The Journal of Laryngology & Otology (2009), ***123*** (Suppl. S31), *24–29.*

doi:10.1017/S0022215109005040

Main Article

Efficacy of autologous fat injection laryngoplasty with an adenoviral vector expressing hepatocyte growth factor in a canine model

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Abstract

Objective: The effectiveness of autologous fat injection laryngoplasty may be reduced by resorption of injected fat tissue. The aim of the present study was to clarify the efficacy of fat injection laryngoplasty using autologous fat plus a replication-defective adenoviral vector expressing hepatocyte growth factor, regarding reduction of injected fat tissue resorption.

Material and methods: Four female beagle dogs were used in this study. After sedation, a direct laryngoscope was introduced to enable visualisation of the larynx. In each dog, harvested autologous fat plus an adenoviral vector expressing hepatocyte growth factor was injected into the right true vocal fold, and harvested fat plus an adenoviral vector expressing no gene was injected into the left true vocal fold. A total laryngectomy was performed one year after the intracordal fat injection. Coronal sections of the resected whole larynges were made and the following parameters assessed using light and electron microscopy: size of fat area; number of vasculoendothelial cells surrounding adipocytes; and shape of injected adipocytes in the vocal fold.

Results: The fat area was significantly larger and the number of vasculoendothelial cells surrounding adipocytes significantly greater in the intracordal fat injection containing adenoviral vector expressing hepatocyte growth factor, compared with the control intracordal fat injection containing adenoviral vector expressing no gene. When viewed under electron microscopy, the injected adipocytes were observed to have grafted better in the intracordal fat injection with hepatocyte growth factor adenoviral vector, compared with the control intracordal fat injection with adenoviral vector expressing no gene.

Conclusions: Injection into the vocal fold of autologous fat containing an adenoviral vector expressing hepatocyte growth factor can reduce subsequent resorption of injected fat.

Key words: Autologous Fat Injection Laryngoplasty; Adenoviral Vector; Hepatocyte Growth Factor; Vasculoendothelial Cell; Adipocytes

Introduction

Fat injection laryngoplasty is a minimally invasive surgical procedure, compared with framework surgery (i.e. type one thyroplasty, arytenoid adduction surgery or arytenoid adduction surgery plus type one thyroplasty), for the treatment of patients with unilateral vocal fold paralysis. Moreover, fat injection laryngoplasty is reliable, has good long-term results and yields stable, satisfactory vocal function in comparison with framework surgery.[1,2]

However, fat injection laryngoplasty is often followed by resorption of the injected fat. A larger quantity of autologous fat than needed is often injected into the vocal fold in order to compensate for resorption. In a series of 71 patients undergoing fat injection laryngoplasty, only 70–80 per cent subsequently showed normal aerodynamic parameters and acoustic analysis.[3] In addition, if the patient's body mass index (BMI) is high, there are risks associated with resorption of injected fat tissue.[4] Therefore, it is desirable to reduce resorption of injected fat tissue following fat injection laryngoplasty.

The aim of the present study was to assess the efficacy of fat injection laryngoplasty using autologous fat plus a replication-defective adenoviral vector expressing hepatocyte growth factor, regarding reduction of subsequent injected fat tissue resorption. Hepatocyte growth factor is associated with tissue regeneration, mitogenesis, angiogenesis, anti-apoptosis and anti-fibrotic activities in various cells.[5,6] The introduction of hepatocyte growth factor was expected to stimulate angiogenesis and therefore to reduce resorption of injected fat tissue.

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Material and methods

Recombinant adenoviral vectors

As described previously, we generated and prepared a replication-defective adenoviral vector expressing hepatocyte growth factor, and a control adenoviral vector expressing no gene. The former viral vector encoded human hepatocyte growth factor downstream of the transcriptional control of a modified chicken beta-actin promoter, with a cytomegalovirus immediate early enhancer.[7–9]

Animal studies

Intracordal injection of autologous fat plus adenoviral vector. The study was approved by the institutional animal research committee. Four female beagle dogs weighting 9.6 to 11.5 kg were used.

The four dogs were sedated with an initial intravenous injection of propofol (6 mg/kg), followed by maintenance administration of propofol (0.2 to 0.5 mg/kg per minute) during surgery.

