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# Technique for Performance and Evaluation of Parapharyngeal Hypophysectomy in Mice

**Janet B Hoff,[1,*] Robert Dysko,[2] Sumiko Kurachi,[3] and Ko Kurachi[3]**

**Mice at our institution were hypophysectomized to evaluate the effects of growth hormone on the expression of a transfected human factor IX gene. The hypophysectomy was performed in-house by using a parapharyngeal approach modified from previously published surgical techniques. Modifications included: 1) choice of ketamine–xylazine and isoflurane for anesthesia, with butorphanol for postoperative analgesia; 2) use of a V-trough for positioning mice correctly and consistently; 3) selection of increasing sizes of dental burrs to create a foramen in the cranial base through which the pituitary gland was removed; and 4) disuse of a tracheotomy for airway patency. In addition, verification of successful gland removal was assessed by measuring major urinary protein (MUP) in the urine; presence of MUP indicated incomplete hypophysectomy. This assessment enabled antemortem determination of surgical success by using a single urine collection. Each of these modifications contributed to the success of the surgical procedure. We had a safe and reliable anesthetic regimen, consistent positioning of the surgical patient, and smooth and rapid penetration of the cranium. In our experience, the tracheotomy described in previous techniques was unnecessary, as the mice tolerated brief periods of apnea (approximately 5 sec maximum) while the trachea was retracted. Here we seek to provide details that will assist those interested in learning this technique and that will reduce the number of mice needed for practice. Other applications include a method of evaluating the production of growth hormone without euthanizing the animal.**

**Abbreviations:** GH, growth hormone; MUP, major urinary proteins

One means to study the physiologic functions of growth hormone is to surgically remove the pituitary gland (hypophysectomy) and assess the resultant effects during the postsurgical period. The function of hormones produced by the pituitary gland has often been evaluated in rodents using surgical hypophysectomy.[6,10] In fact, several commercial production facilities provide hypophysectomized rodents of various stocks or strains to research institutions.[4] As an alternative to surgical removal, the pituitary gland can be irradiated,[2] or growth hormone function can be blocked pharmacologically.[2,5] Ultimately, we chose surgical hypophysectomy because the adverse endocrine consequences of pituitary removal were better understood than those resulting from radiological or pharmacological treatment, and the small quantities of growth hormone produced elsewhere in the body[11,15] were unlikely to affect the experiment. Although hypophysectomized rodents are commercially available, fragile genetically manipulated strains likely would be exponentially stressed by both recovery after a surgical procedure and subsequent shipment from the production facility to a research institution. Furthermore, commercial production facilities typically perform surgical manipulations on in-house strains only; they do not routinely accept mice from other sources for surgical procedures. With the continued creation of transgenic and 'knock-out' mutational mouse strains, there is increased rationale for hypophysectomy procedures to be performed by the research laboratory staff at the home facility rather than by the commercial vendor.

The pituitary gland is located immediately within the ventral aspect of the cranium. The gland consists of anterior, intermediate, and neural lobes, but it appears as a single pale-pink elliptical gland. For postmortem removal of the gland, the dorsal skull is incised, and the brain is removed to reveal the pituitary gland in the bony depression (sella turcica) between the conspicuous trigeminal nerves. This approach cannot be used for survival hypophysectomy; an alternate approach must be used.

There are 2 established approaches for hypophysectomy in rodents: transauricular and parapharyngeal. The parapharyngeal approach accesses the pituitary gland through the floor of the bony cranium. Smith described the parapharyngeal approach for rats in 1930,[14] and Selye and colleagues noted that mice tolerated the parapharyngeal approach better than rats did.[12] In 1955, Lostroh and Jordan described an improved procedure for parapharyngeal hypophysectomy in the mouse.[8] For our study, we followed the basic technique of Lostroh and Jordan[8] but with several modifications, including a more secure method of animal stabilization (V-trough), the use of 2 sizes of dental burrs, and the use of a preanesthetic cocktail (ketamine–xylazine) combined with isoflurane administered through a precision vaporizer. In addition, we chose not to perform a tracheotomy, as previously described for this procedure;[8] we found that we could avoid this invasive technique because the mice tolerated periodic tracheal manipulation.

