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THE EFFECT OF LOCALLY APPLIED TGF-BETA3 ON WOUND HEALING AND STENOSIS DEVELOPMENT IN TRACHEAL SURGERY

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Abstract

Background: Tracheal stenosis constitutes one of the most frequently seen problems in thoracic surgery. Although many treatment modalities to prevent fibroblast proliferation, angiogenesis, or inflammation that causes tracheal stenosis have been attempted, an effective method has not yet been found. In this study, a TGF-beta3/chitosan combination was used for that purpose.

Methods: A film-shaped slow-releasing preparation containing TGF-beta3 with basal substance of chitosan was made. Thirty albino Wistar rats were divided into three groups. A full-layer vertical incision was made in the anterior side of the trachea of each rat between the 2nd and 5th tracheal rings. The tracheal incision was sutured. Group A was evaluated as the control group. In Group B, a chitosan-based film substance was placed on the incision line. In Group C, a chitosan- TGF- β3 combination slow-release film-coated substance was placed on the incision line. The rats were sacrificed on the thirtieth day, and their tracheas excised by cutting between the lower edge of the thyroid cartilage and the upper edge of the 6th tracheal ring together with the esophagus. Epithelialization, fibroblast proliferation, angiogenesis, inflammation and collagen levels were evaluated histopathologically by the same histopathologist.

Results: It was not found that there were statistically significant differences between the three groups. Cold abscesses were observed at the incision area in both the TGFB3/chitosan combination and chitosan groups. These were thought to have formed due to the chitosan.

Conclusion: As this was the first experiment in the literature to use this type of TGF-beta3 formulation, it is intended to change the formulation and perform this study again with a different TGF-beta3-chitosan preparation.

Keywords: TGF-beta3, trachea, stenosis, surgery

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Introduction

Various treatment methods have been developed for the trachea benign and malignant lesions. Bronchoscopic dilatation, stent placement, laser, surgical resection and reconstruction are among these treatment methods. The most important cause of treatment failure following surgical resection and tip-to-tip anastomosis is stenosis development (1-3).

Several additional methods have been applied in order to increase the efficacy of the surgical treatment. Antibiotics and corticosteroids are frequently used for this purpose. In addition to these, a slow-release 5-florourasil triamcinolone compound, mitomycin-C, hyperbaric oxygen, carnitine, and halofuginon are applied during treatment. There have been investigations into various wound healing regulator agents. In recent years, many studies have been conducted to evaluate the effects of Transforming Growth Factor Beta1 (TGF-β1), TGF-beta2 (β2) and TGF-beta 3 (β3) on wound healing. TGF-β1, β2 and β3 are cytokines that are known for their efficacy at every level of wound healing (4). It is propounded in the studies that TGF-β3 decreases scar tissue while TGF-β1 and TGF-β2 increase collagen synthesis (1). This study aims to determine the role of locally applied TGF-β3 in preventing trachea stenosis after trachea surgery.

Material and Methods

This study was carried out at the Experimental Medicine Research and Application Center of the Faculty of Medicine of Kocaeli University with the approval of the Animal Testing Ethics Committee of the Faculty of Medicine of Kocaeli University, dated 22.02.2011 and numbered 2/1-2011.

By melting it in the substance of base chitosan, Recombinant Human TGF-beta3 (R&D Systems®, Albio), which is protected by a cold chain, was made into a film layer and 5mm x 5mm-dimensioned, slow-release preparation. The intention of this adaptation was that TGF-beta3 would be effective at every level of wound healing.

Thirty male albino Wistar rats, each weighing between 250-300 grams (average weight: 280 grams) were used in the study. Rats were in three groups: A, B, and C. Each group contained

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10 rats. The A, B and C groups were defined as Control, Base (Chitosan) and Active ingredient + base (TGF-β3 + Chitosan).

Rats were anaesthetized with an application of 90 mg/kg ketamine hydrochloride (Ketalar® 10 ml flakon, Pfizer) and 10 mg/kg xylazine hydrochloride (Rompun® 50 ml 2% flakon, Bayer). Rats were left naturally breathing during the operation. A vertical skin incision of approximately 3 cm and extending from the upper border of the thyroid cartilage under maxilla at midline to incisura jugularis was made. The skin and subcutaneous were bypassed and muscles were structured as laryngotracheal by being excluded laterally (figure 1). A vertical incision of approximately 0.5 cm was made at the front of the 2nd and 5th tracheal rings of all the rats by including three cartilages (figure 1). In each rat, the membranous part of the trachea was preserved. The tracheal incision was sutured with 4/0 polyglactin 910 (Vicryl, Ethicon, Belgium).

