Received: 20 April 2021 | Accepted: 2 June 2021

DOI: 10.1002/lio2.602

ORIGINAL RESEARCH

Laryngoscope Investigative Otolaryngology

Effects of corticosteroids vs halofuginone on vocal fold wound healing in an ovine model

Jacqueline Allen MD, FRACS ORL HNS

Department of Surgery, University of Auckland, Auckland, New Zealand

Correspondence
Jacqueline Allen, Department of Surgery, University of Auckland, Park Rd, Grafton, Auckland 1010, New Zealand.
Email: jeallen@voiceandswallow.co.nz

Funding information
Linsell Richards Fund for Education

Abstract

Objectives: To evaluate antifibrotic effects of corticosteroids and halofuginone, a small molecule inhibitor of Smad3, in an ovine model of vocal fold (VF) injury.

Methods: Thirty sheep, using a paired study design, underwent controlled right VF injury by biopsy and then were treated with either no treatment, oral dexamethasone, intralesional triamcinolone, or oral halofuginone. Larynges were evaluated for histological evidence of fibrosis, immunohistochemical presence of Smad3, and vibratory parameters. Outcomes were compared across treatment groups.

Results: Following injury, VF collagen density decreased in both halofuginone-treated and dexamethasone-treated sheep but not in triamcinolone treated sheep. A significant difference was noted between halofuginone and triamcinolone treated sheep (27.8% vs 37%, $P = .017$). Elastin was preserved postinjury by halofuginone treatment in contrast with all steroid treated animals where significant loss of elastin was noted ($P < .05$). Smad3 staining was up-regulated at all injury sites compared to normal left VFs however halofuginone and dexamethasone treatment reduced Smad3 activity significantly whereas triamcinolone treatment did not ($P < .05$). Ex-vivo stroboscopic evaluation demonstrated mucosal wave in all excised larynges with a normalized glottal gap less than 3, suggesting adequate glottal closure.

Conclusions: VF injury in an ovine model results in a wound response able to be modified by Smad3 inhibitor, halofuginone, with benefit to vibratory function. Halofuginone treated sheep demonstrated reduced collagenization of lamina propria with greater elastin density after injury, than sheep treated with either steroid medication. These data support this pathway as a suitable target for manipulation to prevent or reverse fibrosis in the glottis and restore voice quality.

Level of Evidence: NA.

KEYWORDS
animal model, collagen, elastin, halofuginone, phonation, Smad3, steroids, vocal fold fibrosis, vocal fold scar

This manuscript was accepted for presentation at the 2021 ABEA COSM virtual meeting, April 8, 2021.

1 | INTRODUCTION

Vocal fold (VF) fibrosis is currently an insoluble problem sometimes resulting in permanent voice changes. While prevention of injury is the best strategy, in many cases, poor wound healing or injury occurs despite the best surgical efforts or as a consequence of necessary treatment. VF wound healing follows the same phases as wound healing in other body tissues, however the outcome of healing may be very different, due to inability to reconstitute the unique 3D microarchitecture of the VFs.[1-5] However, if timely intervention can be applied, the healing process may sometimes be manipulated positively. Frequently corticosteroid medication are utilized to modulate fibrosis and reduce scar formation. These medications are cheap and freely available. In the larynx, localized steroid injections reduce systemic side effects and have been used to treat glottic disease and subglottic stenoses.[6]

Glucocorticoids exert effects by binding the intracellular glucocorticoid receptor (GR) resulting in direct DNA binding, altering transcriptional behavior. They may also modify nuclear signaling molecules, which indirectly affect DNA transcription or participate in nongenomic interactions with other receptors or proteins such as organelle membranes.[7] Dexamethasone is a potent glucocorticosteroid usually given for its anti-inflammatory effects but also as a mitigator of fibrosis. Overall, the effect of glucocorticoid presence is to reduce inflammatory processes but they also inhibit proliferation of lymphocytes and fibroblasts.[7] VF fibroblasts (VFFs) constitutively express the GR, which is down-regulated when exogenous dexamethasone is applied.[8] There is also GR expression in the epithelium, capillaries and stroma of the VFs.[8]

Dexamethasone is 30× more potent than hydrocortisone due to greater affinity for binding the GR, with a three-times longer half-life (12 vs 36-72 hours).[9] It decreases fibroblast proliferation, reduces collagen synthesis triggered by the TGF-β1 pathway and reduces extracellular matrix (ECM) turnover.[7-9] A previous study revealed decreased collagen deposition in an injured rabbit VF model after dexamethasone injection.[9] Significant depression of collagen gene expression was noted.[9] In a clinical study, Wang et al identified 22 patients that developed VF fibrosis following microlaryngeal excisional surgeries.[10] These patients were treated with transoral or transnasal injections of combined dexamethasone (5 mg/mL) and triamcinolone (10 mg/mL) [1:1 mix] under local anaesthesia. Outcome assessment by videostroboscopy, cepstral peak prominence, and Voice Handicap Index-10 scores showed significant improvements in three quarters of subjects postinjection.[10] Mortensen and Woo utilized methylprednisolone for patients exhibiting postoperative VF scar, granuloma or benign VF lesions. They treated 34 patients, with 82% demonstrating subjective improvement in voice.[11]

