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Impaired mammalian sperm function and lower phosphorylation signaling caused by the herbicide Roundup® Ultra Plus are due to its surfactant component.

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Abstract:	The use of worldwide glyphosate-based herbicide Roundup ® is growing and to date its effects on mammalian spermatozoa are controversial. This study aims to investigate the functional impact of in vitro exposure of pig spermatozoa to low concentrations of Roundup ® Ultra Plus (RUP), similar to those present as environment contaminants, to its active ingredient glyphosate, and to the non-active component, surfactant POEA. Pig spermatozoa were incubated in Tyrode's basal medium (TBM) or Tyrode's complete medium (TCM) (1h at 38.5 °C) with several RUP dilutions or equivalent concentrations of glyphosate or POEA. RUP treatment causes a significant dilution-dependent decrease in sperm motility, a significant increase in plasma membrane disorganization and reduction in GSK3 b phosphorylation (TBM) and in two PKA substrates (TBM and TCM), whereas does not affect sperm viability or mitochondrial membrane potential (MMP). Equivalent glyphosate concentrations do not affect any functional sperm parameters. However, POEA concentrations equivalent to RUP dilutions mimic all RUP sperm effects: decrease sperm motility in a concentration-dependent manner, increase sperm plasma membrane lipid disorder and significantly inhibit GSK3 b phosphorylation (TBM) and two PKA substrates without affecting sperm viability or MMP. In summary, low concentrations RUP herbicide cause sperm motility impairment without affecting sperm viability. This adverse effect could be likely due to a detrimental effect in the plasma membrane lipid organization and to inhibition of phosphorylation of both, GSK3 b and specific PKA substrates. Importantly, our results indicate that negative effects of low RUP concentrations in pig spermatozoa function are likely caused by the surfactant included in its formulation and no by its active ingredient glyphosate.

Leonardo Brito, DVM, PhD, DACT

Associate Editor of *Theriogenology*

May 20th, 2021

Dear Associate Editor:

Please find enclosed our revised article Ref. No THERIO-D-21-00274, entitled "Impaired mammalian sperm function and lower phosphorylation signaling caused by the herbicide Roundup® Ultra Plus are due to its surfactant component". On April 21, you informed us that the paper has been found acceptable for publication and also requested us to consider the modifications detailed in the Reviewer´s comments.

During this time, we have performed some experiments and modified the manuscript as requested. We have included the responses point-to-point to all individual comments raised by the two reviewers. Moreover, the manuscript has been modified accordingly.

We consider that after this revision the manuscript has significantly improved and hope that can be now suitable for publication in Theriogenology.

Yours sincerely,

M. Julia Bragado, Ph. D

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Replies to Reviewer #1:

- **Referee states that "data on GLY effect on viability, plasma membrane lipid disorganization and mitochondrial membrane potential must be added at least as a supplementary file".**

Answer: As requested, we have included the results obtained after incubation of boar spermatozoa in TBM or TCM medium with different concentrations of glyphosate at 38.5 °C. Data regarding sperm viability, mitochondrial membrane potential and plasma membrane lipid organization are now shown in the Supplementary Data and cited in Results Section (page 13, line 322).

- Referee states that plasma "membrane integrity" is used referring to the results obtained with M540/ YoPro1 creating some confusion with the evaluation of sperm viability (SYBR-14/PI). Its suggestion is to specify the staining used talking about membrane integrity or to use sperm membrane lipid disorder - lipid organization/membrane permeability for data obtained with M540/ YoPro-1.

Answer: We have addressed this issue though the manuscript and changed the term "membrane integrity" by "membrane lipid disorder or membrane lipid organization" (abstract lines 61 and 65; Discussion line 464 and Conclusions line 517)

- Referee asked: "Were spermatozoa capacitated at the end of the incubation period in capacitating medium? No specific comment on the effect on capacitation process is present in the discussion".

Answer: The aim of this work was to study the possible effect of RUP or its components, GLY or surfactant, in boar sperm quality, focusing mainly sperm motility.

Also, the incubation time at which we have detected sperm effects in motility is 1 h. Previously (Hurtado de Llera et al., BBA-Biomembranes 2013), we have concluded that in order to experimentally induce the capacitation process in boar spermatozoa is necessary to incubate longer than 1 h, at least under the same experimental conditions used in this work. This is the reason why we have not measured any cell parameter indicative of sperm capacitation status in this work as both, the short incubation time and the research aim, were not suitable for study boar sperm capacitation. Moreover, the rationality of the boar sperm incubation in two mediums, non-capacitating or capacitation medium, is based in the hypothesis that the effect of RUP or its components might be affected by the presence of sperm stimuli, as BSA, bicarbonate or calcium, in the incubation medium. Therefore, we have used the TCM as the medium that includes those stimuli and compared to TBM as the medium where these stimuli are absent in order to compare their effects in RUP action. To clarify this issue, we have included a sentence in the discussion section mentioning that sperm capacitation has not been addressed under the experimental conditions used in this work and therefore we cannot make any assumptions about RUP or its components in this functional sperm process (Discussion page 20, lines 470-472).

- **Referee states: ”It is unlikely that such high concentrations of glyphosate or formulated glyphosate would reach porcine spermatozoa in vivo. Even if it is clearly declared that pig spermatozoa were used as an in vitro model to investigate toxicity, it should be mentioned that the observed effects are unlikely to occur in livestock production unless pigs are directly consuming the formulated herbicide product”.**

Answer: In this in vitro work we have used glyphosate concentrations that are much lower (100-400 times) than those recommended for agriculture purposes. The concentrations of RUP or its components, glyphosate or surfactant POEA, evaluated in the present work can be therefore considered as environment contaminants or remnants amounts after agriculture use. In fact, this was our aim, not to study the direct effect of RUP at concentrations recommended for its agriculture use, but to evaluate in a well-validated in vitro cell model its effects as possible contaminants that remain in the environment after its massive use and to whose lower amounts the boar might be somehow exposed.

We consider that this explanation makes clear this issue, but if the referee still thinks that we should include the suggested sentence (…observed effects are unlikely to occur in livestock production) we have no problem at all to include it if necessary and in order to clarify this matter.

SPECIFIC COMMENTS

> Line 29. TBM and TCM. Provide full name.

Answer: The full names have been included

> Lines 38-40. Reduce plasma membrane integrity. ….without affecting sperm viability. This statements sound in contradiction. "reduce plasma membrane integrity" should be changed into "increase sperm membrane lipid disorder/permeability".

Answer: The suggested change has been done

> Line 42-43. See above.

Answer: The suggested change has also been done.

> Line 73. No effects.

Answer: This error has been amended.

> Line 81. "360 µg/mL". Please check. Anifandis et al. (2018) in their work investigated the effect of 0.36 µg/mL glyphosate.

Answer: We thank the reviewer and have corrected the error in the numbers of line 82 (new version) and also 3 lines further referring to “10000 times lower than those recommended for agriculture” (line 85 of highlighted version).

> Lines 87-88. "at concentrations similar to those investigated in human spermatozoa". Please check. Nerozzi et al (2020) evaluated the effects of sperm exposure to 0, 5, 25, 50, 100 and 360 µg/mL glyphosate.

Answer: We have checked it and modified accordingly.

> Line 133. "Tyrode's complete medium (TCM), a spermatozoa-capacitating medium". No evaluation on capacitation status was performed. Add data on capacitation status or add references.

Answer: As mentioned before in the comments to this referee, we have not evaluated any sperm capacitation parameter as it was not our aim and also because the incubation time in this work is insufficient to achieve capacitation in boar spermatozoa, at least in under experimental conditions. Therefore, as suggested, we have added a reference.

> Lines 158-159. Check POEA concentrations, it should be: 0.0008%, 0.0004% and 0.0002% respectively.

Answer: We have corrected the error in the concentrations numbers

> Lines 258-259. "None of the RUP dilutions studied has a significant effect on sperm viability". In Fig. 1 viability with RUP 5 and 10 in TBM are statistically different. Please check.

Answer: We have carefully checked Figure 1 and there was an error showing asterisks. This has been corrected in the new Figure 1 showing that none of the RUP dilutions studied has a significant effect on sperm viability.

> Lines 274 and 277. "sperm stimulant medium" and "non-stimulant medium". Sperm capacitating medium and sperm non-capacitating medium.

Answer: The suggested changes have been performed.

> Lines 280-282. Please check. RUP 0,01% in TCM significantly reduced LIN and WOB (table 1).

Answer: This sentence has been modified to include this statistical result. New sentence states: “However, sperm incubation with different RUP dilutions does not modify other sperm motility coefficients analyzed showed in Table 1, except for linearity coefficient (LIN) or wobble movement coefficient (WOB), which are significantly reduced by incubation with RUP 0.01%”.

> Lines 289-290. (Data not shown). Data on GLY effect on viability, plasma membrane lipid disorganization and mitochondrial membrane potential must be added at least as a supplementary file.

Answer: As suggested, we have now included glyphosate effects in boar sperm viability, plasma membrane lipid disorganization and mitochondrial membrane potential as a supplementary file.

> Line 296. "Figure 1C". Figure 1 lower histograms or add letters in the figure.

Answer: The suggested change has been done.

> Line 299. "The in vitro effects". Add "on sperm motility".

Answer: The suggested change has been done.

> Lines 317-318. Looking at the table 1, POEA induced a significant decrease of STR in TBM and a significant decrease of LIN, STR, WOB in TCM.

Answer: These data regarding statistical differences due to POEA have been included. New sentences states: “Regarding other spermatozoa motility coefficients, POEA incubation (0.0004%) significantly decreased STR in TBM and at 0.0008% significantly reduced LIN, STR and WOB in TCM (Table 1).

> Lines 326 and 331. Figure 8A and figure 8B. Add letters in the figure.

Answer: Same issue was requested by this referee for Figure 1C (line 296 of highlighted version) and it has been modified accordingly; therefore, we have decided to add in the text the words “ upper histograms” or “lower histograms” wherever the text is referring to Figure 8 and also in Figure 9 (lines 345 and 347 of highlighted version) and Figure 10 (lines 358 and 366 of highlighted version).

> Line 339. "RUP significantly reduces GSK3α/β phosphorylation in spermatozoa incubated in TBM". Looking at the Figure 9, RUP significantly reduced only GSK3 β phosphorylation.

Answer: This suggestion has been addressed and the new sentence states: “Sperm treatment for 1h at 38.5 ºC with 0.01% RUP reduces GSK3α/β phosphorylation in spermatozoa incubated in both, TBM and TCM (Figure 9 histograms), although reduction was statistically significant only for GSK3β phosphorylation after incubation in TBM (Figure 9 right histograms)”.

> Lines 354-356-357. Figure 10A and figure 10B. Add letters in the figure.

Answer: The suggested change has been done.

> Line 366. "inconclusive". Quite strong.

Answer: We have addressed it changing this word by “little literature” (line 373 highlighted version)

> Line 383. "GLY (360 µg/mL) in human [16]". Please check

Answer: We have amended the error in the glyphosate concentration. New sentence states as follow: “ ... and also its equivalent concentration of GLY (0.36 µg/mL) in human [16] or a higher concentration in pig spermatozoa (360 µg/mL)” (lines 390 and 391 of highlighted version).

> Figure 7. Right panel. The dot plots of POEA seem to be both in TCM.

Answer: The referee is right and we have changed the dot plots in this Figure to show the correct data.

Reviewer #2:

The reviewer found this paper interesting and the experiments are overall convincing. Moreover, referee raised the following 2 points to improve:

1. Please explain why two different mediums were used.

Answer: The aim of this work was to study the possible effect of RUP or its components, GLY or surfactant, in boar sperm quality, focusing mainly in sperm motility. It is well known that sperm motility is highly dependent of the stimuli present in the extracellular (or incubation) medium. Moreover, the rationality of the boar sperm incubation in two different media, non-capacitating or capacitation medium, is based in the hypothesis that the effect of RUP, or its components GLY or POEA, might be affected by the presence in the incubation medium of sperm stimuli, as bicarbonate or calcium. Therefore, we have used the TCM as the medium that includes those stimuli and TBM as the medium where these stimuli are absent in order to compare their effects in RUP action under these two experimental conditions that we know are affecting motility.

2. Figure 9, the total GSK3alpha and beta should also be examined.

Answer: As requested, we have included the results of western blot analyzing GSK3alpha and beta under experimental conditions studied in Figure 9. The new Figure 9 shows the images of western blot under TBM (left images) or TCM conditions (right images), being the lower images those corresponding to the analysis of GSK3alpha and beta levels.

Impaired mammalian sperm function and lower phosphorylation signaling caused by the herbicide Roundup® Ultra Plus are due to its surfactant component.

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Running Title: The Roundup® surfactant POEA inhibits pig sperm motility

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ABSTRACT

The use of worldwide glyphosate-based herbicide Roundup® is growing and to date its effects on mammalian spermatozoa are controversial. This study aims to investigate the functional impact of *in vitro* exposure of pig spermatozoa to low concentrations of Roundup® Ultra Plus (RUP), similar to those present as environment contaminants, to its active ingredient glyphosate, and to the non-active component, surfactant POEA. Pig spermatozoa were incubated in Tyrode's basal medium (TBM) or Tyrode's complete medium (TCM) (1h at 38.5 °C) with several RUP dilutions or equivalent concentrations of glyphosate or POEA. RUP treatment causes a significant dilution-dependent decrease in sperm motility, a significant increase in plasma membrane disorganization and reduction in GSK3β phosphorylation (TBM) and in two PKA substrates (TBM and TCM), whereas does not affect sperm viability or mitochondrial membrane potential (MMP). Equivalent glyphosate concentrations do not affect any functional sperm parameters. However, POEA concentrations equivalent to RUP dilutions mimic all RUP sperm effects: decrease sperm motility in a concentration-dependent manner, increase sperm plasma membrane lipid disorder and significantly inhibit GSK3β phosphorylation (TBM) and two PKA substrates without affecting sperm viability or MMP. In summary, low concentrations RUP herbicide cause sperm motility impairment without affecting sperm viability. This adverse effect could be likely due to a detrimental effect in the plasma membrane lipid organization and to inhibition of phosphorylation of both, GSK3β and specific PKA substrates. Importantly, our results indicate that negative effects of low RUP concentrations in pig spermatozoa function are likely caused by the surfactant included in its formulation and no by its active ingredient glyphosate.

Key words: pig spermatozoa, herbicide Roundup, motility, glyphosate, surfactant.

1. Introduction

Glyphosate, *N*-(phophonomethyl) glycin, is the active compound of the commercial Roundup® herbicide, which possess a broad spectrum and is among the most used worldwide in agriculture. Not only its use is massive but also is growing about 20% every year. The consequences of the wide use of glyphosate-based herbicides have become a big concern for human and animal health as they gravely contaminate the environment, including soil, water and ecosystems, and therefore represent a serious risk. The research about its potential harmful effects has recently been addressed in different mammals species [1-3], where glyphosate can act as an endocrine disruptor at low doses, impairing hormones physiological role [2, 4]. In this regard, even low Roundup® concentrations that are considered as herbicide residues cause effects on cell structure (cytoplasmic damage) and function in hepatoma tissue culture cells [5].

The commercial Roundup® formulation includes not only the active compound glyphosate, but also other non-active ingredients, such as detergents that function as tensioactive molecules. In this regard, it has been demonstrated that Roundup® herbicide has more negative effect than glyphosate alone in human cells [6-9] including placental cells [10-12] and embryonic cells [10, 11].

In the male reproductive system of mammals, some initial works show controversial results, raising issues about the reproductive toxicity of this herbicide. Thus, Williams et al. [13] found no definitive evidence that glyphosate negatively affected human reproductive physiology. Additionally, Cassault-Meyer et al. [14] described abnormal sperm morphology just in two particular days after 8 days of acute glyphosate exposure in rats, whereas no effects were observed neither in sperm concentration, viability or motility. A meta-analysis about effects on sperm

concentration in rodents concludes that glyphosate exposure decreased sperm concentration and therefore, glyphosate is toxic for male rodent´s reproductive system [1]. More recently it has been demonstrated that *in vitro* treatment of human spermatozoa with Roundup® at concentration of 1 µg/mL, causes a rapid and adverse effect on sperm motility, probably due to a concomitant mitochondrial deregulation [15]. Same authors reported later that its active ingredient glyphosate at 0.36 µg/mL, which is the concentration equivalent to 1 µg/mL Roundup®, causes also a rapid *in vitro* effect in human spermatozoa, decreasing progressive motility and blocking mitochondrial activity after 1 h exposure [16]. Interestingly, the concentrations of herbicide used in these studies are 10000 times lower than those recommended for use in agriculture. A recent study conducted in pig spermatozoa has investigated the impact of pure glyphosate and Roundup® on sperm function and survival [3] at concentrations higher to those investigated in human spermatozoa [15, 16]. These authors conclude that while both, glyphosate and Roundup®, have an adverse effect on male gametes, Roundup® is more toxic than its main active ingredient, glyphosate. It is clear that more research on the male gamete consequences of Roundup® herbicide exposure is required for several reasons: i) the glyphosate-based herbicides and glyphosate concentrations that decreased motility resulted also cytotoxic for spermatozoa [3], making impossible to really elucidate the negative impact of glyphosate-based herbicides and its active compound to sperm physiology, ii) more importantly, the individual effects of the different components included in the formulation of the commercial glyphosate-based herbicides, especially the surfactants, have not been addressed in the same study in parallel to the Roundup® and glyphosate in mammalian spermatozoa. Therefore, the aim of the present work is to investigate the functional impact of *in*

vitro exposure of mammalian spermatozoa to the herbicide Roundup®, to its active ingredient glyphosate, as well as to the non-active ingredient the detergent POEA (the main claimed adjuvant by the manufacturer) at relatively low Roundup® concentrations (100 times lower than recommended for agricultural use). These concentrations are similar to those present as environment contaminants that might affect sperm function, as motility, but do not result cytotoxic for spermatozoa. For this purpose, we have used pig spermatozoa as a validated *in vitro* mammalian cell model to investigate cell toxicity [3, 17, 18] and also a well-demonstrated cell model in sperm physiological studies for its successful translation into human assisted reproduction techniques [19].