In Group A, the tracheal incision region was only sutured, and Group A was evaluated as the control group. In Group B, a chitosan film layer was placed as a base substance on the tracheal incision region. In Group C, a 5mm x 5mm dimensioned- slow-release film layered-1µ TGF-β3 preparation was placed into the sutured region (figure 1).

On the thirtieth day, all the rats in Groups A, B, and C were killed with the application of a high-dose isoflurane (İsofludem®, Dem) via inhalation anesthesia apparatus. The tracheas of each rat were removed, with the esophagus, by cutting the upper edge of the thyroid cartilage and the lower part of the 6th tracheal ring. During the study, all surgical operations were performed with the same surgical instruments by the same person to maintain standardization.

The rats' tracheas were separately numbered for each group for microscopic examination and then subject to blind examination by a pathologist who has experienced especially on pulmonary system and in this kind of studies in our hospital. For the microscopic examination, incisions were fixed with 10% neutral formaldehyde.

In the histopathologic examination, epithelialization, inflammation, angiogenesis, fibroblast and collagen parameters were reviewed. All parameters were qualified as none (-), mild (+), moderate (++), a lot (+++) and excessive (++++). In the course of microscopic evaluation, the

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parameters, which had been evaluated with the signs of "+" and "-", were converted into numerical values of 0, 1, 2, 3, 4 for statistical evaluation (table 1).

For the statistical analysis of the study's findings, the SPSS (Statistical Package for Social Sciences) for Windows 13.0 program was used. The *Pearson Chi-Square Test* and *Trend Chi-Square Test* was used for statistical evaluation of the epithelialization, inflammation, angiogenesis, fibroblast, collagen and complication results of the three groups. Results were evaluated at 95% confidence interval, $p<0.05$ significance level and at 99% confidence interval, $p<0.01$ severe significance level.

Results

The rats had been monitored up to termination for respiratory distress, stridor and nutritional disorder. In the first week of the study, two rats from group C (TGF-β3), and one rat from Group B (chitosan) died of general malignancy related to nutritional disorder. While no other abnormal situation was encountered over the following two weeks, one rat from Group C (TGF-β3), one rat from Group B (chitosan), and one rat from Group A (Control Group) died. No new subjects were added because the number of subjects was sufficient statistically. After a 30-day surveillance, seven rats in Group C (TGF-β3), eight rats in Group B (chitosan), and nine rats in Group A (Control Group) were killed.

Massive lesions at different dimensions were palpated in the tracheal incision regions of four subjects from Group C, the combination group of TGF-β3/chitosan, and five subjects from Group B, the chitosan group (figure 2). A complication of the massive lesions was that they were paratracheal-located lesions. There were no complications encountered in the control group. In a histopathologic examination, it was found that the lesions of Group B and C were similar to each other in terms of their characteristics. After a histopathological examination, the lesions were identified as cold abscesses (figure 2).

We aimed to provide a preparation, which could release TGF-beta3 slowly to affect the all levels of wound healing. Before the study, we used chitosan in rats. We did not observe any cold abscess formation. But in the study, cold abscess formation was observed in both chitosan and chitosan + TGF-beta3 group. As we know there are no any slow-releasing preparations. We don't give-up to use TGF-beta3 and in future studies we will try to make the best TGF-beta3 slow-releasing preparation.

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We also had experience of that model of tracheal surgery before and did not see any abscess formation in those studies, we did not use any antibiotics either. In this study, we did not observe any infection symptoms in rats. There were neither local erythema nor heat in the surgical area. There was also no sign of infection in pathological studies, so we did not take specimen for culture. The skin wounds healed without any complication so we thought they were cold abscess due to the foreign body reactions.

The Ki-Kare test was used in order to determine whether there is a statistically significant difference between the TGF-β3/chitosan combination group (Group C), the chitosan group (Group B) and the control group (Group A). From the point of complications, the difference between the groups was found as statistically significant.