Triamcinolone acetonide is also a corticosteroid, with much longer tissue dwell duration than dexamethasone. This is due to the particularized formulation, which results in a white appearance with multiple small particles visible in suspension. Two different concentrations are commonly available—10 or 40 mg/mL. Cho et al preferred use of triamcinolone in a study of steroid injection at the time of microsurgical removal of benign VF pathology.[12] They chose triamcinolone for its longer dwell time and depot effect and were able to detect white residue subepithelially in the VF at 1 to 2 months postsurgery in injected patients. Reduced likelihood of recurrent pathology and decrease in persistent dysphonia was noted in this group.[12]

An alternative approach to improving wound healing can be undertaken by manipulating small signaling molecules that act downstream of TGF-β in the fibrotic cascade. The Smad family of signaling molecules are intracellular messengers that transduce messages from the cell surface to the nucleus and are activated by binding of ligand to the transforming growth factor beta receptor (TGFβ-R). When TGF-β binding occurs, Smad2 and 3 are phosphorylated and then can bind co-Smad4.[13-16] The trimeric molecule then translocates to the nucleus and the whole complex attaches to Smad binding elements (SBEs) on DNA. Transcription of genes, particularly collagen genes, can then be initiated.

Halofuginone is a small-molecule, specific inhibitor of Smad3. Halofuginone prevents phosphorylation of Smad3 thereby preventing translocation of the trimeric Smad molecule to the nucleus, and preventing transcription of COL1A1 gene product (scar collagen).[17-19] Halofuginone has been demonstrated to reduce bleomycin-induced pulmonary fibrosis in rats, liver cirrhosis, muscle fibrosis in muscular dystrophies (including in Phase Ib human trials), skin fibrosis in chronic graft vs host disease-afflicted mice and tight skin mice (a model of scleroderma) as well as in human subjects with scleroderma and fibrous adhesions postsurgery.[18,20,21] Additional restrictions are seen in MMP-2 and TIMP-1 expression (ECM regulator molecules), and halofuginone blockade of COL1A1 transcription is amplified if TGF-β is present.[20] Halofuginone also prevents fibroblast to myofibroblast transition, thereby preventing collagen synthesis[18] and appears to attenuate IL-17 function resulting in anti-inflammatory benefit. It has also demonstrated anti-angiogenic effects that have been used in an anti-tumoural role.[21-24] Currently halofuginone is not yet commercially available.

This study was designed to evaluate and compare the effects of corticosteroid treatment vs halofuginone treatment on VF wound healing in an ovine laryngeal injury model to determine whether halofuginone treatment was noninferior to current standard of care medical therapies.

2 | METHODS

Ethical consent for this study was obtained (University of Auckland, AEC00259, 2018). Thirty adult dry ewes older than 2 years were selected and acclimatized for 7 days within the animal laboratory, while being fed on sheep pellet chow. The sheep were housed in individual temperature-controlled pens within the Large Animal Laboratory and given daily feed with *ad libitum* water.

All sheep underwent an initial surgical procedure during which biopsy from the right VF was performed using a 2 mm cup forceps under general anaesthesia. At 1 month postbiopsy all the sheep were humanely euthanased (pentobarbital 300, 80 mg/kg; Southern Veterinary Supplies, Hamilton, New Zealand) and a total laryngectomy also excising a segment of trachea, was performed.

Twenty-four larynges were stored in Carnoy's fixative (absolute ethanol, chloroform, and acetic acid glacial), while six larynges were snap frozen in liquid nitrogen and stored in −80°C for vibratory examination.

The sheep were divided into four groups. In Group 3 animals, immediate injection of triamcinolone was performed into the site of the biopsy. Visible inflation of the VF was seen at the injection site using the rigid endoscope as visual guide during injection. Following initial biopsy surgery, the other animals were administered test medications as listed below by group designation. Oral test medications were delivered by drenching gun. No tolerance issues were identified with any medications administered.

2.1 | Sheep groups

Group 1. Untreated sheep (n = 2)*. VF biopsy taken but no antifibrotic medications were given.

Group 2. Sheep treated with halofuginone 20 mg per oral (by drenching gun) three times weekly for 4 weeks (12 doses) (n = 16).

