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Review

Does exposure to glyphosate lead to an increase in the micronuclei frequency? A systematic and meta-analytic review

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HIGHLIGHTS

- Systematic meta-analytical review correlating glyphosate exposure and micronuclei.
- Groups exposed to glyphosate formulations have increased formation of micronuclei.
- Significant difference among glyphosate (GLY) and its commercial formulations.
- Difference in MN formation among different exposure routes of GLY.
- Difference in MN formation among different groups of vertebrates.

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ABSTRACT

Glyphosate-based herbicides are among the most used pesticides worldwide. Reviews on the safety of glyphosate have been conducted by several regulatory agencies and researches centers, many times with contradictory results. This study is a systematic meta-analytical review of experimental studies on the relationship between exposure to the glyphosate (GLY) and its formulations with the formation of micronuclei (MN) to establish a quantitative estimate of the environmental risks. The natural logarithm (ln) of the estimated response ratio was calculated from 81 experiments. A meta-analysis was performed on the complete data set, and individual meta-analyses were conducted after stratification by test system, class of vertebrate, exposure route, gender, endpoints, type of literature, formulation, GLY dose and exposure time. A forest plot showed an overall positive association between GLY exposure and its formulations and MN, corroborated by the cumulative effects size. Different responses were observed on mammalian and non-mammalian. Interesting results was noticed in exposure route where oral administration of GLY presented no significance. Exposure by intraperitoneal injection presented the highest MN formation. Pure GLY caused fewer effects than to commercial mixtures, but both presented mutagenic effects. The studies with males presented significant responses, while studies with females were not significant. The cumulative effects size was not clearly related to GLY dose, and was negatively related to exposure time. It can be attributed to different test systems, exposure routes and protocols analyzed. In conclusion, our results support the hypothesis that exposure to GLY and its formulations increases the frequency of MN formation.

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1. Introduction

Glyphosate [N-(phosphonomethyl) glycine] (GLY) is one of the main pesticides that have been discovered to date and is the most globally commercialized pesticide for the non-selective control of weeds (Baylis, 2000; Monsanto, 2005). This systemic herbicide inhibits the growth of plants by interfering in the production of the aromatic amino acids phenylalanine, tyrosine and tryptophan, which causes a reduction in protein synthesis (Faus et al., 2015).

Current agricultural activities are highly dependent on the use of glyphosate-based commercial formulations, and this has become even more true in recent years because more than 75% of genetically modified plants have been formulated to tolerate high levels of glyphosate (Vera-Candioti et al., 2013). The formulations of glyphosate-based herbicides are complex and variable mixtures – adjuvants and surfactants are added to the active ingredient (GLY) with the objective of increasing its absorption and effectiveness (Baylis, 2000). Unfortunately, surfactants can present toxicity many times greater than GLY, making the formulated product much more toxic than the isolated active ingredient (Vera-Candioti et al., 2013). The specific original Roundup® (RU) formulation was composed by 41% isopropylamine glyphosate salt and surfactant (15.4% a polyethoxylated tallowamine). Nowadays, it is no longer sold in many markets, and other glyphosate formulations with different compositions are sold under the Roundup® brand name, with different glyphosate forms, concentrations and surfactant systems (Kier and Kirkland, 2013a). Despite the great number of benefits of the use of pesticides in agriculture, such as GLY, these agrochemicals can be dangerous if not used appropriately, and many of them pose a potential risk due to their contamination of foods, water and air (WHO, 1994). The great use and ubiquity of this GLY-based products increases the need for toxicological studies that determine the level of environmental risks of these products and their effects on non-target organisms (Borggaard and Gimsing, 2008). In this regard, numerous studies have been performed in recent years with different test systems to evaluate the harmful effects of GLY, both alone and in its commercial formulations, but the results of these studies are highly conflicting.

On the one hand, glyphosate-based herbicides are very effective in the control of undesired vegetation and are described by their manufacturers as having low toxicity and good environmental compatibility (Cox, 1998), and they are believed to be less toxic than other pesticides. Nonetheless, other studies have shown that GLY is moderately persistent in water under low light conditions and it is also highly persistent in the dark (Mercurio et al., 2014). It can potentially contaminate rivers, surface waters and soil, in which the detection levels of the herbicide is increased proportionally to the dosage of applications. Likewise, the flow increased by rain causes the transport of the herbicide from the direct area of influence to downstream sites (Peruzzo et al., 2008). A recent study shows that GLY can induce the growth of human breast cancer cells via estrogen receptors, and also tumor promoting activity in mice (George et al., 2010; Thongprakaisang et al., 2013).

Pesticide and its residues are subjected to chemical reactions with environmental reagents from the very beginning. The main reactions in the environment include oxidation, reduction, and nucleophilic displacements in biomolecules such as DNA (Crosby, 1982), and for this reason the genotoxicity of pesticides is a worldwide concern. The genotoxic and mutagenic effects of GLY and RU have been studied in different manners (Grisolia, 2002; Li and Long, 1988; Mañas et al., 2009; Poletta et al., 2009; Seiler, 1977 among many others), and these studies have generated some contradictory results. According to Williams et al. (2000), there is no *in vitro* or *in vivo* evidence that RU causes direct damage to DNA, indicating that it and its components do not present risks in regard to somatic or heritable mutations in humans. Similar results were obtained in a genetic mutation test with *Salmonella typhimurium* and in a mammalian cell culture study (Wildeman, 1982). Additionally, Li and Long (1988) performed an *in vitro* DNA synthesis test in rat hepatocytes to examine the genotoxicity of GLY and reported no DNA damage; they also reported that GLY did not cause DNA damage in the bone marrow of rats using a chromosome aberration test. In the same manner, other studies have found that neither GLY nor RU caused an increase in the frequency of micronuclei and chromosomal aberrations in rats after *in vivo* exposure to these pesticides (Dimitrov et al., 2006; Rank et al., 1993). Many interesting results from several databases were compiled in the recent review paper from Kier and Kirkland (2013a). As pointed by authors, negative results for *in vitro* gene mutation and a majority of negative results for chromosomal effect assays in mammalian cells have provided evidences that glyphosate is not typically genotoxic for these endpoints in mammalian systems. Mixed

results were observed for micronucleus assays of GLY-based formulations in non-mammalian systems. Reports of positive results for DNA damage endpoints indicate that glyphosate and its formulations tend to elicit DNA damage effects at high or toxic dose levels, but this can be due to cytotoxicity or to the surfactants present in complex commercial mixtures (Kier and Kirkland, 2013a).

The individual study by Bolognesi et al. (1997) observed that both pure GLY and RU had DNA-damaging activity in the forms of DNA single-strand breaks and a significant increase in chromosomal alterations *in vivo* and *in vitro*. In the same study, weak genotoxic activity was evident for RU (Bolognesi et al., 1997). Positive results for *in vivo* DNA adducts in rats and chromosomal aberrations in the onion *Allium cepa* have also been demonstrated for RU, but not for GLY (Peluso et al., 1998; Rank et al., 1993). Other studies have shown that RU induces an increase in micronuclei (MN) and DNA damage in goldfish (Çavas and Könen, 2007) and *Tilapia rendalli*, but not in rats (Grisolia, 2002). A more recent study indicated that RU can be significantly harmful to the DNA of fish, even with exposure to extremely low realistic levels (parts per billion – μg/L) for short period of time (Ghisi and Cestari, 2013).

The micronucleus (MN) test is one of the most well-established and commonly used methods for evaluating the mutagenic effects of a wide spectrum of compounds. The MN test shows great potential because it can be executed rapidly, is relatively inexpensive and is a good indicator of chemical contamination in organisms. Micronuclei are small masses of chromatin that are found outside the main nucleus of cells, and they originate from chromosome breaks or malfunction of the mitotic fuse during nuclear division (Fenech, 2007). During cell division, entire chromosomes or partial chromosomes that were not incorporated into the main nucleus of the daughter cell, appear as small, round, dark structures, with the same appearance and refraction as the nuclear material (Fenech, 2007). Although there is a basal level of spontaneous formation of micronuclei in most of the species (Mañas et al., 2009), the exposure of organisms to clastogenic substances, such as some pesticides, have been shown to increase the frequency of micronuclei formation in the laboratory and in field studies (Bombail et al., 2001; Grisolia and Starling, 2001; Guilherme et al., 2010).

