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EFFECTS OF GLYPHOSATE AND ROUNDUP UPON MAMMALIAN GAMETES

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ABSTRACT

The wide use of glyphosate-based herbicides (GBHs) has become a controversial issue due to the potential harmful effects on human health. The neurologic, reproductive, endocrine and gastroenteric toxicity of glyphosate related to chronic exposure seems to be underestimate by official authorities since from recent literature it clearly appears that GBHs formulations for agricultural use can have deleterious effects on population, possibly being more toxic than glyphosate which is their active principle. Commercial formulations, among which Roundup is the most famous one, contain a number of surfactants and adjuvants inside; most of these are patented and not publicly known, therefore they can act differently from glyphosate alone and might strengthen its toxic effect. This could make glyphosate, as pure active substance, and Roundup not the same from a toxic standpoint.

The present study is focused on GBHs reproductive toxicity with a special regard to glyphosate and Roundup impact on male and female mammalian gametes after exposure to concentrations ranging from the one recommended for agricultural use (0.1% Roundup, containing 360 µg/mL glyphosate), to 70-fold lower or more.
Briefly, this PhD thesis includes:

- a study on boar semen quality after 1h and 3h glyphosate and Roundup exposure;
- a study on swine in oocytes after glyphosate and Roundup exposure during IVM period (44 h);
- a preliminary study on stallion semen quality after 1h glyphosate and Roundup exposure.

In the first study, boar semen was added with glyphosate or Roundup at 0-360 µg/mL concentrations and then incubated at 38 °C. After incubation, sperm motility was evaluated with a commercial computer-assisted sperm analysis (CASA) system, while flow cytometry was used to determine sperm viability, acrosome integrity, mitochondrial activity and DNA fragmentation.
Our results suggest that while both glyphosate and Roundup induce toxic effects on mammalian sperm function and survival, Roundup has much more detrimental impact than glyphosate, even at equivalent concentrations of glyphosate. Furthermore, based on our results, it can be hypothesized that the toxic effect of these pesticides on spermatozoa may be linked to an impairment in

mitochondrial activity and a subsequent decrease in ATP production and/or alterations in the redox balance, which impact cell motility and plasma membrane stability. In spite of this, DNA integrity seems not to be altered either by Roundup or pure glyphosate.

In the second study, using an in vitro model of pig oocyte maturation, we examined the impact of both glyphosate and Roundup on female gamete evaluating nuclear maturation, cytoplasmic maturation and developmental competence of oocytes, steroidogenic activity of cumulus cells as well as intracellular levels of glutathione (GSH) and ROS of oocytes. We found that glyphosate and Roundup exposure during IVM detrimentally affect the subsequent developmental ability of embryos, providing further evidence of their potential toxic effect on female reproductive system. Moreover, Roundup at the same glyphosate-equivalent concentrations resulted to be more toxic than pure glyphosate, altering steroidogenesis and increasing oocyte ROS levels.

In the last and preliminary study on the impact of glyphosate or Roundup on stallion semen, the parameters used for boar sperm analysis in the first study and as well as sperm ROS production were evaluated. Moreover, two additional concentrations were tested, a lower (0,5 µg/ml) and a higher one (720 µg/ml). This study was interrupted due to COVID emergency. Notwithstanding, the analysis of three stallion was completed giving some interesting indication.
Data obtained suggest that high doses of Roundup induce toxic effects on stallion sperm function and survival whereas glyphosate doesn't affect semen quality at any concentration. As stressed for swine, Roundup seems to induce damage on spermatozoa in a dose-dependent manner, affecting mitochondrial activity, motility, viability and acrosome integrity. Possibly, the decrease in mitochondrial activity is linked to the same tendence of sperm motility as a disfunction of mitochondria leads to lower levels of ATP, which represents the main engine to spermatozoa motility. It can also be hypothesized that adjuvants present in the commercial formulation act as surface-active agent on the cell membranes, thus causing spermatozoa death and alteration of acrosome membranes. High doses of Roundup also induce a decrease in the percentage of viable spermatozoa with active mitochondria not producing ROS, suggesting that GBHs may exert their toxicity also by impairing oxidative balance.

Therefore, according to our results, we can conclude that GBHs exert a negative impact on both male and female gametes and that Roundup adjuvants enhance glyphosate toxic effects and/or are biologically active in their side-effect. The specific mechanism of action of GBHs remains unclear and needs to be further investigated.

ABBREVIATIONS

ADHD	Attention deficit hyperactivity disorder
ADI	Acceptable Daily Intake
AHS	Agricultural Health Study
ALAT	Alanine aminotransferase
ALP	Alkaline phosphatase
AST	Aspartate aminotransferase
Bfr	German Federal Institute for Risk Assessment (German: Bundesinstitut für Risikobewertung)
CASA	Computer-aided Sperm Analysis
CDC	Centers for Disease Control and Prevention
COCs	Cumulus-oocyte-complexes
DCFH	2’-7’dichlorofluorescin diacetate
DMR	DNA methylation regions
EC_{50}	Half maximal effective concentration
EFSA	European Food Safety Authority
EPA	Enviromental Protection Agency
EPSPS	3-fosfoshikimato 1-carbossiviniltransferasi
F	Filial generation
GBHs	Glyphosate based herbicides
GLY	Glyphosate
GM	Genetically modified
GVBD	Germinal vescicle breakdown
IARC	International Agency for Research on Cancer
IUPAC	International Union of Pure and Applied Chemistry
ISPRA	Istituto Superiore per la Protezione e la Ricerca Ambientale
IVM	In vitro maturation
LD_{50}	Lethal Dose 50
LDH	Lactate Dehydrogenase

Abbreviations

LH	Luteinizing Hormone
LOAEL	Lowest Observed Adverse Effect Level)
MII	Mataphase II
NASS	National Agricultural Statistics Services
NHL	Non-Hodgkin lymphoma
NOAEL	No Observed Adverse Effect Level
OGM	Genetically modified organism
PI	Propidium iodide
R	Roundup
RAR	Renewal assessment report
ROS	Reactive oxygen species
STAR	Steroidogenic acute regulatory protein
TGC	Granulosa cells tumor
WHO	World Health Organization
γGT	Gammaglutammiltransferase

INTRODUCTION

HERBICIDES AND HUMAN HEALTH

Intensive agriculture is a distinctive feature of modern world and it is based on massive use of herbicides, pesticides, and fertilizers (Mesnage and Seralini, 2018). As a result, residues of chemicals such as glyphosate-based herbicides (GBHs) have been accumulating in the soil throughout the years, exposing to toxic risks non only farmers, but also general population (Silva et al., 2018; Gillezau et al., 2019).
Nevertheless, the toxicity of pesticides is still alarmingly underestimated since only the main ingredients are to be regulated and tested for human health, while the surfactants present in the formulations can be undeclared, as it usually happens, and consequently poorly tested, ignoring their role in pesticide effects (Defarge et al.,2018).
Herbicides and pesticides bioaccumulation, along with the introduction in many countries of genetically modified (GM) plants resistant to them, are among the main reasons making our intensive system unsustainable in long terms; if we want to guarantee consumers health protection and avoid the total depletion of our ecosystem resources and biodiversity, we need to find alternative solutions in the near future (Mesnage and Seralini, 2018).
However, the problem goes beyond medical and ecological aspects as it has also social, political, economic, and legal implications. Concerning glyphosate, on which this study focuses, we can point out that it is distributed under the strict control of very few companies among which the main one is Monsanto, that has been recently enlarged by the fusion with Bayer (2018). This monopolization has obviously critical consequences on political decisions related to the herbicide commercialization (Mesnage and Seralini, 2018).

GLYPHOSATE AND GLYPHOSATE-BASED HERBICIDES

Glyphosate (IUPAC chemical name N-phosphonomethilglycine) is the active ingredient of all glyphosate-based herbicides (GBHs). Plants absorb it through foliage and, after several days, they die as glyphosate causes the interruption of essential aromatic amino acids synthesis (Williams et al., 2000; Gomes et al., 2014) by inhibition of 5-enolpyruvylshikimate-3-phosphate synthase, an enzyme

that participate to the Shikimate pathway. Shikimate pathway is a reaction chain that allows the synthesis of essential aromatic amino acids such as phenylalanine, tryptophan, and tyrosine and it is not present in mammals, which should make GBHs safe for humans (Williams et al., 2000; Stenersen, 2004).

GBHs were introduced in 1974 and their use has been amazingly growing up worldwide ever since. They have different applications in agricultural field: they are used in traditional agriculture before planting for weed control, but also after planting in genetically modified glyphosate-resistant crops, which were introduced late in the 90s. Moreover, GHBs are used for desiccation of grain crops, between trees in orchards, and also to keep streets and parks clean in urban areas (Van Bruggen et al., 2018).

GBHs are formulations commonly containing 36–48% glyphosate as active principle, while 10–20% consists of other chemical known as surfactants or adjuvants that can act as membrane disruptors, mitochondrial inhibitors or DNA damaging factors (Peixoto, 2005; Mesnage et al., 2015; Bailey, 2017).

Also heavy metals as arsenic, chromium, cobalt, lead, and nickel are present in numerous herbicides at levels well above admissible ones in water, probably as a result of contamination of formulations due to their manufacturing process. These metals are known to be toxic and to have endocrine disruption proprieties themselves (Defarge et al., 2018).

THE GLYPHOSATE AFFAIR

Intensive use of GBHs on large scale increases the exposing level for farmers, via inhalation and dermal contact, but also for general population that can assume glyphosate by water and food consumption, as demonstrated by environmental contamination (Gillezeau et al., 2019). Therefore, several major concerns have arisen in recent years about harmful side effects of glyphosate for human health. In 2015, the International Agency for Research on Cancer (IARC) classified glyphosate as a "probable human carcinogen" (category 2A) (Guyton et al., 2015; IARC, 2015), starting a dispute against the European Food Safety Agency (EFSA), that claimed that no carcinogenic hazard consisted, considering IARC evidence not reliable (EFSA, 2015).

IARC analyzed several scientific papers finding out "sufficient evidence" of a connection between glyphosate and higher rate of cancer in experimental animals, and "little evidence in humans" (Guyton et al., 2015; IARC, 2015). On the other side, EFSA decision was based mainly on the glyphosate renewal assessment report (RAR), presented by German Federal Institute for Risk

Assessment, which concluded that glyphosate was "unlikely to pose a cancerogenic hazard to humans" (Bfr, 2013). In 2017, the European Commission extended the authorization for glyphosate for another 5 years (EU 2017/2324), agreeing with EFSA opinion and claiming that the overall weight of evidence reported by IARC was not significative in indicating any cancerogenic risk. However, European Commission's decision was very criticized, and many scientists took a stand against it, making GBHs an issue of public concern. Possible conflict of interest emerged as some members of BfR (German Federal Institute for Risk Assessment) and EFSA were found financially connected to the manufacturing companies, which obviously had great financial interests in proving glyphosate safety (Torretta et al., 2018). Eventually, in September 2017, "The Guardian" reported an article that revealed that many sections of the Renewal Assessment Report, prepared by the BfR and used by EFSA, were actually copy-pasted from a study done by Monsanto (Nelson, 2017).

What certainly arises from the "glyphosate affair" is the need of renewing the system that regulates herbicides toxicity trials. Until now, herbicide side effects have been measured by testing the active principle alone, both for acute and chronic toxicity. What is not considered in this way is that commercial formulations also contain many adjuvants that might enhance or alter the effects on the organism (Defarge et al., 2018). As an example of this, GBHs adjuvants are generally considered as inert diluents, but there is a consistence body of literature that claims side effects for these compounds and many studies have demonstrated that commercial formulations, among which Roundup is the most famous one, are far more toxic than glyphosate itself (Defarge et al., 2018; Benachour et al., 2007; Benachour and Seralini, 2009; Richard et al., 2005; Peixoto, 2005; Mesnage et al., 2013; Vanlaeys et al., 2018). This points out that the whole formulation of herbicides has to be taken into account in order to achieve an appropriate and truthful safety evaluation.

GLYPHOSATE EXPOSURE

Glyphosate-containing herbicides are usually applied to crops 2 to 3 times per season to remove weeds or to dry out grain in a process called 'desiccation'. Once applied, glyphosate is absorbed by plants and fruits and its residues can be found in food for over a year or more, even if they are washed, frozen, dried or cooked (Kruger et al., 2016).

Glyphosate and its degradation product AMPA are also adsorbed by the soil, get attached to clay and organic matter, and, consequently, can also be transported by rain erosion and spread by the wind eventually contaminating the surrounding areas (Silva et al., 2018).

Introduction

In USA, where genetically modified glyphosate-resistant crops are common, glyphosate has been measured in river and stream water at levels from 2 to 430 μg/L. Nevertheless, also in Europe, where OGM crops are not allowed, glyphosate has been detected, even if at lower levels. In Germany, Switzerland, Hungary and northeastern Spain glyphosate was found at levels from 0.1 to 2.5 μg/L in samples of surface water, but occasionally levels reached 165 μg/L in France and Denmark (Van Bruggen et al., 2018).
Silva at al., in 2018, published the first large-scale assessment of distribution (occurrence and concentrations) of glyphosate and AMPA in EU agricultural topsoils stating that residues were present in 45% cases from eleven countries, with a maximum concentration of 2 mg/kg.
Concerning residues concentrations in crops products, detected levels can vary from 0.1–100 mg/kg in legumes, 0.1– 25 mg/kg in cereals and rice, 0.1–28 mg/kg in oil seeds and 1– 344 mg/kg in fodder (Van Bruggen et al., 2018).
In Italy, 100 food products coming from our territory as corn flakes, rusks, pasta, and 26 samples of drinking water were analyzed by the magazine Test-Salvagente (2016) finding traces of glyphosate in half of them with levels going from 0.019 mg/kg up to 0.160 mg/kg and AMPA in two samples of potable water at 4.6 and 2.3 μg/L respectively. Considering that the tolerable limit for pesticides in drinking water is 0.5 μ/L, the values found in the research conducted are clearly unacceptable (Torretta et al., 2018).
ISPRA institute (Italian Higher Institute for Environmental Protection and Research) also provided important data on water contamination in Italy which were also published in the "*National Report of Pesticides in Water*" in May 2016 (Paris et al., 2016). The Report assessed the presence of pesticides and contaminants in 63.9% of surface monitoring points (rivers, lakes, streams) and in 31.7% of the groundwater monitoring points, with an increasing trend compared to the previous biennium, about 20% and10% respectively. What's alarming is that in surface waters 21.3% of monitoring points showed glyphosate concentrations above the 0,1 μg/L limit imposed by low to guarantee environmental quality standards (Paris et al., 2016).
All considered, these data highlight a widespread diffusion of contamination by pesticides and herbicides. Residues of these products are very difficult to be kept under control first of all because of their direct use on the ground and secondly because rainfalls determine a faster transport in the surface and underground water bodies, especially in periods as early spring (Torretta et al., 2018)

Considering the ∼100-fold increase in GBH use in the last four decades, all animals and humans are chronically indirectly exposed to glyphosate by eating and drinking. Kruger et al. (2016) results showed that glyphosate is detectable in intestine, liver, muscle, spleen, and kidney tissue.

Nevertheless, there is still no legislation on residues in tissues and organs of food animals and little is known about the influence of glyphosate residues on the quality of animal products.

Humans may be exposed to glyphosate through various routes and a distinction can be made between occupational, para-occupational or environmental exposure. The first group includes workers in the agriculture field such as farmers or pesticide sprayers and also workers involved in the manufacturing and processing of glyphosate. Gardeners, horticulturalists, and forest workers are considered as para-occupationally exposed, whereas normal population is environmentally exposed to glyphosate by food and drink consumption (Myers et al. 2016; Vandenberg et al., 2017).

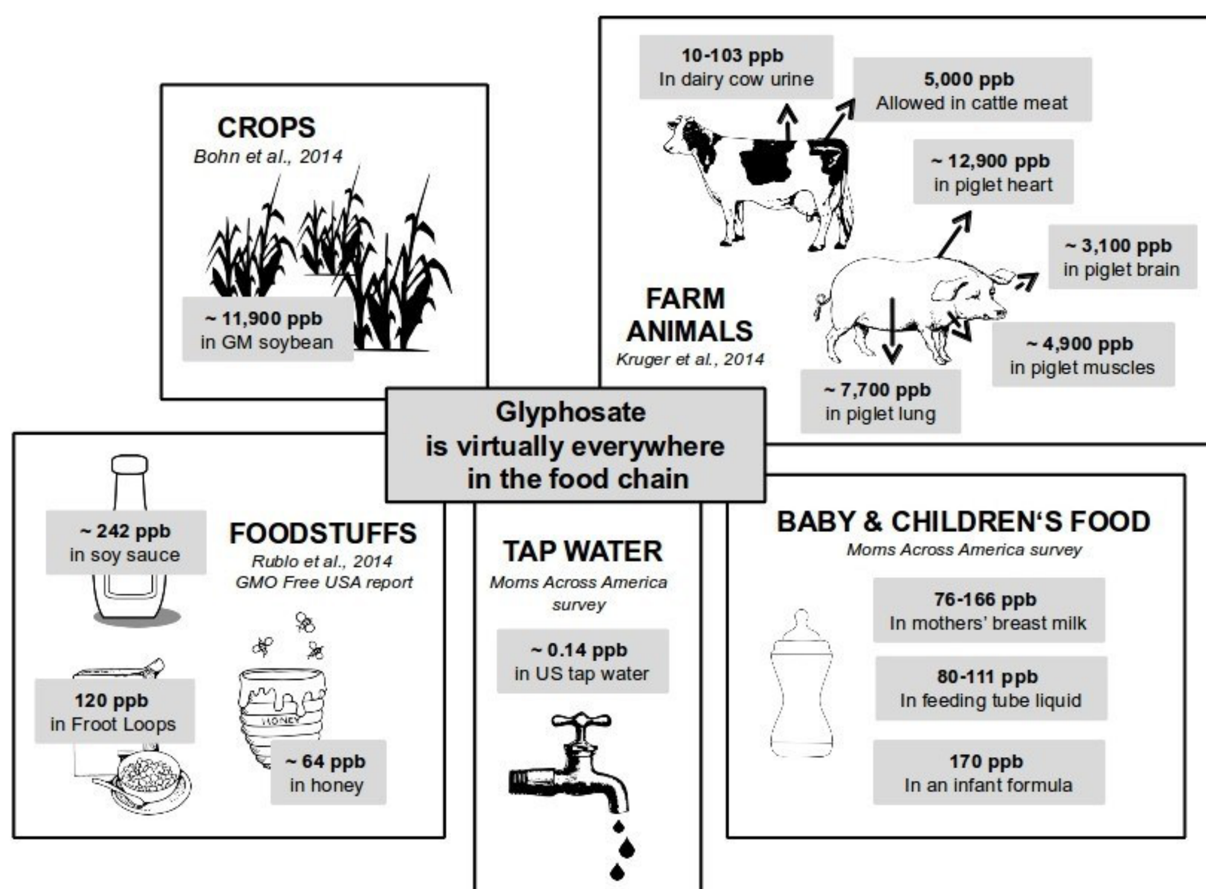

Fig. 1: Different ways and levels of glyphosate exposure for normal population. 1 ppm corresponds to 1.000 mg/L (The Detox Project, Glyphosate in Food & Water).

Unfortunately, it is very difficult to draw conclusions about the real entity of contamination we are exposed to as the few available studies were made in different countries and utilized different methodologies, measurements and approaches (Tarazona, 2017). A recent review on this topic made by Gillezeau et al. (2019) has shown that the mean levels in urine samples among general population

are generally below 4 μg/L, while the average urinary levels in occupationally exposed subjects varies from 0.26 to 73.5 μg/L. That review took into account five studies on occupational and para-occupational exposure, eleven on general population, and three analyzing both.

