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Are glyphosate and glyphosate-based herbicides endocrine disruptors that alter female fertility?

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ABSTRACT

Numerous evidences have alerted on the toxic effects of the exposure to glyphosate on living organisms. Glyphosate is the herbicide most used in crops such as maize and soybean worldwide, which implies that several non-target species are at a high risk of exposure. Although the Environmental Protection Agency (EPA-USA) has reaffirmed that glyphosate is safe for users, there are controversial studies that question this statement. Some of the reported effects are due to exposure to high doses; however, recent evidences have shown that exposure to low doses could also alter the development of the female reproductive tract, with consequences on fertility. Different animal models of exposure to glyphosate or glyphosate-based herbicides (GBHs) have shown that the effects on the female reproductive tract may be related to the potential and/or mechanisms of actions of an endocrine-disrupting compound. Studies have also demonstrated that the exposure to GBHs alters the development and differentiation of ovarian follicles and uterus, affecting fertility when animals are exposed before puberty. In addition, exposure to GBHs during gestation could alter the development of the offspring (F1 and F2). The main mechanism described associated with the endocrine-disrupting effect of GBHs is the modulation of estrogen receptors and molecules involved in the estrogenic pathways. This review summarizes the endocrine-disrupting effects of exposure to glyphosate and GBHs at low or "environmentally relevant" doses in the female reproductive tissues. Data suggesting that, at low doses, GBHs may have adverse effects on the female reproductive tract fertility are discussed.

1. Introduction

The environmental pollution caused by urbanization, agriculture and industrialization has been reported to affect animal and human health (Luque et al., 2018). Throughout their lifespan, both humans and animals are exposed to many of man-generated chemicals, which can have either a positive or a negative impact on their health. These compounds can be found in the environment, food, cosmetics, household items, pharmaceutical products, and many other products (Darbre 2018, 2019). Studies on the possible hormonal activity of some of these compounds have shown that they have the potential to disrupt the endocrine system of many animal species, including humans (Amereh et al., 2020; Tsai et al., 2019). The potential risk of these chemicals can be assessed by means of *in vitro* studies or animal models, including fish, amphibians and mammals (Bergman et al., 2013). Colborn et al. (1997) were pioneers in demonstrating the risks of exposure to chemicals in the environment known as endocrine-disrupting chemicals (EDCs). The World Health Organization (WHO) defined an EDC as "an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny or (sub)populations" (Bergman et al., 2013). Exposure to EDCs can occur via ingestion of water, food and dust, via inhalation of gases and air particles, and via dermal absorption. Once in the organism, EDCs interact with the endocrine system and disrupt normal endocrine function, sexual development, and ultimately reproduction. EDCs exert their actions by triggering genomic mechanisms and non-genomic actions, through the binding to several hormone receptors, including thyroid and, especially, steroid receptors, mainly estrogen (ER) or androgen (AR) receptors (Diamanti-Kandarakis et al., 2009; Gore et al., 2015). These "xenochemicals" (chemicals that are foreign to the body) may possess a range of agonist, partial agonist or antagonist activities dependent on the dose, presence of endogenous receptor ligand, and nature of the structure–activity relationship (interaction with the nuclear hormone receptors) (Hall and Greco, 2019). The exposure to EDCs in pregnant mothers may reach the developing fetus and exposure

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Abbreviations

AMPA	aminomethylphosphonic acid
AR	androgen receptors
As	Arsenic
Bmp2	bone morphogenetic protein 2
Co	Cobalt
COUP-TFII	COUP transcription factor 2
Cr	Chromium
Cyp19a1	cytochrome P450, family 19, subfamily A, polypeptide 1
DOHaD	Developmental Origins of Health and Disease
EDCs	endocrine-disrupting chemicals
EPA	Environmental Protection Agency
ER	steroid receptors
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
ERE	estrogen response element
Foxa2	forkhead box protein A2
FSHR	Follicle Stimulating Hormone Receptor
GBHs	glyphosate-based herbicides
GnRH	Gonadotropin-releasing hormone
Hoxa10	homeobox a10
IARC	International Agency for Research on Cancer
IGFBP-3	insulin-like growth factor binding protein 3
LHR	luteinizing hormone receptor
Ni	Nickel
NOAELs	no-observed-adverse-effects level
Pb	lead
PND	post-natal day
POEA	polyethoxylated amine;
PR	progesterone receptor
3β-HSD	3β-hydroxysteroid dehydrogenase
WHO	World Health Organization
Wnt	wingless-type MMTV integration site family

during early life has been shown to disrupt the normal development of reproductive tissues and may predispose to diseases in adulthood (Rattan and Flaws, 2019). Moreover, the exposure to these chemicals neonatally can negatively impact the reproductive health of future generations and cause transgenerational effects on reproduction in both males and females (Brehm and Flaws, 2019). The mechanisms of action by which EDCs transmit adverse effects to future generations include epigenetic modifications (Rattan and Flaws, 2019). Despite several evidences on the potential adverse effects of EDCs on reproduction, the molecular mechanisms underlying these effects are not completely understood.

Although there are more than 80,000 chemicals in commercial use, the risk of most of them has not yet been assessed. In 2015, by means of the ToxCast program, the Endocrine Disruptor Screening Program of the U.S. Environmental Protection Agency (EPA) made efforts to demonstrate the estrogenic and antiestrogenic activities of more than 1800 chemicals (US EPA, 2015). Among the chemicals that highly contaminate the environment and for which their toxicity has not yet been completely assessed, we can mention all the agrochemicals. Moreover, for many of these agrochemicals, there is an absence of evidence of human and animal levels of exposure. With the development of herbicide-tolerant soybeans, corn and cotton in 1996, the use of glyphosate and glyphosate-based herbicides (GBHs) has increased dramatically over the past two decades. With an estimation of more than 800 million kg sprayed around the globe in 2014, GBHs are among the most used agrochemicals in the world (Benbrook, 2016). In Argentina, glyphosate is the most commonly used herbicide, with around 180–200 million liters applied every year (Aparicio et al., 2013). Currently existing information on the safety of glyphosate and/or its formulations is controversial, suggesting that glyphosate or its adjuvants are responsible for the adverse impacts on human, animal and ecological health (Meftaul et al., 2020; Vandenberg et al., 2017). However, most studies involve exposures over long periods, high doses, or the use of glyphosate alone, which is not a real situation regarding environmental contamination. This review aims to analyze and summarize the current literature that has evaluated the effects of low or "environmentally relevant" doses of glyphosate and GBHs acting as EDCs, mainly regarding evidences obtained in the female reproductive tissue. We present enough data suggesting that, at low doses, GBHs may have adverse effects on the development of the female reproductive tract and fertility.

2. Glyphosate and glyphosate-based herbicides (GBHs)

2.1. Formulations, surfactants, exposed species and human exposure

The first GBH, Roundup®, was introduced by Monsanto in the early 1970s. Glyphosate kills weeds and grasses by inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate synthase, involved in the biosynthesis of aromatic compounds in plants and microorganisms, and was thus considered safe to animals and humans. For this, and other aforementioned reasons, GBHs are the herbicides most frequently applied worldwide (Mertens et al., 2018). However, after the WHO's International Agency for Research on Cancer (IARC) re-classified glyphosate as "probably carcinogenic to humans" based on a small number of epidemiological studies following occupational exposures (Guyton et al., 2015; Myers et al., 2016), concerns about the carcinogenic properties of GBHs have increased.

