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*Experimental Study / Deneysel Çalışma*

# Effects of transforming growth factor-beta-1 neutralizing antibody and transforming growth factor-beta-3 on the development of tracheal stenosis

## *Transforme edici büyüme faktörü-beta-1 nötralizan antikoru ve transforme edici büyüme faktörü-beta-3'ün trakeal darlık gelişimi üzerine etkileri*

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***ABSTRACT***

***Background:*** This study aims to evaluate the effects of transforming growth factor-β3 and neutralizing antibody of transforming growth factor-β1 containing polymeric polycaprolactone film formulations on prevention of stenosis in tracheal surgery.

***Methods:*** The study included 24 male Wistar albino rats (weight 200 g to 250 g). Groups were defined as A) control (n=6); B) blank polymeric polycaprolactone film (n=6); C) transforming growth factor-β3 containing polymeric polycaprolactone film formulation (n=6); and D) transforming growth factor-β1 neutralizing antibody containing polymeric polycaprolactone film formulation (n=6). Approximately a 0.5 cm vertical incision was performed on all rats between the second and fifth tracheal circles. In group A, tracheal incision was only sutured. In groups B, C and D, tracheal incision was sutured and then blank polymeric polycaprolactone film, transforming growth factor-β3 containing polymeric polycaprolactone film formulation and transforming growth factor-β1 neutralizing antibody containing polymeric polycaprolactone film formulation was placed on the tracheal incision, respectively. The rats were sacrificed 30 days after the surgery. Subsequently, tracheas of rats were examined microscopically. Epithelialization, fibrosis, angiogenesis and inflammation statuses were evaluated histopathologically.

***Results:*** The rats that were observed in terms of respiratory distress, stridor, and malnutrition for 30 days did not show any abnormal events. When the groups were evaluated in terms of inflammation, fibrosis, angiogenesis and epithelization, no statistically significant difference was found (p>0.05).

***Conclusion:*** The active forms of transforming growth factor have a considerably short half-life in the tissue and extracted rapidly. Bioactivity may be maintained and controlled release may be provided with preparations to be developed. Further detailed researches are required to evaluate the effect of transforming growth factor-β3 and transforming growth factor-β1 neutralizing antibody on prevention of granulation tissue after tracheal surgery.

***Keywords:*** Stenosis; trachea; transforming growth factor.

***ÖZ***

***Amaç:*** Bu çalışmada transforme edici büyüme faktörü-β3 ve transforme edici büyüme faktörü-β1 nötralizan antikoru içeren polimerik polikaprolakton film formülasyonlarının trakea cerrahisinde darlığın önlenmesi üzerine etkileri değerlendirildi.

***Çalışma planı:*** Çalışmaya 24 erkek Wistar albino sıçan (ağırlık 200 g-250 g) dahil edildi. Gruplar A) kontrol (n=6); B) boş polimerik polikaprolakton film (n=6); C) transforme edici büyüme faktörü-β3 içeren polimerik polikaprolakton film formülasyonu (n=6); D) transforme edici büyüme faktörü-β1 nötralizan antikoru içeren polimerik polikaprolakton film formülasyonu (n=6) olarak tanımlandı. Tüm sıçanlara ikinci ve beşinci trakeal halkalar arasında yaklaşık 0.5 cm'lik vertikal insizyon yapıldı. Grup A'da trakeal insizyon sadece sütüre edildi. Grup B, C ve D'de trakeal insizyon sütüre edildikten sonra trakeal insizyonun üzerine sırası ile boş polimerik polikaprolakton film, transforme edici büyüme faktörü-β3 içeren polimerik polikaprolakton film formülasyonu, transforme edici büyüme faktörü-β1 nötralizan antikoru içeren polimerik polikaprolakton film formülasyonu yerleştirildi. Cerrahiden 30 gün sonra sıçanlar sakrifiye edildi. Sonrasında sıçanların trakeaları mikroskobik olarak incelendi. Epitelizasyon, fibrozis, anjiogenezis ve inflamasyon durumları histopatolojik olarak değerlendirildi.