A procedure for verifying complete removal of the pituitary gland of the mouse, prior to its use in an experimental study and without requiring euthanasia, has not been described previously. Lostroh and Jordan verified completeness of pituitary removal at postmortem examination by using the weights of adrenal glands, ovaries, and uterus, which atrophy after successful hypophysectomy.[8] The authors also examined the hypophyseal fossa for pituitary remnants. We identified a potential verification method based on the action of growth hormone (GH) on hepatic proteins. Major urinary proteins (MUP) are a

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*[1]Center for Integrative Genomics and [2]Unit for Laboratory Animal Medicine, Ann Arbor, Michigan; [3]Age Dimension Research Center, AIST Tsukuba Central 6th site, 1-1-1, Higashi, Tsukuba, Ibaraki, 305-8566, Japan.*
*[*]Corresponding author. Email: jhlvt@umich.edu*

**Table 1.** Key items needed

| Equipment item | Manufacturer | Purpose |
|---|---|---|
| Dental drill with high-speed hand piece and foot switch (Star – 430K) | Star Dental Lancaster, PA | Drive the dental burrs |
| #2 and #4 round dental burrs | Meer Dental Ann Arbor, MI | Create a foramen in the ventral cranium |
| Air compressor with 2-horsepower, 4-gallon tank | Sears, Roebuck Hoffman Estates, IL | Provide compressed air to power the dental drill |
| Dissecting microscope with 0.5–3.0× magnification | Cambridge Instruments Inc. Auburn, NY | View the ventral surface of the cranium |
| Fiberoptic light | Foster Auburn, NY | Facilitate visualization of surgical site |
| Vacuum pump with 5–10 pounds of vacuum pressure | Gomco Surgical Buffalo, NY | Remove the pituitary from the cranium |
| Germinator dry sterilizer | Cellpoint Scientific Rockville, MD | Sterilize instruments |
| Rechargeable cordless animal hair clippers (series 8900) | Wahl Clipper Sterling, IL | Remove the fur over the surgical site |
| Two 4 1/2-in. splinter forceps with fine points | Henry Schein Port Washington, NY | Separate tissue so that it can be removed from the surgical site |
| Pin holder with chuck | Fine Scientific Tools Foster City, CA | Hold wire loop that retracts the trachea |
| Dental spatula | Fine Scientific Tools Foster City, CA | Scrape periosteal tissue from surgical area |
| Wound clips and wound clip applicator | Stoelting Wood Dale, IL | Close skin |
| Operating board | Custom made | Restrain mouse |
| '2–finger hook' elastic stays (332X series) | Lone Star Medical Products Houston, TX | Retract salivary glands |
| Cellulose sponge on a polypropylene handle (Weck-Cel surgical spear) | Solan Ophthalmic Products Jacksonville, FL | Absorb blood and fluid from hypophysectomy site |

family of α2-microglobulin-related proteins that are produced and secreted by the liver and excreted by the kidneys into the urine.[16] Production of MUP has been shown to be controlled by GH.[9] 'Little' mice, a strain that is GH-deficient, have very low urine levels of MUP.[9] Injections of GH into Little mice elevate urine MUP levels to those of normal male mice.[9] Because hypophysectomy removes the primary endogenous source of GH, we expected urine levels of MUP to decrease postsurgically to levels similar to those observed in the Little mice.

We chose surgical hypophysectomy as a method to study the role of growth hormone on transgenic mice carrying a mutant human factor IX gene. Factor IX is a circulating serine protease precursor that functions as a key blood coagulation factor. Hemophilia B is a sex-linked recessive hemophilia that is caused by an absence of, or abnormal, factor IX. The Leyden form of hemophilia B is an interesting variant in that factor IX levels in affected males gradually increase once they reach puberty, and the disease abates.[3] Single-nucleotide mutations within a 60-nucleotide region of the factor IX gene (nucleotides –40 to +20) result in development of the Leyden phenotype.[7] A breeding colony of mice transfected with a mutant human factor IX gene was established at our institution. In order to study the influence of GH on the postpubertal induction of factor IX gene expression and the amelioration of the clinical disease, GH was eliminated from mice in this colony.

