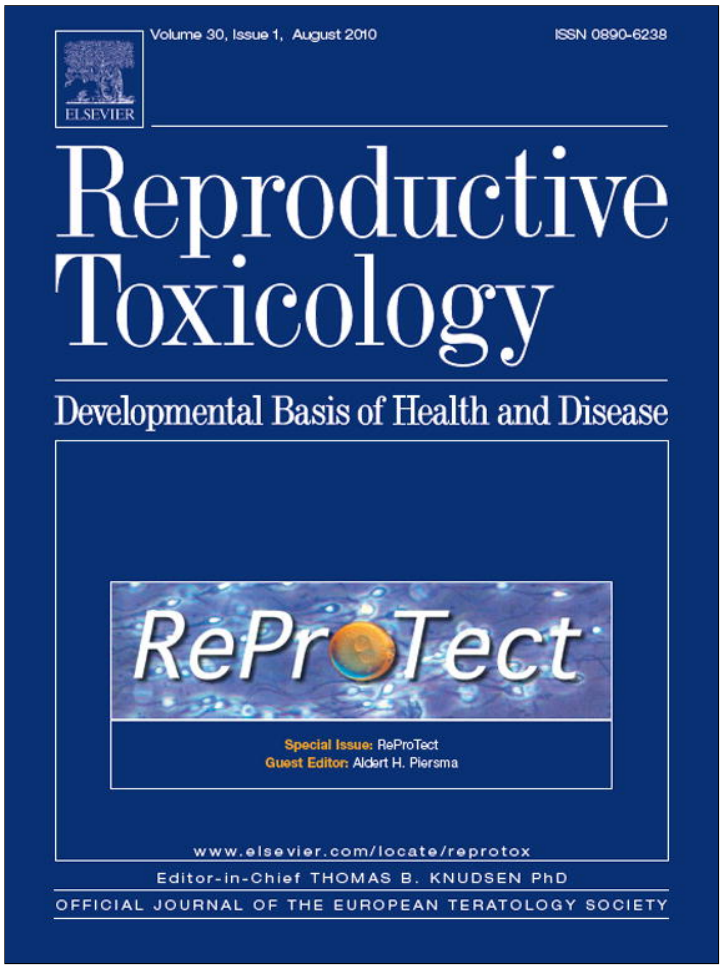

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Quality assessment of a placental perfusion protocol

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ABSTRACT

Validation of in vitro test systems using the modular approach with steps addressing reliability and relevance is an important aim when developing in vitro tests in e.g. reproductive toxicology. The *ex vivo* human placental perfusion system may be used for such validation, here presenting the placental perfusion model in Copenhagen including control substances. The positive control substance antipyrine shows no difference in transport regardless of perfusion media used or of terms of delivery ($n = 59$, $p < 0.05$). Negative control studies with FITC marked dextran correspond with leakage criteria (<3 ml h^{-1} from the fetal reservoir) when adding 2 ($n = 7$) and 20 mg ($n = 9$) FITC-dextran/100 ml fetal perfusion media. Success rate of the Copenhagen placental perfusions is provided in this study, including considerations and quality control parameters. Three checkpoints suggested to determine success rate revealed that 15% of the cannulated placentae received in one year ($n = 202$) were successfully perfused.

1. Introduction

Maintenance of normal fetal and placental development is critical for healthy pregnancy outcome. Endogenous and exogenous substances including chemicals and medicines may adversely interfere with fetal development. The fetus undergoes rapid growth and organ development, has incomplete metabolic capacities and is therefore very vulnerable to exogenous compounds. During pregnancy, the placenta separates fetal and maternal circulations, transporting nutrients but also potentially hazardous compounds. Fetal exposure assessment is therefore an important part of fetal risk assessment, which has become increasingly relevant as epidemiological data are linking adverse health outcomes with fetal exposures. Animal models are available; however, large interspecies differences in placental morphology and gestation make it difficult to extrapolate results from animals to humans [1]. Human data regarding fetal exposure are only available from terminated pregnancies or limited clinical studies performed with drugs on diagnosed pregnant women just before birth.

The *ex vivo* human placental perfusion of an isolated cotyledon is an experimental model used to investigate human placental transfer and placental metabolism. Using exogenous compounds important information for the assessment of fetal exposure can be obtained. As human placentae are used after delivery, the studies impose no risk on mother or child and pose only a limited number of ethical dilemmas. All personnel in contact with the placenta and samples are vaccinated against tetanus and hepatitis A and B to eliminate some infection risks in handling human material. The study model allows collection of samples from fetal, maternal and placental compartments contributing new information obtained from structurally intact human tissue. The placenta develops throughout pregnancy, and the human term placenta has the shortest distance between fetal and maternal blood vessels compared to earlier stages of pregnancy. Transport of potentially hazardous compounds across the term placenta is thus considered a worst case scenario with respect to fetal exposure [2].

Abbreviations: BSA, bovine serum albumin; DON, deoxynivalenol; FITC-dextran, fluorescein isothiocyanate labeled dextran; FM ratio, fetomaternal concentration ratio, the fetal concentration at a time point divided by the maternal concentration at the same time point; HE, hematoxylin; HSA, human serum albumin; IQ, 2-amino-3-methylimidazo[4.5-f]quinoline; PBDE, poly brominated diphenyl ethers; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; RBC, red blood cells; TCDD, 2,3,7,8-tetrachlorodibenzodioxin.

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This methodological paper describes the *ex vivo* placental perfusion system established in our laboratory in Copenhagen. The effect caused by different perfusion media on the transfer of the positive control substance antipyrine was investigated, and also the possible difference in transport through placentae from vaginal births versus caesarean sections. The transfer from fetal to maternal reservoir of the negative control substance FITC-dextran added to the fetal perfusion medium was compared in successful versus leaky perfusions. Success rates are presented for placental perfusions of different duration performed in Copenhagen during the last years with a description of the development of the method since it was first established in 2004. Technical improvements to ensure the viability of the placental lobule and collection of data to compare with the in vivo situation are described.

The *ex vivo* human placental perfusion model is an alternative to animal studies which may potentially reduce and replace animal testing. In the research program ReProTect addressing reliability and relevance in developing in vitro tests in reproductive toxicology, validation of in vitro test systems in accordance with the modular approach is an important goal [3]. The Work Package 2 of ReProTect covers the implantation area and within this work package, the *ex vivo* placental perfusion model may be used for validation in Modules 1, 2, 3 and 4 covering the test definition, within-laboratory variability, transferability, and between-laboratory variability, respectively [4].

2. Materials and methods

2.1. Placentae

Only placentae from uncomplicated pregnancies and births are used in the perfusion model. We obtain the placentae immediately after vaginal birth or elective caesarean section at Copenhagen University Hospital, Denmark. Informed written consent is obtained from the mother. The project is approved by the Ethical Committees in the Municipalities of Copenhagen and Frederiksberg (KF 01-145/03 + KF(11) 260063) and the Danish Data Protection Agency.

