



## NOTE

Surgery

# Visual and histological evaluation of the effects of trafermin in a dog oronasal fistula model

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**ABSTRACT.** The standard procedure to treat oronasal fistula in dogs requires tooth extraction to close the fistula; hence, the subject would lose its tooth. In this study, trafermin was applied to four dog models with oronasal fistula to investigate the periodontal tissue regenerative effects of trafermin in the treatment without tooth extraction. A fistula was created along the palatal side of each upper canine tooth. One of the fistulae was filled with trafermin, whereas that on the contralateral side was left unfilled as a control. The results showed a significant decrease in the non-calcified periodontal tissue volume on the trafermin side after the fourth week. In addition, oronasal fistula closure was visually and histologically confirmed at the eighth week on the trafermin side of all four models.

**KEY WORDS:** dog, oronasal fistula, periodontal regeneration, recombinant human basic fibroblast growth factor, trafermin

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Oronasal fistula in dogs is a disease in which the subject's oral and nasal cavities are connected due to a substantial loss of alveolar bone caused by the progress of maxillo-dental periodontitis. Self-healing of the disease is challenging because of the constant passage of food or fluid through the pathway formed by oronasal fistula, and the most effective method to treat the fistula is to close it with the gum flap by tooth extraction [5, 6, 9, 13, 15, 23, 26]. However, tooth loss, especially of canine teeth, where oronasal fistula most often occurs, would pose appearance issues or functional problems; sometimes, the owner does not agree with the treatment. Therefore, conservative treatment without tooth extraction is implemented, but the recurrence rate is relatively high for radical treatment [16].

Currently, periodontal tissue regeneration treatment is implemented for periodontal tissue loss in dogs with severe periodontitis, and clinical applications of artificial bones [3] or enamel matrix proteins [25] have been reported. In the case of human dental treatment, clinical application of trafermin, which contains *Escherichia coli*-derived gene-modified recombinant human basic fibroblast growth factor (rh-bFGF) as an active ingredient of periodontal tissue regenerative agents, has been recently employed [8, 27]. Trafermin has been histologically confirmed with evident regeneration of alveolar bone, cementum, and periodontal membrane [1, 10, 11, 21] in marginal periodontitis in dog models. It has also been confirmed with a significant decrease in the attachment loss in cases of spontaneous periodontitis cases [24].

As mentioned above, the number of cases of clinical application of trafermin in dogs with periodontitis are gradually increasing. In this study, therefore, we experimentally prepared canine oronasal fistula models to apply trafermin and investigate its periodontal tissue regeneration effect visually and histologically.

The present study was approved by the Institutional Animal Care and Use Committee at the Gifu University Faculty of Applied Biological Sciences (approval number: 2019-176).

This study used 4 beagles (male, aged 12 months old and weighing 10.6 kg to 12.5 kg) that seemed to be clinically healthy. All dogs were housed individually and fed the same dry diet (Special Support Sensitive Joint, ROYAL CANIN JAPON, Inc., Tokyo, Japan) once daily with free access to water during the study period.

All test subjects were anesthetized with propofol (PropoFlo, DS Pharma Animal Health Co., Ltd., Osaka, Japan) by intravenous injection (6 mg/kg), and endotracheal intubation was performed to maintain the anesthetic condition with a mixture of pure oxygen

