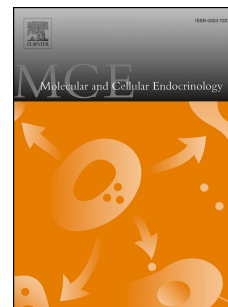


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Are glyphosate and glyphosate-based herbicides endocrine disruptors that alter female fertility?

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25 **Abstract**

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

46

47 **Abbreviations**

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

62 **Outline**

63 1- Introduction

64 2- Glyphosate and glyphosate-based herbicides (GBHs)

65 2.1 Formulations, surfactants, exposed species and human exposure

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67 3- Mechanisms of action: *in vitro* studies68 4- *In vivo* effects in animal models

69 4.1 Effects in fish and chicken

70 4.2 Effects on the ovary of rats, mice and ewe lambs

71 4.3 Effects on pregnancy outcome and genital tracts of rats, mice and ewe lambs

72 5- Evidences that support the hypothesis that glyphosate and GBHs are endocrine
73 disruptors

74 6- Conclusions

75 ***1- Introduction***

76 The environmental pollution caused by urbanization, agriculture and industrialization
77 has been reported to affect animal and human health (Luque et al. 2018). Throughout
78 their lifespan, both humans and animals are exposed to many of man-generated
79 chemicals, which can have either a positive or a negative impact on their health. These
80 compounds can be found in the environment, food, cosmetics, household items,
81 pharmaceutical products, and many other products (Darbre 2018; Darbre 2019). Studies
82 on the possible hormonal activity of some of these compounds have shown that they
83 have the potential to disrupt the endocrine system of many animal species, including
84 humans (Amereh et al. 2020; Tsai et al. 2019). The potential risk of these chemicals can
85 be assessed by means of *in vitro* studies or animal models, including fish, amphibians
86 and mammals (Bergman et al. 2013). Colborn et al. (1997) were pioneers in
87 demonstrating the risks of exposure to chemicals in the environment known as
88 endocrine-disrupting chemicals (EDCs). The World Health Organization (WHO)
89 defined an EDC as “an exogenous substance or mixture that alters function(s) of the
90 endocrine system and consequently causes adverse health effects in an intact organism,
91 or its progeny or (sub)populations” (Bergman et al., 2013). Exposure to EDCs can occur
92 via ingestion of water, food and dust, via inhalation of gases and air particles, and via
93 dermal absorption. Once in the organism, EDCs interact with the endocrine system and
94 disrupt normal endocrine function, sexual development, and ultimately reproduction.
95 EDCs exert their actions by triggering genomic mechanisms and non-genomic actions,
96 through the binding to several hormone receptors, including thyroid and, especially,
97 steroid receptors, mainly estrogen (ER) or androgen (AR) receptors (Diamanti-
98 Kandarakis et al. 2009; Gore et al. 2015). These “xenochemicals” (chemicals that are
99 foreign to the body) may possess a range of agonist, partial agonist or antagonist

100 activities dependent on the dose, presence of endogenous receptor ligand, and nature of
101 the structure–activity relationship (interaction with the nuclear hormone receptors) (Hall
102 and Greco 2019). The exposure to EDCs in pregnant mothers may reach the developing
103 fetus and exposure during early life has been shown to disrupt the normal development
104 of reproductive tissues and may predispose to diseases in adulthood (Rattan and Flaws
105 2019). Moreover, the exposure to these chemicals neonatally can negatively impact the
106 reproductive health of future generations and cause transgenerational effects on
107 reproduction in both males and females (Brehm and Flaws 2019). The mechanisms of
108 action by which EDCs transmit adverse effects to future generations include epigenetic
109 modifications (Rattan and Flaws 2019). Despite several evidences on the potential
110 adverse effects of EDCs on reproduction, the molecular mechanisms underlying these
111 effects are not completely understood.

112 Although there are more than 80,000 chemicals in commercial use, the risk of most of
113 them has not yet been assessed. In 2015, by means of the ToxCast program, the
114 Endocrine Disruptor Screening Program of the U.S. Environmental Protection Agency
115 (EPA) made efforts to demonstrate the estrogenic and antiestrogenic activities of more
116 than 1,800 chemicals (US EPA 2015). Among the chemicals that highly contaminate
117 the environment and for which their toxicity has not yet been completely assessed, we
118 can mention all the agrochemicals. Moreover, for many of these agrochemicals, there is
119 an absence of evidence of human and animal levels of exposure. With the development
120 of herbicide-tolerant soybeans, corn and cotton in 1996, the use of glyphosate and
121 glyphosate-based herbicides (GBHs) has increased dramatically over the past two
122 decades. With an estimation of more than 800 million kg sprayed around the globe in
123 2014, GBHs are among the most used agrochemicals in the world (Benbrook 2016). In
124 Argentina, glyphosate is the most commonly used herbicide, with around 180–200

125 million liters applied every year (Aparicio et al. 2013). Currently existing information
126 on the safety of glyphosate and/or its formulations is controversial, suggesting that
127 glyphosate or its adjuvants are responsible for the adverse impacts on human, animal
128 and ecological health (Meftaul et al. 2020; Vandenberg et al. 2017). However, most
129 studies involve exposures over long periods, high doses, or the use of glyphosate alone,
130 which is not a real situation regarding environmental contamination. This review aims
131 to analyze and summarize the current literature that has evaluated the effects of low or
132 “environmentally relevant” doses of glyphosate and GBHs acting as EDCs, mainly
133 regarding evidences obtained in the female reproductive tissue. We present enough data
134 suggesting that, at low doses, GBHs may have adverse effects on the development of
135 the female reproductive tract and fertility.

136

137 **2- Glyphosate and glyphosate-based herbicides (GBHs)**

138 *2.1. Formulations, surfactants, exposed species and human exposure*

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

149 In the world market, there are several formulations of GBHs, which differ
150 mainly in the content of surfactants, the most common of which over the last years have
151 been ethoxylated amines. The addition of surfactants to the active compound promotes
152 the penetration and stabilization of glyphosate in plants. In addition, arsenic (As), cobalt
153 (Co), chromium (Cr), nickel (Ni) and lead (Pb), several of which are known to be
154 EDCs, are present in numerous herbicide formulations, at levels well above admissible
155 ones in water (Defarge et al. 2018). A comparative cytotoxicity study of surfactants in
156 human cell lines concluded that alkyl polyglucosides, which are high-quality nonionic
157 surfactants, are the least toxic compounds, followed by polyethoxylated alkyl phosphate
158 ethers, which are quaternary ammonium surfactants, and finally by POE-tallow amines,
159 which are the most toxic (Defarge et al. 2016). However, some occupational and food
160 risk assessments have shown that there are no significant human health issues
161 associated with the use of POEA as surfactants in glyphosate products (Martens et al.
162 2019). The first generation of POEA surfactants (POE-tallow amines) in Roundup® are
163 markedly more toxic than glyphosate and thus pose higher risks to human health,
164 especially among heavily-exposed applicators (Defarge et al. 2016). In some cases, the
165 absence of compositional data may cause problems in the reproducibility of the
166 experiment because, in formulated GBH products with the same commercial name, the
167 mixture of chemicals could vary among different countries (Mesnage et al. 2019).

168 In Argentina, the percentage of land destined to agricultural production has
169 increased significantly in recent years, mainly due to new technologies, including
170 modern irrigation, pesticides, chemical fertilizers, conservation tillage and the
171 expansion of soybean crops. This habitat fragmentation and the constant exposure to
172 pesticides and GBHs affect different species (Burella et al. 2018; Dornelles and Oliveira
173 2016), including the main agricultural insect pollinator, the honey bee (*Apis mellifera*)

174 (Vazquez et al. 2018). In the last years, the effects of glyphosate either alone or in
175 formula have been analyzed in several species such as fish, lambs, caimans, mice and
176 rats (Alarcón et al. 2019a; Albanil Sanchez et al. 2019; Ingaramo et al. 2016; Pham et
177 al. 2019; Szepanowski et al. 2018; Varayoud et al. 2017). Regarding animal feed,
178 residues of glyphosate have been found in soybean and maize, both of which play an
179 important role in animal nutrition (Poppe et al. 2019). Indeed, a study comparing 18
180 commercial animal feeds from different companies has recently demonstrated that every
181 product contained detectable glyphosate residues in a range between 78.3 and 2140
182 $\mu\text{g}/\text{kg}$, which results in an exposure of animals 4-12 times higher than that of humans
183 per kg basis (Zhao et al. 2018).

184 Despite the widespread use of glyphosate, data on the potential human
185 exposures during common occupational uses are limited. The occupational exposure of
186 amenity horticulturalists to GBHs has shown that contamination is usually greater than
187 that reported in environmental studies (Connolly et al. 2018). Very high concentrations
188 of glyphosate and its main metabolite, aminomethylphosphonic acid (AMPA), may
189 occur in airborne particulate matter, which can be inhaled by humans and animals
190 (Bento et al. 2017). In this context, the data of urine levels is a powerful tool in
191 biomonitoring studies to estimate human exposure (Conrad et al. 2017; Krüger et al.
192 2014). Some occupational biomonitoring studies have analyzed pooled urine samples
193 collected over a 24-h period, providing an estimate of the average exposure over this
194 sampling period (Acquavella et al. 2004; Mesnage et al. 2012), whereas others have
195 analyzed a spot urine sample as a marker of the 24-h exposure (Connolly et al., 2017).
196 Several researchers have found that the detectable concentrations of glyphosate in
197 urines collected from farmers and their families during the study period were in the
198 range of $<0.1\text{--}233\text{ ng/mL}$, with the highest systemic dose estimated at 0.004 mg/kg

199 (Acquavella et al. 2004; Jauhiainen et al. 1991; Thongprakaisang et al. 2013). The
200 systemic dose, which is calculated as the amount of glyphosate excreted in urine
201 divided by each individual's body weight, is an integrated measure of the amount of a
202 substance absorbed per kilogram of body weight that provides a basis to compare
203 human exposures with levels of toxicological significance (Acquavella et al. 2004).
204 Parvez et al. (2018) evaluated the exposure of pregnant women to GBHs by direct
205 measuring in urine samples and found that >90% of them had detectable glyphosate
206 levels, which were higher in women living in rural areas, and that these levels correlated
207 significantly with shortened pregnancy lengths. In Thai women, Kongtip et al. (2017)
208 demonstrated high maternal (0.2–189.1 ng/mL) and umbilical cord (0.2–94.9 ng/mL)
209 serum concentrations of glyphosate.