2.2. Dually perfused placenta method and sample analysis

2.2.1. Setup

The *ex vivo* placental perfusion system was first described and developed by Panigel and later modified and refined by Schneider and other research groups [5,6]. The perfusion model developed in Copenhagen has been described previously [1,7,8], and is adapted from the models described by Schneider and Huch [9] and by Myllynen et al. [10]. The model is described here in as much detail as needed to present the quality and success criteria reported later.

The perfusion equipment consists of a perfusion chamber (manufactured at the University of Copenhagen), two peristaltic roller pumps, two magnetic stirring devices, tubing, plastic sondes, fittings, and a maternal and fetal reservoir as seen in Fig. 1. A vascular unit of the placenta is perfused by simultaneous perfusion of the fetal and maternal circulation of one villous tree. The fetal flow is established by cannulation (Flocare Pur Sondes-MP, Ch 5/50 + Ch 6/60) of a chorionic artery and vein pair supplying one cotyledon with Krebs Ringer. If there is no leakage, the cotyledon is cut from the placenta and gently set in the perfusion chamber, which is moved to a heated (37 °C) flowbench (Holm-Halby, Denmark). The maternal circulation is established by inserting two blunt cannulae in the intervillous space. Maternal venous outflow is led back into the maternal reservoir through a connecting tube. If fetal vessels are still intact, the Krebs Ringer media is replaced by perfusion buffer and recirculated.

2.2.2. Media

The fetal and maternal perfusion media (each 100 ml) consists of Krebs Ringer supplemented with glucose and heparin in short perfusions of 2.5 h or cell culture medium (RPMI 1640, Panum Institute, University of Copenhagen) supplemented with 1 ml L-glutamine (200 mM), penicillin and streptomycin (1%) when perfusing for 4 h or more. Tissue preparation is always performed with supplemented Krebs Ringer. The Krebs Ringer buffer is bubbled with 95% O_2/5% CO_2 for about 15 min until the pH is in the range: 7.2–7.4, after which heparin (5000 IU/ml, Copenhagen University Hospital Pharmacy, 5 ml/l medium) and glucose (2.61 g/l) are added. To aid dissolution of lipophilic study compounds in the perfusion media, physiological levels of human serum albumin (HSA) (fetal: 40 mg/ml and maternal: 30 mg/ml HSA (20% solution, product # 109697, CSL Behring Gmbh, dialysed in Krebs Ringer)) are added. As the pregnant woman has a lower blood concentration of HSA compared to the fetal level, the maternal reservoir contains less HSA. The higher concentration of HSA in the fetal medium further mimics the higher viscosity (hematocrit) of fetal blood and reduces loss of fluid from the fetal reservoir due to higher pressure in the closed fetal circuit. The higher viscosity in the perfusion medium is otherwise created by the addition of dextran (Sigma–Aldrich) (fetal: 3.0 g/100 ml, maternal: 0.85 g/100 ml. The volume of media is chosen in relation to the need to minimize use of test substance and to imitate realistic exposures, providing sufficient fluid to supply the placenta with nutrients and keeping an even flow through pumps and tubing.

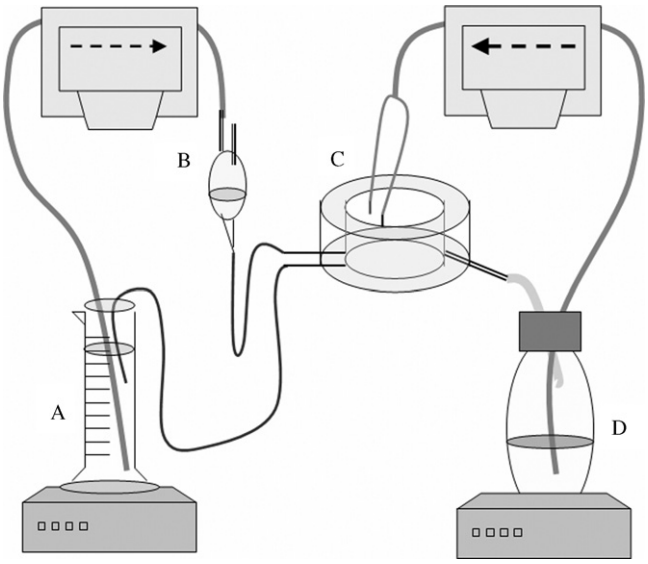

Fig. 1. The placental perfusion setup in Copenhagen. The fetal reservoir (A) is on the left hand side and the maternal (D) on the right hand side and placed on magnetic stirring devices. The fetal inflow tubing contains a bubble trap (B). Above are the peristaltic pumps and in the middle is the perfusion chamber (C) depicted without the placenta lobule. The whole system is located in a heated flow bench which keeps it at 37 °C.

2.2.3. Washing procedure and system adherence test

Since some test substances are known to adhere to red blood cells, the amount of red blood cells is reduced by a washing procedure prior to initiation of the recirculation of the maternal perfusion fluid. The placental lobule is bathed in a container with Krebs Ringer buffer and gently massaged while still attached to the infusion bag. After connection to the perfusion pump, the first 100 ml of maternal venous out flow is discarded. The fetal system is rinsed thoroughly during cannulation. To investigate the concentration of RBC in the media after performing the washing procedure, hemoglobin was measured on samples from a perfusion after performing the washing procedure.

System adherence tests, which are perfusions without a cotyledon, perfusing only the maternal circulation and the perfusion chamber, were introduced as a part of the standard procedure before initiating perfusions with new unlabeled study compounds. Perfusion fluid with antipyrine, study compound and 3 ml umbilical cord blood is recirculated and sampled at planned time points to investigate the adherence of the study compound.

2.2.4. Flow and oxygenation

During the perfusion, the perfusion media is circulated by peristaltic pumps to obtain a flow of 3 and 9 ml/min in fetal and maternal circulations, respectively. The fetal solution is gassed with 95% N_2/5% CO_2, and the maternal solution is gassed with 95% O_2/5% CO_2 throughout the perfusion. Prior to perfusion, a preperfusion period is performed to establish transfer of O_2 from maternal to fetal perfusion fluid. If oxygen transfer as assessed by measurement of higher pO_2 in fetal outflow than fetal inflow is not detected, the maternal cannulae are replaced to a position that provide O_2 transfer before initiating the perfusion. In preperfusion the pO_2 reaches the desired levels (10–15 kPa in fetal and 25–35 kPa in maternal solution), and a minimum of gassing is required to maintain these levels throughout the perfusion. Consequently, foaming caused by gassing the medium containing HSA is not a critical issue.

2.2.5. Control substances

Antipyrine (Aldrich-Chemie, Steinheim, Germany) is included as a positive control of transport across the placental membranes and is added to the maternal reservoir at a final concentration of 100 μg/ml. Antipyrine is a small hydrophilic molecule which diffuses passively across the placenta in a flow dependent manner. The fetal/maternal concentration ratio (FM) of antipyrine at individual time points after initiation of the perfusion is applied to test the overlap between maternal and fetal circulation within the perfused cotyledon and to compare perfusions using

different flow rates. The FM ratio of antipyrine must be greater than 0.75 within 2.5 h perfusion in order to consider the perfusion as successful. FITC-dextran with an average molecular weight of 40 kDa (Sigma–Aldrich, St. Louis, MO) is included as a negative control to verify the integrity of the fetal capillary. FITC-dextran is added to the fetal reservoir at a final concentration of 200 μg/ml.

