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Short communication

# The teratogenic potential of the herbicide glyphosate-Roundup® in Wistar rats

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**Abstract**

The aim of this study was to assess the teratogenicity of the herbicide glyphosate-Roundup® (as commercialized in Brazil) to Wistar rats. Dams were treated orally with water or 500, 750 or 1000 mg/kg glyphosate from day 6 to 15 of pregnancy. Cesarean sections were performed on day 21 of pregnancy, and number of corpora lutea, implantation sites, living and dead fetuses, and resorptions were recorded. Weight and gender of the fetuses were determined, and fetuses were examined for external malformations and skeletal alterations. The organs of the dams were removed and weighed. Results showed a 50% mortality rate for dams treated with 1000 mg/kg glyphosate. Skeletal alterations were observed in 15.4, 33.1, 42.0 and 57.3% of fetuses from the control, 500, 750 and 1000 mg/kg glyphosate groups, respectively. We may conclude that glyphosate-Roundup® is toxic to the dams and induces developmental retardation of the fetal skeleton.


*Keywords:* Teratogenicity; Herbicide-Roundup®; Glyphosate; Polyoxyethyleneamine; Rats; Skeletal alterations

## 1. Introduction

Glyphosate is the active ingredient of Roundup®, marketed as a non-selective, broad spectrum, post-emergence herbicide. It is used to control weeds in emerged grasses, broad-leaf weeds, pastures and rice, corn and soy plantations (Smith and Oehme, 1992). Technical glyphosate has been tested for acute, chronic and reproductive toxicity such as embryotoxicity and/or teratogenicity.

Acute studies have reported that products containing glyphosate and polyoxyethyleneamine (the surfactant) may be more toxic than glyphosate alone (Adam et al., 1997). The $LD_{50}$ of oral acute glyphosate for rats and rabbits ranges from 4 to 6 g/kg (Atkinson, 1985), while the $LD_{50}$ of the polyoxyethyleneamine for rats ranges from 1 to 2

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g/kg (Sawada et al., 1988). Two chronic administration studies using technical glyphosate were conducted on rats, one in 1979–1981 and the other in 1988–1990 (WHO, 1994): the animals received 3, 10 and 32 mg/kg per day in the first study and 100, 410 and 1060 mg/kg per day in the second. In the first study a significant increase in the incidence of interstitial cell tumors was observed in rat testes; however, the absence of the same effect with the higher doses used in the second study was the basis to exclude glyphosate from the carcinogenic category (WHO, 1994).

Teratogenicity studies showed that oral administration of high doses of technical glyphosate during pregnancy (3500 mg/kg to Charles River COBS CD rats from day 6 to 19 of pregnancy and 350 mg/kg to rabbits from day 6 to 27 of pregnancy) caused maternal toxicity. The major effects observed were mortality of the dams, and increased number of fetuses with reduced ossification of sternebra (WHO, 1994). Since the acute toxicity of glyphosate is increased when the substance is combined with polyoxyethyleneamine, the aim of the present study was to assess the potential teratogenic effects (reproductive toxicity protocols: segment II; EPA, 1996) of subchronic administration of Roundup®, as marketed in Brazil (36% glyphosate and 18% polyoxyethyleneamine) to Wistar rats.

## 2. Materials and methods

### 2.1. Chemicals

The Roundup® formulation (lot: BS 1096/98, Monsanto of Brazil) consisting of 360 g/l glyphosate (*N*-phosphonomethylglycine) and 18% (w/v) polyoxyethyleneamine (the surfactant) was used. The solutions of the Roundup® formulation were prepared by the addition of appropriate volumes of distilled water.

### 2.2. Animals

Adult male and virgin female Wistar rats (90 days old, 200–280 g) bred in the animal facilities of our own Department were used. All breeding phases and all experiments conformed to the rules of the Ethics and Animal Experimentation Committee of our institution. All animals were housed in polyethylene ($65 \times 25 \times 15$ cm) home cages, with sawdust-covered floors. Animals were maintained in a colony room at $22 \pm 2$ °C under conditions of controlled humidity and on a 12 h-light/dark cycle (lights on from 09:00 to 21:00 h), with free access to standard laboratory rat chow and water.

### 2.3. Mating

Three female rats were placed in a cage with one male during the dark period (between 21:00 and 09:00 h) for mating. On the subsequent morning (09:00 h), vaginal smears were obtained from all females and examined. Females showing sperm were housed in individual cages, and this was considered the 0 day of pregnancy (Paumgartten et al., 1997).