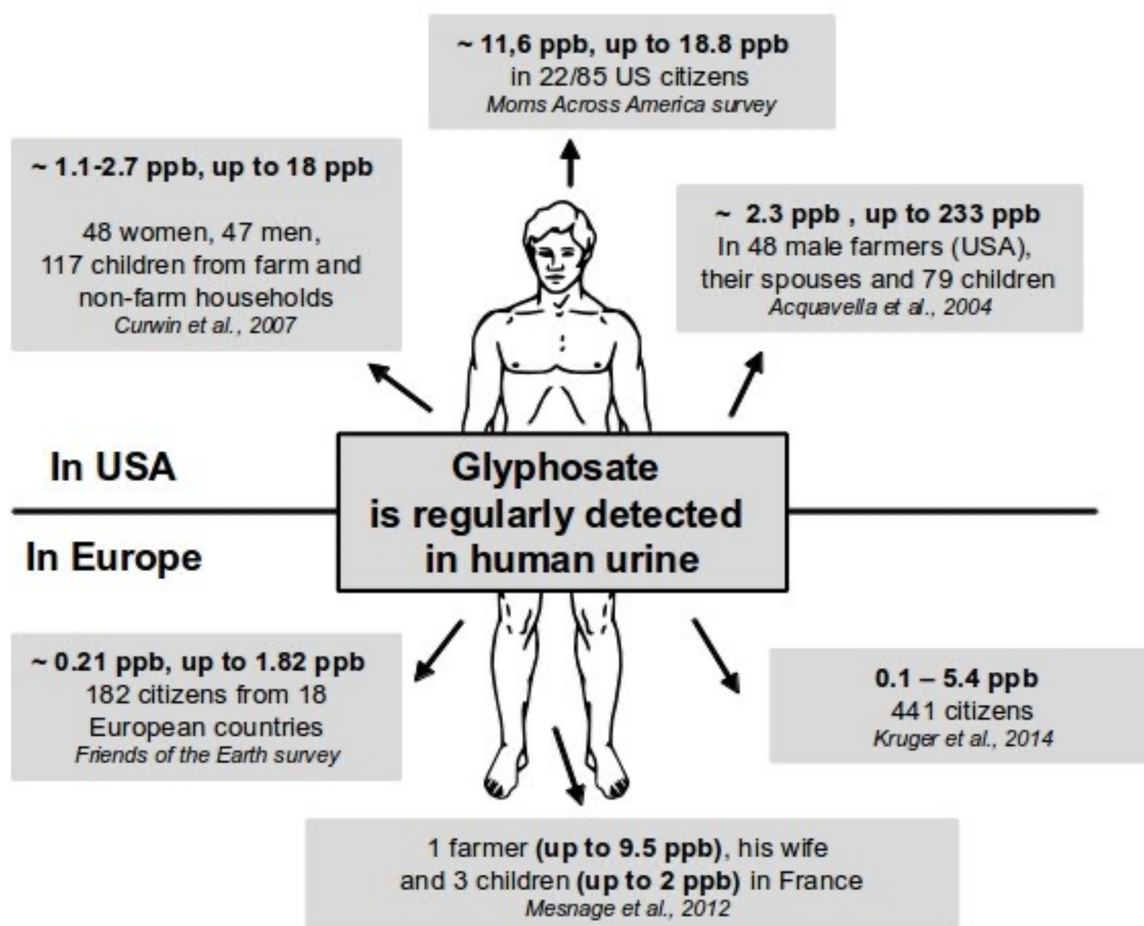

Fig. 2: Glyphosate levels detected in human urine in USA vs Europe (The Detox Project, Glyphosate in Food & Water).

Further epidemiological studies, with systematic and homogeneous data collection, are needed to have an accurate risk assessment and to give an answer to the current questions of glyphosate safety under debate by health and environmental agencies around the world.

Introduction

GBHs AND THEIR TOXIC EFFECTS

Basing on oral, inhalation and dermal LD_{50} estimated, EPA (US Enviromental Protection Agency) has classified glyphosate as a low toxicity substance (EPA, 1993).

TOXICITY CLASSIFICATION – GLYPHOSATE				
	High Toxicity	Moderate Toxicity	Low Toxicity	Very Low Toxicity
Acute Oral LD_{50}	Up to and including 50 mg/kg (≤ 50 mg/kg)	Greater than 50 through 500 mg/kg (>50-500 mg/kg)	**Greater than 500 through 5000 mg/kg (>500-5000 mg/kg)**	**Greater than 5000 mg/kg (>5000 mg/kg)**
Inhalation LC_{50}	Up to and including 0.05 mg/L (≤0.05 mg/L)	Greater than 0.05 through 0.5 mg/L (>0.05-0.5 mg/L)	**Greater than 0.5 through 2.0 mg/L (>0.5-2.0 mg/L)**	**Greater than 2.0 mg/L (>2.0 mg/L)**
Dermal LD_{50}	Up to and including 200 mg/kg (≤200 mg/kg)	Greater than 200 through 2000 mg/kg (>200-2000 mg/kg)	**Greater than 2000 through 5000 mg/kg (>2000-5000 mg/kg)**	Greater than 5000 mg/kg (>5000 mg/kg)
Primary Eye Irritation	Corrosive (irreversible destruction of ocular tissue) or corneal involvement or irritation persisting for more than 21 days	Corneal involvement or other eye irritation clearing in 8 – 21 days	**Corneal involvement or other eye irritation clearing in 7 days or less**	**Minimal effects clearing in less than 24 hours**
Primary Skin Irritation	Corrosive (tissue destruction into the dermis and/or scarring)	Severe irritation at 72 hours (severe erythema or edema)	Moderate irritation at 72 hours (moderate erythema)	**Mild or slight irritation at 72 hours (no irritation or erythema)**

The highlighted boxes reflect the values in the "Acute Toxicity" section of this fact sheet. Modeled after the U.S. Environmental Protection Agency, Office of Pesticide Programs, Label Review Manual, Chapter 7: Precautionary Labeling. http://www.epa.gov/oppfead1/labeling/lrm/chap-07.pdf

Table 1: Compounds toxicity classification after different types of direct exposure, based on signs gravity. Glyphosate profile is yellow labelled. US Environmental Protection Agency, Office of Pesticide Programs (EPA, 2018): "Label Review Manual". Chapter 7: Precautionary Statements.
https://www.epa.gov/sites/production/files/2018-04/documents/chap-07-mar-2018.pdf

Despite this, animals and humans directly exposed to GBHs, occasionally displayed gastroenteric symptoms and dermatological and respiratory problems, probably as a consequence of the adjuvants present in commercial formulations (Connolly et al., 2019; Camacho and Mejía, 2017). Skin exposure to ready-to-use concentrated glyphosate formulations can cause irritation and spray mist may cause oral or nasal discomfort and throat irritation. Eye exposure may lead to mild conjunctivitis and superficial corneal injury (Henderson et al., 2010). However, death has been reported only after deliberate overdose (Bradberry, 2004).

Relating to chronic toxicity, it has to be stressed that there are many studies linking the increasing glyphosate environmental contamination to many human chronic diseases, including different forms

of cancer, liver and kidney pathologies, neurological syndromes and endocrine and reproductive alterations (Bailey et al., 2017; Samsel and Seneff, 2013).

According to glyphosate regulatory assessment (EFSA, 2015), 100 mg/kg bw/d in rats and at 50 mg/kg bw/d in rabbits represent the overall long-term NOAEL (No Observed Adverse Effect Level), while the overall LOAEL (Lowest Observed Adverse Effect Level) is 350 mg/kg bw/d. The current U.S. EPA reference dose (RfD) for glyphosate is 1.75 mg /kg bw/day; in contrast, the current EU ADI is fixed at 0.5 mg/kg bw/day (taking into account the lowest NOAEL and a safety factor of 100), more than 5-fold lower than U.S. RfD (Henderson et al., 2010).

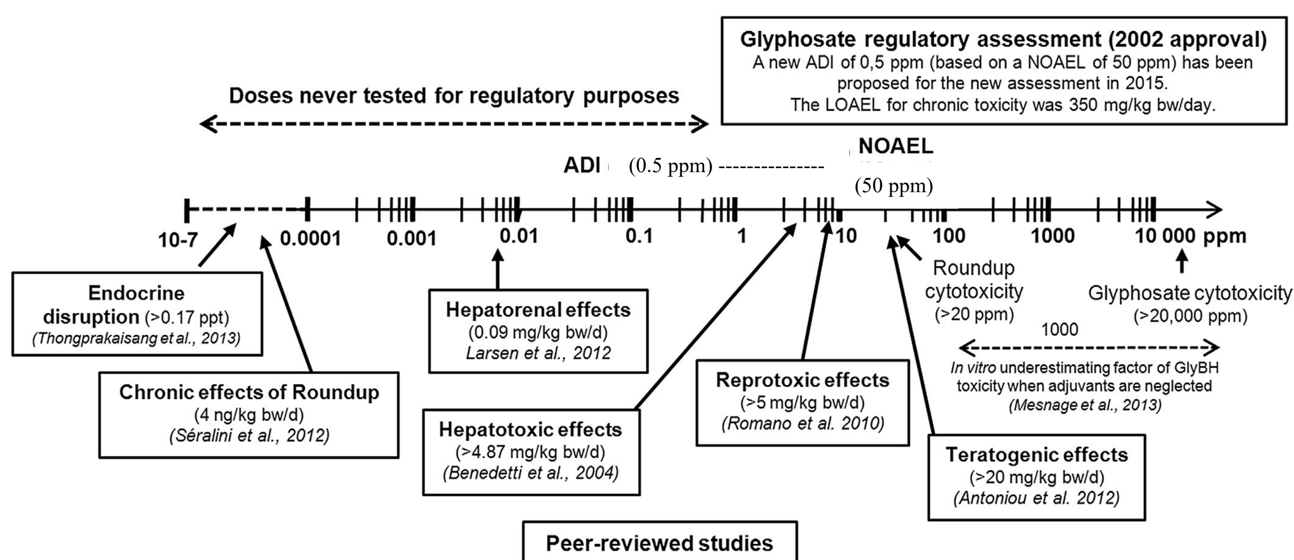

Fig.3: The top line of the figure explains the regulatory tests and assessments driving glyphosate commercial authorizations. Glyphosate has never been tested at the ADI. The middle line represents regulatory limits for glyphosate alone established in the EU. The bottom line represents findings of toxicity below regulatory limits that were dismissed (Mesnage et al., 2013).

NEUROTOXICITY

Different mechanisms could correlate glyphosate to neurotoxicity and lately several studies had associated mental diseases such as Attention-deficit/hyperactivity disorder (ADHD), Alzheimer, Parkinson, and autism to GBHs exposure (Garry et al., 2002; Wan and Lin, 2016; Nevison, 2014; Swanson et al., 2014).

Firstly, AMPA, the main glyphosate metabolite, acts like a selective agonist for non-NMDA glutamic acid receptors in the central nervous system, being able to decrease acetylcholinesterase activity. If

acetylcholine breakdown is insufficient, nerve impulses are not switched off and this can bring serious neurological disorders (Kwiatkowska et al., 2014).

Secondly, glyphosate is very similar to glycine and, as a consequence, it can antagonize glycine uptake and protein biosynthesis, impairing cellular proliferation (Li et al., 2013) and altering neuronal transmission (Mesnage et al., 2015).
Taking into account the studies published so far, in vitro addition of high dose of glyphosate (4000 mg/L) for 24 h seems to alter neural cell development and axon growth (Coullery et al., 2016). Hernandez-Plata et al. (2015) have also shown how in vivo acute intraperitoneal injections of 150 mg/kg bw/d glyphosate bring hypoactivity in rats. Moreover, chronic exposure of pregnant rats to Roundup by drinking water (0.36% or 3600 mg/L) leads to depressive-like behavior as a consequence of glutamate excitotoxicity and decreased acetylcholinesterase activity in the hippocampus (Cattani et al., 2017).
Finally, oxidative stress induced by glyphosate on brain tissues has been proved. Treated rats at 10 mg/kg bw/d showed an increase in lipid peroxidation, protein carbonylation, and nitrite formation with a decrease in alpha-tocopherol; they also showed a loss of mitochondrial trans- membrane potential and cardiolipin content in the substantia nigra (Astiz et al., 2009).

GASTROENTERIC TOXICITY

In a work published in 2013, Samsel and Seneff assessed the existence of a relationship between glyphosate and the development of gastroenteric chronic diseases suggesting that intestinal bacteria are the fundamental components damaged by glyphosate. Monsanto company has always supported the safety of the molecule for humans and animals as it inhibits 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which has an important role only in plants. However, this is not entirely correct, since the enzyme is also contained in some bacteria, especially in intestinal ones within the human and animal body. Glyphosate could therefore be able to destroy the microbiome present in the digestive system and to inhibit the absorption of vitamins, minerals, and even some proteins (Samsel et al., 2013). Furthermore, exposure levels are growing drastically as well as the rate of diseases associated to grain and cereals, such as celiac disease (Torretta et al., 2018). A specific evaluation, provided by CDC (Centers for Disease Control and Prevention, Atlanta, GA, USA), and NASS (National Agricultural Statistics Services, Washington, DC, USA) highlighted the relationship between the diagnosis of celiac disease and glyphosate applications in the United States (Samsel et al., 2013). Moreover, Mao et al. (2018) highlighted that GBHs exposure at doses considered safe can

induce the modification of gut microbiota in growing Sprague Dawley rats, particularly before the onset of puberty.

HEPATIC AND KIDNEY TOXICITY

Glyphosate has been classified by the World Health Organization (WHO) as a compound "unlikely to present acute hazard in normal use". Cases of severe intoxication are only present after high dose ingestions (Lee and Choi, 2017). More attention has to be put on chronic toxicity.

Liver and kidney injury after GBHs exposure is caused by the reduction of respiratory activity and the consequent increase in oxidative stress, which reflects in the increase of alkaline phosphatase (ALP) or aspartate aminotransferase (AST) and alanine aminotransferase (ALAT) (Mesnage et al., 2015).

In vitro comparative studies between impact of glyphosate and Roundup (concentrations of 0,5; 1; 2 and 5 mM) on isolated rat liver mitochondria by Peixoto et al. (2005) showed how even on mitochondrial respiration, membrane potential and enzymatic activities Roundup formulations are more toxic than the glyphosate alone.

Seralini et al. (2014) performed a 2-year-long in vivo study using groups of 10 Spraguee Dawley rats, which were administered with 0.1 ppb of a Roundup formulation in drinking water (admissible concentration of GBHs residues in drinking water) and signs of hepatorenal toxicities, as well as urine and blood biochemistry disturbances, were revealed at the 15th month. In another study, glyphosate exposure of rats at 0.09 mg/kg bw/d (0.7 ppm in drinking water) caused an increase in glutathione levels and enhanced glutathione peroxidase activity in liver and kidneys (Larsen et al., 2012). Roundup induced the activation of xenobiotic-metabolizing enzyme after a 90-day exposure of 0.7 ppm dissolved in water (Larsen et al., 2014); disruption of the same enzymes was observed at levels as low as 0.1 ppb in drinking water for a life-long exposure (Seralini et al., 2014).

In 2018, Saleh et al. study showed clearly that 15 days daily administration of Roundup (25, 50 and 100 mg/kg bwt) causes histopathological and biochemical alterations on the liver of albino rats, Levels of ALT showed an increase in the serum of treated groups compared to those of controls yet at 25 mg/kg bwt while AST enzymes AST enzymes were altered from 100 mg/kg bwt. These biochemical alterations were histologically matched to an increase in connective tissue, disarrangement in parenchymal cells, mononuclear cell infiltration, many apoptotic hepatocytes and many focal necrotic areas. Also, an increase in the number of Kupffer cells with deposition of lipofuscin pigments and a remarkable collection of inflammatory cells adjacent to some blood vessels

and invading the hepatic tissue was noticeable. These changes were more intensified in the case of high dose (100 mg/kg bwt).
A recent similar study by Tazdaït et al. (2020) exposed rats to 269.9 mg/kg b.w Roundup for 30 days and highlighted an increase in serum hepatic and renal markers: AST, ALT, ALP, LDH, γGT, bilirubin, urea, and creatinine.

CARCINOGENICITY

After analyzing evidence collected until 2015, IARC included glyphosate in category 2A substances, as a "probable human carcinogen". Even though not all the considered studies revealed a positive association with a higher rate in tumors, there was certainly strong evidence that glyphosate and its metabolite AMPA can operate through two well-known mechanisms of human carcinogenicity i.e. genotoxicity (DNA damage) and oxidative stress. IARC considered this evidence along with a few positive findings enough to declare it as a hazard (Guyton et al. 2015; IARC 2015).
As already said, IARC position was strongly opposed by other regulatory authorities EFSA, BfR and EPA (BfR 2013; EFSA 2015; EPA 2016).
Aside from the manufacturing companies' financial interests in stating the glyphosate safety and the potential implication of some members of the regulatory authorities (Torretta et al., 2018), the two diametrically opposed conclusions on glyphosate carcinogenicity can be based on the following main points.
Firstly, EFSA and EPA based their judgments on studies performed with pure glyphosate, while IARC took also into account assays, studies and reviews on GBHs formulations and on AMPA, giving them considerable importance (Benbrook, 2019). Secondly, EFSA and EPA mostly relied on registrant-commissioned, unpublished regulatory studies, whereas IARC mainly took in consideration peer-reviewed studies. Moreover, many positive evidence reported by IARC derived from testing doses which are far higher than the one general population is exposed to; especially the epidemiological studies considered situations of high level exposure that occur in agricultural areas among farmers. On one side, EFSA and EPA stated that regulatory judgements on pesticide cancer risk should be based upon an assessment of general population under dietary exposure, therefore the abovementioned studies considered by IARC should not be taken into account as they don't reflect normal conditions but specific scenarios that lead to much higher dermal exposures for humans. Hence, the IARC' weight-of-evidence judgement that GBHs are "probably carcinogenic to humans" could eventually be applied only to occupationally exposed population (Benbrook, 2019). Finally, statistical relevance of many studies considered by IARC was questioned because of the small number

of cases and relying on the fact they didn't take into account other possible environmental factors of carcinogenicity (Sorahan, 2015).

Since IARC vs EFSA debate in 2015 (see 'The glyphosate affair' Chapter), many other papers have been published supporting (Portier, 2015; Portier et al., 2016; Richmond, 2018; Székács et al., 2018) or criticizing (Williams et al., 2016; Tarazona, 2017) IARC conclusions.

Some of the main studies that brought positive or negative evidence of glyphosate carcinogenicity are reported below.

With reference to animals, in 2000 Williams et al. published a review that collected genotoxicity studies available so far. Basing on the strong preponderance of data showing no effects on chromosome and gene mutations in "in vivo" mammalian assays, the authors concluded that glyphosate was not mutagenic or genotoxic as a consequence of interaction with DNA. The few results which were an exception were considered as a consequence of toxicity rather than DNA reactivity (Williams et al., 2000).

In 2013, Kier and Kirkland published a second review analyzing 66 in vitro and in vivo genotoxicity assays in different species. Most of the studies performed with pure glyphosate were negative, indicating that glyphosate does not have a direct DNA-reactive mechanism, thus supporting the conclusion of no genotoxicity asserted by Williams et al. (2000). Anyway, some opposing results were observed for commercial formulations and that was linked to a possible genotoxic effect of surfactants (Kier and Kirkland, 2013).

In 2014, Seralini et al. published the first long term study (2 years) emulating chronic exposure, administrating the whole Roundup formulation to rats at environmentally levels from 0.1 ppb by water or feeding. The results obtained suggested severe toxicity signs on liver and kidney, a tumorigenic hormone-dependent effect on mammary and pituitary glands in treated females, plus an increasing incidence of large palpable tumors in males (mostly in kidney and skin) (Seralini et al., 2014). IARC monograph (2015) also highlighted a higher rate of kidney, liver and pancreatic tumors in rats exposed to GBHs.

In human, the results on GBHs carcinogenicity from epidemiological studies are controversial. In 2008, Eriksson et al. claimed a significant risk of Non-Hodgkin lymphoma (NHL) associated with GBH use confirming the results of other studies that had already suggested a link with hairy-cell leukemia (HCL), a rare NHL variant (Nordstrom et al., 1998; Hardell et al., 2002). In 2014, evidence of carcinogenicity in humans was also supported by a meta-analysis on occupational exposure to agricultural pesticides by Schinasi and Leon, who confirmed a positive association between glyphosate and non-Hodgkin lymphoma subtypes (Schinasi and Leon, 2014).

In spite of this, in 2018, an analysis on more than five thousand applicators of GBHs made by the Agricultural Health Study and sponsored by the US EPA showed no statistically significant association with cancer at any site (AHS, 2018). However, a 2.4-fold increase of acute myeloid leukemia was registered among applicators in the highest exposure quartile (Andreotti et al., 2018).
In the latest meta-analysis, dated 2019, Zhang et al. included the 2018 *Agricultural Health Study* (AHS) cohort update along with other five case-control studies and reported that the overall meta-relative risk (meta-RR) of NHL in GBH-exposed individuals was increased by 41%.
Epidemiological studies published so far are few and don't follow a similar methodological approach; that's why it is very difficult to give a valid interpretation of the data provided (Sorahan, 2015).
If we take into account also in vitro studies, Kwiatkowska et al. (2017) showed that the exposure of human peripheral blood cells to glyphosate (at moderate to high concentrations from 85 to 1690 mg/L) resulted in DNA damage in leucocytes and decreased DNA methylation (at 42 mg/L glyphosate). DNA damage was also revealed during an epidemiologic study among Brazil soybean workers (Benedetti et al., 2013) and also among Ecuadorian and Argentine occupationally exposed population (Paz-y-Mino et al., 2007; Manas et al., 2009). Changes in DNA methylation due to glyphosate can alter the balance between cancerous cell proliferation and programmed cell death (Hervouet et al., 2013); for this reason, IARC proposed genotoxicity and oxidative stress as the supporting mechanistic evidence of glyphosate possible carcinogenicity (Tarazona, 2017).
In 2013, another in vitro study by Thongprakaisang et al. outlined that glyphosate can promote the growth of estrogen-dependent human mammary breast cancer cells starting from 0.1 ppt. Notwithstanding this information, it is worth to say that in 2005 an investigation on breast cancer among farmer wives found no link with GBHs usage (Engel et al., 2005).

