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Original Contribution

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



Vera Lúcia de Liz Oliveira Cavalli^a, Daiane Cattani^a, Carla Elise Heinz Rieg^a,
Paula Pierozan^b, Leila Zanatta^a, Eduardo Benedetti Parisotto^c, Danilo Wilhelm Filho^c,
Fátima Regina Mena Barreto Silva^a, Regina Pessoa-Pureur^b, Ariane Zamoner^{a,*}

^a Departamento de Bioquímica and Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, 88040-970 Florianópolis, Santa Catarina, Brazil

^b Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil

^c Departamento de Ecologia e Zoologia, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, 88040-970 Florianópolis, Santa Catarina, Brazil

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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 $^{45}\text{Ca}^{2+}$ 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.

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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

* Corresponding author. Fax: +55 48 3721 9672.

E-mail addresses: ariane.zamoner@ufsc.br, arianezps@gmail.com, arianezamoner@yahoo.com (A. Zamoner).

Ca²⁺ intracellular stores and/or increased permeability of the biomembranes to this ion. Intracellular Ca²⁺ 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 Ca²⁺ 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). [⁴⁵Ca]CaCl₂ (sp act 321 kBq/mg Ca²⁺) 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/cm², 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% CO₂:95% 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 ⁴⁵Ca²⁺ uptake and cell viability, as described below.

⁴⁵Ca²⁺ uptake

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

(122 mM NaCl, 3 mM KCl, 1.2 mM MgSO₄, 1.3 mM CaCl₂, 0.4 mM KH₂PO₄, 25 mM NaHCO₃) for 15 min in a Dubnoff metabolic incubator at 34 °C, pH 7.4, and gassed with O₂:CO₂ (95:5; v/v). Further, the medium was exchanged with fresh KRb buffer and the whole testis or Sertoli cells were preincubated again with or without channel blockers, antioxidants, or kinase inhibitors for 15 min before the pesticide addition and during the entire incubation period. The following drugs were used: nifedipine (10 μM), flunarizine (1 μM), U73122 (30 μM), LY294002 (10 μM), PD98059 (30 μM), SB239063 (10 μM), Bapta-AM (50 μM), dantrolene (50 μM), H89 (10 μM), Ro 31-8220 (20 μM), Trolox, or ascorbic acid. After that, the medium was changed to fresh KRb buffer with 0.1 μCi/ml ⁴⁵Ca²⁺ and incubated for 30 min in the absence (control) or presence of glyphosate–Roundup (treated groups) ranging from 0.72 to 360 ppm (corresponding to 0.00072 to 0.360 g/L, respectively). Extracellular ⁴⁵Ca²⁺ was thoroughly washed off with a washing solution containing 127.5 mM NaCl, 4.6 mM KCl, 1.2 mM MgSO₄, 10 mM HEPES, 11 mM glucose, 10 mM LaCl₃, pH 7.3 (30 min in washing solution). The presence of La³⁺ during the washing stage was found to be essential to prevent the release of the intracellular ⁴⁵Ca²⁺ [42]. After washing, tissue slices or cell cultures were digested and homogenized with 0.5 M NaOH solution, and 100 μl aliquots were placed in scintillation fluid and counted in a LKB rack β liquid scintillation spectrometer (Model LS 6500; multi-purpose scintillation counter, Beckman Coulter, Boston, MA, USA), and 5-μl aliquots were used for protein quantification as described by Lowry and colleagues [43].

Measurement of lactate dehydrogenase (LDH) activity

After incubation of the testes or Sertoli cells in the absence or presence of the glyphosate–Roundup at nominal concentrations ranging from 0.72 to 360 ppm for 30 min, the incubation medium was collected for determination of extracellular LDH activity by a spectrophotometric method. The estimation of LDH activity was carried out by measuring the oxidation of NADH and the results were expressed as U/L/mg of protein.

α-[¹⁴C]Methylaminoisobutyric acid ([¹⁴C]MeAIB) accumulation

For amino acid accumulation experiments, rat testis were preincubated in KRb buffer for 30 min in a Dubnoff metabolic incubator at 34 °C, pH 7.4, and gassed with O₂:CO₂ (95:5; v/v). The testes were then incubated in fresh KRb buffer for 60 min. [¹⁴C]MeAIB (3.7 kBq/ml) was added to each sample during the incubation period [44]. Glyphosate–Roundup at 36 ppm (0.036 g/L) was added to incubation medium for the last 30 min of incubation. After incubation, the slices were lysed in 0.5 M NaOH, the protein concentration was determined [43], and 25-μl aliquots of tissue and external medium were placed in scintillation fluid and counted in a Beckman β liquid scintillation spectrometer (Model LS 6500; multi-purpose scintillation counter, Beckman Coulter) for radioactivity measurements. The results were expressed as the tissue/medium (T/M) ratio: cpm/ml tissue fluid per cpm/ml incubation medium.

Antioxidant enzyme assays

For enzymatic activity, testes from control or Roundup-treated groups were preincubated for 15 min in KRb buffer followed by incubation with or without 36 ppm glyphosate–Roundup for 30 min. After incubation, the tissue was homogenized in cold 0.1 M Tris buffer, pH 8.5 (10% homogenate w/v) to determine γ-glutamyltransferase (γGT) activity or in 0.2 M Tris buffer, pH 7.4, to quantify glucose-6-phosphate dehydrogenase (G6PD) activity. Sample aliquots were saved for total protein determinations [43].

To determine CAT, SOD, GR, GPx, and GST activities, after treatment with 36 ppm glyphosate–Roundup for 30 min the testes were homogenized in a cold buffer containing 20 mM sodium phosphate, pH 7.4, 0.1% Triton, and 150 mM NaCl (1:20 w/v). The determinations were performed using the supernatant after centrifugation of the homogenate (5000g for 5 min).

γ-Glutamyltransferase assay

γGT activity was measured by using the method previously described by Orłowski and Meister [45], using L-γ-glutamyl p-nitroanilide as substrate and glycylglycine as the acceptor molecule. Aliquots of the tissue homogenate prepared as described above were incubated with the enzymatic substrate. The reaction was allowed to proceed for 60 min at 37 °C and the enzymatic reaction was stopped by addition of acetic acid. The absorbance of the samples was determined in a plate reader (Tecan Infinite 200 Pro) at 530 nm. The results were expressed as U/L/μg protein.

Glucose-6-phosphate dehydrogenase assay

For measuring the G6PD activity, aliquots of tissue homogenate were incubated in the presence of NADP⁺ leading to the oxidation of glucose 6-phosphate to 6-phosphogluconate. The NADPH produced was measured in a kinetic mode for 10 min. The results were calculated by assessing the increase in the optical density per minute (slope) of the sample against the “slope” of standard G6PD enzyme activity. The G6PD assay kit was kindly provided by Intercientífica (São José dos Campos, SP, Brazil).

Catalase activity

The CAT activity was determined in tissue homogenates according to the method described by Aebi [46], which is based on measuring the decreased absorbance in a 10 mM hydrogen peroxide solution at 240 nm for 30 s. The enzyme activity was expressed as mmol min/g.

Superoxide dismutase activity

The SOD activity was analyzed according to the method described by Misra and Fridovich [47] and modified by Boveris et al. [48]. The reaction is based on epinephrine oxidation (pH 2.0 to pH 10.2), which produces superoxide anion and adrenochrome, which is measured at 480 nm. A unit of SOD is defined as the amount of enzyme that inhibits the speed of oxidation of adrenalin by 50% and the result expressed in U SOD/g.