This study evaluated the relationship between exposure to glyphosate (in different formulations) and micronuclei formation frequency through a systematic review of the literature. Using these data in a meta-analytic study, we aimed to furnish a quantitative estimate of the environmental risk of GLY pesticides. Our hypotheses were the following: (i) The damage rate is expected to be higher in the exposed experimental groups than in the control groups, independent of the chemical formulations of GLY; (ii) GLY will present less mutagenicity than the complex commercial mixture; (iii) different test systems and class of vertebrates will present different responses in MN formation after GLY exposure and its formulations; (iv) there are differences among genders; (v) different responses are expected according to the exposure route; (vi) different responses are observed in counting of all erythrocytes or only polychromatic cells; (vii) The damage rate is expected to increase with exposure time; (vii) The damage rate is expected to increase with dose; and (ix) Publication bias is not expected.

2. Materials and methods

2.1. Identification and selection of studies

A search of the electronic databases in "ISI Web of Knowledge®" (http://apps.webofknowledge.com/) and "Science Direct" (http://www.sciencedirect.com/) was conducted. The search was limited to references from 1975 (when the micronucleus test to evaluate genetic damage caused by chemical substances was first described) to June 1st, 2014 and used combinations of the following words: micronucleus, micronucleus test, glyphosate and Roundup®. The reference lists of relevant publications were reviewed to identify additional relevant references. In addition, the "Biblioteca Digital Brasileira de Teses e Dissertações (http://bdtd.ibict.br/), the "Networked Digital Library of Theses and Dissertations" (http://www.ndltd.org/find) and "Cybertesis" (http://www.cybertesis.cl/n-mundo.html) were search with the same key words both in Portuguese, English and Spanish to find dissertations and thesis to find non-peer reviewed references (gray literature). Unpublished regulatory studies were obtained in online supplementary material in papers by Kier and Kirkland (2013a) and were categorized as non-peer reviewed.

All titles and abstracts obtained from the initial search were read to determine the relevance of each publication for the subject of our study. The criteria used to select publications were the following: (1) refers to the formation of micronuclei after exposure to glyphosate; and (2) presents data as means with standard error (SE) or standard deviation (SD) and sample size, the glyphosate formulation used and the exposure time and dose of the glyphosate pesticide. In some cases, the authors of the studies were consulted to obtain additional information or clarification. Studies with insufficient data to determine an estimate of the effects size of glyphosate and the associated confidence interval were excluded.

2.2. Data extraction

A structured table of the data from all of the selected studies was created, with the following information included: citation of authors and year of publication, and tested organism; tested system (mammalian or non-mammalian specie); exposure route; gender; MN endpoints; GLY formulation; literature type; time (in days); dose (mg L^{-1} or mg Kg^{-1}); number of individuals in control group (NC) and experimental group (NE); mean of the control group (XC), mean of the experimental group (XE), standard deviation of the control group (SDC) and standard deviation of the experimental group (SDE).

For inclusion in the meta-analysis, the study must have had an experimental group and an individual control group. For studies that tested more than one exposure time or dose, but only one control group, we opted to: (1) compare the data of the control group to the mean of the data of the experimental groups (exposure time) – the mean variance was adopted as well; or (2) compare the data of the control group with the data of the highest dose tested in the study (dose). The exposure times for all studies were converted to days. The doses were converted to mg/L or mg/kg body weight of product applied to the organism. Specifically in the study by Poletta et al. (2009) with caiman eggs, the highest concentrations of GLY were used divided by the average weight of the eggs – to keep the pattern mg/kg. When the paper presented standard errors (SE), they were converted to SD by formula $SD = SE \times \sqrt{n}$. When both polychromatic erythrocytes and normochromatic MN were presented, only the polychromatic data were used in order to follow the pattern.

The studies were classified according to the literary source from which they were obtained, gray literature (studies not reviewed by peers) and articles reviewed by peers. They were categorized also by the tested product: pure GLY or commercial mixture (Roundup and other brands) and according to the specific formulation based in the commercial name followed by percentage of GLY, if applicable; or only GLY plus its percentage.

2.3. Meta-analytic methodology

A detailed description of the data analysis methodology can be found in Rosenberg et al. (Rosenberg et al., 2000). Thus, only a brief description of the methodology is included here. All calculations were performed using the MetaWin 2.1 program. Meta-analytic viability depends on obtaining an estimate of the effects size for each study (Cooper and Hedges, 1994; Rosenberg et al., 2000). The effect of interest in the present article was the effect exerted by glyphosate on micronuclei formation in various organisms.

We used the response ratio (R) to evaluate the effects size, which consisted of the ratio of the means of the experimental group in relation to those of the control group. We used the natural logarithm of this measure (lnR) because it had preferable statistical properties (Hedges et al., 1999). All methods that estimate the effects size (means, standard deviation, and sample size) vary from $-\infty$ to $+\infty$, where zero is the absence of a difference between the experimental group and the control; negative values indicate that the control group had a higher value than the experimental group; and positive values indicate that the experimental group had a higher value than the control group (Hedges et al., 1999).

The cumulative effects size represents the global dimension of the effect present in every study. When the calculated confidence interval (CI) of the cumulative effects size does not include zero, the cumulative effects size is considered significantly different from zero (e.g., in the present case, a significant CI would indicate that exposure to glyphosate has a significant effect on micronuclei formation). Studies with means and SD equal zero were not included in the statistical analysis, because no effects size could be calculated to them.

2.4. Evaluation of heterogeneity

In addition to the cumulative effects size, it is necessary to determine if the sizes of a set of effects are homogeneous. Thus, we tested the total heterogeneity of the samples, Qt, against a chi-square distribution with $n-1$ degrees of freedom, using the null hypothesis that all of the effects are equal (Hedges et al., 1999). A significant Qt indicates that the variance between the effects size is greater than what was expected by simple sampling error and that other variables are influential.

2.5. Categorical data (group of organisms; test systems; exposure route; gender; MN endpoint; GLY formulation; type of literature)

To verify differences between the results in each category we calculated the global cumulative effects size, E+, and identified its confidence interval (Rosenberg et al., 2000). For each group in every category, E+ represents an estimate of the real cumulative effects size for that study set, which is considered significant if its confidence interval does not bracket zero. An analysis of the difference between the groups was performed observing the values of homogeneity (Q), $Q_{between}$ and Q_{inside}. A significant $Q_{between}$ indicates that there are differences in the cumulative effects size between the groups. A significant Q_{inside} indicates that heterogeneity exists between the effects size that cannot be explained by the model.

2.6. Continuous data (GLY dose/exposure time vs. micronucleus formation)

We examined the relationships between the dose data and exposure times to GLY and its formulations and the formation of micronuclei using a continuous meta-analysis model (Greenland, 1987; Hedges et al., 1999). A weighted least-square regression analysis model was performed to determine relationships between the effects size (lnR) and the independent variables (final dose of GLY and exposure time). This regression model uses the data of the effects size (lnR), the weight of the studies (Var(lnR)) and the independent variable values. A significant coefficient of regression (slope) indicates that the independent variable explains some of the variation in the effects size. Similar to the categorical data model, the total heterogeneity explained by the regression model can be divided into $Q_{between}$ (or Q of the regression) and Q_{inside} (or residual Q). The same criteria used for the categorical data was employed for the data interpretation. The data of final dose of GLY to plot in analysis were obtained by the dose informed in each literature, multiplied by the percentage of GLY in the product used.

2.7. Publication bias

Publication bias is the selective publication of favorable or statistically significant results. To minimize the publication bias resulting from the non-publication of small studies with negative results in peer-reviewed journals, we included articles that were written in Portuguese and Spanish, and theses and dissertations from large universities. We also obtained references not reviewed by peers, and from sponsored regulatory studies.

To exploit distribution data for examining publication bias, we used a funnel plot that indicated the effects size and their variance for all studies. We also performed the Spearman rank correlation coefficient analysis, where a significant correlation between E^* and n (number of individuals) indicates a publication bias, with the effects size being greater in one direction (e.g., positive effect). The fail-safe numbers was calculated too (Rosenberg et al., 2000).

3. Results

3.1. General view of the literature: selection of the references and characteristics of the study

A total relevant number of studies were obtained from the "ISI Web of Knowledge" and "Science Direct" databases and from databases of theses and dissertations. Repeated studies were excluded from the sampling. In addition, a large number of studies were excluded for the following reasons: (i) defined dose of exposure was not presented; (ii) micronucleus test was not performed and (iii) insufficient data for the meta-analyses.

