The Effects of Cryotherapy on Vocal Fold Healing in a Rabbit Model

Ting Gong, MD; Chi Zhang, MD, PhD; Jing Kang, MD; Zhewei Lou, MD; Austin Scholp, BS; Jack J. Jiang, MD, PhD

Objectives/Hypothesis: Cryotherapy has been shown to be a scarless treatment modality for dermal lesions; however, there are limited data addressing the effect of cryotherapy on vocal fold tissue. The aim of this study was to clarify the effectiveness of cryotherapy for prevention of postsurgical vocal fold scarring.

Study Design: Prospective animal study in rabbits.

Methods: The lamina propria of 20 rabbit vocal folds was bilaterally stripped, followed by randomized unilateral cryotherapy. Five larynges were harvested for real-time polymerase chain reaction (RT-PCR) analysis at 1 day, 3 days, and 7 days postinjury. The remaining five were harvested for histologic analysis at 3 months. Images of the healing phase were recorded by laryngoscopy. Analyses of RT-PCR for cyclooxygenase (COX)-2, interleukin (IL)-6, collagen I, collagen III, matrix metalloproteinase 1 (MMP1), transforming growth factor β (TGFβ1), a smooth muscle actin (α-SMA), and hyaluronan synthase 1 (HAS1) were completed. Histological samples were completed for collagen and hyaluronic acid analysis.

Results: RT-PCR results revealed that higher expressions of HAS1 and MMP1 and lower expressions of COX-2, IL-6, collagen I, collagen III, TGFβ1, and α-SMA were observed, and histological examination showed significantly increased hyaluronic acid, decreased deposition, and more organized configuration of collagen in injury with the cryotherapy cohort compared with the injury cohort.

Conclusions: Cryotherapy can inhibit the inflammatory reaction and simulate a fetal healing environment in extracellular matrix synthesis to regenerate vocal fold tissue with less fibrosis. Histological results showed that cryotherapy achieves a mature healing result with less scar, which tends to return to normal. In summary, the findings of this study suggest that administration of cryotherapy at the time of injury has the potential to minimize vocal fold scarring.

Key Words: Cryotherapy, vocal fold scarring, fetal healing, extracellular matrix.

Level of Evidence: NA

INTRODUCTION
Vocal fold scarring observed following vocal fold surgery is caused by the unbalanced production of extracellular matrix (ECM) components in the lamina propria (LP). Scarring is the single greatest cause of a poor voice after vocal fold surgery, leading an intractable loss of tissue viscoelasticity critical for vocal fold vibration, which can lead to various degrees of dysphonia. A robust inflammatory environment following injury can disrupt ECM metabolism, yield scar formation, and ultimately increase tissue stiffness. Adult wound healing is commonly categorized into three overlapping phases of inflammation, proliferation, and remodeling, with the formation of scar as the end product of tissue injury. Conversely, fetal wounds display a process of regeneration with reduced scar formation, characterized by minimal inflammation, rapid re-epithelialization, and reduced collagen formation. Knowledge of this fetal wound-healing mechanism seems to likely be beneficial in achieving scarless healing of vocal folds. Treatments of vocal fold scarring remain challenging due to difficulty in restoring the inherent architecture of the LP; thus, the prevention of vocal fold scarring is of great importance.