A subcutaneous injection of buprenorphine hydrochloride (0.05 mg/kg) provided analgesia. Autologous fat was harvested from the abdominal subcutaneous fat tissue by liposuction. In two dogs, only 0.5 ml of autologous fat was harvested because they had little subcutaneous fat tissue. In the other two dogs, 1.0 ml autologous fat was harvested.

A direct laryngoscope was then introduced to enable visualisation of the larynx.

In two dogs, adenoviral vector expressing hepatocyte growth factor (4.6×10^9 particles) was injected into the right vocal fold, together with 0.5 ml autologous fat, via a 19 G needle designed for endolaryngeal microsurgery. Adenoviral vector expressing no gene (4.6×10^9 particles) was injected into the left vocal fold together with 0.5 ml autologous fat, as a control.

In the other two dogs, adenoviral vector expressing hepatocyte growth factor (4.6×10^9 particles) was injected into the right vocal fold together with 1.0 ml autologous fat. Adenoviral vector expressing no gene (4.6×10^9 particles) was injected into the left vocal fold together with 1.0 ml autologous fat, as a control.

Histopathological analysis. The four dogs were humanely sacrificed 12 months after the initial intracordal autologous fat injection. The whole larynges were removed and fixed in 10 per cent formalin and dehydrated in graded concentrations of ethanol.

The bilateral vocal folds of the removed larynges were sectioned in a coronal plane into four pieces and embedded in paraffin. Haematoxylin and eosin stain and factor VIII stain (N1505; Dako, Tokyo, Japan) were used for each section. Haematoxylin and eosin staining was used to investigate the size of the fat tissue injection area. Each fat tissue area was measured with a light microscope, using Win Foof photoanalytical software. In addition, the total fat tissue area of the four sections of each vocal fold was measured for each dog. We then compared the total fat tissue area of the 16 sections of all four dogs' right vocal folds with that of the 16 sections of all four dogs' left vocal folds, using the variance component model to evaluate correlation among eight repeated measures within each dog.

Factor VIII staining was used to investigate angiogenesis around adipocytes. The number of vasculoendothelial cells surrounding adipocytes was counted at five different sites in each section, under light microscopy ($\times 400$). The total number of vasculoendothelial cells at 80 sites within the right vocal folds of all four dogs was compared with that at 80 sites within the left vocal folds of all four dogs, using Poisson regression with generalized estimating equation (GEE) estimation in order to determine the correlation within each dog.

For scanning electron microscopy, small specimens of injected adipocytes within the vocal fold were fixed in 2.5 per cent glutaraldehyde at 4°C for 2 hours. After rinsing with cacodylate buffer solution, specimens were postfixed in 2 per cent osmium tetroxide with cacodylate buffer solution at 4°C for 2 hours. This was followed by dehydration in a graded series of ethanol, immersion in *tert*-butyl alcohol and drying by the *tert*-butyl alcohol freezing method. Specimens were then sputter-coated with gold and examined under a Hitachi S-800 scanning electron microscope (Hitachi, Tokyo, Japan). The shape of adipocytes from the right and left vocal folds was compared.

Results

Bilateral coronal sections of a canine larynx one year after injection of 1.0 ml autologous fat are shown in Figure 1. The size of the fat tissue area in the right vocal fold, with adenoviral vector expressing hepatocyte growth factor, appears large in comparison with that in the left vocal fold, with adenoviral vector expressing no gene (i.e. control). Table I compares the fat tissue areas for 16 sections of right vocal fold (receiving hepatocyte growth factor viral vector) versus 16 sections of left vocal fold (receiving control viral vector), for all four dogs. The fat tissue area in the right vocal fold was statistically wider than that in the left vocal fold.

Figure 2 shows vasculoendothelial cells surrounding adipocytes. More vasculoendothelial cells were observed in the right vocal fold than in the left vocal fold. Table II compares vasculoendothelial cell results for 80 sites in the right vocal fold (receiving hepatocyte growth factor viral vector) versus 80 sites in the left vocal fold (receiving control viral vector), for the four dogs. The total number of vasculoendothelial cells surrounding the adipocytes was significantly larger in sites injected with autologous fat plus adenoviral vector expressing hepatocyte growth factor, compared with sites injected with autologous fat plus control adenoviral vector expressing no gene.

