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Transplacental transfer and metabolism of diuron in human placenta

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ABSTRACT

Diuron is a broad-spectrum phenylurea derived herbicide which is commonly used across the globe. Diuron is toxic to the reproductive system of animals and carcinogenic to rat urothelium, and recently found to be genotoxic in human cells. In *in vivo*, it is metabolized predominately into 3-(3,4-dichlorophenyl)-1-methyl urea (DCPMU) in humans and 3-(3, 4-dichlorophenyl)urea (DCPU) in animals. Information on diuron toxicokinetics and related toxicity in human placenta is absent. We have investigated the toxicokinetics of diuron in *ex vivo* human placental perfusion and in *in vitro* human placental microsomes and human trophoblastic cancer cells (BeWo). Diuron crossed human placenta readily in placental perfusion. Furthermore, diuron was metabolized into DCPMU in perfused placenta and in *in vitro* incubations using microsomes from placentas of smokers. In incubations with placental microsomes from non-smokers, and in BeWo cells, metabolism to DCPMU was detected but only with the highest used diuron concentration (100 μM). Diuron metabolism was inhibited upon addition of α-naphthoflavone, a CYP1A1 inhibitor, underscoring the role of CYP1A1 in the metabolism. In conclusion, it is evident that diuron crosses human placenta and diuron can be metabolized in the placenta to a toxic metabolite *via* CYP1A1. This implicates *in vivo* fetal exposure to diuron if pregnant women are exposed to diuron, which may result in fetotoxicity.

1. Introduction

Exposure to xenobiotics is unavoidable during pregnancy and practically all chemicals cross human placenta and reach the fetus to some extent. Pregnant women are exposed to a wide variety of chemicals including chemicals in tobacco smoke or contaminated drinking water and medicinal drugs. Prenatal exposure to chemicals may result in detrimental effects in the fetus which may extend to adulthood (For reviews see, Vähäkangas, 2011; Burton et al., 2016; Padmanabhan et al., 2016). Fetotoxicity of xenobiotics is determined by a number of factors, including physicochemical characteristics of the chemical, health status of the pregnant mother and intensity, length and timing of the exposure, as well as the physiological characteristics of the placenta which vary significantly during gestation.

Pesticides are major contaminants in food and water (Kim et al., 2017). Of these, diuron (3-(3,4-di-chlorophenyl)-1,1-dimethylurea) is a broad spectrum phenylurea derived herbicide. It is used to control the vegetation of both broadleaf and grassy weeds (APVMA, 2011). People, including pregnant women, can be exposed to diuron orally either by consumption of contaminated food or *via* drinking water. Although diuron has been banned in many parts of Europe, the widespread and massive use of diuron across the globe as well as the high stability and persistence in aquatic environment has led to contamination of water in different geographical areas in Europe and America (Ensminger et al., 2013; Palma et al., 2015; Poulier et al., 2015; Ansanelli et al., 2016; Ccanccapa et al., 2016). For instance, in France diuron has been found at 1.2 μg/l in environmental samples (Akcha et al., 2016).

Diuron is toxic to aquatic organisms. Among these toxic effects, it has been found that environmentally relevant concentrations of diuron are toxic to reproductive system and developmentally toxic to oysters and fish (Akcha et al., 2012; Behrens et al., 2016). Diuron has relatively low acute toxicity in mammalians (USEPA, 2003). Subchronic toxicity studies in animals have shown toxicity in multiple target organs including kidney, urinary bladder and spleen (Domingues et al., 2012; Fernandes et al., 2012; Da Rocha et al., 2013). Urinary bladder and kidney cancers in rats and mammary gland cancers in mice are the basis of US Environmental Protection Agency classification of diuron as a known/likely human carcinogen (USEPA, 1997). Also, according to ECHA, diuron is regarded as toxic to aquatic species with lifelong detrimental effects. Upon chronic exposure, it might initiate cancer and cause organ damage (ECHA, 2018). Diuron has been shown to reduce the viability of human choriocarcinoma BeWo cells, implicating

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cytotoxicity in human placenta. In addition, it has been found that diuron is genotoxic to human breast carcinoma MCF7 cells (Huovinen et al., 2015).

Data on diuron metabolism is very scarce and there is no information on diuron related toxicity in humans. Diuron is extensively metabolized *in vivo* in animals yielding several toxic metabolites. The predominant metabolite in rats after high doses of diuron has been 3-(3,4-dichlorophenyl)urea (DCPU) followed by 3-(3,4-dichlorophenyl)-1-methyl urea (DCPMU) (Da Rocha et al., 2013). Da Rocha et al. (2013) characterized the mechanisms of urothelial cytotoxicity induced by diuron in rats. They concluded that metabolism of diuron to DCPU is required for cancer development in urothelial cells. While DCPU was the main metabolite in the urine of rats, both DCPU and DCPMU were cytotoxic, DCPMU being the most potent of the metabolites of diuron. In the only study in human, postmortem samples taken from a person exposed to massive doses of diuron, it was completely metabolized, predominately into DCPMU followed by DCPU in smaller amount (Van Boven et al., 1990).

Xenobiotics cross the placenta *via* passive diffusion, facilitated transport where no energy is required, or active transport requiring energy (Ganapathy et al., 2000; Vähäkangas and Myllynen, 2009). In addition, several metabolizing enzymes are found in human placenta but in low levels compared to liver (Hakkola et al., 1998; Myllynen et al., 2009). Although many xenobiotic metabolizing CYP enzymes have been detected at mRNA level in human placenta, most of them seem not to be functional. For instance, CYP1A1 has been found to be functionally active only in placentas from smokers (Vähäkangas et al., 1989). Recombinant CYP1A1/2 has been demonstrated to be responsible for the metabolism of diuron into DCPMU (Abass et al., 2007). This gives an idea that diuron could be metabolized in human placenta forming a toxic metabolite if the mother is a smoker. Metabolism of some xenobiotics has been demonstrated in human placental perfusion and *in vitro* studies. For instance, we have shown that benzo (a)pyrene (Vähäkangas et al., 1989; Karttunen et al., 2010) and aflatoxin B1 (Partanen et al., 2010) are metabolized in human placental microsomal incubation and perfusion, resulting in toxic metabolites.