Cryotherapy, also referred to as cryosurgery or cryoablation, is the localized application of extremely cold temperatures for medical treatments including eliminating lesions or relieving physical suffering. The use of cold temperatures in medicine dates back to various ancient cultures (e.g., Greece, Persia, and the Roman Empire), and it is still used today. The practical use of cold with modern equipment for therapeutic purposes has been carried out during the last century and now is widely used in clinical practice. The earliest reports using cryosurgery can be traced back to 1850, when James Arnott pioneered the use of iced saline to treat uterine and breast cancer. Modern cryosurgery grew in 1960 when Cooper pioneered the use of cryoablation to treat breast cancer. Modern cryosurgery grew in 1960 when Cooper improved the equipment to carry out a cryothalamectomy for abnormal movement disorders. The improvements allowed for precise application of cryosurgical treatment deep in the body. Since 1990, with the improvement in cryosurgical equipment and imaging techniques to
intraoperatively monitor the tissue-freezing process, there has been much research focused on the mechanism of cryotherapy on tissue and on new clinical applications. Since the 1970s, cryosurgery has been used to treat various laryngeal diseases including papilloma,11,12 carcinomas,12,13 subglottic hemangioma,14,15 and glottic and subglottic stenosis.16,17 Cryotheraphy is often used as a primary or salvage therapy but it has been gaining recognition as a method to improve the healing process and as an adjuvant therapy for solid tumors for its preservation of fiber framework. A research study by Knott et al.18 focused on the process of glottic healing and scarring in canine models and found less and better-organized collagen formation as well as decreased keratinization, resulting in an improvement in glottic function after adjuvant cryotherapy when compared with CO2 laser surgery alone. In a clinical trial19 by the same research team, similar results were seen where patients with early-stage glottic carcinoma underwent CO2 laser resection with adjuvant cryotherapy and showed significant improvement in subjective voice quality.

Cryotherapy has been shown to be a scarless treatment modality for dermal lesions. Minor freezing injury causes an inflammatory response, which has some therapeutic uses, but severe freezing kills cells and results in destruction, which is the prime requirement for treating tumors, producing coagulation necrosis in the frozen tissue in the days after thawing. In vocal fold surgery, lesions on vocal folds are expected to be ablated without destroying the LP. It has been established that collagen loses its structural integrity in the coagulation of protein that occurs with thermal damage. Conversely, cryotherapy can protect the ECM from being devitalized and preserve the integrity of the extracellular architecture. An intact ECM allows cells to migrate and is important to the regenerative regrowth potential of the tissues. In this way, minimal scarring is an advantage of cryotherapy.

We hope the use of cryotherapy in wound healing may be of great significance to voice outcomes in vocal fold surgery. However, to date, the molecular mechanism for this improved wound repair and tissue regeneration has not been identified, and there has been sparse research on vocal fold healing and vocal outcome after cryotherapy. Therefore, we studied the effects of cryotherapy on the healing process of vocal fold injuries. The current study builds on previous work that suggests that cryotherapy may hold significant therapeutic potential in scar prevention and regeneration in the LP of the vocal fold.

MATERIALS AND METHODS

Surgical Procedure

This study was approved by the institutional review board of the Eye, Ear, Nose, and Throat Hospital of Fudan University, Shanghai, China. Animals were cared for in accordance with established institutional guidelines. A total of 23 New Zealand White rabbits with similar physical conditions and body weights (male, 2.5–3.2 kg) were used. Twenty rabbit vocal fold lamina propria were bilaterally stripped down to the thyroarytenoid muscle by microlaryngeal cup forceps and followed by randomized unilateral cryotherapy. All stripping was performed by a single surgical team to maintain consistency. Five larynges were harvested for real-time polymerase chain reaction (RT-PCR) analysis at each of three time points (1 day, 3 days, and 7 days postsurgery). The remaining five larynges were harvested for histologic analysis at 3 months. Three untreated rabbits served as normal controls for histologic analysis. Rabbits were anesthetized intramuscularly with ketamine hydrochloride (40 mg/kg), diazepam (2 mg/kg), and xylazine hydrochloride (4 mg/kg). Atropine sulfate (0.05 mg/kg) was injected to reduce the secretion of saliva and sputum in the laryngeal lumen. Topical anesthetic in the form of 1% tetracaine chloride was applied to the larynx. The rabbit larynx was visualized with aid of a 0° rigid laryngoscope (Zhejiang Tiansong Medical Instrument Co., Ltd, Hangzhou, China) coupled to an apparatus consisting of a camera (Sony α7S; Sony, Tokyo, Japan), lens (Karl Storz 593-A/N and SOLIGOR 2X to fit Pentax; Karl Storz, Tuttinglen, Germany), adapter (transferring M42 to E mount), portable light source (Karl Storz 11301 D4, LED light; Karl Storz), and normal light source (Stryker X6000, cold light; Stryker, San Jose, CA). Laryngeal photos were taken at each time point.

