Cryotherapy Has Antifibrotic and Regenerative Effects on Human Vocal Fold Fibroblasts

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Objectives/Hypothesis: Vocal fold scarring remains a major treatment challenge, and scar prevention without residual lesions remains a dilemma. Cryotherapy has shown cosmetic outcomes on skin lesions with minimal scarring. The aim of this study was to clarify the beneficial effects of cryotherapy for the prevention and the treatment of vocal fold scarring.

Study Design: In vitro.

Methods: Primary cultures of human vocal fold fibroblasts (VFFs) were used in this study. Myofibroblast differentiation was stimulated by transforming growth factor β1 (TGF-β1). We mimicked the cryotherapy effect on vocal fold healing in vivo by freezing VFFs ± TGF-β1 in vitro. The influence of freezing on cell viability, proliferation, migration, and contractile properties were analyzed. The expression of collagen I, collagen III, fibronectin, TGF-β1, matrix metalloproteinase 1 (MMP1), hyaluronan synthase 1 (HAS1) were investigated by real-time polymerase chain reaction (RT-PCR), and the expression of alpha smooth muscle actin (α-SMA) and decorin were investigated by RT-PCR and Western blot.

Results: Freezing was found to modify extracellular matrix (ECM) synthesis and differentiation of VFFs. Expression of collagen I, collagen III, fibronectin, α-SMA, and TGF-β1 was downregulated, and MMP1 was upregulated in VFFs + TGF-β1 (myofibroblast) by freezing. HAS1 and decorin were upregulated in both VFFs ± TGF-β1 by freezing. Freezing VFFs + TGF-β1 (myofibroblast) with fast thawing had a lower expression of α-SMA when compared with slow thawing. Freezing reduced the migration and collagen contraction of VFFs + TGF-β1 (myofibroblast).

Conclusion: Cryotherapy induces antifibrotic and regenerative ECM alterations in VFFs. These data provide insight into the prevention and the treatment of vocal fold scarring with cryotherapy in phoniatrics.

Key Words: Cryotherapy, vocal fold fibroblasts, extracellular matrix, antifibrotic, regenerative.

Level of Evidence: NA

INTRODUCTION

Vocal fold scarring continues to pose a significant clinical challenge, which is characterized by disordered extracellular matrix (ECM) in the lamina propria (LP). This is typically recognized by increased collagen deposition, poorly organized collagen orientation, and decreased proteoglycan density.1 Vocal fold scarring will reduce voice quality and can even result in a significant dysphonia that is difficult to treat.2 Vocal fold fibroblasts (VFFs) are responsible for ECM synthesis and supporting the LP. When attack happens on the vocal folds, VFFs may exhibit an unbalanced production of ECM components, and the most extreme response to injury is scarring. One of the early steps in wound healing involves the recruitment of fibroblasts to the site of injury.1–3 Therefore, two issues must be addressed for preventing vocal fold scarring: 1) the aberrant synthetic phenotype of the VFFs after injury and 2) the regeneration of the LP with organization and orientation of novel collagen fibers. The above issues should move toward normalization to achieve the restoration of the pliability and viscoelastic properties of the vocal fold.4–6

