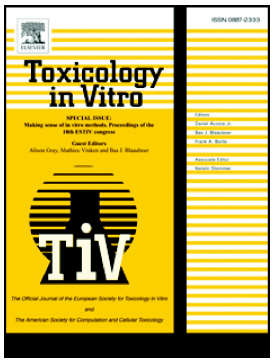

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IN VITRO EFFECTS OF GLYPHOSATE AND ROUNDUP ON SERTOLI CELL PHYSIOLOGY

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ABSTRACT

Roundup (R), a formulation that contains glyphosate (G) as the active ingredient, is a commonly used nonselective herbicide that has been proposed to affect male fertility. It is well known that an adequate Sertoli cell function is essential to maintain germ cell development. The aim of the present study was to analyze whether G and R are able to affect Sertoli cell functions, such as energy metabolism and blood-testis barrier (BTB) integrity. Sertoli cell cultures from 20-day-old rats were exposed to 10 and 100 ppm of G or R, doses which do not decrease cell viability. Neither G nor R caused impairment in lactate production or fatty acid oxidation. G and R decreased Transepithelial Electrical Resistance, which indicates the establishment of a Sertoli cell junction barrier. However, neither G nor R modified the expression of claudin11, ZO1 and occludin, proteins that constitute the BTB. Analysis of cellular distribution of claudin11 by immunofluorescence showed that G and R induced a delocalization of the signal from membrane to the cytoplasm. The results suggest that G and R could alter an important function of Sertoli cell such as BTB integrity and thus they could compromise the normal development of spermatogenesis.

KEYWORDS: Sertoli cells, Glyphosate, Roundup, Blood-testis barrier, claudin11

INTRODUCTION

Roundup, a formulation that contains glyphosate as the active ingredient and polyoxyethyleneamine (POEA) as the surfactant agent, is the major herbicide used worldwide. It is marketed as a nonselective, broad-spectrum, post-emergence herbicide used to control weeds in crops particularly on genetically modified plants that have been designed to tolerate it (Smith and Oehme, 1992). Glyphosate prevents the development of plants by inhibiting the enzyme enolpyruvylshikimate phosphate synthase (EPSPS) and interfering with the production of essential aromatic aminoacids (Boocock and Coggins, 1983). As this enzyme is not expressed by any member of the animal kingdom, the actions of glyphosate have been postulated to be present exclusively in plants (Franz *et al.*, 1997; Williams *et al.*, 2000). However, collateral effects in the animal kingdom have been observed. Particularly, it has been proposed that glyphosate might act as an endocrine disruptor that can affect male fertility. In *in vitro* studies, it has been shown that Roundup inhibits steroidogenesis by disrupting Steroidogenic Acute Regulatory (StAR) protein expression in MA-10 Leydig cell line (Walsh *et al.*, 2000). In addition, Roundup can also lead to alterations in aromatase mRNA levels and activity of the enzyme in a placental cell line and in human HEK293 cells (Benachour *et al.*, 2007; Richard *et al.*, 2005) and in HepG2 cells Roundup inhibits the expression of androgen and estrogen receptors (Gasnier *et al.*, 2009). The reproductive toxicity of Roundup/glyphosate has also been studied in vivo. In rats, Dallegrave *et al.* (2007) have shown that exposure to glyphosate-Roundup in utero and during lactation may induce significant adverse effects on the reproductive system of male Wistar rats at puberty and during adulthood. Romano *et al.* (2010) showed that exposure to the herbicide alters testosterone levels and testicular morphology and Cassault-Meyer *et al.* (2014) showed alterations in aromatase levels in testis and in sperm nuclear quality. In adult rabbits, Yousef *et al.* (1995) also reported reduced ejaculate volume and sperm concentration and increased abnormal and dead sperm. In

mice, Pham *et al.* (2019) showed that perinatal exposure to glyphosate affects spermatogenesis by reducing testosterone and the number of spermatogonia and spermatozoa. Noticeably, possible impairment of Sertoli cell function, which may be partly responsible for the adverse effects provoked by the herbicide on male reproduction, has not been analyzed so far.

In adult mammalian testis, spermatogenesis takes place in the seminiferous tubule where Sertoli cells, which provide structural and nutritional support to germ cells, constitute the main component of the blood-testis barrier (BTB) (Dym and Fawcett, 1970; Setchell and Waites, 1975; Yan and Cheng, 2005). By the presence of a BTB two compartments —basal and adluminal— can be recognized in the seminiferous tubules. The majority of the germ cells is situated in the adluminal compartment —not receiving blood nutrients or other circulation products— and depends on substances produced by Sertoli cells to fuel their metabolism (Regueira *et al.*, 2015a; Regueira *et al.*, 2017; Riera *et al.*, 2001). In this context, an adequate metabolic function of Sertoli cells is essential to maintain germ cell development. Beyond the nutritional support, Sertoli cell production of different substances supplies a precise and distinct microenvironment for successful meiosis and spermatogenesis (Mruk and Cheng, 2015; Russell, 1978). This particular microenvironment is maintained by the presence of the BTB, which is regulated by an array of intriguingly coordinated signaling pathways and molecules (Lui et al., 2003; Wong and Cheng, 2005). It is worth mentioning that impairment of the BTB leads to disruption of spermatogenesis and that some negative effects of several environmental toxicants on male reproductive functions have been attributed to a perturbation of BTB integrity (Gao et al., 2015). Few studies have assessed the effects of Roundup or glyphosate on Sertoli cell function, particularly on metabolism and barrier integrity, and data are not conclusive.

The detection of pesticide residues in the agricultural workers homes (Curwin *et al.*, 2005), the presence of these residues and their metabolites in food (Cox and Surgan, 2006) and in the urine

of families living near country areas (Curwin *et al.*, 2007) show that there is a risk of environmental exposure. Despite the obvious benefits of pesticides, their extensive use has posed problems for both the environment and human health. Therefore, searches for mechanisms by which pesticides could interfere with normal cell functions are of great interest. The aim of the present study was to analyze whether Roundup and/or glyphosate can affect Sertoli cell functions, such as energy metabolism and BTB integrity, which are essential to maintain spermatogenesis.

MATERIALS AND METHODS

Materials

[2,6-^{3}H]-2-deoxy-D-glucose (2-DOG) and [9,10*(n)*-^{3}H] palmitic acid were purchased from NEN (Boston, MA, USA). Culture media, glyphosate and all other drugs and reagents were purchased from Sigma-Aldrich (St Louis, MO, USA). The glyphosate formulation used in this work was the formulation available on the market called Roundup Full II (Monsanto Argentina S.A.I.C.), which contains 54% w/v acid glyphosate.

Sertoli cell (SC) isolation and culture

Twenty-day-old Sprague-Dawley rats (Rattus norvegicus) were obtained from the Animal Care Laboratory, Facultad de Ciencias Veterinarias, Buenos Aires, Argentina. Animals were killed by CO_2 asphyxiation according to protocols for animal laboratory use following the principles and procedures outlined in the National Institute of Health Guide for Care and Use of Laboratory Animals. The protocol was approved by the Comité Institucional de Cuidado y Uso de Animales de Laboratorio (CICUAL) from the Hospital de Niños "Dr. Ricardo Gutiérrez". SC were isolated as previously described (Gorga *et al.*, 2017). Decapsulated testes were digested with 0.1% w/v

collagenase and 0.006% w/v soybean trypsin inhibitor in Hanks' balanced salt solution (HBSS) for 5 min at room temperature by manual agitation. The enzymatic action was stopped by dilution with four volumes of HBSS. Seminiferous tubules were collected by sedimentation and washed twice with HBSS. Then seminiferous tubules were cut with a razor and submitted to 1 M glycine -2 mM EDTA (pH 7.4) treatment for 10 min to remove peritubular cells. At the end of the incubation period nine volumes of HBSS were added and a 30 min sedimentation was performed. The washed tubular pellet was then digested again with 0.1% w/v collagenase and 0.006% w/v soybean trypsin inhibitor in HBSS for 10 min at room temperature by continuous pipetting. The enzymatic action was stopped by dilution with four volumes of HBSS. The cell suspension was collected by centrifugation at 200 x g for 3 min. The cell suspension was diluted with HBSS and submitted to a 10 min sedimentation to remove germ cells. The pellet containing SC was filtered through a nylon mesh and SC was recovered by centrifugation at 200 x g for 3 min. SC were resuspended in culture medium which consisted of a 1:1 mixture of Ham's F-12 and Dulbecco's modified Eagle's medium, supplemented with 10 mM HEPES, 100 IU/ml penicillin, 2.5 µg/ml amphotericin B, 1.2 mg/ml sodium bicarbonate, 10 µg/ml transferrin, 5 µg/ml insulin, 5 µg/ml vitamin E and 4 ng/ml hydrocortisone. SC were cultured on 6-, 24- or 96-multiwell plates (5 µg DNA/cm^2), on Matrigel-coated cell culture inserts (15 µg DNA/cm^2) placed on 24-multiwell plates or on glass coverslips coated with laminin at 34°C in a mixture of 5% CO_2:95% v/v air.

