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Low concentrations of glyphosate alone affect the pubertal male rat meiotic step: an *in vitro* study

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## Abbreviations

Trans-epithelial electrical resistance: TEER; checkpoint kinase 2: CHK2; DEAD-box helicase 4: VASA; GDNF family receptor alpha 1: GFR $\alpha$ 1; testis specific histone 2B: TH2B; Stathmin 1: STMN1; Transition Protein 1: TP1; Transition Protein 2: TP2; Protamine 3: PRM3; Androgen receptor: AR; Estrogen receptor alpha: ER $\alpha$ ; Estrogen receptor beta: ER $\beta$ .

## **Abstract**

Glyphosate is the most used herbicide in the world. Controversial studies exist on its effect on the male reproductive system. We used the validated BioAlter® model to test the effects of low concentrations of Glyphosate. Pubertal rat seminiferous tubules were treated with Glyphosate 50 nM, 500 nM, 5 µM or 50 µM over a 3-week culture period. The Trans-Epithelial Electrical Resistance was not modified by any of the concentrations. The decrease of Clusterin mRNAs suggested that glyphosate would target the integrity of Sertoli cells. The decrease of the numbers of germ cells from day 14 onward highlighted the chronic effect of glyphosate at 50 nM, 500 nM or 5 µM. No consistent effect of glyphosate was observed on the numbers of spermatogonia or on their specific mRNA levels. However, those low concentrations of glyphosate targeted young spermatocytes and middle to late pachytene spermatocytes resulting in a decrease of the numbers of round spermatids, the direct precursors of spermatozoa. This study underlines that the effect of a toxicant should be also studied at low doses and during the establishment of the blood-testis barrier.

**Keywords:** Glyphosate, low concentrations, meiosis, *in vitro* study

## Introduction

Glyphosate dates from the 1970s and has become the most used herbicide in the world (Benbrook, 2016). Glyphosate has been mixed with other chemicals to constitute widely used glyphosate-based herbicides. Among them Roundup® has been used in agricultural fields and home gardens (Gillezeau et al., 2019). There is increasing interest in potential chronic effects of formulated glyphosate and its degradation products as they accumulate in the environment (Bai and Ogbourne, 2016; Battaglin et al., 2014). Glyphosate concentrations vary from 2 to 430 µg/L (11.8 nM to 2.5 µM) in river water and stream water in the USA (Battaglin et al., 2009, 2005; Coupe et al., 2012; Mahler et al., 2017). In Europe, glyphosate has also been detected in various waters (from 0.1 to 2.5 µg/L i.e 0.6 nM to 14.8 nM) (Poiger et al., 2017; Skark et al., 1998). Higher levels (up to 165 µg/L i.e 976 nM) were found in France (Villeneuve et al., 2011) and Denmark (Rosenbom et al., 2015).

Human beings may be exposed to glyphosate through food and drinking water (Bai and Ogbourne, 2016). Glyphosate was detected in human urine samples. Its concentrations were higher among subjects in the USA (mean 2–3 µg/L i.e 11.8-17.6 nM) with maximum up to 233 µg/L i.e 1.4 µM) than in Europe (mean: 1 µg/L i.e 5.9 nM and maximum 5 µg/L i.e 29.5 nM) (Niemann et al., 2015).

There is a growing interest in toxicology of male reproduction because human semen quality has declined worldwide over the last decades (Carlsen et al., 1992; Geoffroy-Siraudin et al., 2012a; Le Moal et al., 2014; Olesen et al., 2018). In addition, semen quality may be a fundamental biomarker of overall male health (Eisenberg et al., 2014; Jensen et al., 2009).

Controversial *in vivo* studies exist on the effect of glyphosate alone on male reproductive system. Two studies have shown that glyphosate affects spermatogenesis (*i*) in mice by

decreasing the number of spermatozoa and the plasma levels of testosterone (Pham et al., 2019), (ii) in rabbits resulting in a decline in ejaculate volume and sperm concentration (Yousef et al., 1995). By contrast, Li's group has shown that glyphosate does not affect male reproduction in rat (Dai et al., 2016) and Svingen's team found no significant effect of glyphosate on testes or testosterone synthesis in rats (Johansson et al., 2018). There are only very few *in vitro* studies on the effect of glyphosate alone on primary cultures of testicular cells. Séralini's team tested glyphosate on mature rat fresh testicular cells and showed that within 24-48 hours glyphosate was essentially toxic to Sertoli cells (Clair et al., 2012). The group of Meroni showed that glyphosate could alter the blood-testis barrier which is essential for spermatogenesis, but only at a high concentration: 100 ppm ie 100 µg/ml (588 µM) (Gorga et al., 2020). Toxic effects on sperm progressive motility but not on sperm DNA integrity were induced in human by glyphosate alone at 360 µg/L (2.1 µM), a concentration that greatly exceed environmental exposures in Europe (Anifandis et al., 2018).

The purpose of the present *in vitro* study is to investigate the effect of low doses of glyphosate alone at 50 nM (8.45 ng/ml), 500nM (84.5 ng/ml), 5µM (845 ng/ml) or 50 µM (8.45 µg/ml), in our 20-22-day-old rat seminiferous tubule culture model, called BioAlter®. These concentrations of glyphosate were selected on the basis of the mean plasma concentrations despite they may be somewhat different from the concentrations present in the seminiferous tubules. These concentrations are partially overlapping the levels found in occupational workers (11.73 ng/ml i.e 69 nM) (Pan et al., 2017), or in patients with acute self-poisoning reported as asymptomatic (2.9 µg/ml i.e 17 µM) or suffering from minor to moderate symptoms (40.8 µg/ml i.e 243 µM) (Roberts et al., 2010). BioAlter® mimics the *in vivo* situation and has been carefully validated from a physiological (Geoffroy-Siraudin et al., 2010; Perrard et al., 2010, 2003) and a toxicological points of view (Goldstein et al., 2016),

and is relevant to investigate the mechanisms of action on spermatogenesis of heavy metals (Carette et al., 2013; Geoffroy-Siraudin et al., 2012b, 2010) pesticides or pollutants either separately or in cocktail (Ali et al., 2014; Carette et al., 2015; Durand et al., 2020, 2017; Pisani et al., 2016) during a 3-week culture period. In addition, BioAlter® decreases the number of sacrificed rats by 20 to 30 compared to *in vivo* studies; in agreement with the European REACH directive.