## Materials and Methods

**Animals.** We hypophysectomized 20 male mice in this study. The mice were C57BL/6 × SJL hybrids transgenic for the human coagulation factor IX. Male mice were chosen to establish an effect on 1 sex and because the human coagulation factor IX gene is on the X chromosome. The mice were housed at the University of Michigan, in facilities accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. This study was approved by the institutional animal use and care committee to ensure that animals were well cared for and treated humanely. The mice used in this study were fed a commercial rodent chow (LabDiet 5008, PMI Nutrition International, Brentwood, MO) and provided municipal tap water ad libitum via bottle. They were housed in either stainless steel boxes with pine shavings for bedding or polycarbonate boxes with wire-bar lids (no filter tops) on corncob bedding (Bed-o'-cobs, The Andersons, Maumee, OH). The mice in this facility were not maintained in a specific pathogen-free state, and sentinels from the room historically had been positive serologically for mouse hepatitis virus and rotavirus. Sentinels were negative for Sendai virus, pneumonia virus of mice, minute virus of mice, mouse parvovirus, Theiler's murine encephalomyelitis virus, reovirus 3, *Mycoplasma pulmonis*, lymphocytic choriomeningitis virus, mouse adenovirus, ectromelia virus, K virus, polyoma virus, and pinworm species.

**Equipment.** Major equipment items and their manufacturers are listed in Table 1.

**Anesthesia and patient preparation.** Mice were preanesthetized with 0.5 ml/kg of an anesthetic cocktail consisting of ketamine (Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (Akorn, Decatur, IL) corresponding to 37.5 mg/kg ketamine and 2.5 mg/kg xylazine, administered intraperitoneally.

**Figure 1.** The V-trough was made from a 5.0 × 7.5-cm sheet of aluminum that was 1.5 mm thick. From the top 1/3 of the aluminum sheet, we removed a 2.5 × 1.9-cm section from each corner. From the middle third of the piece, a 2.5 × 1.2-cm section was removed from each edge. The bottom 1/3 of the aluminum sheet was left as is, thereby leaving a single 7.5-cm piece of aluminum that was 1.2 cm wide at the top 1/3, 2.5 cm wide at the middle 1/3, and 5.0 cm wide at the bottom 1/3. At the center, the sheet then was bent up at a 45°-angle and then down again at a 45°-angle for the lateral 'wings' of the bottom 1/3 of the piece.

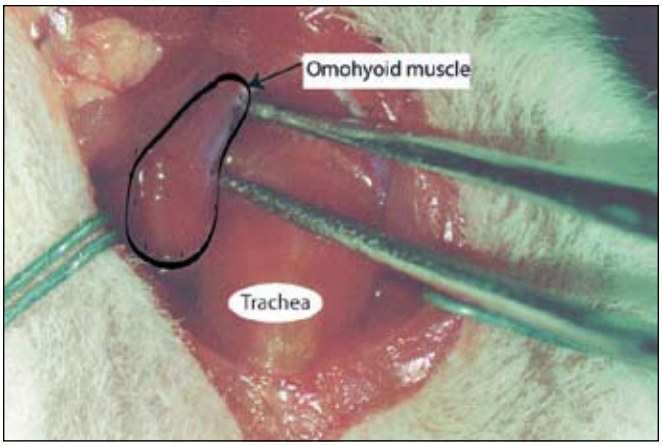


**Figure 2.** The omohyoid muscle was separated from the trachea so that it could be retracted cranially and laterally.

The cocktail was prepared by mixing 3 ml (300 mg) ketamine with 1 ml (20 mg) xylazine. This anesthesia cocktail provided enough sedation to immobilize the mouse so that he could be positioned precisely—level from the tip of the nose to the sternum—on a specially designed V-trough. A nosecone then was placed on the nose, and anesthesia was maintained with 1% to 2% of vaporized isoflurane in 100% oxygen for the duration of the surgical procedure.

The V-trough was made from a 5.0 × 7.5-cm piece of aluminum that was 1.5 mm thick (Figure 1). The head was held in position with a loop of wire attached to the inside of the nosecone, so that just the nose and mouth were inside the nosecone, leaving the pharyngeal incision site easily accessible. The pedal reflex was monitored to ensure adequate depth of anesthesia. The front feet were restrained laterally by using retractors fashioned of pipe cleaners and rubber bands and attached to the sides of a rodent surgical board. The tail was directed caudally and secured with a piece of tape. To maintain body temperature during surgery, a latex glove filled with warm water was placed over the tail and around the sides of the mouse.

For optimum performance of the procedure a small wedge (for example, a 2.5 × 2.5-cm piece of cardboard) was placed under the V-trough below the mouse's head and neck. The exact size and location of the neck support varied, depending on the size of the mouse and the position of the mouse's head in relation to the nosecone. Most importantly, the tip of the nose and the sternum had to be level (essentially forming a straight line parallel to the operating board), and the dorsal aspect of the cervical region had to be resting on a solid surface.