2. Material and Methods

2.1. Chemical and sources

Roundup® Ultra Plus from Monsanto Europe (Ambers, Belgium); glyphosate potassium salt and M540 were from Sigma-Aldrich (St Louis, MO, USA); polyethoxylated tallow amine (POEA) from Dr. Ehrenstorfer GmbH (Augsburg, Germany); Propidium iodide (PI), SYBR-14, Yo-Pro-1 and 5,5',6,6'–tetrachloro-1,1',3,3' tetraethylbenzymidazolyl carbocyanine (JC-1) probes from Thermo Fisher Scientific (Waltham, MA, USA); *DC* ™ Protein Assays and 2x Laemmli Sample Buffer from Bio-Rad (Hercules, CA, USA); Intercept® (TBS) blocking buffer, IRDye® 800RD and 680RD secondary antibodies from LI-COR Biotechnology (Bonsai Lab, Alcobendas, Spain). Furthermore, the anti-phospho (Ser/Thr) PKA Substrate (#9624) and anti-phospho (Ser21/9) GSK3α/β (#9331) and total GSK3α (#9338) and GSK3β (#9332) polyclonal antibodies were from Cell Signaling Technology, Inc. (Beverly, MA, USA); the anti-α-tubulin antibody (TU-02, #SC-8035)

was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All reagents used to prepare incubation media were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Spermatozoa incubation media

Tyrode's basal medium (TBM; 96 mM NaCl, 4.7 mM KCl, 0.4 mM $MgSO_4$, 0.3 mM NaH_2PO_4, 5.5 mM glucose, 1 mM sodium pyruvate, 21.6 mM sodium lactate, 20 mM HEPES, 5 mM EGTA and 0.02% PVA) was prepared and used as the non-capacitating medium. A variant of TBM was made omitting EGTA and PVA and adding 1 mM $CaCl_2$, 15 mM $NaHCO_3$ and 3 mg/mL BSA, then it was equilibrated with carbogen (5% CO_2/95% O_2) and termed Tyrode's complete medium (TCM), a spermatozoa-capacitating medium [20]. All media were prepared on the day of use and adjusted to pH 7.45 with an osmolarity of 290-310 mOsm kg^{-1}.

2.3. Boar semen collection and experimental treatment of spermatozoa.

Sperm samples from Duroc boars (2-4 years old) were commercially obtained from a regional porcine company (Tecnogenext, S.L, Mérida, Spain), without any requirement of approval from the animal research review board of the University of Extremadura. All boars were housed in individual pens in an environmentally controlled building (15-25 °C) according to Regional Government and European regulations and received the same diet. Fresh ejaculates were collected with the gloved hand technique and stored at 17 °C before use in the laboratory. In order to minimize individual boar variations, samples from up to 3 animals were pooled using semen from no less than 12 boars in different combinations. Only semen pools with at least 80% morphologically normal spermatozoa were used. Semen was centrifuged at 900 *g* for 4 minutes, washed with phosphate-buffered saline (PBS) and spermatozoa were placed in TBM or TCM medium to a final concentration of 30 x 10^6 spermatozoa mL^{-1}.

Depending on each experimental procedure, 0.5 or 1.5 mL of spermatozoa samples containing 30 × 10^6 spermatozoa mL^{-1} were incubated during 1 h at 38.5 °C in the absence (control) or presence of Roundup® Ultra Plus (RUP) that was previously diluted 1/10000, 1/20000 and 1/40000 times, yielding final RUP concentrations (v/v) of 0.01%, 0.005% and 0.0025%, respectively. The commercially available Roundup® Ultra Plus contains 36% (w/v) of the active ingredient glyphosate (GLY) and 6% (w/v) of the surfactant, polyoxyethylene amine (POEA). The above-mentioned dilutions of the commercial RUP yield final GLY concentrations of 164, 82 and 41 µM and final POEA concentrations of 0.0008%, 0.0004% and 0.0002%, respectively. These concentrations of the active and non-active ingredients of RUP, which resulted after different RUP dilutions, are so-called equivalent concentrations of RUP and were used for the treatment of pig spermatozoa.

When spermatozoa were incubated in TBM, incubation was performed in absence of air, whereas TCM treatment was performed in a humidified atmosphere of 5% CO_2/95% air atmosphere In order to minimize possible experimental variations, all the different experimental treatments were carried out in each of the semen pools.

All experiments were performed in accordance with European, national and Regional guidelines and regulations.

2.4. Evaluation of spermatozoa motility

After incubation with RUP or its different components, 2 µl of spermatozoa sample were placed in a 38.5 ºC pre-warmed counting chamber with 20 µm depth (Leja®, Nieuw-Vennep, The Netherlands). Spermatozoa images were take using a microscope equipped with a 10X negative-phase contrast objective, with a heated

stage, and a CCD camera that takes 25 consecutive digitalized images obtained during 1 s form at least 4 different fields and 300 spermatozoa per sample [21]. Digitalized images were analyzed using a Computer Assisted Semen Analysis system, specifically the ISAS® system (Integrated Semen Analysis System, Proiser R+D, Paterna, Valencia, Spain). Sperm motility parameters and coefficients: motile spermatozoa (percentage of spermatozoa with an average path velocity > 10 μm/s), progressive motile spermatozoa (percentage of spermatozoa with a straightness coefficient > 80%), VCL (curvilinear velocity in μm/s), VSL (straight-line velocity in μm/s), VAP (average path velocity in μm/s), LIN (linearity coefficient in %), STR (straightness coefficient in %) and WOB (wobble coefficient in %).

2.5. Flow cytometry analysis

Flow cytometry analysis was performed using an ACEA NovoCyte® flow cytometer (ACEA Biosciences, Inc., San Diego, CA, USA) equipped with a three detection channels for blue laser (488 nm): BL-1 (530 ± 30 nm band pass filter); BL-2 (572 ± 28 nm band pass filter) and BL-4 (675 ± 30 nm band pass filter) and a detection channel for a red laser (640 nm): BL-3 (660 ± 20 nm band pass filter). Flow cytometry experiments and data analyses were performed using ACEA Novo Express® software (ACEA Biosciences, Inc., San Diego, CA, USA). Fluorescence data were represented in a logarithmic scale.

2.5.1. Analysis of spermatozoa viability by flow cytometry

As described previously [21], fluorescent staining using SYBR-14 and propidium iodide (PI) was performed to measure sperm viability. Briefly, 5 μl of SYBR-14 (2 μM) and 10 μl of PI (240 μM) were added to 100 μl of spermatozoa (30×10^6 cells mL^{-1}) diluted with 400 μl of PBS, until a final concentration of 20 nM for SYBR-14 and 5 μM for PI. Then, the samples were incubated for 15 min at room temperature

(RT) in darkness and analyzed in the flow cytometer. After excitation at 488 nm, SYBR-14 fluorescence was detected using a 530 ± 30 nm band pass filter and PI fluorescence using 675 ± 30 nm band pass filter. Results of viable spermatozoa were expressed as the average of the percentage of $SYBR14^+$ and PI^- spermatozoa ± standard error of the mean (SEM).

2.5.2. Analysis of sperm mitochondrial membrane potential (ΔΨm) by flow cytometry

As described previously, fluorescent staining using the specific probe JC-1 was used as mitochondrial membrane potential marker [21]. The experimental procedure consists of diluting 100 µl of spermatozoa (30×10^6 cells mL^{-1}) in 400 µl of PBS containing 0.9 µM of JC-1, mixed an incubated at 38.5 °C for 30 min. The fluorescence values were collected on both channels BL-1 (JC-1 monomer) and BL-2 (JC-1 polymer) the results were expressed in percentage of spermatozoa with high mitochondrial membrane potential (high ΔΨm) with respect to the total number of spermatozoa analyzed.

2.5.3. Evaluation of the degree of sperm plasma membrane lipid organization by flow cytometry

Fluorescent staining using the probes merocyanine M540 was used as a membrane lipid fluidity marker, and YoPro-1, as a marker of changes in plasma membrane permeability (commonly associated with cell death), was performed as previously described [20]. Briefly, 100 µl of spermatozoa (30×10^6 cells mL^{-1}) were diluted in 400 µl of PBS containing 75 nM of Yo-Pro-1 and 6 µM of M540 and incubated at 38 °C for 15 min. Then, remixed before flow cytometry analysis. The fluorescence values of probes Yo-Pro-1 and M540 were collected on both BL-1 and

224 BL-2 channels, respectively. Labelled spermatozoa were categorized as i) viable
225 cells (Yo-Pro-1^-, $M540^+$), and ii) non-viable cells (Yo-Pro-1^+). Results are expressed
226 as the geometric mean of relative fluorescence intensity (RFI) of viable
227 spermatozoa ± SEM.

228 *2.6. Analysis of boar spermatozoa phosphorylated proteins by western blotting*

229 Spermatozoa (1.5 mL) were centrifuged at 10000 rpm for 1 min at RT, washed in
230 PBS and centrifuged again. Pellet was resuspended in 90 µl of Laemmli Sample
231 Buffer (2X), incubated for 10 min in constant rotation and then centrifuged at 10,000
232 g for 10 min. The protein concentration of the supernatant was determined using a
233 Bio-Rad DC Protein Assay. After protein concentration analysis, 2-mercaptoethanol
234 (2.5% v/v) was added to the sperm lysates before heating for 5 min at 95 °C and
235 store at -20 ºC.

236 Sperm proteins (10 µg) were resolved using 10% SDS-PAGE. After
237 electrophoresis, proteins were transferred to nitrocellulose membranes at 380 mA
238 for 2.5 hours, then were blocked for 1 hour using Intercept® (TBS) blocking buffer
239 containing 0.2% Tween-20. Membranes were then incubated at 4 °C overnight
240 using anti-phospho-GSK3α/β (1:1.000) or anti-phospho-PKA-substrates (1:1.000) or
241 anti-α-tubulin (1:5.000) antibodies. The membranes were then washed and
242 incubated with the appropriate secondary antibody IRDye® 800RD or 680RD as
243 indicated by de manufactured. Fluorescent was detected using an Odyssey Fc
244 Imaging System (LI-COR Biotechnology), and bands were quantified using the
245 Image Studio™ software from Li-COR.

246 *2.7. Statistical analysis*

247 In order to show if the differences are statistically significant between the
248 different treatment or concentrations, hypothesis tests were carried out. Data were

analyzed for normal distribution with a Kolmogorov-Smirnov test and for homoscedasticity with a Levene test. Differences were determined by a parametric test, as one-way analysis of variance (ANOVA) followed by post-hoc Tukey. All data are shown as the mean ± Standard Error of the Mean (SEM). All analyses were performed using SPSS v19 for Windows software (SPSS Inc. Chicago, IL). Statistical significances were set at *p* values lower than 0.05.

3. Results

3.1. Effect of Roundup® Ultra plus (RUP) on pig sperm viability and motility.

Initially, we studied pig spermatozoa viability after treatment with different concentrations of Roundup® Ultra plus, RUP, (0.0025%, 0.005% and 0.01%) in two different incubation media (TBM and TCM) for 1 h at 38.5 ºC (Figure 1). None of the RUP dilutions studied has a significant effect on sperm viability either in non-capacitating (TBM) or capacitating (TCM) medium when compared to the control (absence of RUP).

The *in vitro* effect of RUP in sperm motility was evaluated using the same experimental conditions as above (0.0025%, 0.005% and 0.01% of RUP in both media during 1 h at 38.5 ºC) using the ISAS® software. As observed in Figures 2A and 3A, RUP exposition leads to a clear concentration-dependent reduction in the percentages of motile (Figure 2A) and progressive motile spermatozoa (Figure 3A) independently of the incubation media. This reduction is statistically significant at 0.01% of RUP dilution in both media, TBM and TCM (Figures 2A and 3A).

We also evaluated the effect of RUP on spermatozoa velocities such as the curvilinear velocity VCL (Figure 4A), the straight-line velocity VSL (Figure 5A) and the average velocity path VAP (Figure 6A) in TBM and TCM. As observed, RUP treatment leads to clear reduction in spermatozoa velocities in both media with a

stronger reduction in TCM than in TBM (Figures 4A, 5A and 6A). Thus, sperm incubation with RUP for 1h at 38.5ºC in a sperm capacitating medium (TCM) causes a concentration-dependent reduction in any sperm velocity, that is statistically significant at RUP dilutions of 0.01% and 0.005% (right panels of Figure 4A, 5A and 6A). Incubation with RUP in a non-capacitating medium (TBM) causes a significant reduction only at a RUP dilution of 0.01% in the straight-line speed (Figure 5A) and average velocity path (Figure 6A).

However, sperm incubation with different RUP dilutions does not modify other sperm motility coefficients analyzed in Table 1, except for linearity coefficient (LIN), or wobble movement coefficient (WOB), which are significantly reduced by incubation with RUP 0.01% in TCM.

3.2. Effect of glyphosate on sperm motility and functional parameters.

In order to know whether the negative effect observed after RUP in sperm motility might be due to its active ingredient glyphosate (GLY), we incubated pig spermatozoa in the presence of the concentration range (41, 82 and 164 µM) that is present in the diluted formulations of RUP used. None of these GLY concentrations had a significant effect on sperm motility (Figures 2B, 3B, 4B, 5B, 6B and Table 1) or viability or plasma membrane lipid disorganization or mitochondrial membrane potential (Supplementary Data) either in TBM or TCM after incubation 1 h at 38.5 ºC.

3.3. Effect of surfactant polyoxyethylene amine (POEA) on sperm motility.

We next investigated whether the reduction in sperm motility caused by RUP might be due to its non-active ingredient, the surfactant polyoxyethylene amine (POEA). Therefore, we incubated pig spermatozoa with those POEA concentrations (0.0002%, 0.0004% and 0.0008%) that result when diluting the herbicide RUP as mentioned above concentrations. As observed in Figure 1 lower histograms, none

of the POEA dilutions have a significant effect on sperm viability either in TBM or TCM after 1 h of incubation at 38.5 ºC.

The *in vitro* effects on sperm motility of different dilutions of the surfactant POEA were evaluated in spermatozoa under same experimental conditions (1h of incubation at 38.5 °C in TBM or TCM). The surfactant POEA causes a clear and statistically significant decrease in the percentage of total motile spermatozoa, independently of the incubation media (Figure 2C). Thus, only about 15% of spermatozoa remain motile after 0.0004% POEA treatment in TBM (left) or after 0.0008% in TCM (right). As observed (Figure 2C), the inhibitory effect in the percentage of motile spermatozoa caused by POEA is greater than the observed with RUP (Figure 2A) in any medium. The incubation of spermatozoa with POEA surfactant also causes a clear reduction in the percentage of progressive motile spermatozoa (Figure 3C) in a dilution-dependent manner. This reduction is potent and almost blocked progressive motility, as only about 5% of spermatozoa remain progressive motile after treatment with 0.0004% POEA in TBM or 0.0008% POEA in TCM (Figure 3C). This inhibition of progressive motile spermatozoa was statistically significant when 0.0004% and 0.0008% POEA dilutions were used either in TBM or in TCM.

A dilution-dependent negative effect of POEA treatment in motility can be also observed in any sperm velocity studied. Thus, VCL (Figure 4C), VSL (Figure 5C) and VAP (Figure 6C) are significantly reduced in the presence of 0.0008% POEA, in both TBM and TCM medium. Regarding other spermatozoa motility coefficients, POEA incubation (0.0004%) significantly decreased STR in TBM and at 0.0008% significantly reduced LIN, STR and WOB in TCM (Table 1).

3.4. Comparative effects of RUP and POEA on other sperm functional parameters.

Incubation of spermatozoa under same experimental conditions with different dilutions of RUP and its equivalent concentrations of POEA have no a significant effect on mitochondrial membrane potential, either in TBM or TCM (Figure 7) at any dilutions studied.

On the other hand, exposure of pig spermatozoa during 1 h at 38.5 ºC to RUP induced a dose-dependent increase in the percentage of spermatozoa showing plasma membrane lipid disorganization (Figure 8 upper histograms) either in TBM (left) or TCM (right). This greater sperm lipid disorganization is statistically significant at 0.01% RUP in TBM. Similarly, when sperm samples are exposed to POEA at concentrations that are equivalents of RUP, it is observed also a significantly increase in the percentage of spermatozoa showing plasma membrane lipid disorganization (Figure 8 lower histograms) either in TBM at 0.0004% and 0.0008% POEA (left) or TCM at 0.0008% POEA (right).

3.5. Comparative effects of RUP, GLY and the surfactant POEA in the intracellular signalling pathways mediated by PKA and glycogen synthase kinase 3 (GSK-3) in spermatozoa.

In order to compare effects in relevant sperm signaling pathways, pig spermatozoa were incubated with equivalent dilutions of RUP (0.01%), GLY (164 µM) or POEA (0.0008%). Sperm treatment for 1h at 38.5 ºC with 0.01% RUP reduces GSK3α/β phosphorylation in spermatozoa incubated in both, TBM and TCM (Figure 9 histograms), although reduction was statistically significant only for GSK3β phosphorylation after incubation in TBM (Figure 9 right histograms). However, sperm incubation with GLY concentration that is equivalent to the obtained within RUP 0.01% has not any effect on GSK3α/β phosphorylation either

348 in TBM or TCM (Figure 9 histograms). Interestingly, sperm treatment with the
349 surfactant POEA concentration (0.0008%) that is equivalent to the obtained within
350 RUP 0.01% causes a similar effect to RUP on sperm GSK3α/β phosphorylation
351 either in TBM or TCM. Thus, POEA leads to a clear although non-significant
352 reduction on GSK3α phosphorylation (Figure 9 left histograms) and a significant
353 decrease on GSK3β phosphorylation (Figure 9 right histograms).