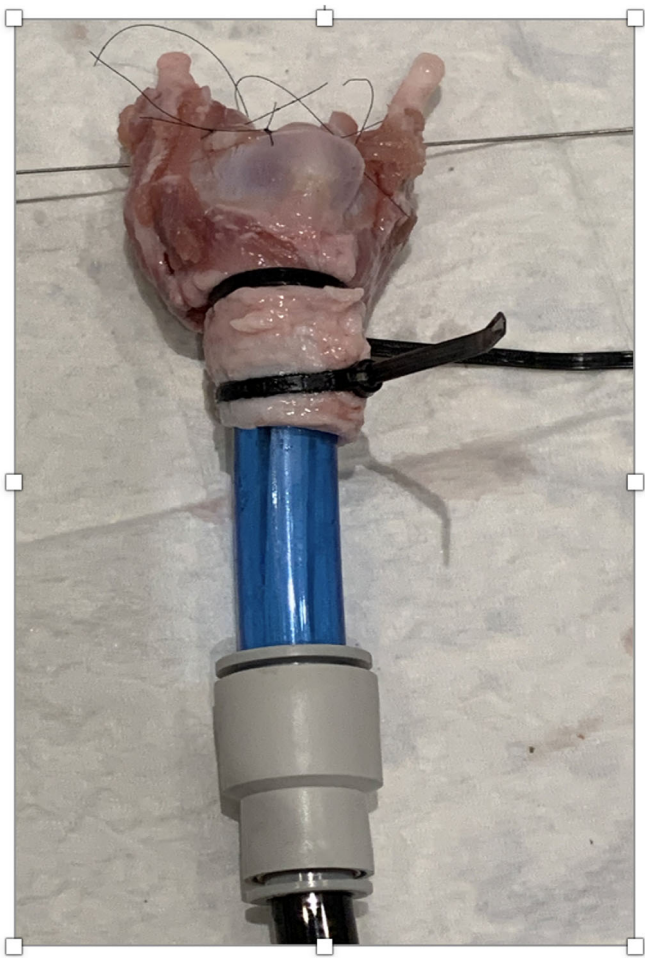

FIGURE 1 Photograph of excised ovine larynx attached to air supply tubing with partial removal of supraglottic tissue and spinal needle adducting arytenoids prior to stroboscopic recording

Group 3. Sheep treated with intrachordal injection of triamcinolone (Kenacort A40, Aspen Pharmaceuticals Healthcare Logistics, New Zealand, 40 mg/mL, 0.5 to 1 mL injected) at the time of VF biopsy (single dose) (n = 6).

Group 4. Sheep treated with 5 days of per oral postoperative dexamethasone (Aspen Pharmaceuticals Healthcare Logistics, New Zealand) (8 mg/2 mL added to 8 mL normal saline; five doses; n = 6).

(*Only two control sheep were added to this study as the Animal Ethics Committee recommended reducing animal numbers and evaluating data from a prior study including seven control sheep with the same right VF injury.[25])

After 1 month in fixative, the larynges were removed from fixation and sagittally sectioned at the midline then dissected removing the left and right VFs from vocal process to anterior commissure, cassetting each side separately. Specimens were embedded in paraffin wax then 5 μm sections were cut. Sections were prepared with four different staining techniques and one immunohistochemical staining technique. Haematoxylin and eosin staining was chosen to assess epithelial thickness and cellular infiltrate. Picrosirius red was utilized to assess collagen content and fibrosis and orcein staining was used to assess elastin presence. Smad3 detection was performed using antibody binding techniques (see below).

TABLE 1 Raw data for Sirius Red/collagen percentage in injured and noninjured vocal folds

Treatment	Mean percentage (SD)	*P* value
Control		
Left	47.6 (8.8)	**.04**
Right	30.4 (8)	
Halofuginone		
Left	42.9 (9.4)	**.0000009**
Right	27.8 (9.5)	
Dexamethasone		
Left	36.9 (7.5)	**.05**
Right	34.0 (10)	
Triamcinolone		
Left	39.1 (10.4)	.7
Right	37.3 (8)	
Students *t*-test comparisons		
Control R vs Halo R[a]	30.5 vs 27.8	.52
Control R vs Dexa R[a]	30.5 vs 34	.51
Control R vs Tri R[a]	30.5 vs 37.3	.14
Halo R vs Dexa R[a]	27.8 vs 34	.17
Halo R vs Tri R[a]	27.8 vs 37.3	**.017**
Halo R inj vs Halo R normal[a]	27.8 vs 44	**.002**
Dexa R inj vs Dexa R normal[a]	30 vs 43	.30
Tri R inj vs Tri R normal[a]	37.3 vs 51	**.05**

Note: Significant *P*-values are bolded.
Abbreviations: Dexa, dexamethasone; Halo, halofuginone; Inj, injury; L, left; R, right; Tri, triamcinolone.
[a]Groups compared using Student's *t*-test.

2.2 | Antibody staining

For Smad3 detection polyclonal rabbit antibody ab84177 (Abcam, Cambridge, UK) in a 1:100 dilution in Nocacastra IHC diluent (RE7133) was used as no sheep-specific antibody was available . For antigen retrieval a Novolink Polymer Detection system was used (Leica Biosystems, Newcastle-upon-Tyne, UK). This contains peroxidase block, protein block, Novolink polymer, DAB chromogen, Novolink DAB substrate buffer and haematoxylin. Briefly, sections were cut at 5 μm and washed in xylene then absolute alcohol. After water wash, they were immersed in tris-buffered saline (TBS) then heat mediated (HIER) antigen retrieval in TRIS-EDTA pH 9, buffer was undertaken. Slides were further washed in TBS then blocked with peroxidase for 5 minutes, washed in TBS and blocked by protein block for a further 5 minutes. Slides were then exposed to 150 μL of antibody for 60 minutes at room temperature. After TBS wash they were incubated with Novolink polymer system for 30 minutes. A DAB stain was performed with 50 μL of DAB in 1 mL buffer with a counterstain with haematoxylin performed after washing. Slides were dehydrated and mounted in DPX (distyrene, plasticizer, xylene; Sigma Aldrich, Auckland, NZ).

All prepared slides were examined under microscopy on a Leica DMR microscope (Leica Microsystems, Wetzlar, Germany) and photographs taken with a digital sight cooled camera and Nikon NIS Elements system (Nikon, Tokyo, Japan) at 10× and 25× power.

For assessment of fibrosis, Fiji color deconvolution was utilized to separate color images and then to calculate the percentage collagen density per red slide based on color intensity using 8-bit images and manual thresholding. A similar process was used to count elastin density on selected orcein stained slides without need for color deconvolution. Finally, Smad3 staining in a region of interest (ROI, 900 × 500 microns) was assessed after color deconvolution using the Fiji haematoxylin-DAB automated threshold and then creating 8-bit photomicrographs. The percentage of ROI containing Smad3 stain was measured.

