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Prenatal exposure to diabetes or cigarette smoke and postnatal sensitivity to cigarette smoke

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We use this protocol and it's working

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Abstract

Prenatal exposure to maternal diabetes and cigarette smoke have been linked with negative respiratory consequences for offspring, including increasing risk for asthma and chronic obstructive pulmonary disease (COPD). This project aims to explore how prenatal factors influence offspring sensitivity to cigarette smoke later in life, as a surrogate for COPD risk. Downstream indicators of lung function, inflammation, and tissue for molecular analysis were collected. All the procedures for this study were done in accordance with University of Manitoba Animal Ethics (#21-011). In keeping with the 3R's of animal research, a single control group was used in this study, exposures and data were collected at the same time as the experimental groups. In this way, the control mice act as a relevant comparator for multiple prenatal exposures without necessitating separate control groups. There are four prenatal exposure groups generated from this protocol: Control (low-fat diet and room air), Maternal Diabetes (high-fat diet and room air), Maternal Smoking (low-fat diet and cigarette smoke), and Maternal Diabetes with Smoking (high-fat diet and cigarette smoke). The two aims of this project were to address 1) whether individual prenatal factors influence offspring sensitivity to cigarette smoke, 2) if prenatal exposure interact to influence influence cigarette smoke sensitivity.

Guidelines

All the procedures for this study were done in accordance with University of Manitoba Animal Ethics (#21-011)



Materials

Animals:

- C57BL/6NJ mice (Jackson Laboratory Strain #005304)
- Female: 5 weeks of age
- Male (breeding): 7-8 weeks of age

Consumables:

- Low-fat (10% kcal) food (Research Diets #D12450B)
- High-fat (45% kcal) food (Research Diets #D12451)
- Glucose
- Distilled water
- Kentucky reference cigarettes (3R4F)
- 26G-Needles
- Sodium pentobarbital
- 20-gauge polyethylene catheter
- Saline
- 1mL syringe
- 2mL Eppendorf tube
- RNA-later (Invitrogen)
- Liquid nitrogen
- FACS buffer (PBS+ 0.5% BSA)
- RPMI Media (SIGMA)
- ACK Buffer ($\text{NH}_4\text{Cl} + \text{KHCO}_3 + 0.5\text{M EDTA} + \text{ddH}_2\text{O}$)

Equipment

- Blood glucose monitor (Contour® Next, model #7359)
- Blood glucose test strips (Contour® Next strips 100x)
- Scale
- Dissecting Scissors
- Scireq inExpose® and Smoking Robot with Whole Body Chamber
- Scireq flexiVent®
- Refrigerated centrifuge

Software:

- Flexiware (Version 7.6)

Protocol materials

 Low-fat 10% kcal diet **Research Diets Inc Catalog #D12450B**

 High-fat 45% kcal diet **Research Diets Inc Catalog #D12451**

Study Groups for this Protocol

1

		Maternal Diet	
		Low-Fat	High-Fat
Maternal Exposure	Room Air	Control	MD
	Cigarette Smoke	CS	MD+CS

Prenatal backgrounds generated by the exposure protocols presented. CS - Cigarette Smoke, MD - Maternal Diabetes. Both male and female offspring used for downstream measures of lung health.

Maternal Diabetes Exposure Model

18h

2 Diet Exposure

- 2.1 Use six-week-old female mice (C57BL/6NJ) for diet exposure. Randomly assign mice into groups.

1. Maximum 4 female mice per cage.

2. Add environmental enrichments to the cage.
- 2.2 Record initial body weight of mice as week 0.
- 2.3 Add 250g of high-fat diet (

 High-fat 45% kcal diet **Research Diets Inc Catalog #D12451**

) into MD group cages to induce body weight gain and glucose intolerance as per published diet induced maternal diabetes model.



Citation

Pereira TJ, Moyce BL, Kereliuk SM, Dolinsky VW (2015)

. Influence of maternal overnutrition and gestational diabetes on the programming of metabolic health outcomes in the offspring: experimental evidence.

<https://doi.org/10.1139/bcb-2014-0141>

LINK

2.4 Add 250g of low-fat diet (

 Low-fat 10% kcal diet **Research Diets Inc Catalog #D12450B**) to the control cages.

