**Tema:**

## TÉCNICAS DE PERFUSIÓN HEPÁTICA EN RATA

**Fechas: 12 – 13 abril 2011**

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Hola a todos,

En mi Centro hay un investigador que obtiene microsomas de hígado de rata adultas, aproximadamente de 2-3 meses de edad. Me comenta si sería posible "decolorar" el hígado mediante una perfusión in situ de sacarosa 0.23 M a través de la vena porta. Yo no lo he hecho nunca. No sé exactamente a que se refiere, yo he perfundido con PFA ratones, pero era bajo anestesia y a través del corazón.

¿Alguien tiene experiencia en esta técnica? ¿Me podría explicar como hacerla? O bien, decirme dónde puedo obtener información de dicho procedimiento.

Muchas gracias!

Un saludo

Itziar

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Hola Itziar,

Yo lo hago con cierta frecuencia, pero no por porta , sino por aorta.

Supongo que cuando se refiere a "decolorar", hace referencia al color que adquiere el hígado cuando se perfunde bien (marrón claro).

El "truco", para adquirir una buena perfusión es:

1º Heparinizar al animal.

2º Velocidad de flujo de perfusión de aproximadamente 46 ml/minuto.

3º Mantener latido cardiaco (necesitas ventilación mecánica). Se puede hacer sin ventilación, pero entran en distres respiratorio, aparece edema pulmonar (esto en principio no te afectaría), pero mi experiencia es que no quedan tan bien los riñones ni los hígados.

4º Usar un catéter del 16G (para meter por aorta a la altura de la salida cardiaca).

5º Inmediatamente antes o después de introducir el catéter en aorta, cortar la aurícula derecha y cava ascendente en tórax.

Normalmente, para ratas de 300 grs. suelo emplear 70-90 ml de suero salino, Perfadex o Eurocollins para la perfusión. Por cierto, yo los empleo fríos (4-6ºC) pero no se si en tu caso eso es importante o no.

Bueno, espero haberte ayudado. Si necesitas algo más dame un toque al teléfono y te comento con más detalle.

Saludos

Jose L.

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Estimados,

adjunto protocolos sobre perfusión hepática en rata.

Espero que les sirva.

Un abrazo.

Micaela.

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Perfusion Protocol

Preparation for Perfusion: Fixative =4% PFA Prepare fresh on day of use or the night before and keep at 4oC.

4% Paraformaldehyde

3.8% Sodium Borate

Add 40g of paraformaldehyde to 800ml of water and heat to 60oC while stirring. Add 38g of sodium borate, paraformaldehyde will not dissolve until it is added. Cool off to 4oC. Adjust pH to 9.5 with glacial acetic acid.

4% PFA with 10% sucrose=

In a 100ml cylinder add 10g of Sucrose, than add 4% PFA to 100ml

Perfusion

* Anesthetize animal, open thoracic cavity, expose heart and visualize ascending aorta. Insert 18-gauge needle (butterfly) through left ventricle into ascending aorta- if possible, hard to tell sometimes. Clamp cannula into place with would clip or small clamp and then snip right atrium. (I find it's easier - on mice- to hold needle in place, because clamp gets in the way).

* Slowly perfuse animal with about 40-50ml room temperature saline (it's recommended to use a pump here, but I use 50 ml syringe, and perfuse slowly) until fluid is clear.

* Change to cold 4% PFA and slowly perfuse animal. When the animalâ€™s forelimbs are fully extended reduce perfusion speed, use about 50ml of fixative (for a 30g mouse).

* After perfusion is complete, decapitate the animal and quickly remove the brain using a caudal approach. Place brain in 4% PFA with 10% sucrose at 4oC for overnight. (The 4%PFA with sucrose will post-fix brain and cryoprotect tissue at the same time.) Some recommend to post-fix in 4% PFA-only, overnight, then cryoprotect with 30% sucrose for another 24 hours.=20

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Protocol for Perfusion-Fixation of the Rat

By: Tim Goble

Introduction: Many neuroscience protocols require you to perfuse and fixate a brain via the circulatory system. The rationale behind this process is that fixation is necessary to stabilize the proteins or peptides in the brain in order to allow for subsequent antibody binding. The rationale of the solutions as well as how to make solutions for this protocol are outlined in the 'Protocol for Making Solutions for Perfusion and Fixation of the Rat'. This protocol will give a step by step outline of a perfusion procedure as well as rationale for the techniques used.

