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A GLYPHOSATE-BASED FORMULATION BUT NOT GLYPHOSATE ALONE ALTERS HUMAN PLACENTAL INTEGRITY

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ABSTRACT

Glyphosate (G)-based herbicidal formulations, such as the most commonly used one, Roundup (R), are major pesticides used worldwide on food and feed. Pregnant women may thus be frequently exposed to R compounds. These are composed of G, which is declared as the active principle, and other products contained in formulations, named formulants, which have been declared as inerts and diluents by the manufacturers. These formulants have, in fact, been demonstrated to be much more toxic than G, in particular to placental and embryonic human cells. In this work, we thus compared the effect of G and R, using placental perfusion *ex vivo*. R, but not G alone, was demonstrated to alter the placental permeability of a known small model molecule, antipyrine. Similar results were observed for the fetal venous flow rate. The transfer of G alone increases with time, but is significantly decreased in presence of its formulants. The perfusion of R provokes a destruction of fetal vessels, as demonstrated by immunohistochemistry. Formulants obviously alter the fetal-placental circulation and placental integrity according to time of exposure. Therefore, G does not appear to be the main toxic agent of R. Formulants, although undeclared, include polyoxyethanolamines, PAHs, or heavy metals, and may be responsible for this toxicity. These compounds are also present in other pesticides. The progressive blood flow reduction due to the toxic compounds of formulations may diminish the nutrient supply to the fetus, alter the development, and may enhance the poisoning effects. Although these are preliminary results, they could at least partially explain some adverse pregnancy outcomes in mothers exposed to pesticides or other environmental pollutants. The debate on glyphosate alone is proven insufficient for the understanding of the toxicity.

Key Words: Glyphosate, Roundup, formulants, toxicity, placenta, human

Abbreviations

A Antipyrine; BSA bovine serum albumin; FITC Fluoresceine isothiocyanate; FTR Fetal transfer rate; G Glyphosate; GBH Glyphosate-based herbicidal formulations; GM genetically modified; PAH polycyclic aromatic

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hydrocarbons; PBS phosphate-buffered saline; PFA perfluoroalkoxy; POEA polyethoxylated alkylamines; R Roundup; SDHI succinate dehydrogenase inhibition.

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INTRODUCTION

Glyphosate-based herbicidal formulations (GBH), such as the major one known as Roundup (R), are the most commonly used pesticides worldwide (Benbrook, 2016[1]). They are used on large areas of crops that are genetically modified (GM) to be tolerant to R (ISAAA, 2019). Their residues are thus consumed by intensively farmed animals fed with GM soy or corn (Seralini, ESE, 2020[2]). The chemicals from R also more directly enter the human food chain, since wheat and or other cereal crops (for instance, used for beer), are treated with it before harvest (Malalgoda et al., 2020[3]); these contaminate rivers and other water sources (Carles et al., 2019[4]). G as a marker has even been measured in human urine and tissues (Connolly et al., 2019[5]); its formulants are also measured in human urine (Mesnage et al., 2021[6]). Pregnant women may thus be frequently exposed to R compounds. These are composed of G, declared as the active principle, and other products of formulations, known as formulants (Fig.1), which have been declared as inerts and diluents by the manufacturers but have in fact been demonstrated to be much more toxic than G (Richard et al., 2005[7]; Seralini et al., 2015[8]), in particular to placental and embryonic human cells (Benachour and Seralini, 2009[9]). At lower non-cytotoxic levels, R formulations have been shown to be endocrine disrupters (Defarge et al., 2016[10]), to a much greater extent than G alone.

These formulants have been carefully analyzed and include a family of polyethoxylated alkylamines (POEA), as well as detergents, oxidized petroleum residues, and heavy metals such as arsenic, cobalt, chromium, nickel, and lead (Defarge et al., 2018[11]) (Fig. 1). This list is not exhaustive, but the full list is unknown, since the formulants have been classified as inerts or not declared by the manufacturers. Polycyclic aromatic hydrocarbons (PAH) have even been recently found in R formulations which are not G-based, but instead contain acetic or pelargonic acids (Seralini and Jungers, 2020).