2.2.6. Perfusion conditions

Every 30 min, the O_2 tension, the pH, and the glucose and lactate concentrations are measured by an ABL715 blood gas analyzer (Radiometer, Denmark) in the maternal and fetal perfusion media and in the fetal venous outflow. pH adjustments are made using 1 M HCl or 1 M NaOH when pH deviate from 7.2 to 7.4. The inflow of gasses is increased or decreased when pO_2 levels deviate from the desired levels. Initially glucose levels are 10 mmol/l in both Krebs Ringer and cell culture media. Glucose levels are kept above 5 mmol/l by adding 100 μl glucose solution (2.61 g/10 ml H_2O) with intervals defined by the control measurements.

2.2.7. Sampling

The first samples are collected before adding test substances. Hereafter samples are collected after adding test and control substances (time point: 0 min), at 2 min after adding test substance (perfusion start) and at ten further time points depending on perfusion length. Different sample volume, sample frequency or tissue extraction methods may be applied depending on the physicochemical properties of the test substances and the analytical method needed to quantify the compound.

2.2.7.1. Dissolution of lipophilic substances. To dissolve test substances that are lipophilic or in other ways insoluble in water in the maternal perfusion media prior to initiation of the perfusion study, the test substance and the control substances are mixed in approximately 30 ml of the final perfusion media (maternal outflow collected during preperfusion) for at least 20 min. When returned into the remaining maternal media the initial time point sample (0 min) is taken before the onset of the perfusion. The negative control substance is added to the fetal reservoir at the initial time point and a sample is taken out immediately.

2.2.7.2. Sample preparation. The samples are centrifuged for 5 min at 4000 × *g*, and the supernatant is transferred to a vial. In case of perfusing a radioactively labeled test substance 2 ml of Ecoscint (XR LS-372) scintillation liquid (BN instruments A/S) is added to 200 μl of supernatant, and another 200 μl of supernatant is stored at −20 °C until antipyrine detection by HPLC. Substance binding to the red blood cells is studied in pellets from maternal and fetal samples collected in separate vials after centrifugation. The pellets are then centrifuged again, and the supernatant is replaced with 200 μl fresh media in which the pellet is resuspended and 2 ml scintillation liquid is added.

2.2.7.3. Tissue samples. Tissue samples (1 g) are taken from the placenta before the perfusion, and from an area within the cotyledon (clearly perfused white area) and an unperfused area surrounding the cotyledon (still red) after the end of perfusion. All tissue samples are taken within the central part of the perfused cotyledon to avoid tissue in contact with the surface area.

These samples are cut into small pieces and extracted in 3 ml of acetonitrile (J.T. Baker) for at least 72 h. After the extraction, the tissue is removed, and 4 ml of scintillation liquid is added before scintillation counting. The remaining placental tissue (unperfused tissue, perfused cotyledon, and surrounding tissue) is fixed in 4% formalin. As a further success criterion, the perfused tissue can be examined histologically for perfusion injuries. By use of different staining techniques the exact location of the adhering or accumulating test substance can be visualized.

2.2.8. Analysis

Radioactivity is detected by liquid scintillation (Liquid Scintillation Analyzer, TRI-CARB 2300TR, Packard), and a calibration curve is included in each test round. Antipyrine is detected on a LaChrom HPLC system equipped with a C-18 column and a SecurityGuard precolumn as described elsewhere [8,11]. FITC-dextran is detected by a POLARstar Galaxy fluorescence reader (BMG Labtechnologies, Offenburg, Germany). Briefly, samples are diluted in PBS + 1% BSA, added to black MaxiSorp microtiter plates (NUNC, Roskilde, Denmark), and fluorescence is measured using excitation filter 495-10 and emission filter 520-p. Gain adjustment is performed on the standard with the highest concentration of FITC-dextran. Hemoglobin content is measured on a spectrometer (Unicam UV 300 spectrometer, Cambridge, UK) diluted 1:1 in phosphate buffer, analyzed by absorbance measurements at scan speed 240 nm/min, at the Department of Clinical Biochemistry, Copenhagen University Hospital.

2.3. Histopathology

Immediately after each study, representative samples of placental tissue from the perfused areas and the remaining parts of the non-perfused placenta are fixed in 4% formalin. The placental tissue is examined grossly and representative samples from umbilical cord, membranes, non-perfused and perfused areas are submitted and embedded in paraffin. The paraffin embedded tissue is sectioned by use of a microtome to 7 μm slices. These are placed on routine glass slides and colored with eosine and hematoxylin (HE). Two identical sets of HE slides are prepared, and the morphology is evaluated by two different pathologists, both specialized and experienced in the field of placental and perinatal pathology. The non-perfused and perfused areas are evaluated for any pathological process by use of a scoring system developed for this use based upon basic principles in the updated literature on placental pathology [12,13]. The perfused areas are also evaluated for signs of successful perfusion and tissue damage.

2.4. Calculations

2.4.1. Fetal/maternal concentration ratio and permeability rate

The FM ratio enables comparison between perfusions and between studies using different placentae and test-substance concentrations. An indicative measure of the permeability coefficient is estimated from the initial linear part of the relationship between perfusion time and the FM ratio. The indicative permeability rate represents the initial rate of transfer from maternal to fetal circulation, whereas the FM ratio illustrates the final level of exchange at the end of perfusion.

2.4.2. Statistics

The FM ratios are compared at each time point by a two tailed student *t*-test, and differences are deemed statistically significant if $p < 0.05$.

All data are presented as the mean ± SD, unless otherwise stated.

2.4.3. Mass balance after end of perfusion

The mass balance of the added test substance is calculated by pooling the recovered test substance (counts or mass) in all the known reservoirs and samples at the end of perfusion and comparing this to the known added amount of test substance [8].

In the recovery equation below, C_M = counts in the maternal compartment at the end of perfusion, V_M = volume of the maternal compartment at the end of perfusion, V_A = volume analyzed in the scintillation counter, C_F = counts in the fetal compartment at the end of perfusion, V_F = volume of the fetal compartment at the end of perfusion, C_j = counts in each sample removed during the perfusion from both maternal and fetal compartments for samples 1 through *n*, V_j = volume of each sample removed during the perfusion, C_C = counts from the cotyledon sample, M_{CT} = total mass of the perfused cotyledon, M_{CA} = mass of the cotyledon sample analyzed in the scintillation counter, C_T = counts in the surrounding tissue sample, M_{TT} = total mass of the surrounding tissue, M_{TA} = mass of the sample of the surrounding tissue analyzed in the scintillation counter, C_I = initial counts in the maternal compartment after adding the test substance, and V_I = initial volume of the maternal compartment.

$$\text{Recovery} = \frac{\left(C_M \cdot V_M/V_A + C_F \cdot V_F/V_A + \sum_{j=1}^{n} C_j \cdot V_j/V_A + C_C \cdot M_{CT}/M_{CA} + C_T \cdot M_{TT}/M_{TA}\right)}{C_I \cdot V_I/V_A} \times 100\%$$

2.5. Protocol development – historical considerations

Human placental perfusions are now performed by several research groups using very similar systems. However, perfusion systems can vary in terms of cannulation method, isolation procedure, perfusion fluid, oxygenation, perfusion flow, and control measurements. Table 1 summarizes the differences in the perfusion setups used by four research groups in this field [8,14–16].