***Bulgular:*** Otuz gün boyunca solunum sıkıntısı, stridor ve beslenme bozukluğu açısından izlenen sıçanlarda anormal bir durum görülmedi. Gruplar enflamasyon, fibrozis, anjiyogenezis ve epitelizasyon açısından değerlendirildiğinde istatistiksel olarak anlamlı farklılık bulunmadı (p>0.05).

***Sonuç:*** Transforme edici büyüme faktörünün aktif formları dokuda oldukça kısa yarılanma ömrüne sahiptir ve hızla uzaklaştırılmaktadır. Yeni geliştirilecek preperatlar ile bioaktivite korunabilir ve kontrollü salınım sağlanabilir. Transforme edici büyüme faktörü-β3'ün ve transforme edici büyüme faktörü-β1 nötralizan antikorlarının trakea cerrahisi sonrasında granülasyon dokusunu önleme etkisini değerlendirmek için daha ileri detaylı araştırmalara gerek vardır.

***Anahtar sözcükler:*** Darlık; trakea; transforme edici büyüme faktörü.

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Tracheal stenosis leads to constriction in the trachea due to the formation of hypertrophic scars during tissue healing after endotracheal intubation, tracheostomy, and tracheal surgery. It is associated with various factors and can create serious clinical problems. The clinical treatment of tracheal stenosis is surgery.[1] Recurrent stenosis after surgical procedures are observed in the airway and may cause serious problems that require further surgery.[1,2] To improve the effectiveness of the surgical treatment, various methods have been attempted, although an effective treatment has not been developed. Recently, many studies have investigated the effects of transforming growth factor-beta 1 (TGF)-β1, TGF-β2, and TGF-β3 on wound healing. Many studies have indicated that high levels of TGF-β3 reduces scarring in embryos.[3,4] In this study, we aimed to evaluate the effects of TGF-β3 and neutralizing antibody of TGF-β1 containing polymeric polycaprolactone (PCL) film formulations on prevention of stenosis in tracheal surgery.

## MATERIALS AND METHODS

This *in vivo* experimental study, which was carried out in the experimental research laboratory of the Faculty of Medicine between February 2012 and February 2014, included 24 male Wistar albino rats (weight 200 g to 250 g). The study was approved by the Kocaeli University Ethics Committee and complied with the Guidelines for the Care and Use of Experimental Animals. The study was conducted in accordance with the principles of the Declaration of Helsinki.

Polymeric film formulations were prepared as PCL (MW: 65 kDa, Sigma Aldrich, Steinheim, Germany) film layers (5x5mm) containing either TGF-β3 (Escherichia coli human recombinant 1 µg, Cloud-Clone Corp., Houston, TX, USA) or anti-TGF-β1 (mouse anti-TGF β1, 50 µg, Abcam, Inc., Cambridge, MA, USA). These polymeric formulations were protected by a cold chain.

The rats were divided into four groups defined as: A) control (n=6); B) blank PCL film (n=6); C) TGF-β3 containing PCL film formulation (n=6); and D) TGF-β1 neutralizing antibody containing PCL film formulation (n=6).

Each rat was anesthetized with intramuscular injection of 90 mg/kg ketamine hydrochloride (Ketalar® 10 mL vial, Pfizer, Istanbul, Turkey) and 10 mg/kg xylazine hydrochloride (Rompun® 50 mL 2% vial, Bayer, İstanbul,. Turkey) combination. The rats were allowed to breathe spontaneously during the operation. Approximately a 3 cm vertical skin incision was made, extending from the thyroid cartilage to the incisura jugularis. Then, a 0.5 cm full layer vertical incision was made to all rats between the second and fifth tracheal circles. The membranous portion of the trachea was preserved in all rats (Figure 1). The tracheal incision was sutured with 4/0 polyglactin 910 (Vicryl, Ethicon, Brussels, Belgium).

In group A, tracheal incision was sutured and no film formulation was placed on these rats. In group B, C and D, tracheal incision was sutured and then blank PCL film formulation (5×5 mm), TGF-β3 containing PCL film formulation (5×5 mm), and TGF-β1 neutralizing antibody containing PCL film formulation (5×5 mm) were placed on the tracheal incision, respectively.