Glutathione peroxidase activity

The GPx activity was analyzed by using the method described by Flohé and Günzler [49]. This method is based on *tert*-butylhydroperoxide reduction via oxidation of GSH to GSSG, catalyzed by GPx, and subsequent regeneration of GSH by the enzyme GR with oxidation of NADPH at 340 nm. Therefore, the rate of oxidation of NADPH is proportional to the activity of the GPx in the sample. The enzyme activity was expressed as μmol min/g.

Glutathione reductase activity

The GR activity was determined by the method of Carlberg and Mannervik [50], which measured the rate of NADPH oxidation at 340 nm due to the formation of GSH, from GSSG, by the action of GR present in the sample. The unit of enzyme activity was expressed as μmol min/g.

Glutathione S-transferase activity

The GST activity was measured using the methodology described by Habig et al. [51]. In this protocol, 1-chloro-2,4-dinitrobenzene (CDNB; as substrate for GST) was used. In this reaction, GST promotes the CDNB–GSH conjugation. This reaction was spectrophotometrically

monitored for 60 s at 340 nm. The enzyme activity was expressed as $\mu\text{mol min/g}$.

Reduced glutathione assay

Reduced glutathione (GSH) was determined according to Beutler et al. [52], using the reagent DTNB (5,5'-dithiobis-2-nitrobenzoic acid). After being centrifuged at 5000g for 5 min, the supernatants from the acid extracts [12% trichloroacetic acid (TCA), 1:10 w/v] were added to 2.5 mM DTNB in 0.2 M sodium phosphate buffer, pH 8.0, and the formation of the yellow thiolate anion was immediately measured at 412 nm. Determinations were expressed in $\mu\text{mol/g}$.

Quantification of lipoperoxidation levels

The endogenous lipid peroxidation was evaluated in the testes by detection of substances that react with thiobarbituric acid (TBARS), particularly malondialdehyde, according to the method described by Bird and Draper [53]. Briefly, the homogenate was precipitated with 12% TCA followed by incubation in buffer (60 mM Tris-HCl, pH 7.4, 0.1 mM diethylenetriaminepentaacetic acid) and 0.73% thiobarbituric acid, at 100 °C, for 60 min. After cooling, the samples were centrifuged (5 min at 10,000g) and the absorbance of the chromophore was measured at 535 nm. The values were expressed in nmol TBARS/g.

Protein carbonyl assay

The oxidative damage to proteins by carbonylation was determined by the method described by Levine et al. [54]. Soluble protein was reacted with 10 mM 2,4-dinitrophenylhydrazine in 2 M hydrochloric acid for 1 h at room temperature in the dark and precipitated with 20% TCA. After centrifugation (11,000g for 5 min) the protein pellet was washed three times by resuspension in ethanol/ethyl acetate (1/1). Proteins were then solubilized in 6 M guanidine hydrochloride in 20 mM potassium phosphate and centrifuged at 14,000g for 5 min to remove any trace of insoluble material. The carbonyl content was measured spectrophotometrically at 370 nm. The total protein concentration was determined by the method of Lowry et al. [43] and the protein carbonyl concentration was expressed in $\mu\text{mol/mg protein}$.

Western blot analysis

In some experiments testes were treated with or without 36 ppm Roundup for 30 min, then total tissue extracts were processed to Western blot analysis. Testes were homogenized in 300 μl of a lysis solution containing 2 mM EDTA, 50 mM Tris-HCl, pH 6.8, 4% (w/v) SDS, and protein concentration was determined by the method of Lowry et al. [43] using serum bovine albumin as the standard. Then, the samples were dissolved in a 25% (v/v) solution containing 40% glycerol, 5% mercaptoethanol, 50 mM Tris-HCl, pH 6.8, and boiled for 3 min. Equal protein concentrations were loaded onto 10% polyacrylamide gels and analyzed by SDS-PAGE and transferred to nitrocellulose membranes for 1 h at 15 V in transfer buffer (48 mM Trizma, 39 mM glycine, 20% methanol, and 0.25% SDS). The nitrocellulose membranes were washed for 10 min in Tris-buffered saline (TBS; 0.5 M NaCl, 20 mM Trizma, pH 7.5), followed by 2 h incubation in blocking solution (TBS plus 5% defatted dried milk). After incubation, the blot was washed twice for 5 min with TBS plus 0.05% Tween 20 (T-TBS) and then incubated overnight at 4 °C in blocking solution containing the following antibodies: anti-ERK1/2, anti-phospho-ERK1/2, anti-p38MAPK, anti-phospho-p38MAPK (diluted 1:2000). The blot was then washed twice for 5 min with T-TBS and incubated for 2 h in

TBS containing peroxidase-conjugated anti-rabbit IgG 1:2000. In addition, we used the following controls in the antibody experiments: primary antibody only, secondary antibody only, and negative control (lacking the sample containing the antigen of interest). These controls defined the specificity and sensibility of the antibody. The blot was washed twice again for 5 min with T-TBS and twice for 5 min with TBS. The blot was then developed using an enhanced chemiluminescence kit. β -Actin immunoprotein was used as a protein loading control. Western blots were quantified by scanning the films and determining optical densities with OptiQuant version 02.00 software (Packard Instrument Co.).

Statistical analysis

The results are means \pm SEM. When multiple comparisons were performed, evaluation was done using one-way ANOVA followed by Bonferroni multiple comparison test or Student *t* test. Differences were considered to be significant when $p < 0.05$.

Results

Involvement of Ca^{2+} on the mechanism of acute Roundup-induced testis toxicity

Initially, rat testes were exposed to glyphosate-Roundup at concentrations ranging from 0.72 to 360 ppm, corresponding to 0.00072 to 0.36 g/L, respectively, and $^{45}\text{Ca}^{2+}$ uptake was investigated. It is important to emphasize that Roundup is used in agricultural work at dilutions ranging from 10,000 to 20,000 ppm (10 to 20 g/L), concentrations much higher than those described in our results. Results showed that Roundup exposure for 30 min increased the $^{45}\text{Ca}^{2+}$ uptake at doses ranging from 7.2 to 36 ppm glyphosate; however, 360 ppm Roundup lead to an important decrease in $^{45}\text{Ca}^{2+}$ influx (Fig. 1A). To investigate whether the alterations in $^{45}\text{Ca}^{2+}$ uptake were related to cell death, LDH release from the rat testis was measured. Interestingly, we observed an apparent link between $^{45}\text{Ca}^{2+}$ uptake and LDH release at 36 ppm (0.036 g/L) Roundup (Fig. 1B). Otherwise, at the higher Roundup dose (360 ppm), the LDH release was higher, despite the decreased $^{45}\text{Ca}^{2+}$ uptake (Fig. 1B). These findings strongly suggest that necrotic cell death could be directly related to Ca^{2+} toxicity up to 36 ppm of the pesticide. However, at very high concentrations (360 ppm), more complex mechanisms leading to necrotic cell death in the rat testis seem to be elicited by this xenobiotic. Therefore, in this study, we were interested in investigating some mechanisms underlying Ca^{2+} toxicity in rat testis exposed to 36 ppm Roundup.

