

# Comparative Study of Collagen Density, Fibroblast, and Neovascularization in Tracheal Defect Reconstruction with Primary Repair and External Oblique Muscle Aponeurosis Patch (A Novel Study with New Zealand Rabbit)

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## Abstract

**Background:** The lack of ideal materials and the high incidence of complications make tracheal defect reconstruction challenging. The use of aponeurosis as a surgical patch and flap in reconstruction is not uncommon, but no studies had been conducted to analyse its use in tracheal defect reconstruction, though its effectivity in wound healing was significant in reconstruction of other organs.

**Objective:** This study aims to compare collagen density, fibroblast expression, and neovascularization in tracheal defect reconstruction using primary suture and using external oblique muscle aponeurosis patch.

**Methods:** This randomized control trial is a laboratory experimental study using male New Zealand white rabbits. Randomization was carried out with permuted block randomization into a control group (primary suture) and a treatment group (reconstruction with external oblique muscle aponeurosis patch). Macroscopic and histopathological assessments were carried out 14 days after surgery.

**Results:** We got homogeneous samples in both groups (9 samples each) in terms of age ( $p = 0.763$ ), weight before procedure ( $p = 0.347$ ), and weight after procedure ( $p = 0.536$ ). Macroscopically, no wound dehiscence, pus, or inflammation at the surgical site in either the control or treatment groups. Histopathological analysis showed that there were no differences in collagen density ( $p = 1.00$ ), fibroblast expression ( $p = 0.414$ ), and neovascularization ( $p = 0.558$ ) between the two groups.

**Conclusion:** It can be concluded that there are no differences in collagen density, fibroblast expression, and neovascularization in New Zealand rabbits with tracheal defect using primary suture and using external oblique muscle aponeurosis patch.

**Keywords:** tracheal defect, tracheal reconstruction, aponeurosis patch, external oblique muscle aponeurosis

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## Introduction

Tracheal injury, which can be caused by trauma, disease, or iatrogenic, is a medical condition that requires special attention due to its complex mechanism. In routine medical procedures such as intubation with an endotracheal tube (ETT), tracheal trauma is rare, but serious and potentially life-threatening. The incidence of tracheal trauma is estimated at 1/20,000 (0.00005%) intubations. Tracheal stenosis can also occur, which is a complication of tracheostomy and is ultimately realized too late. Most patients are asymptomatic although symptoms can include shortness of breath on exertion, and progress to wheezing, stridor and cyanosis.<sup>1</sup>

However, until now, reconstruction of tracheal defects is still a challenge for surgeons, because there is no material that is considered ideal for the trachea and there are many long-term complications after reconstruction.<sup>1</sup> The lack of ideal materials and the high incidence of complications make tracheal defect reconstruction challenging. The use of aponeurosis as a surgical patch and flap in reconstruction is not uncommon, but no studies had been conducted to analyse its use in tracheal defect reconstruction, though its effectivity in wound healing was significant in reconstruction of other organs. Many of the above give rise to the idea that the aponeurosis patch has the advantage of its unique characteristics, which is hoped that it can reduce the risk and incidence of tracheal stenosis after reconstruction procedures.

## Methods

### Research Design

This research was an experiment all laboratory study, using a randomized control trial design conducted in Animal Laboratory, Department of Biochemistry, Faculty of Medicine, Universitas Airlangga. Thirty New Zealand male white rabbits aged 6 to 9 months with bodyweight between 1900-2500 grams were obtained and the protocol was approved by Animal Care and Use Committee from Faculty of Veterinary, Universitas Airlangga, Indonesia (*certificate number: 2.KE.072.04.2018*). Rabbits were acclimatized for 7 days, then divided into control and treatment groups and permuted block randomization was applied. In the control

group, the tracheal wounds of the rabbits were done with primary suture, while in the treatment group, the wounds were covered with aponeurosis patch of musculus obliquus externus. After 14 days, collagen density was examined in the area of the surgical wound.