Taking all into account, we can conclude that the issue of glyphosate carcinogenicity is still very controversial. To improve solid evidence and give a reliable hazard assessment, it is clear that GBHs formulation, and not pure glyphosate, should be tested, recognizing the effects of adjuvants as well as the ones due to pure glyphosate. Additionally, a human biomonitoring program for glyphosate and its metabolite is strongly suggested. Finally, it is important to collect more epidemiological studies, especially on occupationally exposed workers, by using the same scientific approach method (Vanderberg et al., 2017; Benbrook, 2019).

GBHs IMPACT ON REPRODUCTION

Industrialized countries have seen a sharp increase in the infertility rate, especially among men. Average sperm concentration dropped from 113 x 10^6 to 66 x 10^6 spz/ml only in the past half century and about 20% of young men (one out of five) between the ages of 18 and 25 in Europe have sperm counts below the WHO (World Health Organization) reference level of 20 x 10^6 spz/ml. In the same way, semen quality has been affected and incidence of TGC (testicular germ cell cancer) has worryingly increased (Wan Ho, 2014).

Toxicants as pesticides are blamed to be among the environmental factors associated to infertility, as well as imbalanced diet, obesity, exposure to smoke and air pollution (Foster, 2008). An age-independent decline in testosterone levels has been recorded over the last decades in the United States, thus supporting this thesis. Alarmingly, this tendency has begun in the 90s just after the introduction of genetically modified (GM) crops and the subsequent increase in glyphosate herbicides use (Wan Ho et al., 2014).

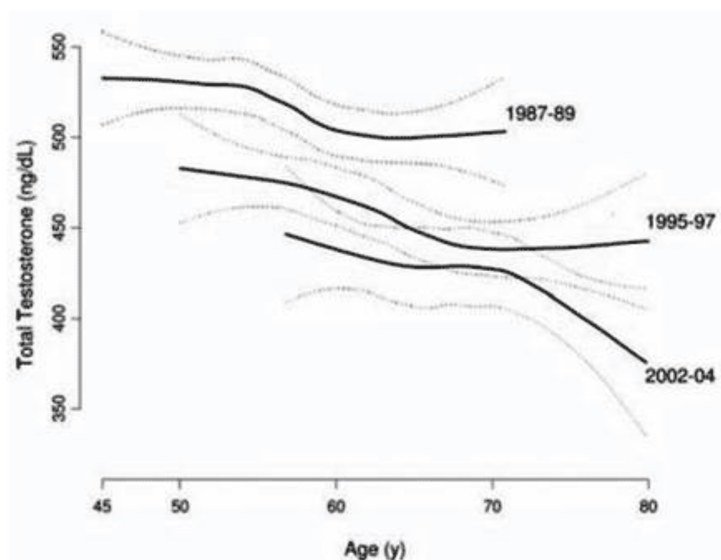

Fig.4: Decline of testosterone among American men in the last decades (Wan Ho et al., 2014).

On the contrary, available biomonitoring data provides evidence of extremely low environmental glyphosate exposures and gives no solid proof of a linkage with adverse reproductive effects

(Williams et al., 2012). Nonetheless, it has to be said that existing epidemiological studies have many methodological limitations consisting mainly in a lack of valid quantitative measurements (urine and/or blood levels) of glyphosate and of solid estimation of GBHs exposure. Therefore, many negative findings cannot be considered reliable (De Araujo, 2016).

Up today, an increasing number of published in vivo studies on animals are claiming that GBHs exposure at environmental levels could represent a real risk for infertility impacting on both the male and the female reproductive system (Foster, 2008). The underneath mechanism for infertility problems could be that glyphosate at low doses acts as an endocrine disruptor in mammals, causing imbalance in redox system and altering hormonal function at levels lower than the cytotoxic one (Kwiatkowska et al., 2013, Defarge et al., 2018). Therefore, GBHs bioaccumulation may have serious implications (Dallegrave et al., 2007).

Male reproductive abnormalities linked to GBHs include delayed onset of puberty, behavioral alterations, testis pathology, reduced sperm production, testis cells apoptosis as well as reduced testosterone production (Clair et al., 2012; Romano et al., 2012; Hernandez-Plata et al., 2015; Cai et al., 2017; Cattani et al., 2017). As to female pathologies, uterine abnormalities, altered ovarian steroidogenesis and implantation pathology were reported (Ingaramo et al., 2016; Perego et al., 2017; Milesi et al.2018) and birth defects were also detected in rodents (Garry et al., 2002).

The topic of glyphosate implications on reproduction became even more important after the publication in 2019 of the first study on the glyphosate potential transgenerational impacts on successive generations in absence of continued direct glyphosate exposure, by epigenetic-bases mechanisms (combination of molecular factors and processes around DNA that regulate genome activity independently from DNA) (Kubsad et al., 2019). After directly exposing gestating F0 generation female rats to glyphosate (half the NOAEL - 25 mg/kg bw/d during days 8 to 14 of pregnancy), F0, F1, F2 and F3 generation offspring were aged to 1 year and euthanized for pathology and sperm epigenetic analysis (Kubsad et al., 2019). A negligible impact of pathologies on F0 generation as well as on F1 generation offspring was recorded; in contrast, in F2 and F3 transgenerational offspring there was a significant increase in pathologies such as prostate disease, obesity, kidney disease, ovarian disease, mammary gland tumors and parturition abnormalities. Sperm analysis showed differential DNA methylation regions (DMRs) between F1, F2 and F3 generations and some of these DMR associated genes were involved in the pathologies recorded. Authors finally assumed that the main pathologies seen in glyphosate lineage offspring generations might reflect some of the problems that afflict human population nowadays, as the dramatic increase in obesity or the higher rate in parturition abnormalities such as premature birth and infant abnormalities (Kubsad et al., 2019). The evidence of onset toxicity in adults through epigenetic-based

mechanisms was confirmed by recent study of Smith et al. (2019) that using a fish model (Japanese medaka - *O. latipes*) showed how Roundup and its active ingredient glyphosate may induce developmental, reproductive and epigenetic effects such as changes in the expression of reproductive genes.

GBHs AND MALE FERTILITY

The precise mechanisms ruling the effects of GBHs on male reproductive system remains unclear but is probably linked to the glyphosate action as endocrine disruptor and oxidant factor. Specifically, Roundup alters serum steroid hormone level by downregulating STAR protein expression, whose function is to transfer cholesterol from the outer to the inner mitochondrial membrane, where the cytochrome P450 enzyme initiates the synthesis of steroid hormones (Walsh et al., 2000). Moreover, Roundup inhibits P450 aromatase activity in Leydig cells (Richard et al., 2005) and thus the conversion of cholesterol in pregnenolone. Roundup impairment of steroidogenesis implies that of every 100 molecules of cholesterol available for transport to the inner mitochondrial membrane, only 10 molecules actually reach the P450scc enzyme and of these 10 molecules only 3 are converted to pegnenolone and then to testosterone (Walsh et al., 2000).
Many other studies support GBHs endocrine disruptor activity suggesting other mechanisms besides the aforementioned, i.e. impairing aromatase gene expression, altering transcriptional activities of both androgen and estrogen receptors (Gasnier et al., 2009), interacting with the active site of aromatase enzyme and also altering estrogen-regulated genes expression (Richard et al., 2005; Benachour et al., 2007; Thongprakaisang et al., 2013). All this eventually leads to disfunctions in spermatogenesis. Secondly, GBHs seem to impact on oxidative stress promoting Ca^{2+} overload and antioxidant defenses depletion; they might also contribute to Sertoli cell disruption and spermatogenesis dysfunction (Cavalli et al., 2013).

Furthermore, heavy metals such as arsenic, chromium, cobalt, lead and nickel (identified by mass spectrometry) were found in numerous pesticides at levels well above the admissible ones in water, probably as a result of contamination of formulations during the manufacturing process. These metals are known to be toxic and endocrine disruptors as well (Defarge et al.,2018).
GBHs impact on male fertility has been largely investigated. Romano et al. (2012) found that maternal exposure to 50 mg/kg bw/d glyphosate disturbed the masculinization process of male rat offspring increasing testosterone, estradiol and LH serum concentrations, altered sperm production,

induced early onset of puberty and promoted sexual behavioral changes. Another in vivo study on 50, 150 and 450 mg/kg bw/day Roundup exposure during pregnancy and lactation produced in male offspring rats a decrease in sperm production, an increase in the percentage of abnormal spermatozoa and a dose-related decrease in the testosterone serum levels (Dallegrave et al., 2007). Romano et al. (2010) assessed that administration of 5 mg/kg bw/d Roundup limited to prepubertal period, decreased serum testosterone concentrations and induced changes in testicular morphology in rats. Finally, the Ramazzini Institute pilot study in 2019 demonstrated that rats exposed to Roundup at ADI dose (1,75 mg/kg bw/day), from prenatal period to adulthood, underwent endocrine effects and alteration of reproductive developmental parameters, in particular showing a significant increase in anogenital distance which is a marker of prenatal endocrine disruption (Manservisi et al., 2019). Concerning acute toxicity, Cassault-Mayer et al. (2014) showed that Roundup 5mg/L exposure for 8-days of 60-day-old male rats caused alteration of aromatase activity, a rise of abnormal sperm morphology and nuclear structural protein damages, but no modification in concentration or motility. Other studies confirmed the same negative effects: repeated application of glyphosate at relatively low doses of 5 mg/kg (Abarikwu et al., 2015) or a single application at a high dose of 500 mg/kg (Dai et al., 2016) have affected male fertility in adult rats.

Fish represents a primary animal model for fertility research due to the cheap and easy maintenance in laboratories of these animals, their short reproductive cycle period and their close similarity to mammals in reproductive regulation systems (Veldman et al., 2008). Moreover, water is one of the most polluted substrates by pesticide; therefore, fish are constantly exposed to these compounds by nature thus making it ideal to be taken as animal model for toxicological studies (Carvan et al., 2007). Lopes et al. (2014) demonstrated that zebrafish exposed to 0.5 mg/L to glyphosate, after 24h showed a decrease in sperm quality, mitochondrial activity and sperm motility and an increase of membrane and DNA damage. Also Jenynsia multidentata sperm motility and concentration were disturbed by exposure to similar sublethal concentrations of glyphosate and Roundup (Hued et al., 2012; Sanchez et al., 2017).

Toxic effects of environmental concentrations of Roundup (130 and 700 µg L^{-1}) on sperm quality were confirmed by Harayashiki et al. (2013) in the guppy Poecilia hiki vivipara, with alterations in plasma membrane integrity, mitochondrial functionality, DNA integrity, motility, motility period, and sperm cell concentration. Finally, Gonçalves et al. (2018) demonstrated that low concentrations of glyphosate-based herbicide impaired Astyanax lacustris sperm viability at 300 µg/L, a concentration that is within legal limits in US water bodies whereas motility was impaired yet at 50 µg/L.

According to the few in vitro experiments carried out on human semen, glyphosate at the dose of 0,36 mg/L caused a significant reduction of progressive motility after 1 h incubation whereas 1 mg/L Roundup caused, after 1 h of incubation, a drop in sperm progressive motility and a depression of mitochondrial activity (Anifandis et al., 2017; Anifandis et al., 2018). A very recent study by Ferramosca et al. (2121) claim that glyphosate negatively affects mitochondrial respiration efficiency starting from concentration of 100 nM. In presence of 10 nM DHT, the negative effect of glyphosate was visible already at the concentration of 0.1 nM (Ferramosca et al., 2121). This is an intriguing aspect, since suggests that sex steroid hormones could enhance glyphosate effect on mitochondrial function.

GBHs AND FEMALE FERTILITY

Even if males seem to be more sensitive to GBH exposure (de Melo et al., 2020), disruption endocrine effects at low doses have been reported in females as well. The main evidence stressed out in literature is an alteration in steroid production at concentrations much lower than the agricultural dilutions. Several authors stressed that aromatase activity, required for bioconversion of androgens into estrogens, can be affected in human embryonic and placental cells by different ways: glyphosate acts as a disruptor of cytochrome P450 aromatase activity, impairs aromatase gene expression, interacts with the active site of the enzyme and also causes alterations of estrogen-regulated genes (Richard et al., 2005; Benachour et al., 2007; Thongprakaisang et al., 2013). The decrease in estrogen production is far more evident with GBHs than with glyphosate alone, possibly due to a supportive role of formulants in inhibiting aromatase function (Mesnage et al., 2013; Defarge et al., 2016).
Gasnier et al. (2009) reported a decrease in progesterone levels in response to GBHs sub-agricultural doses exposure which explains the inhibition in treated cell lines of cholesterol transfer in the mitochondria, where aromatase protein and estrogen and androgen receptors are held. Nevertheless, Perego and al. (2017) evaluated bovine ovarian granulosa and teca cell proliferation as well as the production of progesterone, androgens and estrogens to determine glyphosate effects at low doses on ovarian follicles. Granulosa cells proliferation and steroidogenesis were strongly inhibited by 10 mg/ml GBH, but no effects were reported when glyphosate alone at the same concentrations was tested. Those authors therefore suggested that GBHs at concentrations much below agriculture use and authorized residues in food or feed, act as endocrine disruptors (Perego et al., 2017).
Moreover, the effects of GLY at different doses (2, 4 and 16 µg/mL) on swine granulosa cell steroidogenesis and oxidative stress were evaluated by Gigante et al. (2018), Their results showed an

inhibition on cellular growth, 17-β estradiol production and antioxidant activity while, on the other side, progesterone and ROS production increased.

Anyway, according to present literature, evidence of GBHs impact on estrogen production is still controversial. Thongprakaisang et al. (2013) found out that pure glyphosate improves estrogen receptors (ERs) transcriptional activity and their expressions, suggesting an estrogenic activity of low and environmentally relevant concentrations of glyphosate. This finding is although in contradiction with another study by Gasnier et al. (2009) who found that a glyphosate-based herbicide inhibited the transcription of estrogen receptors in HepG2 cells, whereas there was no significative difference with pure glyphosate. This discrepancy may be due to cell types as Thongprakaisang et al. (2013) used E2 targeted cells like breast cancer cells and different results may also be due to different concentration tested, which were higher for Gasnier et al. (2009) study.

Focusing on other fertility parameters as oocytes maturation, Zhang et al. (2019) study on mouse oocytes revealed that exposure to 500 µM glyphosate reduced rates of germinal vesicle breakdown (GVBD) and first polar body extrusion. Moreover, after 14 h of exposure, metaphase II (MII) displayed abnormalities in spindle morphology and DNA double-strand breaks. Finally, glyphosate exposure negatively impacted mitochondria membrane potential while promoting ROS production and early apoptosis.

In 2020, Yahfoufi et al. investigated the tolerance of both mouse oocytes and embryos to 0 – 300 µM glyphosate. Their results confirmed that glyphosate exposure impairs metaphase II mouse oocyte by disrupting microtubule structure (anomalous pericentrin formation, spindle fiber destruction and disappearance) and causing defective chromosomal alignment. Other consequences highlighted by the authors were the substantial depletion of intracellular zinc bioavailability and accumulation of reactive oxygen species. Similar effects were found in embryos. Induction of oxidative stress and apoptosis were also observed in bovine embryos cultured in presence of Roundup (Cai et al. 2020).

Furthermore, in vitro studies showed that GBHs exposure at low doses can lead not only to endocrine disfunction but also to cellular mutagenic and toxic effects on cells involved in reproduction such as embryonic, fetal, placental and umbilical cord vein cells (Benachour and Séralini, 2009; Benachour et al., 2007; Richard et al., 2005).

Nowadays, several studies have tested the effects of GBHs on female reproductive tract in vivo.

Seralini et al. (2014) evidenced, among the results obtained from their study, the endocrine disrupting effect of Roundup on estrogen/androgen balance starting at 0.1 ppb dose, causing hormone-dependent mammary tumors and pituitary and sex hormone disruption in females linked to chronic exposure. Ingaramo et al., in 2016, published a scientific work where neonatal female rats received 2 mg/kg

bw/day of a GBHs on days 1, 3 and 7 after birth and at 3 months age they were mated to evaluate reproductive performance. The authors detected a significantly higher rate of fetus resorptions, probably due to a direct action on uterus rather than on ovarian activity as no alterations in steroid production or ovulation rate were detected. The subchronic 60 days exposure of female Wizard rats to 0.175 ml/day GBH Kalach 360 SL (corresponding to 315 mg /kg/day glyphosate), impaired folliculogenesis, ovary development and estrogen secretion while promoted oxidative stress (Hamdaoui et al., 2018). Not only endocrine disruption signs were reported, but also ovary damage. Cell necrosis, vacuolisation of follicles, dissociation of oocytes from granulosa cells and several atretic follicles were reported as histological findings (Hamdaoui et al., 2018). Ren et al. (2019) aimed to investigate the toxic effects on pregnant mice and their fetuses during pregnancy. Mice were orally administered 0.5% pure glyphosate and Roundup equivalent solution from gestation day 1 to 19 and, after that, ovaries and serum were collected. Glyphosate treated pregnant females showed a decrease in body weight gain and ovary histopathological alterations as increased atretic follicles, interstitial fibrosis and decreased number of mature follicles. Serum concentrations of both progesterone and estrogen were significantly impacted and there were also interferences on hypothalamic-pituitary-ovarian axis activity. Furthermore, oxidative stress was observed. With regard to litters, the sex ratio was also altered by prenatal exposure. The latest Ramazzini Institute pilot study (2019) showed a testosterone increase in female rats exposed to Roundup at ADI dose from prenatal period to adulthood, and also an alteration of reproductive developmental parameters, such as increase of anogenital distance (marker of prenatal endocrine disruption) and delay of first estrous (Manservisi et al., 2019).

Finally, Milesi et al. (2018) evaluated the reproductive performance of F1 and F2 offspring, originated from F0 female rats exposed to 2 mg/kg bw/day of GBHs during pregnancy. The only parameter with a negative score in F1 was the number of implantation sites, while F2 offspring showed lower fetal weights and lengths, higher incidence of early parturition and occurrence of congenital anomalies.

Studies performed using fish as reproductive model uphold the thesis of negative impact of GBHs on female fertility. Armiliato et al. (2014) reported in Zebrafish *Danio rerio* exposed to 0,065 mg /L glyphosate changes in ovary structure associated to an overexpression of Steroidogenic Factor-1 (SF-1) in oocytes.

Relating to effects on embryos, a recent study published by Zebral et al. in 2018, confirmed that Roundup negatively impacted fish reproduction in *Austrolebias nigrofasciatus as* 0.36 mg a e./L. Roundup treated fish produced fewer but larger embryos with lower tolerance to heat. It has to be kept in mind that concentrations in water surfaces are regularly around 0.01 mg/L in the environment

and during occasional peak contamination events they can reach 0.5 mg/L. Much higher doses induce clearly acute toxicity, as shown by Webster et al. (2013) who tested both 10 mg / L Roundup (glyphosate acid equivalent) and glyphosate alone in *Danio rerio* Zebrafish and highlighted an increase in early embryo mortality and premature hatching. In 2007, Soso et al., had already suggested that exposure to sublethal concentration (3,6 mg/L) of glyphosate in water was deleterious to *Rhamdia quelen* reproduction, altering steroid profiles and egg viability.

Mottier et al. study, published in 2013, provided the first data for both embryotoxicity and metamorphosis tests conducted in a marine bivalve Pacific oyster, Crassostrea gigas. According to the authors' opinion glyphosate and AMPA can be considered "slightly toxic", whereas the two commercial formulations can be classified as "moderately toxic". The EC50 values were much lower for commercial formulations than for glyphosate alone.

The available results on GBH effects on the female reproductive tract by using animal models are interesting because they suggest a link with the reproductive problems observed in women living in rural zones with massive use of herbicides. For example, in a study performed on an Ontario Farm Population, women provided information on spontaneous abortions and it was demonstrated that preconception exposure to glyphosate increased the risk of abortions (Arbuckle *et al*. 2001).

ALTERNATIVE SOLUTIONS TO GLYPHOSATE

Clearly, to give a critical opinion on GBHs use and its consequences on population is not easy. To suggest practicable alternatives is also a really complex manner as obviously a good solution should have a good efficacy and should be both ecologically and economically sustainable.

However, it is good to make a critical analysis and try to take strategic choices with the aim of preserving our health. At present, other compounds that can compete with glyphosate for costs and efficacy don't exist, therefore the only alternative is to change the type of agriculture introducing new techniques to manage unwanted herbs (Torretta et al., 2018).

For domestic use and for urban areas and road maintenance this problem can be approached in a quite practicable way by using natural methods of weed control, such as:

- Preventive measures: regularly clean the garden and avoid the accumulation of fine materials by covering the empty cracks or microspaces in which it could easily accumulate.

- Soil processing: working the soil and cutting the green surfaces thus eliminating unwanted herbs and preventing their regrowth
- Use of natural substances such as cooking salt, diluted vinegar (around 10%) and products based on herbal extracts.

For agricultural fields the question gets harder; some alternatives to glyphosate exist, but inevitably lead to a cost increase. Here below, a short description of the most interesting practical applications:

Pyroherbicide: direct physical control over weeds, fungal diseases, and insects by using direct flame. The application time is as such that it does not involve the carbonization of the vegetables, but only a rapid increase in temperature that damages the cells outer membrane leading the plat to die within 1–3 days.
Regarding the environmental impact, pyroherbicide is not dangerous, as it releases only carbon dioxide and water vapor, and it doesn't cause any alteration of the color of the soil on which it is used.