Mechanisms of acute Roundup-induced $^{45}\text{Ca}^{2+}$ uptake: Ca^{2+} channels and signaling pathways

The following experiments were carried out at 36 ppm Roundup, a concentration able to induce the peaked $^{45}\text{Ca}^{2+}$ uptake and necrotic cell death. Fig. 2A shows that Roundup-induced $^{45}\text{Ca}^{2+}$ uptake occurred through L-type voltage-dependent Ca^{2+} channels (L-VDCs), as demonstrated by using nifedipine (L-VDC blocker). The role of the high intracellular Ca^{2+} levels in the Roundup-triggered $^{45}\text{Ca}^{2+}$ uptake was evidenced by using Bapta-AM (cell-permeative Ca^{2+} chelator) and dantrolene (inhibitor of ryanodine receptors). Co-incubation of Roundup and each drug was able to prevent the $^{45}\text{Ca}^{2+}$ uptake, suggesting dependence on intracellular Ca^{2+} levels and Ca^{2+} release mediating the effects of the pesticide (Fig. 2A). The use of dantrolene allowed us to set the implication of ryanodine receptors in the mechanism of action of Roundup. To further clarify the mechanisms involved in Roundup-mediated Ca^{2+} influx, the contributions of phospholipase C (PLC),

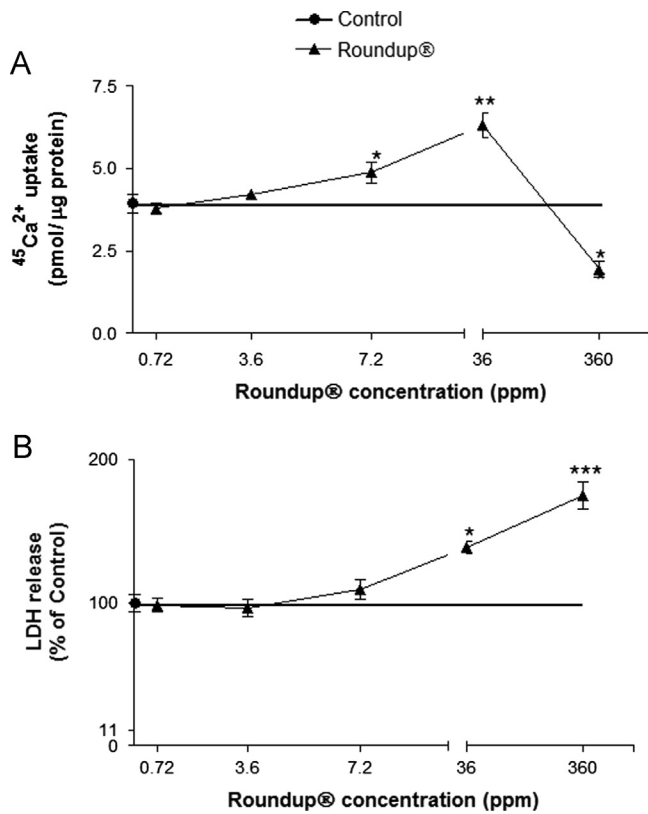


Fig. 1. Dose–response curve of Roundup (A) for $^{45}\text{Ca}^{2+}$ uptake and (B) for LDH release in immature rat testes. Rat testes were preincubated for 15 min and then incubated in the presence of 0.1 mCi/ml $^{45}\text{Ca}^{2+}$ with or without Roundup at various concentrations, 0.72 to 360 ppm, corresponding to 0.00072 to 0.36 g/L. Values are means \pm SEM of eight animals. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with control group.

protein kinase C (PKC), and protein kinase A (PKA) to the pesticide effect were verified by using 30 μM U73122, 20 μM Ro 31–8220, and 10 μM H89 (PLC, PKC, and PKA inhibitors, respectively). Results showed that U73122 was able to totally prevent the effect of Roundup on Ca^{2+} uptake, whereas Ro 31–8220 partially prevented the Ca^{2+} influx, evidencing a PLC- and PKC-dependent mechanism, respectively. On the other hand, H89 was ineffective at preventing such effect, suggesting that PKA activation is not involved in the mode of action of this pesticide (Fig. 2B).

The participation of other protein kinases, such as phosphatidylinositol 3-kinase (PI3K), and MAPK signaling pathways (MEK/ERK and p38MAPK) in the mechanism of action of 36 ppm Roundup was investigated by using the specific inhibitors LY294002 (10 μM), PD 98059 (30 μM), and SB239063 (10 μM), respectively. The outcomes showed that all the inhibitors used totally prevented the stimulatory effect of the pesticide on $^{45}\text{Ca}^{2+}$ uptake (Fig. 2C), suggesting that the ability of Roundup to increase $^{45}\text{Ca}^{2+}$ uptake could be associated with activation of several kinase pathways. Altogether, these findings demonstrate a role for PLC/PKC, PI3K, ERK, and p38MAPK in Ca^{2+} influx induced by the pesticide in rat testes.

To verify whether the effects observed with Roundup could be ascribed to glyphosate, the main active component of the pesticide, $^{45}\text{Ca}^{2+}$ uptake was measured in the presence of 36 ppm (0.036 g/L) glyphosate (in the absence of adjuvant/surfactant). Results showed increased $^{45}\text{Ca}^{2+}$ uptake induced by glyphosate. Moreover, the glyphosate-induced Ca^{2+} influx was partially prevented by nifedipine (Fig. 2D), suggesting a role for glyphosate in the toxicity of the pesticide.

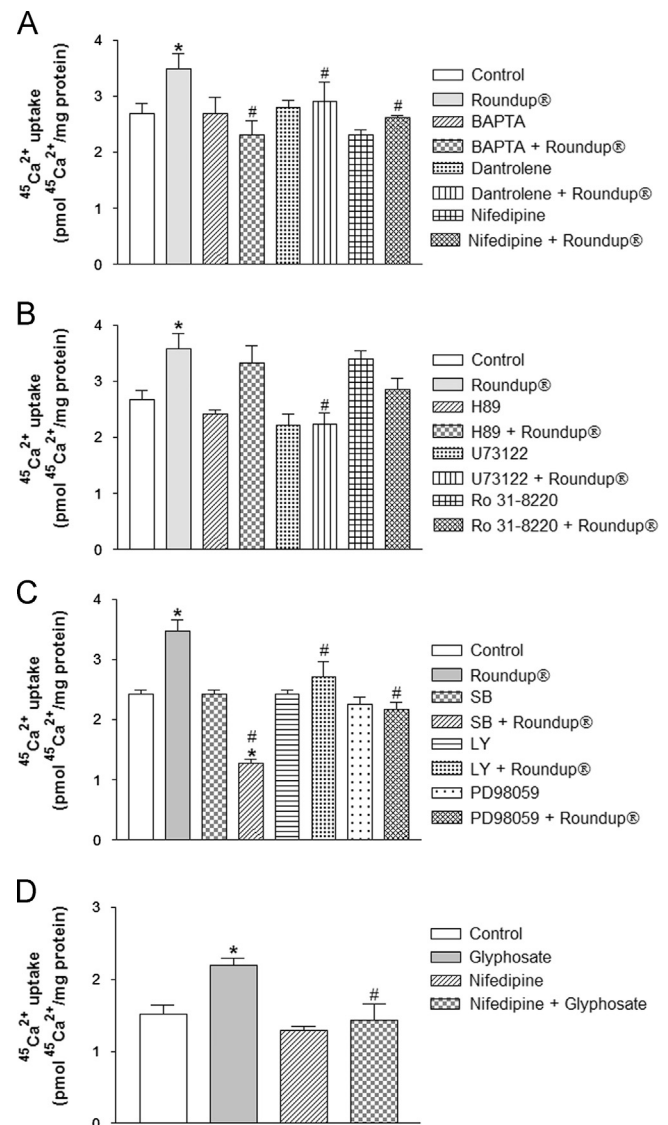


Fig. 2. Involvement of calcium channels (VDCCs), intracellular calcium levels, and kinase pathways in $^{45}\text{Ca}^{2+}$ uptake in immature rat testis. Testes were preincubated for 15 min with or without (A) 10 μM nifedipine (L-VDCC blocker), 50 μM Bapta-AM (intracellular calcium chelator), or 50 μM dantrolene (ryanodine calcium channel blocker); (B) 10 μM H89 (PKA inhibitor), 10 μM U73122 (PLC inhibitor), or 20 μM Ro 31–8220 (PKC inhibitor); or (C) 10 μM SB 239063 (p38 MAPK inhibitor), 10 μM LY 294002 (PI3K inhibitor), or 10 μM PD 98059 (MAPK inhibitor). After that, the testes were incubated with or without 36 ppm (0.036 g/L) Roundup (A, B, and C) for 30 min (incubation) in the presence of 0.1 mCi/ml $^{45}\text{Ca}^{2+}$. (D) Instead of Roundup, testes were incubated with glyphosate (0.036 g/L) after preincubation with 10 μM nifedipine. Values are means \pm SEM of eight animals expressed as a percentage of control. * $p < 0.01$ compared with control group. # $p < 0.01$ compared with Roundup or glyphosate group.

