Impact of glyphosate and its formulation Roundup® on stallion spermatozoa

IMPACT OF GLYPHOSATE AND ITS FORMULATION ROUNDUP® ON STALLION SPERMATOZOA

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ABSTRACT

The growing and widespread use of glyphosate-based herbicides (GBHs) has raised an intense public debate about the impact of environmental contamination on animal and human health, including male fertility. The aim of this study was to deepen the impact of glyphosate (Gly) and GBHs on mammalian sperm investigating the effect of in vitro exposure of stallion spermatozoa to Gly and to its commercial formulation Roundup® (R).

Spermatozoa were incubated at 37°C with different Gly or R concentrations (from 0.5 to 720 µg/mL Gly or R at the same Gly-equivalent concentrations). After 1h of incubation motility, viability, acrosome integrity, mitochondrial activity and ROS production were assessed.

Gly, at all the concentrations tested, did not induce any detrimental impact on the sperm quality parameters evaluated. Conversely, R starting from 360 µg/mL (Gly-equivalent dose) significantly ($P<0.05$) decreased total and progressive motility, viability, acrosome integrity, mitochondrial activity and the percentage of live spermatozoa with intact mitochondria not producing ROS.

Our results indicate that the commercial formulation R is more toxic than its active molecule Gly and that the negative impact on stallion sperm motility might be likely due to a detrimental effect mainly at membrane and mitochondrial level and, at least in part, to redox unbalance. Moreover, based on the data obtained, it can be hypothesized a species-specificity in sperm sensitivity to Gly and GBHs as horse spermatozoa were negatively influenced at higher concentrations of R compared to those reported in literature to be toxic for human and swine male germ cells.

KEYWORDS

Stallion sperm, glyphosate, Roundup, motility, sperm membranes, mitochondrial activity, ROS

1. INTRODUCTION

Glyphosate, N-(phosphonomethyl)glycine, is the most widely used herbicide worldwide which primarily acts by specifically inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) that is fundamental for the biosynthesis of essential aromatic amino acids in plants [1]. This biosynthetic pathway (shikimate pathway) is absent in mammalian cells and thus glyphosate (Gly) has been considered to be safe to humans and animals by regulating agencies and several researchers [2–9]. Despite this, the widespread use of glyphosate-based herbicides (GBHs) has raised an intense public debate about the impact of environmental contamination on animal and human health for both its potential carcinogenic and non-carcinogenic effects, such as nervous, digestive, endocrine and reproductive system adverse effects [10–15].

GBHs, among which the popular commercial formulation Roundup® (R), include the active ingredient Gly and co-formulants that strengthen its efficiency by promoting toxicity and improving the plant absorption of the herbicide. GBHs additives are generally considered to be 'inert diluents' by manufacturers and are generally classified as confidential for regulatory purposes; however, a consistent body of literature demonstrates that commercial formulations are far more toxic than Gly itself [12,15–23].

Concerning the impact of GBHs on male reproduction both in vitro and in vivo studies reported conflicting findings. Several authors concluded that the use of Gly and GBHs pose no or limited risk of adverse effects on human or animal male reproduction [1,24].

However, in rats GBHs have been reported to induce an alteration of androgen signalling causing a pro-androgenic or an anti-androgenic effect depending on the timing of exposure [13,25–28]. The activity of GBHs as environmental endocrine disruptor has been confirmed in vitro by several authors. In rat, a decrease of testosterone due to low pesticide (Gly and R) concentrations (1ppm) has been reported by Clair et al.[29]. The mechanism by which GBHs perturb testosterone synthesis seems to be due to the inhibition of steroidogenic acute regulatory protein (STAR) expression in Leydig cells [30,31]. Furthermore, Gly, and more seriously GBHs, have been reported to be cytotoxic for testicular cells. Signs of GBHs toxicity in both Leydig and Sertoli cells have been reported in vitro [22,29] and de Liz Oliveira Cavalli et al. [32] observed that the acute exposure of Sertoli cells to R at low doses (36µg/ml) induces Ca^{2+}-mediated toxicity, oxidative imbalance, and depletion of the antioxidant defense systems.

Moreover, in vivo GBHs exposure may lead to a reduction of male fertility by impairing the sperm quality and/or reducing sperm concentration. Rat exposure to R has been reported to induce an increase of morphologically abnormal sperm and a decreased sperm nuclear quality without adverse effects on viability, motility and sperm concentration [33]. In contrast, other authors suggest that sperm concentration in rodents can be negatively influenced by GBHs [25,34,35].

Recently some in vitro studies were carried out to explore the direct effects of both technical-grade glyphosate and R on mammalian sperm.