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Since all the above-mentioned chemicals are themselves toxic to human placental and embryonic cells, as previously underlined (Benachour and Seralini, 2009[9]), and also in vivo to mammals (Seralini et al., 2014[12]), we investigated in this work the differential toxicity between G and R directly to the human placenta, and the consequent placental transfer through this natural barrier *ex vivo*.

An increasing number of birth defects (Mai et al., 2019[13]), adverse pregnancy outcomes (Ventura et al., 2012[14]) and (among other diseases) childhood cancers (Patel et al., 2020[15]) are reported in the world and are being linked to environmental pollution. Moreover, the debate on R in the scientific literature has been mostly centered on G. We thus compared the effect of G and R on the most sensitive interface between mother and child as a preliminary approach.

MATERIALS AND METHODS

Chemicals and chemical analyses

As a model, R GT plus containing 45% G (450 g/L), market authorization 2020448 from Monsanto Company has been used and diluted in phosphate-buffered saline (PBS) at 1 ppm. G (N-phosphonomethyl glycine, G, CAS 1071-83-6) was obtained from Sigma–Aldrich, Saint Quentin Fallavier, France. G is assayed by liquid chromatography followed by double mass spectrometry (Seralini and Douzelet, 2017[16]), together with its main metabolite aminomethyl phosphonic acid (AMPA). In R, formulants POEA and metals have been previously measured (Mesnage et al., 2013[17]) and naphtalene as a marker for PAH has been evidenced in R in this study via the DIN 38407-39 method, using gas chromatography and mass spectrometry (Seralini and Jungers, 2020[18]).

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Human tissues and ethics

Full-term placentas (37-40 weeks of gestation) from uneventful pregnancies were immediately collected after vaginal delivery or caesarean section from Port-Royal Obstetric Department (Paris, France). The enrolled patients did not receive any medication, except for epidural analgesia or oxytocin during labor, if necessary, and did not present any vascular disease complications, such as diabetes mellitus, preeclampsia, or intrauterine growth restriction. The placentas were obtained following informed patient written consent and approval from our local ethics committee (CPP paris Ile-de-France 3, N-18-05, Paris, France).

Placental perfusion

Collected placentas were perfused in a double circuit according to a validated method as previously described (Ceccaldi et al., 2009[19]). Maternal and fetal solutions were prepared with Earle medium containing 25 g/L of bovine serum albumin (Euromedex, Souffelweyersheim, France). The pH of the maternal and fetal compartments were adjusted to 7.4 ± 0.1 and 7.2 ± 0.1, respectively. Perfusion experiments were started within 20 min after delivery. After a visual examination to confirm the vascular integrity of both maternal and fetal sides, a distal branch of a fetal artery and its associated vein that were supplying a selected cotyledon were cannulated. The fetal circulation was established at a flow rate of 6 mL/min to ensure a balance between arterial and fetal venous flow. Placentas with evidence of vascular leakage were discarded. On the maternal side, the perfused area progressively whitened, which allowed visualization of the chosen cotyledon. After a wash step of 10 min, the maternal perfusion was initiated by inserting two catheters into the intervillous space on the maternal side. The maternal circulation was established at a flow rate of 12 mL/min. Parameters such as perfusion pressure in the fetal vasculature and potential fluid leakage from the fetal circulation were monitored to check the

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technical process. After an additional wash step of 5 min, a freely diffusing marker, Antipyrine (A) (final concentration 20 mg/L, CAS 60-80-0, from Sigma-Aldrich, Saint Quentin Fallavier, France) was used as a positive control for all perfused placentas. A (Kar, 2005[20]) is a small molecule which crosses the placental barrier without tissue accumulation. Usually, A is used as a marker to validate the model of placental permeability; it shows the overlap between the maternal and fetal circulations. Then FITC-Dextran was used as a negative control (final concentration 660 µg/mL, CAS 60842-46-8, from Sigma-Aldrich, Saint Quentin Fallavier, France). FITC-Dextran is a large molecule that does not pass through the placental barrier and thus proves its integrity. G at 1ppm alone, or in R formulation, was subsequently added in the same compartment. The perfusion maximal duration was 240 min. Samples from maternal circulation and venous fetal circulation were collected each 30 min following A transfer; and at 5, 120 and 240 min in order to monitor G concentrations. At the end of perfusion, all the samples were stored at -80°C until analysis.