Lumen narrowing was seen in all groups. We preferred to compare microscopic findings between the groups. We did not calculate the tracheal lumens but all tracheal sections were prepared from the narrowest part of the tracheas.
Besides we did not examined tracheal stenosis directly because of the short study time. We aimed to evaluate wound healing and look for epithelialization, fibroblast proliferation, angiogenesis, inflammation and collagen levels those were thought to form tracheal stenosis.

In histopathological examination, the Ki-Kare test was used in order to evaluate whether there is a statistically significant difference between the TGF-β3/chitosan combination group (Group C), the chitosan group (Group B) and the control group (Group A) in terms of epithelialization, inflammation, angiogenesis, fibroblast and collagen parameters levels. For all parameters' levels, statistically significant differences could not be found between the groups ($P>0.05$). Results of histopathologic examinations were shown in table1,2,3,4.

Discussion

Trachea surgery is performed as curative method in most cases although it is sometimes difficult in terms of the anatomic and technical aspects. Tracheal resection is frequently applied for tracheal tumors, stenosis, trauma, congenital anomaly and vascular lesions. The primary and most important complication in surgical treatments is recurrent stenosis related to formation of granulation tissue (3).

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Some treatments are applied in order to avoid the formation of granulation tissue and any one of the three stages of wound healing (5). Treatment methods according to these stages are listed below:

1. Inflammation phase: antibiotics and corticosteroids, hyperbaric oxygen therapy,
2. Proliferation phase: antibiotics and corticosteroids, mitomycin-C, combination of 5-flourouracil and triamcinolone acetate, carnitine, hyperbaric oxygen therapy,
3. Maturation phase: halofuginone, beta-aminopropionitrile, colchicine, penicillamine, N-acetyl-L-cysteine.

Research was conducted for new treatment methods to prevent granulation formation of the patients with tracheal stenosis. In the study conducted with estrogen and progesterone, Liman et. al. showed that sex hormones prevent massive collagen and fibroblast proliferation of wound healing after trachea surgery (6).

Mitomycin-C, used for tracheal stenosis treatment for the first time in 1998, is the most-investigated drug (5). Although it is not clear how mitomycin-C prevents the fibroblast activity, there are some findings showing that mitomycin-C and fibroblast activity are pressed depending on apoptosis. Also, it is stated that mitomycin-C makes wound healing slower at the inflammation and proliferation stages by decreasing bFGF (basic fibroblast growth factor) and TGF- β1 (transforming growth factor β1) levels (7).

There are studies showing that TGF-β3, which was used in this study, is confidently used in topical form for the treatment of hypertrophic scar tissues of skin. Hirsnberg et.al. indicated with their study that the gel form of TGF-β3 had been used topically for bedsores and it was very effective (8). Saquier CA. et al. researched permeability and the stability of TGF-β3 used topically on oral mucosa (9). They demonstrated that topical TGF-β's tissue permeability on oral mucosa was very successful, and sufficient concentration was obtained for target tissue.

As it is understood from the effect mechanism of mitomycin-C, superfamily TGF-β3 is an anabolic cytokine that has an important role in wound healing and shaping scars. TGF-β1 and TGF-β2 cause fibrosis by inhibiting collagen production of fibroblasts and ECM destruction of metalloproteases. Although TGF-β1 is profibrotic, TGF-β3 has an antifibrotic effect. According to their studies of an experimental model of rat wounds, Shah et al. identified a decline-recession in the control group's collagen depots in the wound area as a result of TGF-

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β1 and the TGF-β2 neutralizing antibody and exogen TGF-β3 practices (10). TGF-β3 is used in this study mainly because it affects all stages of wound healing, especially the proliferative phase.

Loewen et al. conducted research by traumatizing the cricoid cartilage on rats and applied 1µ TGF-β3 on one group and 0.18µ TGF-β3 on another group (11). They compared the control group with these groups. The results of the study indicated that the group given 1µ TGF-β3 showed the best epithelization and the other groups did not show a significant change. No significant difference could be found among the three groups for inflammation level.