In vitro exposure to 0.36 mg/L pure Gly exerted an adverse effect on human sperm progressive motility but not on DNA integrity after 1 h incubation whereas 1 mg/L R (corresponding to 0.36 mg/L pure Gly) caused a drop in sperm progressive motility and a depression of mitochondrial activity [16,36]. The negative effect of Gly on human sperm mitochondria has been confirmed by Ferramosca et al. [37] who reported a negative effect of Gly starting from concentration of 100 nM on mitochondrial respiration efficiency. Two recent studies investigated the direct impact of pure Gly and R on boar spermatozoa; Nerozzi et al. [38] reported that Gly at 360 µg/mL significantly decreased sperm motility, viability, mitochondrial activity and acrosome integrity while the same parameters were affected by R at very lower Gly-equivalent concentrations suggesting that the commercial formulation is far more toxic than its main component, Gly. Torres-Badia et al. [21] confirmed the higher toxicity of R compared with pure Gly and suggested that the adverse effect on sperm motility observed at 0.01% R dilution can be due to the surfactant polyoxyethyleneamine (POEA, the main claimed adjuvant by the manufacturer) as all negative effects induced by R were mimicked by POEA concentrations equivalent to those present in the commercial formulation.

Up to now no data are available in literature on the possible effects of Gly and GBHs on stallion spermatozoa.

On these bases, the objective of this study was to investigate the effects of different concentrations of Gly and R on stallion sperm, from 0.5 to 720 µg/mL Gly or from 0.000139% to 0.2% R (equivalent to 0.5 and 720 µg/mL Gly respectively), evaluating different quality parameters: motility, viability, acrosome integrity, mitochondrial activity and ROS production. All R concentrations are expressed as Gly-equivalent concentration.

2. MATERIALS AND METHODS

Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich (Saint-Louis, MO, USA) except Roundup® Bioflow (Monsanto Europe N.V., Anversa, Belgium) containing 360 g/L of glyphosate acid in the form of 486 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).

2.1. Semen collection and experimental treatment

Stallion semen was obtained from five stallions of proven fertility ranging in age from 5 to 19 years (12.8±6.1 years, mean±SD). The stallions were housed at AUB-INFA (Italy). The week before starting the experiment, one ejaculate was collected from all stallions for five consecutive days to deplete extragonadal sperm reserves. Three ejaculates were collected from each stallion (n = 15 ejaculates). Semen was collected on a phantom in the presence of estrous mare using an artificial vagina (Missouri model; Minitube, Tiefenbach, Germany) with an inner liner (Minitube) and in-line filter (Animal reproduction System, Chino, CA, USA) .

Sperm concentration was determined by NucleoCounter® SP-100™ (Chemotec, Denmark). The semen was extended at 50×10^6 sperm/mL with pre-warmed (37°C) Kenney extender (glucose 49 g/L, skimmed milk powder 24 g/L, penicillin G potassium salt 0.627 g/L, streptomycin sulfate 1 g/L) [39] and brought to the lab within 1h.

Aliquots of semen were centrifuged, resuspended at 30×10^6 sperm/mL in Tyrode's medium (20 mM HEPES, 5 mM Glucose, 96 mM NaCl, 15 mM NaHCO3, 1mM Na-Pyruvate, 21.6 Na-Lactate, 2 mM CaCl2*2H2O, 3.1 mM KCl, 0.4 mM MgSO4*7H2O, 0.3 mM NaH2PO4*H2O, 0.3% BSA) [40] and incubated in a water bath at 37°C with Gly (0, 0.5, 5, 50, 100, 360 and 720 µg/mL) or R at concentrations equivalent to the Gly ones.

After 1 h of incubation, samples were assessed for sperm motility, viability, acrosome integrity, mitochondrial activity, and ROS production, as described below.

2.2. *Motility assessment*

Three microliters of semen samples were loaded onto a pre-warmed (37°C) Leja 20 micron four chamber slide (IMV Technologies, Piacenza, Italy) and six fields per chamber were analysed by a computer-assisted sperm analyzer (CASA; IVOS v. 12, Hamilton Thorne Inc., Denver, MA, USA), using the following settings [41]: 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), amplitude of lateral head displacement (ALH) and beat cross frequency (BCF).

2.3. *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 sperm/mL to 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 far 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 SYBR14, FITC-PSA, CM-H2DCFDA (chloromethyl derivative of 2',7'-dichlorodihydrofluorescein diacetate) 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, 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 at a "LOW" flow rate.

2.4. Sperm membrane integrity (SYBR14/PI)

Sperm viability was assessed by determining the membrane integrity using two separate fluorochromes SYBR-14 and PI (LIVE/DEAD Sperm Viability Kit; Molecular Probes, Invitrogen, Milan, Italy) [42]. 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-14+/PI-).

