Review

Glyphosate and the key characteristics of an endocrine disruptor: A review

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HIGHLIGHTS

- Glyphosate is the active component of the most commonly used herbicide in the world.
- There is conflicting evidence regarding the effects of glyphosate in the endocrine system.
- This is the first review that consolidates the mechanistic evidence on glyphosate as endocrine-disrupting chemical (EDC).
- Glyphosate satisfies at least 8 key characteristics of an EDC.
- Prospective cohort studies are needed in order to elucidate whether glyphosate is an EDC.

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ABSTRACT

Glyphosate is a large-spectrum herbicide that was introduced on the market in 1974. Due to its important impact on the crop industry, it has been significantly diversified and expanded being considered the most successful herbicide in history. Currently, its massive use has led to a wide environmental diffusion and its human consumption through food products has made possible to detect it in urine, serum, and breast milk samples. Nevertheless, recent studies have questioned its safety and international agencies have conflicting opinions about its effects on human health, mainly as an endocrine-disrupting chemical (EDC) and its carcinogenic capacity. Here, we conduct a comprehensive review where we describe the most important findings of the glyphosate effects in the endocrine system and asses the mechanistic evidence to classify it as an EDC. We use as guideline the ten key characteristics (KCs) of EDC proposed in the expert consensus statement published in 2020 (La Merrill et al., 2020) and discuss the scopes of some epidemiological studies for the evaluation of glyphosate as possible EDC. We conclude that glyphosate satisfies at least 8 KCs of an EDC, however, prospective cohort studies are still needed to elucidate the real effects in the human endocrine system.

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1. Introduction

Glyphosate, (*N*-phosphonometylglycine, CAS Number: 1071-83-6) is a herbicidal derivative of the amino acid glycine, it was first synthesized by Henri Martin in 1950 while working for a small swiss pharmaceutical company called Cilag. Twenty years later, in Monsanto company, the organic chemist John Franz discovered that glyphosate had a potent herbicide capacity (Myers et al., 2016). Consequently, this compound was registered in the United States Environmental Protection Agency (EPA) under brand name Roundup®, for control of nonselective weed (Franz et al., 1997; Malik et al., 1989). Two decades later, the introduction of glyphosate-resistant crops (GRCs), principally corn, soy, canola, and sugar beet, greatly increased its use in agriculture (Duke, 2018; Swanson et al., 2014). Therefore, becoming the major herbicide worldwide (Duke and Powles, 2008).

Currently, glyphosate is used as an active component in many formulations known as Glyphosate-Based Herbicides (GBHs) employed mainly for inhibiting the growth of around 100 species of weeds and 60 species of perennial weed plants in industrial and residential settings (Dill et al., 2010). Glyphosate is present in a variety of chemical forms, such as isopropylamine, diammonium, ammonium, dimethylammonium, and potassium salt, which provides solubility without affecting its properties as active ingredient. In addition, various adjuvants enhance the uptake and translocation of the active ingredient in plants and improve its herbicidal properties (Bradberry et al., 2004). These adjuvant compounds have been proposed to enhance the cytotoxic properties of glyphosate (Székács et al., 2014).

GBH are today used in 140 countries becoming one of the world's leading agrochemical (Soukup et al., 2020; Woodburn, 2000). Due to its large use in the most varied sectors of agriculture and urban environments, GBH has been widespread in the environment. In fact, since the late 1970's, the volume of GBH applied has increased around 100-fold and several reports claim that trace levels of glyphosate can be found widely in soil, foodstuffs, air, and water as well as in human serum, breast milk and urine (Demonte et al., 2018; IARC, 2017; Mercurio et al., 2014; Mörtl et al., 2013; Niemann et al., 2015; Philipp Schledorn, 2014; Simonetti et al., 2015; Steinborn et al., 2016; Yoshioka et al., 2011). Indeed, recent studies have detected glyphosate occurrence on beer and kids breakfast cereals, suggesting that exposure is not only occupational (Jansons et al., 2018).

Even though, agencies such as the European Food Safety Authority (EFSA), EPA and U.S. National Cancer Institute have declared no evidence of the potential interaction of glyphosate with endocrine pathways or carcinogenic effects (Andreotti et al., 2018; EFSA, 2017; U.S. EPA, 2015), their use has been either restricted or banned in a lot of countries. This decision has been made due to recent evidence that suggests that GBH possess certain characteristic as an endocrine disruptor and probable carcinogen (Guyton et al., 2015; IARC Working Group, 2015; Leon et al., 2019; Myers et al., 2016). Therefore, its classification as an endocrine disruptor and/or carcinogen compound is still unclear.

In this review, we summarize the main reports related to glyphosate as a possible endocrine disruptor, based on the ten key characteristics of EDCs recently proposed (La Merrill et al., 2020). Finally, we discuss the scopes of some epidemiological studies and their implications for the evaluation of glyphosate to classify as possible EDC.

2. Data collection method

For this review article, all publications up to 2020 were searched in MEDLINE (through PubMed), Web of Science, and SCOPUS. To identify studies addressing glyphosate as an endocrine disruptor; articles not written in English were excluded.

3. Chemical properties and mechanism of glyphosate

Chemically, glyphosate is a relatively simple molecule classified as an organophosphorus compound, specifically a phosphonic acid resulting from the formal oxidative coupling of the methyl group of methyl phosphonic acid with the amino group of glycine (Kim et al., 2019). It is an analog of the natural amino acid glycine with a basic amino group and a phosphate group strongly ionized, thus is a very polar and amphoteric molecule. Structurally, it lacks of chemical groups able to form a stable binding with DNA and according to Deductive Estimation of Risk from Existing Knowledge (DEREK), it does not present a risk of chromosomal damage or mutagenicity (Kier and Kirkland, 2013).

The glyphosate molecule can exist in different ionic states in aqueous solution depending on the pH, whose dissociation constants, pKa1, pKa2, pKa3 and pKa4 are 2.0, 2.6, 5.6, and 10.6, respectively (Stalikas and Konidari, 2001). In plants, studies with [^{14}C]glyphosate have shown a fast capacity to be absorbed following application through leaves and stem surfaces (Duke and Powles, 2007; Kirkwood et al., 2000), thus, it is translocated from

the leaf via the phloem to the same tissues that are metabolic rich in sucrose. Afterward, it concentrates on the meristem tissue (Franz et al., 1997).

According to EPA, the glyphosate molecule is relatively stable to chemical and photo decomposition (U.S. EPA, 1993). On heating, it decomposes producing toxic fumes that include nitrogen oxides and phosphorus oxides (IARC, 2017). In soil and water, the main route for their degradation is soil microbial action, where is metabolized by two major pathways, one of them by a glyphosate oxidoreductase that generates aminomethylphosphonic acid (AMPA) and glyoxylate (Duke, 2011). Another but minor pathway of degradation, is via conversion to glycine (only by Pseudomonas sp. Strain LBr) (Jacob et al., 1988). Furthermore, abiotic factors such as ultraviolet radiation, peroxide oxidation, and mineral oxidation constitute the third mode of glyphosate degradation in the environment (Duke, 2011). Thus, in soil, the half-life of glyphosate ranges between 2 and 197 days, where the soil type and climate conditions also determine their persistence. In water, the median half-life varies from a few to 91 days (Tomlin C, 2006). It has been described that glyphosate has a low vapor pressure $(5.7 \times 10^{-8}$ Pa at 25 °C), implying that the volatilization of soils is not an important form of dissipation (U.S. EPA, 1993).

The mechanism by which glyphosate kills plants and bacteria is through the binding and inhibition of the activity of the enzyme enolpiruvylshikimate-3-phosphate synthase (EPSPS). The EPSPS enzyme acts at the beginning of the shikimic acid pathway, essential for the synthesis of aromatic amino acids, hormones and many other important plant metabolites in algae, higher plants, bacteria, and fungi (Maeda and Dudareva, 2012). Given the absence of the shikimic acid pathway in animals, EPSP synthase is a suitable target for the development of antimicrobial agents against bacterial, parasitical, and fungal pathogens.

