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Impact of chlorpyrifos on human villous trophoblasts and chorionic villi

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ABSTRACT

Placental barrier regulates maternal-fetal interchange protecting the baby from damage caused by substances found in the uterine environment or circulating in the vascular system. Organophosphate (OP) pesticides are a paramount group of environmental pollutants used in intensive agriculture for protection against diseases and pests. While many studies have reported an increased risk of pregnancy alterations in pregnant women exposed to OPs, few have analyzed the effects caused by these pesticides in the placenta.

Herein, we evaluated the effects of chlorpyrifos (CPF), one of the most widely used OP insecticides, on human placenta using *in vitro* and *ex vivo* exposure models. Villous cytotrophoblast cells isolated from normal human term placentas maintained their cell viability, differentiated into syncytiotrophoblast-like structures, and increased the expression of β-hCG, ABCG2, and P-gp in the presence of CPF at concentrations of 10 to 100 μM. The same doses of CPF induced marked changes in chorionic villi samples. Indeed, CPF exposure increased stroma cell apoptosis, altered villi matrix composition, basement membrane thickness, and trophoblastic layer integrity. Histomorphological and ultrastructural alterations are compatible with those found in placentas where maternal-placenta injury is chronic and able to impair the placental barrier function and nutrient transport from mother to the fetus.

Our study shows that placental *ex vivo* exposure to CPF produces tissue alterations and suggest that human placenta is a potential target of CPF toxicity. In addition, it highlights the importance of using different models to assess the effects of a toxic on human placenta.

1. Introduction

Placenta is a highly complex organ that allows and regulates continuous exchanges between the mother and the fetus. It supplies nutrients and oxygen to the fetus and removes waste products back into the mother. Therefore, it acts as the lung, kidney, and digestive system of the infant. It also plays a major role in the production of hormones like human chorionic gonadotrophin (hCG), estrogen, and progesterone. Furthermore, placenta protects the baby from damage caused by substances found in the uterine environment or consumed by the mother, such as pollutants endocrine disruptors, alcohol, or drugs. Most of these important functions take place in the chorionic villi covered by the syncytiotrophoblast (STB), a continuous epithelial layer bathed in maternal blood. Between this layer and the basement membrane are the mononuclear proliferative villous cytotrophoblast cells (vCTBs). The trophoblastic basement membrane separates the trophoblast from the villous stroma composed of connective tissue containing fibroblasts, smooth muscle cells, specialized macrophages, and the fetal vessels surrounded by the intravillous matrix (Ji et al., 2013).

Environmental pollutants may modulate the delicate balance of interactions involved in placental formation and function (Myllynen and Vahakangas, 2013). Impaired placental development and function are associated not only with pregnancy pathologies but also with adult life diseases in the unborn child (Rogers and Velten, 2011; Guttmacher et al., 2014). Consequently, it is critical to investigate whether environmental toxicants may affect the placenta to prevent fetal health risk.

Pesticides are a family of compounds which have brought many benefits to mankind in the agricultural, industrial, and health areas, although their toxicities in both humans and animals have always been

Abbreviations: ABCG2, ATP binding cassette subfamily G member 2; CPF, chlorpyrifos; hCG, human chorionic gonadotrophin; *MDR1*, multi-drug resistance 1 gene; OP, Organophosphate; P-gp, P-glycoprotein 1; PSG, pregnancy specific glycoprotein; STB, syncytiotrophoblast; vCTB, villous cytotrophoblast.

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a concern (Mostafalou and Abdollahi, 2017). Organophosphorus compounds or organophosphates (OPs) form a large group of chemicals pollutants. Following the restrictions on organochlorine pesticides because of their adverse effects on human health and the environment, as well as their high persistence, OPs have become the most commonly used pesticides (Elersek and Filipic, 2011).

An increased risk of spontaneous abortions, intrauterine growth restriction, and premature births have been reported in pregnant women exposed to OP pesticides (Eskenazi et al., 2004; Levario-Carrillo et al., 2004a,b; Whyatt et al., 2005). Others demonstrated that *in uterus* exposure to OPs affects neurodevelopment and psychomotor indices of the individual in his childhood (Eskenazi et al., 2007; Rauh et al., 2011; Androutsopoulos et al., 2013; Zhang et al., 2014). Nevertheless, the toxic effects of OPs on gestation, fetal development, and placentation remain controversial (Eaton et al., 2008).

OPs and their metabolites have been detected in placenta, amniotic fluid, and umbilical cord. Epidemiological studies amongst pregnant women living in rural areas exposed to OPs revealed several molecular alterations in the placenta. They included modifications in the enzymatic activity of acetylcholinesterase, carboxylesterase, catalase, and PI-4 kinase; modulation in the expression of IL-13, arginase, and ornithine decarboxylase; as well as in the mitochondrial complex IV activity and steroidogenic function (Souza et al., 2004, 2005; Bulgaroni et al., 2013; Chiapella et al., 2014; Rivero Osimani et al., 2016).