354 We also studied whether the herbicide RUP, its active ingredient GLY and the
355 surfactant POEA, could be affecting the sperm PKA signalling pathway by
356 investigating the phosphorylation of its downstream substrates. As observed in the
357 Figure 10 (histograms), the sperm treatment with herbicide RUP (0.01%) causes a
358 clear and significant reduction in the phosphorylation of the bands called II and III
359 corresponding to some PKA substrates detected in spermatozoa, either in TBM
360 (Figure 10A) or TCM (Figure 10B). Interestingly, sperm treatment with the
361 surfactant POEA (0.0008%) leads to a similar effect to the RUP, decreasing the
362 phosphorylation level of PKA substrates bands II and III, either in TBM (Figure 10A)
363 or TCM (Figure 10B). By contrary, sperm treatment with RUP or POEA in any
364 medium does not significantly affect other bands such as those called I and IV
365 (Figure 10 histograms).

366 However, the sperm incubation in TBM or TCM with the RUP active ingredient
367 GLY does not modify at all the phosphorylation levels of the PKA substrates
368 detected (Figure 10 histograms).

369 **4. Discussion**

370 Due to its worldwide, massive and growing use, the toxic effects of glyphosate-
371 based herbicides have been investigated in several animal species and cell models.
372 However, little literature exists to date about the functional impact of this type of

herbicides in mammalian reproductive cells, compared to that existing in fish reproductive gametes, and, particularly, in the male gamete.

This work demonstrates the adverse impact of Roundup® Ultra Plus and its non-active ingredient the surfactant POEA, but no glyphosate in mammalian sperm function using pig spermatozoa as a *in vitro* cell model to study contaminants in male reproductive effects. Pig spermatozoon has been reported as a well-validated *in vitro* cell model not only for cell toxicity studies [3, 17, 18] but also for physiological studies successfully translated to human assisted reproduction techniques [19].

The herbicide RUP causes a clear inhibition of the motility in pig spermatozoa at concentrations comparable to those present as environment contaminants, as they are much lower (100 times) than those recommended for agriculture. Our results are in agreement with previous studies demonstrating that Roundup® effects in mammalian spermatozoa, at lower concentrations than those used as herbicide, lead to an inhibition of mammalian sperm motility [3, 15, 16]. Thus, Roundup® treatment at 1 µg/mL rapidly decreased motility in human spermatozoa [15] and also its equivalent concentration of GLY (0.36 µg/mL) in human [16] or a higher concentration in pig spermatozoa (360 µg/mL) [3].

The reduction of mammalian sperm motility caused by low concentrations of RUP occurs in any incubation medium evaluated, either in non-stimulant medium as TBM or sperm stimulant medium, TCM. However, this adverse effect of low concentrations of RUP cannot be attributed to its active ingredient, GLY, as the incubation of pig spermatozoa under same experimental conditions with GLY concentrations (9-36 µg/mL), which are equivalent to those contained in the RUP concentrations evaluated in this work, do not affect at all sperm motility, the lipid

398 organization of plasma membrane, GSK3β and PKA signalling pathways or viability
399 (this later are data not shown) in any medium. This lack of effect of GLY in sperm
400 motility or viability is in agreement with results from a recent work by Nerozzi et al.
401 [3] in pig spermatozoa at low GLY concentrations (range 5-50 µg/mL), which are
402 comparable to those used in this work. However, it is reported that higher
403 concentrations of GLY (360 µg/mL), that are 10 times greater than the maximum
404 concentration used in the present work, not only caused also a decrease in sperm
405 motility in human [16] and pig [3], but also in mitochondrial membrane potential,
406 acrosome integrity and sperm viability in pig spermatozoa [3]. These mentioned
407 studies, using 360 µg/mL GLY and the present work using 10 times lower GLY
408 concentrations, suggest the idea of a clear concentration-dependent effect of GLY
409 on mammalian spermatozoa, where low concentrations of GLY closer to
410 environmental exposures do not affect at all mammalian sperm motility whereas
411 greater GLY concentrations that exceeds environmental exposures impairs sperm
412 motility and result toxic [3, 16].

413 Interestingly, the present work clearly demonstrates that the detrimental effects
414 of low concentrations of RUP in sperm function can be attributed to its non-active
415 component, POEA, as exposure to this non-ionic surfactant at RUP equivalent
416 concentrations, mimics RUP effect and leads to an inhibition of sperm motility in a
417 similar extent than that caused by RUP. Additionally, the surfactant POEA triggers a
418 concentration-dependent disorganization of lipids at the sperm plasma membrane
419 in a similar way as occurs with equivalent RUP concentrations, suggesting that the
420 inhibition of sperm motility caused by RUP can be due, at least in part, by altered
421 sperm plasma membrane lipid organization induced by the surfactant POEA. To our
422 knowledge, the potential action of other herbicide ingredients besides GLY, such as

surfactants, has not been studied so far in mammalian spermatozoa. However, it has been generally reported that the most used surfactant in GLY-based herbicides, POEA, induces membrane damages in different mouse cell types leading to a high cytotoxicity [22]. Additionally, in human umbilical, embryonic and placental cells, POEA has demonstrated also to induce damage in cell membranes that causes changes in cell permeability [10] and in other human cell lines this surfactant disturbs the integrity of the membrane and alters cellular respiration processes [7].

The reduction in sperm motility is not due to a cytotoxic impact or by any potential side effect triggered by RUP or POEA in pig spermatozoa that might compromise sperm vitality, as sperm viability remains unaffected by RUP or POEA under same experimental conditions and equivalent concentrations. This lack of RUP effect in sperm viability agrees with the previous report in pig spermatozoa only at the lowest Roundup® concentrations that they used, below 50 µg/mL of GLY equivalent concentration [3], which are in a similar range as those evaluated in this work. However, at Roundup® concentrations equivalent to 50 µg/mL of GLY and higher, a dose-dependent decrease in sperm viability is described [3], reinforcing the idea that the glyphosate-based herbicides adverse effects in mammalian spermatozoa viability are concentration-dependent.

Additionally, the inhibition of pig sperm motility cannot be attributed to a detrimental effect of RUP or POEA in the sperm mitochondrial membrane potential. Thus, although pig sperm mitochondrial activity is slightly decreased under some conditions (RUP in TCM or after 0.0008% POEA), this effect is not statistically significant. Also, a slight effect of Roundup® in mitochondrial activity has been reported by Nerozzi et al.[3] in pig spermatozoa although in their case this effect resulted statistically significant.

As we have not addressed boar sperm capacitation in this work, we cannot make any assumptions about the possible effects of RUP or its components in this functional sperm process.

Regarding intracellular signalling pathways that might be altered by RUP treatment in spermatozoa, our results point out to an inhibition of GSK3α/β phosphorylation cascade and also to a selective inhibition of phospho-PKA substrates (so-named substrates II and III). Interestingly, both signalling cascades are unaffected by equivalent concentrations of GLY but totally reproducible when using POEA equivalent concentrations. To date, there are no previous studies about the intracellular signalling affected by RUP in spermatozoa. As GSK3α (but not GSK3β) and PKA pathways are demonstrated to be regulating pig sperm motility [23, 24], our results suggest that the impairment in sperm motility caused by POEA and subsequently by RUP is likely due to an inhibition of the phosphorylation of both kinases pathways. However, more experiments are needed to clarify the individual contribution of each particular pathway to the detrimental effects of RUP low concentrations in mammalian spermatozoa, especially GSK3β, whose contribution to pig sperm motility has not been established yet and also which are the specific substrates that are selectively inhibited by PKA.

5. Conclusions

In summary, this work demonstrates that i) low concentrations of the herbicide RUP similar to those present as environment contaminants impairs pig sperm motility without affecting sperm viability. This work might indicate a solid evidence linking ambient exposure to RUP and concomitantly to its non-active ingredient POEA at relatively low concentrations comparable to contaminants, with adverse reproductive effects, in particular, in mammalian spermatozoa; ii) the RUP adverse

effect on sperm motility cannot be attributed to its active ingredient GLY, but to its non active compound, the surfactant POEA; iii) the impairment in sperm motility caused by RUP might be likely due to a detrimental effect of its ingredient POEA at the plasma membrane causing a loss of membrane lipid organization; iv) RUP adverse effects in pig spermatozoa are likely mediated by a POEA-triggered inhibition of two phosphorylation pathways that control sperm motility: GSK3β and/or by a selective inhibition of PKA that particularly affects specific substrates.

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7. Author contributions

Torres-Badia M, Solar S and Gomez A performed the experiments. Torres-Badia M also contributed to data curation, formal analysis, interpretation of data and wrote the draft manuscript. Martin-Hidalgo D and Hurtado de Llera A contributed to data curation and critically revised the manuscript. Garcia-Marin L.J, Gonzalez-Fernandez L and Bragado M. J conceptualized and designed the study, analyzed

and supervised the results, wrote the paper and contributed to funding acquisition. All authors read and approved the final version of the manuscript.

8. Declaration of competing interest

The authors declared that they have not any competing financial and/or non-financial interests in relation to the present work.

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575 **Figure Legends**

576 **Figure 1. Effects of Roundup® Ultra plus (RUP) and surfactant**
577 **polyoxyethylene amine (POEA) in pig spermatozoa viability.** *Left panel:*
578 spermatozoa were incubated in TBM or TCM for 1 h at 38.5 ºC in the absence or
579 presence of different concentrations of RUP (upper histograms) or POEA (lower
580 histograms). This experiment was performed 5 times (n = 5) and the results are
581 expressed as the mean of the percentage of SYBR14-positive and PI-negative
582 spermatozoa ± standard error of the mean (SEM). No statistical differences were
583 found. *Right panel:* Representative two-dimensional SYBR-14 fluorescence versus
584 PI fluorescence dot plots for sperm samples incubated in absence or presence of
585 RUP (0.01%) and POEA (0.0008%).

586 **Figure 2. Effects of Roundup® Ultra plus (RUP), glyphosate and surfactant**
587 **polyoxyethylene amine (POEA) in the percentage of motile spermatozoa.** Pig
588 spermatozoa were incubated 1 h in TBM or TCM at 38.5 °C in the absence or
589 presence of different concentrations of RUP (A), glyphosate (B) or POEA (C). The
590 percentage of motile spermatozoa was evaluated by ISAS® system. Each
591 experiment was performed 6 times (n = 6) and results are expressed as the mean
592 of the percentage of total spermatozoa ± SEM. Statistical differences are shown
593 with * ($P < 0.05$).

Figure 3. Effects of Roundup® Ultra plus (RUP), glyphosate and surfactant polyoxyethylene amine (POEA) in the percentage of progressive motile spermatozoa. Pig spermatozoa were incubated 1 h in TBM or TCM at 38.5 °C in the absence or the presence of indicated concentrations of RUP (A), glyphosate (B) and POEA (C). Progressive motility was evaluated by ISAS® system. Each experiment was performed 6 times (n = 6) and results are expressed as the mean of the percentage of progressive spermatozoa ± SEM. Statistical differences are shown with * ($P < 0.05$).

Figure 4. Effects of Roundup® Ultra plus (RUP), glyphosate (GLY) and surfactant polyoxyethylene amine (POEA) in the curvilinear velocity (VCL) of pig spermatozoa. Spermatozoa were incubated in TBM or TCM for 1 h at 38.5 °C in the absence or presence of indicated concentrations of RUP (A), glyphosate (B) and POEA (C). Sperm VCL was evaluated by ISAS® system, the curvilinear velocity (VCL) is expressed as µm/s. Each experiment was performed 6 times (n = 6) and results are expressed as mean ± SEM. Statistical differences are shown with * ($P < 0.05$).

Figure 5. Effects of Roundup® Ultra plus (RUP), glyphosate (GLY) and surfactant polyoxyethylene amine (POEA) in the straight-linear velocity (VSL) of pig spermatozoa. Spermatozoa were incubated 1 h in TBM or TCM at 38.5 °C in the absence or presence of indicated concentrations of RUP (A), glyphosate (B) and POEA (C). Sperm VSL was evaluated by ISAS® system, the straight-linear velocity (VSL) is expressed as µm/s. Each experiment was performed 6 times (n = 6) and results are expressed as mean ± SEM. Statistical differences are shown with * ($P < 0.05$).

Figure 6. Effects of Roundup® Ultra plus (RUP), glyphosate (GLY) and surfactant polyoxyethylene amine (POEA) in the average velocity (VAP) of pig spermatozoa. Spermatozoa were incubated 1 h in TBM or TCM at 38.5 °C in the absence or presence of indicated concentrations of RUP (A), glyphosate (B) and POEA (C). Sperm VAP was evaluated by ISAS® system, the average velocity (VAP) is expressed as μm/s. Each experiment was performed 6 times (n = 6) and results are expressed as mean ± SEM. Statistical differences are shown with * ($P < 0.05$).

Figure 7. Effects of Roundup® Ultra plus (RUP) and surfactant polyoxyethylene amine (POEA) in mitochondrial membrane potential (ΔΨm) of pig spermatozoa. *Left panel:* Spermatozoa were incubated for 1 h in TBM or TCM at 38.5 ºC in the absence or presence of different concentrations of RUP (upper graph) or POEA (lower graph). Results are expressed as the mean of the percentage of spermatozoa exhibiting relative higher ΔΨm from the total sperm cells analysed ± SEM. Each experiment was performed 5 times (n = 5). No statistical differences were found ($P > 0.05$). *Right panel:* Representative two-dimensional JC-1 monomer fluorescence versus JC-1 polymer fluorescence dot plots for sperm samples incubated in absence or presence of RUP and POEA.

Figure 8. Effects of Roundup® Ultra plus (RUP) and surfactant polyoxyethylene amine (POEA) in plasma membrane lipid organization of pig spermatozoa. *Left panel:* Spermatozoa were incubated in TBM or TCM for 1 h at 38.5 °C in the absence or presence of indicated concentrations of RUP (upper graph) and POEA (lower histograms). Each experiment was performed at least 5 times (n = 5). Results are expressed as the geometric mean ± SEM of relative fluorescence intensity (RFI) of M540 fluorescence/Yo-pro-1 negative. Statistical

differences are shown with * ($P < 0.05$). *Right panel:* Representative two-dimensional M540 fluorescence versus Yo-pro-1 fluorescence dot plots (upper panel) and flow cytometry histograms for M540 fluorescence of Yo-pro-1 negative spermatozoa (middle and lower panels) for sperm samples incubated in absence or presence of the indicated concentrations of RUP and POEA.

Figure 9. Effects of Roundup® Ultra plus (RUP), glyphosate (GLY) and surfactant polyoxyethylene amine (POEA) in the phosphorylation of GSK-3 on pig spermatozoa. Spermatozoa were incubated in TBM (left) or TCM (right) for 1 h at 38.5 °C in the absence or the presence of herbicide RUP (0.01%), and the equivalent concentrations of its ingredients GLY (164 µM) and the surfactant POEA (0.0008%). *Upper panel:* Sperm proteins (10 µg) were analysed by western blotting using anti-phospho GSK3α/β as primary antibody. Each experiment was performed 5 times and representative films are shown. Loading controls using GSK3α and β antibodies (lower films) were performed for each experiment. Arrows indicate the cross-reactive sperm bands corresponding to phosphorylated forms of GSK3α and GSK3β (upper images) and GSK3α and β (lower images). *Lower panel:* Densitometry analysis of GSK3α and GSK3β bands is shown and values are expressed as the mean ± SEM of arbitrary units. Statistical differences are shown with * ($P < 0.05$).

Figure 10. Effects of Roundup® Ultra plus (RUP), glyphosate (GLY) and surfactant polyoxyethylene amine (POEA) in the phosphorylation of PKA-substrates on pig spermatozoa. Spermatozoa were incubated in TBM (left) or TCM (right) for 1 h at 38.5 °C in the absence or the presence of herbicide RUP (0.01%), and the equivalent concentrations of its ingredients GLY (164 µM) and the surfactant POEA (0.0008%). Sperm proteins (10 µg) were analysed by western

668 blotting using anti-phospho-PKA-substrates as primary antibody. Each experiment
669 was performed 5 times and representative films are shown. Loading controls using
670 anti-α-tubulin antibody (lower films) were performed for each experiment in the
671 same membrane. Arrows indicate cross-reactive bands (I-IV) of sperm
672 phosphorylated proteins that are substrates of PKA. *Lower panel:* Densitometry
673 analysis of I-IV bands is shown and values are expressed as mean ± SEM of
674 arbitrary units. Statistical differences are shown with * ($P < 0.05$).
675

676 **Table**

677 **Table 1. Effects of Roundup® Ultra plus (RUP), glyphosate and surfactant**
678 **polyoxyethylene amine (POEA) in pig spermatozoa motility coefficients.**

Buffer	Treatment	Concentration	LIN (%)	STR (%)	WOB (%)
TBM	RUP	0%	61.5±3.5	88.13±1.95	69.55±2.52
		0.0025%	60.1±4.6	86.48±2.53	69.05±3.41
		0.005%	60.3±5.5	86.15±2.72	69.40±4.18
		0.01%	46.4±7.0	74.05±5.66	60.80±5.01
	Glyphosate	0	59.8±2.0	88.10±0.87	67.80±1.67
		41μM	58.3±2.6	86.72±1.53	67.00±1.84
		82μM	62.6±1.6	89.28±0.66	70.07±1.34
		162μM	61.2±2.2	88.52±1.50	69.07±1.35
	POEA	0%	57.5±2.3	85.82±1.96	66.88±1.48
		0.0002%	60.2±2.3	86.42±1.47	69.68±1.62
		0.0004%	39.9±3.4	70.42±2.08*	56.30±3.59
		0.0008%	48.1±9.4	67.52±6.25*	69.23±5.07
TCM	RUP	0%	79.8±0.7	93.67±0.80	85.15±0.41
		0.0025%	78.8±1.1	92.25±0.95	85.43±0.74
		0.005%	78.4±0.5	92.78±0.78	84.57±0.87
		0.01%	72.4±1.9*	90.35±1.07	67.88±12.01*
	Glyphosate	0	80.0±2.4	94.32±0.96	84.70±1.72
		41μM	76.4±3.0	92.15±1.54	82.73±2.07
		82μM	78.5±2.2	92.47±1.31	84.77±1.26
		162μM	76.8±2.7	91.55±1.68	83.73±1.64
	POEA	0%	77.0±1.9	92.12±0.98	83.50±1.32
		0.0002%	76.5±1.4	92.20±1.08	82.92±0.86
		0.0004%	69.0±6.1	88.92±3.12	76.77±4.65
		0.0008%	38.3±8.3*	61.32±9.40*	59.53±5.47*

679

680 Pig spermatozoa were incubated in TBM or TCM at 38.5 °C in the absence or
681 presence of indicated concentrations of RUP, glyphosate or POEA. Sperm
682 kinematic parameters were evaluated by ISAS® system: linearity (LIN, in%);
683 straightness (STR, in %) and wobble movement coefficient (WOB, in %). This

684 experiment was performed 6 times (n = 6) and values are expressed as the mean ±
685 SEM. Statistical differences from their own control are shown with * ($P < 0.05$).
686

Supplementary data: Effects of glyphosate in boar spermatozoa viability, mitochondrial membrane potential (ΔΨm) and plasma membrane lipid organization.

