Adipose-derived Mesenchymal Stem Cells Improve the Healing of Tracheoesophageal Fistula in a Beagle Model

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Research

Keywords: Tracheoesophageal fistula, Mesenchymal stem cells, animal model

Posted Date: January 18th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-146858/v1

Abstract

Background Tracheoesophageal fistula (TEF) is still a devastating clinical problem with high mortality. New clinical strategies were developed to sustain survival time. Mesenchymal stem cells were applied in many clinical wound healing fields. This study aims to investigate the main effect of stem cell to TEF.

Material and Method We established a beagle model with TEF by punching the trachea and esophagus membrane and suturing. The beagles were divided into three groups (group 1 = 1, group 2 = 6, group 3 = 6). Group 2 and 3 received a TEF building operation. Group 3 were injected 2 ml stem cells (10^6 per animal), and group 2 injected saline water with same volume. Group 1 did not receive any intervention. All animals receipted total parental nutrition. The closure degree of fistula tissue was observed by bronchoscope and post-mortem after 35 d.

Result Morphologic and histopathologic changes of fistulas were assessed by gross and endoscopic observation. The fistulas diameter was measured. In 35 d postoperatively, group 2 showed that 3 animals died for acute smother, 2 animal died for severe chronic pneumonia and 1 animal with consistent fistula. Group 3 showed that 2 animals fully closed, 3 animals fistula diameter significantly decreased, and 1 animal died for acute smother. In autopsy result, group 2 animals showed severer pneumonia degree than group 3 animals in 35 d.

Conclusion The transplantation of stem cells can promote healing degree of TEF without any complications and relieve pneumonia at the same time.

Introduce

1. **Tracheoesophageal fistula**

Tracheoesophageal fistula (TEF) is a disabling and devastating clinical problem with high mortality in pulmonary and critical care medicine. The majority of fistulas origin from malignant diseases including tracheal cancer and esophageal cancer[1]. The injury by tracheal intubation after mechanical ventilation is another reason of iatrogenic TEF[2]. Despite various therapeutic efforts like surgery, endoscopic, and radiotherapeutic strategies, the median survival time is 35 d according to a past research[1]. The patients may die within 3~4 months without treatment timely[3-5].

2. **Main clinical strategies**

Recently, the clinical strategies include kinds of surgeries and endoscopic interventions, the patients are still confronted with poor prognosis. Endoscopic strategies are useful in small fistula (<1cm) with mild infection by using plastic endoprostheses or cuffed plastic stents[3, 6, 7], and the fistula (>1cm) with severe local infection should be surgeon according to precious studies. Hence, surgery is the common clinical strategy for current TEF. However, traditional surgical procedure may cause large drainage wound, severe pain, and slow healing, and complicated operative procedure leads to a high rate of postoperative

complications and mortality[8, 9]. Other alternative operation needs the autologous flaps and other material with adequate blood supply[4, 5, 10]. The availability of pedicled flap is limited by the guaranteed blood supply. Therefore, the TEF need the breakthrough in clinical strategies. Among the new cures, stem cell transplantation is a research highlight.

Material And Method

Animal

Six male beagles 3~4 years old, 8~11 kg were obtained from *Gangdong laboratory animals monitoring institute.* These animals were divided into three groups: group 1 (a blank group, n=1 each, normal animal), group 2 (a control group, n=6 each, TEF operation and saline water injection), group 3 (an experimental group, n=6 each, TEF operation and stem cell injection). These animals were randomly divided into three groups and numbered (group 1: 1101, group 2: 2101-2106 and group 3: 3101-3106).

Mesenchymal stem Cells

Mesenchymal stem cells were selected adipose-derived mesenchymal stem cells (AMSCs) from *Saliai Stem Cell Science and Technology Co.LTD*. To confirm the AMSCs maintain their phenotypic characteristic after growth in culture. The MSCs were tested for cell-surface marker via flow cytometry (Beckeman-Coulter, Danvers, MA, https://www.beckmancoulter.com) and found positive for CD73, CD90, CD105 and negative for CD34, CD45, BL and HLA-DR.