Glyphosate is recognized to have low toxicity in non-target species, including humans, since it is not metabolized and it is excreted mainly unchanged through the urine. (Williams et al., 2000). However, analysis of serum from glyphosate-poisoned patients and urine analysis of occupationally exposed workers, have been found trace levels of AMPA that could be hypothesized to come from the product of gut microbial metabolism (Conrad et al., 2017; Zhang et al., 2020). Furthermore, a recent research on mice, concluded that glyphosate can also be metabolized in high concentrations in liver cells, producing reactive metabolites, such as glyoxylate, that lead to severe metabolic defects (Ford et al., 2017). Thus, the current available data suggest that glyphosate metabolism in humans is minimal and may be driven primarily by intestinal bacteria and possibly by liver cells to produce AMPA.

3.1. Human exposure

Long-term use of glyphosate worldwide has led to rising the human exposure, mainly through contaminated food consumption (Myers et al., 2016). The presence of glyphosate in food is due to the high thermal stability of its molecule, which elicit their accumulation in crops and thus their easy transfer to plant-based foods (Narimani and Da Silva, 2020) (Gillezeau et al., 2019). Taking this into account, the Food and Agriculture Organization of the United Nations (FAO) in 2004, regulated the amount of glyphosate that can be consumed daily without an appreciable health risk, setting an acceptable daily intake (ADI) at 1 mg/kg of body weight (bw) (WHO/FAO, 2004). The Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 2016 reaffirmed this value for glyphosate and its metabolites concluding that was not necessary to establish an acute reference dose (ARfD) given the low glyphosate toxicity (FAO & WHO, 2016). Furthermore, the maximum residue limits (MRL) were stablished in the different kinds of foods, which ranged

between 0.025 and 2 mg/kg for the majority of vegetables, however for some grains and oils MRL is above 30 mg/kg (Agostini et al., 2020; FAO/WHO, 2016). On the other hand, the EFSA in 2015 recommend that the ADI and ARfD for glyphosate and its metabolites be 0.5 mg/kg bw/day, while the acceptable operator exposure level (AOEL) must be 0.1 mg/kg bw/day (EFSA, 2015).

In order to assess whether the population could be exposed to the acceptable glyphosate levels stated by FAO and EFSA, several researchers in the last decades have directed their efforts to determine the concentrations of glyphosate in various types of food. A study from Argentina carried out on soybean plants reported that glyphosate residues ranged from 1.9 to 4.4 mg/kg in leaves and stems, while in grains from 0.1 to 1.8 mg/kg, which are below the currently acceptable limits established by regulatory entities (Arregui et al., 2004). Most recent studies, recapitulated the detection of high levels of glyphosate residues in soy-based products. In Brazil for instance, glyphosate was detected with an arithmetic mean (MA) of 0.19 mg/kg, ranging from 0.03 mg/kg to 1.08 mg/kg and AMPA with an AM of 0.05 mg/kg in a range from 0.02 mg/kg to 0.17 mg/kg (Rodrigues and de Souza, 2018). In another study from Switzerland, cereals such as wheat and pulses were analyzed among others, with resulting values of 0.13 and 0.17 mg/kg respectively (Zoller et al., 2018). Honey samples from the USA also showed glyphosate detection, where 27% of the samples had values above the limit of quantification, with a mean of 118 ppb (Berg et al., 2018).

Although it is common to find studies reporting glyphosate detection in the literature, the values are mainly below acceptable limits and reveal almost no detection in milk, meat or fish, suggesting that the main routes of human exposure are plant foods rather than those of animal origin. However, despite the detection levels are below of the regulatory doses recommended by the FAO/EFSA, we cannot rule out that the use of strict vegetarian diets with contaminated food that may result in a potential risk to human health.

It is known that food is the main active source of human consumption of glyphosate and its detection in different spheres of the environment has generated concern about the possible risks of a reiterative human exposition (Myers et al., 2016). In fact, numerous environmental analysis suggest that glyphosate detection is highly frequent in ground and surface water with a median concentration ranging from 0.03 to 1.41 μg/L (Poiger et al., 2017; Rendón-Von Osten and Dzul-Caamal, 2017; Struger et al., 2015). Regarding atmospheric pollution, a recent study made with air samples taken from Provence-Alpes-Côte-d'Azur region, France, reported glyphosate levels with a 7% of detection frequency, ranging from 0% to 23% in the different locations analyzed and a maximum concentration of 1.04 ng/m^3 (Ravier et al., 2019). Another study from Brazil, reported high glyphosate levels in all air samples evaluated, with values between 0.002 and 0.144 μg/m^3 (mean of 0.055 μg/m^3) in rural zones, while in urban zones ranged from 0.009 to 2.576 μg/m^3 (mean of 1.006 μg/m^3) (Maria et al., 2019). Therefore, these recent reports from environmental sources demonstrate the presence of glyphosate in the biosphere that also contribute to human exposure along with food.

Urinary levels of glyphosate metabolites are markers generally used to assess the degree of both occupational and non-occupational exposure. In according with Williams et al. (2016) the prevalence rate and mean concentration of glyphosate in human urine increased notably between 1993 and 2016 from 0.00001 to 0.001 mg kg BW^{-1} d^{-1}. A revision of different recent studies based on collected samples from people non-ocupationally exposed, demonstrated high variation in the detection frequency and concentration of glyphosate. The highest frequencies were

reported in a recent study from Denmark that reported 100% detection of glyphosate and AMPA in a population of 13 mothers and 14 children, with a mean of 1.28 μg/L (range: 0.49−3.22) in mothers, whilst in Children a mean of 1.96 μg/L (range: 0.85−3.31) (Knudsen et al., 2017). In another study recently published, glyphosate was detected in urine samples in the 92.5% of the cases of healthy lactating women from USA (mean: 0.28 ± 0.38 μg/L, with AMPA following the same pattern. However, in the breast milk samples glyphosate was not detected (Mcguire et al., 2016). On the other hand, in 50 healthy adults from Ireland, glyphosate was detected in 10 cases (20%), with a median concentration of 0.87 μg/ L in a range from 0.80 to 1.35 μg/L (Connolly et al., 2018).

In summary, glyphosate is present in the environment and the general population is exposed to it through several pathways, mainly the consumption of plant-based food. Although the frequency of detection and concentrations found from non-occupationally exposed population shows high variability between studies, the current trend towards a high degree of exposure suggests that a review of the endocrine disrupting properties of glyphosate is needed.

4. Endocrine-disrupting chemicals

The endocrine disruptor chemicals (EDCs) (Damstra et al., 2002; Strauss and Williams, 2009) were reported for the first time in the 90′s, when a series of publications suggested that some chemical commonly used in pesticides, cosmetics, detergents, and even in toys, could have the capacity to disrupt the connection between hormones and their receptors (Lear et al., 1997). At present, the best-known EDCs include pharmaceuticals compounds, industrial solvents, plastics, and pesticides. Additionally, some natural compounds commonly consumed from vegetables, as phytoestrogens, can also act as EDCs (Kuiper et al., 1998).

Regarding glyphosate, several authors have tried over approximately 30 years to evaluate its role as EDC using *in vitro, in vivo,* and epidemiological approaches. However, despite the evidence shown below, there is not a consensus about the hazard implications in the human endocrine system.

Given the lack of a systematic method to integrate data to help to identify EDC hazards, recently it has been recognized ten functional properties of agents that alter hormone action (La Merrill et al., 2020). These are known as the "key characteristics (KCs) of EDCs", which provide a structure for searching and organizing the relevant literature on mechanistic information in support of an evaluation of an EDC. According to La Merrill et al. (2020), the KCs comprise heterogeneous features of EDCs related to their ability to interfere with regulatory process in the hormonal physiology. The first KC states that an EDC can *interact with or activate hormone receptors*, which include to those compounds that, either through a direct binding or mediated by a second messenger, can associate with and/or turn on the hormone receptors, leading to its inappropriate activity. Thus, substances that act like "hormone mimics" could be considered as an EDC by this mechanism (Lee et al., 2013). Nevertheless, it has been described EDCs that, through similar interaction mechanisms, lead to opposite effects (Tabb and Blumberg, 2006). In this case, they have been grouped into a second KC, named "*antagonization of hormone receptors*". Thereby, compounds that block the hormone effects by a receptor-mediated way can be considered to possess this KC of EDCs.