Buffer	Treatment	Viability (%)	Higher **ΔΨm (%)**	Plasma membrane lipid disorder (RFI)
TBM	Control	87.2±1.2	82.6±2.7	91.8±32,3
	Glyphosate (41µM)	86.5±1.7	83.1±2.2	109.2±29.2
	Glyphosate (82µM)	87.3±1.4	86.1±2.9	99.4±29.1
	Glyphosate (164µM)	87.2±1.4	84.2±3.3	100.3±33.2
TCM	Control	63.7±12.8	66.4±5.6	122.7±6.4
	Glyphosate (41µM)	64.5±12.2	69.7±2.4	125.8±8.1
	Glyphosate (82µM)	64.4±12.6	69.9±6.4	121.9±8.2
	Glyphosate (164µM)	64.5±12.8	59.0±4.2	119.9±10.8

Pig spermatozoa were incubated in TBM or TCM at 38.5 °C in the absence or presence of different concentrations of glyphosate. This experiment was performed 5 times (n = 5) and the results are expressed as the mean ± standard error of the mean (SEM) of the percentage of SYBR14-positive and PI-negative spermatozoa (viability) or percentage of spermatozoa exhibiting relative higher ΔΨm from the total sperm cells analysed (mitochondrial membrane potential) or the geometric mean of relative fluorescence intensity (RFI) of M540 fluorescence/Yo-pro-1 negative (plasma membrane lipid organization). No statistical differences were found.

Highlights

> Low concentrations of the herbicide Roundup impair pig sperm motility without affecting sperm viability.

> Negative effects of low Roundup concentrations are caused by the surfactant included in its formulation but no by its active ingredient glyphosate.

> Roundup adverse effects are likely due to a detrimental effect of surfactant at the plasma membrane integrity.

> Roundup negative effects are at least partially mediated by inhibition of phosphorylation signaling pathways that control pig sperm motility: GSK3α/β and PKA.

Impaired mammalian sperm function and lower phosphorylation signaling caused by the herbicide Roundup® Ultra Plus are due to its surfactant component.

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Running Title: The Roundup® surfactant POEA inhibits pig sperm motility

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ABSTRACT

The use of worldwide glyphosate-based herbicide Roundup® is growing and to date its effects on mammalian spermatozoa are controversial. This study aims to investigate the functional impact of *in vitro* exposure of pig spermatozoa to low concentrations of Roundup® Ultra Plus (RUP), similar to those present as environment contaminants, to its active ingredient glyphosate, and to the non-active component, surfactant POEA. Pig spermatozoa were incubated in Tyrode's basal medium (TBM) or Tyrode's complete medium (TCM) (1h at 38.5 °C) with several RUP dilutions or equivalent concentrations of glyphosate or POEA. RUP treatment causes a significant dilution-dependent decrease in sperm motility, a significant increase in plasma membrane disorganization and reduction in GSK3β phosphorylation (TBM) and in two PKA substrates (TBM and TCM), whereas does not affect sperm viability or mitochondrial membrane potential (MMP). Equivalent glyphosate concentrations do not affect any functional sperm parameters. However, POEA concentrations equivalent to RUP dilutions mimic all RUP sperm effects: decrease sperm motility in a concentration-dependent manner, increase sperm plasma membrane lipid disorder and significantly inhibit GSK3β phosphorylation (TBM) and two PKA substrates without affecting sperm viability or MMP. In summary, low concentrations RUP herbicide cause sperm motility impairment without affecting sperm viability. This adverse effect could be likely due to a detrimental effect in the plasma membrane lipid organization and to inhibition of phosphorylation of both, GSK3β and specific PKA substrates. Importantly, our results indicate that negative effects of low RUP concentrations in pig spermatozoa function are likely caused by the surfactant included in its formulation and no by its active ingredient glyphosate.

Key words: pig spermatozoa, herbicide Roundup, motility, glyphosate, surfactant.

1. Introduction

Glyphosate, *N*-(phophonomethyl) glycin, is the active compound of the commercial Roundup® herbicide, which possess a broad spectrum and is among the most used worldwide in agriculture. Not only its use is massive but also is growing about 20% every year. The consequences of the wide use of glyphosate-based herbicides have become a big concern for human and animal health as they gravely contaminate the environment, including soil, water and ecosystems, and therefore represent a serious risk. The research about its potential harmful effects has recently been addressed in different mammals species [1-3], where glyphosate can act as an endocrine disruptor at low doses, impairing hormones physiological role [2, 4]. In this regard, even low Roundup® concentrations that are considered as herbicide residues cause effects on cell structure (cytoplasmic damage) and function in hepatoma tissue culture cells [5].

The commercial Roundup® formulation includes not only the active compound glyphosate, but also other non-active ingredients, such as detergents that function as tensioactive molecules. In this regard, it has been demonstrated that Roundup® herbicide has more negative effect than glyphosate alone in human cells [6-9] including placental cells [10-12] and embryonic cells [10, 11].

In the male reproductive system of mammals, some initial works show controversial results, raising issues about the reproductive toxicity of this herbicide. Thus, Williams et al. [13] found no definitive evidence that glyphosate negatively affected human reproductive physiology. Additionally, Cassault-Meyer et al. [14] described abnormal sperm morphology just in two particular days after 8 days of acute glyphosate exposure in rats, whereas no effects were observed neither in sperm concentration, viability or motility. A meta-analysis about effects on sperm

concentration in rodents concludes that glyphosate exposure decreased sperm concentration and therefore, glyphosate is toxic for male rodent´s reproductive system [1]. More recently it has been demonstrated that *in vitro* treatment of human spermatozoa with Roundup® at concentration of 1 µg/mL, causes a rapid and adverse effect on sperm motility, probably due to a concomitant mitochondrial deregulation [15]. Same authors reported later that its active ingredient glyphosate at 0.36 µg/mL, which is the concentration equivalent to 1 µg/mL Roundup®, causes also a rapid *in vitro* effect in human spermatozoa, decreasing progressive motility and blocking mitochondrial activity after 1 h exposure [16]. Interestingly, the concentrations of herbicide used in these studies are 10000 times lower than those recommended for use in agriculture. A recent study conducted in pig spermatozoa has investigated the impact of pure glyphosate and Roundup® on sperm function and survival [3] at concentrations higher to those investigated in human spermatozoa [15, 16]. These authors conclude that while both, glyphosate and Roundup®, have an adverse effect on male gametes, Roundup® is more toxic than its main active ingredient, glyphosate. It is clear that more research on the male gamete consequences of Roundup® herbicide exposure is required for several reasons: i) the glyphosate-based herbicides and glyphosate concentrations that decreased motility resulted also cytotoxic for spermatozoa [3], making impossible to really elucidate the negative impact of glyphosate-based herbicides and its active compound to sperm physiology, ii) more importantly, the individual effects of the different components included in the formulation of the commercial glyphosate-based herbicides, especially the surfactants, have not been addressed in the same study in parallel to the Roundup® and glyphosate in mammalian spermatozoa. Therefore, the aim of the present work is to investigate the functional impact of *in*

vitro exposure of mammalian spermatozoa to the herbicide Roundup®, to its active ingredient glyphosate, as well as to the non-active ingredient the detergent POEA (the main claimed adjuvant by the manufacturer) at relatively low Roundup® concentrations (100 times lower than recommended for agricultural use). These concentrations are similar to those present as environment contaminants that might affect sperm function, as motility, but do not result cytotoxic for spermatozoa. For this purpose, we have used pig spermatozoa as a validated *in vitro* mammalian cell model to investigate cell toxicity [3, 17, 18] and also a well-demonstrated cell model in sperm physiological studies for its successful translation into human assisted reproduction techniques [19].

2. Material and Methods

2.1. Chemical and sources

Roundup® Ultra Plus from Monsanto Europe (Ambers, Belgium); glyphosate potassium salt and M540 were from Sigma-Aldrich (St Louis, MO, USA); polyethoxylated tallow amine (POEA) from Dr. Ehrenstorfer GmbH (Augsburg, Germany); Propidium iodide (PI), SYBR-14, Yo-Pro-1 and 5,5',6,6'–tetrachloro-1,1',3,3' tetraethylbenzymidazolyl carbocyanine (JC-1) probes from Thermo Fisher Scientific (Waltham, MA, USA); *DC* ™ Protein Assays and 2x Laemmli Sample Buffer from Bio-Rad (Hercules, CA, USA); Intercept® (TBS) blocking buffer, IRDye® 800RD and 680RD secondary antibodies from LI-COR Biotechnology (Bonsai Lab, Alcobendas, Spain). Furthermore, the anti-phospho (Ser/Thr) PKA Substrate (#9624) and anti-phospho (Ser21/9) GSK3α/β (#9331) and total GSK3α (#9338) and GSK3β (#9332) polyclonal antibodies were from Cell Signaling Technology, Inc. (Beverly, MA, USA); the anti-α-tubulin antibody (TU-02, #SC-8035)

was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All reagents used to prepare incubation media were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Spermatozoa incubation media

Tyrode's basal medium (TBM; 96 mM NaCl, 4.7 mM KCl, 0.4 mM $MgSO_4$, 0.3 mM NaH_2PO_4, 5.5 mM glucose, 1 mM sodium pyruvate, 21.6 mM sodium lactate, 20 mM HEPES, 5 mM EGTA and 0.02% PVA) was prepared and used as the non-capacitating medium. A variant of TBM was made omitting EGTA and PVA and adding 1 mM $CaCl_2$, 15 mM $NaHCO_3$ and 3 mg/mL BSA, then it was equilibrated with carbogen (5% CO_2/95% O_2) and termed Tyrode's complete medium (TCM), a spermatozoa-capacitating medium [20]. All media were prepared on the day of use and adjusted to pH 7.45 with an osmolarity of 290-310 mOsm kg^{-1}.

2.3. Boar semen collection and experimental treatment of spermatozoa.

Sperm samples from Duroc boars (2-4 years old) were commercially obtained from a regional porcine company (Tecnogenext, S.L, Mérida, Spain), without any requirement of approval from the animal research review board of the University of Extremadura. All boars were housed in individual pens in an environmentally controlled building (15-25 °C) according to Regional Government and European regulations and received the same diet. Fresh ejaculates were collected with the gloved hand technique and stored at 17 °C before use in the laboratory. In order to minimize individual boar variations, samples from up to 3 animals were pooled using semen from no less than 12 boars in different combinations. Only semen pools with at least 80% morphologically normal spermatozoa were used. Semen was centrifuged at 900 *g* for 4 minutes, washed with phosphate-buffered saline (PBS) and spermatozoa were placed in TBM or TCM medium to a final concentration of 30 x 10^6 spermatozoa mL^{-1}.

Depending on each experimental procedure, 0.5 or 1.5 mL of spermatozoa samples containing 30 × 10^6 spermatozoa mL^{-1} were incubated during 1 h at 38.5 °C in the absence (control) or presence of Roundup® Ultra Plus (RUP) that was previously diluted 1/10000, 1/20000 and 1/40000 times, yielding final RUP concentrations (v/v) of 0.01%, 0.005% and 0.0025%, respectively. The commercially available Roundup® Ultra Plus contains 36% (w/v) of the active ingredient glyphosate (GLY) and 6% (w/v) of the surfactant, polyoxyethylene amine (POEA). The above-mentioned dilutions of the commercial RUP yield final GLY concentrations of 164, 82 and 41 µM and final POEA concentrations of 0.0008%, 0.0004% and 0.0002%, respectively. These concentrations of the active and non-active ingredients of RUP, which resulted after different RUP dilutions, are so-called equivalent concentrations of RUP and were used for the treatment of pig spermatozoa.

When spermatozoa were incubated in TBM, incubation was performed in absence of air, whereas TCM treatment was performed in a humidified atmosphere of 5% CO_2/95% air atmosphere In order to minimize possible experimental variations, all the different experimental treatments were carried out in each of the semen pools.

All experiments were performed in accordance with European, national and Regional guidelines and regulations.

2.4. Evaluation of spermatozoa motility

After incubation with RUP or its different components, 2 µl of spermatozoa sample were placed in a 38.5 ºC pre-warmed counting chamber with 20 µm depth (Leja®, Nieuw-Vennep, The Netherlands). Spermatozoa images were take using a microscope equipped with a 10X negative-phase contrast objective, with a heated

stage, and a CCD camera that takes 25 consecutive digitalized images obtained during 1 s form at least 4 different fields and 300 spermatozoa per sample [21]. Digitalized images were analyzed using a Computer Assisted Semen Analysis system, specifically the ISAS® system (Integrated Semen Analysis System, Proiser R+D, Paterna, Valencia, Spain). Sperm motility parameters and coefficients: motile spermatozoa (percentage of spermatozoa with an average path velocity > 10 μm/s), progressive motile spermatozoa (percentage of spermatozoa with a straightness coefficient > 80%), VCL (curvilinear velocity in μm/s), VSL (straight-line velocity in μm/s), VAP (average path velocity in μm/s), LIN (linearity coefficient in %), STR (straightness coefficient in %) and WOB (wobble coefficient in %).

2.5. Flow cytometry analysis

Flow cytometry analysis was performed using an ACEA NovoCyte® flow cytometer (ACEA Biosciences, Inc., San Diego, CA, USA) equipped with a three detection channels for blue laser (488 nm): BL-1 (530 ± 30 nm band pass filter); BL-2 (572 ± 28 nm band pass filter) and BL-4 (675 ± 30 nm band pass filter) and a detection channel for a red laser (640 nm): BL-3 (660 ± 20 nm band pass filter). Flow cytometry experiments and data analyses were performed using ACEA Novo Express® software (ACEA Biosciences, Inc., San Diego, CA, USA). Fluorescence data were represented in a logarithmic scale.

2.5.1. Analysis of spermatozoa viability by flow cytometry

As described previously [21], fluorescent staining using SYBR-14 and propidium iodide (PI) was performed to measure sperm viability. Briefly, 5 μl of SYBR-14 (2 μM) and 10 μl of PI (240 μM) were added to 100 μl of spermatozoa (30×10^6 cells mL^{-1}) diluted with 400 μl of PBS, until a final concentration of 20 nM for SYBR-14 and 5 μM for PI. Then, the samples were incubated for 15 min at room temperature

(RT) in darkness and analyzed in the flow cytometer. After excitation at 488 nm, SYBR-14 fluorescence was detected using a 530 ± 30 nm band pass filter and PI fluorescence using 675 ± 30 nm band pass filter. Results of viable spermatozoa were expressed as the average of the percentage of $SYBR14^+$ and PI^- spermatozoa ± standard error of the mean (SEM).

2.5.2. Analysis of sperm mitochondrial membrane potential (ΔΨm) by flow cytometry

As described previously, fluorescent staining using the specific probe JC-1 was used as mitochondrial membrane potential marker [21]. The experimental procedure consists of diluting 100 µl of spermatozoa (30×10^6 cells mL^{-1}) in 400 µl of PBS containing 0.9 µM of JC-1, mixed an incubated at 38.5 °C for 30 min. The fluorescence values were collected on both channels BL-1 (JC-1 monomer) and BL-2 (JC-1 polymer) the results were expressed in percentage of spermatozoa with high mitochondrial membrane potential (high ΔΨm) with respect to the total number of spermatozoa analyzed.

2.5.3. Evaluation of the degree of sperm plasma membrane lipid organization by flow cytometry

Fluorescent staining using the probes merocyanine M540 was used as a membrane lipid fluidity marker, and YoPro-1, as a marker of changes in plasma membrane permeability (commonly associated with cell death), was performed as previously described [20]. Briefly, 100 µl of spermatozoa (30×10^6 cells mL^{-1}) were diluted in 400 µl of PBS containing 75 nM of Yo-Pro-1 and 6 µM of M540 and incubated at 38 °C for 15 min. Then, remixed before flow cytometry analysis. The fluorescence values of probes Yo-Pro-1 and M540 were collected on both BL-1 and

BL-2 channels, respectively. Labelled spermatozoa were categorized as i) viable cells (Yo-Pro-1^-, $M540^+$), and ii) non-viable cells (Yo-Pro-1^+). Results are expressed as the geometric mean of relative fluorescence intensity (RFI) of viable spermatozoa ± SEM.

2.6. Analysis of boar spermatozoa phosphorylated proteins by western blotting

Spermatozoa (1.5 mL) were centrifuged at 10000 rpm for 1 min at RT, washed in PBS and centrifuged again. Pellet was resuspended in 90 µl of Laemmli Sample Buffer (2X), incubated for 10 min in constant rotation and then centrifuged at 10,000 g for 10 min. The protein concentration of the supernatant was determined using a Bio-Rad DC Protein Assay. After protein concentration analysis, 2-mercaptoethanol (2.5% v/v) was added to the sperm lysates before heating for 5 min at 95 °C and store at -20 ºC.

