Introduction

Foetal growth and development is extremely dependent on placenta functionality, as the major role of the placenta is to exchange oxygen, nutrients, hormones and waste products between the mother and the foetus. In addition, the placenta has an important role in hormone synthesis and metabolism of compounds that regulate and maintain pregnancy (Ahokas and Anderson, 1987). The placenta develops from embryonic tissue and by the end of the third gestation week embryonic blood circulates through the capillaries of the villi (Khong and Pearce, 1987). The barrier function of the placenta membrane depends on molecular size, polarity, lipid solubility and protein binding of the substances. Both active transport mechanisms and facilitated transport have been described (Syme et al., 2004). Substances able to cross the placenta have the potential to cause adverse effects directly on foetal development or placenta function. Substances not able to cross the placenta may harm the foetus through effects on the mother, e.g. toxicity.

The first evidence of reproductive toxicity caused by a foetal exposure from maternal intake was the thalidomide disaster in 1957-1961. Pregnant women from approximately 46 countries worldwide were prescribed thalidomide as a safe sedative. Given between the 34th and 50th day of pregnancy, thalidomide may exert teratogenic effects seen as skeletal malformation especially of the limbs. These malformations were reported in more than 10,000 babies who survived the exposure. When investigated further, the mechanism of action was specific to humans, but was later confirmed in a second animal species. As a result of this disaster the demands on testing of drugs to be used during pregnancy were increased (Botting, 2002; Brent, 2004).

In the early 1970s it was reported that prenatal alcohol exposure can cause mental retardation, facial malformations, prenatal and/or postnatal growth retardation (Riley and McGee, 2005; West and Blake, 2005). In 1971 the effect of the synthetic non-steroidal estrogen diethylstilbestrol (DES) on the reproductive system became evident. The drug was prescribed to prevent mis-
carriage and other pregnancy complications but, as an unknown teratogenic effect, it also caused carcinomas in the vagina and cervix in young women and malformation of reproductive organs in girl and boy offspring (Palmlund, 1996; Swan, 2000). This led to a broader definition of the concept of reproductive effects, including not only functional and cognitive effects seen at birth, but also effects seen later in life caused by foetal exposure. It is now well established that maternal smoking (Brown et al., 1988; Habek et al., 2002; Philipp et al., 1984) and maternal exposure to methyl mercury (Hamada et al., 1997), lead (Banks et al., 1997) and environmental chemicals such as polychlorinated biphenyls (Schantz, 1996; Tilson et al., 1990) may cause adverse developmental defects in the offspring.

Tools for foetal risk assessments

Risk characterisation of foetal development from maternal exposure to chemicals and pharmaceuticals is most commonly based on results from animal studies, sometimes supported by in vitro studies or more seldom by epidemiological studies. The OECD test guidelines for reproductive toxicity testing include a prenatal developmental study (TG 414), one-generation study (TG 415), two-generation study (TG 416) and a reproduction/developmental toxicity screening test (TG 421 + TG422) (http://ecb.jrc.it/testing-methods/, 2005). These studies are designed to provide dose-response relationships concerning the toxic effect of prenatal exposure on the pregnant test animal, on the developing organism in the uterus, and the effect on the integrity and performance of the male and female reproductive systems after one or two exposed generations, see table 1. Fertility, growth, malformations, and survival are typical effect endpoints in the offspring after administration of very high doses to the pregnant dam. The studies are designed to induce toxicity but not mortality in the pregnant animals at the highest dose level. The observed foetal toxicity is either caused by a direct developmental effect or a maternally mediated effect. Analysis of the distribution of compounds in and between the maternal and foetal compartments is not required in all instances. However, applying the OECD guideline regarding toxicokinetics (B.36), where pregnant animals are sometimes requested, may provide such information (tab. 1).