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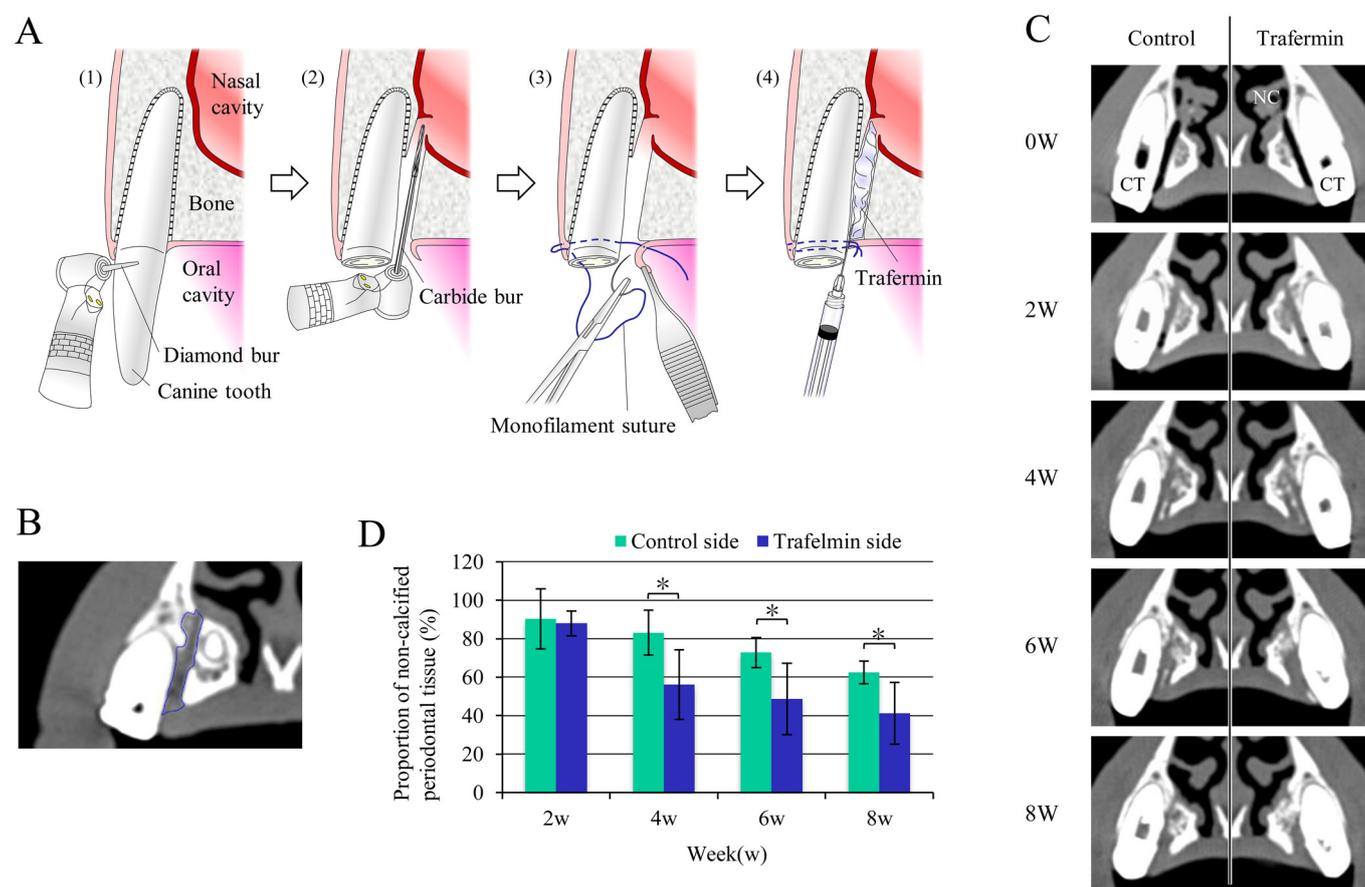
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and 2% isoflurane (Isoflu, DS Pharma Animal Health Co., Ltd.). Scaling was performed on all teeth, the oral cavity was thoroughly rinsed, and infraorbital nerve block anesthesia was performed with a mixture of lidocaine (2 mg/kg, Xylocaine, AstraZeneca K.K., Osaka, Japan) and bupivacaine (1 mg/kg, Marcain, AstraZeneca K.K.). The oronasal fistula models were created using a dental carbide bur (DEN Zekrya Bur, length: 28 mm, Jianguyin Gaofeng Tools Co., Ltd., Jianguyin, PR China), as shown in Fig. 1A (1–2). The palate and gum mucous membranes at the fistula were closed with a 5–0 monofilament suture (ETHICON PDS-II®, Johnson & Johnson, Co., Ltd., Tokyo, Japan) as shown in Fig. 1A: (3), and either the left or right side was randomly filled with trafermin (Regroth® Dental Kit 1,200 µg, 2,553 µg/ml, Kaken Pharmaceutical Co., Ltd., Tokyo, Japan) and marked as the trafermin side, and the other side was filled with the substrate of Regroth®, namely 3% hydroxypropyl cellulose solution (150–400 cP, FUJIFILM Wako Pure Chemical Corp., Osaka, Japan) and marked as the control side, as shown in Fig. 1A: (4). The average amount of rh-bFGF injected into the bone defect was 373 µg.