Preoperative preparation

The surgical procedure was performed under general anesthesia using intravenous injection of ketamin (10 mg/kg) and thialazine hydrochloride (1 mg/kg). as soon as the dogs lost consciousness an intravenous line was established. Anesthesia ventilation uses isoflurane (1%~5%) maintain anesthesia. All animals were intubated and received supplemental oxygen via the tube (30 ml/kg, 15/min). during the operation the dogs had electrocardiographic monitoring.

TEF model construction

The anesthetized animal was placed on the table. The skin incision was mad parallel to the medial margin of the left sternocleidomastoid muscle until the trachea was reached from a lateral approach. The trachea was resected completely from the cricoid cartilage down to the jugulum, the esophagus was also dissected from the connective tissue. Then the trachea was rotated 90° to the right to expose the membranous wall of trachea. A hole was burned by an electrotome into the membranous part, a corresponding puncture was burned into the anterior wall of the esophagus. The mediocre punch was used to expend and form a fistula 8 mm long and 8 mm wide in trachea and esophagus. Then the margin of the esophageal puncture was sutured to the margin of the tracheal fistula. This resulted in a communicating fistula between trachea and esophagus. The subcutis and cutis were closed with 3.0

Prolene sutures. The all animals received 1.0 g Ampicillin sodium as a prophylactic antibiotic after surgery immediately (fig 1).

Stem cell transplantation

During the operation, all group 3 animals were injected 1 x 10^6 AMSCs 2 ml into the tracheal membrane and esophageal submucosa. All group 2 animals were injected equal saline water instead in the same place.

Postoperative care

For the first 48 h after surgery, the dogs received analgesia with intramuscular Flunixin meglumine 0.5 g/d.

Each week after operation, the bronchoscope is conducted to ensure that there is not hematocele or secretion in trachea. The fistula is observed at same time.

The oral intake was forbidden in group 2 and 3 during the postoperative period. The total parenteral nutrition (TPN) was applied in two group. During this period each animal was monitored rigorously. The symptoms like chocking, vomiting, cough aggravate and even smother should be recorded. The group 1 animal use the normal nutrition. The other aspects of animal care are same in three group

Euthanasia and observational indexes

The animal was euthanized by cutting the femoral artery to avoid the interference of blood congestion in lung at 35 d. the animal died before 35 d should record the death time and analysis the death reasons. The gross fistula tissues were cut for the describe of fistula parameter in the following section after euthanasia and accidental death.

Fistula parameter

After harvesting the fistula form the animal, the trachea and esophagus tissues are opened longitudinally. The diameter of fistula was measured by vernier caliper. The change of the diameter was assessed in three groups.

Histology

Tissue sections of fistula were immediately dissected using a scalpel into pieces and incubation in 4% paraformaldehyde, embedded in paraffin, serially sectioned and stained with Masson dye for light microscope.

Statistical analysis

SPSS statistics software v25.0 was used for data analysis. Quantitative data are expressed as the mean ± standard deviation (SD).

Results

Symptom, nutrition and survival time

The operation procedure was performed successfully in all 12 beagles. In postoperative period, each TEF animal present symptoms like cough, vomiting and tracheal chocking which induced by the reflux of gastric content especially.

The survive of animal was present in the figure 1. Three control group animals and one experimental animal (2101, 2103, 2106, 3102) were died in the first postoperative week (fig 1). The fistulas in animal died in first week were not formed according to the autopsy because the TEF in these animals was maintained by the surgical suture rather than fibrotic fistula wall between trachea and esophagus (Fig 2). During the rest experimental period, two animals of control group were died for the pulmonary inflammation, and rest animals in experimental group were survived. These animals (control 1 and experimental 5) were euthanized by cutting the femoral artery in 35 d.

