



# Laser surface modification of decellularized extracellular cartilage matrix for cartilage tissue engineering

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## Abstract

The implantation of autologous cartilage as the gold standard operative procedure for the reconstruction of cartilage defects in the head and neck region unfortunately implicates a variety of negative effects at the donor site. Tissue-engineered cartilage appears to be a promising alternative. However, due to the complex requirements, the optimal material is yet to be determined. As demonstrated previously, decellularized porcine cartilage (DECM) might be a good option to engineer vital cartilage. As the dense structure of DECM limits cellular infiltration, we investigated surface modifications of the scaffolds by carbon dioxide (CO<sub>2</sub>) and Er:YAG laser application to facilitate the migration of chondrocytes inside the scaffold. After laser treatment, the scaffolds were seeded with human nasal septal chondrocytes and analyzed with respect to cell migration and formation of new extracellular matrix proteins. Histology, immunohistochemistry, SEM, and TEM examination revealed an increase of the scaffolds' surface area with proliferation of cell numbers on the scaffolds for both laser types. The lack of cytotoxic effects was demonstrated by standard cytotoxicity testing. However, a thermal denaturation area seemed to hinder the migration of the chondrocytes inside the scaffolds, even more so after CO<sub>2</sub> laser treatment. Therefore, the Er:YAG laser seemed to be better suitable. Further modifications of the laser adjustments or the use of alternative laser systems might be advantageous for surface enlargement and to facilitate migration of chondrocytes into the scaffold in one step.

**Keywords** Cartilage · Chondrocytes · Surface modification · Tissue engineering · Er:YAG laser · Carbon dioxide laser

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## Introduction

Functional defects and deformations of nasal structures as a result of trauma, tumor, or congenital lesions mostly demand complex reconstruction surgeries. Due to the fact that cartilage lacks an intrinsic regeneration capacity because of its high differentiation level and the slow metabolism of the tissue, as well as for the lack of a distinct vascularization [1], the reconstruction of such defects is of outstanding concern in otorhinolaryngology. Standard multistage surgical procedures include the harvesting of septal, auricular, or rib cartilage [2–4] which might cause additional donor-site morbidity, transplant degeneration, as well as unsatisfying cosmetic results [5, 6]. Sometimes sufficient tissue for harvesting might not be available due to prior trauma or surgery.

Tissue engineering provides a chance to solve these problems. Multiple materials and techniques have been developed [7–12], but until today none has been convincing due to the complex requirements demanding shape

stability, biocompatibility, and appropriate mechanical properties of the scaffold material. Scaffolds of decellularized extracellular matrix (ECM) seem to provide optimal conditions for tissue regeneration due to its three-dimensional framework which closely resembles the original native cartilage including substructural properties and the presence of bioactive molecules which can induce e.g., cell migration and differentiation. Furthermore, an appropriately prepared ECM scaffold is biodegradable and does not provoke a significant adverse response of the immune system [11, 12]. Therefore, decellularized porcine cartilage (DECM) is a promising material for nasal defect reconstruction. Above all, it has further advantages, as it is infinitely available, individually shapeable, and allows even allogenic or xenogenic chondrocytes to migrate and form native, stable cartilage [13, 14]. Laser application is a standard technique in numerous medical fields and is also commonly used in head and neck surgery. In addition to cutting techniques in tumor surgery, lasers are also used for cartilage reshaping and surface modification of bioimplants [1, 15–17]. The latter increases the biocompatibility and osseointegration of several medical implants [15]. Previous research demonstrated migration of chondrocytes into DECM scaffolds and their redifferentiation, which is accompanied by production of novel extracellular matrix proteins [13, 14]. However, it takes several weeks until the scaffold is completely filled with chondrocytes. Consequently, we were interested in speeding up this process. Therefore, the aim of this study was to investigate whether laser treatment would enlarge the surface of DECM scaffolds and enhance migration of chondrocytes inside the scaffolds. We compared the effect of a carbon dioxide (CO<sub>2</sub>) laser and an Er:YAG laser, as they are standard instruments in surgical treatment, easily operated and can be used at low cost. Both ablative lasers are preferred for most indications in the head and neck region and have been previously investigated for their positive ablation effects on cartilage tissue [18, 19].