### 2.4. Treatment

Sixty pregnant rats were divided into four groups ($n = 15 \pm 1$ per group). The control group received distilled water and the experimental groups received 500, 750 or 1000 mg/kg glyphosate-Roundup® diluted in water. The dosing regimen was based on no observed adverse effect level (NOAEL) for developmental toxicity in rats (maternal and fetal effects), which was 1000 mg/kg glyphosate (Williams et al., 2000).

Treatments were administered orally by gavage in a volume of 10 ml/kg from day 6 up to 15 of pregnancy, defined as the critical period for the structural development span of the embryonic stage for rats (WHO, 2001).

### 2.5. Cesarean section

Each dam was submitted to cesarean section 21 days after the beginning of pregnancy. The animals were anesthetized with a combination of 5 mg/kg xylazine (2% xylazine chloride; Virbac®) and 90 mg/kg ketamine (5% ketamine chloride; Vetanarcol®) injected intramuscularly (Allen et al., 1998). The abdomen was incised, the gravid

uterus was removed and weighed with its contents. The number of living and death fetuses and implantation sites was recorded. The heart, lungs, liver, spleen and kidneys of the dams were removed and weighed. The fetuses were examined and the prevalence of teratogenic characteristics (reproductive toxicity protocols: segment II; EPA, 1996) was determined. Body weight, gender and external malformations were recorded. The fetuses were numbered with a marker and fixed in 5% formalin for future skeletal analysis.

### 2.6. Maternal variables

Body weight was recorded daily during pregnancy, with the value recorded on day 0 (sperm-positive smear) being considered as 100%. The differences in weight observed during the experiment were expressed as relative weight gain. Food and water intake was recorded every 3 days throughout pregnancy. Food and water consumption during each 3-day period (grams and milliliter, respectively) were related to body weight during the same period for the determination of relative consumption.

Maternal toxicity was characterized by decreased relative weight (expressed as percentage of body weight), decreased water or food intake, decreased relative weight of the organs (expressed as percentage of body weight) after cesarean section, and occurrence of death during pregnancy. Reproductive indices including embryo resorptions, number of corpora lutea, implantation sites and pups, and pup viability were also assessed (EPA, 1996).

### 2.7. Fetal variables

The fetal variables recorded were body weight (g), sex ratio (male/female), occurrence of external malformations, and skeletal alterations. After the tissue had been cleared with trypsin and the bones stained with alizarin (Taylor and VanDyke, 1985), skeletal alterations were evaluated according to the atlas of rat skeletal anomalies in (Chahoud, 1997).

### 2.8. Statistical analysis

Parametric data, expressed as mean $\pm$S.E.M., were analyzed by repeated measure ANOVA or one-way ANOVA, followed by the Duncan test when appropriate. The non-parametric data, expressed as proportion or percentage, were analyzed by the $\chi^2$-test. Differences were considered to be statistically significant when $P < 0.05$.

## 3. Results

### 3.1. Maternal variables

The total weight gain (mean $\pm$S.E.M.) was $106.4 \pm 9.0$, $84.9 \pm 7.0$, $107.4 \pm 9.5$ and $102.2 \pm 8.3$ g for the control, 500, 750 and 1000 mg/kg glyphosate groups, respectively ($P = 0.693$, one-way ANOVA). These results, which are illustrated in Fig. 1, showed that the expected weight gain occurred in all groups during pregnancy. Although the group receiving the highest dose showed a decrease in body weight during the treatment period, this difference was not statistically significant. Fifty percent of the dams treated with 1000 mg/kg glyphosate died between day 7 and 14 of

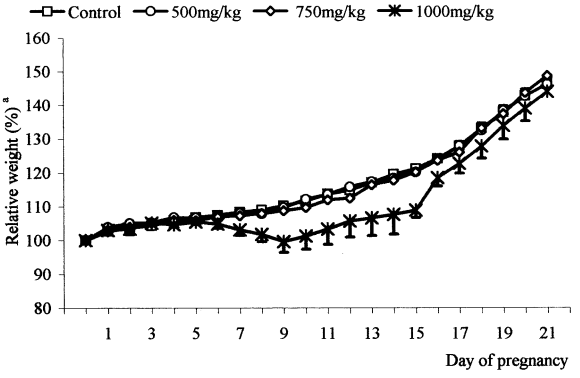


Fig. 1. Relative weight gain of the dams (with respect to day 0 which represented 100%) treated with water (control), 500, 750 or 1000 mg/kg of glyphosate-Roundup® from day 6 to 15 of pregnancy. Data are reported as mean $\pm$S.E.M. *, Significant difference among days 16, 17, 18, 19, 20 and 21 (Duncan test). Significant difference among days ($P < 0.001$, repeated measure ANOVA). No significant difference among groups ($P > 0.05$, repeated measure ANOVA), and no interactions between treatment and days.

pregnancy. No deaths were observed with the lower doses.