Endoscope

Each week after surgery, the bronchoscope was performed to confirming the location and healing degree of tracheal fistula. (picture 3)

On the aspect of secretion, excess white foamy secretion was covered the fistula and diffused in whole trachea during the early postoperative stage (1~2 week) in all groups.

The difference occurred at the 14 d postoperatively. In the control group, the viscous secretion appeared in the upper trachea and the accumulation of sputum in low lobes was observed during the later postoperative stage (3~5 week). on the contrary, in the experimental group, the volume of secretion and sputum was decreased in the 3 week after surgery, and sustain a condition with few reflux and secretion during the 4~5 week postoperatively (table 1).

The healing degree of fistula was consistent with the volume of secretion. In the control group, there is little change about fistula diameter under bronchoscope. In the early postoperative stage (1~2 week), the margin fistula appeared amount of necrotic tissue in both groups. In the 3 week, the diameter of fistula in group 3 was decreased gradually especially in the animal 3104 and 3105 (table 2). On the contrary, the fistula in group 2 was still unchanged. This diversity was sustained during the rest period of experimental. The difference was confirmed by the diameter measured from the fistula tissue after autopsy.

In pneumonia, the open fistula leads to the contamination in lower lobs in animals from control group (table 4). This result also can be confirmed by the follow-up autopsy result. The lung in animal 2102

present a severe inflammation in almost whole lung, and the lung in animal 3104 was in a healthy condition (fig 3).

Histology

As shown in the fig 4, the fistula models were successfully established with fused constructions, as confirmed by Masson staining and gross examination of fistula tissue.

The diameters of each fistula were presented in figure 1. In group 3, two fistulas were closure (3104, 3105), the rest fistulas show the tendency of decreasing the diameter. In group 2, there is little change in fistula diameter.

In open fistula, the border between trachea and esophageal was well fused together. The border of epithelium was formed in the open fistula in the group 2 and 3. It was not founded in the closed fistulas in group 3 (fig 4).

There was little secretion in trachea in group 3 compared with group 2 (fig 5).

Discussion

In this study, we aimed to investigate the morphological changes of repairing fistula with transplanting AMSCs into fistula tissue in an animal model of TEF. Results showed that the AMSCs group received more benefit than control group in healing and inflammation aspects. The use of AMSCs led to less mortality, less rate of pulmonary infection and better degree of closure.

Mesenchymal stem cells (MSCs), which are referred to as stromal progenitor cells are self-renewing and expandable stem cells. MSCs from amount sites, including bone marrow and adipose tissue, may have immunomodulatory properties, thereby indicating a possible use for MSCs in regenerative medicine and tissue engineering[11].

In contrast to bone marrow-derived stem cells, adipose-derived stem cells are abundant in adipose tissue and are easy to harvest and expand in vitro, which makes them a describe cell type for regenerative cell therapy[12].

In our result, all animals present severe symptoms like acute cough, airway chocking, and vomiting in the first postoperative week. The mortality caused by refluxing of gastric contents was different in two group (fig 1). There are not any researches have been reported yet. Combining the subsequent results about pulmonary infection. we suspect the stem cell may reduce the risk of postoperative and reflux mortality in the first week.

There were many reports that MSCs have been used to cure, including bone repair, cardiovascular disease, plastic surgery, idiopathic pulmonary fibrosis, chronic liver injury, acute renal injury, and so on[13]. However, few studies using in vivo animal models had provided evidence on healing effects of MSCs in