210 2.2. Metabolism and its potential impact on the toxicity of glyphosate and GBHs

211 Although several reports have claimed no significant transformation of
212 glyphosate to AMPA *in vivo* (EFSA 2015; Niemann et al. 2015; Williams et al. 2000),
213 Ford et al. (2017) demonstrated the formation of AMPA and glyoxylate in livers from
214 glyphosate-treated mice. These authors showed that approximately 4% of the
215 glyphosate levels detected in the mouse liver are metabolized to glyoxylate (Ford et al.
216 2017). Glyphosate metabolism may depend on the physiology of the animal. At our
217 lab in lambs exposed to GBH during their first postnatal days, we found no AMPA
218 serum levels, suggesting a limited metabolism of glyphosate into AMPA by intestinal
219 microbial action, considering that the rumen of lambs exhibits limited functional
220 development on post-natal day (PND) 15 (Alarcón et al. 2019a). Although the acute
221 toxic effects of glyphosate and AMPA on mammals are low, there are animal data
222 raising concern of the health effects associated with chronic ultra-low doses of these
223 compounds, related to their accumulation in the environment (Van Bruggen et al. 2018).

224 Anadon et al. (2009), for example, found that the bioavailability of glyphosate by oral
225 administration in rats is 23.21%, whereas, in ewe lambs, we have recently found that the
226 levels of glyphosate in serum and the effects on the female reproductive tract in orally
227 and subcutaneously GBH exposed animals were similar (Alarcón et al 2019). Several
228 experiments have demonstrated that the estrogenic potential and toxicity of glyphosate
229 are different from those of GBHs and polyethoxylated amine (POEA) (De Almeida et
230 al. 2018; Mesnage et al. 2017; Perego et al. 2017a; Perego et al. 2017b; Richard et al.
231 2005).

232 Regarding other issues not directly related to reproduction, Davoren and Schiestl
233 (2018) suggested that glyphosate has the potential to affect the human gut microbiome
234 profile and function, leading to decreased pathogen defense and inflammation, both in
235 the intestine and systemically. They suggested that the adjuvant surfactants and
236 emulsifiers present in GBHs would contribute to microbiome disruption more than
237 glyphosate alone (Davoren and Schiestl 2018). This represents another pathway through
238 which glyphosate and GBHs could induce adverse effects related to inflammation and
239 immune response that may affect reproduction.

240

241 **3- Mechanisms of action: *in vitro* studies**

242 Many of the mechanisms by which the neonatal exposure to glyphosate or
243 GBHs affects the female reproductive tract are not known but the few studies regarding
244 this issue have been done *in vitro*. However, most of the effects of glyphosate and
245 GBHs shown *in vitro* have not been able to be reproduced in *in vivo* experiments.
246 Several of the *in vitro* studies on glyphosate and GBHs have been performed using
247 cancer cell lines that are estrogen-responsive (HEC1A, MCF-7, and T47D-KBluc cell
248 lines) or the estrogen-insensitive line MDA-MB-231. HEC1A cells are endometrial

249 cancer cells that express the wild-type form of estrogen receptor alpha (ER α). Since
250 estrogen induces the proliferation of these cells (Castro-Rivera and Safe 1998), De
251 Almeida et al. (2018) demonstrated a non-monotonic reduction in the viability of
252 HEC1A cells exposed to pure glyphosate (75-500 $\mu\text{g/mL}$). These authors also found
253 that exposure to the GBH Wipeout $\text{\textcircled{R}}$ (75, 125 and 250 $\mu\text{g/mL}$) induces proliferative
254 effects and DNA damage in both HEC1A and MDA-MB-231 cells. Based on the
255 differential toxicities of the GBHs Roundup $\text{\textcircled{R}}$ and Wipeout $\text{\textcircled{R}}$ in human whole blood and
256 HEC1A cells, these authors determined that the adjuvants and/or glyphosate impurities
257 were potential contributing factors of toxicity (De Almeida et al. 2018). They also found
258 that, in human whole blood, glyphosate and Roundup $\text{\textcircled{R}}$ led to similar non-monotonic
259 toxicological profiles, evidenced by a significant reduction in cell viability ($P \leq 0.01$) at
260 pure glyphosate and Roundup $\text{\textcircled{R}}$ concentrations of 10 and 50 $\mu\text{g/mL}$ and no decreased
261 cell viability at higher concentrations (500 $\mu\text{g/mL}$). Finally, they found that Wipeout $\text{\textcircled{R}}$
262 led to a monotonic reduction in cell viability from a threshold concentration of 50
263 $\mu\text{g/mL}$ (De Almeida et al. 2018). In human peripheral blood mononuclear cells,
264 (Martinez et al. 2007) demonstrated that the cytotoxicity of Roundup $\text{\textcircled{R}}$ was higher than
265 that of glyphosate alone, because the concentration of Roundup $\text{\textcircled{R}}$ to cause 50%
266 mortality of the cells was 30 times lower than that of pure glyphosate. These results
267 support the concept that the additives in commercial formulations play a significant role
268 in the toxicity attributed to GBHs.

269 In HepG2 cells, which are the best characterized human liver cell line and are
270 used to study xenobiotic toxicity, Gasnier et al. (2009) determined glyphosate
271 cytotoxicity with three assays (Alamar Blue, MTT, ToxiLight), genotoxicity by the
272 comet assay, and anti-estrogenic effects (on ER α , ER β) and anti-androgenic effects (on
273 the androgen receptor (AR) by using gene reporter tests. They also checked androgen to

274 estrogen conversion by aromatase activity and mRNA. At sub-agricultural doses, all
275 these parameters were disrupted with all formulations within 24 h. In HepG2 cells, the
276 active formulation R400 (from 2 parts per million (ppm)) inhibited the transcriptional
277 activities on both estrogen receptors. These effects were more dependent on the
278 formulation than on the glyphosate concentration. In addition, in MDA-MB453-kb2
279 cells, these authors observed decreased AR and aromatase transcription from 0.5 ppm of
280 GBH and inhibited enzymatic activity from 10 ppm (Gasnier et al. 2009). In human
281 ovarian and prostate cancer cells, Li et al. (2013) demonstrated that glyphosate and
282 AMPA can inhibit proliferation and promote apoptosis. One of the mechanisms by
283 which glyphosate would inhibit cell proliferation is through the enzyme serine
284 hydroxymethyltransferase, a major source of intracellular glycine (Mesnage et al. 2015).
285 Other *in vitro* studies have shown that exposure of human lymphocytes to different
286 glyphosate concentrations does not cause effects on their proliferation/mitotic index
287 (Santovito et al. 2018).

288 Regarding the effects of glyphosate on ovarian tissue and stem cell
289 differentiation, Gigante et al. (2018) studied swine granulosa cells and found that, at
290 different doses (0.2, 4 and 16 $\mu\text{g}/\text{mL}$), glyphosate decreased the growth of granulosa
291 cells (by bromodeoxyuridine incorporation and ATP production) and estrogen
292 production and increased progesterone and nitric oxide secretion, all of which suggest
293 that the *in vivo* ovarian function may be affected. On the other hand, by using granulosa
294 cells from bovine ovary, Perego et al. (2017a) found different results between exposure
295 to glyphosate and Roundup®. While Roundup® (10 and 300 $\mu\text{g}/\text{mL}$) dramatically
296 decreased the number of granulosa cells and estrogen and progesterone production,
297 glyphosate alone had no effect (Perego et al. 2017a). In cow granulosa cells, Wrobel
298 (2018) reported that pure glyphosate stimulates the secretion of estrogen, while GBHs

299 and pure glyphosate increase oxytocin and decrease progesterone secretion from luteal
300 cells. All these *in vitro* studies with bovine ovarian cells suggest that glyphosate and/or
301 GBHs may affect, at least in part, the female reproductive system via direct action on
302 the ovarian function.

303 Richard et al. (2005) tested the toxicity of glyphosate and Roundup® and their
304 possible ability to act as EDCs in human placental JEG3 cells. Their study showed that
305 Roundup® interacts with the active site of the enzyme aromatase and decreases its
306 activity and mRNA expression. Besides, they demonstrated that glyphosate is toxic
307 within the lowest concentrations used in agriculture, and that this effect increases with
308 higher concentrations and longer times as well as in the presence of Roundup®
309 adjuvants (Richard et al. 2005). On the other hand, (Thongprakaisang et al. 2013)
310 observed that, in the range of concentrations described in the environment and at levels
311 to which humans and domestic animals are exposed, glyphosate increases the
312 proliferation of the hormone-dependent breast cancer T47D cell line, acting via ER α . In
313 this cell line, glyphosate exhibits estrogenic activity and interferes with normal estrogen
314 signaling, probably through the activation of an estrogen response element (ERE) since
315 these responses can be blocked by the ER antagonist ICI 182780 (Thongprakaisang et
316 al. 2013). Mesnage et al. (2017) reported that glyphosate (≥ 10 $\mu\text{g}/\text{mL}$) promotes the
317 proliferation of estrogen-dependent MCF-7 human breast cancer cells and increases the
318 expression of an ERE-luciferase reporter gene (ERE-luc) in T47D-KBluc cells, the
319 latter of which can be blocked by the estrogen antagonist ICI. In MCF-7 cells, they
320 found a weak and unstable interaction (compared to estrogen) between glyphosate and
321 ER α and that glyphosate could activate this receptor although at relatively higher
322 concentrations than estradiol (Mesnage et al. 2017). Based on their results, these authors
323 proposed the hypothesis that glyphosate exerts its effects in a ligand-independent

324 pathway via the cAMP-dependent protein kinase A, which modulates the balance
325 between cell proliferation and apoptosis (Mesnage et al. 2017). They also showed that,
326 at the concentrations at which glyphosate causes estrogenic effects, glyphosate also
327 causes apoptosis and disturbance in liver cell mitochondrial respiration. Finally, they
328 found that commercial GBH formulations or their adjuvants alone did not exhibit
329 estrogenic effects acting through ER α (Mesnage et al. 2017).