Figure 3 shows scanning electron microscopy views of the right and left vocal folds. Adipocyte diameter was longer and adipocyte density greater in the right vocal fold (receiving adenoviral vector expressing hepatocyte growth factor), compared with the left

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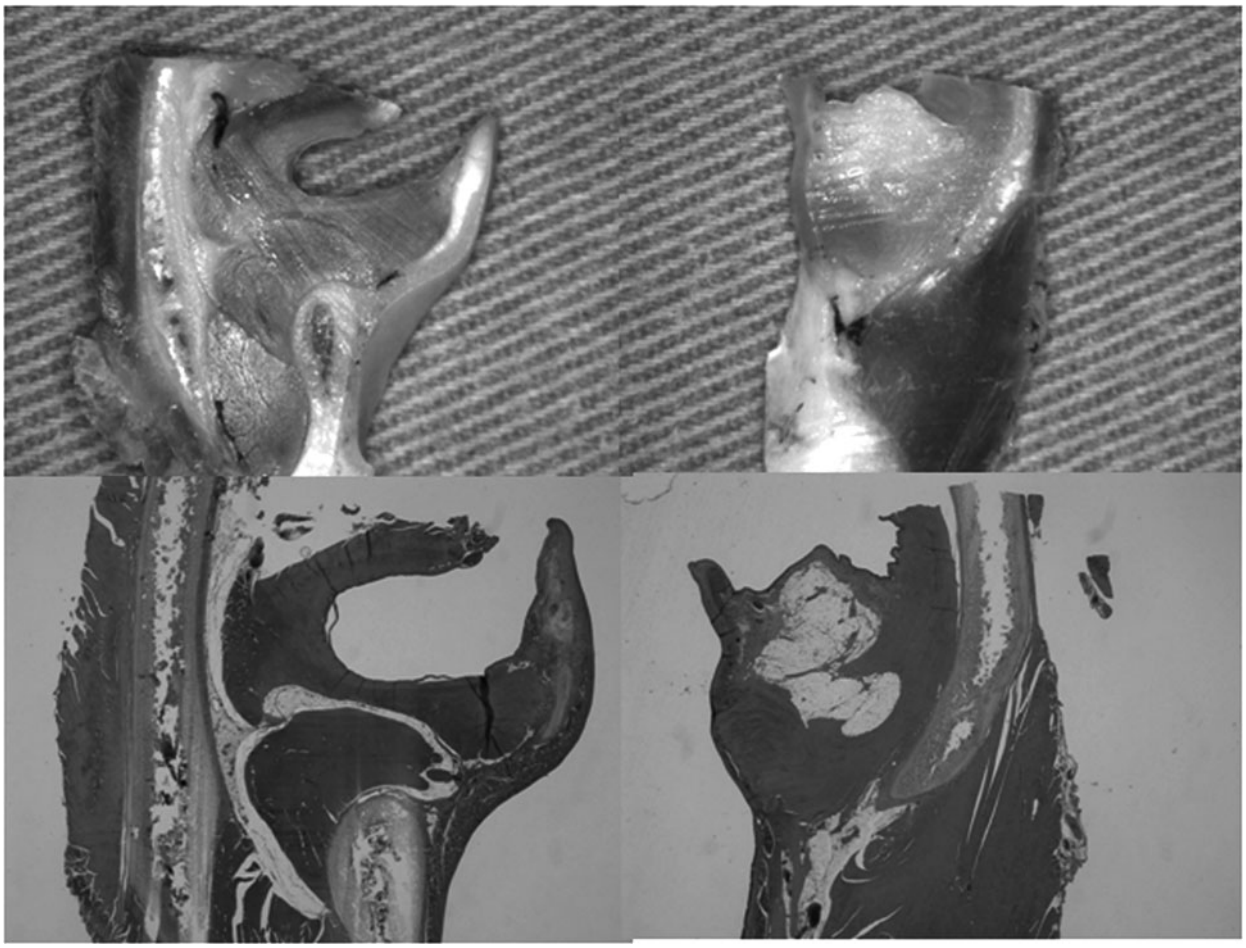

FIG. 1

(a) Coronal section of the right vocal fold (right) and left vocal fold (left). (b) Equivalent sections stained with H&E. The size of the fat tissue area in the right vocal fold (receiving adenoviral vector expressing hepatocyte growth factor) is wider than that in the left vocal fold (receiving adenoviral vector expressing no gene).

vocal fold (receiving adenoviral vector expressing no gene).

