The Laryngoscope

Fibrin Gel Suspended Autologous Chondrocytes as Cell-based Material for long-term Injection Laryngoplasty

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Objectives/Hypothesis: Injection laryngoplasty of materials for unilateral vocal-fold paralysis has shown various results regarding the long-term stability of the injected material. We evaluated a fibrin-gel based cell suspension with autologous chondrocytes *in-vitro* and *in-vivo* as long-term-stable vocal-fold augmentation material in an animal model.

Study Design: This study compises an *in vitro* cell-culture part as well as an *in vivo* animal study with New Zealand White Rabbits.

Methods: In in-vitro experiments, auricular chondrocytes harvested from 24 New Zealand White Rabbits cadavers were cultivated in pellet cultures to evaluate cartilage formation for 4 weeks using long-term-stable fibrin gel as carrier. Injectability and injection volume for the laryngoplasty was determined in-vitro using harvested cadaveric larynxes. In-vivo 24 Rabbits were biopsied for elastic cartilage of the ear and autologous P1 cells were injected lateral of one vocal cord into the paraglottic space suspended in a long-term-stable fibrin gel. Histologic evaluation was performed after 2, 4, 12, and 24 weeks.

Results: During 12-week pellet culture, we found extracellular matrix formation and weight-stable cartilage of mature appearance. *In-vivo*, mature cartilage was found in two larynxes (n = 6) at 4 weeks, in four (n = 6) at 12 weeks, and in five (n = 6) at 24 weeks mostly located in the paraglottic space and sometimes with spurs into the vocalis muscle. Surrounding tissue was often infiltrated with inflammatory cells. Material tended to dislocate through the cricothyroid space into the extraglottic surrounding tissue.

Conclusions: A cell-based approach with chondrocytes for permanent vocal-fold augmentation has not previously been reported. We have achieved the formation of structurally mature cartilage in the paraglottic space, but this is accompanied by difficulties with dislocated material, deformation of the augmentation, and inflammation.

Key Words: Unilateral vocal cord paralysis, injection laryngoplasty, vocal cord augmentation, chondrocytes.

Level of Evidence: N/A

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INTRODUCTION

Injection laryngoplasty has been shown to be a valuable treatment option for different pathologies[1] causing glottal insufficiency. Most experience has been gained in the field of vocal fold augmentation for unilateral vocal fold paralysis, with the first injection being reported in 1911.[2]

Vocal fold paralysis with its diverse etiologies[3–5] can present as partial or complete immobility of the vocal fold with (partial) damage to the recurrent laryngeal nerve.[6–8] Therapeutic options are voice therapy,[4] phonosurgery, or combinations thereof. The aim of surgical treatment is to shift the paralyzed vocal fold closer to the midline enabling glottal closure during phonation.[8] To achieve midline contact, two surgical therapy options exist: transcutaneous laryngeal framework surgery, which pushes the paralyzed vocal fold to the glottal midline (medialization thyroplasty), and endoscopic augmentation of the paralyzed vocal cord, which involves injecting various materials into the vocal folds (injection laryngoplasty, undertaken at the doctor's office under local anesthesia or during intubation narcosis).[8,9] For temporary vocal cord relining recommended materials in clinical use are hyaluronic acid[10–12], calcium hydroxyapatite,[13] or collagen.[14] Unilateral paralysis for more than 12 months usually persists. In cases of persistent permanent glottal insufficiency, more permanent and less absorbable materials such as autologous fat[15,16] or a thyroplasty are preferred treatments[5,17] to spare the patient recurrent interventions every few months. Compared with thyroplasty, injection laryngoplasty is a fast and relatively low-risk intervention, but its durability is affected by the major deficit of all the injectable

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materials presently in use, namely their physical resorption over time. [18–20]

The aim of this study was to evaluate a cell-based approach with autologous auricular chondrocytes suspended within long-term-stable fibrin gel as a possibly durable material for injection laryngoplasty.

MATERIALS AND METHODS

All experiments were performed in the laboratory and animal facilities of the Klinikum rechts der Isar, Technical University of Munich according to the relevant animal protection act guidelines and a license was granted by the Bavarian governments' ethical committee (55.2–1-54-2531-92-10) for 24 New Zealand White rabbits. All animals were kept in isolation for the duration of the experiment and were given food and water ad libitum.

Prior to the cell-based injection laryngoplasty in this rabbit model, the injection volume and the cell number per 0.1 ml injection volume were established *in vitro* to guarantee an optimal experimental set up and to allow comparisons of *in vitro* cartilage formation and *in vivo* results.

In Vitro Evaluation of Cartilage Formation

Fresh cadavers of New Zealand White Rabbits from the animal facility were biopsied for elastic auricular cartilage of the ear (surgically removing any remaining perichondrium), and larynxes were harvested. Chondrocytes were isolated following standard protocol (see below), expanded in monolayer culture, and evaluated for cartilage formation in pellet culture using a long-term-stable fibrin-thrombin solution as carrier gel. In addition, injectability (passage of the mixture through the cannula) and injection volumes of the chondrocyte-thrombin-fibrin mix were tested on cadaveric larynxes to optimize the laryngoplasty procedure for the *in vivo* experiments.

Cell Isolation and Monolayer Cell Culture

A total of 2 cm^2 auricular cartilage biopsies were harvested from fresh cadavers for *in vitro* experiments or autologously for injection purposes *in vivo* in a sterile manner. A standard protocol for chondrocyte isolation was followed.[21] Detailed protocols can be found in the Supporting information.