Calculation of placental parameters

Fetal venous flow rates were standardized to the value at the beginning of the perfusion. At the equilibrium, the fetal circulation was established at a flow rate of 6 mL/min to ensure a balance between arterial and fetal venous flow (at this step, the value of the fetal venous flow rate corresponds to 100%). Standard parameters were calculated according to the formulas of Challier (1985)[21]. Placental transfer was estimated for two parameters: the FTRs of A and of G. The FTR was calculated as follows: FTR = (Cf / Cm) x 100, where Cf is the venous fetal concentration of a molecule and Cm is the maternal concentration of the same molecule. The result is given with a percentage. A maximum value of FTR was reached after 15 min of perfusion; this value must be stable until the end of the perfusion. FTR of A had to be over 20%, as described by Challier (1985)[21].

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Immunohistochemistry

Immunohistochemistry was performed on the placental cotyledon used for perfusion. At the end of each perfusion, the cotyledon was fixed in 4% PFA overnight at 4°C, washed in PBS 1X. About 5 mm^3 of tissue was embedded in 4% (w/v) agarose (Electran, low melting ref 444153H, VWR) and tissue sections (150 µm and 200 µm) were realised using a vibratom. Sections were permeabilized with 1% Triton X-100 for one hour and then saturated with 3% IgG-free BSA, 7% goat serum, human IgG (final concentration: 12.5 µg/mL), 0.01% Triton X100 for 4 hours at room temperature. Tissue sections were incubated under agitation overnight at 4°C, with anti-CD31 antibody at 2 µg/ml (M0823, dako) for endothelial cells labelling, anti-CK7 antibody at 1µg/ml (SAB4501652, Sigma) for trophoblast labelling in 1%. PBS BSA. After washing in 0.05% PBS Triton, sections were incubated for 2 hours under agitation at room temperature with the appropriate fluorochrome-conjugated secondary antibody (1:500, Alexa Fluor 488 or 546 in PBS BSA 1%). Nuclei were stained with DAPI (0.2 µg/mL) and the sections mounted with Fluoromount-G mounting fluid (#FP-483331, Interchim). Acquisitions were made with a Leica SP8 confocal microscope and images analysed with Leica software.

Statistics

Statistical analyses were performed using GraphPad Prism 9.1. All results are expressed as means ± standard errors of the means (SEM). Data were analyzed for significant differences using one-way analysis of variance (ANOVA), followed by Friedman test for multiple comparisons. Relationships between variables were assessed using the Pearson correlation coefficient ρ. A two-sided p-value < 0.05 was considered statistically significant.

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RESULTS

Transplacental transfer of A and fetal venous flow in presence of G alone or with its formulants (R).

Two control parameters were used to validate the perfusion experiments: a positive control with A that should evidence at least a transfer rate of 20% (Challier, 1985[21]), as was the case here, and a negative control with FITC-Dextran, that does not transfer when there is placental integrity. No FITC-Dextran was detected in the fetal circulation, whatever the treatment. The experiences were validated; the 3 placentas were successfully perfused for each condition: A control alone, or together with G alone at 1 ppm, or with the same concentration of 1 ppm of R or GBH, i.e. G in mixture with its formulants, declared as inert diluents by the manufacturer (Fig. 2). The study of the mean transfer rate of A is described in Fig. 2A according to the 3 treatments. For control placentas and G alone, we showed that a maximum value of FTR was reached and remained stable during all the perfusions. The FTR calculated for A in presence of 1 ppm of G was always below the control but not statistically different from control.

For placentas perfused with GBH, we observed that the FTR decreased slightly from 30 to 120 min. Then the FTR decreased significantly to reach a value below the threshold of 20% from 150 min. Similar results were observed for the fetal venous flow rate. It decreased in the same time during the perfusion with G in R (Fig. 2B) and correlated positively (Fig. 2C, r = 0.56) with the A FTR, indicating a loss of placental function through venal flow decrease to the fetus.

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G transplacental transfer rate alone or with its formulants (R) in comparison to A.

We observe in Fig. 3A that after 5 min, the transfer of G alone or associated with its formulants (R) is already detectable. After 120 min of perfusion, the transfer of G alone or associated with its formulants (R) is similar. After 240 min the transfer of G alone is significantly higher (approximately 5-fold) than after 5 min ($p= 0.049$). However after 240 min the transfer of G is decreased in presence of its formulants. In Fig. 3B, a very significant correlation is shown between the transfer rates of G and A, whatever the time of perfusion. Fig. 3C demonstrates that there is no such correlation between the transfer of G in formulants and A.

