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The use of a unique co-culture model of fetoplacental steroidogenesis as a screening tool for endocrine disruptors: The effects of neonicotinoids on aromatase activity and hormone production

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ABSTRACT

Estrogen biosynthesis during pregnancy is dependent on the collaboration between the fetus producing the androgen precursors, and the placenta expressing the enzyme aromatase (*CYP19*). Disruption of estrogen production by contaminants may result in serious pregnancy outcomes. We used our recently developed *in vitro* co-culture model of fetoplacental steroidogenesis to screen the effects of three neonicotinoid insecticides on the catalytic activity of aromatase and the production of steroid hormones. A co-culture of H295R human adrenocortical carcinoma cells with fetal characteristics and BeWo human choriocarcinoma cells which display characteristics of the villous cytotrophoblast was exposed for 24 h to various concentrations of three neonicotinoids: thiacloprid, thiamethoxam and imidacloprid. Aromatase catalytic activity was determined in both cell lines using the tritiated water-release assay. Hormone production was measured by *ELISA*. The three neonicotinoids induced aromatase activity in our fetoplacental co-culture and concordingly, estradiol and estrone production were increased. In contrast, estriol production was strongly inhibited by the neonicotinoids. All three pesticides induced the expression of *CYP3A7* in H295R cells, and this induction was reversed by co-treatment of H295R cells with exogenous estriol. *CYP3A7* is normally expressed in fetal liver and is a key enzyme involved in estriol synthesis. We suggest that neonicotinoids are metabolized by *CYP3A7*, thus impeding the 16α-hydroxylation of fetal DHEA(-sulfate), which is normally converted to estriol by placental aromatase. We successfully used the fetoplacental co-culture as a physiologically relevant tool to highlight the potential effects of neonicotinoids on estrogen production, aromatase activity and *CYP3A7* expression during pregnancy.

1. Introduction

The use of *in vitro* models in toxicology has significantly enhanced our understanding of the mechanisms by which chemicals cause adverse effects in humans and wildlife. However, to mimic the interactions that occur *in vivo* is a challenge when whole animal or human studies are not possible. Well thought-out *in vitro* models, such as the use of co-culture models, are promising approaches to study the communication between different cell types in a more complex context. As example, a co-culture using primary human mammary fibroblasts and MCF-7 (epithelial breast cancer cells) was developed by (Heneweer et al., 2005) to study intercellular interactions in breast cancer. More recently, we developed a co-culture model that reproduces the steroidogenic fetoplacental unit and can be used to evaluate the impacts of endocrine disruptors on this delicate aspect of fetoplacental communication (Hudon Thibeault et al., 2014; Hudon Thibeault et al., 2017).

During pregnancy, the fetoplacental unit plays an important endocrine role, ensuring, amongst others, estrogen (estrone, estradiol and estriol) biosynthesis. Estrogens are required in several physiological processes during pregnancy, such as the formation of the syncytiotrophoblast and regulation of uteroplacental blood flow (Yashwanth et al., 2006). Maternal cholesterol is converted to androgen precursors in the fetus by the action of several enzymes, such as cytochrome P450 17 (*CYP17*), sulfotransferase 2A1 (*SULT2A1*) and steroid 16α-hydroxylase (*CYP3A7*). In the placenta, *CYP19* (aromatase) is responsible for the final step in estrone, estradiol and estriol biosynthesis (Leeder et al., 2005; Rainey et al., 2002). Alone, the placenta cannot produce estrogens *de novo*, as it needs the steroid precursors synthesized

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by the fetus (for complete steroidogenesis pathway, see Fig. 7 in Results section). Estriol is uniquely produced during pregnancy, and its synthesis requires correct functioning of the fetoplacental unit (Mucci et al., 2003). Thus, a disruption in biosynthesis of estrogens such as that of estriol may adversely alter development and influence important indicators of fetal health like birth weight and head circumference (Kaijser et al., 2000; Troisi et al., 2003). Moreover, decreased free estriol in maternal serum has been associated with growth retardation, reduced Apgar scores and postnatal complications in a control study of 869 women (Gerhard et al., 1986).