One originality of this work is that the effect of glyphosate could be studied: (i) at the level of the blood-testis barrier; (ii) on the numbers of Sertoli cells and of each germ cell type (spermatogonia, spermatocytes, secondary spermatocytes and round spermatids which are the direct precursors of spermatozoa); (iii) on the levels of transcripts specific of either the blood-testis barrier proteins, or of Sertoli cells, or of germ cells (spermatogonia, pachytene spermatocytes, round spermatids) and of steroid hormone receptors.

## **Material and methods**

### **1. Animals**

Experiments were performed with 20-22-day-old male Sprague-Dawley rats (Charles River, Saint-Germain-Nuelles, France) having undergone no treatment. The experimental protocol was designed in compliance with recommendations of the European Economic Community (EEC) (86/609/EEC) for the care and use of laboratory animals. Testes from four 20-22-day-old rats were pooled in every culture experiment.

### **2. Preparation and culture of seminiferous tubule segments**

Pooled seminiferous tubule segments from 4 to 6 rats were cultured in a serum-free culture medium as previously described (Hue et al., 1998; Staub et al., 2000). The culture medium

consisted of 15 mM HEPES-buffered F12/DMEM supplemented with antibiotics, 1.2 g/L NaHCO<sub>3</sub>, 10 µg/ml insulin, 10 µg/ml transferrin, 10<sup>-4</sup> M vitamin C, 10 µg/ml vitamin E, 10<sup>-7</sup> M testosterone, 3.3 × 10<sup>-7</sup> M retinoic acid, 3.3 × 10<sup>-7</sup> M retinol, 10<sup>-3</sup> M pyruvate (all from Sigma), and 50 ng/ml NIH (Bethesda, MD) porcine FSH (lot #AFP 5600; obtained through NIDDK, Rockville, MD). Some cultures were performed in the absence of testosterone. A stock solution of glyphosate (Sigma-Aldrich, St. Quentin Fallavier, France) 25 mM (4.23 mg/ml) was prepared in water, then diluted in the culture medium in order to obtain the final concentrations 50 µM (8.45 µg/ml), 5 µM (845 ng/ml), 500 nM (84.5 ng/ml) and 50 nM (8.45 ng/ml) of glyphosate in the basal compartment of the bicameral chambers of culture. In the wells containing the treated cells, glyphosate was added to the culture medium from day 2 onward. Basal media (with or without glyphosate) were renewed every 2 days. At selected days of culture, cells were detached from the culture dishes and isolated with trypsin for determining cell number, and cell viability by trypan blue exclusion or cell flow cytometry analysis.

### **3. Trans-epithelial electrical resistance**

Trans-epithelial electrical resistance (TEER) measurement was performed with an EVOM2 (World Precision Instruments, Florida, USA) (Durand et al., 2017). The culture was equilibrated at room temperature before the assay. The TEER was calculated according to the following equation:  $TEER = (R_{total} - R_{control}) \cdot A$  ( $\Omega \text{ cm}^2$ ), where  $R_{total}$  is the resistance measured,  $R_{control}$  is the resistance of the control insert (insert alone with the culture medium), and  $A$  is the surface area of the insert.

### **4. Immunolabeling of cultured cells for flow cytometric analysis**

Cultured cells from seminiferous tubules were fixed in 70% ethanol. To distinguish germ

cells from somatic cells, the latter are mainly Sertoli cells as rat possess only one layer of peri-tubular/myoid cells (Maekawa et al., 1996), cells were immunolabeled with a monoclonal antibody against vimentin (clone V9; DAKO SA, Trappes, France) that was conjugated with a green fluorochrome, Alexa 488, by using a Zenon Mouse labeling kit (Life Technologies, Cailloux-sur-Fontaines, France) as previously described (Durand et al., 2017; Perrard et al., 2009). Then, cells were analyzed using an Attune Acoustic Focusing Cytometer (Applied biosystems) as described (Carette et al., 2015; Durand et al., 2017). Acquisition and analysis were performed using the Attune Cytometric Software. The vimentin-positive somatic/Sertoli cells and the vimentin-negative tetraploid, diploid and haploid germ cells were separated with the bivariate analysis of DNA content/vimentin. Then, the bivariate linear forward light scatter and linear side angle light scatter analyses allowed the identification of the different populations of cells (Durand et al., 2017; Godet et al., 2000; Perrard et al., 2009).

## **5. RT-qPCR**

Total RNA was extracted from cultured rat seminiferous tubules cells using TRI-REAGENT (Euromedex, Souffelweyersheim, France) according to the manufacturer's protocol. All samples were treated by DNase I before the reverse transcription; first strand cDNA was synthesized from 2 µg RNA with Super Script III in the presence of oligodT and Random Primer (all from Thermo Fisher Scientific, Villebon sur Yvette, France). Realtime PCR was performed in a Step One Plus instrument (Applied biosystems) by using SYBR-Green (Qiagen, Courtaboeuf, France). Primers sequences are presented in Table 1. Mitochondrial ribosomal proteins S18 and S16 mRNAs were used as housekeeping gene controls.



## **6. Statistical analyses**

Statistical analyses were performed using Graphpad and R softwares. Data are presented as the means  $\pm$  S.E.M. Data were analysed by paired Student's t-test to compare treated cells with their corresponding controls. When required, values were logarithmically transformed to eliminate heterogeneity of variance and achieve a reasonable assumption of normal distribution (Bland and Altman, 1996)  $P < 0.05$  was considered significant.

## **Results**

### **1. Effect of glyphosate on the TEER**

Seminiferous tubules segments were cultured for 23 days in the absence or presence of 50 nM, 500 nM, 5  $\mu$ M or 50  $\mu$ M of glyphosate. Under control conditions, the TEER increased from 12 to 95 ohm.cm<sup>2</sup> between day 2 and day 12 of culture and slightly decreased down to 59 ohm.cm<sup>2</sup> on day 23 (Fig. 1). This high TEER should be associated with the presence of tight junctions between the Sertoli cells, the main component of the blood-testis barrier (Carette et al., 2015; Linlin et al., 2013). The TEER of seminiferous tubules culture was not modified at any concentration of glyphosate.

For further studies only the lower concentrations (50 nM, 500 nM and 5  $\mu$ M) of glyphosate were chosen.

