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Chapter 14

Assessment of placental transport function in studies of disease programming

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Abstract

Environmental conditions during pregnancy affect fetal growth and development and programme the offspring for poor future health. These effects may be mediated by the placenta, which develops to transfer nutrients from mother to the fetus for growth. The ability to measure the uni-directional maternofetal transfer of non-metabolisable radio-analogues of glucose and amino acid by the placenta *in vivo* has thus been invaluable to our understanding of the regulation of fetal growth, particularly in small animal models. Herein, I describe the method by which *in vivo* placental transfer function can be quantified in the mouse, an animal model widely used in studies of *in utero* disease programming.

Keywords: placenta, amino acid, glucose, transport, nutrient allocation, intrauterine growth restrition, overgrowth

1 Introduction

In mammals, the main determinant of growth and development *in utero* is the placenta. The placenta constitutes the interface between the mother and her fetus which is responsible for controlling the amount of nutrients and oxygen transferred. In human pregnancies, abnormal birth weight is associated with changes in placental transporter capacity, which suggests that the placenta is a key mediator of alterations in fetal growth [**1, 2**]. Indeed, in animal models, placental supply capacity is modified in response to environmental and hormonal challenges in the mother and appears to link maternal perturbations to changes in fetal growth and offspring outcome [**3-6**]. Moreover, placental transport capacity adapts dynamically to both fetal signals of nutrient demand and maternal signals of nutrient availability to ensure appropriate allocation of available resources [**7-11**]. Therefore, by assessing placental transport capacity we are able to better understand the regulation of fetal growth and identify programming mechanisms.

This chapter aims to describe how the transfer of nutrients from mother to the fetus by the placenta may be quantified *in vivo*. The technique involves injecting radiolabelled non-metabolisable substrates into the maternal circulation and then assessing the clearance rates across the placenta over time in relation to the accumulation of radiolabel in the fetus. The method has largely been used in studies of mouse, rat and guinea pig pregnancy and has been most widely used to measure the placental transfer of glucose and amino acid, which are indicative of facilitated diffusion and active transport function, respectively [**9, 12-32**]. However, this method has also been used to examine the *in vivo* transplacental transport of radioactive sodium and calcium [**33-36**] and the passive permeability characteristics of the placenta for solute flux, using radioactive inert hydrophilic substrates [**12, 37, 38**]. Thus, by substituting the radiolabelled substrate or tracer used, this method can also be applied to study other transport systems *in vivo* [**39**]. Herein, the method for simultaneous measurement of the uni-directional placental transfer of ^{3}H-methyl-D glucose (^{3}H-MeG) and ^{14}C-amino isobutyric acid (^{14}C-MeAIB), an amino acid analogue principally transferred by the System A transporters [**40**], is described for the mouse.

2 Materials

2.1 Specialised equipment

1. Warming/heating pad to maintain the dam's body temperature.
2. Table lamp.
3. Sensitive balance to accurately measure weight of conceptuses.
4. Incubator set at 55°C.
5. Liquid scintillation counter.
6. Liquid dispensing pump for scintillation fluid.
7. Refrigerated centrifuge.

2.2 Materials and Reagents

1. Small weigh boats.Stainless steel sterile scapel blades (size #21).
2. Single-edge razor blades.
3. 27 gauge needles 0.4 mm x 20 mm.
4. 25 gauge needles 0.5 mm x 16 mm.
5. Polythene tubing (800/100/200, I.D. 0.58 mm, OD 0.96 mm).
6. Benchcote.
7. Cotton bud tips.
8. 1 mL syringes.
9. Ethylenediaminetetraacetic acid (EDTA)-coated tubes.
10. 1.5 mL eppendof tubes.
11. 15 mL screw cap tubes.
12. 5 mL plastic scintallation vials.
13. AS Scintlogic Scintillation fluid (LabLogic; Sheffield, UK)
14. ^{14}C-MeAIB (NEN NEC-671; specific activity 1.86 GBq/mmol).
15. ^{3}H-MeG (NEN NEC-377; specific activity 2.1 GBq/mmol).
16. Hypnorm (fentanyl citrate, 0.315 mg/mL and fluanisone, 10 mg/mL).
17. Hypnovel (midazolam, 10 mg/2mL).
18. Sterile water for injection.
19. Biosol (National Diagnostics; Atlanta, GA, USA).
20. Sterile physiological saline 0.9% NaCl.
21. 70% Ethanol.