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

335

336 ***4- In vivo effects in animal models***

337 *4.1 Fish and chicken*

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

342 Studying the effect of glyphosate in zebrafish (*Danio rerio*), Armiliato et al.
343 (2014) found a significant increase in the diameter of oocytes, raising the concern on the
344 effects of glyphosate on fish reproduction. Recently, Smith et al. (2019) showed that
345 Roundup® and its active ingredient glyphosate can induce developmental, reproductive,
346 and epigenetic effects on Japanese medaka fish. In addition, in the testes of these fish
347 exposed to 0.5 mg/L Roundup® or glyphosate, the expression of FSHR was
348 significantly reduced, whereas in the ovaries the expression of FSHR remained

349 unchanged (Smith et al. 2019). In zebrafish (*Danio rerio*) embryos, glyphosate
350 treatment inhibited the activity of carbonic anhydrase, caused production of reactive
351 oxygen species, especially in branchial regions, triggered cellular apoptosis and induced
352 several types of malformations, including pericardial edema, yolk sac edema, spinal
353 curvature and body malformation in a dose-dependent manner (Sulukan et al. 2017).

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

359 *4.2 Effects on the ovary of rats, mice and ewe lambs*

360 Within the ovary, signaling between granulosa and theca cells is essential to
361 support follicle development, oocyte development, and ovulation. Moreover, the
362 ovarian follicle can be considered a very fragile micro-environment where several
363 interactions between hormones, growth factors, the oocyte and its surrounding somatic
364 cells are essential to generate a fully competent oocyte. Disruption of this finely tuned
365 balance can lead to an incomplete germ cell nest breakdown (multi-oocyte follicles)
366 (Pepling 2012), alter the activation of follicles with an increase of atresia (Monniaux
367 2018) or anovulation (Petro et al. 2012). Due to this multiple interaction and
368 complexity, ovaries and follicles are excellent targets to investigate endocrine-
369 disrupting effects. However, the effects of the exposure to glyphosate or GBHs on
370 ovarian function and stem cell differentiation are still largely unknown. In female rats
371 exposed to GBHs (Paraquat and/or Roundup®) during the first days of pregnancy,
372 Almeida et al. (2017) found lower weight ovaries and a decrease in the number of
373 corpora lutea. In mice exposed *in utero* to glyphosate a decreased ovarian weight and

374 histopathological alterations, increased atretic follicles, interstitial fibrosis and
375 decreased mature follicles on gestational day 19 was described (Ren et al. 2018). Serum
376 levels of progesterone and estrogen were significantly altered following glyphosate
377 exposure together with changes in the expression of Gonadotropin-releasing hormone
378 (GnRH), luteinizing hormone receptor (LHR), Follicle Stimulating Hormone Receptor
379 (FSHR), 3β -hydroxysteroid dehydrogenase (3β -HSD) and cytochrome P450, family 19,
380 subfamily A, polypeptide 1 (Cyp19a1) genes at the hypothalamic-pituitary-ovarian axis;
381 in the ovary oxidative stress increased (Ren et al. 2018). Similar results were observed
382 by Hamdaoui et al. (2018) in female rats exposed to sub-chronic doses of the GBH
383 Kalach 360 SL showing impaired folliculogenesis, altered ovary development,
384 decreased estrogen secretion, oxidative stress and altered ovarian morphology. Results
385 suggesting that this GBH can induce endocrine-disrupting effects.

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

396 The developmental origins of health and disease (DOHaD) paradigm posits that,
397 during development, there are sensitive windows in which tissue formation and function
398 can be modified by environmental stressors (such as glyphosate or GBHs), which can

399 lead to increased susceptibility to adverse health outcomes across the life course (Luque
400 et al. 2018). All the *in vivo* studies performed in animal models previously commented
401 regarding the histophysiology of the ovary and follicles suggest that, when the exposure
402 to GBHs or glyphosate occurs during development, these compounds may induce
403 endocrine-disrupting effects observed long after the EDC exposure has ended.

404 *4.3 Effects on pregnancy outcome and genital tracts of rats, mice and ewe lambs*

405 Estrogens and xenoestrogens probably exert most or all of their effects through a
406 specific receptor and the effects depend on the dose, time, and probably the duration of
407 exposure; such receptors are present in the brain, pituitary, gonads, and accessory sex
408 organs at one or another time during fetal, prepubertal, or adult life (Toppari et al.
409 1996).

410 Even though most of the endocrine disruptive effects of glyphosate and GBHs
411 have been demonstrated in males (Dallegrave et al. 2007; Pham et al. 2019; Romano et
412 al. 2012; Romano et al. 2010), some recent studies have also shown adverse effects on
413 development and functionality of the female reproductive tract. In the uterus of ewe
414 lambs, we have recently found decreased cell proliferation but no alterations in the
415 histomorphology after early postnatal oral or subcutaneous exposure to GBH (Alarcón
416 et al. 2019a). In addition, in subcutaneously GBH-exposed ewe lambs, a decreased
417 uterine cell proliferation in association with an increased expression of the insulin-like
418 growth factor binding protein 3 (IGFBP-3) gene and p27 protein and a deregulated
419 expression of ER α and PR was found (Alarcon et al. 2020). Furthermore, GBH
420 exposure decreased the expression of Wnt5a and forkhead box protein A2 (Foxa2) in
421 glandular epithelium, Wnt7a and homeobox a10 (Hoxa10) in subepithelial stroma, and
422 β -catenin in luminal and glandular epithelia (Alarcon et al. 2020). The wingless-type
423 MMTV integration site family members (Wnt) are a group of signal transduction

424 pathways of morphogenetic proteins that participate in female reproductive physiology
425 and control essential developmental processes, such as embryonic patterning, cell
426 growth, migration, and differentiation (Hayashi et al. 2011). In the endometrium,
427 estrogen induces Wnt signaling in both an ER-dependent and an ER-independent
428 manner (Susheelamma et al. 2018). Diethylstilbestrol, a synthetic estrogen, in the mouse
429 endometrium represses Wnt7a expression, causing a range of uterine defects similar to
430 those seen in Wnt7a knockout mice (Fan et al. 2012; Miller et al. 1998). Estrogens also
431 up-regulate both ER and progesterone receptor (PR) gene expression in the uteri of
432 several species (Ing and Tornesi 1997).

433 In adult ovariectomized rats, Varayoud et al. (2017) found that neonatal
434 exposure to a low dose of GBH (0.5 mg GBH/kg/day) did not affect the uterine weight
435 or epithelial proliferation but led to an increase in the luminal epithelial cell height. This
436 suggests that GBH modulates the expression of estrogen-sensitive genes and that the
437 uterine gene expression of ER α , ER β and PR is deregulated (Varayoud et al. 2017).
438 Guerrero Schimpf et al. (2017) found that neonatal exposure of female rats to GBH (2
439 mg/kg/day) increased cell proliferation in the uterine luminal and stromal compartments
440 on PND8 and altered the expression of proteins involved in the differentiation of uterine
441 organogenesis. ER α was induced in the uterine stromal compartment on PND8 and
442 downregulated in the luminal epithelial compartment on PND21 (Guerrero Schimpf et
443 al. 2017). In a further study, these authors found that early postnatal exposure of female
444 rats to GBH led to an enhanced sensitivity of the adult uterus to an exogenous treatment
445 with estrogen and to histomorphological and molecular changes associated with uterine
446 hyperplasia and a deregulation of uterine genes like Wnt7a, ER α , PR and Hoxa10
447 (Guerrero Schimpf et al. 2018). Moreover, rats exposed to the same doses of GBH (i.e.
448 2 mg/kg/day) during the neonatal period showed fertility failures (Ingaramo et al. 2017;

449 Ingaramo et al. 2016). The main long-term alterations observed were associated with
450 low PR expression associated with the downregulated expression of COUP transcription
451 factor 2 (COUP-TFII, also known as Nr2f2) and bone morphogenetic protein 2 (Bmp2)
452 mRNA and the increase in Hoxa10 and the cell proliferation at the implantation site
453 (Ingaramo et al. 2016).

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

471 Dechartres et al. (2019) observed that the treatment of rats with glyphosate and
472 Roundup® did not alter litter characteristics such as length, weight, and sex ratio,
473 whereas Ren et al. (2018) demonstrated that prenatal exposure of mice to pure

474 glyphosate influenced the sex ratio of litters. Milesi et al. (2018) exposed pregnant rats
475 (F0) to GBH (200 mg/kg/day) along pregnancy and found that this exposure impaired
476 the reproductive performance of F1 females and induced structural congenital anomalies
477 (conjoined fetuses and abnormally developed limbs) in the F2 offspring.

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

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

491

492 ***5- Evidences that support the hypothesis that glyphosate and GBHs are endocrine*** 493 ***disruptors***

494 The safety of glyphosate and GBHs has been intensely debated. The “low-dose
495 effects” of estrogenic agonists in mammals have generated considerable concern (Luque
496 et al. 2018). The term “low-dose effects” is defined as the biological changes that occur
497 at environmentally relevant exposure levels or at doses that are lower than those
498 reported in the standard toxicity testing guidelines of the EPA. Moreover, the

499 estrogenicity of the dose effects of estrogenic agonists varies significantly among
500 species, as well as between *in vivo* and *in vitro* tests. In the regulatory field regarding
501 chemicals with endocrine-disrupting properties in humans and wildlife, the WHO
502 defines them as those that may have a) adverse effects, b) endocrine activity, and c)
503 plausible mechanistic links between the observed endocrine activity and adverse effects
504 (Serra et al. 2019).