Solutions:

0.5% Sodium Nitrite in 0.1M PBS

10% Sucrose in 0.1M PBS

4% Paraformaldehyde in 0.1M PBS

Water
Urethane Anesthetic (30%)

Preparation: Rat (similar procedures apply to other small mammals)

Equipment: (Re-usable)
Tubing Clamps (2)
Tygon Tubing
I.V. Administration Set
Formalin Waster Container
Roto-Motor Tool
Sucrose

Tools: (Washed and Kept in Tool Kit) Pliers
Blunted 18-G needle
Scalpel
Small Sharp-tipped Scissors
Medium Sharp/Blunt Surgical Scissors
Small Rib Splitter
Large Hemostat Forcep
Bone Rongeur
Spatula
Small Stir Bar
Magnetic Stir Bar Remover

Materials: (Disposable)
3-5 cc syringe
23-27 G 1 in needle
Disposable Scalpel Blade
Paper Towels
Specimen Bottle

Materials: (Washed After Use)
1L Aspirator Bottle with Base Glass Hose- Connector
Small Guillotine
Funnel
Animal Steel Cage Lid with Feeding Trough
Medium plastic pan with high walls
Large Sink
Stir Plate

Prepare Perfusion Pump: First you need to prepare your gravity-driven perfusion system. There are manufacture's peristaltic perfusion pumps with variable-speed delivery rates that can substitute for around $1000 dollars, but gravity works just as well.

Time: 1 hour

• Cut and attach a 2 in piece of Tygon tubing onto the glass hose connector at the base of the 1L Aspirator Bottle, wetting the tubing to ease application and ensure a tight seal (Make sure you use a tubing size that fits snugly on the flask!)
• Attach a circular tubing clamp over the Tygon tubing and the glass hose connector (Use pliers to attach tubing clamp, holding the Tygon tubing in place. Be careful not to break the glass fitting!)
• Attach I.V. Administration Drip Set to other end of Tygon tubing
• Attach a circular tubing clamp over the Tygon tubing and the I.V Administration Set (Use pliers to attach tubing clamp. Be careful not to damage I.V. Set!)
• Prepare a Blunted 18-G needle using a Roto-Motor Tool with Sanding Blade. Cut the needle so it is approximately 1 inch long. Use the Roto-Motor Tool to ensure optimal opening and blunted tip (Use commercially available blunt tipped 18-G if available!)
• Place Blunted 18-G needle on other end of I.V. Administration Set
• Pour water into the Aspirator Bottle
• Place the bottle at least 2 feet above working level so gravity will pull solutions through
• Open the adjustable roll clamp all the way open (Note: If water flows from the Bottle through the I.V Set out your blunt needle with no leaks, your equipment works! IF NOT, squeeze the drip bulb on the IV Set to get water flowing. IF NOT, check to make sure the shutoff and roll clamps are open and that your blunt needle is not clogged).

Prepare Perfusion Site: Make sure that you have adequate light and adequate room by a sink in your laboratory. (Note: For the Thompson Lab, perfusions are NEVER done in the main lab in MP2.232, to prevent contamination of the slice preparation environment. Refer to Protocol for Making Solutions for reason.) Fully preparing your perfusion siteand laying out all needed items beforehand, is critical to a successful perfusion. If you have to run to the refrigerator for a solution or if you have to dig for a tool, that could be missing…, you lose critical time and might end up with a bad perfusion.