TEF. There are several researches about the efficacy of various approaches on TEF animal model (fig 2). In term of survival time, the surgery retains a longest survival rate and time.in Yang et al study[14], all TEF animals received a long survival time up to 56 d. it is longer than survival time of stent and stem cell. In the research of Wagner[15], the healing effects of covered stent, the longest survival time was 36 d which still need an additional tracheal stent. The longest survival time by one esophageal stent was only 30 d. Our survival day was 35 d in stem cell group, lower than Yang but longer than Wagner. In the rate of pneumonia, the aspiration pneumonia occurred in Wagner and our study after the first postoperative week compared with the surgery study. In our experiment, the pulmonary infection presents a severe consolidation in even whole lungs (upper lobes and lower lobes) in the control group. On the contrary, the contamination was concentrated in the upper lobes in the experimental group. The current researches about stem cells have showed the relative curative effect on the pneumonia. But we believe the fistula closure effect from stem cells is the main motivation to avoid pulmonary infection. The open fistula leads to the persistence of reflux of gastric, which lead to the infection in upper lobs in the early postoperative stage (1~2 week). In the middle stage, the closure of fistula prevents the erosion in lung caused by the reflux in the experimental group (3 week). This procedure was not occurred in the control group, which lead the severe lesion in the lower lobs in the later stage. The infection which involved in whole lung bring about the death of two animals (2102, 2014) in control group.

Although there are not reports on the healing research of TEF by stem cells, there are many cases for the bronchopleural fistula on the stem cell in the healing of the bronchial stumps.

The earliest report of stem cell in treatment of trachea fistula is form Agero et al in 2008[16]. he reported a case that a bronchopleural fistula patient was used autologous adipose-derived stem cell suspended in fibrin glue to close the fistula successfully. That is the first report on the treatment of tracheal fistula by autologous cell therapy. Prudencio et al[17] reported that two patients with bronchopleural fistula received a successful closure, and no treatment -related adverse reactions recurred in the 3 year follow up. Francesco et al[18] reported that bone marrow-derived stem cells were effectively closes bronchopleural fistula in animal model by extraluminal fibroblast proliferation and collagenous matrix development, which is similar with our results. In 2015, he reported the succeed in tracheobronchial dehiscence after stem cell infusion[19]. In 2016, Johnathon et al[20] combined the bio-absorbable mesh and autologous stem cell, and the stem cell-seeded matrix graft was used to repair a multiply recurrent postpneumonectomy bronchopleural fistula successfully. That is the first application of combination with bio-material and stem cell in tracheal fistula therapy. In the meantime, the difficulty spot we met is also that stem cells lack adhesive carrier in fistula transplantation. Partial stem cells injected into submembrane were leaked into outside. The leak of cells might be the reason that the animals in group 3 present a different healing degree. According to our previous research, the cardiac septal defect occlude (Amplazer occlude) has an eminent load capacity of various cells in vitro. However, the occlude alone could not have a long-term curative effect to fistula in our precious animal study. The difficulty in epithelialization and growth of miscellaneous bacteria are the limitation for the new occlude. Hence, the

stem cells might ameliorate the problem according to the good epithelialized result from our research. The combination may be another new clinical strategy of the treatment of TEF.

In the current study, we reported the various effects of MSCs transplantation on TEF such as following: (1) reduce the death risk in early stage of TEF; (2) relieve the pneumonia and reduce the accumulation of secretion; (3) accelerate the closure of TEF in some degree; (4) increase in collagen synthesis because of fibroblast from stem cell.

In conclusion, this was the first study to use locally transplanted cell therapy for the healing of TEF. AMSCs therapy significantly accelerated the healing degree of TEF. That may be a potential therapeutic strategy to acquired TEF if combine the appropriative stent which can load stem cell efficiently.

Abbreviations

tracheoesophageal fistula - TEF

mesenchymal stem cells - MSCs

adipose-derived mesenchymal stem cells - AMSCs

total parenteral nutrition - TPN

Declarations

Availability of Data and Materials

All data, models, and code generated or used during the study appear in the submitted article.

Acknowledgements

1 am very grateful to my mentor and all participants in this research.

Declarations

All authors promise the truth of all data, models and code generated or used during the study appear in the submitted article. All data appeared in article were not contain individual person`s data. All data in submitted article were still not published. All authors agree to publish the submitted article.