The fur was removed from the ventral surface of the neck by using animal clippers. A surgical scrub was performed using alternating sponges soaked in chlorhexidine solution (Nolvasan, Fort Dodge Animal Health, Fort Dodge, IA) and alcohol. A 12.5 × 17.5-cm sterile drape was placed over the mouse; this size of drape provided an area on either side of the mouse on which to place sterile surgical instruments.

**Hypophysectomy procedure.** On the ventral surface of the neck, a 1-cm midline incision was made with sharp iris scissors. The skin was carefully dissected away from the connective tissue. Using two 4 1/2-in. splinter forceps with fine points (one in each hand), the surgeon gently separated the salivary glands at the center and retracted them by using '2-finger hook' elastic stays (332X series, Lone Star Medical Products, Houston, TX). Retraction of the salivary glands revealed the trachea and omohyoid muscles. The omohyoid muscles are located on both sides of the trachea in the cranial aspect of the incised area and are distinguishable by their teardrop shape. One of the omohyoid muscles (typically on the animal's right) was separated from 1 side of the trachea using the splinter forceps (Figure 2). The muscle was then retracted cranially and laterally. If the lateral branches of the jugular vein became visible, they were retracted laterally. The trachea was retracted for short periods of time with a wire loop inserted into a pin holder (Figure 3). Tracheal retraction enabled visualization of the ventral surface of the cranium (Figure 4). A dental spatula then was used to scrape a 4- to 6-mm section of periosteal tissue away from the cranium directly beneath the trachea.

The surgical site is surrounded by veins, arteries, and nerves. During manipulations of tissue in this area, it was vitally important to concentrate on the center of the ventral surface only and not to drift in either direction. When the periosteal tissue was removed, the occipitosphenoidal synchondrosis (suture line) appeared as a faint blue line (Figure 5A and 5B).

To penetrate the ventral bony cranium, a high-speed dental drill with a foot switch (Star – 430K, Star Dental, Lancaster, PA) was used, pressurized by a 2-horsepower 4-gallon air compressor (Sears, Roebuck, Hoffman Estates, IL). A #2 dental burr was inserted in the dental drill and placed on the center of the suture line. Using alternating firm and gentle pressure, a small hole was made through the suture line. Drilling was terminated when the burr created a foramen through the cranial wall or when soft cream-colored tissue became apparent around the dental burr (Figure 6). The size of the foramen then was increased by drilling in the same site with a #4 burr. At this point the pituitary gland was accessible for removal. By using a modified 16-gauge needle (beveled point filed off) connected to a vacuum pump, the pituitary gland gently was suctioned out of the cranium. It is vitally important that the needle is passed over the foramen several times and that no direct negative pressure is applied to the foramen. A small cellulose sponge on a polypropylene handle (Weck-Cel surgical spear, Solan Ophthalmic Products, Jacksonville, FL) was used to absorb any bleeding that occurred. Once the gland was removed, isoflurane administration was

**Figure 3.** Wire loop inserted into the pin holder; this instrument was used for tracheal retraction.

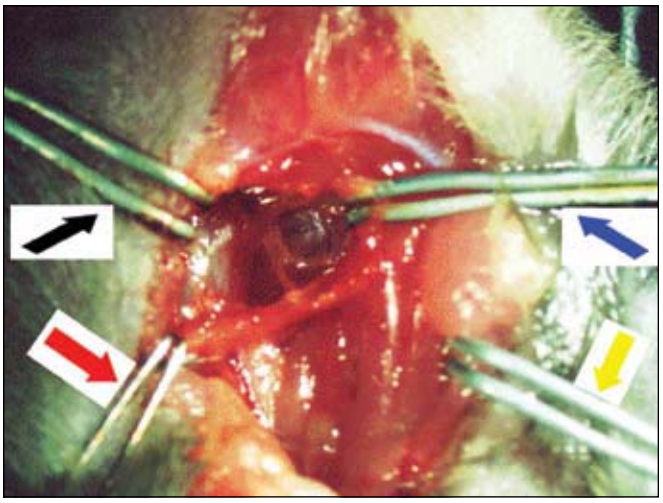

**Figure 4.** The trachea was retracted to visualize the base of the cranium. The blue arrow points to the wire loop (shown in Figure 3) that is retracting the trachea; the black arrow points to the retractor holding the omohyoid muscle and skin; the yellow arrow points to the retractor holding the skin; and the red arrow points to the retractor holding the skin, jugular vein, and connective tissue.

discontinued, and the mouse was maintained on 100% oxygen for 3 to 5 min. The trachea, muscles, and salivary glands were restored to their anatomic locations and the skin incision closed with wound clips. The entire procedure took about 25 min to complete.