Time: 15 min

• Again, place your gravity perfusion setup around 2 ft above the sink, but close enough to change solutions and sturdy enough so it won't fall or spill solutions during the perfusion
• Obtain an Animal Steel Cage Lid with Feeding Trough. Place the lid upright, trough side toward the back of the sink, on top of the sink basin. (Note: If the cage lid does not fit on top of the basin, obtain a dish-drying rack or other item to place under the cage lid for optimal fit)
• Spread out paper towels in a close location to the cage lid in your working area
• Place your surgical tools on the paper towels (Note: This makes your tools easier to see and also gets you into the habit of putting your tools out before you need them!!!) This includes your syringe with needle, Urethane, scalpel with disposable scalpel blade, both scissors, hemostat forceps, rongeur and funnel
• Place your medium plastic pan into the base of the sink

Prepare Perfusion Solutions: By now, you have already made your solutions and have made a batch of paraformaldehyde within the last 48 hr. They are all sitting in the refrigerator and it's time to get them optimal to perfuse. Most opinions state that room temp is a reasonable temp for solutions. Body temperature is too warm and will degrade your fixative and 4C (refrigerator) is too cold and may induce some thermal shock. Most professionals also say they don't have definitive studies to prove this. Some use body temperature for initial buffer rinse, room temperature for a fixative and a cold post-rinse sucrose solution.

Time: 20 min

• Fill the plastic pan that is in the base of the sink with hot water to make a water bath
• Place your Sodium Nitrite, Paraformaldehyde, and Sucrose solutions in the water bath, loosen the lids (solution will expand with high temp), and warm up for 5-10 minutes
• Empty the water you had running through your perfusion pump
• Place 300-500mls of 0.5% Sodium Nitrite is 0.1M PBS into the perfusion pump using the funnel
• Let the Sodium Nitrite running at full speed through the IV Set to ensure sodium nitrite is running through the pump with zero air bubbles and zero plain water
• Play with the drip rate by changing the roll clamp. The optimal drip rate for the initial Sodium Nitrite is around 20ml/min or around 3 drops in the IV bulb/second
• Make a marking on the adjustable roll clamp at 3 drops/sec for a quick reference to the appropriate rate when opening
• Remove the other solutions from the water bath, empty the water bath, and place solutions in your working space so you can change the solutions when the time comes

Anesthesia and Placement of Perfusion Needle into the Right Ventricle: Urethane is a carcinogenic anesthetic. The advantages of urethane is that it can be administrated by several parenteral routes, produces a long-lasting effects. Urethane results in significant alterations of renal hemodynamics, also causing reproductive problems. (CAUTION!!! The Protection of Research Subjects and Animal Care Committee state that urethane represents a significant health risk and reproductive hazard to laboratory personnel. Urethane can be absorbed through the skin, been known to cause many different cancers)

Time: 1 to 1 ½ hr

• Draw approximately 1cc/100g of urethane into your syringe
• Recap needle until ready for use
• Prepare rat by administering a lethal dose of urethane
• Monitor the rat until the point when the rat fails to respond to tail pinching, eye reflex, and whisker twitching (The heart needs to still be beating)
• Place the rat, belly up – tail towards you, into the trough part of the cage lid
• Using the scalpel, make a surgical cut along the midline to expose the rib cage, from abdomen to neck
• Using the scalpel, make a surgical cut along the diaphragm
• Using the Sharp/Blunt Surgical Scissors, insert the sharp tip 1-inch into the base of the rib cage, close the scissors to cut through the rib cage (Note: Be careful. You risk cutting the heart or lungs if you stick your scissors too far into the rat. This cut is a starter cut into the ribs)
• Turn the Sharp/Blunt Surgical Scissors over to the Blunt tip and insert into the starter cut you just made

• Make small ½ inch cuts, angling the scissors parallel with the rat, all the way through the rib cage
• Open the thoracic cavity by using the rib splitter or by manually cracking the rib cage open
• Clear away the connective tissue around the heart (Note: The heart should still be beating!!)
• Insert the 18-G needle about 1 inch into the Apex (tip) of the left ventricle of the heart going toward the top where the ascending aorta connects (Note: See the addendum for a cross-section of the rat's heart. It will help!!)
(Note: You may use a bulldog clamp to secure the needle in the heart. This can also tear up the heart and cause many problems)
• Be careful not to pull out the needle throughout the surgery
• Open the adjustable roll clamp to previously marked rate (Sodium Nitrite)
• Look for the right atrium (It will be on your left side, will be a darker colored flap)
• Using the small sharp tipped scissors cut a slit into the right atrium (Blood will flow out) (Note: Cutting the atrium may be delayed until it has become expanded by the introduction of the perfused solution)
• Perfuse with Sodium Nitrite for 3-5 minutes

NOTE: At this time, you may clamp the descending aorta using the hemostat forceps. This will speed up the perfusion process, only perfusing the top half of the body.