After a thorough scanning, 41 references were selected, 24 of these references were obtained from Online Supplementary Material in Kier and Kirkland (2013b). These 41 references provided 93 data sets with control and experimental groups. Table 1 presents summarized data from the 93 control-experimental studies used in this analysis, including authors, year of publication, test system, exposure route, gender, MN endpoint, glyphosate formulation, literature source, GLY doses, exposure times, number of individuals, and estimate of the effects size (lnR) and its variance [Var(lnR)]. Of 93 experiments, 12 of them had mean of control and treated group as zero, so in these the lnR and Var(lnR) could not be calculated. Consequently, 81 experiments were used in meta-statistical analysis.

In Table 1 above, we can observe that 61 experiments were performed with mice, 24 with fish, 5 with alligators, 2 with amphibian, and only one with onion, summarizing 32 non-mammalian and 61 mammalian test systems. Among the 61 studies with mice, 15 were exposed to GLY by oral form and 49 by peritoneal injection. Of all studies, the most applied exposure route was oral administration, used in exactly half of the studies (46); 26 studies used immersion in water with GLY diluted (22 of them with fishes) and, 17 used intraperitoneal injection. Regarding gender, many studies did not inform the sex of individual, and 14 studies

Table 1
Study summary from relevant publications dealing with GLY and its derivatives exposure and micronucleus formation. NC = number of control individuals; NE = number of experimental individuals (contaminated); XC = control average; XE = experimental group average; SDC = control Standard Deviation; SDE = experimental Standard Deviation; lnR = response ratio natural log; Var(lnR) = variance of lnR; Liter = literature type; PR = peer reviewed; GL = gray literature (not peer reviewed). Note: the data are classified according the dose.

No.	Reference; specie (group)	Test syst.[a]	Rt[b]	Gender[c]	End-points[d]	Formulation[e]	Lit[f]	Time (day)	Dose (mg L^{-1}/ mg Kg^{-1})[g]	NC	NE	XC	XE	SDC	SDE	lnR	Var(lnR)
1	Poletta et al., 2011; *C. latirostris* (Crocodylia)	no	sp.	both	er.	RU66.2%GLY	PR	81	19800	7	6	2.61	5.57	1.138	1.519	0.7580	0.0395
2	Poletta et al., 2011; *C. latirostris* (Crocodylia)	no	sp.	both	er.	RU66.2%GLY	PR	78	19,800	8	7	1.08	4.46	0.537	0.688	1.4182	0.0343
3	Jensen, 1991, mice	mm	or.	M	pc.	GLY98.6%	GL	2	5000	5	5	1.5	1.23	0.700	0.567	−0.1957	0.0858
4	Jensen, 1991, mice	mm	or.	F	pc.	GLY98.6%	GL	2	5000	5	5	1.2	1.33	0.300	0.7	0.1054	0.0676
5	Suresh, 1993; Swiss albino mice	mm	or.	M	pc.	GLY96.8%	GL	2	5000	10	5	6.7	8.80	5.500	1.8	0.2726	0.0758
6	Suresh, 1993; Swiss albino mice	mm	or.	F	pc.	GLY96.8%	GL	2	5000	10	5	4.9	10.40	2.700	4.9	0.7526	0.0748
7	Fox and Mackay, 1996, 1996; CD-1® (ICR) BR (mice)	mm	or.	M	pc.	GLY95.6%	GL	1	5000	5	5	1.6	2.10	0.800	1.6	0.2719	0.1661
8	Fox and Mackay, 1996, 1996; CD-1® (ICR) BR (mice)	mm	or.	F	pc.	GLY95.6%	GL	1	5000	5	5	1.4	2.10	0.700	1.6	0.4055	0.1661
9	Fox and Mackay, 1996; CD-1® (ICR) BR (mice)	mm	or.	M	pc.	GLY95.6%	GL	2	5000	5	5	1.7	2.10	1.300	1.9	0.2113	0.2807
10	Fox and Mackay, 1996; CD-1® (ICR) BR (mice)	mm	or.	F	pc.	GLY95.6%	GL	2	5000	5	5	0.7	0.80	0.600	0.8	0.1335	0.3469
11	Gava, 2000; Swiss albino mice	mm	i.p.	M	pc.	GLY61.27%	GL	2	3024	5	5	0.6	0.70	0.500	1	0.1542	0.5471
12	Gava, 2000; Swiss albino mice	mm	i.p.	F	pc.	GLY61.27%	GL	2	3024	5	5	0.4	0.70	0.500	1	0.5596	0.7207
13	Jones, 1999; CD-1 mice	mm	or.	M	pc.	GLY59.3%	GL	1	2000	5	5	0.2	0.90	0.450	0.42	1.5041	1.0561
14	Jones, 1999; CD-1 mice	mm	or.	M	pc.	GLY59.3%	GL	2	2000	5	5	0.8	0.90	0.970	0.96	0.1178	0.5216
15	Honarvar (2005); NMRI mice	mm	or.	M	pc.	GLY97.73%	GL	1.5	2000	5	5	0.9	1.20	0.600	0.85	0.2877	0.1892
16	Honarvar (2005); NMRI mice	mm	or.	F	pc.	GLY97.73%	GL	1.5	2000	5	5	0.7	0.85	0.800	0.8	0.1942	0.4384
17	Honarvar, 2008; NMRI mice	mm	or.	M	pc.	GLY99.1%	GL	1	2000	5	5	0.7	0.70	0.700	0.4	0.0000	0.2653
18	Honarvar, 2008; NMRI mice	mm	or.	M	pc.	GLY99.1%	GL	2	2000	5	5	0.7	0.80	0.600	0.6	0.1335	0.2594
19	Flügge, 2009; CD rats	mm	or.	M	pc.	GLY98.8%	GL	1	2000	5	5	0.8	0.60	0.600	0.4	−0.2877	0.2014
20	Flügge, 2009; CD rats	mm	or.	F	pc.	GLY98.8%	GL	1	2000	5	5	0.9	0.40	0.200	0.4	−0.8109	0.2099
21	Flügge, 2009; CD rats	mm	or.	M	pc.	GLY98.8%	GL	2	2000	5	5	1.0	0.80	0.900	0.4	−0.2231	0.2120
22	Flügge, 2009; CD rats	mm	or.	F	pc.	GLY98.8%	GL	2	2000	5	5	1.1	0.40	0.700	0.4	−1.0116	0.2810
23	Erexson, 2003a; Crl:CD-1®(ICR)BR mice	mm	or.	M	pc.	GLY36.6%	GL	1	2000	5	5	0.3	0.70	0.200	0.4	0.8473	0.1542
24	Erexson, 2003a; Crl:CD-1®(ICR)BR mice	mm	or.	M	pc.	GLY36.6%	GL	2	2000	5	5	0.00	0.60	0.000	0.4	–	–
25	Erexson, 2003b; Crl:CD-1®(ICR)BR mice	mm	or.	M	pc.	GLY65.2%	GL	1	2000	5	5	0.5	0.40	0.200	0.2	−0.2231	0.0820
26	Erexson, 2003b; Crl:CD-1®(ICR)BR mice	mm	or.	M	pc.	GLY65.2%	GL	2	2000	5	5	0.1	0.20	0.100	0.1	0.6931	0.2500
27	Erexson, 2006; CD-1®(ICR)BR mice	mm	or.	M	pc.	GLY30.3%	GL	1	2000	5	5	0.9	0.80	0.200	0.1	−0.1178	0.0130
28	Erexson, 2006; CD-1®(ICR)BR mice	mm	or.	M	pc.	GLY30.3%	GL	2	2000	5	5	0.4	0.30	0.200	0.2	−0.2877	0.1389
29	Xu, 2008a; Hsd:ICR(CD-1) mice	mm	or.	M	pc.	GLY38.7%	GL	1	2000	5	5	0.4	0.10	0.200	0.2	−1.3863	0.8500
30	Xu, 2008a; Hsd:ICR(CD-1) mice	mm	or.	M	pc.	GLY38.7%	GL	2	2000	5	5	0.00	0.50	0.000	0.5	–	–
31	Xu, 2008b; CD-1®(ICR)BR mice	mm	or.	M	pc.	GLY31.1%	GL	1	2000	5	5	0.2	0.80	0.300	0.9	1.3863	0.7031
32	Xu, 2008b; CD-1®(ICR)BR mice	mm	or.	M	pc.	GLY31.1%	GL	2	2000	5	5	0.3	0.50	0.400	0.4	0.5108	0.4836
33	Xu, 2009a; CD-1®(ICR)BR mice	mm	or.	M	pc.	GLY71.6%	GL	1	2000	5	5	0.3	0.20	0.300	0.4	−0.4055	1.0000
34	Xu, 2009a; CD-1®(ICR)BR mice	mm	or.	M	pc.	GLY71.6%	GL	2	2000	5	5	0.5	0.20	0.900	0.3	−0.9163	1.0980
35	Xu, 2009b; CD-1®(ICR)BR mice	mm	or.	M	pc.	GLY38.5%	GL	1	2000	5	5	0.5	0.30	0.000	0.3	−0.5108	0.2000
36	Xu, 2009b; CD-1®(ICR)BR mice	mm	or.	M	pc.	GLY38.5%	GL	2	2000	5	5	0.2	0.30	0.300	0.4	0.4055	0.8056
37	Xu, 2009c; Hsd:CD-1®(ICR)BR mice	mm	or.	M	pc.	GLY30.9%	GL	1	2000	5	5	0.5	0.30	0.400	0.3	−0.5108	0.3280
38	Xu, 2009c; Hsd:CD-1®(ICR)BR mice	mm	or.	M	pc.	GLY30.9%	GL	2	2000	5	5	0.5	0.00	0.500	0	–	–
39	Negro Silva, 2009; Swiss mice	mm	or.	M	pc.	GLY28.7%	GL	2	2000	6	6	0.6	0.60	0.200	0.2	0.0000	0.0370
40	Flügge, 2010a; NMRI mice	mm	or.	M	pc.	TropM48,36% GLY	GL	1	2000	5	5	1.8	0.80	0.800	0.3	−0.8109	0.0676
41	Flügge, 2010a; NMRI mice	mm	or.	F	pc.	TropM48,36% GLY	GL	1	2000	5	5	1.3	1.40	0.600	1.1	0.0741	0.1661
42	Flügge, 2010a; NMRI mice	mm	or.	M	pc.	TropM48,36% GLY	GL	2	2000	5	5	1.3	2.10	0.400	0.4	0.4796	0.0262
43	Flügge, 2010a; NMRI mice	mm	or.	F	pc.	TropM48,36% GLY	GL	2	2000	5	5	1.6	1.90	0.900	0.7	0.1719	0.0904
44	Flügge, 2010b; Crl (CD)(SD) rat	mm	or.	M	pc.	GLY75.7%	GL	1	2000	5	5	1.1	1.20	0.700	0.6	0.0870	0.1310
45	Flügge, 2010b; Crl (CD)(SD) rat	mm	or.	F	pc.	GLY75.7%	GL	1	2000	5	5	2.0	1.80	1.200	0.6	−0.1054	0.0942
46	Flügge, 2010b; Crl (CD)(SD) rat	mm	or.	M	pc.	GLY75.7%	GL	2	2000	5	5	1.2	0.40	0.300	0.2	−1.0986	0.0625
47	Flügge, 2010b; Crl (CD)(SD) rat	mm	or.	F	pc.	GLY75.7%	GL	2	2000	5	5	1.1	0.60	1.000	0.7	−0.6061	0.4375
48	Negro Silva, 2011; Swiss mice	mm	or.	M	pc.	GLY49.935%	GL	2	2000	6	6	0.8	0.60	0.200	0.2	−0.2877	0.0289
49	Dimitrov et al., 2006; C57BL mice	mm	or.	M	pc.	RU??	PR	2.5	1080	8	8	0.5	0.54	0.339	0.294	0.0770	0.0947
50	Durward, 2006; albino Crl:CD-1TM(ICR)BR mice	mm	i.p.	M	pc.	GLY95.7%	GL	1	600	7	7	0.6	1.90	0.600	0.7	1.1527	0.1622
51	Durward, 2006; albino Crl:CD-1TM(ICR)BR mice	mm	i.p.	M	pc.	GLY95.7%	GL	2	600	7	7	1.0	0.90	1.200	1.1	−0.1054	0.4191
52	Marques, 1999; Swiss albino mice	mm	i.p.	M	pc.	GLY95.49%	GL	2	562.5	5	5	0.4	0.40	0.500	0.9	0.0000	1.3250
53	Marques, 1999; Swiss albino mice	mm	i.p.	F	pc.	GLY95.49%	GL	2	562.5	5	5	0.8	0.60	0.800	0.5	−0.2877	0.3389