2.5. Acrosomal and plasma membrane integrity analysis (FITC-PSA/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 PI (2.4mM stock solution) [43].

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 PSA-FITC 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 quadrant; 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.

2.6. *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 can selectively enter into 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 in 500 µL of 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 [38].

2.7. *Cellular ROS production (CM-H2DCFDA; propidium iodide; MitoTracker deep red)*

CM-H2DCFDA is a non-fluorescent agent that accumulates in the cell cytoplasm due to deacetylation and emits green fluorescence, detected by FL1 photomultiplier, upon oxidation by H_2O_2 being converted to the highly fluorescent 2',7'-dichlorofluorescein (DCF). This staining was coupled with PI that stains spermatozoa with disrupted plasmalemma (dead spermatozoa) emitting red/orange fluorescence detected by the FL3 photomultiplier [41]. Mitotracker Deep red (MT) was included to assess the mitochondrial integrity as described in 2.3, being excited by the red diode laser and detecting the fluorescence with the FL-4 photomultiplier.

Sperm samples were diluted in 500 µL of Tyrode's medium and stained with 2.5 µL CM-H2DCFDA(in DMSO, 50 µM final concentration), 2.5 µL PI (in water, 2.4 µM final concentration), and 2.5 µL MT (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 intact or not 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 either intact or non-intact mitochondria: spermatozoa with intact mitochondria producing ROS (DCF^+MT^+), spermatozoa with intact mitochondria not producing ROS (DCF^-MT^+), spermatozoa with non-intact mitochondria producing ROS (DCF^+MT^-), spermatozoa with non-intact mitochondria not producing ROS (DCF^-MT^-).

2.8. 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 ± SEM 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.

3. RESULTS

No significant differences between control and all the Gly concentrations tested for the sperm motility parameters assessed were observed, except for a decrease of VAP and VCL induced by 720 µg/mL Gly (Fig.1, Tab. 1).

Conversely, R starting from 360 µg/mL (Gly-equivalent dose) significantly ($P<0.05$) decreased, compared to control, both total and progressive motility (Fig. 1). In addition, as shown in Table 1, exposure to R caused a detrimental impact on some kinematic parameters namely VAP, VSL and VCL at concentrations ≥ 360 µg/mL (Gly-equivalent dose) and ALH at the concentration of 720 µg/mL.

While the percentage of viable spermatozoa (SYBR14+/PI-) was not affected by Gly at all the concentrations tested, a decrease in sperm viability was observed after R exposition at concentrations ≥ 360 µg/mL ($P<0.05$) (Fig. 2A). Moreover, R exposure also induced a significant ($P<0.01$) decrease in the percentages of acrosome-intact live spermatozoa (PNA-FITC^{-}/PI^{-}) after incubation with concentrations ≥ 360 µg/mL Gly-equivalent (Fig. 2B).

Similarly, addition of semen samples with R ≥ 360 µg/mL GLY-equivalent significantly ($P<0.05$), reduced the percentage of spermatozoa with high mitochondrial membrane potential (HMMP cells) as assayed by JC-1 stain (Fig. 2C); the effect was not present in Gly-treated samples.

Regarding ROS production, the percentage of live spermatozoa with intact mitochondria producing ROS (DCF^{+}MT^{+}) showed a non-significant tendency ($P=0.15$) to increase when spermatozoa were exposed to R rather than Gly (Fig. 3A). However, while the percentage of live spermatozoa with intact mitochondria not producing ROS (DCF^{-}MT^{+}) was not influenced by Gly, this parameter significantly ($P < 0.05$) decreased when semen samples were exposed to R at concentrations equal or higher than 360 µg/mL Gly-equivalent (Fig. 3B).

No difference due to Gly and R exposition were observed regarding the percentage of all live spermatozoa producing ROS (DCF^{+}MT$^{+/-}$) (Fig. 3 C).

4. DISCUSSION

A consistent body of literature suggests that Gly and GBHs have negative effects on human and animal health and fertility.

The purpose of this work was to deepen the impact of Gly and GBHs on mammalian sperm evaluating the effects of both pure Gly and R exposure on stallion spermatozoa by assessing motility, viability, acrosome integrity, mitochondrial activity as well as ROS production after 1h incubation at 37°C.

Total and progressive sperm motility were not affected by Gly at the concentration tested. In humans the adverse effect of Gly at low concentration (0.36 µg/mL) on progressive sperm motility after 1h of incubation has been reported by Anifandis et al. [16], while in swine total and progressive motility has been reported to be affected at a much higher Gly concentration (360 µg/mL) [38]. The absence of negative effect on stallion spermatozoa even at a concentration of 720 µg/mL suggests a species-specific sensitivity to Gly exposure; anyway, it has to be taken into account that, while in this study and that on boar sperm [38] motility assessment was performed through a CASA system, human sperm motility was evaluated under a phase-contrast microscope according to WHO 2010 guidelines [16].