Chlorpyrifos (CPF) is one of the most widely used OP insecticides in the world as an active ingredient in a wide variety of commercial formulations (Peris-Sampedro et al., 2014; Solomon et al., 2014). According to its acute toxicity, WHO classifies CPF as moderately dangerous (Type II). Although its residential use has been banned since 2001 in many countries, including Argentina in 2009, it is extensively used in agricultural settings for pest control due to its relative low cost and broad spectrum of activity. In this sense, contamination by drift during the application or by unintentional release represents a potential risk for aquatic biota and humans, especially in the farm community. CPF has been shown to be relatively safe in adult animals, however concerns about its safety in children and in *in utero* exposure have raised since several reports indicate it as a possible risk factor for neurodevelopmental disorders in children at exposure concentrations lower than those required to inhibit acetilcholinesterase (AchE) (Venerosi et al., 2012; Androutsopoulos et al., 2013). Most studies in animals and humans on the toxicity of OPs published from 2000 refer to CPF, reflecting the interest to further study about its possible toxic effects (Saunders et al., 2012). *In vitro* experiments performed in trophoblast cell lines indicated that CPF induces apoptosis in JAR cells, alters the expression of genes relevant to placental function, disturbs redox balance, and triggers antioxidant defense mechanisms as well as endoplasmic reticulum stress in JEG-3 cells (Saulsbury et al., 2008; Ridano et al., 2012; Chiapella et al., 2013; Reyna et al., 2017). However, little is known about the effects of OP pesticides on human placental tissue structure, on vCTB cell viability and differentiation as well as on gene expression.

In this work, we aimed to evaluate the effects of the OP insecticide CPF on human placenta in two different exposure models. On one hand, we studied cell viability, differentiation, and gene expression in primary vCTBs isolated from normal human term placentas exposed to CPF. On the other hand, in *ex vivo* assays we examined histological features of chorionic villi explants cultured in the presence of CPF.

2. Materials and methods

2.1. Human placental vCTB and explant cultures

Normal human term placentas (37–41 weeks of gestation) were obtained from unidentified anonymous patients and processed within 30 min after caesarean delivery. The study had the approval of the local Advisory Committee of Biomedical Research in Humans and the Human Studies Committee of the Hospital Privado of Córdoba, Argentina.

After removal of the cord, amniochorion, and decidual layer, villous tissue was sampled from the maternal-fetal interface. The tissue was cut into small pieces of approximate 1 mm^3 free of calcifications, infarctions, clots, fibrosis, and visible vasculature and washed thoroughly with 154 mmol/L NaCl. Subsequently, explants were placed in 24 well plates at a ratio of 3 explants per well in 1 mL of DMEM-F12 (Invitrogen) supplemented with 10% v/v fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin/0.1 mg/mL streptomycin) and were cultivated at 37 °C and 5% CO_2.

vCTBs were purified from placental tissue according to the protocol of Kliman et al. (1986), with the modifications previously described (Angeletti et al., 2008). Isolated cells were plated in keratinocyte growth medium (KGM, Invitrogen) supplemented with 10% v/v FBS, antibiotics (100 U/mL penicillin/0.1 mg/mL streptomycin) and with 5 ng/mL of recombinant human epidermal growth factor (Invitrogen).

Purity of primary cultures was evaluated by cytokeratin 7 immunostaining (CK7, Dako) and nuclei counterstaining with Höechst 33342 dye (2 μg/mL) (Molecular Probes). Approximately 95–97% of the cells were CK7 positive (Supplementary Fig. 1A). Aliquots of 1.5×10^6 vCTB cells were plated on each well of 6-well plates in 2 mL of culture medium, or 4.5×10^4 cells on each well of 96-well plates in 100 μL of culture medium. Complete medium was changed every 24 h. The fusion index, which represents the percentage of fusion events in a cell population, was calculated as: (number of nuclei in STB-like structures − number of STB-like structures) / (total number of nuclei) × 100 (Pidoux et al., 2015). At least 500 nuclei in each experiment and condition were quantified.

2.2. CPF treatments

CPF (O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate, CAS#2921–88-2) with a purity of 99.5% was purchased from the Sigma Chemical Company (St. Louis, MO, USA) and was prepared as a 0.25 M stock solution in dimethylsulfoxide (DMSO). CPF was diluted to the working concentration with media and added to cell cultures immediately before treatments. The final DMSO concentrations did not exceed 0.04% v/v.

Placental explants and vCTB cells were exposed to CPF in concentrations up to 100 μM after 8 h or 3 h of initial culture in complete media (with 10% FBS and antibiotics), respectively. Preliminary experiments performed with placental explants maintained in culture without CPF for different time periods confirmed that chorionic villi conserved its normal tissue histology after 48 h. Therefore, explants were treated with CPF or vehicle for 36 h. Isolated vCTB cells were treated for 19 or 61 h to address CPF effects on the initial and advanced phase of *in vitro* differentiation, respectively. Controls without and with different DMSO concentrations were also performed (data not shown).

2.3. Cell viability/cytotoxicity assay

Cell viability was evaluated using the metabolic dye 3-(4,5-dimethlthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT). The assays were carried out with 4.5×10^4 vCTB cells in each well of 96-well plates treated or not with CPF during 61 h. Results are expressed as percentage of cell viability relative to 0.04% v/v DMSO (control).

Acridine orange (AO/*N*,*N*,*N*′,*N*′-tetramethylacridine-3,6-diamine) and ethidium bromide (EB) staining were performed in vCTBs exposed to CPF during 19 or 61 h. After treatments, cells were washed with phosphate buffered saline (PBS) and 20 μL of a 1/10 dilution (in PBS) of AO stock (100 μg/mL) were added and cells were incubated during