Rats were not administered antibiotic or analgesic medications till their sacrification. All rats were sacrificed by high dose inhaled isoflurane (Isofludem®) 30 days after the surgery. Following sacrification, the tracheas together with esophaguses of all animals were excised from the upper edge of the thyroid cartilage to the end of the sixth tracheal circle. For histopathological examination, the samples were especially collected from the constricted parts of the trachea due to scar formation. Each trachea was randomly numbered and examined histopathologically in terms of epithelialization,

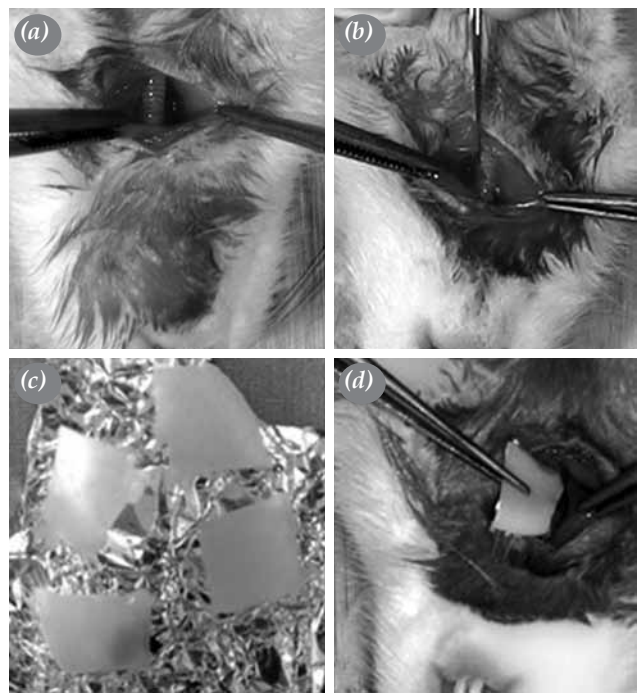


**Figure 1. (a)** Appearance of trachea after dissecting strap muscles laterally. **(b)** Tracheal incision. **(c)** Preparations. **(d)** Placement of preparation into incision line.

fibrosis, angiogenesis and inflammation. Sections for microscopic examination were fixed in 10% neutral formaldehyde. Results were presented as none (-), mild (+), moderate (++), high (+++) or excessive (++++).

### Statistical analysis

Statistical analyses were performed using the IBM SPSS version 20.0 (IBM Corp., Armonk, NY, USA) software package. Variables were presented as frequencies (percentages). Differences between the groups were evaluated using the Fisher's exact test and the Monte Carlo simulation analysis for categorical variables. A *p* value of <0.05 was considered statistically significant.

## RESULTS

The rats were observed in terms of respiratory distress, stridor, and malnutrition for 30 days; no abnormal events occurred. After the 30-day period, all rats were sacrificed. No antibiotics were administered and no local erythema or increased temperatures in the surgical areas were observed. Wound healing occurred without any complications. No signs of infection were detected by the pathological examination.

Our aim was to develop a formulation that released TGF-β3 slowly to have an effect on wound healing. In the present study, we used PCL and did not observe this.

The groups were examined in terms of severity of inflammation; the most severe inflammation was seen in group B (42.9%) and then in group A (28.6%). The least inflammation was seen in group C (14.3%) and group D (14.3%) (Table 1). The results were not significant when evaluated statistically (p>0.05).

When the groups were evaluated in terms of fibrosis, maximum fibrosis was observed in group A (100%), while minimum fibrosis was observed in group D (40%), with no statistically significant difference between the groups (p>0.05) (Table 2).

An evaluation of groups in terms of angiogenesis revealed excessive angiogenesis in groups A (33.3%), B (33.3%), and C (33.3%). Mild angiogenesis was observed only in group A (100%) (Table 3). There was no statistically significant difference between groups in terms of angiogenesis (p>0.05).

An examination of groups in terms of severity of epithelization showed that epithelization was better in group A, with no statistically significant difference (p>0.05) (Table 4).