Exposure during pregnancy to contaminants such as heavy metals, pesticides, polychlorinated biphenyls (PCBs) and phthalates have been linked to fetal growth retardation (Siddiqui et al., 2003), spontaneous abortions, learning disabilities (Hu, 1991; Abadin et al., 1997), reduced birth weight, preterm birth (Jacobson et al., 1990) and disruption of reproductive development (Mylchreest et al., 2000; Honma et al., 2002; Foster, 2006). It is not surprising that exposure to certain chemicals during pregnancy can lead to adverse pregnancy and birth outcomes, since *in utero* development is a critical window of vulnerability of the embryo (Bellinger, 2013). The fetoplacental co-culture model of steroidogenesis developed in our laboratory (Hudon Thibeault et al., 2014) allows us to study chemicals that may impair estrogen biosynthesis or that of other key placental hormones such as β-human chorionic gonadotropin (β-hCG), potentially leading to serious pregnancy complications (Albrecht and Pepe, 1999; Albrecht et al., 2000; Svedas et al., 2002). We have reported earlier that prochloraz, a widely-used fungicide, and norfluoxetine, a selective serotonin-reuptake inhibitor, strongly inhibited aromatase activity and estrogen production in our fetoplacental co-culture model (Hudon Thibeault et al., 2014; Hudon Thibeault et al., 2017).

Neonicotinoids are some of the most widely used insecticides in the world. For example, thiamethoxam and clothianidin were both in the top 10 most sold insecticides in Canada in 2010 (Health Canada, 2014). By 2012, neonicotinoids were applied to 11 million hectares in Canada, representing >216,000 kg of active neonicotinoid (Main et al., 2014). Neonicotinoids are mostly used as seed coatings on the vast majority of crops, fruits and vegetables. Moreover, their physicochemical characteristics (K_{ow} and pK_a) explain their systemic properties and their distribution throughout the entire plant (Bonmatin et al., 2015; Simon-Delso et al., 2015). For this reason, neonicotinoid insecticides also target pollinators, mammals and humans. The scientific community is increasingly accepting that exposure to these insecticides partially explains the worldwide decline in honeybees populations (Decourtye et al., 2004; Girolami et al., 2009; Henry et al., 2012; Goulson, 2013). Neonicotinoid insecticides are also persistent in the environment. Half-lives in soil vary and can reach 1250 days for imidacloprid (Main et al., 2014). Because of their persistence and repeated application, it is expected that neonicotinoids will continue to accumulate in soil (Stokstad, 2013). A recent study analyzed neonicotinoid levels in surface waters from 136 wetlands across Saskatchewan, Canada. Clothianidin and thiamethoxam concentrations were detected in the majority of water samples, reaching concentrations as high as 3110 ng/L (Main et al., 2014). Moreover, human populations are also exposed to neonicotinoids through diet. A study conducted in Boston, Massachusetts, analyzed neonicotinoid residues in honey, fruits and vegetables purchased in local grocery stores. Imidacloprid was the most frequently detected neonicotinoid in the samples. At least one neonicotinoid was detected in all the tested fruits and vegetables. Also, in 72% of fruits and 45% of the tested vegetables, two or more neonicotinoids were detected, with concentrations reaching 100.7 ng/g (Chen et al., 2014). Furthermore, a study conducted with a cohort of 147 farm workers from northeastern Japan evaluated the presence of neonicotinoid metabolites in urine. A metabolite of the neonicotinoid dinotefuran, 3-furoic acid, was detected in 100% of the samples at concentrations as high as 0.13 μM (Nomura et al., 2013). Moreover, the concentrations of 6-chloronicotinic acid, a metabolite of imidacloprid and thiacloprid, reached concentrations of 0.05 μM (Nomura et al., 2013).