2.3 | Statistical analysis

Data are expressed as means and standard errors or standard deviations, with 95% confidence intervals and level of significance set at $P < .05$. Two-sided tests were employed for intergroup comparisons and studied with nonparametric testing using the independent samples Kruskal-Wallis Test with Bonferroni correction for multiple tests and the Mann-Whitney *U* test for paired side comparisons. Statistical analyses were performed using SPSS statistics for Mac (SPSS 23.0, IBM Corp, Armonk, NY).

TABLE 2 Raw data for Orcein/elastin density measurements in injured vs noninjured vocal folds

Treatment	Mean (SD)	*P* value
Control L	23 (7.7)	.89
Control R	28.9 (7.4)	
Halo L	23.8 (9.0)	.146
Halo R	21.68 (7.9)	
Dexa L	27.74 (5.9)	**.000018**
Dexa R	5.86 (4.9)	
Tri L	23.5 (6.1)	**.021**
Tri R	14.81 (4.6)	
Students *t*-test comparisons		
Halo R vs Dexa R[a]		**.00002**
Halo R vs Tri R[a]		**.049**
Control R vs Halo R[a]		.48
Control R vs Dexa R[a]		**.00016**
Control R vs Tri R[a]		**.025**
Control L vs Halo L[a]		.64
Control L vs Dexa L[a]		.17
Control L vs Tri L[a]		.9

Note: Significant *P*-values are bolded.
Abbreviations: Dexa, dexamethasone; Halo, halofuginone; Inj, injury; L, left; R, right; Tri, triamcinolone.
[a]Groups compared using Student's *t*-test.

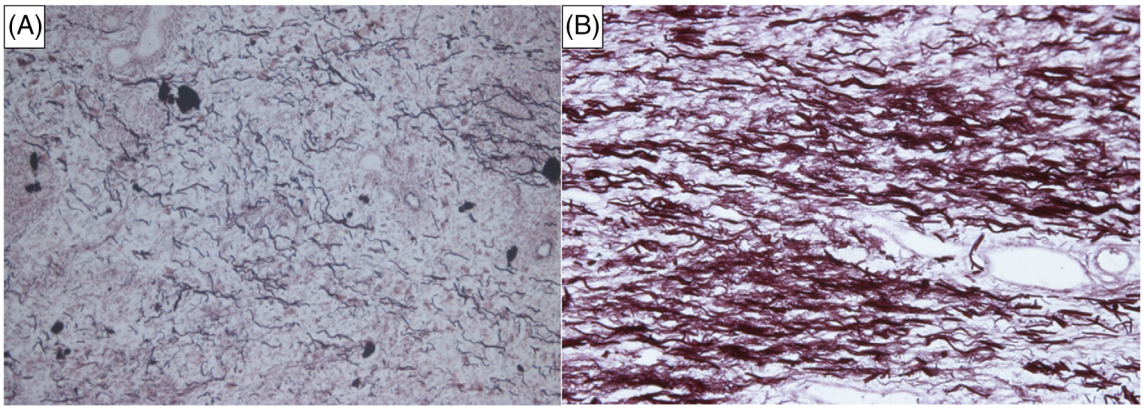

FIGURE 2 A, Photomicrograph of Right (injured) vocal fold, dexamethasone treated, orcein stained tissue at ×10 magnification, demonstrating sparsely distributed, black-stained elastin fibrils in haphazard arrangements in the superficial lamina propria. (Black dots are artifactual). B, Photomicrograph of right vocal fold, halofuginone treated, orcein stained tissue at ×25 magnification, demonstrating purple-brown stained elastin fibres diffusely spread through the lamina propria

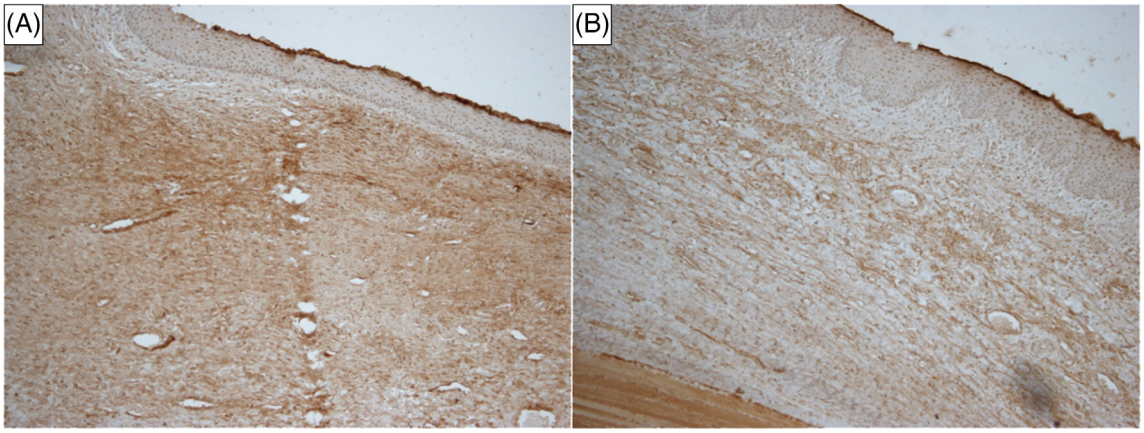

FIGURE 3 Right vocal fold in halofuginone treated animal demonstrating Smad3 staining at the injury site (A) vs noninjured site (B) with decreased brown-orange stain seen in normal noninjured vocal fold