Fibrin - Thrombin Solution

Fibrin (Sigma) was dissolved in Trasylol [Aprotinin Solution (Sigma) 1 mg/ml in 0.9% NaCl (Braun)] 100 mg/ml. Thrombin (from human plasma, Sigma) was dissolved in 100 ml thrombin buffer (20 mM CaCl Solution, Pharmacy MRI) with 5 U/ml buffer. Both components were mixed 1:1.

In Vitro Pellet Culture

Pellet culture was performed with P1 cells after one passage by using the fibrin-thrombin gel as a matrix. 2.5 x 10^6 cells were centrifuged to form a pellet in a microcentrifuge (MZ001.SP, Kisker Biotech) for 30 seconds and dispersed in 50 µl thrombin solution. This mix was pipetted into 8 mm x 8 mm sterile glass rings (Cloning-Zylinder, Hilgenberg) and mixed with 50 µl fibrin solution. After a 45-minute incubation, the glass rings were detached, and the constructs were cultivated in six-well plates under standard conditions for 0, 7, 14, 21, and 28 days (n = 8).

Death-Life Assay

Vitality in pellet cultures was evaluated weekly with a propidium iodine (PI, Sigma-Aldrich) and fluorescein diacetate (FDA, Sigma-Aldrich) stain. PI was dissolved in water at 1 mg/ml and FDA in acetone at 25 mg/ml (Pharmacy, MRI). Aliquots of 500 µl of each solution were combined and diluted with 39 ml Phosphate Buffered Saline (PBS) and pellets were incubated in this mixture for 30 seconds in the dark. Afterward, the construct was evaluated under the fluorescence microscope using UV-light. In the vital cell, FDA is incorporated, and the cells appear green. Necrotic cells stain fluorescent red with PI.

Quantification of Glycosaminoglycan Content

For the pellet culture as well as the native cartilage a 1,9-Dimethyl-Methylene Blue zinc chloride double salt (DMMB) assay was performed after papain digestion (45.2 mg/ml, Worthington) for 16 hour at 60°C. Glycosaminoglycan content (GAG) was quantified by photometric analysis after the addition of 1,9-dimethylmethylene blue solution (Pharmacy, MRI) to a standard chondroitin sulfate solution (Pharmacy, MRI) with linear regression.

DNA Quantification

DNA quantification of the pellet culture as well as the native cartilage was performed with the Hoechst 33258 Assay as previously published.[22]

Animal Model

A total of 20 male and four female New Zealand White rabbits (2500 g weight) were biopsied for auricular cartilage under narcosis with propofol and a local anesthetic gel. The biopsy region was 1 x 2 cm in size, and the cartilage was harvested after separation of the overlying skin and perichondrium, which was carefully lifted and separated from the cartilage and left in situ. This step is crucial to avoid contamination of the biopsied cartilage sample with perichondrial cells. After harvest, the defect was sutured with 5–0 ethilon, and sutures were removed during the second surgery to spare the animal any additional stress. After 14 days in monolayer culture, P1 cells were subsequently injected into the paraglottic space of one vocal fold. For the injection, as for the pellet culture, we used P1 cells at a concentration of 2.5 x 10^6 cells per 100 µl fibrin/thrombin solution and a volume of 200 µl by using a custom-designed holding device for a neonatal laryngoscope (Storz 8576 E Size 1), a 30° extra thin rigid endoscope (Ø 2.7 mm, 18 cm length, Eickenmeyer), and insulin syringes attached to an angled tonsil canule (90°, Eisenhut) (Fig. 1).

Both surgeries were performed under general anesthesia without intubation, but sufficient self-ventilation by using a combined approach involving premedication with Metamizol (Novalgin®, 175 mg/animal) orally, followed by the initiation of narcosis with Propofol (Fresenius Kabi 1%, 8–15 mg/kg, i.v.), and then an anesthetic combination of S-Ketamine (17 mg/kg, i.m.) and Medetomidine (Domitor®, 0.25 mg/kg, i.m.) injected into the quadriceps muscle. The antagonizing step with Atipamezol (Antisedan®, 1.25 mg/kg, s.c.) was performed no earlier than 30 minutes after the intramuscular injection. In addition, during the injection laryngoplasty, the local anesthetic Xylocain (Astra Zeneca) was used as a spray.

Postoperatively, all animals were checked multiple times a day and treated with Meloxicam (Metacam®, 0.1–0.2 mg/kg, p.o.) for 3 days and with Enrofloxican (Baytril®, 7.5 mg/kg, p.o.)

At the end of the experiment, at 2, 4, 12, or 24 weeks after laryngoplasty, the animals were killed under i.v. Propofol narcosis with Pentobarbital-Natrium (Narcoren®, 100 mg/kg, i.v.). Larynxes were harvested and fixed in formalin for subsequent histological evaluation.

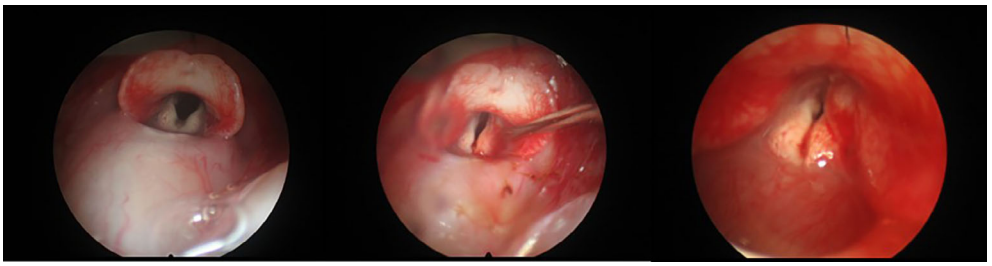

Fig. 1. View through the 30° rigid endoscope. Visualization of the Larynx, Injection of the chondrocyte-fibrin-thrombin mixture with a 90° angled cannula, and post-augmentation control.