We preferred anterior incision not a full tracheal resection. Because we tried to make damage to trachea but we did not want to intubate the animals. Full tracheal resection might be needed to intubate. We preferred the more easy way to damage the trachea. Anterior incision, which extended 2 to 5 cartilages, provided more damaged tissue area for us. We also made a preparation of TGF-beta3 and we thought the preparation should cover the injury site completely in that way. We tried to avoid esophageal injury and complications depending upon esophageal trauma.

In this study, the dosage of TGF-β3 was determined in accordance with literature and slow-release preparations containing TGF-β3 in the dose of 1µ were prepared. TGF-β3 was combined with chitosan in order to prepare a slow-release preparation. Chitosan was preferred because it is widely-used in the pharmaceutical area and in the preparation of various dosage forms such as microspheres, nanoparticles, microcapsules, and film and tablet formations.

Also, chitosan was preferred as a base substance because of its use in the biomedical area, its degradability, toxicity, and hemostatic functions. In addition, it accelerates wound healing, and has antimicrobial, antiviral and hypocholesterolamic activities, and it is concordant with the body. That the complication of abscess development was not observed in the control group but in the chitosan combination group and chitosan group indicated that the cause could be the chitosan. However, this issue should be examined through further research, as there have been no reported complications regarding chitosan.

In our study also it was aimed to make a slow-releasing preparation with chitosan base substance and affect the all levels of wound healing. We thought that foreign body reactions

caused another inflammation and prevented TGF-beta3 releasing. TGF-beta3 is in form of powder. Preparing solution could be possible but it could not be effective to provide all coverage of the injured area. It would disperse into the neck tissue. That's why we preferred to make a film preparation with chitosan. In addition there is not another preparation form of TGF-beta3 suitable like that. Therefore we are planning to make a new experimental study using a different slow-releasing form.

The results of this study are not in accordance with the literature. TGF-β3 was not found effective for wound healing. Contrary to the hypothesis, the reducing effect on collagen and fibrosis could not be observed on airway wound healing. An abscess that had been caused by chitosan could prevent TGF-β3 from penetrating target tissue. There were excessive collagen formation and tracheal lumen narrowing. We may not say that TGF-beta3 was ineffective, but we can say that we could not show the effect of TGF-beta3 because those abscess formations. They might affect the wound healing process. We would like to prepare another slow-releasing preparation in future. But if somebody else would like to use chitosan, this complication should be known.

There are two stages in the tissue reaction against slow-release film layered preparations. In the first stage, fibrous capsule encircle the preparation. In the second stage, foreign body reaction occurs in the surroundings of microcapsules. Those events occur faster than degradation of the material. Spaces between microcapsules of slow-release film material are filled with macrophages and fibroblasts, which are the basic elements of granulation formation. Fibroblasts produce collagen and fibrous tissue is formed. In those circumstances, very small number of effective microcapsules is left. In the last stage, vascular capillaries disappear and fibrous capsule is constricted. Collagen fibers become compact and the remaining effective microcapsules are trapped. Because of these histopathologic changes, we believe that TGF-beta3 did not reach the injured tissue effectively in our study.

Consequently, the slow-release preparation-TGF-β3, not been encountered in the literature, was first used in tracheal surgery. It was thought that sufficient tissue concentration could not be achieved because of abscess caused by the chitosan base substance. However, this study is suggestive of further research about TGF-β3 to prevent granulation tissue formation after tracheal surgery.

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Table 1: Distribution of inflammation in-groups and among groups

Table 2: Distribution of collagen in-groups and among groups

Table 3: Distribution of epithelium regeneration in-groups and among groups

Table 4: Distribution of angiogenesis in-groups and among group

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FIGURE LEGENDS

Figure 1: Appearance of trachea after dissecting strap muscles laterally (a). Tracheal incision (b). Suturing incision of trachea (c). Placement of TGF-β3/chitosan preparation onto the incision line (d). Appearance of the surgical field after placing preparation (e).

Figure 2: Appearance of the mass palpated in the suture line in surgical field (a). Abscess formation over the trachea (b). Cross-section view of trachea and abscess (green arrow: abscess, red arrow: tracheal incision line) (c). Microscopically appearance of abscess and trachea (HEx40) (green arrow: abscess, red arrow: tracheal incision line) (d).