Cryo-Surgery Procedure

The cryo-surgery was performed using a liquid nitrogen-based cryoablation unit (Ningbo Senscure Biotechnology Co., Ltd, Zhejiang, China). The circulating nitrogen gas expands into the cryoprobe and creates a reduction in temperature. We are able to regulate the liquid nitrogen release and control the freeze time through the control system. We designed the catheter containing the cryoprobe specifically for targeting the vocal fold. A thermocouple probe, which is used to monitor the tissue temperature, connected to a thermometer (A28852; AZ Instrument, Taichung, Taiwan) was mounted on the tip of the cryoprobe (Fig. 1). Temperatures can be consistently achieved in the wound surface during freeze–thaw schedules utilized in this study. Cryosurgical regime was between 15 and 20 seconds where temperatures reached about −30 to −40°C.

Gene Expression Analysis

To analyze changes of the ECM and inflammation factors in the acute healing phase, we measured mRNA expression of: collagen I (COL1), collagen III (COL3), TFPI, metalloproteinase 1 (MMP1), MMP9, hialuronan synthase 1 (HAS1), cyclooxygenase-2(COX-2), and interleukin-6 (IL-6). Total RNA was extracted using TRIzol Reagent (CWBio, Beijing, China). The quality and concentration of RNA were measured using an A260/A280 ratio. Then, we used PrimeScript RT Master Mix (Takara, Kyoto, Japan) to synthesize complementary DNA. RT-PCR was performed via the use of SYBR Green chemistry (Takara) with Applied Biosystem 7500 System (Life Technologies, Carlsbad, CA). Primers were designed and synthesized (Sangon Biotech, Shanghai, China). Amplification of glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. Gene expression was calculated using the comparative threshold cycle (2−ΔΔCT) method, all relative to the injury cohort at day 1.

Histological Examination

Five larynges at 3 months after surgery and three normal control larynges were harvested for histological analysis. Specimens were embedded in paraffin blocks and then sectioned at 4-μm thick along the coronal axis of the larynx. Next, slides were stained with hematoxylin and eosin, Masson’s trichrome (for

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Many experiments have confirmed that cryotherapy on skin lesions achieves healing with a lack of scarring.\cite{10,23,25} Freezing can destroy cells, but spares the connective tissue matrix. The resistance to freezing damage of collagen fibers in skin is the basis for this favorable healing. The remaining scaffolds will become the wound-healing bed in which cells infiltrate the scaffold and proliferate to help the wound healing without wound contraction compared to the untreated vocal fold (Fig. 2f).

**Gene Expressions**

Gene expressions in the acute healing process are shown in Figure 3. Cryotherapy downregulated the expression of inflammation factors with significant differences between all time points in IL-6 and at 3 days in COX-2. The expression of TGFβ1 reduced at 3 days with cryotherapy. Cryotherapy also downregulated the expression of collagen (COL1 and COL3), with significance occurring at 3 days and 7 days, whereas cryotherapy significantly upregulated the expression of the MMP1 at all time points. Lower expressions of α-SMA was found with cryotherapy, with higher expressions of HAS1 at all time points.

**Histological Changes of the Lamina Propria**

**Collagen.** The collagen in the normal vocal folds is loosely distributed with an ordered arrangement in the LP (Fig. 4A, Masson’s row). At 3 months, in the injury cohort (Fig. 4B, Masson’s row), collagen was distributed irregularly in all regions of the LP, and the intensity significantly increased when compared with the normal control (P < .0001). In the injury with cryotherapy cohort (Fig. 4C, Masson’s row), a significant reduction of the amount of collagen was seen in all regions of the LP when compared with the injury cohort (P < .001), whereas only a small increase was seen when compared to the normal control (P < .05).