Ethics

All animals were designed to minimize pain or discomfort to the animal. This protocol was approved by the IACUC of *Gangdong laboratory animals monitoring institute.* The number of IACUC is IACUC2020132.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors

Author contributions

All authors contributed to the conception of the study;

Yuchao Wang perform the experiment, data collected, data analyses and wrote the manuscript;

Shifang Yang performed the data analyses and modified manuscript;

Pingping Chen and Hanyi Xu contributed to modified manuscript mainly;

Jinghua Cui performed some experiment and modified manuscript.

Jing Li contributed significantly to analysis and manuscript preparation;

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Tables

Due to technical limitations, table 1-4 is only available as a download in the Supplemental Files section.

Figures

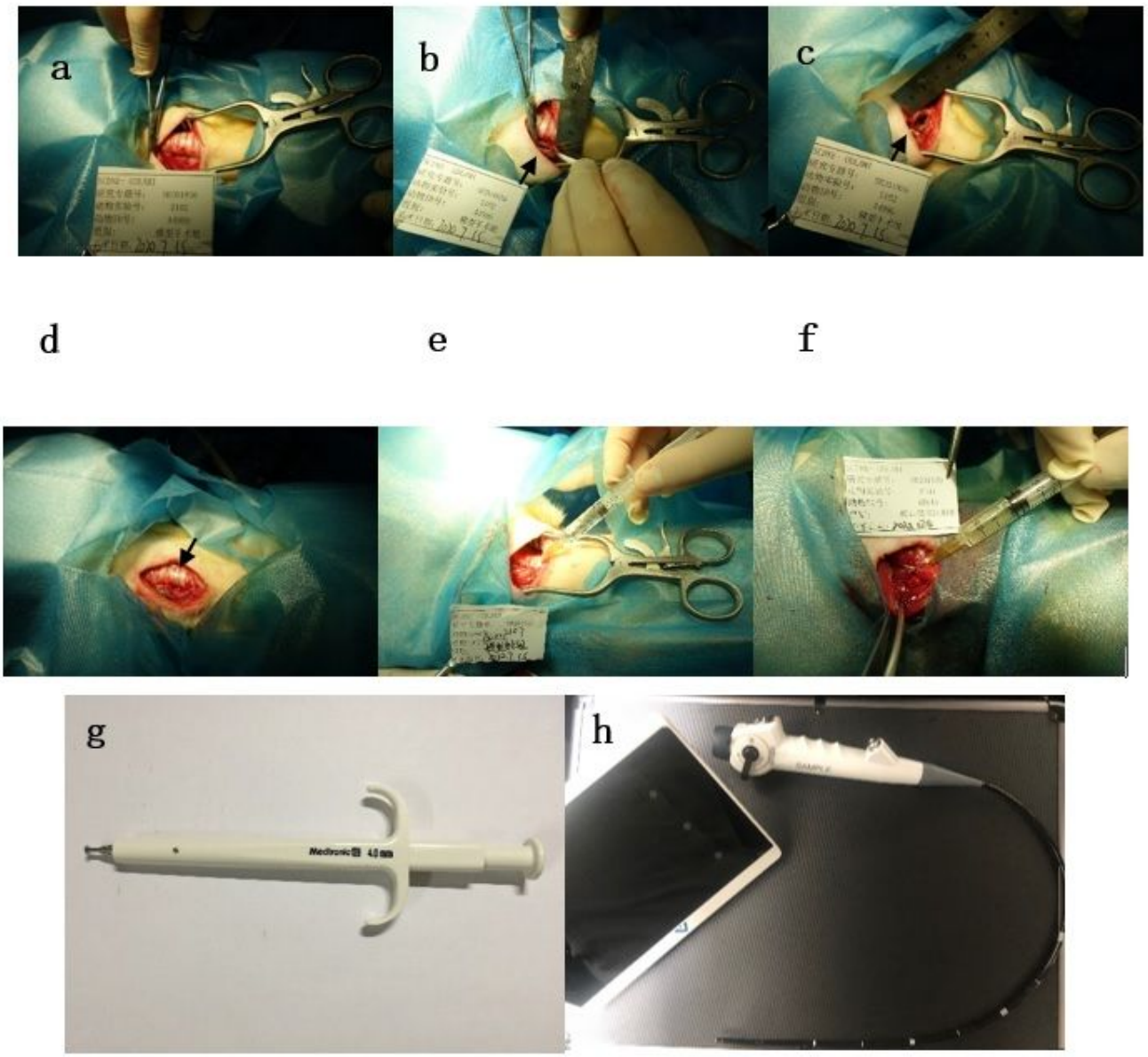

Figure 1

operation procedure and involved apparatuses. a. cervical median incision was made and exposed the trachea and esophagus; b and c. the black arrow point trachea and esophagus fistulas, 8 mm; d. black arrow point the stitched fistula; e. the control group (group 2) injected the saline water; f. the experimental group (group3) injected the AMSCs; g. the mediocre punch we used, limited by the type, we try to multiple punch; h. the bronchoscope we used.

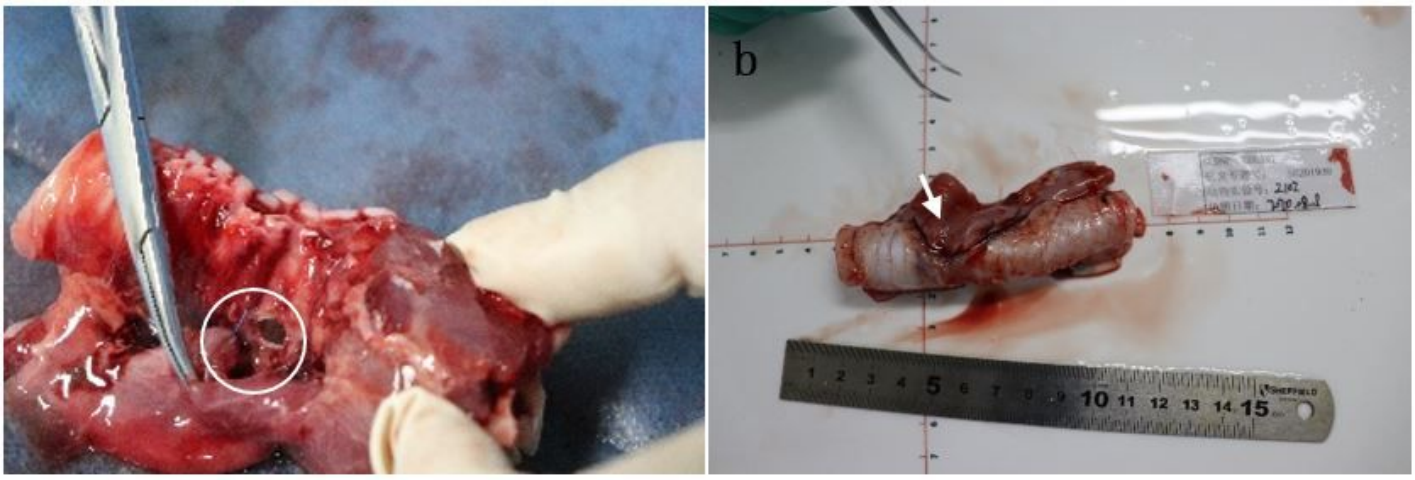

Figure 2

different constructure between fistula. a. fistula from animal 2101. Due to it died in the first week, the fistula was not fibrotic, the stitch maintained the fistula. b. fistula from animal 2102. The animal was survived 35 d, the fistula was maintained by the fibrotic construction between trachea and esophagus.