In the world market, there are several formulations of GBHs, which differ mainly in the content of surfactants, the most common of which over the last years have been ethoxylated amines. The addition of surfactants to the active compound promotes the penetration and stabilization of glyphosate in plants. In addition, arsenic (As), cobalt (Co), chromium (Cr), nickel (Ni) and lead (Pb), several of which are known to be EDCs, are present in numerous herbicide formulations, at levels well above admissible ones in water (Defarge et al., 2018). A comparative cytotoxicity study of surfactants in human cell lines concluded that alkyl polyglucosides, which are high-quality nonionic surfactants, are the least toxic compounds, followed by polyethoxylated alkyl phosphate ethers, which are quaternary ammonium surfactants, and finally by POE-tallow amines, which are the most toxic (Defarge et al., 2016). However, some occupational and food risk assessments have shown that there are no significant human health issues associated with the use of POEA as surfactants in glyphosate products (Martens et al., 2019). The first generation of POEA surfactants (POE-tallow amines) in Roundup® are markedly more toxic than glyphosate and thus pose higher risks to human health, especially among heavily-exposed applicators (Defarge et al., 2016). In some cases, the absence of compositional data may cause problems in the reproducibility of the experiment because, in formulated GBH products with the same commercial name, the mixture of chemicals could vary among different countries (Mesnage et al., 2019).

In Argentina, the percentage of land destined to agricultural production has increased significantly in recent years, mainly due to new technologies, including modern irrigation, pesticides, chemical fertilizers, conservation tillage and the expansion of soybean crops. This

habitat fragmentation and the constant exposure to pesticides and GBHs affect different species (Burella et al., 2018; Dornelles and Oliveira, 2016), including the main agricultural insect pollinator, the honey bee (*Apis mellifera*) (Vazquez et al., 2018). In the last years, the effects of glyphosate either alone or in formula have been analyzed in several species such as fish, lambs, caimans, mice and rats (Alarcón et al., 2019; Albanil Sanchez et al., 2019; Ingaramo et al., 2016; Pham et al., 2019; Szepanowski et al., 2018; Varayoud et al., 2017). Regarding animal feed, residues of glyphosate have been found in soybean and maize, both of which play an important role in animal nutrition (Poppe et al., 2019). Indeed, a study comparing 18 commercial animal feeds from different companies has recently demonstrated that every product contained detectable glyphosate residues in a range between 78.3 and 2140 μg/kg, which results in an exposure of animals 4–12 times higher than that of humans per kg basis (Zhao et al., 2018).

Despite the widespread use of glyphosate, data on the potential human exposures during common occupational uses are limited. The occupational exposure of amenity horticulturalists to GBHs has shown that contamination is usually greater than that reported in environmental studies (Connolly et al., 2018). Very high concentrations of glyphosate and its main metabolite, aminomethylphosphonic acid (AMPA), may occur in airborne particulate matter, which can be inhaled by humans and animals (Bento et al., 2017). In this context, the data of urine levels is a powerful tool in biomonitoring studies to estimate human exposure (Conrad et al., 2017; Krüger et al., 2014). Some occupational biomonitoring studies have analyzed pooled urine samples collected over a 24-h period, providing an estimate of the average exposure over this sampling period (Acquavella et al., 2004; Mesnage et al., 2012), whereas others have analyzed a spot urine sample as a marker of the 24-h exposure (Connolly et al., 2018). Several researchers have found that the detectable concentrations of glyphosate in urines collected from farmers and their families during the study period were in the range of <0.1–233 ng/mL, with the highest systemic dose estimated at 0.004 mg/kg (Acquavella et al., 2004; Jauhiainen et al., 1991; Thongprakaisang et al., 2013). The systemic dose, which is calculated as the amount of glyphosate excreted in urine divided by each individual's body weight, is an integrated measure of the amount of a substance absorbed per kilogram of body weight that provides a basis to compare human exposures with levels of toxicological significance (Acquavella et al., 2004). Parvez et al. (2018) evaluated the exposure of pregnant women to GBHs by direct measuring in urine samples and found that >90% of them had detectable glyphosate levels, which were higher in women living in rural areas, and that these levels correlated significantly with shortened pregnancy lengths. In Thai women, Kongtip et al. (2017) demonstrated high maternal (0.2–189.1 ng/mL) and umbilical cord (0.2–94.9 ng/mL) serum concentrations of glyphosate.

2.2. Metabolism and its potential impact on the toxicity of glyphosate and GBHs

Although several reports have claimed no significant transformation of glyphosate to AMPA *in vivo* (EFSA, 2015; Niemann et al., 2015; Williams et al., 2000), Ford et al. (2017) demonstrated the formation of AMPA and glyoxylate in livers from glyphosate-treated mice. These authors showed that approximately 4% of the glyphosate levels detected in the mouse liver are metabolized to glyoxylate (Ford et al., 2017). Glyphosate metabolization may depend on the physiology of the animal. At our lab in lambs exposed to GBH during their first postnatal days, we found no AMPA serum levels, suggesting a limited metabolism of glyphosate into AMPA by intestinal microbial action, considering that the rumen of lambs exhibits limited functional development on post-natal day (PND) 15 (Alarcón et al., 2019). Although the acute toxic effects of glyphosate and AMPA on mammals are low, there are animal data raising concern of the health effects associated with chronic ultra-low doses of these compounds, related to their accumulation in the environment (Van Bruggen et al., 2018). Anadon et al. (2009), for example, found that the bioavailability of glyphosate by oral administration in rats is 23.21%, whereas, in ewe lambs, we have recently found that the levels of glyphosate in serum and the effects on the female reproductive tract in orally and subcutaneously GBH exposed animals were similar (Alarcón et al., 2019). Several experiments have demonstrated that the estrogenic potential and toxicity of glyphosate are different from those of GBHs and polyethoxylated amine (POEA) (De Almeida et al., 2018; Mesnage et al., 2017; Perego et al. 2017a, 2017b; Richard et al., 2005).

Regarding other issues not directly related to reproduction, Davoren and Schiestl (2018) suggested that glyphosate has the potential to affect the human gut microbiome profile and function, leading to decreased pathogen defense and inflammation, both in the intestine and systemically. They suggested that the adjuvant surfactants and emulsifiers present in GBHs would contribute to microbiome disruption more than glyphosate alone (Davoren and Schiestl, 2018). This represents another pathway through which glyphosate and GBHs could induce adverse effects related to inflammation and immune response that may affect reproduction.