## DISCUSSION

Tracheal resection is a widely used method for the treatment of tracheal tumors, stenosis, trauma, and

**Table 1. Distribution of inflammation in groups and among groups**

| Inflammation | Group A (Control) | | Group B (Blank PCL) | | Group C (TGF-ß3) | | Group D (TGF-ß1 antibody) | | *Total* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | n | % | n | % | n | % | n | % | n | % |
| Mild (+) | | | | | | | | | | |
| Subjects | 1 | | 1 | | 1 | | 1 | | 4 | |
| Inflammation | | 25.0 | | 25.0 | | 25.0 | | 25.0 | | 25.0 |
| Group | | 16.7 | | 16.7 | | 16.7 | | 16.7 | | 16.7 |
| Moderate (++) | | | | | | | | | | |
| Subjects | 1 | | 1 | | 2 | | 2 | | 6 | |
| Inflammation | | 16.7 | | 16.7 | | 33.3 | | 33.3 | | 100.0 |
| Group | | 16.7 | | 16.7 | | 33.3 | | 33.3 | | 25.0 |
| High (+++) | | | | | | | | | | |
| Subjects | 2 | | 1 | | 2 | | 2 | | 7 | |
| Inflammation | | 28.6 | | 14.3 | | 28.6 | | 28.6 | | 100.0 |
| Group | | 33.3 | | 16.7 | | 33.3 | | 33.3 | | 29.2 |
| Excessive (++++) | | | | | | | | | | |
| Subjects | 2 | | 3 | | 1 | | 1 | | 7 | |
| Inflammation | | 28.6 | | 42.9 | | 14.3 | | 14.3 | | 100.0 |
| Group | | 33.3 | | 50.0 | | 16.7 | | 16.7 | | 29.2 |
| *Total* | | | | | | | | | | |
| Subjects | 6 | | 6 | | 6 | | 6 | | 24 | |

PCL: Polymeric polycaprolactone; TGF: Transforming growth factor.

**Table 2. Distribution of fibrosis in groups and among groups**

| Fibrosis | Group A (Control) | | Group B (Blank PCL) | | Group C (TGF-ß3) | | Group D (TGF-ß1 antibody) | | *Total* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | n | % | n | % | n | % | n | % | n | % |
| Mild (+) | | | | | | | | | | |
| Subjects | 2 | | 0 | | 1 | | 2 | | 5 | |
| Fibrosis | | 40.0 | | 0.0 | | 20.0 | | 40.0 | | 100.0 |
| Group | | 33.3 | | 0.0 | | 16.7 | | 33.3 | | 20.8 |
| Moderate (++) | | | | | | | | | | |
| Subjects | 3 | | 5 | | 3 | | 3 | | 14 | |
| Fibrosis | | 21.4 | | 35.7 | | 21.4 | | 21.4 | | 100.0 |
| Group | | 50.0 | | 83.3 | | 50.0 | | 50.0 | | 58.3 |
| High (+++) | | | | | | | | | | |
| Subjects | 0 | | 1 | | 2 | | 1 | | 4 | |
| Fibrosis | | 0.0 | | 25.0 | | 50.0 | | 25.0 | | 100.0 |
| Group | | 0.0 | | 16.7 | | 33.3 | | 16.7 | | 16.7 |
| Excessive (++++) | | | | | | | | | | |
| Subjects | 1 | | 0 | | 0 | | 0 | | 1 | |
| Fibrosis | | 100.0 | | 0.0 | | 0.0 | | 0.0 | | 100.0 |
| Group | | 16.7 | | 0.0 | | 0.0 | | 0.0 | | 44.2 |
| *Total* | | | | | | | | | | |
| Subjects | 6 | | 6 | | 6 | | 6 | | 24 | |

PCL: Polymeric polycaprolactone; TGF: Transforming growth factor.

congenital anomalies. However, the formation of granulation tissue can lead to recurrent stenosis, the most significant complication of surgical treatments. Following injury, the healing process begins, and consists of three steps: inflammation and migration, proliferation, and remodelling and maturation.[5] The proliferation time may vary between two and seven days. Specific conditions of the patient and the size