505 The first evidences that suggested that glyphosate may be an endocrine-
506 disrupting chemical came from studies in male reproduction (Anifandis et al. 2018; Dai
507 et al. 2016; Johansson et al. 2018; Pham et al. 2019; Romano et al. 2012; Romano et al.
508 2010; Vanlaeys et al. 2018). In male mice, Dai et al. (2016) and Pham et al. (2019)
509 found that glyphosate and GBHs were able to cause endocrine-disrupting effects on
510 male reproduction at low doses. In many of these studies, the endocrine-disrupting
511 effects observed could be attributed to glyphosate itself and/or to the additives in the
512 formulations (Benachour and Seralini 2009; Martini et al. 2016; Richard et al. 2005;
513 Vanlaeys et al. 2018). The different endocrine-disrupting effects caused by different
514 formulations with glyphosate suggest that the effects could be exerted by other
515 constituents of the formulation besides the active principle (several of which are
516 unknown) (Defarge et al. 2016; Jacques et al. 2019). Defarge et al. (2016) tested the
517 endocrine-disrupting effects of the co-formulants in six GBHs and found inhibition of
518 aromatase at levels lower than the lowest agricultural dilution recommended. These
519 results may suggest that the action of glyphosate on aromatase could explain, at least in
520 part, some of the effects on reproduction found in *in vivo* experiments (Richard et al.
521 2005).

522 Both *in vitro* and *in vivo* studies have suggested that glyphosate and GBHs act as
523 xenoestrogens through ERE activation (Lorenz et al. 2019; Thongprakaisang et al.

524 2013). In rats, perinatal exposure to GBH induce decreased DNA methylation in the
525 uterine site of the ER α gene, that might participate in the transcriptional upregulated
526 expression of total ER α mRNA (Lorenz et al. 2019). As mentioned previously,
527 glyphosate showed estrogenic activity and interferes with estrogen signaling, probably
528 through the activation of an estrogen response element (ERE) since these responses can
529 be blocked by the ER antagonist ICI 182780 (Thongprakaisang et al. 2013). Exposure to
530 a GBH causes long-term epigenetic disruption of the uterine ER α gene, which could be
531 associated with the GBH-induced implantation failures.

532 *In vitro* studies showing higher toxicity of GBHs *versus* glyphosate may explain
533 the numerous *in vivo* results with GBHs not seen with glyphosate alone (Defarge et al.
534 2016; Johansson et al. 2018; Vandenberg et al. 2017). It is worth mentioning that
535 glyphosate alone and commercial formulations might affect different endpoints or have
536 effects at different ages (Manservigi et al. 2019; Pham et al. 2019; Ren et al. 2018)
537 (Table 2). The latter might also contribute to the controversy on the toxicity of
538 glyphosate *versus* GBHs; further *in vivo* studies must be conducted to evaluate the
539 effects of glyphosate alone, GBHs and different adjuvants. The emerging field of omics,
540 which refers to the large-scale data-rich biological measurement of the genome, might
541 contribute to identifying individual risk and susceptibility of disease through targeted
542 biomarkers that reconstruct past exposure and predict future risk (Messerlian et al.
543 2017). These new technologies will advance our understanding on the impact of
544 agrochemicals as endocrine disruptors on animal and human health.

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

547

548 **6- Conclusions**

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

567 If used safely, GBHs could be extremely useful for the agricultural industry.
568 However, there are evidences that the misuse of these compounds has led to adverse
569 consequences, including the contamination of soils and rivers and the accumulation of
570 residues in the food chain. This contamination has led non-target species, including
571 humans, to be exposed to these compounds. Currently, the use of glyphosate as GBHs is
572 increasing worldwide and the environmental contamination levels show high
573 concentrations of these compounds, with levels significantly higher in countries or areas

574 where agriculture is more intense. As described before, it has been demonstrated that
575 animals (like rats, ewe lambs, cows) neonatally exposed to glyphosate and/or GBHs
576 show altered ovarian and uterus development. These effects in turn alter tissue
577 morphology and functioning, suggesting adverse effects on future fertility. Increasing
578 trends in fertility failures in different species, including humans, could be related, in
579 many cases, to the effects of EDCs present in the environment. Due to the high abortion
580 rates observed in women living in agricultural and rural areas, the use of pesticides and
581 their potential effects create strong concern. The advances in research about the effects
582 of glyphosate are of absolute interest to the world population due to the massive use of
583 this compound worldwide. *In vitro* studies in mammalian cells describing the
584 mechanisms of glyphosate have shown that glyphosate and GBHs interact with ER α ,
585 affecting signaling pathways involved in the control of cell proliferation (Figure 1).
586 Studies have also shown that glyphosate and GBHs affect the normal expression of
587 aromatase. It is important to point out that most of the studies focused on the endocrine-
588 disrupting effects of glyphosate in endocrine-dependent tissues (ovary and uterus)
589 presented in this review suggest mechanisms at very low concentrations, which would
590 be undetected in traditional toxicology studies. It is clear that several studies have
591 shown that glyphosate is able to cause endocrine disruption alone or in its formulations
592 depending on the levels and time of exposure. In the environment, however, we are
593 exposed not only to pure glyphosate but also to different formulations and pesticide
594 mixes. Since most of the formulations are different regarding their adjuvants, it is
595 difficult to assert which ones are more dangerous than glyphosate alone. Moreover,
596 some formulations are patent protected, so their components are unknown. Thus, it
597 would be important to reduce the danger through the use of more harmless adjuvants. In
598 addition, beyond the fact that adjuvants may have effects, the adverse health effects

599 reported by the exposure of glyphosate in its pure form or in combination should be
600 taken in consideration by regulatory agencies to establish better criteria on the safe use
601 of this substance and to tailor health prevention strategies.

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

612

613 REFERENCES

- 614 Acquavella JF, Alexander BH, Mandel JS, et al. (2004) Glyphosate biomonitoring for farmers
615 and their families: results from the Farm Family Exposure Study. *Environmental*
616 *health perspectives* 112(3):321-6 doi:10.1289/ehp.6667
- 617 Alarcón R, Ingaramo PI, Rivera OE, et al. (2019a) Neonatal exposure to a glyphosate-based
618 herbicide alters the histofunctional differentiation of the ovaries and uterus in
619 lambs. *Molecular and cellular endocrinology* 482:45-56
620 doi:10.1016/j.mce.2018.12.007
- 621 Alarcon R, Rivera O, Ingaramo PI, et al. (2020) Neonatal exposure to a glyphosate-based
622 herbicide alters the uterine differentiation of prepubertal ewe lambs. *Environmental*
623 *Pollution* doi:https://doi.org/10.1016/j.envpol.2020.114874
- 624 Albanil Sanchez JA, da Costa Klosterhoff M, Romano LA, De Martinez Gaspar Martins C
625 (2019) Histological evaluation of vital organs of the livebearer *Jenynsia multidentata*
626 (*Jenyns, 1842*) exposed to glyphosate: A comparative analysis of Roundup((R))
627 formulations. *Chemosphere* 217:914-924 doi:10.1016/j.chemosphere.2018.11.020
- 628 Almeida LL, Teixeira AAC, Soares AF, et al. (2017) Effects of melatonin in rats in the initial
629 third stage of pregnancy exposed to sub-lethal doses of herbicides. *Acta*
630 *histochemica* 119(3):220-227 doi:10.1016/j.acthis.2017.01.003
- 631 Amereh F, Babaei M, Eslami A, Fazelipour S, Rafiee M (2020) The emerging risk of exposure
632 to nano(micro)plastics on endocrine disturbance and reproductive toxicity: From a
633 hypothetical scenario to a global public health challenge. *Environ Pollut* 261:114158
634 doi:10.1016/j.envpol.2020.114158

