimpf et al., 2017; Milesi et al., 2012, 2015; Ingaramo et al., 2016a, 2017; Alarcon et al., 2019). In our previous studies we evaluated the effects of the active principle endosulfan but not the commercial formulation. In addition, the most important aim of this study was the evaluation of the co-exposure to both pesticides (MIX group) using its commercial formulations. First, we investigated the acute effects of them in the uterus of rats on PND8. During first postnatal days the rodent uterus is not fully developed or differentiated and have high sensitivity to chemical compounds, thus a brief exposure may produce permanent uterine morphological or functional changes (Spencer et al., 2012; Zama and Uzumcu, 2010). Interestingly, MIX exposure induced deleterious uterine effects on differentiation evidenced by increased stromal and myometrial thickness and an increase in the incidence of LEH, a morphological alteration of luminal epithelium. The results were similar between MIX and GBH treated rats. We previously showed that the exposure to GBH induced LEH; however, there are not reports about effects of a co-exposure to EF. The EF group, however, did not show uterine morphologic alterations as a consequence of exposure to the commercial formulation. It is worth noting that we had not found morphologic alterations in previous studies using the active principle endosulfan (Milesi et al., 2012). The results indicate a predominant effect of GBH related to EF effects when the pesticides were co-administered. A similar effect on luminal epithelium was also described with other EDCs like tamoxifen, toremifene and DES (diethylstilbestrol) (Sa et al., 2019; Branham et al., 1988), indicating a high sensitivity of uterine epithelial cells to EDC exposure. In addition, a higher area of stroma (myometrial and subepithelial stroma) was detected after MIX exposure showing that morphological changes are detected in different uterine cellular compartments. All of them are functionally important to long-term events during adulthood and could predispose to consequences on fertility and/or development of neoplastic lesions.

The expression of key proteins for uterine development could be affected after exposure to EDCs during high sensitivity periods. Different studies indicate that a disruption of expression of these proteins modified the normal development of the uterus and produced long-term consequences. Steroid receptors (such as, ERα and PR) and homeobox proteins (such as Hoxa10) have been modified after exposure to EDCs (Bromer et al., 2009; Kitajewski and Sassoon, 2000; Shanle and Xu, 2011). All the treatments evaluated in the present study affects the expression of Hoxa10, a key protein that regulate anterior-posterior and radial patterning of the müllerian duct, affecting the normal uterine development. Altered expression of Hoxa10 during development results in uterine anomalies, as seen in mice exposed to DES and bisphenol A (Block et al., 2000; Varayoud et al., 2008b). In addition, mice and rats exposed to DES showed many uterine malformations, characterized by luminal and glandular squamous metaplasia, endometrial hyperplasia and increased risk of leiomyomas. (Kitajewski and Sassoon, 2000; Vigezzi et al., 2016). Similar to these results, in our work, a brief exposure to a mixture of both pesticides during development lead to endometrial hyperplasia. In a previous work we detected that bisphenol A affected the normal expression of Hoxa10 during development with long term consequences on fertility (Varayoud et al., 2008b, 2011). Steroid hormones receptors, like Hoxa10, are critical proteins during organogenetic and functional uterine differentiation (Taylor et al., 1997). Different endocrine-signaling pathways related with progesterone/PR and 17β-estradiol/ERα, regulate both the normal development and the implantation and decidualization process during