Sperm proteins (10 µg) were resolved using 10% SDS-PAGE. After electrophoresis, proteins were transferred to nitrocellulose membranes at 380 mA for 2.5 hours, then were blocked for 1 hour using Intercept® (TBS) blocking buffer containing 0.2% Tween-20. Membranes were then incubated at 4 °C overnight using anti-phospho-GSK3α/β (1:1.000) or anti-phospho-PKA-substrates (1:1.000) or anti-α-tubulin (1:5.000) antibodies. The membranes were then washed and incubated with the appropriate secondary antibody IRDye® 800RD or 680RD as indicated by de manufactured. Fluorescent was detected using an Odyssey Fc Imaging System (LI-COR Biotechnology), and bands were quantified using the Image Studio™ software from Li-COR.

2.7. Statistical analysis

In order to show if the differences are statistically significant between the different treatment or concentrations, hypothesis tests were carried out. Data were

analyzed for normal distribution with a Kolmogorov-Smirnov test and for homoscedasticity with a Levene test. Differences were determined by a parametric test, as one-way analysis of variance (ANOVA) followed by post-hoc Tukey. All data are shown as the mean ± Standard Error of the Mean (SEM). All analyses were performed using SPSS v19 for Windows software (SPSS Inc. Chicago, IL). Statistical significances were set at *p* values lower than 0.05.

3. Results

3.1. Effect of Roundup® Ultra plus (RUP) on pig sperm viability and motility.

Initially, we studied pig spermatozoa viability after treatment with different concentrations of Roundup® Ultra plus, RUP, (0.0025%, 0.005% and 0.01%) in two different incubation media (TBM and TCM) for 1 h at 38.5 ºC (Figure 1). None of the RUP dilutions studied has a significant effect on sperm viability either in non-capacitating (TBM) or capacitating (TCM) medium when compared to the control (absence of RUP).

The *in vitro* effect of RUP in sperm motility was evaluated using the same experimental conditions as above (0.0025%, 0.005% and 0.01% of RUP in both media during 1 h at 38.5 ºC) using the ISAS® software. As observed in Figures 2A and 3A, RUP exposition leads to a clear concentration-dependent reduction in the percentages of motile (Figure 2A) and progressive motile spermatozoa (Figure 3A) independently of the incubation media. This reduction is statistically significant at 0.01% of RUP dilution in both media, TBM and TCM (Figures 2A and 3A).

We also evaluated the effect of RUP on spermatozoa velocities such as the curvilinear velocity VCL (Figure 4A), the straight-line velocity VSL (Figure 5A) and the average velocity path VAP (Figure 6A) in TBM and TCM. As observed, RUP treatment leads to clear reduction in spermatozoa velocities in both media with a

stronger reduction in TCM than in TBM (Figures 4A, 5A and 6A). Thus, sperm incubation with RUP for 1h at 38.5ºC in a sperm capacitating medium (TCM) causes a concentration-dependent reduction in any sperm velocity, that is statistically significant at RUP dilutions of 0.01% and 0.005% (right panels of Figure 4A, 5A and 6A). Incubation with RUP in a non-capacitating medium (TBM) causes a significant reduction only at a RUP dilution of 0.01% in the straight-line speed (Figure 5A) and average velocity path (Figure 6A).

However, sperm incubation with different RUP dilutions does not modify other sperm motility coefficients analyzed in Table 1, except for linearity coefficient (LIN), or wobble movement coefficient (WOB), which are significantly reduced by incubation with RUP 0.01% in TCM.

3.2. Effect of glyphosate on sperm motility and functional parameters.

In order to know whether the negative effect observed after RUP in sperm motility might be due to its active ingredient glyphosate (GLY), we incubated pig spermatozoa in the presence of the concentration range (41, 82 and 164 μM) that is present in the diluted formulations of RUP used. None of these GLY concentrations had a significant effect on sperm motility (Figures 2B, 3B, 4B, 5B, 6B and Table 1) or viability or plasma membrane lipid disorganization or mitochondrial membrane potential (Supplementary Data) either in TBM or TCM after incubation 1 h at 38.5 ºC.

3.3. Effect of surfactant polyoxyethylene amine (POEA) on sperm motility.

We next investigated whether the reduction in sperm motility caused by RUP might be due to its non-active ingredient, the surfactant polyoxyethylene amine (POEA). Therefore, we incubated pig spermatozoa with those POEA concentrations (0.0002%, 0.0004% and 0.0008%) that result when diluting the herbicide RUP as mentioned above concentrations. As observed in Figure 1 lower histograms, none

of the POEA dilutions have a significant effect on sperm viability either in TBM or TCM after 1 h of incubation at 38.5 ºC.

The *in vitro* effects on sperm motility of different dilutions of the surfactant POEA were evaluated in spermatozoa under same experimental conditions (1h of incubation at 38.5 °C in TBM or TCM). The surfactant POEA causes a clear and statistically significant decrease in the percentage of total motile spermatozoa, independently of the incubation media (Figure 2C). Thus, only about 15% of spermatozoa remain motile after 0.0004% POEA treatment in TBM (left) or after 0.0008% in TCM (right). As observed (Figure 2C), the inhibitory effect in the percentage of motile spermatozoa caused by POEA is greater than the observed with RUP (Figure 2A) in any medium. The incubation of spermatozoa with POEA surfactant also causes a clear reduction in the percentage of progressive motile spermatozoa (Figure 3C) in a dilution-dependent manner. This reduction is potent and almost blocked progressive motility, as only about 5% of spermatozoa remain progressive motile after treatment with 0.0004% POEA in TBM or 0.0008% POEA in TCM (Figure 3C). This inhibition of progressive motile spermatozoa was statistically significant when 0.0004% and 0.0008% POEA dilutions were used either in TBM or in TCM.

A dilution-dependent negative effect of POEA treatment in motility can be also observed in any sperm velocity studied. Thus, VCL (Figure 4C), VSL (Figure 5C) and VAP (Figure 6C) are significantly reduced in the presence of 0.0008% POEA, in both TBM and TCM medium. Regarding other spermatozoa motility coefficients, POEA incubation (0.0004%) significantly decreased STR in TBM and at 0.0008% significantly reduced LIN, STR and WOB in TCM (Table 1).

3.4. Comparative effects of RUP and POEA on other sperm functional parameters.

Incubation of spermatozoa under same experimental conditions with different dilutions of RUP and its equivalent concentrations of POEA have no a significant effect on mitochondrial membrane potential, either in TBM or TCM (Figure 7) at any dilutions studied.

On the other hand, exposure of pig spermatozoa during 1 h at 38.5 ºC to RUP induced a dose-dependent increase in the percentage of spermatozoa showing plasma membrane lipid disorganization (Figure 8 upper histograms) either in TBM (left) or TCM (right). This greater sperm lipid disorganization is statistically significant at 0.01% RUP in TBM. Similarly, when sperm samples are exposed to POEA at concentrations that are equivalents of RUP, it is observed also a significantly increase in the percentage of spermatozoa showing plasma membrane lipid disorganization (Figure 8 lower histograms) either in TBM at 0.0004% and 0.0008% POEA (left) or TCM at 0.0008% POEA (right).

3.5. Comparative effects of RUP, GLY and the surfactant POEA in the intracellular signalling pathways mediated by PKA and glycogen synthase kinase 3 (GSK-3) in spermatozoa.

In order to compare effects in relevant sperm signaling pathways, pig spermatozoa were incubated with equivalent dilutions of RUP (0.01%), GLY (164 µM) or POEA (0.0008%). Sperm treatment for 1h at 38.5 ºC with 0.01% RUP reduces GSK3α/β phosphorylation in spermatozoa incubated in both, TBM and TCM (Figure 9 histograms), although reduction was statistically significant only for GSK3β phosphorylation after incubation in TBM (Figure 9 right histograms). However, sperm incubation with GLY concentration that is equivalent to the obtained within RUP 0.01% has not any effect on GSK3α/β phosphorylation either

in TBM or TCM (Figure 9 histograms). Interestingly, sperm treatment with the surfactant POEA concentration (0.0008%) that is equivalent to the obtained within RUP 0.01% causes a similar effect to RUP on sperm GSK3α/β phosphorylation either in TBM or TCM. Thus, POEA leads to a clear although non-significant reduction on GSK3α phosphorylation (Figure 9 left histograms) and a significant decrease on GSK3β phosphorylation (Figure 9 right histograms).

We also studied whether the herbicide RUP, its active ingredient GLY and the surfactant POEA, could be affecting the sperm PKA signalling pathway by investigating the phosphorylation of its downstream substrates. As observed in the Figure 10 (histograms), the sperm treatment with herbicide RUP (0.01%) causes a clear and significant reduction in the phosphorylation of the bands called II and III corresponding to some PKA substrates detected in spermatozoa, either in TBM (Figure 10A) or TCM (Figure 10B). Interestingly, sperm treatment with the surfactant POEA (0.0008%) leads to a similar effect to the RUP, decreasing the phosphorylation level of PKA substrates bands II and III, either in TBM (Figure 10A) or TCM (Figure 10B). By contrary, sperm treatment with RUP or POEA in any medium does not significantly affect other bands such as those called I and IV (Figure 10 histograms).

However, the sperm incubation in TBM or TCM with the RUP active ingredient GLY does not modify at all the phosphorylation levels of the PKA substrates detected (Figure 10 histograms).

4. Discussion

Due to its worldwide, massive and growing use, the toxic effects of glyphosate-based herbicides have been investigated in several animal species and cell models. However, little literature exists to date about the functional impact of this type of

herbicides in mammalian reproductive cells, compared to that existing in fish reproductive gametes, and, particularly, in the male gamete.

This work demonstrates the adverse impact of Roundup® Ultra Plus and its non-active ingredient the surfactant POEA, but no glyphosate in mammalian sperm function using pig spermatozoa as a *in vitro* cell model to study contaminants in male reproductive effects. Pig spermatozoon has been reported as a well-validated *in vitro* cell model not only for cell toxicity studies [3, 17, 18] but also for physiological studies successfully translated to human assisted reproduction techniques [19].

The herbicide RUP causes a clear inhibition of the motility in pig spermatozoa at concentrations comparable to those present as environment contaminants, as they are much lower (100 times) than those recommended for agriculture. Our results are in agreement with previous studies demonstrating that Roundup® effects in mammalian spermatozoa, at lower concentrations than those used as herbicide, lead to an inhibition of mammalian sperm motility [3, 15, 16]. Thus, Roundup® treatment at 1 µg/mL rapidly decreased motility in human spermatozoa [15] and also its equivalent concentration of GLY (0.36 µg/mL) in human [16] or a higher concentration in pig spermatozoa (360 µg/mL) [3].

The reduction of mammalian sperm motility caused by low concentrations of RUP occurs in any incubation medium evaluated, either in non-stimulant medium as TBM or sperm stimulant medium, TCM. However, this adverse effect of low concentrations of RUP cannot be attributed to its active ingredient, GLY, as the incubation of pig spermatozoa under same experimental conditions with GLY concentrations (9-36 µg/mL), which are equivalent to those contained in the RUP concentrations evaluated in this work, do not affect at all sperm motility, the lipid

organization of plasma membrane, GSK3β and PKA signalling pathways or viability (this later are data not shown) in any medium. This lack of effect of GLY in sperm motility or viability is in agreement with results from a recent work by Nerozzi et al. [3] in pig spermatozoa at low GLY concentrations (range 5-50 μg/mL), which are comparable to those used in this work. However, it is reported that higher concentrations of GLY (360 μg/mL), that are 10 times greater than the maximum concentration used in the present work, not only caused also a decrease in sperm motility in human [16] and pig [3], but also in mitochondrial membrane potential, acrosome integrity and sperm viability in pig spermatozoa [3]. These mentioned studies, using 360 μg/mL GLY and the present work using 10 times lower GLY concentrations, suggest the idea of a clear concentration-dependent effect of GLY on mammalian spermatozoa, where low concentrations of GLY closer to environmental exposures do not affect at all mammalian sperm motility whereas greater GLY concentrations that exceeds environmental exposures impairs sperm motility and result toxic [3, 16].

Interestingly, the present work clearly demonstrates that the detrimental effects of low concentrations of RUP in sperm function can be attributed to its non-active component, POEA, as exposure to this non-ionic surfactant at RUP equivalent concentrations, mimics RUP effect and leads to an inhibition of sperm motility in a similar extent than that caused by RUP. Additionally, the surfactant POEA triggers a concentration-dependent disorganization of lipids at the sperm plasma membrane in a similar way as occurs with equivalent RUP concentrations, suggesting that the inhibition of sperm motility caused by RUP can be due, at least in part, by altered sperm plasma membrane lipid organization induced by the surfactant POEA. To our knowledge, the potential action of other herbicide ingredients besides GLY, such as

surfactants, has not been studied so far in mammalian spermatozoa. However, it has been generally reported that the most used surfactant in GLY-based herbicides, POEA, induces membrane damages in different mouse cell types leading to a high cytotoxicity [22]. Additionally, in human umbilical, embryonic and placental cells, POEA has demonstrated also to induce damage in cell membranes that causes changes in cell permeability [10] and in other human cell lines this surfactant disturbs the integrity of the membrane and alters cellular respiration processes [7].

The reduction in sperm motility is not due to a cytotoxic impact or by any potential side effect triggered by RUP or POEA in pig spermatozoa that might compromise sperm vitality, as sperm viability remains unaffected by RUP or POEA under same experimental conditions and equivalent concentrations. This lack of RUP effect in sperm viability agrees with the previous report in pig spermatozoa only at the lowest Roundup® concentrations that they used, below 50 µg/mL of GLY equivalent concentration [3], which are in a similar range as those evaluated in this work. However, at Roundup® concentrations equivalent to 50 µg/mL of GLY and higher, a dose-dependent decrease in sperm viability is described [3], reinforcing the idea that the glyphosate-based herbicides adverse effects in mammalian spermatozoa viability are concentration-dependent.

Additionally, the inhibition of pig sperm motility cannot be attributed to a detrimental effect of RUP or POEA in the sperm mitochondrial membrane potential. Thus, although pig sperm mitochondrial activity is slightly decreased under some conditions (RUP in TCM or after 0.0008% POEA), this effect is not statistically significant. Also, a slight effect of Roundup® in mitochondrial activity has been reported by Nerozzi et al.[3] in pig spermatozoa although in their case this effect resulted statistically significant.

As we have not addressed boar sperm capacitation in this work, we cannot make any assumptions about the possible effects of RUP or its components in this functional sperm process.

Regarding intracellular signalling pathways that might be altered by RUP treatment in spermatozoa, our results point out to an inhibition of GSK3α/β phosphorylation cascade and also to a selective inhibition of phospho-PKA substrates (so-named substrates II and III). Interestingly, both signalling cascades are unaffected by equivalent concentrations of GLY but totally reproducible when using POEA equivalent concentrations. To date, there are no previous studies about the intracellular signalling affected by RUP in spermatozoa. As GSK3α (but not GSK3β) and PKA pathways are demonstrated to be regulating pig sperm motility [23, 24], our results suggest that the impairment in sperm motility caused by POEA and subsequently by RUP is likely due to an inhibition of the phosphorylation of both kinases pathways. However, more experiments are needed to clarify the individual contribution of each particular pathway to the detrimental effects of RUP low concentrations in mammalian spermatozoa, especially GSK3β, whose contribution to pig sperm motility has not been established yet and also which are the specific substrates that are selectively inhibited by PKA.

5. Conclusions

In summary, this work demonstrates that i) low concentrations of the herbicide RUP similar to those present as environment contaminants impairs pig sperm motility without affecting sperm viability. This work might indicate a solid evidence linking ambient exposure to RUP and concomitantly to its non-active ingredient POEA at relatively low concentrations comparable to contaminants, with adverse reproductive effects, in particular, in mammalian spermatozoa; ii) the RUP adverse

effect on sperm motility cannot be attributed to its active ingredient GLY, but to its non active compound, the surfactant POEA; iii) the impairment in sperm motility caused by RUP might be likely due to a detrimental effect of its ingredient POEA at the plasma membrane causing a loss of membrane lipid organization; iv) RUP adverse effects in pig spermatozoa are likely mediated by a POEA-triggered inhibition of two phosphorylation pathways that control sperm motility: GSK3β and/or by a selective inhibition of PKA that particularly affects specific substrates.

6. Acknowledgements

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7. Author contributions

Torres-Badia M, Solar S and Gomez A performed the experiments. Torres-Badia M also contributed to data curation, formal analysis, interpretation of data and wrote the draft manuscript. Martin-Hidalgo D and Hurtado de Llera A contributed to data curation and critically revised the manuscript. Garcia-Marin L.J, Gonzalez-Fernandez L and Bragado M. J conceptualized and designed the study, analyzed

and supervised the results, wrote the paper and contributed to funding acquisition. All authors read and approved the final version of the manuscript.

8. Declaration of competing interest

The authors declared that they have not any competing financial and/or non-financial interests in relation to the present work.

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Figure Legends

Figure 1. Effects of Roundup® Ultra plus (RUP) and surfactant polyoxyethylene amine (POEA) in pig spermatozoa viability. *Left panel:* spermatozoa were incubated in TBM or TCM for 1 h at 38.5 ºC in the absence or presence of different concentrations of RUP (upper histograms) or POEA (lower histograms). This experiment was performed 5 times (n = 5) and the results are expressed as the mean of the percentage of SYBR14-positive and PI-negative spermatozoa ± standard error of the mean (SEM). No statistical differences were found. *Right panel:* Representative two-dimensional SYBR-14 fluorescence versus PI fluorescence dot plots for sperm samples incubated in absence or presence of RUP (0.01%) and POEA (0.0008%).