In animal testing, the foetal exposure is not investigated in detail, as no confirmatory studies on the presence or distribution of compound in foetal tissue are required. An adverse effect in the foetus is considered an indication of foetal exposure. Human placental perfusion can provide information about transplacental transfer, placental metabolism, storage, acute toxicity and potential role of transporters, vascularisation and foetal exposure. It is beneficial to use human placenta tissue, as extrapolations from animal to human are bypassed. This is very important as the human placenta is unique in structure and only resembles placentas from certain monkey species like baboons (Enders and Blankenship, 1999). The human placenta is hemomonochorial where the foetal tissue is in direct contact with maternal blood and villous where the placenta is divided into small vascular

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N = number of pregnant animals per dosing and control group.
TG 422 = combined repeated dose toxicity study with the reproductive/developmental toxicity screening test.
units, i.e. cotyledons (Faber et al., 1992; Leiser and Kaufmann, 1994). The use of human tissue in placenta perfusion can help overcome the differences in kinetics, placenta structure, sensitivity, duration of gestation and background levels. It carries potential to replace or reduce the number of animals used for toxicological testing. Results from the human placenta perfusion test system may provide important knowledge on transplacental transfer of new chemical substances as well as on environmental exposures of hazardous compounds in humans.

The aim of this presentation is to describe the placenta perfusion method established at the University of Copenhagen to study the transplacental transfer and placental storage of environmental compounds. The system allows us to collect, and determine the concentration of compound in umbilical cord blood, maternal and paternal blood samples, providing useful samples for biomonitoring studies in families.

It is also possible to determine endpoints such as placental metabolism, placental transporters, and the presences of specific biomarkers in blood and placenta samples. Placental perfusion will be developed in the integrated projects ReProTect and NewGeneris. The data on placental transfer will be included in another ReProTect workpackage exploiting data for QSAR consideration (Hareng et al., 2005).

Placenta perfusion

The placenta perfusion system was first described and developed by Panigel and later modified by Schneider and other research groups to enable more systematic studies (Panigel, 1985; Schneider and Huch, 1985). Several research groups have subsequently used similar system to investigate the placental transfer and metabolism of different compounds, especially pharmaceutical drugs as sulindac, oxcarbazepine, lamotrigine and theophylline administered to the maternal compartment (Ala-Kokko et al., 1997; Lampela et al., 1999; Myllynen et al., 2001; Myllynen et al., 2003; Nanovskaya et al., 2002; Omarini et al., 1992; Pienimaki et al., 1995).

Materials and methods

Chemicals

Antipyrine 98% was purchased from Aldrich-Chemie (Steinheim, Germany), p-acetopenetidide 97% (phenacetin) from Acros organics (Geel, Belgium), dextran from Leuconostoc mesenteroides from Sigma-Aldrich (Steinheim, Germany), and heparin 5000 IE/ml from SAD (Copenhagen, Denmark). Potassium chloride, potassium dihydrogen phosphate, calcium chloride (CaCl₂·H₂O), magnesiumsulphate (MgSO₄·7H₂O) and sodium hydrogen carbonate were manufactured by Merck (Darmstadt, Germany). Sodium chloride was from J. K. Baker (Deventer, Holland) and hydrochloric acid 6N from Bie & Berntsen (Roedovre, Denmark). D(+) -Glucose monohydrate, phosphoric acid 85%, and methanol were purchased from Applichem (Biochemia, Darmstadt, Germany). All solvents were of gradient grade used for high-performance liquid chromatography.

Instruments

The maternal and foetal circulation is driven by two essential roller pumps (Watson Marlow SciQ 323E/D). Another roller pump (MasterFlex easy-load 3 model 77800-50) serves to retain buffer in the perfusion chamber. The perfusion chamber is handmade of Plexiglas material at the University of Copenhagen. Tygon R3603 tubing is used (Saint-Gobain). The lidded reservoirs are placed on common stirring devices. The tissue is homogenised by a Turax homogeniser (T25 basic, 8mm). An eight canal Powerlab System (ADInstruments, Oxfordshire, UK) is connected to two flow-thru oxygen electrodes (Microelectrodes, New Hampshire, USA), a pH meter, and a temperature amp from ADInstruments and an on-line computer software programme (see fig. 1).