On the other hand, stallion sperm total and progressive motility were significantly reduced in a dose dependent manner after 1h R exposure starting from 360 µg/mL Gly-equivalent (corresponding to 0.1% R dilution). This represents a quite high concentration, that is however 10 times lower than the lowest concentration used for agricultural purposes. Nonetheless, also with regard to R effect on spermatozoa, both human and boar sperm has been reported to be more sensitive than stallion one. In fact, a significant decrease of human sperm progressive motility has been observed after exposure to 1mg/mL R (corresponding to a Gly-equivalent concentration of 0.36 mg/mL) [36], while boar sperm total and progressive motility has been reported to be impaired by R concentrations around ten time lower than that found to be detrimental for stallion sperm [21,38]. As mentioned above, the discrepancy between the results obtained in those studies and ours could be due to a species-dependent sensitivity to R exposure. Anyway, our results on stallion spermatozoa confirm that commercial formulations are far more deleterious than the pure active molecule Gly, as already recorded in boar sperm [21,38].

In a similar way, while stallion sperm viability and acrosome integrity were not impaired by Gly at the concentration tested, both these parameters were worsened at R concentrations ≥360 µg/mL. Again, lower concentrations of R (100 µg/mL) have been reported to exert a detrimental effect on

boar sperm viability and acrosome integrity, while pure Gly induced a negative impact at higher concentrations (360 μg/mL) [38]. Moreover, low R concentration (0.01%) and equivalent concentration POEA (non-ionic surfactant present in some R commercial formulation) have been reported to cause lipid disorganization of boar sperm plasma membrane suggesting a major role of this last molecule on membrane detrimental effect [21]. All these results are in agreement with studies on mouse fibroblast-like and human cells lines reporting that adjuvants of GBHs are not inert diluents but can induce cellular toxicity [19,44]. Torres-Badia [21] suggested that the sperm membrane damage due to the surfactant present in GBHs can be, at least in part, the cause of the inhibition of boar sperm motility and that the inhibition of GSK3α/β phosphorylation cascade induced by both R and POEA can have a role in the impairment of sperm motility.

Mitochondrial perturbation has been reported after R exposure at low concentration in human and pig sperm (respectively 0.36 and 25 μg/mL) and after Gly exposure at higher concentration in boar spermatozoa (360 μg/mL) [36,38]. In case of Gly, Ferramosca et al. [37] observed an impairment of human sperm mitochondrial functionality by decreasing oxygen consumption starting from concentration of 100 nM. The more serious mitochondrial detrimental effect of GBHs compared to pure Gly has also been demonstrated in an immature mouse Sertoli TM4 cell line, in which a reduction of the mitochondrial succinate dehydrogenase activity was observed, as well as in isolated rat liver mitochondria, in which an impairment of mitochondrial respiration, membrane potential and enzymatic activities has been recorded [22,45]. Our results are in agreement with those observations as R, even if at higher concentrations (360 μg/mL) compared to human and swine spermatozoa, caused a reduction of the percentage of stallion sperm with high mitochondrial membrane potential (HMMP), whereas Gly induced only a non-significant tendency to a reduction of this parameter; therefore, also in stallion spermatozoa surfactants present in GBHs seems to potentiate and/or cause the mitochondrial dysfunction induced by the commercial formulation.

Stallion spermatozoa preferentially rely on mitochondrial oxidative phosphorylation (OXPHOS) to produce ATP to sustain sperm motility and maintain membrane integrity [46–51].

Therefore, it can be hypothesized that the detrimental effect exerted by R on stallion spermatozoa may be liked to both a direct effect on sperm membrane and to a perturbation of mitochondrial activity that, in turn, may cause a decrease in ATP production.

As mitochondrial activity is closely related to the production of reactive oxygen species (ROS) and Gly and GBHs have been reported to increase in vitro ROS levels in female gamete and embryos

[20,52,53] as well as in different human cell lines [18,54], we evaluated stallion sperm ROS production by CM-H2DCFDA/PI/MitoTracker deep red stain.

According to the results obtained, the percentage of live spermatozoa with intact mitochondria producing ROS (DCF^+MT^+) and that of all live spermatozoa producing ROS ($DCF^+MT^{+/-}$), showed a non-significant tendency to increase when spermatozoa were exposed to R rather than Gly. On the other hand, the percentage of live spermatozoa with intact mitochondria not producing ROS (DCF^-MT^+) significantly decreased after exposure to R ≥ 360 μg/mL. At this purpose, it is not yet clear whether ROS production can be retained as a merely negative event or not: on one hand, referring to the findings of Gibb et al. [55], it could be important that ROS are produced by spermatozoa to get fertile ejaculates. On the other hand, as reported by Peña et al. [46], the amount of ROS produced by spermatozoa should not exceed the antioxidant capacity of the cells, because they can turn dangerous for sperm structures (membrane, DNA, proteins) and, consequently, functions.