Table 1 (*continued*)

No.	Reference; specie (group)	Test syst.[a]	Rt[b]	Gender[c]	End-points[d]	Formulation[e]	Lit[f]	Time (day)	Dose (mg L^{-1}/ mg Kg^{-1})[g]	NC	NE	XC	XE	SDC	SDE	lnR	Var(lnR)
54	Bolognesi et al., 1997; Swiss CD1 mice	mm	i.p.	M	pc.	RU30.4%GLY	PR	0.5	450	6	6	0.75	2.65	0.460	0.8	1.2622	0.0779
55	Mañas et al., 2009; Balb C mice	mm	i.p.	both	er.	GLY96%	PR	2	400	5	5	3.8	13.0	0.800	3.5	1.2299	0.0234
56	Grisolia, 2002; Swiss mice	mm	i.p.	both	pc.	RU48%GLY	PR	2	200	8	8	1.5	0.80	0.800	1	−0.6286	0.2309
57	Nascimento and Grisolia, 2000; mice	mm	i.p.	n.i.	pc.	RU?	PR	2	200	6	6	1.5	0.80	0.050	0.01	−0.6286	0.0002
58	Grisolia, 2002; *Tilapia rendalli* (fish)	no	i.p.	n.i.	er.	RU48%GLY	PR	4	170	9	7	0.4	3.20	0.400	1.1	2.0794	0.1280
59	Nascimento and Grisolia, 2000; *O. niloticus* (fish)	no	i.p.	n.i.	er.	RU?	PR	4	170	8	8	0.8	0.50	0.200	0.1	−0.4700	0.0128
60	Bosch et al., 2011; *Odontophrynus cordobae* (Amphibia)	no	im.	both	er.	RU48%GLY	PR	5	100 a.i	5	5	0.4	0.88	0.180	0.33	0.7885	0.0686
61	Bosch et al., 2011; *Rhinella arenarum* (Amphibia)	no	im.	both	er.	RU48%GLY	PR	5	100 a.i	5	5	0.3	0.46	0.090	0.16	0.4274	0.0422
62	Costa, 2008; Swiss albino mice	mm	i.p.	M	pc.	GLY98%	GL	1	62.5	5	5	0.00	0.30	0.000	0.7	–	–
63	Costa, 2008; Swiss albino mice	mm	i.p.	F	pc.	GLY98%	GL	1	62.5	5	5	0.00	0.00	0.000	0	–	–
64	Prasad et al., 2009; Swiss albino mice	mm	i.p.	M	pc.	RU>41%GLY	PR	3	50	5	5	1.18	8.48	0.067	0.2012	1.9722	0.0008
65	Prasad et al., 2009; Swiss albino mice	mm	i.p.	M	pc.	RU>41%GLY	PR	2	50	5	5	1.10	8.25	0.022	0.0894	2.0149	0.0001
66	Prasad et al., 2009; Swiss albino mice	mm	i.p.	M	pc.	RU>41%GLY	PR	1	50	5	5	1.24	6.86	0.022	0.0894	1.7106	0.0001
67	Zoriki Hosomi, 2007; Swiss mice	mm	or.	M	pc.	GLY98.01%	GL	2	30	6	6	0.6	1.40	0.300	0.4	0.8473	0.0553
68	González et al., 2013; *C. latirostris* (Crocodylia)	no	im.	n.i.	er.	RU66.2%GLY	PR	60	26	12	12	0.43	2.09	0.450	0.935	1.5811	0.1081
69	Poletta et al., 2009; *C. latirostris* (Crocodylia)	no	t.a.	n.i.	er.	RU66.2%GLY	PR	66.5	24.07	10	12	1.86	7.75	0.822	3.152	1.4271	0.0333
70	Çavas and Könen, 2007; *Carassius auratus* (fish)	no	im.	n.i.	er.	RU48%GLY	PR	6	15 a.i	5	5	3.0	18.70	1.923	1.252	1.8299	0.0831
71	Çavas and Könen, 2007; *Carassius auratus* (fish)	no	im.	n.i.	er.	RU48%GLY	PR	4	15 a.i	5	5	2.88	16.50	1.252	2.638	1.7456	0.0429
72	Çavas and Könen, 2007; *Carassius auratus* (fish)	no	im.	n.i.	er.	RU48%GLY	PR	2	15 a.i	5	5	3.17	12.20	1.073	2.817	1.3477	0.0336
73	Poletta et al., 2009; *C. latirostris* (Crocodylia)	no	t.a.	n.i.	er.	RU66.2%GLY	PR	69	14.81	9	10	2.09	5.83	0.630	3.194	1.0259	0.0401
74	Cavalcante et al., 2008; *Prochilodus lineatus* (fish)	no	im.	n.i.	er.	RU41%GLY	PR	4	10	9	12	0.18	0.11	0.330	0.277	−0.4925	0.9024
75	Cavalcante et al., 2008; *Prochilodus lineatus* (fish)	no	im.	n.i.	er.	RU41%GLY	PR	1	10	9	12	0.07	0.05	0.150	0.173	−0.3365	1.5102
76	Cavalcante et al., 2008; *Prochilodus lineatus* (fish)	no	im.	n.i.	er.	RU41%GLY	PR	0.25	10	8	10	0.00	0.00	0.000	0	–	–
77	Moreno, 2011; *Prochilodus lineatus* (fish)	no	im.	n.i.	er.	GLY96%	GL	4	5	4	5	0.00	0.10	0.000	0.224	–	–
78	Moreno, 2011; *Prochilodus lineatus* (fish)	no	im.	n.i.	er.	RUT48%GLY	GL	4	5	4	5	0.00	0.00	0.000	0	–	–
79	Moreno, 2011; *Prochilodus lineatus* (fish)	no	im.	n.i.	er.	GLY96%	GL	1	5	6	6	0.10	0.10	0.245	0.244	0.0000	2.0000
80	Moreno, 2011; *Prochilodus lineatus* (fish)	no	im.	n.i.	er.	RUT48%GLY	GL	1	5	6	6	0.00	0.00	0.000	0	–	–
81	Moreno, 2011; *Prochilodus lineatus* (fish)	no	im.	n.i.	er.	GLY96%	GL	0.25	5	6	5	0.00	0.00	0.000	0	–	–
82	Moreno, 2011; *Prochilodus lineatus* (fish)	no	im.	n.i.	er.	RUT48%GLY	GL	0.25	5	6	5	0.00	0.00	0.000	0	–	–
83	Ferraro, 2009; *Rhamdia quelen* (fish)	no	im.	both	er.	RU48%GLY	GL	10	3.16	20	20	2.65	3.30	1.950	2.0733	0.2194	0.0468
84	Ferraro, 2009; *Cyprinus carpio* (fish)	no	im.	both	er.	RU48%GLY	GL	10	3.16	20	20	2.90	3.95	1.590	2.593	0.3090	0.0366
85	Ferraro, 2009; *Astyanax sp.* (fish)	no	im.	both	er.	RU48%GLY	GL	10	3.16	20	20	4.1	4.83	2.220	3.233	0.1639	0.0371
86	Guilherme et al., 2010; *Anguilla anguilla* (fish)	no	im.	n.i.	er.	RU30.8%GLY	PR	3	0.116	6	6	0.00	0.00	0.000	0		
87	Guilherme et al., 2010; *Anguilla anguilla* (fish)	no	im.	n.i.	er.	RU30.8%GLY	PR	1	0.116	6	6	0.60	0.80	0.980	1.20	0.2877	0.8196
88	Ghisi and Cestari, 2013; *Corydoras paleatus* (fish)	no	im.	both	er.	RU48%GLY	PR	6	0.00667	20	21	2.80	3.475	2.587	4.010	0.2159	0.1061
89	Rossi et al., 2011; *Astyanax* sp. (fish)	no	im.	n.i.	er.	RU48%GLY	PR	4	0.0069834	12	12	8.083	19.33	4.542	13.425	0.8721	0.0665
90	Francabandeira, 2007; *O. niloticus* (fish)	no	im.	n.i.	er.	RU48%GLY	GL	97	0.005	6	6	0.70	3.70	1.200	1.9	1.6650	0.5337
91	Francabandeira, 2007; *O. niloticus* (fish)	no	im.	n.i.	er.	RU48%GLY	GL	151	0.005	6	6	0.70	4.30	0.500	1.4	1.8153	0.1027
92	Francabandeira, 2007; *O. niloticus* (fish)	no	im.	n.i.	er.	RU48%GLY	GL	171	0.005	6	6	0.3	2.3	0.5	2.5	2.0369	0.6599
93	Krüger, 2009; *Allium cepa* (Plant)	no	im.	vegetal	plant	Glifosato48% GLY	GL	2	0.004	6	6	0.4	14	0.5	2	3.5553	0.2638