Our setup has been developed and refined to ease the experimental procedures and reduce the number of technical failures. The perfusion chamber has been developed in four steps from a technically advanced chamber with many smaller parts to a more simple and easy to handle chamber (Fig. 2), and the cannulation method was changed from using short stump cannulae to long slim neonatal feeding tubes.

Initially, antipyrine kinetics alone was used as a control measurement. A Powerlap ADInstruments capable of measuring and monitoring signals from six channels was then purchased to monitor pH, temperature, pressure in fetal circulation, and oxygen pressure in fetal vein, fetal artery, and maternal reservoir. Studies with phthalate monoesters [17,18] and phytoestrogens were performed with the Powerlap surveillance system. However, the oxygen microelectrodes were fragile and required frequent and time consuming calibrations, and the instrument was therefore replaced by an ABL5 blood and gas analyzer capable of measuring pH, pO_2 and pCO_2. Studies with pesticides [11]; the poly aromatic hydrocarbon: benzo(a)pyrene [8], nitrosodimethylamine (NDMA), mentioned in Annola et al. [19] and the heterocyclic amines: PhIP and IQ were performed with these control measurements. At present an ABL715 blood and gas analyzer capable of measuring pH, pO_2, pCO_2, glucose and lactate is in use [8]. Studies with bisphenol A, DON, brominated flame retardants PBDE 47, PBDE 99 and PBDE 209, and PCB 52 and PCB 180 have been performed using the ABL715 control apparatus, and henceforth glucose and lactate measurements during the perfusion are available.

The recirculation system has also been improved over time. The silicone tubing system initially used was replaced by an inert Tygon PVC-free tubing system. Previously, simple lidded glass flasks were used as both fetal and maternal reservoir. A glass flask with aperture screw cap and a silicone membrane lid is now used as the

Table 1
Differences in setup and quality criteria between different groups using the human *ex vivo* placenta perfusion model.

Parameter	Laboratory of Schneider [7]	Laboratory of Miller [8]	Laboratory of Vähäkangas [9]	Laboratory of Knudsen [10]
Cannulation	Fetal: metal cannulae fastened with ligatures, maternal: 4–5 blunt metal cannulae.	Fetal: umbilical catheters, maternal: 2 catheters inserted on maternal side.	Fetal: neonatal feeding tubes fastened using the amnion, maternal: 2 blunt cannulae.	Fetal: neonatal feeding tubes sutured to the tissue, maternal: 2 blunt cannulae.
Isolation procedure and perfusion chamber	Clamping, 8 cm diameter	Clamping	Clamping	Cutting, 9 cm diameter
Perfusion fluid	Blood free buffer based on Earle solution added dextran (10 g/l), HSA (2 g/l), glucose (2 g/l), heparin (2.5 IU/l). Addition of tissue culture medium (NCTC-135) for long term perfusions.	M199 tissue culture medium added dextran (fetal: 30 g/l, maternal: 7.5 g/l), glucose (2 g/l), heparin (25 IU/ml) and gentamycin and Bactrim (50 mg/l)	RPMI 1640 cell culture medium with dextran (2 g/l), BSA (2 g/l), heparin (25 IU/ml), sodium pyruvate (1 mM), non-essential amino acid solution (10 ml/l), penicillin–streptomycin (25 U/ml) and L-glutamine (8 nM)	Krebs Ringer added glucose, 1 mL Pen-Strep, 25 IU/mL, 30 g/L HSA in maternal perfusate and 40 g/L HSA in fetal. For long term perfusions: RPMI 1640 cell culture medium added 58.4 mg L-glutamine
Oxygenation	Maternal: 95% O_2/5% CO_2 or atmospheric air, fetal: 95% N_2/5% CO_2 oxygenators	Maternal: 95% O_2/5%CO_2, fetal: 95% N_2/5% CO_2 oxidizing method unknown	Maternal: 95% O_2/5% CO_2, fetal: 95% N_2/5% CO_2 oxygenators	Maternal: 95% O_2/5% CO_2, fetal: 95% N_2/5% CO_2 bubbled directly into media.
Oxygen tension 1 mmHg = 0.1 3 kPa	Not stated	Fetal: 3–9–7.8 kPa	Maternal: 20–30 kPa, fetal: 10–15 kPa	Maternal: 20–30 kPa, fetal: 10–15 kPa
Heating	Warm water circuit surrounding chamber and reservoirs.	Warm water bath surrounding perfusion chamber.	Warm water circuit surrounding chamber and some tubes.	Setup in heated flow bench.
Fetal pressure measurements	20–30 mmHg	<70 mmHg	70 mmHg	None
Perfusion flow	Maternal: 12–20 ml/min, fetal: 6–8 ml/min			Maternal: 9–12 ml/min, fetal: 3–3.5 ml/min
Metabolic measurements	Glucose and oxygen consumption, lactate production, protein synthesis (hPl, SP-1, hCG and PAPP-A)	Human chorionic gonadotropin (hCG), lactate, glucose, oxygen consumption	Glucose consumption	Glucose and oxygen consumption, lactate production
Quality control of perfusion	Leak of fetal media (<4 ml/h), antipyrine, pressure in fetal circuit	Leak of fetal media (<2 ml/h), pressure in fetal circuit	Leak of fetal media (<3 ml/h), antipyrine, pressure in fetal circuit	Leak of fetal media (<3 ml/h), antipyrine (FM > 0.75), stable fetal flow, oxygen transfer, pH (7.2–7.4).

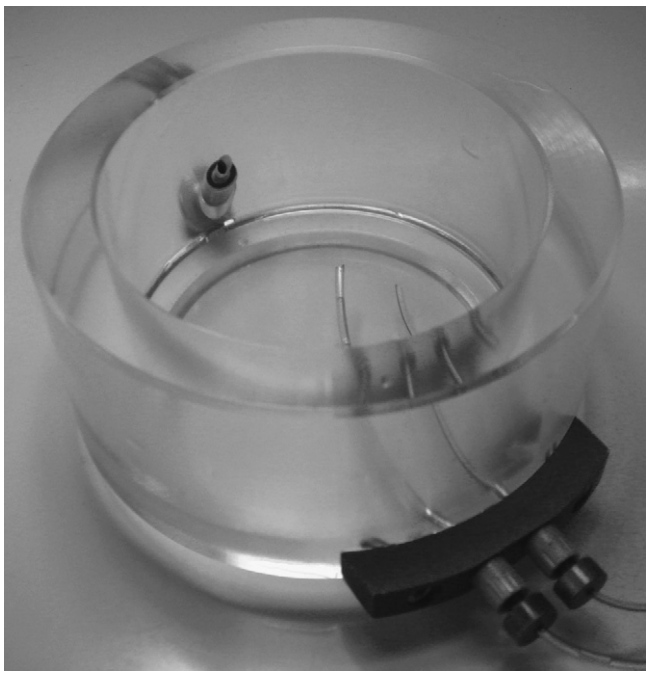

Fig. 2. Perfusion chamber used by Knudsen et al. [3]. The finally applied chamber with a solid surface and a gravity exit of maternal perfusion media in the wall of the perfusion chamber.

maternal reservoir, and a measuring cylinder is used as the fetal reservoir making it possible to monitor the fetal volume loss during perfusion. Further, a bubble trap has been introduced in the fetal circulation to eliminate the risk of transferring air into the fetal circulation.