Supporting the involvement of the MAPK pathway in Roundup-induced toxicity to testicular cells, Western blot analysis showed activated/phosphorylated ERK1/2 and p38MAPK in the prepubertal rat testis acutely exposed to pesticide (Fig. 3).

Effects of antioxidants in Roundup-induced $^{45}\text{Ca}^{2+}$ uptake and in cell viability

To verify the involvement of depleted nonenzymatic oxidative defenses on Roundup-induced $^{45}\text{Ca}^{2+}$ uptake and necrotic cell death, the antioxidants ascorbic acid (vitamin C) and Trolox (stable form of vitamin E) were used. Results showed that both antioxidants prevented the effect of the pesticide on $^{45}\text{Ca}^{2+}$ uptake,

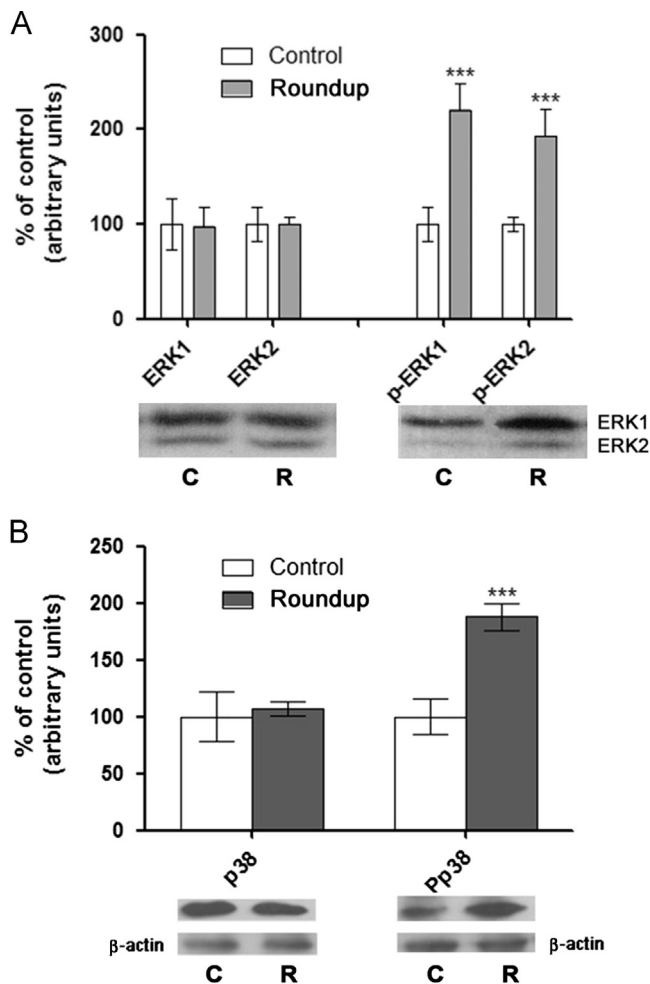


Fig. 3. Effect of Roundup on (A) ERK1/2 and (B) p38MAPK activation in rat testis. After 15 min preincubation, rat testes were incubated with Roundup (36 ppm or 0.036 g/L during 30 min). Tissue was lysed and the total and phosphorylated levels of ERK1/2 and p38MAPK were determined by Western blot. Data are reported as means \pm SEM of six animals in each group and expressed as % of control. Statistically significant differences as determined by one-way ANOVA followed by Tukey–Kramer multiple comparison test are indicated: *** $p < 0.0001$ compared with control group. Representative immunoblots are shown. p-ERK, phospho-ERK; Pp38, phospho-p38.

suggesting the contribution of oxidative events triggered by Roundup in testicular cell toxicity (Fig. 4A). Moreover, Trolox totally prevented, whereas ascorbic acid only partially prevented, the LDH release by Roundup (Fig. 4B). We, therefore, assessed other biochemical parameters to better evaluate the consequences of Roundup exposure on oxidative damage in rat testicular cells.

Effects of acute Roundup exposure on biochemical parameters involved in oxidative damage

To study the oxidative damage in the Roundup-exposed testis, some biomarkers of oxidative damage were assessed. The content of TBARS, which are an indicator of lipid peroxidation, was significantly increased, as well as protein carbonyl levels, an indicator of oxidative damage to proteins, in the Roundup groups compared to controls (Figs. 5A and B).

Once we established the participation of oxidative events in the mechanism of toxicity of Roundup, we sought to determine the enzymatic and nonenzymatic antioxidant defenses in Roundup-treated rat testis. Results showed that exposure to the pesticide led to decreased GSH levels. In addition, the activities of the enzymes involved in glutathione metabolism, G6PD, GR, GPx, GST, and γ GT,

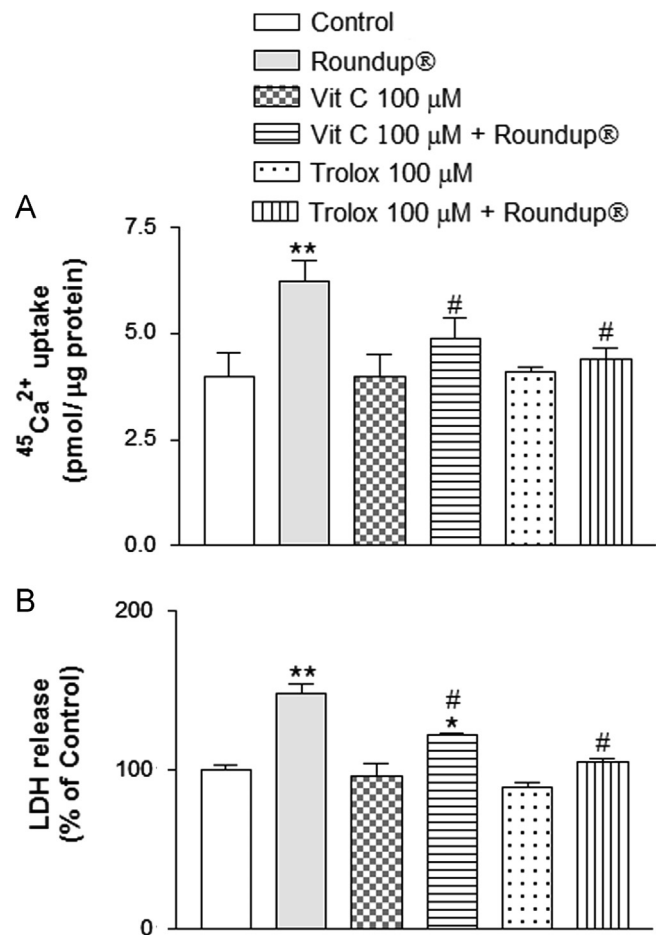


Fig. 4. Involvement of oxidative stress on Roundup-induced $^{45}\text{Ca}^{2+}$ uptake and cell death in rat testis. Testes were preincubated for 20 min in the presence or absence of the antioxidants 100 μM Trolox and 100 μM ascorbic acid. After that, the tissue was incubated with or without 36 ppm or 0.036 g/L Roundup plus the antioxidants for 30 min. Data are reported as means \pm SEM of eight animals in each group and expressed as % of control. Statistically significant differences from controls, as determined by one-way ANOVA followed by Bonferroni multiple comparison test, are indicated: * $p < 0.05$ and ** $p < 0.01$ compared with control group. # $p < 0.05$ compared with Roundup group.

were significantly higher in Roundup-treated rats than in controls. Roundup-exposed rat testis also presented higher CAT and SOD activities (Fig. 6) compared to controls.