Figure 2. Effects of Roundup® Ultra plus (RUP), glyphosate and surfactant polyoxyethylene amine (POEA) in the percentage of motile spermatozoa. Pig spermatozoa were incubated 1 h in TBM or TCM at 38.5 °C in the absence or presence of different concentrations of RUP (A), glyphosate (B) or POEA (C). The percentage of motile spermatozoa was evaluated by ISAS® system. Each experiment was performed 6 times (n = 6) and results are expressed as the mean of the percentage of total spermatozoa ± SEM. Statistical differences are shown with * ($P < 0.05$).

Figure 3. Effects of Roundup® Ultra plus (RUP), glyphosate and surfactant polyoxyethylene amine (POEA) in the percentage of progressive motile spermatozoa. Pig spermatozoa were incubated 1 h in TBM or TCM at 38.5 °C in the absence or the presence of indicated concentrations of RUP (A), glyphosate (B) and POEA (C). Progressive motility was evaluated by ISAS® system. Each experiment was performed 6 times (n = 6) and results are expressed as the mean of the percentage of progressive spermatozoa ± SEM. Statistical differences are shown with * ($P < 0.05$).

Figure 4. Effects of Roundup® Ultra plus (RUP), glyphosate (GLY) and surfactant polyoxyethylene amine (POEA) in the curvilinear velocity (VCL) of pig spermatozoa. Spermatozoa were incubated in TBM or TCM for 1 h at 38.5 °C in the absence or presence of indicated concentrations of RUP (A), glyphosate (B) and POEA (C). Sperm VCL was evaluated by ISAS® system, the curvilinear velocity (VCL) is expressed as µm/s. Each experiment was performed 6 times (n = 6) and results are expressed as mean ± SEM. Statistical differences are shown with * ($P < 0.05$).

Figure 5. Effects of Roundup® Ultra plus (RUP), glyphosate (GLY) and surfactant polyoxyethylene amine (POEA) in the straight-linear velocity (VSL) of pig spermatozoa. Spermatozoa were incubated 1 h in TBM or TCM at 38.5 °C in the absence or presence of indicated concentrations of RUP (A), glyphosate (B) and POEA (C). Sperm VSL was evaluated by ISAS® system, the straight-linear velocity (VSL) is expressed as µm/s. Each experiment was performed 6 times (n = 6) and results are expressed as mean ± SEM. Statistical differences are shown with * ($P < 0.05$).

Figure 6. Effects of Roundup® Ultra plus (RUP), glyphosate (GLY) and surfactant polyoxyethylene amine (POEA) in the average velocity (VAP) of pig spermatozoa. Spermatozoa were incubated 1 h in TBM or TCM at 38.5 °C in the absence or presence of indicated concentrations of RUP (A), glyphosate (B) and POEA (C). Sperm VAP was evaluated by ISAS® system, the average velocity (VAP) is expressed as μm/s. Each experiment was performed 6 times (n = 6) and results are expressed as mean ± SEM. Statistical differences are shown with * ($P < 0.05$).

Figure 7. Effects of Roundup® Ultra plus (RUP) and surfactant polyoxyethylene amine (POEA) in mitochondrial membrane potential (ΔΨm) of pig spermatozoa. *Left panel:* Spermatozoa were incubated for 1 h in TBM or TCM at 38.5 ºC in the absence or presence of different concentrations of RUP (upper graph) or POEA (lower graph). Results are expressed as the mean of the percentage of spermatozoa exhibiting relative higher ΔΨm from the total sperm cells analysed ± SEM. Each experiment was performed 5 times (n = 5). No statistical differences were found ($P > 0.05$). *Right panel:* Representative two-dimensional JC-1 monomer fluorescence versus JC-1 polymer fluorescence dot plots for sperm samples incubated in absence or presence of RUP and POEA.

Figure 8. Effects of Roundup® Ultra plus (RUP) and surfactant polyoxyethylene amine (POEA) in plasma membrane lipid organization of pig spermatozoa. *Left panel:* Spermatozoa were incubated in TBM or TCM for 1 h at 38.5 °C in the absence or presence of indicated concentrations of RUP (upper graph) and POEA (lower histograms). Each experiment was performed at least 5 times (n = 5). Results are expressed as the geometric mean ± SEM of relative fluorescence intensity (RFI) of M540 fluorescence/Yo-pro-1 negative. Statistical

differences are shown with * ($P < 0.05$). *Right panel:* Representative two-dimensional M540 fluorescence versus Yo-pro-1 fluorescence dot plots (upper panel) and flow cytometry histograms for M540 fluorescence of Yo-pro-1 negative spermatozoa (middle and lower panels) for sperm samples incubated in absence or presence of the indicated concentrations of RUP and POEA.

Figure 9. Effects of Roundup® Ultra plus (RUP), glyphosate (GLY) and surfactant polyoxyethylene amine (POEA) in the phosphorylation of GSK-3 on pig spermatozoa. Spermatozoa were incubated in TBM (left) or TCM (right) for 1 h at 38.5 °C in the absence or the presence of herbicide RUP (0.01%), and the equivalent concentrations of its ingredients GLY (164 μM) and the surfactant POEA (0.0008%). *Upper panel:* Sperm proteins (10 μg) were analysed by western blotting using anti-phospho GSK3α/β as primary antibody. Each experiment was performed 5 times and representative films are shown. Loading controls using GSK3α and β antibodies (lower films) were performed for each experiment. Arrows indicate the cross-reactive sperm bands corresponding to phosphorylated forms of GSK3α and GSK3β (upper images) and GSK3α and β (lower images). *Lower panel:* Densitometry analysis of GSK3α and GSK3β bands is shown and values are expressed as the mean ± SEM of arbitrary units. Statistical differences are shown with * ($P < 0.05$).

Figure 10. Effects of Roundup® Ultra plus (RUP), glyphosate (GLY) and surfactant polyoxyethylene amine (POEA) in the phosphorylation of PKA-substrates on pig spermatozoa. Spermatozoa were incubated in TBM (left) or TCM (right) for 1 h at 38.5 °C in the absence or the presence of herbicide RUP (0.01%), and the equivalent concentrations of its ingredients GLY (164 μM) and the surfactant POEA (0.0008%). Sperm proteins (10 μg) were analysed by western

blotting using anti-phospho-PKA-substrates as primary antibody. Each experiment was performed 5 times and representative films are shown. Loading controls using anti-α-tubulin antibody (lower films) were performed for each experiment in the same membrane. Arrows indicate cross-reactive bands (I-IV) of sperm phosphorylated proteins that are substrates of PKA. *Lower panel:* Densitometry analysis of I-IV bands is shown and values are expressed as mean ± SEM of arbitrary units. Statistical differences are shown with * ($P < 0.05$).

Table

Table 1. Effects of Roundup® Ultra plus (RUP), glyphosate and surfactant polyoxyethylene amine (POEA) in pig spermatozoa motility coefficients.

Buffer	Treatment	Concentration	LIN (%)	STR (%)	WOB (%)
TBM	RUP	0%	61.5±3.5	88.13±1.95	69.55±2.52
		0.0025%	60.1±4.6	86.48±2.53	69.05±3.41
		0.005%	60.3±5.5	86.15±2.72	69.40±4.18
		0.01%	46.4±7.0	74.05±5.66	60.80±5.01
	Glyphosate	0	59.8±2.0	88.10±0.87	67.80±1.67
		41μM	58.3±2.6	86.72±1.53	67.00±1.84
		82μM	62.6±1.6	89.28±0.66	70.07±1.34
		162μM	61.2±2.2	88.52±1.50	69.07±1.35
	POEA	0%	57.5±2.3	85.82±1.96	66.88±1.48
		0.0002%	60.2±2.3	86.42±1.47	69.68±1.62
		0.0004%	39.9±3.4	70.42±2.08*	56.30±3.59
		0.0008%	48.1±9.4	67.52±6.25*	69.23±5.07
TCM	RUP	0%	79.8±0.7	93.67±0.80	85.15±0.41
		0.0025%	78.8±1.1	92.25±0.95	85.43±0.74
		0.005%	78.4±0.5	92.78±0.78	84.57±0.87
		0.01%	72.4±1.9*	90.35±1.07	67.88±12.01*
	Glyphosate	0	80.0±2.4	94.32±0.96	84.70±1.72
		41μM	76.4±3.0	92.15±1.54	82.73±2.07
		82μM	78.5±2.2	92.47±1.31	84.77±1.26
		162μM	76.8±2.7	91.55±1.68	83.73±1.64
	POEA	0%	77.0±1.9	92.12±0.98	83.50±1.32
		0.0002%	76.5±1.4	92.20±1.08	82.92±0.86
		0.0004%	69.0±6.1	88.92±3.12	76.77±4.65
		0.0008%	38.3±8.3*	61.32±9.40*	59.53±5.47*

Pig spermatozoa were incubated in TBM or TCM at 38.5 °C in the absence or presence of indicated concentrations of RUP, glyphosate or POEA. Sperm kinematic parameters were evaluated by ISAS® system: linearity (LIN, in%); straightness (STR, in %) and wobble movement coefficient (WOB, in %). This

experiment was performed 6 times (n = 6) and values are expressed as the mean ± SEM. Statistical differences from their own control are shown with * ($P < 0.05$).

Credit Author Statement

Torres-Badia M, Solar S and Gomez A performed the experiments. Torres-Badia M also contributed to data curation, formal analysis, interpretation of data and wrote the draft manuscript. Martin-Hidalgo D and Hurtado de Llera A contributed to data curation and critically revised the manuscript. Garcia-Marin L.J, Gonzalez-Fernandez L and Bragado M. J conceptualized and designed the study, analyzed and supervised the results, wrote the paper and contributed to funding acquisition. All authors read and approved the final version of the manuscript.

Figure 1 New

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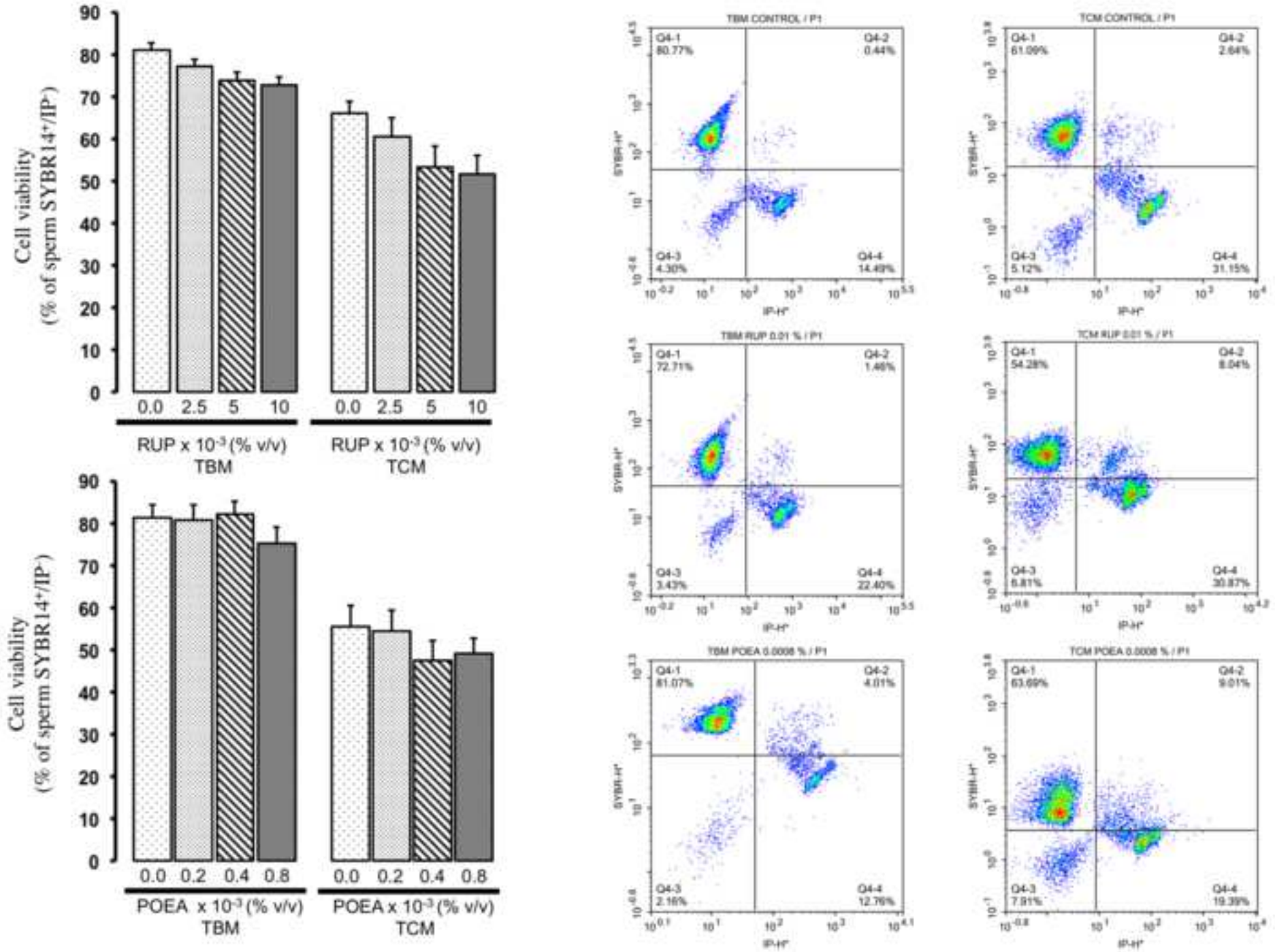

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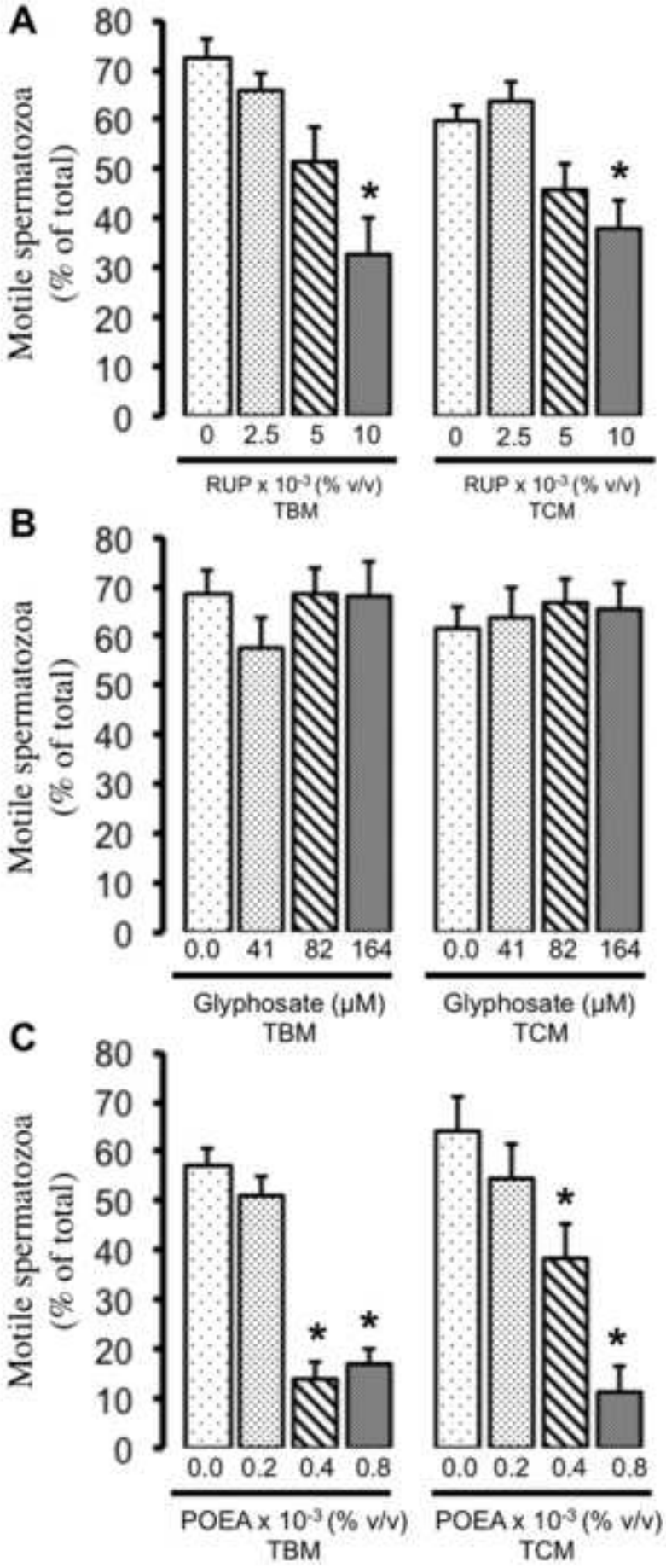