Adhesion tests and background studies were introduced when working with phthalate monoesters, as phthalates can adhere to laboratory utensils or liberate from PVC in laboratory equipment. The system adherence test performed without placental tissue in the system can be used to quantify the amount of circulating test substance during perfusions. In perfusions with substances prone to adhere to the system, the system adherence test can reveal a loss of circulating substance, often with the greatest loss within the first few minutes of perfusion. An example of this is seen in Fig. 3, showing the adherence of bisphenol A. Often substances adhere or accumulate in the placental tissue. This is revealed by a loss of substance from the maternal circulation without finding the substance transported through to the fetal side. Sometimes extraction of tissue samples shows that the test substance is located in the tissue, and histological examination may show the exact location of test substance in the tissue. When testing environmentally occurring substances that are unlabeled, a gain in substance can be found due to accumulation in tissue during pregnancy. Our studies with blank perfusions with no added substances showed the liberation of phthalates from the placental tissue when perfused [18].

2.6. Success rate

At the recent International Placenta Perfusion Meeting in Kuopio, Finland (June 2009), the participants, including professors Kirsi Vähäkangas and Henning Schneider, agreed to report the success rate in new publications (Vähäkangas et al. personal communication) as the amount of placentae (%) having passed one of the three checkpoints in relation to the number of placentae cannulated:

- Checkpoint 1: cannulation and decision to proceed by isolating the cotyledon.
- Checkpoint 2: preperfusion with oxygen transfer and no leak of fetal perfusion media.
- Checkpoint 3: successful perfusion by the success criteria stated; antipyrine FM ratio above 0.75 and volume loss from fetal reservoir no more than 3 ml/h.

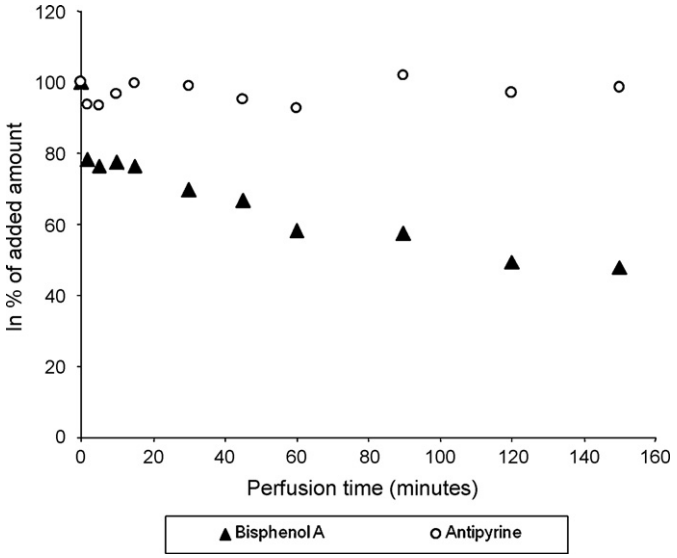

Fig. 3. Adhesion test of the test substance bisphenol A and the positive control substance antipyrine. Bisphenol A adheres to the system indicated by a rapid drop within the first 2 min perfusion, and a continued drop in concentration throughout the perfusion. Antipyrine remains at the level of added substance as it does not adhere to the system.

3. Supply of placentae

The Obstetric Clinic at The Copenhagen University Hospital has around 4000 births a year. Approximately 25% of the births are caesarean sections of which half are performed as planned elective caesarean sections. Every Wednesday 3–6 pregnant women with uncomplicated pregnancies are giving birth by planned elective caesarean section. Due to planning and the easy access to placental tissue within working hours, this is our preferred placental perfusion day. The day before elective caesarean sections, the pregnant women are gathered at the hospital at an information meeting, where we inform them about the placental perfusion study and obtain the informed signed consents. Placentae from planned elective caesarean sections are generally available faster compared to placentae from vaginal births, and the placentae have not undergone the contractions and trauma during labor. The form of delivery and its effect on the placental tissue is debatable. However, we have experienced that placentae from caesarean sections are more often damaged by the manual removal of the placenta from the deciduas, resulting in unsuccessful cannulation due to leakage.

The placentae are usually available 10–15 min after the birth of the child, in extreme cases the placenta is received after a longer time period. The umbilical cord blood is collected (for background levels in studies with unlabeled study compounds), supplemented Krebs Ringer buffer injected into fetal arteries, and the placenta transported to the laboratory. Within 30–45 min from birth of the child, the fetal circulation is re-established. Umbilical cord blood is spun down immediately.

4. Results

4.1. Positive control

Antipyrine data from 59 perfusions with 13 different substances (bisphenol A, dimethoat, miconazol, glyphosat, methiocarb, tebuconazol, benzoic acid, caffeine, benzo(α)pyrene, TCDD, daidzein, biochanin A and genestein) were collected. The FM ratios of the perfusions from placentae delivered by caesarean sections and vaginal births were compared (Fig. 4). No significant difference was found between the two groups. In eight of the perfusions (bisphenol A and benzo(α)pyrene) the media contained physiological levels of albumin. No significant difference in FM ratio with and without physiological levels of albumin in the perfusion media was found. Similarly no difference was found in antipyrine transfer between perfusions using Krebs Ringer buffer and cell culture media as perfusion solution, respectively. These results indicate that the transplacental transport of antipyrine is not affected by the type of delivery or perfusion media used, and is an appropriate substance to use as positive reference of contact between fetal and maternal perfused tissue.

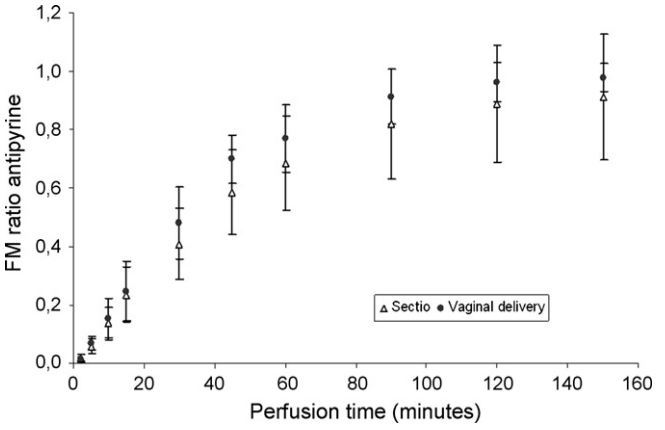

Fig. 4. The FM ratio of antipyrine in a total of 60 perfusions. The graph compares the FM ratio (mean ± SD) of antipyrine in placentae from vaginal deliveries ($n = 20$) and cesarean sections ($n = 40$).