No myoid cell contamination was revealed in the cultures when an immunoperoxidase technique was applied to SC cultures using a specific antiserum to smooth muscle α actin. Remaining cell contaminants were of germ cell origin and this contamination was below 5% after 48 h in culture as examined by phase contrast microscopy.

Culture conditions

SC were allowed to attach for 48 h in the presence of insulin and medium was replaced at this time with fresh medium without insulin. Treatment with glyphosate (G) and Roundup (R) was performed with variable doses and for variable periods of time. Cells incubated for 48 h with 10, 100 and 1000 ppm of G and R harvested on day five were used to evaluate cell viability and LDH leakage. The cells treated for 48 h with 100 ppm of G and R were harvested on day five and used to evaluate *GLUT1, FAT/CD36* and *CPT1* mRNA levels, glucose uptake and fatty acid (FA) oxidation and the 48 h-conditioned media were utilized to evaluate lactate production. For western blot studies, cells cultured for 4 days under basal conditions and pretreated for 30 min with 10 and 100 ppm of G or R were used. To quantify Transepithelial Electrical Resistance (TER), SC were cultured at high cell density (15 µg DNA/cm^2, corresponding to 1.2×10^6 cells/cm^2) on Matrigel-coated (1:6 dilution with F12/DMEM v/v) cell culture inserts (Millicell HA inserts) (Millipore, Billerica, MA, USA) placed on 24-multiwell plates. On day 3 in culture testosterone was added and TER across SC monolayer was recorded every 24 h in culture. On day 5, when the tight junction barrier had been formed (Lui *et al.*, 2001), different doses of G or R were added and TER was recorded until day 8. To study the distribution and localization of claudin11, the cells were cultured on glass coverslips coated with laminin and treated with 100 ppm G or R in the presence or absence of testosterone for 48 h and harvested on day 5.

Evaluation of Sertoli cell energetic metabolism

Energetic metabolism in SC has been considered to have features of its own. Lactate, produced by SC, provides the energetic substrate to germ cells in the adluminal compartment (Grootegoed *et al.*, 1984; Jutte *et al.*, 1983). Consequently, it has been postulated that SC utilizes FA as their energy source. In this context, lactate production, glucose uptake, FA oxidation and the expression of genes involved in these processes were evaluated.

a) Lactate determination

Conditioned media obtained from cells cultured in 24-multiwell plates were used to determine lactate production. Lactate was measured by a standard method involving conversion of NAD^+ to NADH (Gorga *et al.*, 2017).

b) Measurement of 2-deoxyglucose (2-DOG) uptake

Glucose transport was studied using the uptake of the labeled non-metabolizable glucose analogue 2-DOG on cells cultured in 24-multiwell plates as previously described (Gorga *et al.*, 2017).

c) Fatty acid oxidation assay

FA oxidation was performed measuring the release of ^3H_2O to the incubation medium from [^{3}H]-palmitate on SC cultured in 24-multiwell as previously described (Regueira *et al.*, 2015b).

Evaluation of BTB function

As mentioned in the introduction SC supply germ cells with a microenvironment preserved by the BTB. The main component of the BTB is the presence of tight junctions between neighboring SC. In order to evaluate BTB function, Transepithelial Electrical Resistance (TER), claudin11 cellular distribution and the expression of proteins that participate in tight junction assembly were evaluated.

a) Transepithelial Electrical Resistance (TER) measurement

The establishment of the SC junction barrier was assessed daily from day 3 to day 8 by measurement of TER across the SC monolayer by a Millicell electrical resistance system (Millipore), as described previously (Perez *et al.*, 2014). Briefly, a short (~2 sec) 20-µA pulse of current was passed through the epithelial monolayer between 2 silver-silver chloride electrodes and electrical resistance was measured. Electrical resistance was then multiplied by the surface area of the

insert to yield the area of resistance in ohms.cm^2. The net value of electrical resistance was then computed by subtracting the background, which was determined by Matrigel-coated cell-free inserts. Each time point had quadruplicate bicameral units. This experiment was run four times on different batches of cells.

b) Immunofluorescent (IF) detection of claudin11 protein

Monolayers were fixed with methanol for 10 min at −20°C. After washes with PBS, cells were permeabilized with 0.1% Triton X-100 in PBS for 30 min at room temperature. After 3 washes with PBS for 1 min each, the cells were blocked with 5% bovine serum albumin (BSA). Then, the coverslips were incubated with a 1:50 dilution of polyclonal antibody against claudin11 (Zymed Lab. Inc.) in PBS overnight at 4°C. After 3 washes with PBS for 1 min each, coverslips were incubated with an anti-rabbit IgG fluorescein isothiocyanate (FITC)-conjugated (1:25; Vector Laboratories, Burlingame, CA, USA). For negative controls, primary antibodies were replaced by PBS. Finally, the coverslips were washed 3 times with PBS for 1 min each, mounted in buffered glycerine and observed using an Axiophot fluorescent microscope with epi-illumination (Carl Zeiss Inc., Oberkochen, Germany).

RT-Real-time PCR (RT-qPCR)

The expression of genes that participate in energetic metabolism (*GLUT1, FAT/CD36* and *CPT1)* and in BTB organization (*occludin*, *claudin11* and *ZO-1)* was evaluated by RT-qPCR.

Total RNA was isolated from SC cultured in 6-multiwell plates with TRI Reagent (Sigma-Aldrich). The amount of RNA was estimated by spectrophotometry at 260 nm. RT was performed on 2 µg RNA at 42°C for 50 min with a mixture containing 200 U MMLV reverse transcriptase enzyme, 125 ng random primers and 0.5 mM dNTP Mix (Invitrogen, Argentina Ltda).

Real-time PCR was performed by a Step One Real Time PCR System (Applied Biosystems, Warrington, UK). The specific primers for RT-qPCR are shown in Table 1. Amplification was carried out as recommended by the manufacturer: 25 µl reaction mixture containing 12.5 µl of SYBR Green PCR Master mix (Applied Biosystems), the appropriate primer concentration and 1 µl of cDNA. The relative cDNA concentrations were established by a standard curve using sequential dilutions of a cDNA sample. The data were normalized to HPRT1. The amplification program included the initial denaturation step at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 sec, and annealing and extension at 60°C for 1 min. Fluorescence was measured at the end of each extension step. After amplification, melting curves were acquired and used to determine the specificity of PCR products. The comparative ΔΔCt method was used to calculate relative gene expression.

Western blot analysis

Cells cultured in 6-multiwell plates were washed once with PBS at room temperature. Then, 200 µl of PBS containing 2 µl of protease inhibitor cocktail (P-8340; Sigma-Aldrich), 1 mM NaF, 1 mM EGTA, 1 mM EDTA, 50 nM okadaic acid and 2 mM PMSF was added to each well. Cells were then placed on ice and disrupted by ultrasonic irradiation. Western blot analysis was performed as previously described (Riera *et al.*, 2007). Membranes were probed with antibodies that allow specific recognition of total Akt and mTOR, phosphorylated p38-MAPK and ERK1/2 (Cell Signaling Technology, Inc., Danvers, MA, USA), claudin11 (Zymed Lab. Inc.), androgen receptor and GAPDH (Santa Cruz Biotechnology, Inc, USA). A 1:1000 dilution of primary antibodies, as indicated by the manufacturer, was used. For chemiluminescent detection of the blots, a commercial kit from Cell Signaling Technology was used. The intensities of the autoradiographic bands were estimated by

densitometry scanning using NIH Image Software (Scion Corporation). Levels of the corresponding total Akt, mTOR and GAPDH served as loading controls.