Other common feature of some EDCs is the capacity to disrupt the receptor content in endocrine cells (Lee et al., 2013), which is described in a third KC: *"EDCs can alters hormone receptor expression"*. Given that hormone receptor level as well as its localization are key to define the hormone activity, and any compound that

change these properties will produce severe defects in the hormonal physiology (Grimm et al., 2002). Hence, this characteristic involves those substances that can modulate the abundance of hormonal receptors through a transcription-mediated mechanism or by altering their cellular localization. On the other hand, EDCs can not only exert their action through hormone receptors, but can also affect their signaling (Lee et al., 2013), which has been grouped into a fourth KC that states: *"EDCs can alter signal transduction in hormone-responsive cells"*. Among the most important events altered by EDCs in this context are the interruption of interactions with co-regulatory factors such as activators and repressors, post-translational modifications, the activity of second messengers and enzymes. Thus, the change in any of these events can lead to a remodeling in signal transduction with a consequent activation or attenuation of molecular pathways in endocrine cells.

Epigenetic modifications also were included as a common feature of some EDCs. Thus, KC5 states: *"EDCs induce epigenetic modifications in hormone-producing or hormone-responsive cells"*. In according with Plunk and Richards (2020), EDCs can be exerts its effects in hormone-sensitive cells or producer cells by three epigenetic mechanisms, such as: chromatin modifications, DNA methylation, and expression of non-coding RNA. Thereby, substances that lead to these changes in endocrine cells could be considered EDC by this mechanism.

Another mechanism usually found in some EDCs is the ability *to alter hormone synthesis* (Lee et al., 2013). In fact, some pesticides have the property of causing hormonal imbalances by altering intracellular transport, changing vesicular dynamics or cell secretion. Thus substances that induce disruption in these processes satisfy the sixth KC and can be considered as EDC. Additionally to the hormone synthesis, some EDCs "*alter hormone transport across cell membranes"*. This seventh KC take into account some EDC that can disrupt the movement of hormones through the membrane altering the intracellular transport, vesicle dynamics or cellular secretion (Villar-Pazos et al., 2017).

Others EDCs have shown the property to *"alter hormone distribution or circulating levels of hormones"*, which have been grouped as an eighth KC. The hormone levels are finely regulated by synthesis and release process in the endocrine cells (Hiller-Sturmhöfel and Bartke, 1998). However, some EDC can induce change in its plasma levels through the change of blood protein levels or its binding capacity (Gore et al., 2015). These defects can induce *"alterations in hormone metabolism or clearance"* (KC9). Circulating hormones are removed from the blood by different mechanisms, such as metabolic processing, binding with tissues, and excretion. Therefore, compounds with properties to alter any of these processes are considered part of this KC and usually recognized as EDC (Gore et al., 2015).

Finally, the last KC established by Le Merril et al. (2020) are the phenotypic changes induced by some EDCs, thus KC10 states: *"EDCs can alter the fate of hormone-producing or hormone-responsive cells"*. In this case, disrupting or promoting differentiation, proliferation, migration or cell death during development and adulthood constitute the main evidence of this characteristic, which have been observed by some pesticides (Strong et al., 2015; Zhou et al., 2016).

Although these KCs are common features among some EDCs and are a well way to represent the categories for organizing mechanistic evidence, their use to classify a compound as an EDC should be associated and complemented with other evidences, including epidemiological data and experimental approaches (La Merrill et al., 2020).

5. Glyphosate: perspectives from the ten key characteristics of an EDC

5.1. It interacts with or activates hormone receptors

Since all hormones can bind to specific receptors, any interaction of environmental substances or xenobiotics that disrupt the activity of these receptors can lead to negative effects of the endocrine function (Diamanti-Kandarakis et al., 2009). Different mechanisms of interaction and alteration of hormonal receptors by EDCs have been described: some can mimic the interaction between endogenous hormones and cellular receptors stimulating their activity (i.e., receptor agonism), and others can lead to inhibition of the formation of receptor—hormone complexes (i.e., receptor antagonism) (Evans, 2011).

The majority of the receptors that are targeted by EDCs are nuclear hormone receptors (NHRs) (Combarnous Yves, 2019). These receptors are part of a family of ligand-regulated transcription factors, and are activated by steroid hormones, such as estrogen, progesterone, and various other lipid-soluble signals. Once are activated these can induce long-term effects in their target cells. NHR family include the androgen receptor (AR), glucocorticoid receptor (GR), progesterone receptor (PR), mineralcorticoid receptor (MR), estrogen receptor (ER)α, and ERβ (Sever and Glass, 2013). On the other hand, EDCs can also disturb hormone membrane receptors, eliciting signaling pathways and short-term acute responses (Diamanti-Kandarakis et al., 2009). One of the best-known EDC that interacts with hormone receptors is Bisphenol A (BPA), a compound found in many hard plastics and hygiene products, which has a high affinity for ERα eliciting its activity even at very low concentrations (Calaf et al., 2020; Liu et al., 2018).

Throughout the last 20 years, several reports have evaluated the capacity of glyphosate to interact with hormone receptors, mainly with ERα, ERβ and AR. Kojima et al. (2004), evaluated estrogenic and androgenic activities in 200 pesticides, using a reporter gene assay in Chinese hamster ovary cells (CHO cells) developed by the same group above (Kojima et al., 2003). The results for glyphosate showed neither agonist nor antagonist activity, in the range of concentrations from 10^{-8} to 10^{-5} M. Three years later, through a DNA microarray and confirmation by q-RT-PCR, it was demonstrated that MCF7 cells treated with glyphosate at 0.00023% induces an alteration of estrogen-regulated gene expression at 18 h (Hokanson et al., 2007). Therefore, opening the hypothesis that glyphosate could act as an EDC in human cells, through a molecular mechanism that would induce an inappropriate ER activation.

A subsequent analysis using gene reporter assays, showed that glyphosate does not affect the ERα neither ERβ, but disrupt AR transcriptional activity in a range of non-toxic concentration at 24 h of exposition (Gasnier et al., 2009). In this study, were also assessed four Roundup® formulations which induced an anti-estrogenic activity on ERα, ERβ and AR. (Gasnier et al., 2009). These results were later evaluated by Thongprakaisang et al. (2013), who through reporter assays, demonstrated that glyphosate at 1 mM induces estrogenic activity in a breast cancer cell line (T47D). Further, this effect was blocked by the ER antagonist ICI182780, suggesting the possibility that glyphosate behaves like a xenoestrogen. However, in the wide range of E2 concentration from 10^{-12} to 10^{-6} M, glyphosate behaved as an ER antagonist (Thongprakaisang et al., 2013). Finally, Mesnage et al. (2017), using the similar cell models and a robust set of additional experiments, demonstrated that glyphosate but no other components present in GBH, induces ERα activation at high concentrations (1×10^{3} μg/mL) in T47D cells under exposure for 24 h.

It is quite interesting to note that although glyphosate is a simple molecule with low molecular weight, it is capable of activate ERα. The molecular mechanism of this activation is still unknown, but different hypotheses can be approached from a biochemical perspective: Structurally, the ERα is composed of different functional domains with specific roles. The ligand-binding domain (LBD), is a hormone-binding pocket composed by 11 α-helices, that maintains a sizeable ligand-binding cavity at the narrower end of the domain. In addition, this LBD grants a high hydrophobic environment (Kumar et al., 2011). In this cavity, some glutamine and arginine residues are critical, because allow hydrogen bonds formation with hydroxyl groups at positions 7 and 13 of E2, promoting a proper targeting. Therefore, hydroxyl groups in ligands and hydrophobic interactions at this site seem key for binding and recognition (Brzozowski et al., 1997). Although the most effective ER-ligands possess a phenolic hydroxyl group, it has been described also a binding with not phenolic compounds. Additionally, molecules that contain hydroxyl groups separated by a rigid hydrophobic binding region are susceptible to interaction (Ascenzi et al., 2006). The dynamic of glyphosate-ER interactions assessed by Mesnage et al. (2017) predicts an unstable interaction (−4.10 kcal/mol), much lower than expected for estradiol (−34.88 kcal/mol) or Bisphenol A (−23.77 kcal/mol) suggesting that ER activation does not involve a direct interaction within LBD (Mesnage et al., 2017). Thus, it is possible that glyphosate can trigger signaling pathways upstream ER, such as MAPK or PI3K-mTOR, by an independent-ligand mechanism. Likewise, cellular processes such as apoptosis or proliferation, induced by glyphosate can activate ER pathways indirectly. However, these hypotheses have not been proved yet.

In summary, the current evidence indicates that glyphosate can favor hormonal receptor activity, particularly ERα by stimulating their transcriptional activation and therefore promoting phenotype changes in breast cell line models. Nevertheless, the molecular mechanism of interaction is unknown.