**Postoperative animal care.** Post-operatively, all mice were given butorphanol (2 mg/kg) and 2 ml lactated Ringer's solution subcutaneously. Body temperature was maintained by placing a heat lamp at a safe distance over the mouse. Mice typically recovered completely from the anesthetic within 30 to 60 min. A laboratory animal technician monitored mice for any adverse affects, including loss of appetite, dehydration, swelling, and lethargy. The laboratory animal care technician for this study determined that mice did not need additional doses of postoperative pain medication. Because more emphasis has been placed on postoperative pain management in rodents since this study was conducted (1998), mice might have received a lower dose of butorphanol more often, such as every 4 h for the first 24 to 72 h postoperatively. Typically, mice that survived the first 72 h postsurgery continued to do well for the duration of the study. If normal posture and behavior did not return within 72 h, the mouse was euthanized by carbon dioxide asphyxiation.

After hypophysectomy, mice were given drinking water supplemented with 10% glucose for the duration of the study (3 mo). Glucose water was provided to reduce the possibility of hypoglycemia that can occur upon removal of the pituitary gland. This hypoglycemia is a result of the loss of both adrenocorticotropic hormone and GH. These pituitary hormones increase blood glucose concentrations and counterbalance the effects of pancreatic insulin.[13,14] Thus, their absence can lead to fasting hypoglycemia in hypophysectomized rodents not provided with glucose-supplemented drinking water. A commercial laboratory that performs hypophysectomy on mice provides 5% glucose or sucrose in the drinking water as standard postsurgical diet manipulation;[4] Adams[1] suggests administration of a 5% glucose solution intraperitoneally for 3 d.

**Measurement of mouse MUP.** To assess for the presence or absence of MUP, 5-µl samples of urine were collected at 10 d postsurgery (to eliminate any effects from residual hormone). Samples were collected simply by picking up a mouse and collecting urine, as they typically urinate upon handling. Occasionally, mice would have to be handled several times to collect sufficient urine. Samples were collected at 0900 from an unoperated control mouse and the hypophysectomized mice. Urine samples were heated with sample buffer at 99 °C for 3 min and applied to a 12% denaturing polyacrylamide gel at a pH of 8.8. Electrophoresis was performed at 130 V for 20 to 30 min until the bromphenol blue indicator ran down. The gel was stained with Coumassie Brilliant Blue G250 dye for 20 min and then destained (Figure 7). MUP, when present, appeared as a single band at the approximately 20 kDa-level.[9]

## Results

Of the first 20 mice (75%) that underwent the surgical procedure and were allowed to recover, 15 survived for the duration of the study (3 mo). The 5 mice that did not survive died within 24 h after the surgical procedure. No necropsies were performed to determine cause of death. Of the 15 surviving mice, 10 (67%) were completely hypophysectomized as determined by the lack of MUP excretion, another 4 mice (27%) were partially hypophysectomized (intermediate levels of MUP), and the remaining mouse was considered to have had a completely unsuccessful surgery (normal MUP excretion). The numbers presented are representative of the 1st group of mice that were allowed to recover and that survived for the duration of this study. The mortality rate likely will decrease and the number of successful complete hypophysectomies increase as surgeons gain more experience in performing the technique.

## Discussion

Here we describe a novel method of anesthesia and postoperative pain management, as well as a detailed description of surgical techniques, intended to facilitate investigators interested in learning this procedure. Previous publications describing a technique for performing hypophysectomy in mice are more than a decade old, and in the interim, improvements in anesthetics and analgesics for rodents and published illustrations have been made. We found it valuable to begin by practicing on mice that had already been euthanatized. Once trained on these mice, we proceeded to perform the surgical procedure on mice anesthetized with 1.0 ml/kg of a ketamine–xylazine cocktail (300 mg ketamine combined with 20 mg xylazine), and we euthanized