Biological Agriculture: method of cultivation based only on the use of natural substances already present in nature. The aim of biological agriculture is to create a system at low cost, with low environmental impact, that does not need any type of external human operation (mechanical, thermal, chemical, extractive, etc.) and that preserve biodiversity. In order to achieve these objectives, very specific rules are followed:

- organic crops are rotated to ensure a more efficient use of resources;
- the use of pesticides, herbicides, synthetic chemicals, and genetically modified organisms is prohibited;
- the farms are organized in a closed cycle, so that the farms supply the agricultural fertilizers and the agriculture supplies the food for the animals;
- mulching is practiced to cover the ground, avoiding the growth of weeds;
- green manure is used, that is the sowing of some plants, such as clover, spinach, etc., which once flowered and buried improve the fertility of the soil (Torretta et al., 2018).

Hydroponic Agriculture: Hydroponics is the process of growing plants without the use of soil, delivering plant nutrients directly through watering. One of the greatest benefits of growing plants hydroponically is that it requires just 1/5 of the land needed for the same amount of plants being cultivated on farmland.

Moreover, hydroponic growing actually uses only 10% of the water that is typically needed to grow plants in traditional soil. Finally, most hydroponic growing is done in greenhouses or other structures and this condition leads to greatly reduced pesticide use, as seeds don’t blow in and germinate in adjacent soil (Woodard, 2019).

OBJECTIVES

The present work aimed to study the impact of glyphosate and Roundup exposure on mammalian gametes.

In the first study pig spermatozoa were used as an “in vitro” model to investigate which is the real impact of pure glyphosate and its most known commercial formulation, Roundup, on sperm function and survival. With this purpose, fresh commercial boar semen doses were incubated at 38 ºC for 3 h with different concentrations (0, 5, 25, 50, 100 and 360 µg/mL) of either glyphosate or Roundup (glyphosate equivalent concentrations). After 1 h and 3 h of incubation, sperm quality parameters (sperm motility, viability, mitochondrial membrane integrity, acrosome integrity and DNA fragmentation) were evaluated.

The objective of the second study was to characterize the impact of glyphosate and Roundup (0, 5, 10, 100, 200 and 360 µg/mL glyphosate and glyphosate equivalent concentrations respectively) on female gamete using an “in vitro” model of pig oocyte maturation (IVM) by evaluating nuclear maturation, cytoplasmic maturation and developmental competence of oocytes, steroidogenic activity of cumulus cells as well as intracellular levels of glutathione (GSH) and ROS of oocytes.

Finally, in the third study we tested glyphosate and Roundup effects on stallion semen to investigate whether the species could be considered as a variable factor. Experimental design was similar to the one used for paper 1, with few adjustments as the addition of a lower (0,5 µg/ml) and a higher concentration tested (720 µg/ml). Moreover, we assayed sperm ROS production as an additional index for sperm quality. All the evaluations were performed after 1h of incubation at 38°C.

The first two set of experiments are reported in Paper 1 and 2 of the Paper Compendium.

Paper 1. Nerozzi C., Recuero S., Galeati G., Bucci D., Spinaci M., Yeste M. “Effects of Roundup and its main component, glyphosate, upon mammalian sperm function and survival”. Scientific Reports. 2020; doi: 10.1038/s41598-020-67538-w.

Paper 2. Spinaci M., Nerozzi C., Tamanini C., Bucci D., Galeati G. “Glyphosate and its formulation Roundup impair pig oocyte maturation”. Scientific Reports. 2020; doi: 10.1038/s41598-020-68813-6.

Objectives

The Material and Methods and Results of the third set of experiments, that represents a preliminary study, are reported at the end of Paper Compendium

PAPER COMPENDIUM

PAPER 1

Effects of Roundup adjuvants, rather than its main component glyphosate, detrimentally impact mammalian sperm function and survival

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Effects of Roundup and its main component, glyphosate, upon mammalian sperm function and survival

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The wide use of glyphosate-based herbicides (GBHs) has become a matter of concern due to its potential harmful effects on human health, including men fertility. This study sought to investigate, using the pig as a model, the impact of pure glyphosate and its most known commercial formulation, Roundup, on sperm function and survival. With this purpose, fresh commercial semen doses were incubated with different concentrations (0–360 µg/mL) of glyphosate (GLY; exp. 1) or Roundup, at the equivalent GLY concentration (exp. 2), at 38 °C for 3 h. Glyphosate at 360 µg/mL significantly ($P < 0.05$) decreased sperm motility, viability, mitochondrial activity and acrosome integrity but had no detrimental effect at lower doses. On the other hand, Roundup did significantly ($P < 0.05$) reduce sperm motility at ≥ 5 µg/mL GLY-equivalent concentration; mitochondrial activity at ≥ 25 µg/mL GLY-equivalent concentration; and sperm viability and acrosome integrity at ≥ 100 µg/mL GLY-equivalent concentration as early as 1 h of incubation. In a similar fashion, GLY and Roundup did not inflict any detrimental effect on sperm DNA integrity. Taken together, these data indicate that, while both glyphosate and Roundup exert a negative impact on male gametes, Roundup is more toxic than its main component, glyphosate.

Abbreviations

CASA	Computer-assisted sperm analysis
JC1	5,5′,6,6′-Tetrachloro-1,1′,3,3′tetraethylbenzimidazolylcarbocyanine iodide
GLY	Glyphosate
GBHs	Glyphosate-based herbicides
MMP	Mitochondrial membrane potential
PI	Propidium iodide
PNA	Lectin from *Arachis hypogaea*
3Rs	Replacement, reduction and refinement
SCSA	Sperm chromatin structure assay

Glyphosate (GLY) is the active ingredient of all glyphosate-based herbicides (GBHs), including the most famous commercial formulation, Roundup (R). Despite GBHs being currently used worldwide, not only does this massive usage represent a risk for farmers but also for the general population, as environmental contamination with glyphosate affects water and food consumption[1,2]. For this reason, the use of GBHs has become a matter of concern for public health, and much debate has been raised about their potential carcinogenicity and negative impact on neurologic, gastroenteric, endocrine and reproductive systems[3–9].

Previous research has confirmed that, at low doses, glyphosate acts as an endocrine disruptor in mammals altering hormonal function[10,11]. In particular, it has been suggested that GBHs can interfere with steroidogenesis

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in different ways, such as downregulating the expression of the steroidogenic acute regulatory protein (STAR), disrupting cytochrome P450 aromatase[10–12], impairing the expression of oestrogen-regulated genes, or interacting with both androgen and oestrogen receptors[6,10–11,13]. Furthermore, GBHs have been purported to induce redox imbalance, causing Ca^{2+} overload and depletion of the antioxidant defence systems[14,15]. Since they often contain traces of heavy metals that can reach up to 80 ppb, such as arsenic, chromium, cobalt, lead and nickel, GBHs are also known to be cytotoxic and act as endocrine disruptors[16].

Concerning the effects on male fertility, Anifandis et al. found that addition of 0.36 μg/mL glyphosate causes a significant reduction in progressive motility of human spermatozoa after 1 h incubation[17]. Furthermore, Clair et al. reported that glyphosate at 1,800 μg/mL is cytotoxic for testicular germ cells and, to a lesser extent, for Leydig cells[18]. It is worth mentioning that the aforementioned concentration is half of the one utilised to dilute the herbicide. Remarkably, signs of endocrine disruption, such as a decrease of 35% in testosterone serum levels, are detected at much lower glyphosate concentration (0.36 μg/mL)[18]. Using the zebrafish as a model, Lopes et al. reported that 24 h after adding feeding with glyphosate, there was a reduction in sperm motility (5 mg/mL) and in mitochondrial activity and DNA integrity (10 mg/mL)[19].

Most of the research conducted in the last years has been focused on commercial glyphosate formulations, since they seem to exert more detrimental side-effects than glyphosate alone, possibly because formulants are not inert compounds at all[10,13,20–27]. As far as Roundup is concerned, it has been demonstrated that very low concentrations of this commercial product alter steroidogenesis[12], and that it induces a notable cytotoxic effect on all testicular rat cells after incubation at 0.1% (corresponding to 360 μg/mL glyphosate) for 24 h[18]. This concentration is ten times lower than that recommended for agricultural use. According to the only in vitro experiment conducted thus far, incubation of human sperm with 1 μL/mL Roundup (corresponding to a glyphosate concentration of 0.36 μg/mL) for 1 h causes a drop in progressive motility and a depletion in mitochondrial activity[17]. Moreover, in vivo studies conducted in a fish species (*Jenynsia multidentata*) showed that exposure to Roundup, at a corresponding glyphosate concentration of 0.5 μg/mL, for 24 h decreases sperm motility and concentration[28]. In rats, exposure to 5 μg/mL Roundup for 8 days increases the proportions of morphologically abnormal spermatozoa and alters nuclear integrity[29]. In addition, when rats are exposed to Roundup during the prepubertal period, there is a decrease in serum testosterone levels and changes in testicular morphology occur[30,31]. Perinatal exposure leads to an increase in serum concentrations of testosterone, oestradiol and luteinizing hormone, inducing an early onset of puberty and sexual behavioural changes in the male offspring[32]. When the time of exposure lasts from the perinatal period to lactation, male offspring rats suffer from a drop in daily sperm production and show morphological abnormalities[33]. Finally, Roundup administered from the prenatal period to adulthood at an acceptable daily intake (ADI) dose of 1.75 mg/kg of body weight per day is enough to induce an alteration in rat reproductive developmental parameters, such as the increase of anogenital distance, which is a marker of prenatal endocrine disruption[34].

Against this background, the aim of this work was to investigate, for the first time in the same study, the effects of different concentrations of glyphosate and Roundup on mammalian sperm following exposure for 1 h and 3 h at 38°. We used the pig as a model, and tested concentrations ranging from either 360 μg/mL GLY or 0.1% Roundup (equivalent to 360 μg/mL GLY), which seem to be cytotoxic according to Clair et al.[18], to 70-fold lower. All Roundup concentrations are expressed as GLY-equivalent concentration.

Results

Experiment 1: Effects of glyphosate on sperm quality and functional parameters. Compared to the control, addition of 360 μg/mL glyphosate significantly ($P<0.05$) decreased total and progressive motility, viability, mitochondrial activity and acrosome integrity after 1 h and 3 h of incubation at 38 °C. In contrast, no significant differences between the control and lower glyphosate concentrations were observed. On the other hand, DNA fragmentation analysis showed no differences ($P>0.05$) between the control and treatments after either 1 h or 3 h of incubation at 38 °C (Figs. 1, 2).

Experiment 2: Effects of Roundup on sperm parameters. As shown in Fig. 3, total sperm motility was significantly ($P<0.05$) reduced in all Roundup concentrations (≥ 5 μg/mL GLY-equivalent) after 1 h and 3 h of incubation at 38 °C. In the case of progressive sperm motility, exposure to Roundup at concentrations ≥ 25 μg/mL GLY-equivalent significantly ($P<0.05$) decreased this parameter after 1 h of incubation at 38 °C. In addition, concentrations ≥ 5 μg/mL GLY-equivalent showed significantly ($P<0.05$) lower progressive sperm motility than the control after 3 h of incubation at 38 °C. Therefore, there was a dose-dependent impact of Roundup upon total and progressive sperm motility (Fig. 3). In addition, not only did Roundup have detrimental effects on total and progressive sperm motility, but also on kinematic parameters, as shown in Supplementary Table 2.

On the other hand, percentages of viable spermatozoa ($SYBR14^+/PI^-$) significantly ($P<0.05$) decreased when semen samples were exposed to Roundup at concentrations equal or higher than 100 μg/mL GLY-equivalent. This reduction, which was observed after 1 h of incubation at 38 °C, persisted at 3 h (Fig. 4). As expected, exposure to Roundup also led to a significant ($P<0.05$) decrease in the percentages of acrosome-intact spermatozoa after 1 h (≥ 100 μg/mL GLY-equivalent) and 3 h of incubation at 38 °C (≥ 50 μg/mL GLY-equivalent; Fig. 4).

Addition of semen samples with Roundup ≥ 25 μg/mL GLY-equivalent significantly ($P<0.05$) reduced the percentage of spermatozoa with high MMP after 1 h of incubation at 38 °C. Furthermore, exposure to Roundup ≥ 50 μg/mL GLY-equivalent significantly ($P<0.05$) decreased the percentage of spermatozoa with high MMP after 3 h of incubation at 38 °C (Fig. 4). In a similar fashion to that observed for sperm motility, viability, acrosome integrity and mitochondrial activity decreased in a dose-dependent manner. Finally, Roundup, like glyphosate, had no effect on sperm DNA integrity.

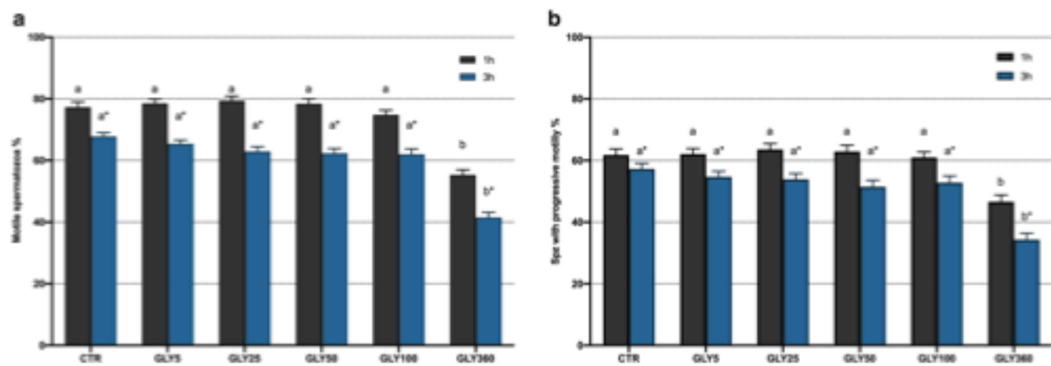

Figure 1. Effects of exposure to 0, 5, 25, 50, 100 and 360 µg/mL glyphosate on total (**a**) and progressive (**b**) sperm motility evaluated through CASA system. Different letters (*a*, *b*) represent significant ($P < 0.05$) differences between treatments. (*) represents significant ($P < 0.05$) differences between incubation times within a given treatment. CTR: control, sperm sample without addition of glyphosate; Spz: spermatozoa. Data are shown mean ± SEM.

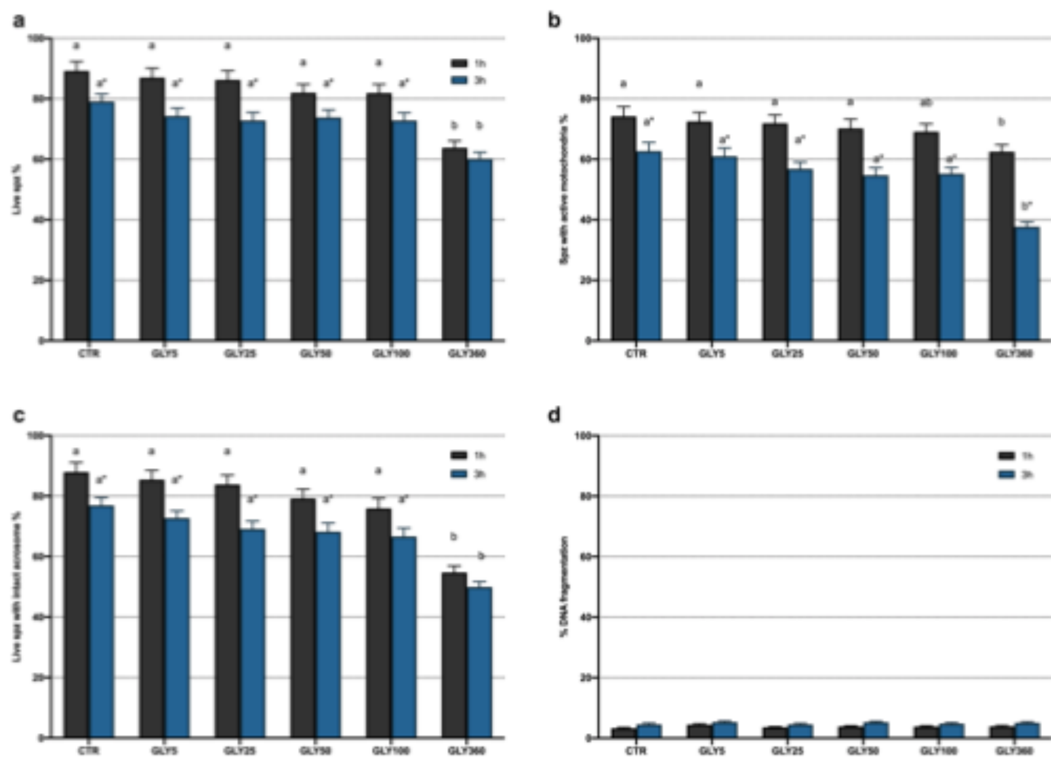

Figure 2. Effects of exposure to 0, 5, 25, 50, 100 and 360 µg/mL glyphosate on viability (**a**), percentage of spermatozoa with high mitochondrial membrane potential (**b**), acrosome integrity (**c**) and DNA fragmentation (**d**). Different letters (*a*, *b*) represent significant ($P < 0.05$) differences between treatments. (*) represents significant ($P < 0.05$) differences between incubation times within a given treatment. CTR: control, sperm sample without addition of glyphosate; Spz: spermatozoa. Data are shown mean ± SEM.

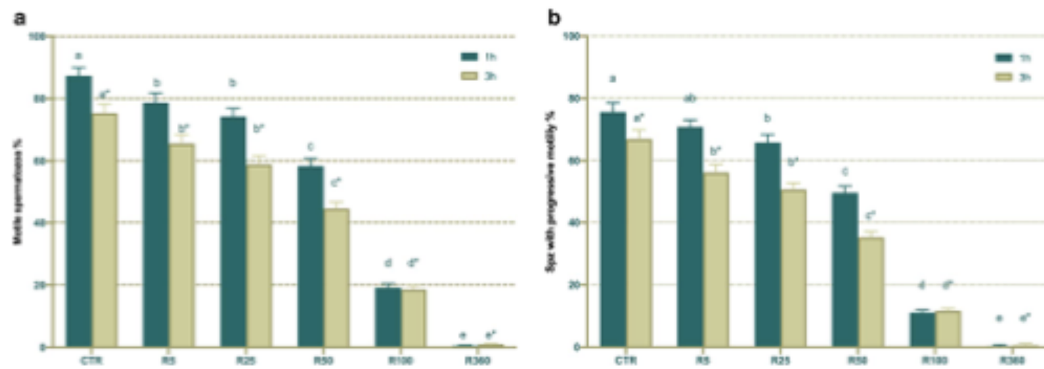

Figure 3. Effects of exposure to Roundup (at concentrations equivalent to glyphosate: 0, 5, 25, 50, 100 and 360 µg/mL) on total (**a**) and progressive (**b**) motility acquired by CASA system. Different letters (*a, b*) represent significant ($P<0.05$) differences between treatments. (*) represents significant ($P<0.05$) differences between incubation times within a given treatment. CTR: control, sperm sample without addition of Roundup; Spz: spermatozoa. Data are shown mean ± SEM.

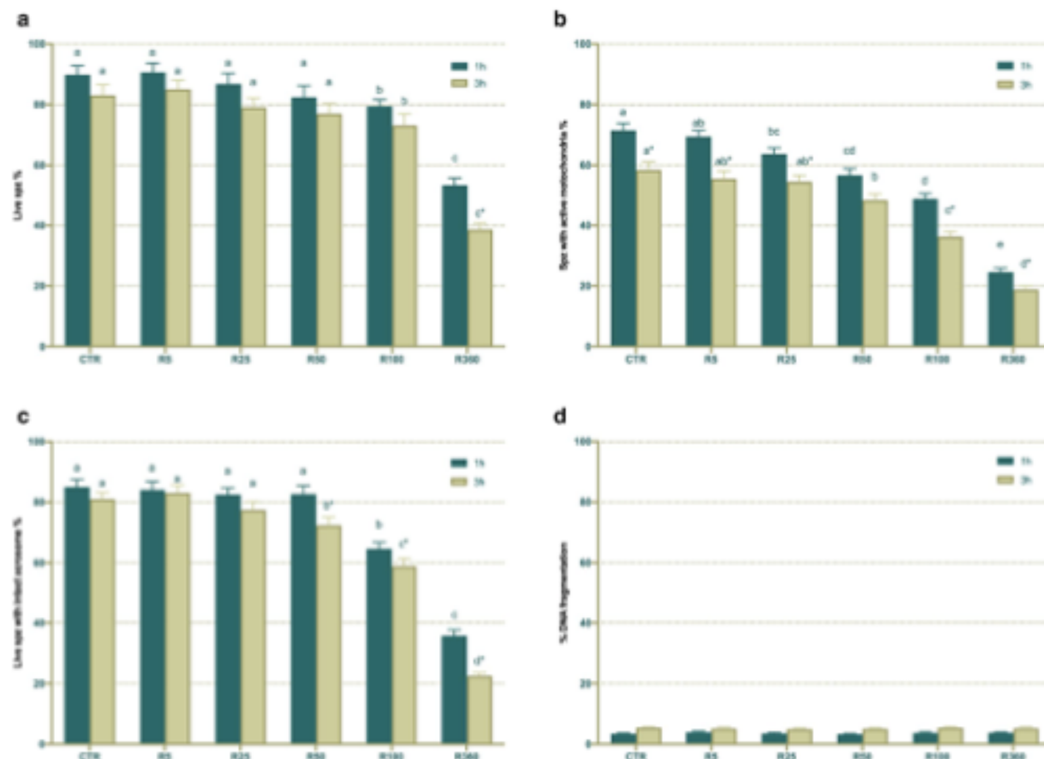

Figure 4. Effects of exposure to Roundup (at concentrations equivalent to glyphosate: 0, 5, 25, 50, 100 and 360 µg/mL) on sperm viability (**a**), percentage of spermatozoa with high mitochondrial membrane potential (**b**), acrosome integrity (**c**) and DNA fragmentation (**d**). Different letters represent significant ($P<0.05$) differences between treatments. (*) represents significant ($P<0.05$) differences between incubation times within a given treatment. CTR: control, sperm sample without addition of Roundup; Spz: spermatozoa. Data are shown mean ± SEM.