For pain control, the subjects were administered butorphanol (0.2 mg/kg; Vetorphale, Meiji Seika Pharma Co., Ltd., Tokyo, Japan) by intravenous injection on the day of surgery. During the post-operative days 1 to 3, meloxicam (Metcam Oral Solution, Boehringer Ingelheim Animal Health Japan Co., Ltd., Tokyo, Japan) was administered orally (0.2 mg/kg on day 1, and 0.1 mg/kg on days 2 and 3).

In addition, cefalexin (Larixin, FUJIFILM Toyama Chemical Co., Ltd., Tokyo, Japan) was orally administered (20 mg/kg, bid) as an antibacterial agent until day 7, and the subjects were fed the same dry diet mentioned earlier, softened by soaking in warm water for a week after the surgery.

A series of computerized tomography (CT: Alexion TSX-034A, Toshiba Medical Systems, Tochigi, Japan) scanning (with



**Fig. 1.** A: Preparation of oronasal fistula model and trafermin application. (1) The crown of the upper canine tooth was cut through with a diamond bur to enable the dental carbide bur to cut the fistula into the nasal cavity, and the canine tooth was shortened by severing the vital pulp and implementing pulp capping. (2) The alveolar bone on the upper canine palatal side was cut deep enough to allow the dental carbide bur to reach the nasal cavity. (3) The fistula on the oral side was closed by suturing the palatal mucosa with the gingival mucosa with monofilament suture. (4) The bone defect was filled with a sufficient quantity of trafermin through the gap in the sutured palatal mucosa. B: Setting region of interest (ROI). The non-calcified periodontal tissue (<345 HU) was set as the ROI (area indicated by blue line) in all slices with 0.5 mm thickness in the CT images. C: Changes in the CT images. Fistula reaching the nasal cavity was created on both sides immediately after surgery (0w). Findings indicating tissue calcification was not observed at the second week (2w), but after the fourth week (4w), the trafermin side began to show an increase in calcified periodontal tissue, which eventually closed the fistula at sixth–eighth weeks (6–8w), while fistula closure was not observed on the control side. CT: canine teeth, NC: nasal cavity. D: Proportion of non-calcified periodontal tissue (n=4) in the CT images. No significance was observed on either side in the second week after surgery (2w). After the fourth week (4w), the trafermin side began to show a significant decrease in the proportion of non-calcified periodontal tissue. \*:  $P < 0.05$ .

0.5-mm slice thickness) was conducted under sedation immediately after surgery and 2, 4, 6, and 8 weeks after surgery. The subjects were placed under sedation by intravenous injection of medetomidine (30 µg/kg, Domitor, Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan), midazolam (0.15 mg/kg, Dormicum, Astellas Pharma Inc., Tokyo, Japan), and butorphanol (0.1 mg/kg). They were awoken by intravenous injection with atipamezole hydrochloride (0.15 mg/kg, Atipame injection, Kyoritsu Seiyaku Co., Tokyo, Japan) which was injected intravenously immediately after the CT scanning. The DICOM image files obtained by CT scanning were evaluated by loading to the image processing application software, Osirix (Pixmeo SARL, Switzerland), with non-calcified periodontal tissue set as the region of interest (ROI). Since the mean CT pixel value of human cancellous bone is 344.45 HU [19], tissue images below 345 HU were categorized as non-calcified periodontal tissue (Fig. 1B), and the volume of which was measured in the ROI set on relevant slices of the defective parts of the same side. The proportion of non-calcified periodontal tissue volume of each subject was calculated by dividing the volumes measured at 2, 4, 6, and 8 weeks after surgery with those measured immediately after surgery to evaluate the level of calcification in the periodontal tissue. In addition, the CT images up to the eighth week after surgery were examined for the closure of oronasal fistula by calcified periodontal tissue. The Student's *t*-test was conducted to compare the proportions of non-calcified periodontal tissue volume at the trafermin and control sides, and the Fisher's exact probability test was implemented to confirm the closure of oronasal fistula with calcified periodontal tissue. Values of  $P < 0.05$  were considered statistically significant.