3. Mechanisms of action: *in vitro* studies

Many of the mechanisms by which the neonatal exposure to glyphosate or GBHs affects the female reproductive tract are not known but the few studies regarding this issue have been done *in vitro*. However, most of the effects of glyphosate and GBHs shown *in vitro* have not been able to be reproduced in *in vivo* experiments. Several of the *in vitro* studies on glyphosate and GBHs have been performed using cancer cell lines that are estrogen-responsive (HEC1A, MCF-7, and T47D-KBluc cell lines) or the estrogen-insensitive line MDA-MB-231. HEC1A cells are endometrial cancer cells that express the wild-type form of estrogen receptor alpha (ERα). Since estrogen induces the proliferation of these cells (Castro-Rivera and Safe, 1998), De Almeida et al. (2018) demonstrated a non-monotonic reduction in the viability of HEC1A cells exposed to pure glyphosate (75–500 μg/mL). These authors also found that exposure to the GBH Wipeout® (75, 125 and 250 μg/mL) induces proliferative effects and DNA damage in both HEC1A and MDA-MB-231 cells. Based on the differential toxicities of the GBHs Roundup® and Wipeout® in human whole blood and HEC1A cells, these authors determined that the adjuvants and/or glyphosate impurities were potential contributing factors of toxicity (De Almeida et al., 2018). They also found that, in human whole blood, glyphosate and Roundup® led to similar non-monotonic toxicological profiles, evidenced by a significant reduction in cell viability ($P \leq 0.01$) at pure glyphosate and Roundup® concentrations of 10 and 50 μg/mL and no decreased cell viability at higher concentrations (500 μg/mL). Finally, they found that Wipeout® led to a monotonic reduction in cell viability from a threshold concentration of 50 μg/mL (De Almeida et al., 2018). In human peripheral blood mononuclear cells (Martinez et al., 2007), demonstrated that the cytotoxicity of Roundup® was higher than that of glyphosate alone, because the concentration of Roundup® to cause 50% mortality of the cells was 30 times lower than that of pure glyphosate. These results support the concept that the additives in commercial formulations play a significant role in the toxicity attributed to GBHs.

In HepG2 cells, which are the best characterized human liver cell line and are used to study xenobiotic toxicity, Gasnier et al. (2009) determined glyphosate cytotoxicity with three assays (Alamar Blue, MTT, ToxiLight), genotoxicity by the comet assay, and anti-estrogenic effects (on ERα, ERβ) and anti-androgenic effects (on the androgen receptor (AR) by using gene reporter tests. They also checked androgen to estrogen conversion by aromatase activity and mRNA. At sub-agricultural doses, all these parameters were disrupted with all formulations within 24 h. In HepG2 cells, the active formulation R400 (from 2 parts per million (ppm)) inhibited the transcriptional activities on both estrogen receptors. These effects were more dependent on the formulation than on the glyphosate concentration. In addition, in MDA-MB453-kb2 cells,

these authors observed decreased AR and aromatase transcription from 0.5 ppm of GBH and inhibited enzymatic activity from 10 ppm (Gasnier et al., 2009). In human ovarian and prostate cancer cells, Li et al. (2013) demonstrated that glyphosate and AMPA can inhibit proliferation and promote apoptosis. One of the mechanisms by which glyphosate would inhibit cell proliferation is through the enzyme serine hydroxymethyltransferase, a major source of intracellular glycine (Mesnage et al., 2015). Other *in vitro* studies have shown that exposure of human lymphocytes to different glyphosate concentrations does not cause effects on their proliferation/mitotic index (Santovito et al., 2018).

Regarding the effects of glyphosate on ovarian tissue and stem cell differentiation, Gigante et al. (2018) studied swine granulosa cells and found that, at different doses (0.2, 4 and 16 μg/mL), glyphosate decreased the growth of granulosa cells (by bromodeoxyuridine incorporation and ATP production) and estrogen production and increased progesterone and nitric oxide secretion, all of which suggest that the *in vivo* ovarian function may be affected. On the other hand, by using granulosa cells from bovine ovary, Perego et al. (2017a) found different results between exposure to glyphosate and Roundup®. While Roundup® (10 and 300 μg/mL) dramatically decreased the number of granulosa cells and estrogen and progesterone production, glyphosate alone had no effect (Perego et al., 2017a). In cow granulosa cells, Wrobel (2018) reported that pure glyphosate stimulates the secretion of estrogen, while GBHs and pure glyphosate increase oxytocin and decrease progesterone secretion from luteal cells. All these *in vitro* studies with bovine ovarian cells suggest that glyphosate and/or GBHs may affect, at least in part, the female reproductive system via direct action on the ovarian function.

Richard et al. (2005) tested the toxicity of glyphosate and Roundup® and their possible ability to act as EDCs in human placental JEG3 cells. Their study showed that Roundup® interacts with the active site of the enzyme aromatase and decreases its activity and mRNA expression. Besides, they demonstrated that glyphosate is toxic within the lowest concentrations used in agriculture, and that this effect increases with higher concentrations and longer times as well as in the presence of Roundup® adjuvants (Richard et al., 2005). On the other hand (Thongprakaisang et al., 2013), observed that, in the range of concentrations described in the environment and at levels to which humans and domestic animals are exposed, glyphosate increases the proliferation of the hormone-dependent breast cancer T47D cell line, acting via ERα. In this cell line, glyphosate exhibits estrogenic activity and interferes with normal estrogen signaling, probably through the activation of an estrogen response element (ERE) since these responses can be blocked by the ER antagonist ICI 182780 (Thongprakaisang et al., 2013). Mesnage et al. (2017) reported that glyphosate (≥10 μg/mL) promotes the proliferation of estrogen-dependent MCF-7 human breast cancer cells and increases the expression of an ERE-luciferase reporter gene (ERE-luc) in T47D-KBluc cells, the latter of which can be blocked by the estrogen antagonist ICI. In MCF-7 cells, they found a weak and unstable interaction (compared to estrogen) between glyphosate and ERα and that glyphosate could activate this receptor although at relatively higher concentrations than estradiol (Mesnage et al., 2017). Based on their results, these authors proposed the hypothesis that glyphosate exerts its effects in a ligand-independent pathway via the cAMP-dependent protein kinase A, which modulates the balance between cell proliferation and apoptosis (Mesnage et al., 2017). They also showed that, at the concentrations at which glyphosate causes estrogenic effects, glyphosate also causes apoptosis and disturbance in liver cell mitochondrial respiration. Finally, they found that commercial GBH formulations or their adjuvants alone did not exhibit estrogenic effects acting through ERα (Mesnage et al., 2017).

A recent study using ER+ and ER-breast cancer cell lines demonstrated that GBHs (but not AMPA) affect several pathways related to DNA damage repair, base excision repair, nucleotide excision repair and mismatch repair (Stur et al., 2019). Other authors have suggested that glyphosate would induce cell apoptosis by activating caspases 3 and/or 7 (Benachour and Seralini, 2009; Clair et al., 2012).

4. In vivo effects in animal models

4.1. Fish and chicken

Despite all the *in vitro* studies previously mentioned, most of the effects described for glyphosate and GBHs have not been able to be reproduced in *in vivo* experiments. Another significant issue regarding glyphosate toxicity is the effects of low or "environmental relevant" doses.

Studying the effect of glyphosate in zebrafish (*Danio rerio*), Armiliato et al. (2014) found a significant increase in the diameter of oocytes, raising the concern on the effects of glyphosate on fish reproduction. Recently, Smith et al. (2019) showed that Roundup® and its active ingredient glyphosate can induce developmental, reproductive, and epigenetic effects on Japanese medaka fish. In addition, in the testes of these fish exposed to 0.5 mg/L Roundup® or glyphosate, the expression of FSHR was significantly reduced, whereas in the ovaries the expression of FSHR remained unchanged (Smith et al., 2019). In zebrafish (*Danio rerio*) embryos, glyphosate treatment inhibited the activity of carbonic anhydrase, caused production of reactive oxygen species, especially in branchial regions, triggered cellular apoptosis and induced several types of malformations, including pericardial edema, yolk sac edema, spinal curvature and body malformation in a dose-dependent manner (Sulukan et al., 2017).