## Methods

### DECM scaffolds

Fresh porcine nasal septal cartilage (pNSC) from adult 7- to 8-month-old animals was obtained from a local abattoir. After the removal of the perichondrium cylindrical biopsies of 5 mm in diameter and 1 mm thickness were punched out and prepared for further treatment. The samples underwent a wet chemical process [13, 14] in order to remove cells and glycosaminoglycans (GAGs) and to obtain a pure collagen matrix

without immunogenic proteins. The scaffolds were stored in a sterile NaCl solution (0.9%) at  $-24\text{ }^{\circ}\text{C}$  until further use.

### Chondrocytes from human nasal cartilage

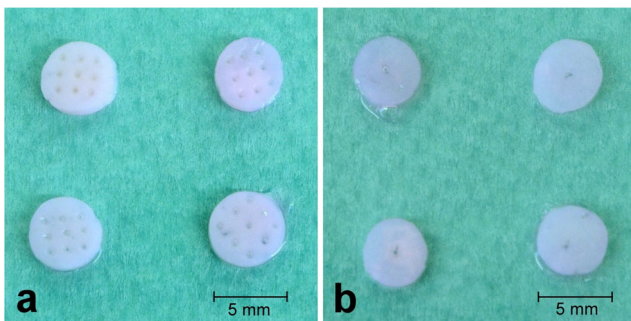
Human nasal septal cartilage was obtained during routine nasal surgery (septumplasty or septorhinoplasty) in the Department of Otorhinolaryngology, Head and Neck Surgery, Ulm University Medical Center. The donor age ranged from 19 to 39 years, with an average of  $26 \pm 11.27$  ( $n = 3$ ). All donors involved in this project signed an Informed Consent, approved by the University of Ulm Ethical Committee (Ethical application number 152/08). The cartilage was used for harvesting human primary nasal chondrocytes (hpch). The isolation of the chondrocytes was performed as formerly described [13, 14]. In short, cartilage was placed in digestion medium (DMEM/HAMsF12 + 0.5% Gentamicin) containing collagenase type II (0.3%; Worthington) for 16 h at  $37\text{ }^{\circ}\text{C}$  in a shaking water bath. The cell suspension was centrifuged, and the total cell number, as well as vitality, was determined by trypan exclusion. Hpch were seeded with an initial density of  $0.5 \times 10^4$  cells  $\text{cm}^{-2}$  and cultured in standard medium (DMEM/HAMsF12 + 10% FBS + 0.5% Gentamicin) until a confluence of 80 to 90% was reached. Cells were amplified over one passage. The cells were subsequently cryopreserved before further use for seeding experiments on scaffolds in order to develop a clinically applicable protocol. Only cells in passage 2 were used in this study.

### Laser surface modification

Surface modification of the decellularized porcine scaffolds was performed using a commercially available CO<sub>2</sub> laser (AcuPulse, Lumenis GmbH, Germany) with a wavelength of 10.600 nm and an Er:YAG laser (SupErb XL, Baasel, Germany) with a wavelength of 2.940 nm. The CO<sub>2</sub> laser was used in single pulse mode (0.3 mm focus diameter, 8 W) with a pulse duration of 0.03–0.06 s. Nine cavities per scaffold were generated by a sequence of 5–10 short pulses (Fig. 1). The absorbed dose per cavity was 1.2 to 4.8 J. The Er:YAG laser was used in single pulse mode (1 mm focus diameter, total energy 0.5 J) with a pulse duration of 0.1–1.0 ms. One cavity was created per sample by a sequence of 10 shots (Fig. 1). Twenty-eight modified scaffolds were produced with each laser and cryopreserved in sterile NaCl solution (0.9%) until further use.

### Seeding and 3D culture on collagenous biomatrix

Hpch were thawed and amplified in monolayer culture for 4 days until 80–90% confluence was reached. The laser-modified scaffolds were thawed and sterilized in 80% ethanol for 1.5 h and air-dried under sterile conditions at room



**Fig. 1** Macroscopic aspects of laser-modified DECM scaffolds. DECM scaffolds from decellularized porcine cartilage treated by **a** CO<sub>2</sub> laser and **b** Er:YAG laser. Due to the different focus diameters of the laser beam scaffolds were treated nine times by the CO<sub>2</sub> laser and once by the Er:YAG laser

temperature. Subsequently, the sterile scaffolds were incubated in standard medium for 24 h at 37 °C and 5% CO<sub>2</sub> to ensure rehydration and equilibration. Scaffolds were seeded with hpch ( $1 \times 10^6$  cells/scaffold) afterwards as published previously [13, 14]. 3D-culture was performed in a chondrocyte differentiation medium (NH Chondro Diff medium; Miltenyi) supplemented with 0.5% gentamicin. The scaffolds were analyzed on days 14, 28, and 42 after seeding.