**Table 3. Distribution of angiogenesis in groups and among groups**

| Angiogenesis | Group A (Control) | | Group B (Blank PCL) | | Group C (TGF-ß3) | | Group D (TGF-ß1 antibody) | | *Total* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | n | % | n | % | n | % | n | % | n | % |
| Mild (+) | | | | | | | | | | |
| Subjects | 1 | | 0 | | 0 | | 0 | | 1 | |
| Angiogenesis | | 100.0 | | 0.0 | | 0.0 | | 0.0 | | 100.0 |
| Group | | 16.7 | | 0.0 | | 0.0 | | 0.0 | | 44.2 |
| Moderate (++) | | | | | | | | | | |
| Subjects | 1 | | 3 | | 2 | | 3 | | 9 | |
| Angiogenesis | | 11.1 | | 33.3 | | 22.2 | | 33.3 | | 100.0 |
| Group | | 16.7 | | 50.0 | | 33.3 | | 50.0 | | 37.5 |
| High (+++) | | | | | | | | | | |
| Subjects | 3 | | 2 | | 3 | | 3 | | 11 | |
| Angiogenesis | | 27.3 | | 18.2 | | 27.3 | | 27.3 | | 100.0 |
| Group | | | | 33.3 | | 50.0 | | 50.0 | | 45.8 |
| Excessive (++++) | | | | | | | | | | |
| Subjects | 1 | | 1 | | 1 | | 0 | | 3 | |
| Angiogenesis | | 33.3 | | 33.3 | | 33.3 | | 0.0 | | 100.0 |
| Group | | 16.7 | | 16.7 | | 16.7 | | 0.0 | | 12.5 |
| *Total* | | | | | | | | | | |
| Subjects | 6 | | 6 | | 6 | | 6 | | 24 | |

PCL: Polymeric polycaprolactone; TGF: Transforming growth factor.

**Table 4. Distribution of epithelium regeneration in groups and among groups**

| Epithelium | Group A (Control) | | Group B (Blank PCL) | | Group C (TGF-ß3) | | Group D (TGF-ß1 antibody) | | *Total* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | n | % | n | % | n | % | n | % | n | % |
| Mild (+) | | | | | | | | | | |
| Subjects | 0 | | 1 | | 0 | | 0 | | 1 | |
| Epithelium | | 0.0 | | 100.0 | | 0.0 | | 0.0 | | 100.0 |
| Group | | 0.0 | | 16.7 | | 0.0 | | 0.0 | | 4.2 |
| Moderate (++) | | | | | | | | | | |
| Subjects | 1 | | 3 | | 4 | | 4 | | 12 | |
| Epithelium | | 8.3 | | 25.0 | | 33.3 | | 33.3 | | 100.0 |
| Group | | 16.7 | | 50.0 | | 66.7 | | 66.7 | | 50 |
| High (+++) | | | | | | | | | | |
| Subjects | 3 | | 2 | | 2 | | 2 | | 9 | |
| Epithelium | | 33.3 | | 22.2 | | 22.2 | | 22.2 | | 100.0 |
| Group | | 50.0 | | 33.3 | | 33.3 | | 33.3 | | 37.5 |
| Excessive (+++) | | | | | | | | | | |
| Subjects | 2 | | 0 | | 0 | | 0 | | 2 | |
| Epithelium | | 100.0 | | 0.0 | | 0.0 | | 0.0 | | 100.0 |
| Group | | 33.3 | | 0.0 | | 0.0 | | 0.0 | | 8.33 |
| *Total* | | | | | | | | | | |
| Subjects | 6 | | 6 | | 6 | | 6 | | 24 | |

PCL: Polymeric polycaprolactone; TGF: Transforming growth factor.