2.5 **Weekly:**

- Record food consumption by weighing remaining food and replenish to 250g.
- Record body weight.

Safety information

Monitor and record body condition, behaviour, and physical signs of distress in each mouse.

2.6 At week 6 of diet, perform glucose tolerance test (GTT)  [go to step #3](#)



2.7 Maternal diets continue throughout the breeding, pregnancy and until the offspring get weaned at three-weeks of age.



3 Glucose Tolerance Test

3.1 Fast mice overnight (18 hrs) before GTT. Fasting starts in the evening prior to the test date (around 4 pm). Remove food from cage, keep water bottles overnight during fasting hours.

18h



Weigh the food and save it to give back to the mice after GTT.

Note

If food is present in bedding of the cage, move animals to a new cage to ensure no food is consumed during fasting.

- 3.2 Freshly prepare 10% glucose solution.
Weigh 1g of D-Glucose and dissolve it in 10 mL of distilled water.
Mix well by vortexing.
Filter sterilize 10% glucose solution using 0.22 µm syringe filter.



- 3.3 Weigh mouse and record body weight for dose calculation. Calculate dose of glucose for injection (10 µL/g).



Dose Calculation:

$$\text{Volume of 10\% Glucose (}\mu\text{L)} = 10 \mu\text{L/g} \times \text{Body weight of mouse (g)}$$

Prepare 1mL syringes and 26G needle with calculated volume of glucose, removing air bubbles.

- 3.4 Using lance supplied with glucose monitor, lightly poke mouse tail along side to generate small drop of blood for testing.



Alternatively, perform blood sampling from saphenous vein.

Safety information

Do not put significant pressure on the tail, this process should be painless and not require manipulation of the tail.

- 3.5 Measure fasting/basal blood glucose level at time 'ZERO'.

Note

Blood glucose can be measured using commercially available glucose meters and test strips. For example:

- Contour® Next, model #7359
- Contour® Next strips 100x

- 3.6 Perform intraperitoneal injection (IP) with appropriate volume of glucose solution.

Safety information

1. Restrain the mouse.
2. Identify the appropriate IP injection site.
3. Insert needle at 45 degree angle into the right lower abdominal quadrant.
4. Before injection, pull back the plunger to make sure the needle has not penetrated through bladder, Bowel or blood vessel.
5. Inject and return mouse back to the cage.

- 3.7 Measure and record blood glucose levels for each mouse at specific time intervals post injection:
10, 20, 30, 60 and 120 minutes, using blood from tail vein or saphenous vein.
- 3.8 Return mice back to their cages with food and environmental enrichments to the cages.
- 3.9 Plot time (minutes) versus blood glucose level (mmol/L) to assess glucose tolerance.

Expected result

Blood glucose concentration should spike 10-20 minutes post IP injection and return to baseline within 120 minutes.

Maternal Cigarette Exposure Model

50m

- 4 Begin cigarette smoke exposure in female mice 2-weeks prior to breeding. If paired with maternal diet exposures, this would be week 4 of the diet exposure.
- 4.1 Have a spare, foreign cage available to act as the room air exposure for control animals.
- 4.2 Split female mice into two groups:
1. Control room air exposure (foreign cage)
 2. Cigarette smoke (CS) exposure.
- 4.3 Expose CS female mice to whole body CS using Scireq In-Expose smoking robot (SCIREQ, Montreal, QC, Canada) as follows:
- 1. Use standard 3R4F research cigarettes (University of Kentucky, Lexington, KY).**
 - 2. Use 10 cigarettes for 50 minutes per exposure.**
 - 3. CS exposure twice daily (Total 20 cigarettes per day), 5 days per week.**
 - 4. Use flow rate of 2 L/min and one puff per minute, with 5 puffs per cigarette.**
 - 5. Confirm lighting of cigarettes in smoking robot.**



6. Smoke according to ISO 3308 Standard for mainstream smoke.

- 4.4 Expose control female mice to room air in a spare foreign cage, for the same length of time to mimic the stress associate with the exposure.
- 4.5 Cover mouse foreign cages/smoke chamber with a green cloth during exposures to reduce animal stress and anxiety.
- 4.6 After each cigarette smoke exposure return mice back to their cages with food, water and environmental enrichments to the cages.
- 4.7 **Weekly:**
 - Record food consumption by weighing remaining food and replenish to 250g as described in step #2.5.
 - Record body weight.