- 635 Anadon A, Martinez-Larranaga MR, Martinez MA, et al. (2009) Toxicokinetics of glyphosate
636 and its metabolite aminomethyl phosphonic acid in rats. *Toxicology letters*
637 190(1):91-5 doi:10.1016/j.toxlet.2009.07.008
- 638 Anifandis G, Katsanaki K, Lagodoti G, et al. (2018) The Effect of Glyphosate on Human
639 Sperm Motility and Sperm DNA Fragmentation. *International journal of*
640 *environmental research and public health* 15(6) doi:10.3390/ijerph15061117
- 641 Aparicio VC, De Geronimo E, Marino D, Primost J, Carriquiriborde P, Costa JL (2013)
642 Environmental fate of glyphosate and aminomethylphosphonic acid in surface
643 waters and soil of agricultural basins. *Chemosphere* 93(9):1866-73
644 doi:10.1016/j.chemosphere.2013.06.041
- 645 Armiliato N, Ammar D, Nezzi L, Straliotto M, Muller YM, Nazari EM (2014) Changes in
646 ultrastructure and expression of steroidogenic factor-1 in ovaries of zebrafish *Danio*
647 *rerio* exposed to glyphosate. *Journal of toxicology and environmental health Part A*
648 77(7):405-14 doi:10.1080/15287394.2014.880393
- 649 Benachour N, Seralini GE (2009) Glyphosate formulations induce apoptosis and necrosis in
650 human umbilical, embryonic, and placental cells. *Chemical research in toxicology*
651 22(1):97-105 doi:10.1021/tx800218n
- 652 Benbrook CM (2016) Trends in glyphosate herbicide use in the United States and globally.
653 *Environmental sciences Europe* 28(1):3 doi:10.1186/s12302-016-0070-0
- 654 Bento CPM, Goossens D, Rezaei M, et al. (2017) Glyphosate and AMPA distribution in wind-
655 eroded sediment derived from loess soil. *Environ Pollut* 220(Pt B):1079-1089
656 doi:10.1016/j.envpol.2016.11.033
- 657 Bergman A, Heindel JJ, Kasten T, et al. (2013) The impact of endocrine disruption: a
658 consensus statement on the state of the science. *Environmental health perspectives*
659 121(4):A104-6 doi:10.1289/ehp.1205448
- 660 Brehm E, Flaws JA (2019) Transgenerational Effects of Endocrine-Disrupting Chemicals on
661 Male and Female Reproduction. *Endocrinology* 160(6):1421-1435
662 doi:10.1210/en.2019-00034
- 663 Burella PM, Odetti LM, Simoniello MF, Poletta GL (2018) Oxidative damage and antioxidant
664 defense in *Caiman latirostris* (Broad-snouted caiman) exposed in ovo to pesticide
665 formulations. *Ecotoxicology and environmental safety* 161:437-443
666 doi:10.1016/j.ecoenv.2018.06.006
- 667 Cassault-Meyer E, Gress S, Seralini GE, Galeraud-Denis I (2014) An acute exposure to
668 glyphosate-based herbicide alters aromatase levels in testis and sperm nuclear
669 quality. *Environmental toxicology and pharmacology* 38(1):131-40
670 doi:10.1016/j.etap.2014.05.007
- 671 Castro-Rivera E, Safe S (1998) Estrogen- and antiestrogen-responsiveness of HEC1A
672 endometrial adenocarcinoma cells in culture. *The Journal of steroid biochemistry*
673 *and molecular biology* 64(5-6):287-95
- 674 Clair E, Mesnage R, Travert C, Seralini GE (2012) A glyphosate-based herbicide induces
675 necrosis and apoptosis in mature rat testicular cells in vitro, and testosterone
676 decrease at lower levels. *Toxicology in vitro : an international journal published in*
677 *association with BIBRA* 26(2):269-79 doi:10.1016/j.tiv.2011.12.009
- 678 Colborn T, Dumanoski D, Myers JP (1997) *Our Stolen Future: Are We Threatening Our*
679 *Fertility, Intelligence, and Survival? A Scientific Detective Story.* . New York: Plume
- 680 Connolly A, Basinas I, Jones K, et al. (2018) Characterising glyphosate exposures among
681 amenity horticulturists using multiple spot urine samples. *International journal of*
682 *hygiene and environmental health* 221(7):1012-1022 doi:10.1016/j.ijheh.2018.06.007
- 683 Conrad A, Schroter-Kermani C, Hoppe HW, Ruther M, Pieper S, Kolossa-Gehring M (2017)
684 Glyphosate in German adults - Time trend (2001 to 2015) of human exposure to a
685 widely used herbicide. *International journal of hygiene and environmental health*
686 220(1):8-16 doi:10.1016/j.ijheh.2016.09.016

- 687 Cha J, Bartos A, Park C, et al. (2014) Appropriate crypt formation in the uterus for embryo
688 homing and implantation requires Wnt5a-ROR signaling. *Cell reports* 8(2):382-92
689 doi:10.1016/j.celrep.2014.06.027
- 690 Dai P, Hu P, Tang J, Li Y, Li C (2016) Effect of glyphosate on reproductive organs in male rat.
691 *Acta histochemica* 118(5):519-26 doi:10.1016/j.acthis.2016.05.009
- 692 Dallegre E, Mantese FD, Oliveira RT, Andrade AJ, Dalsenter PR, Langeloh A (2007) Pre- and
693 postnatal toxicity of the commercial glyphosate formulation in Wistar rats. *Archives*
694 *of toxicology* 81(9):665-73 doi:10.1007/s00204-006-0170-5
- 695 Darbre PD (2018) Overview of air pollution and endocrine disorders. *International journal of*
696 *general medicine* 11:191-207 doi:10.2147/IJGM.S102230
- 697 Darbre PD (2019) The history of endocrine-disrupting chemicals. *Current Opinion in*
698 *Endocrine and Metabolic Research* 7:26-33
- 699 Davoren MJ, Schiestl RH (2018) Glyphosate-based herbicides and cancer risk: a post-IARC
700 decision review of potential mechanisms, policy and avenues of research.
701 *Carcinogenesis* 39(10):1207-1215 doi:10.1093/carcin/bgy105
- 702 De Almeida LKS, Pletschke BI, Frost CL (2018) Moderate levels of glyphosate and its
703 formulations vary in their cytotoxicity and genotoxicity in a whole blood model and
704 in human cell lines with different estrogen receptor status. *3 Biotech* 8(10):438
705 doi:10.1007/s13205-018-1464-z
- 706 Dechartres J, Pawluski JL, Gueguen MM, et al. (2019) Glyphosate and Glyphosate-based
707 herbicide exposure during the peripartum period affects maternal brain plasticity,
708 maternal behavior and microbiome. *Journal of neuroendocrinology*:e12731
709 doi:10.1111/jne.12731
- 710 Defarge N, Spiroux de Vendomois J, Seralini GE (2018) Toxicity of formulants and heavy
711 metals in glyphosate-based herbicides and other pesticides. *Toxicology reports*
712 5:156-163 doi:10.1016/j.toxrep.2017.12.025
- 713 Defarge N, Takacs E, Lozano VL, et al. (2016) Co-Formulants in Glyphosate-Based Herbicides
714 Disrupt Aromatase Activity in Human Cells below Toxic Levels. *International journal*
715 *of environmental research and public health* 13(3) doi:10.3390/ijerph13030264
- 716 Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, et al. (2009) Endocrine-disrupting
717 chemicals: an Endocrine Society scientific statement. *Endocrine reviews* 30(4):293-
718 342 doi:10.1210/er.2009-0002
- 719 Dornelles MF, Oliveira GT (2016) Toxicity of atrazine, glyphosate, and quinclorac in bullfrog
720 tadpoles exposed to concentrations below legal limits. *Environmental science and*
721 *pollution research international* 23(2):1610-20 doi:10.1007/s11356-015-5388-4
- 722 Dunlap KA, Filant J, Hayashi K, et al. (2011) Postnatal deletion of Wnt7a inhibits uterine
723 gland morphogenesis and compromises adult fertility in mice. *Biology of*
724 *reproduction* 85(2):386-96 doi:10.1095/biolreprod.111.091769
- 725 EFSA (2015) Conclusion on the peer review of the pesticide risk assessment of the active
726 substance glyphosate. *EFSA Journal* doi:https://doi.org/10.2903/j.efsa.2018.5263
- 727 Fan X, Krieg S, Hwang JY, et al. (2012) Dynamic regulation of Wnt7a expression in the
728 primate endometrium: implications for postmenstrual regeneration and secretory
729 transformation. *Endocrinology* 153(3):1063-9 doi:10.1210/en.2011-1826
- 730 Fathi MA, Abdelghani E, Shen D, et al. (2019) Effect of in ovo glyphosate injection on
731 embryonic development, serum biochemistry, antioxidant status and
732 histopathological changes in newly hatched chicks. *Journal of animal physiology and*
733 *animal nutrition* doi:10.1111/jpn.13181
- 734 Ford B, Bateman LA, Gutierrez-Palominos L, Park R, Nomura DK (2017) Mapping Proteome-
735 wide Targets of Glyphosate in Mice. *Cell chemical biology*
736 doi:10.1016/j.chembiol.2016.12.013

- 737 Gasnier C, Dumont C, Benachour N, Clair E, Chagnon MC, Seralini GE (2009) Glyphosate-
738 based herbicides are toxic and endocrine disruptors in human cell lines. *Toxicology*
739 262(3):184-91 doi:10.1016/j.tox.2009.06.006
- 740 Gigante P, Berni M, Bussolati S, et al. (2018) Glyphosate affects swine ovarian and adipose
741 stromal cell functions. *Animal reproduction science*
742 doi:10.1016/j.anireprosci.2018.05.023
- 743 Ginsberg GL, Pullen Fedinick K, Solomon GM, et al. (2019) New Toxicology Tools and the
744 Emerging Paradigm Shift in Environmental Health Decision-Making. *Environmental*
745 *health perspectives* 127(12):125002 doi:10.1289/EHP4745
- 746 Gore AC, Chappell VA, Fenton SE, et al. (2015) EDC-2: The Endocrine Society's Second
747 Scientific Statement on Endocrine-Disrupting Chemicals. *Endocrine reviews* 36(6):E1-
748 E150 doi:10.1210/er.2015-1010
- 749 Guerrero Schimpf M, Milesi MM, Ingaramo PI, Luque EH, Varayoud J (2017) Neonatal
750 exposure to a glyphosate based herbicide alters the development of the rat uterus.
751 *Toxicology* 376:2-14 doi:10.1016/j.tox.2016.06.004
- 752 Guerrero Schimpf M, Milesi MM, Luque EH, Varayoud J (2018) Glyphosate-based herbicide
753 enhances the uterine sensitivity to estradiol in rats. *The Journal of endocrinology*
754 doi:10.1530/JOE-18-0207
- 755 Guyton KZ, Loomis D, Grosse Y, et al. (2015) Carcinogenicity of tetrachlorvinphos, parathion,
756 malathion, diazinon, and glyphosate. *The Lancet Oncology* 16(5):490-1
757 doi:10.1016/S1470-2045(15)70134-8
- 758 Hall JM, Greco CW (2019) Perturbation of Nuclear Hormone Receptors by Endocrine
759 Disrupting Chemicals: Mechanisms and Pathological Consequences of Exposure. *Cells*
760 9(1) doi:10.3390/cells9010013
- 761 Hamdaoui L, Naifar M, Rahmouni F, et al. (2018) Subchronic exposure to kalach 360 SL-
762 induced endocrine disruption and ovary damage in female rats. *Archives of*
763 *physiology and biochemistry* 124(1):27-34 doi:10.1080/13813455.2017.1352606
- 764 Hayashi K, Erikson DW, Tilford SA, et al. (2009) Wnt genes in the mouse uterus: potential
765 regulation of implantation. *Biology of reproduction* 80(5):989-1000
766 doi:10.1095/biolreprod.108.075416
- 767 Hayashi K, Yoshioka S, Reardon SN, et al. (2011) WNTs in the neonatal mouse uterus:
768 potential regulation of endometrial gland development. *Biology of reproduction*
769 84(2):308-19 doi:10.1095/biolreprod.110.088161
- 770 Hou X, Tan Y, Li M, Dey SK, Das SK (2004) Canonical Wnt signaling is critical to estrogen-
771 mediated uterine growth. *Mol Endocrinol* 18(12):3035-49 doi:10.1210/me.2004-0259
- 772 Ing NH, Tornesi MB (1997) Estradiol up-regulates estrogen receptor and progesterone
773 receptor gene expression in specific ovine uterine cells. *Biology of reproduction*
774 56(5):1205-15 doi:10.1095/biolreprod56.5.1205
- 775 Ingaramo PI, Guerrero Schimpf M, Milesi MM, Luque EH, Varayoud J (2019) Acute uterine
776 effects and long-term reproductive alterations in postnatally exposed female rats to
777 a mixture of commercial formulations of endosulfan and glyphosate. *Food and*
778 *chemical toxicology : an international journal published for the British Industrial*
779 *Biological Research Association* 134:110832 doi:10.1016/j.fct.2019.110832
- 780 Ingaramo PI, Varayoud J, Milesi MM, et al. (2017) Neonatal exposure to a glyphosate-based
781 herbicide alters uterine decidualization in rats. *Reprod Toxicol* 73:87-95
782 doi:10.1016/j.reprotox.2017.07.022
- 783 Ingaramo PI, Varayoud J, Milesi MM, Schimpf MG, Munoz-de-Toro M, Luque EH (2016)
784 Effects of neonatal exposure to a glyphosate-based herbicide on female rat
785 reproduction. *Reproduction* 152(5):403-15 doi:10.1530/REP-16-0171
- 786 Jacques MT, Bornhorst J, Soares MV, Schwerdtle T, Garcia S, Avila DS (2019) Reprotoxicity of
787 glyphosate-based formulation in *Caenorhabditis elegans* is not due to the active