Placental histology after 240 min perfusion of G or R.

In control placentas, as well as the ones treated with G perfusions (Fig. 4A and B), there was no visible alteration of fetal vessels after 240 min perfusion; a normal structure was observed. This was demonstrated on both cytotrophoblast and syncytiotrophoblast aspects, as evidenced by differential stainings. On Fig. 4C, a destruction of fetal vessels was noticed, showing the effect of R perfusion. The presence of formulants was the only difference. Thus the formulants alter the placental circulation and integrity, according to time of exposure.

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DISCUSSION

To our knowledge, this is the first study on G and R placental transfer performed in an *ex vivo* human perfusion model. The main mechanism of placental transfer is passive diffusion. The transfer rate across the barrier is determined by the physicochemical properties of the drug, such as lipid solubility, polarity, molecular weight, protein binding, and ionisation. The addition of a marker that undergoes only passive diffusion, such as A, into the maternal circulation can be used to measure tissue membrane permeability. Since A does not bind to proteins and does not accumulate in placental tissue, its transfer rate depends only on fetal and maternal flows, which should be constant during normal perfusion.

The addition of FITC-Dextran as a negative control can be used to ensure that placental tissue is preserved without transcellular passage of this large molecular marker.

Since G alone, as a comparably small sized molecule, did not modify statistically the normal transfer of A, we can conclude that G at a relatively high level (1 ppm), found at ppb levels in humans (Connoly et al., 2019[5]), is not the main toxic agent on this parameter. However, when G is perfused with its formulants used in the commercial mixture called R, the fetal transfer of small molecules such as A is affected from 150 min. Usually, the balance of flows in the fetal circuit was controlled throughout the perfusion procedure. Given the fact that the fetal artery flow was known (induced by the peristaltic pump), if a difference was noticed between the fetal artery and the fetal vein, it was possible to conclude that the balance of flows was incorrect (leading to a failure of placental transfer). In this case, this may represent a fetal progressive deprivation from nutrients and essential life factors. Polyoxyethanolamines,

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PAHs or heavy metals may thus be responsible for the toxicity examined in this study. This deleterious effect may begin slightly from 1h but become significant after 2.5h. We also demonstrated *in vitro* that fetal and embryonic human cells were sensitive to these pesticide formulants after a few hours (Richard et al., 2005[7]; Benachour and Seralini, 2009[9]). This was demonstrated by an inhibition of mitochondrial succinate dehydrogenase (SDHI), damaging cell respiration, membrane and nuclei integrities, promotion of apoptosis, and, at lower levels, endocrine disruption. *In vivo*, naphtalene for instance can cause hemolytic anemia, respiratory failure, methemoglobinemia, and hepatic and renal injuries in newborn infants, as well as oxidative stress (Sahni et al., 2019[22]). POEA may also by itself induce adverse mammalian reproductive changes (Dallegrave et al., 2007[23]; Vanlaeys et al 2018[24]). Heavy metals can have similar effects, as is widely known. Maternal exposure to glyphosate formulants that are also used for other pesticides (Defarge et al., 2018[11]; Seralini and Jungers, 2020[18]) may be chronic during pregnancy. We demonstrate here that the placental permeability, even for small molecules, is disrupted at the ppm level in a few hours, but we previously demonstrated that this represents an optimization of the chronic ppb effects of the formulants that become visible *in vivo* after a few weeks or months (Seralini et al., 2014[12]). All these formulants may present detersive, disruptive and destructive effects on placental membranes. The fetal venous flow rate is consequently diminished afterwards, as demonstrated here. Fetal circulation and respiration can be affected by these impacts.

In the following results, we clearly observe that the transfer of G alone is possible and may increase with time, possibly resulting in G bioaccumulating in the fetal compartment. Then toxic effects may not be excluded after a chronic exposure, even without the formulants. However, again, R does not behave as G. The presence of the formulants may provoke a specific degradation of the

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human placenta. The permeability is highly altered. A venal collapsus was checked histologically for this reason. It was confirmed in our present results. The progressive blood flow reduction due to the toxic compounds of formulations may diminish the nutrient supply to the fetus and enhance the poisoning of the embryo. Depending on the time and duration of exposure, this may impact or modify development of the fetus during pregnancy, including organ formation. These impacts may potentially cause malformation of a newborn or even a fetal death.