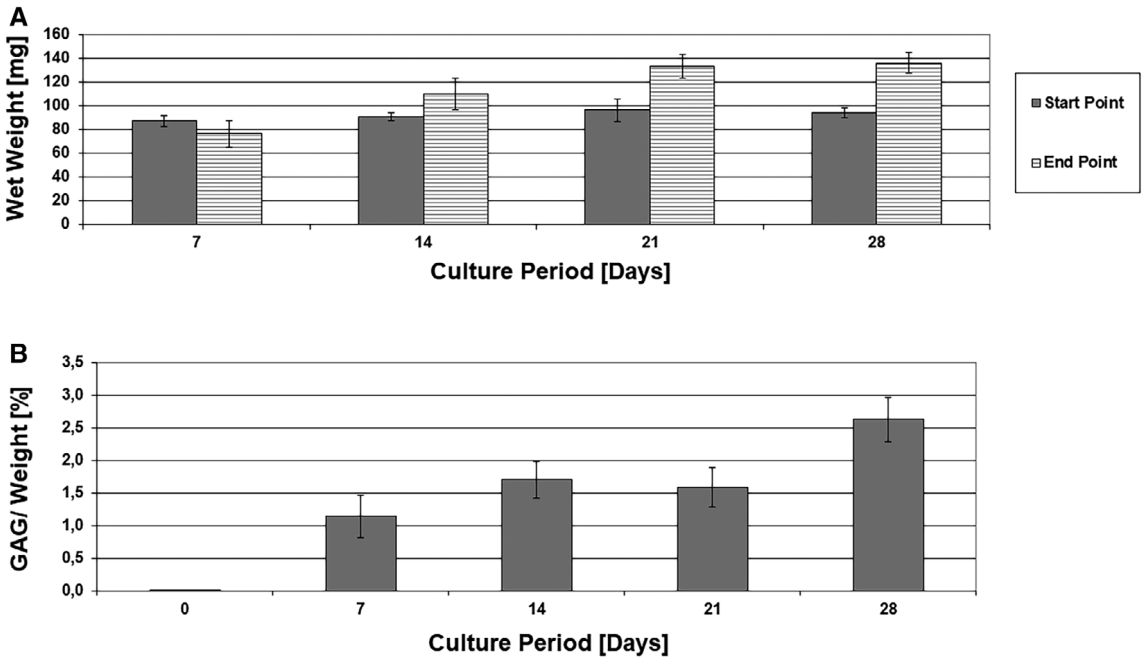

Fig. 2. A, Wet Weight for Pellet Culture with significant weight gain for distinct time points of 3-D culture. Median Weight at Starting Point: 91.85 ± 6.22 mg, Day 7: 76.25 ± 11.26, Day 14:109.87 ± 13.02 mg, Day 21: 132.98 ± 10.12 mg, Day 28: 136.02 ± 8.50 mg. Empty Control constructs showed no overall weight changes (data not shown). n = 6. B, Increase in the GAG Content of the Pellet Culture in Relation to the Weight Gain for distinct Time Points of 3-D Culture. On Day 0 the baseline signal of the fibrin-thrombin gel was evaluated (0.11 ± 0.02 μg/mg). An empty control construct without cells served to correct the baseline weight for the weekly harvest and was subtracted from the weight. Already on day 7 a GAG-content of 11.41 ± 3.27 μg/mg was found. Almost as a linear trend, day 14 showed 17.00 ± 2.84 μg/mg and day 28 peaked with 26.25 ± 3.39 μg/mg. Only on day 21, we found comparable values to day 14 with 15.84 ± 3.02 μg/mg. n = 6.

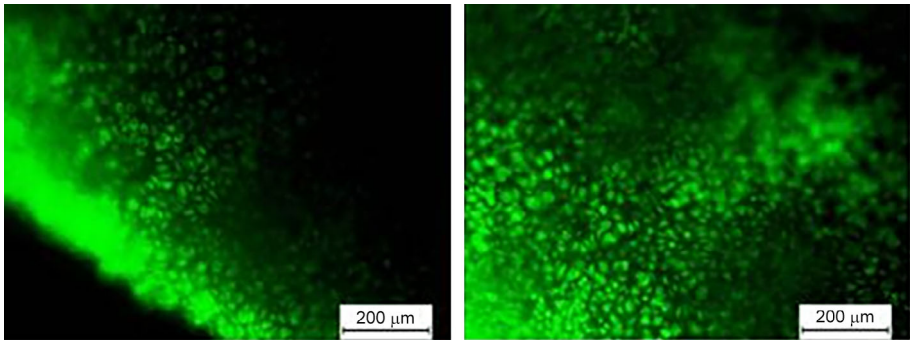

Fig. 3. Viability (PI-FDA Stain) within the 3-D Pellet Construct throughout the culture period (exemplary picture of Day 21). Life cells fluorescent in green (FDA, intracellular) and dead cells red (PI). Left picture shows the edge of the pellet, right picture the center area. Magnification 100x.