### Sample Preparation and Surgical Procedure

A total of 30 New Zealand male white rabbits aged 6 to 9 months with bodyweight between 1900-2500 grams were acclimatized for 7 days. Then the randomization was done by permuted block randomization into two groups, namely the treatment group of 15 rabbits and the control group of 15 rabbits. After being acclimatized for 7 days, the rabbits were given treatment under the influence of general anesthesia using intramuscular ketamine at a dose of 20-40 mg/kg body weight. The front neck's fur of each rabbit was shaved and disinfected using povidone iodine 10%. All rabbits from both groups (control and treatment groups) were wounded on the 8th ring of trachea an area of 0.6 x 0.6 cm<sup>2</sup> using a scalpel number 15. In the control group, tracheal defect was closed with simple interrupted sutures using 6-0 vicryl thread. In the treatment group, the treatment involves taking aponeurosis patch from musculus obliquus externus extra by shaving the right side of the abdomen, and disinfect the area using povidone iodine, do the incision using a scalpel number 15 until the aponeurosis patch of musculus obliquus externus has shown, and take the area in size of 1x1cm and saved in a place contained of NaCl 0.9% before used. Defect was closed using vicryl 6-0 thread, and skin was sutured using nylon 3-0 thread. Tracheal defect was closed using the aponeurosis patch of musculus obliquus externus in size of 1.5 bigger than before, and put it aside of trachea with simple interrupted sutures using vicryl 6-0 thread. Daily antibiotics (penicillin 50,000 units/kg) intramuscularly were given after the surgery for five days.

Specimens were taken from the area of 1 cm cranio-caudal tracheal surgery involving healthy (0.5 cm) and pathological (0.5 cm) visible tissue, 0.5 cm medio-lateral width, covering the entire thickness of the trachea. Then the specimens were put in a 10% neutral buffered

### ***Collagen Density Evaluation***

Histopathology evaluation was performed using Masson's trichrome staining and collagen density was assessed with a binocular light microscope (Olympus CX 41) with 100x magnification for 1 field of view. Collagen density was assessed using scoring:

Score 0: There are no collagen fibers in the wound area

Score 1: The density of collagen fibers in the wound area is low

Score 2: The density of collagen fibers in the wound area is moderate

Score 3: The density of collagen fibers in the wound area is tight

Score 4: The density of collagen fibers in the wound area is very tight

### ***Fibroblast Measurement***

Specimens were taken from the area of 1.2 cm cranio-caudal tracheal surgery involving healthy (0.6cm) and pathological (0.6cm) visible tissue, 0.6cm medio-lateral width, covering the entire thickness of the trachea. Then the specimens were put in a 10% neutral buffered formalin solution and sent for paraffin blocks and histopathological examination using hematoxylin and eosin stain. The number of fibroblast was assessed with a binocular light microscope with 400x magnification in 3 fields of view.

### ***Neovascularization Evaluation***

Specimens was put in neutral buffered formalin 10% and sent for paraffin blocks. The specimens were cut in width of 5 micrometer and performed using hematoxylin and eosin staining. Specimens were evaluate based on the neovascularization that classified into 3 groups, such as:

1+: bad neovascularization (up to 10 neovascularizations)

2+: moderate neovascularization (11-20 neovascularizations)

3+: advanced neovascularization (21-more neovascularizations)

### **Statistical Analysis**

The characteristic data taken was in the form of nominal data. The data of collagen, fibroblast, and neovascularization in histopathology was taken in the form of ordinal data and compared with the treatment group using Mann-Whitney U nonparametric test.

## **Results**

### **1. Sample Characteristic**

The research was conducted on 18 New Zealand White Rabbit rabbits in the laboratory animal unit of the Faculty of Veterinary Medicine, Airlangga University, which were then divided into 9 control groups and 9 treatment groups. It was found that the age of the control sample ranged from 6-8.5 months with a mean of 7.05 months, a median of 7 months, and a standard deviation of 0.88. Meanwhile, the age of the treatment sample ranged between 6-9 months with a mean of 7.2 months and a median value of 7.5 months, with a standard deviation of 1.03. The results of the analysis of the age variable did not show a statistically significant difference between the control group and the treatment group ( $p = 0.763$ ). The body weight of rabbits before the procedure in the control group had a mean of 2,202.2 g with a standard deviation of 104.53 g and a median value of 2,190 g. The weight before the procedure in the treatment samples had a mean of 2,084.44 g with a standard deviation of 95.23 g and a median value of 2,035 grams.

There is no difference statistically significant in the results of the weight variable analysis before the intermediate action control group and treatment group ( $p = 0.347$ ). The control sample had an average weight after the procedure of 2,084.4 g with a standard deviation of 78.64 g and a median value of 2,035 g. The weight after the procedure in the treatment samples had a mean of 2,043.3 g with a standard deviation of 81.12 g and a median value of 2,025 g. The results of the analysis of weight variables after the procedure between the control group and the treatment group did not show any differences which is statistically significant ( $p = 0.536$ ).