5.2. It antagonizes hormone receptors

Some pesticides, such as dichlorodiphenyltrichloroethane (DDT), inhibit hormonal activity by blocking the receptor, inhibiting their function and contributing to impaired hormonal feedback (Lemaire et al., 2004). Regarding glyphosate, there is no evidence associated with the antagonistic capacity of hormonal receptors.

5.3. It alters hormone receptor expression

Hormone receptor expression is important as well as the hormone itself, since its localization and abundance determine the magnitude of hormone activity and the cellular response. The disruption of hormone-receptor expression pattern is a typical feature of EDCs. Although, no all EDCs show this characteristic, it is broadly seen in the animal-models subjects (Diamanti-Kandarakis et al., 2009).

In this context, to evaluate the glyphosate effect, in vitro studies have been conducted in different cell line models. Thongprakaisang et al. (2013) determined the effect of glyphosate on ER expression levels in human breast cancer cell lines. Such results showed that glyphosate at a wide range of concentrations (1×10^{-7} to 1×10^{-12} M) altered the levels of ERα and ERβ proteins after 6 h of exposure in a concentration-dependent manner, while at 24 h of exposure only ERα showed a significant induction at the highest glyphosate concentration (Thongprakaisang et al., 2013). Another study, but in primary Leydig cells, reported no changes in AR either ER mRNA levels after 24 h of exposition of glyphosate at different percentage of dilutions (1×10^{-1} to 1×10^{-3}) (Clair et al., 2012).

Analysis in vivo has been an important tool for evaluating hormone receptor disruption caused by glyphosate exposure. In many of these it has been observed altered the ER levels, either in pre or

postnatal exposures (Ingaramo et al., 2016). In 2017 a study analyzed the effect of 2 mg/kg bw of GBH injected subcutaneously every 48 h, on the expression of proteins involved in uterine organogenic differentiation of neonatal rats. Results described induction of ERα in the subepithelial stroma on a postnatal day (PND) 8, and a down-regulation in the luminal epithelial cells of GBH-exposed animals on PND21 (Guerrero et al., 2017). On the other hand, it showed a notable increase in the progesterone receptor (PR) expression in both, the luminal epithelium and the stromal compartments (Guerrero et al., 2017). These results were similar in another study that found an increase in ERα expression at PND60 in GBH-exposed Wistar male rats under the same conditions (2 mg/kg, every 48 h) (Altamirano et al., 2018).

Afterward, the same group evaluated the effects of perinatally GBH exposition in female rats during the preimplantation period. The authors found that a dose of 350 mg of glyphosate/kg bw/day provided through fed, induces the increasing of ERα mRNA expression in the uterus, relative to control groups (Lorenz et al., 2019). In contrast, another similar analysis reported a decreased expression of ER and PR levels in the uterine glands of neonatal rats after GBH exposure (2 mg/kg/day of glyphosate) on PND 1, 3, 5 and 7 (Ingaramo et al., 2016).

Taken together, these results demonstrated an eventual role of glyphosate in ER expression disruption; however, further *in vitro* and *in vivo* studies using pure glyphosate are needed to provide more physiological relevant evidence.

5.4. It alters signal transduction in hormone-responsive cells

Many EDCs interplay with endocrine regulations through factors that mediate responses of a receptor (Combarnous and Diep, 2019). This leads to modifying the signaling pathway but without a direct interaction with the hormonal receptor.

Currently, there is little evidence on the implications of glyphosate in altering intracellular signaling pathways in hormone-responsive cells. The most important findings are on ER positive cholangiocarcinoma cells, whose acute exposition of glyphosate at low concentrations (1×10^{-7} to 1×10^{-11} M) induces ER/ERK1/2 signaling pathway and alters the expression levels of several proteins, such as ERK, cyclin D1 and cyclin A (Sritana et al., 2018). Similarly, in cancer breast cell lines, it was showed a deregulation of eleven canonical pathways after 48-h exposure with GBH at 1.1 mM glyphosate (0.05%), mainly in pathways related to cycle and DNA damage repair. Additionally, it also induced the expression of proliferative signaling-related proteins including ERα, VEGFR2, pERK, PI3K(p85), and PCNA (Stur et al., 2019).

On the other hand, in Sertoli cells from prepubertal rats, treatments at 0.036 g/L of glyphosate or GBH (36 ppm) for 30 min, were associated with a significant disruption of Ca^{2+} homeostasis and an activation of multiple stress-response pathways that led to Sertoli cell death disruption. Although, it is important to note that in this case the effects were study to explore the molecular mechanisms underlying acute glyphosate toxicity, the concentrations used (10 times more dilute than recommended for herbicide action) were highly toxic (De Liz Oliveira et al., 2013).

5.5. It induces epigenetic modifications in hormone producing or hormone-responsive cells

Epigenetic means genetic regulation by factors different from the DNA sequence of an organism (Samanta et al., 2017). Thus, epigenetic changes are characterized as "any long-term gene function change that persists even when the initial trigger is long gone and does not imply a change in the gene sequence or structure" (Alavian-Ghavanini and Rüegg, 2018). In other words,

epigenetics can switch genes on or off and determine which proteins are transcribed. Many types of epigenetic processes have been identified, they include methylation, acetylation, phosphorylation, ubiquitylation, and sumolyation (Weinhold, 2006).

A long time ago, a hypothesis emerged suggesting that some EDCs can induce epigenetic changes (Rakitsky et al., 2000). Currently, these effects have been well documented, where BPA and DES are some examples (Bhan et al., 2014). However, the exact mechanisms by which they interfere with epigenetic marks are not fully understood.

Evidence suggests that glyphosate could be associated with epigenetic modifications in hormone-producing cells. In the non-neoplastic breast epithelial MCF-10 A cells, it was found that treatments with low dose (10^{-11} M) every three to four days over 21 days, promoted a global DNA hypomethylation through ten-eleven translocations (TET) enzymes (Duforestel et al., 2019). Interestingly, the authors of this study also demonstrated that glyphosate treatment may predispose breast cells to tumorigenesis through epigenetic reprogramming. With the same purpose, another report (Lorenz et al., 2019) evaluated whether pregnant Wistar rats orally exposed to GBH (350 mg of glyphosate/kg bw/day) from GD9 until the end of weaning (on lactational day (LD) 21), imprint uterine epigenetic modifications during the preimplantation stage. The results showed a long-term epigenetic disruption in one of the five ERα promoters, (O promoter), specifically a marked decrease in DNA methylation, as well as an increase in histone H4 acetylation and histone H3 methylation. Consequently, all these epigenetic changes induced to an increase in ERα mRNA expression and possibly to implantation failures (Lorenz et al., 2019). In the same way, a different report evaluated the effects of developmental exposure to GBH (3,7 and 352 glyphosate mg/kg bw/day) on mammary gland growth and development in pre- and post-pubertal male Wistar rats. The results revealed hypermethylation of the CpG islands of ERα promoters, which was associated with lower ESR1 expression. The authors sustained that this epigenetic disturbance could be due to the molecular mechanism behind the altered mammary gland development observed after GBH exposure (Gomez et al., 2019).

The epigenetic changes induced by glyphosate observed in hormone-producing or hormone-responsive cells were reported not only by direct exposure but also through trans generational assays. In recent work, pregnant Sprague Dawley rats (F0 generation) transiently exposed to 25 mg/kg bw/day of glyphosate, during days 8–14 of gestation, producing negligible impacts on the directly exposed F0 or F1 generation offspring (Kubsad et al., 2019). In contrast, dramatic increases in pathologies in the F2 and F3 were observed. Additionally, sperm from F1, F2, and F3 were found to have differential DNA methylation regions (DMRs) in genes or promoters associated with transcription, signaling, metabolism, receptors, and cytokines (Kubsad et al., 2019). Therefore, according to this work, glyphosate appears to have a low risk for direct exposure but promotes generational epigenetic changes.

Noncoding RNAs (ncRNAs) play an important role in transcription regulation and are sometimes considered an epigenetic mechanism (Aristizabal et al., 2019). One class of noncoding RNAs are the microRNAs (miRNAs) which are short (~22 nucleotides in length), single-stranded, RNAs that post-transcriptionally control gene expression via either translational repression or mRNA degradation (Cai et al., 2009). A recent study assessed the effects of GBH treatment on the miRNA expression in prefrontal cortex from mouse offspring. In this study the animals were subjected to orally exposure to an equivalent of 50 mg of glyphosate/kg/day during pregnancy and lactation. The results indicated that 53 miRNAs were differentially expressed, of which 11 were involved in brain development and neurodevelopmental disorders. Therefore, the

authors hypothesized that de-regulated expression of miRNAs may be involved in the mechanism of glyphosate-induced neurotoxicity (Ji et al., 2018).

