Pro Gradu Thesis

Transplacental Transfer of Perfluorinated Compounds

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ABBREVIATIONS

PFOS- Perfluorooctane Sulfonate

PFOA- Perfluorooctanoic Acid

HCAs- Heterocyclic Amines

ABC- ATP Binding Cassette

BRCP – Breast Cancer Resistance Protein

ATP- Adenosine Tri-Phosphate

PFCs- Perfluorinated Compounds

MDR- Multi Drug Resistance

IUGR- Intra Uterine Growth Resistance

DNA- Deoxyribonucleic Acid

CYP450- Cytochrome P450

BPA- Bisphenol A

OAT – Organic Anion Transporter

ATCC- American Type Culture Collection

mRNA- Messenger ribonucleic acid

cDNA- Complementary De-ribonucleic acid

RT-PCR- Reverse Transcriptase-Polychromatose Chain Reaction

ABCG2- ATP-Binding Cassette, Sub-Family G (WHITE), Member 2 (Junior Blood Group)

ABC- ATP Binding Cassette.

MRPs- Multi Drug Resistance associated proteins.

BW- Body Weight

HCG- Human Chorionic Gonadotropin

FITC-, Fluorescein Isothiocyanate 1

FM-ratio- Fetal to Maternal concentration ratio

JEG-3- Human placental choriocarcinoma cell line

JAr- Human choriocarcinoma cells

RPMI- Roswell Park Memorial Institute

FBS- Fetal Bovine Serum

DMSO- Dimethyl Sulfoxide

EDTA- Ethylenediaminetetraacetic acid

PMSF- Phenylmethylsulfonyl Fluoride

PVDF- Polyvinylidenfluorid

HPLC- High-performance Liquid Chromatography

Ph- Numeric scale used to specify the acidity or alkalinity of an aqueous solution.

Pco2- Partial pressure of Carbondioxide

TCDD- Tetrachlorodibenzo-p-dioxin

PCB52- Polychlorinated biphenyls 52

ABSTRACT

Humans are exposed to chemical carcinogens and endocrine disruptors for instances through environment and diet they consume. Special attention should be paid to pregnant mothers in whom consumption of any harmful compounds can lead to adverse effects in a new born baby as it is believed that these compounds pass through the placenta. The developing foetus is vulnerable to toxic and teratogenic effects and the prenatal exposure of chemicals might lead to developmental changes or even increase in the risk of developing cancer later in life.

The aim of this study was to systematically evaluate the transplacental transfer of perfluorinated compounds using ex-vivo placental perfusion methodology. Placental perfusion techniques are currently used to study not only the organ functions but also the transplacental transfer and placental metabolism of different compounds. In addition BeWo choriocarcinoma cell line to study the effect of PFOS and PFOA on ABCG2 transporter expression.

Several placentas were perfused with PFOA and PFOS, but only 2 perfusions fulfilled the criteria for successful perfusion. It was shown that both PFOS and PFOA cross placenta although the transfer rate is relatively slow and clearly slower than with reference compound antipyrine which crosses placenta by passive diffusion.

In conclusion our results suggest that foetuses are exposed to PFOS and PFOA which is in line with biomonotoring studies. Furthermore, neither PFOS nor PFOA significantly affected ABCG2 transporter expression in BeWo cells.

1 INTRODUCTION

Perfluorinated chemicals (PFCs) are a chemical family of organic compounds consisting of a carbon backbone fully surrounded by fluorine, that makes them resistent to heat, acid or other forces that typically break down chemical compounds. They are used in several industrial branches as well as in a large range of consumer products. It consists of properties such as chemically inert, non-wetting, very slippery, nontoxic, non-stick, highly fire resistant, extreme temperature ratings and highly weather resistant. As a result they are applied in fluoropolymer coated cookware, sports clothing, extreme weather military uniforms, food handling equipment, medical equipment, motor oil additives, fire fighting foams, paint and ink as well as water repellent products. (Posner S. 2010)

PFCs gradually break down in the environment and are often characterized as persistent. There is a widespread wildlife and human exposure to several PFCs, including perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS). More research is needed to fully understand all sources of human exposure, but people are most likely exposed to these compounds by consuming PFC-contaminated water or food, or by using products that contain PFCs. (Posner S. 2010)

In animal studies, some PFCs interfere normal endocrine activity; reduce immune function; cause adverse effects on multiple organs, including the liver and pancreas; and also cause developmental problems in rodent offspring exposed in the womb. Data from some human studies suggests that PFCs may also have effects on human health, while other studies have failed to find absolute links. Additional research in animals and in humans is needed to better understand the potential adverse effects of PFCs for human health. (Posner S. 2010)

In the foetal stage of life, humans are necessarily exposed to a large variety of chemicals and food borne carcinogens. Consequently, there is an increasing concern about fetal and neonatal safety as a result of exposure to foreign compounds. Although there is information about the placental transfer of some foreign compounds and their metabolic activation in human placenta, for most

compounds such knowledge does not exist. (Lankas et al. 1998, Dietrich et al. 2003)

The outcome of pregnancy depends on maintenance of normal fetal and placental development. The fetal development may be adversely affected by exogenous substances including chemicals and medicines. The foetus is vulnerable to exogenous compounds as it undergoes rapid growth and organ development, and has incomplete metabolic capacities. Placenta separates fetal and maternal circulation. It serves to transport nutrients and oxygen, produces hormones to support the pregnancy and helps in excretion of various metabolites and carbon dioxide. (Myllynen and Vähäkangas 2012).

The different mechanisms for transplacental transfer are passive diffusion, active transport, facilitated diffusion, filtration and pinocytosis. Passive diffusion is an important mechanism for transplacental transfer of xenobiotics. The transplacental transfer by this process depends upon physicochemical properties of xenobiotics such as molecular weight, pKa and lipid solubility. The compounds with a molecular weight of over 500D are generally transferred incompletely across the placenta. The transplacental transfer is also modified by transporter proteins and xenobiotic metabolizing enzymes. Both the brush-border (apical membrane) facing maternal blood and basolateral membrane close to fetal capillaries of syncytiotrophoblast express various transporter proteins. These transporters may either facilitate or slow down the transport of xenobiotic. (Myllynen et al. 2012).

The expression of the transporters are found to differ in apical and basal membranes leading to polarized transport across the placenta. The presence of transporters in fetal capillaries makes this process even more complex. The fetal exposure to xenobiotics may be modified for instance by ATP binding cassette (ABC) transporters. Also, placental metabolism may modify fetal exposure to xenobiotics. Ex vivo human placental perfusion of an isolated cotyledon model can be used to investigate human placental transfer and placental metabolism. As human placenta is surplus tissue after delivery, the studies using delivered placentas impose no risk on mother or child and pose only a limited number of ethical dilemmas. (Mathiesen et al. 2010)

2 LITERATURE REVIEW

2.1 Human placental transfer

It is usually acknowledged that some drugs and chemicals endure teratogenic potential. It is documented that at least to some extent, most of the drugs cross the placenta. Nowadays, there is insufficient information available of the pharmacokinetics of drugs in the feto-placental unit. Comprehensive information about drug transport across the placenta would be appreciated for the development of safe and effective treatments. For reasons of safety, human studies on placental transfer are inhibited to a limited number of drugs. Several in vitro methods for the study of placental transfer have been developed over the past decades. The placental perfusion method is the only experimental method that has been conducted to study human placental transfer of substances in organized placental tissue. (Myöhänen et al. 2011)