All subject dogs were euthanized at the eighth week after surgery by intravenous injection overdose of pentobarbital (100 mg/kg, Somnopentyl, Kyoritsu Seiyaku Co.). Both upper canine teeth, along with the surrounding alveolar bone and gum, were extracted from each dog, and then all of extracted samples were fixed for a week in 10% phosphate-buffered formalin solution and decalcified for 3 weeks in 10% formic acid solution. Then, the samples were vertically cross-sectioned at three parts, namely mesial, central, and distal angles, and paraffin embedded. Subsequently, thin sliced sections of 5-µm thickness were prepared in the cheek-palate direction. The prepared sections were stained with hematoxylin-eosin and examined by optical microscopy to compare the trafermin side with the control side for the closure of fistula by the bridge formation of neonatal bone in the oronasal fistula models, and a series of Fisher's exact probability tests were conducted to confirm the samples with values of  $P < 0.05$  being considered significant.

All dogs were confirmed from the CT images to have communication between the oral and nasal cavities due to bone loss along the palatal side of the canine teeth in all regions that had been surgically treated; all of the dogs were confirmed to be suitable as oronasal fistula models (Fig. 1C, Supplementary Fig. 1: average defect volume on trafermin side, 0.146 cm<sup>3</sup>; average on control side; 0.138 cm<sup>3</sup>). Although calcification in the defective periodontal tissues was not observed on either side of the trafermin and control in the CT images obtained at the second week after surgery (Fig. 1C: 2w), the trafermin side began to show evident calcification 4 weeks after the surgery, suggesting bone regeneration (Fig. 1C: 4–8w). Fistula closure due to calcification of periodontal tissue on the trafermin side was confirmed in one case at the fourth week (25%), three cases at the sixth week (75%), and all cases at the eighth week (100%), respectively, while no cases were confirmed during the whole period on the control side, showing a significant difference at the eighth week (Table 1).

As for the fluctuation in the proportion of non-calcified periodontal tissue volume, the trafermin side showed a significant decrease at weeks 4, 6, and 8 after the surgery (Fig. 1D).

Histological findings indicated that the bone defect area on the trafermin side was observed with the presence of bridging of neonatal bone in the fibrous tissues, confirming the closure of the oronasal fistula (Fig. 2A). On the contrary, the bone defect area on the control side was observed with the growth of neonatal bone at the defect area, but their bridging or closure was not confirmed; most of the defect area was filled with fibrous tissue and the oronasal fistula was not closed (Fig. 2B). Histological evaluation confirmed closure of the oronasal fistula by bridging of neonatal bone in all cases on the trafermin side, while none was observed in all cases on the control side (Table 2).