TABLE 3 Raw data for Smad3 Region of Interest (ROI) percentage area in injured and noninjured vocal folds

Treatment	Mean (SD)	*P* value
Control L	37.8 (6.9)	.44
Control R	42.9 (3.3)	
Halo L	28.4 (9.8)	**.005**
Halo R	38.3 (10.1)	
Dexa L	25.9 (12.6)	.4
Dexa R	33.2 (14.5)	
Tri L	32.33 (11.1)	**.013**
Tri R	45 (5.6)	
Students *t*-test comparisons		
All right treated VFs vs right control VFs[a]		**.047**
Negative control vs left VFs[a]		**.0002**
Negative control vs right VFs[a]		**<.0001**
Halo R vs Dexa R[a]		.32
Halo R vs Tri R[a]		**.034**
Dexa R vs Tri R[a]		**.05**

Note: Significant *P*-values are bolded.
Abbreviations: Dexa, dexamethasone; Halo, halofuginone; Inj, injury; L, left; R, right; Tri, triamcinolone.
[a]Groups compared using Student's *t*-test.

2.3.1 | Ex-vivo phonatory examination

To evaluate VF vibratory function following injury and treatment, excised larynges were first snap-frozen in liquid nitrogen, then slow thawed in saline when needed. Supraglottic tissue including the epiglottis, and part of the thyroid cartilage was excised and the soft tissue was sutured to the remaining cartilage margin, to prevent interference with visualization of the true VF mucosal wave. VFs were adducted using a spinal needle passed through the arytenoids. The trachea was connected to outlet tubing from a compressor system providing conditioned (100% humid, 35°C) air at a rate of 2 to 2.5 L/s to produce true VF oscillation (Figure 1). Glottic motion was recorded on a stroboscopy system (KayPentax 9310HD stroboscope, Pentax VNL-1190 videoendoscope; Pentax, Japan) and analyzed.

2.3.2 | Normalized glottal gap

Mizuta and colleagues reported examination of *ex vivo* canine larynges and used the normalized glottal gap (NGG) to assess closure.[26] NGG was calculated by measuring the area at the glottis during closed phase and normalizing this by use of the length of the glottis in the following formula:

$$NGG = a/L^2 \times 100$$

(a = area, L = VF length from anterior commissure to vocal process).

Fang et al utilized the NGG to assess patients with unilateral VF paralysis and glottal insufficiency, prior to and after lipoinjection augmentation.[27] They identified an NGG >10 as correlating with aspiration, and following VF injection augmentation, resolution of aspiration in the 20 patients studied, with corresponding decrease in NGG to less than 3.[27]

3 | RESULTS

All the sheep tolerated biopsy procedures and medications without difficulty and there were no sheep deaths. Sheep gained an average of 8.15 kg (±3.6 SD) during the housing period. VF wounding demonstrated a predictable and consistent pattern, which was responsive to administered medication.

3.1 | Collagen density

Collagen density *decreased* at all injury sites and differed significantly between injured and noninjured sides (R vs L) in all except triamcinolone-treated sheep. Overall, the untreated and halofuginone-treated animals exhibited collagen levels that decreased by more than one third (35%,

TABLE 4 Quantitative date from ex-vivo stroboscopic examinations of excised larynges

Sheep	Treatment	Vibration characteristics	NGG (ratio units)	Closure	F0 (Hz)
Sheep 13	Triamcinolone	Mucosal wave present, reduced amplitude on right volume deficit	1.93	Incomplete	170
Sheep 14	Triamcinolone	Mucosal wave present, near normal amplitude	0.31	Complete	135
Sheep 15	Dexamethasone	Mucosal wave reduced on right, phase asymmetry, large volume deficit, supraglottic tissue vibration	0.25	Incomplete	No F0
Sheep 16	Dexamethasone	Mucosal wave present, near normal amplitude	1.2	Complete	180
Sheep 17	Halofuginone (20 mg)	Mucosal wave present, near normal amplitude	2.7	Incomplete	168
Sheep 18	Halofuginone (20 mg)	Mucosal wave present, near normal amplitude	2.7	Complete	194

Abbreviation: F0, fundamental frequency.

P <.05) whereas those treated with dexamethasone demonstrated an 8% reduction and with triamcinolone only a 4% reduction compared to non-injured VFs. Between group comparisons by Students t-tests revealed a significant difference in collagen density between halofuginone-treated animals and triamcinolone-treated animals ($P = .017$). There was no significant difference between halofuginone- vs dexamethasone-treated animals (27.8% vs 34%, $P = .17$) [Table 1].

3.2 | Elastin density

Orcein staining revealed dramatic loss of elastin in injured VFs treated by steroid, (P <.02) whereas halofuginone treatment protected elastin concentration (21.68 [halofuginone] vs 5.86 [dexamethasone], $P = .00$; 21.68 [halofuginone] vs 14.81 [triamcinolone], $P = .021$; Figure 2A,B). Elastin presence in the halofuginone treated group was equivalent to noninjured VF and to nontreated sheep (21.68 [halofuginone] vs 28.9 [nontreated], $P = .48$; Table 2).