Several decades ago, it was believed that placenta protects foetus from harmful effects of xenobiotic. The thalidomide tragedy evidently showed the susceptibility of the foetus. At present it is known that many chemicals and pharmaceutical drugs can pass the placenta easily and rapidly. According to the studies, human exposure to carcinogens in the perinatal period of life increase cancer incidence both in children and young adults. Not only this, but also cancer incidence may increase later in life. (McBride et al. 1961)

ATP binding cassette (ABC) transporters limit the uptake of toxins, and may modulate their toxic and carcinogenic effects. They act as bouncers in cells effluxing their substrates out of the cells. In addition, they limit the bioavailability of toxins and pharmaceuticals. They are widely expressed in barrier organs of many organisms. Identical to many genes, genes of ABC transporters are polymorphic, and some variants have more restricted substrate specificity or modified expression levels. Multidrug resistance (MDR) is a common problem in anticancer drug treatment. However, ABC transporter expression can also be induced by environmental chemicals. Furthermore, hormones and other

pharmacological and toxicological agents modify the expression and function of ABC transporters. (Prouillac & Lecoeur 2010; Ni & Mao 2011)

2.2 Structure and development of human placenta during pregnancy

The placenta has several fascinating and critical functions. It mediates implantation and establishes the interface for nutrient and gas exchange between the maternal and fetal circulation. Moreover, it initiates maternal recognition of pregnancy and alters the local immune environment. Furthermore, it alters maternal cardiovascular and metabolic functions through the production of paracrine and endocrine hormones. The consequences of abnormalities in any one of these functions includes poor pregnancy outcome. This ranges from the mild intrauterine growth restriction to the severe implantation failure and embryonic, fetal or perinatal death. (Padubidri & Anand 2006)

The function of the placenta during early gestation is primarily to intervene implantation of the embryo into the uterus. Secondarily, to produce hormones that induces maternal recognition of pregnancy. The term used to describe the means by which the lifespan of the corpus luteum is extended, preventing the end of the ovarian cycle. (Padubidri et al. 2006)

The placenta is derived from two major cell types. The trophoblast cell lineage originates from the trophoectoderm at the blastocyst stage. The stromal and vascular components of the placenta are derived from the allantois. It grows out from the embryo proper to establish contact with the overlying trophoblast layer. Villous development initiates only at points of chorioallantoic attachment and this process is particularly vulnerable to perturbation by genetic epigenetic or nutritional influences. The cellular and molecular mechanisms underlying the development of the placenta are best understood in the mouse as a result of studies involving experimental embryology, culture trophoblast stem cells and analysis of mouse mutants that have defects in placental development. In addition to cell intrinsic mechanisms, placental development is also highly regulated by oxygen levels and the availability of nutrients in the maternal circulation. This means that the basic pattern of placental development can be altered by environmental

influences and moreover, that placental development can attempt to compensate for other defects. (Singh 2001)

2.2.1 The structure of human placenta

The main function of placenta is to assist in uptake of nutrition, elimination of waste and exchange of gases via the mother's blood supply. In humans, the average term placenta is 22 cm in diameter 2.5 cm in thickness (enourmous thickness at the centre and become thinner peripherally) and has an average weight of 470 gram (Benirschke K and Kaufmann P, 1999). It connects to the foetus by an umbilical cord of approximately 55-60 cm in length. Umbilical cord consists of two arteries and one vein. It inserts into the chorionic plate. Vessel branch covers the surface of placenta and further divide to form a network covered by a thin layer of cells. This results in formation of villous tree structures. There are two surfaces, maternal and fetal. The surface that attach umbilical cord is maternal surface and the other is fetal surface. (Padubidri et al. 2006)

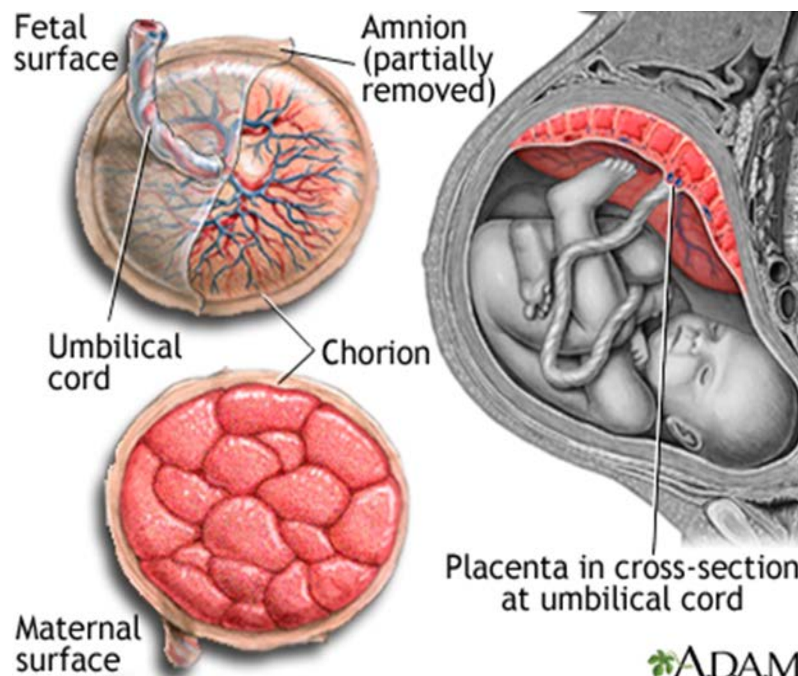

Fig 1: Anatomy of human placenta. (Medline Plus 2015)

Humans have a haemonochorial villous placenta. The exchange between maternal and fetal circulations take place in the chorionic villus. The villus consists of fetal capillary, stroma and trohphoblast layer. In human placenta there are two types of trophoblastic cells. They are mononuclear cells called cytotrophoblasts and multinucleate cells called syncytiotrophoblast. Fetal and maternal circulations are divided by two cell layers, syncytiotrophoblasts and fetal capillary endothelium at term. In human placenta maternal blood comes into direct contact with synsytiotrophoblast/trophoblast cells. The trophoblast layer in contact with maternal blood is a single synsytiotrophoblast layer with underlying cytotrophoblasts. (Padubidri et al. 2006)

2.2.2 The development of placenta

Trophoblast cells which later form the main type of placental cells are present already at blastocyst stages as the outer layer. Before implantation trophoblast consists of two layers, outer syncitiotrophoblast(syncitium) and the inner cytotrophoblast. The trophoblast forms 8 celled structures that develop to form chorionic villi. Later chorionic villi proliferate and become placenta by 10th to 12th weeks of period of gestation. Trophoblast functions by establishing communication between mother and embryo. It also provides the embryo with nutrition at the early embryonic stage. By the end of 12th day cytotrophoblast invaginates into the syncitium to form a finger like projection called primary villi. Then at the end of 16th day extraembryonic mesoderm lines the trophoblast and invaginates primary villi to form secondary villi. Finally in the 21st day mesoderm develops vascular structures that invade in secondary villi to form tertiary villi. By the end of third week, embryo is connected to tertiary villi with the help of mesenchymal stalk. This stalk later develops to become umbilical cord. Furthermore, fetomaternal circulation establishes after the development of placenta. (Malek 2013; Padubidri et al. 2006)

Chorion is attached to decidua basalis and at the placental site. The number, size and complexity of chorionic villi increase to from chorion frondosum. Other than the placental site, chorion atrophies and form chorion layer. The placental villi

grow like a tree between basal plate of decidua and the chorionic plate. Placenta circulation gets well established at 20th weeks and it is 500ml/min at term. The composition of the trophoblastic layer changes during pregnancy. During the first trimester, the villi have a nearly complete cytotrophoblast layer underneath the syncytiotrophoblast layer. On the other hand, in the late pregnancy the cytotrophoblast layer becomes discontinuous. The materno-fetal diffusion distance decreases significantly as gestation advances, decreasing from 50–100 µm in the second month to 4–5 µm at term. (Padubidri et al. 2006; Malek 2013)

