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Original scientific article

A modified technique for thymectomy in adult mouse with no ventilation support

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Summary

Thymectomy in adult mice, a method to eliminate newly produced naïve T cells, has proved valuable in various immunological studies. However, pneumothorax is the primary cause of high mortality while preforming the traditional thymectomy technique. Although modified techniques utilizing mechanical ventilation support can reduce the occurrence of pneumothorax, it may increase operational complexity and cause ventilation-induced lung injury, which could interfere with subsequent investigations by causing inflammation and a series of immune responses. To solve this problem, we developed a novel technique using pleural ligations to replace ventilation support before thymus removal. Our method not only reduced the incidence of pneumothorax but also caused less disturbance to the immune system. No thymic residue was found by postoperative autopsy and a distinct decrease of T cells in peripheral blood was detected by flow cytometry analysis. The mortality rate was 3.3%, which is comparable to the ventilation-support technique, yet the new procedure is simpler and faster. This technique provides both reliability and simplicity for thymectomy in adult mice.

Introduction

Thymectomy has been widely used as an effective method for studying the function of the thymus and T cells in various fields, such as infection, transplantation and tumor immunity etc. As distinct from thymectomy in neonatal mice and genetically engineered mice such as SCID and nude mice, in which T cells are depleted at a very young age, thymectomy in adult mice can create a unique immune status at a selected time at which no newborn T cells are released to the circulation (*Miller, 1965*). In mice, naïve T cells are almost exclusively sustained by thymus output (*den Braber et al., 2012*). Thus, this method may not only help researchers to manipulate the T cell pool according to their interests, but also be an effective tool to study the role of the thymus under different pathological conditions.

The original technique of thymectomy in adult mice was described in 1963, which mainly used suction to remove thymus lobes (*Sjodin et al., 1963*). However, there were two shortcomings that rendered this method unsatisfactory. Firstly, suction may not assure complete removal of the thymus, because suction through the constrictive tube could tear the thymus into pieces. Secondly, the mortality rate was high, up to 5-30% (*Castro JE, 1974*; *DeMatteo et al., 1995*; *Vrisekoop et al., 2008*). Pneumothorax was the primary cause of death followed by hemorrhage of the heart or mediastinal vessels (*DeMatteo et al., 1995*). Our experience is that about 20-30% of mice succumb to this procedure (unpublished data). To overcome this, researchers developed a method using intubation and mechanical ventilation to prevent respiratory failure (*DeMatteo et al., 1995*). Despite the mortality rate decreasing to 3-6%, this modified method not only prolonged the operating time, increased complexity and added experimental cost, but also resulted in unexpected negative consequences such as ventilation lung injury. The aim of the present study was to develop a new method, which provides complete removal of thymus tissue, as well as improving animal survival with minimal side effects.

Materials and Methods

Mice. Thirty C57BL/6J male mice (6-8 weeks old, weighing 19-21g) were purchased from the Institute of Experimental Animals, Chinese Academy of Medical Science (Beijing, China). To avoid fighting, mice from the same litter were housed in groups of five in polypropylene cages (290×178×160mm) with sufficient specific pathogen free (SPF) soft wood shavings (GB14924.3-2010, HFK bioscience, Beijing, China). Cotton wool was added to the shavings to help the mice nesting, and disposable cardboard boxes were provided for hiding and occupation, which might also prevent aggressive behavior. Mice were given *ad libitum* access to double-distilled water and commercial SPF pelleted food (GB14924.3-2010, HFK bioscience, Beijing, China). All animals were kept at a room temperature of 24±2°C and 50±10% relative humidity on a 12h light/dark photoperiod. All procedures performed on animals were approved by the Animal Care Research Ethics Committee of the Capital Medical University of China.

Equipment. Stereomicroscope (*Zeiss Stemi 2000-C, Oberkochen, Germany*), 6-0 silk braided non-absorbable suture (Ethicon, Johnson-Johnson, NJ, USA), and