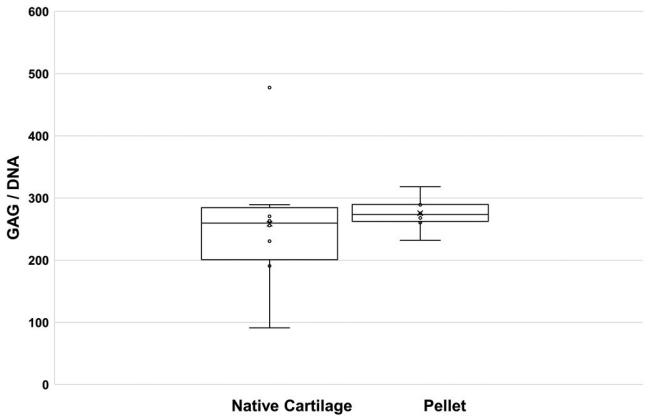

Fig. 4. GAG/DNA Content as Sign for the Quality of the Pellet on Day 28 compared to Native Cartilage Native cartilage had a median content of 263.40 GAG/DNA (91.26–477.58) while the median content was 275.00 GAG/DNA (232.06–318.17) for the pellet. n = 6.

Histology for in vitro and in vivo experiments

Pellet cultures were harvested, fixed for 24 h in 3.7% formalin (Pharmacy, MRI), and dehydrated. Native cartilage and larynxes were decalcified for 1 to 7 days with 20% ethylenediaminetetraacetic acid-solution (Pharmacy, MRI) and dehydrated. Afterward, the tissue was embedded in 60°C paraffin (100% Roti-Plast, Carl Roth) and subsequently cut at a thickness of 7 µm.

Hematoxylin–Eosin staining and cartilage specific Safranin-O and Alcian Blue staining visualizing the GAG-content were performed in a standardized manner, as previously published.[23] Elastica van Giesen staining was performed to evaluate the content of elastic fibrils, following standard protocols.[24]

RESULTS

Pellet Culture

A total of 40 pellet constructs with $2.5*10^6$ chondrocytes per 0.1 ml Fibrin-Thrombin gel were harvested at 0, 7, 14, 21, and 28 days for further analysis. Wet weight showed a significant gain over the culture period as shown in Figure 2A with a significant increase in GAG/weight content (Fig. 2B) and a stable vitality within the constructs (Fig. 3) over the time of culture with no significant changes in DNA content (data not shown). Cell number calculated based on DNA content was below the