In this experiment, we used recombinant bFGF derived from a human sequence. Previous studies have shown that human bFGF is effective in canine regenerative therapy [10, 11, 14]. In addition, the high degree of conservation (98%) of the amino acid sequences of human and canine bFGF suggests that the two have equivalent functional properties (Supplementary Fig. 2). However, Edamura *et al.* [4] recently succeeded in purifying recombinant canine bFGF protein, and in future it would be worthwhile to test its efficacy and any differences in adverse effects. Trafermin is a periodontal tissue regeneration agent containing rh-bFGF as its active ingredient. It regenerates periodontal tissues by producing vascular endothelial growth factor, bone morphogenetic protein 2, and transforming growth factor-β1 from marrow stromal cells, periodontal ligament stem cells, and their precursor cell lines [2, 7, 12, 17, 18, 20]. When trafermin was applied to one-, two-, and three-wall alveolar bone defects experimentally created in dogs or on grade 2 furcation involvement, periodontal tissue regeneration commenced by regenerating alveolar bone, cementum, and

**Table 1.** Oronasal fistula closure in the CT image (n=4)

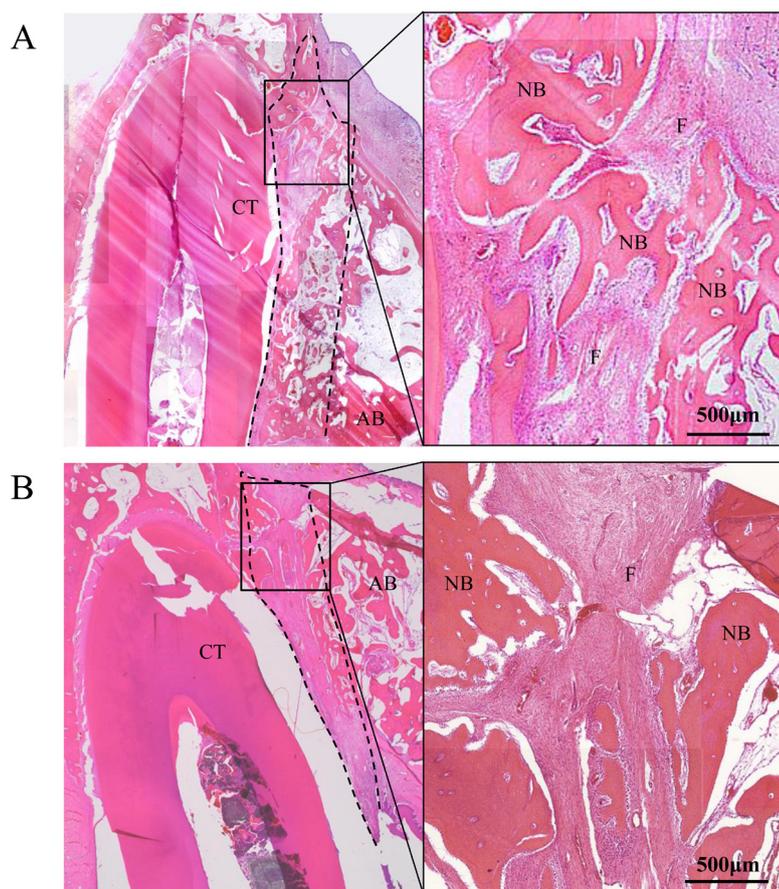
	Pre	2W	4W	6W	8W
Trafermin side	0	0	1	3	4*
Control side	0	0	0	0	0

The closure of the oronasal fistula with calcified periodontal tissue was confirmed in all cases on the trafermin side by the eighth week; none was observed on the control side in all cases. \* $P=0.029$ .

**Table 2.** Oronasal fistula closure in the histological evaluation (n=4)

	Oronasal fistula closure	
	+	-
Trafermin side	4	0
Control side	0	4

The histological evaluation at the eighth week after surgery showed closure of the oronasal fistula by bridging of neonatal bone in all cases at the trafermin side, while none was observed on the control side ( $P=0.029$ ).