2.3 General aspects of placental transfer

Most chemicals cross placenta at least to some extent using passive diffusion. The important properties determining the placental transfer by passive diffusion are molecular weight, pKa, lipid solubility and protein binding. In addition to passive diffusion, compound may cross the placenta via active transfer, facilitated diffusion, phagocytosis, and pinocytosis. The rate of passive diffusion may change as pregnancy proceeds. As mentioned earlier diffusion distance shortens as pregnancy proceeds. The materno-fetal diffusion distance decreases significantly as gestation advances, decreasing from 50–100 µm in the second month to 4–5 µm at term. (Myllynen et al. 2012)

There is increase in maternal serum albumin concentration from 25 g/l at 12 weeks to 35 g/L in term pregnancy. In the first trimester albumin concentration in fetal serum is much lower than in maternal serum. The fetal serum albumin concentration increases linearly as gestation advances reaching about 20% higher than albumin concentration in maternal serum at term. Thus, there may be change in the transport of albumin bound drugs across placenta in term pregnancy compared to earlier stages. (Myllynen et al. 2012)

2.4 Transporter proteins and Transplacental transfer

ATP-binding cassette (ABC) transporters are a large group of membrane proteins, expressed in wide range of tissues. They are active protein pumps using ATP as the source of energy and have endogenous substrates. Some of them also transport xenoboitics and take part in tissue defence in for instance placenta. For instance, ABCG2 is an efflux transporter localised in the apical membrane of the placental microvillous membranes, transferring its substrates from the placenta syncytiotrophoblast back to the maternal circulation. ABCG2 has been shown to decrease placental transfer of its substrates. (Prouillac & Lecoeur 2010; Ni & Mao 2011; Myllynen et al. 2008)

Both trophoblastic cells and fetal capillary endothelium express transporter proteins. In syncytiotrophoblast, transporters are expressed both in apical facing maternal blood and basolateral membrane close to fetal capillaries where they transfer compounds in and out of syncytiotrophoblast. Transporter proteins are known to interact with xenobiotics such as pharmaceutical drugs and environmental contaminants. Efflux transporters decrease the concentration of xenobiotics in cells whereas uptake transporters increase it. So far, over 30 drug transporters have been found from the placenta belonging to several transporter super families. Based on function and localization efflux transporters may either increase or decrease fetal exposure. (Myllynen 2012; Ni and Mao 2011).

P-glycoprotein (ABCB1 gene product, MDR1) is one of the well-known transporters that prevent the entry of xenobiotic into the cells. P-glycoprotein is the first discovered and the best characterized of drug efflux transporters. In the placenta, it plays role in the regulation of drug disposition to the foetus. P-glycoprotein is expressed in human placental trophoblasts from the first trimester to term. In animal experiment the placental P-glycoprotein has been shown to protect the developing embryo and fetus from toxic substances and thus prevent teratogenesis and fetal toxicity (Lankas et al. 1998; Smit et al. 1999). In addition to p-gp other placental efflux transporters such as selected multidrug resistance associated proteins (MRPs; ABCCs) and the breast cancer resistant protein

(BCRP; ABCG2) may have similar function (Myllynen, Pasanen & Pelkonen 2004; Novotna, Pavek & Staud 2006).

Interestingly, transporter proteins expression changes dynamically during pregnancy. For example, p-glycoprotein (ABCB1) expression in the apical membrane of syncytiotrophoblast decreases as gestation advances (Myllynen et al. 2012).

2.4.1 Xenobiotic metabolizing enzymes in human placenta

Placental metabolism may also affect fetal exposure to xenobiotics. Compared to the liver, the activities of xenobiotic metabolizing enzymes in human placenta are generally low. Still placental metabolism may lead to metabolites with toxic potential compared to the parent compound. For instance, human placenta can metabolize retinoids to both more and less toxic metabolites and benzo pyrene to active metabolites binding to DNA. The expression of xenobiotic metabolizing enzymes is dependent on the developmental stage of the placenta. Quite a few enzymes like CYP (Cytochrome P450) are expressed at the mRNA level, especially during the first trimester. (Vähäkangas and Myllynen 2009)

Trophoblastic cells in human placenta express xenobiotic metabolizing enzymes. Xenobiotic metabolizing enzymes are found in the placenta throughout the pregnancy. However, only few of them have been shown functionally active. CYP1A1 is expressed in the placenta and it is inducible for instance by smoking. Also some conjugative enzymes are expressed in the placenta. The enzymes glutathione-S-transferase, glucuronyltransferase as well as sulfotransferase are active from the first trimester and N-acetyltransferase in term placenta. The enzymes glutathione-S-transferase, glucuronyltransferase as well as sulfotransferase are active from the first trimester and N-acetyltransferase in term placenta. (Vähäkangas et al. 2009)

2.5 Perfluorinated compounds

2.5.1 Introduction to perfluorinated compounds

There are emerging concerns about potential effects on child health and development of early-life exposure to substances such as brominated flame retardants, perfluorinated compounds, phthalates and phenols (including bisphenol A). Perfluorochemicals (PFCs) are a group of chemicals which are used to make fluoropolymer coatings in variety of products like clothing, furniture, adhesives, food packaging, heat-resistant and non-stick cooking surfaces, surface coating and the insulation of electrical wire. This coating resists heat, oil, stains, grease, and water. They have long half-lives to persist in the environment. These PFCs reach the human body through the ingestion of contaminated foodstuffs and/or drinking water. PFCs have been detected in fish, meat, milk products, and plants, for instance grains. (Casas et al. 2012)

2.5.2 Toxicokinetics

The animal experiments have shown that PFC uptake can occur by oral, inhalation, or dermal exposure. (Kennedy 1985; Kennedy et al. 1986; Kudo and Kawashima 2003). Once absorbed in the body, it distributes predominantly to the liver and plasma, and to a lesser extent the kidney and lungs. PFCs are not metabolized in human body. Two of the most common PFASs, perfluorooctane sulfonate (PFOS) and perfluorooctanoate (PFOA), have median half-lives in human blood of approximately 2–5years. (Bartell et al. 2010; Olsen et al. 2007).

A recent study suggests that the biological half-life of PFOA in humans is 4.37 years based on study of occupationally exposed workers. (Kudo & Kawashima 2003). PFOA is excreted in both urine and faeces. PFOA is excreted into urine by active tubular secretion, and certain organic anion transporters (OATs) that are thought to be responsible for the secretion. (Stahl et al. 2011).

Faecal excretion is also important in the elimination of PFOA. Some studies had suggested that PFOA undergoes enterohepatic circulation resulting in reduced amounts of faecal excretion. The studies have also demonstrated that these compounds are not freely permeable to blood brain barrier. (Harada et al. 2007)

2.5.3 Evidences from animal studies

Qin et al. 2010, found that the binding strength of perfluorinated compounds increased with the increasing chain length of the perfluorinated compound to bovine serum albumin. According to Austin et al. (2003) and Seacat et al (2003) liver to serum ratio for PFOS is about 2.5. PFOA is mainly distributed in the liver but as soon as level in liver reaches 4 mg/kg then it spills into blood or other tissues. The low-dosage PFOA (0.041 mg/kg BW) was injected intravenously in rats, and found that a larger proportion of the substance was found in the liver (52%) (Kudo et al. 2007). Some studies suggest that these PFCs might undergo beta oxidation in liver to form more toxic compounds than original compound. (Martin et al. 2009) As PFCs is bound to protein, the glomerular filtration rate is low. However, they are secreted actively via organic anion transporter (OAT) (Kudo et al. 2002).