Discussion

Fat injection laryngoplasty was first reported by Mikaelian *et al.* in 1991.[10] This procedure is widely used because it appears to be a reasonable, safe alternative to framework surgery with high patient acceptance, which potentially offers long-term stability. Good results for the procedure have been reported by many authors.[11]

Furthermore, fat injection laryngoplasty is a minimally invasive procedure compared with framework surgery (i.e. type one thyroplasty, arytenoid adduction surgery, or arytenoid adduction surgery plus type one thyroplasty), for the treatment of patients with unilateral vocal fold paralysis. Fat injection

TABLE I

VOCAL FOLD FAT TISSUE AREA BY VIRAL VECTOR TYPE, FOR INDIVIDUAL AND COMBINED DOGS

Viral vector type	Fat tissue area (pixels/inch^2)		Test value (df)	*p*
	Mean	SD		
*1 dog**				
Ad.CA-HGF	1 866 234	976 880	3.21075 (3)	$<0.049^{\dagger}$
Ad.dE1.3	308 322	9947		
4 dogs‡				
Ad.CA-HGF	466 558	325 597	6.01 (3)	$<0.0092^{**}$
Ad.dE1.3	77 081	46 908		

*Total of 20 sites (five sites in each of four coronal laryngeal sections). ‡Total of 80 sites (five sites in each of four coronal laryngeal sections, for four dogs). †Paired *t*-test; ***t*-test based on variance component model. Df = degrees of freedom; SD = standard deviation; Ad.CA-HGF = adenoviral vector expressing HGF (used in right vocal fold); Ad.dE1.3 = adenoviral vector expressing no gene (used in left vocal fold)

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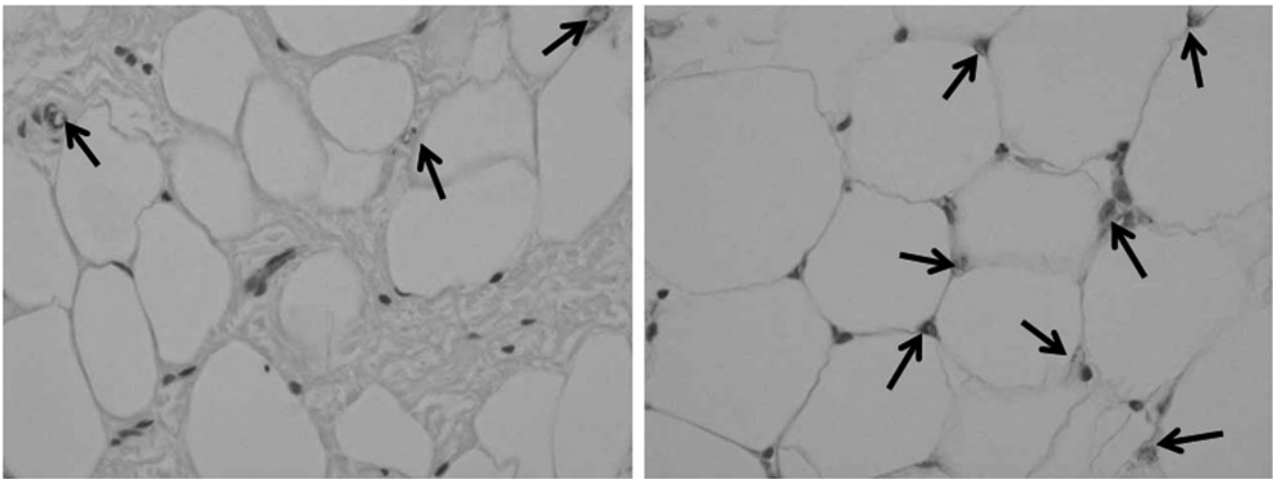

FIG. 2

Light microscopy photomicrographs showing vasculoendothelial cells (arrow) surrounding adipocytes in (a) the right vocal fold (receiving autologous fat plus adenoviral vector expressing hepatocyte growth factor) and (b) the left vocal fold (receiving autologous fat plus adenoviral vector expressing no gene). More vasculoendothelial cells were observed in the right vocal fold compared with the left vocal fold.

laryngoplasty has been found to result in more satisfactory post-operative vocal function, compared with framework surgery.[2]