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Table 1: distribution of inflammation in-groups and among groups

INFLAMMATION		TGF-B3 (Group C)	CHITOSAN (Group B)	CONTROL (Group A)	TOTAL
(+)	**Number of subjects**	**3**	**4**	**4**	**11**
	Inflammation %	27,3%	36,4%	36,4%	100,0%
	Group %	42,9%	50,0%	44,4%	45,8%
(++)	**Number of subjects**	**2**	**1**	**2**	**5**
	Inflammation %	40,0%	20,0%	40,0%	100,0%
	Group %	28,6%	12,5%	22,2%	20,8%
(+++)	**Number of subjects**	**1**	**3**	**2**	**6**
	Inflammation %	16,7%	50,0%	33,3%	100,0%
	Group %	14,3%	37,5%	22,2%	25,0%
(++++)	**Number of subjects**	**1**	**0**	**1**	**2**
	Inflammation %	50,0%	,0%	50,0%	100,0%
	Group %	14,3%	,0%	11,1%	8,3%
Total	**Number of subjects**	7	8	9	24

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Table 2: distribution of collagen in-groups and among groups

COLLAJEN		TGF-B3 (Group C)	CHITOSAN (Group B)	CONTROL (Group A)	TOTAL
(-)	**Number of subjects**	**1**	**1**	**7**	**9**
	Collagen %	11,1%	11,1%	77,8%	100,0%
	Group %	14,3%	12,5%	77,8%	37,5%
(+)	**Number of subjects**	**2**	**4**	**0**	**6**
	Collagen %	33,3%	66,7%	,0%	100,0%
	Group %	28,6%	50,0%	,0%	25,0%
(++)	**Number of subjects**	**4**	**2**	**0**	**6**
	Collagen %	66,7%	33,3%	,0%	100,0%
	Group %	57,1%	25,0%	,0%	25,0%
(+++)	**Number of subjects**	**0**	**1**	**2**	**3**
	Collagen %	,0%	33,3%	66,7%	100,0%
	Group %	,0%	12,5%	22,2%	12,5%
Total	**Number of subjects**	**7**	**8**	**9**	**24**

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Table 3: distribution of epithelium regeneration in-groups and among groups

EPITHELIUM		TGF-B3 (Group C)	CHITOSAN (Group B)	CONTROL (Group A)	TOTAL
(-)	**Number of subjects**	**0**	**1**	**0**	**1**
	Epithelium %	,0%	100,0%	,0%	**100,0%**
	Group %	,0%	12,5%	,0%	**4,2%**
(+)	**Number of subjects**	**4**	**1**	**2**	**7**
	Epithelium %	57,1%	14,3%	28,6%	**100,0%**
	Group %	57,1%	12,5%	22,2%	**29,2%**
(++)	**Number of subjects**	**2**	**3**	**4**	**9**
	Epithelium %	22,2%	33,3%	44,4%	**100,0%**
	Group %	28,6%	37,5%	44,4%	**37,5%**
(+++)	**Number of subjects**	**1**	**3**	**3**	**7**
	Epithelium %	14,3%	42,9%	42,9%	**100,0%**
	Group %	14,3%	37,5%	33,3%	**29,2%**

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Table 4: distribution of angiogenesis in-groups and among group

ANGIOGENESIS		TGF-β3	CHITOSAN	CONTROL	TOTAL
(-)	**Number of subjects**	**0**	**0**	**1**	**1**
	Angiogenesis %	,0%	,0%	100,0%	100,0%
	Group %	,0%	,0%	11,1%	4,2%
(+)	**Number of subjects**	**3**	**3**	**4**	**10**
	Angiogenesis %	30,0%	30,0%	40,0%	100,0%
	Group %	42,9%	37,5%	44,4%	41,7%
(++)	**Number of subjects**	**2**	**2**	**3**	**7**
	Angiogenesis %	28,6%	28,6%	42,9%	100,0%
	Group %	28,6%	25,0%	33,3%	29,2%
(+++)	**Number of subjects**	**2**	**3**	**1**	**6**
	Angiogenesis %	33,3%	50,0%	16,7%	100,0%
	Group %	28,6%	37,5%	11,1%	25,0%
Total	**Number of subjects**	**7**	**8**	**9**	**24**