### **2. Macroscopic Analysis of Specimens**

Specimens were evaluated 14 days after surgery. In the area of defect was not found any of wound

dehiscence, pus, or inflammation process at the surgical site (trachea or abdomen). In the control group, we got midline scar incision with suture using vicryl 6.0 without any leakage, active bleeding, hematoma, pus, or even granulomas. There is a stenosis of trachea in a level of incision that sutured. In the treatment group, we got midline scar incision with suture using vicryl 6.0 without any leakage, active bleeding, hematoma, pus, or even granulomas, and without any stenosis in a site that covered by aponeurosis patch of musculus obliquus externus.

### 3. Histopathological Analysis

#### Collagen Density

Collagen density examination using simple staining cannot differentiate between collagen type 1 and type 3, so the assessment is done in general. This collagen density is documented in 5 difference fields of view. In a control group, we obtained homogen results of stage 1 collagen (15:15; 100%). In a treatment group, we obtained some variation of results, which is 5 samples are consist of stage 1 collagen, 3 samples are consist stage 2 collagen, and 1 sample is consist of stage 3 collagen.

Mean of collagen in a control group was 1.0 +/- 0,0, meanwhile in treatment group was 1.56 +/- 0.72. In this study, the collagen density difference test described by the collagend ensityscore was carried out using the Mann-Whitney U test because the type of collagen density score data is ordinal data. The result of the Mann-Whitney U test was  $p > 0.05$  (1.00) (Table 5.3), indicating that the difference of collagen density in the control group (primary suture) compared to the treatment group (aponeurosis patch of musculus obliquus externus) was not statistically significant. Based on these results it was concluded that the group given aponeurosis patch of muculus obliquus externus had a lower collagen density than the polypropylene mesh group, but it was not significantly different.

#### Fibroblast Measurement

The fibroblast expression is documented in 5 difference fields of view. In control group with 9 samples of specimens, 5 samples had stage 1 fibroblast (5/9; 55.5%), 3 samples had stage 2 fibroblast (3/9; 33.3%), and 1 sample had stage 3 fibroblast (1/9;

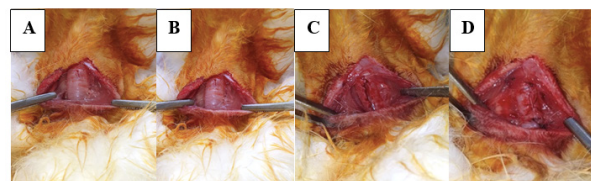
11.1%). Difference results happened in treatment group, with the highest stage of fibroblast which is stage 3 happened in 5 samples, 2 samples had stage 2 fibroblast, and 1 sample for stage 1 (Table 5.4).

Mean of fibroblast is 0.56 +/- 0.72 in control group, and 1.44 +/- 0.72 in treatment group. The result of the Mann-Whitney U test was  $p > 0.05$  (0,414) (Table 5.3), indicating that the difference of collagen density in the control group (primary suture) compared to the treatment group (aponeurosis patch of musculus obliquus externus) was not statistically significant.

#### Neovascularization

The fibroblast expression is documented in 5 differencefields of view. In control group with 9 samples of specimens almost had stage 0 neovascularization (7/9; 77.8%). Only 1 sample of specimen had stage 1 neovascularization (1/9; 11.1%). In the treatment group, 5 samples had stage 3 neovascularization, 3 samples had stage 1 neovascularization. Mean of neovascularization is 0.33 +/- 0.7 in control group, and 1.44 +/- 0.72 in treatment group. The result of the Mann-Whitney U test was  $p > 0.05$  (0,558) (Table 5.3), indicating that the difference of collagen density in the control group (primary suture) compared to the treatment group (aponeurosis patch of musculus obliquus externus) was not statistically significant.

In this study, the characteristics of the research sample could influence the results of the study. Therefore, the study sample was made as homogeneous as possible to avoid bias. The experimental animals were selected from the same place (Pusat Veterinaria Farma) and given the same food. The statistical tests T-test on body weight and the Mann-Whitney U test on the age of rabbits gave the results that the weight and age of the rabbits, although varied but still homogeneous, so there was no bias from differences in body weight and age of the rabbits.



**Figure 1. Rabbit's trachea in control group, a) Normal trachea; b) Tracheal insicion; c)widening the tracheal insicion; d)defect closure with primary suture.**

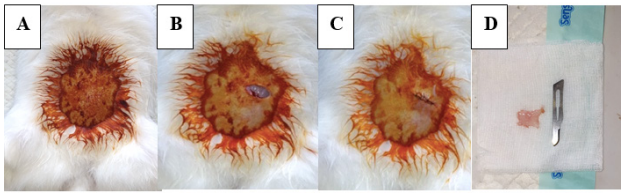


Figure 2. Aponeurosis patch of musculus obliquus externus; a) Desinfection; b) Incise the abdomen until the aponeurosis is revealed; c) close the defect with primary suture.