## 2. Effect of glyphosate on cell viability and on the numbers of Sertoli cells and of germ cells

Figure 2 shows the changes in the numbers of total cells, Sertoli cells, and of germ cells in 4 different control cultures. As expected (Staub et al., 2000, Perrard et al., 2003), the number of each type of tubular cells varied according to the number of seeded total cells (Fig. 2) as may vary also the yield of each step of the spermatogenic differentiation, as it does *in vivo*. Hence, in every experiment, this was the same population of tubular cells which was exposed (treated cells) or not (control cells) to glyphosate (paired samples). Therefore, in the following figures, the results were expressed as a percent of the values obtained with the corresponding control cells (Carette et al., 2015). The proportion of viable cells ranged between 91% and 99% of total cells during the whole culture period and was not affected by 50 nM, 500 nM or 5  $\mu$ M of glyphosate, at any day of culture (data not shown). Neither the numbers of total cells nor the numbers of Sertoli cells were modified by glyphosate at any day of culture (Fig. 3A, 3B). The numbers of total germ cells were decreased by glyphosate at 50 nM or 500 nM on day 14 ( $p \leq 0.05$  to  $p \leq 0.01$ ) and at 50 nM or 5  $\mu$ M on day 21 ( $p \leq 0.05$ ). The numbers of spermatogonia were not modified but decreased for the lower concentration at the end of the culture ( $p \leq 0.05$ ). The numbers of young spermatocytes were decreased only at the end of the culture by the two higher concentrations ( $p \leq 0.05$ ), while the numbers of middle to late pachytene spermatocytes were decreased (or tended to be decreased) by glyphosate at 500 nM ( $p \leq 0.05$ ) or 5  $\mu$ M ( $p = 0.06$ ) on day 14 and by the 3 concentrations on day 21 ( $p \leq 0.05$ ,  $p = 0.08$  and  $p \leq 0.01$  for 50nM, 500 nM and 5  $\mu$ M respectively). The numbers of secondary spermatocytes were decreased (or tended to be decreased) by the 3 concentrations on day 14 ( $p \leq 0.05$ ,  $p = 0.1$  and  $p = 0.06$  for 50nM, 500 nM and 5  $\mu$ M respectively and only by the higher concentration at the end of the culture ( $p \leq 0.05$ ). The numbers of round spermatids were decreased (or tended to be decreased) by the 3 concentrations all along the cell culture ( $p$

= 0.06 to  $p \leq 0.01$  on day 7,  $p = 0.1$  to  $p \leq 0.05$  on day 14 and  $p \leq 0.05$  to  $p \leq 0.01$  on day 21 (Fig. 3B).

### 3. Effect of glyphosate on the relative levels of some “specific” mRNAs

In order to complete the flow cytometric analyses, some mRNA levels were measured in seminiferous tubules cultured for 7, 14, or 21 days in the absence or presence of glyphosate. For CHK2, VASA, GFR $\alpha$ 1 and  $\alpha$ 6 Integrin which are specific of spermatogonia (Bartkova et al., 2001; Phillips et al., 2010), for TH2B and STMN1 which are more specific of pachytene spermatocytes and for TP1, TP2 and PRM3 which are specifically expressed by round spermatids (Marret et al., 1998; Schluter and Engel, 1995), the numbers of transcripts were expressed by % of spermatogonia, % of 4C germ cells and % of round spermatids respectively (Durand et al., 2020). Indeed, the relative cell composition of the cultures changes according to the day of sampling, as it does *in vivo* during development (Almstrup et al., 2004). Hence, the measured levels of a specific mRNA depend on both the amount of mRNA produced by the specific cells and the proportion of these cells in the sample. Taking into account these two parameters provide more significant data (Plotton et al., 2006, 2005).

CHK2, VASA,  $\alpha$ 6 Integrin and GFR $\alpha$ 1 mRNA levels were not modified by glyphosate treatment at any day (data not shown). But, as shown in Fig. 4, STMN1 mRNAs were increased on day 7 and on day 21 by glyphosate at 5  $\mu$ M ( $p \leq 0.01$ ), whereas TH2B mRNAs were not modified by glyphosate treatment at any day. TP1 mRNAs were increased on days 7 and 14 by the higher concentration ( $p \leq 0.05$  to  $p \leq 0.01$ ), and on day 21 at 500 nM  $p \leq 0.05$ ). TP2 mRNAs were increased by glyphosate at 5  $\mu$ M on days 7 and 14 ( $p \leq 0.05$ ) whereas PRM3 mRNAs were increased at this concentration only on day 7 ( $p \leq 0.05$ ) (Fig. 5). In order to determine if the effect of glyphosate on the mRNAs levels of TP1, TP2 or PRM3 mRNAs

was due to an androgen-like effect, as we found for carbendazim (Carette et al., 2015), seminiferous tubules were cultured without or with glyphosate at 5  $\mu$ M in the absence of testosterone in the culture medium for 7 days, since the maximum effect of glyphosate 5  $\mu$ M in the presence of testosterone was observed on Day 7. In the absence of testosterone, the mRNAs levels of TP1, TP2 or PRM3 were not modified by glyphosate (data not shown). To determine if glyphosate alters Sertoli cell function, mRNA levels of Clusterin (Plotton et al., 2005) and Vimentin (Franke et al., 1979; Kopecky et al., 2005), specific of Sertoli cells, were measured. For these two genes the numbers of transcripts were expressed by % of sertoli cells. As shown in Fig. 6, the vimentin mRNA levels were never modified by glyphosate treatment at any day, while Clusterin mRNA levels were decreased (or tended to be decreased) by the 3 concentrations at the end of the culture ( $p \leq 0.01$  for 50 nM and 500 nM and  $p = 0.1$  for 5  $\mu$ M).

In order to complete the study of the blood–testis barrier, mRNAs levels of Connexin 43, Occludin, TJP1, Claudin 11 and Claudin 4 were measured in the presence or absence of glyphosate. The transcripts of these five genes were not modified by glyphosate treatments at any day of culture (data not shown).

Because spermatogenesis is an androgen- and estrogen-regulated process the levels of AR, ER $\alpha$ , ER $\beta$  and aromatase mRNAs were further determined (Fig. 7). For AR the numbers of transcripts were expressed by % of Sertoli cells. Because ER $\alpha$ , ER $\beta$  and aromatase are expressed by both Sertoli cells and germ cells their mRNA transcripts were not corrected by any cell population. ER $\alpha$  mRNA levels were decreased only by 500 nM glyphosate at the end of the culture ( $p \leq 0.01$ ), and aromatase mRNAs were decreased only by 50 nM glyphosate on day 14. AR and ER $\beta$  mRNA levels were never modified by glyphosate treatment.