3.3 | Smad3 staining

Smad3 is typically up-regulated at an injury site due to TGF-β receptor binding. All injury sites demonstrated increased Smad3 activity (Figure 3, Table 3). There was no significant difference in Smad3 activity seen between halofuginone-treated and dexamethasone-treated VFs ($P = .32$) however there was significantly greater Smad3 activity detected in triamcinolone-treated VFs compared to halofuginone- or dexamethasone-treated animals ($P = .034$ and .05, respectively; Table 3).

3.4 | Ex-vivo phonatory function

Ovine larynges produced consistent oscillation following treatment and healing, confirming that histological findings were reflected in functional phonation. Stroboscopic recordings made were evaluated using the normalized glottal gap. The NGG was under 3 (range 0.25-2.7) for all specimens suggesting satisfactory glottal closure and fundamental frequency was measurable in all but one specimen (range 135-194 Hz; Table 4).

4 | DISCUSSION

Novel approaches to management of VF scar are needed. Much progress has been made in understanding the molecular pathways involved in healing of VF wounds, affording us new targets for manipulation.[13,28-32] This study compared effects of commonly used corticosteroid medications to a novel small-molecule collagen type IA inhibitor, halofuginone, in VF wound healing. Multiple parameters were included to assess the relationship of histological findings to phonatory function in this model.

Changes in the VF secondary to injury were confirmed including reduction of epithelial thickness, reduction in collagen density at 1 month and loss of elastin (matching previously published data in this model).[25] Both halofuginone and dexamethasone moderated collagen loss but halofuginone treatment was also protective against elastin depletion. In addition, Smad3 binding in both halofuginone and dexamethasone-treated sheep was significantly less prominent than in triamcinolone-treated sheep suggesting modulation of the Smad3 fibrotic pathway.

Halofuginone inhibits phosphorylation of Smad3, preventing nuclear translocation and collagen gene transcription. However, it also exerts other anti-fibrotic effects including restriction of MMP-2 and TIMP-1 expression (ECM regulator molecules), and reduction of IL-17 release from T_H17 cells. IL-17 is a potent inflammatory cytokine and blockade of its action may help limit general cellular infiltration at the wound site, moderating the amplification of fibrotic pathways. This acts as a negative feedback loop to diminish fibrogenic processes. Halofuginone also prevents fibroblast to myofibroblast transition, thereby preventing collagen synthesis[18] and has the ability to modify *existing* fibrosis, probably through the MMP and TIMP pathways.[33] This is unusual and highlights the diverse actions that this drug might have.[33]

The appeal of halofuginone is amplified by the ability to administer the medication in a variety of formulations. Applying the drug directly to cells, such as through topical cream in systemic sclerosis patients or the tight skin mouse model, effectively reduced skin fibrosis almost to the same extent as delivering the medication intraperitoneally to treat radiation-induced limb contracture in mice, or per oral to mitigate liver fibrosis in a rat model.[20,33-35] Abramovitch et al also demonstrated halofuginone

benefit on the rat brain tumor model in both intraperitoneal *and* oral administration routes with greater restriction of tumor growth in orally treated animals.[21,23] Two studies have examined halofuginone in the larynx. Yoon et al created a rabbit posterior glottic stenosis model by stripping epithelium, and then applied halofuginone to the area.[36] Halofuginone reduced scarring and granulation tissue compared to untreated animals.[36] Eliashar et al used halofuginone to mitigate subglottic stenosis in a canine model.[37] They report a marked benefit of halofuginone oral therapy in preventing subglottic stenosis at 3 months compared to control animals, and also inhibition of growth of cultured fibroblasts when exposed to halofuginone which was comparable to mitomycin C inhibition.[37]

Differences in Smad3 staining between halofuginone-treated, injured VFs and those treated with dexamethasone or triamcinolone were expected but were not confirmed in this study. This may reflect the ability of glucocorticoids to inhibit TGF-β upstream of the Smad signaling process or to reduce overall presence of fibroblasts contributing to collagen gene expression and transcription. A diminished Smad3-staining pattern would therefore be found in corticosteroid-treated sheep as well as those given halofuginone. It is also possible that the doses administered to sheep were insufficient to achieve efficacy in inhibition or that the timing of IHC staining and evaluation was outside the window in which Smad3 transcription was most active.

Histological and vibratory findings suggest that halofuginone may be able to address multiple aspects of VF microarchitecture and function following injury. Stroboscopic examination of halofuginone-treated larynges demonstrated near-normal wave and NGG below 3, despite volume deficits at the biopsy site. Halofuginone contributes to restoration of the VF laminar structure which can then support improved phonatory function. Our data indicate halofuginone treatment is noninferior to dexamethasone or triamcinolone treatment for VF injury modulation.

4.1 | Limitations

Mechanism of action of the medications differ, although all exert nuclear effects on cells. Animal dosing regimens for these medications are not standardized or validated. Dose regimens were selected based on currently utilized dosing in clinical practice in humans. It is possible that doses were either too low or too high, unduly altering tissue response. The level of vocal use by sheep may not match human vocal loads and this may influence healing patterns. Sheep age may affect healing responses and sheep diet differs from human diet. These factors may influence wound healing. Collection of specimens at 28 days may have reduced detection of antibody staining or tissue repair processes, and earlier and/or sequential sampling would be required to evaluate change over time. Use of rabbit antibody for Smad3 detection in sheep specimens may have affected antibody binding although testing of control specimens showed expected staining patterns.