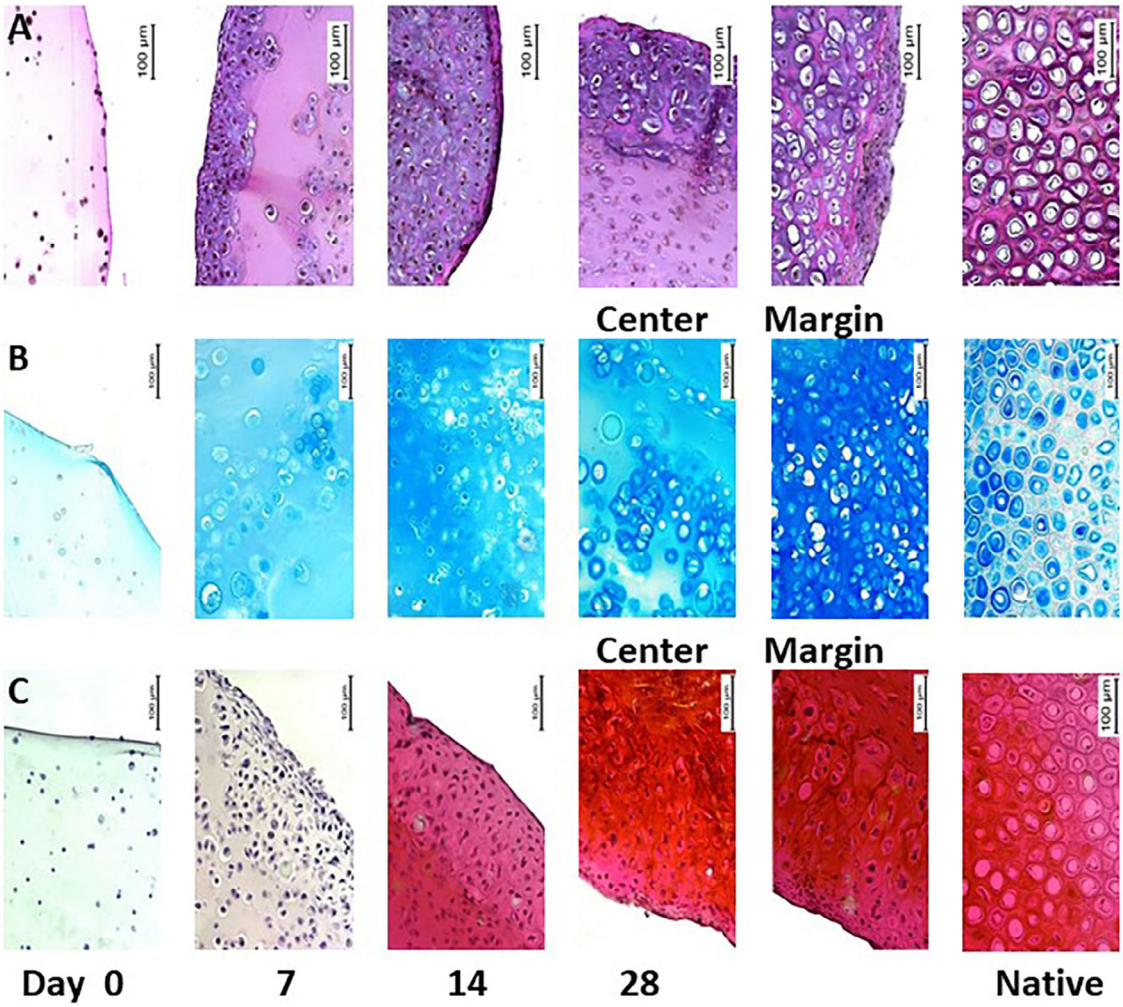

Fig. 5. Pellet Structure and extracellular Matrix in A, Elastica van Giesson B, Alcian Blue Stain C, Safranin-O stain at different time points compared to native auricular cartilage of a New Zealand White Rabbit.

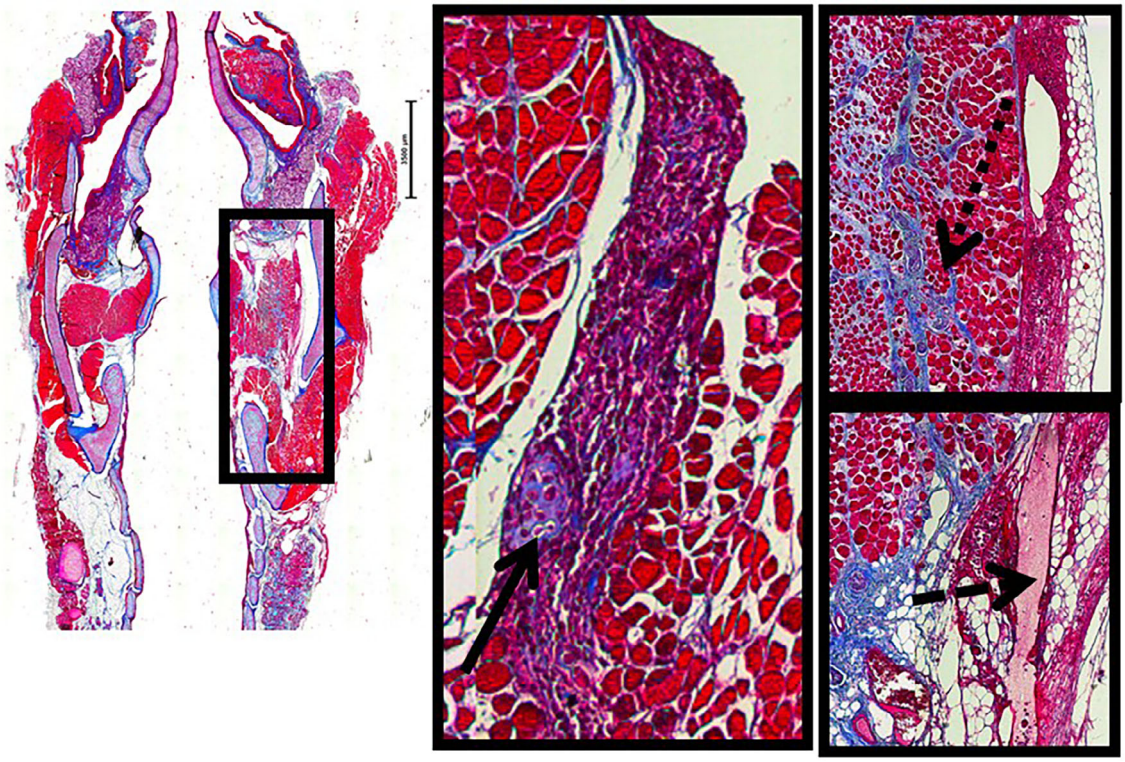

Fig. 6. Inflammatory Processes, first Signs of Cartilage Formation and Fibrotic Changes 2 weeks after injection laryngoplasty. Black box indicated the injected side; full arrow shows new formation of cartilage within fibrous changes, dotted arrow indicates fibrous changes within the vocalis muscles and lined arrow remaining fibrin-thrombin gel.

initial injected value at all time points. From the 2.5×10^6 injected cells, $1.6 \pm 0.25 \times 10^6$ were measured at time point "0," directly after pellet formation. Following an initial decrease in cell number on day 7 to $1.3 \pm 0.19 \times 10^6$, we found an increased number of $1.8 \pm 0.24 \times 10^6$ on day 14. This number stayed constant on day 21 ($1.8 \pm 0.3 \times 10^6$) and day 28 ($1.7 \pm 0.32 \times 10^6$).

After 4 weeks of pellet culture, satisfactory extracellular matrix was found, comparable with the native cartilage (Fig. 4) concerning the GAG/DNA content and as demonstrated by Alcian Blue and Safranin-O stains (Fig. 5). An increased color intensity in the Alcian Blue and Safranin-O stains correlates with the glycosaminoglycan and proteoglycan content of the constructs and demonstrates the increasing content and extracellular matrix deposition of the neo-cartilage in the pellets compared to the native cartilage biopsy, although cells tended to migrate toward the edges because of the nutrition gradient (Fig. 5). The long-term-stable fibrin-gel lasted for at least 4 weeks. Thus, the material and cell concentration showed promising results for an *in vivo* approach.