Although these are preliminary results, they could at least partially explain some pregnancy outcomes in mothers exposed to pesticides or other environmental pollutants. Since these formulants are undeclared, this may be a socially relevant issue for the assessment of the toxicity of pesticides. Based on our results, the debate on glyphosate alone is insufficient for that scientific understanding.

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Declaration of competing interest

The authors declare that they have no competing financial interests or relationships that could influence this work.

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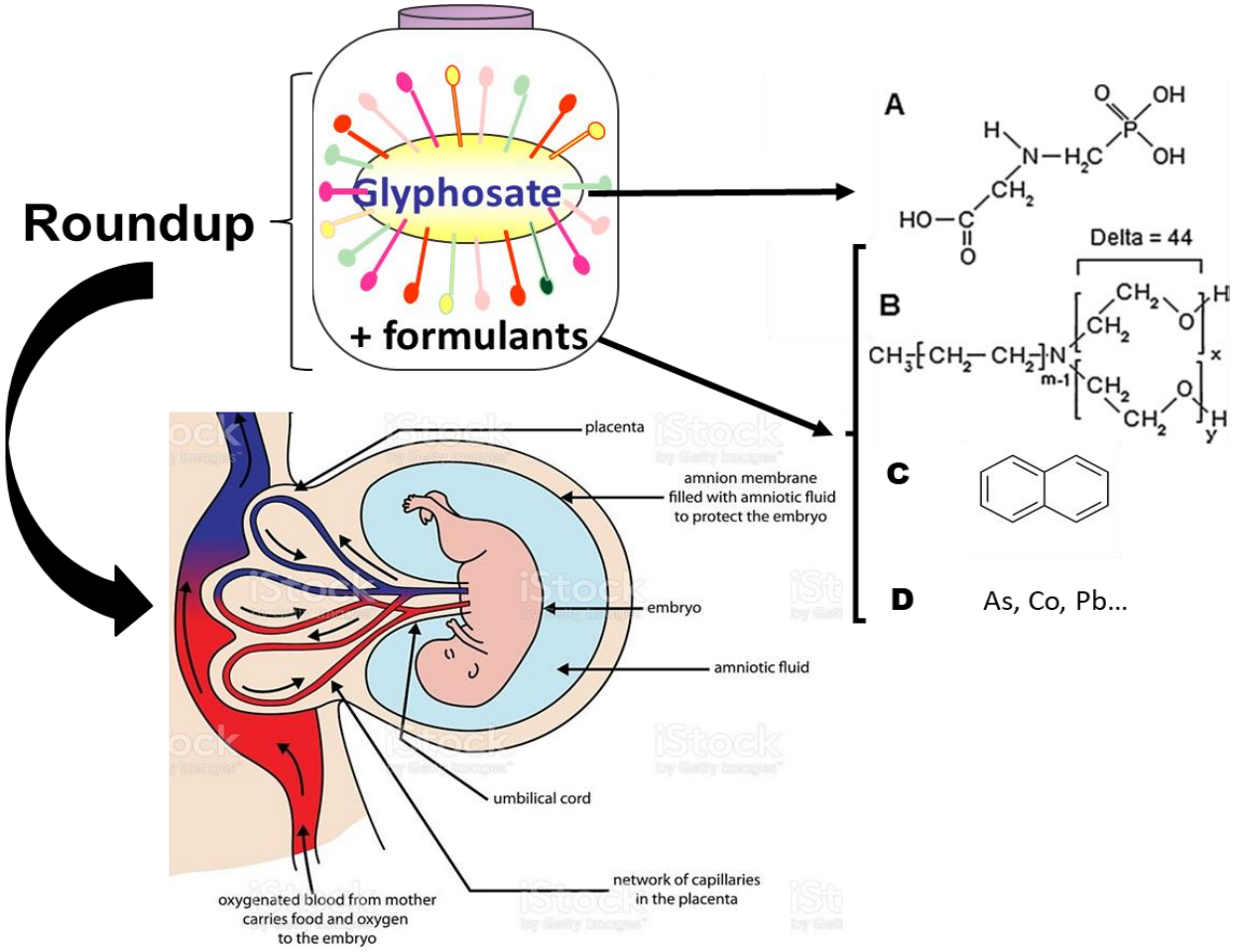
FIGURES