On the other hand, another group that subjected ICR mice to drinking water containing 0.38% glyphosate (1% Roundup®) from ED 14 to PND 7, it was found an aberrant expression of circular RNAs (circRNAs) in the hippocampus, suggesting its potential role in glyphosate-induced neurotoxicity (Yu et al., 2018). Although these studies are not directly implicated in hormone-producing or hormone-responsive cells it is not possible to assume that it is an endocrine disruptor demonstration. Therefore, the possibility that glyphosate can produce a similar effect through ncRNAs on the neuronal development is still open.

5.6. It alters hormone synthesis

Many molecules can exert an endocrine-disrupting effect, not only by directly interfering with hormone receptors but also by affecting the endogenous enzymes that catalyze hormone biosynthesis. Frequently, Such molecules are simple and different in their chemical structure from those of hormones since they do not compete with hormones at the receptor level (Combarnous Yves, 2019).

StAR protein plays a key role in the transfer of cholesterol into the mitochondrial membrane, which is needed for the initial stages of steroid synthesis in the adrenal glands and gonads (New et al., 2014). In 2000, a group of eight pesticides was evaluated in regard to their capacity for inducing alterations in steroid hormones biosynthesis. The results demonstrated that Roundup® treatment for 2 h at 25 μg/mL inhibited steroidogenesis by disrupting StAR protein expression in tumor Leydig cell line (mouse). In the study, it was also shown that Roundup® did not alter 3p-hydroxysteroid dehydrogenase (3 P-HSD) enzyme activity which converts pregnenolone to progesterone (Walsh et al., 2000). Surprisingly, it reduced cytochrome P450 side-chain cleavage (P450scc) activity, the enzyme that converts cholesterol to pregnenolone. Pure glyphosate did not alter steroid production at any dose tested (0−100 g/mL) indicating that at least another component of the formulation is required to disrupt steroidogenesis (Walsh et al., 2000). Subsequently, these results were confirmed through in vivo experiments in 2015, where a complex mechanism was reported in which the treatment of up to 50 mg/kg bw/day of Roundup® for 14 days reduced the levels of endogenous adrenocorticotropic hormone (ACTH) acting on the hypothalamic adrenal pituitary (HPA) axis. This effect led to a down regulation in cyclic adenosine monophosphate StAR phosphorylation dependent of (cAMP)/PKA pathway as well as a reduction in corticosterone synthesis in the adrenal tissue (Pandey and Rudraiah, 2015).

In summary, this evidence suggests that Roundup®, but not glyphosate pure, can alter the biosynthesis of the sexual hormones, all these processes mediating direct and indirect mechanisms through enzyme inhibition, altering the HPA axis respectively.

The cytochrome P450 (CYP) is a superfamily of monooxygenase enzymes highly conserved and have a pivotal role in the clearance of various compounds, including hormone synthesis, metabolism and breakdown (Manikandan and Nagini, 2017); in mammals, oxidize steroids, fatty acids, xenobiotics. A study conducted in 1998 demonstrated that glyphosate inhibited CYP enzymes in plants, particularly CYP71B1 (Lamb et al., 1998) through a mechanism of inhibition that involve binding the nitrogen group of glyphosate to the heme pocket in the enzyme, (IC50 of 12 μM). Later, similar results were obtained in wheat CYP71C6v1 (Xiang et al., 2005). Given these evidences in plant CYP and accumulative reports about glyphosate effect on estrogen signaling, it could be speculated that the herbicide may exert a direct action on CYP aromatase, the

enzyme responsible for estrogen synthesis. Interestingly, in 2005 a study observed that glyphosate acted as a disruptor of mammalian aromatase activity, by interacting with the active site of the purified enzyme in concentrations 100 times lower (0.036 g/L) than the recommended in agriculture (3.6 g/L). Additionally, the effects of glyphosate were facilitated by the Roundup® formulation (Richard et al., 2005). This report was the first evidence that demonstrated the direct effects of glyphosate as a molecule, upon the hormonal biosynthesis by inhibiting an enzyme. A few years later, the same group confirmed the aromatase disruption by Roundup from 0.01% (with 210 μM glyphosate) for 24 h, but now using human microsomes derived from placental cells and human embryonic kidney cells (293). Interestingly, the authors observed that aromatase inhibition was in a temperature-dependent manner (Benachour et al., 2007). Similarly, Gasnier et al. (2009), observed that various glyphosate formulations, including Roundup®, interrupt aromatase activity in human liver HepG2 cells from 10 ppm (nontoxic concentration). In primary Leydig cells exposed at the same concentration, significant increases in aromatase mRNA levels were observed after glyphosate treatment (Clair et al., 2012).

Currently, accumulative evidence in an animal model is claiming that glyphosate is associated with reproductive inefficiency, including embryo loss, uterus development and birth defects (Ren et al., 2018). Thus, these adverse effects occurring in the pregnancy period may have their basis in the dysfunction of progesterone or estrogens biosynthesis. In summary, glyphosate and Roundup® have adverse effects on steroid hormone production and the mechanism might be through affecting different proteins involved in the biosynthesis, among them, StAR, CYP aromatase and P450scc.

5.7. It alters hormone transport across cell membranes

Lipid-derived hormones, such as steroid hormones migrate through the phospholipid bilayer membranes of the endocrine cell. At the target cell, these hormones are released from the transport protein and diffuse across the lipid bilayer. Other types of hormones (such as amine, peptide, protein and thyroid hormones) are not lipid-derived, therefore they cannot diffuse through the plasma membrane and are released through vesicles from the endocrine cell. These hormones bind to specific receptors on the outer surface of the plasma membrane, resulting in activation of a signaling pathway in the target cell and any of these transport mechanisms can be altered by EDCs (Diamanti-Kandarakis et al., 2009).

Although, it does not exist direct evidence related to hormone transport disruption across cell membranes by glyphosate, a study determined whether GBH disrupt the hypothalamic-pituitary-thyroid (HPT) axis, revealing an indirect mechanism (de Souza et al., 2017). Basically, the authors exposed female pregnant Wistar rats to a solution containing Roundup® diluted in water in doses of 5 and 50 mg/kg bw/day from GD18 to PND5. Subsequently, male offspring were euthanized at PND 90, where blood and tissues samples from the hypothalamus, pituitary, liver, and heart were collected for hormonal evaluation and mRNA analyses of genes related to thyroid hormone (TH) function. Such results revealed a disruption in the HPT axis in vivo and a reduction in the expression of genes encoding thyroid hormones transporters, such as the Slc16a2 gene (that codifies to monocarboxylate transporter 8, mct8) and Slco1c1 (that codifies to organic anion transporter1 C1, Oatp1c1) in the hypothalamus. Although, no significant difference in TH, T3, and T4 levels was detected, the disordered expression of Slc16a2 may reduce TH uptake in hypothalamic cells, explaining at least in part, the disruption of HPT axis observed in these animals (de Souza et al., 2017). Even though, this article does not show a direct role of glyphosate for disrupting the hormone transport,

neither a mechanism at a molecular level, it establishes a correlation between GBH exposure and the decrease of hormone transporters, explaining the reduction of the normal functions in hormone-dependent cells leading to serious endocrine disorders.

Fig. 1 shows the effects of glyphosate and its derivatives on different hormone-producing or hormone-sensitive cells. In summary, glyphosate can favor hormonal receptor activity (1), particularly ERα, stimulating their transcriptional activation (Hokanson et al., 2007; Mesnage et al., 2017; Thongprakaisang et al., 2013); (2) disrupting the levels of ERα and ERβ proteins levels (Altamirano et al., 2018; Guerrero et al., 2017; Lorenz et al., 2019; Thongprakaisang et al., 2013); inducing ER/ERK1/2 signaling pathway in cholangiocarcinoma cells (Sritana et al., 2018), deregulating canonical pathways in cancer breast cell lines (Stur et al., 2019), disrupting Ca^{2+} homeostasis in Sertoli cells (De Liz Oliveira et al., 2013) (3); promoting a global DNA hypomethylation in normal breast cell lines (Duforestel et al., 2019) (4); exerting adverse effects on steroid hormone production, specifically, acting as a disruptor of mammalian CYP aromatase activity (Richard et al., 2005) and CYP P450 side-chain cleavage (Walsh et al., 2000) (5) and altering thyroid hormones transport across cell membranes through a reduction in the expression of hormones transporters, such as mct8 and Slco1c in hypothalamic (de Souza et al., 2017) (6).