Some of the PFCs are also excreted through faeces. The animal studies have also demonstrated that the toxicity of PFCs in various body parts and functions. They were found to cause immunotoxicity, hypothyroidism, liver cirrhosis, neurotocity, skin and eye irritation in acute exposure and have teratogenic potential. (Stahl et al. 2011).

2.5.4 Prenatal Effects

The correlation between concentrations of PFOS and PFOA in cord blood and concentrations in maternal serum at the time of delivery were demonstrated in many studies. (Gutzkow et al. 2012, Monroy et al. 2008, Inoue et al. 2004). It was found that, compared to the maternal samples, cord samples had 1.4–4 fold lower median concentration for all the PFCs measured. And the predominant ones in cord samples are PFOS and PFOA as in the maternal samples. (Gutzkow et al. 2012). Thus, PFCs are considered to cross the placental barrier. This was also shown in animal studies (Hinderliter et al. 2005)

The adverse effects of PFCs in newborns and children is still not clear. However, an inverse correlation between PFOS and PFOA in relation to birth weight, ponderal index and head circumference have been reported. (Apelberg et al. 2007; Chen et al. 2012)

A review by Bellinger suggests that, there is only limited evidence that higher prenatal exposures of PFOA and PFOS cause adverse neuropsychological outcomes in children (Bellinger 2013).

2.6 EXPERIMENTAL MODELS TO STUDY FETAL EXPOSURE

People are exposed to chemicals due to medications and life styles factors and so do pregnant women. Numerous chemical factors are present in the environment and the exposure cannot be avoided. Placenta is the organ that is responsible for fetal nutrition and connects the fetus to the mother. In risk assessment placental toxicokinetics plays an essential role in determining the amount of toxins that reaches the fetus. Mathematical methods for placental transfer are under development but still far away from clinical practice. (Hewitt et al. 2007, Giaginis et al. 2009). Another aspect that appears in these kind of studies is ethical aspect. Because of the ethical reasons, number of methods of studies of fetal exposure to xenobiotics and pharmaceuticals is restricted.

2.6.1 Human Placental Perfusion

Human placental tissue can be kept alive for several hours after the delivery in a placental perfusion system. Perfused placental studies show that placental vital functions recover from anoxia which is consequence of delivery. The perfused placenta transports amino acids, and glucose to the fetal circulation and placental lactogen is released to maternal circuit and steroids to both circulations (Dancis et al. 1980, Dancis 1985).

After delivery, placental becomes waste tissue and thus the use placenta in human placental perfusion experiments does not affect the treatment of the mother or newborn in anyway. First perfusion methods for studying the placental transfer were documented in 1920s and were whole placenta perfusions with only fetal side being perfused through cord veins. Currently, the dual perfusion of a single placental cotyledon is commonly used. In this method, placental peripheral artery and vein are cannulated on the fetal side of placenta and the maternal circulation is performed by pushing the cannules into the intervillous space. In dual recirculating placental perfusion, maternal and fetal circulation are separated and closed.

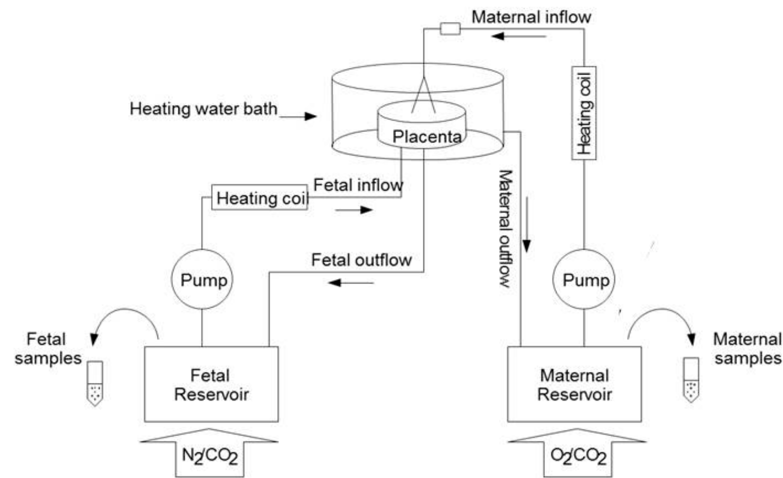

Figure 2. Schematic presentation of the placental perfusion equipment (Myllynen et al. 2008b)

Circulation can also be open, where perfusion media pass only once in the placental tissue (Schneider et al. 1985). Placental transfer kinetics can be measured by taking samples in different time points from both circulations. The appearance of the compound to fetal circulation and disappearance from maternal circulation is studied. In addition to study compounds, the transfer should be monitored by reference compounds. Antipyrine and insulin have been used as reference compounds; they pass placenta by passive diffusion in flow dependent manner.

During the perfusion, leak from fetal circulation to maternal flow is the best marker for placental viability. Leak suggests that, syncytiotrophoblast layer has been damaged and probable rupture in microvillous membranes. Also placental lactate and human chorionic gonadotropin (hcg) hormone production and metabolic enzyme activities can be measured to evaluate the vability. Viable tissue also consumes oxygen and glucose (Dancis et al. 1985).

The major disadvantage of the human this method is that placental perfusion with term placenta can be demonstrated at the end of the pregnancy. Placental metabolic activity and transporter expression vary during pregnancy. And also the number of placental cell layers decrease within advancing gestation (Huppertz 2008). Thus, there may be changes in placental transfer efficiency at different stages of pregnancy.

Human placental perfusion of single cotyledon with separate maternal and fetal circulations was originally introduced by Panigel (1962).Later it was further developed by the groups of Schneider et al. (1985) and Miller et al. (1985). Placental perfusion is the only experimental model to study human transplacental transfer in organised human placental tissue. So far there are no standardized criteria for ex vivo human placental perfusion. Placentas from both vaginal births and C-sections can be used for the perfusion. Ex vivo placental perfusion may be either nonrecirculating (open/single-pass) or recirculating (closed) perfusion. (Myllynen et al. 2012)

Various markers are used to monitor placental viability during the perfusion and the selection of markers may vary between research groups. Minimal loss of fetal perfusate is the main marker of a successful perfusion. Volume loss of 2-3 from the fetal circulation is usually considered acceptable. Recently, a loss greater than 3ml/hour has been associated with higher transfer of control substance FITC dextran in successful perfusion. This compound does not cross placental membranes due to its high molecular weight if maternal-fetal interface is intact. Antipyrine and other reference compound such as creatinine are used to confirm the overlap between maternal and fetal circulations. These markers could be used to normalize placental transfer between perfusions. Other markers used for tissue viability include pH oxygen consumption, net oxygen transfer, glucose consumption, lactate production and tissue morphology after perfusion. Production of hormones such as human chorionic gonadotropin and, human placental lactogen is also a acceptable marker for placental tissue viability. Although placentas from 27-40 weeks have been perfused, the challenging perfusion method is usually applied to term placenta. Thus, the major limitation of this technique is that it does not provide data on placental transfer in the first and

second trimester placentas. Furthermore, the success rate is relatively low. The success rate, counting from the placentas are enough to be placed into the perfusion equipment, is about 50 percent. Therefore, the number of placentas needed for experiments are relatively higher than the actual number of successful experiments. (Myllynen et al. 2012)

As an isolated organ system, placental perfusion naturally does not reflect the in-vivo pharmacokinetic balance of the entire maternal-placental-fetal system. In-vivo maternal pharmacokinetics affects the amount of a xenobiotic available for placental transfer. Furthermore, serum albumin concentrations differ between maternal and fetal circulations change dynamically during pregnancy (Myllynen et al. 2012).