### Histological and immunohistochemical analyses

For histological and immunohistochemical evaluation, the seeded scaffolds, either laser treated or not, were fixed in 3.5–3.7% neutral buffered formalin solution (Fischar) and embedded in paraffin. Paraffin sections (4 μm) of tissue samples were generated and incubated at 56 °C over night. Subsequently, sections were rehydrated and stained with haematoxylin and eosin for visualization of cell distribution and morphology, as well as with Alcian blue (AB) to detect the presence of acidic sulfated GAG.

For immunohistochemical detection of collagen type II sections were deparaffinized and rehydrated. As pretreatment and antigen retrieval, two digestion steps, each 15 min at 37 °C, were performed: first 1% hyaluronidase (Sigma-Aldrich, St. Louis, MO, USA) in phosphate-buffered saline (PBS) and subsequently 0.2% pronase (Merck, Darmstadt, Germany). For visualization of newly synthesized aggrecan, slides were incubated in  $0.5 \text{ U ml}^{-1}$  chondroitinase ABC (Sigma-Aldrich) in PBS for 30 min at room temperature. Aggrecan as well as collagen type II were detected by using the LSAB+ System-HRP (DAKO) according to manufacturer's instructions.

The primary antibody against aggrecan (Serotec, Clone 7D4, Germany) was diluted 1:100, and collagen type II antibody (II-II6B3, Developmental Studies Hybridoma Bank, USA) was diluted 1:400. Subsequently, the primary antibody solutions were added to the sections and incubated for 2 h at RT (room temperature) in a humidified box.

### Cytotoxicity testing using hpch

To determine possible cytotoxic effects due to laser application on vital cells seeded on the decellularized cartilage a standardized test method for biological evaluation of medical devices (ISO 10993-5:2009 (E)) was used.

Hpch served as test cells. Cryopreserved hpch were thawed and seeded with a density of  $0.5 \times 10^4$  cells  $\text{cm}^{-2}$  in culture flasks with standard medium (DMEM/HAMsF12 + 10% FBS + 0.5% Gentamicin). The culture was kept at 37 °C and 5% CO<sub>2</sub> until 80–90% confluence was reached. The medium was changed three times a week.

Liquid extracts of laser-processed DECM scaffolds (either treated with CO<sub>2</sub> or Er:YAG laser) were produced by incubation of laser-treated DECM scaffolds in standard culture medium for 24 h at 37 °C and 5% CO<sub>2</sub> under aseptic conditions. The culture medium served as an extraction vehicle due to its potential to extract polar and nonpolar substances. Cultivation medium with DMSO (10%) served as positive control, while cell culture tested and inert ThinCert PET membranes (3 mm; Greiner Bio-one) served as negative control.

Human primary chondrocytes were detached, seeded in 96-well cell culture plates with a density of  $1 \times 10^4$  cells per well, and incubated for 24 h at 37 °C and 5% CO<sub>2</sub> to allow cell adherence. The medium was removed, and the cells were incubated in the DECM extracts with 1 or 10% FBS or in the control solutions (100 μl) for 24 h. Extracts were removed afterwards, and the MTS solution (20 μl; Promega G3581) mixed with medium was added to each well. The cells were cultured for 2 h under standard culture conditions. The absorbance was measured photometrically at a wavelength of 490 nm. Absorbance lower than 40% was classified as cytotoxic, absorbance between 40 and 70% as slightly cytotoxic, and absorbance higher than 70% was graded as not cytotoxic.

Statistical analysis for determination of cytotoxic effects after laser treatment was conducted using SigmaPlot® 11.2 software (Systat Software GmbH, Germany). The Kruskal-Wallis one-way analysis of variance on ranks was applied to evaluate the significance (level of significance  $\alpha = 0.05$ ) of in vitro cytotoxicity tests. For pairwise multiple comparison procedures, the Tukey Test was used.

### Scanning electron microscopy and transmission electron microscopy

To visualize the scaffolds' morphology and the collagen microstructure after CO<sub>2</sub> and Er:YAG laser application scanning electron microscopy (SEM) and transmission electron microscopy (TEM) of DECM treated with CO<sub>2</sub> and Er:YAG laser were performed. The scaffolds were fixed with 0.1 M phosphate buffer (pH 7.3) containing 2.5% glutaraldehyde and 1% saccharose for 2 h at RT, rinsed in PBS and postfixed in 2% osmium tetroxide for 2 h at RT. Samples were subsequently

washed in PBS and dehydrated in an increasing alcohol series. Scaffolds (each  $n = 2$ ) were critically point-dried for SEM examination, sputtered with gold-palladium (Au-Pd, 20 nm), and studied using a Zeiss DSM 962 SEM.