Minimal scarring is an advantage of cryotherapy. The preservation of the structure frame from cryotherapy, including the collagen fibers’ and fibroblasts’ resistance to freezing, is critically important to wound healing.7–9 Keloid and hypertrophic scars benefit from cryotherapy with better cosmetic results.10–12 The mechanism of resembling normal healthy tissue by cryosurgery can be elucidated from two perspectives: 1) modifying scar fibroblast differentiation toward a normal phenotype and production of organized novel collagen fibers12,13 and 2) the preserved structure frame acts as a scaffold for cellular regeneration in the absence of wound contraction.8,10 Knott et al. reported that vocal fold healing in a canine model with adjuvant cryotherapy resulted in less and better-organized novel collagen formation and an improvement in glottal function when compared with CO2 laser surgery alone.11 Another study found patients with early-stage glottic carcinoma who underwent CO2 laser resection with adjuvant cryotherapy were associated...
with a significant improvement in subjective voice quality.\textsuperscript{15} From the above findings, we speculate that cryotherapy may be helpful in the treatment of vocal fold lesions for the prevention of vocal fold scarring. The benefits of cryotherapy have been shown in treating different lesions of different tissues (skin,\textsuperscript{16} trachea,\textsuperscript{17} esophagus,\textsuperscript{18} bladder\textsuperscript{19}) where there is a preservation of the fiber framework and a reduction in scar tissue formation. In laryngology, cryosurgery has been used since the 1970s to treat various laryngeal diseases, including papilloma,\textsuperscript{20–21} carcinoma,\textsuperscript{21,22} and subglottic hemangioma.\textsuperscript{23,24} Cryosurgery also has been used to treat glottic and subglottic stenosis,\textsuperscript{25,26} which has shown the same scarless results as on dermal lesions. Based on the above, cryotherapy holds promise for use on vocal fold lesions to prevent or minimize scar formation and further treat vocal scarring in phonomicrosurgery. No study has been done to investigate the effects of cryotherapy on VFFs. Our first step was to establish a model that studies the effects of freezing on VFFs in vitro to mimic the effect of cryotherapy on vocal fold healing process in vivo.

**MATERIALS AND METHODS**

**VFF Cultures**

Normal human vocal fold tissue from one patient who underwent total laryngectomy for reasons unrelated to the vocal folds in the Department of Otolaryngology–Head and Neck Surgery at the Eye, Ear, Nose, and Throat Hospital of Fudan University in Shanghai, China was used to culture VFFs. The vocal fold mucosa was judged to be normal without any evidence of disease by the attending surgeon. Informed consent was obtained from the patient. Primary fibroblasts were cultivated and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS). All investigations were carried out in passages four to nine. Differentiation to myofibroblasts was stimulated using media of 2.5% FBS + 10 ng/mL recombinant human transforming growth factor β1 (TGF-β1) (0117209-1; PeproTech, Rocky Hill, NJ) and 2.5% FBS without TGF-β1 as normal fibroblasts (no differentiation stimulation) for 5 days. VFFs were cultured on 6-well plates for scratch assay and real-time polymerase chain reaction (RT-PCR). For Western blot analysis, 10-cm dishes were used. Neighboring cells were trypsinized, freezing was applied, and they were plated on the same-size culture dish. Cells were harvested 24 hours after freezing for RT-PCR and at 48 hours for Western blot. When cells reached confluence after freezing, scratch assays were conducted. For the cell viability and collagen contraction assays, cells were cultured on 96-well plates and 24-well plates, respectively.

**Application of Freezing on VFFs**

To apply freezing on VFFs, a model was developed according to the following requirements: VFFs ± TGF-β1 were shock-exposed to precooled ethanol (in a −80°C refrigerator) for 1.5 minutes in a sterile 1.5 mL Eppendorf tube filled with 1 mL of cell suspension and subsequently thawed fast in an electro-thermostatic water cabinet (37°C) or thawed slowly at room temperature (25°C). Using a thermocouple connected to a thermometer (AZ8832, AZ Instrument, Taichung City, Taiwan) to detect the temperature inside the Eppendorf tube, we observed the freezing temperature on cells reached −30°C. The cells were divided into six groups: VFFs (N), VFFs freezing with fast thawing (F), and with slow thawing (R); VFFs + TGF-β1 (T), VFFs + TGF-β1 freezing with fast thawing (TF), and with slow thawing (TR).

**Cell Viability Assay**

We measured cell viability of VFFs ± TGF-β1 after freezing and VFFs ± TGF-β1 without freezing as the control at each time point. After freezing, cells (1 × 10\(^5\) cells/mL counted before freezing) were plated on 96-well microtiter plates (100 μL/well) in triplicates. At 6, 24, 48, and 72 hours, viable cells were measured using the Cell Counting Kit-8 (CCK-8; Biotech Well, Shanghai, China). The viable cells convert WST-8 tetrazolium (water-soluble tetrazolium salt) in the dye solution into formazan. The absorbance of solubilized formazan was then read at 450 nm using a microplate reader.