Cytotoxicity

A cell viability test (MTT assay) was performed in cells cultured in 96-multiwell using a commercial kit (CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay; Promega Corporation). Cell cytotoxicity was determined by measuring the activity of LDH enzyme leaked from the cytosol of damaged cells into the medium as previously described (Riera *et al.*, 2007). Results were expressed as the percentage of activity detected in the media over the sum of the activities in the media and in cells.

Statistical analysis

All experiments were run in triplicates and repeated 3-4 times. One way ANOVA and post hoc analysis using Tukey-Krämer's multiple comparisons test were performed using InfoStat versión 2016 (Grupo InfoStat, FCA, UNC, Argentina). P values < 0.05 were considered statistically significant.

RESULTS

Effects of glyphosate and Roundup on SC cell viability

SC cultures were exposed for 48 h to glyphosate (G) and Roundup (R) at concentrations ranging from 10 to 1000 ppm, corresponding to 0.01 to 1 g/L respectively. These concentrations were selected to perform the study because besides being found in some human urine samples (Krüger *et al.*, 2014), they are 10-1000 times lower than the recommended herbicide concentration (10000 ppm) in agriculture practice. Cell viability was analyzed by MTT assay and by measuring LDH leaked

from the cytosol of damaged cells into the medium. The highest dose tested for R (1000 ppm) caused a cell death (Figure 1). Therefore, in the present investigation destined to analyze G and R effects on SC functions only doses of 10 and 100 ppm were utilized.

Effects of glyphosate and Roundup on SC energetic metabolism

Energetic metabolism in SC has been considered to have features of its own. Studies on SC glucose metabolism have shown that these cells actively metabolize this sugar, but the vast majority is converted to lactate (Robinson and Fritz, 1981). Lactate production by SC provides the energetic substrate to germ cells in the adluminal compartment (Grootegoed *et al.*, 1984; Jutte *et al.*, 1983). Consequently, SC cannot rely on glucose for its own energy requirements and it has been postulated that this cell type utilizes FA as their energy source. In this metabolic context, mechanisms by which pesticides could interfere with SC lactate production and FA oxidation are relevant to comprehend some causes of spermatogenesis abnormalities due to pollutants exposure. SC cultures were exposed for 48 h to 100 ppm of G and R. Figure 2 shows the results obtained for lactate production, glucose uptake and *GLUT1* mRNA levels. The exposure to G or R did not modify lactate production neither glucose uptake nor *GLUT1* expression. On the other hand, figure 3 shows the results obtained for FA oxidation, *FAT/CD36* and *CPT1* mRNA levels. Again, no variations in the parameters analyzed were observed after G or R exposure.

Effects of glyphosate and Roundup on blood-testis barrier integrity

As mentioned in the introduction SC supply germ cells with a microenvironment preserved by the BTB. The main component of the BTB is the presence of tight junctions between neighboring SC. The establishment of these junctions between SC in culture was assessed daily from day 3 to day 8 by measuring TER across the SC monolayer. When SC were plated, junctions begin to assemble

and an increase in TER is observed. On day 5, when SC completed barrier assembly, G or R were added to the culture medium. The addition of 100 ppm of G or 10 and 100 ppm of R produced a significant decline in TER (Figure 4 and Table 2).

Considering that it had been demonstrated that p38-MAPK and ERK1/2 signaling pathways were involved in the disruption of BTB integrity by xenobiotics, the possible alteration of these pathways by G or R treatment was evaluated. Figure 5B shows that R increased P-p38-MAPK and P-ERK1/2 levels. Figure 5A shows that G did not modify P-p38-MAPK and P-ERK1/2 levels at any dose tested.

We next evaluated possible effects of G or R on the expression of intercellular junction proteins such as claudin11, occludin and ZO-1. Figure 6 shows that 100 ppm G or R treatment did not modify *claudin11*, neither *occludin* nor *ZO-1* mRNA levels. Finally, claudin11 protein levels and cellular localization of claudin11 was evaluated in SC monolayers. Figure 7A shows that claudin11 protein levels were not modify by G or R treatment. Figure 7B shows that claudin11 was detected at the zone of contact between adjacent cells, in a linear and continuous pattern that delineated cell borders in basal conditions. Addition of 100 ppm G or R induced redistribution of claudin11 since immunofluorescence became discontinuous and was redistributed from the cell surface in to the cytoplasm.

Effects of glyphosate and Roundup on testosterone regulation of blood-testis barrier integrity

It is well known that testosterone is the main regulator of BTB function, and that G or R can act as endocrine disruptors. In order to elucidate a possible mechanism responsible for adverse effects of G or R, we decided to evaluate whether herbicides can interfere with androgen action in BTB. Figure 8A and 8B shows that 100 ppm G or R treatment did not modify androgen receptor mRNA or protein levels. Figure 8C and Table 3 shows that testosterone increased TER and that the

addition of 100 ppm G or R provoked a significant decline in testosterone-stimulated TER values. Finally figure 9 shows that, similar to what was observed under basal conditions, 100 ppm G or R treatment induced a redistribution of claudin11 from cell membrane to cytoplasm under the presence of testosterone.

DISCUSSION

Over the last 60 years a progressive decrease in male reproductive function has been observed. Epidemiological and experimental studies suggest that one of the main causes is exposure to environmental toxicants. As previously stated, several studies have shown that G or R can potentially cause adverse effects in male reproduction (Cai *et al.*, 2017; Dallegrave *et al.*, 2007; Pham *et al.*, 2019; Romano *et al.*, 2010; Yousef *et al.*, 1995). Despite the compelling documented evidence proving the existence of adverse effects on testis function, little is known about direct effects on Sertoli cell function and the possible mechanisms involved.

Primary cultures of Sertoli cells are a good and reliable model to assess the direct effects of xenobiotic exposure on this cell type (Liu *et al.*, 2018; Lu *et al.*, 2016; Reis *et al.*, 2015). Regarding G and its commercial formulation R, initial studies on Sertoli cell cultures utilizing doses from 1 to 10000 ppm of G and R were performed. In these experiments, treatment with 1000 ppm R produced Sertoli cell apoptosis with a maximal effect in 24 h-incubation period. However, in the same study G did not induce apoptosis in isolated Sertoli cells (Clair *et al.*, 2012). Other studies, which measured apoptosis and/or cell viability, have also demonstrated that several human cell lines are more sensitive to R than to G (Benachour and Seralini, 2009; Defarge *et al.*, 2016; Gasnier *et al.*, 2009; Mesnage *et al.*, 2014; Richard *et al.*, 2005). Coincidentally, the results presented herein show that 1000 ppm R decreases cell viability while G does not. These differences on the actions of both agents can be interpreted by the presence of various adjuvants in R. These

adjuvants change human cell permeability and amplify toxicity by G, which may explain the differences observed between R and G effects on Sertoli cell viability (Mesnage *et al.*, 2019). As we were focused on analyzing possible effects on Sertoli cell function, in this study we utilized 10 and 100 ppm doses which do not decrease cell viability.

As mentioned in the introduction, two essential functions of mature Sertoli cells are the provision of nutrients to germ cells and the maintenance of a favorable microenvironment for spermatogenesis. As for Sertoli cell nutritional function, it has been well documented that Sertoli cell glycolysis provides lactate to satisfy germ cell energy demands while FA oxidation supplies energy to fulfill Sertoli cell energetic demands. Consequently, it can be predicted that an alteration in Sertoli cell metabolism can lead to a perturbation in normal germ cell development. The results obtained in the present investigation show that neither G nor R modify lactate production, glucose uptake and *GLUT1* expression in Sertoli cells. Additionally, the results show that G and R do not modify FA oxidation and *FAT/CD36* and *CPT1* expression, proteins that are essential for FA utilization. Altogether, the results presented herein lead us to conclude that G and R effects on testicular function are not mediated by impairment of Sertoli cell metabolism. Noticeably, other reproductive toxicants, including phthalate esters (Williams and Foster, 1989), nitro-benzene (Allenby *et al.*, 1990), gossypol (Monsees *et al.*, 1998b) and certain heavy metal ions (Monsees *et al.*, 1998a; Yu *et al.*, 2019), alter Sertoli cell lactate production and show that toxicant effects strongly depend on the chemical nature of the toxicant.