The expected steady increase in food intake (Fig. 2) and water intake (Fig. 3) occurred in all groups during pregnancy.

No significant difference in relative weight of the maternal organs was observed among the experimental groups ($P > 0.05$, one way ANOVA; Table 1). Likewise, the number of fetuses, corpora lutea, implantation sites and embryo resorption was similar for all groups ($P > 0.05$, one way ANOVA or $\chi^2$-test; Table 2).

### 3.2. Fetal variables

Concerning the fetal variables, the mean ($\pm$ S.E.M.) weight of the fetuses, expressed in grams, was $5.1 \pm 0.03$ for the control group and $5.0 \pm 0.03$, $5.1 \pm 0.03$ and $5.1 \pm 0.05$ for the 500, 750 and 1000 mg/kg glyphosate groups, respectively. There was no significant difference among groups ($P = 0.889$, one way ANOVA).

The male:female sex ratio for the fetuses was 0.94:1 for the control group and 1.06:1, 1.01:1, and 1.5:1 for the groups that received 500, 750 and 1000 mg/kg glyphosate, respectively. Although the group treated with the highest dose presented the highest sex ratio, no statistically significant differences were observed among groups ($P = 0.724$, $\chi^2$-test).

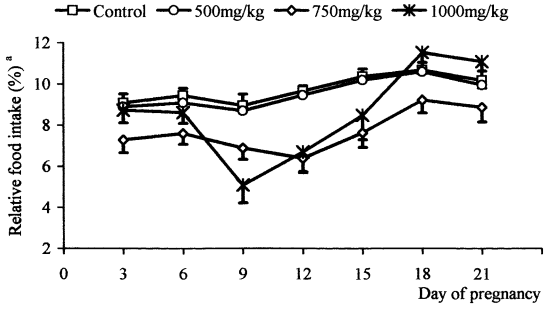


Fig. 2. Relative food intake of the dams treated with water or 500, 750 or 1000 mg/kg of glyphosate-Roundup® from day 6 to 15 of pregnancy. Data are reported as mean $\pm$ S.E.M. *, Significant difference among days 9, 15 and 18 (Duncan test). Significant difference among days ($P < 0.001$, repeated measure ANOVA). Significant interactions between treatment and 3-day period ($P < 0.001$, repeated measure ANOVA, Duncan test). No significant difference among groups ($P > 0.05$, repeated measure ANOVA).

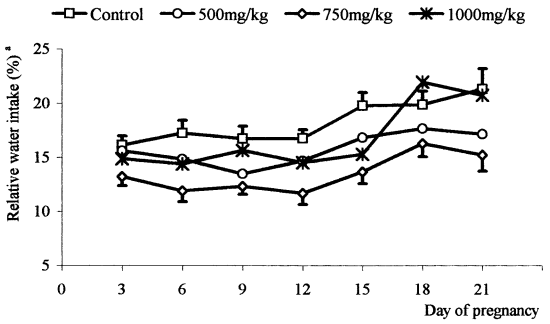


Fig. 3. Relative water intake of the dams treated with water or 500, 750 or 1000 mg/kg of glyphosate-Roundup® from day 6 to 15 of pregnancy. Data are reported as mean $\pm$ S.E.M. *, Significant difference among days 15, 18 and 21 (Duncan test). Significant difference among days ($P < 0.001$, repeated measure ANOVA). Significant interactions between treatment and 3-day period ($P < 0.001$, repeated measure ANOVA, Duncan test). No significant difference among groups ($P > 0.05$, repeated measure ANOVA).

Only two glyphosate groups showed fetuses with external malformations, with rates of 2.0% in the low dose group (500 mg/kg) and 0.6% in the medium dose group (750 mg/kg). The majority of the animals showed anasarca, except for one fetus in the 750 mg/kg group whose development stopped at the beginning of pregnancy and which was not reabsorbed.

There were no significant differences in external malformation rates among the four groups studied ($P = 0.170$, $\chi^2$-test).