Safety information

Monitor and record body condition, behaviour, and physical signs of distress in each mouse during exposures.

- 4.8 Maternal CS exposure continues throughout breeding, pregnancy and until the offspring get weaned at three-weeks of age.

Note

Offspring are never directly exposed to cigarette smoke (ie. never put into the smoke exposure chamber). They are only ever exposed indirectly through pregnancy or during breastfeeding (ie. third hand exposure).

Breeding and Offspring Weaning

- 5 On week 6, following completion of the diet and CS exposure, and confirmation of glucose intolerance, breed mice to generate offspring.
- 5.1 Use 7-8 weeks old C57BL/6NJ male mice for breeding. Breeders are purchased from Jackson Laboratory.

**Note**

Maintain male breeders on a standard research chow diet, except when they paired with females for breeding purpose.

- 5.2 For mating, house two females with one male per cage.
- 5.3 House mice together for 10 days, moving male mice to separate cage on day 10. Keep two female mice together in the same cage.
- 5.4 Check each morning for the presence of a vaginal plug to confirm mating.
- 5.5 Track gestational weight gain every other day to confirm their pregnancy status. Plot gestational day versus gestational weight gain (g) to confirm their pregnancy.

Expected result

With a weight gain exceeding 4 grams indicates successful pregnancy.

Citation

Heyne GW, Plisch EH, Melberg CG, Sandgren EP, Peter JA, Lipinski RJ (1970)
. A Simple and Reliable Method for Early Pregnancy Detection in Inbred Mice.
Journal of the American Association for Laboratory Animal Science : JAALAS.

<https://doi.org/>

LINK

- 5.6 *Optional:* If females are not pregnant, you can choose to rebreed them with the same male for additional 10 days.

Note

This should be noted for downstream data analysis as mice will be exposed to diet and CS for longer than non-rebred mice.





- 5.7 Near the end of gestation, but before females give birth, separate two females into individual cages. Provide fresh water and food.
- 5.8 Check the cages every morning for litters. Take care not to disturb the litters.
- 5.9 After pups are born, cull litters to a maximum of six per litter. Use excess pups to collect tissues (Ex: milk spots and lung tissues) for future downstream analysis.
- 5.10 Keep pups with dam for 3-weeks until weaning.

Note

Diet and CS exposures continue throughout weaning as noted in 2.7 and 4.8

- 5.11 At weaning (3-weeks of age) separate offspring from dam and house according to sex. Males and females in separate cages.

Note

Weigh the weaned offspring and record their body weights and sex.

- 5.12 At this point introduce offspring to a standard research chow diet. Continue diet until they reach 8-weeks of age.

Note

During this 5-week period mice are not exposed to any experimental interventions. NO cigarette smoke exposures and NO specific diet interventions.

Adolescent Cigarette Smoke Exposure - Offspring

- 6 Record the body weights of each mouse at 8-weeks of age.
- 6.1 Split each cage into two more cages.
 - 1) Control group, exposed to room air.
 - 2) Cigarette smoke group.

- 6.2 Cigarette smoke group undergo whole body cigarette smoke exposure for 50 minutes, twice daily, four days. Perform exposures using Scireq inExpose smoking robot as explained in [➡ go to step #4.3](#)
- 6.3 Expose control mice to room air in a spare foreign cage, for the same length of time to mimic the stress associate with the exposure.
- 6.4 Cover mouse foreign cages/smoke chamber with a green cloth during exposures to reduce animal stress and anxiety.
- 6.5 After each cigarette smoke exposure return mice back to their cages with food, water and environmental enrichments to the cages.
- 6.6 Outcomes (lung function and sample collection) are performed on day 5, ~18-hours after last CS exposure.

Lung Function

- 7 Lung function is measured using the Scireq flexiVent system ~18 hours after the last cigarette smoke exposure.

Note

Prior to measuring lung function in mice, complete recommended setup and calibration as per Scireq guidelines.

