

Video Article

Complete Thymectomy in Adult Rats with Non-invasive Endotracheal Intubation

Victoria R. Rendell¹, Charles Giamberardino², Jie Li², M. Louise Markert^{2,3}, Todd V. Brennan¹

¹Department of Surgery, Duke University Medical Center

²Department of Pediatrics, Duke University Medical Center

³Department of Immunology, Duke University Medical Center

Correspondence to: Todd V. Brennan at todd.brennan@duke.edu

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Abstract

Thymectomy in neonatal rodents is an established and reliable procedure for immunological studies. However, in adult rats, complications of hemorrhage and pneumothorax from pleural disruption can result in a significant mortality rate. This protocol is a simple method of rat thymectomy that utilizes a mini-sternotomy and endotracheal intubation. Intubation is accomplished with a non-invasive and easily reproducible method and allows for positive pressure ventilation to prevent pneumothorax and a controlled airway that allows sufficient time for careful thymus dissection to minimize pleural disruption. A 1.5 cm sternal incision decreases contact with mediastinal vessels and pleura, while still providing full visualization of the thymus. Following exposure of the mediastinum, the thymus is removed by blunt dissection under magnification. The pleural space is then sealed by suture closure of the pre-tracheal muscles followed by the application of surgical glue. The thorax is then closed by suture closure of the sternum, followed by suture closure of the skin. All thymectomies were complete as evidenced by immunohistochemical (IHC) staining of mediastinal tissue, and absence of naïve T-cells by flow cytometry, and the procedure had a 96% survival rate. This method is suitable when complete thymectomy with minimal complications is desired for further immunological studies in athymic adult rats.

Video Link

The video component of this article can be found at <http://www.jove.com/video/52152/>

Introduction

Since the early 1960s, the thymus has been recognized for its critical role in the development of central immunological tolerance. Rodent thymectomy has proven to be an essential procedure in defining the role of the thymus in lymphocyte differentiation, self-tolerance, and immunotolerance in the setting of allograft transplantation and tumor metastasis. Removal of the rat thymus allows for studies involving T-cell depletion or the adoptive transfer of defined T cell populations without the re-emergence of native naïve T cells.

Thymectomies in neonatal rodents can be accomplished using a suction technique with reliable outcomes¹. In adult rats, this technique is associated with an approximate 20% mortality rate and frequently results in an incomplete thymectomy². To consistently achieve complete thymectomy in adult rats, open exposure of the mediastinum through a median sternotomy is required. However, this procedure is associated with complications that include tracheal injury, hemorrhage and pneumothorax leading to an overall mortality rate ranging from 1.5 - 6%²⁻⁴.

Over the last two decades improvements in thymectomy techniques have decreased perioperative complications and have improved survival rates. Endotracheal intubation allowing for positive pressure ventilation has lessened pneumothorax rates⁵. Methods of intubation previously described range from open exposure to the trachea to less invasive methods using direct vocal cord visualization. Complications associated with the intubation procedure include tracheal injury, vocal cord rupture, unintended esophageal intubation, and hemorrhage resulting from cardiac puncture or laceration of the superior vena cava. In addition, close proximity of the lower thymic lobes to the pleural lining can result in pneumothorax.

Here we describe a technique of thymectomy through a minimally invasive 2 cm skin incision following a simple method of endotracheal intubation using a blunted-end angiocatheter and transcutaneous tracheal illumination. The thymectomy involves a 1.5 cm sternotomy and a three-layer closure with surgical glue application to seal the mediastinum and minimize the incidence of hemorrhage and respiratory complications. This method reliably results in complete thymectomy as evidenced by the disappearance of CD4⁺ and CD8⁺ naïve T cells following thymectomy and the absence of thymic tissue on IHC staining. Operative times and peri-procedural mortality are kept to a minimum.

Protocol

NOTE: All experimental procedures involving the use of rats were done in accordance with protocols approved by the Animal Care and Use Committee of Duke University.

