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Original Contribution

Roundup disrupts male reproductive functions by triggering calcium-mediated cell death in rat testis and Sertoli cells

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ABSTRACT

Glyphosate is the primary active constituent of the commercial pesticide Roundup. The present results show that acute Roundup exposure at low doses (36 ppm, 0.036 g/L) for 30 min induces oxidative stress and activates multiple stress-response pathways leading to Sertoli cell death in prepubertal rat testis. The pesticide increased intracellular Ca^{2+} concentration by opening L-type voltage-dependent Ca^{2+} channels as well as endoplasmic reticulum IP_3 and ryanodine receptors, leading to Ca^{2+} overload within the cells, which set off oxidative stress and necrotic cell death. Similarly, 30 min incubation of testis with glyphosate alone (36 ppm) also increased $^{45}Ca^{2+}$ uptake. These events were prevented by the antioxidants Trolox and ascorbic acid. Activated protein kinase C, phosphatidylinositol 3-kinase, and the mitogen-activated protein kinases such as ERK1/2 and p38MAPK play a role in eliciting Ca^{2+} influx and cell death. Roundup decreased the levels of reduced glutathione (GSH) and increased the amounts of thiobarbituric acid-reactive species (TBARS) and protein carbonyls. Also, exposure to glyphosate–Roundup stimulated the activity of glutathione peroxidase, glutathione reductase, glutathione *S*-transferase, γ-glutamyltransferase, catalase, superoxide dismutase, and glucose-6-phosphate dehydrogenase, supporting downregulated GSH levels. Glyphosate has been described as an endocrine disruptor affecting the male reproductive system; however, the molecular basis of its toxicity remains to be clarified. We propose that Roundup toxicity, implicated in Ca^{2+} overload, cell signaling misregulation, stress response of the endoplasmic reticulum, and/or depleted antioxidant defenses, could contribute to Sertoli cell disruption in spermatogenesis that could have an impact on male fertility.

Brazil is the world largest consumer of pesticides. This leadership might lead to a large number of health problems for occupationally exposed workers, their families, and the environment [1]. Glyphosate [*N*-(phosphonomethyl)glycine] formulations are widely used herbicides in agriculture worldwide. Although glyphosate is described as the primary active ingredient present in Roundup (Monsanto Co., St. Louis, MO, USA), this commercial formulation has greater side effects than glyphosate alone [2,3]. Moreover, it has been demonstrated that toxic events triggered by Roundup might be due to synergistic effects between glyphosate and other formulation products [4–6]. The adjuvants are considered inert; however, the polyethoxylated tallow amine (POEA) is toxic and could facilitate glyphosate penetration through plasmatic membranes and consequently potentiate its action and toxicity [3,7–10]. In this context, Mesnage et al. [11] demonstrated POEA as one of the active ingredients of Roundup formulations inducing human toxicity, triggering necrosis by disrupting cell membranes around its critical micellar concentration, rather than glyphosate, that leads to apoptosis. Moreover, previous studies have supported the possibility that mixtures of glyphosate and surfactant can aggravate cellular damage, inducing cell death [4–6,8,9,12–17]. Also, the surfactant nonylphenol, an important environmental contaminant, alters Ca^{2+} homeostasis and leads to Sertoli cell apoptosis [18]. Glyphosate alone or Roundup was found to induce significant changes in cellular antioxidant status leading to glutathione depletion, enzymatic disorders, and increased lipid peroxidation in keratinocytes [14,19].

The cytotoxicity provoked by several toxic agents is associated with the loss of intracellular Ca^{2+} homeostasis. The imbalance in Ca^{2+} physiology is believed to be associated with misregulation of

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Ca^{2+} intracellular stores and/or increased permeability of the biomembranes to this ion. Intracellular Ca^{2+} overload can underlie mitochondrial dysfunctions, which involve several molecular events, including activation of signaling pathways in addition to reactive oxygen species (ROS) overproduction that could culminate in cell death [17,20–22].

Nowadays, an important challenge concerning the deleterious effects of pesticides in occupationally exposed agricultural workers is the high prevalence of reproductive dysfunctions observed in this population [23–26]. Glyphosate is supposed to be specific on plant metabolism; however, side effects in animals and humans have been claimed. In this context, glyphosate might act as an endocrine disruptor affecting the male reproductive system, because it can lead to alterations in aromatase activity and expression [8], estrogen-regulated genes [27], and testosterone levels [10,16]. Moreover, Roundup, the commercial formulation of glyphosate, disrupts spermatogenesis and causes loss of fertility, reinforcing its toxicity to testicular cells. Also, in the MA-10 Leydig tumor cell line, Roundup inhibits steroidogenesis by disrupting the expression of the StAR proteins [12]. In addition, Dallegrave and colleagues [28] have demonstrated that glyphosate–Roundup exposure during pregnancy and lactation did not induce maternal toxicity in Wistar rats, but induced adverse reproductive effects in male offspring rats, including decreased daily sperm production during adulthood, increased percentage of abnormal sperms, and decreased testosterone serum level at puberty. Conversely, the authors observe only a vaginal canal-opening delay in exposed female offspring. Taken together, these data strongly suggest Roundup as an endocrine disruptor affecting mainly male reproduction. However, the precise mechanisms underlying the effects of this pesticide on male reproductive tissue remain unclear. Although long-term toxicity of Roundup to animal tissues has been largely described [29], acute exposure to this pesticide is claimed to be toxic to fish [30]. Nevertheless, little information is available on the acute toxicity of low doses of Roundup to mammalian tissues, especially to the reproductive human male system.