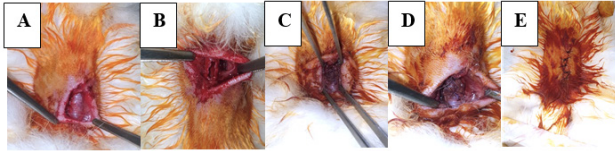


Figure 3. Rabbit's trachea in treatment group; a) Normal trachea; b) Tracheal incision; c&d) close the defect with aponeurosis patch of musculus obliquus externus; e) skin closure

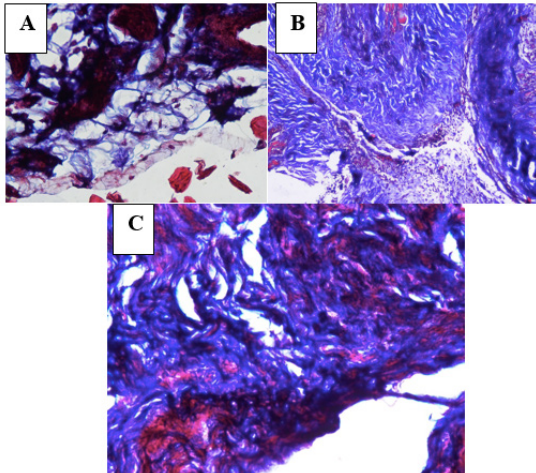


Figure 4. Collagen density; a) low density; b) medium density; c) high density

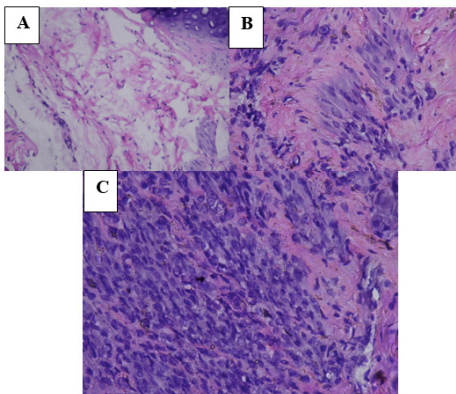


Figure 5. Fibroblast measurement; a) Low density; b) medium density; c) high density

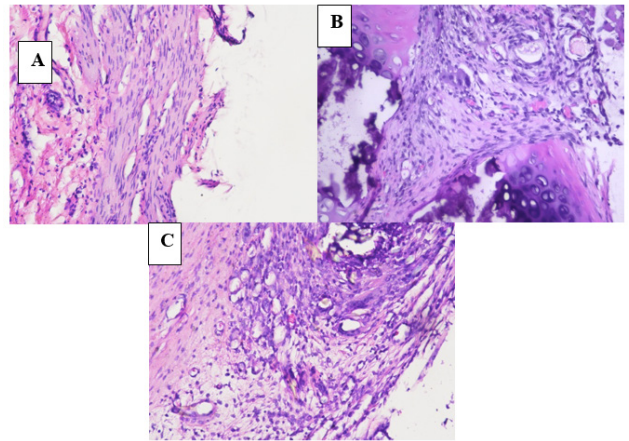


Figure 6. Neovascularization; a) Low density; b) medium density; c) high density

Table 1. Collagendensity score

	Collagen (Mean)	P Value
Control	1,0	1,00
Treatment	1.56	

Table 2. Fibroblast Measurements

	Fibroblast (Mean)	P Value
Control	0.56	0.414
Treatment	1,44	

Table 3. Fibroblast Measurements

	Neovaskularization (Mean)	P Value
Control	0.33	0.558
Treatment	1.44	

### Discussion

Until now, there have been no studies that evaluate the reconstruction of tracheal defects using aponeurosis patches to compare the results of this study. Some other autologous tissues that have been tested for tracheal replacement include fascia, cartilage, dermis grafts, omentum, periosteum, perichondrium, buccal mucosa and auricular cartilage, dura mater, urinary bladder mucosa, periosteum, and jejunal patches as free grafts, as well as the latissimusdorsiintercostals, trapezius muscle, periosteum, buccal mucosa, and turbinate cartilage as vascularized flaps.<sup>2</sup>

In this study, 30 rabbits were used on the experiment where we divide 15 rabbits to each

group (control and treatment groups). The inclusion are the age of each rabbits were 6 months years old to nine months years old, average weight between 1900 grams to 2500 grams, and has no anatomical abnormality. We divided the sample using permuted block randomization. For the experiment, we manipulate the trachea by make an full thickness incision at 8<sup>th</sup> trachea ring with the size of each incision 0,6cm x 0,6cm<sup>2</sup>. For aponeurosis patch, we use the aponeurosis of musculus obliques externusd extra and the aponeurosis were transplanted to the trachea and sutured using continues suture technique with vicryl 6-0. Each sample were collected at 14<sup>th</sup> days and sent to be examined.