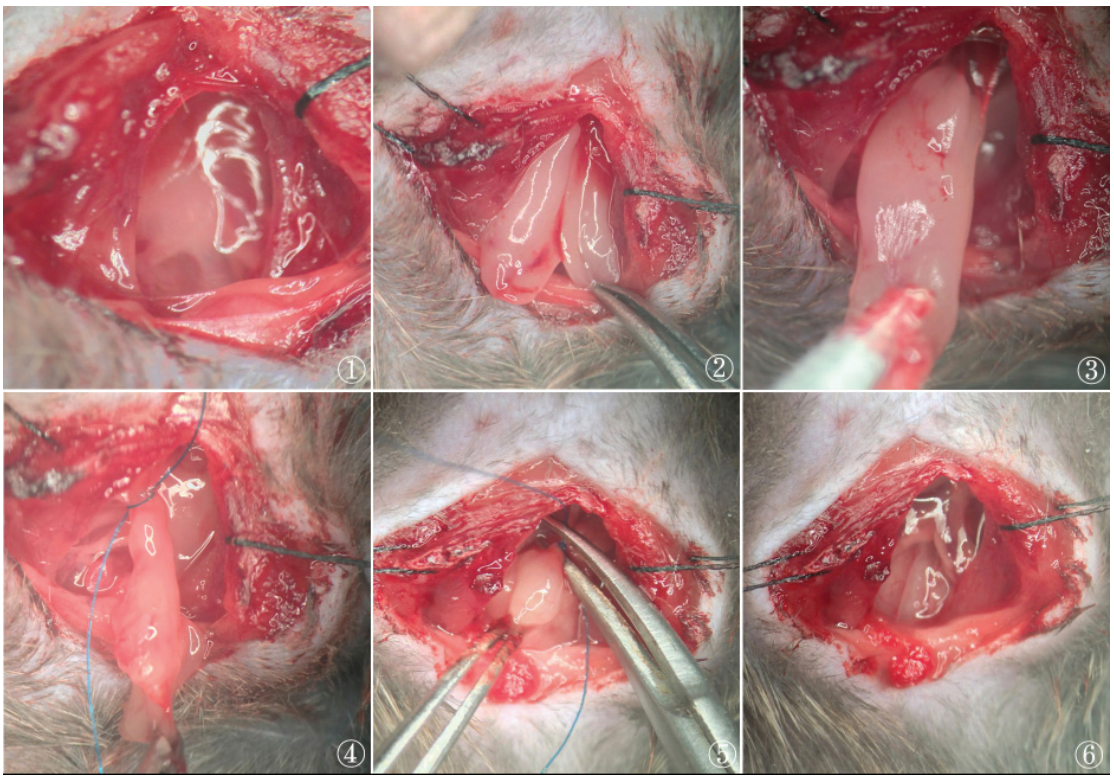

Figure 1. Procedure of pleura ligation and thymectomy in adult mice (10×). (1) The thymus was exposed after sternotomy. (2) Sternum was suspended and two thymus lobes were separated by dissecting their membrane. (3) The right lobe was gently pulled out, and the bottom edge and nourishing blood vessels were clearly visible. (4) A loose knot was set at the edge ready to ligate the pleura. (5) After the ligation, the complete right lobe was cut and removed. (6) Both thymus lobes were removed.

7-0 polypropylene suture (Ethicon, Johnson-Johnson, NJ, USA).

Procedure of thymectomy in adult mouse. All operations were performed in a special disinfected operating room. Surgical instruments were sterilized and disposable sterile gloves were used during the operation. Mice were anesthetized by intraperitoneal injection of narcotic fluid (ketamine 100mg/kg, 10mg/kg xylazine 0.1ml/10g), and placed on a dissecting board in supine position. The feet of the mice were fixed with tape to keep a stable position during the operation. The skin of neck and upper chest was clipped and disinfected with 70% alcohol. Under a stereomicroscope, a 15-20mm skin incision was made along the anterior median line from the suprasternal notch to the level of the third rib. Then, the suprasternal fossa was exposed by dividing the superficial fascia and moving the submaxillary glands aside. A central sternal incision was made to the level of the second rib using microdissection scissors. In order to prevent pneumothorax and hemorrhage, this incision was made strictly along the midline with the scissor tips tilting towards the dorsal aspect of the sternum (Figure1, step 1). The bisected sternum was retracted laterally with 6-0 silk braided sutures implanted on the bilateral edges in order to expose the mediastinum. Suturing was performed from interior to exterior to avoid punctures inside the chest cavity. Such punctures might easily have led to pneumothorax, even hemorrhage and organ damage (Figure1, step 2). Two white thymus lobes were exposed. Each lobe was enveloped separately in a sack-like thin membrane connected to the pleura, which was hard to discern. Careful dissection was performed to open the sack and separate the lobe from its membrane without damaging the pleura. The right lobe was gently held and drawn by iris forceps and the inferior pole of the lobe was exposed (Figure1, step 3). To perform pleura ligation at the bottom of the thymus lobe, a 7-0 polypropylene suture loose knot was made in advance to avoid making risky movements inside the chest cavity while tying knots. Then the circle was set at the caudal edge of the lobe (Figure1, step 4). After the pleura and the nourishing blood vessels of the lobe were ligated, the entire lobe was cut along the edge and removed carefully under clear vision (Figure1, step 5). The left lobe of the thymus was removed in a similar manner. The chest cavity was checked for thymic remnants and accessory injuries before being sealed with 6-0 silk suture lines, which were formerly used for sternal retraction (Figure1, step 6). After the skin had been approximated with single silk sutures or wound clips, the incision was cleaned with 0.9% physiological saline. Following thymectomy, animals were given fentanyl citrate (2.5μg/kg, sc.) and placed in clean cages in groups of five with warming lights until they recovered from anesthesia. Mice were kept for 14 days until euthanized for examination.