Hatching defects, histological alterations and biochemical imbalance were also reported in chicks by Fathi et al. (2019). The study consisted in evaluating newly hatched chicks where the eggs were exposed to glyphosate or GBH (Roundup®). The authors reported a decrease in the hatchability rate in chicks treated with Roundup® (Fathi et al., 2019).

4.2. Effects on the ovary of rats, mice and ewe lambs

Within the ovary, signaling between granulosa and theca cells is essential to support follicle development, oocyte development, and ovulation. Moreover, the ovarian follicle can be considered a very fragile micro-environment where several interactions between hormones, growth factors, the oocyte and its surrounding somatic cells are essential to generate a fully competent oocyte. Disruption of this finely tuned balance can lead to an incomplete germ cell nest breakdown (multi-oocyte follicles) (Pepling, 2012), alter the activation of follicles with an increase of atresia (Monniaux, 2018) or anovulation (Petro et al., 2012). Due to this multiple interaction and complexity, ovaries and follicles are excellent targets to investigate endocrine-disrupting effects. However, the effects of the exposure to glyphosate or GBHs on ovarian function and stem cell differentiation are still largely unknown. In female rats exposed to GBHs (Paraquat and/or Roundup®) during the first days of pregnancy, Almeida et al. (2017) found lower weight ovaries and a decrease in the number of corpora lutea. In mice exposed *in utero* to glyphosate a decreased ovarian weight and histopathological alterations, increased atretic follicles, interstitial fibrosis and decreased mature follicles on gestational day 19 was described (Ren et al., 2018). Serum levels of progesterone and estrogen were significantly altered following glyphosate exposure together with changes in the expression of Gonadotropin-releasing hormone (GnRH), luteinizing hormone receptor (LHR), Follicle Stimulating Hormone Receptor (FSHR), 3β-hydroxysteroid dehydrogenase (3β-HSD) and cytochrome P450, family 19, subfamily A, polypeptide 1 (Cyp19a1) genes at the hypothalamic-pituitary-ovarian axis; in the ovary oxidative stress increased (Ren et al., 2018). Similar results were observed by Hamdaoui et al. (2018) in female rats exposed to sub-chronic doses of the GBH Kalach 360 SL showing impaired folliculogenesis, altered ovary development, decreased estrogen secretion, oxidative stress and altered ovarian morphology. Results suggesting that this GBH can induce endocrine-disrupting effects.

Experiments performed at our lab in a model of ewe lambs exposed to low doses of GBH from birth to PND15 (Alarcón et al., 2019) demonstrated an increase in the number of atretic follicles and a decrease in the mRNA levels of both FSHR and Growth/differentiation factor 9, suggesting the promotion of growth arrest in developing follicles. These GBH-exposed ewe lambs also exhibited an increased incidence of multi-oocyte follicles (Alarcón et al., 2019), an end-point demonstrated to be a sensitive endocrine-disrupting end-point common in other species (such as rats, mice, and caimans) and with different EDCs such as bisphenol A, genistein, diethylstilbestrol, and ethinylestradiol (Alarcón et al., 2019; Jefferson et al., 2006; Rivera et al., 2011; Rodriguez et al., 2010; Stoker et al., 2008).

The developmental origins of health and disease (DOHaD) paradigm posits that, during development, there are sensitive windows in which tissue formation and function can be modified by environmental stressors (such as glyphosate or GBHs), which can lead to increased susceptibility to adverse health outcomes across the life course (Luque et al., 2018). All the *in vivo* studies performed in animal models previously commented regarding the histophysiology of the ovary and follicles suggest that, when the exposure to GBHs or glyphosate occurs during development, these compounds may induce endocrine-disrupting effects observed long after the EDC exposure has ended.

4.3. *Effects on pregnancy outcome and genital tracts of rats, mice and ewe lambs*

Estrogens and xenoestrogens probably exert most or all of their effects through a specific receptor and the effects depend on the dose, time, and probably the duration of exposure; such receptors are present in the brain, pituitary, gonads, and accessory sex organs at one or another time during fetal, prepubertal, or adult life (Toppari et al., 1996).

Even though most of the endocrine disruptive effects of glyphosate and GBHs have been demonstrated in males (Dallegrave et al., 2007; Pham et al., 2019; Romano et al. 2010, 2012), some recent studies have also shown adverse effects on development and functionality of the female reproductive tract. In the uterus of ewe lambs, we have recently found decreased cell proliferation but no alterations in the histomorphology after early postnatal oral or subcutaneous exposure to GBH (Alarcón et al., 2019). In addition, in subcutaneously GBH-exposed ewe lambs, a decreased uterine cell proliferation in association with an increased expression of the insulin-like growth factor binding protein 3 (IGFBP-3) gene and p27 protein and a deregulated expression of ERα and PR was found (Alarcón et al., 2020). Furthermore, GBH exposure decreased the expression of Wnt5a and forkhead box protein A2 (Foxa2) in glandular epithelium, Wnt7a and homeobox a10 (Hoxa10) in subepithelial stroma, and β-catenin in luminal and glandular epithelia (Alarcón et al., 2020). The wingless-type MMTV integration site family members (Wnt) are a group of signal transduction pathways of morphogenetic proteins that participate in female reproductive physiology and control essential developmental processes, such as embryonic patterning, cell growth, migration, and differentiation (Hayashi et al., 2011). In the endometrium, estrogen induces Wnt signaling in both an ER-dependent and an ER-independent manner (Susheelamma et al., 2018). Diethylstilbestrol, a synthetic estrogen, in the mouse endometrium represses Wnt7a expression, causing a range of uterine defects similar to those seen in Wnt7a knockout mice (Fan et al., 2012; Miller et al., 1998). Estrogens also up-regulate both ER and progesterone receptor (PR) gene expression in the uteri of several species (Ing and Tornesi, 1997).

In adult ovariectomized rats, Varayoud et al. (2017) found that neonatal exposure to a low dose of GBH (0.5 mg GBH/kg/day) did not affect the uterine weight or epithelial proliferation but led to an increase in the luminal epithelial cell height. This suggests that GBH modulates the expression of estrogen-sensitive genes and that the uterine gene expression of ERα, ERβ and PR is deregulated (Varayoud et al., 2017). Guerrero Schimpf et al. (2017) found that neonatal exposure of female rats to GBH (2 mg/kg/day) increased cell proliferation in the uterine