## Discussion

The aim of our study was to investigate the possible impact of low concentrations of glyphosate alone on pubertal rat spermatogenesis by using our validated rat seminiferous tubule culture model, BioAlter®. It is important to note that our model decreases the inter-animal variations existing between the rats, in *in vivo* studies, since in every experiment, this is the same population of tubular cells which was exposed (treated cells) or not (control cells) to glyphosate (paired samples). For the first time, our study has shown that glyphosate alone has a detrimental effect on the meiotic step.

The absence of an effect of glyphosate on the TEER is in agreement with the *in vitro* study of Gorga et al. which did not find any alteration of the TEER, in cultured 20-day-old rat Sertoli cells, after a short (5 days) treatment by glyphosate at 10 ppm ie 10 µg/ml (58.8 µM), which is very close to our highest tested concentration for TEER, 8.45 µg/ml (50 µM) (Gorga et al., 2020). But, it is important to underline that our culture system, BioAlter®, is closer to the *in vivo* situation than that of Gorga et al, since we culture Sertoli cells together with germ cells. In addition, BioAlter® allows studying the chronic effect of a toxicant over a 3 week culture period on Sertoli cells and on the germ cell types (from spermatogonia to round spermatids).

Under control condition, the decrease of the number of germ cells from day 14 onward was mainly due to the decrease of the numbers of young spermatocytes and of middle to late pachytene spermatocytes. This fact would mean that the replenishment of the 4C cell pool became less efficient after several days of culture. Indeed, seminiferous tubules were cultured in a chemically defined medium containing testosterone which is necessary for the meiotic step (Smith and Walker, 2014), but which leads to the inhibition of spermatogonial

differentiation (Meistrich and Shetty, 2003) inducing the depletion of the pool of 4C cells. In addition, the decrease of the 4C cell pool could also be due to their apoptosis. Indeed, the proportions of apoptotic 2C and 4C cell in BioAlter® were rather close to those observed during puberty of the rat (Perrard et al., 2003). Nevertheless, as explained above, this is the same population of tubular cells which was exposed or not to glyphosate.

The decrease of the numbers of total germ cells by the 3 concentrations of glyphosate from day 14 onward shows its chronic effect, and is due to the disruption of the meiotic step leading to a decrease of the numbers of young spermatocytes, middle to late pachytene spermatocytes and secondary spermatocytes resulting in the decrease of round spermatids. In addition, it might be noted that the numbers of round spermatids were already decreased from day 7 onward (i.e as soon as they appeared) (Staub et al., 2000), which could be due to a blockage of the two meiotic division. To our knowledge, no *in vitro* or *in vivo* study exists on the effects of glyphosate alone on the meiotic step. Nevertheless, two previous studies have shown that the two herbicides, Roundup and Pondmaster, which contain glyphosate as the active chemical, target spermatocytes: (i) Kale et al have shown that Roundup® and Pondmaster induce a very high frequency of lethals in larval spermatocytes by using the *Drosophila* sex-linked recessive lethal mutation assay (Kale et al., 1995); (ii) the study of Jiang et al. has shown that an excessive apoptosis of Kunming mice male germ cells, mainly spermatocytes and spermatids, occurs after Roundup® exposure (Jiang et al., 2018).

The decrease of the round spermatid numbers we observed is in agreement with two previous *in vivo* studies: (i) Nardi et al. have shown a decrease in round spermatids in prepubertal rats receiving soy milk supplemented with 100 mg/kg glyphosate (Nardi et al., 2017); (ii) Pham et al. have shown a decrease in spermatozoa by a treatment of prepubertal mice with glyphosate at 0.5 and 5 mg/kg/day (Pham et al., 2019). By contrast, two other *in*

*in vivo* studies showed either no effect on testes in adult rats exposed to glyphosate at levels up to 25 mg/kg bw/day (Johansson et al., 2018), or a decrease of the total sperm production only at the dose of 500 mg/kg/day (Dai et al., 2016). It is important to note that these last two teams used sexually mature rats while Nardi et al., like us, worked with rats at the critical age of puberty (Nardi et al., 2017).

Previous *in vivo* studies have shown that glyphosate at 5 mg/kg/day decreases the numbers of undifferentiated spermatogonia (Pham et al., 2019), and that Roundup and Pondmaster induce a very high frequency of lethals in larval spermatocytes (see above) and in spermatogonia (Kale et al., 1995). In our study, no consistent effect of glyphosate was observed on the number of spermatogonia, or on CHK2, VASA,  $\alpha 6$  Integrin and GFR $\alpha 1$  mRNA levels; this might be due to the lower concentrations we used. No correspondence between the diets of the rats and a serum level of glyphosate was indicated in the above cited *in vivo* studies, which leads to an impossibility in the comparison of our results with those of *in vivo* studies.

The young spermatocyte population is composed of leptotene, zygotene spermatocytes and pachytene spermatocytes from stages XIV to IV whereas middle to late pachytene spermatocytes are from stages V to XIII of spermatogenesis (Godet et al., 2000; Perrard et al., 2003). TH2B mRNAs are expressed by pachytene spermatocytes from stages III-XIV onward with higher levels in stages VIII to X whereas STMN1 mRNA levels are roughly similar between stages III to X (Marret et al., 1998). The fact that STMN1 mRNAs were increased on days 7 and 21 by glyphosate at 5  $\mu$ M while the numbers of young spermatocytes were not modified or decreased on days 7 and 21 respectively, might suggest an effect of glyphosate on the regulation of STMN1 mRNAs. Likewise, glyphosate could regulate TH2B mRNAs which were not modified at the end of culture despite the numbers of young spermatocytes and of middle to late pachytene spermatocytes were decreased.

Despite the decrease in the numbers of round spermatids by glyphosate, TP1, TP2 and PRM3 mRNA levels were sometimes increased by glyphosate at 5  $\mu$ M or 500 nM. As, we have previously shown that testosterone increases the amount of TP1 mRNAs transcribed by round spermatids *in vitro* (Vigier et al., 2004), the hypothesis of an androgen like effect of glyphosate could have been suggested. But, no effect of glyphosate was found on the mRNA levels of TP1, TP2 or PRM3 in the absence of testosterone in the medium. Nevertheless, the increase of TP1, TP2 or PRM3 mRNA levels by glyphosate 500 nM or 5  $\mu$ M in the presence of testosterone might suggest an effect of glyphosate on the regulation of those mRNAs. Moreover, no modification in the levels of AR or Er $\alpha$  or Er $\beta$  mRNAs was observed.