Human ex-vivo placental perfusion may also be used to characterize the factors causing inter-individual variation in transplacental transfer. The expression of ABCG2 protein in perfused placenta correlates with fetal to maternal concentration ratio (FM- ratio) of PhIP but not with the FM-ratio of IQ. It suggests that, ABCG2 could modify fetal exposure to PhIP (Myllynen et al. 2008; Immonen et al. 2010).

2.6.2 Placental transfer in vivo

Placental transfer is studied in vivo in maternal blood and cord blood samples collected after delivery (Myllynen et al. 2003). And also, accumulation of chemicals in placental tissue is studied after delivery (Ala-Kokko et al. 1998). The concentration in amniotic fluid can be measured at the delivery or from left over samples taken for the clinical purposes (Myllynen et al. 2007). The concentration of pharmaceuticals is quantified from the serum samples both from maternal and cord blood.

Naturally, environmental chemicals or drugs cannot be administered to the mother due to ethical reasons, excluding the drugs that are essential for the mothers'

health. The concentrations of compounds that mothers are exposed from environmental sources can be measured from blood samples. This data provides information about the exposure levels of pregnant women as well as about placental transfer. However, the single quantitation of the concentration in maternal and the fetal blood does not give any information about how long does it take to achieve equilibrium between the mother and fetus. (Myllynen et al. 2003)

2.6.3 Cell culture models for human placenta

Both continuous and primary cell lines originating from placenta can be used to study placental function in vitro. One of the most commonly used models are BeWo cells which are a placental choriocarcinoma cell line. The b30 sub clone of these cells can be grown on permeable membranes in bicameral chambers to form confluent cell layers that enables rate of both nutrient uptake into the cells from the apical surface and efflux from the basolateral membrane to be determined.

Besides BeWo cells, other cells such as JEG-3 and JAR have been widely used to study the fate and effects of xenobiotics in the placental cells. They can be used to study various aspects of transplacental transfers such as cellular uptake or efflux of xenobiotic form the cells. They may also be used to study the effect of xenobiotics on the expression of transporter proteins. However, ABCB1 expressions in all three cell lines is much more lower compared to primary trophoblast isolated from term placentas, which is the limitations for these models. ABC2 protein expression has been reported to be low in the BeWo and JAr cells. While ABC1 expression is higher in BeWo and JAr cells than in primary trophoblasts. BeWo, JEG-3 and JAr cells also express xenobiotic metabolizing enzymes. In relation to human placenta, CYP1A1 enzyme is present and inducible in BeWo and JEG-3 cell lines. (Myllynen et al. 2012)

3. AIMS OF THE STUDY

The aim of study is to appreciate the effect of PFCs on placental transporter expression using BeWo choriocarcinoma cell lines. Also, placental transfer of PFCs using ex vivo placental perfusion methodology was studied.

4 METHODS

4.1 Cell culture experiments using BeWo choriocarcinoma cell line

BeWo choriocarcinoma cells were purchased from American type culture collection (ATCC). Growth media was prepared by mixing RPMI 1640 (90ml), FBS (10ml), Glutamax (1ml) and Penicillin streptomycin (1ml) (all from Gibco, Paisley, Scotland). When plating cells for the exposure cell, cell culture bottle containing cells was taken from the incubator. Media was removed from the bottle and the cells were washed twice with 10ml of PBS. Trypsinization was done with 1ml of Trypsin (Gibco, Paisley, Scotland) for approximately 5-7 minutes.

After that, they were collected in 50 ml Falcon's tube. It was centrifuged at 1000 revolution per minutes and one Eppendorf's tube was filled with 990µl of PBS at the same time. Supernatant was removed. Cells were dissolved in 1ml of growth media cells were counted under microscope. 10µl of cell suspension was added in 990µl of 1xPBS in Eppendorf's tube. 10µl was taken in Burker chamber for counting. Cells were counted in all chambers and average was calculated.

Cells were seeded in a density of 1 x 10^6 cells/ml medium. After adjustment period cells were exposed for 48 hours to 1µM to 10 µM. Concentrations of PFOS and PFOA (both from Sigma-Aldrich) for protein analyses. For mRNA expression studies cells were exposed to 1 µM PFOS of PFOA with or without folic acid (1 mg/ml Sigma-Aldrich) or folic acid alone (1 mg/ml) for 48 hours. In each series control cell were exposed to vehicle (always <0.5% DMSO).

4.2 mRNA Isolation

Total RNA was isolated from BeWo cells using Tri-Reagent (Invitrogen, Paisley, 233 Scotland, UK) according to the manufacturer`s instructions as follows. Cells were collected in Tri-Reagent and stored at -70 ºC until RNA isolation. Ethanol solution (75%) was prepared by mixing nuclease free water with ethanol. Samples were thawed at room temperature. 200µl of chloroform (without isoamylalcohol) was added in each sample, caps of each tube was tightened and shaken vigorously for 15 seconds. The mixture was then incubated at room temperature for five minutes. The tubes containing mixture were centrifuged at 12000 revolutions per minute for 15 minutes at 4°C. The aqueous phase of the tubes was transferred to fresh tubes. 500µl of Isopropanol was added per ml of TRI reagent solution used for sample homogenization. The tubes were put on vortex for five seconds and shaked vigorously and samples were incubated at room temperature for five minutes. The tubes were centrifuged at 12000 revolutions per minute for eight minutes at 4°C. Supernatant was removed carefully from the tubes without disturbing the white pellets. 75% ethanol was added to each sample to wash the RNA pellets. Mixtures were centrifuged at 7500 rpm for five minutes at 4°C. Ethanol was removed completely without disturbing the pellets. Later, fine tipped pipettes were used for complete removal of ethanol. RNA pellets were dried in air approximately three minutes. The RNA pellets were mixed with nuclease free water and solution was shaked few times through the pipette tip, kept in hot block for 10 minutes and solution was then analysed. Samples were stored at 4°C for immediate mRNA concentration analysis and for long term storage stored at -70°C.

4.3 cDNA conversion and RT-PCR

Synthesis of cDNA was performed using p(dN)6 random primers (Roche) and M-MLV reverse transcriptase (Promega). Reverse transcription polymerase chain reaction (RT-PCR) was used to detect mRNA expression levels. mRNA expression of ABCG2 was analyzed by RT-PCR method using Taqman chemistry (Table 1) on an ABI 7300 sequence detection system (Applied biosystem Foster City , CA, USA) with FastStart Universal Probe master (Rox, Roche, Mannheim, Germany) and the following PCR program 1)50 C 2:00 min 2) 95C 10:00 min 3)95C 15s, 60C 1:00 min(x40). The results were normalised to 18S RNA quantified from the sample using the method.

Table 1.The forward (F) and reverse (R) primers for real time quantitative RT-PCR (Myllynen et al. 2008)

Gene	Primers	Fluorogenic probe
ABCG2	(F)CAATGGGATCATGAAACCTGG	TCAACGCCATCCTGGGACCCA
	(R)AACGAAGATTTGCCTCCACCT	
18s	(F)TGGTTGCAAAGCTGAAACTTAAAG	CCTGGTGGTGCCCTTCCGTCA
	(R)AGTCAAATTAAGCCGCAGGC	

4.4 Protein isolation and immunoblotting

4.4.1 Protein isolation

The membrane proteins were isolated by scraping and lysing the cells into homogenization buffer (10 mM Tris-HCl pH 7.0, 5mM EDTA, 250 mM sucrose with protease inhibitors: 100 µg/ml PMSF, 1 µg/ml Antipain, 1 µg/ml Aprotinin and 1 µg/ml Pepstatin A) followed by centrifugation at 900 x g for 10 minutes at +4 °C. After that the supernatant was centrifuged at 100 000 x g for 60 minutes at

+4 °C. Membrane precipitate was dissolved into buffer (10 mM Tris-HCl pH 7.4, 1 % Triton X-100, 1 % SDS, 0.25 % MEGA-10, with protease inhibitors: 100 µg/ml PMSF, 1 µg/ml Antipain, 1 µg/ml Aprotinin and 1 µg/ml Pepstatin A) and incubated at +4 °C for 1 hour. Insoluble cell material was removed by centrifuging 14 000 x g for 10 minutes at +4 °C and supernantant containing membrane proteins was stored at temperature of -70 °C.