- 788 ingredient only. Environ Pollut 252(Pt B):1854-1862
789 doi:10.1016/j.envpol.2019.06.099
- 790 Jauhainen A, Rasanen K, Sarantila R, Nuutinen J, Kangas J (1991) Occupational exposure of
791 forest workers to glyphosate during brush saw spraying work. American Industrial
792 Hygiene Association journal 52(2):61-4 doi:10.1080/15298669191364334
- 793 Jefferson W, Newbold R, Padilla-Banks E, Pepling M (2006) Neonatal genistein treatment
794 alters ovarian differentiation in the mouse: inhibition of oocyte nest breakdown and
795 increased oocyte survival. Biology of reproduction 74(1):161-8
796 doi:10.1095/biolreprod.105.045724
- 797 Johansson HKL, Schwartz CL, Nielsen LN, et al. (2018) Exposure to a glyphosate-based
798 herbicide formulation, but not glyphosate alone, has only minor effects on adult rat
799 testis. Reprod Toxicol 82:25-31 doi:10.1016/j.reprotox.2018.09.008
- 800 Kongtip P, Nankongnab N, Phupanchaoensuk R, et al. (2017) Glyphosate and Paraquat in
801 Maternal and Fetal Serums in Thai Women. Journal of agromedicine 22(3):282-289
802 doi:10.1080/1059924X.2017.1319315
- 803 Krüger M, Schledorn P, Schrödl W, Hoppe H, Lutz W, Shehata A (2014) Detection of
804 Glyphosate Residues in Animals and Humans. Journal of Environmental & Analytical
805 Toxicology 4
- 806 Li Q, Lambrechts MJ, Zhang Q, et al. (2013) Glyphosate and AMPA inhibit cancer cell growth
807 through inhibiting intracellular glycine synthesis. Drug design, development and
808 therapy 7:635-43 doi:10.2147/DDDT.S49197
- 809 Lorenz V, Milesi MM, Schimpf MG, Luque EH, Varayoud J (2019) Epigenetic disruption of
810 estrogen receptor alpha is induced by a glyphosate-based herbicide in the
811 preimplantation uterus of rats. Molecular and cellular endocrinology 480:133-141
812 doi:10.1016/j.mce.2018.10.022
- 813 Luque E, Muñoz-de-Toro M, Ramos J (2018) Estrogenic Agonist. In: Skinner MK (ed)
814 Encyclopedia of Reproduction (Second Edition). Academic Press, Oxford.:753-759
815 doi:http://dx.doi.org/10.1016/B978-0-12-801238-3.64416-1
- 816 Manservisi F, Lesueur C, Panzacchi S, et al. (2019) The Ramazzini Institute 13-week pilot study
817 glyphosate-based herbicides administered at human-equivalent dose to Sprague
818 Dawley rats: effects on development and endocrine system. Environmental health : a
819 global access science source 18(1):15 doi:10.1186/s12940-019-0453-y
- 820 Martens MA, Bleeker MS, Leopold VA, Farmer DR (2019) Toxicology and human health risk
821 assessment of polyethoxylated tallow amine surfactant used in glyphosate
822 formulations. Regulatory toxicology and pharmacology : RTP
823 doi:10.1016/j.yrtph.2019.03.014
- 824 Martinez A, Reyes I, Reyes N (2007) [Cytotoxicity of the herbicide glyphosate in human
825 peripheral blood mononuclear cells]. Biomedica : revista del Instituto Nacional de
826 Salud 27(4):594-604
- 827 Martini CN, Gabrielli M, Brandani JN, Vila Mdel C (2016) Glyphosate Inhibits PPAR Gamma
828 Induction and Differentiation of Preadipocytes and is able to Induce Oxidative Stress.
829 Journal of biochemical and molecular toxicology 30(8):404-13 doi:10.1002/jbt.21804
- 830 Meftaul IM, Venkateswarlu K, Dharmarajan R, et al. (2020) Controversies over human health
831 and ecological impacts of glyphosate: Is it to be banned in modern agriculture?
832 Environ Pollut 263(Pt A):114372 doi:10.1016/j.envpol.2020.114372
- 833 Mertens M, Hoss S, Neumann G, Afzal J, Reichenbecher W (2018) Glyphosate, a chelating
834 agent-relevant for ecological risk assessment? Environmental science and pollution
835 research international 25(6):5298-5317 doi:10.1007/s11356-017-1080-1
- 836 Mesnage R, Benbrook C, Antoniou MN (2019) Insight into the confusion over surfactant co-
837 formulants in glyphosate-based herbicides. Food and chemical toxicology : an
838 international journal published for the British Industrial Biological Research
839 Association 128:137-145 doi:10.1016/j.fct.2019.03.053

- 840 Mesnage R, Defarge N, Spiroux de Vendomois J, Seralini GE (2015) Potential toxic effects of
841 glyphosate and its commercial formulations below regulatory limits. *Food and*
842 *chemical toxicology : an international journal published for the British Industrial*
843 *Biological Research Association* 84:133-53 doi:10.1016/j.fct.2015.08.012
- 844 Mesnage R, Moesch C, Grand R, et al. (2012) Glyphosate Exposure in a Farmer's Family.
845 *Journal of Environmental Protection* 3(9):1001-1003 doi:10.4236/jep.2012.39115.
- 846 Mesnage R, Phedonos A, Biserni M, et al. (2017) Evaluation of estrogen receptor alpha
847 activation by glyphosate-based herbicide constituents. *Food and chemical toxicology*
848 *: an international journal published for the British Industrial Biological Research*
849 *Association* 108(Pt A):30-42 doi:10.1016/j.fct.2017.07.025
- 850 Messerlian C, Martinez RM, Hauser R, Baccarelli AA (2017) 'Omics' and endocrine-disrupting
851 chemicals - new paths forward. *Nature reviews Endocrinology* 13(12):740-748
852 doi:10.1038/nrendo.2017.81
- 853 Milesi MM, Lorenz V, Beldomenico PM, Vaira S, Varayoud J, Luque EH (2019) Response to
854 comments on: Perinatal exposure to a glyphosate-based herbicide impairs female
855 reproductive outcomes and induces second-generation adverse effects in Wistar
856 rats. *Archives of toxicology* doi:10.1007/s00204-019-02609-0
- 857 Milesi MM, Lorenz V, Pacini G, et al. (2018) Perinatal exposure to a glyphosate-based
858 herbicide impairs female reproductive outcomes and induces second-generation
859 adverse effects in Wistar rats. *Archives of toxicology* 92(8):2629-2643
860 doi:10.1007/s00204-018-2236-6
- 861 Miller C, Degenhardt K, Sassoon DA (1998) Fetal exposure to DES results in de-regulation of
862 *Wnt7a* during uterine morphogenesis. *Nature genetics* 20(3):228-30
863 doi:10.1038/3027
- 864 Monniaux D (2018) Factors influencing establishment of the ovarian reserve and their effects
865 on fertility. *Proceedings of the 10th International Ruminant Reproduction*
866 *Symposium (IRRS 2018)*
867 doi:10.21451/1984-3143-AR2018-0011
- 868 Myers JP, Antoniou MN, Blumberg B, et al. (2016) Concerns over use of glyphosate-based
869 herbicides and risks associated with exposures: a consensus statement.
870 *Environmental health : a global access science source* 15:19 doi:10.1186/s12940-016-
871 0117-0
- 872 Niemann L, Sieke C, Pfeil R (2015) A critical review of glyphosate findings in human urine
873 samples and comparison with the exposure of operators and consumers. *Journal für*
874 *Verbraucherschutz und Lebensmittelsicherheit* 10(1):3-12
875 doi:https://doi.org/10.1007/s00003-014-0927-3
- 876 Parvez S, Gerona RR, Proctor C, et al. (2018) Glyphosate exposure in pregnancy and
877 shortened gestational length: a prospective Indiana birth cohort study.
878 *Environmental health : a global access science source* 17(1):23 doi:10.1186/s12940-
879 018-0367-0
- 880 Pepling ME (2012) Follicular assembly: mechanisms of action. *Reproduction* 143(2):139-49
881 doi:10.1530/REP-11-0299
- 882 Perego MC, Caloni F, Cortinovis C, et al. (2017a) Influence of a Roundup formulation on
883 glyphosate effects on steroidogenesis and proliferation of bovine granulosa cells in
884 vitro. *Chemosphere* 188:274-279 doi:10.1016/j.chemosphere.2017.09.007
- 885 Perego MC, Schutz LF, Caloni F, Cortinovis C, Albonico M, Spicer LJ (2017b) Evidence for
886 direct effects of glyphosate on ovarian function: glyphosate influences
887 steroidogenesis and proliferation of bovine granulosa but not theca cells in vitro.
888 *Journal of applied toxicology : JAT* 37(6):692-698 doi:10.1002/jat.3417