Because, on one hand, the ethical issues associated with *in vivo* studies in pregnant mothers (Halkoaho et al., 2010) and, on the other hand, the anatomical and histological uniqueness of human placenta (Myllynen and Vähäkangas, 2013), it is imperative to use human placental *in vitro* models for fetal and placental exposure assessment of new drugs and toxic chemicals. The most important experimental model for fetal exposure is human placental perfusion as it retains the integrity of human tissues and closely imitates the physiological conditions (For reviews see, Myllynen and Vähäkangas, 2013; Etwel et al., 2014; Gohner et al., 2014). In the light of absence of data on transfer kinetics and metabolism of diuron in human placenta, as well as the paucity of data from animal studies it is worthwhile to investigate the fate of diuron in human placenta. Accordingly, in this study, the transplacental transfer and metabolism of diuron were studied in human placental perfusion, microsomal incubations and human trophoblastic cancer cells.

2. Materials and methods

2.1. Human tissues and ethical aspects

Ethical aspects related to donation of placentas have been previously identified and no major ethical problems exist when placentas are collected anonymously with a written informed consent from the volunteering mothers after being given information (Halkoaho et al., 2010, 2011). Approval from the official ethics committee of the Northern Savo Central Hospital District (54/2007, 30.5.2007) has been granted. For perfusion studies, two term placentas from healthy non-smoking mothers and one from a smoking mother (10 cigarettes per day) were collected right after cesarean section after written informed consent. Information on duration of pregnancy, weight of the placenta, and time of delivery were recorded without personal identification. Human placental samples from 6 smoking and 4 non-smoking mothers were also gained from the KuBiCo Kuopio Birth Cohort (www.kubico.fi). Ethical permission for the collection in KuBiCo cohort was achieved by the Research Ethics Committee of Hospital District of Central Finland in Jyväskylä, Finland (15.11.2011). Human liver samples were obtained from the Oulu University Hospital *via* BD Biosciences Discovery Labware. The ethics committee of the medical faculty of the University of Oulu had granted the approval for collection of the liver samples (January 21, 1986).

2.2. Human placental perfusion

The method used was a dual re-circulating human placental perfusion of a single cotyledon perfused through both fetal and maternal sides separately (Pienimäki et al., 1995; Karttunen et al., 2015). Shortly, after flushing the placenta through umbilical cord with heparinized Krebs-Ringer-phosphate buffer within 10 min after delivery, a peripheral placental lobule with no macroscopic trauma was prepared and attached to the perfusion apparatus. After pre-perfusion for 30 min to restore the physiological conditions, diuron (1 μM) (Sigma-Aldrich, Japan, purity 98%) and the reference compound, antipyrine (25 μg/ml) (Sigma-Aldrich, Switzerland), were added into the maternal circulation. The flow rate of perfusion in the fetal side was maintained at 3 ml/min and in the maternal side at 9 ml/min. Samples were taken from both maternal and fetal sides at designated time points and centrifuged at 12000 × g for 15 min, and supernatant stored at −20 °C. Placental tissue before and after perfusion were snap-frozen in liquid nitrogen and kept at −80 °C. Criteria for a successful perfusion were antipyrine kinetics and leak from fetal to maternal circulation less than 3 ml/ hour (explained in detail, Karttunen et al., 2015). Also, diuron was perfused in maternal side of the perfusion apparatus with no placenta, in order to study whether diuron binds to the tubing of the system. A unidirectional transfer from the maternal to the fetal circulation, and metabolism of the studied compounds in the placental tissue were investigated.

Recovery of diuron in the placental perfusions was calculated by the following formula: Total recovery $= \frac{100X\ (Fa + Ma + TIS + SAM)}{TOT}$, where Fa is the amount of diuron in the fetal perfusate, and Ma is the amount of diuron in the maternal perfusate at the end of the perfusion. TIS is the quantity of diuron accumulated in tissue, SAM is the amount of diuron lost when taking samples from the perfusion medium and TOT is the total amount added in the perfusion.

2.3. BeWo cell culture and treatments

Human placental trophoblastic (BeWo) cells were cultured in RPMI 1640 as described earlier (Huovinen et al., 2015). The cells were seeded in 6 well plates, at an approximate density of 400 000 cells per well. After 24 h, when about 70% confluent monolayer was formed, the cells were exposed for 24 h to 1, 10 or 100 μM diuron and equal amount of the solvent DMSO (0.1%, Sigma-Aldrich, USA) in controls. A sample of 100 μl was taken from culture medium and stored at −80 °C.

2.4. In vitro incubations

Preparation of microsomal and cytosolic tissue fractions were carried out as explained previously (Partanen et al., 2010). The metabolism of diuron was studied in human placental microsomes and, for comparison, in human liver microsomes. About 0.5 mg protein of human placental or liver microsomes were added to the reaction mixture (0.1 M Tris-HCL buffer, pH 7.4, 2 mM NADPH). The concentration of diuron was 2 or 100 μM in placental microsomal incubations and 2 μM in liver microsomal incubations with or without 10 μM α-naphthoflavone (α-NF, Sigma-Aldrich, Germany). The duration of