5.8. It alters hormone distribution or circulating levels of hormones

Once outside the cell, some hormones as lipid-derived hormones bind to carrier proteins that keep them soluble in the bloodstream. However, peptide hormones, due to their high polarity can be soluble and freely transported in the serum. Some EDCs, such as Diethylstilbestrol (DES) or Bisphenol A (BPA) have demonstrated to modify hormone levels such as testosterone and Sex Hormone Binding Globulin (SHBG), respectively leading to severe endocrine dysregulations (Gore et al., 2015; Kitahara et al., 1999; Zhang et al., 2016; Zhou et al., 2013).

Given that sexual development is modulated by hormones and consequently highly sensitive to exogenous substances as EDCs, studies about glyphosate and their relationship with hormone levels are done with rats exposed during late gestational and early postnatal days. The fetal period is a critical stage of sexual hypothalamic differentiation since high aromatase levels direct the conversion of circulating testosterone into estradiol, determining the gender and behavior in adults (Bakker et al., 2002; Romano et al., 2012).

The results of the studies on glyphosate and its relationship with hormone distribution vary depending on several factors in the experimental settings that include the stage of exposure (pre or postnatal), doses administrated, time of exposure, GBH type and administration (oral, subcutaneously). For example, in male Wistar rats treated with Roundup (450 mg/kg glyphosate) during pregnancy (21−23 days) and lactation (21 days), it was observed a decrease in the serum testosterone level at puberty (Dallegrave et al., 2007). Similarly another group (Romano et al., 2010) demonstrated a substantial reduction in serum testosterone concentrations and shifts in testicular morphology of male Wistar rats treated with different Roundup® dilutions, ranging from 5 to 250 mg/kg, during the pre-pubertal period. In contrast, under gestational maternal glyphosate exposure, the male offspring showed an increase in estradiol and testosterone serum concentrations at PND 60.

Another similar study showed markedly altered serum concentrations of both progesterone and estrogens orally administered with pure glyphosate solution 0.5% and GBH at 0.5%. During 19 days in pregnant mice. Specifically, the mice presented diminished serum progesterone and elevated serum estrogen concentration along with changes in the expression of GnRH, LHR, FSHR, 3β-HSD and Cyp19a1 genes at the hypothalamic-pituitary-ovarian axis (Ren et al., 2018). On the other hand, no effects were observed in 17 beta-estradiol (E2) and testosterone serum levels and androgen receptor expression in both PND21 and PND60 in Wistar male rats injected subcutaneously every 48 h with 2 mg GBH/kg bw from PND 1 to PND7 (Altamirano et al., 2018). The same results were observed when glyphosate was given continuously at doses of 5, 50, 500 mg/kg during 5 weeks by lavage in sexually mature (56-day-old) Sprague-Dawley (SD) rats (Dai et al., 2016).

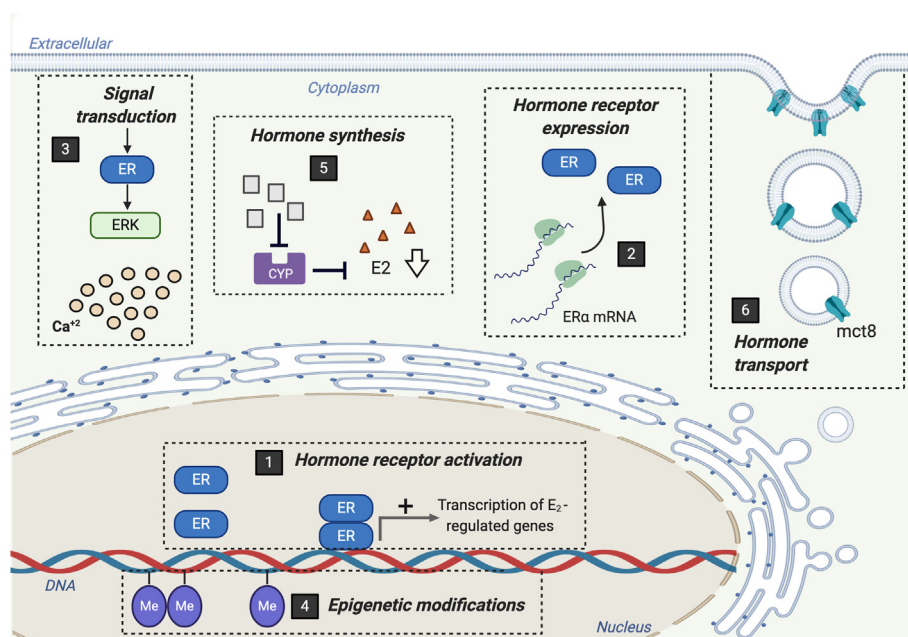On the other hand, glyphosate and its formulation, Roundup®

Fig. 1. Summary of the evidences related to the effects of glyphosate and its derivatives on different hormone-producing or hormone-sensitive cells.

were reported to decrease testosterone hormone secretion into the culture medium after 24 h of exposure at non-cytotoxic concentrations (1×10^{-4} to 1×10^{-2} of percentage of dilution) in primary Leydig cells (Clair et al., 2012). However, in a murine cell model (BLTK1 cells) that expresses all the necessary enzymes required for testosterone biosynthesis and metabolism, glyphosate at 300 μM after 4 h did not affect the testosterone levels, suggesting the lack of steroidogenic effects (Forgacs et al., 2012).

One of the most recent studies at large scale was conducted by the Ramazzini Institute, where SD rats were subjected to GBH orally administered for 13 weeks at 1.75 mg/kg bw/day, the acceptable daily intake defined by the US EPA (Mao et al., 2018). The main result showed that after glyphosate exposure, from the prenatal period to adulthood, there was no a statistically significant increase of TSH in plasma of male rats. However, it was observed a marked and significant increase in Roundup-treated males compared to control. On the other hand, it was an increase in total testosterone levels in animals from the 13-week cohort compared to control as well as altered reproductive developmental parameters in female offspring; particularly, androgen-like effects, including a statistically significant increase of AGDs in both males and females (Mao et al., 2018). In summary, this study determined that GBH induced adverse effects on hormone concentrations and reproductive development.

Regarding GBH effects on thyroid hormones, a recent study showed that female Wistar rats sub-chronically exposed to two doses of GBH equivalent to 126 and 315 mg of Glyphosate/Kg had a decrease in free triiodothyronine (FT3) and thyroxine (FT4), which was associated with an increase of TSH in the plasma level. Additionally, the authors found a decrease in levels of estrogen. All these hormonal alterations led to hypothyroidism and a disruption in the skeletal bone in Wistar rats (Hamdaoui et al., 2020).

Taken together, all these differences in the findings could have an explanation in the experimental design used in each case; therefore, more exhaustive epidemiological studies that consider variables such as exposure times and doses are required. In conclusion, glyphosate and GBH are not harmless and can modify the hormone concentration in animal models, therefore, it satisfies this "key characteristic of EDCs".

5.9. It alters hormone metabolism or clearance

A fine controlled synthesis and release process regulates the hormonal concentration in the blood. Hormones are eliminated from circulation by different pathways that include, metabolic processing by the tissues, binding with the tissues, and excretion (liver or kidneys). All these mechanisms are referred to as "the hormonal clearance". Some EDCs, like DES, have been shown to alter the hormonal clearance (Troisi et al., 2018). Regarding glyphosate, there is no evidence of its impact on hormonal metabolism or clearance.

5.10. It alters the fate of hormone-producing or hormone-responsive cells

Hormones regulate key cellular processes such as proliferation, migration, apoptosis, and differentiation. Thus, any external influence that alters these processes may have consequences at the physiological level, eliciting disturbance in the development, growth and the usage and storage of energy among others process. For instance, some EDCs such as DES or BPA have shown effect on cellular differentiation, leading to severe injuries on the development and growth (Markey et al., 2001; Okada et al., 2001).