For TEM analysis, the samples (each  $n = 2$ ) were embedded in EPON, and ultrathin sections of 70 nm were made. Sections were stained with lead citrate and examined in a Zeiss EM 10 (Zeiss) at 80 kV. Image acquisition and processing were conducted with EM-Menu 4 software (TVIPS).

## Results

### Laser treatment efficiently increases the surface area of the scaffolds

After laser application, deep stable channels were macroscopically visible in the DECM scaffolds (Fig. 1). The laser treatment resulted in channels with varying diameters due to the different focus diameters of the lasers. The CO<sub>2</sub> laser created much smaller channels than the Er:YAG laser. The shape of the channels was inverse pyramidal for both the CO<sub>2</sub> and Er:YAG laser. The channels widely remained in shape when kept in NaCl. Therefore, the surface area of all scaffolds increased significantly. In comparison to the untreated scaffolds stability and handling of the scaffolds appeared not relevantly altered after laser treatment, no matter whether the CO<sub>2</sub> or the Er:YAG laser had been used.

### Cellular migration is reduced after laser treatment

As demonstrated by Alcian blue staining (Fig. 2) and by REM (Fig. 5), the CO<sub>2</sub> laser introduced v-shaped channels in the DECM, whereas the channels after Er:YAG laser treatment were broader and bowl-shaped. Only in one of the Er:YAG treated scaffolds the channel passed through the whole scaffold. Furthermore, histological staining demonstrated vital chondrocytes on the scaffolds surface after 14, 28, and 42 days in 3D culture, no matter whether treated with the CO<sub>2</sub> or Er:YAG laser or unmodified. On all three DECM scaffolds cells were able to produce extracellular matrix (Fig. 2). Significantly more cells were observed inside the cavities made by the lasers than on the untreated surfaces. Cell numbers obviously increased over time. Chondrocytes were able to migrate into the inner parts of the matrices in untreated areas or scaffolds. In laser created channels, only single cells were able to migrate through the laser formed surface with progressing culture time (Fig. 2f, i).

Aggrecan staining most clearly revealed areas of compacted matrix adjacent to the borders of the channels which were created by the different lasers, most pronounced after treatment with the CO<sub>2</sub> laser (Fig. 3). Consequently, chondrocytes migrated much faster into the untreated scaffold

surface areas than in the laser treated zones. The compacted rim of the CO<sub>2</sub> laser-treated channels did not show any sign of migration of cells or matrix protein into that area. On average, the compacted regions were about 80 to 100 μm thick. In contrast, compacted zones in the Er:YAG treated scaffolds were not clearly visible. Generally, Alcian blue as well as the immunohistochemical aggrecan staining revealed that migration of the chondrocytes into the laser treated areas was quite a rare event.

Immunohistochemical stainings indicated the production of collagen type II and aggrecan by the chondrocytes (Fig. 3). They demonstrated that the chondrocytes which dedifferentiated during monolayer expansion were able to redifferentiate and produce cartilage specific ECM.