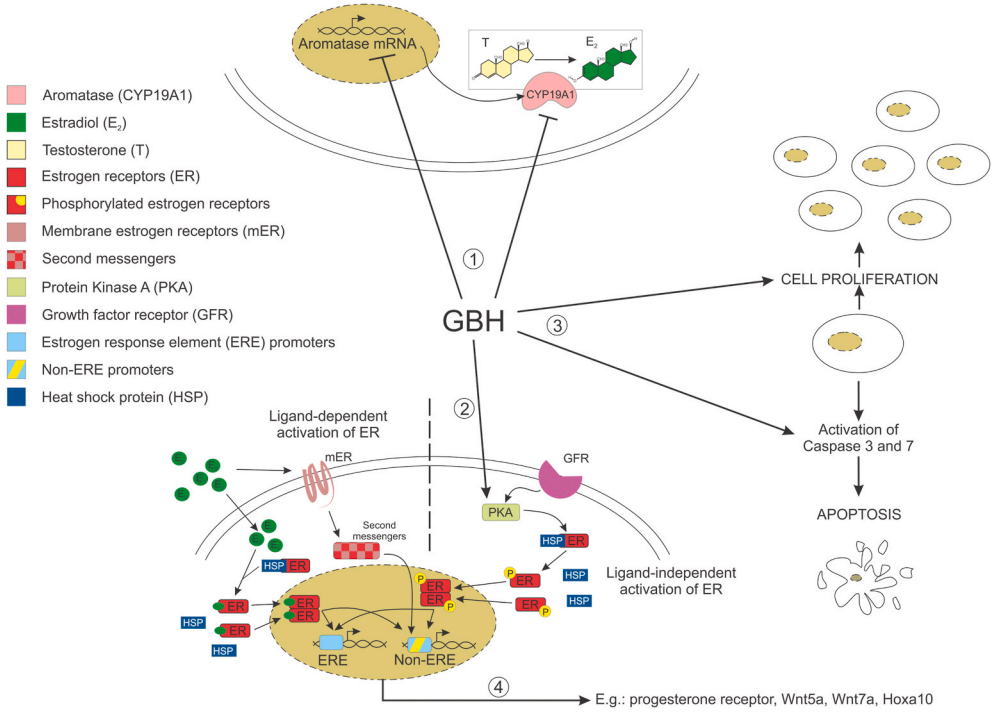

Fig. 1. Schematic representation of the reported effects of the *in vitro* and *in vivo* exposure to glyphosate or glyphosate-based herbicides (GBHs). 1) The exposure to glyphosate or GBHs inhibits the transcription of the aromatase gene and the enzymatic activity of aromatase (Cassault-Meyer et al., 2014; Gasnier et al., 2009; Richard et al., 2005). 2) The exposure to glyphosate or GBHs induces estrogen-like effects by activating estrogen receptor (ER) through a ligand-independent manner by activating protein kinase A (PKA), which in turn induces ER phosphorylation, modulating its transcriptional activity by binding to estrogen response elements (ERE) or non-ERE promoter sequences of target genes (Mesnage et al., 2017). 3) Depending on the cell type or glyphosate nature (formulation) or concentration, the exposure to glyphosate or GBHs stimulates cell proliferation (Guerrero Schimpf et al., 2017; Mesnage et al., 2017; Thongprakaisang et al., 2013) or cell apoptosis by activating caspases 3 and/or 7 (Benachour and Seralini, 2009; Clair et al., 2012). 4) The exposure to glyphosate or GBHs induces the expression of estrogen-dependent proteins like progesterone receptor, Wnt7a or Hoxa10 (Guerrero Schimpf et al., 2017; Ingaramo et al., 2017; Mesnage et al., 2017).

Table 1
Studies performed *in vitro* and *in vivo* to investigate the effects of glyphosate and glyphosate-based herbicides (GBH) in females.

Species	Reported effect	Doses of glyphosate or GBHs	Way and Time of exposure	Authors
Cows	At 10 and 300 μg/mL of RU: decreased number of GCs and the production of E2 and P4. At 1 μg/mL of RU + FSH + IGF1: increased cell number and steroid hormone. At 10 μg/mL of RU + FSH: decreased cell number and steroid hormone production depending on IGF1 addition. At 10 μg/mL of RU + FSH: increased E2 production.	1, 10 and 300 μg/mL	*In vitro*; 48 h	Perego et al. (2017a)
	At 5 μg/mL of GLY + FSH + IGF1: decreased GC number and E2 production. At 0.5 μg/mL of GLY + FSH + IGF1: only decreased GC number. At 1.7 μg/mL of GLY: increased GC proliferation.	0.5, 1.7 and 5 μg/mL	*In vitro*; 48 h	Perego et al. (2017b)
	At 10 ng/mL of GLY: increased E2 secretion from GCs. At 10 ng/mL of GLY or RU: decreased P4 secretion from LCs. RU but not GLY: increased mRNA expression and OT synthesis in LCs. GLY and RU: increased OT secretion from LCs. GLY decreased PGF2α, whereas RU decreased PGF2α and PGE2 secretion from endometrial cells.	0.11 and 10 ng/mL	*In vitro*; 24, 48 or 72 h	Wrobel (2018)
Estuarine crab (*Neohelice granulata*)	Lower muscle glycogen content and increased glycemia. Decreased ovarian vitellogenin content. Higher proportion of reabsorbed vitellogenic oocytes.	0.01 and 0.2 mg/L	Adults; 90 days through tank water	(Canosa et al., 2018)
Zebrafish (*Danio rerio*)	Increased diameter of previtellogenic I and vitellogenic oocytes. Higher expression of steroidogenic factor-1 in ovaries.	65 μg/L	Adults; 15 days through tank water	Armiliato et al. (2014)
	Decreased egg production and gonadosomatic index. Delayed embryo development and hatching. Decreased Cyp19a1 and Esr1 expression in the ovary.	0.01, 0.5, and 10 mg/L	Adults; 21 days through tank water	(Uren Webster et al., 2014)
Mice (ICR)	Decreased body weight gain and ovary and liver weight. Increased atretic follicles and interstitial ovarian fibrosis and decreased mature follicles. Alteration of the hypothalamus-pituitary-ovarian axis and disrupted E2 and P4 hormone secretion. Increased oxidative stress. Altered sex ratio of fetuses.	0.5% w/v of GLY or RU	Adults; From GD1 to GD19 through drinking water	Ren et al. (2018)
Rats (Wistar)	Increased number of resorption sites. Decreased expression of uterine estrogen and progesterone receptors. Downregulation of COUP-TFII and Bmp2 mRNA and increased expression of HOXA10 and proliferation in implantation sites of adult rats.	2 mg/kg/day	Neonatal period; PND1, 3, 5 and 7 through s.c. injection	Ingaramo et al. (2016)
	Increased incidence of luminal epithelial hyperplasia and of the stromal and myometrial thickness. Deregulation of the uterine expression of ERα, PR and Hoxa10 in prepubertal rats.	2 mg/kg/day	Neonatal period; PND1, 3, 5 and 7 through s.c. injection	Guerrero Schimpf et al. (2017)
	Increased uterine LEH. Deregulation of uterine protein expression of ERα, ERβ and PR. Decreased mRNA expression of C3, ERα and PR.	0.5, 5, or 50 mg/kg/day	Adults; 3 days through s.c. injection	Varayoud et al. (2017)
	Altered uterine expression of Wnt5a and β-catenin at PND8 and 21. Uterine deregulation of Wnt5a, Wnt7a, β-catenin, Dkk1 and sFRP4.	2 mg/kg/day	Neonatal period; PND1, 3, 5 and 7 through s.c. injection	Ingaramo et al. (2017)
	Increased sensitivity of the uterus to estradiol treatment. Higher LEH and stromal cell density. Uterine hyperplasia and abnormal endometrial glands.	2 mg/kg/day	Neonatal period; PND1, 3, 5 and 7 through s.c. injection	Guerrero Schimpf et al. (2018)