2.3 Instruments

1. Small curved, serrated forceps for holding catheter in vessel.
2. Curved serrated forceps for dissection.
3. Blunt dissecting scissors.
4. Dressing/operating sharp scissors.
5. Scapel blade holder.
6. 3-sided, small needle file.
7. Pair of mosquito forceps.
8. Seeker needle.
9. Pipettes and tips (10-1000 uL).

3 Methods

3.1 Catheters preparation

1. Stretch the polythene tubing to around 10 cm (leave the first 1 cm un-stretched).
2. Using a single edge razor blade, cut the stretched polythene tubing in such a way that both ends (about 0.5 cm) are un-stretched.
3. With the aid of the mosquito forceps, break off the hub of one 27 gauge needle.
4. To another 27 gauge needle, break off the pointed tip.
5. Using the needle file, file down the broken off end of each needle (**see Note 1**).
6. With the aid of mosquito forceps, insert the broken-off edge of each needle into opposite ends of the polythene tubing (**see Note 2**).
7. Test the catheter with sterile water and ensure that the water is expelled afterwards.

3.2 Radioactive isotope (see Note 3)

1. Create a stock of ^{3}H-MeG (**see Note 4**) and ^{14}C-MeAIB, each at a concentration of 3.5 μCi/100 uL in sterile physiological saline (0.13 MBq).
2. Prepare a 1:1 mixture of 3.5 μCi of ^{3}H-MeG and 3.5 μCi ^{14}C-MeAIB of and store at -20°C in a lockable freezer.

3. Prepare sufficient radioactive isotope for the batch of experiments to be performed (**see Note 5**).

3.3 Anaesthetic preparation

1. Prepare anaesthetic in the following ratio: 1 part hypnorm to 1 part hypnovel and 2 parts sterile water (1:1:2) (**see Note 6**).

3.4 Time-mating of mice (see Note 7)

1. Order in adult mice from your prefered supplier and allow them to acclimitase in your animal facility for 1 week (**see Note 8**).
2. Time mate female mice by placing 1 or 2 females in each stud male cage in the late afternoon (**see Note 9**).
3. The next day, between 0800 and 1000 h check for the presence of a copulatory plug in the entrance to dam's genital tract (the day of plug indicates day 1 of pregnancy).
4. Females can either remain or be removed from the stud male cage (**see Note 10**).

3.5 Placental transport assay (see Notes 11 and 12)

1. On the day of pregnancy when placental transfer function will be assessed, weigh the dam.
2. Prepare the room by turning on the heating pad, covering the entire bench (and heating pad) with benchcote.
3. Label tubes and allow radioactive isotope mixture (containing 3.5 μCi of ^{3}H-MeG and 3.5 μCi of ^{14}C-MeAIB) to thaw at room temperature.
4. Draw up 200 μL of the radioactive isotope mixture into the catheter.
5. Induce anaethesia in the dam using ip injection of hypnorm-hypnovel solution (**see Note 13**).
6. Check the dam for reflexes by firmly squeezing her foot and place her on her back, on the heat pad to ensure body temperature is maintained.
7. Clean the neck area of the animal with a 70% ethanol.
8. Expose the maternal jugular vein (**Fig. 1**) using a scapel blade fitted to a holder to make a 1.5-2 cm vertical incision in the skin of the neck, ~0.5 cm from midline.

9. Then, using the blunt scissors and small curved serrated forceps slowly use blunt dissection to clear the skin and expose the jugular vein.
10. The fat pad needs to be slowly pushed/cleared away from the vessel using a cotton tip bud so that ~0.5-1 cm of the vessel can be easily observed (**see Note 14**).
11. Apply a little pressure on the distal end of the vessel using your finger or cotton tip bud to help it to bulge.
12. Then slowly insert the catheter into the vessel, keeping the insertion shallow/superficial to prevent injection through the vessel, into the underlying interstitium.
13. Once in place, hold the catheter in the vessel using the small curved serrated forceps and slowly infuse in the radioactive isotope into the dam over ~15 s (**see Notes 15 and 16**).
14. Slowly withraw catheter and quicly place cotton tip bud over the vessel entrance to stop blood or isotope leaking out and immediately start the timer counting up.
15. Schedule 1 kill the dam at 1-4 min after tracer injection (**see Notes 17 and 18**).
16. When approaching the time to kill the dam, open the dam's chest cavity and then cut through the top of the heart using the operating/dressing sharp scissors.
17. Rapidly collect the exanguinated blood using the 1 mL syringe and dispense into a labelled pre-chilled EDTA tube, shake and keep on ice.
18. Turn dam over and use cervical dislocation to ensure death of the animal.
19. Open the peritoneal cavity and count the number of viable and dead/resorbing conceptuses in each uteine horn before removing the uterus from the dam.
20. Separate each conceptus into its own small weigh boat.
21. Then, dissect each fetus from its placenta and fetal membranes.
22. After drying on tissue paper, weigh each fetus and placenta and then move fetus into a new weigh boat and decapitate (**see Note 19**).
23. Mince the fetus in the weigh boat using a scapel blade and scrape entire sample into a 15 mL screw cap tube.
24. To the 15 mL tubes containing minced fetuses, add 2 mL or 4 mL of biosol for studies on days 15-16 and days 18-19 of pregnancy, respectively (**see Note 20**).
25. Manually shake the fetal samples to ensure the entire minced fetus is immesered in the biosol.
26. Incubate samples at 55°C for 1 week to ensure complete homogenisation/solubilisation of fetal tissue and release of radioactivity (**see Note 21**).