1. Preparation of the Tracheal Intubation Cannula

1. Cut off the needle end of a 2 inch 14 G angiocatheter needle with cutting pliers.
2. Pinch the lumen of the needle closed with needle-nosed pliers.
3. Cut the pinched section down to the edge of the open-lumen portion with cutting pliers, and then cut the edges on both sides of the remaining end at 30 - 45° angle to the needle edge.
4. Place the bottom 3 - 4 mm of the cannula into the open area of straight slip-joint pliers and pull upwards until a slight upward curve has been formed at the end.
NOTE: This will facilitate directing the catheter into the trachea (**Figure 1**).
5. Use fine sandpaper to smooth down the edges at the end of the cannula.
6. Return the blunted instrument into the angiocatheter tubing. Ensure that the tubing is slightly shorter than the blunted needle.

2. Pre-Surgical Procedures

1. Set up the site for the procedure by placing an operating microscope set to 10X magnification over the operating area.
2. Place a warming pad on the operating area and cover with a clean absorbent pad.
3. Set up a heart rate and blood oxygenation monitor near the surgical field.
4. Weigh the rat for weight-based medication dosing (e.g., postoperative analgesia, antibiotics, or lymphocyte depleting antibody).
NOTE: Typical adult rats weigh between 350 - 450 g.
5. Sedate the rat with vaporized 3% isoflurane-O₂ (3 L/min) using an induction chamber vented to a waste anesthetic gas scavenging system containing activated charcoal. Allow 5 min for anesthesia induction to provide deep anesthesia necessary for endotracheal intubation.
6. Administer Carprofen or Meloxicane (4.4 mg/kg) subcutaneously to the rat prior to proceeding with intubation and surgery.
7. Use electric clippers to shave the neck and chest of the anesthetized rat prior to intubation.

3. Intubation

1. Prepare the intubation setup by rubbing a small amount of lubrication (e.g., K-Y gel) on the end of the intubation cannula. Turn on the ventilator to begin the flow of isoflurane gas through the connector tubing.
2. Transfer the rat to the intubation apparatus and suspend the rat on the metal bar by its upper incisor teeth (**Figure 2A**).
NOTE: Certain intubation apparatuses may be purchased online. The model shown here is homemade using an acrylic sheet molded around sections of a 2 x 4 inch lumbar piece using a butane torch, and reinforcements are attached with chloroform. The bar (or metal wire) is attached by drilling holes over masking tape placed on the side of the acrylic to prevent splintering or cracking.
3. Trans-illuminate the rat's neck by positioning a flexible high-intensity light source 1 - 2 cm from the ventral surface of the neck (**Figure 2B**).
4. Use a pair of student standard pattern forceps to pull the tongue gently upwards and to the side of the bottom teeth. Grip the tongue between the thumb and forefinger of one hand while placing the flat inside surface of one side of the forceps against the lower end of the tongue. Press ventrally to expose the epiglottis and aperture of the larynx (**Figure 2C**).
5. Visualize the vocal cords and guide the upturned, blunted end of the intubation cannula anteriorly through the open glottis into the trachea until the hub of the angiocatheter touches the incisors.
6. Remove the metal stylet, and attach the anesthesia tubing to the angiocatheter opening to begin ventilation with isoflurane.
NOTE: The typical oxygen flow rate to the ventilator is 3 L/min with 3% isoflurane.
 1. Set the ventilator at a rate of 60 respirations/min in a volume controlled ventilator mode that achieves a pressure of ~12 - 14 mmHg. Use a positive end-expiratory pressure (PEEP) of 3 cm H₂O.
7. Observe bilateral chest wall expansion in sync with the ventilator to ensure proper placement of the endotracheal tube.
8. Apply veterinarian-recommended eye ointment to the rat's eyes to prevent dryness while under anesthesia.
9. Apply veterinarian ointment on the rat's eyes to prevent dryness while under anesthesia.
10. Secure the endotracheal tube to the head of the rat with a strip of cloth tape. Ensure a firm connection between the endotracheal tube and the anesthetic inflow tubing.
11. Attach the blood oxygenation and heart rate monitor to the rat's foot and begin monitoring. Confirm proper anesthetization by confirming there is no reaction to toe-pinch.