Glyphosate at 50 nM or 500 nM induced a decrease of Clusterin mRNAs which is a good marker of the amount of RNA of Sertoli cell origin in rat testis samples at day 20 or later (Plotton et al., 2005). This result might suggest that Glyphosate induced some alteration of the function of Sertoli cells. Two previous *in vitro* studies reported a harmful effect of glyphosate on Sertoli cells: (i) a deleterious impact of glyphosate at 3.6  $\mu$ g/ml (21.3  $\mu$ M) (i.e 42.6 to 426 fold higher than our tested concentrations) was shown on the immature mouse Sertoli cell line (TM4) (Vanlaeys et al., 2018); (ii) Glyphosate at concentrations ranging from some human urine and environment to agricultural levels [1 ppm ie 1  $\mu$ g/ml (5.92  $\mu$ M), similar to our 5  $\mu$ M concentration, to 10000 ppm ie 10000  $\mu$ g/ml (59.2 mM), 11840 fold higher than our higher tested concentration] was essentially toxic on cultured mature rat Sertoli cells within 24-48 hours (Clair et al., 2012). Since Sertoli cells are essential for spermatogenesis, some alteration of their function by glyphosate could contribute to the disruption of the meiotic stage that we observed (see above).

The absence of modification of the mRNAs levels of Connexin 43 (the predominant testicular gap junction protein) (Pointis and Segretain, 2005), and Occludin, TJP1, Claudin 11



and Claudin4 (four tight junction proteins) (Mruk and Cheng, 2015) is in line with the absence of effect of glyphosate on the TEER. These last results suggest that the main target of glyphosate would be the function of Sertoli cell and not the Sertoli cell junctions at the blood-testis barrier level.

**Conclusion:** The present study clearly highlights the chronic effects of low concentrations of glyphosate on prepubertal rat seminiferous tubules. These low concentrations of glyphosate target (i) some function of the Sertoli cells, as observed with the decrease in clusterin mRNAs, and (ii) the meiotic step resulting in a decrease of the number of round spermatids, the direct precursors of spermatozoa. This study underlines that the effect of a toxicant should be also studied at low doses and during the establishment of the blood-testis barrier.

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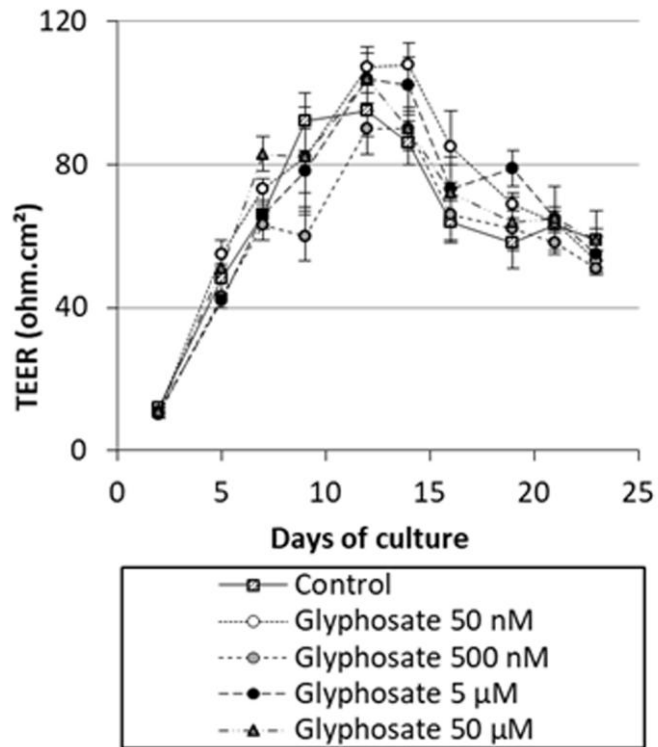
<https://doi.org/10.1080/03601239509372951>

**Table 1.** Sequence of the primers used for RTqPCR

	fwd-5'	rev-5'
CHK2	TGCTGGAGCCTAGGAGTCAT	TCATGTGCTCATCCTGAAGC
VASA	GATGCACCACCGGCAATTTT	TTCCCAGACCCTGTTTGAGC
$\alpha$ 6 integrin	TCGGTCTCTGGAGTCGCTAA	GCCGTGCCGAGGTTTTTAAG
GFR $\alpha$ 1	CGGGTCAAGAACAAGCCTCT	CACCAGCGAGACCATCCTTT
STMN1	AAGGATCTTTCCCTGGAGGA	TTCTCCTCTGCCATTTTGCT
TH2B	GATGCCGCGAAGAGAGTTAC	GGTCGAGCGCTTGTTGTAAT
TP1	CGACCAGCCGCAAATAAAG	ATCATCGCCCCGTTTCTCTAC
TP2	GGCCTCAAAGTCACACCAAT	CTTGCTCACTTTCCCTTCCA
PRM3	GTGGCCTGTGTGAGTCAAGA	CCTCAGCACCATCTTGCTTT
Clusterin	TTCATTCCCTCCAGTCCAAG	CACGAGAGGGGACTTCTGAG
Vimentin	AGATCGATGTGGACGTTTCC	CACCTGTCTCCGGTATTCGT
AR	GTGTCGTCTCCGAAATGTT	GGAATCAGGCTGGTTGTTGT
ER $\alpha$	CATCGATAAGAACCGGAGGA	AAGGTTGGCAGCTCTCATGT
ER $\beta$	AAAGTAGCCGGAAGCTGACA	CTCCAGCAGCAGGTCATACA
Aromatase	TCTCCAGATTCGGCAGCAAG	ATAAGAACAGGGCCCCGTCAG
16S	AGTCTTCGGACGCAAGAAAA	AGCCACCAGAGCTTTTGAGA
18S	ATCATCTTCCATGCCCCATA	AAGGTTACCCGACAACAAC

## Figure Legends

Figure 1:



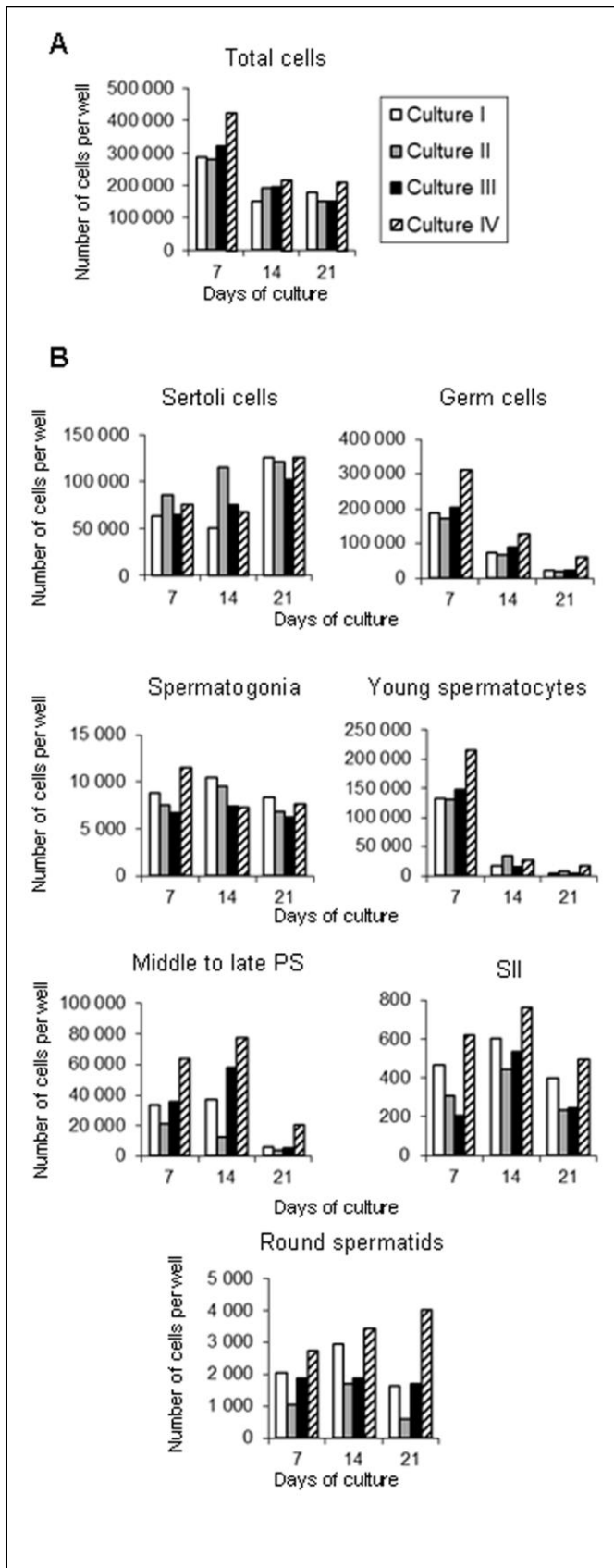
Effects of glyphosate alone on the TEER of rat seminiferous tubules in culture.

Seminiferous tubule segments were cultured in the absence (—■—) or in the presence of 50 nM (---○---) or 500 nM (—●—) or 5 µM (—●—) or 50 µM glyphosate (---▲---).

Results are means ± SEM of 6 wells per day for each condition.

Results are representative of two different experiments.

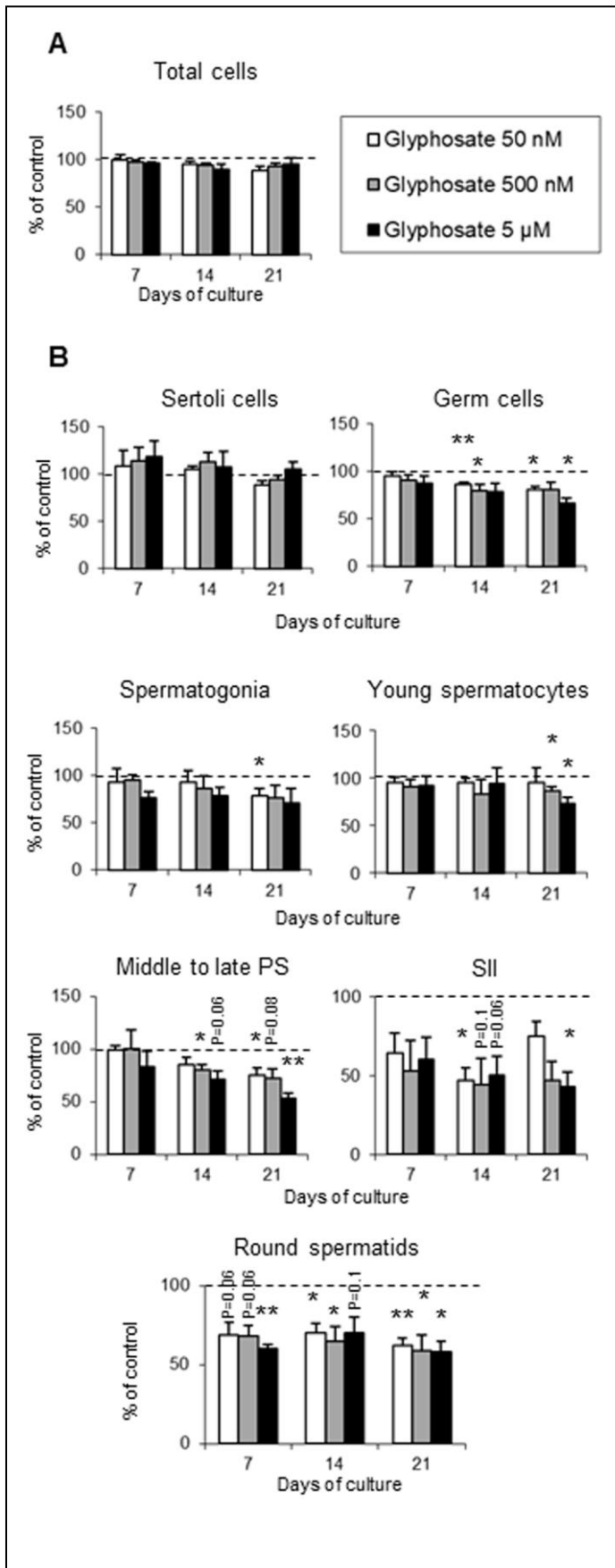
**Figure 2:**



Changes in the numbers of total cells, Sertoli cells, and of germ cells in control cultured seminiferous tubule segments.