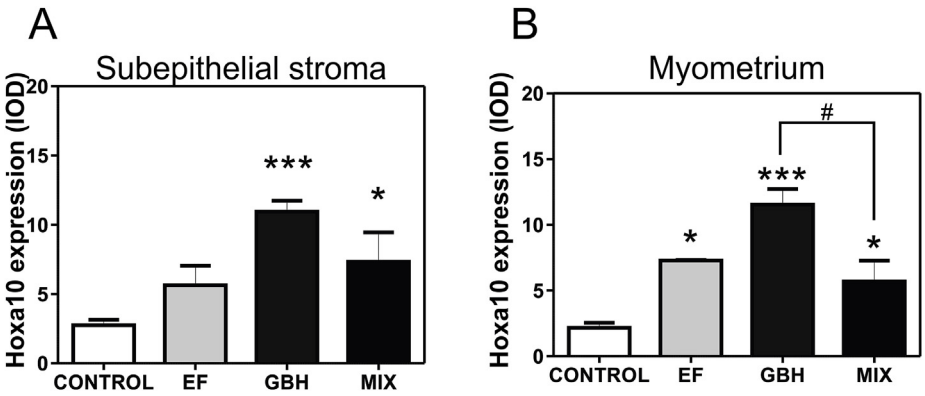

Fig. 5. Effects of neonatal GBH, EF and a mixture of these compounds exposure, on Hoxa10 protein expression in uterus of rats on PND8. Quantification of Hoxa10 immunostaining in subepithelial stroma and myometrium (A–B) is expressed as the IOD. Hoxa10 immunostaining is increased in the subephitelial stroma and myometrium of GBH and MIX-exposed and myometrium of EF rats. Each column represents the mean ± SEM (n = 8 per group). *, P < 0.05 vs. The control group; ***, P < 0.001 vs. The control group; #, P < 0.05 vs GBH group.

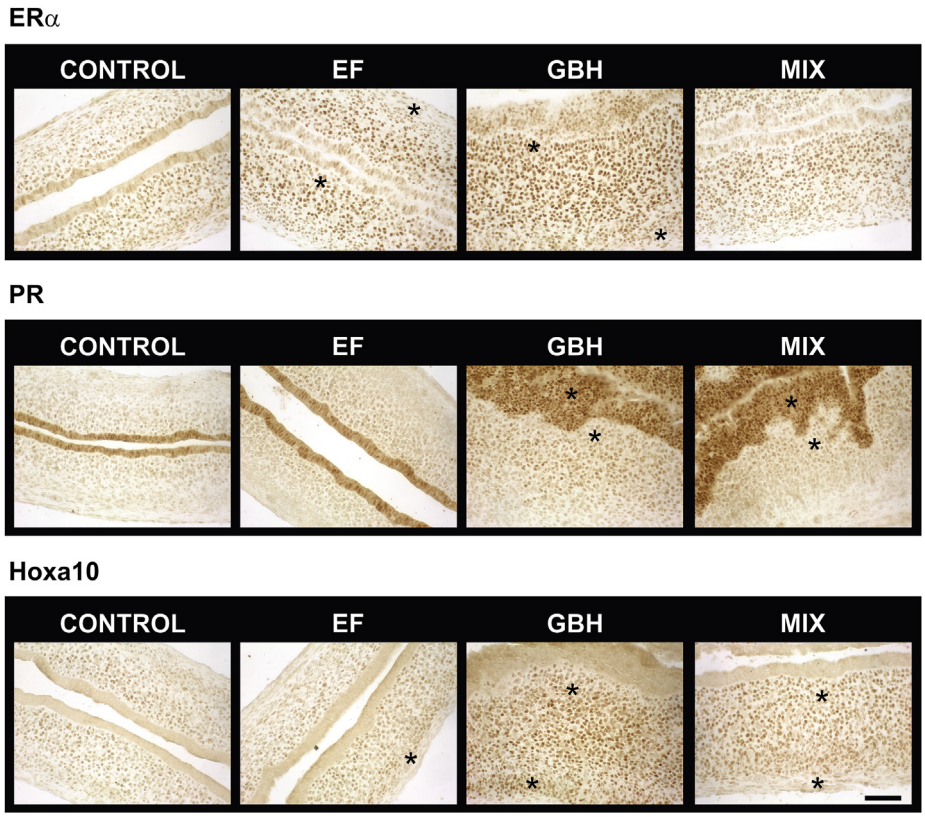

Fig. 6. Representative photomicrographs of ERα, PR and Hoxa10 expression by IHC on sections of the uterus of rats on PND8. Asterisks: significant differences. Scale bar: 50 μm.

gestation (Wetendorf and DeMayo, 2012; Hewitt et al., 2016). The morphologic and molecular alterations detected with the co-administration of the pesticides might lead to reproductive anomalies, such as infertility and early pregnancy loss, and could promote the development of uterine malformations and/or even neoplasia.