Post-operation assessment. To test the success of the thymectomy, autopsy and flow cytometry analysis were performed 14 days after the operation. With the help of narcotic fluid, mice were deeply anesthetized and blood samples were collected from the orbital sinus into heparinized tubes. Then, mice were euthanized by cervical dislocation. After the diaphragm had been dissected, the thoracic cavity was exposed under a stereomicroscope in order to search for thymic residues. In case of small thymic residues invisible to the naked eye, we harvested all soft tissue from the original position of the thymus and stained the cells with fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD45, PE-conjugated CD8 and APC-conjugated CD4 antibodies (BD Bioscience, San Diego, CA, USA). We next detected the influence of thymectomy on peripheral blood. After erythrocyte lysis with BD Pharm Lyse solution (BD Bioscience, Sparks, MD, USA), blood cells were stained with phycoerythrin (PE)-conjugated anti-mouse CD8 and allophycocyanin (APC)-conjugated anti-mouse CD4 antibodies (BD Bioscience, San Diego, CA, USA). All cells were examined tested by a BD FACSCalibur flow cytometer (BD, CA, USA). Flow cytometry data were analyzed using Flowjo (7.6.5, Tree-Star Inc., OR, USA) and Prism (6.0c, GraphPad Software Inc., CA, USA). *Data were analyzed by using the Student t test. $P<0.05$ was defined as significant.*

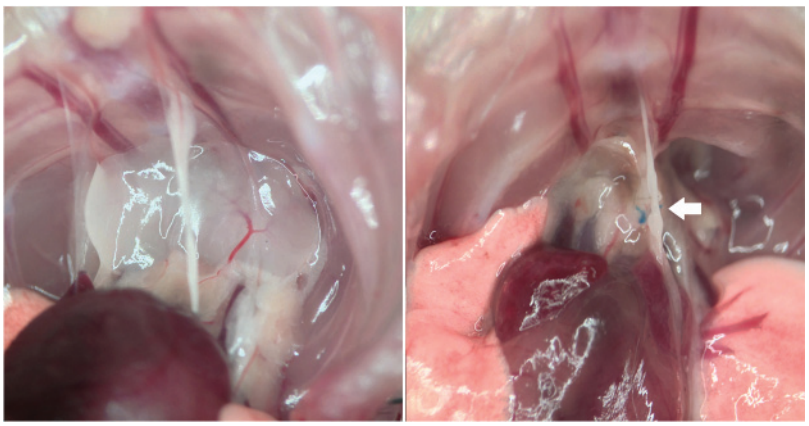

Figure 2. Results of postoperative autopsy (10×). Compared with wild type mouse (left), thymectomized mouse (right) showed no residue of thymus in the mediastinum. In addition, two blue knots were still visible (white arrow).

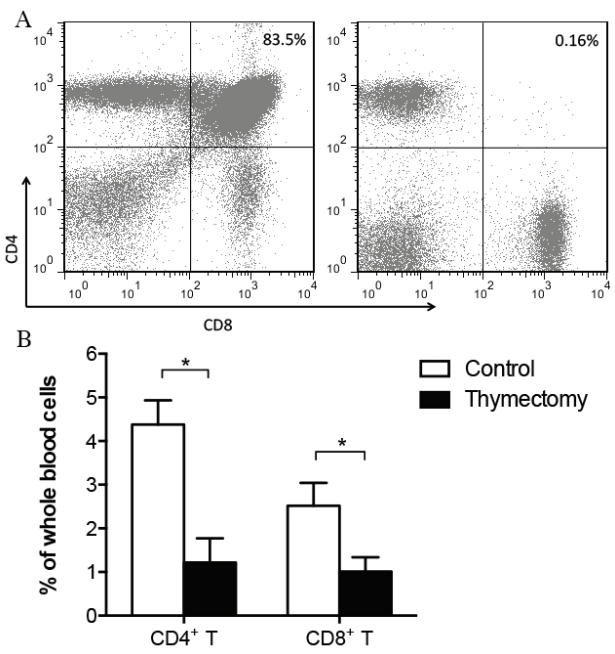

Figure 3. A. Compared to wild type mouse (left), thymectomized mouse showed no group of $CD4^+$ and $CD8^+$ double positive T cells (right).
B. Fourteen days after thymectomy, significant decreases in CD4+ T cells and CD8+ T cells were observed in peripheral blood ($P<0.01$).