In a current study from our laboratory, we noticed that horse spermatozoa with intact mitochondria do not tend to produce high amount of cytoplasmic ROS (H_2O_2, measured by DCF fluorescence) even when the mitochondrial function (in particular, electron transport chain) is impaired with specific inhibitors (Unpublished data). Interestingly, in that study we noticed that in live spermatozoa with intact mitochondria, the subpopulation with low ROS production tended to decrease significantly when complex I and III were inhibited as well as when the proton gradient was disrupted. This trend is present also in this study, at high R doses; therefore, it could be possible that high doses of R impair the inner mitochondrial membrane integrity, thus resulting in an impaired proton gradient or rushed complexes.

Anyhow, the recorded results suggest that R may increase sperm oxidative stress and induce the alteration of the redox balance, but not in a way to be the main mechanism of its toxicity.

In conclusion, based on the data obtained, it can be hypothesized a species-specificity in sperm sensitivity to Gly and GBHs as horse spermatozoa were negatively influenced at higher concentrations of R compared to human and swine, while Gly did not impair the parameter assessed. These results, moreover, confirm that GBHs compounds other than the active molecule, can boost Gly toxic effect and/or exert an intrinsic toxicity and therefore should be considered and tested as active ingredients. Finally, the deterioration in stallion sperm motility induced by R might be likely due to a detrimental effect at membrane and mitochondrial level and, at least in part, to redox unbalance.

AUTHORS CONTRIBUTION

MS, CN and DB designed the work. CN and BM conducted the experiments. MS and DB wrote the manuscript. DB and OP performed the statistical analysis. OP, GM and GG critically revised the work. All authors discussed the results and contributed to the final manuscript.

Declaration of interest

None of the authors have conflict of interest to declare.

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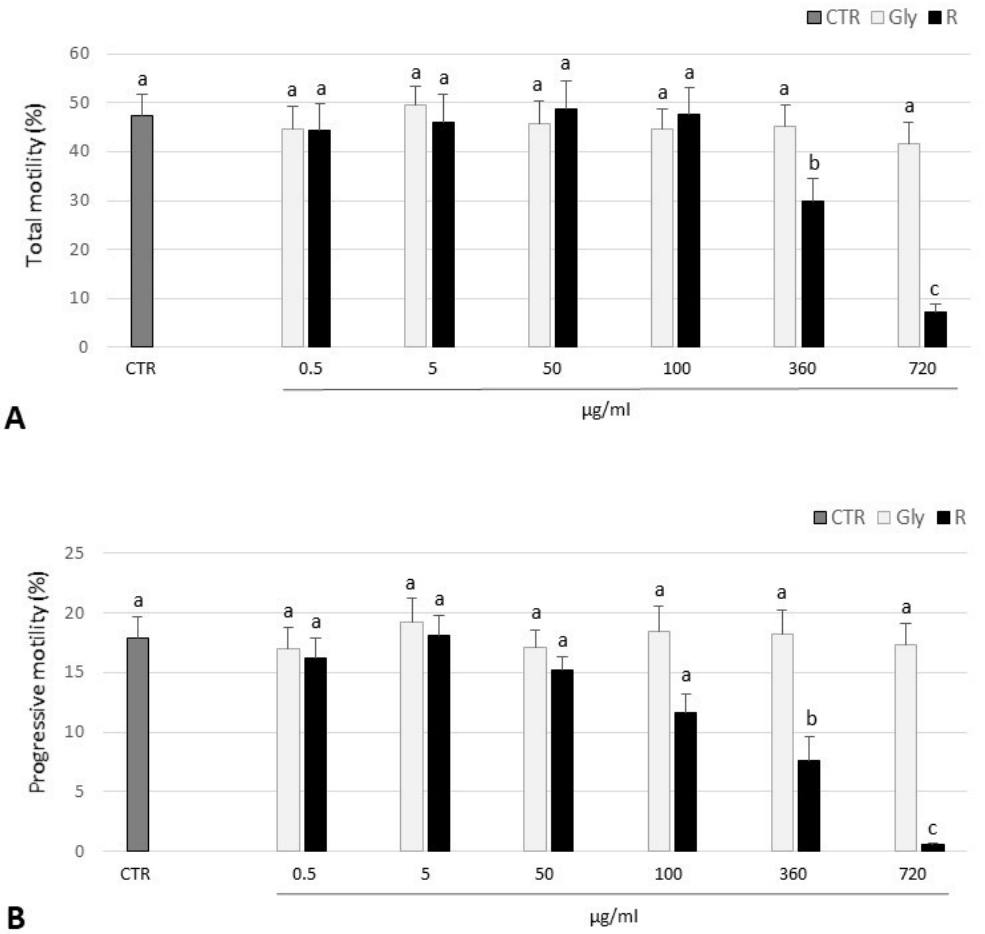