4. Thoracotomy and Thymectomy

1. Follow standard aseptic technique for the entirety of the procedure.
 1. Clean the working area and operating table and disinfect with a 70% ethanol solution.
 2. Use sterile surgical gloves during the procedure, and autoclave all instruments and materials used during the procedure.
 3. Apply providone-iodine to the entire chest area and allow it to dry. Then clean the surface of the skin with 70% ethanol on gauze. Cover the rat with clear plastic wrap cutting a hole to expose the sterile operating field.
2. Identify the suprasternal notch in the upper thoracic region. Make a 2 cm midline incision through the skin starting 2 - 3 mm above the suprasternal notch and extending distally midline along the sternum using blunt-tipped Shea scissors.

3. Perform a 1.5 cm median sternotomy from the suprasternal notch again using blunt-tipped Shea scissors. Keep the bottom edge of the scissors just underneath the sternum and advance slowly.
4. Insert a small Alm retractor just underneath the separated sternum and open to reveal the pre-tracheal strap muscles (sternohyoid and sternothyroid muscles). Separate the pre-tracheal strap muscles using blunt Graefe forceps. NOTE: At this point, the trachea can be seen, and the intubation tube should be visualized inside the trachea.
5. Place the prongs of the small Alm retractor underneath the separated strap muscles and sternum. Open the retractor to expose the superior aspect of the thymus.
6. Use fine Dumont forceps to release the lateral edges of the thymus tissue and expose the lower thymic lobes.
7. Pull the thymus gently superiorly into the open incision site, taking care to avoid contact with the superior vena cava, subclavian and carotid vessels, and to minimize disruption of the delicate pleural lining between the thymus and the lungs.
8. As the thymic vessels are dissected and revealed, use micro-scissors to divide them. Use a cotton swab to hold pressure on the vessels for hemostasis if needed.
9. Deliver the lower thymic lobes into the incision and then sharply lyse the posterior attachments. Remove the intact thymus and carefully inspect the removed thymus for any missing sections.
NOTE: Small thoracic lymph nodes may be visualized surrounding the thymus and are often difficult to differentiate from thymic tissue. These will be discrete round nodes with a similar appearance to thymic tissue but will not be in continuity with the thymus.
10. Remove the Alm retractor and close the sternohyoid and sternothyroid muscles with two interrupted 5-0 Maxon sutures.
11. Apply 2 drops of surgical grade cyanoacrylate tissue adhesive across the sutures to seal the mediastinum under positive pressure ventilation. NOTE: This will decrease the incidence of pneumothorax and hematoma.
12. Close the sternum with two interrupted 4-0 silk sutures on a cutting needle. Insert the needle through gaps between the ribs, taking care to guide the needle just under the sternum to avoid the underlying muscle layer.
13. Close the skin layer with a running 4-0 Nylon suture. Discontinue the isoflurane at this point to shorten the post-operative anesthesia recovery period.
14. Clean the incision site and surrounding skin with saline-moistened gauze.
15. Apply several drops of Bupivacaine (0.25%) for local anesthesia, followed by 1 - 2 drops of cyanoacrylate glue over the incision to seal the incision.
16. Continue to ventilate until the rat shows signs of independent respiratory effort and begins to move its extremities. Extubate the rat and allow it to recover under close observation in a cage placed on a warming pad.
17. Administer Buprenorphine (0.01 - 0.05 mg/kg) subcutaneously to the rat immediately post-operatively and repeat every 8-12 hr for 48 hr post-operatively (normally 5 total doses). At 24 and 48 hr post-operatively, administer Carprofen or Meloxicane (4.4 mg/kg) subcutaneously (3 total doses including the pre-operative dose).
18. Continue to monitor until the rat is moving around the cage. At any signs of respiratory distress, perform re-intubation and re-exploration of the chest.
 1. Do not leave an animal unattended until it has regained sufficient consciousness to maintain sternal recumbency.
 2. Do not return an animal that has undergone surgery to the company of other animals until fully recovered.
19. Remove the skin layer sutures 1 week post-operatively.