Animal Model

Four animals had to be excluded from this study because of spontaneous hip dislocation, a false pregnancy, an unclear bowel infection with bowel obstruction, and one adverse event during augmentation attributable to a vagal-triggered reflex bradycardia with asystole and unsuccessful resuscitation. In all these cases, standard veterinarian treatment was provided.

In the remaining animals, mature cartilage was found in none of three animals at 2 weeks. Only traces of new cartilage formation were discerned in this group, and mainly inflammatory processes were observed together with leucocyte infiltration and fibrous tissue changes, plus remaining traces of the fibrin-thrombin gel. Material seemed to partly dislocate outside of the paraglottic space through the cricothyroid space. The inflammatory processes partly included the vocalis muscle (Fig. 6).

In 40% (two of five) of the 4-week larynxes, 66% (four of six) of the 12-week larynges, and ~80% (five of six) of the 24-week larynxes, cartilage formation was, however, observed was mostly located in the paraglottic space, along the thyroid cartilage, and sometimes with spurs into the vocalis muscle (Fig. 7). The surrounding tissue was often still infiltrated with inflammatory cells but to a much lesser extend as in the 2-week group. Material tended to dislocate through the cricothyroid space into the extraglottic surrounding tissue, extruding via the laryngeal box (Fig. 8). Because of the irregular shapes of the newly formed cartilage and the location next to the thyroid cartilage, volume measurements were not possible, and results between the animals were not easily comparable (Fig. 7). Compared with the pellet culture,

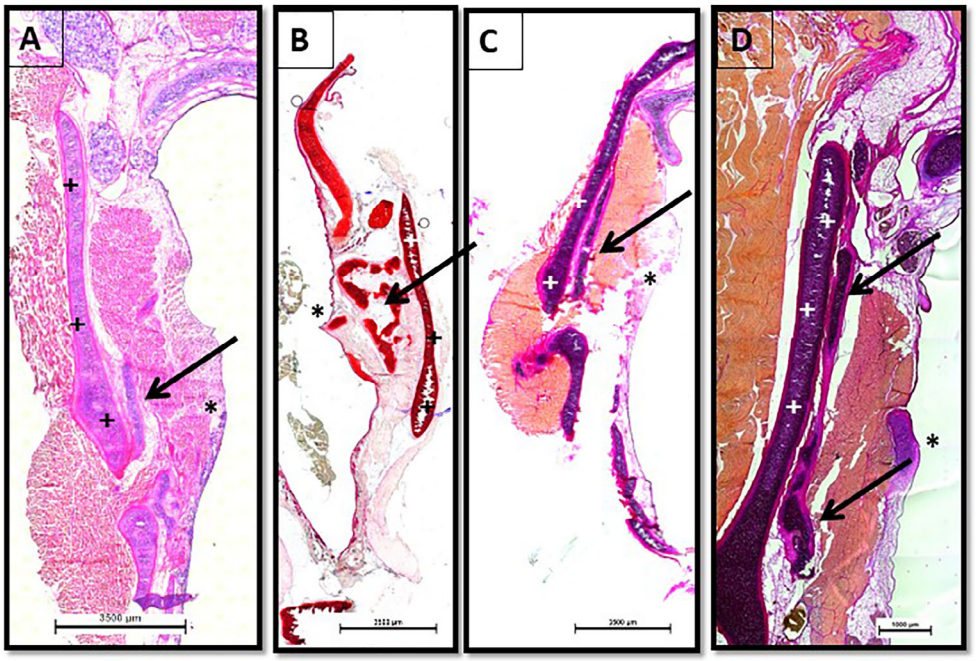

Fig. 7. Cartilage Formation after A, 4 weeks; B, 12 weeks; and C and D, 24 weeks. * = Vocal Fold; + = Thyroid Cartilage.