5 | CONCLUSIONS

VF injury in an ovine model results in a wound response able to be modified by Smad3 inhibitor, halofuginone, with benefit to vibratory function. These data support this pathway as a suitable target for manipulation to prevent or reverse fibrosis in the glottis and restore voice quality. Halofuginone treatment is noninferior to corticosteroid treatment in its ability to modulate VF wound healing.

ACKNOWLEDGEMNTS

Thank you to Ms. Sharon Waldvogel-Thurlow for providing histological processing expertise and Ms. Maree Schollum and team who cared for the animals.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ORCID

Jacqueline Allen https://orcid.org/0000-0002-3302-7045

BIBLIOGRAPHY

1. Wynn TA, Ramalingam TR. Mechanisms of fibrosis: therapeutic translation for fibrotic disease. *Nat Med*. 2012;18(7):1028-1040. https://doi.org/10.1038/nm.2807.
2. Hammond TH, Gray SD, Butler J, Zhou R, Hammond E. Age- and gender-related elastin distribution changes in human vocal folds. *Otolaryngol - Head Neck Surg*. 1998;119:314-322. https://doi.org/10.1016/S0194-5998(98)70071-3.
3. Gray SD, Titze IR, Alipour F, Hammond TH. Vocal fold extracellular matrix and its biomechanical influence. Part I: the fibrous proteins. *Natl Cent Voice Speech Status Prog Rep*. 1999.
4. Gray SD, Alipour F, Titze IR, Hammond TH. Biomechanical and histologic observations of vocal fold fibrous proteins. *Ann Otol Rhinol Laryngol*. 2000;109(1):77-85. https://doi.org/10.1177/000348940010900115.
5. Velnar T, Bailey T, Smrkolj V. The wound healing process: an overview of the cellular and molecular mechanisms. *J Int Med Res*. 2009;37(5):1528-1542. https://doi.org/10.1177/147323000903700531.
6. Franco RA, Husain I, Reder L, Paddle P. Awake serial intralesional steroid injections without surgery as a novel targeted treatment for idiopathic subglottic stenosis. *Laryngoscope*. 2018;128:610-617. https://doi.org/10.1002/lary.26874.
7. Rafii B, Sridharan S, Taliercio S, et al. Glucocorticoids in laryngology: a review. *Laryngoscope*. 2014;124(7):1668-1673. https://doi.org/10.1002/lary.24556.
8. Zhou H, Sivasankar M, Kraus DH, Sandulache VC, Amin M, Branski RC. Glucocorticoids regulate extracellular matrix metabolism in human vocal fold fibroblasts. *Laryngoscope*. 2011;121(9):1915-1919. https://doi.org/10.1002/lary.21920.
9. Kosinski AM, Pothen JM, Panitch A, Sivasankar MP. Dexamethasone controlled release on TGF-β1 treated vocal fold fibroblasts. *Ann Otol Rhinol Laryngol*. 2015;124(7):572-578. https://doi.org/10.1177/0003489415570933.
10. Te Wang C, Hsu YC, Liao LJ, Huang TW. Assessment of patient outcomes after adjuvant vocal fold steroid injection for fibrosis after microlaryngeal surgery. *JAMA Otolaryngol - Head Neck Surg*. 2019;145(9):811-816. https://doi.org/10.1001/jamaoto.2019.1682.
11. Mortensen M, Woo P. Office steroid injections of the larynx. *Laryngoscope*. 2006;116(10):1735-1739. https://doi.org/10.1097/01.mlg.0000231455.19183.8c.