4.2. Negative control

Perfusions were performed with 2 mg and 20 mg FITC-dextran added to the maternal or the fetal reservoir, and transport was analyzed in both leaky and successful perfusions. Our aim was to find a more sensitive leakage marker than fetal reservoir volume loss and to validate the cut off value of <3 ml/h loss from the fetal reservoir in a successful perfusion.

Results from 7 perfusions containing 2 mg FITC-dextran/100 ml fetal medium and 4 perfusions containing 20 mg FITC-dextran/100 ml fetal medium showed that successful perfusions with total volume losses <3 ml/h had FITC-dextran levels in the same range as autofluorescence (<3%) in maternal reservoirs. Unsuccessful perfusions with fetal volume loss >3 ml/h had FITC-dextran levels in maternal medium >20% of the concentration initially present in fetal chamber after 6 h of perfusion. The correlation between the FITC-dextran concentrations in maternal perfusion media and the perfusion time up to 6 h of perfusion is illustrated in Fig. 5. The fluorescence in maternal media after successful perfusions equaled autofluorescence. Autofluorescence was high compared to the maximum fluorescence in fetal perfusion media from the beginning, possibly hiding early leakage (autofluorescence not shown in the figure).

When adding FITC-dextran to the maternal media, no FITC-dextran could be detected in the fetal reservoir during the perfusion regardless of the success of the perfusion or leakage of fetal media. This indicates a bulk flow in leaky perfusions going only in one direction from fetal to maternal reservoir.

4.3. Perfusion conditions

Table 2 shows perfusion conditions from our most recent perfusions lasting 2.5 and 4 h. These parameters are reported to ensure stable and uniform conditions throughout perfusion and between different perfusion experiments. The time of the birth of the baby

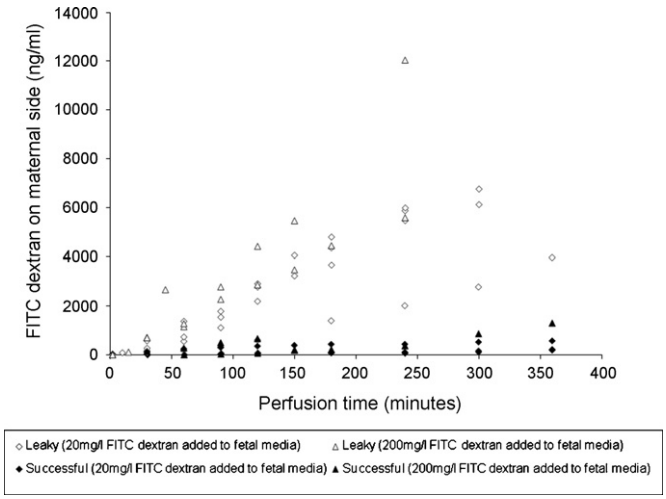

Fig. 5. FITC-dextran (20 and 200 mg/l added to the fetal reservoir) fluorescence measurements during successful ($n = 5$) versus leaky ($n = 6$) perfusions. Leaky perfusions are perfusions with a loss of fluid from fetal reservoir exceeding 3 ml h^{-1}.

and the time of cannulation are noted, as a measure of placental dormant time. Instead of the birth time of the baby, birth or clamping of the placenta may be more relevant, but generally the birth time of the baby is recorded by the hospital, and in caesarean sections the birth of the baby and the placenta differ by few minutes.

Red blood cells are mostly seen in high amounts in the maternal perfusate if no washing/rinsing procedure is done. The fetal vessels in the cotyledon are rinsed through during the initial cannulation procedure and rarely contain great amounts of red blood cells. When the washing procedure is performed, the hemoglobin content is 37–63 μM in maternal samples; in fetal samples: around 0.6 μM. The normal value of hemoglobin in whole blood from females is 7.4–9.9 mM [20].

4.4. Success rate

Table 3 shows the number of placentae received in one year (22nd May 2008–21st May 2009) and the amount that reached each of the three checkpoints. As the table shows, a majority (92%) of the placentae was successfully cannulated, placed in the chamber and tested in the system. After transfer to the system, only 21% of the placentae had a transfer of O_2 and no fetal leak, and therefore the perfusion was started by addition of the test substance. Of the perfusions to which test substance was added, 72% were successful; corresponding to 15% of all the placentae received resulting in successful perfusions. In two of the placentae, two cotyledons from each were successfully perfused resulting in four successful perfusions performed in two placentae. As different substances were analyzed during the year, we have data from perfusions lasting 2.5, 4 and 6 h. The success rate of the three different lengths of perfusion differed with the 6-h perfusion having the highest success rate, and the 4-h perfusion having the lowest.

4.5. Histopathology

Twenty six placentae were evaluated histologically, and 16 were perfused for 6 h, 3 for 4 h, 1 for 3.5 h, and 6 for 2.5 h. Different

Table 2
Perfusion variables in 2.5- and 4-h perfusions.

Parameter		2.5-h perfusion ($n = 9$)	4-h perfusion ($n = 10$)
Volume loss (ml)[a]	Maternal reservoir	1.2 ± 6.5	3.7 ± 6.5
	Fetal reservoir	4.7 ± 4.6	5.0 ± 3.2
Volume loss (ml/h)	Fetal reservoir	1.36 ± 1.38	1.28 ± 0.91
pO_2 (kPa)	Maternal reservoir	37.6 ± 14.8	33.1 ± 10.4
	Fetal reservoir	13.5 ± 2.1	12.8 ± 2.1
	Fetal outflow	16.5 ± 2.7	15.5 ± 2.3
pH	Maternal reservoir	7.22 ± 0.14	7.20 ± 0.15
	Fetal reservoir	7.34 ± 0.06	7.35 ± 0.07
	Fetal outflow	7.34 ± 0.07	7.36 ± 0.07
Glucose (mmol/l)	Maternal reservoir	6.3 ± 1.2	6.4 ± 1.4
	Fetal reservoir	7.8 ± 1.1	6.6 ± 1.3
	Fetal outflow	7.5 ± 1.1	6.5 ± 1.3
Lactate (mmol/l)	Maternal reservoir	10.6 ± 3.1	6.4 ± 1.4
	Fetal reservoir	4.0 ± 2.1	6.6 ± 1.3
	Fetal outflow	5.0 ± 2.3	6.5 ± 1.3
Lactate produced during perfusion (mmol/l)	Maternal reservoir	4.1 ± 1.8	12.3 ± 3.5
	Fetal reservoir	3.4 ± 1.7	9.2 ± 3.5
Fetal flow rate (ml/min)		3.0 ± 0.2	3.0 ± 0.2
Time (min)[b]		30.6 ± 8.1	30.1 ± 12.5
Preperfusion time (min)		56.9 ± 18.0	42.3 ± 8.7
Caesarean section		All	4 out of 10

[a] The samples removed during the perfusion are subtracted volume loss calculations, sample volume is not replaced.
[b] Time from delivery of child to cannulation of the placenta in the laboratory.