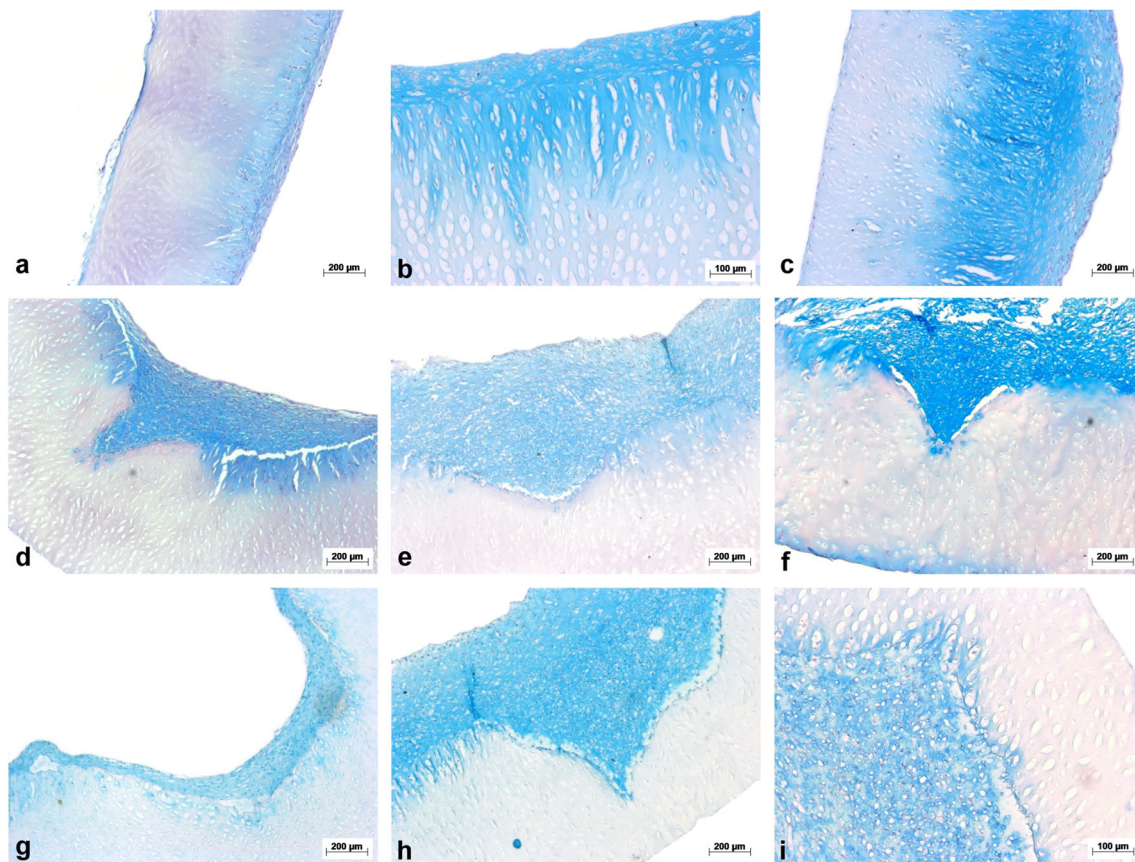
### Laser treatment does not induce cytotoxic effects

Since the laser treatment of the decellularized cartilage might potentially lead to the formation of cytotoxic factors, which could affect the metabolism and growth of chondrocytes, cytotoxicity testing was conducted. The absence of cytotoxic effects of the DECM scaffolds on cellular growth has been demonstrated in previous studies [14].

Overall, our results demonstrated no cytotoxic effect of the DECM scaffolds treated with either CO<sub>2</sub> or Er:YAG laser (Fig. 4). The positive control clearly confirmed the cytotoxic effect of 10% DMSO, with cell viability ranging between  $17.6 \pm 3.04$  to  $25.89 \pm 1.10\%$ , whereas the negative control using ThinCert PET membranes revealed regular cell growth. There was, however, a significant difference in viability observed between the extracts from CO<sub>2</sub> laser and Er:YAG laser-treated scaffolds when using only 1% FBS, with a significantly lower viability of Er:YAG laser-treated scaffolds. The addition of only 1% FBS makes cells more susceptible to potentially harmful substances. Still, both vitalities are clearly beyond the threshold for slightly cytotoxic effects in the range of non-cytotoxic effects (Fig. 4).

### The surface of DECM is compacted after laser treatment

The examination of the DECM scaffolds treated with CO<sub>2</sub> and Er:YAG laser by transmission electron microscopy clearly demonstrated the change of the microstructure due to the laser application. Figure 5b and e visualize the aspect of the untreated DECM scaffolds. Predominantly fibrils of collagen type II and some single fibrils of collagen type I are arranged in different directions. The fibrils show the typical crossbanding. In contrast, the collagen fibrils in the regions which were treated by laser application were coarse. The microstructure of the ECM was abolished, and ECM seemed to be compacted. The loss of the crossbanding was obvious. These alterations are demonstrated in both DECM scaffolds



**Fig. 2** Alcian blue staining (AB) of DECM scaffolds treated with CO<sub>2</sub> or Er:YAG laser. AB staining of control scaffolds (a–c) without laser modification on days 14, 28, and 42 exhibit a distinct migration of chondrocytes into the central parts of the scaffolds by visualizing new synthesized GAGs. DECM scaffolds treated with CO<sub>2</sub> laser (d–f) reveal a

decreased chondrocyte migration to the inner areas of the scaffolds matrix. Sections g–i show the Er:YAG-modified scaffolds on days 14 to 42. Findings are similar to those of CO<sub>2</sub> laser-treated scaffolds. Cells are not able to migrate into the scaffolds matrix. Only after 42 days, a few migrating cells were detected (i)