Different results have shown that glyphosate and endosulfan have endocrine disruptive effects (Gasnier et al., 2009; Soto et al., 1994; Li et al., 2013; Richard et al., 2005; Andersen et al., 2002; Thongprakaisang et al., 2013). These pesticides could alter the endocrine system, causing histological and endocrine problems in reproductive parameters (Varayoud et al., 2017; Ren et al., 2018; Guerrero Schimpf et al., 2017; Milesi et al., 2017, 2018; Manservisi et al., 2019). Some effects of glyphosate or its formulations were observed in male rats, and their reproductive toxicities are related to the alteration of ERα expression in mammary gland, reduction in testosterone and estradiol synthesis, as well as, alteration in sperm production during adulthood (Romano et al., 2010; Dallegrave et al., 2007; Altamirano et al., 2018). In female rats we previously demonstrated that exposure to GBH altered the uterine ERα expression and promoted luminal epithelial hyperplasia at the prepubertal period, and post-implantation losses at adulthood (Guerrero Schimpf et al., 2017; Ingaramo et al., 2016b, 2017; Varayoud et al., 2017; Lorenz et al., 2019). Endosulfan exposure, meanwhile, alter proteins expression related to development such as, ERα and Hoxa10, with long-term adverse consequences on fertility evidenced by pre-implantation embryo losses (Milesi et al., 2012, 2015, 2017). In male schoolchildren (10–19 years of age) of a village, where endosulfan had been aerially sprayed for more than 20 years has been demonstrated that endosulfan may delay sexual maturity and interfere with sex hormone synthesis (Saiyed et al., 2003).

The effects of different EDCs on fertility using different models of exposures have been previously described. (Curtis et al., 1999; Greenlee et al., 2003; Gray and Ostby, 1998; Schug et al., 2011). In previous works, we detected that the postnatal exposure to EF or GBH induced subfertility using a rat model, however the effect of co-administration of these pesticides was not previously evaluated (Ingaramo et al., 2016a, 2017; Milesi et al., 2015). We showed that both pesticides affected the rat fertility producing alterations in different stages of gestation. GBH produced post-implantation embryo loss in adult female rats associated with an altered decidualization response and a defective differentiation/proliferation uterine process (Ingaramo et al., 2016b, 2017). On the other hand, the active principle endosulfan affects the endocrine pathways necessary to the implantation process and reduced the number of implantation sites (Milesi et al., 2015). In the present work, the long-lasting effects of EF were similar to our previously work, reported with the active principle endosulfan. However, when we determined the long-lasting effects of the co-administration of pesticides, the results showed that animals experimented subfertility evidenced by a higher number of post-implantation losses. The effects detected on MIX group were similar to GBH group, indicating a predominant effect of glyphosate in the MIX group. All these results indicate that both pesticides, independently if there were administered alone or in combination, produced subfertility in rats.

Formulations of pesticides contain adjuvants, which are often kept confidential and are called inerts components by the manufacturing companies, plus a declared active principle, which is usually tested alone (Mesnage et al., 2014). It also has even demonstrated the toxic effects and endocrine disrupting properties of the formulations were mostly due to the formulants and not to the active principle alone (Defarge et al., 2016, 2018; Richard et al., 2005; Wan et al., 2005). Several heavy metals detected in most formulations, in particular arsenic, cobalt, chromium, nickel and lead, have endocrine disrupting effects (Defarge et al., 2018). According to (Mesnage et al., 2014) regulatory tests should be also performed with the formulations of pesticides to better estimate health risks.