**Hyaluronic acid.** In the injury cohort (Fig. 4B, Alcian blue row), we observed a statistically significant reduction of the amount of HA when compared with the normal control (Fig. 4A, Alcian blue row) (P < .01). When we compare the injury cohort with the cryotherapy cohort (Fig. 4C, Alcian blue row), there is an increase of the amount of HA, which is statistically significant (P < .05). The cryotherapy-treated vocal folds appeared similar to the normal control (P > .05).

**RESULTS**

**Changes of Vocal Fold Morphology**

The vocal fold injuries were created bilaterally (Fig. 2a), and the cryotherapy was conducted unilaterally (Fig. 2b). Acute vocal fold wound-healing processes were observed at 1 day, 3 days, and 7 days after injury. Finally, scar formations were observed after 3 months. At 3 days, the injury side (left) showed granulation tissue with hyperemia and edema, whereas the cryotherapy treated side (right) showed pseudomembrane formation with less inflammation (Fig. 2d). At 1 day and 7 days, no visible difference in inflammation were seen on vocal fold healing with or without cryotherapy (Fig. 2c,e). At 3 months, a more normal regenerative vocal fold (right) appeared with cryotherapy. The surface of the vocal fold tended to be smoother, with fewer irregularities and contraction compared to the untreated vocal fold (Fig. 2f).

**Statistical Analysis**

For statistical analysis of the data we used Graphpad Prism 7.04 (GraphPad Software, La Jolla, CA). Paired-samples t tests were used to determine statistically significant changes in gene expression in injured vocal fold samples with or without cryotherapy at different time points. One-way analysis of variance followed by Tukey’s post hoc test was used to compare values among cohorts for histological densitometry. All data are depicted as the mean ± standard deviation. P values of < .05 were considered to indicate statistical significance.
contraction, providing a space that is suitable for the induction of tissue regeneration. Collagen is replaced slowly after low-temperature injury, in marked contrast to rapid replacement observed following burning. Wound contraction was also marked after burning. These effects did not occur after freezing. Additionally, cryotherapy has been shown to have positive effects on the synthetic phenotype of skin fibroblasts toward normalization, which may help to create a controlled process of wound healing in case of overexpression of collagens and fibronectin. Furthermore, the use of adjuvant cryotherapy in wound healing is associated with a decreased inflammatory infiltrate and more organized and less dense final collagen histoarchitecture. All of the above indicate a good healing process brought by cryotherapy, but the precise etiology of how cryotherapy effects healing remains unclear.

**Scarless Healing**

Acute inflammation plays a central role in initiating and controlling the wound-healing process. When less inflammation occurs, wound healing may proceed better and with reduced scarring, or even completely without scarring. For instance, mice without macrophages that failed to raise an immune inflammatory response to dermal wounding healed scarlessly and more rapidly. The anti-inflammatory effects of cryotherapy were demonstrated here in decreased expression levels for inflammatory cytokines IL-6 and COX-2. IL-6 increases collagen production and plays a role in scarring, and it has been shown to be strongly upregulated during the inflammatory phase of healing. Increases in IL-6 expression after injury is transient in fetal wounds but prolonged in adult wounds. COX-2 induces scarring and fibroblast proliferation in fetal skin healing, and lower levels of COX-2 have been found to result in scarless healing in murine skin models, suggesting that COX-2 elevation results in scar formation. The treatment managing acute inflammation response may attenuate scarring and promote better wound healing in the vocal folds. α-SMA is a well-accepted marker of myofibroblast differentiation that is responsible for overdeposition of collagen and contraction during wound healing. The expression of α-SMA was also suppressed by cryotherapy in the acute inflammatory phase. The mature healing scar at 3 months also showed a more normally regenerated vocal fold with less contraction with cryotherapy. This is consistent with what is seen during the healing of the freeze-injured skin. TGFβ1 is a profibrotic cytokine in wound scarring and a potential target for antiscarring gene therapy, and its stimulated models have been successfully used to provide insight into fibrogenesis and suggest novel strategies for modulation of wound healing in different tissue.
Fig. 3. Effects of cryotherapy on extracellular matrix components and inflammation factors (gene expression, mRNA). (A, B) COL1 and COL3: cryotherapy downregulated the collagen expression at 3 days and 7 days. (C) TGFβ1: cryotherapy downregulated the TGFβ1 expression at 3 days. (D) MMP1: cryotherapy upregulated the MMP1 expression all the time points. (E) α-SMA: cryotherapy downregulated the α-SMA expression at all the time points. (F) IL-6: cryotherapy downregulated the IL-6 expression at all time points. (G) COX-2: cryotherapy downregulated the COX-2 expression at 3 days. (H) HAS1: cryotherapy upregulated the HAS1 expression at all time points (*P < .05, **P < .01, ***P < .001, ****P < .0001). One day, 3 days, 7 days = the dates after injury with or without cryotherapy. α-SMA = α smooth muscle actin; COL1 = collagen I; COL3 = collagen III; COX-2 = cyclooxygenase-2; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; HAS1 = hyaluronan synthase 1; I = injury on vocal fold; I+C = injury + cryotherapy on vocal fold; IL-6 = Interleukin 6; MMP1 = matrix metallopeptidase 1; TGFβ1 = transforming growth factor β1.
expression of TGFβ1 occurred in keloids after cryotherapy treatment. Our results also showed that cryotherapy suppressed TGFβ1 expression. We speculate that cryotherapy may inhibit the TGFβ1 fibrotic pathway to curb excessive inflammatory response in the healing process.