Concerning the total percentage of skeletal alterations, the groups exposed to glyphosate-Roundup® showed significant differences compared with control ($P < 0.001$, $\chi^2$-test). The percentage of altered fetuses was 15.4, 33.1, 42.0, and 57.3 for the control and the 500, 750, and 1000 mg/kg glyphosate groups, respectively. The most frequent skeletal alterations observed were incomplete skull ossification and enlarged fontanel (Table 3), which occurred more frequently in the experimental groups compared with control ($P < 0.001$, $\chi^2$-test).

Supernumerary and misshapen ribs, unossified sternebra, and short femur occurred in 0.6% of the

Table 1
Relative weight of the organs of dams treated with water, or 500, 750 or 1000 mg/kg glyphosate-Roundup® from day 6 to 15 of pregnancy

| Organs | Control | Glyphosate-Roundup® (mg/kg) | | |
|---|---|---|---|---|
| | | 500 | 750 | 1000 |
| Number of dams | 15 | 15 | 16 | 07 |
| Heart (%) | 0.30±0.012 | 0.35±0.008 | 0.33±0.008 | 0.34±0.021 |
| Lungs (%) | 0.49±0.024 | 0.55±0.044 | 0.53±0.019 | 0.47±0.031 |
| Liver (%) | 4.57±0.130 | 4.73±0.130 | 4.89±0.100 | 5.11±0.190 |
| Right kidney (%) | 0.30±0.009 | 0.33±0.009 | 0.31±0.005 | 0.33±0.008 |
| Left kidney (%) | 0.30±0.008 | 0.31±0.020 | 0.31±0.006 | 0.32±0.010 |
| Spleen (%) | 0.23±0,009 | 0.24±0.012 | 0.22±0.009 | 0.22±0.010 |

Data are reported as mean±S.E.M. based on the percentage of the organ weight in relation to total body weight. There was no significant difference among groups ($P > 0.05$, one way ANOVA) for each organ evaluated.

control animals, but were not observed in the glyphosate groups.

The general occurrence of incomplete ossification and bipartite sternebra was higher in the 500 mg/kg glyphosate group; humerus incomplete ossification and zygomatic fusion were only noted in this group (rates of 2.7 and 0.7%, respectively). Some skeletal alterations occurred only in fetuses from dams treated with 750 mg/kg glyphosate. The rate of misshapen sternebra was 11.7% ($P < 0.001$, $\chi^2$-test, compared with the other groups), and the rate of bent ileum and agenesis of fore paw phalanges were 2.5 and 1.9%, respectively ($P > 0.05$, $\chi^2$-test). Parietal extra ossification, occipital and cervical incomplete ossification, short mandible, fused ribs and bent clavicle were observed in 1.2% of the animals ($P > 0.05$, $\chi^2$-test). Misshapen zygomatic, misaligned or dumbbell cervical vertebrae, wavy sternum, irregular clavicle, misshapen clavicle, misshapen scapula, radius/ulna incomplete ossification, unossified metacarpal and bent femur occurred at a rate of 0.6% ($P > 0.05$, $\chi^2$-test).

Misshapen atlas and other cervical and thoracic vertebrae appeared in 1.3% of the fetuses from 1000 mg/kg glyphosate-treated dams ($P > 0.05$, $\chi^2$-test) but were not observed in any other group.

Table 3 presents the percentage of skeletal alterations that occurred in two or more of the groups analyzed in this study. Bipartite interparietal and unossifled hind phalanges were significantly more frequent in the 500 and 750 mg/kg glyphosate groups ($P < 0.001$, $\chi^2$-test, compared with control). Incomplete ossification of squama and absence of caudal vertebrae appeared more frequently in the 750 and 1000 mg/kg glyphosate groups ($P < 0.001$, $\chi^2$-test, compared with control).

Table 2
Reproductive indices of the dams treated with water or 500, 750 or 1000 mg/kg glyphosate-Roundup® from day 6 to 15 of pregnancy

| Reproductive indices | Control | Glyphosate-Roundup® (mg/kg) | | |
|---|---|---|---|---|
| | | 500 | 750 | 1000 |
| Number of dams | 15 | 15 | 16 | 07 |
| Number of fetuses | 154 | 148 | 162 | 75 |
| Corpora lutea (mean±S.E.M.) | 11.4±0.5 | 11.6±0.6 | 10.1±0.8 | 12.9±0.9 |
| Implantation sites (mean±S.E.M.) | 10.5±0.7 | 09.8±0.8 | 11.6±0.4 | 11.4±1.1 |
| Embryo resorption (%) | 2.4 | 3.3 | 2.6 | 3.8 |
| Fetuses/dams (mean±S.E.M.) | 10.3±0.8 | 09.7±0.9 | 10.1±0.9 | 10.7±1.1 |

There was no significant difference among groups ($P > 0.05$, $\chi^2$-test or one way ANOVA, according the analyzed data).