(*continued on next page*)

Table 1 (*continued*)

Species	Reported effect	Doses of glyphosate or GBHs	Way and Time of exposure	Authors
	Increased uterine cell proliferation and deregulated expression of ERα, ERβ, Wnt7a and β-catenin mRNA and protein expression.			
	Decreased body and ovary weights. Decreased surface area of secondary and tertiary follicles. Increased percentage of atretic follicles. Increased malondialdehyde and advanced oxidation protein products. Decreased catalase, superoxide dismutase and glutathione peroxidase.	126 mg/kg or 315 mg/kg	Adults; 60 days through oral administration	Hamdaoui et al. (2018)
Rats (Wistar)	F1 females (*in utero* exposed) showed lower number of implantation sites. F2 (F1 offspring) showed delayed growth in association with lower fetal weight and length and higher placental weight. F2 showed structural congenital anomalies.	3.7 and 352 mg/kg/day	Adults; from GD9 to LD21 through pellet chow	Milesi et al. (2018)
	ERα increased uterine expression. Epigenetic changes in the O-promoter of uterine ERα.	3.7 and 352 mg/kg/day	Adults; from GD9 to LD21 through pellet chow	Lorenz et al. (2019)
Ewe lambs (Friesian)	Decreased primordial follicles and increased transitional and primary follicles. Increased small antral atretic follicles and MOFs incidence. Increased proliferation of granulosa and theca cells and decreased FSHR and GDF9 mRNA expression in the ovary. Decreased cell proliferation in the uterus.	2 mg/kg/day	Neonatal period; from PND1 to 14 through oral administration or sc injection	Alarcon et al. (2019)

GCs: granulosa cells; E2: estradiol; P4: progesterone; FSH: follicle-stimulating hormone; IGF1: insulin-like growth factor; RU: GBH Roundup; GLY: glyphosate; OT: oxytocin; LCs: luteal cells; PGF2α: prostaglandin F 2α; PGE2: prostaglandin E2; Cyp19a1: aromatase gene; Esr1: estrogen receptor α gene; COUP-TFII: chicken ovoalbumin upstream promoter transcription factor 2; Bmp2: bone marrow protein 2; Hoxa10: homeobox a10; ERα: estrogen receptor α; PR: progesterone receptor; C3: complement component 3; Wnt5a: wingless-type MMTV integration site family member 5a; Wnt7a: wingless-type MMTV integration site family member 7a; Dkk1: dickkopf-related protein 1; sFRP4: secreted frizzled-related protein 4; LEH: luminal epithelial height; PND: postnatal day; GD: gestational day; LD: lactational day; MOF: Multi-oocyte follicles.

luminal and stromal compartments on PND8 and altered the expression of proteins involved in the differentiation of uterine organogenesis. ERα was induced in the uterine stromal compartment on PND8 and downregulated in the luminal epithelial compartment on PND21 (Guerrero Schimpf et al., 2017). In a further study, these authors found that early postnatal exposure of female rats to GBH led to an enhanced sensitivity of the adult uterus to an exogenous treatment with estrogen and to histomorphological and molecular changes associated with uterine hyperplasia and a deregulation of uterine genes like Wnt7a, ERα, PR and Hoxa10 (Guerrero Schimpf et al., 2018). Moreover, rats exposed to the same doses of GBH (i.e. 2 mg/kg/day) during the neonatal period showed fertility failures (Ingaramo et al. 2016, 2017). The main long-term alterations observed were associated with low PR expression associated with the downregulated expression of COUP transcription factor 2 (COUP-TFII, also known as Nr2f2) and bone morphogenetic protein 2 (Bmp2) mRNA and the increase in Hoxa10 and the cell proliferation at the implantation site (Ingaramo et al., 2016).

In mice, the administration of estrogen up-regulates uterine Wnt5a mRNA expression (Hou et al., 2004). However, exposure of mice to estrogen or ER agonists during critical developmental periods inhibits endometrial adenogenesis associated with reduction or suppression of Wnt proteins, like Wnt5a and Wnt7a (Dunlap et al., 2011; Hayashi et al., 2011; Yin and Ma, 2005). Interestingly, the temporal and spatial expression of Wnt genes also plays a critical role during implantation and decidualization in mice. Particularly, the dysregulation of Wnt5a has been associated with increasing numbers of resorption sites during gestation (Cha et al., 2014; Hayashi et al., 2009). We have found that the neonatal exposure of prepubertal rats to GBH increased the expression of both Wnt5a and Wnt7a (Ingaramo et al., 2017). In the uterus of pregnant adult rats neonatally exposed to GBH, we found a decrease in Wnt5a, a result suggesting that this could be the mechanism involved in the increased incidence of fetal resorptions (Ingaramo et al., 2017). Almeida et al. (2017) exposed rats from gestational day (GD) 1 to GD7 to 500 mg/kg of Roundup® and showed decreased implantation sites and increased pre-implantation losses in association with more oxidative stress and altered uterine histology (decreased luminal and glandular epithelial heights and number of endometrial glands).

Dechartres et al. (2019) observed that the treatment of rats with glyphosate and Roundup® did not alter litter characteristics such as length, weight, and sex ratio, whereas Ren et al. (2018) demonstrated that prenatal exposure of mice to pure glyphosate influenced the sex ratio of litters. Milesi et al. (2018) exposed pregnant rats (F0) to GBH (200 mg/kg/day) along pregnancy and found that this exposure impaired the reproductive performance of F1 females and induced structural congenital anomalies (conjoined fetuses and abnormally developed limbs) in the F2 offspring.

While most studies related to pesticides have addressed the effects of each individual chemical, it is very important to provide information about the effects of mixtures of pesticides because mixtures represent more realistic scenarios to mimic the environmental exposure. Recently, we reported that co-administration of a GBH with a commercial formulation of endosulfan (Thionex®) causes acute uterine effects and long–term deleterious reproductive effects that are similar to those induced by the GBH alone (Ingaramo et al., 2019). Generally, when the exposure levels of the chemicals within a mixture are in the range of the no-observed-adverse-effects levels (NOAELs) or below, and the components of the mixture have different modes of toxic action, no additive or potentiating interactions were observed (Ingaramo et al., 2019).

Table 1 summarizes the adverse effects so far described of glyphosate or GBH exposure on the female reproductive tract, whereas Fig. 1 summarizes all the *in vitro* and *in vivo* cellular mechanisms of action of glyphosate described so far.

Table 2
Similarities and divergences between the effects of pure glyphosate and those of glyphosate-based herbicides (GBHs)*.