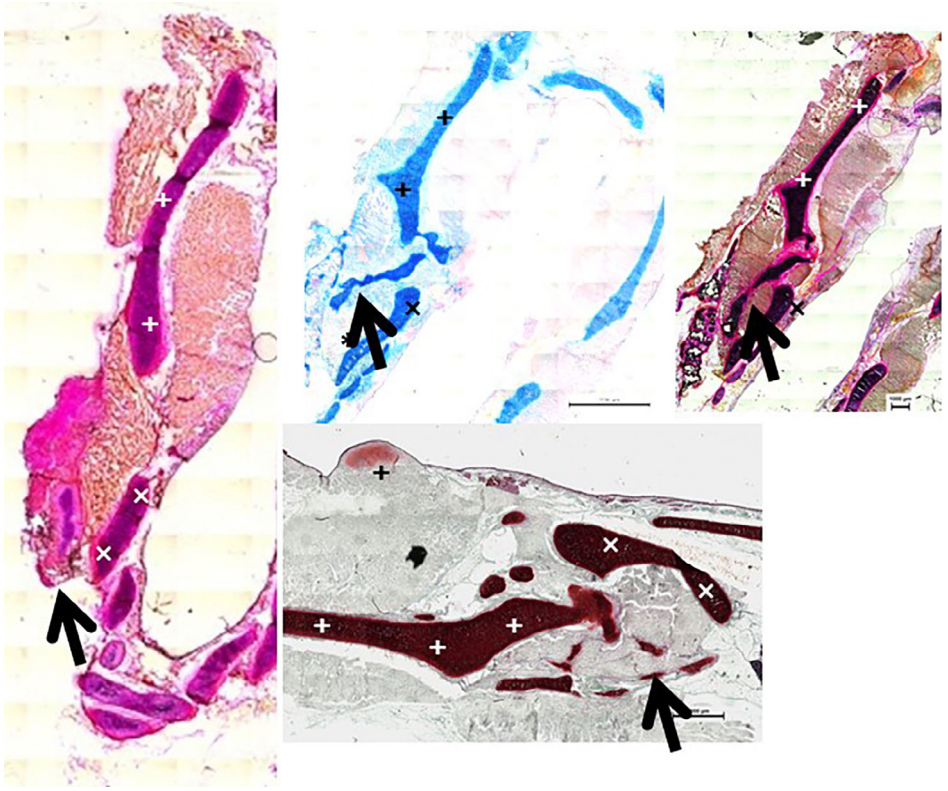

Fig. 8. Cartilage Extrusion through the Cricothyroid Space outside of the laryngeal box, surrounded by fibrous tissue. Cartilage formation also appears patchy and discontinuous (→). * = Vocal Fold; + = Thyroid Cartilage; × = Cricoid Cartilage.

however, cartilage formation appeared to emerge much later *in vivo* and to a lesser extent.

DISCUSSION

Injection laryngoplasty is one of the therapy options for acute and chronic vocal fold paresis and paralysis causing a glottal insufficiency increasingly performed in outpatient settings under local anesthesia or full narcosis.[9] Multiple injectable materials, each one having its own advantages and disadvantages, have been employed.[19] Hyaluronic acid has so far been the most frequently used material. Its easy injectability and good biocompatibility, plus its favorable biomechanical properties regarding vocal fold oscillation have often been promulgated.[20] One great disadvantage is its degradability within only a few months and thus short-term nature of its augmentation. For this reason, hydroxyapatite has been introduced for vocal cord injection and is increasingly used.[25] Although its better durability and volume stability, often for a few years, is favorable for persistent vocal fold paralysis with a glottic gap, several adverse reports of foreign body reactions and granuloma formation,[26,27] and even implant migration together with dislocation of the material[28] and an unfavorable biomechanical aspect attributable to the stiffness of the material regarding phonation[29] have appeared in the literature. One material that was thought to combine favorable biomaterial and biomechanical characteristics and endurance properties was autologous fat. However, to date, the outcome has been quite variable with respect to long-term volume stability.[18,30,31] Autologous fascia was also introduced in the 1990s but it has not become widely established despite long-term results seeming to show promise. This might be attributable to the increased effort in processing the harvested fascia or fear of donor-site morbidity.[32] A new material recently invented with the intention of use in vocal fold augmentation is a silk-protein microparticle-based filler. This novel material has provided favorable results in *in vitro* studies and *in vivo* in a canine model.[28,33] Its long-term volume and form stability, plus its biomechanical properties *in vivo*, have yet to be tested, with inflammation representing a possible problem.[28] Minced cartilage has also been introduced as an option for long-term augmentation, but the authors have also found foreign body reaction and degradability on its use.[34]

To our knowledge, an autologous chondrocyte cell-based approach to injection laryngoplasty for vocal fold augmentation *in vivo* in an animal model has not yet been published. We hypothesized that an approach to an injection-based long-term vocal fold medialization approach using chondrocytes with their capability to form a stable extracellular matrix might become an alternative to the laryngeal framework technique.

The elastic ear cartilage is a suitable donor site for chondrocytes because of its large availability and its ease of harvest under local anesthesia.[35] Furthermore, the use of autologous cells should prevent foreign body reaction and possible inflammation. There is a vast amount of literature about autologous chondrocyte transplantation in the field of orthopedic surgery and joint repair,[36–38] which is already in clinical use and additionally supports this assumption. For the autologous chondrocyte transplantation usually, primary or passaged chondrocytes are injected into a cartilage defect using a carrier gel.[36–38] Since dedifferentiation of chondrocytes during monolayer passage occurs early in culture and redifferentiation to the status of the primary cell is still often not achieved, passage number should be kept to minimum.[39] Thus, a balance between passage number to gain a certain cell number needed for the injection and the defect size of the harvest site has to be established. In addition, since chondrocytes are a tissue of little cell–cell contact and great amounts of extracellular matrix, usually a carrier gel is used for redifferentiation purposes serving as 3-D matrices[39] or for an injection. As for the carrier gel in this experimental set-up the choice of a fibrin-thrombin mixture was for two reasons. For one, this material could in the future be gained autologous from the patient when the cartilage biopsy is taken, thus providing a purely autologous approach. This would reduce the risk of allergic reactions f.e. to collagen or hyaluronic acid, which have been reported before[40–42] or prevent foreign body reactions as reported about hydroxyapatit.[43,44] Second, the material had proven to provide the chondrocytes a favorable, mid-term stable matrix with a balanced absorption/ degradation versus replacement with deposition of extracellular matrix.[45,46] Plus, it is injectable for a short period of time when freshly mixed together.

Our *ex vivo* experiments using chondrocytes after one passage and a fibrin-thrombin mix as 3-D matrix produced satisfying results regarding cell number, cartilage formation, and volume stability over 4 weeks. Using P1 cells we kept dedifferentiation during monolayer expansion to a minimum while also considering the defect size of the harvest site with pellet culture. Even though the cell and matrix distribution tended to display a nutrient gradient, as has previously been described by other authors[45,47,48] the histologic analysis showed mature cartilage formation without any signs of cell contamination by other cell types (i.e., cells of the perichondrium, which had been surgically removed prior to processing the cartilage biopsy). Based on these encouraging *ex vivo* findings, we transferred the experiment to an *in vivo* setting.