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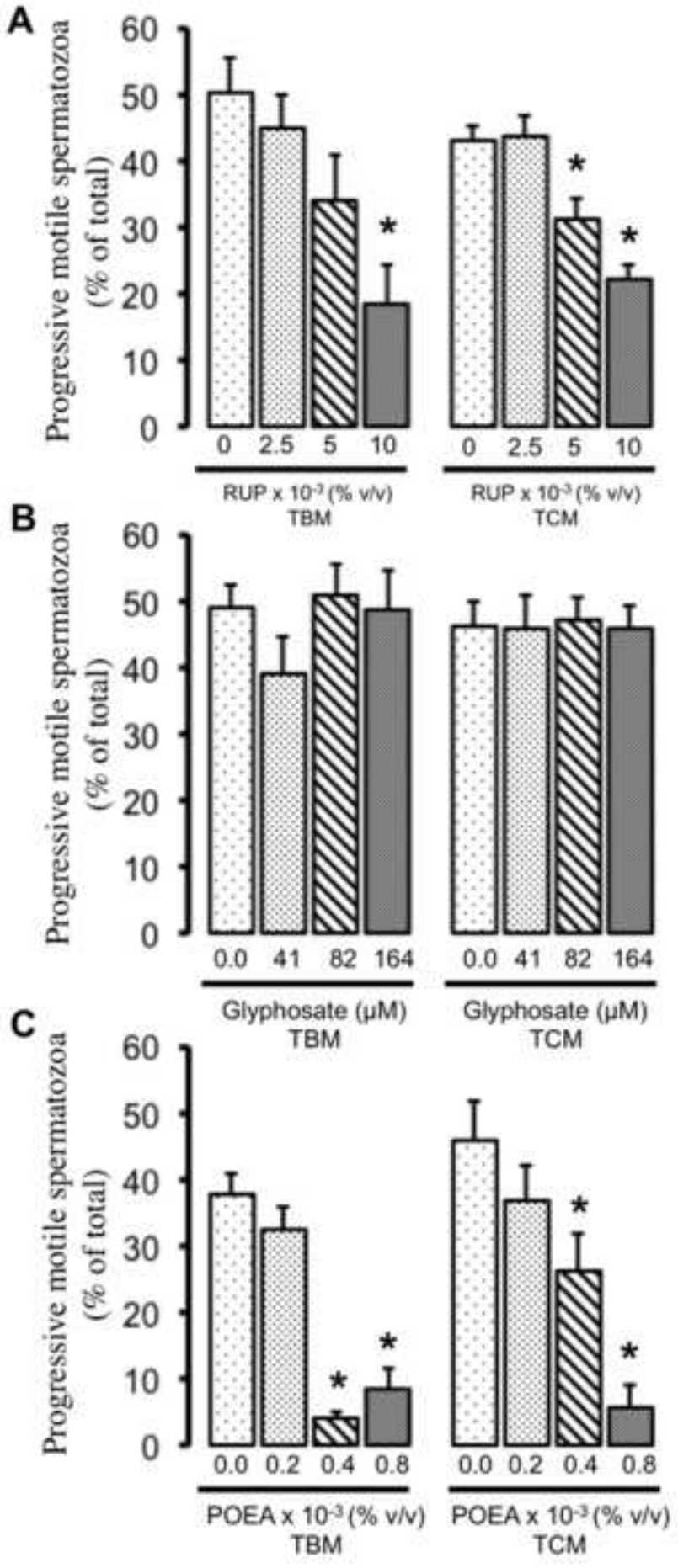

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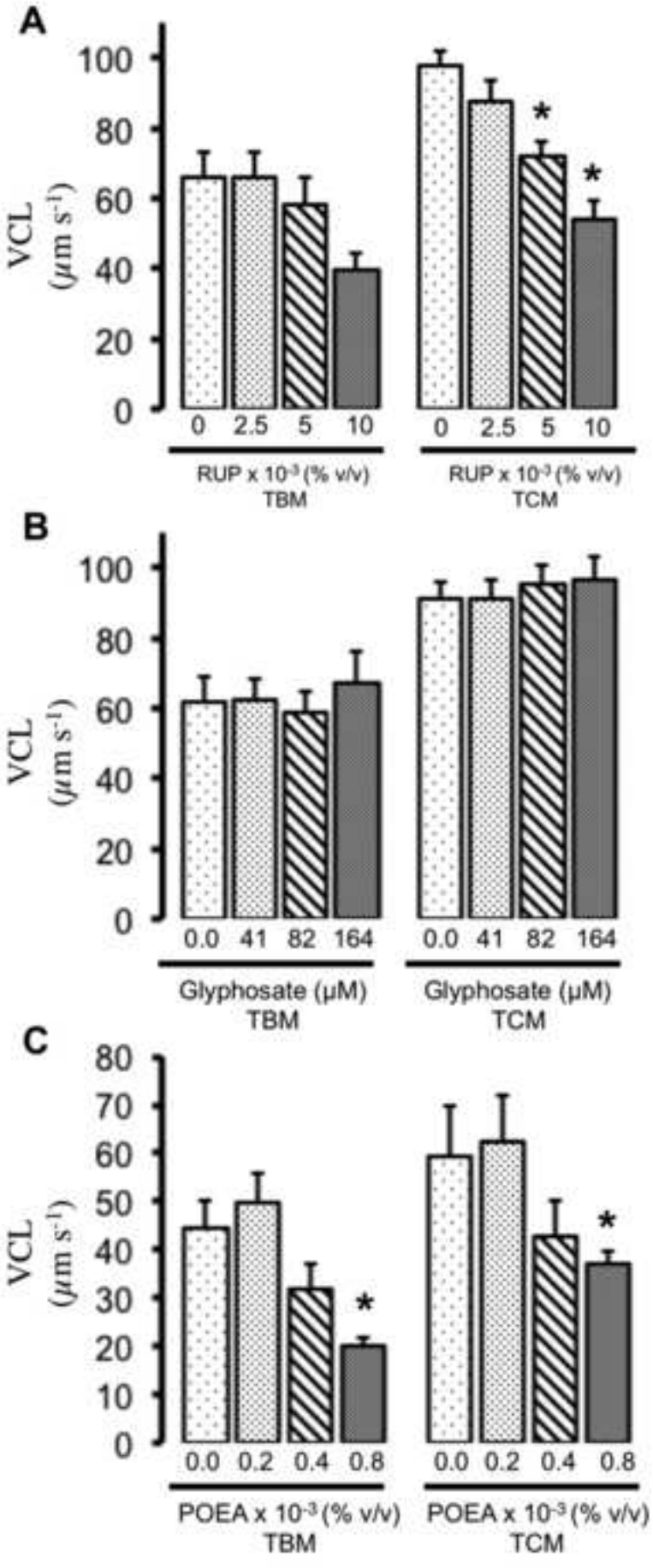

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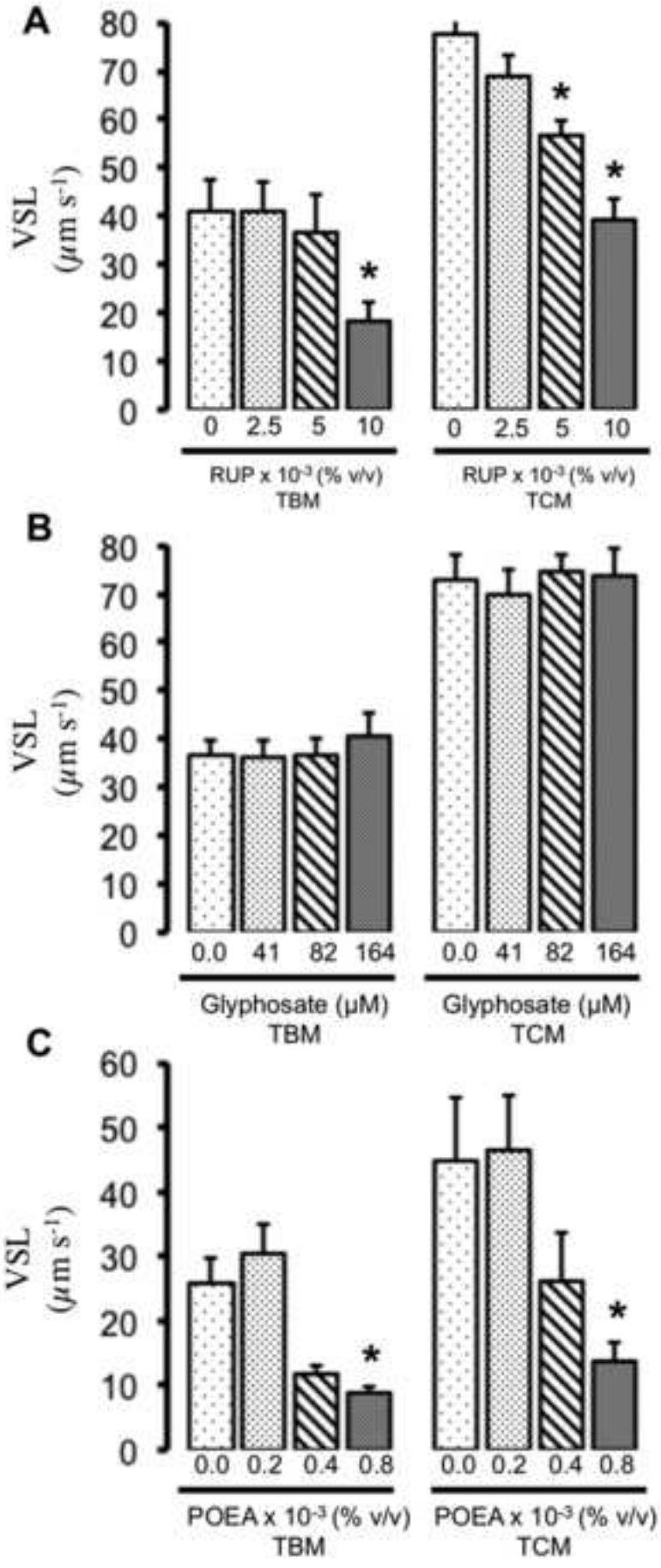

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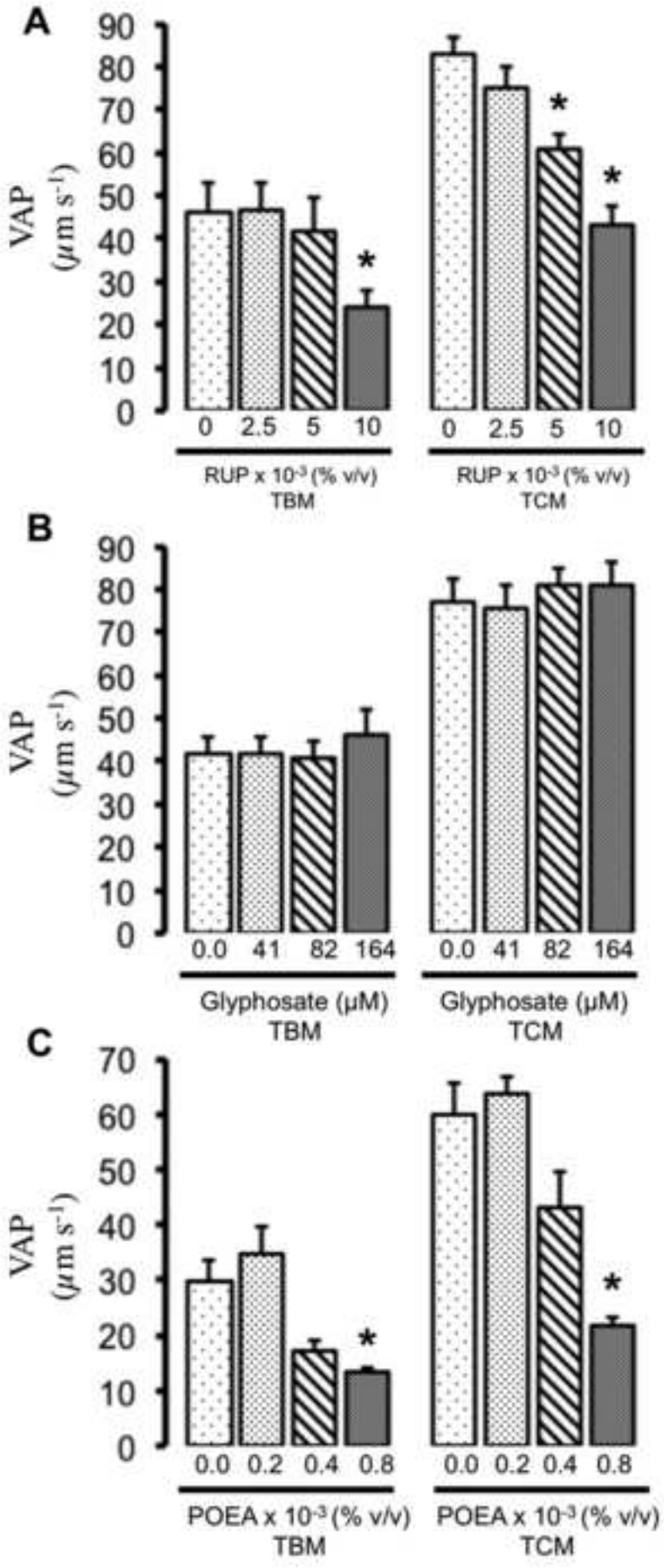

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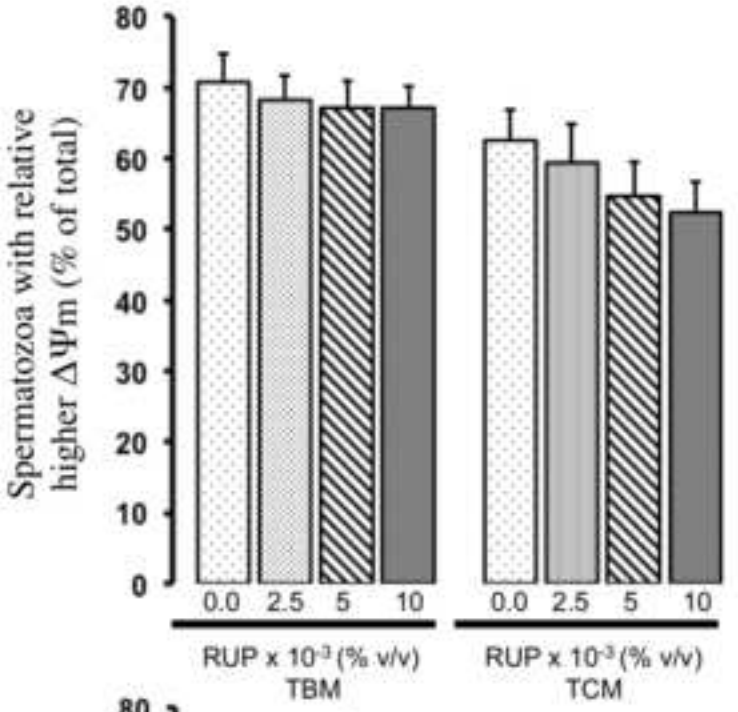