Studies in vitro on hormone-producing or hormone-responsive cells have demonstrated direct effects of glyphosate mainly on

cell proliferation (George and Shukla, 2013; Lin and Garry, 2000; Mesnage et al., 2017; Sritana et al., 2018; Thongprakaisang et al., 2013) and apoptosis (Benachour and Séralini, 2009; Clair et al., 2012; Li et al., 2013; Stur et al., 2019). The first study revealed that glyphosate can induce cell proliferation of the MCF7 cell line in a range of 10^{-5} M to 10^{-4} M, the same concentrations where it was observed estrogenic effects, thus it was the first evidence suggesting that glyphosate can act as an EDC through a molecular mechanism involving ER activity (Lin and Garry, 2000). Similar results were confirmed afterward in the uterus of ovariectomized adult rats, where GBH at 50 mg/kg/day for 3 consecutive days alters estrogen-dependent gene and protein expression but without affecting the wet weight of the uterus (Varayoud et al., 2017). These studies opened the way for further evaluation, such as, whether glyphosate can affect during early developmental periods, like embryonic, fetal, neonatal, childhood, and puberty. Today, it is known that glyphosate, specially GBH causes alterations during the whole life of the exposed individual and sometimes in its descendants.

The Ramazzini Institute revealed the results of a pilot study aimed to evaluate whether exposure to GBH at the dose of 1.75 mg/kg bw/day of glyphosate is considered to be safe on the development and endocrine system across different life stages in SD rats. The findings showed that GBH exposure induced endocrine effects from prenatal to adulthood and altered reproductive developmental parameters in male and female SD rats. Specifically, inducing androgen-like effects, including a significant increase of anogenital distance (AGD) in both males and females, and a delay of first estrous and increased testosterone in females. In addition, the group noted that commercial Roundup® Bioflow formulation was more aggressive than pure glyphosate (Mao et al., 2018).

In the last century, the first toxicological report in vivo on xenobiotics showed sexual disorders exerted by glyphosate and others herbicides (Yousef et al., 1995). Later, other studies analyzing maternal exposure during pregnancy and lactation in different animal models showed that GBH disturbs several developmental and reproductivity parameters in F1 offspring. Among them the studies are demonstrated developmental retardation of the fetal skeleton (Dallegrave et al., 2003), disruption in the skeletal bone associated to an aspect of osteoporosis (Hamdaoui et al., 2020), promotion of behavioral changes (Romano et al., 2012), alteration in uterine decidualization (Guerrero et al., 2017), and the differentiation of the ovaries and uterus in lambs (Alarcón et al., 2019) as well as post-implantation embryo loss (Guerrero et al., 2017).

In this sense, special attention is given to the findings in alterations produced in the mammary gland of pre- and post-pubertal rats, where it was demonstrated that GBH-treated groups exhibited greater development of male mammary gland such as, a higher number of terminal end buds (Altamirano et al., 2018) and a higher percentage of hyperplastic ducts (Zanardi et al., 2020). However, the rats showed a less developed mammary gland, accompanied by a lower proliferation index with other doses (Gomez et al., 2019).

Zebrafish (Danio rerio) serves as a useful model to study the effect of drugs on early development via morphological, biomechanics, behavioral and physiological areas since they breed readily and their transparency enables the visualization of fluorescently labeled tissues (Roper and Tanguay, 2018). Thus, several studies on glyphosate and its implications for development have been carried out on the zebrafish model. Some experiments have shown that exposure to glyphosate did no induce apparent changes in general morphology of reproductive system (Armiliato et al., 2014). On the other hand, the fertilization rate did not change but oocytes significantly increased in diameter and reduced egg production, affecting equally fish reproduction (Uren Webster et al., 2014). In
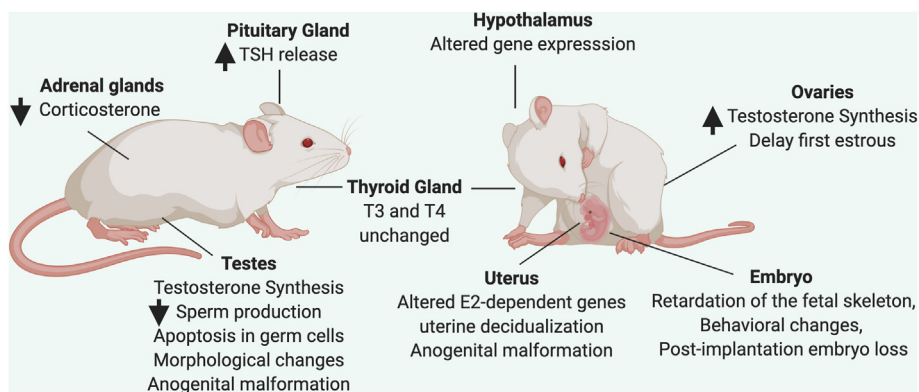
J.P. Muñoz, T.C. Bleak and G.M. Calaf

Fig. 2. Summary of the evidence on the effects of glyphosate and its derivatives in *in vivo* models.

another study, it was determined a decreased ocular distance for larvae zebrafish exposed at 0.5 mg/L of glyphosate, as well as a significant impairment in memory and a reduction in aggressive behavior (Bridi et al., 2017). Likewise, the results of Zhang et al. (2017) demonstrated a delay in the epiboly process and a decrease in body length, eye and head area after glyphosate treatment at concentrations higher than 10 mg/L (Zhang et al., 2017). These results were corroborated by Sulukan et al. (2017) who showed that apoptosis induced by glyphosate at 1 mg/mL during embryologic development, caused several types of malformations including pericardial edema, yolk sac edema, spinal curvature and body malformation in a dose-dependent manner (Sulukan et al., 2017).

Finally, several studies on diverse animal models such as bovine, cows, pigs, and lizards suggest associations between exposures to GBH and adverse outcomes in development, pregnant and reproduction process. However, such studies did not show a direct relationship or a specific mechanism that depicts a specific role of glyphosate (Canosa et al., 2019; Gigante et al., 2018; Mestre et al, 2019, 2020; Wrobel, 2018).

In summary, the evidence shows that exposure to glyphosate or GBH in different animal models at different stages of development is associated with several physiological changes, especially in the mammary gland, reproductive system, and skeletal bone formation, suggesting an active role of glyphosate in altering the fate of hormone-producing or hormone-sensitive cells.

Fig. 2 presents the evidence on the effects of glyphosate and its derivatives in *in vivo* models. In male rats, GBH lead to a reduction of corticosterone synthesis in the adrenal tissue (Pandey and Rudraiah, 2015), an increase in plasma TSH concentration from the pituitary gland and a marked disruption of testosterone levels (Mao et al., 2018; Romano et al., 2010). Moreover, GBH exposure from the prenatal period to adulthood altered reproductive developmental parameters, inducing a significant increase of anogenital distance (AGD). In female rats, the GBH exposure in distinct stages is associated with increased testosterone levels, developmental retardation of the fetal skeleton (Dallegrave et al., 2003), disrupting behavioral changes (Romano et al., 2012), skeletal bone (Hamdaoui et al., 2020), uterine decidualization (Guerrero et al., 2017), differentiation of the ovaries and uterus in lambs (Alarcón et al., 2019) and post-implantation embryo loss (Guerrero et al., 2017).

6. Epidemiological perspective

Given the vast evidence that suggests that offspring of pesticide appliers have increased risks of reproductive disorders and birth anomalies (Garry et al, 1989, 1992, 1996; Giwercman et al., 1993; Lipkowitz et al., 1992), numerous epidemiological studies have been addressed in order to know whether glyphosate exerts similar effects on these physiological process. However, due to methodological difficulty related to this type of study, such as the need of quantitative results, the paucity of cases and accuracy in the period of exposure lead to small number of useful reports to evaluate a direct implication of glyphosate like EDC. Therefore, in the epidemiological analysis, were considered only articles that include a thorough analysis of the effects of glyphosate according to inclusion criteria described by De Araujo et al. (2016). In according to them, only epidemiological reports regarding reproductive or developmental effects that specifically come from GBH expositions were considered, excluding thus studies that consider GBHs in conjunction with other compounds.