Figure 1



Legend: <u>Roundup composition containing glyphosate and its formulants tested through human placenta.</u>
Roundup R is composed of a declared active principle glyphosate G (A) and other products (formulants) in the commercialised herbicide product, and evidenced more recently, such as polyethoxylated alkylamines POEA (B), naphtalene (C), and heavy metals (D). The transfer of R (with formulants) and G (without formulants) through the placenta *ex vivo* are studied here.

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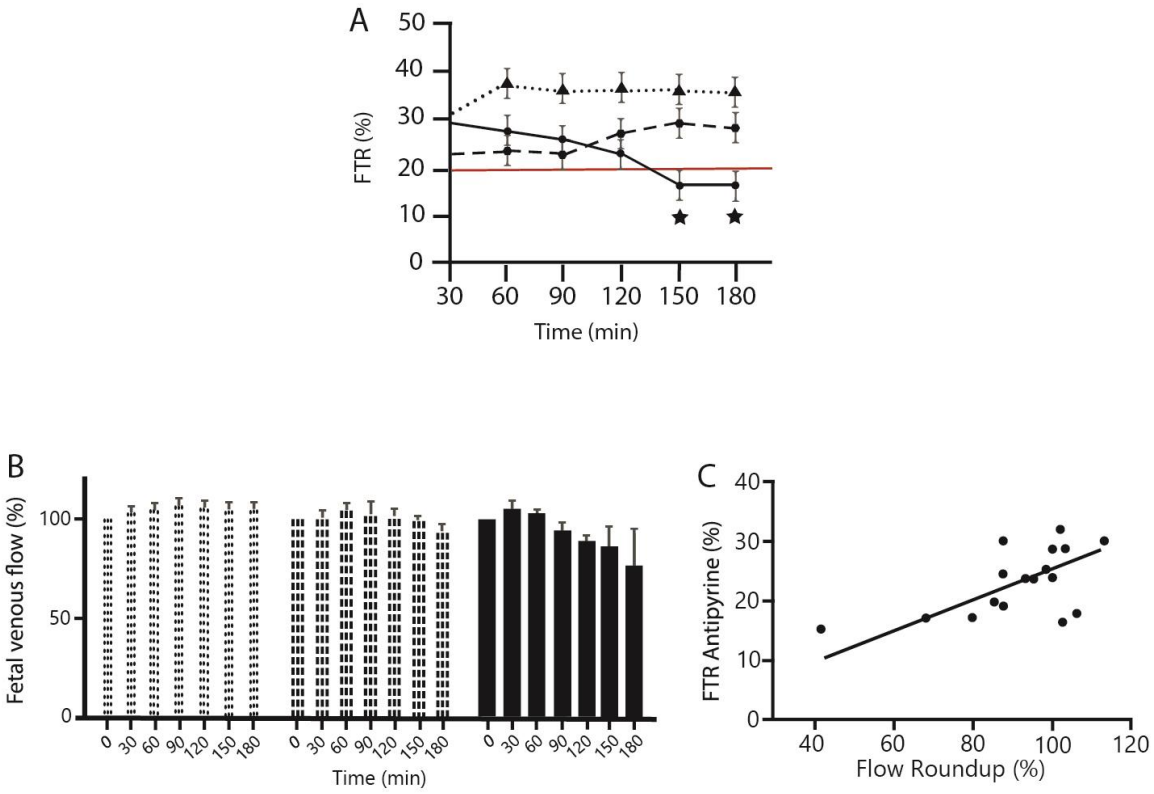
Figure 2



Legend: <u>Fetal transfer rate and fetal venous flow of antipyrine in presence of glyphosate alone or with its formulants.</u>

(A) Effect of 1 ppm G alone or in R compared to antipyrine fetal transfer rate (FTR). *Significant differences, for 150 min $p<0.04$, for 180 min $p<0.02$. Small dots: control, dashed line: G alone, bold curve: R. Red line, minimal normal FTR.

(B) Effect of 1 ppm G alone or in R on fetal venous flow during placental perfusion. Control is in light bars, grey bars are for G alone, R treatments correspond to black bars.