27. Centrifuge maternal blood at 3000 x g for 10 min at 4°C and recover plasma into an eppendorf tube (**see Note 22**).
28. Them determine counts in maternal plasma, add 198 uL biosol, 2 uL plasma and 4 mL scintillation fluid to a scintillation vial.
29. Cap the vial and then shake.
30. Prepare triplicates of each plasma sample and also prepare a background sample which contains 200 uL biosol and 4 mL scintillation fluid.
31. Allow all samples to sit in the dark to allow chemiluminence to dissipate and then determine ^{3}H and ^{14}C content using a liquid scintillation counter (**see Notes 23 and 24**).
32. To determine counts in homogenised fetuses, add 250 uL or 500 uL of fetal homogenate from days 15-16 and days 18-19 of pregnancy, respectively, to a scintillation vial.
33. Add 4 mL scintillation fluid, cap the vial and then shake.
34. Prepare duplicates of each fetal homogenate and prepare a background vial which replaces the volume of fetal homogenate with biosol.
35. Allow samples to sit in the dark to allow chemiluminence to dispate and then determine ^{3}H and ^{14}C content using a liquid scintillation counter (**see Notes 23 - 25**).

3.6 Calculations and data analysis

1. Subtract the background value from the mean maternal plasma and fetal counts.
2. Using the maternal plasma counts, create a one phase exponential decay curve for ^{3}H and ^{14}C (**see Note 26 and Fig. 2**).
3. Values should be in disintegrations per min (DPM) per μL maternal plasma.
4. Calculate the weight specific clearance of each tracer (K_{mf}) by the placenta using the equation:

$$K_{mf} = N_X / W \int_0^X Cm\ (t)dt$$

Where N_X is the radioactivity (DPM) in the fetus at time of death ($_X$)

W is the weight of the corresponding placenta in grams and $\int_0^X Cm\ (t)dt$ is the integral (area under curve) of the first order exponential decay curve of maternal plasma radioactivity (Cm, DPM per μL) with time, up to the time of death [12]

(Values should be presented as placental clearance of ^{3}H-MeG and ^{14}C-MeAIB expressed as µL/min/g placenta)

5. Use radioactive counts in each fetus to calculate the amount of radioisotope transferred per gram of fetus (also known as fetal accumulation) using the following formula:

   [A x (Vb + fetal weight in grams) / Vf)] / weight of fetus in g

   Where A = DPM in fetal sample
   Vb = volume of biosol used to homogenize sample (eg 2 mL for samples on day 16 of pregnancy)
   Vf = volume of fetal homogenate counted (eg 0.25 mL for samples on day 16 of pregnancy)
   (Values should be presented as fetal accumulation of ^{3}H-MeG and ^{14}C-MeAIB expressed as DPM/g fetus) (**see Notes 27 and 28**)

4 Notes

1. File at right-angles to the needle and then around the edge of the break. Careful filing is essential to make sure there are no rough edges or splinters that can damage the polythene tubing (and make leaks in the catheter).
2. If necessary, use the seeker needle to widen the opening of the polythene tubing. Make sure that a seal is made between the needle and tubing; that the needle reaches down to the stretched part of the catheter.
3. Ensure all the regulatory procedures are in place, including appropriate training of individuals, to undertake ^{3}H and ^{14}C radioactive work in your workplace. Always abide by local, environmental and instutional policies for working and disposing of radioactive subtances.
4. The ^{3}H-MeG is often suplied in ethanol. If possible, purchase ^{3}H-MeG in a concentrated form, so that once it is diluted there is only very little residual ethanol. The half life of ^{3}H is 12.3 years. A decay correction may need to be applied when preparing new new batches of ^{3}H-MeG.