Logistics

The placentas are obtained from births delivered at the Danish National Hospital by elective Caesarean section. The weekday before delivery, the Elective Section Team at the hospital, composed of midwife, obstetrician and anaesthesia nurse, meets with the pregnant woman. On occasion of this meeting, information about the placenta perfusion study is given and written material is handed out. The material contains a short summary of the study, a detailed declaration of informed consent, a questionnaire on maternal data and exposure, and a folder from the ethics committee about patient rights when participating in scientific experiments. Further information on the study is available on the homepage (www.pubhealth.ku.dk/placenta/). If the pregnant woman wants to participate, a signed declaration of informed consent is given and a questionnaire is filled out and returned before hospitalisation for Caesarean section. Coordi-

Fig. 1: Schematic presentation of the Placenta perfusion system
nation with the midwife makes it possible to collect placentas immediately after birth.

Placenta
Immediately after delivery the placenta is weighed. Umbilical blood is drawn into a VenoJect sodium heparin tube. 25 ml freshly prepared 37°C warm *Krebs-Ringer buffer (NaCl, 121 mM; NaHCO₃, 24.9 mM; KCl, 3.0 mM; KH₂PO₄, 0.4 mM; CaCl₂, 1.3 mM; MgSO₄, 0.58 mM) with added heparin (25,000 IU/l) and glucose (9 mmol/l), adjusted to pH 7.2-7.4 with HCl, is injected into umbilical arteries and vein. The placenta is carried to the laboratory in an insulated box. Upon arrival, the placenta is inspected and remarks on size, ruptures, infarcts, and condition in general are noted.

Placental perfusion method
The dual recirculation placenta perfusion system at the University of Copenhagen was, with modifications, adapted from the University of Oulu, Finland (Myllynen et al., 2003). Briefly, a foetal artery-vein pair supplying a well-defined cotyledon is cannulated using Luer stub adapters of size 20G and 18G, respectively. *Krebs-Ringer buffer is manually injected into the foetal circulation, the cotyledon is isolated and connected to the pump system to ensure that inflow equals outflow. A tissue sample from beside the removed cotyledon is taken, cut into small pieces, homogenised and stored at -20°C. The cotyledon is placed in the perfusion chamber with the maternal side up and two Luer stub adapters (size 18G) are gently inserted into the intervillous space 5-7 mm below the maternal surface. The perfusion chamber enables the cotyledon to be wetted in maternal perfusate.

The perfusion buffer in both the maternal and foetal reservoirs is *Krebs-Ringer buffer (200ml) with 8.5 g/l and 30 g/l dextran added, respectively. *Krebs-Ringer buffer is manually injected into the foetal circulation, the cotyledon is isolated and connected to the pump system to ensure that inflow equals outflow. A tissue sample from beside the removed cotyledon is taken, cut into small pieces, homogenised and stored at -20°C. The cotyledon is placed in the perfusion chamber with the maternal side up and two Luer stub adapters (size 18G) are gently inserted into the intervillous space 5-7 mm below the maternal surface. The perfusion chamber enables the cotyledon to be wetted in maternal perfusate with a minimum of evaporation due to the small entrances for tubing representing arteries and veins. The cotyledon is preperfused for 30 minutes to restore adequate oxygen to the tissue and to ensure stable foetal venous outflow. Antipyrine and compound is added on the maternal side and samples are drawn from both reservoirs after 0, 15, 30, 60, 90, 120, and 150 min. The volume collected is replaced with perfusion buffer.

The perfusion buffer in both the maternal and foetal reservoir is *Krebs-Ringer buffer (200ml) with 8.5 g/l and 30 g/l dextran added, respectively. The foetal perfusate is constantly gassed with 95% N₂/ 5% CO₂ and the maternal perfusate is gassed with 95% O₂/ 5% CO₂. The perfusion flow rate is 3.5 ml/min in the foetal circulation and 12 ml/min in the maternal circulation.