Discussion

At present, there is a consistent body of literature suggesting that GBHs have negative effects on human health. However, it remains to be elucidated whether it is the main component of these products, i.e. glyphosate, or other compounds present in the commercial formulation of GBHs, which are the most toxic for the organism. In addition, although the detrimental effects of GBHs and glyphosate have been studied in animal models, with special reference to endocrine disruption, less attention has been paid on the effects of these products on mammalian sperm quality. For this reason, this study was the first to evaluate the effects of exposing mammalian spermatozoa to pure glyphosate or one of its most commercial formulations (Roundup) at different concentrations (0, 5, 25, 50, 100 and 360 μg/mL glyphosate, or glyphosate equivalent doses in the case of Roundup). We utilised the pig as a model, since not only is this animal species important for agriculture, but also for its use in biomedical research, due to its anatomical and physiological similarities with the human[35,36]. Moreover, and according to the 3Rs principle, sperm from mammalian species other than rodents have been demonstrated to have the potential to serve as a useful in vitro screening test for reproductive toxicology[37].

Sperm motility, sperm viability, acrosome integrity, mitochondrial membrane potential, and DNA integrity were evaluated after 1 h and 3 h of incubation at 38 °C. On the one hand, while we observed that pure glyphosate decreased sperm motility (both total and progressive), viability and mitochondrial membrane potential, this only occurred at the highest concentration (360 μg/mL) and after both 1 h and 3 h of incubation. In contrast, no effects were observed at lower concentrations. Whilst the detrimental impact of exposing mammalian spermatozoa to glyphosate has also been reported in humans, our results differ from those obtained by Anifandis et al., as they found that progressive sperm motility decreased at a much lower concentration (0.36 μg/mL) after 1 h of incubation[17]. This discrepancy could be due to a species-specific sensitivity to glyphosate exposure and/or could be ascribed to the different procedures for sperm motility assessment; indeed, while we evaluated sperm motility through a CASA system, Anifandis et al.[17] assessed human semen motility according to WHO 2010 guidelines under a phase-contrast microscope. With regard to DNA fragmentation, we found that glyphosate samples did not differ from the control, which in this case did agree with Anifandis et al.[17].

The negative impact on quality and functional parameters was much more apparent when spermatozoa were exposed to Roundup. This finding confirmed previous research on human JEG3 and embryonic kidney 293 cells indicating that commercial formulations are far more deleterious than glyphosate itself[16,38]. Moreover, we observed that Roundup was detrimental for sperm in a concentration-dependent manner, as total sperm motility was impaired at the lowest concentration (5 μg/mL) and progressive sperm motility was significantly reduced at ≥ 25 μg/mL. These results agree with those of Anifandis et al.[39], who found that progressive sperm motility in humans is negatively influenced by the presence of 1 μg/mL Roundup (corresponding to a glyphosate concentration of 0.36 μg/mL). Herein, however, we found that detrimental effects occur at higher doses in pig sperm, possibly due, again, to a species-dependent sensitivity to Roundup exposure and/or to the different methods used to assess sperm motility[17]. Moreover, and in agreement with Anifandis et al.[39], the decrease in mitochondrial membrane potential was concomitant with that of total sperm motility, as incubation with 25 μg/mL Roundup for 1 h reduced mitochondrial activity. The detrimental impact of GBHs on mitochondrial activity has already been demonstrated in *Caenorhabditis elegans*, as exposing these animals to 3% TouchDown (expressed as % glyphosate; TouchDown Hitech, formulation of 52.3% glyphosate) leads to a decrease in mitochondrial respiration, oxygen consumption and ATP production, and an increase in intracellular levels of hydrogen peroxide[40]. These toxic effects have been suggested to be exerted through the impairment of the electron transport chain function via inhibition of Complex II (succinate dehydrogenase)[40,41]. Moreover, Roundup has been demonstrated to reduce the activity of mitochondrial succinate dehydrogenase in an immature mouse Sertoli TM4 cell line at a concentration as low as 0.001%, with heavier toxic effect than glyphosate alone[42]. By studying rat liver mitochondria, Peixoto et al. demonstrated the capacity of GBHS to alter mitochondrial bioenergetics at concentrations ≥ 5 mM glyphosate[43]. Our results are in agreement with the aforementioned studies and confirm that the compounds present in commercial herbicides may potentiate mitochondrial perturbation. However, it is worth mentioning that, in this study, spermatozoa considered to exhibit high mitochondrial membrane potential included heterogeneous sperm populations. These sperm populations went from cells with very high mitochondrial membrane potential, which showed orange fluorescence when stained with JC1, and with high or intermediate membrane potential, in which JC1 emitted in both orange and green along the mid-piece. Therefore, further studies should also evaluate the impact of glyphosate and Roundup on the separate sperm populations that show different green and orange fluorescence intensities following JC1-staining.

With regard to sperm viability, 100 μg/mL Roundup significantly reduced the percentages of $SYBR14^{+}/PI^{-}$ spermatozoa after 1 h of incubation at 38 °C. These results were in accordance with those obtained following evaluation of acrosome integrity, as percentages of spermatozoa exhibiting a non-intact acrosome were higher after 1 h and 3 h of exposure to 100 and 50 μg/mL Roundup. Previous studies confirmed the negative impact of Roundup on cell viability. Richard et al. reported its toxicity for human placental JEG3 cells at a concentration of 0.4% after 1 h of incubation[10], and Clair et al. found signs of Roundup toxicity in both Leydig and Sertoli rat cells with a 0.1% concentration after 24 h[18]. The same result obtained by Clair et al. was confirmed by Vanlaeys et al. in an immature mouse Sertoli TM4 cell line TM4[42].

It is worth noting that all sperm quality and functional parameters affected by the exposure to Roundup showed a clear dose-dependent trend. Moreover, as observed by Anifandis et al. in human sperm[39], most of the induced damage was already apparent after 1 h of incubation at 38 °C, suggesting that the negative effects induced by Roundup could occur rapidly during the first hour of exposure. This quick action of Roundup, which contrasts with that of glyphosate alone, was already observed following 90-min exposure of human embryonic kidney cells (HEK 293) to different glyphosate-based herbicides, including two different Roundup formulations (Roundup WeatherMAX and Roundup Classic) that are very similar to Roundup Bioflow[16].

To the best of our knowledge, this study represents the first evaluation and comparison of biological activities of glyphosate and Roundup on mammalian spermatozoa. Overall, our results suggest that, while both glyphosate and Roundup induce toxic effects on mammalian sperm function and survival, Roundup has much more detrimental impact than glyphosate, even at equivalent concentrations of glyphosate. Therefore, the fact that Roundup is more toxic than pure glyphosate itself, causing more severe alterations than this active principle, confirms the hypothesis that formulants present in commercial products either boost glyphosate toxicity or are harmful themselves[10,13,20–27]. Based on these results, not only should the perniciousness of glyphosate be evaluated when handling glyphosate-based herbicides, but also that of the other compounds. Therefore, whether bioaccumulation of these formulants, which are petroleum-derivatives, may have serious implications and cause chronic toxicity needs to be investigated. This is crucial given the growing concerns on the impact and safety of glyphosate and glyphosate-based herbicides.

On the other hand, one should bear in mind that, while this was a toxicological study testing high glyphosate and Roundup doses, environmental concentrations and levels in serum and urine recorded in recent publications are far lower than those tested herein[44,45]. Moreover, the biotransformation process that the compound undergoes inside the organism should also be taken into consideration, since glyphosate can be partially degraded prior to reaching male germ cells, which would make it less cytotoxic. In spite of all the aforementioned, it is clear from this study that the large use of glyphosate formulations, especially Roundup, may entail a risk for male fertility; hence, further research aimed at clarifying the effects and toxicity of each compound is much warranted.

Based on our results, it can be hypothesized that the toxic effect of these pesticides may be linked to an impairment in mitochondrial activity and a subsequent decrease in ATP production and/or alterations in the redox balance, which impact cell motility and plasma membrane stability. In spite of this, DNA integrity seem not to be altered either by Roundup or pure glyphosate. At present, the mechanism of action of GBHs remains unclear and needs to be investigated further.

In conclusion, the consequences of the massive use of glyphosate remains a matter concern for human health and food quality. Through using the mammalian spermatozoon, we found that both glyphosate and Roundup detrimentally affect cell function and survival, the latter being much more toxic than the former. This indicates that GBHs components other than glyphosate damage spermatozoa and may have a detrimental effect on fertilizing ability. Therefore, and in order to address the concerns on the use of GBHs properly, we suggest that all components present in the commercial formulation of glyphosate should also be tested individually. In addition, further research should address how each of these GBHs components damages the sperm cell.

Methods

Reagents. Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich (Saint-Louis, MO, USA). The commercial formulation of glyphosate, Roundup Bioflow (containing 0.36 g/mL of glyphosate acid in the form of isopropylamine salts of glyphosate, 41.5%; water, 42.5%; and surfactant, 16%), was purchased from Monsanto Europe N.V. (Anversa, Belgium).

All fluorochromes were purchased from Invitrogen Molecular Probes (Thermo Fisher Scientific, Waltham, MA, USA) and diluted with dimethyl sulfoxide (DMSO).

Seminal samples. A total of 10 different ejaculates coming from 10 separate Pietrain boars were used. Animals, aged between two and three years old, were collected by the hand-gloved method. Boar studs were healthy, stabled in climate-controlled buildings (Servicios Genéticos Porcinos, S.L., Roda de Ter, Spain), and fed an adjusted diet, with water being provided ad libitum. After collection, sperm-rich fractions were diluted to a final concentration of 30×10^6 spermatozoa/mL with a commercial extender (Duragen, Magapor; Egea de los Caballeros, Zaragoza, Spain) and cooled to 17 °C. Ninety mL commercial doses were brought to our laboratory in about 45 min, inside a thermal container at 17 °C. It is worth mentioning that, since authors purchased seminal doses from the aforementioned local farm that operates under commercial, standard conditions, they did not manipulate any animal. Therefore, specific authorization from an Ethics Committee was not required to conduct this study.

Experimental design. This work consisted of two separate experiments. In the first one, we investigated the impact of different glyphosate (GLY) concentrations (0, 5, 25, 50, 100 and 360 µg/mL) on sperm quality and functional parameters (30×10^6 spermatozoa/mL). In the second experiment, pig semen was added with commercial glyphosate-based herbicide Roundup (R) at concentrations equivalent to the glyphosate ones tested in the first experiment. All Roundup treatments are expressed as glyphosate equivalent concentrations.

In each experiment, semen was added with glyphosate or Roundup at the aforementioned concentrations and then incubated at 38 °C for 3 h. After 1 h and 3 h of incubation, sperm motility, viability, mitochondrial membrane integrity, acrosome integrity and DNA fragmentation were evaluated.

Evaluation of sperm motility. Sperm motility was evaluated with a commercial computer-assisted sperm analysis (CASA) system (Integrated Sperm Analysis System V1.0; Proiser, Valencia, Spain), following the settings described by Yeste et al.[46]. Briefly, samples were incubated at 38 °C for 5 min and a 5-µL drop was subsequently placed onto a pre-warmed Makler chamber. For every treatment, three replicates of 1,000 spermatozoa each were analysed. A sperm cell was considered to be motile when its average path velocity (VAP) was higher than 10 µm/s, and progressively motile when its straightness (STR) was higher than 45%.

Analysis of sperm parameters with flow cytometry. Flow cytometry was used to determine sperm viability, acrosome integrity, mitochondrial activity and DNA fragmentation, and information in this section is

given according to the recommendations of the International Society of Flow Cytometry[47]. In all assessments, sperm concentration was adjusted to 1×10^6 spermatozoa/mL in a final volume of 0.5 mL, stained with fluorochromes and evaluated through a Cell Laboratory QuantaSC cytometer (Beckman Coulter, Fullerton, CA, USA). Sheath flow rate was set at 4.17 µL/min; electronic volume (EV) and side scatter (SS) were recorded in a linear mode (in EV vs. SS dot plots) for a minimum of 10,000 events per replicate. The analyser threshold was adjusted on the EV channel to exclude cell debris (particle diameter < 7 µm) and aggregates (particle diameter > 12 µm). Each parameter was evaluated twice for every treatment at both evaluation times. EV, SS, FL1, FL2 and FL3 were collected in List-mode Data files and cytometric histograms and dot plots were analysed with Lab QuantaSC MPL Software (version 1.0; Beckman Coulter).

Evaluation of sperm viability. Sperm viability was assessed by using two fluorescent probes, SYBR14 and Propidium Iodide (PI), included in the LIVE/DEAD Sperm Viability Kit[48]. Following staining with 0.5 µL SYBR14 (final concentration: 100 nM) for 10 min at 38 °C in darkness, and then with 2.5 µL PI (final concentration: 12 µM) at the same conditions for 5 min, samples were analysed using two filters FL1 (SYBR14 detection) and FL3 (PI detection) and three different sperm populations were identified: (a) viable, green-stained spermatozoa ($SYBR14^+/PI^-$); (b) non-viable, red-stained spermatozoa ($SYBR14^-/PI^+$) and (c) non-viable spermatozoa that were stained both green and red ($SYBR14^+/PI^+$). Debris, non-stained particles appeared in the lower left quadrant ($SYBR14^-/PI^-$) and were subtracted from the total number of spermatozoa.

Evaluation of acrosome integrity. Acrosome integrity was determined through staining with the lectin from *Arachis hypogaea* (PNA) conjugated with FITC (fluorescein isothiocyanate; PNA-FITC) and PI. Five hundred µL of each sperm sample was incubated with 0.5 µL PNA-FITC (final concentration: 1.25 mg/mL) and 2.5 µL PI (final concentration: 12 µM) for 10 min at 38 °C in darkness. Spermatozoa were then evaluated with the flow cytometer and two categories were distinguished: (a) viable spermatozoa with intact acrosome and plasma membrane ($PNA\text{-}FITC^-/PI^-$), and (b) spermatozoa that had damaged their plasma membrane and/or their acrosome, which included $PNA\text{-}FITC^+/PI^-$, $PNA\text{-}FITC^+/PI^+$, $PNA\text{-}FITC^-/PI^+$ populations. Debris, non-stained particles found in SYBR14/PI staining (i.e. $SYBR14^-/PI^-$) were subtracted from the $PNA\text{-}FITC^-/PI^-$ population and the other percentages were recalculated.

Evaluation of mitochondrial membrane potential. Mitochondrial membrane potential (MMP) was evaluated with 5,5′,6,6′-tetrachloro-1,1′,3,3′tetraethylbenzimidazolylcarbocyanine iodide (JC1). Briefly, after incubating 500-µL aliquots with 0.5 µL JC-1 (final concentration: 3 µM) for 30 min at 38 °C in darkness, two sperm populations were distinguished: (a) spermatozoa with high MMP, which mainly emitted at the orange light spectrum as in these cells the stain aggregates and changes its fluorescence from green to orange; this population appeared in the upper half of FL1/FL2 dot-plots; and (b) spermatozoa with low MMP, which only emitted at the green light spectrum, as JC1 maintains its monomer status; this second population appeared in the lower half of FL1/FL2 dot-plots.

Evaluation of DNA fragmentation. DNA fragmentation was assessed using the Sperm Chromatin Structure Assay (SCSA) test as modified by Morrell et al.[49]. Sperm samples were diluted to a final concentration of 2×10^6 spermatozoa/mL with a buffer solution (TNE: 0.15 M NaCl, 0.01 M Tris–HCl, 1 mM EDTA, pH = 7.4). Two hundred µL of this solution were added with 400 µL of an acid-detergent solution (80 mM HCl, 150 mM NaCl, and 0.1% Triton X-100; pH = 1.2) on ice. After 30 s, samples were added with 1.2 mL acridine orange (AO) and incubated on ice for further 3 min. Afterwards, spermatozoa were evaluated with FL1 and FL3 filters for green and red fluorescence, respectively, and the following parameters were recorded: (a) percentage of DNA fragmentation (%DFI), which corresponded to the ratio between red (ssDNA) fluorescence and red (ssDNA) + green (dsDNA) fluorescence; (b) standard deviation of DFI; and (c) mean fluorescence intensity of ssDNA (Mean DFI).

Statistical analyses. Statistical analyses were performed using a statistical package (IBM SPSS for Windows version 25.0; IBM Corp., Armonk, NY, USA). Data were first tested for normality and homogeneity of variances through Shapiro–Wilk and Levene tests, respectively. When required, data (x) were transformed using arcsine square root (arcsin $\sqrt{x}$) before a general mixed model, in which the between-subjects factor was the treatment (GLY or R concentrations) and the within-subjects factor was the incubation time at 38 °C (1 or 3 h), was run. Pair-wise comparisons were made with post-hoc Sidak test. When no transformation attained normal distribution and homoscedasticity, Scheirer–Ray–Hare and Mann–Whitney tests were used as non-parametric alternative models. In all cases, data are shown as mean ± standard error (SEM) and the minimal level of significance was set at $P \leq 0.05$.

Data availability

Data are available from the authors on reasonable request.

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Author contributions

Conceptualization: G.G., D.B., M.S. and M.Y.; methodology: C.N. and S.R.; validation: G.G, D.B, M.S. and M.Y.; formal analysis: C.N. and S.R.; investigation: C.N. and S.R.; resources: M.S. and M.Y.; data curation, C.N.; writing-original draft preparation, C.N.; writing-review and editing: G.G., D.B., M.S. and M.Y.; supervision: G.G., D.B., M.S. and M.Y.; project administration: M.S. and M.Y.; funding: M.S. and M.Y. All authors have read and agreed to the published version of the manuscript.

Competing interests

The authors declare no competing interests.

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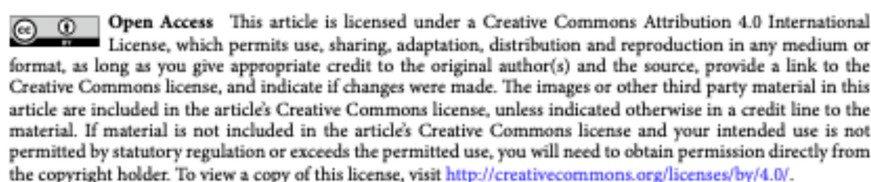

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PAPER 2

Glyphosate and its formulation Roundup impair pig oocytes maturation

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Glyphosate and its formulation Roundup impair pig oocyte maturation

Marcella Spinaci✉, Chiara Nerozzi, Car lo Tamanini, Diego Bucci & Giovanna Galeati

Glyphosate, formulated as glyphosate-based herbicides (GBHs) including the best-known formulation Roundup, is the world's most widely used herbicide. During the last years, the growing and widespread use of GBHs has raised a great concern about the impact of environmental contamination on animal and human health including potential effect on reproductive systems. Using an in vitro model of pig oocyte maturation, we examined the biological impact of both glyphosate and Roundup on female gamete evaluating nuclear maturation, cytoplasmic maturation and developmental competence of oocytes, steroidogenic activity of cumulus cells as well as intracellular levels of glutathione (GSH) and ROS of oocytes. Our results indicate that although exposure to glyphosate and Roundup during in vitro maturation does not affect nuclear maturation and embryo cleavage, it does impair oocyte developmental competence in terms of blastocyst rate and cellularity. Moreover, Roundup at the same glyphosate-equivalent concentrations was shown to be more toxic than pure glyphosate, altering steroidogenesis and increasing oocyte ROS levels, thus confirming that Roundup adjuvants enhance glyphosate toxic effects and/or are biologically active in their side-effect and therefore should be considered and tested as active ingredients.

Glyphosate (Gly), or *N*-(phosphonomethyl)glycine, is a non-selective herbicide widely used worldwide to control weeds[1]. Gly is commonly applied as part of glyphosate-based herbicides (GBHs), which include the popular commercial formulation Roundup (R), in which adjuvants enhance the herbicidal properties.