Individual values of four different cultures (I-IV) are presented. (A) Changes in the number of total cells. (B) Changes in the numbers of Sertoli cells and of germ cells. Germ cells are: spermatogonia, young spermatocytes, middle to late pachytene spermatocytes (PS), secondary spermatocytes (SII) and round spermatids.

**Figure 3:**



Effects of glyphosate alone on the numbers of total cells, Sertoli cells and of germ cells in seminiferous tubule segments cultured in the absence (control) or presence of 50 nM or 500 nM or 5 μM glyphosate.

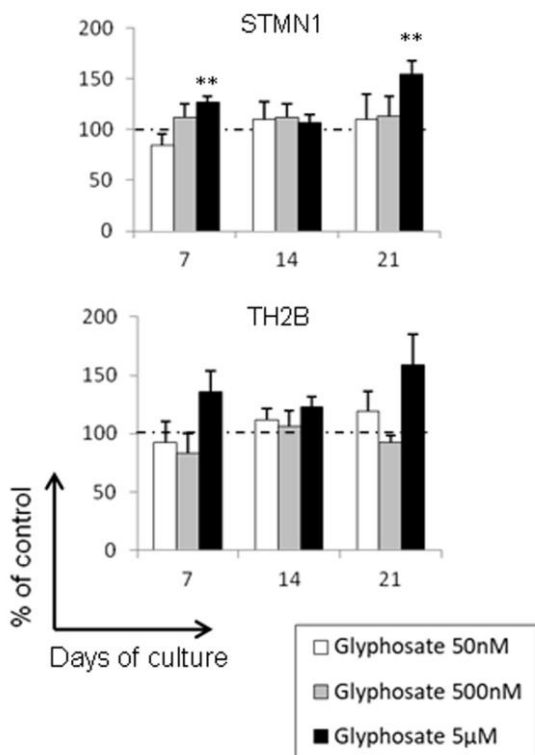
As the number of seeded cells varied from one culture to another, results were expressed relatively to the numbers of each of the different cell types under control conditions which were given as an arbitrary value of 100. Each value is the mean ± SEM of 4 cultures.

(A) Changes in the number of total cells

(B) Changes in the numbers of Sertoli cells and of germ cells. Germ cells are: spermatogonia, young spermatocytes, middle to late pachytene spermatocytes (PS), secondary spermatocytes (SII) and round spermatids.

\* p<0.05; \*\* p<0.01 vs control.

**Figure 4:**



**Figure 4:**

Changes in the levels of STMN1 or TH2B mRNA-related qPCR products in seminiferous tubule segments cultured in the absence (control) or presence of 50 nM or 500 nM or 5 µM glyphosate.

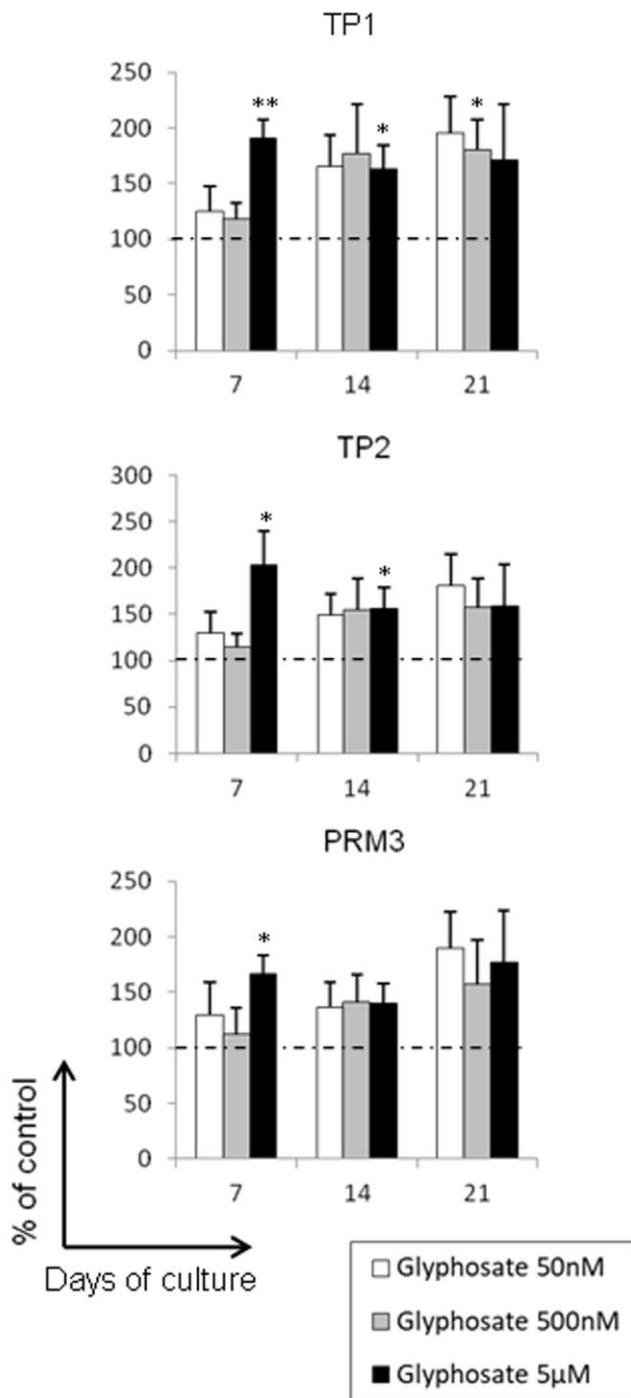
The numbers of transcripts were expressed by % of 4C cells in the cultures.

Results are expressed as a percentage of values obtained from cells cultured under control conditions. Each value is the mean  $\pm$  SEM of 4 cultures.

\*\* p < 0.01 vs control.