[a] Teste system: no, non-mammalian; mm, mammalian.
[b] Rt, Route of exposure: sp., spraying; or., oral; i.p., intraperitoneal injection; im., immersion.
[c] gender: M, male; F, female; n.i., not identified; both, male and female, without segregation of results.
[d] endpoints: er., erythrocytes; pc., polychromatic erythrocytes.

[e] Formulation: Ru, Roundup; GLY, glyphosate; RUT, Roundup Transorb.
[f] Literature type: PR, peer-reviewed reference; GL, non peer-reviewed reference.
[g] Dose informed in each reference: a.i., active ingredient.

used exclusively females. Almost half studied males, justifying that there was no difference between sexes in the range finding test. About MN endpoint, 32 studies counted micronucleated erythrocytes and 60 informed polychromatic cells.

The forest plot of all results showed that the grand mean of lnR is a positive value (mean effects size E+ = 1.37), and this was also observed in most of the studies individually (Fig. 1). This indicates that the groups exposed to glyphosate formulations generally have greater rates of formation of micronuclei than the non-exposed groups.

3.2. Magnitude of the global effects of exposure to glyphosate versus micronuclei frequency

Based on the analysis of all of the studies collectively, exposure to glyphosate was found to cause an overall effect on micronuclei formation (X^2 *p-value* = 0.00000; df = 80). The cumulative effects size presented a positive value indicating that the experimental groups had higher value than the control groups (E+ = 1.37), with a confidence interval that did not include zero (95% CI = 1.3563 to 1.3807), clearly significant.

3.3. Evaluation of total heterogeneity

The total heterogeneity of the samples (Qt = 25869.79) was significant when tested against a X^2-distribution (d.f. = 80; p = 0.000). A significant Qt indicates that the variance among effect sizes is greater than expected by sampling errors and it implies that other explanatory variables should be investigated. One possible explanation for this result is that there may be some underlying structure data. So we tested the data set against categorical and continuous factors.

3.4. Incorporating categorical factors

The data set were categorized by test system (mammalian × non-mammalian) and group of organisms, where the non-mammalian were individualized in different classes of vertebrates. In this last categorization, onion was not included, because groups with fewer than 2 valid studies were eliminated from the analysis. In Fig. 2A, we can see significant differences on mammalian and non-mammalian responses (Q between p = 0.000). The mammalians presented the mean effects size E+ = 1.379 (95% CI = 1.366 to 1.391), while the mean effects size in the non-mammalian was E+ = 0.740 (95% CI = 0.641 to 0.840). In the categorization by class of vertebrates, significant differences were found in comparison of all data (Q between = 224.349, p = 0.000). In Fig. 2B, we can see the clear formation of two groups: crocodilians (E+ = 1.210) are very close to mice (E+ = 1.379) and form a statistically homogeneous group (p = 0.066). On the other hand, fish (E+ = 0.518) and amphibian (E+ = 0.565) form another separated group also statistically homogenous (p = 0.7838), with less mean effects sizes.

The data set was categorized by exposure route as well (Fig. 3A). Significant differences on methods of GLY application were found (Q between = 879.774, p = 0.000). The highest mean effects size was shown in intraperitoneal injections (E+ = 1.396) and it means that the highest difference between control and experimental group was observed in this exposure route, with a significant difference (95% CI = 1.383 to 1.410). Topic application and spraying were the specific methods used exclusively in alligators' eggs, and both showed positive means (respectively, E+ = 1.245 and E+ = 1.111). Immersion showed E+ = 0.895 and also a significant difference among treatments (95% CI = 0.758 to 1.033). In this categorization, it is interesting to observe that the oral exposure presents no significant difference between control and treated groups (E+ = −0.003; 95% CI = −0.101 to 0.095).