Study	Glyphosate	GBHs
In vitro	Reduction in cell viability at concentrations of 10 and 50 μg/mL. No effects on the number of granulosa cells or estrogen and progesterone production.	Reduction in cell viability at concentrations of 10 and 50 μg/mL. Roundup® decreases the number of granulosa cells and estrogen and progesterone production.
	No decreased cell viability at 500 μg/mL; initial toxicity at 1000 μg/mL. Glyphosate is more genotoxic than Wipeout® formulation and has a genotoxic effect similar to that of Roundup® in the HEC1A cell line. In human ovarian and prostate cancer cells, it inhibits proliferation and promotes apoptosis.	No decreased cell viability at 500 μg/mL; initial toxicity in cell viability at 800 μg/mL. Roundup® has a genotoxicity similar to that of glyphosate, and Wipeout® formulation is less genotoxic than glyphosate. In human blood mononuclear cells, the cytotoxicity of Roundup® is 30 times higher than that of glyphosate alone.
	It increases oxytocin and decreases progesterone secretion from luteal cells. Estrogenic effects via ERα at higher doses compared to estrogen. The estrogenic effects may be ligand-independent.	Increased oxytocin and decreased progesterone secretion from luteal cells. GBH did not show ERα-activity at a concentration equivalent to that of glyphosate.
In vivo	Mice exposed *in utero* to glyphosate show decreased ovarian weight and histopathological alterations, increased atretic follicles and interstitial fibrosis, and decreased mature follicles on gestational day 19. Glyphosate has low effects on microbiome disruption. In mice, prenatal exposure influenced the sex ratio of litters. In rats, the exposure did not alter litter characteristics such as length, weight, and sex ratio.	In female rats, GBH exposure during the first days of pregnancy induces lower weight ovaries and decreases the number of corpora lutea. GBHs contribute to microbiome disruption. In rats, the exposure did not alter litter characteristics such as length, weight, and sex ratio.

* Data were obtained from Almeida et al., (2017); Davoren and Schiestl (2018); De Almeida et al., (2018); Dechartres et al., (2019); Gasnier et al., (2009); Gigante et al., (2018); Li et al., (2013); Martinez et al., (2007); Mesnage et al., (2017); Perego et al., (2017a) & b; Ren et al., (2018); Santovito et al., (2018); and Wrobel (2018).

5. Evidences that support the hypothesis that glyphosate and GBHs are endocrine disruptors

The safety of glyphosate and GBHs has been intensely debated. The "low-dose effects" of estrogenic agonists in mammals have generated considerable concern (Luque et al., 2018). The term "low-dose effects" is defined as the biological changes that occur at environmentally relevant exposure levels or at doses that are lower than those reported in the standard toxicity testing guidelines of the EPA. Moreover, the estrogenicity of the dose effects of estrogenic agonists varies significantly among species, as well as between *in vivo* and *in vitro* tests. In the regulatory field regarding chemicals with endocrine-disrupting properties in humans and wildlife, the WHO defines them as those that may have a) adverse effects, b) endocrine activity, and c) plausible mechanistic links between the observed endocrine activity and adverse effects (Serra et al., 2019).

The first evidences that suggested that glyphosate may be an endocrine-disrupting chemical came from studies in male reproduction (Anifandis et al., 2018; Dai et al., 2016; Johansson et al., 2018; Pham et al., 2019; Romano et al. 2010, 2012; Vanlaeys et al., 2018). In male mice, Dai et al. (2016) and Pham et al. (2019) found that glyphosate and GBHs were able to cause endocrine-disrupting effects on male reproduction at low doses. In many of these studies, the endocrine-disrupting effects observed could be attributed to glyphosate itself and/or to the additives in the formulations (Benachour and Seralini, 2009; Martini et al., 2016; Richard et al., 2005; Vanlaeys et al., 2018). The different endocrine-disrupting effects caused by different formulations with glyphosate suggest that the effects could be exerted by other constituents of the formulation besides the active principle (several of which are unknown) (Defarge et al., 2016; Jacques et al., 2019). Defarge et al. (2016) tested the endocrine-disrupting effects of the co-formulants in six GBHs and found inhibition of aromatase at levels lower than the lowest agricultural dilution recommended. These results may suggest that the action of glyphosate on aromatase could explain, at least in part, some of the effects on reproduction found in *in vivo* experiments (Richard et al., 2005).

Both *in vitro* and *in vivo* studies have suggested that glyphosate and GBHs act as xenoestrogens through ERE activation (Lorenz et al., 2019; Thongprakaisang et al., 2013). In rats, perinatal exposure to GBH induce decreased DNA methylation in the uterine site of the ERα gene, that might participate in the transcriptional upregulated expression of total ERα mRNA (Lorenz et al., 2019). As mentioned previously, glyphosate showed estrogenic activity and interferes with estrogen signaling, probably through the activation of an estrogen response element (ERE) since these responses can be blocked by the ER antagonist ICI 182780 (Thongprakaisang et al., 2013). Exposure to a GBH causes long-term epigenetic disruption of the uterine ERα gene, which could be associated with the GBH-induced implantation failures.

In vitro studies showing higher toxicity of GBHs *versus* glyphosate may explain the numerous *in vivo* results with GBHs not seen with glyphosate alone (Defarge et al., 2016; Johansson et al., 2018; Vandenberg et al., 2017). It is worth mentioning that glyphosate alone and commercial formulations might affect different endpoints or have effects at different ages (Manservisi et al., 2019; Pham et al., 2019; Ren et al., 2018) (Table 2). The latter might also contribute to the controversy on the toxicity of glyphosate *versus* GBHs; further *in vivo* studies must be conducted to evaluate the effects of glyphosate alone, GBHs and different adjuvants. The emerging field of omics, which refers to the large-scale data-rich biological measurement of the genome, might contribute to identifying individual risk and susceptibility of disease through targeted biomarkers that reconstruct past exposure and predict future risk (Messerlian et al., 2017). These new technologies will advance our understanding on the impact of agrochemicals as endocrine disruptors on animal and human health.

Fig. 2 Summarizes the *in vivo* studies so far performed in rats and ewe lambs, following pre- and/or postnatal exposure to GBHs.