Regarding lipid metabolism, it has been demonstrated that 24 h exposure to 5000 ppm G or R induce lipid droplet accumulation in the Sertoli cell line TM4 cytoplasm. This increase in lipid droplet accumulation was interpreted as storage of potentially deleterious lipophilic formulants in the cytoplasm of TM4 cells and was considered a sign of the cytotoxic effect (Vanlaeys *et al.*, 2018). An alternative explanation for the above results would be a modification in lipid

metabolism, however, the authors did not explore this possibility. We have explored the latter possibility and the results presented herein show that 100 ppm G and R, doses that do not affect Sertoli cell viability, do not alter FA oxidation.

It is worth mentioning that Sertoli cells, in addition to the nourishment provided to germinal epithelia, supply germ cells with a suitable microenvironment for successful meiosis and completion of spermatogenesis (Russell, 1978; Russell *et al.*, 1989). This microenvironment is sustained by the BTB, whose main components are the tight junctions between neighboring Sertoli cells (Dym and Fawcett, 1970; Setchell and Waites, 1975; Yan and Cheng, 2005). The BTB is highly dynamic and is regulated by an array of intriguingly coordinated signaling pathways and molecules (Lui *et al.*, 2003). Several studies have shown that many environmental toxicants, such as cadmium, bisphenol A, fluoride and sulfur dioxide exert their effects by targeting Sertoli and germ cell junctional proteins, as well as the permeability of the BTB (Li *et al.*, 2009b; Siu *et al.*, 2009; Zhang *et al.*, 2016). TER is a widely accepted quantitative technique that measures the integrity and permeability of BTB *in vitro*. This *in vitro* model has been widely used by several studies related to the field of testicular toxicants (Byers *et al.*, 1986; Janecki *et al.*, 1992; Li *et al.*, 2001; Okanlawon and Dym, 1996). The results presented in this investigation show that 100 ppm G and 10 and 100 ppm R decrease TER after 24 and 48 h-treatment. Notable, the disrupting effect of G on the permeability of other barriers had already been observed. It was shown that exposure to 10 mg/ml (10000 ppm) G reduces TER and increases permeability to mannitol in Caco-2 and IEC-18 intestinal cell lines (Vasiluk *et al.*, 2005). In addition, 1 and 10 µM G treatment (0.16 and 1.6 ppm) decreases TER and increases permeability to fluorescein in iPSC-derived brain microvascular endothelial cells cell line (BMECs) (Martinez and Al-Ahmad, 2019). Results from other authors and our own let us postulate that one of the mechanisms by which G exerts toxic effects is related to disruption of barrier properties in different important organs such as intestine, brain and testis.

It has been shown that p38-MAPK and ERK1/2 pathways play central roles in the dynamics of BTB. For example, TGFb3-induced physiological effect on Sertoli cell BTB dynamics is mediated via the p38-MAPK pathway (Lui *et al.*, 2003). As for toxicant effects, it has been demonstrated that the polychlorinated biphenyls (PCBs), such as congener Aroclor1254, and the perfluorooctane sulfonate (PFOS) can disrupt the BTB integrity by activating the p38-MAPK pathway (Jia *et al.*, 2017; Qiu *et al.*, 2013). Additionally, it has been shown that Biphenol A and mono (2-ethylhexyl) phthalate (MEHP) alter BTB by activating ERK1/2 pathway (Chiba *et al.*, 2012; Thuillier *et al.*, 2009). The present investigation shows that R increases P-p38-MAPK and P-ERK1/2 levels. Therefore, it might be suggested that R decreases TER through a p38-MAPK and ERK1/2 dependent pathways. However, the same reasoning cannot be applied to the effects of G considering that it lowers TER while it does not modify P-p38-MAPK and P-ERK1/2 levels. Hence, it seems that there is no direct relationship between P-p38-MAPK and P-ERK1/2 levels and TER levels, at least as a consequence of G and R exposure. We then decided to look for further mechanisms that may be involved in the disruption of BTB integrity by exposure to G and R.

The next set of experiments was devoted to analyze possible alterations in the expression of some tight junction proteins, such as occludin, claudin11 and ZO-1, in response to G or R. An increased expression of these proteins, at the time when the junctions are assembled as manifested by a stable TER across the Sertoli cell epithelia, was demonstrated (Lui *et al.*, 2001; Wong *et al.*, 2000). It is worth mentioning that certain agents that perturb BTB permeability, such as cytokines or toxicants, alter the expression of cell junction proteins (Chiba *et al.*, 2012; Chung *et al.*, 2001; Kaitu'u-Lino *et al.*, 2007; Lui *et al.*, 2001; McCabe *et al.*, 2016; Perez *et al.*, 2014; Ramos-Trevino *et al.*, 2017). The results obtained in the present investigation show that neither occludin nor claudin11 or ZO-1 expression was modified by G or R treatment.

Finally, we decided to investigate whether the cellular localization of a tight junction protein such as claudin11 could shed some light on the mechanism underlying the decrease in TER. Claudin11 is a key molecule that provides functional integrity to the BTB. It is located in functional Sertoli cell tight junctions and its intracellular distribution pattern changes when gonadotropins are suppressed, coincident with a dysfunctional barrier (McCabe *et al.*, 2016). Claudin11 null mice are sterile highlighting the importance of this protein for BTB integrity (Gow *et al.*, 1999). It is worth mentioning that in men with testicular disorders such as intraepithelial neoplasia, hypospermatogenesis, spermatogenic arrest, and Sertoli cell only testes, claudin11 is located in the cytoplasm, away from the tight junctions (Fink *et al.*, 2009; Haverfield *et al.*, 2013; Nah *et al.*, 2011) reinforcing the idea that localization of claudin11 in cytoplasm can be considered a sign of BTB dysfunction. In *in vitro* studies, a direct relationship between TER and localization of claudin11 in membrane junctions has been observed (McCabe *et al.*, 2016; Qiu *et al.*, 2013). The results presented herein show that although G and R treatment does not modify claudin11 protein levels, a redistribution of claudin11 from membrane to cytoplasm is observed. This alteration in the distribution of claudin11 may be interpreted as the result of an increase in membrane protein recycling from cell surface to cytoplasm. This last result may explain, at least in part, the effects of G and R in the integrity of BTB and the deleterious effect of these toxicants at testicular level. Disruption of the Sertoli cell permeability barrier by loss of usual distribution patterns of other junctional proteins such as occludin, ZO-1, and Cx43 induced by other toxicants has also been demonstrated (Fiorini *et al.*, 2004; Li *et al.*, 2009a; Qiu *et al.*, 2013) and these results indicate that redistribution of these proteins can also contribute to alter barrier permeability. Further studies will be necessary to definitively assign a role to this phenomenon in the alteration of BTB dynamics by G o R.

Several lines of evidence obtained from in vivo and in vitro approaches highlight the importance of testosterone in the regulation of BTB assembly and function. In vivo studies showed that reestablishment of testicular androgen levels by hCG or testosterone treatment leads to a restoration of claudin11 localization and BTB function (Haverfield *et al.*, 2013; McCabe *et al.*, 2012; McCabe *et al.*, 2010). In vitro studies showed that Sertoli cell permeability barrier formation and claudin11 localization are promoted by androgen treatment (Kaitu'u-Lino *et al.*, 2007). Furthermore, numerous studies describe G or R as endocrine disruptors. On the one hand, it was demonstrated that in vivo treatment of drakes with R decreases the expression of the androgen receptor (AR) in Sertoli cells (Oliveira *et al.*, 2007) while in rats, G treatment does not modify AR expression in the same cell type (Dai *et al.*, 2016). On the other hand, it was shown that in vivo treatment of rats decreases serum testosterone levels (Romano *et al.*, 2010), and furthermore, it was demonstrated that the effect on testosterone levels may be attributed to a decrease in the expression of STAR in Leydig cells (Walsh *et al.*, 2000). Therefore, it is tempting to speculate that G or R effects on BTB integrity can be partially attributed to the interference with androgen action. The results presented herein show that neither G nor R treatment modified androgen receptor mRNA and protein levels. They also show that the effects of the herbicides on TER and on claudin11 localization in the presence of testosterone were similar to those observed under basal conditions. Thus, G and R are able to disrupt BTB function in the presence of testosterone. Further in vivo experiments will be necessary to determine a possible role of G or R as endocrine disruptors.