4.4.2 Immunblotting

This technique is widely known as western blotting and it analyzes and detects specific protein in the given sample of cells or tissue. Gel electrophoresis is used for separating the proteins according to size and proteins are transferred to PVDF or nitrocellulose membrane. After that selected proteins are detected using antibodies specific to target protein.

The reagents required for the preparation of gel are 1.5 M Tris-HCl, Ph 8.8, 0.5 M Tris-HCl, pH 6.8, 10% (w/v) SDS stock, Acrylamide/Bis (30% stock), 10% freshly prepared ammonium persulfate, TEMED and distilled water. Once the gels were prepared they were either used immediately to run the sample or they were stored in refrigerator in a moist condition covered with plastic. The use of gloves was mandatory before the preparation of gels. Tris-HCl, TEMED and Acrylamide solutions was stored in the fridge and were protected from light. SDS, ammonium persulfate and distilled water were stored in room temperature. While mixing the reagents, APS and TEMED were added to other reagents before polymerization of gel. Approximately 3.45 ml of gel was pipetted and topped with distilled water. It was left for 45 to 60 minutes for polymerization. Once the lower gel was ready, distilled water was removed from the top and with the help of filter paper. Approximately, 2.5 ml of Stacking gel was added on top of upper edge of glasses and comb inserted immediately. It was left for 30 to 45 minutes for polymerization.

The proteins samples are thawed in ice and mixed with the sample buffer (20 μg of protein/sample). Samples were denaturized by incubating samples at least 20 min at room temperature after which samples were separated using gel electrophoresis as follows. Comb from the apparatus were taken off and wells were washed with distilled water. Cassettes were inserted in to the chamber and were made sure that the cassette was not leaking from the chamber to the lower chamber. Running buffer was prepared by mixing 70 ml of 5 times running buffer (contents of running buffer) in 280 ml of distilled water. Upper chamber was filled with the running buffer in such a way that surface was above the edge of smaller glass. Wells were washed with running buffer. Samples were loaded to the wells and are proteins were separated at 200 volt for 30 to 45 minutes.

Once the samples were run, buffer was poured in the basin and gel cassettes are gently opened. Small glasses were detached from large glasses in such a way that gel remains on top of smaller glasses. Gels were then equilibrated in transfer buffer) for 15 minutes. At the same time PVDF membrane were cut and incubated in methanol for 15 seconds, in distilled water for 2 minutes and in transfer buffer for 10 minutes. Special attention was paid so that gel didn't touch PVDF membrane while incubating in transfer buffer. The proteins were transferred to PVDF membrane using. Biorad paper (Extra thick filter paper 7, 5*10cm*60mm) was dipped into transfer buffer and rolled to remove the air bubbles. PVDF Membrane was put above the biorad paper followed by gel and again biorad paper in the top. They were again rolled off to remove the air bubbles. Biorad semidry immunoblotter was used to transfer proteins from gels to PVDF membranes (30 minutes 10 V max 300 mA). PVDF membranes were then handled protein side up from now onwards.

Membranes were washed with distilled water for five minutes and non-specific binding sites were blocked by using TBS-Tween in 5 % non fat dry milk at room temperature for 3 hours. Membranes were again washed with TBS-Tween in room temperature in a shaker. Primary antibody in TBS-2.5% milk maintained at -20 degree is added. ABCG2/BCRP primary antibody was purchased from Millipore (Clone BXP-21, MAB4146). For every experiment, Sodium Potassium ATPase (Abcam, Cambridge, UK) was used as a loading control. For all antibodies anti-

mouse IgG (Cell Signalling Technologies) was used as the secondary antibody. Proteins were detected with ECL+ chemiluminescence kit (GE Healthcare, Buckinghamshire, UK) and visualized digitally by FUJI LAS-3000 reader. The band intensities were quantitated by densitometry using QuantityOne-software (BioRad).

5 PLACENTAL PERFUSION

Placentas were obtained within 10-15 minutes after delivery. Donating placenta for research purposes after delivery does not affect the management of mother or newborn in anyway because placenta becomes waste tissue after delivery. Krebs-Ringer phosphate-bicarbonate buffer with heparin (Leo Pharma, Malmö, Sweden) was injected to umbilical cord veins to prevent coagulation in maternity ward and placenta was carried to Department of Pharmacology and Toxioclogy in 0.9% saline solution. In the research laboratory, circulation was established in one placental lobe by cannulating the artery and vein using neonate feeding tubes. When the outflow from the vein was confirmed to be similar to inflow to the artery, the placental lobe was placed in the perfusion apparatus. Maternal flow rate was 9 ml/min and fetal 3 ml/min throughout the perfusion. Perfusion solutions were gassed with membrane oxygenator using technical air (20 % CO_2/ 80 N_2) on maternal circulation and 95 % N_2/5 % CO_2 in fetal circulation. The perfusion medium used was RPMI1640 cell culture medium without phenol red supplemented with non-essential amino acids (Gibco, Paisley, Scotland), L-glutamine (Gibco), heparin (25 IU/ml, 166 Leo Pharma, Malmö, Sweden) and human albumin 2 mg/ml (20g/100 ml, Sanquin, Netherlands). The volumes of perfusates were 200 mL in maternal and 120 mL in fetal circulation. In order to give placenta time to recover from hypoxia placentas were perfused for 30 minutes without study substances. The study compounds were added to maternal circulation (1000 ng/ml of PFOS or PFOA) simultaneously with reference compound antipyrine. Antipyrine is used to confirm the overlap between maternal and fetal circulations. Placentas were perfused for 4 hours after addition

of study compounds. Samples were collected from both maternal and fetal circulations during the perfusion. Glucose concentrations were measured using Contour blood sugar measurement device 190 (Bayer, Leverkusen, Germany). Perfusion pH and pO2 were monitored with Stat profile pHOX Basic (Nova biomedical, Waltham, 193 USA). As per requirement, pH values were adjusted to physiological levels using 0.5 M HCl. Criteria for successful perfusion were leak less than 4 ml/h from fetal to maternal circulation, normal pH, production of hCG, glucose consumption and antipyrine transfer.

5.1 Ethical Issues in Placental Perfusions

Ethical committee of the Northern Ostroothnia Hospital District has approved the use of placentas in the placental perfusion studies. During the placental perfusion, study written informed consent were obtained from all the mothers donating their placentas for the study purpose. The collection of placenta does not affect the management of delivery or newborn in anyway because after delivery placenta is surplus tissue.

5.2 Analytical methods for antipyrine, PFOS and PFOA

Antipyrine analysis was done by HPLC as previously described using phenacetin as an internal standard (Immonen et al. 2010). PFOS and PFOA concentrations were measured at National Institute of Health and Welfare using LC-ESI-MS/MS as described earlier (Koponen et al. 2013).

5.3 Calculations and statistical analyses

Fetal to maternal concentration ratio was used to describe placental transfer of PFOS, PFOA and antipyrine.

FM ratio= Fetal sample concentration/Maternal sample concentration

Statistical analyses were done using Student's t-test. P-values < 0.5 were considered significant.