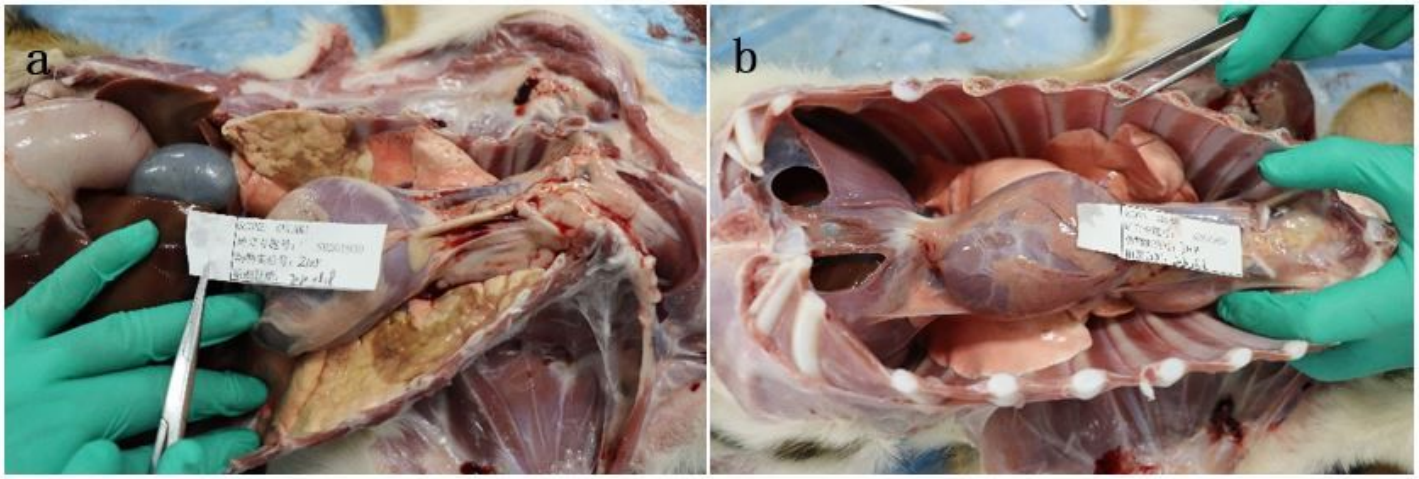

Figure 3

the pneumonia condition. a. animal 2105, the contamination was diffused whole lung, only little right lobs appear healthy condition. b. animal 3104, the contamination was limited in the upper lobs, and most of the lung remain healthy condition.