Effect of Roundup on neutral amino acid transport

Results showed that 36 ppm Roundup led to downregulation of Na^+ -coupled [^{14}C]MeAIB accumulation (Fig. 7).

Effects of antioxidants on acute Roundup-induced $^{45}\text{Ca}^{2+}$ uptake and LDH release in Sertoli cells

To investigate Sertoli cells as a target of Roundup action within the testis, we examined the effects of the pesticide on $^{45}\text{Ca}^{2+}$ uptake and LDH release in primary cultures. Sertoli cell cultures from 30-day-old rats were exposed to Roundup at concentrations ranging from 0.72 to 360 ppm, and the influx of $^{45}\text{Ca}^{2+}$ was measured. Results showed that in Sertoli cells exposed to 36 ppm Roundup for 30 min, both $^{45}\text{Ca}^{2+}$ influx and LDH release were increased (Figs. 8A and B). Moreover, 360 ppm of the pesticide was able to increase $^{45}\text{Ca}^{2+}$ uptake and drastically decrease LDH release (Figs. 8A and B). It is important to emphasize that according to those results obtained in whole testis, 36 ppm

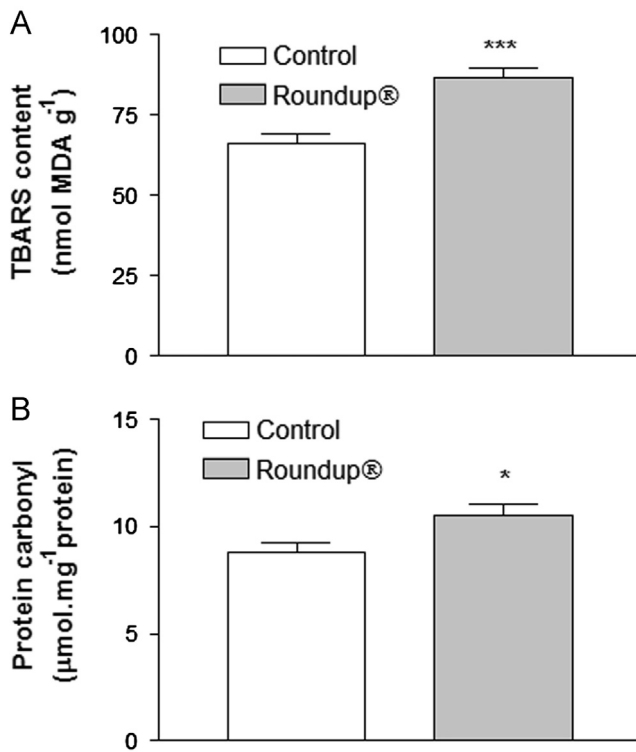


Fig. 5. Effect of Roundup on lipid peroxidation and protein carbonyl levels in immature rat testis. Testes were incubated in the presence or absence of 36 ppm or 0.036 g/L Roundup for 30 min. Data from TBARS measurement of lipid peroxidation and protein carbonyl levels are reported as means \pm SEM of six animals from each group. Statistically significant differences from controls, as determined by Student's *t* test, are indicated. **p* < 0.01, ****p* < 0.0001.

Roundup was also able to peak $^{45}\text{Ca}^{2+}$ uptake and provoke necrotic cell death in cultured Sertoli cells. Moreover, the decreased Sertoli cell $^{45}\text{Ca}^{2+}$ uptake at 360 ppm Roundup (Fig. 8A) was also coincident with the most prominent LDH release (Fig. 8B), as previously observed in whole testis (Fig. 1). These results strongly suggest that Sertoli cells could be one of the main targets of Roundup toxicity within rat testis.

To evaluate the role of depleted oxidative defenses, new experiments were carried out in cultured Sertoli cells supplemented with ascorbic acid and Trolox. Interestingly, in agreement with the results obtained in whole testis, Trolox (Figs. 9A and B) and also ascorbic acid (Figs. 9C and D) prevented Ca^{2+} overload and cell death in Sertoli cells in culture. We further assayed the effects of co-incubation of Trolox and ascorbic acid in Roundup-treated cells. The combination of both antioxidants used at physiological concentrations (75 μM ascorbic acid plus 50 μM Trolox) prevented both Ca^{2+} overload and necrotic cell death induced by Roundup. On the other hand, 150 μM ascorbic acid plus 75 μM Trolox were able to induce a per se effect on $^{45}\text{Ca}^{2+}$ uptake and necrotic cell death. Moreover, this effect was not modified by Roundup exposure (Figs. 9E and F).

Discussion

In this study we shed light on the molecular mechanisms underlying the acute toxicity of Roundup in the prepubertal male reproductive system. Acute Roundup exposure to low doses induces L-VDCC-mediated Ca^{2+} influx and cell death. These events might be prevented, at least in part, by antioxidants. Activation of Ca^{2+} influx and necrotic cell death are dependent on PLC/PKC,

PI3K, and MAPK signaling pathways. The PLC/IP₃ pathway together with ryanodine Ca^{2+} channels promotes Ca^{2+} release from the endoplasmic reticulum, contributing to Ca^{2+} overloading. Activated enzymatic systems including SOD, CAT, GPx, GR, G6PD, and GST support the decreased GSH levels found, whereas γGT affects GSH synthesis/turnover from extracellular amino acids. Depleted antioxidant defenses could underlie enhanced lipid and protein oxidation. The oxidative damage could misregulate cell function, culminating in necrotic cell death (Fig. 10).

It is important to note that the mechanisms of Roundup-mediated toxicity in prepubertal rat testis were dependent on the concentration of the pesticide. In this context, Roundup concentrations up to 36 ppm provoked Ca^{2+} overload, redox imbalance, disruption of cell signaling pathways, and necrotic cell death in rat testis. Otherwise, at a 10-fold higher ppm dose (360 ppm), the mechanisms underlying Roundup toxicity seemed to be independent of Ca^{2+} influx. Our data demonstrate the complexity of the dose-dependent toxicity of this pesticide and suggest that apoptosis could not be a response to an acute insult with low doses of the pesticide. These results are in agreement with previous studies showing that the mechanisms of Roundup toxicity changed around the critical micellar concentration of the surfactants [11].

Also, it is important to note that in this study we demonstrate that glyphosate–Roundup concentrations 10-fold more diluted than that recommended for herbicide action are highly toxic for humans. Our data contribute to evidence of the high risk of handling this formulation, mainly in childhood and puberty, and the consequences for life.