6.1. Evidence on reproductive effects

The Ontario Farm Family Health Study (OFFHS) has provided valuable data from retrospective studies to assess the potential adverse effects of commonly used pesticides on pregnancy. In the first report published in 1997, the Canadian census of agriculture served as the sampling frame for the selection of farms. Thus, the selection was based on residence (on or near the farm), and whether the age of female partners was 44 years or younger at the time of the interview. Male farm activities from 3 months before conception were evaluated in relation to reproductivity parameters in their female couples, such as small-gestational-age births, miscarriage and preterm delivery. The results showed that among the 1.898 couples with complete data chemical activities were not associated with miscarriage, neither associations were found between farm chemicals and small-gestational-age births or altered sex ratio (Savitz et al., 1997). Two years later, the same group evaluated whether exposure to 13 different pesticides, was associated with an altered fecundability and longer time to pregnancy considering a universe of 2.946 couples. These results showed that 6 of 13 categories of pesticides that were evaluated (among them glyphosate) were associated with a decrease in fecundability when women were exposed to activities related to pesticide use. Moreover, no apparent association among reproduction parameters and pesticide type was observed. In contrast, when only men were engaged in pesticide activities, 3 class of pesticides (among them glyphosate) were related to an increase in fecundability (17−30%).

Regarding time-to-pregnancy, a non-significant association was found between glyphosate use by farmers (Curtis et al., 1999). Finally, in 2001, another retrospective cohort study performed by OFFHS in 2.110 Canadian farm women, revealed that preconception exposures to GBH were associated with a moderate rise in the risk

of early abortion (<20 weeks) and an elevated risk of late abortion, regardless the time in which the exposure occurred (Arbuckle et al., 2001).

In another retrospective cohort study, it was assessed the association between glyphosate applied by aerial spray and time to pregnancy. The analysis compared 2.592 fertile Colombian women from 5 different regions with different use patterns of pesticides. One region with organic crops (without glyphosate spraying) was used as a reference. Results showed no association between glyphosate use and delayed time to pregnancy among different regions (Sanin et al., 2006).

On the other hand, a prospective study was carried out in Indiana in 2018. The aim of that study was to assess the association between glyphosate exposure in pregnancy and shortened gestational length. For this purpose, urine samples from 71 pregnant women and residential drinking water were obtained as a direct measurement of glyphosate exposure. The results showed that women who lived in rural areas had higher glyphosate levels, and were significantly correlated with shortened gestational lengths ($r = -0.28$, $p = 0.02$) (Parvez et al., 2018).

6.2. Evidence on birth defects

Given the evidence supporting a causal relationship between maternal glyphosate exposure and offspring birth defects, several epidemiological studies have focused on analyzing whether this pattern is also applied in humans with childhood health disorders. In a case-control study published in the late 1990's, it was evaluated whether the father's exposure to some pesticides, before conception or during the first trimester of pregnancy, influence the development of selected congenital defects in their descendants. Through dichotomous exposure analysis (absent, present) it was shown that only parental exposure to pyridyl derivatives is statistically representative for risk of congenital malformations (adjusted OR 2.77, 95% CI 1.19–6.44). However, parental exposure to GBH showed that was not associated with the risk of malformations in offspring (García et al., 1998).

A cross-sectional retrospective study conducted by Garry et al. (2002) evaluated birth defects in 1.532 children from 695 farm families who worked as pesticide applicators in the Red River Valley of Minnesota, USA. The study reported that the birth defect rate was 31.3 per 1.000 births and that 43% of children (6 of 14) who had attention deficit disorder were from parents who had used GBH. Therefore, the use of GBH shows certain association with neurobehavioral disorders (OR: 3.6, CI: 1.3–9.6). In the same context, another study evaluated the two most common subtypes of neural-tube defects (NTDs), anencephaly and spina bifida regarding the maternal exposure to 59 different pesticides during the month of conception. The data was collected from two birth cohorts born in California from 1987 to 1991 and it was analyzed by unconditional logistic regression. Additionally, each pesticide was evaluated in both single- and multiple-pesticide models that when it was analyzed by the regression method with a multiple-pesticide model there were no association among NTDs and glyphosate use. Interestingly, when a single pesticide model of conventional regression analysis was evaluated, the results showed a significant association between proximity to glyphosate exposure and NTD ($OR = 1.5$; 1.0–2.4) (Rull et al., 2006), suggesting that NTD risk was associated with maternal exposure of glyphosate applications.

A similar study was conducted by Yang et al. (2014) who examined whether early gestational exposures to GBH due to maternal residential proximity to pesticide sprayed crops, were associated with an increased risk of NTD. The data was provided from the population derived from the San Joaquin Valley, California (1997–2006) and shown no association between glyphosate and

NTD. Similarly, this population study was used to explore whether early gestational exposures to pesticides were associated with the risk of gastroschisis (Shaw et al., 2014). They used the same criteria (maternal residential proximity) when 156 newborns with gastroschisis were evaluated, of which 30 were from mothers exposed to glyphosate. These authors found no association between gastroschisis and maternal glyphosate exposure by using logistic regression 785 cases of babies without birth defects (as controls).

On the other hand, a cross-sectional study was conducted with the OFFHS data (Arbuckle et al., 1999), to evaluate the relationship between gestational exposures to glyphosate and the health results of the offspring, which included, persistent cough or bronchitis, asthma, and allergies or hay fever. This retrospective study considered a total of 5853 pregnancies and, despite its limitations, showed no statistical association between pesticide exposure during pregnancy and adverse health outcomes in offspring (Weselak et al., 2007).

In the same line, a study from the Agricultural Health Study evaluated the association between maternal pesticide exposure with birth weight in the offspring (Sathyanarayana et al., 2010). The authors analyzed 27 individual pesticides (among them glyphosate) on 2246 farm women. Results showed that the mean birth weight for infants was 3586 g ($\pm$546 g) and 3% of the infants had low weight. Therefore, there were no statistically significant associations between birth weight loss and glyphosate-related activities during early pregnancy.

In summary, some articles suggest a risk of miscarriages, decrease in fecundability and neurological behavioral disorders in the descendant in a significant manner. However, considering all of these studies certain methodological limitations must be taken into consideration, for instance, retrospective perspective studies with no quantitative data about time or dose of glyphosate exposure. Thus, in the current scenario prospective cohort studies are needed with quantitative estimations of exposure, to better elucidate the effects of glyphosate and GBH in the endocrine system. In fact, some authors have suggested that current safety standards for GBH must be modernized and may fail to protect public health and the environment (Vandenberg et al., 2017).

7. Conclusions and future perspectives

Here, the mechanistic evidence associated with glyphosate as an endocrine disruptor according to the ten key characteristics of EDCs were analyzed for the first time. In addition, the main epidemiological reports regarding the possible association between glyphosate exposure and the high risk of adverse reproductive outcomes and birth defects in the progeny were summarized. The evidence from some epidemiological studies show that women exposed to glyphosate increase the risk of late miscarriages and a decrease in fecundability.

In the animal phenotype (rodents mainly), glyphosate exposure during pregnancy is associated with an increase of anogenital distance in both males and females, delay of first estrous and increased testosterone levels in the female. Further, GBH disturbs several developmental and reproductive parameters in progeny, such as retardation of the fetal skeleton. In addition, it promotes other effects such as disruption in the skeletal bone associated to an aspect of osteoporosis, behavioral changes, uterine decidualization, as well as the differentiation of the ovaries and uterus in lambs and post-implantation embryo loss (Fig. 2).

Mechanistic data showed that glyphosate exhibited eight of the ten KCs of an EDC: glyphosate 1) can favor hormonal receptors activity, particularly ERα, stimulating their transcriptional activation in breast cell line models. 2) disrupts the levels of ERα and ERβ. 3) induces deregulation of eleven canonical pathways in cancer

breast cell lines.4. It induces epigenetic modifications in normal breast cell lines. 5) has adverse effects on steroid hormone production (estrogens and testosterone). 6) alters thyroid hormones transport across cell membranes. 7) modify the hormone concentration, such as estrogen and testosterone in animal models. 8) alter the proliferation rate of breast cell lines (Fig. 1).

Thus, it can be concluded that glyphosate behaves like an EDC by altering hormonal activity which induces defects in the reproductive process and progeny. However, new prospective studies in humans are needed in order to confirm these conclusions.

Authorship contribution statement

Juan P. Muñoz: Conceptualization, Formal analysis, Investigation, Writing - review & editing. Tammy C. Bleak: Resources, Writing - review & editing, Visualization, Investigation. Gloria M. Calaf: Conceptualization, Methodology, Writing - review & editing, Supervision, Data curation, Funding acquisition.

Ethics approval and consent to participate

Not applicable.

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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