- 7.1 Weigh and record body weight of each mouse.
- 7.2 Anesthetize mice using sodium pentobarbital (90 mg/kg, i.p).
- 7.3 Make a small incision in the skin of the neck and expose the trachea using forceps and scissors.
- 7.4 Carefully insert a 20-gauge polyethylene catheter into the trachea and connect animal to the flexiVent small animal ventilator via catheter.
- 7.5 Ventilate mice with a tidal volume of 10 ml/kg of body weight, at a rate of 150 breaths per minute.



- 7.6 Capture desired lung function outcomes on flexiVent. This can include PV-Loops, inspiratory capacity, total lung resistance (Rrs), Compliance (C), Elastance (E), Newtonian resistance (Rn), Tissue damping (G), and Tissue elastance (H).

Note

Refer to Scireq measurement guidelines for direction.

- 7.7 Remove mouse from flexiVent and euthanize for sample collection.

Note

Keep catheter in trachea for sample collection.

Bronchoalveolar Lavage (BAL) Collection

10m

- 8 Label 2 mL collection tubes and keep them on ice.
- 8.1 Prepare ice-cold Phosphate buffered saline (PBS) and load syringes with 1mL of PBS.
- 8.2 Gently and with consistent pressure, instill PBS into lung through the tracheal cannula.
- 8.3 After instillation, gently aspirate. Repeat instillation and aspiration one more time to collect lung lavage fluid.

Safety information

Avoid forceful or uneven instillation to prevent lung damage.

Expected result

You can expect to typically recover ~80% of the instilled volume.



8.4 Repeat this wash for one more time using 1 mL of ice-cold PBS. Collect and pool both washes and keep them on ice.

8.5 Measure and record the total recovered BAL volume.

8.6 Centrifuge all BAL samples.  1100 rpm, 4°C, 00:10:00

10m

8.7 Transfer cell-free supernatant to a new 2 mL vial and make 250-500 µL aliquots. Store these aliquots at  -80 °C for future analysis.

Note

Careful not to disturb the cell pellet.

8.8 Resuspend the cell pellet in 1 mL of FACS buffer (PBS with 0.5% Bovine Serum Albumin, BSA).

8.9 Use 10 µL of cell suspension to count total cells/mL using hemocytometer.

Expected result

Expect an average of ~50,000 cells per mL in the control group.

8.10 Remaining cell pellet can be used for differential cell analysis by flow cytometry.

Tissue Collection

9 Pinch and lift the abdominal skin with forceps, then cut through it to expose the rib cage. Pull the skin aside to keep fur away from the body.

9.1 With the rib cage exposed, make a small abdominal incision below the diaphragm and ribs.



- 9.2 Using fine scissors, make a small cut in the abdominal artery. Collect blood using a sterile syringe, as needed.
- 9.3 Cut through the ventral rib cage, starting from diaphragm and continuing up to thoracic cavity.
- 9.4 Using scissors and forceps, carefully reveal the lungs.

Note

Take care to avoid puncturing the lungs.

- 9.5 Gently separate the left and right lung using small, sharp scissors.

9.6 **Left lung lobes (DNA & Protein)**

- 9.7 Mince the left lung lobes into smaller pieces using sterile scissors.

- 9.8 Transfer tissue into 2 mL collection tubes.

- 9.9 Immediately snap-freeze in liquid nitrogen.

- 9.10 Store samples at  -80 °C until use for DNA and protein extraction.

Note

Avoid repeated freeze-thaw cycles.

9.11 **Right lung lobes (RNA)**

- 9.12 Mince the right lung lobes into smaller pieces using sterile scissors.



9.13 Place tissue into a 2 mL tube containing 1 mL of RNAlater[®] solution (Invitrogen, Thermo Fisher Scientific).

9.14 Keep samples at  4 °C for 24 hrs.

9.15 Transfer tubes to  -80 °C for long term storage until use for RNA extractions.

Note

Avoid repeated freeze-thaw cycles.

Citations

Step 5.4

Heyne GW, Plisch EH, Melberg CG, Sandgren EP, Peter JA, Lipinski RJ. A Simple and Reliable Method for Early Pregnancy Detection in Inbred Mice.

<https://doi.org/>

Step 5.5

Heyne GW, Plisch EH, Melberg CG, Sandgren EP, Peter JA, Lipinski RJ. A Simple and Reliable Method for Early Pregnancy Detection in Inbred Mice.

<https://doi.org/>

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