(C) Correlation between antipyrine fetal transfer rate and fetal venous flow for R. Values given are raw data. Significant correlation $p<0.0102$; $r = 0.58$.

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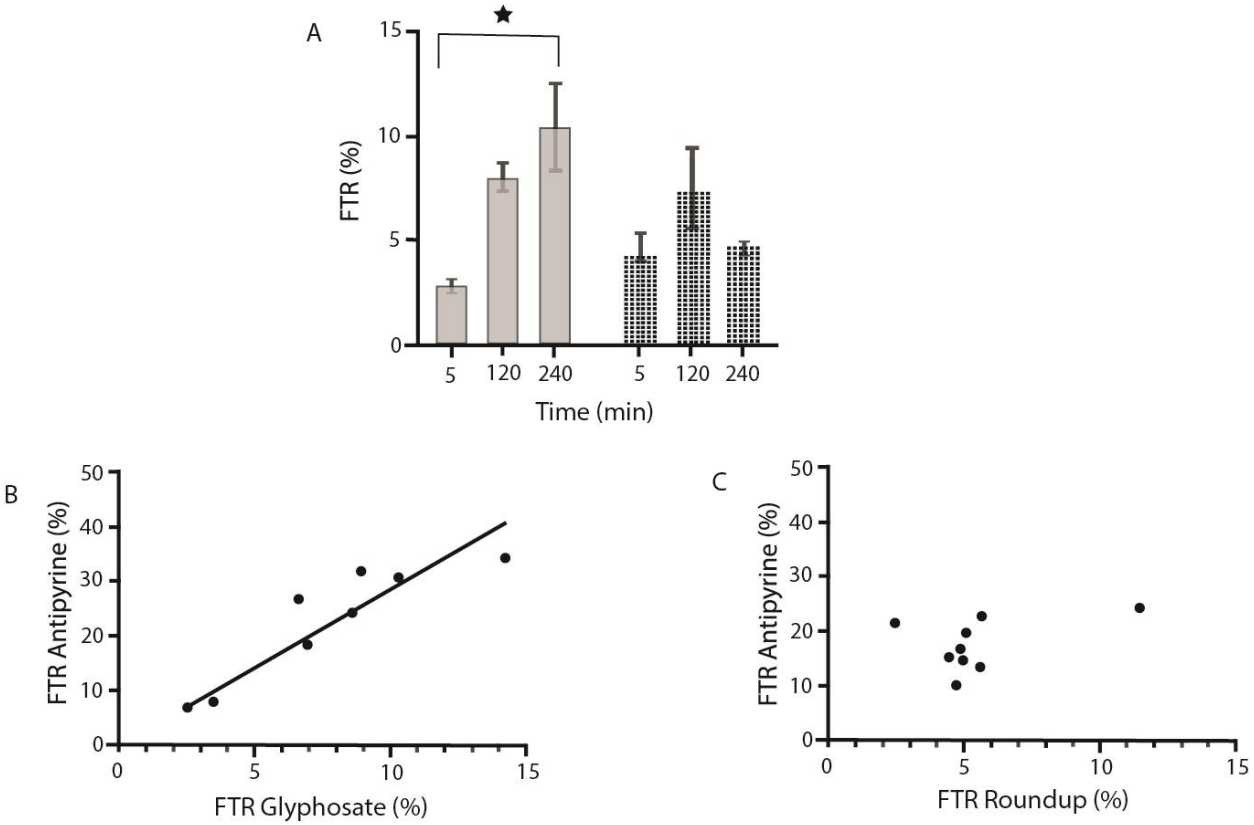
Figure 3



Legend: <u>Fetal transfer rate of G alone or with its formulants in R and correlations with antipyrine.</u>

(A) **G transplacental transfer rate.** G is measured by LC-MS-MS, together with its metabolite AMPA; values are given as mean ±SEM. * Significant difference p=0.049. In grey: G alone; in dotted bars: G with formulants corresponding to R.

(B) **Correlation between A transfer rate and G transfer rate**. r= 0.9039; p=0.0021.

(C) **No real correlation between A transfer rate and R transfer rate**. r= 0.403; p=0.2811.