ROS generation might be due to either physiological or pathological conditions. Enzymatic and nonenzymatic antioxidants are essential to maintaining the redox status and serve as a defense against ROS [31]. In this context, when present at high levels, ROS play an essential role in the pathogenesis of many reproductive processes, considering their potential toxic effects to sperm quality and function. In addition, excessive ROS generation may induce DNA damage, accelerating germ cell death and causing decreased sperm counts. Altogether, these events could be associated with male infertility [32–34]. Environmental contaminants are known to modulate the antioxidant defense system and to cause oxidative stress in various species and cell types [6,19,35,36]. Our research group has previously demonstrated that the activity of antioxidant enzymes [superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione *S*-transferase (GST)], as well as reduced glutathione (GSH) levels, could be affected by endocrine diseases, such as hypo- and hyperthyroidism, leading to oxidative stress in immature rat testis [37], thereby showing the participation of the endocrine system in the redox potential of Sertoli cells. However, the effects of Roundup on oxidative stress and antioxidant defenses in the testis remain to be clarified.

Therefore, we selected acute exposure of immature rat testis to low doses of this pesticide as a model of toxicity to the male reproductive system. In this study we investigated the molecular basis of the toxicity of this xenobiotic, focusing on the role of Ca^{2+} homeostasis, misregulation of signaling pathways, and oxidative damage in the whole rat testis and in Sertoli cells in culture.

Materials and methods

Chemicals

Nifedipine; 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl ester) (Bapta-AM); *N*-[2-(*p*-bromocinnamylamino) ethyl]-5-isoquinoline sulfonamide (H89); bisindoylmaleimidine IX, 2-{1-[3-(amidinothio)propyl]-1*H*-indol-3-yl}-3-(1-methylindol-3-yl)maleimide ethanesulfonate salt (Ro 31–8220); Trolox [(±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid]; L-ascorbic acid; flunarizine; dantrolene sodium salt; Dulbecco′s modified Eagle′s medium (DMEM); Ham′s F12 medium; penicillin; streptomycin; kanamycin and amphotericin B; serum replacement 3; bovine pancreas deoxyribonuclease (DNase type I); hyaluronidase (type I-S); trypsin; soybean trypsin inhibitor; sodium pyruvate; D-glucose; Hepes; and sodium bicarbonate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Collagenase-dispase and bovine serum albumin were from Roche Diagnostics (Indianapolis, IN, USA). [^{45}Ca]$CaCl_2$ (sp act 321 kBq/mg Ca^{2+}) and Optiphase Hisafe III biodegradable liquid scintillation were purchased from PerkinElmer (Boston, MA, USA). Anti-p44/42 mitogen-activated protein kinase (MAPK) (anti-ERK1/2), anti-phospho-p44/42 MAPK (anti-phospho-ERK1/2), anti-p38MAPK, and anti-phospho-p38MAPK antibodies were from Cell Signaling Technology (Danvers, MA, USA). The herbicide Roundup Original (Homologation No. 00898793), containing glyphosate 360 g/L, is a commercial formulation registered by the Brazilian Ministry of Agriculture, Livestock, and Supply (Ministério da Agricultura, Pecuária e Abastecimento). The Immobilon Western chemiluminescence horseradish peroxidase substrate was obtained from Millipore. All other chemicals were of analytical grade.

Animals

Wistar rats were bred in an animal house and maintained in an air-conditioned room (about 21 °C) with controlled lighting (12-h/12-h light/dark cycle). Pelleted food (Nuvital, Nuvilab CR1, Curitiba, PR, Brazil) and tap water were available ad libitum. All animal procedures were carried out in accordance with ethical recommendations of the Brazilian Veterinary Medicine Council and the Brazilian College of Animal Experimentation (Protocol CEUA/PP00471).

Primary Sertoli cell culture

Some experiments were carried out in Sertoli cells from 30-day-old Wistar rats. Rats were killed by decapitation, and testes were removed and decapsulated. Sertoli cells were obtained by sequential enzymatic digestion as previously described by Dorrington et al. [39]. Sertoli cells from 30-day-old rat testis were seeded at the concentration of 650,000 cells/cm^2, in 24-well culture plates (Falcon, Deutscher, Brummath, France) and cultured for 72 h in Ham′s F12/DMEM (1/1) supplemented with serum replacement 3, 2.2 g/L sodium bicarbonate, and antibiotics (50,000 IU/L penicillin, 50 mg/L streptomycin, 50 mg/L kanamycin) and fungicide (0.25 mg/L amphotericin B), in a humidified atmosphere of 5% CO_2:95% air at 32 °C. Three days after plating, residual germ cells were removed by a brief hypotonic treatment using 20 mM Tris–HCl (pH 7.2) [40,41]. Cells were washed with phosphate-buffered saline, and fresh Ham′s F12/DMEM (1/1) was added. On day 5 after plating, cells were used to study the effects of Roundup on $^{45}Ca^{2+}$ uptake and cell viability, as described below.

$^{45}Ca^{2+}$ uptake

Whole testis or Sertoli cells in culture from 30-day-old male rats were preincubated in Krebs Ringer-bicarbonate (KRb) buffer