Figure 1. Effects of exposure to 0.5, 5, 50, 100, 360 and 720 µg/mL glyphosate (Gly) or Roundup (R) at glyphosate-equivalent doses on total (A) and progressive (B) sperm motility evaluated through CASA system.

CTR: control, sperm sample without addition of Gly or R.

Data are shown as mean ± SEM of 15 replicates (5 stallions, 3 ejaculates each stallion). Different letters represent significant (P<0.05) differences.

Treatment	VAP (µm/s)	VSL (µm/s)	VCL (µm/s)	ALH (µm)	BCF (Hz)	STR (%)	LIN (%)
CTR	91.88±5.3 ^{ab}	53.80±3.4 ^{a}	177.33±6.8 ^{ab}	6.73±0.2 ^{a}	42.04±0.8 ^{a}	60.33±2.8 ^{a}	33.00±1.6 ^{abc}
Gly 0.5 µg/ml	98.47±5.7 ^{a}	58.68±3.9 ^{a}	188.29±8.6 ^{a}	6.98±0.2 ^{a}	41.54±1.1 ^{a}	61.33±3.0 ^{a}	30.83±2.8 ^{abc}
Gly 5 µg/ml	96.56±4.9 ^{ab}	56.32±3.2 ^{a}	182.18±7.3 ^{a}	6.63±0.3 ^{a}	42.88±0.7 ^{a}	60.50±3.3 ^{a}	33.75±1.5 ^{ab}
Gly 50 µg/ml	89.38±5.7 ^{abc}	54.05±2.9 ^{a}	168.96±8.6 ^{ab}	6.41±0.3 ^{a}	42.29±0.7 ^{a}	57.00±5.0 ^{a}	35.08±1.2 ^{ab}
Gly 100 µg/ml	93.74±4.5 ^{ab}	58.02±3.0 ^{a}	173.68±7.9 ^{ab}	6.51±0.4 ^{a}	42.76±0.7 ^{a}	63.25±3.7 ^{a}	35.92±1.9 ^{a}
Gly 360 µg/ml	92.88±3.4 ^{ab}	56.90±3.1 ^{a}	172.33±5,7 ^{ab}	6.18±0.4 ^{a}	42.81±0.6 ^{a}	62.83±3.3 ^{a}	35.83±1.5 ^{ab}
Gly 720 µg/ml	84.77±3.9^{bc}	51.79±2.9 ^{a}	157.56±5.8 ^{b}	5.81±0.3 ^{a}	42.26±0.8 ^{a}	64.36±4.3 ^{a}	36.09±2.0 ^{a}
R 0.5 µg/ml	96.42±3.6 ^{ab}	55.61±2.9 ^{a}	183.77±5.6 ^{ab}	6.77±0.3 ^{a}	42.23±0.7 ^{a}	59.56±3.3 ^{a}	33.00±1.4 ^{ab}
R 5 µg/ml	94.06±6.3 ^{ab}	54.76±3.8 ^{a}	176.96±8.2 ^{ab}	6.69±0.3 ^{a}	40.43±1.3 ^{a}	60.56±3.1 ^{a}	33.78±1.8 ^{ab}
R 50 µg/ml	99.19±4.8 ^{ab}	55.09±2.8 ^{a}	188.31±8.6 ^{ab}	7.06±0.5 ^{a}	42.34±1.0 ^{a}	57.38±3.3 ^{a}	31.88±1.5 ^{abc}
R 100 µg/ml	93.70±6.6 ^{abc}	45.49±3.3 ^{ab}	186.60±9.4 ^{ab}	7.25±0.5 ^{a}	42.56±1.4 ^{a}	51.63±2.8 ^{a}	26.50±1.4 ^{bc}
R 360 µg/ml	77.60±5.3 ^{c}	38.46±5.6 ^{bc}	160.56±6.7 ^{b}	6.44±0.4 ^{a}	42.38±1.1 ^{a}	52.75±3.2 ^{a}	26.75±2.7 ^{abc}
R 720 µg/ml	51.80±4.5 ^{d}	20.64±2.7 ^{c}	131.06±7.1 ^{c}	3.92±0.7 ^{b}	41.40±0.3 ^{a}	44.40±2.0 ^{a}	19.20±1.8 ^{c}

Table 1. Effects of exposure to 0.5, 5, 50, 100, 360 and 720 µg/mL glyphosate (Gly) or Roundup (R) at glyphosate-equivalent doses on kinematic parameters assessed by CASA system. CTR: control, sperm sample without addition of Gly or R; VAP: average path velocity; VSL: straight line velocity; VCL: curvilinear velocity; ALH: amplitude lateral head displacement; BCF: beat cross frequency; STR: straightness; LIN: linearity.