6 RESULTS

6.1 Viability criteria for the ex vivo placental perfusion method

During the experiment two successful perfusions were made. In these perfusions the leak from fetal to maternal circulation was 2.23-2.38 ml/h (acceptable level < 3ml/h). Placentas consumed glucose and oxygen as expected (table 2). pH values were also acceptable in one of the perfusion and from another data was not available. HCG production was observed in both perfusions suggesting that the tissue was viable.

Table 2. Criteria for successful perfusion

Parameter	PFOS-perfusion	PFOA-perfusion
Leak (ml/h)	2,38	2,23
Glucose consumption (µmol/g of tissue/h)	2,5	41,4
hCG production (mIU/g/h)	12,7	35,0
pH at the beginning (fetal artery)	7,521	NA
pH at the beginning (fetal vein)	7,62	NA
pH at the beginning (maternal artery)	7,464	NA
pH at the beginning (maternal vein)	7,474	NA
pH at the 4 h (fetal artery)	7,451	NA
pH at the 4 h (fetal vein)	7,425	NA
pH at the 4 h (maternal artery)	7,491	NA
pH at the 4 h (maternal vein)	7,348	NA
Oxygen consumption (mL/min/kg)	0,77	NA
Oxygen transfer (mL/min/kg of tissue)	0,04	NA

6.2 Transplacental kinetics of PFOS and PFOA

During 4 hours of human placental perfusions, both PFOS and PFOA cross placenta. Although, the transfer rate was relatively slow (Figure 3) and clearly slower than with reference compound antipyrine which crosses placenta by passive diffusion (Figure 4). Fetal to maternal concentration ratios at the end of the perfusions were 0.29 for PFOS and 0.24 for PFOA. PFOS concentration at the end of perfusions was 138.4 ng/ml on fetal and 480.3 ng/ml on maternal circulation.

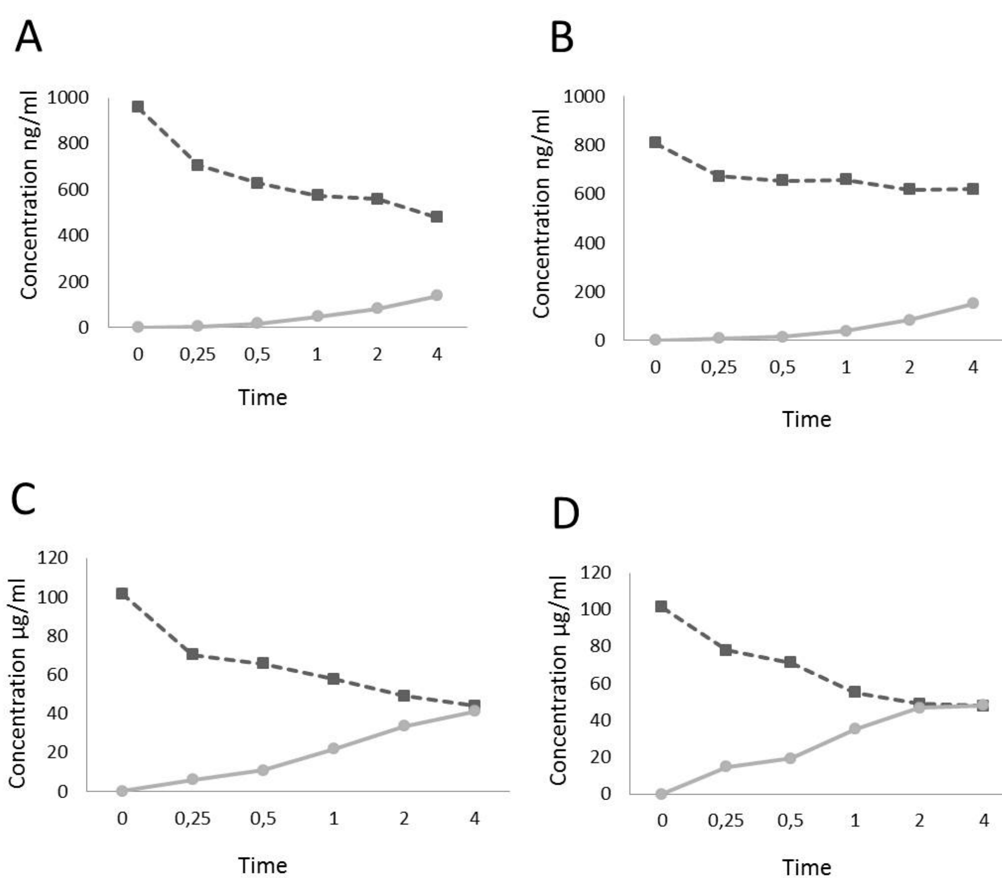

Figure 3. Placental transfer of PFOS, PFOA and reference compound antipyrine antipyrine. A) Placental transfer of PFOS. B) Placental transfer of PFOA C) Placental transfer of reference compound antipyrine in PFOS perfusion. D) Placental transfer of reference compound antipyrine in PFOA perfusion.

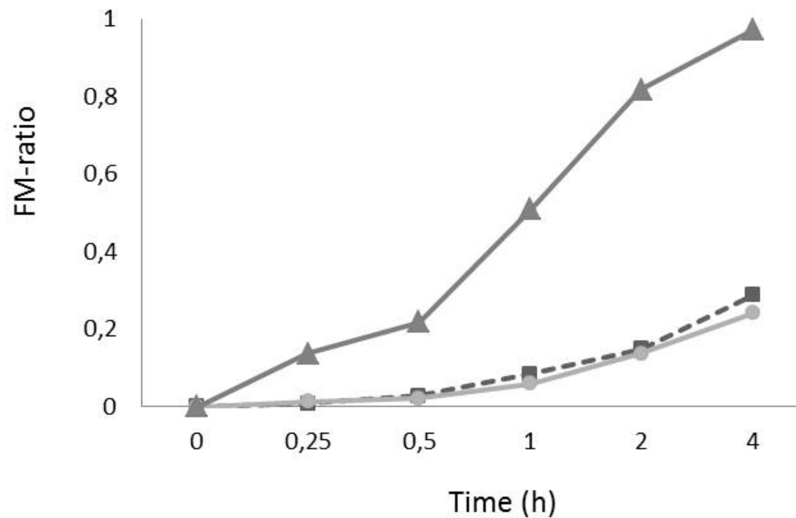

Figure 4. Transplacental transfer of PFOS (dotted line), PFOA (solid light gray line) and antipyrine (solid dark gray line).

6.3 PFOA/PFOS and transporter expression

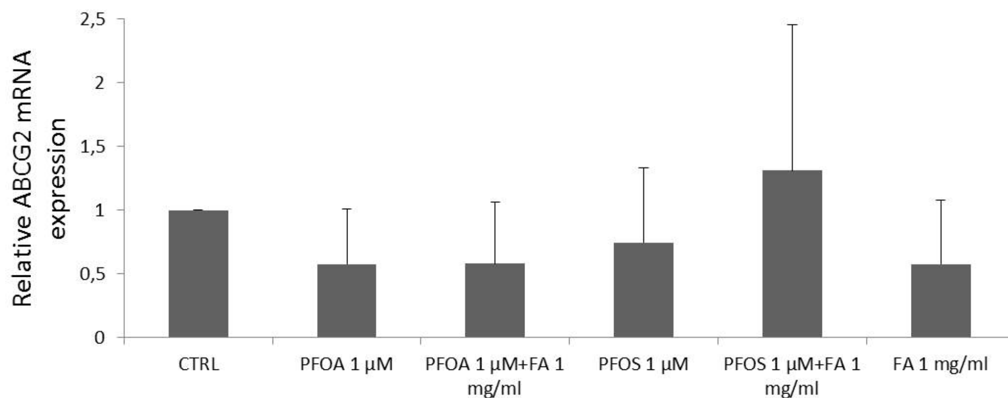

Figure 5. mRNA expression of ABCG2 after 48 hour expsosure to PFOA, PFOS and folic acid (FA). n=6 in each group, data are mean±SD. No statistically significant differences ($p>0.05$; Student's t-test)

PFOS and PFOA did not statistically significantly down-regulates mRNA expression of ABCG2 although there was a small trend after exposure to PFOA. Protein expression of ABCG2 was studied after 48 h exposure to PFOS or PFOA

using immunoblotting. Neither of the compounds affected protein expression of ABCG2 transporter (Figure 6).