12. Cho JH, Kim SY, Joo YH, Park YH, Hwang WS, Sun D. Il. Efficacy and safety of adjunctive steroid injection after microsurgical removal of benign vocal fold lesions. *J Voice*. 2017;31(5):615-620. https://doi.org/10.1016/j.jvoice.2017.01.003.
13. Paul BC, Rafii BY, Gandonu S, et al. Smad3: an emerging target for vocal fold fibrosis. *Laryngoscope*. 2014;124(10):2327-2331. https://doi.org/10.1002/lary.24723.
14. Davidson JM. Perspective Smad about elastin regulation. *Am J Resp Cell Mol Biol*. 2002;26(2):164-166.
15. Branski RC, Barbieri SS, Weksler BB, et al. Effects of transforming growth factor-β1 on human vocal fold fibroblasts. *Ann Otol Rhinol Laryngol*. 2009;118(3):218-226. https://doi.org/10.1177/000348940911800310.
16. Flanders KC. Smad3 as a mediator of the fibrotic response. *Int J Exp Pathol*. 2004;85(2):47-64. https://doi.org/10.1111/j.0959-9673.2004.00377.x.
17. Pines M, Nagler A. Halofuginone: a novel antifibrotic therapy. *Gen Pharmacol*. 1998;30:445-450. https://doi.org/10.1016/S0306-3623(97)00307-8.
18. McLaughlin NP, Evans P, Pines M. The chemistry and biology of febrifugine and halofuginone. *Bioorganic Med Chem*. 2014;22:1993-2004. https://doi.org/10.1016/j.bmc.2014.02.040.
19. Pines M. Targeting TGFβ signaling to inhibit fibroblast activation as a therapy for fibrosis and cancer: effect of halofuginone. *Expert Opin Drug Discov*. 2008;3(1):11-20.
20. McGaha TL, Phelps RG, Spiera H, Bona C. Halofuginone, an inhibitor of type-I collagen synthesis and skin sclerosis, blocks transforming-growth-factor-β-mediated Smad3 activation in fibroblasts. *J Invest Dermatol*. 2002;118(3):461-470. https://doi.org/10.1046/j.0022-202x.2001.01690.x.
21. Abramovitch R, Dafni H, Neeman M, Nagler A, Pines M. Inhibition of neovascularization and tumor growth, and facilitation of wound repair, by Halofuginone, an inhibitor of collagen type I synthesis. *Neoplasia*. 1999;1:321-329. https://doi.org/10.1038/sj.neo.7900043.
22. Assis PA, De Figueiredo-Pontes LL, Lima ASG, et al. Halofuginone inhibits phosphorylation of SMAD-2 reducing angiogenesis and leukemia burden in an acute promyelocytic leukemia mouse model. *J Exp Clin Cancer Res*. 2015;34:65. https://doi.org/10.1186/s13046-015-0181-2.
23. Abramovitch R, Itzik A, Harel H, Nagler A, Vlodavsky I, Siegal T. Halofuginone inhibits angiogenesis and growth in implanted metastatic rat brain tumor model - an MRI study. *Neoplasia*. 2004;6:480-489. https://doi.org/10.1593/neo.03520.
24. de Jonge MJA, Dumez H, Verweij J, et al. Phase I and pharmacokinetic study of halofuginone, an oral quinazolinone derivative in patients with advanced solid tumours. *Eur J Cancer*. 2006;42(12):1768-1774. https://doi.org/10.1016/j.ejca.2005.12.027.
25. Allen J. Response of an ovine laryngeal injury model to a novel fibrosis inhibitor. *ANZ J Surg*. 2017;87(4):266-270. https://doi.org/10.1111/ans.13852.
26. Mizuta M, Hirano S, Ohno S, Kanemaru SI, Nakamura T, Ito J. Restoration of scarred vocal folds using 5 amino acid-deleted type hepatocyte growth factor. *Laryngoscope*. 2014;124(3):E81-E86. https://doi.org/10.1002/lary.24413.
27. Fang TJ, Li HY, Tsai FC, Chen IH. The role of glottal gap in predicting aspiration in patients with unilateral vocal paralysis. *Clin Otolaryngol Allied Sci*. 2004;29(6):709-712. https://doi.org/10.1111/j.1365-2273.2004.00876.x.
28. Hiwatashi N, Bing R, Kraja I, Branski RC. Stem cell-mediated paracrine signaling alters fibroplasia in human vocal fold fibroblasts in vitro. *Ann Otol Rhinol Laryngol*. 2017;126:581-588. https://doi.org/10.1177/0003489417716186.
29. Hiwatashi N, Hirano S, Suzuki R, et al. Comparison of ASCs and BMSCs combined with atelocollagen for vocal fold scar regeneration. *Laryngoscope*. 2016;126(5):1143-1150. https://doi.org/10.1002/lary.25667.
30. Kishimoto Y, Hirano S, Suehiro A, et al. Effect of exogenous hepatocyte growth factor on vocal fold fibroblasts. *Ann Otol Rhinol Laryngol*. 2009;118(8):606-611. https://doi.org/10.1177/000348940911800813.
31. Fishman JM, Long J, Gugatschka M, et al. Stem cell approaches for vocal fold regeneration. *Laryngoscope*. 2016;126(8):1865-1870. https://doi.org/10.1002/lary.25820.
32. Hiwatashi N, Bing R, Kraja I, Branski RC. Mesenchymal stem cells have antifibrotic effects on transforming growth factor-β1-stimulated vocal fold fibroblasts. *Laryngoscope*. 2017;127(1):E35-E41. https://doi.org/10.1002/lary.26121.
33. Turgeman T, Hagai Y, Huebner K, et al. Prevention of muscle fibrosis and improvement in muscle performance in the mdx mouse by halofuginone. *Neuromuscul Disord*. 2008;18:857-868. https://doi.org/10.1016/j.nmd.2008.06.386.
34. Bruck R, Genina O, Aeed H, et al. Halofuginone to prevent and treat thioacetamide-induced liver fibrosis in rats. *Hepatology*. 2001;33:379-386. https://doi.org/10.1053/jhep.2001.21408.
35. Pines M, Snyder D, Yarkoni S, Nagler A. Halofuginone to treat fibrosis in chronic graft-versus-host disease and scleroderma. *Biol Blood Marrow Transplant*. 2003;9:417-425. https://doi.org/10.1016/S1083-8791(03)00151-4.
36. Yoon YH, Rha KS, Koo BS, Park JY, Kim YM, Park YH. The preventive effect of halofuginone on posterior glottic stenosis in a rabbit model. *Otolaryngol - Head Neck Surg*. 2008;1(139):94-99. https://doi.org/10.1016/j.otohns.2008.03.007.
37. Eliashar R, Ochana M, Maly B, Pines M, Sichel JY, Nagler A. Halofuginone prevents subglottic stenosis in a canine model. *Ann Otol Rhinol Laryngol*. 2006;115(5):382-386. https://doi.org/10.1177/000348940611500511.

How to cite this article: Allen J. Effects of corticosteroids vs halofuginone on vocal fold wound healing in an ovine model. *Laryngoscope Investigative Otolaryngology*. 2021;6(4):786–793. https://doi.org/10.1002/lio2.602