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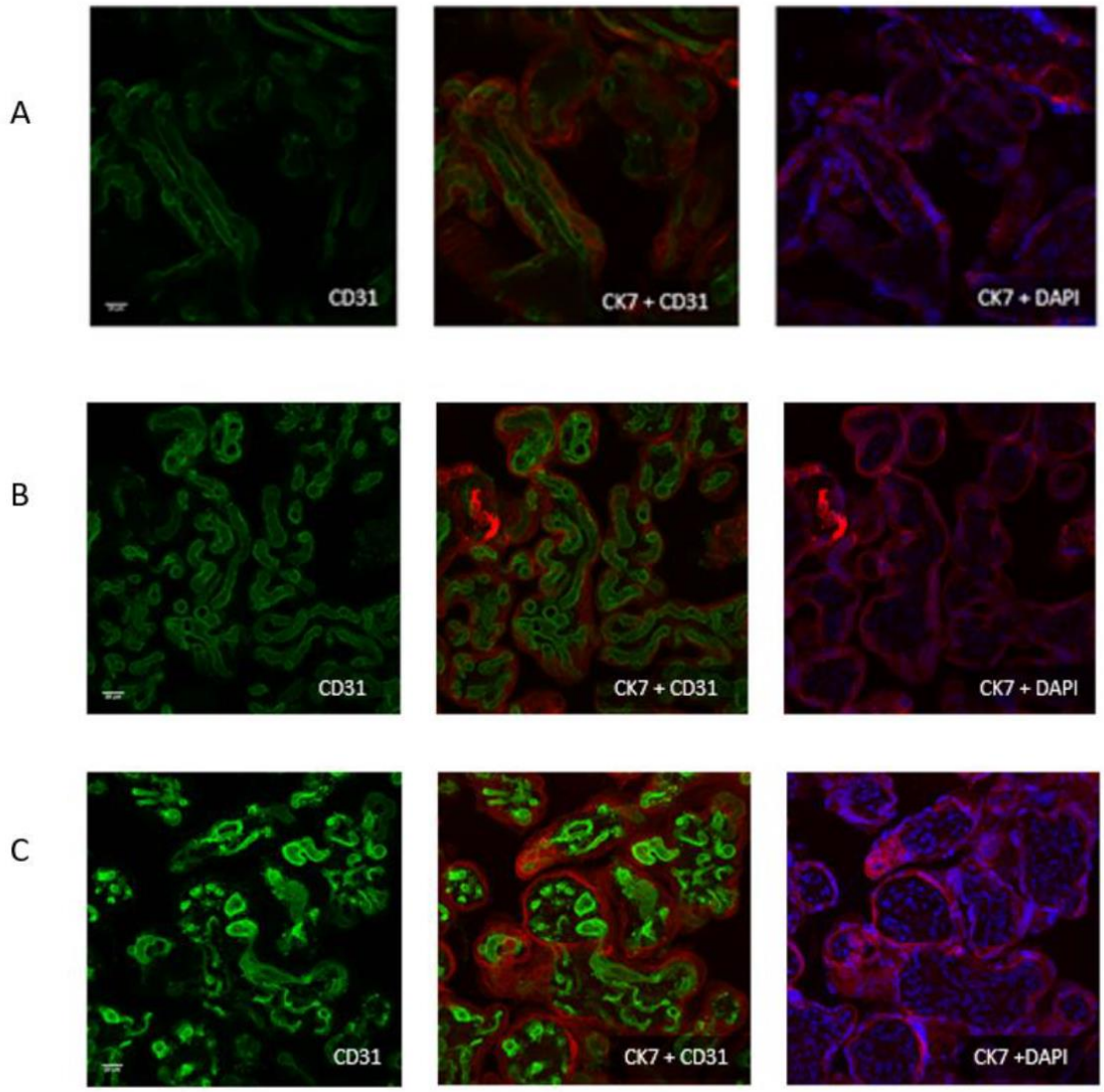
Figure 4.

Legend: Histological sections of perfused cotyledons.



Acquisitions were performed on 240 min-perfused placentas with a Leica SP8 confocal microscope (40X/1.3) in immersion. Anti-CD31 antibodies were used for endothelial cells and thus cytotrophoblast labelling, anti-CK7 antibodies for syncytiotrophoblast labelling, and nuclei were stained with DAPI. a control, b after G perfusion, c after R perfusion.

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