Data represent the mean ± SEM of 15 replicates (5 stallions, 3 ejaculates each stallion). Different letters represent significant (P<0.05) differences within each column.

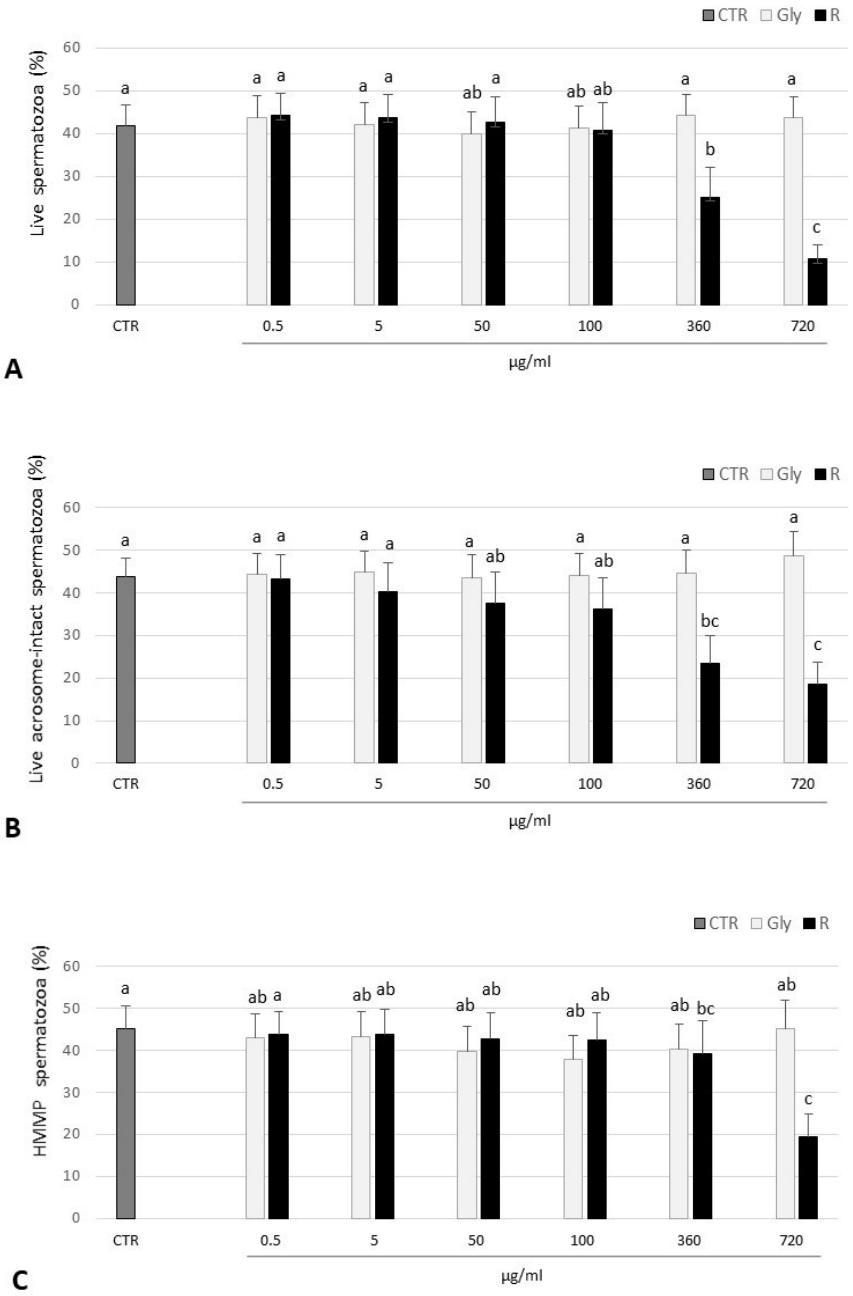

Figure 2. Effects of exposure to 0.5, 5, 50, 100, 360 and 720 µg/mL glyphosate (Gly) or Roundup (R) at glyphosate-equivalent doses on viability (A), acrosome integrity (B) and percentage of spermatozoa with high mitochondrial membrane potential (HMMP)(C).