The inflammatory response involving the above factors (COX-2, IL-6, TGFβ1, α-SMA) in vocal fold injury will produce collagen deposition and distortion of its organization. Increased collagen deposition is known as a common histological finding in a scarred vocal fold. In our rabbit model of vocal fold wound healing, cryotherapy decreased the expression of collagens (COL1 and COL3) whereas it increased the level of MMP1, which is a metalloproteinase degrading collagen, in the acute inflammation phase, and a corresponding result in mature healing process was seen histologically in terms of the relative density of collagens. Moreover, the spatial distribution of collagen appeared to be similar to that of control. Decreasing hyaluronic acid (HA) deposition is another common histological finding in a scarred vocal fold. HA is thought to prevent scar formation as it creates a fetal-like environment.

HA is abundant in fetal tissue, and scarless fetal regeneration throughout the entire wound repair process. HA is resistant to being destroyed at −30°C to −35°C. In vivo experience indicates that not all cells die after freezing to end temperatures that are lower than the lethal one. It is hard to relate accurately the severity of freezing to the injury produced at a cellular level. Further studies...
are expected to supervise the temperature more precisely to avoid destruction of the surrounding tissues. Cryotherapy itself is a tool introducing cold to a treatment target and must be used with great care and caution. To that end, continued investigation regarding the optimal parameters for this tool is warranted to optimize treatment paradigms and to fully understand the physiology underlying the inherent tissue alterations associated with the cold temperature. Second, the rabbit animal model is chosen to investigate vocal fold wound healing because it possesses a histology of the LP similar to that of humans. Three months in the life of a rabbit is similar to several years in humans, therefore, our animal study based on 3 months was able to demonstrate the long-term effect of cryotherapy on the mature wound-healing phase. However, the translation of findings from the rabbit model to human vocal fold is tenuous. It is sometimes misleading to assume that results of study designed to investigate wound healing in experimental animals can be applied to humans. Third, it is likely that critical biochemical events occur in the acute period, and the limited time points involved in the current study likely fail to capture the whole picture of the wound-healing process. Regardless, these data are the first to attempt to elucidate the molecular effect of cryotherapy on the injured vocal fold in the healing process.

CONCLUSION

Results from this study provide evidence to support the hypothesis that cryotherapy contributes to wound healing by simulating an environment similar to what is seen in the fetal wound-healing environment for minimal scarring. The findings of this study suggest that the application of cryotherapy at the time of injury has the potential to prevent vocal fold scarring, and this feature may make cryotherapy a promising therapy in vocal fold surgery, with a resultant improvement in glottic function leading to a better voice quality.

BIBLIOGRAPHY