During the last years, the growing and widespread use of GBHs has raised a great concern about the impact of environmental contamination on animal and human health. Human and animal Gly exposure may occur through various routes such as food and drinking water, skin contact or by inhalation[2,3]. Only a small amount of Gly is metabolized by mammals, while the majority is excreted unmodified by urine in which Gly residues have been detected in both humans[2,4] and animals as rats[5], cows[6,7], rabbits[7], dogs and cats[8].

The possible risk associated with Gly exposure to human and animal health is a matter of an intense public debate for both its potential carcinogenic and non-carcinogenic effects, including potential adverse effects on nervous, digestive, endocrine and reproductive systems[9–14]. However, findings of both in vitro and in vivo studies are conflicting and several authors concluded that Gly is safe at levels below regulatory permissible limits[15–22].

Nevertheless, GBHs have been clearly demonstrated to exert their effects through a chemical endocrine disruption; in fact, they have been shown to impair the androgen/estrogen balance[23,24], thus determining an endocrine disarray in cell lines (e.g. [25,26]). Furthermore, R exposure in rats has been demonstrated to interfere with both steroidogenic enzymes and reproductive health[27,28]. It has been suggested that the modification in reproductive hormone concentrations induced by GBHs could be due to changes in the number and activity of Leydig cells and modification of steroidogenic acute regulatory protein (StAR) or aromatase levels and activity[28,29,30]. According to another study, R seems to exert an inhibitory effect at the hypothalamic-pituitary level and to disrupt cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway and corticosterone synthesis in the adrenal gland[31]. Moreover, Gly and R have been demonstrated to impair bovine and swine granulosa cells growth and steroid production[32–34]. In female rat and mice, Gly and GBHs have been reported to cause hormonal imbalances, oxidative stress and alterations in folliculogenesis including an increase of atretic follicles[35,36]. Recently Yahfoufi et al.[37] reported that Gly exposure of mature mouse oocyte (MII) induces spindle fibre destruction, disturbance in chromosomal alignment, depletion of intracellular zinc bioavailability and ROS

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	0	5	10	100	200	360
Gly (μg/mL)						
MII (%)	91.1 ± 7.1	91.9 ± 6.6	88.7 ± 5.2	88.0 ± 7.8	92.1 ± 5.1	90.9 ± 5.8
Oocytes (n°)	272	266	259	282	259	253
Roundup (μg/mL Gly eq)						
MII (%)	92.8 ± 7.6	91.8 ± 4.1	90.9 ± 6.9	90.3 ± 6.8	91.3 ± 6.6	92.8 ± 4.1
Oocytes (n°)	264	239	250	264	255	261

Table 1. Effect of Gly and Roundup on percentage of oocytes in metaphase II at 44 h of culture. Data represent the mean ± SD of six replicates repeated in different experiments.

	0	5	10	100	200	360
Gly (μg/mL)						
Penetration rate	75.6 ± 4.0	73.3 ± 5.2	73.5 ± 8.1	78.2 ± 5.8	78.1 ± 5.2	72.7 ± 5.0
Monospermy rate	65.3 ± 12.2	62.8 ± 9.7	51.3 ± 12.6	60.2 ± 15.6	63 .2 ± 13.1	53 .5 ± 6.4
Male pronuclear formation	99.4 ± 1.5	99.4 ± 1.6	98.5 ± 2.4	99.4 ± 1.5	99.3 ± 1.7	98.5 ± 2.5
Oocytes (n°)	284	284	243	221	244	262
Roundup (μg/mL Gly eq)						
Penetration rate	76.0 ± 5.2	75.4 ± 4.8	74.5 ± 10.1	75.6 ± 8.7	68.8 ± 10.0	69.0 ± 8.9
Monospermy rate	54.4 ± 11.8	56.5 ± 9.9	63.7 ± 14.2	55.4 ± 11.1	66.9 ± 11.0	56.9 ± 12.5
Male pronuclear formation	100.0 ± 0.0	98.7 ± 2.0	98.8 ± 2.9	97.2 ± 3.3	98.7 ± 2.1	99.5 ± 1.3
Oocytes (n°)	267	227	233	254	228	242

Table 2. Effect of Gly and Roundup addition during IVM on fertilization rate, monospermy rate and on the ability of oocytes to sustain male pronucleus formation after in vitro fertilization. Data represent the mean ± SD of six replicates repeated in different experiments.

accumulation. Similar effects were found in mouse embryos exposed to Gly during embryo culture[37]. Induction of oxidative stress and apoptosis were also observed in bovine embryos cultured in presence of R[38].

However, very few data are available in literature on possible effects of Gly and GBHs on mammal oocyte maturation, which prepare oocyte for fertilization events and affect early embryonic development[39]. Zang et al.[40] observed that Gly interferes with in vitro mouse oocyte maturation impairing nuclear maturation, generating oxidative stress and inducing DNA damage and early apoptosis.

On these bases, the objective of this study was to characterize the impact of Gly and R on female gamete using an "in vitro" model of pig oocyte maturation (IVM) evaluating nuclear maturation, cytoplasmic maturation and developmental competence of oocytes, steroidogenic activity of cumulus cells as well as intracellular levels of glutathione (GSH) and ROS of oocytes. We tested concentrations ranging from either 360 μg/mL Gly or 0.1% Roundup (containing 360 μg/mL Gly) to 70-fold lower on the basis of previous in vitro studies on reproductive tissues and gametes[25,30,33,40,41].

Results

Effect of Gly and R on nuclear and cytoplasmic maturation.

When COCs were matured in presence of Gly at 0, 5, 10, 100, 200 and 360 μg/mL or R at the same Gly-equivalent doses, no significant variations in the proportion of oocytes completing nuclear maturation showing a MII nuclear morphology were recorded (Table 1).

Gly or R addition during oocyte maturation at all the concentrations tested did not influence, after IVF with frozen-thawed semen, the percentage of penetrated oocytes, monospermic oocytes and percentage of penetrated oocytes with at least one male pronucleus (Table 2).

While the presence of Gly during IVM at all the doses tested (0, 200, 360 μg/mL) did not affect the cleavage rate, it caused a significant ($p < 0.01$) reduction of the percentage of oocytes that developed to blastocyst stage at the higher concentration (360 μg/mL). Moreover, a significant decrease in the mean number of blastomeres per blastocyst was observed starting from Gly 200 μg/mL ($p < 0.05$) (Fig. 1, left panel).

R did not influence the cleavage rate; however, at all the concentrations tested (200 and 360 μg/mL Gly-equivalent) it induced a significantly lower blastocyst rate ($p < 0.05$ and $p < 0.001$ for R 200 and R 360, respectively) and mean number of blastomeres per blastocyst ($p < 0.01$ and $p < 0.001$ for R 200 and R 360 respectively) compared to control (Fig. 1, right panel).

Effect of Gly and R on cumulus cell steroidogenesis.

Basal steroid production by COCs after 22 and 44 h of culture is shown in Figs. 2 and 3.

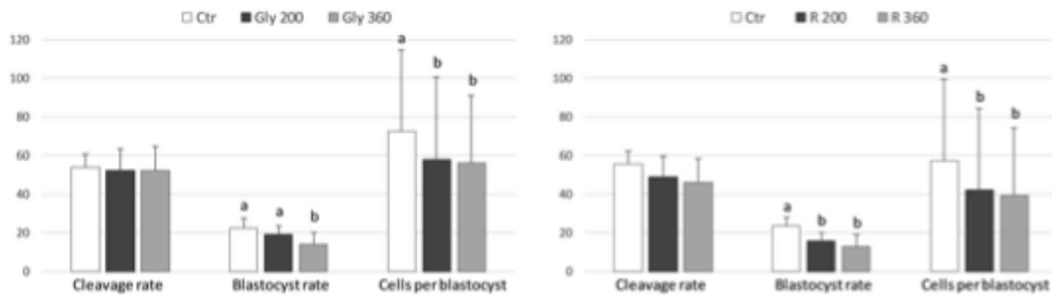

Figure 1. Effect of Gly (left panel) and R (right panel) addition during IVM on cleavage rate, blastocyst rate and blastomere number per blastocyst. Data represent the mean ± SD of five replicates repeated in different experiments. Different letters represent significant difference for $P < 0.05$ between treatments.

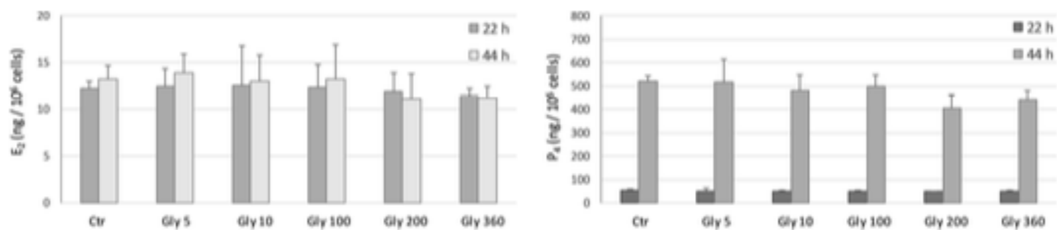

Figure 2. Effect of Gly (0, 5, 10, 100, 200, 360 μg/mL) on E2 (left panel) and P4 (right panel) production by porcine cumulus cells after 22 h and 44 h of culture. Data represent mean ± SD of 4 independent experiments.

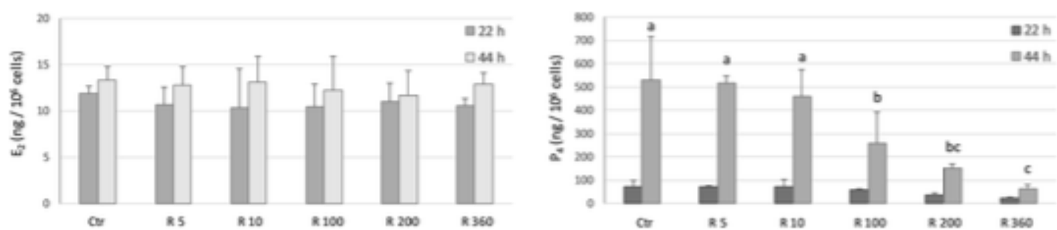

Figure 3. Effect of R (0, 5, 10, 100, 200, 360 μg/mL Gly-equivalent) on E2 (left panel) and P4 (right panel) production by porcine cumulus cells after 22 h and 44 h of culture. Data represent mean ± SD of 4 independent experiments. Different letters on the same bar type represent significant difference for $P < 0.01$ between treatments.

The production of P4 was significantly higher ($p < 0.0001$) at 44 h of culture compared to 22 h irrespective of Gly and R concentrations.

E2 and P4 production was not affected by Gly exposure in none of the 2 days of culture (Fig. 2).

None of the R concentrations tested induced any effect on E2 production, in either the first and the second day of culture (Fig. 3, left panel), and on P4 production after 22 h (Fig. 3, right panel).

After 44 h of culture, R inhibited P4 production in a dose dependent manner starting from 100 μg/mL ($p < 0.0001$) (Fig. 3, right panel).

Effect of Gly and R on GSH and ROS levels. The oocyte GSH levels were not statistically influenced by the exposure during IVM to Gly or R at the concentrations of 100, 200 and 360 μg/mL (Fig. 4).

While Gly presence during in vitro maturation did not modify intracellular ROS levels (Fig. 5, left panel), R at the highest concentration tested (360 μg/mL Gly-equivalent) significantly increased intracellular ROS levels ($p < 0.01$) (Fig. 5, right panel).

Discussion

The purpose of this study was to evaluate the impact of Gly and R exposure on female gamete using an in vitro model of pig oocyte maturation.

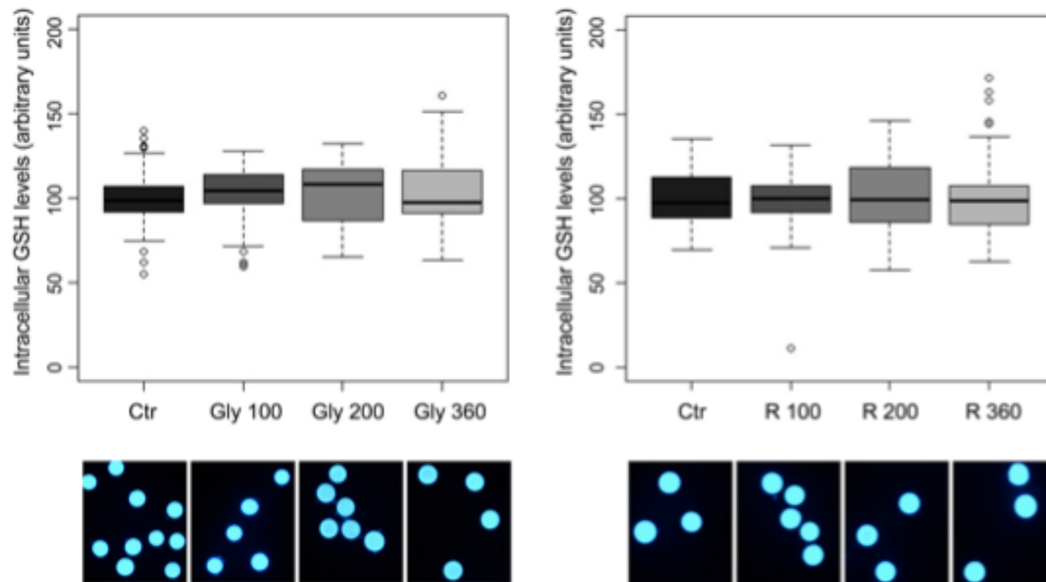

Figure 4. Upper panel. Box plots for intracellular GSH levels of oocytes matured in presence of Gly (left panel) and R (right panel). Oocytes were dyed with CellTracker Blue. Central lines represent median; boxes represent 25–75 percentile; whiskers represent minimum and maximum; dots represent outliers. The experiment was replicated 5 times with 15–20 oocytes each time. Lower panel. Representative epifluorescent microphotographic images of porcine oocytes matured in presence of Gly (left panel) and R (right panel) stained with CellTracker Blue to detect intracellular GSH levels.

Pig is an important species not only for agriculture, but also for research as a biomedical animal model due to anatomical and physiological similarity compared to human[42,43]. Moreover, according to 3Rs principle, the use of female gametes from non rodent-species, such as farm animals, has been considered to serve as useful in vitro screening test for reproductive toxicology[44].

As a first step of this study we investigated the effect of Gly and R exposure during IVM on nuclear maturation of pig oocytes. None of the concentrations tested (5, 10, 100, 200 and 360 μg/mL Gly or Gly-equivalent doses for R) modified the percentage of oocytes reaching MII stage compared to control. These results are in contrast with the only study performed up to now on the effect of Gly exposure during IVM on nuclear maturation of mammalian oocytes[40]. In that study, 200 and 500 μM Gly (33.8 and 84.5 μg/mL respectively) decreased the proportion of polar body extrusion of mouse oocytes due to misaligned chromosomes and abnormal spindle morphology; the authors suggested that Gly toxicity on mouse oocytes could be mediated by the increase of intracellular ROS levels.

The discrepancy between Zang et al.[40] and our results could be due to a species-dependent sensitivity to Gly and/or it can be ascribed to different cultural conditions: Zang et al.[40] matured mouse oocytes in M2 medium, while, in our model, pig IVM was performed in NCSU 37 medium supplemented with cysteine and β-mercaptoethanol, molecules known to reduce pig oocyte ROS levels, and 10% porcine follicular fluid (pFF), endowed with high radical scavenging activity elicited from SOD isoenzymes[45–48]; these supplementations, increasing the antioxidant activity of the system, may have masked the potential Gly toxic effects on nuclear maturation. In fact, it has been recorded that cell damage encompassed by ROS are difficult to detect in pig oocytes cultured in a medium supplemented with 10% pFF, even in the presence of ROS generated by the hypoxanthine-xanthine oxidase system[47]. Anyway, it must be kept in mind that in vivo oocytes are normally protected from the harmful effects of ROS by anti-oxidant enzymes which are present in the follicular fluid[49] and therefore it can be assumed that our culture system is capable of closely mimicking the in vivo environment during oocyte maturation. In our pig model, a significant increase of the intracellular levels of ROS with R at the concentration of 360 μg/mL, but not with pure Gly, was recorded; this increase, however, was not as dramatic as that recorded in mouse oocyte after Gly exposure[40].

In order to evaluate the potential toxic effect of Gly and R exposure on cytoplasmic maturation, as a first step, fertilization parameters and oocytes ability to decondense sperm head and sustain male pronucleus formation after in vitro fertilization were evaluated. As observed for nuclear maturation, no detrimental effect of all the Gly and R doses tested on these parameters was recorded. Adequate oocyte GSH levels are needed in order to reduce protamine disulfide bonds that represent the first step in the induction of sperm nuclear decondensation and hence male pronucleus formation after in vitro fertilization[50]. The maintained sperm nuclear decondensing

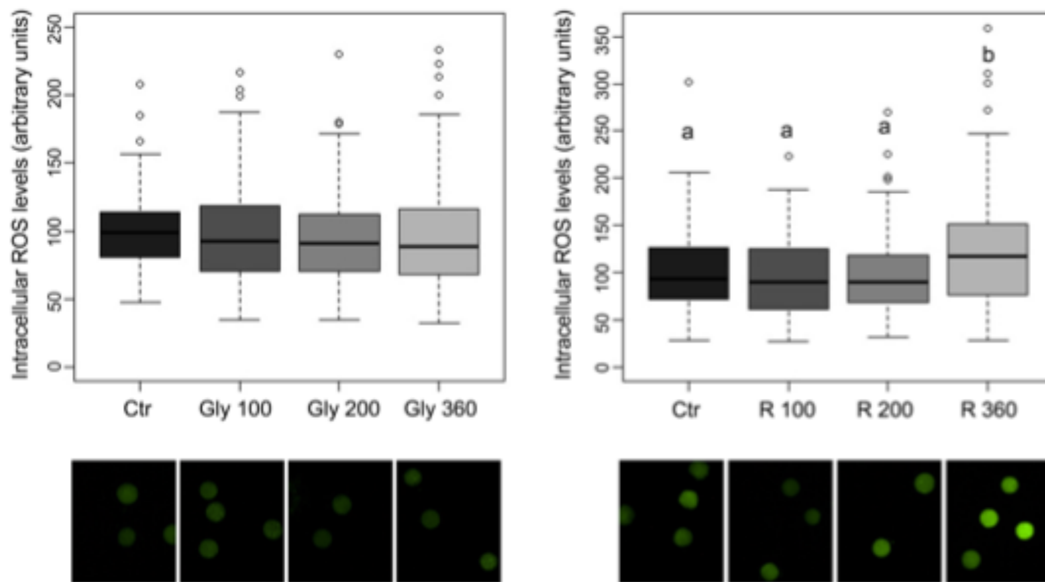

Figure 5. Upper panel. Box plots for intracellular ROS levels of oocytes matured in presence of Gly (left panel) and R (right panel). Oocytes were dyed with H2DCFDA. Central line represents median; boxes represent 25–75 percentile; whiskers represent minimum and maximum; dots represent outliers. Different letters within same graph represent significant difference for $P < 0.05$ between treatments. The experiment was replicated 5 times with 15–20 oocytes each time. Lower panel. Representative epifluorescent microphotographic images of porcine oocytes matured in presence of Gly (left panel) and R (right panel) stained with H2DCFDA to detect intracellular ROS levels.

ability of the exposed oocytes agree well with the absence of any effect of either Gly or R on intracellular GSH levels which was recorded in this study. Even if an inverse relationship between oocytes intracellular ROS and GSH levels has been observed by many authors[45–48], in this study, R 360 significantly increased the intracellular levels of ROS; it is likely that this rise was not so strong to significantly reduce oocyte GSH levels and, in turn, to impair oocyte decondensing activity.

The developmental competence of exposed oocytes after IVF was used as a further parameter of proper cytoplasmic maturation. Even the highest doses tested of both Gly and R had no effect on embryo cleavage. By contrast, R, and to a lesser extent Gly, induced a dose dependent reduction of both blastocyst rate and blastomere number per blastocyst. Therefore, oocyte exposure to R and Gly during IVM impaired the acquisition of a proper cytoplasmic maturation leading to a reduction of developmental competence, even if pesticides were not more present during embryo culture. This toxic effect was more evident with R compared to Gly, thus suggesting a synergistic effect provoked by the adjuvants present in the commercial formulation.

Concerning the effects on cumulus cell steroidogenesis, while Gly did not induce any alteration of E2 and P4 levels, R decreased P4 (but not E2) production. In our system, P4 secretion by COCs dramatically increased during the second half of culture, as already observed[48,51,52], likely due to cumulus cell differentiation-luteinization and R was markedly effective in inhibiting this P4 increase as P4 produced after 48 h of culture was significantly lower in R treated groups, starting from 100 μg/mL, as compared to control. Similarly, treatment of bovine granulosa cells with Gly (10 and 300 μg/mL) had no effect on P4 and E2 production[33] while R (10 μg/mL) dramatically decreased steroid levels (P4 and E2). Other researches carried out by Gigante et al.[34] on swine granulosa cell showed that Gly (0.2, 4 and 16 μg/mL) induced a significant inhibitory effect on granulosa cell E2 secretion, viability and proliferation; in contrast, P4 secretion was stimulated at all tested concentrations. It must be considered that the researches carried out by Perego et al.[33] and Gigante et al.[34] were performed on plated granulosa cell with the addition of testosterone or androstenedione as estradiol precursors while our model, consisting of cumulus cell-oocyte complexes, is completely different. In fact, cumulus cells and mural granulosa cells are phenotypically/functionally different: cumulus cells play an essential role in the normal growth and development of the oocyte, while mural granulosa cells primarily exert an endocrine function and support follicle growth[53]. Moreover, evidence exists that oocyte plays an active role in determining the fate of follicle somatic cells; results obtained by Coskun et al.[54] demonstrated that porcine oocytes secrete molecule(s) that inhibits steroid production by cumulus and granulosa cells. Therefore, based on these information we cannot strictly compare our results on steroidogenesis with those obtained by Perego et al.[33] and Gigante et al.[34].