CTR: control, sperm sample without addition of Gly or R.

Data are shown as mean ± SEM of 15 replicates (5 stallions, 3 ejaculates each stallion). Different letters represent significant (P<0.05) differences.

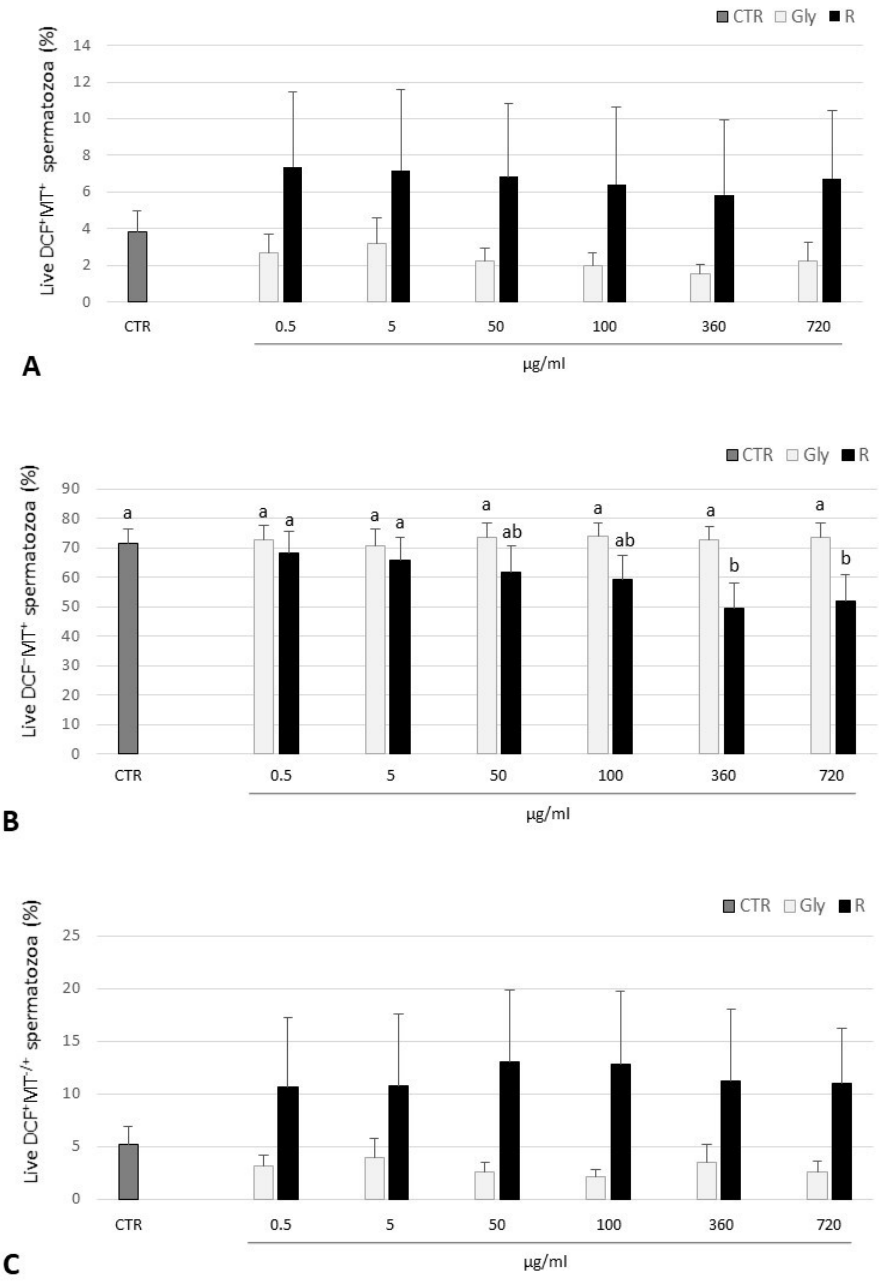

Figure 3. Effects of exposure to 0.5, 5, 50, 100, 360 and 720 µg/mL glyphosate (Gly) or Roundup (R) at glyphosate-equivalent doses on the percentage of live spermatozoa with intact mitochondria not producing ROS ($DCF^{+}MT^{+}$) (A), the percentage of of live spermatozoa with intact mitochondria not producing ROS ($DCF^{-}MT^{+}$) (B) and the percentage of all live spermatozoa producing ROS ($DCF^{+}MT^{+/-}$) (C). Different letters (a, b) represent significant (P<0.05) differences.

CTR: control, sperm sample without addition of Gly or R.

Data are shown as mean ± SEM of 15 replicates (5 stallions, 3 ejaculates each stallion). Different letters represent significant (P<0.05) differences.