After the end of perfusion the cotyledon is weighed, cut into small pieces, homogenised, and stored at -20°C. The volume of perfusion liquid in the maternal and foetal chambers is measured. Samples are placed on ice and within 30 minutes the blood cells are precipitated by centrifugation at 4,000 x g for 5 minutes. The supernatant is stored at -20°C until analysis; 0.2 ml is needed for the antipyrine analysis.

Safety concerns
No information is a priori available about the health status of the pregnant woman and all tissues are considered potentially infected with e.g. hepatitis and HIV. Thus, laboratory personnel handling placentas are vaccinated with Hepatitis B and all procedures are performed with personal protection including gloves, glasses and laboratory coats.

Analysis of antipyrine
200 µl supernatant is added to 200 µl H₃PO₄ (0.5 M) with phenacetin added (10 µg/ml) as internal standard. Antipyrine and phenacetin are analysed using a reverse phase LaChrom HPLC system equipped with an L-7100 pump, an L7200 autosampler, an D-7000 interface, a L-7300 column oven and a L-7400 UV detector (Merck, Hitachi). The stationary phase is a C18 column (NUCLEOSIL C-18, ODS, 20 x 4.6 mm, 5 µm particles) with a SecurityGuard precolumn (Phenomenex C-18, ODS, 4 mm L x 3.0 mm ID). The mobile phase is a degassed methanol/water (45/55 v/v) solution adjusted to a flow rate of 1 ml/min. Injection volume is 25 µl, oven temperature is 30-32°C and absorbance is detected at 254 nm. A calibration graph of antipyrine (0, 0.5, 1.5, 10, 25 µg/ml) and phenacetin is constructed with *Krebs Ringer as matrix.

Ethics
Placenta perfusion studies cause minimal ethical problems, partly because the experiments are non-invasive – causing no harm to mother or child - and partly because placentas are normally discarded and incinerated after birth. The use of placenta and umbilical cord blood in scientific research requires a signed and informed consent from the mother. To obtain maternal and paternal blood, a similar declaration is required from both the mother and the father. Much time must be scheduled to inform participants properly and straightforwardly about their contribution to the study and the study in general.

Results
Antipyrine
Antipyrine crosses the placenta by passive diffusion; therefore it is a good functionality marker of the placental perfusion system. In figure 2, the transfer of antipyrine from the maternal to the foetal compartment is shown. Within 150 minutes, 40 µg/ml antipyrine diffused from maternal to foetal chamber and an equilibrium between maternal and foetal circulation was almost established. In figure 3, the feto-maternal ratio (FM) of the transplacental passage from the same experiment is shown. The FM ratio is the concentration ratio of antipyrine in foetal and maternal perfusate.

Controlling physiological experimental conditions
When perfusions comply the following demands, they are evaluated as successful:
- Foetal venous outflow stable within 7.0 ± 0.2 ml per 2 min.
- Volume loss from foetal circulation < 20 ml after end perfusion.
- The FM ratio of antipyrine transfer > 0.7 after 150 minute of perfusion.
- The transfer of oxygen from maternal to foetal circulation has to be sufficient pO₂ (foetal vein) >> pO₂ (foetal artery).
- Temperature in foetal perfusate 37 ± 2°C.
-pH in foetal perfusate within 7.2-7.4 ± 0.1.
-Time from birth of child to first cannulation < 30 minutes.

Conclusions

The human placenta perfusion system is a reliable and feasible supplement to the existing animal tests used for human foetal risk assessments, as this test system gives useful information on placental transfer, placental storage and metabolism in the placenta tissue, without the need for extrapolation to a different species. Results from human perfusion studies can improve the human foetal exposure assessment, providing important information on placental toxicokinetics. Such data are seldom included in reproductive toxicity studies in animals. The human placental perfusion system is a supplement to animal reproductive testing to be further developed within the integrated project ReProTect.

References


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