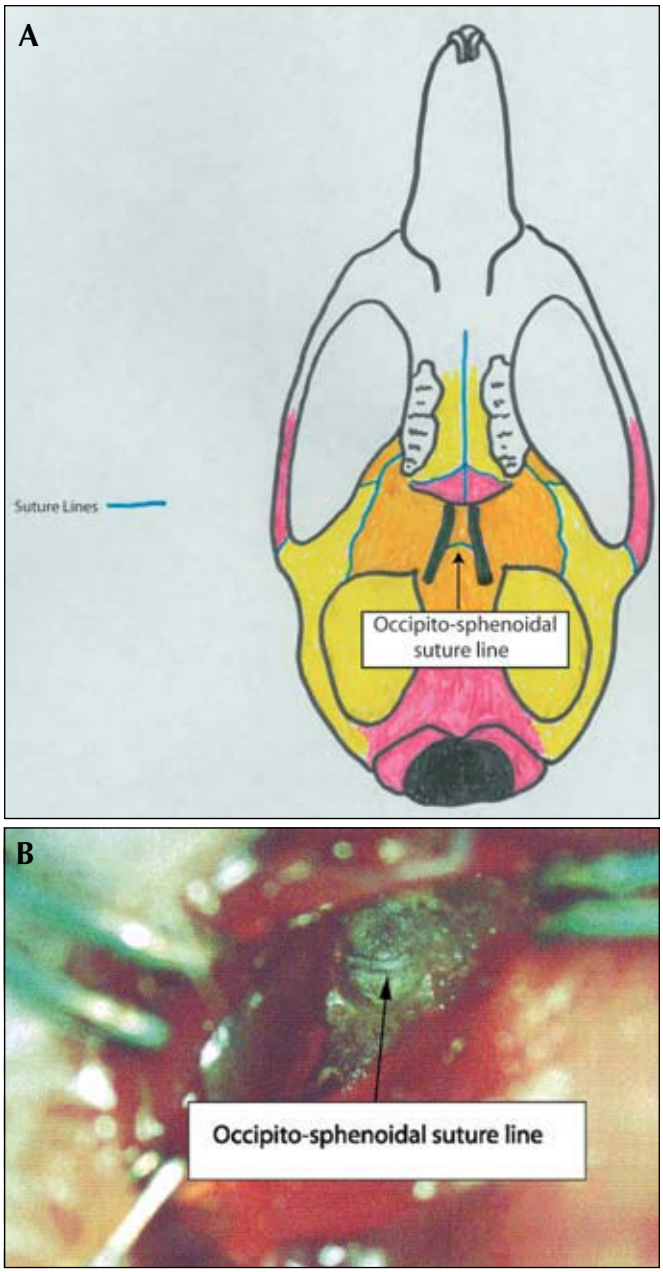


**Figure 5.** (A) A sketch of the ventral surface of the mouse skull modified from Popesko and colleagues, 1990. An arrow indicates where the suture line is located. (B) A photograph of the suture line during the surgical procedure. An arrow points to the suture line.

the mice immediately at the conclusion of the practice surgeries. To maximize the use of mice already in the facility and to avoid the purchase of mice solely for practice, we used naive mice (no previous surgical manipulation) scheduled for euthanasia. Only after we felt comfortable performing the technique did we allow any mice to recover from the anesthetic.

While developing and practicing the surgical technique, we found that cannulating the trachea for airway control, as described by Lostroh and Jordan,[8] caused unnecessary trauma and did not improve the survival rate. Mice on this study were maintained on isoflurane and oxygen during surgery by using a rodent-sized nosecone over the nose and mouth. The ability to precisely control anesthetic depth according to the mouse's individual response to anesthesia yielded a lower mortality rate than when the surgery was performed using

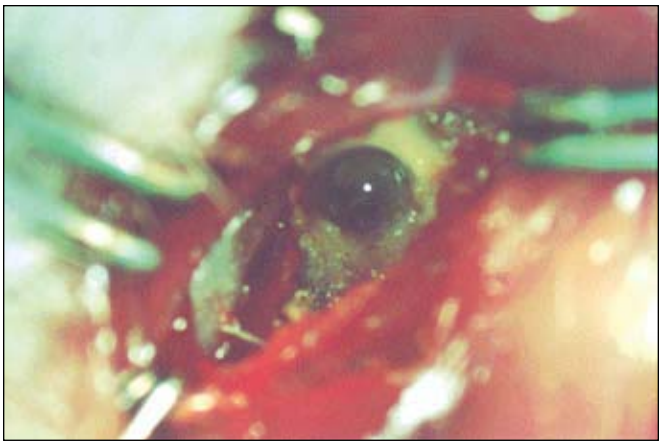

**Figure 6.** Cream-colored tissue became apparent around the hole created by the dental burr, indicative of penetration into the cranium.