Representative Results

This procedure was performed on adult Lewis rats ($n = 26$). The mean operation time was 15 ± 3 min. There was no intraoperative mortality. The mean intubation time – from placement of rat on the intubation apparatus to establishment of ventilation – was 45 ± 5 sec. 24 rats had a full recovery from the operation with no evidence of respiratory difficulties or hemorrhage through post-operative day (POD) 14. One rat developed respiratory difficulty on POD 4 and underwent re-operation to explore the mediastinum. The rat was found to have a collapsed lung lobe. Following re-expansion of the lung, the mediastinum was re-sealed with surgical glue under positive pressure ventilation and the chest was re-closed. The rat recovered with fully with no further respiratory abnormalities. One rat died on POD 7 of unknown causes.

The post-thymectomy mediastinum was inspected for retained thymus and the removed thymus tissue was visually inspected for missing sections and later examined histologically. At necropsy, thoracic tissue was examined by hematoxylin-eosin (H&E) stain. Immunohistochemistry (IHC) for cytokeratin was then performed to differentiate remnant thymic tissue from thoracic lymph nodes (LN), focusing particularly on sections with higher levels of nuclear staining by H&E as is normal for thymus tissue. Cytokeratin staining was performed using rabbit pan anti-cytokeratin as the primary ab, followed by biotinylated goat anti-rabbit IgG and Vectastain Elite ABC Kit Slides were counter-stained with haematoxylin prior to microscopy evaluation. Thymic tissue can be distinguished from thoracic LNs by denser nuclear staining on H&E (**Figure 3A & 3B**) and a characteristic lacy pattern of cytokeratin staining, which differs from the absence of cytokeratin staining seen in LNs (**Figures 3C & 3D**).

Peripheral blood samples obtained from euthanized rats were analyzed for persistent depletion of naïve T cells. Briefly, red blood cells were lysed with ACK lysing buffer and peripheral blood leucocytes (PBLs) were washed twice with PBS 2% FBS prior to stained with antibodies for 30 min at 4°C . PBLs were then fixed with 4% w/v paraformaldehyde in neutral pH-buffered saline prior to analysis by multi-color flow cytometry. Total T-cells were identified by staining PBMCs (peripheral blood mononuclear cells) with anti-CD45+ and CD3+, and naïve T cells were identified by staining with anti-CD45RC, anti-CD62L and either anti-CD4 or anti-CD8. The percentages of each T cell subpopulation were multiplied by the absolute lymphocyte count (obtained by Duke Vet Clinical Diagnostic Lab) to determine cell counts. Representative Naïve CD4⁺ and CD8⁺ T-cell and total T-cell counts are shown in **Figure 4**. Thymectomized rats maintained overall T-cell counts compared to control rats but demonstrated loss of the naïve T-cell populations.



Figure 1. Design of the intubation cannula. The cannula is formed by blunting the end of the stylet of a 14 G x 2 inch angiocatheter. The distal 3 mm of the stylet is slightly bent to guide the catheter ventrally to facilitate tracheal intubation. **(A)** Side view of metal stylet. **(B)** Side view of angiocatheter over stylet. **(C)** Magnified view of blunted tip of stylet.