In recent years, a growing number of studies have evaluated the endocrine disrupting potential of neonicotinoid insecticides. We demonstrated that two neonicotinoids (thiacloprid and thiamethoxam) induce aromatase expression in a promoter-specific manner *in vitro*, targeting promoters known to be overexpressed in breast cancer (Caron-Beaudoin et al., 2016). Moreover, Bal et al. (2012) found that male rats exposed to imidacloprid (2 mg/kg/day) through diet showed increased apoptosis and fragmentation of seminal DNA. In female rats exposed to the same neonicotinoid (20 mg/kg/day), Kapoor et al. (2011) noted decreased ovarian weight and altered levels of follicle stimulating hormone and progesterone.

In this study, we used our recently developed fetoplacental co-culture model as a screening tool to determine the effects of three widely used neonicotinoid insecticides on steroidogenesis in the human fetoplacental unit, and more precisely, on aromatase activity, estrogen production and *CYP3A7* expression (key enzyme in the fetal production of the estriol precursor 16α-hydroxyDHEA(-sulfate). Our previous work showed that two neonicotinoid insecticides, thiacloprid and thiamethoxam, induced *CYP19* expression and aromatase activity at environmentally relevant concentrations in human H295R adrenocortical carcinoma cells displaying characteristics of the fetal adrenal cortex and which represents the fetal compartment of our fetoplacental co-culture (Caron-Beaudoin et al., 2016). Therefore, we hypothesized that neonicotinoids may also disrupt the production of estrogens within the fetoplacental unit.

2. Materials and methods

2.1. Chemicals

All pesticides were purchased from Sigma-Aldrich (St-Louis, MO) (thiacloprid, Pestanal 37905, purity > 99%; thiamethoxam, Pestanal 37924, purity > 99%; imidacloprid, Pestanal 37894, purity > 99%). All neonicotinoids were dissolved in dimethylsulfoxide (DMSO) as 30 or 100 mM stock solutions.

2.2. Feto-placental co-culture

The feto-placental co-culture (Hudon Thibeault et al., 2014) consists of H295R adrenocortical carcinoma and BeWo choriocarcinoma cells. H295R cells have the characteristics of the fetal adrenocortex (Gazdar et al., 1990; Staels et al., 1993) as well as that of fetal liver (Hudon Thibeault et al., 2014) and reflect the steroidogenesis that would occur in the fetal compartment. BeWo cells are a well documented model of the placental trophoblast (Ellis et al., 1990; Nampoothiri et al., 2007). This co-culture model of the fetoplacental unit is capable of *de novo* production of estrogens, including the unique pregnancy estrogen estriol, under our experimental conditions (Hudon Thibeault et al., 2014). Briefly, BeWo (ATCC no. CCL-98) and H295R (ATCC no. CRL-2128) cells were cultured separately in their respective recommended media. BeWo cells were cultured in DMEM/F-12 without phenol red (Catalog no. 11039021, Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% fetal bovine serum (FBS; Hyclone, Tempe, AZ). H295R were cultured in DMEM/F-12 (Catalog no. 11039021, Thermo Fisher Scientific, Waltham, MA), supplemented with 2.5% Nu Serum (BD Biosciences, Mississauga, ON, Canada) and 1% ITS + Premix (BD Biosciences, Mississauga, ON, Canada). Once confluent, BeWo and H295R cells were trypsinized, and H295R cells were seeded in 24-well plates at a concentration of 2.5×10^4 cells/well, whereas BeWo cells were seeded in transwell inserts (Corning Life Sciences, Corning, NY) at a concentration of 1.25×10^4 cells/insert, After 24 h, the co-culture was assembled by adding the inserts containing BeWo cells to the 24-well plates containing H295R cells. The original culture media were removed and replaced with co-culture media which contained DMEM/F-12 without phenol red, supplemented with 2.5% stripped Nu Serum, 1% ITS + Premix and 1% stripped FBS, and the various concentrations of the neonicotinoids or