Each of sample blocks were stained using Masson's trichrome stain at day 14<sup>th</sup> and examined using Olympus CX 14 microscope.<sup>3</sup> Collagen density were scored by 0-4 as 0 : no visible collagen ; 1 : low density of collagen at site ; 2 : moderate density of collagen at site ; 3 : high collagen density at site ; and 4 : very tight collagen density at site.<sup>6</sup> Fibroblast expression were examined by using hematoxylin and eosin stain and counted by using three different field of view.<sup>4-5</sup> Neovascularization were examined using histopathological scoring system as 1+ : bad neovascularization (none to 10 new vascular); 2+ : moderate vascularization (11-20 new vascular) ; 3+ : abundant (21 or more new vascular).<sup>7</sup>

This study revealed, based on characteristics, there were no significant differences between the control group and the treatment group in terms of age and weight before and after the procedure ( $p > 0.05$ ). This shows that the sample in this study is homogeneous, so it has a low potential for bias. Analysis of the distribution of collagen in the two groups showed that there was no statistically significant difference between the two groups ( $p > 0.05$ ) even though the mean value of collagen levels in the treatment group was higher than the mean value of collagen levels in the control group. This result broke the research hypothesis that the group of rabbits with the external oblique muscle aponeurosis patch had better collagen density compared to the group of rabbits that only used the primary suture method.

Fibroblast levels in both groups also showed similar results, where it was found that the general mean value of fibroblast levels in the treatment group

was higher than the general mean value of fibroblast levels in the control group, but the analysis showed that there were no statistically significant differences in the two groups. ( $p > 0.05$ ). This result also contradicts our hypothesis that fibroblast expression would be found higher in the treatment group with the oblique muscle aponeurosis patch external.

Similar results were also found in the analysis of the distribution neovascularization. The general mean value of the level of neovascularization in treatment group higher than the control group, however analysis showed no statistically significant differences in either group ( $p > 0.05$ ). This result invalidates our treatment group hypothesis with patches of external oblique muscle aponeurosis having neovascularization which was better than the control group that only used the method primary sewing.

The use of fascia as a patch includes, among other things, reconstruction of wall defects abdomen after tumor resection or incisional abdominal hernia infected.<sup>8-10</sup> Abdominal wall reconstruction with autologous fascia transplantation is possible uses the anterior layer of the rectus sheath, the aponeurosis of the muscle external oblique as in this study, and the fascia lata patch, but the fascia patch. The fascia lata is most commonly used because it is larger and stronger. Hamilton et al. (1963) analyzed fascia lata patches taken 2 years post-insertion, and Microscopy revealed a thick, wavy collagen layer accompanied by a number of nourishing blood vessels.<sup>12</sup> Another study in 2012 assessed the use of the anterior aponeurosis of the rectus sheath as a graft to close the abdominal wall in rabbits. Histological analysis 7 days post operative procedures in this study showed an inflammatory process with collagen and maturing myofibroblasts, varying from incipient to moderate. The number of blood vessels appears reduced, with inflammatory cells visible generally plasma cells and macrophages. There were no signs of acute rejection either dehiscence, herniation, cellulitis, abscesses or hematomas in wounds.<sup>12</sup> In accordance with the findings of gross specimen analysis after patch use on.

In this study, there was no significant morbidity and patch tissue. Macroscopically it appears sturdy and integrated with the tracheal tissue. However, this study is not free from several limitations. One

of The basic thing is that the number of samples is small with limited time, So drawing conclusions from these results can be said to be not optimal. Another limitation of this study is that only histopathological assessment was carried out by an anatomical pathologist. Histopathological data were analyzed semiquantitatively, so it can give rise to bias which should be minimized by assessment by two or more anatomical pathologists.

**Ethical Clearance:** Taken from Animal Care and Use Committee from Faculty of Veterinary, Universitas Airlangga, Indonesia.

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**Conflict of Interest:** There is no conflict of interest in this study

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