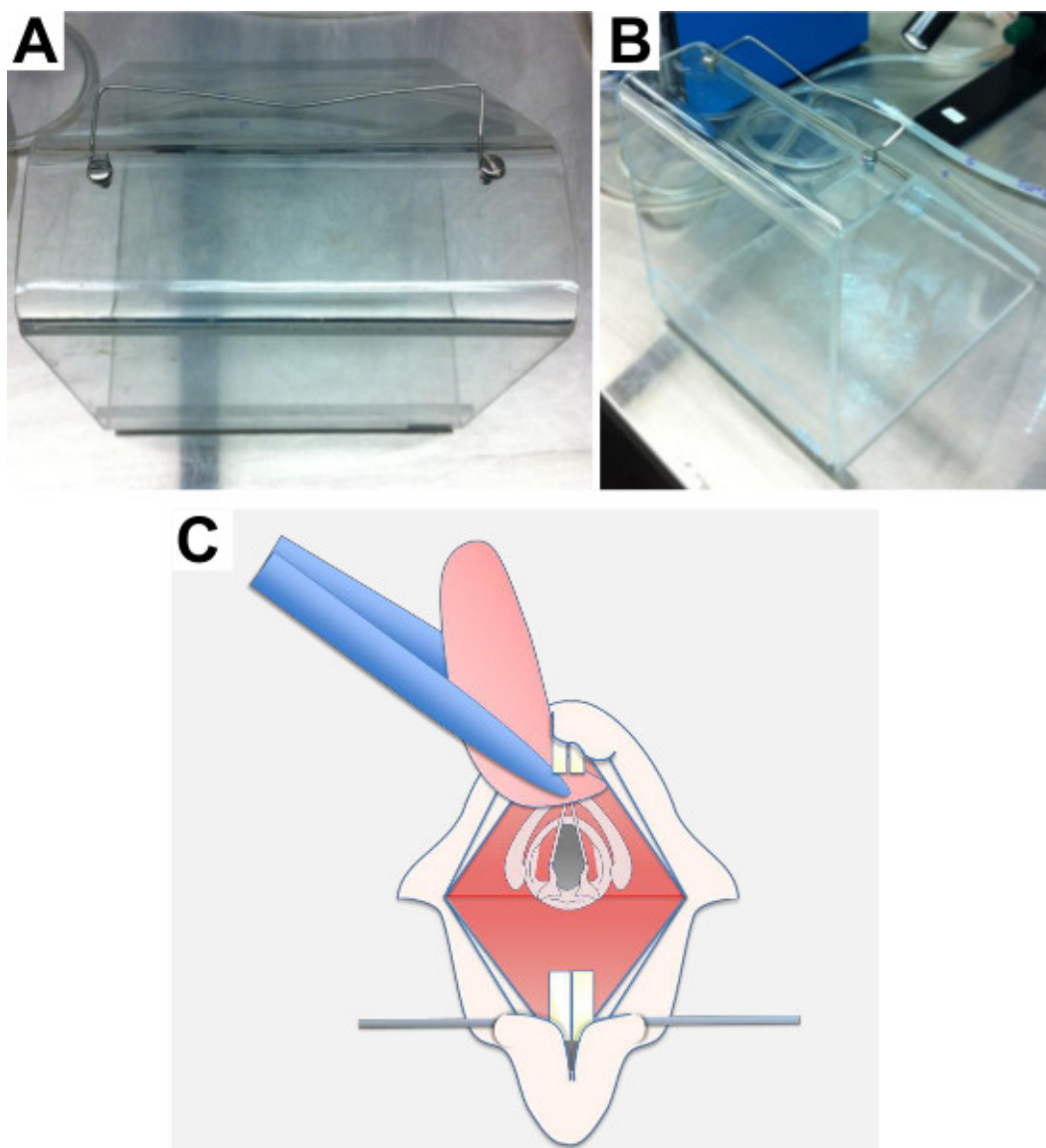


Figure 2. A sloped intubation platform is used to position the rat for intubation. The rat is suspended from the metal bar of the intubation platform by its upper incisor teeth. A light source will then be positioned ventral to the rat's neck to trans-illuminate the pharynx. Forceps are used to hold the tongue and expose the aperture of the larynx. **(A)** Top view and **(B)** side view of intubation platform. **(C)** Diagram of the visualization of the glottis after suspension of the rat and exposure of the larynx. The larynx in the diagram is from a slide purchased from Motifolio.