injectable anesthetics alone. Inhalant anesthesia also has the postsurgical benefit of a rapid recovery time, although the novel approach of using preanesthetics, such as the cocktail described in the anesthesia and subject preparation section of this report, prolonged the effects of anesthesia so that mice did not recover too quickly. Premedicated mice recovered more slowly and appeared to be less stressed (in light of their body posture and normal movement) than mice anesthetized with isoflurane alone.

To produce hypophysectomized mice that recovered comfortably, it was imperative that the amount of bleeding was minimized and that the dental burr was not advanced into the cranial vault. The ventral bony cranium is surrounded by veins and arteries. A steady hand is needed to avoid major hemorrhage. By using a smaller (#2) burr initially, cutting through the bone is quick and easy. Subsequently, the #4 burr can readily enlarge the hole created by the #2 burr. Both of the burrs we chose are smaller than the #5 burr used by Lostroh and Jordan.[5] We had found that too much pressure was needed to push the #5 burr through the bone and that it was difficult to keep the burr out of the cranial vault after bony penetration, a development that could result in immediate death. Alternating firm and gentle pressure on the dental drill also aids in keeping the burrs out of the cranial vault. Complications also can occur if the suction needle is placed directly over the foramen; doing so can result in rupturing the membrane that separates the pituitary from the brain proper. Extensive hemorrhage also can result if care is not taken when working near the branches of the jugular vein. A very slight movement in the hands can result in laceration of these delicate vessels. The advantages of V-trough placement and removal of the tracheotomy procedure are more difficult to quantify. However, we felt that these modifications also contributed to procedure success. In fact, adherence to the many details of this surgical procedure (both original and our modifications) is very important as it will reduce variables and decrease the number of animals needed for practice.

Examining the MUP level after surgery enables the investigator to maintain mice for as long as needed with confidence that the surgical removal of the gland was successful. Urine collection is very simple to achieve in mice and requires no technical expertise. MUP was decreased for partially hypophysectomized mice and was undetectable for completely hypophysectomized mice. If the pituitary gland was removed completely, the mouse no longer excreted MUP. This assay enabled us to determine the completeness of the hypophysectomy without euthanizing mice

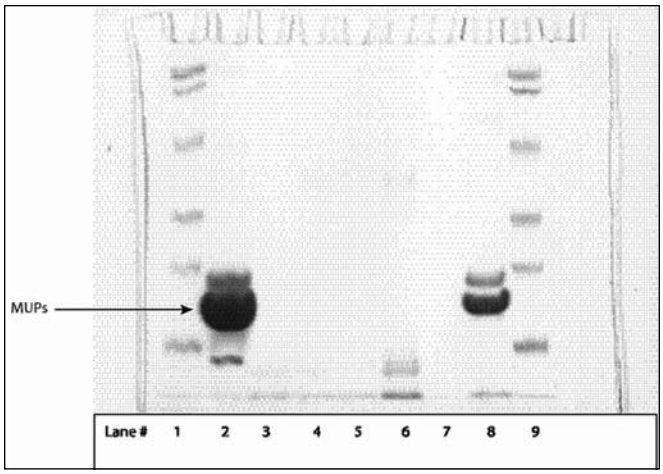


**Figure 7.** Denaturing polyacrylamide gel demonstrating the 20-kDa band corresponding to major urinary protein (lane 2); this band is absent in samples from 5 hypophysectomized mice (lanes 3 through 7) but present in the sample from one mouse with incomplete hypophysectomy (lane 8). Lanes 1 and 9, molecular weight markers.

or measuring serum concentrations of pituitary hormones. It is possible that the reliability of the assay could be substantiated in future studies. Correlation of MUP results with GH levels or histologic evaluation of the surgical site would have been scientifically valuable.

As unique transgenic and mutant mice, bred in research institutions, become more popular models in studies of growth hormone (or other pituitary hormones), the need for investigators to become trained in this surgical technique increases. The procedure described is not uncomplicated and requires the purchase of some expensive equipment, but it may be the only method available to assess pituitary endocrine functions in certain genetically manipulated strains of mice.

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