In concerns to its viscoelasticity autologous chondrocytes had previously been shown to be a promising material by one of the authors in a porcine cadaver study.[48] Although we did find neo-cartilage formation of mature cartilage *in vivo* after 4, 12, and 24 weeks we can neither make a statement regarding the volume stability nor predict the form, volume, and localization of any neo-cartilage formed.

With regard to their biomechanical properties, chondrocytes within a fibrin-thrombin gel have been favorably evaluated in a porcine cadaver larynx model[48] studied by using a plate rheometer. Plate rheometers have commonly been used to evaluate injectable material with regard to the biomechanical property of viscoelasticity.[48–51] The results obtained are, however, controversial from an engineering perspective, since the plate rheometer was employed in determinations made on mixed tissues, whereas it was designed for pure materials, and thus, a linear skin rheometer would be the

more favorable method of analysis. We need to evaluate whether these properties persist after cartilage formation and the deposition of extracellular matrix by the chondrocytes. Minced cartilage has nevertheless been positively tested previously on patients[35] but showed resorptive processes and degradation of the injected minced cartilage pieces,[52,53] which makes *in vivo de-novo* cartilage formation an interesting option for long-term-stable augmentation.

One surprising histological finding was the accompanying inflammation process that we found. This was comparable to that observed by Cakmak et al., who reported accompanying foreign-body and inflammation processes in the neocartilage formed by chondrocytes of various origins and fibrin glue of various concentrations injected into various locations in a model based on New Zealand White rabbits.[54] Moon et al. used polyacrylamide hydrogel, calcium hydroxyapatite, or a hyaluronic acid derivate for vocal cord augmentation in New Zealand White rabbits, and also experienced inflammatory processes for all materials tested.[55] Lee et al. reported empty lacuna and inflammation processes in a canine model using minced autologous cartilage for vocal fold augmentation.[34,52,53] Through these inflammatory processes leading to increased resorption, especially in the rabbit model, the problem of volume stability seems to be unsolved for most injected materials.[56–58] In our experiment, inflammation might have been triggered by the non-autologous fibrin-thrombin gel, but it could also have been the injection into the sensitive vocal fold system itself, as inflammation has been experienced by other authors with diverse materials.[53,56–58] These inflammatory reactions indicate that new and different injectables, maybe containing locally active anti-inflammatory agents need to be sought as a substrate for the chondrocytes. Since the inflammatory processes observed decreased between week two and four substantially, we concluded that the lack of remaining fibrin-thrombin mix in the 4 weeks larynxes (while the pellet culture was volume stable for 4 weeks) might be owed to the inflammation caused either by the carrier or the injection itself.

One more unfavorable finding was the diversity of shapes of the neo-cartilage formation we found. In many cases, the neo-cartilage almost mimicked the thyroid cartilage, nearly forming a second layer to it. Since this has been observed by other authors too, working with diverse injectables with this animal model,[58,59] regardless of using a vocal fold palsy-model or not, this might be owed to the injection site (lateral vs. medial of the vocalis muscle) or the (lack of) rigidity of the injected material as well as the volume we injected. We chose a 200 µl injection volume since we had successfully previously evaluated it on cadaver larynxes and it was set to allow us to visualize a discrete medialization of the injected vocal fold as an immediate visible control, at the same time minimize swelling or reduction of the glottis gap diameter, which might cause respiratory difficulties and still provide a sufficient enough volume for histologic evaluation. Besides unfavorable slim shapes of neo-cartilage we also experienced material extrusion through the cricothyroid space. Both findings might be owed to the lateral injection site but also to excess volume or excess pressure applied during the injection. Mau et al. reported that particularly laterally placed boluses extruded through the cricothyroid space in their cadaver studies on human larynxes.[60,61] One other explanation might be of a technical nature: because of the minute dimensions of the rabbit larynx (7 mm diameter), the tolerance for the correct positioning of the needle was very small. In addition, excess pressure might have had to be applied in order to inject the material,[62] thus leading to the above shown extrusion material and neo-cartilage formation outside the laryngeal box.

In the future, we consider working with a larger animal model to reduce technical difficulties such as extrusion of the injected material due to excess pressure or spacious limitations. We plan on using an autologous fibrin-thrombin gel for the autologous chondrocytes as opposed to the non-autologous one used in this work, possibly adding additional anti-inflammatory agents to minimize inflammatory processes. Localization of the injected material and its volume stability could then be re-evaluated, possibly using micro-CT imaging at various time points. Viscoelasticity can, as far as we know, only be evaluated *ex vivo* by using a high-speed camera and a complex *ex vivo* set up with air flow through the larynx. An *in vivo* experimental set-up to assess the viscoelasticity at several time points in the same animal during the formation of cartilage would be a great innovation to address these issues.

CONCLUSION

We have shown that autologous elastic chondrocytes represent an interesting cell-based approach for mid- or long-term injection laryngoplasty in the future. We have further shown mature cartilage formation *in vivo* in the larynx of New Zealand White rabbits but have faced some difficulties in regard to the shape and formation of the neo-cartilage and the extrusion of neo-cartilage outside the laryngeal box. Nevertheless, an autologous approach to minimize foreign body and inflammatory reactions with a cell source that is easy to reach and process, such as auricular cartilage, is worth considering; further research is, therefore, needed.

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