5. This will eliminate introducing variation from preparing stocks on different days.
6. This anaesthetic combination is most effective when prepared fresh on the day. Excess can be stored at $4^{o}C$ for use within the week.
7. Accurate timing of pregnancy is required as the nutrient transfer capacity of the placenta changes with gestational age.
8. Ordered females should be older than 7 weeks and males older than 10 weeks to ensure they are sufficiently reproductively mature.
9. To increase chance of mating, females should be placed in the stud cage when they are in estrous.
10. Dams that are pregnant should start gaining weight from day 10 of pregnancy.
11. Make sure ethical approval for the proposed regulated procedures on mice (administration of substances) have been attained from the government and local committees.
12. This procedure has been performed on mice that are at day 15 or later in pregnancy.
13. Typically, 400 uL is given to mice at day 16 of pregnancy and 600 uL to those on day 19 of pregnancy. The hypnorm-hyponvel combination rapidly induces anaesthesia with minimal cardiovascular depression, and thus minimal changes in uterine blood flow.
14. The jugular will be surround by a fat pad (the size of the fat pad depends on maternal age and environmental manipulation).
15. If the first attempt does not work, apply pressure using cotton tip bud to the jugular vein to stop any bleeding. Then try the jugular vein on the other side of the animal.
16. You can practice performing the procedure by injecting physiological saline or a coloured non-toxic substance.
17. Between 1 and 4 min, there is minimal backflux of radioisotope from fetus to mother. Make sure that the average time for each experimental group is 2 min.
18. Weigh catheter before and after tracer injection to ensure that 200 uL has been successfully administered to the dam. Exclude animal from placental transfer analysis if volume injected varies by 10% or more than intended.
19. Yolk sacs or placentas can be taken for DNA extraction and sexing the fetuses, if required.
20. Placentas can also be minced and then digested in biosol (2 mL) to determine radiolabel accumulation. Just note that due to large amounts of blood in the placenta, samples may have high levels of chemiluminenesce which interferes with the

discrimination of the ^3H and ^{14}C channels. Appropriate run programs should be developed in consultation with technical support for the scintillation counter.

21. To speed up the solubilisation process, samples can be vortexed each day or placed in an incubator with a shaking element.
22. Plasma can be stored at -20°C or immediately analysed for radioactivity content.
23. The length of time required to allow chemiluminence to dissipate in the samples needs to be determined in consultation with technical support for the liquid scinillation counter.
24. During measurement of both maternal plasma and fetal radioactivity, simultaneously run quench-adjusted standards with samples to discern between the ^3H and ^{14}C channels. This should be done in consultation with technical support for the liquid scintillation counter.
25. If placental samples were homogenised in biosol, add 250 uL of the homogenate to 4 mL scintillation fluid. Prepare duplicates of each sample and determine ^3H and ^{14}C content using a liquid scintillation counter.
26. Prepare a radioisotope clearance curve for each tracer at each gestational age studied.
27. If the surface area of the placenta functioning in maternofetal exchange has been determined stereologically using the method described by Coan et al., [41], then transfer of each radiolabel per unit of surface area can be estimated yielding the estimated μL/min/cm^2 of placental exchange surface area [19].
28. If ^3H and ^{14}C were counted in placental samples, placental accumulation of ^3H-MeG and ^{14}C-MeAIB can also be determined using the formula:

   [A x (Vb + placental weight in grams) / Vp)] / weight of the placenta in grams
   Where A = DPM in placental sample
   Vb = volume of biosol used to homogenize sample (ie 2 mL)
   Vf = volume of placental homogenate counted (ie 0.25 mL)
   (Values should be presented as placental accumulation of ^3H-MeG and ^{14}C-MeAIB expressed as DPM/g placenta)

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Figure Legends

Fig. 1 Photo of an anaesthetised pregnant dam, showing the jugular vein and fat pad cleared away (work was conducted in the University of Cambridge Animal Facility abiding by the UK Home Office Animals (Scientific Procedures) Act 1986 and local ethics committee). Prior to incision, the neck area is normally cleaned with 70% ethanol.

Fig. 2 An example of a one phase exponential decay curve for a radioisotope. Each data point represents counts in a plasma sample from a single mouse dam. In larger species that have a greater blood volume, like the rat and guinea pig, repeated sampling of the dam is possible and a radioactive isotope clearance curve can be generated for each animal.

Figure 1

Figure 2