**Fig. 2.** A: Tissue image on the trafermin side. The area circled by the dotted line between the canine tooth (CT) and palatal alveolar bone (AB) was the prepared bone defect. Bridging of the neonatal bone (NB) was confirmed within the fibrous tissues (F) in the defected area, closing the oronasal fistula. B: Tissue image on the control side. The area circled by the dotted line between the canine tooth (CT) and palatal alveolar bone (AB) was the prepared bone defect. The formation of the neonatal bone (NB) was confirmed, but no bridging was observed. Most of the tissues in the defected area were fibrous tissue (F), and the oronasal fistula was not closed.

periodontal membrane [1, 10, 11, 21], showing a significant decrease in the attachment loss in dogs with spontaneous marginal periodontitis [24]. Since periodontitis-induced oronasal fistula mostly occurs at the palatal side of the upper canine root [16], an oronasal fistula was artificially created in a particular region in the model for this study. The CT images and histological observations confirmed maxillary bone defects in the area from the palatal to the nasal mucosa with complete penetration between them, indicating that the oronasal fistula model prepared in this study was ideal for the study of treatment methods for oronasal fistula (Fig. 1C, Supplementary Fig. 1). The model also showed no significant difference between both sides in non-calcified periodontal tissue volumes immediately after the surgery, suggesting that the model was suitable for comparative investigation of the treatment effect in the trafermin side with the control side. Although we used human HU values to set ROIs for cancellous bone [19] in dogs, non-calcified tissue was clearly distinguishable from cancellous bone (Fig. 1B and 1C) when these values were used.

The volume proportions of non-calcified periodontal tissue in the oronasal fistula model showed a significant decrease in the trafermin side at the fourth, sixth, and eighth week after surgery in the CT images (Fig. 1D). These results suggest that trafermin promotes calcification in periodontal tissue defects in the oronasal fistula model, as well as in the commonly observed cases of marginal periodontitis. It also indicates that a minimum 4-week period is required to evaluate the effect of trafermin on oronasal fistula from CT images. The attachment loss at 2 months after trafermin application was evaluated in cases of spontaneous periodontitis [24], and the results of this study suggest that the treatment effect in the model can be evaluated by the CT image findings much earlier than that of control. In contrast to that report, which evaluated the effects of trafermin only in terms of attachment loss [24], here we were able to demonstrate these effects experimentally by using both imaging and histological analysis. The closure of the oronasal fistula by calcified periodontal tissue regeneration was confirmed by CT images of all cases (100%) on the trafermin side at the eighth week after surgery, while none was confirmed on the control side (Table 1). It was also confirmed histologically in all cases of trafermin side with oronasal fistula closure by bridging with neonatal bone, while none was confirmed on the control side (Table 2). The closure of the oronasal fistula with neonatal bone formation can improve the regeneration of connective tissue adhesion, mitigate palatal mucosa degeneration, or defect of periodontal tissue by application of trafermin twice at the defect site. If we set an observation period longer than 8 weeks in this experiment, the second application of trafermin would allow most of the applied agent to remain at the lesion, and further periodontal tissue regeneration could be anticipated.

As discussed above, the CT image and histological findings in this experiment suggested the treatment validity of trafermin application on oronasal fistula. The model used in this study was different from the cases of periodontitis-induced oronasal fistula encountered in actual medical practice in small animals, in that infections at the root surfaces or propagation of oral bacteria should be present in the latter; both of them could be the inhibiting factors for periodontal tissue regeneration [22]. However, it has been reported that improvement of the clinical manifestation in spontaneous periodontitis cases has been observed in the study

of trafermin application [24], and a sufficient level of efficacy should be expected in the oronasal fistula cases as well, if trafermin would be applied after complete removal of infected tissues while practicing thorough curettage. Here, we used 12-month-old experimental dogs. However, because dogs of a wide range of ages are affected by periodontitis [7], the use of dogs of one age in an oronasal fistula model for bone regeneration experiments was a limiting condition. Although we have experimentally proved the efficacy of trafermin in oronasal fistulas, it is still necessary to prove this efficacy in spontaneous cases in various age groups.

In conclusion, our study provides CT imaging and histological evidence of the therapeutic effects of trafermin in oronasal fistulas. Trafermin, as used in this study, should be considered as a proven new therapeutic agent for the conservative treatment of oronasal fistulas without tooth extraction in dogs.

POTENTIAL CONFLICTS OF INTEREST. The authors declare that they have no competing interests.

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