In categorization by gender (Fig. 3B), the highest mean effects size was presented in males, with a significant difference between treatments (E+ = 1.833; 95% CI = 1.819 to 1.847). Similar results were obtained in studies with both sexes (E+ = 0.674; CI = 0.523 to 0.825). It is evident in this analysis that there are significant differences between females and males, including different responses obtained by each one. In females E+ = 0.088 and 95% CI = −0.153 to 0.328 − no significant CI, *i.e.*, did not present difference between control and groups exposed to GLY. The studies that did not identify

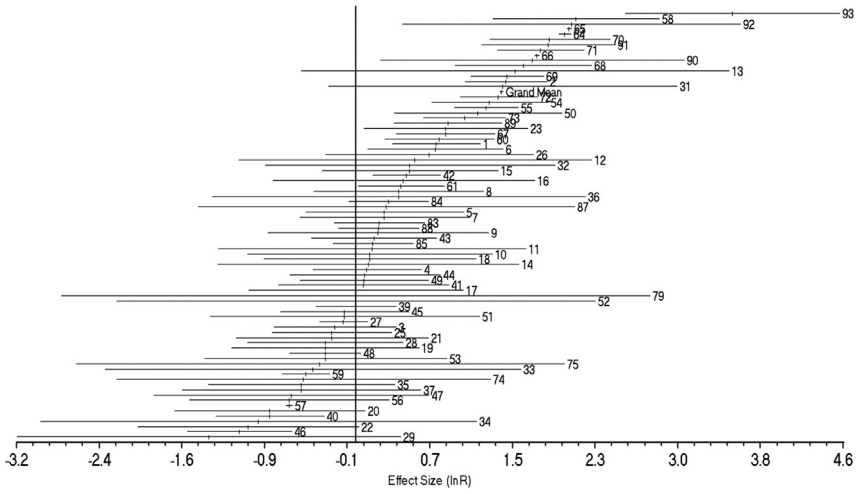

Fig. 1. Forest plot of studies evaluating micronucleus frequency in glyphosate exposure, arranged by effects size. Note: estimator of response ratio (effects size − lnR) and 95% confidence interval (CI) of each experiment included in the meta-analysis are presented. The number beside the bars represents the reference number of each experiment as in Table 1. Grand Mean is the overall mean effects size of all studies.

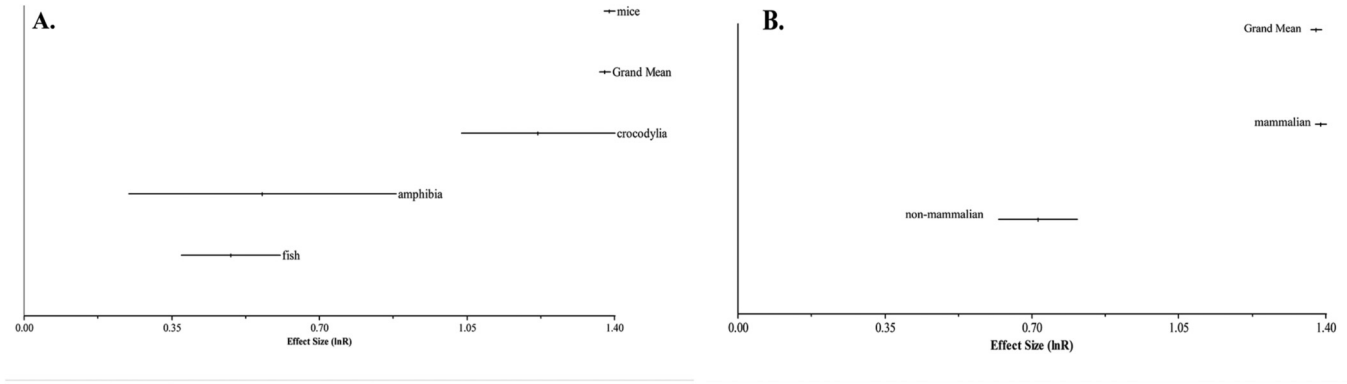

Fig. 2. Forest plot representing the categorization by (A) group of vertebrate; and (B) test system. The bars represent means of the effects size and 95% confidence interval. Grand Mean is the overall mean effects size of all studies.

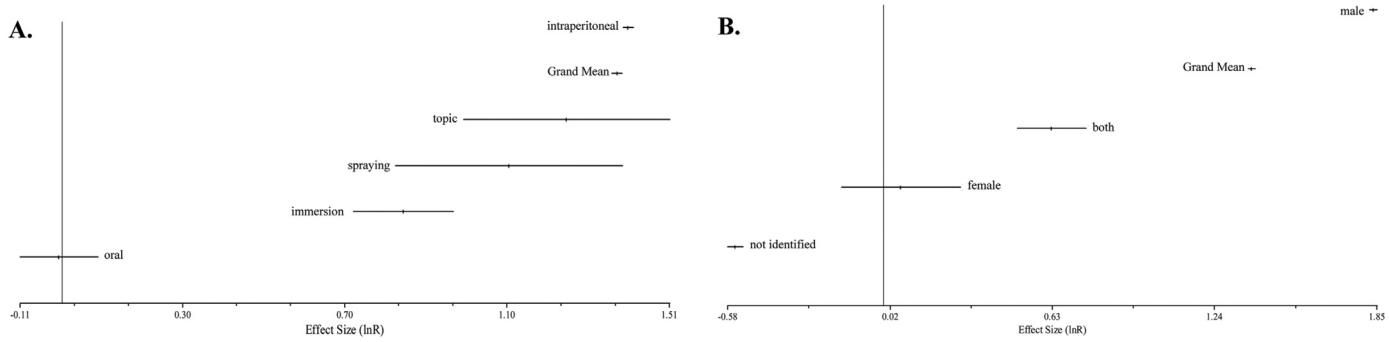

Fig. 3. Forest plot representing the categorization by (A) exposure route; and (B) gender. The bars represent means of the effects size and 95% confidence interval. Grand Mean is the overall mean effects size of all studies.

the gender of individuals presented a negative significant mean (E+ = −0.557; 95%CI = −0.587 to −0.527).

The data were structured by MN endpoints, as shown in Fig. 4. There are differences between mean effects size: erythrocytes (E+ = 0.762) and polychromatic cells (E+ = 1.379), but both presented the same results of overall response – their confident limits do not bracket zero, i.e. there are differences between treated and control groups.

The formulations were separated in application of pure glyphosate or the complex mixture in commercial formula – Roundup and related products. There were 46 experiments testing pure GLY and 35 with commercial mixtures (30 with Roundup®, 4 TropM® and 1 Glifosato®). In Fig. 5A, we can see that the mean effects size for Roundup and commercial mixture (E+ = 1.388) was higher than the mean for Glyphosate exposure (E+ = 0.121). Both categories show significant results: Roundup (95% IC = 1.375 to 1.400) and GLY (0.021–0.221).

Stratification by type of literature was significant (d.f. = 1; $Q_{between}$ = 842.781; p = 0.000), *i.e.*, there was difference between peer-reviewed studies and non-peer-reviewed studies (Fig. 5B). The highest mean was observed in peer-reviewed literature (E+ = 1.394) in comparison to non-peer reviewed ones

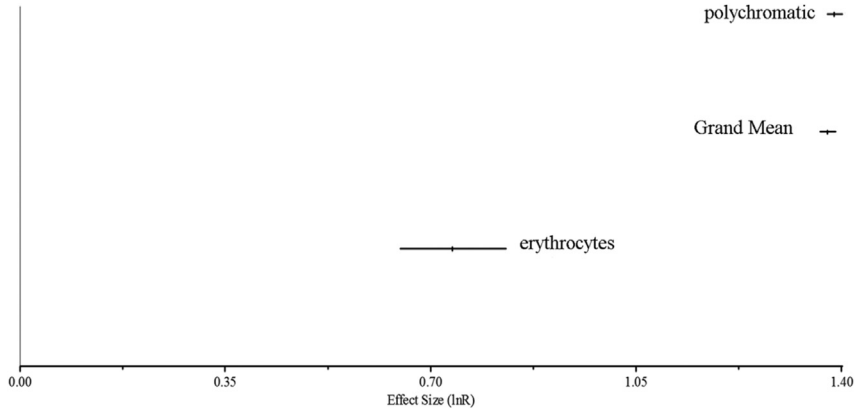

Fig. 4. Forest plot of data set categorized by endpoints. The bars represent means of the effects size and 95% confidence interval. Grand Mean is the overall mean effects size of all studies.