**Scratch Wound Healing Assay**

The migration of VFFs was assessed with a scratch assay as described by Branski et al.\textsuperscript{27} Briefly, cells were seeded in pre-marked six-well plates and grown to reach a confluent monolayer. Gentle scratch was performed along the longitudinal axis of each well using a 200-μL pipette tip, followed by a phosphate-buffered saline (PBS) wash to remove detached cells. The cells on the edge of the wounds would move toward the opening to close the scratch until new contacts between cells were established. This may mimic the migration of fibroblasts to the injury site to some extent. Digital images were captured of each well immediately after the scratch procedure and again 24 hours later, by using the plate markings as landmarks to ensure that photographs were identically positioned. Scratch areas were measured in pixels using ImageJ software (National Institutes of Health, Bethesda, MD). The following formula was used to quantify migration: initial area − final area = Δarea/24 hours.

**Gene Expression Analysis**

To analyze changes of the ECM, we measured mRNA expression of: collagen I (COL1), collagen III (COL3), fibronectin, decorin, TGF-β1, matrix metalloproteinase 1 (MMP1), and hyaluronan synthase 1 (HAS1). To analyze the differentiation of myofibroblasts, alpha smooth muscle actin (α-SMA) was measured. Total RNA was extracted using TRIzol Reagent (CWbio Co., Ltd., Beijing, China). The quality and concentration of RNA was measured using an A260/A280 ratio. Then, we used PrimerScript RT Master Mix (Takara Bio Inc., Singap, Japan) to synthesize complementary DNA (cDNA). The RT-PCR was performed via the use of SYBR Green chemistry (Takara Bio Inc.) with Applied Biosystems’ 7500 system (Thermo Fisher Scientific, Waltham, MA). Primers were designed and synthesized (Sangon Biotech, Shanghai, China). Amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Gene expression was calculated using the comparative threshold cycle (ΔΔCT) method.

**Western Blot**

Proteins were extracted in radioimmunoprecipitation assay buffers and were quantified using a bicinchoninic acid assay method. Equivalent amounts of proteins were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels to perform electrophoresis, and then were transferred to a polyvinylidene difluoride membrane. After blocking in 5% bovine serum albumin (BSA), membranes were incubated with primary antibodies decorin (1:1000; ab175404, abcam, Cambridge, MA),...
α-SMA (1:2000; ab5694, abcam), and fibronectin (1:1000; ab6328, abcam) overnight at 4°C. Each membrane was probed with a horseradish peroxidase-coupled goat anti-rabbit or goat antimal mouse secondary antibody (1:2000; Jackson ImmunoResearch, West Grove, PA) for 2 hours. The immunoblots were detected by enhanced chemiluminescence kit and visualized by x-ray imaging. Data were quantified via densitometry using ImageJ. The protein expression for each sample was normalized against the expression of GAPDH as a loading control.

**Cell Viability and Proliferation**

**RESULTS**

**Statistical Analyses**

Comparisons of cell viability analyses were made using two-way analysis of variance (ANOVA) with repeated measures. For other analyses, one-way ANOVA was used. If the P value was less than the significance level α = .05, post hoc comparisons were performed via the Tukey method. Analysis was performed using GraphPad Prism 7.04 (GraphPad Software, La Jolla, CA). All data were expressed as mean ± standard error. All experiments were repeated at least three times.

**RESULTS**

**Cell Viability and Proliferation**

There was no difference in the viability of VFFs ± TGF-β1 at the measured time points (Fig. 1A). The procedure showed a 15% inhibition of viability after freezing after 6 hours of incubation followed by a gradual increase in viability from 6 hours to 48 hours in both VFFs ± TGF-β1. At 48 hours, all groups including the control reached the same level of viability (Fig. 1B,C). No difference in viability was seen in the different thawing groups (Fig. 1B,C).