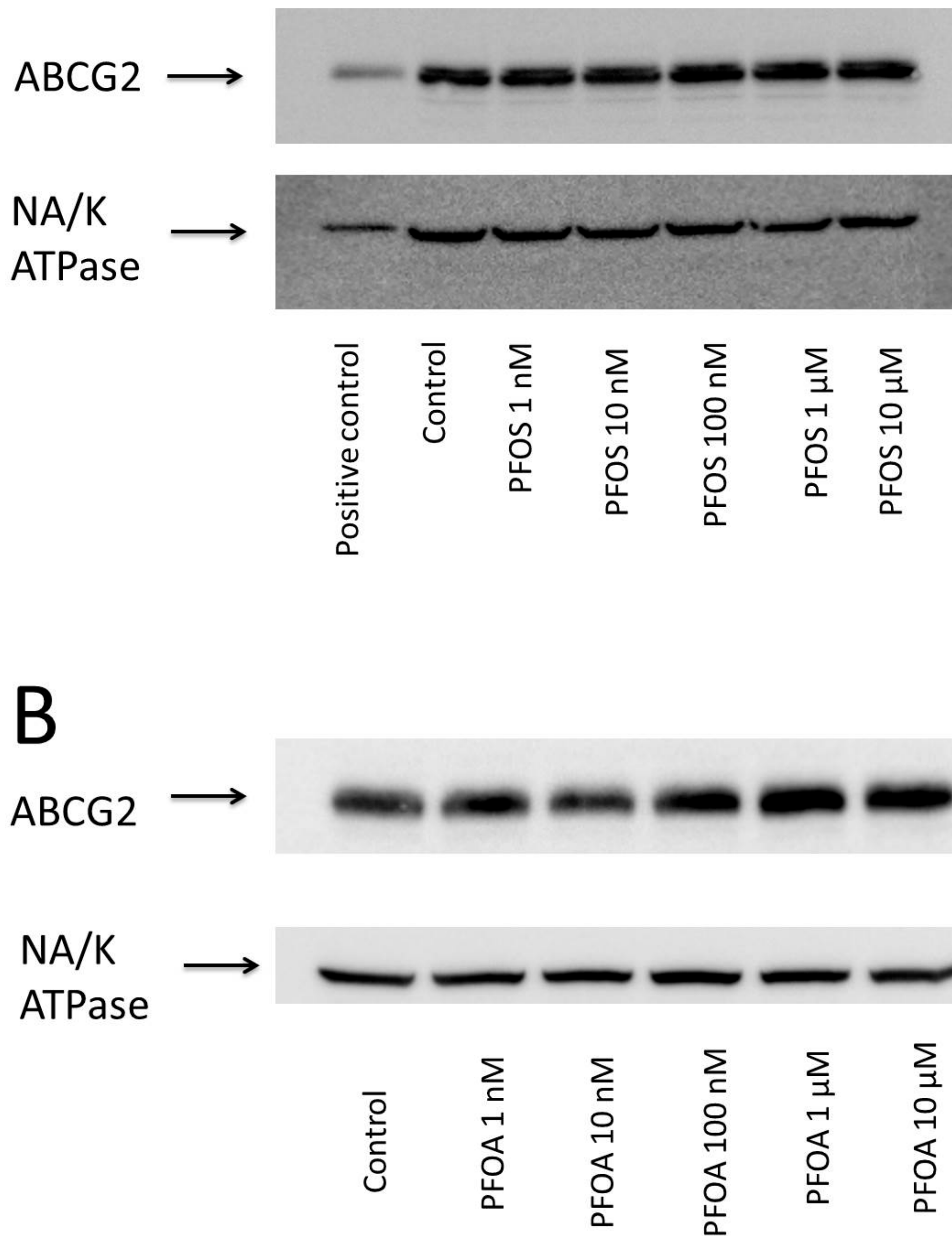

Figure 6. ABCG2 protein expression in BeWo cells after 48 hour exposure to 1 nM to 10 µM PFOS (A) or PFOA (B). A representative immunoblot is shown. Each well contained 20 µg/protein and Na/K APTase was detected as loading control. Membrane protein from MCF-7/MTX cells was used as a positive control.

7 DISCUSSION

According to Hutson et al. (2011) human placental perfusion is able to accurately predict in vivo transfer of chemicals after modeling. Transfer rate for PFOA and PFOS is relatively slow and FM- ratio stays below 1. At the end of perfusion, fetal concentrations of PFOS and PFOA are 25-30% of maternal concentrations which agrees quite well with biomonitoring data. In biomonitoring studies, the average PFOS concentrations in cord blood *in vivo* have usually been reported to be in between 30 and 60 % of the maternal concentrations (Monroy et al. 2008; Midasch et al. 2007; Inoue et al. 2004; Gutzkow et al. 2012). PFOA seems to be transferred across placenta in slightly higher rate during biomonitoring studies than suggested by single perfusion in this study. Compared to other contaminants, PFOS and PFOA are transferred slightly faster than TCDD but slower than PCB52 while, bisphenol A crosses placenta relatively faster (Mose et al. 2012).

Human placenta expresses transporter which interact with environmental chemicals**.** Depending on function and localization, these transporters may either increase or decrease transplacental transfer. Perfluornated compounds are known substrates for OAT-transporters (Nakagawa 2009). Placenta expresses OAT4 (Ugele 2003) and based on localization and function. OAT4 may restrict transplacental transfer of perfluorinated compounds and explain at least partially the low transfer rate observed in study.

ABC transporters are believed to have fetoprotective functions. Pharmacological inhibition of ABCB1 or ABCG2 may increase fetal exposure to pharmaceutical drugs (Lankas et al., 1998; Jonker et al., 2000; Mölsä et al., 2005). Recently, human placental ABC transporter polymorphisms have been shown to modify fetal exposure to methyl mercury (Llop et al. 2014)

Furthermore, several environmental compounds are known to affect placental transporters. For instance, cadmium inhibits ABCG2 (Kummu et al. 2012), aflatoxin down regulates ABCG2 while ABCC2 and OAT4 expressions were upregluated in mRNA level (Huuskonen et al. 2013), PFOS and PFOA inhibit the ABC transporters ABCG2, ABCC1, ABCC4 and ABCB1 in transporter-overexpressing membrane vesicles (Dankers et al. 2013).

However, even if it seems that PFOS and PFOA affect ABCG2 transporter function according to the results they do not alter ABCG2 protein expression.

8 CONCLUSIONS

Preliminary results presented in MSc thesis suggest that foetuses are exposed to PFOS and PFOA that was in line with biomonotoring studies. Furthermore, neither PFOS nor PFOA significantly affected ABCG2 transporter expression in BeWo cells. Further studies focusing on the significance of OAT4 transporter in fetal exposure to perfluornated compounds are needed. Only two perfusions were made and for conclusive results higher number of perfusion will be needed.

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