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Glyphosate affects swine ovarian and adipose stromal cell functions

Paolo Gigante, Melissa Berni, Simona Bussolati, Francesca Grasselli, Stefano Grolli, Roberto Ramoni, Giuseppina Basini*

Dipartimento di Scienze Medico-Veterinarie, Università di Parma, Via del Taglio 10, 43126, Parma, Italy

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ABSTRACT

Although Glyphosate (GLY) is a widely used pesticide, its effects on ovarian function and stem cell differentiation are still largely unknown. Therefore, as a contribution on this subject, the present work reports an investigation of the *in vitro* effects of GLY on swine granulosa cells and adipose stromal cells (ASCs).

The effect of GLY at different doses (0.2, 4 and 16 μg/mL) was evaluated on granulosa cells growth (BrDU incorporation and ATP production), steroidogenesis (17-β estradiol and progesterone secretion) and redox status (superoxide and nitric oxide production and non-enzymatic scavenging activity). GLY has been shown to inhibit cell growth, 17-β estradiol and non-enzymatic scavenging activity and to increase progesterone and nitric oxide secretion ($P < 0.05$). In addition, GLY significantly decreased the viability of ASCs ($P < 0.001$), and inhibited their adipogenic differentiation. These data indicate that GLY alters the main features of granulosa cells and ASCS thus suggesting that GLY could affect both reproductive function and adipose tissues homeostasis.

1. Introduction

An endocrine disruptor substance (ED) is defined by the U.S. Environmental Protection Agency (EPA) as “an agent that interferes with the synthesis, secretion, transport, binding, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development and/or behavior” (Kavlock et al., 1996). This heterogeneous category includes some metals, many industrial chemical products, natural and synthetic hormones, pharmaceutical drugs and pesticides (Marya and Zlatnik 2016). The use of many pesticides has been associated with health and environmental issues, and, as a consequence, some of them have been withdrawn from the market (Zheng et al., 2016).

An important class of EDs is represented by xenoestrogens, compounds that show an estrogenic-like activity, with adverse effects on normal estrogenic signaling by interaction with ERalpha and ERbeta estrogen receptors (Liu et al., 2013). Xenoestrogens are present in fruits and vegetables (phytoestrogens), cosmetics (parabens), plastic containers (BPA, polyvinyl chloride, phthalates), and pesticides (DDT, endosulfan). Among these, glyphosate (GLY) is the most widely used organophosphoric type (Thongprakaisang et al., 2013; Mesnage et al., 2017), which has been detected in food products, e.g. soy grain, canola and barley (Myers et al., 2016), beer and tea (Nagatomi et al., 2013) as well as drinking water (Solomon 2016).

GLY consumption may occur by ingestion, skin contact or by inhalation. The absorbed substance is mainly distributed to the small intestine, colon, kidneys and bones. Only a small amount of GLY is metabolized, while the majority is excreted unmodified by urine. Residues of glyphosate have been detected in human urine samples collected throughout Europe (Niemann et al., 2015).

Different studies suggest that GLY herbicides are potentially harmful to the endocrine system (El-Shenawy 2009; Gasnier et al.,

* Corresponding author.
E-mail address: basini@unipr.it (G. Basini).

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2009). GLY may affect mammalian reproductive activity, in particular pregnancy, or it might interfere with gametogenesis and spermatogenesis in the male (Beuret et al., 2006).

In 2015, the International Agency for Research on Cancer (IARC, 2015), classified GLY as possibly carcinogenic to humans (Group 2 A); on the other hand, in the "Conclusion on the peer review of the pesticide risk assessment of the active substance glyphosate", the European Food Safety Authority (EFSA) has defined the substance as "unlikely carcinogenic to humans" (EFSA, 2015).

In order to assess GLY mechanisms of action several studies have analyzed the effect of the substance on oxidative stress; Gehin et al. (2005) have documented its oxidizing activity in human keratinocytes and similar effects were observed in liver cell line HepG2 (Chaufan et al., 2014).

Recent data have been published about GLY potential role as an endocrine disruptor (Omran and Salama 2016; De Souza et al., 2017). In particular, it has been hypothesized that GLY may act as a disruptor of cytochrome P450 aromatase both in human placental tissue and embryonic liver cells possibly impairing steroid hormones balance, particularly the androgen/estrogen ratio (Benachour et al., 2007; Walsh et al., 2000). Recently, Perego et al., (2017a, b) demonstrated that GLY impairs bovine granulosa cell functions. Moreover, it has been demonstrated that reproduction can be affected in term of birth defects in populations of the Argentine province of Chaco, where rice and soy are abundantly sprayed with the substance (Bianco et al., 2012).