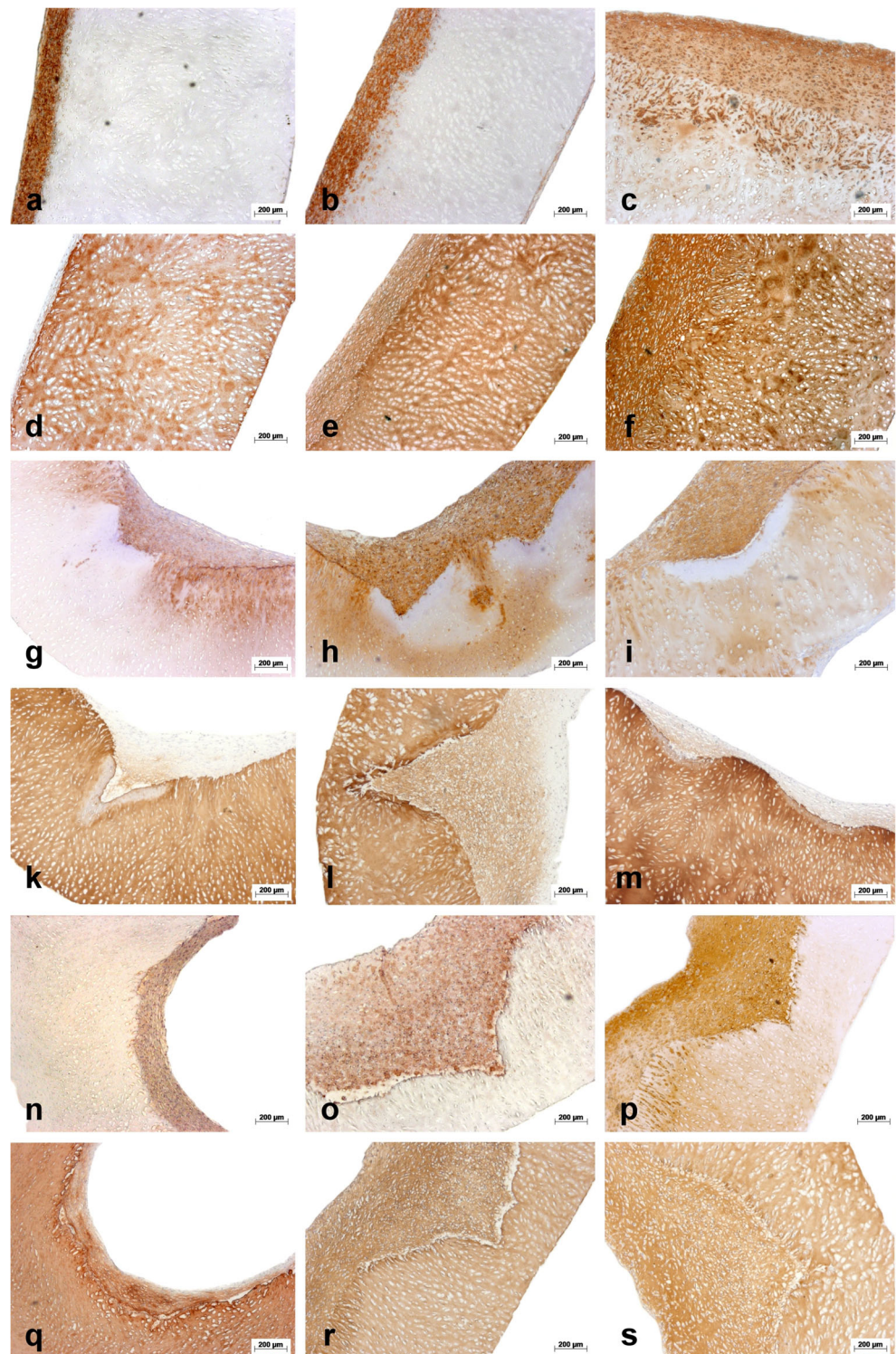
treated with CO<sub>2</sub> or Er:YAG laser in a comparable manner (Fig. 5c, f).