The molecular changes detected on prepubertal uterus could be

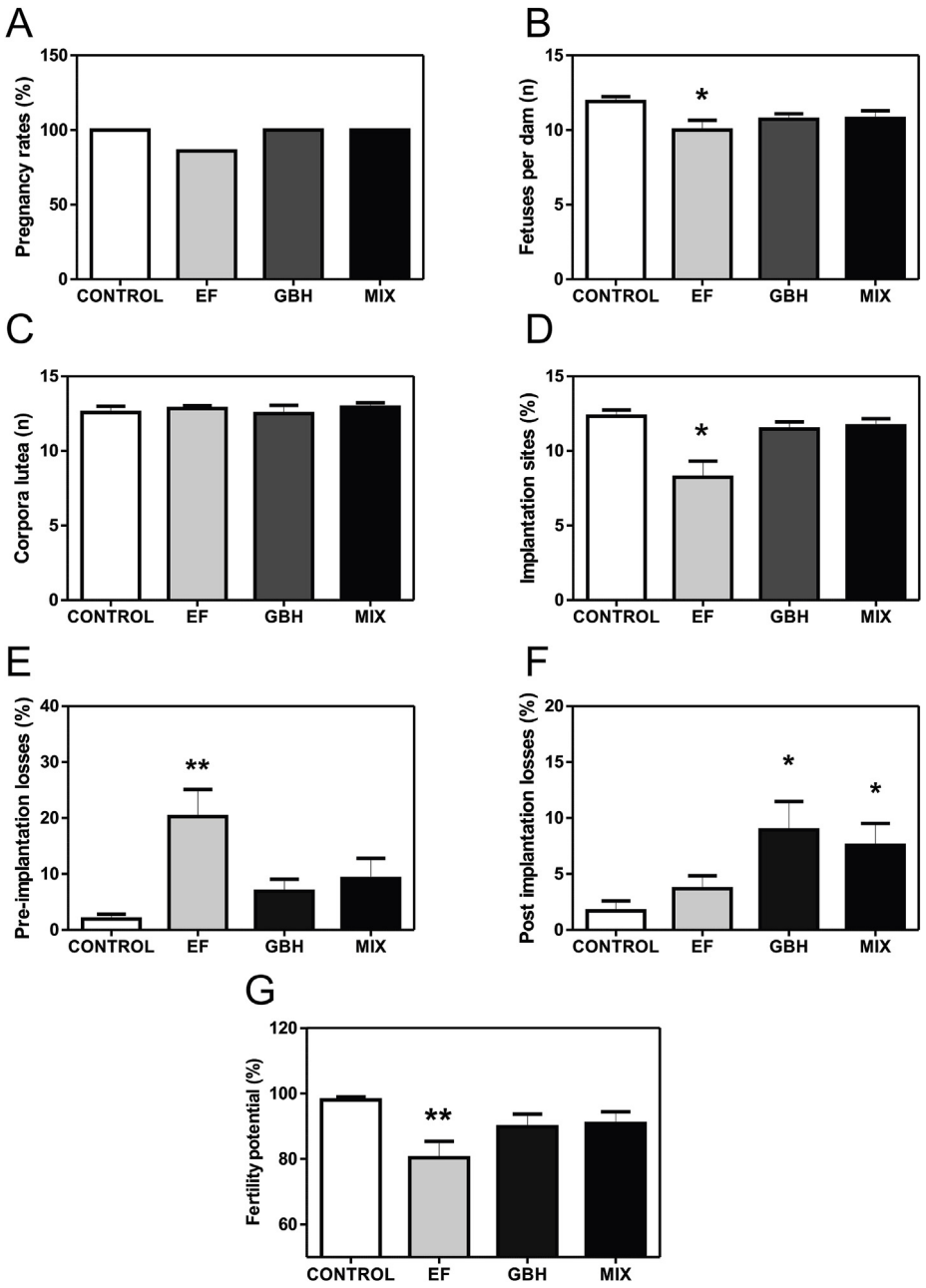

Fig. 7. Reproductive parameters in female rats neonatally exposed to GBH, EF and a mixture of these pesticides. Percentage of pregnancy rates (A); number of fetuses per dam (B); number of corpora lutea (CL) (C) and implantation sites (IS) (D) evaluated on GD19 (each column represents the mean ± SEM). Percentage of pre-implantation (E) and post-implantation (F) loss and the fertility potential (G) on GD19 (results were calculated as described in M&M). *, $P < 0.05$.