Table 3
Percentage of skeletal alterations in fetuses from dams treated with water or 500, 750 or 1000 mg/kg glyphosate-Roundup® from day 6 to 15 of pregnancy

| Regions or structures | Abnormalities (%) | Control | Glyphosate-Roundup® (mg/kg) | | |
|---|---|---|---|---|---|
| | | | 500 | 750 | 1000 |
| Number of fetuses | | 154 (15)[a] | 148 (15)[a] | 162(16)[a] | 75 (7)[a] |
| Skull, general | Incomplete ossification | 10.4 | 29.1* | 38.9* | 56.0* |
| | Enlarged fontanel | 1.9 | 25.7* | 37.0* | 53.3* |
| Interparietal | Bipartite | 0.6 | 18.9* | 4.9* | 0.0 |
| Supraoccipital | Bipartite | 9.7 | 19.6* | 1.2 | 0.0 |
| | Incomplete ossification | 3.2 | 0.0 | 1.2 | 13.3* |
| Premaxilla | Misshapen | 0.0 | 0.7 | 1.2 | 0.0 |
| Maxilla | Short | 0.6 | 0.7 | 0.0 | 1.3 |
| Squama | Incomplete ossification | 0.0 | 0.0 | 3.1* | 2.7* |
| Thoracic vertebrae | Incomplete ossification | 0.6 | 0.0 | 0.6 | 0.0 |
| Lumbar vertebrae | Incomplete ossification | 2.6 | 4.1 | 3.1 | 0.0 |
| | Misshapen | 0.0 | 0.7 | 1.9 | 0.0 |
| Sacral vertebrae | Misshapen | 1.3 | 0.0 | 0.6 | 0.0 |
| | Unossified | 0.6 | 0.0 | 0.6 | 0.0 |
| Caudal vertebrae | Absent | 1.9 | 0.0 | 7.4* | 14.7* |
| | Unossified | 0.0 | 2.0 | 1.9 | 0.0 |
| Ribs | Absent | 1.3 | 2.7 | 3.1 | 4.0 |
| | Incomplete ossification | 1.9 | 2.0 | 5.6 | 4.0 |
| | Bifurcated | 0.0 | 0.7 | 0.6 | 0.0 |
| | Wavy | 0.6 | 2.0 | 4.9* | 0.0 |
| Sternebra | Incomplete ossification | 1.9 | 14.9* | 0.0 | 2.7 |
| | Bipartite | 3.9 | 14.2* | 0.6 | 9.3 |
| Limbs | Incomplete ossification | 0.0 | 0.0 | 17.9* | 1.3 |
| Scapula | Incomplete ossification | 0.6 | 3.4 | 1.2 | 4.0 |
| Metacarpal bones | Incomplete ossification | 1.3 | 1.4 | 0.6 | 2.7 |
| Fore phalanges | Unossified | 5.2 | 2.7 | 4.3 | 1.3 |
| Femur | Incomplete ossification | 3.2 | 3.4 | 13.0* | 0.0 |
| Tibia/fibula | Bent | 0.0 | 1.4 | 2.5 | 0.0 |
| | Incomplete ossification | 2.6 | 2.7 | 11.7* | 8.0 |
| Metatarsal bones | Unossified | 4.5 | 1.4 | 13.6* | 10.7 |
| Hind phalanges | Unossified | 7.1 | 20.9* | 22.2* | 2.7 |
| Ileum | Incomplete ossification | 11.7 | 4.7 | 1.2 | 0.0 |
| Ischium | Incomplete ossification | 4.5 | 2.7 | 9.3 | 0.0 |
| Pubis | Incomplete ossification | 3.9 | 2.7 | 11.1* | 0.0 |

Significant difference compared with the control group ($P < 0.05$, $\chi^2$-test).
[a] Number of dams.