- 889 Petro EM, Leroy JL, Covaci A, et al. (2012) Endocrine-disrupting chemicals in human follicular
890 fluid impair in vitro oocyte developmental competence. *Hum Reprod* 27(4):1025-33
891 doi:10.1093/humrep/der448
- 892 Pham TH, Derian L, Kervarrec C, et al. (2019) Perinatal exposure to glyphosate and a
893 glyphosate-based herbicide affect spermatogenesis in mice. *Toxicological sciences :
894 an official journal of the Society of Toxicology* doi:10.1093/toxsci/kfz039
- 895 Poppe J, Bote K, Merle R, Makarova O, Roesler U (2019) Minimum Inhibitory Concentration
896 of Glyphosate and a Glyphosate-Containing Herbicide in *Salmonella enterica* Isolates
897 Originating from Different Time Periods, Hosts, and Serovars. *European journal of
898 microbiology & immunology* 9(2):35-41 doi:10.1556/1886.2019.00005
- 899 Rattan S, Flaws JA (2019) The epigenetic impacts of endocrine disruptors on female
900 reproduction across generations dagger. *Biology of reproduction* 101(3):635-644
901 doi:10.1093/biolre/ioz081
- 902 Ren X, Li R, Liu J, et al. (2018) Effects of glyphosate on the ovarian function of pregnant mice,
903 the secretion of hormones and the sex ratio of their fetuses. *Environ Pollut* 243(Pt
904 B):833-841 doi:10.1016/j.envpol.2018.09.049
- 905 Richard S, Moslemi S, Sipahutar H, Benachour N, Seralini GE (2005) Differential effects of
906 glyphosate and roundup on human placental cells and aromatase. *Environmental
907 health perspectives* 113(6):716-20
- 908 Rivera OE, Varayoud J, Rodriguez HA, Munoz-de-Toro M, Luque EH (2011) Neonatal exposure
909 to bisphenol A or diethylstilbestrol alters the ovarian follicular dynamics in the lamb.
910 *Reprod Toxicol* 32(3):304-12 doi:10.1016/j.reprotox.2011.06.118
- 911 Rodriguez HA, Santambrosio N, Santamaria CG, Munoz-de-Toro M, Luque EH (2010)
912 Neonatal exposure to bisphenol A reduces the pool of primordial follicles in the rat
913 ovary. *Reprod Toxicol* 30(4):550-7 doi:10.1016/j.reprotox.2010.07.008
- 914 Romano MA, Romano RM, Santos LD, et al. (2012) Glyphosate impairs male offspring
915 reproductive development by disrupting gonadotropin expression. *Archives of
916 toxicology* 86(4):663-73 doi:10.1007/s00204-011-0788-9
- 917 Romano RM, Romano MA, Bernardi MM, Furtado PV, Oliveira CA (2010) Prepubertal
918 exposure to commercial formulation of the herbicide glyphosate alters testosterone
919 levels and testicular morphology. *Archives of toxicology* 84(4):309-17
920 doi:10.1007/s00204-009-0494-z
- 921 Santovito A, Ruberto S, Gendusa C, Cervella P (2018) In vitro evaluation of genomic damage
922 induced by glyphosate on human lymphocytes. *Environmental science and pollution
923 research international* 25(34):34693-34700 doi:10.1007/s11356-018-3417-9
- 924 Serra H, Beausoleil C, Habert R, Minier C, Picard-Hagen N, Michel C (2019) Evidence for
925 Bisphenol B Endocrine Properties: Scientific and Regulatory Perspectives.
926 *Environmental health perspectives* 127(10):106001 doi:10.1289/EHP5200
- 927 Smith CM, Vera MKM, Bhandari RK (2019) Developmental and epigenetic effects of Roundup
928 and glyphosate exposure on Japanese medaka (*Oryzias latipes*). *Aquat Toxicol*
929 210:215-226 doi:10.1016/j.aquatox.2019.03.005
- 930 Stoker C, Beldomenico PM, Bosquiazzo VL, et al. (2008) Developmental exposure to
931 endocrine disruptor chemicals alters follicular dynamics and steroid levels in *Caiman
932 latirostris*. *General and comparative endocrinology* 156(3):603-12
933 doi:10.1016/j.ygcen.2008.02.011
- 934 Stur E, Aristizabal-Pachon AF, Peronni KC, et al. (2019) Glyphosate-based herbicides at low
935 doses affect canonical pathways in estrogen positive and negative breast cancer cell
936 lines. *PloS one* 14(7):e0219610 doi:10.1371/journal.pone.0219610
- 937 Sulukan E, Kokturk M, Ceylan H, et al. (2017) An approach to clarify the effect mechanism of
938 glyphosate on body malformations during embryonic development of zebrafish
939 (*Danio rerio*). *Chemosphere* 180:77-85 doi:10.1016/j.chemosphere.2017.04.018

- 940 Susheelamma CJ, Pillai SM, Asha Nair S (2018) Oestrogen, progesterone and stem cells: the
941 discordant trio in endometriosis? *Expert reviews in molecular medicine* 20:e2
942 doi:10.1017/erm.2017.13
- 943 Szezanowski F, Szezanowski LP, Mausberg AK, et al. (2018) Differential impact of pure
944 glyphosate and glyphosate-based herbicide in a model of peripheral nervous system
945 myelination. *Acta neuropathologica* 136(6):979-982 doi:10.1007/s00401-018-1938-4
- 946 Thongprakaisang S, Thiantanawat A, Rangkadilok N, Suriyo T, Satayavivad J (2013)
947 Glyphosate induces human breast cancer cells growth via estrogen receptors. *Food
948 and chemical toxicology : an international journal published for the British Industrial
949 Biological Research Association* 59:129-36 doi:10.1016/j.fct.2013.05.057
- 950 Toppari J, Larsen JC, Christiansen P, et al. (1996) Male reproductive health and
951 environmental xenoestrogens. *Environmental health perspectives* 104 Suppl 4:741-
952 803 doi:10.1289/ehp.96104s4741
- 953 Tsai MS, Chen MH, Lin CC, Liu CY, Chen PC (2019) Children's environmental health based on
954 birth cohort studies of Asia (2) - air pollution, pesticides, and heavy metals.
955 *Environmental research* 179(Pt A):108754 doi:10.1016/j.envres.2019.108754
- 956 US EPA (2015) Endocrine Disruptor Screening Program (EDSP) Estrogen Receptor Bioactivity.
957 Washington, DC: US Environ Prot Agency [https://www.epa.gov/endocrine-
958 disruption/endocrine-disruptor-screening-program-edsp-estrogen-receptor-
959 bioactivity](https://www.epa.gov/endocrine-disruption/endocrine-disruptor-screening-program-edsp-estrogen-receptor-bioactivity)
- 960 Van Bruggen AHC, He MM, Shin K, et al. (2018) Environmental and health effects of the
961 herbicide glyphosate. *The Science of the total environment* 616-617:255-268
962 doi:10.1016/j.scitotenv.2017.10.309
- 963 Vandenberg LN, Blumberg B, Antoniou MN, et al. (2017) Is it time to reassess current safety
964 standards for glyphosate-based herbicides? *Journal of epidemiology and community
965 health* 71(6):613-618 doi:10.1136/jech-2016-208463
- 966 Vanlaeys A, Dubuisson F, Seralini GE, Travert C (2018) Formulants of glyphosate-based
967 herbicides have more deleterious impact than glyphosate on TM4 Sertoli cells.
968 *Toxicology in vitro : an international journal published in association with BIBRA*
969 52:14-22 doi:10.1016/j.tiv.2018.01.002
- 970 Varayoud J, Durando M, Ramos JG, et al. (2017) Effects of a glyphosate-based herbicide on
971 the uterus of adult ovariectomized rats. *Environmental toxicology* 32(4):1191-1201
972 doi:10.1002/tox.22316
- 973 Vazquez DE, Iliina N, Pagano EA, Zavala JA, Farina WM (2018) Glyphosate affects the larval
974 development of honey bees depending on the susceptibility of colonies. *PloS one*
975 13(10):e0205074 doi:10.1371/journal.pone.0205074
- 976 Viguie C, Chaillou E, Gayraud V, Picard-Hagen N, Fowler PA (2020) Toward a better
977 understanding of the effects of endocrine disrupting compounds on health: Human-
978 relevant case studies from sheep models. *Molecular and cellular endocrinology*
979 505:110711 doi:10.1016/j.mce.2020.110711
- 980 Williams GM, Kroes R, Munro IC (2000) Safety evaluation and risk assessment of the
981 herbicide Roundup and its active ingredient, glyphosate, for humans. *Regulatory
982 toxicology and pharmacology : RTP* 31(2 Pt 1):117-65 doi:10.1006/rtph.1999.1371
- 983 Wrobel MH (2018) Glyphosate affects the secretion of regulators of uterine contractions in
984 cows while it does not directly impair the motoric function of myometrium in vitro.
985 *Toxicology and applied pharmacology* 349:55-61 doi:10.1016/j.taap.2018.04.031
- 986 Yin Y, Ma L (2005) Development of the mammalian female reproductive tract. *Journal of
987 biochemistry* 137(6):677-83 doi:10.1093/jb/mvi087
- 988 Zhao J, Pacenka S, Wu J, et al. (2018) Detection of glyphosate residues in companion animal
989 feeds. *Environ Pollut* 243(Pt B):1113-1118 doi:10.1016/j.envpol.2018.08.100