In summary, this investigation shows that G and R alter the Sertoli cell junction barrier permeability. This study also shows that, at least in part, the loss of location of claudin11 at the interface between neighboring Sertoli cells might be responsible for the disassemble of the

barrier. We postulate that BTB integrity is a sensitive target for the adverse effects of G or R on male reproductive function.

DECLARATION OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Table 1: Rat-specific primers sets for RT-PCR analysis.

Gene	Primer sequence	Product Size (pb)	Accession Number
Glut1	FWD: 5'-GGAGTGTCGGTTTAGGTTGC-3'	98	NM_138827.1
	REV: 5'-GCTGTGAAACGGAGAATGGA-3'		
Fat/Cd36	FWD: 5'-ACCAGGCCACATAGAAAGCA-3'	137	NM_031561.2
	REV: 5'-CACCAATAACGGCTCCAGTAA-3'		
Cpt1	FWD: 5'-GGAACTCAAACCCATTCGTC-3'	113	NM_031559.2
	REV: 5'-GTTGGATGGTGTCTGTCTCT-3'		
Claudin 11	FWD: 5'-TGGTCTCTACCACTGCAAGC-3'	95	NM_053457.2
	REV: 5'-CCAGAACTGAGGCAGCAATC-3'		
Occludin	FWD: 5'-CCACTATGAAACCGACTACACG-3'	73	NM_031329.2
	REV: 5'-ATATTCCCTGAGCCAGTCCTC-3'		
ZO-1	FWD: 5'-CATCTAAACCTCCAAGTGCTTC-3'	132	NM_001106266.1
	REV: 5'-CAATATCTTCAGGTGGCTTCG-3'		
Hprt1	FWD: 5'-AGTTCTTTGCTGACCTGCTG-3'	127	NM_012583.2
	REV: 5'-TTTATGTCCCCCGTTGACTG-3'		

Table 2: Effect of 48-h treatment with G and R on TER

	TER ($\Omega.cm^2$)
Control	92,3 ± 3,1
G 10 ppm	85,5 ± 4,5
G 100 ppm	58,0 ± 1,5*
R 10 ppm	67,8 ± 2,8*
R 100 ppm	62,2 ± 4,9*

SC monolayers were maintained under Basal conditions or treated with 10 or 100 ppm of G or R on day 5 for 48 h. Results are presented as means±SD of four independent experiments (*$p<0.05$).

Table 3: Effect of 48-h treatment with G and R on testosterone regulation of TER across SC

	TER ($\Omega.cm^2$)
Basal	94,7 ± 8,4
T 1µM	144,9 ± 13,2*
T+G 100 ppm	111,5 ± 12,5
T+R 100 ppm	98,0 ± 6,0

SC monolayers were maintained under Basal conditions or stimulated with testosterone (T) since day 3. On day 5, SC monolayers were treated with 100 ppm of G or R for 48 h. Results are presented as means±SD of three independent experiments (*$p<0.05$).

FIGURE LEGENDS

Figure 1: Effect of G and R on SC cytotoxicity.

SC were maintained under Basal conditions or incubated with 10, 100 or 1000 ppm of G or R for 48 h. (A) Cell viability was determined by MTT assay. (B) LDH activity was determined in SC monolayer and in the culture medium. Values represent mean±S.D. of one representative experiment out of three. *p<0.05 versus Basal.

Figure 2: Effect of G and R on lactate production, glucose uptake and on *Glut1* mRNA levels in SC.

SC were maintained under Basal conditions or incubated with 100 ppm of G or R for 48 h. (A) Lactate levels were determined in the conditioned media. (B) Glucose uptake assay (2-DOG uptake) was performed after the 48 h incubation period. (C) Total RNA was extracted and RT-qPCR was performed to detect *Glut1* mRNA levels. The comparative ΔΔCt method was used to calculate relative gene expression. Graphics show pooled data from four independent experiments performed indicating fold variation in mRNA levels relative to Basal.

Figure 3: Effect of G and R on FA oxidation, *FAT/CD36* and *CPT1* mRNA levels in SC.

SC were maintained under Basal conditions or incubated with 100 ppm of G or R for 48 h. (A) Fatty acid oxidation was assessed by measuring $^{3}H_2O$ produced in the incubation medium. (B and C) Total RNA was extracted and RT-qPCR was performed to detect *FAT/CD36* and *CPT1* mRNA levels. The comparative ΔΔCt method was used to calculate relative gene expression. Graphics show pooled data from four independent experiments performed indicating fold variation in mRNA levels relative to Basal.

Figure 4: Effect of G and R on TER across SC.

SC monolayers were maintained under Basal conditions or treated with 10 or 100 ppm of G (A) or R (B) on day 5. TER across SC monolayer was measured from day 3 to 8. Values represent

mean±S.D. of triplicate wells from the same culture of one representative experiment out of four. Asterisks indicate significant differences from basal cultures for each particular day, $p<0.05$.

Figure 5: Effect of G and R on P-p38-MAPK and P-ERK1/2 levels in SC.

SC were maintained under Basal conditions or incubated with 10 or 100 ppm of G (A) or R (B) for 30 min. Western blot analysis was performed utilizing antibodies for phosphorylated p38-MAPK (P-p38-MAPK) and ERK1/2 (P-ERK1/2) or total Akt (T-Akt). Results are representative of 3 independent experiments performed/treatment group.

Figure 6: Effect of G and R on *claudin11, occludin* and *ZO-1* mRNA levels in SC.

SC were maintained under Basal conditions or incubated with 100 ppm of G or R for 48 h. Total RNA was extracted and RT-qPCR was performed to detect *occludin*, *claudin11* and *ZO-1* mRNA levels. The comparative ΔΔCt method was used to calculate relative gene expression. Graphics show pooled data from four independent experiments performed indicating fold variation in mRNA levels relative to Basal.

FIGURE 7: Effect of G and R on claudin11 protein levels and localization in SC.

SC were maintained under Basal conditions or incubated with 100 ppm of G or R for 48 h. A) Western blot analysis was performed utilizing antibodies for claudin11 or total Akt (T-Akt). Results are representative of three independent experiments performed/treatment group. B) Claudin11 was revealed by IF. Bars: 50 µm.

Figure 8: Effect of G and R on androgen receptor (AR) expression and testosterone regulation of TER across SC.

SC were maintained under Basal conditions or incubated with 100 ppm of G or R for 48 h. A) Total RNA was extracted and RT-qPCR was performed to detect AR mRNA levels. The comparative ΔΔCt method was used to calculate relative gene expression. Graphics show pooled data from four independent experiments performed indicating fold variation in mRNA levels relative to Basal. B)

Western blot analysis was performed utilizing antibodies for AR or total Akt (T-Akt). Results are representative of three independent experiments performed/treatment group. C) SC monolayers were maintained under Basal conditions or stimulated with testosterone (T) since day 3. On day 5, SC monolayers were treated with 100 ppm of G or R. TER across SC monolayer was measured from day 3 to 8. Values represent mean±S.D. of triplicate wells from the same culture of one representative experiment out of three. Symbols indicate significant differences for each particular day: *p<0.05 T vs Basal; #p<0.05 T vs T+G; §p<0.05 T vs T+R.

FIGURE 9: Effect of G and R on claudin11 localization in the presence of testosterone in SC.

SC were incubated with 100 ppm of G or R in the presence of testosterone for 48 h. Claudin11 was revealed by IF. Bars: 50 µm.