Get to know your rat's circulating blood volume: The goal of the initial sodium nitrite wash is to clear to vascular system of blood. The mean of circulating blood volume, derived through research, is 64ml/kg, or if your rat weighs 250g = 16ml of blood. At a perfusion rate of 20ml/min, this should be accomplished within 3-5 minutes, with overkill even if your rat weighs 500mls.

• After the solution runs clear, it is time to change solutions to your 4% Paraformaldehyde in 0.1M PBS
• Empty most of the remaining Sodium Nitrite from the Aspirator Bottle (NOTE: Do not empty all the way. DO NOT get air into the tubing!!)
• Using the funnel, pour around 400-500mls of paraformaldehyde into the bottle
• Continue to perfuse around a rate of 20ml/min such that 400mls is perfused over 10-20 minutes (NOTE: When the fixative begins to enter the rat, there may be some twitching of muscles, which provides an indication that the perfusion is proceeding properly. After several minutes, there should be some indication of stiffening of the forelimbs and head.)
• Formalin is a carcinogenic! Place the pan under the rat to catch the formalin dripping from the rat
• Using a funnel, pour 200-300mls of 10% Sucrose in 0.1M PBS into the Bottle and perfuse at the same rat for 5-10 minutes (The purpose of sucrose is to prevent freezing artifacts in the tissue during sectioning)
• While the Sucrose is running through, carefully take the formalin waste and a funnel and place into a formalin waste container (Wash the funnel and pan out with water)
• Prepare a specimen bottle by adding 5mls of 10% Sucrose in 0.1M PBS and 1.5g of sucrose
• Place the small stir bar in the bottle and place of the stir plate
• Turn on the heat to low and the stir rate to a level where you visibly see the bar rotating
• Spin until sucrose is dissolved
• Remove stir bar with large magnetic stir bar remover

Brain Removal: The rat has now been perfused. Take out the needle from the heart, empty the sucrose solution and wash out the bottle/perfusion pump with hot water. Let the water run through the IV Set and needle for a few minutes.

Time: 20 min

• Place the small guillotine in your work area, with some paper towels on the base
• Place the rat's head inside the blades of the guillotine (CAUTION: Do not place your fingers inside the guillotine!!)
• Cut the head off (There is no rush now, take your time)
• With the scalpel, cut the skin by making a lateral incision between the ears to the cut
• Spread the skin
• Using the rongeurs, carefully remove small pieces of the rat's brain (NOTE: TAKE YOUR TIME!! Do not damage the brain by taking chunks of it out with the rongeurs or by using it as leverage to take off the skull)
• Remove until the frontal lobes of the brain are visible
• With a spatula, carefully slide your spatula between the frontal lobes of the brain and the skull
• Bluntly, tear the olfactory bulbs and the cranial nerves as the slowly lift the brain from the skull
• Place the brain in the specimen bottle, and close with a lid (NOTE: The brain will float until the brain has soaked up enough of the 30% sucrose)
• Appropriately label the bottle with the following information; [Rat #], [30% Sucrose in 0.1M PBS], [DATE], [Initials]
• Store in the refrigerator in the undergraduate lab. There are still remnants of fixative that could kill tissue that you want to stay alive.
• Place the solutions back in the refrigerator

• Wash off all the tools, rack lid, guillotine
• Wrap rat and head securely and place in the freezer (When the freezer is pretty full, discretely place them in a trash bag and take them to the freezer in the animal facility)
• Put away all of the tools, wash and clean your work space