The deleterious effects of Roundup to the endocrine system of animals have been previously described by several researchers [12,16,28,55,56]. Also, the consequences of glyphosate exposure to testicular physiology became recognized from initial studies linking this herbicide with alterations in sperm quality, including decline in ejaculate volume, sperm concentration, semen initial fructose, and semen osmolality [24]. In addition, a recent publication from Romano et al. [56] has demonstrated that glyphosate may disturb the masculinization process and promote behavioral changes, as well as histological and endocrine problems to male reproduction.

Our results showed that acute exposure to pure glyphosate, at the same concentration as in Roundup (36 ppm), was able to significantly enhance $^{45}\text{Ca}^{2+}$ uptake, and the mechanisms responsible for such effect seemed to involve L-VDCC. Thus, we could propose a main role for glyphosate in Ca^{2+} overload toxicity of Roundup in prepubertal rat testis; however, the possibility exists that POEA could alter or potentiate the cytotoxicity of the glyphosate and this remains to be determined.

The Roundup-induced Ca^{2+} overload and cell death observed in rat testis were mediated by L-VDCC and IP₃- and ryanodine-mediated Ca^{2+} release, clearly indicating that disruption in Ca^{2+} homeostasis plays a critical role in the toxic effects of this herbicide. More than 2 decades ago, Olorunsogo [57] demonstrated that glyphosate increased mitochondrial membrane permeability to protons and Ca^{2+} , suggesting early on a mechanism for the toxic effect of this herbicide. Calcium may enter the cell through plasma membrane channels following an extracellular signal or be released from the endoplasmic reticulum into the cytosol in response to intracellular messengers. An imbalance in these events can lead to Ca^{2+} overload, one of the earlier steps for eliciting oxidative stress and cellular apoptosis. The normal function of the endoplasmic reticulum is essential for Ca^{2+} signaling, and disturbance of Ca^{2+} homeostasis may affect protein folding and induce endoplasmic reticulum stress [58,59].

The Ca^{2+} -mediated Roundup cytotoxicity in rat testis also involves the activation of kinase cascades including PLC/PKC,

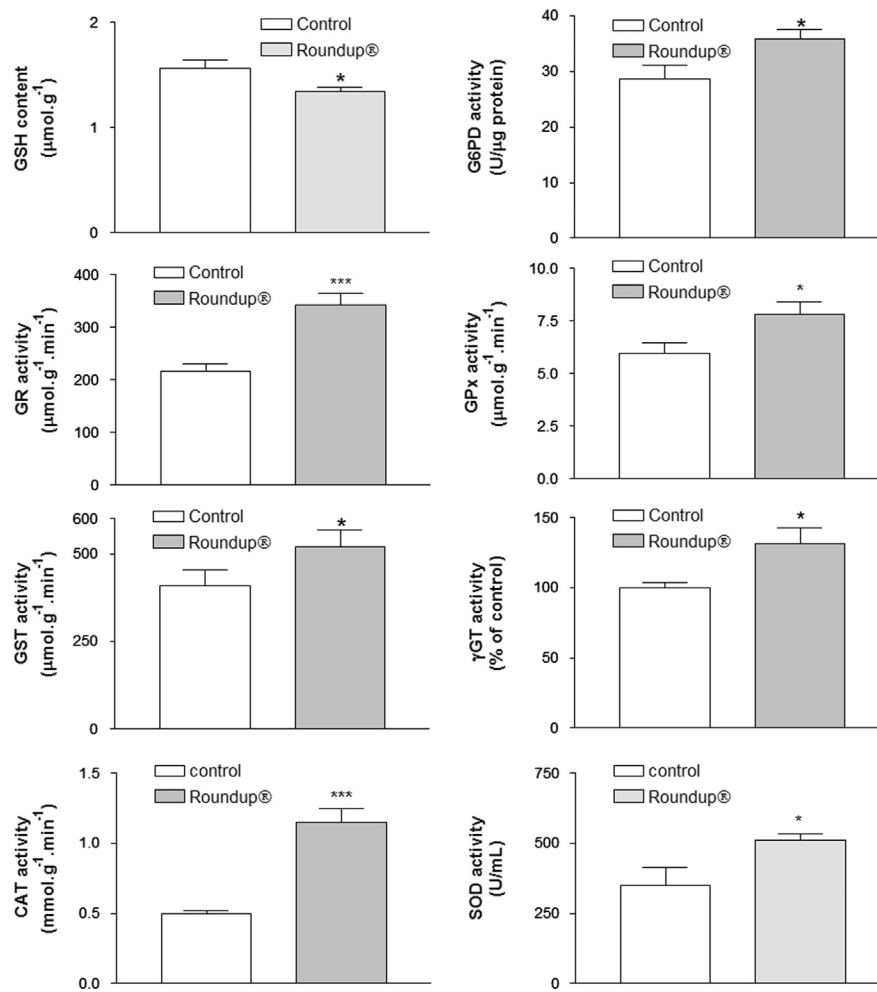


Fig. 6. Effects of Roundup on GSH levels and on the activities of G6PD, GR, GPx, GST, γ GT, CAT, and SOD in immature rat testis. Rat testes were incubated for 30 min in the presence or absence of 36 ppm or 0.036 g/L Roundup. Data are reported as means \pm SEM of eight animals from each group. Statistically significant differences from controls, as determined by Student's *t* test, are indicated. * $p < 0.01$, *** $p < 0.0001$.

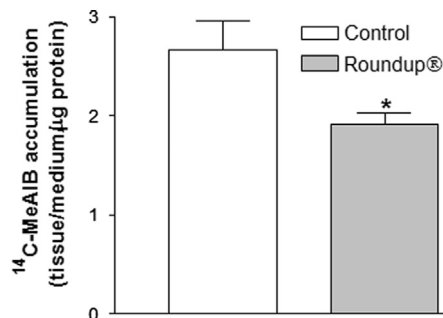


Fig. 7. Effect of Roundup on [¹⁴C]MeAIB accumulation in rat testes. Rat testes were incubated for 30 min in the presence of 3.7 kBq/ml [¹⁴C]MeAIB with or without 36 ppm or 0.036 g/L Roundup. Data are reported as means \pm SEM of eight animals from each group. Statistically significant difference from control, as determined by Student's *t* test, is indicated. * $p < 0.001$.

PI3K, ERK1/2, and p38MAPK. These kinases might be associated with the adaptive response to endoplasmic reticulum stress and/or ROS generation within the testis. Eukaryotic cells respond to extracellular stimuli by recruiting signal transduction pathways, including those involved in Ca²⁺ homeostasis. Signaling pathways orchestrated by MAPK family members (ERK, p38MAPK, and SAPK/JNK) have been associated with hypo- and hyperthyroidism in rat testis [37,38]. However, their physiological roles and regulation are not completely understood. Although architecturally

homologous to the Ras/MAPK pathway, the SAPK/JNK and p38MAPK pathways are not primarily activated by mitogens, but by cellular stress (such as oxidative stress) and inflammatory cytokines, resulting in growth arrest, apoptosis, or activation of immune cells. Moreover, it has been suggested that the p38MAPK pathway primarily inhibits cell growth and promotes either necrotic or apoptotic cell death [60]. Interestingly, under our experimental conditions, apoptotic cell death was not associated with acute Roundup toxicity, taking into account that caspase 3 activity was reduced after Roundup exposure (results not shown), suggesting that apoptosis could not be a response to an acute insult at low doses of the pesticide. Also, hyperphosphorylation of p38MAPK may activate the nuclear factor erythroid 2-related factor 2 (Nrf2), a ROS-activated signal transduction molecule, which can modulate genes encoding the enzymes involved in the antioxidant defense system. Increased p38MAPK phosphorylation associated with a shift of Nrf2 into the nuclear fraction, as well as modulation of antioxidant and proinflammatory signaling pathways, was recently demonstrated in Sertoli cells [61].