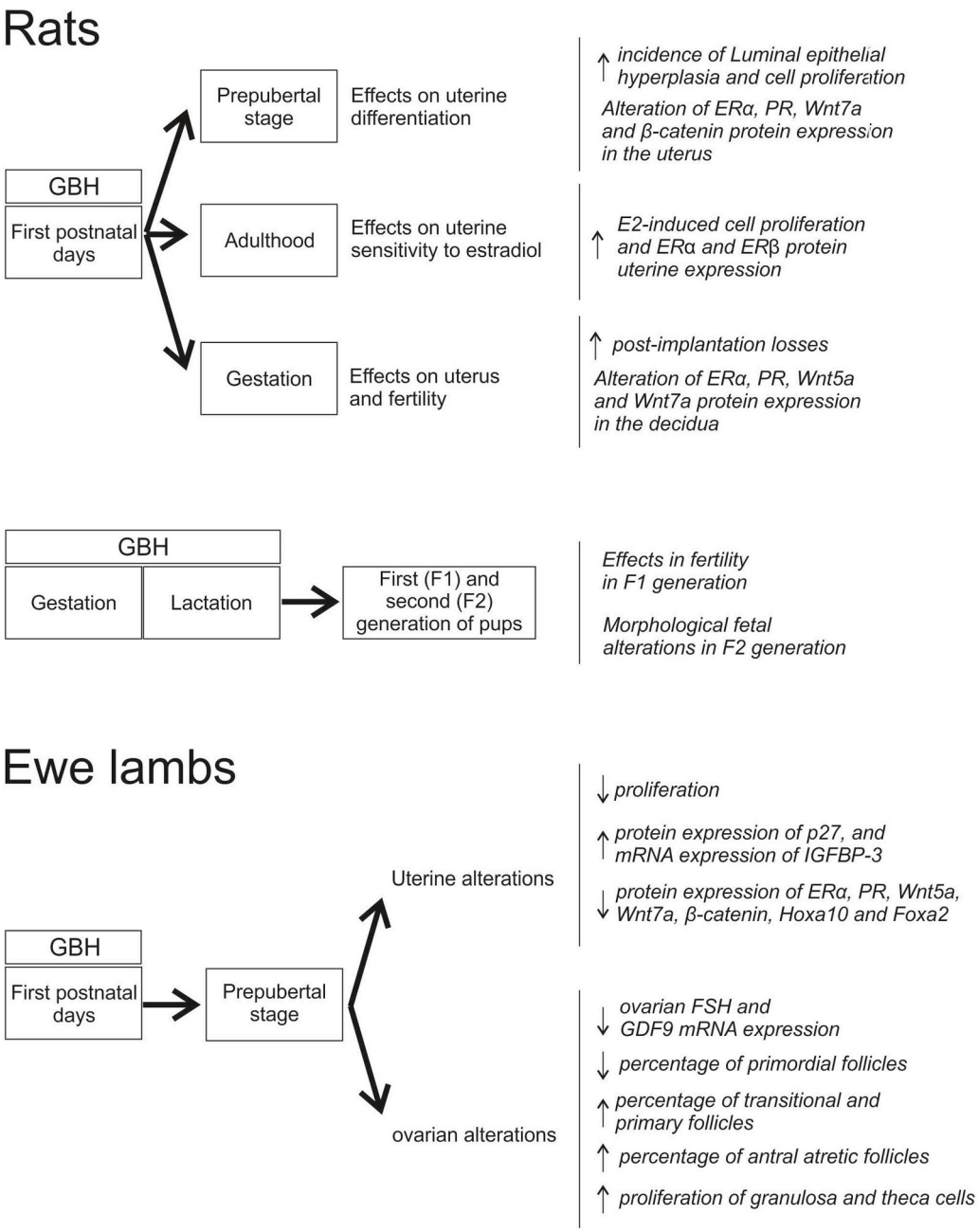

Fig. 2. Summary of *in vivo* studies performed after neonatal (pre- and/or postnatal) exposure of female rats and ewe lambs to glyphosate-based herbicides (GBHs). The arrows begin at the time of exposure and the arrowhead points to the moment when animals were studied. The most relevant experimental results obtained are mentioned in italics. GBH: Glyphosate-based herbicide; IGF1: insulin-like growth factor; ERα: estrogen receptor α; ERβ: estrogen receptor β; Hoxa10: homeobox a10; PR: progesterone receptor; Wnt5a wingless-type MMTV integration site family member 5a; Wnt7a: wingless-type MMTV integration site family member 7a; Insulin-like growth factor binding protein-3; Foxa 2: forkhead box protein A2; GDF9: Growth/differentiation factor 9. References: (Alarcón et al., 2019a; Alarcón et al., 2020; Guerrero Schimpf et al. 2017, 2018; Ingaramo et al. 2016, 2017, 2019; Lorenz et al., 2019; Milesi et al. 2018, 2019; Varayoud et al., 2017).

6. Conclusions

In vitro studies are useful to describe possible mechanisms of action of glyphosate but the implications of the *in vitro* effects on *in vivo* outcomes are difficult to be analyzed. *In vitro* assays exhibit known limitations to simulate *in vivo* metabolism, predict effects in different tissues and across different life stages or predict the influence of chemical properties affecting bioavailability (Ginsberg et al., 2019). The gold standard of safety or risk evaluation for pesticides and environmental contaminants is whole animal toxicity testing. Predicting the possible adverse effects of EDCs on human health is usually difficult due to the wide differences between species in the regulation of endocrine functions and their effects on biological processes (Viguie et al., 2020). However, due to the similarity between sheep and humans, especially regarding gestational and thyroid physiologies and brain ontogeny, sheep constitute a highly appropriate model to evaluate the effects of EDCs (Viguie et al., 2020). Since sheep are grazing animals, they are also useful models to assess the consequences of chronic environmental exposure to "real-life" glyphosate mixtures at different stages of the reproductive life cycle (Viguie et al., 2020). Based on these advantages, at our lab, we are conducting experiments in ewe lambs to test the adverse effects of glyphosate and GBHs and their possible endocrine-disrupting activity, with focus on female reproduction and fertility.

If used safely, GBHs could be extremely useful for the agricultural industry. However, there are evidences that the misuse of these compounds has led to adverse consequences, including the contamination of soils and rivers and the accumulation of residues in the food chain. This contamination has led non-target species, including humans, to be exposed to these compounds. Currently, the use of glyphosate as GBHs is increasing worldwide and the environmental contamination levels show high concentrations of these compounds, with levels significantly higher in countries or areas where agriculture is more intense. As described before, it has been demonstrated that animals (like rats, ewe lambs,

cows) neonatally exposed to glyphosate and/or GBHs show altered ovarian and uterus development. These effects in turn alter tissue morphology and functioning, suggesting adverse effects on future fertility. Increasing trends in fertility failures in different species, including humans, could be related, in many cases, to the effects of EDCs present in the environment. Due to the high abortion rates observed in women living in agricultural and rural areas, the use of pesticides and their potential effects create strong concern. The advances in research about the effects of glyphosate are of absolute interest to the world population due to the massive use of this compound worldwide. *In vitro* studies in mammalian cells describing the mechanisms of glyphosate have shown that glyphosate and GBHs interact with ERα, affecting signaling pathways involved in the control of cell proliferation (Fig. 1). Studies have also shown that glyphosate and GBHs affect the normal expression of aromatase. It is important to point out that most of the studies focused on the endocrine-disrupting effects of glyphosate in endocrine-dependent tissues (ovary and uterus) presented in this review suggest mechanisms at very low concentrations, which would be undetected in traditional toxicology studies. It is clear that several studies have shown that glyphosate is able to cause endocrine disruption alone or in its formulations depending on the levels and time of exposure. In the environment, however, we are exposed not only to pure glyphosate but also to different formulations and pesticide mixes. Since most of the formulations are different regarding their adjuvants, it is difficult to assert which ones are more dangerous than glyphosate alone. Moreover, some formulations are patent protected, so their components are unknown. Thus, it would be important to reduce the danger through the use of more harmless adjuvants. In addition, beyond the fact that adjuvants may have effects, the adverse health effects reported by the exposure of glyphosate in its pure form or in combination should be taken in consideration by regulatory agencies to establish better criteria on the safe use of this substance and to tailor health prevention strategies.

Finally, according with the WHO definition and based on the results commented in the present review, glyphosate and GBHs may have the properties to be EDCs. They cause adverse effects on the ovary and the female reproductive tract, impairing embryo implantation and/or development, even when animals are exposed to low doses. In addition, *in vitro* and *in vivo* results have demonstrated that glyphosate and GBHs inhibit aromatase activity and stimulate estrogenic pathways. All these features allow postulating that there is a link between the endocrine activities of glyphosate/GBHs and the adverse effects on female reproduction. Having in mind that much research is still needed to know the real toxicity effects of glyphosate and GBHs in humans and domestic animals, the “precaution principle” should be taken in consideration.

Competing financial interests

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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