associated with long-term consequences on fertility. The Hoxa10 and PR expression have been related with subfertility in different works (Milesi et al., 2015; Varayoud et al., 2011). Other authors have reported a dose-responsive increase in uterine Hoxa10 expression in 2-week-old mice following in utero BPA exposure (Smith and Taylor, 2007). We previously observed an induction of Hoxa10 by neonatal GBH or endosulfan exposure in prepubertal rats demonstrating that Hoxa10 could be a common target of endocrine disruption (Guerrero Schimpf et al., 2017; Milesi et al., 2012). In addition, in adulthood, animals exposed to endosulfan showed a silencing of Hoxa10 expression while GBH exposed rats exhibit an induction of the homeotic protein associated to an increment in endometrial proliferation. As for PR uterine expression, some discrepancies were observed. While GBH induced PR expression, endosulfan decreased its expression in prepubertal uterus (Milesi et al., 2012; Guerrero Schimpf et al., 2017). Then, PR expression decreased in GBH-exposed rats and increased in endosulfan-exposed rats in adulthood (Ingaramo et al., 2016b; Milesi et al., 2015). In conclusion, the exposure to EDC disrupt Hoxa10 and PR expression during development with long-term consequences during pregnancy (Milesi et al., 2012, 2015; Varayoud et al., 2008b, 2011; Ingaramo et al., 2016b). In accordance with our previous results, in the present work we found altered expression of molecules related to uterine development and fertility. We propose that a similar molecular disruption occurs in MIX group as a possible explanation of the subfertility after the co-treatment with both pesticides.

Recently, was reported the exposure to a complex EDC mixture, modifies reproductive parameters in female mice. The study evaluate the effect of a mixture of three phthalates administered from conception to adulthood, and detected changes in several fertility parameters in exposed females and in their progenie (Patino-Garcia et al., 2018). In

addition, other study found that prenatal exposure to a phthalate mixture caused a life-long impact on the reproduction in male mice, affecting the normal development of reproductive organs, the levels of testosterone and spermatogenesis (Barakat et al., 2019).

While the majority of the studies related to pesticides effects addressed the effect of each chemical, the present work provides valuable information about effects of mixture of two worldwide used pesticides evaluating the short and long-term effects, indicating the novelty of the present study. The combination is worth to study because the mixture represents more realistic scenarios to mimics the environmental exposure to pesticides. It has been reported that risk assessment based on single substances alone can underestimate the risk for adverse effects of exposure to several pesticides (Hass et al., 2017). The complexity of toxicological interactions can lead to unpredictable effects of pesticide mixtures. Interactions on metabolic processes affecting the biotransformation of pesticides seem to be by far the most common mechanism of synergism (Hernandez et al., 2017). Since the limited available empirical evidence suggests that synergisms at low exposure levels are rather rare, and experimentally occurred at unrealistic high concentrations, synergism cannot be predicted quantitatively on the basis of the toxicity of mixture components (Hernandez et al., 2017). Generally, when exposure levels of the chemicals within a mixture are in the range of the NOAELs or below, and the components of the mixture have different modes of toxic action, no additivity and no potentiating interactions are found, indicating the applicability of the basic concept of "simple dissimilar action", which suggests that adverse reactions of the mixture would be unlikely (Hughes and Woods, 2002). Our study could indicate that EF and GBH (at low doses of exposure) could produce a simple dissimilar action, making unpredictable the effects of them.

In conclusion, the effects of mixtures of pesticides with different mechanism of action, such as herbicides and insecticides, are more difficult to predict and understand. The issues involved in examining the effects of chemical mixtures are going to continue to increase in complexity as additional chemicals are introduced (Kienzler et al., 2016). The findings of the present and other studies highlight the need for further studies on the potential reproductive impact of different pesticide mixtures.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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