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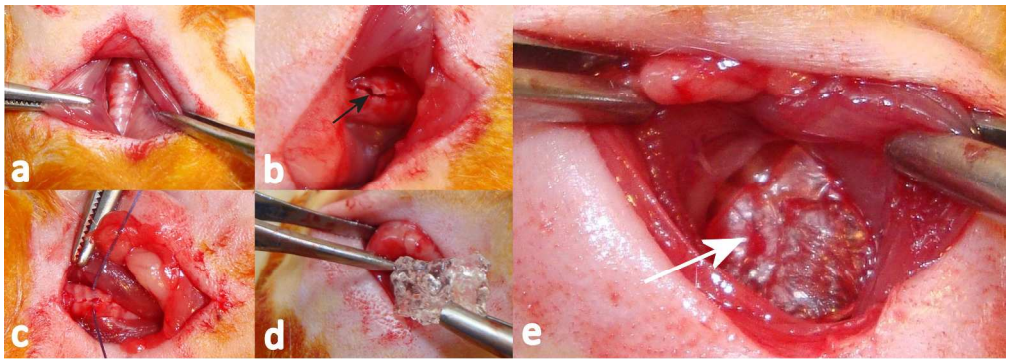

Appearance of trachea after dissecting strap muscles laterally (a). Tracheal incision (b). Suturing incision of trachea (c). Placement of TGF-β3/chitosan preparation onto the incision line (d). Appearance of the surgical field after placing preparation (e).
568x199mm (300 x 300 DPI)

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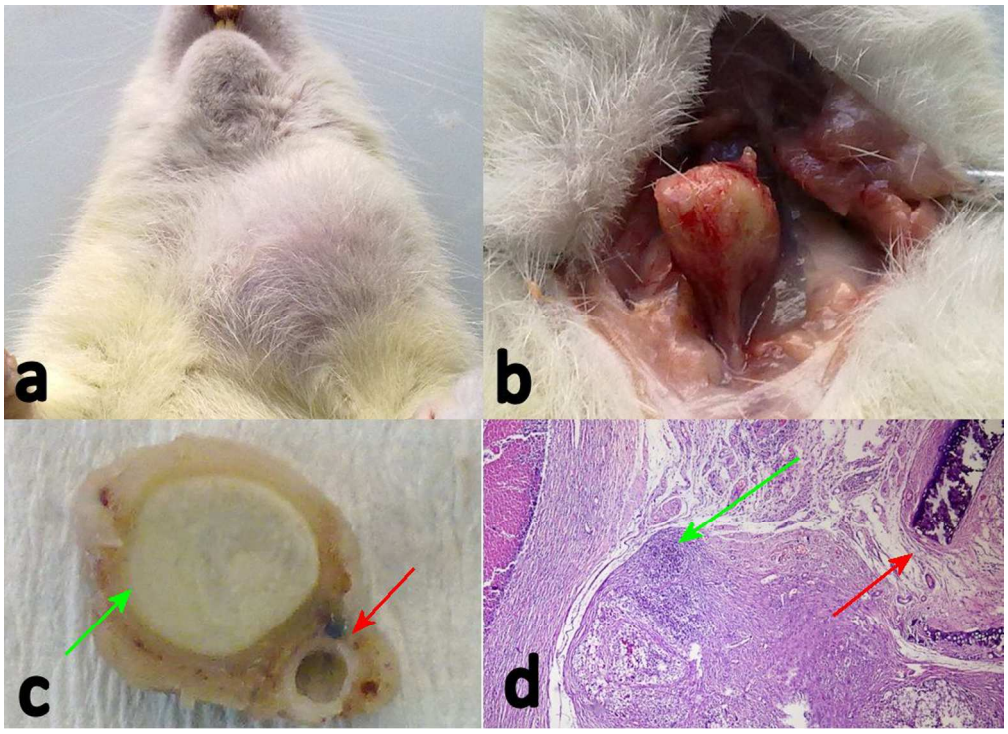

Appearance of the mass palpated in the suture line in surgical field (a). Abscess formation over the trachea (b). Cross-section view of trachea and abscess (green arrow: abscess, red arrow: tracheal incision line) (c). Microscopically appearance of abscess and trachea (HEx40) (green arrow: abscess, red arrow: tracheal incision line (d)
384x277mm (300 x 300 DPI)