HIGHLIGHTS

- Glyphosate (G) and Roundup (R) effects on Sertoli cell function was studied in vitro
- G and R do not modify Sertoli cell lactate production and fatty acid oxidation
- G and R decrease Transepithelial Electrical Resistance
- G and R cause a redistribution of claudin11 from membrane to cytoplasm
- G and R could impair Blood-Testis-Barrier integrity

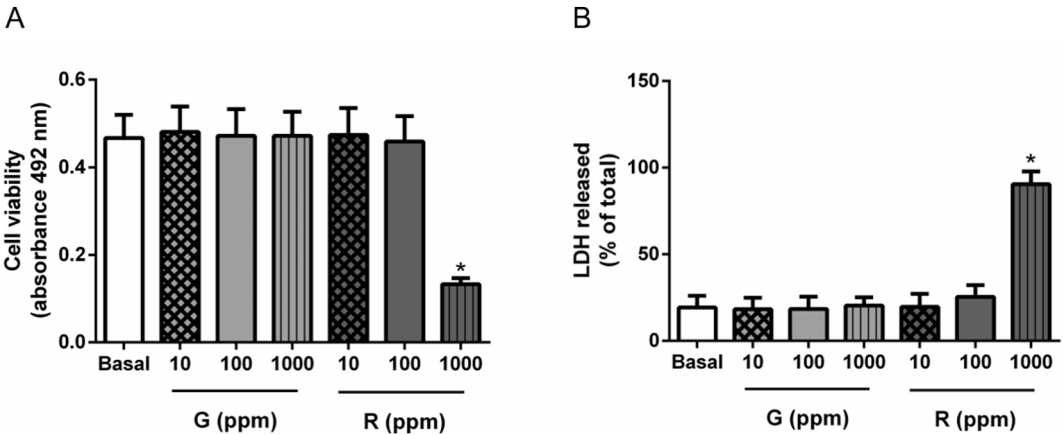
A
Cell viability
(absorbance 492 nm)
0.6
0.4
0.2
0.0
*
Basal
10
100
1000
10
100
1000
G (ppm)
R (ppm)
B
LDH released
(% of total)
150
100
50
0
*
Basal
10
100
1000
10
100
1000
G (ppm)
R (ppm)

Figure 1

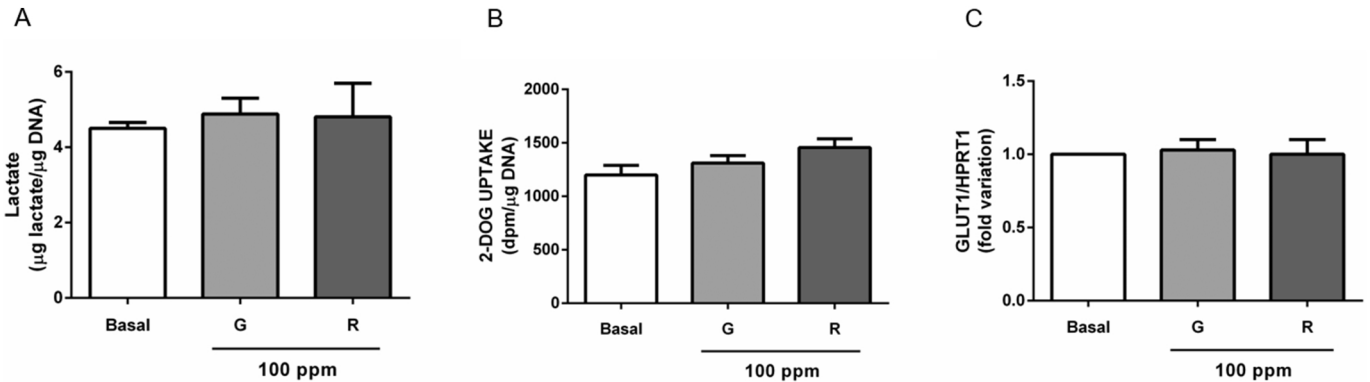
A
Lactate
(µg lactate/µg DNA)
6
4
2
0
Basal
G
R
100 ppm
B
2-DOG UPTAKE
(dpm/µg DNA)
2000
1500
1000
500
0
Basal
G
R
100 ppm
C
GLUT1/HPRT1
(fold variation)
1.5
1.0
0.5
0.0
Basal
G
R
100 ppm

Figure 2

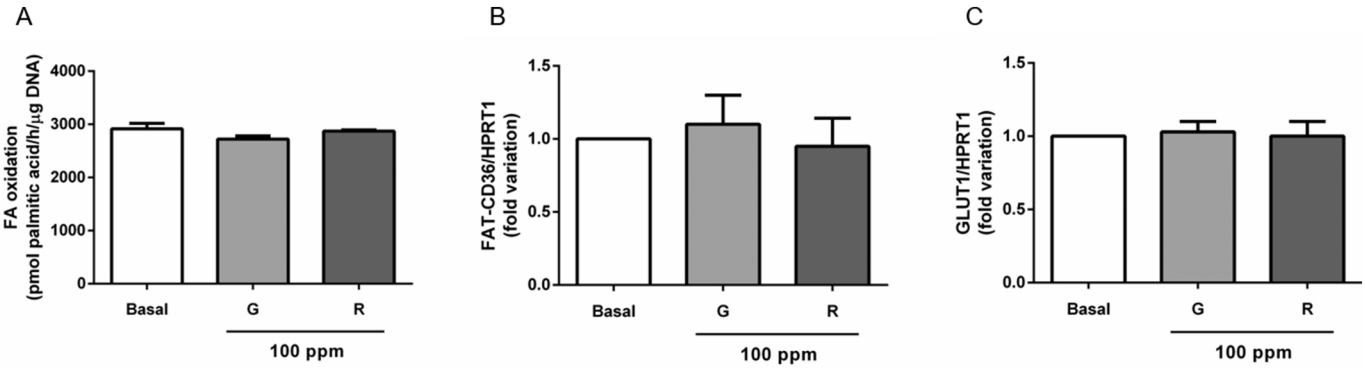
A
FA oxidation
(pmol palmitic acid/h/µg DNA)
4000
3000
2000
1000
0
Basal
G
R
100 ppm
B
FAT-CD36/HPRT1
(fold variation)
1.5
1.0
0.5
0.0
Basal
G
R
100 ppm
C
GLUT1/HPRT1
(fold variation)
1.5
1.0
0.5
0.0
Basal
G
R
100 ppm

Figure 3

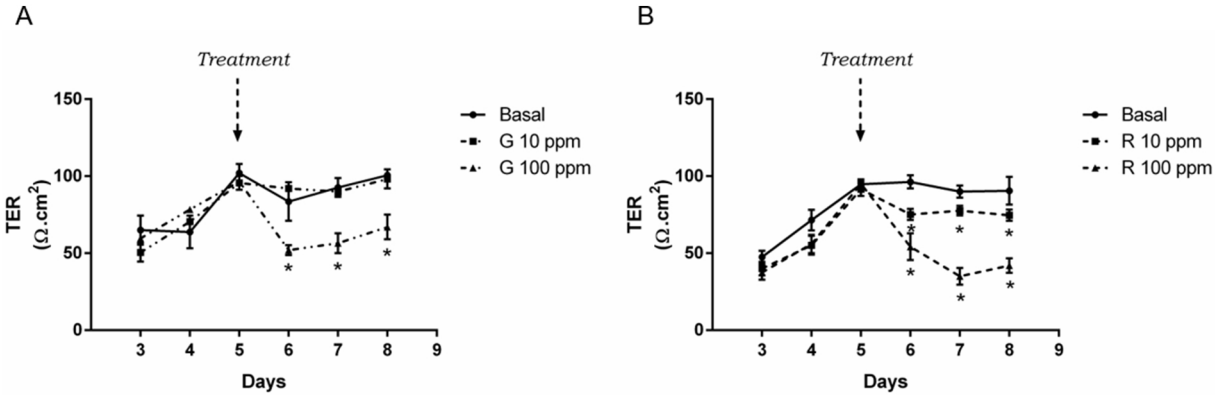
A
Treatment
150
100
50
0
TER
(Ω.cm²)
3
4
5
6
7
8
9
Days
Basal
G 10 ppm
G 100 ppm
B
Treatment
150
100
50
0
TER
(Ω.cm²)
3
4
5
6
7
8
9
Days
Basal
R 10 ppm
R 100 ppm