P4 produced by cumulus complexes has been reported to positively influence porcine oocyte cytoplasmic maturation and to improve developmental competence to the blastocyst stage following IVF[55,56]. Therefore, the more

After 1 h semen was washed twice with BTS and finally resuspended with Brackett and Oliphant's medium[71] supplemented with 12% fetal calf serum (Gibco, Invitrogen, Italy) and 0.7 mg/mL caffeine (IVF medium). Forty-five to fifty oocytes freed from cumulus cells were washed twice in IVF medium and transferred to 500 μL of the same medium containing 0.25×10^6 sperm/mL. After 1 h of gamete coincubation, oocytes were transferred to fresh IVF medium previously equilibrated under 5% CO_2 and cultured for 17 h until fixation as above described (total number of oocytes examined 2,747).

Parameters evaluated were: penetration rate (number of oocytes penetrated/total inseminated), monospermy rate (number of oocytes containing only one sperm head-male pronucleus/total fertilized) and the ability of oocytes to sustain male pronucleus formation.

Degenerated and immature oocytes were not counted.

(*b*) *developmental competence of embryos after 7 days of in vitro culture.*

Based on the results obtained from in vitro fertilization, a set of experiments was carried out to evaluate the effect of Gly and R exposure during IVM on embryonic development of oocytes after IVF. At the end of maturation period in presence of different concentrations of Gly (0, 200 and 360 μg/mL) or R at the same Gly-equivalent doses, oocytes were coincubated with frozen-thawed spermatozoa for 1 h as described above, washed twice in IVF medium and incubated 3 h in the same medium. Then presumptive zygotes were washed twice in NCSU-23[68] and cultured in 500 μL of the same medium. On Day 5 post-fertilization, 250 μL of the medium were replaced with fresh pre-equilibrated NCSU-23 containing 20% (v/v) FCS to reach a final FCS concentration of 10% (v/v). At Day 7 post-fertilization, percent of blastocysts and number of blastocyst nuclei were determined by fixing and staining embryos as above described for oocytes (total number of fertilized oocytes 2,634). Embryos with at least 20 blastomeres and a clearly visible blastocoel were considered as blastocysts.

Evaluation of cumulus cell steroidogenesis. IVM media of both the first and the second day of culture of COCs in presence of Gly (0, 5, 10, 100, 200 and 360 μg/mL) or R at the same Gly-equivalent doses, were collected, centrifuged at 900×*g* for 5 min and the supernatants were stored at − 20 °C until assayed for progesterone (P4) and estradiol-17β (E2) by validated radioimmunoassays[52]. At the end of the maturation period, cumulus cells were counted using a Thoma's hemocytometer, after being freed from matured oocytes by gentle repeated pipetting. For P4, the intra- and interassay coefficients of variation were 6.8% and 10.1%, respectively; assay sensitivity was 4.4 pg/tube. The intra- and interassay coefficients of variation for E2 were 5.4% and 10.5%, respectively; assay sensitivity was 1.7 pg/tube. Steroid concentrations are expressed as ng/10^6 cells.

Detection of GSH and ROS levels. Intracellular GSH and ROS levels of oocytes at the end of maturation period in presence of Gly (0, 100, 200 and 360 μg/mL) or R at the same Gly-equivalent doses, were determined using 4-chloromethyl-6.8-difluoro-7-hydroxycoumarin (CellTracker Blue; CMF2HC; Invitrogen, Italy) or 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA; Invitrogen) respectively as previously described[36]. From each treatment group, oocytes were incubated in the dark for 30 min at 39 °C in PBS/0.1% (wt/vol) PVA supplemented with 10 μM H2DCFDA or 10 μM CellTracker Blue. Following incubation, the oocytes were washed in PBS/0.1% (wt/vol) PVA, placed into 10-μL droplets, and fluorescence was evaluated under a Nikon Eclipse E 600 epifluorescence microscope (Nikon Europe BV, Badhoeverdop, The Netherlands). The fluorescence images were analysed with Image J software (public domain). Relative oocyte fluorescence was measured by normalizing the oocyte fluorescence with the background and with each oocyte area. Five independent experiments were performed (GSH samples, n = 747 oocytes; ROS samples, n = 804 oocytes).

Statistical analyses. Statistical analyses were performed using R (version 3.4.0)[72]. Values are expressed as mean ± standard deviation (SD) and level of significance was at $p < 0.05$.

Data on nuclear maturation, IVF trials, blastocyst formation and cumulus cell steroidogenesis were analysed using a general linear model with binomial distribution and a Tukey post-hoc test was subsequently run to determine differences between treatments.

Data on blastomere number were analysed using a Poisson distribution and a Tukey post-hoc test was subsequently run to determine differences between treatments.

Data on GSH and ROS intracellular levels, after being tested for normality and homogeneity of variances through Shapiro–Wilk test, were analysed using Non-parametric Kruskal–Wallis Test and Wilcoxon test was subsequently used to assess differences between treatments.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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serious negative effects of R compared to the pure molecule on oocyte developmental competence can possibly be due, at least in part, to the observed perturbation induced by R on P4 production by cumulus cells. Anyway, the mechanism through which R exerts its effects on cumulus cell steroidogenesis needs further investigations.

The decrease in P4 production by cumulus cells recorded in presence of R could have contributed to the increase in intracellular ROS levels induced by R 360. Previous studies, in fact, demonstrated that progesterone possesses antioxidant properties that are blocked by RU486, a P4 receptor antagonist[57].

All these results support the hypothesis that surfactants/adjuvants present in GBHs are responsible for the increased toxicity of Gly[30,58] or exert an intrinsic toxicity inducing membrane disruption[30,59], apoptosis[60], inhibition of mitochondrial respiration[30,59,61] and DNA damage[62].

To the best of our knowledge, the present study is the first work describing the effects of Gly and R on pig oocytes maturation.

Our results indicate that the exposure to Gly and its commercial formulation R during IVM, even if it does not affect nuclear maturation and embryo cleavage, impairs oocyte developmental competence in term of blastocyst rate and cellularity. Moreover, R at the same Gly-equivalent concentrations resulted to be more toxic than pure Gly, altering steroidogenesis and increasing oocyte ROS levels, thus confirming that R adjuvants enhance Gly toxic effects and/or are biologically active in their side-effect and therefore should be considered and tested as active ingredients.

Glyphosate concentrations detected in human urine has been reported to be at ng/mL levels with higher level in specifically exposed individuals[22,63–65]; mean levels of 0.26 μg/mL (range < 0.020–17.2 μg/mL) in occupationally exposed workers have been recently reported[66]. These concentrations are far lower than those observed to be toxic in this study. Blood glyphosate levels recorded in human acute intoxications were 61 μg/mL (range 0.6–150 μg/mL) and 838 μg/mL respectively in mild–moderate and severe intoxication cases[67], concentrations of the order of magnitude of those that were toxic in our study.

Obviously, the effects induced by Gly and formulants should be lower in vivo than in culture, and in vitro methods cannot provide the information that can be derived from in vivo tests. Nevertheless, in vitro maturation of pig oocytes, that can be obtained in large number from ovaries collected at the slaughterhouse, can be used as a reliable model to screen toxic agent for female gamete allowing the reduction of the number of laboratory animals used in vivo accordingly to 3R principles.

The consequences of the massive use of GBHs remain a matter of concern on public health. We found that Gly and R exposure during IVM detrimentally affect the subsequent developmental ability of embryos, providing further evidence of their potential toxic effect on female reproductive system that is worth of a deeper investigation.

Methods

Chemicals. *N*-(Phosphonomethyl)glycine (Glyphosate, Gly, CAS Number 1071-83-6) as well as the other chemicals, unless otherwise specified, were purchased from Sigma-Aldrich (Saint-Louis, MO, USA) except Roundup Bioflow (Roundup Bioflow, Monsanto Europe N.V., Anversa, Belgium) containing 360 g/L of glyphosate acid in the form of 480 g/L isopropylamine salts of glyphosate (41.5%), water (42.5%) and surfactant (16%; chemical name, CAS number and/or exact percentage have been withheld as a trade secret).

Oocytes collection and in vitro maturation (IVM). Ovaries were collected from pre-pubertal gilts at a local slaughterhouse and transported (in 0.9% wt/vol NaCl solution) to the laboratory within 2 h. Cumulus-oocyte complexes (COCs) were aspirated from antral follicles, 3–6 mm in diameter, with a 18-gauge needle fixed to a 10-mL disposable syringe. Intact COCs were selected under a stereomicroscope and only COCs with more than three layers of compact cumulus cells and with uniform cytoplasm were transferred into a petri dish (35 mm, Nunclon, Denmark) prefilled with 2 mL of modified PBS supplemented with 0.4% BSA. After three washes in NCSU 37[68] supplemented with 5 μg/mL insulin, 1 mM glutamine, 0.57 mM cysteine, 10 ng/mL epidermal growth factor (EGF), 50 μM β-mercaptoethanol and 10% porcine follicular fluid (IVM medium), groups of 50 COCs were transferred to a Nunc 4-well multidish containing 500 μL of the same medium per well and cultured at 39 °C in a humidified atmosphere of 5% CO_2 in air. For the first 22 h of in vitro maturation the medium was supplemented with 1.0 mM db-cAMP and 0.12 IU/mL Pluset (Carlier, Italy). For the last 22 h COCs were transferred to fresh maturation medium[69].

Evaluation of nuclear maturation. In order to assess the effect of Gly and R on nuclear maturation, pig COCs were exposed during in vitro maturation period (44 h) to 0, 5, 10, 100, 200 and 360 μg/mL Gly or R at the same Gly-equivalent doses.

At the end of the maturation period the oocytes were denuded by gentle repeated pipetting and then mounted on microscope slides, fixed in acetic acid/ ethanol (1:3) for 24 h and then stained with Lacmoid. The oocytes were observed under a phase contrast microscope in order to evaluate the meiotic stage achieved (total number of oocytes examined 3,124). Oocytes with a nuclear morphology corresponding to metaphase-II stage (MII) were considered mature[70].

Evaluation of cytoplasmic maturation. Cytoplasmic maturation was assessed by evaluating:

(*a*) *insemination parameters and ability of oocytes to sustain male pronucleus formation after in vitro fertilization.*

At the end of the maturation period in presence of Gly (0, 5, 10, 100, 200 and 360 μg/mL) or R at the same Gly-equivalent doses, the oocytes were fertilized with frozen boar semen purchased from a commercial company (Inseme S.P.A., Modena, Italy). Straws were thawed in a waterbath at 37 °C under agitation for 30 s and immediately diluted, at the same temperature, in Beltsville Thawing Solution (BTS) at a dilution rate 1:3.

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Author contributions
M.S. and G.G. designed of the work, conducted the experiments and wrote the manuscript. C.N. conducted the experiments. C.T. critically revised the work. D.B. performed the statistical analysis. All authors discussed the results and contributed to the final manuscript.

Competing interests
The authors declare no competing interests.

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EXPERIMENT 3

Effects of Glyphosate or Roundup exposure on stallion semen quality

MATERIALS AMD MATHODS

Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich (Saint-Louis, MO, USA). The commercial formulation of glyphosate, Roundup Bioflow® (containing 0.36 g/mL of glyphosate acid in the form of isopropylamine salts of glyphosate, 41.5%; water, 42.5%; and surfactant, 16%), was purchased from Monsanto Europe N.V. (Anversa, Belgium).
Stallion semen was obtained from three stallions of proven fertility housed at INFA (Italy). Three ejaculates were collected from each stallion.
Semen was collected on a phantom using an artificial vagina (Missouri, IMV) with an inner liner and in-line filter (Hamilton Thorne Research, Denver, MA, USA). Sperm concentration was determined by NucleoCounter® SP-100™ (Chemotec, Denmark). The semen was extended at 30×10^{6} mL^{-1} with pre-warmed Kenney extender (37°C).
Sperm cells were incubated at 37°C semen with glyphosate (0, 0.5, 5, 50, 100, 360 and 720 µg/mL) or Roundup at concentrations equivalent to the glyphosate ones.
After 1 h of incubation, samples were assessed for sperm motility, viability, acrosome integrity, mitochondrial activity, and ROS production, as described below.

Motility assessment
Sperm motility was assessed with a computer-assisted sperm analyzer (CASA; IVOS v. 12, Hamilton Thorne Inc., Denver, MA, USA), using the following settings: 60 frames/s, minimum contrast of 70 pixels, minimum cell size of 10 µm², slow cells velocity (VSL) threshold of 30 µm/s, slow cell threshold of 20 µm/s, minimum average path velocity (VAP) >30 µm/s and threshold straightness (STR) of 80% for progressive cells. A minimum of 1000 cells was analyzed in at least eight randomly selected fields. Sperm motility endpoints were: proportion of total motile spermatozoa (MOT), proportion of progressive spermatozoa (PM), curvilinear velocity (VCL), average-path velocity (VAP), straight-line velocity (VSL), linearity (LIN), straightness (STR), wobble (WOB), lateral head displacement (ALH), beat cross frequency (BCF).

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Flow cytometry analysis

All reagents for flow cytometry were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Flow cytometry analyses were conducted to evaluate sperm viability associated with mitochondrial activity and mitochondrial ROS production/cellular ROS production or mitochondrial function. In each assay, sperm concentration was adjusted to 1×10^6 mL^{-1} in a final volume of 0.5 mL Tyrode's medium, and spermatozoa were stained with the appropriate combinations of fluorochromes (fluorochromes and final concentrations described below). Samples were run through a FACSCalibur flow cytometer (Becton Dickinson, Milan, Italy) equipped with a 488 nm argon-ion laser and a 635 nm red diode laser. Emission of each fluorochrome was detected by using filters: 530/30 band-pass (green/FL-1), 585/42 band-pass (orange/FL-2), >670 long pass (far-red/FL-3) and 661/16 band-pass (orange for red laser/FL-4). Data were acquired using the BD CellQuest Pro software (Becton Dickinson). Signals were logarithmically amplified, and photomultiplier settings were adjusted to each particular staining method. FL-1 was used to detect green fluorescence from DCFDA and low mitochondrial membrane potential (JC-1 negative); FL-2 was used to detect orange fluorescence for high mitochondrial membrane potential (JC-1 positive); FL-3 was used to detect the red fluorescence from propidium iodide (PI); FL-4 was used to detect the red fluorescence from MitoTracker deep red. Side scatter height (SSC-H) and forward scatter height (FSC-H) were recorded in logarithmic mode (in FSC vs. SSC dot plots), and the sperm population was positively gated based on FSC and SSC while other events were gated out. A minimum of 10,000 sperm events was evaluated per replicate.

Sperm membrane integrity (SYBR14/PI)

Sperm viability was assessed by checking the membrane integrity using two separate fluorochromes SYBR-14 and PI (LIVE/DEAD Sperm Viability Kit; Molecular Probes, Invitrogen, Milan, Italy). SYBR-14 is a membrane-permeable dye, which stains the head of viable spermatozoa in green, while PI is a membrane impermeable dye that only penetrates through disrupted plasma membrane, staining the sperm heads of non-viable cells in red. Aliquots of sperm samples of 500 µL were stained with 5 µL SYBR-14 working solution (final concentration: 100 nM) and with 2.5 µL of PI (final concentration: 12 mM) for 10 min at 37°C in darkness. Viable spermatozoa exhibited a positive staining for SYBR-14 and negative staining for PI (SYBR-14þ/PI-). Single-stained samples were used for setting the voltage gain for FL1 and FL3 photomultipliers.

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Acrosome integrity analysis (PSA-FITC/PI)

Sperm acrosome intactness was assessed by Pisum sativum agglutinin (PSA) conjugated with fluorescein isothiocyanate (FITC) (2.5 mg/mL stock solution; 0.5 mg/mL working solution) coupled with Propidium Iodide (2.4mM stock solution). Aliquots of sperm samples of 500 µL were stained with 10 µL FITC-PSA (final concentration: 10 mg/mL) and with 3 µL PI (final concentration: 14 mM) for 10 min at 37°C in darkness. Four different sperm subpopulations were distinguished: a) viable acrosome-intact spermatozoa were those cells that did not stain with either FITCPSA or PI and appeared in the lower left quadrant of FL1 vs. FL3 plots; b) viable spermatozoa with disrupted acrosome stained only in green with FITC-PSA and were found in the lower right panel; c) non-viable spermatozoa with intact acrosome stained with PI only and appeared in the upper left quadrant; and d) non-viable spermatozoa with disrupted acrosomes were found in the upper right quadrant and stained positively with both stains.

Mitochondrial membrane potential analysis (JC-1)

5,5’,6,6’-tetrachloro-1,1’,3,3’-tetraethylbenzimidazolyl carbocyanine iodide (JC-1) was used to evaluate mitochondrial membrane potential. JC-1 enters the mitochondria, forming multimers (known as J-aggregates) if the membrane potential is high and emits orange fluorescence at 590 nm (detected by the FL-2 photomultiplier). In contrast, when mitochondria have low membrane potential, JC-1 maintains its monomeric form (M-band) and emits green fluorescence at 530 nm (detected by FL-1 photomultiplier).

Sperm samples diluted with Tyrode’s medium were stained with 2,5 µL of JC-1 (in DMSO; 1 µg/mL final concentration). Tubes were incubated at 37°C for 30 min in the dark.

Mitochondria with high membrane potential (HMMP) emit orange fluorescence (higher FL-2), and those with low mitochondrial membrane potential (LMMP) emit green fluorescence (higher FL-1). Cells thus were classified as HMMP or LMMP according to the total amount of orange and green fluorescence. Since the average orange fluorescence also varied between treatments, we also used the MFI (mean fluorescence intensity) of the orange fluorescence in HMMP.

Cellular ROS production (DCFH-DA; propidium iodide; MitoTracker deep red)

DCFH-DA (DCF) is a non-fluorescent agent that accumulates in the cell cytoplasm due to deacetylation and emits green fluorescence upon oxidation by H_2O_2, detected by FL1 photomultiplier. This staining was coupled with propidium iodide (PI) that stains spermatozoa with disrupted plasmalemma (dead spermatozoa) emitting red/orange fluorescence detected by the FL3

photomultiplier. MT was included to assess the mitochondrial function as described in 2.2.1, being excited by the red diode laser and detecting the fluorescence with the FL-4 photomultiplier.
Sperm samples were diluted with Tyrode's medium and stained with 2.5 µL DCFDA (in DMSO, 50 µM final concentration), 2,5 µL propidium iodide (in water, 2.4 µM final concentration), and 2.5 µL Mitotracker Deep red (in DMSO, 100 nM final concentration). Samples were incubated at 37°C for 30 min in the dark.
The intracellular ROS production by viable cells with active or inactive mitochondria was recorded in this analysis. In this study, we used the population of live spermatozoa (ignoring PI^+ events), distinguishing cells with high and low cytoplasmic H_2O_2 generation in the subpopulations with high and low mitochondrial activity: DCF^+MT^+, DCF^-MT^+, DCF^+MT^-, DCF^-MT^-.

Statistical analysis

Data were analyzed using the R statistical environment v. 3.6.2 (The R Foundation for Statistical Computing, Vienna, Austria). Results are presented as the mean ± standard deviation and level of significance was at $p<0.05$. To assess differences between treatments a linear mixed effects model was applied using treatment as a fixed factor and horse as a random factor and a Tukey post hoc test was applied.

RESULTS

No significant differences between control and all the glyphosate concentrations tested were observed for the sperm quality parameters assessed (Fig. 5-7).
On the other hand, Roundup starting from 360 µg/mL (glyphosate-equivalent dose) significantly ($P<0.05$) decreased, compared to control, total and progressive motility, mitochondrial activity, viability and acrosome integrity after 1 h of incubation at 37 ºC as shown in Fig. 5 and Fig. 6. In addition, not only did Roundup have detrimental effects on total and progressive sperm motility, but also on some kinematic parameters, as shown in Table 2.
With regard to ROS production, the percentage of live spermatozoa producing ROS showed a not significant tendency to increase when spermatozoa were exposed to Roundup rather than glyphosate (Fig. 7B). Anyhow, the oxidative status index that showed statistical significance among our results

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was the percentage of live spermatozoa with active mitochondria not producing ROS that decreased starting from 360 µg/mL Roundup exposure (Fig. 7A).