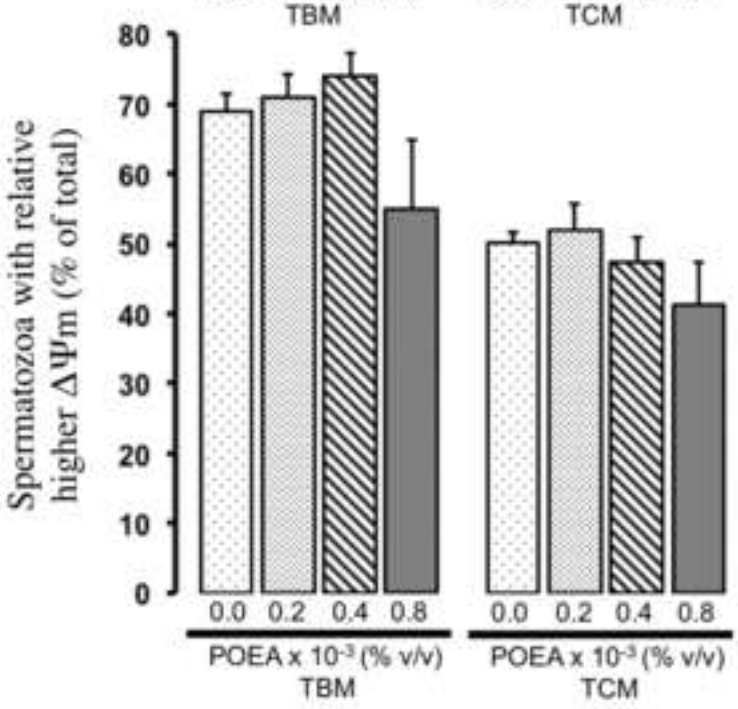

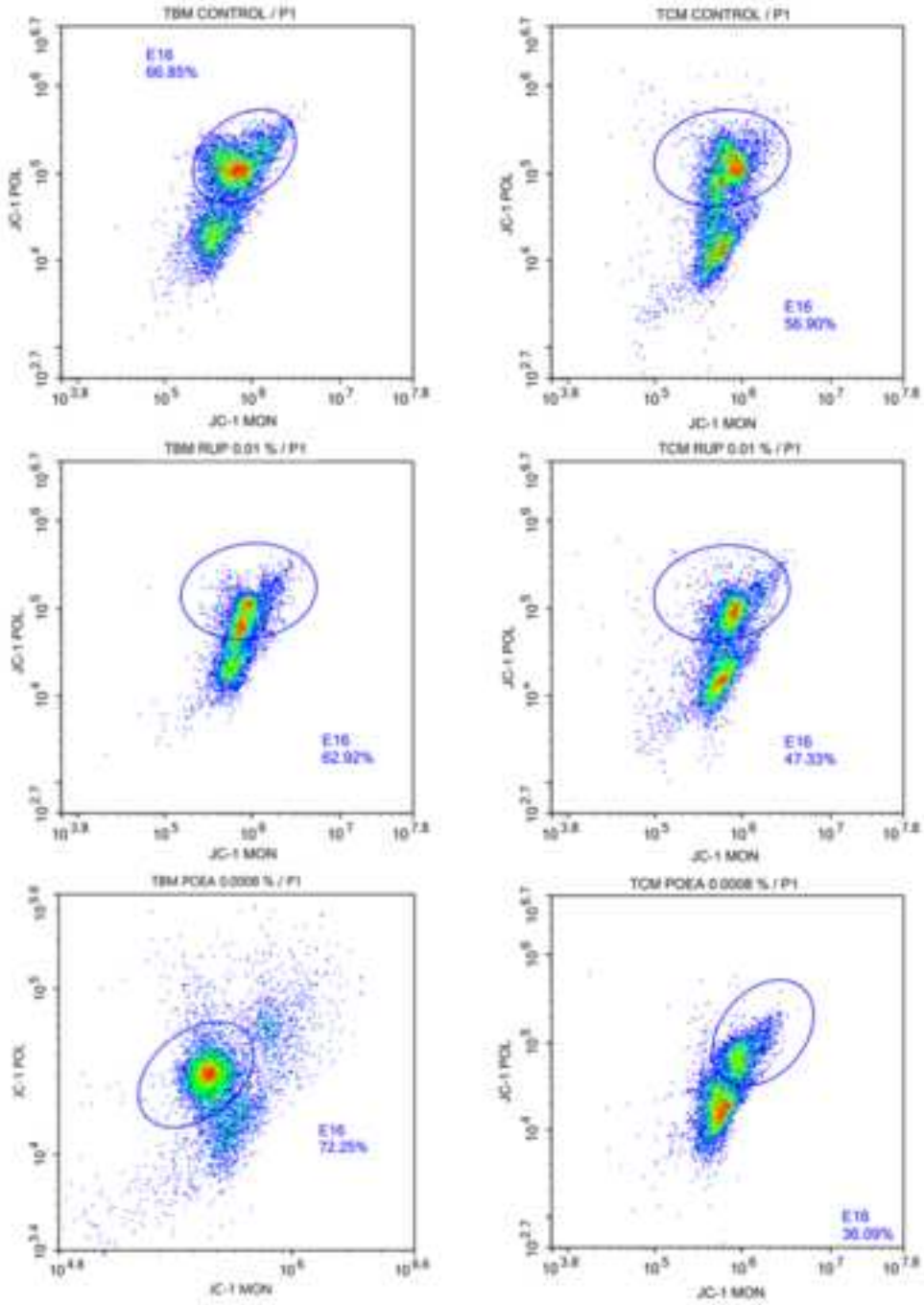

Figure 8

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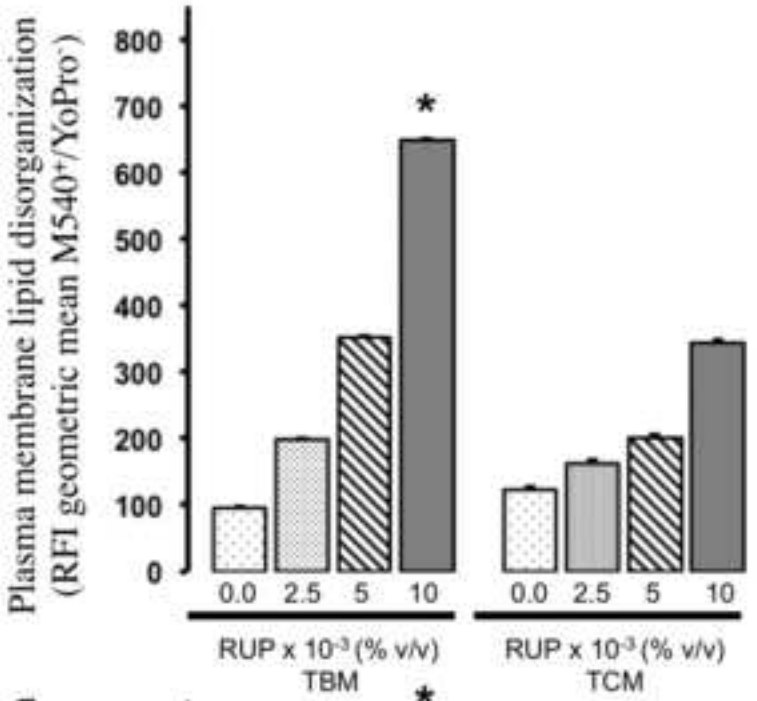

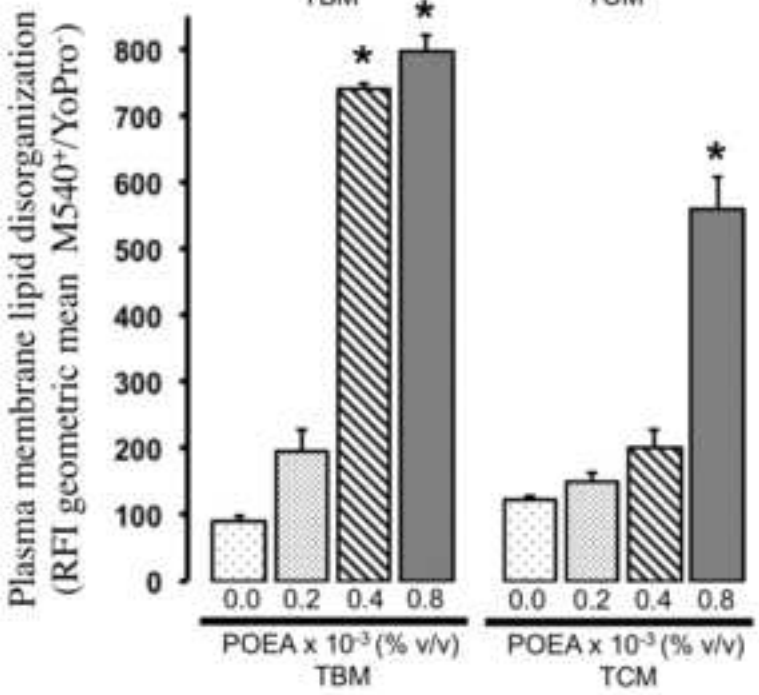

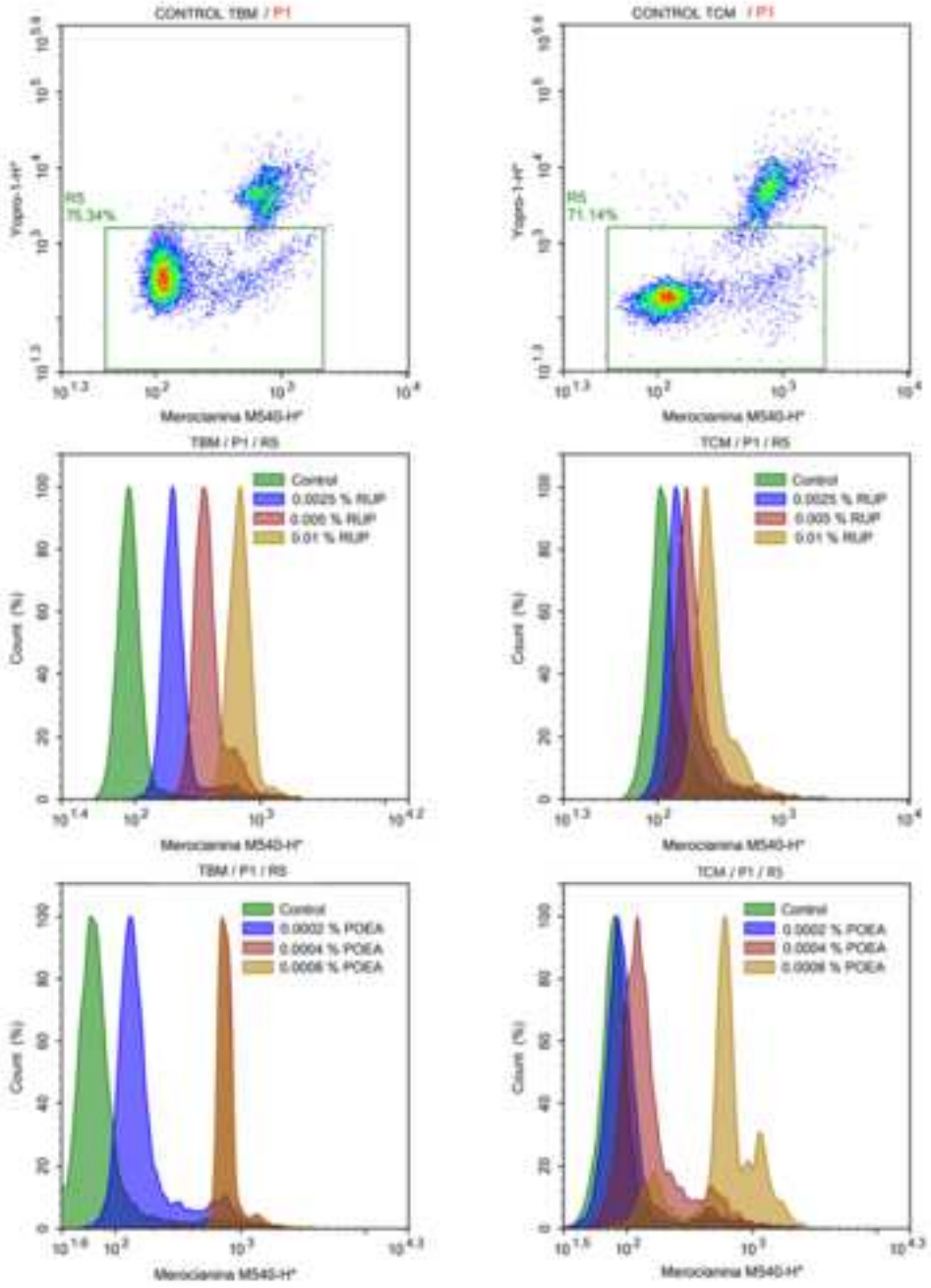

Figure 9 New

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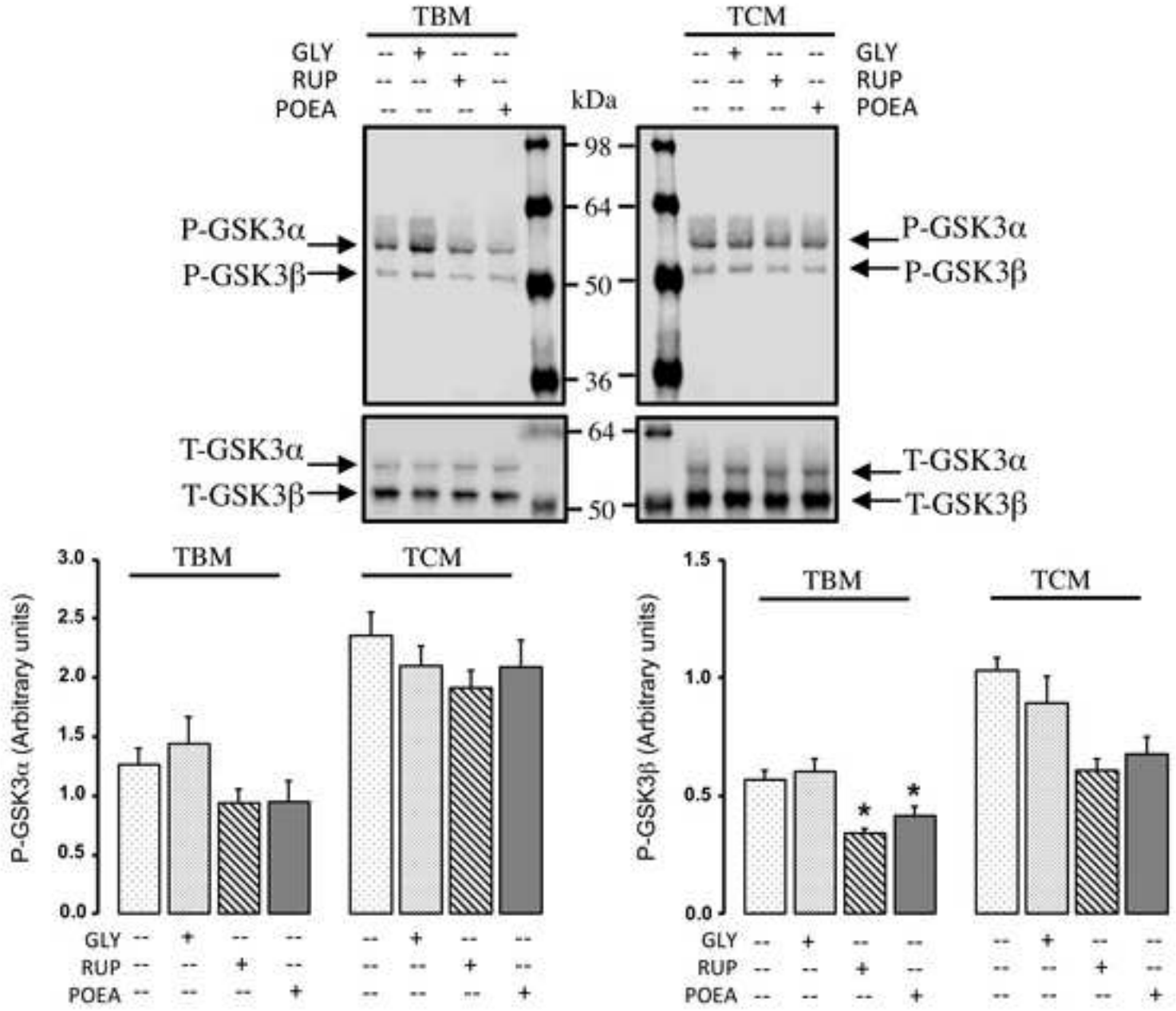

Figure 10 New

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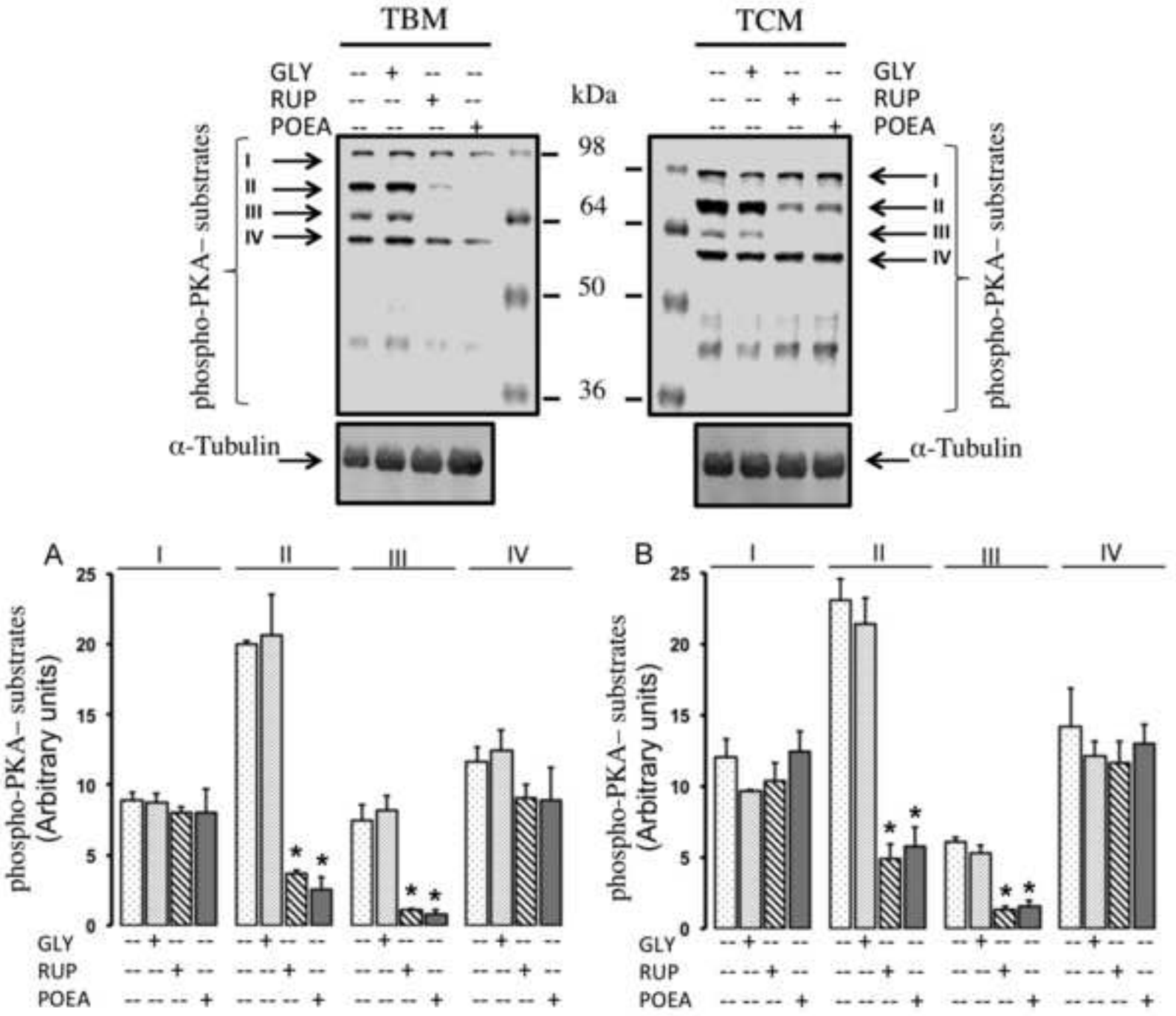

Supplementary data: Effects of glyphosate in boar spermatozoa viability, mitochondrial membrane potential (ΔΨm) and plasma membrane lipid organization.

Buffer	Treatment	Viability (%)	Higher **ΔΨm (%)**	Plasma membrane lipid disorder (RFI)
TBM	Control	87.2±1.2	82.6±2.7	91.8±32,3
	Glyphosate (41µM)	86.5±1.7	83.1±2.2	109.2±29.2
	Glyphosate (82µM)	87.3±1.4	86.1±2.9	99.4±29.1
	Glyphosate (164µM)	87.2±1.4	84.2±3.3	100.3±33.2
TCM	Control	63.7±12.8	66.4±5.6	122.7±6.4
	Glyphosate (41µM)	64.5±12.2	69.7±2.4	125.8±8.1
	Glyphosate (82µM)	64.4±12.6	69.9±6.4	121.9±8.2
	Glyphosate (164µM)	64.5±12.8	59.0±4.2	119.9±10.8

Pig spermatozoa were incubated in TBM or TCM at 38.5 °C in the absence or presence of different concentrations of glyphosate. This experiment was performed 5 times (n = 5) and the results are expressed as the mean ± standard error of the mean (SEM) of the percentage of SYBR14-positive and PI-negative spermatozoa (viability) or percentage of spermatozoa exhibiting relative higher ΔΨm from the total sperm cells analysed (mitochondrial membrane potential) or the geometric mean of relative fluorescence intensity (RFI) of M540 fluorescence/Yo-pro-1 negative (plasma membrane lipid organization). No statistical differences were found.

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