However, autologous fat injection laryngoplasty can sometimes be associated with post-operative resorption of injected fat tissue. A larger quantity of autologous fat than needed is often injected into the vocal fold in order to compensate for resorption.[3] Hsiung reported that over-injection is necessary in order to medialise the vocal fold and to compensate for the anticipated fat absorption.[11]

In a series of 71 patients receiving fat injection laryngoplasty, 70–80 per cent subsequently showed normal aerodynamic parameters and acoustic analysis.[3] While these results are acceptable, there is room for improvement. Furthermore, post-operative resorption of injected fat tissue carries extra risks in patients with a high BMI.[4] Therefore, it is desirable to reduce the resorption rate of injected fat tissue after fat injection laryngoplasty.

TABLE II

VOCAL FOLD VASCULOENDOTHELIAL CELLS (SURROUNDING ADIPOCYTES) BY VIRAL VECTOR TYPE, FOR INDIVIDUAL AND COMBINED DOGS

Viral vector type	Vasculoendothelial cells (n/high power field)		χ^2 (df)	*p*
	Mean	SD		
*1 dog**				
Ad.CA-HGF	134.8	12.0	238.17 (1)	<0.001†
Ad.dE1.3	76.8	11.1		
4 dogs‡				
Ad.CA-HGF	33.7	5.9	238.41 (1)	<0.001†
Ad.dE1.3	19.2	5.2		

*Total of 20 sites (five sites in each of four coronal laryngeal sections for one dog). ‡Total of 80 sites (five sites in each of four coronal laryngeal sections, for four dogs). †Chi-square test based on Poisson model. Df = degrees of freedom; SD = standard deviation; Ad.CA-HGF = adenoviral vector expessing HGF (used in right vocal fold); Ad.dE1.3 = adenoviral vector expressing no gene (used in left vocal fold)

One study has addressed this issue. In an effort to prevent loss of fat volume and generation of additional adipose tissue after intracordal injection of autologous fat, Tamura *et al.* reported the effects of injecting fat together with basic fibroblast growth factor into the vocal folds of 12 dogs.[12] Autologous fat was injected into one vocal fold, and a mixture of autologous fat and gelatin microspheres containing basic fibroblast growth factor and collagen sponge was injected into the other. The vocal folds receiving autologous fat with basic fibroblast growth factor showed fusiform, immature adipocytes in the injected fat eight weeks after injection. The volume of the injected fat was maintained almost completely, even at 24 weeks post-injection. In comparison, the vocal folds receiving only autologous fat showed a marked decrease in the volume of injected fat over time. These results showed that strong vascularisation, occurring in response to basic fibroblast growth factor, prevents the loss of fat volume and the generation of additional adipose tissue, following intracordal injection of autologous fat.

Hepatocyte growth factor was originally identified and cloned as a potent mitogen for hepatocytes.[13,14] It has been reported to have mitogenic, angiogenic, antiapoptotic and antifibrotic effects on various cells.[5,6]

In the current study, hepatocyte growth factor was expected to stimulate angiogenesis around the injected fat tissue in the vocal fold. As expected, the number of vasculoendothelial cells surrounding adipocytes was significantly increased by the addition of hepatocyte growth factor. We surmise that the adipocytes were well supported by such increased vasculature. Furthermore, a large quantity of fat tissue was satisfactorily maintained in association with this