**Figure 5:**



**Figure 5:**

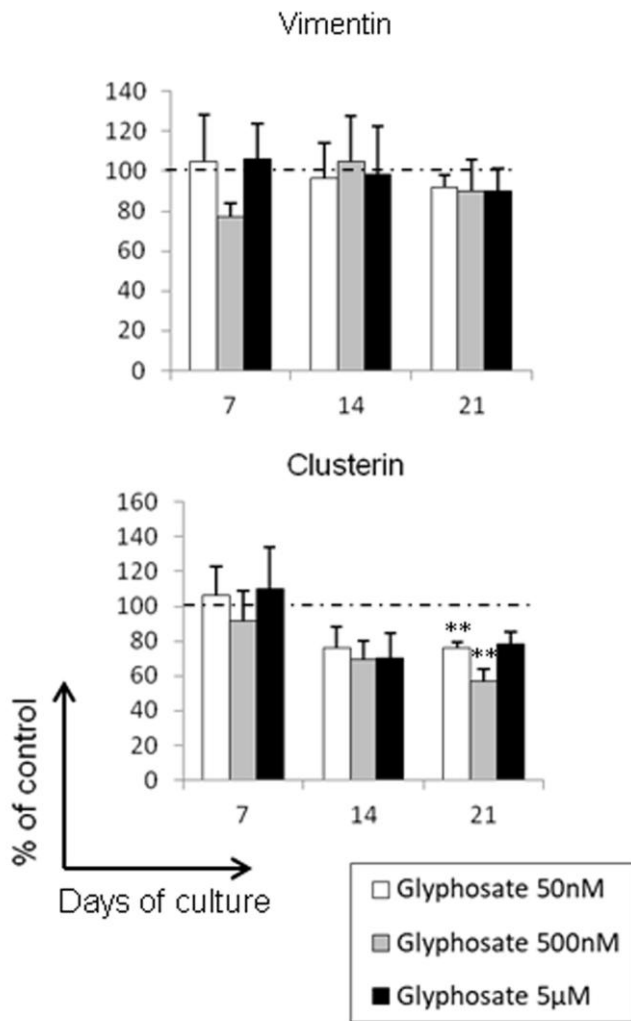
Changes in the levels of TP1, TP2 or PRM3 mRNA-related qPCR products in seminiferous tubule segments cultured in the absence (control) or presence of 50 nM or 500 nM or 5 µM glyphosate.

The numbers of transcripts were expressed by % of 1C cells in the cultures.

Results are expressed as a percentage of values obtained from cells cultured under control conditions. Each value is the mean ± SEM of 4 cultures.

\* p < 0.05; \*\* p < 0.01 vs control.

**Figure 6:**



**Figure 6:**

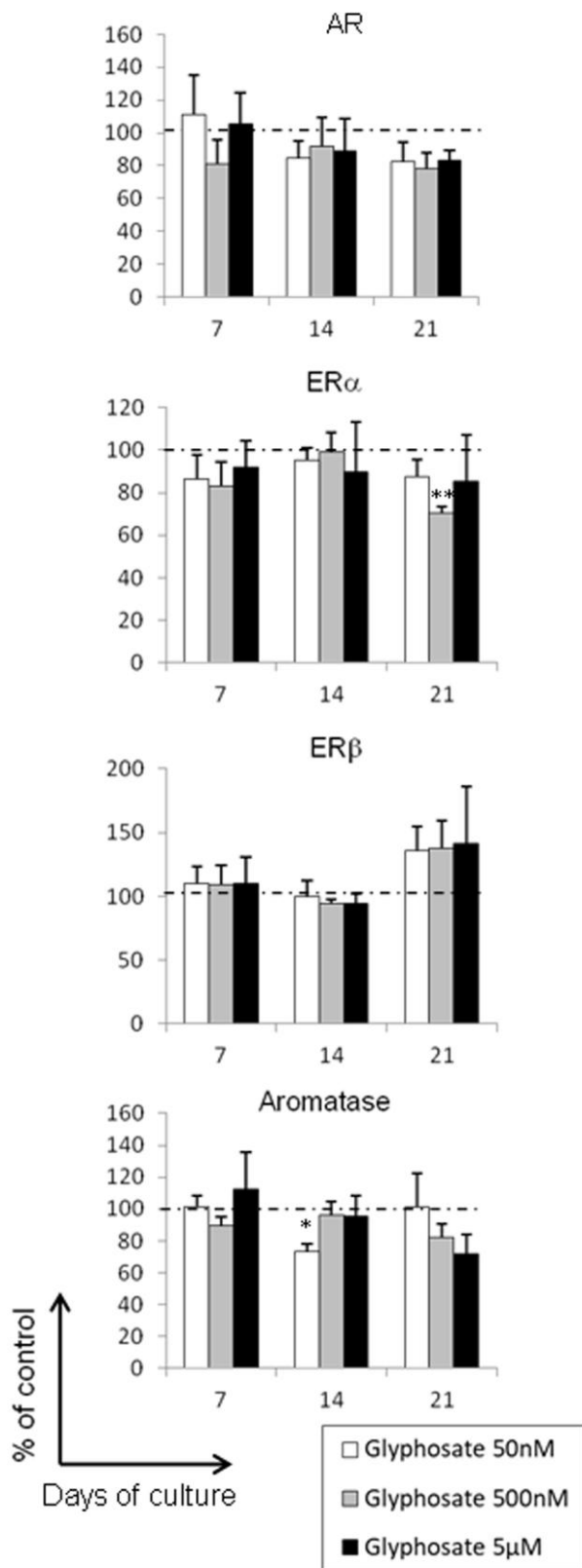
Changes in the levels of Vimentin or Clusterin mRNA-related qPCR products in seminiferous tubule segments cultured in the absence (control) or presence of 50 nM or 500 nM or 5 µM glyphosate.

The numbers of transcripts were expressed by % of Sertoli cells in the cultures.

Results are expressed as a percentage of values obtained from cells cultured under control conditions. Each value is the mean  $\pm$  SEM of 4 cultures.

\*\*  $p < 0.01$  vs control.

**Figure 7:**



**Figure 7:**

Changes in the levels of AR, ER $\alpha$ , ER $\beta$  or Aromatase mRNA-related qPCR products in seminiferous tubule segments cultured in the absence (control) or presence of 50 nM or 500 nM or 5  $\mu$ M glyphosate.

For AR the numbers of transcripts were expressed by % of Sertoli cells. Given that oestrogen receptors ER $\alpha$  and ER $\beta$  and Aromatase are expressed by both Sertoli and germ cells, their mRNA transcripts were not corrected by any cell population.

Results are expressed as a percentage of values obtained from cells cultured under control conditions. Each value is the mean  $\pm$  SEM of 4 cultures.

\*  $p < 0.05$ ; \*\*  $p < 0.01$  vs control.