**Cell Migration**

As shown in Figure 2, VFFs + TGF-β1 (T) had a higher migration than VFFs (N). After freezing, no difference was noted in VFFs (N), whereas in VFFs + TGF-β1 (T), migration was reduced. No difference was seen in the different thawing groups.

**Gene Expressions**

Gene expressions are shown in Figure 3. TGF-β1 upregulated the expression of COL1, COL3, α-SMA, MMP1, and fibronectin, whereas TGF-β1 downregulated the expression of decorin (T compared with N). No change in the expression of COL1, COL3, TGF-β1, MMP1, α-SMA, or fibronectin was observed in VFFs freezing treated with fast (F) or slow (R) thawing when compared with untreated VFFs (N). Lower expression of COL1, COL3, TGF-β1, fibronectin, α-SMA and higher expression of MMP1 were found in VFFs + TGF-β1 freezing treated with fast (TF) or slow (TR) thawing when compared with untreated VFFs + TGF-β1 (T). There was a difference related to thawing rate, as TF had lower α-SMA expression than TR. Higher decorin and HAS1 expression were found both in VFFs ± TGF-β1 after freezing treated compared with untreated, respectively.

**Western Blot**

Western blot results are shown in Figure 4. TGF-β1 significantly increased the expression of α-SMA and decreased the expression of decorin (T compared with N). Lower α-SMA expression was found in VFFs + TGF-β1 freezing treated with fast (TF) or slow (TR) thawing when compared with untreated TGF-β1 + VFFs (T). There was a difference related to thawing rate, as TF had lower α-SMA expression than TR. Higher decorin expression was found both in VFFs ± TGF-β1 after freezing treated compared with untreated, respectively.
**Collagen Contraction**

After edges were liberated from arrays wells, we observed gel contraction at 0 hours (0h) and 24 hours (24h) (Fig. 5). No differences were observed at 0h (without obvious contraction) between any of the groups. At 24h, TGF-β1 significantly increased the gel contraction (T compared with N). Less gel contraction was found in VFFs + TGF-β1 freezing treated with fast (TF) or slow (TR) thawing when compared with untreated TGF-β1 + VFFs (T). There was a difference related to thawing rate as TF had less gel contraction than TR.

**DISCUSSION**

Vocal fold tissue is composed of layered structures including the epithelium, LP, and muscle. Fibrosis in vocal fold scarring is characterized as aberrant fibroblastic activity of VFFs in the LP, including various ECM components, such as fibrous proteins (collagen), glycoproteins (fibronectin), and proteoglycans (hyaluronic acid and decorin). Effective prevention and treatment modalities for vocal fold scarring are lacking because of the difficulty in restoring the structural changes. Cryotherapy is considered a good therapeutic option, offering quality cosmetic results with a low incidence of complications of skin lesions. Some mechanisms relating to the antiscar effects of cryotherapy have been found. One is the preservation of the structure frame acting as a scaffold for regenerating cellular components and slowly replacing collagen in the absence of constriction during wound healing. This is in contrast to a burn injury leaving an obvious scar. Another is modifying scar fibroblasts toward a normal phenotype to produce organized novel collagen fibers. We proposed that cryotherapy's effect on vocal fold wound healing would be similar to the process with dermal connective tissue. To verify the potential value of cryotherapy, we developed a model of controlled freezing on VFFs ± TGF-β1. Considering cryosurgical regimes that involve severe and prolonged freezing of the skin are quite capable of producing obvious scarring, we compared groups with different rates of thawing. This study provides the first evidence of the effects of freezing on the alterations of VFFs. The overall cellular response to freezing supports a normalized outcome induced by the antifibrotic and regenerative effects of cryotherapy.

In our study, freezing-treated VFFs ± TGF-β1 showed a 15% inhibition of viability at 6 hours of incubation followed by a gradual increase in the proliferation from 6 hours to 48 hours, compared with the untreated group. There was no difference between the different thawing groups. This indicates that VFFs resist cryotherapy the same as dermal fibroblasts reported at −30°C to −35°C. Fibroblasts are capable of surviving exposure to rapid freezing at a low temperature, but it is unlikely that epidermal cells are equally resistant. Freezing the full thickness of skin results in more damage to the epidermal cells than to the fibroblasts. It is conceivable that the survival of fibroblasts at the boundary of the cryotherapy region contributes to wound healing on the premise of destroying the targeted lesions. This is ideal for the lesions located in the vocal fold epithelium layer, such as leukoplakia.