On these bases, our first aim was to deepen the knowledge concerning GLY effects on reproduction. Therefore, we chose to isolate swine ovarian granulosa cells, which represent a valid *in vitro* model to study the consequences of chemical contaminant exposure (Grasselli et al., 2010; Basini et al., 2012a; and 2014).

Few studies have reported the potential effect of GLY on adipose tissue, which is increasingly regarded as an endocrine 'additional' organ (Luo and Liu 2016) since it can be involved in the decrease of reproductive efficiency (Liu and Ding 2017). To date, only Martini et al. (2016) have shown GLY effects on adipogenesis, possibly involving an inhibition of peroxisome proliferator-activated receptor gamma (PPARγ) (Martini et al., 2012), which appears to be essential to start the process.

Adipose tissue is dynamic since throughout life the proliferative activity of mesenchymal stromal cells drives a constant remodeling which can modify both its total mass and biological activity. Given that GLY can accumulate into adipose tissue, especially as a consequence to chronic exposition, its effects on ASCs proliferation and viability are worthy of investigation.

Therefore, this study was set up to evaluate the potential effects of GLY in two different endocrine cell models. Firstly, we investigated GLY role on functional activity of granulosa cells. In addition, we also explored its effects in the adipose tissue, a biological matrix which could play a relevant role in the maintenance of reproductive function.

2. Materials and methods

All reagents, unless otherwise indicated, were purchased from Sigma chemical Co Lt (ST. Louis, MO, USA), while the plastics were from Sarstedt AG&Co (Numbrecht, Germany).

2.1. Isolation and culture of granulosa cells

Swine ovaries were collected at a local slaughterhouse, placed in cold phosphate-buffered saline (PBS; 4 °C) supplemented with penicillin (100 Ul/mL), streptomycin (100 Ul/mL) and amphotericin B (2.5 µg/mL), maintained in a freezer bag, and transported to the laboratory within 1 h.

To improve cleaning, samples were immersed for 1 min in ethanol 70% before processing and subjected to further washes with PBS (Basini et al., 2016); cystic or hemorrhagic follicles were discarded. Granulosa cells were harvested by aspiration of follicles in the later state of maturation (> 5 mm) with a 26-gauge needle (Foxcroft and Hunter, 1985; Basini et al., 2016). Cells were then centrifuged at 450xg for 10 min and cell pellet was treated with ammonium chloride 0.17 M at 37 °C for 1 min to remove red blood cells.

Cell number was estimated after vital staining with trypan blue dye (0.4% w/v). Cells were than plated and cultured in a validated serum free system composed by DMEM/Ham's F12 medium supplemented with penicillin (100 µg/mL), amphotericin B (2,5 µg/mL), streptomycin (100 µg/mL), sodium selenite (5 ng/mL) and transferrin (5 µg/mL) (Basini and Tamanini, 2000; Basini et al., 2012a, 2012b), indicated hereafter as CM. The validated CM is useful to maintain granulosa cell characteristics and to avoid luteinization.

At the time of seeding in 96-well plates, cells were treated with GLY (0.2, 4 or 16 µg/mL) and then they were incubated at 37 °C under humidified conditions (5% CO_2) for 48 h. High concentrations of GLY were chosen since their effectiveness has been demonstrated in endocrine cells (Defarge et al., 2016), while the lowest one mimics drinking water contamination (Di Guardo and Finizio, 2018).

2.1.1. Granulosa cell growth

2.1.1.1. Cell proliferation. The BrdU cell proliferation ELISA, (Roche Diagnostic, Indianapolis, In, USA) is an immunological colorimetric assay used for the quantitative analysis of cell proliferation since BrdU incorporated into the newly synthesized DNA of replicating cells is detected by specific antibodies.

Briefly, granulosa cells were plated into 96-well plates (10^4 cells/100 µL CM) and incubated overnight in the presence or absence of GLY (0.2, 4 or16 µg/mL). At the end, plates were centrifuged for 10 min at 400 × *g*; supernatants were discarded, cells were dried using a hair-dryer for about 15 min then they were fixed and DNA was denatured before the addiction of the anti-BrdU antibody, conjugated with horseradish peroxidase (POD). The POD substrate, tetramethyl-benzidine (TMB), upon POD catalyzed oxidation, develops a blue color in a quantity that is proportional to the amount of newly synthesized DNA. The reaction is stopped by the