Journal Pre-proof

992 **LEGENDS TO FIGURES**

993

994 **Figure 1.** Schematic representation of the reported effects of the *in vitro* and *in vivo*
995 exposure to glyphosate or glyphosate-based herbicides (GBHs). 1) The exposure to
996 glyphosate or GBHs inhibits the transcription of the aromatase gene and the enzymatic
997 activity of aromatase (Cassault-Meyer et al. 2014; Gasnier et al. 2009; Richard et al.
998 2005). 2) The exposure to glyphosate or GBHs induces estrogen-like effects by
999 activating estrogen receptor (ER) through a ligand-independent manner by activating
1000 protein kinase A (PKA), which in turn induces ER phosphorylation, modulating its
1001 transcriptional activity by binding to estrogen response elements (ERE) or non-ERE
1002 promoter sequences of target genes (Mesnage et al. 2017). 3) Depending on the cell type
1003 or glyphosate nature (formulation) or concentration, the exposure to glyphosate or
1004 GBHs stimulates cell proliferation (Guerrero Schimpf et al. 2017; Mesnage et al. 2017;
1005 Thongprakaisang et al. 2013) or cell apoptosis by activating caspases 3 and/or 7
1006 (Benachour and Seralini 2009; Clair et al. 2012). 4) The exposure to glyphosate or
1007 GBHs induces the expression of estrogen-dependent proteins like progesterone receptor,
1008 Wnt7a or Hoxa10 (Guerrero Schimpf et al. 2017; Ingaramo et al. 2017; Mesnage et al.
1009 2017).

1010

1011 **Figure 2.** Summary of *in vivo* studies performed after neonatal (pre- and/or postnatal)
1012 exposure of female rats and ewe lambs to glyphosate-based herbicides (GBHs). The
1013 arrows begin at the time of exposure and the arrowhead points to the moment when
1014 animals were studied. The most relevant experimental results obtained are mentioned in
1015 italics. GBH: Glyphosate-based herbicide; IGF1: insulin-like growth factor; ER α :
1016 estrogen receptor α ; ER β : estrogen receptor β ; Hoxa10: homeobox a10; PR:
1017 progesterone receptor; Wnt5a wingless-type MMTV integration site family member 5a;
1018 Wnt7a: wingless-type MMTV integration site family member 7a; Insulin-like growth
1019 factor binding protein-3; Foxa 2: forkhead box protein A2; GDF9:
1020 Growth/differentiation factor 9. References: (Alarcón et al. 2019a; Alarcon et al. 2020;
1021 Guerrero Schimpf et al. 2017; Guerrero Schimpf et al. 2018; Ingaramo et al. 2019;
1022 Ingaramo et al. 2017; Ingaramo et al. 2016; Lorenz et al. 2019; Milesi et al. 2019;
1023 Milesi et al. 2018; Varayoud et al. 2017).

1024

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 $\mu\text{g}/\text{mL}$ of RU: decreased number of GCs and the production of E2 and P4. At 1 $\mu\text{g}/\text{mL}$ of RU + FSH + IGF1: increased cell number and steroid hormone. At 10 $\mu\text{g}/\text{mL}$ of RU + FSH: decreased cell number and steroid hormone production depending on IGF1 addition. At 10 $\mu\text{g}/\text{mL}$ of RU + FSH: increased E2 production.	1, 10 and 300 $\mu\text{g}/\text{mL}$	<i>In vitro</i> ; 48 hours	(Perego et al. 2017a)
	At 5 $\mu\text{g}/\text{mL}$ of GLY + FSH + IGF1: decreased GC number and E2 production. At 0.5 $\mu\text{g}/\text{mL}$ of GLY + FSH + IGF1: only decreased GC number. At 1.7 $\mu\text{g}/\text{mL}$ of GLY: increased GC proliferation.	0.5, 1.7 and 5 $\mu\text{g}/\text{mL}$	<i>In vitro</i> ; 48 hours	(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 $\text{PGF2}\alpha$, whereas RU decreased $\text{PGF2}\alpha$ and PGE2 secretion from endometrial cells.	0.11 and 10 ng/mL	<i>In vitro</i> ; 24, 48 or 72 hours	(Wrobel 2018)
Estuarine crab (<i>Neohelice granulata</i>)	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 (<i>Danio rerio</i>)	Increased diameter of previtellogenic I and vitellogenic oocytes. Higher expression of steroidogenic factor-1 in ovaries.	65 $\mu\text{g}/\text{L}$	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 ER α , 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 ER α , ER β and PR. Decreased mRNA expression of C3, ER α 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)

	Increased uterine cell proliferation and deregulated expression of ER α , ER β , 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)
	F1 females (<i>in utero</i> 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)
	ER α increased uterine expression. Epigenetic changes in the O-promoter of uterine ER α .	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; PGF2 α : prostaglandin F 2 α ; 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; ER α : 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

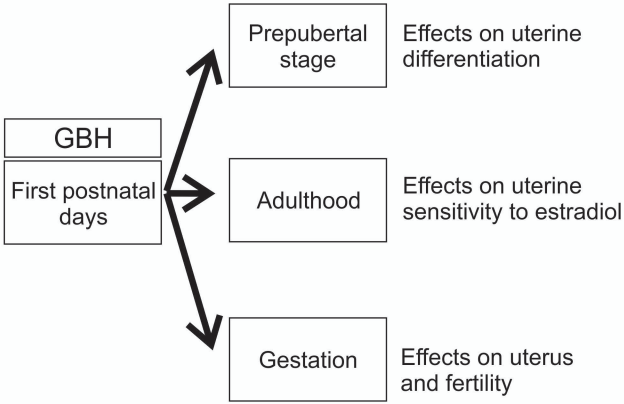
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Table 2: Similarities and divergences between the effects of pure glyphosate and those of glyphosate-based herbicides (GBHs)*.

Study	Glyphosate	GBHs
<i>In vitro</i>	Reduction in cell viability at concentrations of 10 and 50 $\mu\text{g}/\text{mL}$.	Reduction in cell viability at concentrations of 10 and 50 $\mu\text{g}/\text{mL}$.
	No effects on the number of granulosa cells or estrogen and progesterone production.	Roundup® decreases the number of granulosa cells and estrogen and progesterone production.
	No decreased cell viability at 500 $\mu\text{g}/\text{mL}$; initial toxicity at 1000 $\mu\text{g}/\text{mL}$.	No decreased cell viability at 500 $\mu\text{g}/\text{mL}$; initial toxicity in cell viability at 800 $\mu\text{g}/\text{mL}$.
	Glyphosate is more genotoxic than Wipeout® formulation and has a genotoxic effect similar to that of Roundup® in the HEC1A cell line.	Roundup® has a genotoxicity similar to that of glyphosate, and Wipeout® formulation is less genotoxic than glyphosate.
	In human ovarian and prostate cancer cells, it inhibits proliferation and promotes apoptosis.	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.	Increased oxytocin and decreased progesterone secretion from luteal cells.
	Estrogenic effects via ER α at higher doses compared to estrogen. The estrogenic effects may be ligand-independent.	GBH did not show ER α -activity at a concentration equivalent to that of glyphosate.
<i>In vivo</i>	Mice exposed <i>in utero</i> to glyphosate show decreased ovarian weight and histopathological alterations, increased atretic follicles and interstitial fibrosis, and decreased mature follicles on gestational day 19.	In female rats, GBH exposure during the first days of pregnancy induces lower weight ovaries and decreases the number of corpora lutea.
	Glyphosate has low effects on microbiome disruption.	GBHs contribute to 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 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.

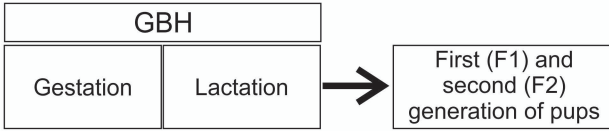
Rats



↑ incidence of Luminal epithelial hyperplasia and cell proliferation
 Alteration of $ER\alpha$, PR, Wnt7a and β -catenin protein expression in the uterus

↑ E2-induced cell proliferation and $ER\alpha$ and $ER\beta$ protein uterine expression

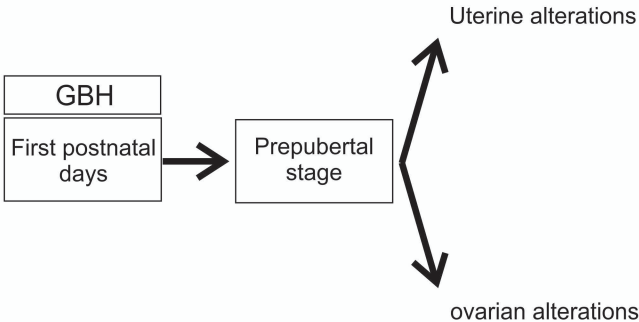
↑ post-implantation losses
 Alteration of $ER\alpha$, PR, Wnt5a and Wnt7a protein expression in the decidua



Effects in fertility in F1 generation

Morphological fetal alterations in F2 generation

Ewe lambs



↓ proliferation
 protein expression of p27, and
 ↑ mRNA expression of IGFBP-3 (unpublished)
 ↓ protein expression of $ER\alpha$, PR, Wnt5a, Wnt7a, β -catenin, Hoxa10 and Foxa2 (unpublished)

↓ ovarian FSH and GDF9 mRNA expression
 ↓ percentage of primordial follicles
 ↑ percentage of transitional and primary follicles
 ↑ percentage of antral atretic follicles
 ↑ proliferation of granulosa and theca cells
 ↓ response to oFSH stimulation (unpublished)