Exogenous TGF-β1 increased VFFs migration, suggesting that TGF-β1 may be chemotactic to VFFs, recruiting them into the injury site. TGF-β1 secreted at sites of injury is thought to activate quiescent mesenchymal cells adjacent to the wound bed, promoting their transdifferentiation into a proliferative, fibrogenic phenotype eventually into contractile myofibroblasts. Freezing reduced the migration capacity of VFFs ± TGF-β1 (myofibroblasts) into the injury site, but did not affect the normal VFFs. This possibly explains the mechanism of cryotherapy preventing the overexpression of ECM proteins such as collagen, potentially reducing scar formation by decreasing the amount of myofibroblasts at the wound site. On the other hand, not affecting the normal VFFs gathering at...
Fig. 3. Effects of TGF β1 and freezing on ECM components (gene expression, mRNA). (A, B) COL1 and COL3: TGF β1 upregulated the collagen expression and freezing downregulated the collagen expression in T. (C) TGF β1: freezing downregulated the TGF β1 expression in T. (D) MMP1: TGF β1 upregulated the MMP1 expression and freezing upregulated it in T. (E) α-SMA: TGF β1 upregulated the α-SMA expression and freezing downregulated it in T, a difference relating to thawing rate that TF had lower expression than TR. (F) Fibronectin: TGF β1 upregulated the fibronectin expression and freezing downregulated it in T. (G, H) decorin and HAS1: TGF β1 downregulated the decorin expression. Both in N and T, freezing upregulated the expression of decorin and HAS1. *P < .05, **P < .01, ***P < .001, ****P < .0001, relative to N. #P < .05, ##P < .01, ###P < .001, ####P < .0001, relative to T. ****Between different thawing rates, P < .0001. α-SMA = alpha smooth muscle actin; COL1 = collagen I; COL3 = collagen III; ECM = extracellular matrix; F = N + freezing with fast thawing; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; HAS1 = hyaluronan synthase 1; MMP1 = matrix metallopeptidase 1; N = VFFs; R = N + freezing with slow thawing; T = VFFs + TGF β1; TF = T + freezing with fast thawing; TR = T + freezing with slow thawing; VFFs = vocal fold fibroblasts.
the injury site may also contribute to the healing process, because having too few fibroblasts would delay the process. However, we only observed the migration at 24 hours. It is unclear what the effect would be in long-term wound healing and when the functional results would actually manifest.

The RT-PCR outcome showed for VFFs + TGF-β1 (myofibroblasts) that freezing downregulated the expression of ECM components (COL1, COL3, and fibronectin), and α-SMA upregulated the expression of MMP1 (which increased collagen degradation). For both VFFs ± TGF-β1, freezing upregulated HAS1 (which secreted hyaluronic acid) and decorin (which contributed to restoring LP viscoelasticity). The ECM change induced by cryotherapy answered two questions about preventing vocal fold scarring and restoring the pliability and viscoelastic properties of vocal fold: 1) Attenuating myofibroblasts differentiation and preventing ECM overdeposition was for modifying the aberrant synthetic phenotype of VFFs (myofibroblasts) to the normalized type. 2) Enhancing decorin and hyaluronic acid expression of fibroblasts was for the regeneration of the LP with organization and orientation of novel collagen fibers.

A myofibroblast, a phenotypic transition expressing α-SMA, is considered to be activated transiently in the wound healing process and is primarily responsible for excessive ECM deposition to the formation of a collagen-rich scar.32,33 α-SMA is a well-accepted marker of myofibroblast differentiation that is responsible for contraction during wound healing.34 Vyas et al. demonstrated that
VFFs were capable of differentiation into myofibroblasts by stimulation from TGF-β1. One important finding of our study was that freezing decreased the expression of α-SMA at the level of genes and protein, and the collagen contraction assay further ascertained this. Based on these results, we made the presumption that cryotherapy appears to attenuate the extent of differentiation to a myofibroblast phenotype.