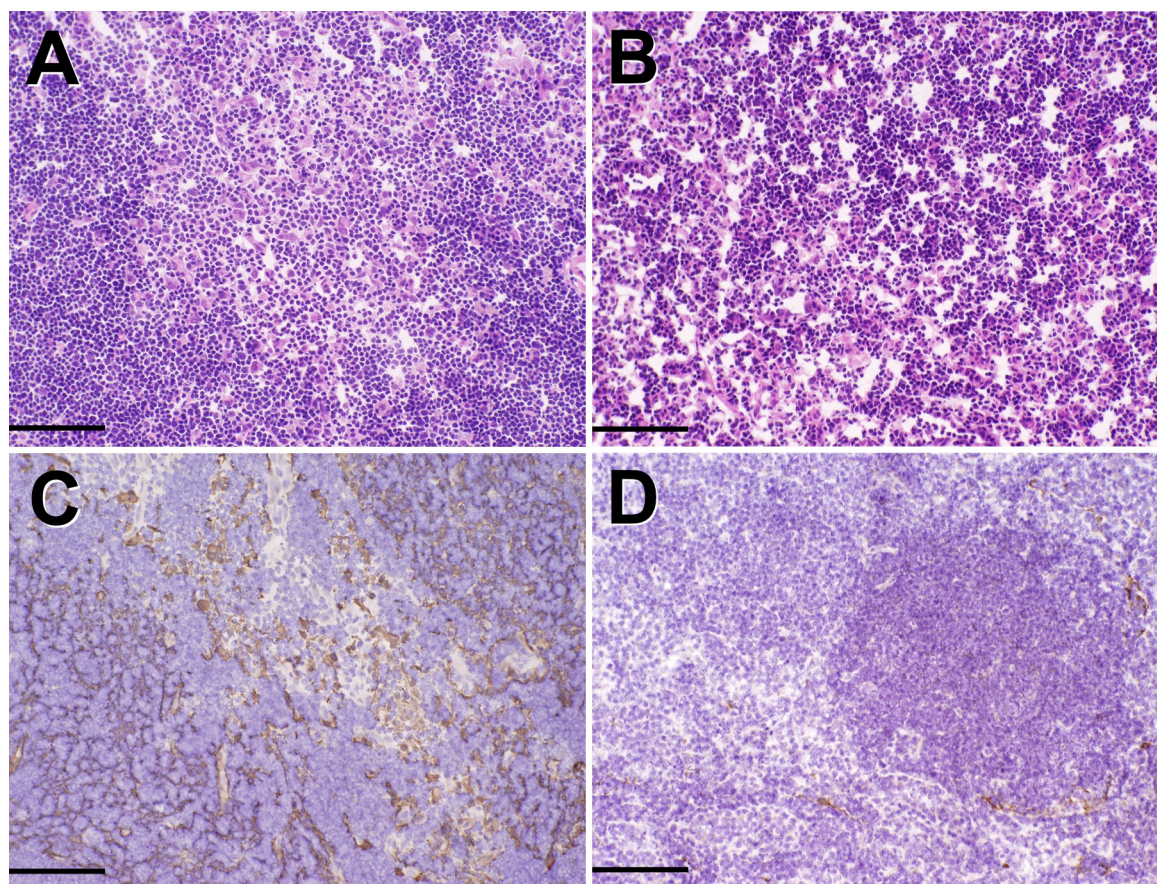


Figure 3. H&E and cytokeratin staining of mediastinal tissue can distinguish thymus from lymph nodes. Hematoxylin and eosin (H&E) staining of a normal thymus (A) and thoracic LN (B). Cytokeratin staining of thymic tissue (C) and a thoracic LN recovered post-thymectomy (D). Magnification is 200X. Bar is 100 μ M. [Please click here to view a larger version of this figure.](#)