Table 3
Success rate of placental perfusions.

	n	% of placentas received reaching checkpoint	% successful perfusions of cotyledons in preperfusion
Placentas received (one-year period)	202		
Checkpoint 1, cannulations in system	185	92%	
Checkpoint 2, preperfusion successful: substance added	43	21%	
Checkpoint 3, successful perfusion (2.5, 4, 6 h)	31 (13, 10, 9)	15% (6%, 5%, 4%)	72% (81%, 67%, 90%)

test substances were used. Microscopic examination of the placental tissue showed mature chorionic villi in all samples. The perfused areas were characterized by emptiness of fetal blood vessels and the intervillous (maternal) space as well as dilatation of the fetal vessels. In 14 of the samples, vacuolization of the cytotrophoblast was observed, and this was interpreted as a sign of tissue damage. Eleven of these were 6-h perfusions. In the first 11 placentae examined, bacteria were observed in the fetal blood vessels in the perfused areas. In some of these areas neutrophil granulocytes were seen in the villi and surrounding intervillous space indicating an active inflammatory process in vitro. The remaining cases (with Pen-Strep added) showed no bacteria or inflammation. In some cases pathological processes were found focally. These included common findings like small intraplacental and retroplacental hematomas, but also rarer entities such as mild chronic villitis and eosinophilic vasculitis.

5. Discussion

Module 1 in the validation process includes test definition as described in Section 2 and discussed below.

5.1. Perfusion conditions

During collection and cannulation, the placenta can be kept at room temperature or lower. In preperfusion the temperature is raised to physiological body temperature (37 °C), and the placenta is perfused with media supplying oxygen, glucose and other nutrients as the metabolism continues. Different approaches have been made by other groups to increase the oxygen supply by using perfusion media with variable oxygen tensions, higher flow rates, perfluorocarbons as artificial oxygen carriers or diluted blood. These supplements and modifications increase the oxygen consumption to a maximum of 33–50% of the physiological oxygen consumption [21,22]. As a higher oxygen tension may lead to hyperoxia, and higher flow rates endanger the integrity of the tissue, few of these methods to improve oxygenation are suitable in placental perfusions. To avoid hyperoxia atmospheric air can be used instead of oxygen mixtures as practiced in the group of Schneider [22].

For perfusions lasting more than 150 min, the addition of Pen-Strep in the perfusion media is necessary to avoid the explosive growth of bacteria in the warm cell culture media. As the placentae is not sterile, microbiotic growth may cause a decrease in the pH and pO_2 and lead to unsuccessful perfusions. Cleaning the glassware by autoclavation eliminates most infections and is recommended. Histological findings of neutrophils in the perfused placental tissue suggest that cellular responses can occur (see Section 4.5).

Since the placenta metabolizes glucose and produces lactate, acidification may occur during the perfusion. Oxygenizers may help to stabilize the pH given that the content of CO_2 in the gas mixture stabilizes the pH via the carbonate buffer. Unsuccessful perfusions seem to have greater glucose consumption indicating some cell distress.

5.2. Success criteria

The most commonly used criteria for successful perfusions are a minimum of leakage from the fetal system and the successful passage of a control substance. The leakage is measured as the amount of fetal fluid lost during the perfusion. As there is a natural loss of fluid from the closed fetal circuit due to evaporation, and due to pressure in the closed fetal circulation, a small loss of fluid is allowed (2–4 ml/h perfusing) [14]. A higher viscosity and osmolarity in the fetal media compared to the maternal media contains the fluid in the fetal reservoir and compensates for some of the loss. Antipyrine is widely used as a positive control substance because of its ability to diffuse freely across the human placenta, and because the placental transfer kinetics of this substance is well known [23,24]. The rate of diffusion of antipyrine may be used to normalize transfer or diffusion of flow limited compounds. It has been suggested to use creatinine which has a slower flow-independent passage for longer lasting perfusions [25] and may serve as baseline for substances limited in their rate of crossing by the characteristics of the membrane.

The studies performed in our laboratory with the negative control substance FITC-dextran added to the fetal compartment, show a fetal to maternal leakage of FITC-dextran in perfusions with a fluid loss greater than 3 ml/h (in perfusions recirculating the fetal media with a flow of 3 ml/min), indicating a true leakage from fetal to the maternal reservoir. This verifies volume loss and FITC-dextran transfer as suitable measurements of fetal membrane integrity during perfusion studies and further justifies the use of volume loss as a reliable leakage parameter. When adding FITC-dextran to the maternal media, no FITC-dextran could be detected in the fetal reservoir regardless of the success of the perfusion or leakage of fetal media. This indicates a bulk flow in leaky perfusions going only in the direction from fetal to maternal reservoir.

The transfer of oxygen is used as a marker of contact between the maternal and fetal perfused areas. However, this marker is most useful at the beginning of the perfusion to resituate the maternal cannulae in case of insufficient contact, as applied by Wier and Miller [26]. The oxygen transfer (difference in oxygen content from media in fetal vein and fetal reservoir) appears more stable throughout perfusion when it is related to the transplacental oxygen gradient (difference in oxygen content in media from fetal and maternal reservoir); since there may be variations in oxygen content in maternal media between perfusions and within the same perfusions due to cotyledon size and regulation of gas flow. The oxygen in maternal reservoir is the source of oxygen for the fetal reservoir, which makes the oxygen level in maternal reservoir a determining factor in oxygen transfer through the placenta, together with cotyledon size and placement of maternal cannulae.

To demonstrate placental viability during perfusions, the oxygen, pH, glucose, and lactate levels are often presented when reporting placental perfusion results. The level of gasses in the fluids and the pH should be kept stable and in range. However, as the measurements often represent the extreme state immediately before corrections are made, some deviation when reporting pH and pO_2 should be allowed. The glucose and lactate concentrations in the perfusion fluids are a measure of a "live, glucose consuming, lactate producing" placenta, but a rapid increase in lactate levels can also indicate "crashing" or an infection. Oxygen and glucose consumption and lactate production are metabolic measurements ensuring tissue viability rather than tissue integrity during perfusion [26] and are not measured in all labs. Besides consumption of oxygen and glucose and lactate production the net synthesis of placental proteins like hCG, hPL and leptin are very sensitive criteria of viability as was shown by comparing dually perfused tissue with cultures of explants from the same placenta [26].

Studies comparing gene expression of different markers of oxidative stress and apoptotic activity indicate that ischemia-reperfusion injuries are induced in placentae undergoing labor compared to placentae from elective cesarean sections [27]. Tissue which has not been subjected to stress of labor and delivery may better reflect the functional steady state of the placenta in late pregnancy. Limited degree of stress on the other hand may serve as a preconditioning effect making the tissue more resistant to ischemic anoxia. How this affects the transfer kinetics of substances through the placenta has not been investigated.