Results

By using legation thymectomy, the mortality rate was 3.3% (1 of 30). Only one animal succumbed to severe pneumothorax during sternotomy. All other mice had successful operations and lived until the endpoint of the experiment with no postoperative complications (such as tachypnea, wound infection etc.). Autopsy confirmed that no residue or regenerated tissue of thymus was observable in the mediastinum (Figure 2, blue polypropylene knots were still visible). No signs of pulmonary or cardiovascular injury were observed. Results from flow cytometry showed that no group of CD4/CD8 double positive cells was found in tissue harvested from the former thymus location (Figure 3A), and a significant decrease of $CD4^+$ T and $CD8^+$ T cells in peripheral blood was found ($P<0.01$) (Figure 3B). Results from both autopsy and flow cytometry indicated a complete removal of thymus. Moreover, the average time of the operation for each mouse was around ten minutes in our laboratory.

Discussion

The primary challenge of thymectomy in adult mice is manipulation of the delicate pleura. Animals can suffer from instant death due to anoxia and mediastinal flutter triggered by the change in intrapleural pressure. In such cases, researchers use mechanical ventilation to support pulmonary function and prevent progression of small pneumothorax (*DeMatteo et al., 1995*). Although this method seems to be a successful solution, the extra apparatus and procedural steps needed may render the whole method too complicated. Moreover, ventilation support could lead to ventilation-induced lung injury (VILI) including collapse of alveolar units, edema and inflammation etc. In particular, VILI has been associated with increased bronchoalveolar lavage fluid (BAL) levels of cytokines such as TNF-α and IL-6, and chemokines such as macrophage inflammatory protein (MIP)-2 (*Chiumello et al., 1999*; *Cheng et al., 2002*). Both impaired pulmonary function and inflammation might adversely affect subsequent immunological experiments. To eliminate these problems, it is necessary to develop an innocuous technique free from ventilation support and with high reliability as well.

We noted that the inferior pole of the thymus lobe was intimately attached to the pleura and cardiac pericardium with several nourishing blood vessels embedded. Because the pleura was more soft and fragile than blood vessels, it was easier to tear while blood vessels were still attached to thymus lobes. A small pleural leakage could quickly be enlarged by chest movement and become fatal. We also observed that in most failed cases, pneumothorax often occurred when the inferior pole of the thymus was pulled or bluntly dissected. In fact, strong or sudden traction of thymus lobes could even lead to aorta and atrium dislocation and rupture. Hence, thymectomy with strong direct traction, such as suction described in the conventional technique, may not be appropriate. Furthermore, the sucking force is difficult to control and the cannula tip needs fine adjustment. Suitable modification of the cannula tip is extremely difficult because the pleura is likely to be involved during suction using a large diameter cannula, yet thymic residue is hard to avoid since a small diameter tip could easily tear tissue into pieces instead of removing the complete thymus.

To maintain the integrity of the pleura, we abandoned suction thymectomy and introduced ligation thymectomy by ligating the pleura at the bottom of thymus lobes. Firstly, ligation could prevent small pleural leakage by tying up the nourishing blood vessels with surrounding pleura, which could greatly minimize the risk of pneumothorax. Secondly, thymus excision is more reliable and safer with ligation, because the edge of the thymus lobe is clearly visible. In this way, complete removal of the thymus is ensured. Post-operation

examination also confirmed that no residue of thymus remained. For better operational sight and handling, we strongly suggest this procedure be performed with the help of a stereomicroscope and microinstruments.

This method of thymectomy in adult mice is as efficient as procedures with ventilation support yet it is simpler, easier and cheaper. More importantly, there are no ventilation-induced injuries to the respiratory system and subsequent immunological disturbance is eliminated. Our method conforms to the concept of the 3Rs (replacement, reduction, refinement) and provides a stable animal model for future immunological investigation.

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Authors declare no competing interests.

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