The pro-oxidant potential of long-term exposure to Roundup has been largely demonstrated in various nontarget organisms, such as fish [62,63] and mammals, leading to hepatotoxicity, nephrotoxicity, lipid peroxidation, and genotoxicity [64]. Also, exposure of a human keratinocyte cell line to glyphosate for 30 min to 24 h evidenced cytotoxic effects, concomitant with oxidative disorders [65]. Accordingly, supplementation with vitamin

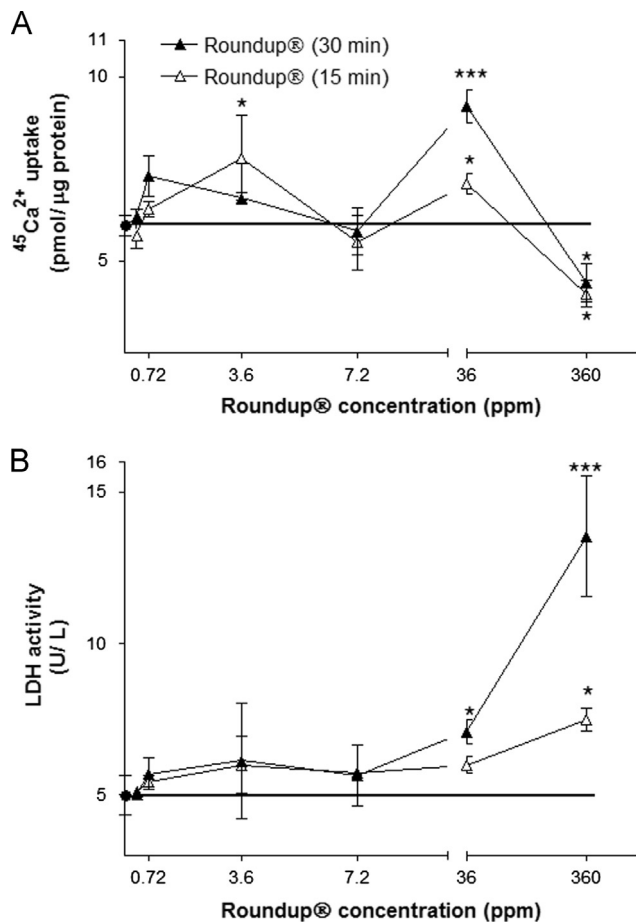


Fig. 8. Dose–response curve of Roundup for (A) $^{45}\text{Ca}^{2+}$ uptake and (B) LDH release in Sertoli cell culture from immature rat testis. Sertoli cells were preincubated for 15 min and then incubated for 15 or 30 min in the presence of 0.1 mCi/ml $^{45}\text{Ca}^{2+}$ with or without 36 ppm or 0.036 g/L Roundup at various concentrations (0.72 to 360 ppm). Values are means \pm SEM of four independent experiments. * $p < 0.01$, *** $p < 0.0001$ compared with control group.

C or E decreased lipid peroxidation in Roundup-treated keratinocytes [14,19], as well as after exposure to other organophosphate pesticides [66]. In line with this, we found that ascorbic acid (vitamin C) and Trolox (an analogue of vitamin E) abolished the Roundup-induced Ca^{2+} influx and necrosis in the testis and in Sertoli cells in culture, corroborating a role for depleted antioxidant defenses in Roundup cytotoxicity.

The Sertoli cell antioxidant system is characterized by relatively high activity of SOD, CAT, GPx, GST, GR, γ GT, catalase, and intracellular GSH levels [37,38,67,68]. The induction of oxidative stress in testicular cells by Roundup was confirmed by decreased GSH levels accompanied by increased TBARS and protein carbonyl contents, indicating a potential risk for oxidative damage in Roundup-treated testis, linking our findings with ROS overgeneration and oxidative damage. Enhancement of TBARS levels by Roundup in rat testis suggests lipid peroxidation, a process of oxidative degradation of polyunsaturated fatty acids resulting in impaired membrane structure and function. Levels of lipid peroxidation and protein carbonyls, indicating possible damage to lipids and proteins, respectively, may be used as biomarkers of herbicide exposure [35,69,70]. Therefore, TBARS and protein carbonyls could be due to the concomitant increase in ROS generation and depleted antioxidant defense systems in Roundup-treated testicular cells.

In line with this, pesticide exposure induced the enzymatic activities of GPx, GR, GST, γ GT, CAT, SOD, and G6PD that could

support GSH depletion. GSH plays an essential role in the testis, providing a defense system against oxidant agents. The complex redox system of GSH consists of GPx, GR, GST, and γ GT. The redox cycling of oxidized glutathione is catalyzed by GR, whereas the supply of NADPH, a major reducing agent for this redox cycling, is provided by G6PD activity [31]. Moreover, GSH conjugates xenobiotics by generating a biotransformation product through a reaction catalyzed by GST [31,71–73]. Considering that this GSH conjugation is one of the first steps in xenobiotic detoxification, the GSH depletion observed under our experimental conditions may be a consequence of its consumption by GST activation. Metabolism of glutathione can also be modulated by the ecto- γ GT, which breaks it down, generating a γ -glutamyl amino acid and cysteinyl-glycine for GSH synthesis. Thus, γ GT causes the degradation of extracellular GSH to provide cells with substrates for de novo GSH synthesis [31]. Our results are in agreement with previous studies reporting a GSH depletion in response to other pesticides and herbicides in different cellular populations, in vitro [14,74,75], and in vivo [76].

Moreover, the membrane-bound enzyme γ GT may be used as a biomarker of Sertoli cell toxicity. Also, testicular cell exposure to the pesticide Roundup stimulated the activity of other antioxidant enzymes such as CAT and SOD. CAT and GPx are both involved in removing H_2O_2 ; however, they must cooperate with other defense systems such as SOD, to render the cells more resistant to oxidative damage [31,77,78]. Taking into account these findings, alterations in the activity of the enzymes involved in GSH metabolism induced by Roundup may reflect a decline in cellular defense against oxidative damage within the testis. Alterations in such antioxidants have been used as an index of unbalanced ROS generation and oxidative stress in physiological systems [79].

The enzyme γ GT is primarily involved in metabolizing extracellular GSH, providing precursor amino acids to be assimilated and reutilized for intracellular GSH synthesis [80]. However, although the γ GT activity was increased in Roundup-treated testis, results showed low cytoplasmic GSH levels, which were probably due to downregulation of the Na^+ -coupled [^{14}C]MeAIB accumulation in testicular cells. It is feasible that the decreased [^{14}C]MeAIB uptake could diminish the amino acid availability for GSH de novo synthesis. Therefore, the decreased GSH levels after exposure to Roundup might be due to its consumption by conjugation via GST activity and/or to its decreased synthesis/turnover.