## 4. Discussion

According to Palmer (1977), almost any type of malformation ever recorded can occur sporadically in any given species, and more than one cause can generate a malformation. Moreover, the definitions of normality and abnormality can be extremely questionable. Consequently, discrepancies among investigators, already evident with obvious malformations, become greatly magnified with less important malformations. As a result, extra information gained by recording more subtle changes can actually hinder the interpretation of data. Furthermore, the nomenclature used to describe observations of fetal and neonatal morphology often varies considerably among laboratories, investigators and textbooks in the fields of teratology and developmental toxicology. In this

study, we evaluated the toxicity of glyphosate-Roundup® as commercialized in Brazil, based on the International Federation of Teratology Societies parameters (Wise et al., 1997) and the atlas of rat skeletal anomalies (Chahoud, 1997).

Lorke (1977) classified the deviations from normal found on the skeleton as individual variations of normal, developmental retardations of the skeleton, and malformations. The individual variations of normal consist of lack of development of the ossification centers to be expected at cesarean section, such as the ossification centers of the terminal phalanges in rats. Development retardations consist of missing ossification centers in bilateral bone structures or the presence of shape and size clearly matching an earlier stage of development. Larger fontanelles and incomplete development of skull bones belong to this class. Malformations consist of the absence of important bones or parts of bones, shortenings, bendings, asymmetry, fusions or clefts.

Signs of delayed ossification in several body structures such as skull, sternebra and limbs (especially hind paw), were observed in the groups receiving all glyphosate-Roundup® doses. Although in the absence of treatment retarded ossification can be related to body weight and/or size (Souza et al., 1997), in this study there was a clear increase in the frequency of this alteration in the glyphosate-Roundup®-treated groups. However, this alteration was not related to body weight or size. The occurrence of multiple alterations was significantly higher in the treated groups compared with control, but did not show a dose-related pattern. However, developmental retardations of the skeleton such as incomplete ossification of skull and enlarged fontanel showed a dose-related response.

According to the WHO (1994), the administration of a dose level equivalent to 3500 mg/kg per day of technical glyphosate to pregnant Charles River COBS CD rats from day 6 to 19 of pregnancy determined an increased incidence of soft stools, diarrhea, breathing rattles, red nasal discharge and reduced activity, increased maternal mortality (24% during the treatment period), growth retardation, increased incidence of early resorptions, decrease of total number of implantations and viable fetuses, and increased number of fetuses with reduced ossification of sternebra (WHO, 1994). Although the precise toxicity mechanism is yet to be clarified, it is known that maternal exposure to agrochemicals such as glyphosate during pregnancy induces a variety of functional abnormalities in the specific activity of the liver enzymes, and in the heart and brain of the pregnant rats and their fetuses (Daruich et al., 2001).

The maternal toxicity observed in this study shows that the formulation commercially sold in Brazil is more toxic than the technical glyphosate, as determined by Adam et al. (1997) and Dallegrave et al. (2002). Both studies suggested that the surfactant present in the commercial formulations is more toxic than glyphosate. Despite the reproductive signs such as early resorptions, reduced implantation sites and reduced number of viable fetuses related by the WHO (1994) for exposure to 3500 mg/kg technical glyphosate, in the present study these effects were not observed at the 1000 mg/kg of glyphosate in the commercial formulation. Probably, because the other components of the formulation directly affected the systemic organ of the dams, the reproductive effects did not show in this study at the 1000 mg/kg of glyphosate. However, a significant increase in the frequency of fetuses exhibiting skeletal alterations was observed in all glyphosate-Roundup®-exposed groups, indicating that these alterations were not directly associated with maternal toxicity and that the effects were probably were related to fetal skeletal development.

The developmental retardations of the skeleton reported in the present study shows that the effect of glyphosate-Roundup® was more marked than that of technical glyphosate (WHO, 1994). The higher maternal toxicity reported here in comparison to that of technical glyphosate is probably related to the presence of other components in the commercial formulation, such as the surfactant polyoxyethyleneamine (Adam et al., 1997; Dallegrave et al., 2002). Despite the fact that the doses used in this study would never expected to correspond to human exposure levels under normal circumstances, as reported by Williams et al. (2000) for glyphosate and polyoxyethyleneamine

in adults or children (margins of exposure = 5420, 3370 and 461 577, respectively), this results shows that the commercial formulation poses an increased potential risk for the rat skeletal system. To better understand this fact, additional studies should be carried out to determine the mechanisms of effects of the commercial formulation on the skeletal system of the fetuses, since humans are also potentially exposed to this commercial formulation.

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