In this paper the type of delivery did not affect the transfer of antipyrine which is a small freely diffusing substance. It would be even more interesting to investigate potential differences in transfer of molecules actively transported in placentae from vaginal births and caesarean sections, respectively, as active transport is a more representative measure of the state of the placenta, rather than free diffusion. Oxidative stress and apoptosis may affect the experimental outcome and success rate of placental perfusions. However, the placenta has an increased tolerance to hypoxia and seems to recover nicely from the ischemic period from birth to re-perfusion in the laboratory [28]. After delivery the placenta presumably enters a state of partial metabolic arrest with energy saving mechanisms and down regulated metabolism, similar to what is observed in hibernating mammals or deep sea diving turtles [22,28,29]. However, further studies are needed to clarify the consequences of partial metabolic arrest upon re-perfusion and extrapolation of the results to the *in vivo* situation.

5.3. Success rate

As the *ex vivo* placenta perfusion model uses term placentae, which are discarded and isolated organs, the method has certain shortcomings. The method requires training as it is technically challenging. Furthermore, it is very time consuming and has a low rate of success. Table 3 demonstrates the success rate for the placental perfusions in Copenhagen over one year. The numbers reflect the challenges of the model, as well as the difficulties with having different lab personnel and equipment and adjustments with new substances. The access to placentae depends on the number of healthy women delivering at the university hospital, acquiring consents, and the absence of other competing projects also collecting placentae. Among these, there was least concern with getting consent, as the placenta is otherwise discarded, and most women are positive towards participating in scientific research [30].

Two of the placentae during the one-year period, produced two successful perfusions each, indicating that the individual structure and handling of a placenta during delivery may affect the probability of a successful perfusion. Differences in cannulating and perfusion techniques between laboratories may affect the success rate. Clamping the unperfused area surrounding the cotyledon induces a risk of damaging the circulation when introducing metal spikes [14–16]. However, cutting induces an even larger risk of destroying the closed fetal circulation [8]. The clamping technique forces the maternal perfusion media to exit the intervillous space through the deciduas. When cutting, the perfusion media can exit the intervillous space by the neighboring intervillous spaces, which may reduce the flow in the intervillous space. However, comparative antipyrine data using the two different methods indicate that the different flow patterns in the intervillous space are not of importance. Successful perfusions are obtained using both methods, but so far it is unknown whether the methods results in the same success rate. The main difference in the cannulation of the intervillous space is the number of tubes representing maternal spinal arteries. Additional tubes provide better perfusion and circulation of the intervillous space, but present a risk of penetrating the fetal vessels. The majority of laboratories use gas mixtures of 95%N_2/5%CO_2 in fetal and 95%O_2/5%CO_2 in maternal circulation. The gas is led into the perfusion media directly [8] or via oxygenizers [14,16]. Directly gassing the media creates the risks of the media foaming and getting air bubbles into the placenta which may disrupt the perfusion. However, oxygenizers may introduce another problem as they often consist of silicone tubing which can bind many study compounds. These different parts of perfusion techniques can be improved individually to increase success rate. Although success rate may vary between laboratories the results from successful perfusions may still be comparable.

The majority of the placentae pass checkpoint 1 of cannulation, but only about 20% of the placentae are still usable after being connected to the system, having maternal cannulae set into the intervillous space and running 30 min of preperfusion (second checkpoint) (Table 3). The period from the first to the second checkpoint could be split in several checkpoints to verify the point of decision whether to terminate the perfusion or add the test substance. There are several critical points: the placentae should be able to sustain the flow when connected to the system, as there is a risk that the pressure generated by the peristaltic pump will cause a leakage in the fetal vessels. Another point is the placement of the maternal cannulae set into the intervillous space which, especially when using multiple maternal cannulae presents a risk of penetrating the fetal vessels. Finally, there should be oxygen transfer, which if not achieved at the first placement of the maternal cannulae, leaves the option of replacement. When adding test substance, it has to be highly probable that the perfusion will fulfill the criteria of being successful, otherwise substance is wasted.

The checkpoints from receiving the placenta to obtaining a successful perfusion may reveal which stage is in need of technical improvement, or help determine unsuccessful perfusions at an earlier stage, saving time and valuable test substance. The checkpoints can hopefully also give an insight into what can be done to obtain a higher success rate.

Table 3 contains data from 2.5-, 4- and 6-h perfusions. The success rate of the different lengths of perfusions differed, with the 6-h perfusion having the highest success rate, and the 4-h perfusion having the lowest. This does not indicate any correlation between success rate and length of perfusion. Some of the longer perfusions last several hours before a leak is present, suggesting that if a cotyledon from a leaky 6-h perfusion was instead perfused with a substance requiring fewer hours of perfusion, the perfusion could have been a successful perfusion of shorter duration. If the leakage in a perfusion is just above the leak criteria for successful perfusions, and the data for the test substance are identical in three or more successful perfusions of the same substance, it is used in exceptional cases to support the existing data and add to statistical significance.

5.4. Recovery

Recovery of added test substance is calculated using samples taken from the perfusion medium during the perfusion and tissue samples taken after perfusion. The samples alone do not give a true picture of substance distribution due to the accumulation or adherence of the substance in tissue, the chamber and tubing. The adherence test, explained in Section 2.2.3, account for the loss of substance due to system adherence. During the perfusion, placenta tissue is bathed in the maternal media, supplied by the maternal cannulae. The cannulae are placed in the cotyledon, which results in a localized higher concentration of substance. The heterogenic distribution of substance makes it difficult to account for the substance in the tissue analyzing only tissue samples, therefore a relatively large margin is allowed.

5.5. Histopathology

The histological examination of the perfused area is an additional quality control of the perfusion. Due to the finding of bacteria in the fetal blood vessels of the perfused areas, addition of antibiotics to the medium in cases of long perfusions was needed. Neutrophil granulocytes in these areas suggested that an active inflammatory process is able to take place in the perfusion system. The examination of the non-perfused areas is an opportunity to examine placentae from pregnancies with a known normal outcome. These placentae are usually not sent for pathological

examination. Minor pathological processes were expected to be found; however, it was surprising to find rarer entities such as chronic villitis and eosinophilic vasculitis. These findings will be discussed in detail in a separate paper. Our findings might suggest that these entities may in milder degrees be more common in normal pregnancies than generally believed.

6. Conclusion

Our studies show comparability in placental perfusions between different laboratories pointing at checkpoints of validating control data, the success criteria of a positive control and minimum fetal leakage. We suggest uniform perfusion conditions regarding controls, sampling and data representation. A method paper with a detailed description of the equipment, different procedural steps and validation should be published from each lab. For publications using the established method the method paper can be cited and furthermore the following elements are suggested:

1. Calculation of success rates by the checkpoints defined and inclusion of checkpoint 3 in publications.
2. Inclusion of antipyrine and eventually creatinine or another actively transported control substance.
3. Frequent measures of leakage, oxygen transport and pH.
4. Histopathological evaluation.

There is a great need for follow up meetings discussing placental perfusion with the objective to establish consensus on various aspects of the method and on the information which should be given in materials and methods section of all manuscripts based on this methodology.

Conflict of interest

None.

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