of the wound determine the proliferation time. After proliferation, re-epithelialization and extracellular matrix formation occur.[6] In the last phase, the wound stabilises and cell proliferation diminish.[7] In normal wound healing, TGF-β oscillation is necessary for keratinocyte migration. In human beings and most mammals, TGF-β has three subtypes (TGF-β1, β2, β3) and these influence the healing process. Due to the high synthesis rate of collagen, scar tissue forms in tissues affected by TGF-β1 and TGF-β2.[7] However, the TGF-β3 isoform suppresses intense collagen production caused by TGF-β1, preventing scar formation.[8] The same may be true in the trachea. In many studies, TGF-β has been delivered locally. Loewen et al.[9] traumatized cricoid cartilage in rats and applied 1 µg TGF-β3 to one group and 0.18 µg TGF-β3 to another. They found that the former group showed improved epithelialization, whereas the other group showed no significant improvement. Shah et al.[10] identified a decline in collagen deposits in a wound area attributable to TGF-β1 and TGF-β2 neutralizing antibodies and exogenic TGF-β3. We used TGF-β3 because it affects all stages of wound healing, particularly the proliferative phase. Gunay et al.[11] reported that platelet rich plasma including growth factors reduce complications and possible tracheal stenosis after surgery.

In active form, TGF-βs are broken down fast and extracted from the tissues. Hence, new drug-delivery systems are needed to maintain their bioactivity and provide controlled release. In our study, TGF-β1 neutralizing antibody and TGF-β3 were loaded into film formulations for three reasons: to prevent enzymatic breakdown, reduce the rate of TGF-β activity, and provide controlled release. Biocompatible polymers such as PCL and poly (lactic-co-glycolic acid) (PLGA) are also used in such polymeric films. In the evaluation of *in vivo* practises, no tissue reactions were encountered in biocompatible and biodegradable polymer preparations (e.g., PCL and PLGA). However, a previous study reported a tissue reaction when using slow-release preparations containing TGF-β3 at a dose of 1 µg combined with chitosan.[12] This decreased the effectiveness of the film formations. Therefore, we used new preparations to ensure tissue compatibility.

In preparing our rats for study, we performed an anterior incision rather than a full tracheal resection because the aim was to damage the trachea without having to intubate the animals. Anterior incision (extending from the second to the fifth tracheal ring) provided a greater area of damaged tissue. We also prepared formulations/patches that covered injured sites completely. We avoided oesophageal injury and complications from oesophageal trauma.

The dose of TGF-β3 that we used was determined based on the literature, and the slow-release preparations contained 1 μg TGF-β3 combined with PCL. This polymer was chosen because it has been used widely in pharmaceutical preparations of various forms, such as microspheres, nanoparticles, microcapsules, films, and tablets. Synthetic polymers tend to be better than natural polymers. They have high purity and non-toxic by-products. They are also easy to produce and their biodegradability can be controlled; hence, they are widely used in the production of drug-release systems. Polyesters such as PCL, polylactic acid, glycolic acid, and copolymers of lactic acid and glycolic acid are the most commonly used synthetic polymers.[13] One of the most important features of PCL is that it can be used in combination with many different polymers. Thus, this polymer can be used in various medical practices.[14,15] Polycaprolactone is a deformable polymer and its biodegradation occurs slowly over a long period of time. It is also widely biocompatible,[16] which is the main reason we chose it for our current study.

The results of this study were not in accordance with the literature. Transforming growth factor-β3 and TGF-β1 neutralizing antibody were not effective for wound healing. Contrary to our hypothesis, reduced collagen levels and fibrosis were not observed during healing. We reached the same results in a previous study in which we used a slow-release alkaline chitosan. However, in the previous study, chitosan caused a tissue reaction. In addition, the TGF active ingredient had not reached a sufficient concentration in the tissue. To enable release of TGF-β3 slowly to have an effect on wound healing without causing cold abscesses, we used PCL and did not observe cold abscesses.

The main limiting factor that negatively affects our study is the 30 days-period limitation for usage of preparates.

In conclusion, the active forms of transforming growth factor-beta growth factors are broken down fast and extracted from the tissue; thus new drug-delivery systems are needed to maintain their bioactivity and provide controlled release. In the present study, the active release time of our preparations was 30 days, which might not be long enough to reach meaningful results. Therefore, there is a need for further research on the use of transforming growth factor-beta-3 for preventing granulation of tissue following tracheal surgery.

**Declaration of conflicting interests**

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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