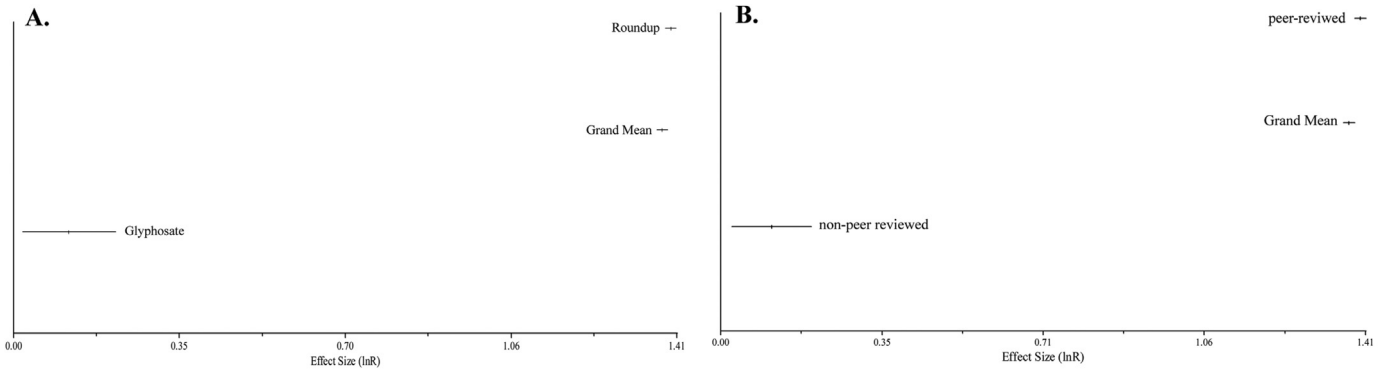

Fig. 5. Forest plot representing the categorization by (A) product tested: Glyphosate or Roundup. Note: in 35 experiments grouped as 'Roundup', four of them used the commercial name TropM® and one was Glifosato®; and (B) type of literature. The bars represent means of the effects size and 95% confidence interval. Grand Mean is the overall mean effects size of all studies.

(E+ = 0.114). Nevertheless similar results were observed in both data set with no CI bracketing zero (peer-reviewed 95%CI = 1.381 to 1.407; non-peer-reviewed 95%CI = 0.027 to 0.202) i.e., for both literature types, the experimental groups had larger values than the control groups.

3.5. Incorporating continuous factors

3.5.1. Relationship between exposure time and the effects size

A negative relationship between exposure time and the effects size could be noticed. The slope (as well as the $Q_{regression}$ heterogeneity) was significant (p = 0.00002), it implies that the independent variable explains a significant portion of the variation in effects size. We can see in Fig. 6A that the inclination is a negative value (slope = −0.0046), indicating that the time is inversely proportional to the mean effects size. The Q_E calculates the amount of residual error heterogeneity, and for this analysis Q_E was significant (p = 0.000), implying that there is still heterogeneity among effects size that was still not explained by the model.

When again we separate by group of vertebrate, we can see that mice + crocodiles presented a significant negative slope (slope = -0.0064; p = 0.00000). On other hand, fish + amphibian presented a significant positive result (slope = 0.0090; p = 0.00001).

3.5.2. Relationship between GLY doses and the effects size

When we plot all data set together, the weighted least-square

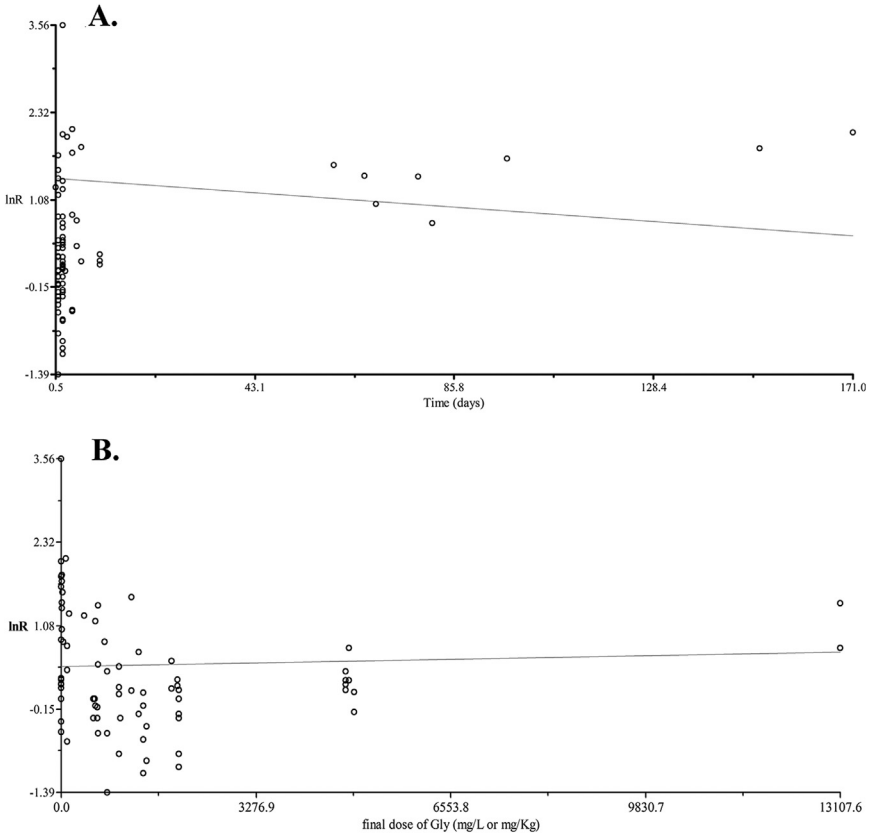

Fig. 6. Regression Graphic showing the relationship between the effects size (lnR) and (A) time; and (B) glyphosate final doses (%GLY × dose).

regression analysis showed that there was no relationship between the effects size and GLY dose (mg/L or mg/kg) because the inclination (as well as the $Q_{regression}$ heterogeneity) was not significant (slope = 0.000; p = 0.1201). Q_E was significant (p = 0.000), indicating that not all heterogeneity was explained by the model (Fig. 6B). So we decided to plot the data in the two homogeneous groups of organisms (mice + crocodiles; and fish + amphibians). When only mice and crocodiles were tested in the regression, the slope was positive and significant (p = 0.00006), indicating a positive correlation between dose and effects size in these organisms. On the other hand, when fish and amphibians were tested, the slope was not significant (p = 0.659), indicating no relation between the real dose of GLY and the effects size.

3.6. Publication bias

Visual inspection of the funnel plot (Fig. 7) did not show any clear asymmetry arising from the lack of studies with smaller effects size. Publication bias tends to skew the shape of the funnel or the points' distribution within the funnel. The Spearman rank correlation coefficient (effect versus sample size), a statistic method to test bias, did not reveal a significant correlation (p = 0.876), indicating that there was no publication bias. The fail-safe numbers were 473.2 (by Orwin's Method) and 89631.1 (by Rosenthal's Method). They are the numbers of non-significant, unpublished or missing studies that would need to be added to meta-analysis to change the results from significant to non-significant. As these numbers are larger in comparison to the number of observed studies, the observed results can be treated as a reliable estimate of the true effect.

4. Discussion

In recent years, a large number of articles have reported the evaluation of damaging effects caused by glyphosate in various organisms using different test systems. However, to the best of our knowledge, this study is the first meta-analysis that combined data about micronuclei formation frequency with the exposure of different organisms to the herbicide glyphosate and glyphosate formulations. We believe that systemic reviews of the literature and meta-analyses of data in the literature (as presented here) are useful tools that help integrate information and increase the understanding of scientific results.

The general results of this meta-analysis suggest a positive association between GLY and its formulations and micronuclei formation, suggesting that they are potentially mutagenic. This conclusion is in agreement with those of some narrative reviews and individual studies (Cox, 1998; Guilherme et al., 2010; Mañas et al., 2009; Poletta et al., 2009). The studies included in this meta-analysis evaluated a variety of species (mice, anurans, fish and crocodiles), and this can be a reason why our data showed significant heterogeneity. Thus, the experiments were categorized in groups of similar taxa, where each group represents a more homogeneous group. The fish were grouped with anurans, and mice formed a group with crocodile, which were statistically homogeneous. However, both groups presented positive results for mutagenicity of GLY.

When the data were grouped in test systems by mammalian or non-mammalian, all mice were grouped in mammalian group and they were statistically different from non-mammalian group, but both with significant difference among control and exposed group to GLY.