Moreover, 720 µg/mL Roundup group showed a significantly lower ($P<0.05$) total sperm motility and a further significantly decrease ($P<0.05$) in live cells with active mitochondria not producing ROS compared to 360 µg/mL stressing a dose-dependent impact of Roundup on these sperm quality indexes (Fig. 5A and Fig. 7A).

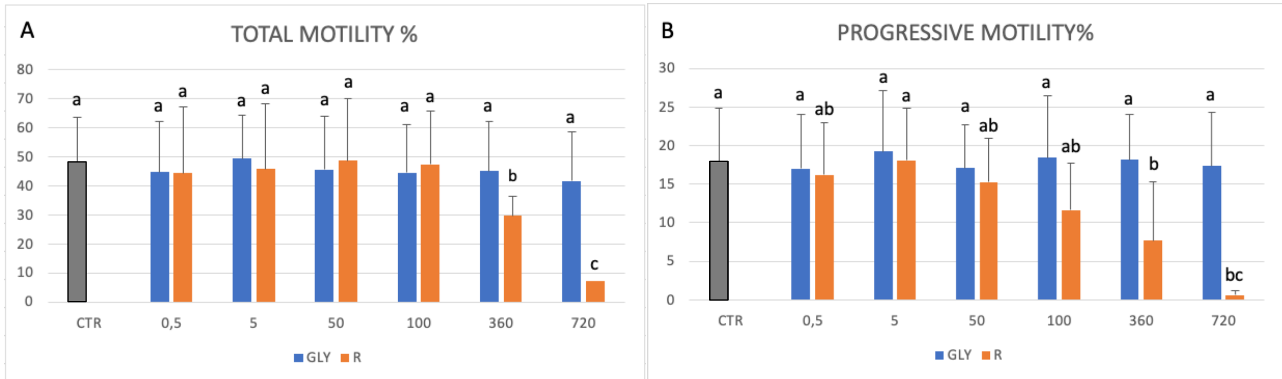

Figure 5. Effects of exposure to 0, 0.5, 5, 50, 100, 360 and 720 µg/mL glyphosate or Roundup (glyphosate-equivalent dose) on total (A) and progressive (B) sperm motility evaluated through CASA system. Different letters represent significant ($P<0.05$) differences.

CTR: control, sperm sample without addition of glyphosate or Roundup. Data are shown as mean ± SD.

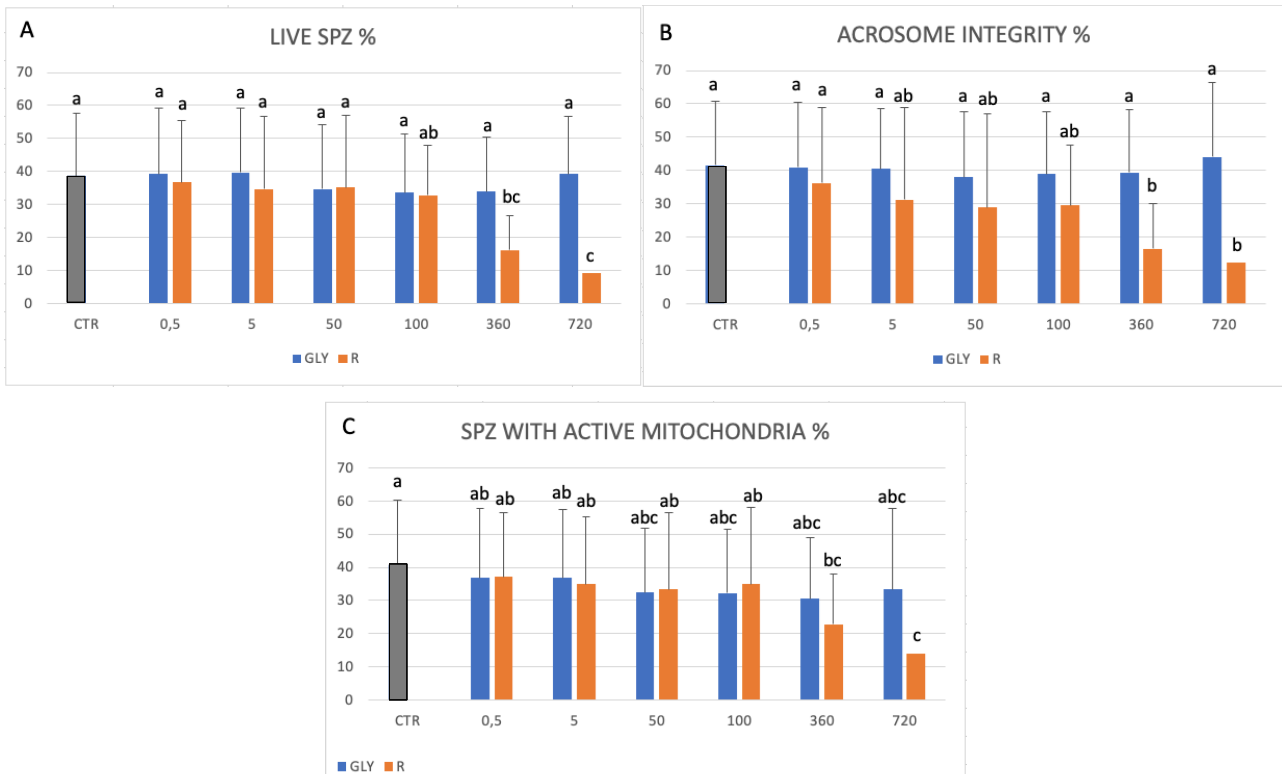

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Figure 6. Effects of exposure to 0, 0.5, 5, 50, 100, 360 and 720 µg/mL glyphosate or Roundup (glyphosate-equivalent dose) on viability (A), acrosome integrity (B) and percentage of spermatozoa with high mitochondrial membrane potential (C). Different letters represent significant (*P*<0.05) differences. CTR: control, sperm sample without addition of glyphosate or Roundup; Spz: spermatozoa; HMMP: spermatozoa with high mitochondrial membrane potential. Data are shown as mean ± SD.

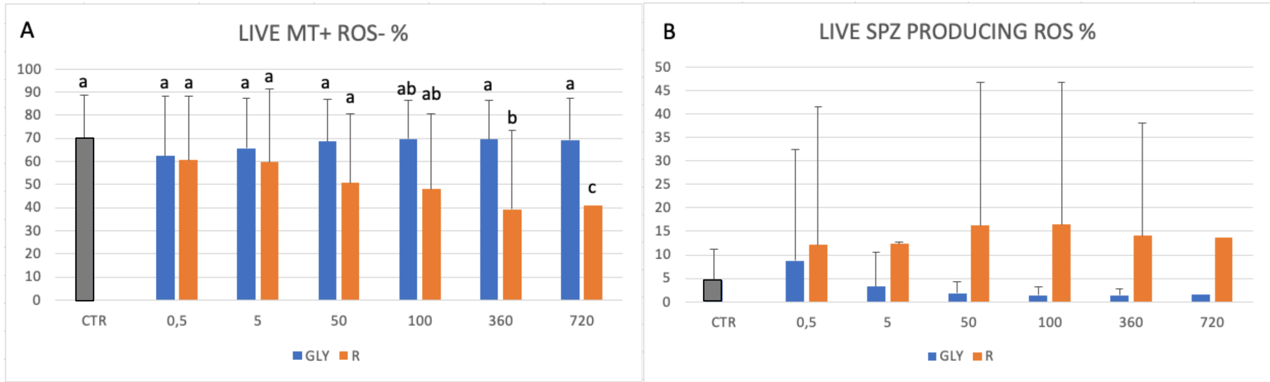

Figure 7. Effects of exposure to 0, 0.5, 5, 50, 100, 360 and 720 µg/mL glyphosate or Roundup (glyphosate-equivalent dose) on percentage of live spermatozoa with active mitochondria not producing ROS (A) and percentage of live spermatozoa producing ROS (B). Different letters (*a, b*) represent significant (*P*<0.05) differences.
CTR: control, sperm sample without addition of glyphosate or Roundup; Spz: spermatozoa; LIVE MT+ ROS-: live spermatozoa with active mitochondria not producing ROS. Data are shown as mean ± SD.

	ALH	BCF	LIN	STR	VAP	VCL	VSL
CTR	6,73 ± 0,75 a	42,04 ± 3,17	33 ± 6,27 abc	60,33 ± 10,84	91,88 ± 20,55 ab	177,33 ± 26,17 ab	53,8 ± 13,35 a
GLY0,5	6,96 ± 1,21 a	41,54 ± 4,35	30,83 ± 10,77 abc	61,33 ± 11,77	98,47 ± 21,99 a	188,29 ± 33,16 a	58,68 ± 15,29 a
GLY5	6,63 ± 1,06 a	42,88 ± 2,74	33,75 ± 5,72 ab	60,5 ± 12,71	96,56 ± 19,04 ab	182,18 ± 28,44 a	56,32 ± 12,43 a
GLY50	6,41 ± 1,19 a	42,29 ± 2,6	33,09 ± 4,56 ab	57 ± 19,18	89,38 ± 21,94 abc	168,96 ± 33,24 ab	54,05 ± 11,08 ab
GLY100	6,51 ± 1,57 a	42,76 ± 2,53	35,92 ± 7,53 a	63,25 ± 14,19	93,74 ± 17,27 ab	173,68 ± 30,5 ab	58,01 ± 11,77 a
GLY360	6,18 ± 1,57 a	42,81 ± 2,23	35,83 ± 5,81 ab	62,83 ± 12,81	92,88 ± 13,05 ab	172,33 ± 21,89 ab	56,9 ± 11,95 a
GLY720	5,81 ± 1,35 a	42,26 ± 3,08	36,09 ± 7,93 a	64,36 ± 16,48	84,77 ± 15,11 bc	157,56 ± 22,49 b	51,79 ± 11,13 ab
R0,5	6,77 ± 1,14 a	42,23 ± 2,78	33 ± 5,48 ab	59,56 ± 12,87	96,42 ± 13,89 ab	183,77 ± 21,64 ab	55,61 ± 11,33 a
R5	6,69 ± 1 a	40,43 ± 5,12	33,78 ± 7,10 ab	60,56 ± 11,86	94,06 ± 24,29 abc	176,96 ± 31,70 ab	54,75 ± 14,53 a
R50	7,06 ± 1,85 a	42,34 ± 3,79	31,88 ± 5,62 abc	57,38 ± 12,68	99,19 ± 18,61 ab	188,31 ± 33.16 ab	55,09 ± 10,68 a
R100	7,25 ± 2 a	42,56 ± 5,44	26,5 ± 5,44 bc	51,63 ± 11,02	93,7 ± 25,66 abc	186,6 ± 36,49 ab	45,49 ± 12,79 ab
R360	6,44 ± 1,7 a	42,38 ± 4,27	26,75 ± 5,32 ab	52,75 ± 12,50	77,6 ± 20,39 c	160,56 ± 25,81 b	38,46 ± 21,75 bc
R720	3,92 ± 2,6 b	41,4 ± 1,21	19,2 ± 7,09 c	44,4 ± 7,83	51,8 ± 17,32 d	131,06 ± 27,49 c	20,64 ± 10,39 c

Table 2. Effects of exposure to 0, 0.5, 5, 50, 100, 360 and 720 µg/mL glyphosate or Roundup (glyphosate-equivalent dose) on kinematic parameters assessed by CASA system. Different letters represent significant (*P*<0.05) differences. CTR: control, sperm sample without addition of glyphosate or Roundup; Spz: spermatozoa; ALH: amplitude head displacement; BCF: beat cross frequency; LIN: linearity; STR: straightness; VAP: average path velocity; VCL: curvilinear velocity; VSL: straight line velocity. Data are shown as mean ± SD.

GENERAL DISCUSSION

The purpose of this project was to evaluate the impact of glyphosate and Roundup exposure on mammals' gametes using an in vitro model.

In vitro studies that use cells derived from animals or cell lines are cheap and simple-to-procure model systems to predict a given chemical toxicity; moreover, they do not provoke ethical issues on killing animals. A major drawback is that they fail to capture the complex environment of a multicellular creature and cannot completely predict the biokinetic profile of a given chemical. On the other hand, in vivo studies provide a complex system, but this doesn't put aside the problem of translatability at all as there are considerable physiological differences between humans and animals that impact absorption, distribution, metabolism and excretion. Moreover, they require advanced personnel training and high maintenance fees, being time and resource intensive biological models (Blaauboer, 1996; Saeidnia et al., 2015). For these reasons, it should be borne in mind that both in vitro and in vivo study cannot represent what exactly happens in human organism, but they can provide us much information.

For the first and second study of this thesis, respectively on semen quality and oocytes maturation after herbicide exposure, gametes were obtained from swine, which is an important animal not only for agriculture, but also for research as a biomedical model due to anatomical and physiological similarity compared to human (Nakamura et al., 2018; Walters et al., 2017). Moreover, according to 3Rs principle, the use of gametes from non-rodent species, such as farm animals, has been considered to serve as a useful in vitro screening test for reproductive toxicology (Santos et al., 2014). Eventually, stallion semen was used for the third preliminary study to evaluate whether the results obtained on boar were confirmed in a different species.

In the first two studies we tested concentrations ranging from either 360 µg/mL glyphosate or 0.1% Roundup (containing 360 µg/mL glyphosate) to 70-fold lower on the basis of previous in vitro studies on reproductive tissues and gametes (Clair et al., 2012; Defarge et al., 2016; Perego et al., 2017; Zhang et al., 2019). In addition, in the third study on stallion semen, we tested a lower (0,5 µg/ml) and a higher concentration (720 µg/ml).

Discussion

With reference to the first study, which evaluated the effects of exposing boar spermatozoa to pure glyphosate or Roundup at different concentrations, it is shown that sperm quality decrease after exposition to high levels of glyphosate or much lower levels of Roundup.

We observed that pure glyphosate impaired boar sperm quality only at the highest concentration tested (360 µg/mL), while the negative impact on semen functional parameters was much more apparent when spermatozoa were exposed to Roundup. The more affected parameters due to Roundup exposure for 1h were sperm progressive motility (starting from 5 µg/mL) and mitochondrial membrane potential (starting from 25 µg/mL), followed by a decrease viability (starting from 100 µg/mL) which was concomitant with a higher percentage of acrosome-reacted spermatozoa. It is worth noting that induced damage was dose-dependent, and it was already apparent after 1 h of incubation at 38 °C, suggesting that the negative effects induced by Roundup could occur rapidly during the first hour of exposure.

Preliminary data on the effects of glyphosate or Roundup exposure on stallion semen confirm the toxic impact of the commercial formulation on spermatozoa. In this case the detrimental effects on quality parameters appeared at higher concentrations of Roundup compared to swine and no alteration due to glyphosate exposure were recorded, at any dose tested.

Mitochondrial activity (as assayed by JC1) and motility were affected starting from 360 µg/mL Roundup, while the same parameters in swine started to decrease yet from 25 µg/mL and 5 µg/mL respectively. In a similar manner, also viability and acrosome integrity of boar sperm were impaired by lower concentration of Roundup (100 µg/mL) compared to stallion (360 µg/mL). What remains unchanged between the two species is that mitochondrial activity and spermatozoa motility seem to be linked, and the same also as viability and acrosome integrity concerned. This can be explained by the fact that an impairment in mitochondrial bioenergetics leads to a decrease of ATP production, which is the main energy source used for promoting spermatozoa tail movements. Moreover, the concomitant decrease of viability and acrosome integrity confirm the probable action of Roundup formulation surfactants on lipid membranes, both cellular and acrosomal (Song et al., 2012). These results are in agreement with the aforementioned study on swine and confirm that the compounds present in commercial herbicides may potentiate mitochondrial perturbation and membrane stability, affecting mammalian sperm function and survival and being more toxic than glyphosate alone. On the other hand, the discrepancy in toxic concentrations of Roundup compared to boar, as well as the fact that stallion spermatozoa are not impaired by glyphosate, can be due to a different species-specific sensitivity. Indeed, it is known that horse spermatozoa have a very stable cytoplasmatic membrane, which make them difficult to be capacitated (Leemans et al., 2019).

Discussion

Preliminary data on stallion included also analysis on ROS production, assessed by staining with DCFDA and MitoTRacker fluorochromes. According to the results obtained, the percentage of live spermatozoa producing ROS seems to increase when spermatozoa are exposed to Roundup rather than glyphosate, even though our results show no statistical significance, whereas the percentage of live spermatozoa with active mitochondria not producing ROS decrease with high concentration of Roundup. Anyhow, the recorded results suggest that Roundup may increase sperm oxidative stress, but not in a way to be the main mechanism of its toxicity.

With reference to the second study of this thesis, which evaluate the impact of glyphosate and Roundup exposure on female gamete, the results showed that nether glyphosate nor Roundup have a negative impact on nuclear maturation of pig oocytes during IVM. Likewise, no detrimental effect of glyphosate and Roundup on fertilization parameters and on oocytes ability to decondense sperm head and sustain male pronucleus formation was recorded. This result agrees well with the absence of any effect on intracellular GSH levels, that are essential to reduce protamine disulfide bonds that represent the first step in the induction of sperm nuclear decondensation and hence male pronucleus formation after in vitro fertilization (Yoshida et al., 1993). Possibly, high concentrations of Roundup significantly increased the intracellular levels of ROS, but not in a way to reduce oocyte GSH levels and, in turn, to impair oocyte decondensing activity. This evidence is sustained by the preliminary data obtained on stallion spermatozoa as also in that case there was a slight imbalance in oxidative status, but not likely to be the main reason of sperm quality impairment.
Moreover, both glyphosate and Roundup had no effect on embryo cleavage but Roundup, and to a lesser extent glyphosate, induced a dose dependent reduction of both blastocyst rate and blastomere number per blastocyst underlying an impairment in cytoplasmic maturation that leads to a reduction of developmental competence, even if pesticides were not more present during embryo culture.
Finally, Roundup also affected steroidogenesis of cumulus cells surrounding oocytes inhibiting P4 increase during IVM. As progesterone possesses antioxidant properties (Yuan et al., 2016), the decrease in P4 production by cumulus cells induced by Roundup could have contributed to the increase in intracellular ROS levels induced by high concentrations of Roundup.

CONCLUSIONS

At present, there is a consistent body of literature suggesting that GBHs have negative effects on human health and fertility, but it remains unclear whether it is the main component of these products, glyphosate, to be toxic for the organism or other compounds present in the commercial formulation.
Overall, our results suggest that, while both glyphosate and Roundup induce toxic effects on mammalian sperm function and survival, Roundup has much more detrimental impact than glyphosate, even at equivalent concentrations of glyphosate. Furthermore, it can be hypothesized that the toxic effect of these pesticides on spermatozoa may be linked to an impairment in mitochondrial activity and a subsequent decrease in ATP production, which impact cell motility. In spite of this, DNA integrity seem not to be altered either by Roundup or pure glyphosate. Anyway, at present, the mechanism of action of GBHs remains unclear and needs to be investigated further.
GBHs impact was confirmed by our studies not only on male gametes, but also on female ones. We found that glyphosate and Roundup exposure during IVM detrimentally affect the subsequent developmental ability of embryos, providing further evidence of their potential toxic effect on female reproductive system. Moreover, even on oocytes Roundup at the same glyphosate-equivalent concentrations resulted to be more toxic than pure glyphosate, altering steroidogenesis and increasing oocyte ROS levels.
The fact that Roundup is more toxic than pure glyphosate itself, causing more severe alterations than this active principle, confirms the hypothesis that formulants present in commercial products either boost glyphosate toxicity or are harmful themselves.
Bioaccumulation of these formulants, which are petroleum-derivatives, needs to be investigated as it may lead to serious implications and cause chronic toxicity. This is crucial given the growing concerns on the impact and safety of glyphosate and glyphosate-based herbicides.
On the other hand, one should bear in mind that this work, as many upon literature, was a toxicological study testing in vitro glyphosate and Roundup doses which are far higher than levels found in serum and urine due to environmental exposure (Niemann et al., 2015; Mills et al., 20017). Glyphosate concentrations detected in human urine has been reported to be at ng/mL levels with higher level in specifically exposed individuals (Soukup et al., 2020; Conrad et al., 2017; Knudsen et al., 2017; Acquavella et al., 2004); mean levels of 0.26 μg/mL (range < 0.020-17.2 μg/mL) in occupationally

exposed workers have been recently reported (Zhang et al. 2020). These concentrations are far lower than those observed to be toxic in this study. Blood glyphosate levels recorded in human acute intoxications were 61 µg/mL (range 0.6–150 µg/mL) and 838 µg/mL respectively in mild–moderate and severe intoxication cases (Zouaoui et al., 2013), concentrations of the order of magnitude of those that were toxic in our study.

Moreover, the biotransformation process that the compound undergoes inside the organism should also be taken into consideration, since glyphosate can be partially degraded prior to reaching germ cells, which would make it less cytotoxic. All this considered, the effects induced by glyphosate and formulants should be lower in vivo than in culture, and in vitro methods cannot provide the information that can be derived from in vivo tests.

In spite of all the aforementioned, it is clear from this study that the large use of glyphosate formulations, especially Roundup, may entail a risk for fertility at least for occupational exposed population; hence, and in order to address the concerns on the use of GBHs properly, further research aimed at clarifying the effects and toxicity of each compound is much warranted.

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