Figure 4

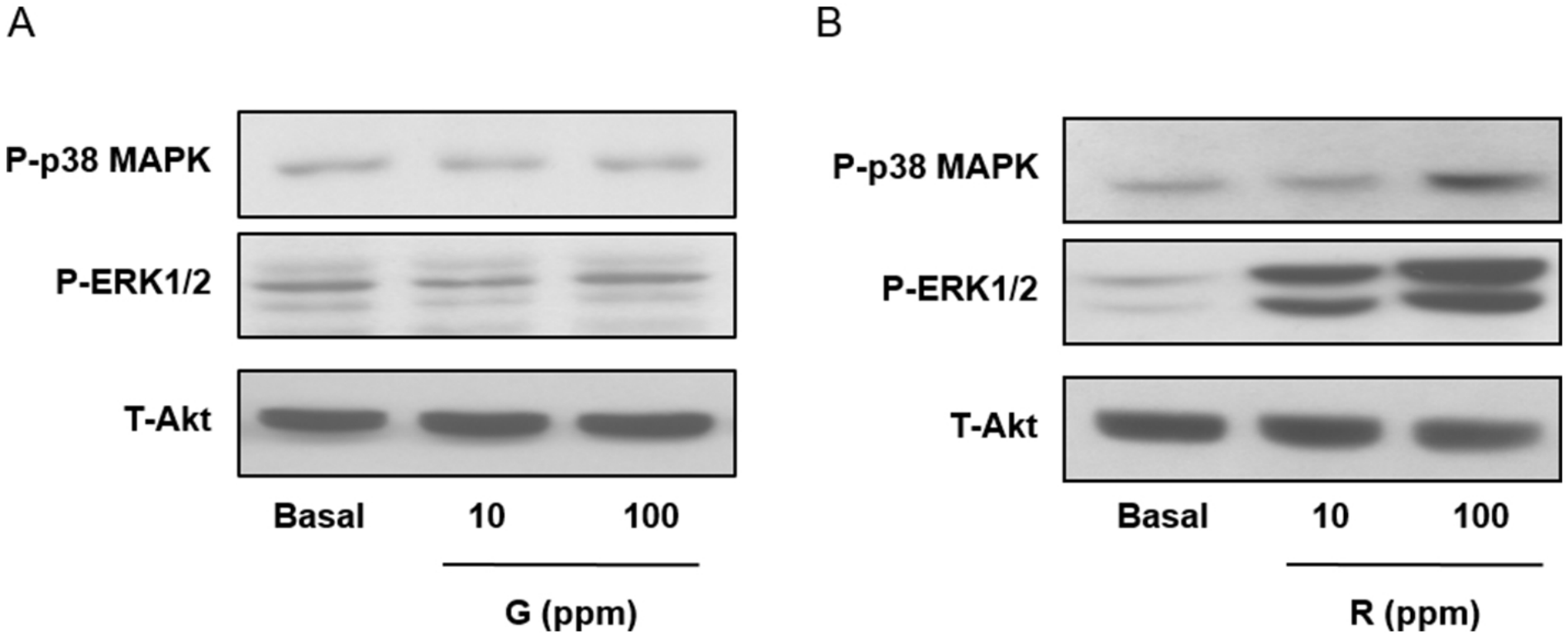
A
P-p38 MAPK
P-ERK1/2
T-Akt
Basal
10
100
G (ppm)
B
P-p38 MAPK
P-ERK1/2
T-Akt
Basal
10
100
R (ppm)

Figure 5

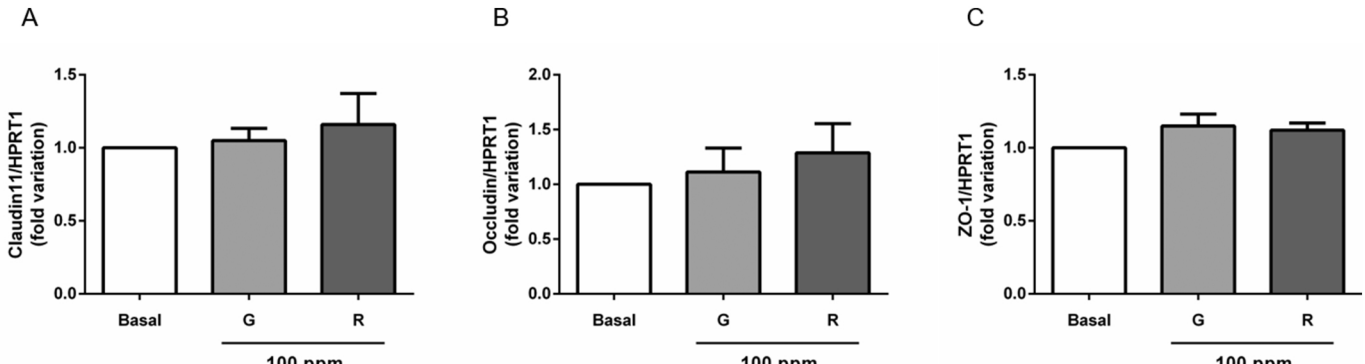
A
Claudin11/HPRT1
(fold variation)
1.5
1.0
0.5
0.0
Basal
G
R
100 ppm
B
Occludin/HPRT1
(fold variation)
2.0
1.5
1.0
0.5
0.0
Basal
G
R
100 ppm
C
ZO-1/HPRT1
(fold variation)
1.5
1.0
0.5
0.0
Basal
G
R
100 ppm

Figure 6

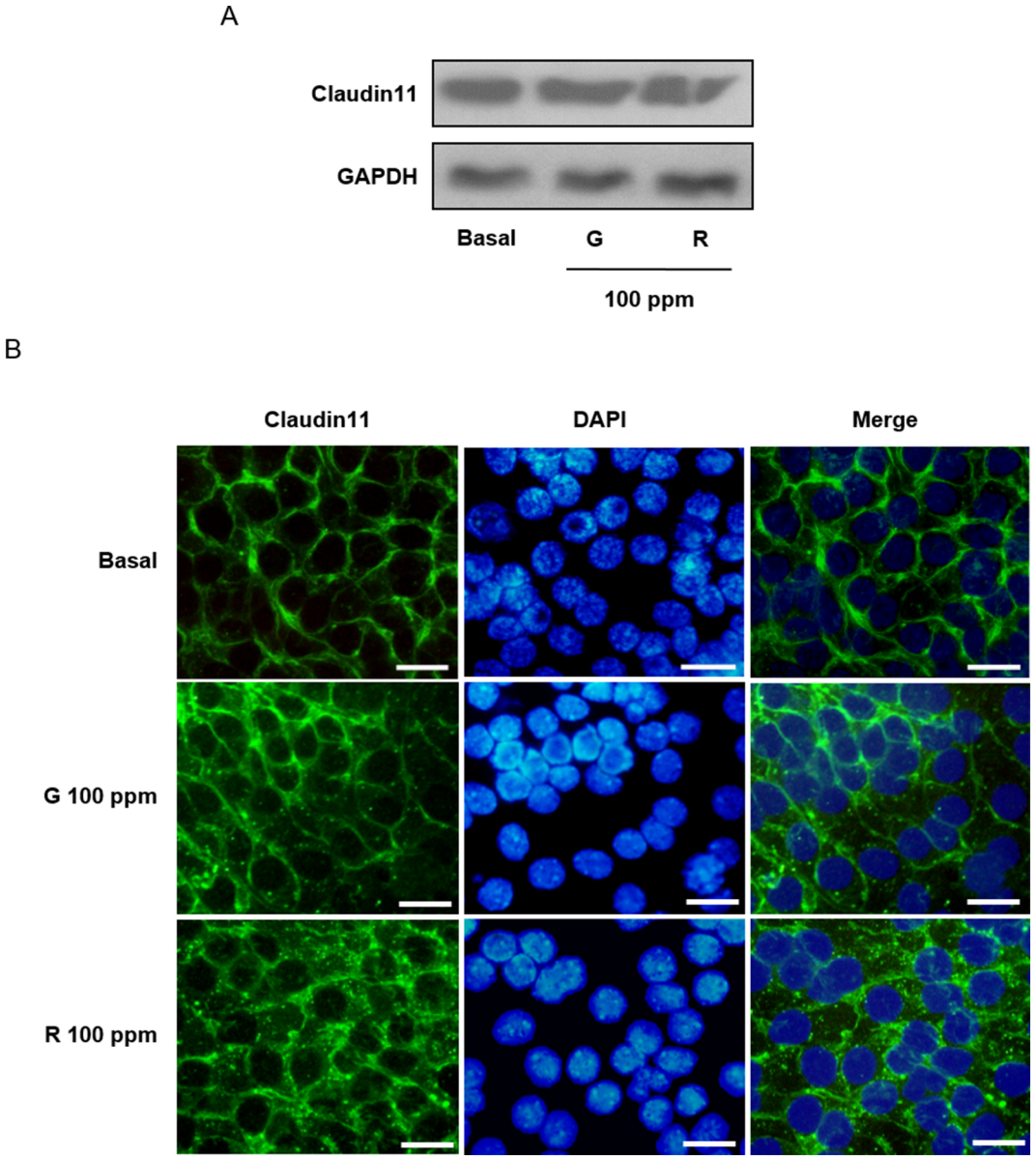
A
Claudin11
GAPDH
Basal
G
R
100 ppm
B
Claudin11
DAPI
Merge
Basal
G 100 ppm
R 100 ppm