The US Environmental Protection Agency (USEPA) has published that more than 400 formulated products containing GLY as their active ingredient have been registered in more than 100 countries, with Roundup® (RU) as the main trademark and sales leader (USEPA, 2009). This product (RU) is so widely used globally that some researchers who have evaluated its toxicity defend the use of the commercial formulation in experiments, as GLY enters the environment through the use of RU (Cavalcante et al., 2008; Ghisi and Cestari, 2013; Poletta et al., 2009). On the other hand, other researchers prefer to use the active ingredient (GLY) alone or its main product of degradation (aminomethylphosphonic acid – AMPA) for experiments. In some cases, researchers test several of these chemical substances at the same time (Moreno, 2011; Rank et al., 1993). Different results may be obtained according to the substance evaluated. In our meta-analysis, we found a significant difference between experiments testing only GLY and experiments with the commercial complex mixture (RU and two other brands). The complex mixture presented higher effects size than GLY, indicating more mutagenic effects. This has already been reported in several studies, and it can be attributed to surfactant products that are added to the commercial products to enable better penetration of GLY through leaf surfaces (Kier and Kirkland, 2013a). Surfactant effects provide a very plausible mechanism for observation of commercial mixtures of GLY inducing DNA damage responses, which can be associated with cytotoxic exposures (Kier and Kirkland, 2013a).

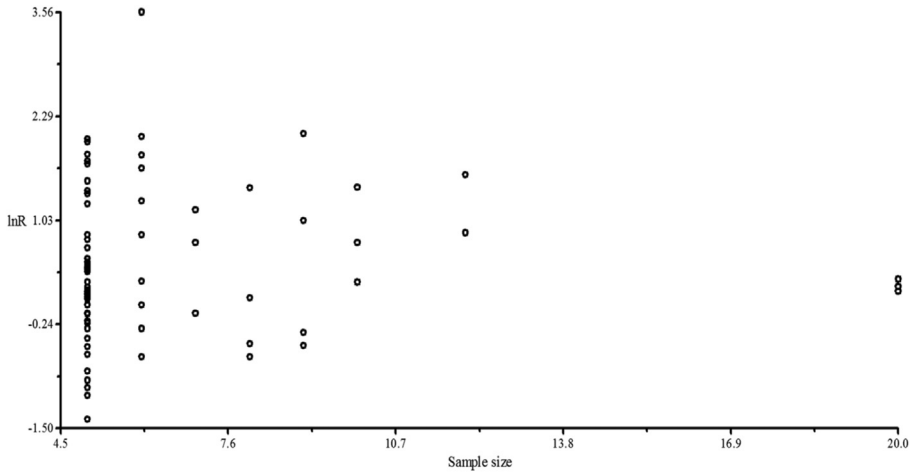

Fig. 7. Funnel plot showing the data distribution in the correlation between the effects size (lnR) and magnitude of the sample in the control group (sample size).

Interesting differences were noticed in responses according to different exposure routes. Exposure by oral methods showed no significant differences between control and treated groups. On the other hand, the highest value was observed in intraperitoneal injection. Some authors and regulatory agencies consider this exposure an unphysiological route and it is not recommended to the safe evaluation of chemicals (Kier and Kirkland, 2013a). The USEPA consider that GLY is of relatively low oral and dermal acute toxicity (USEPA, 1993). The exposure by water immersion with GLY diluted showed an increase in MN formation. This route was tested in fish, crocodiles and frogs, and it can be considered the most typical exposure route for these organisms. Topic application and spraying were methods tested in caiman eggs, and both presented evidences of mutagenicity of sub-chronic exposure to Roundup.

In segregation by gender, from 81 experiments, 53 presented clearly the sex of individual, in which 40 used males; 11 studies used both sexes (without differentiated results by sex) and 17 studies did not identify which sex was used. Several authors, especially in regulatory studies, inform that only males were used because there are no sex difference in toxicity rangefinder (Erexson, 2006, 2003a, 2003b; Xu, 2009b, 2008a, 2008b). However, in our meta-analysis, we found statistic differences on responses of male and females. In this, the females presented no difference for control or treated groups, and males presented a significant difference. In a study by Jasper et al. (2012) with mice exposed orally to RU, males were more responsive than female as well. Males presented an increase in lipid peroxidation at both dosages tested, and a NPSH decrease in the hepatic tissue, whereas in females significant changes in these parameters were observed only at the highest dose rate.

In the categorization by type of cell counted, studies that counted 'polychromatic erythrocytes' presented higher values than those that counted 'erythrocytes'. The use of segregation of immature erythrocytes (polychromatic) is a practice mostly applied for those studies testing mice. As seen in Table 1, all studies that assess polychromatic cells were developed with mice. It is a good practice because the assessment of immature erythrocytes can evaluate more precisely acute exposure (few days) – and the MNs detected can really be attributed to chemical treatment. If all the erythrocytes are counted many of them may have been matured before treatment, underestimating the total number of MN.

Publication bias is a serious concern in meta-analytic studies, and it arises from preferential publication in peer-reviewed journals of studies with results that are positive, statistically significant or have particularly strong effects. This may, for example, lead to an overestimation of a particular effect (Timmer, 2011), producing, inevitably, an imbalance in the scientific literature (Abaid et al., 2007). To minimize this type of bias, authors of systematic reviews do extensive research using large databases and frequently expand their searches to the gray literature (Martin et al., 2005). We also searched the gray literature to obtain complementary data to that of the published, peer-reviewed literature. Non-peer-reviewed references are generally less credible, but in our study, both presented similar results – an increased MN formation in groups exposed to GLY and its formulations. Nevertheless the difference between the two types of literature was remarkable, while references with more positive results are concentrated in peer-reviewed literature, and least means are in non-peer reviewed references. Probably because of the our care for searching peer-reviewed and not-peer reviewed literature, our data did not present publication bias, as seen in the funnel plot shape and in the non-significant Sperman Rank correlation coefficient. The fail-safe numbers were considerable high, and thus, our results can be treated as a reliable estimate of the true effect.

On the other hand, it was not possible to establish a clear relationship between the effects size and GLY real dose when all data set were tested, or when only fish and amphibians were tested. When we grouped only mice and crocodiles, a positive significant relationship between dose and effects size was found, that is, there is an increase in MN formation according to the increase of the real dose of GLY. Our results were similar to those obtained by individual studies from Poletta et al. (2009), González et al. (2013) and Bosch et al. (2011), which found a concentration-dependent effect on the frequency of micronuclei, showing that higher Roundup® doses caused more DNA damage.

In our meta-analysis, the absence of a relationship between the mutagenic data and GLY final concentration found by the linear regression analysis of the complete data set may be attributed to the variability in the responses different genera and species (Bosch et al., 2011). We plotted data of various species belonging to diverse taxonomic groups (mice, anurans, fish and crocodiles), and therefore, different sensitivities were expected. Some of these species probably present a lower response to greater doses and times of exposure. Moreover, we must acknowledge the limitations of the meta-analysis in examining dose response relationships when studies employ such a variety of test systems as well as treatments ranging from external water exposure for aquatic organisms to intraperitoneal injection, and some other variations in laboratory protocols.

A negative relationship between exposure time and effects size was found in our meta-analysis. It means that in the course of exposure time, the MN formation is decreased. It can be explained by the adaptation of detoxification mechanisms and the metabolization of xenobiotics and repair of DNA damage along the time of exposure. Here again, we must consider the variation among different species, and acknowledge the limitation of the meta-analytic method in examining exposure time/effect relationships because of the wide variety of exposures and different micronucleus protocols employed. Some exposures were continuous (e.g. exposure in water for aquatic species) while others were discrete, single or two doses, as gavage or intraperitoneal injection.

5. Conclusion

The present study provides support for the hypothesis that exposure to the pesticide glyphosate and its formulations increases micronucleus formation. In several categorizations, we can see different responses according to the test system, the group of animals tested, and the type of cells analyzed (polychromatic erythrocytes or all erythrocytes). For all categories above, the results were significant to MN formation. In segregation by gender, we found that males are more responsive than females – since females presented no difference between control groups and groups treated with GLY and its formulations. In separation of data in pure GLY or commercial complex mixture, we can see that they affect differently the MN rate, with higher MN formation observed when the mixture is tested. About the exposure routes, the intraperitoneal injection showed the higher mutagenicity, while the oral exposure presented no difference between treatment groups. It was not possible to establish a clear relationship between effects size and exposure dose when all data were considered, but when only mice + crocodiles were analyzed, a positive significant relationship was found. There was negative correlation between MN formation and GLY exposure time, with different responses in different groups of organisms.

Conflict of interest statement

The author's affiliation is as shown on the cover page. The authors are solely responsible for the analyses and preparation of this

manuscript; the opinions and conclusions are those of the authors and are not necessarily those of the sponsoring entity. The authors declare that there are no conflicts of interest. Universidade Estadual de Maringá (UEM) and Universidade Tecnológica Federal do Paraná (UTFPR) are Brazilian universities dedicated to teaching, research and extension of knowledge.

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