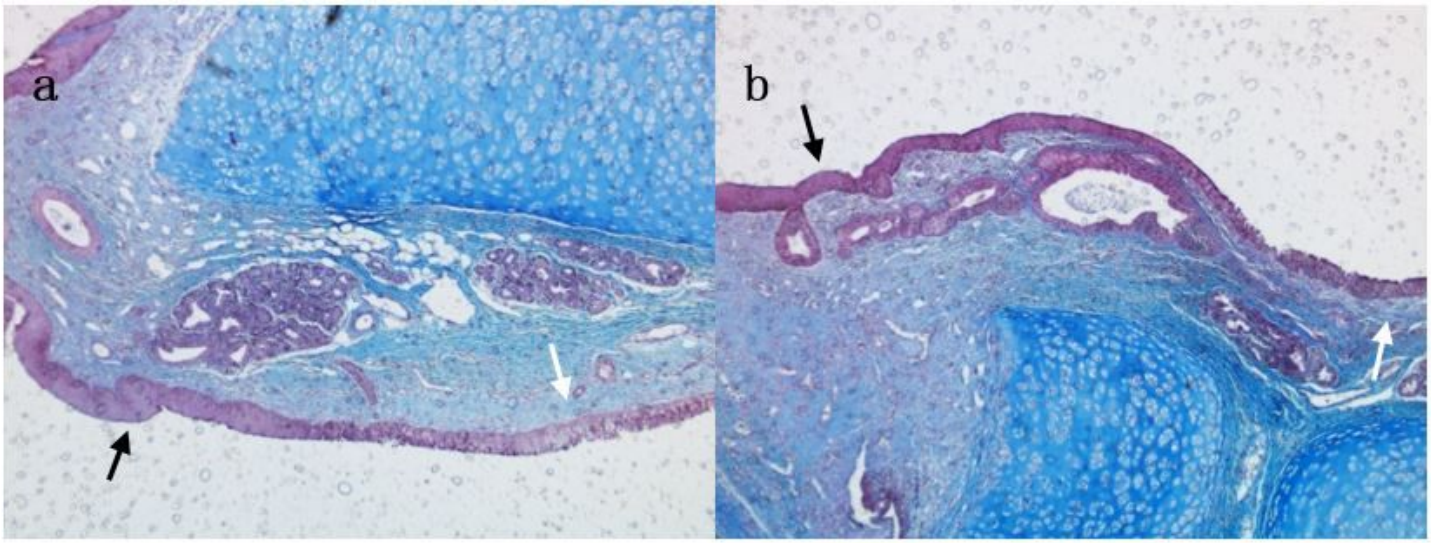

Figure 4

The border between trachea and esophagus. There are two kind of epithelial cells from two group animals. fig 4a. animal 3101; fig 4b. animal 2105. Both animals present the open fistula. In the border between trachea and esophagus, the black arrow point the esophageal epithelial cells and the white arrow point the tracheal epithelial cells.

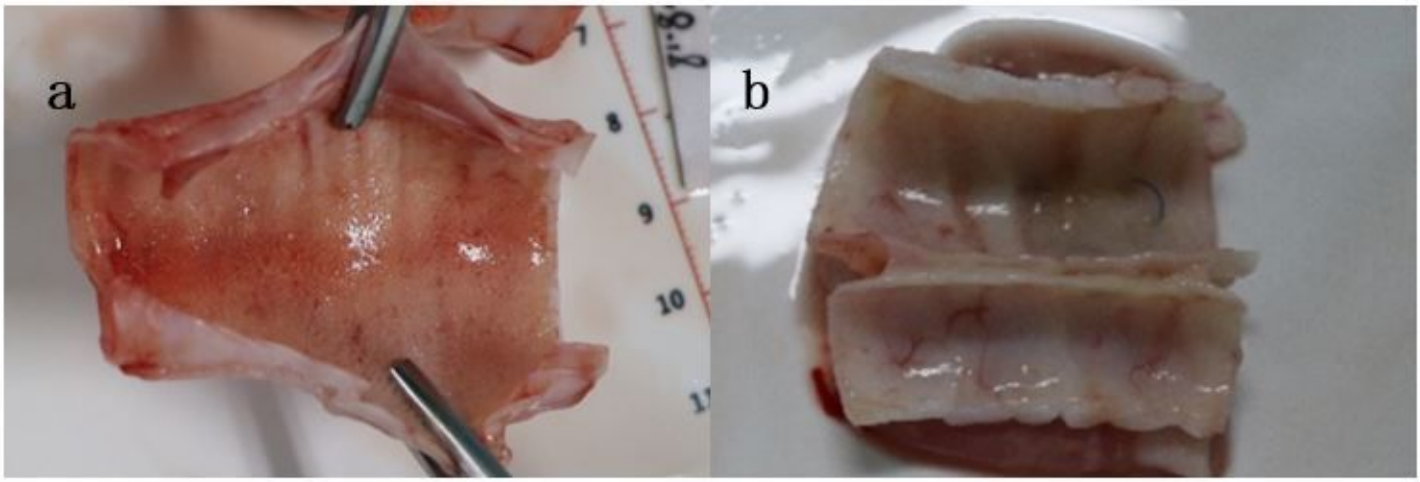

Figure 5

Different secretion condition in animals from group 2 and 3.5a. animal 2102 with much secretion in the trachea; 5b. animal 3104 with few secretion in the trachea.

Supplementary Files

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- Tables.docx