Decorin is a secreted proteoglycan found in the ECM of several tissues. It is a well-known anti-fibrotic and an inhibitor of TGF-β1, which appears to be able to bind type I collagen and TGF-β1 simultaneously. It has been demonstrated to have the beneficial effect of antifibrosis in hypertrophic scars formed after burns and in VFF scars. The anti-fibrotic functions of decorin include not only spacing collagen fibrils appropriately but also regulating collagen fibrillogenesis, causing a decrease in both the diameter and number of fibrils. Decreased levels of decorin have been found in hypertrophic scars, which may account for the poor organization of collagen fibrils and has also been found in vocal fold scars. Our study found that freezing upregulated decorin expression in both VFFs ± TGF-β1 indicated that cryotherapy may have an effect on formation of novel organized collagen in the healing process. TGF-β1 is a profibrotic cytokine in wound scarring and a potential target for antiscarring gene therapy. Because the lower expression of TGF-β1 that occurred in the VFFs + TGF-β1 after freezing was also found in keloids after cryotherapy, we speculate that cryotherapy may inhibit the TGF-β1 fibrotic pathway; further study is needed to verify this.

An interesting finding of our results was that the VFFs + TGF-β1 after freezing with fast thawing expressed less α-SMA and weakened constriction compared with slow thawing. Guan et al. proved that the application of higher thawing temperature to dermal fibroblasts in vitro induces decreased differentiation of myofibroblasts. Thus, application of 37°C as a thawing temperature may weaken the contractile activity generated during wound healing. Regarding thawing rate as one of the important treatment factors in cryotherapy, slow thawing results in more freezing damage that may be adverse to wound healing, whereas a faster thawing phase may cause cellular and vascular damage in the target tissue while protecting the surrounding normal tissue. Considering there was no difference in cell viability between the different thawing groups, we assume that thawing rate has a selective effect on fibroblasts, are damaged either way, but modify VFFs functions into reducing the expression of α-SMA. This suggests that thawing rate plays a role in wound healing and is useful in a cryotherapy procedure. Using the same cooling condition while increasing thawing rate would achieve a better cosmetic result while keeping the same damaging effects for target lesions. Further study needs to be done to determine the mechanism.

Cryotherapy was used as a primary or salvage therapy, but recently there has been increasing recognition of the value of cryotherapy in improving the healing process and as an adjuvant therapy to deal with the solid tumors for its preservation of fiber frame. Our study shows that the change of biomarkers expression was toward an anti-fibrotic alteration and clarified the potential cellular and molecular mechanism of the anti-fibrotic effect of cryotherapy. The cryotherapy effects hold promise in treating vocal fold lesions for preserving voice quality by reducing scar formation or further treating vocal scarring in phonomicrosurgery.

A limitation in the current study is that we did not measure the different extents of freezing, such as a cooling temperature lower than −30°C. Maybe the VFFs can resist a lower temperature, which would be meaningful in the treatment of carcinoma, ensuring total lesion ablation while preserving voice quality. In addition, the mechanisms of fast thawing reduced α-SMA expression over slow thawing significantly enough to warrant further investigation of how different thawing rates contribute to wound treatment.

CONCLUSION

This study investigated the potential of cryotherapy application in phonomicrosurgery. In vitro data indicated that using freezing treatment on VFFs + TGF-β1 (myofibroblast) modulated ECM turnover toward antifibrosis and normalization, attenuated myofibroblast differentiation, reduced cell migration, and weakened the contractile activity. On both VFFs ± TGF-β1, freezing treatment raised LP regeneration (HAS1, decorin). The findings indicate that cryotherapy may play a protective role in scar formation and contribute to lamina propria regeneration. These preliminary data lend further support to the use of cryosurgery as a therapy for prevention and treatment of vocal fold scarring.

BIBLIOGRAPHY