Conclusion

Recent reports demonstrate that many currently used pesticides have the capacity to disrupt reproductive function in animals. Although this reproductive dysfunction is typically characterized by alterations in serum steroid hormone levels, disruptions in spermatogenesis, and loss of fertility, the mechanisms involved in pesticide-induced infertility remain unclear. Particularly, the herbicide Roundup has been described as an environmental endocrine disruptor by inhibiting the steroidogenic acute regulatory protein expression in Leydig cells [12]. Interestingly, glyphosate alone did not alter steroid production, indicating that at least another component of the formulation is required to disrupt steroidogenesis [12]. Our present findings shed light onto additional mechanisms beyond the classical ones, which can contribute to understanding the possible effects of glyphosate–Roundup on the decline of male reproductive functions. We suggest that Ca^{2+} -mediated toxicity, oxidative imbalance, and disrupted signaling mechanisms seem to underlie the acute exposure to low doses of glyphosate–Roundup in prepubertal rat testis, the Sertoli cells being one of the targets for this pesticide. Considering that normal onset of spermatogenesis depends on

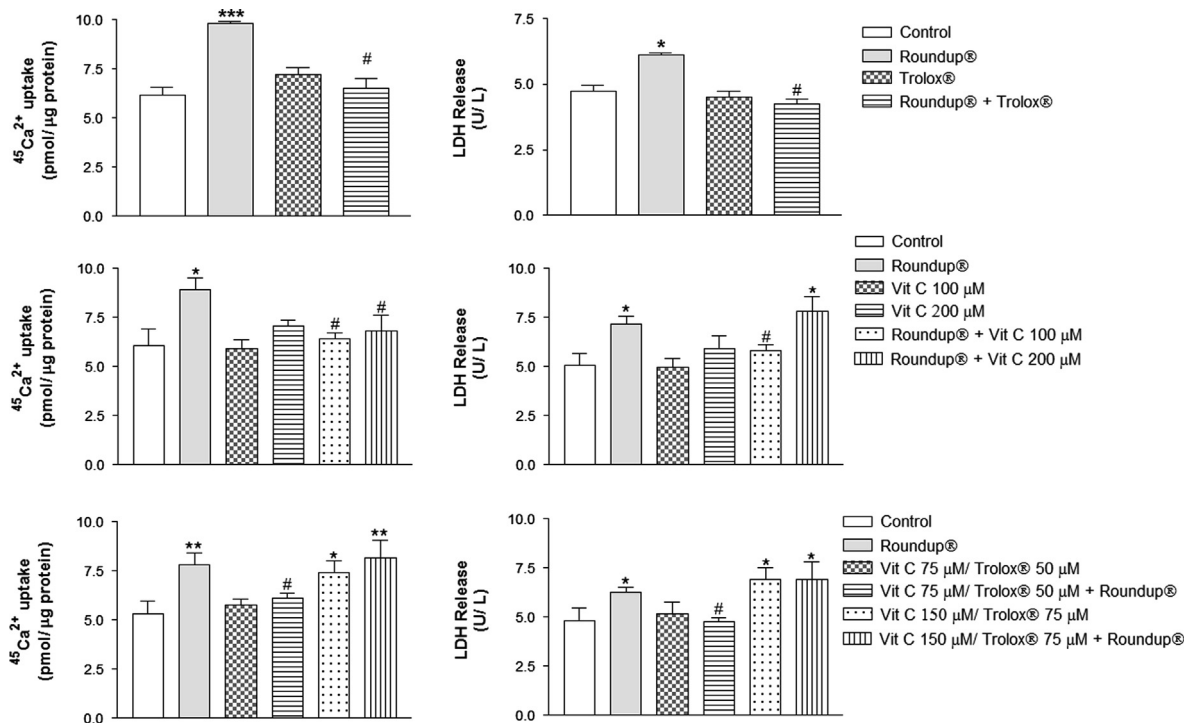


Fig. 9. Prevention by antioxidants of Roundup-induced $^{45}\text{Ca}^{2+}$ uptake and cell death in Sertoli cells from immature rat testis. Sertoli cells were preincubated for 20 min in the presence or absence of Trolox (100 μM), vitamin C (100 or 200 μM), or vitamin C plus Trolox (75 μM Vit C+50 μM Trolox or 150 μM Vit C+75 μM Trolox). After that, the tissue was incubated with or without 36 ppm or 0.036 g/L Roundup in the presence or not of the antioxidant for 30 min. Values are means \pm SEM of four independent experiments. Statistically significant differences from controls, as determined by one-way ANOVA followed by Bonferroni multiple comparison test, are indicated: * $p < 0.01$ and *** $p < 0.0001$ compared with control group; # $p < 0.05$ compared with Roundup group.

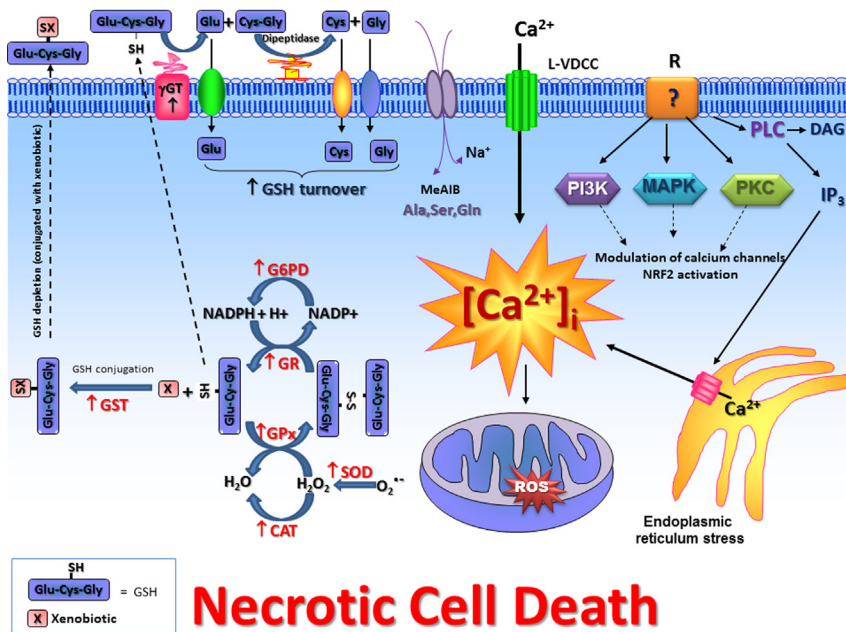


Fig. 10. Proposed mechanism of reproductive toxicity of Roundup to Sertoli cells. Roundup increased intracellular Ca^{2+} concentration by opening voltage-dependent calcium channels (L-VDCC) and endoplasmic reticulum receptors (such as IP_3 and ryanodine), leading to Ca^{2+} overload within the cells, which set off oxidative stress and cell death. Activation of Ca^{2+} influx and necrotic cell death are dependent on PLC/PKC, PI3K, and MAPK signaling pathways. PLC/ IP_3 together with ryanodine Ca^{2+} channels promote Ca^{2+} release from the endoplasmic reticulum, contributing to Ca^{2+} overloading. Roundup exposure induced the activity of the following antioxidant enzymes: glutathione peroxidase (GPx), glutathione reductase (GR), glutathione S-transferase (GST), γ -glutamyltransferase (γGT), catalase (CAT), superoxide dismutase (SOD), and glucose-6-phosphate dehydrogenase (G6PD). Considering that conjugation with GSH is one of the first steps in detoxifying a xenobiotic, the GSH depletion may occur as a consequence of its consumption by the activation of GST. The GSH metabolism can also be modulated by γGT , which breaks it down generating a γ -glutamyl amino acid and cysteinylglycine to the GSH synthesis. Altogether, these events suggest that Roundup compromises the Sertoli cell antioxidant defense system, probably leading to endoplasmic reticulum stress and cell death, which may affect male reproduction. R, Roundup.

Sertoli cell function to support and nourish germ cells, the impairment of these cells may affect male fertility. Altogether, the Ca^{2+} -mediated disturbances by glyphosate–Roundup in

rat testicular cells around 36 ppm, at levels below those to which people working with this herbicide are typically exposed, could contribute to the reproductive outcomes induced by this

formulation observed in male agricultural workers exposed to this pesticide at prepubertal age.

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