## Discussion

Tissue engineering is a promising technology for the reconstruction of multiple tissue defects. Three-dimensional scaffolds are the base for the construction of new vital tissue. The mechanical properties and the surface configuration of the applied biomaterials predominantly determine the quality of the engineered tissue. Various commercially available human and animal originated ECM scaffolds for the reconstruction of different tissue defects, such as dermal, bony or cardiac defects, are known [20]. For the reconstruction of articular cartilage defects, various tissue engineering approaches have already been developed, but although promising they have not been established as the gold standard therapy yet [21]. To reconstruct the cartilaginous nasal skeleton, the used material has to fulfill several requirements, such as a sufficient mechanical stability and

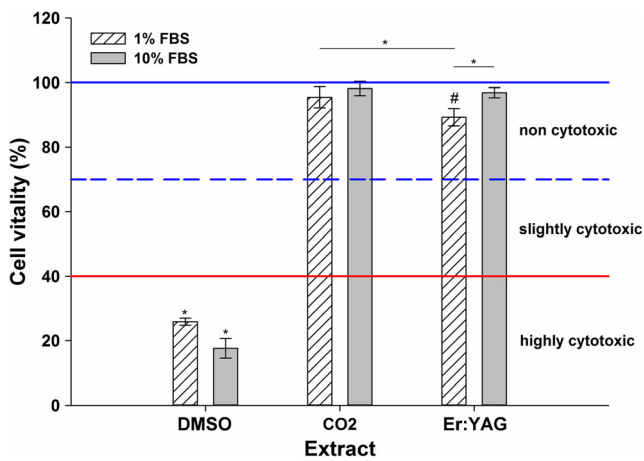
adequate porosity at the same time, a certain degree of flexibility, biocompatibility, and easy malleability. The reconstruction of cartilaginous defects in the head and neck region with autologous cartilage from the ear, nose, or rib is currently considered the clinical gold standard [2, 3, 22]. Possible side effects of the harvesting procedure include scars, pain, and wound infections at the donor site [5, 6, 23]. Allografts, homografts, and alloplastic materials which are alternatively used cause different problems, such as displacement, infection, and extrusion [24, 25]. The use of tissue-engineered cartilage could prevent these problems. The characteristics of the scaffold used play an important role in the engineering of functional cartilage. Recently, DECM has been identified as a potential material for reconstruction of cartilage defects in the head and neck area. The mechanical stability of the material along with its properties to enhance chondrocytic function and support formation of new ECM in vitro and in vivo experiments [13, 14, 26, 27] are essential characteristics for its potential use in the head and neck area. The major drawback of the material, however, seems to be the relatively dense extracellular matrix

**Fig. 3** Aggrecan and collagen type II immunohistochemical staining of CO<sub>2</sub> and Er:YAG treated DECM scaffolds. All scaffolds were examined on day 14 (left column), 28 (middle column), and 42 (right column). The brown coloring of the cell layer on the unlasered DECM (a–c) demonstrates aggrecan production in contrast to the uncolored matrix which lacks aggrecan due to the decellularization process. Likewise chondrocytes produced new collagen type II (d–f). The intensity of the staining, cell number and migration into the scaffold increased with time. Aggrecan production is detectable in CO<sub>2</sub> laser treated scaffolds (g–i) and Er:YAG laser treated scaffolds (n–p) in comparable amounts. Especially CO<sub>2</sub> laser irradiated scaffolds (g–m) revealed a compact zone which impeded chondrocytes from migrating into the matrix. Collagen type II production of the seeded chondrocytes is detected in sections k–m for the CO<sub>2</sub> laser treated scaffolds and in sections q–s for the Er:YAG treated ones



structure which inhibits a fast migration of chondrocytes into the scaffolds. In fact, the migration of the cells into the collagen matrix needs several weeks *in vitro*. With regard to resorption, degradation, and remodeling processes *in vivo*, the migration of the chondrocytes should be accelerated to prevent a loss of mechanical strength.

In this study, we decided to modify the scaffolds' surface by laser application to enlarge the surface of the DECM scaffolds with the intention to speed up the migration of the seeded chondrocytes into the matrix. Own preliminary studies of pricking or cutting the DECM scaffolds to facilitate migration of cells and loosen up the relatively dense matrix structure