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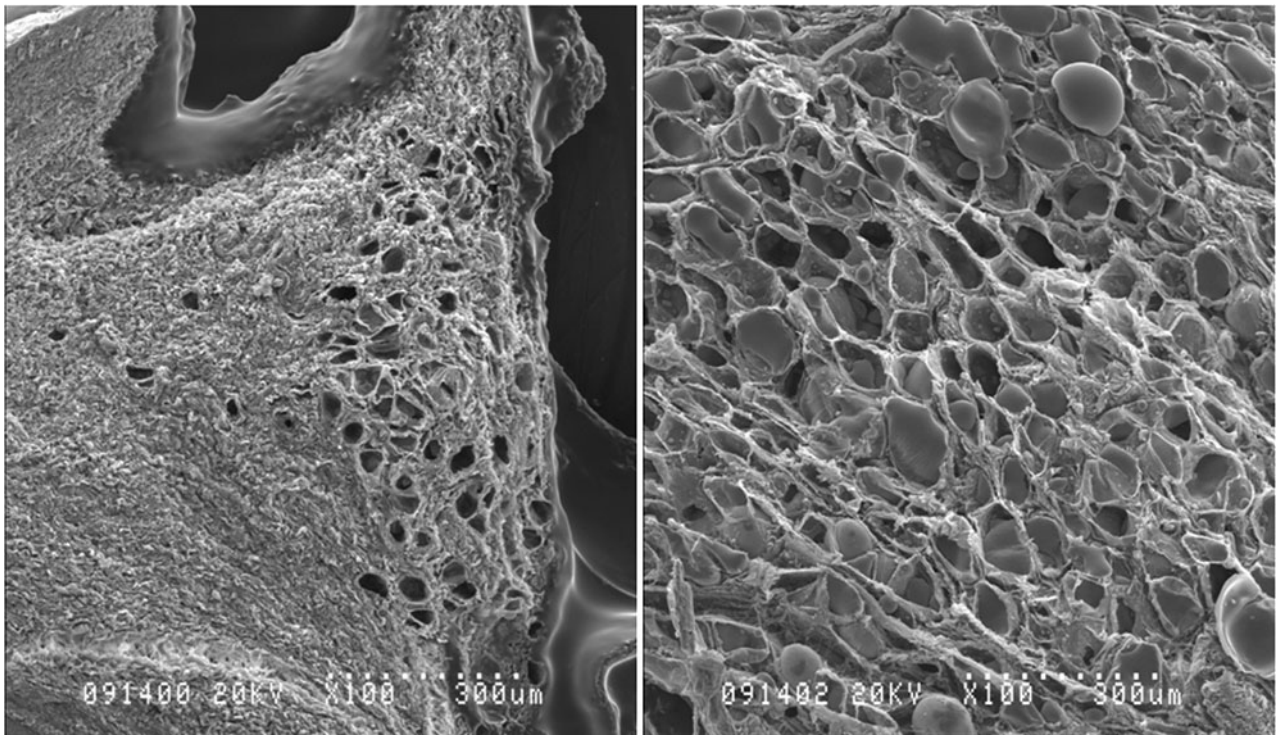

FIG. 3

Fig. 3 Scanning electron microscopy views of injected adipocytes in the (a) right and (b) left vocal folds. Adipocyte diameter and density were greater in the right vocal folds (receiving adenoviral vector expressing hepatocyte growth factor), compared with the left vocal folds (receiving adenoviral vector expressing no gene).

angiogenesis. As a result, the diameter and density of adipocytes were greater in the right vocal fold, which had received adenoviral vector expressing hepatocyte growth factor, compared with the left vocal fold, which had received only control adenoviral vector.

Gene therapy has been explored recently in the context of regenerative medical practice. Such efforts are based fundamentally on the expression of viral vectors to provide sustained release of a specific growth factor from cells using plasmid deoxyribonucleic acid have resulted in lower gene expression in comparison to viral vectors.

Cell transplantation therapy strategy in combination with growth factor has been recently explored in experiments in the context of regenerative medicine, and such previous efforts used administration of recombinant protein or plasmid DNA containing transgene. Although growth factor enhanced beneficial effects of cell transplantation therapy, the crucial issues in the previous approaches are short duration of half-lives of growth factor itself (*e.g.*, a few minutes in the body) and plasmid deoxyribonucleic acid (*e.g.*, a few days), as well as low transduction efficiency and low expression levels in the case of the use of plasmid DNA. In the present study, we for the first time used adenoviral vector system, which usually allows much higher gene transduction efficiency and much longer transgene expression (*e.g.*, several weeks), for introducing growth factor gene into the transplanted cells; in actuality, the present result was promising. In this regard, the novel strategy shown in this study may open up a new way in the field of cell transplantation therapy and regenerative medicine.

Conclusion

This study demonstrated the efficacy of fat injection laryngoplasty using autologous fat plus an adenoviral vector expressing hepatocyte growth factor, in a canine model. However, further preclinical study is necessary in order to carefully assess the clinical applicability of such treatment, including its safety and efficacy.

Acknowledgement

This study was supported in part by a Grant-in-Aid for Scientific Research (#19591997) from the Ministry of Education, Culture, Sports Science and Technology, Japan.

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Dr H Umeno takes responsibility for the integrity of the content of the paper.
Competing interests: None declared