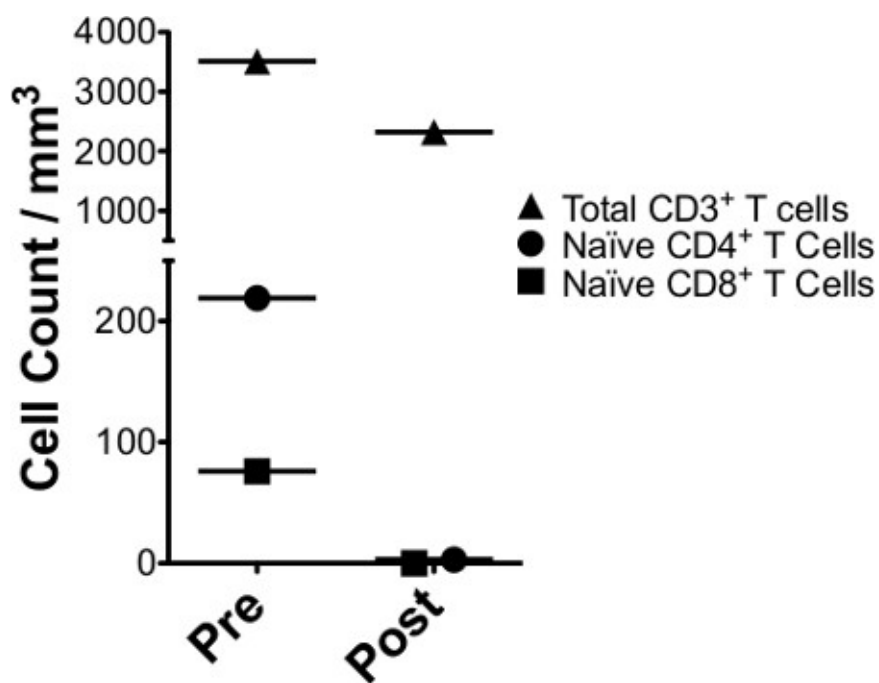


Figure 4. Attrition of peripheral blood naïve T cells post-thymectomy. Total T cell, naïve CD4⁺ T cells and naïve CD8⁺ T cells were quantified from PBLs by flow cytometry in pre-thymectomy (Pre) and rats 4 weeks post-thymectomy (Post). Data are shown for one rat per group and are representative of overall results obtained.

Discussion

The present protocol for complete thymectomy provides a minimally invasive approach with a three layer incision closure with surgical glue application designed to minimize complications. Complete removal of the thymus was demonstrated by the loss of naïve T cells and by IHC staining of mediastinal lymphoid tissue for cytokeratin.

The procedure of adult rat thymectomy has been complicated by mortality rates ranging from 1.5 - 20% due to peri-operative complications, most of which are attributable to the intimate association of the lower lobes of the thymus with the pleura, the heart and the major mediastinal vessels^{2,4}. Open tracheal intubation techniques, where the trachea is punctured, have a 6% mortality rate². The use of non-invasive tracheal intubation has been reported by Na and co-workers; however, survival rates were not reported⁹. Intubation with the cannula prepared as described here with transcutaneous tracheal illumination permits clear visualization of the vocal cords for consistently successful intubations. No advanced equipment is needed for the presented method, which differs from other reported non-invasive techniques^{2,5-8}. Our method of blunting the end of an angiocatheter needle and creating an upward bend at the end makes it easier to avoid inadvertent esophageal intubation and minimizes tracheal trauma. These are improvements to a previously described method of intubation also using a modified angiocatheter⁹.

We find that keeping the sternal incision to a minimum decreases contact with major mediastinal vessels and associated risk of hemorrhage while allowing adequate exposure for complete removal of the thymus. Theoretically, the smaller incision should also lessen post-procedural pain and recovery time. The use of fibrin glue was first reported as a modification of an open tracheal intubation procedure to prevent pneumothorax and airway injury². We have found that the application of a small amount of glue over the sutured pre-tracheal muscles while applying positive pressure adequately seals the pleural cavity and helps to prevent post-operative respiratory complications.

There are a few aspects of the protocol that are important to highlight so the procedure can be performed successfully. It is important to secure the anesthesia tubing with adequate tape because it is possible to disconnect the gas flow during the operation as the surgeon's hands are positioned near the rat's head and visualization is focused only on the surgical site under the microscope. The minimum possible lubrication applied to the cannula prior to intubation prevents the cannula from slipping into the esophagus. During the multi-layer closure, it also helps to apply the cyanoacrylate tissue adhesive through a small gauge needle with a syringe, which allows precise application and prevents accidental over-application of glue.

This procedure does not allow re-vascularization of the removed thymus in a recipient rat, which has been described elsewhere¹⁰. We also did not attempt this procedure in mice, which are often used for immune studies. The most recent descriptions of the thymectomy procedure in adult mice involve either vacuum aspiration of the thymus after exposure, which has high complication rate in adult rats, or a dissection method that is similar to our procedure^{11,12}. The closure is not performed in layers, and anesthesia is achieved by intraperitoneal injection of pentobarbital, which does not mandate intubation. The main limitations of the thymectomy procedure in any rodent are the requirement for an operating microscope, which can be very expensive, and the technical expertise needed to operate under magnification and perform the procedure quickly.

Complete thymectomy in adult rats facilitates immunological studies involving T-cell development, T cell reactivity and tolerance in transplantation and tumor studies, and studies involving T-cell depletion in athymic rats¹³⁻¹⁵.

Disclosures

The authors have nothing to disclose.

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