Figure 7

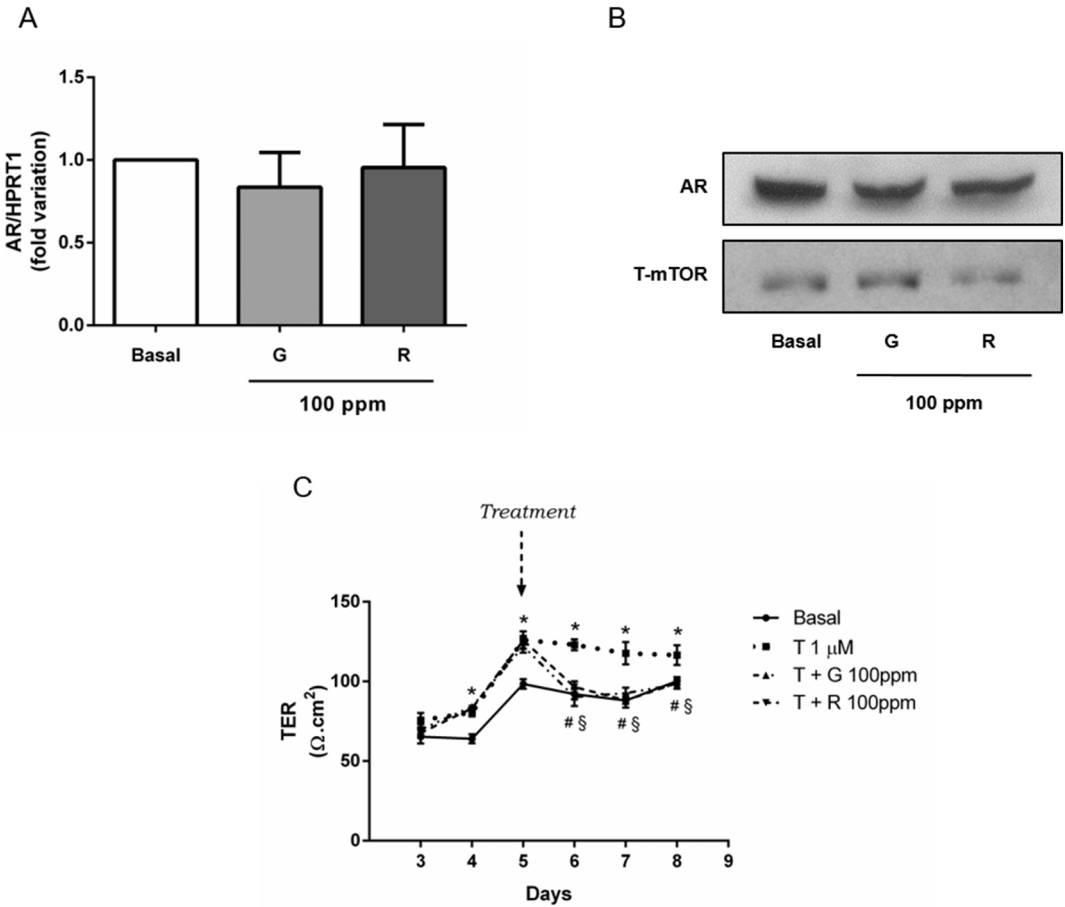
A
AR/HPRT1 (fold variation)
1.5
1.0
0.5
0.0
Basal
G
R
100 ppm
B
AR
T-mTOR
Basal
G
R
100 ppm
C
Treatment
TER (Ω.cm²)
150
100
50
0
3
4
5
6
7
8
9
Days
Basal
T 1 µM
T + G 100ppm
T + R 100ppm

Figure 8

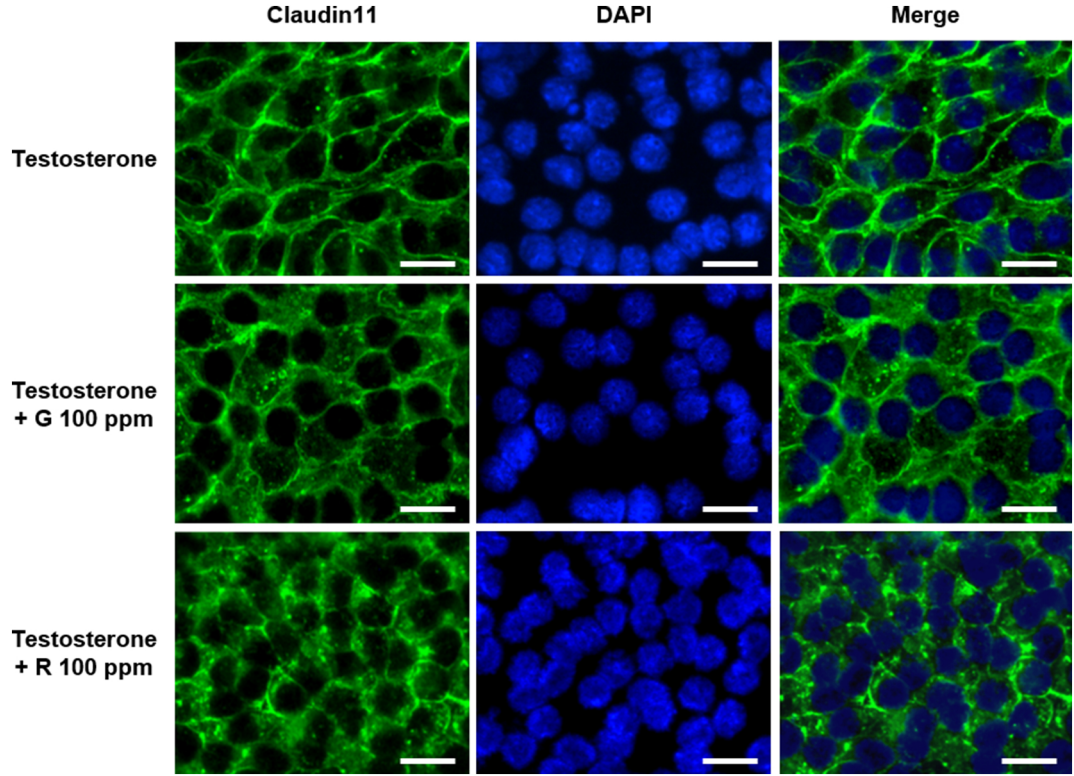
Claudin11
DAPI
Merge
Testosterone
Testosterone
+ G 100 ppm
Testosterone
+ R 100 ppm

Figure 9