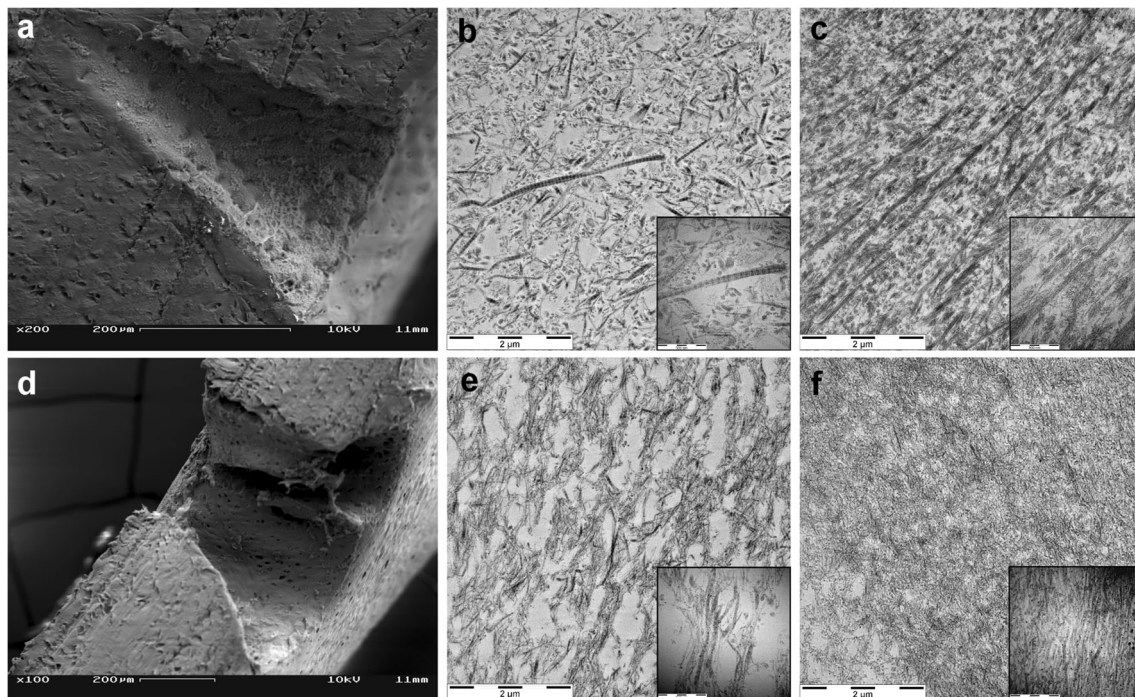


**Fig. 4** Cytotoxicity testing. Results of the positive control (DMSO), CO<sub>2</sub> laser and Er:YAG laser treated scaffolds are set in relation to the negative control corresponding 100% cell vitality. Cytotoxicity testing demonstrated that the laser modification of the scaffolds did not relevantly decrease cell viability in both laser types. However, sensitized cells after serum-starving in 1% FBS had a decreased viability after Er:YAG laser treatment. Still, cell vitality was in the “non-cytotoxic” range in both conditions

showed no positive effect (data not published). The puncture canals or cuts immediately vanished after transferring the scaffolds to the culture medium due to steeping of the collagen matrix. Likewise, variations of the decellularization process to enhance the porosity of the collagen matrix led to a decrease

of the scaffold’s stability and were, with regard to a future application in vivo, no option to accelerate the migration of the chondrocytes into the scaffold (unpublished data).

It is well known that laser surface modification of bioimplants can improve cell adhesion and integration into the surrounding tissue [15, 19]. In the head and neck region, laser application is a common technique especially for transoral tumor surgery of the pharynx and larynx [28], for stapes surgery [29] and some other indications such as the hypertrophy of the inferior nasal turbinates [30] as well as recurrent bleeding in hereditary hemorrhagic telangiectasia [31]. Laser-assisted cartilage reshaping has been recently published as an alternative to standard surgical procedures, applied for the correction of nasal septum deviations or ear malformations with potentially good results [32, 33]. Based on the latter technique, several studies have examined the influence of laser application on cartilage repair: Holden et al. investigated the effects of Nd:YAG laser application on rabbit septal cartilage and found a collagen type II production in the tissue around the laser-treated areas but could not detect collagen type I expression in contrast to the normal wound healing process [34]. He concluded that the healing after laser irradiation must be different from normal wound healing and that the laser application can leave an intact collagen matrix in which chondrocytes are able to recover. Sobol et al. postulated that



**Fig. 5** REM and TEM analyses of laser-modified scaffolds. (a, d) Vertical sections of laser-treated areas using REM. CO<sub>2</sub> laser application (a) results in a v-shaped channel, Er:YAG laser irradiation led to a cylinder-shaped indentation (d). However, the channel surface after CO<sub>2</sub> laser application (a) was clearly more compacted and molten than after Er:YAG laser treatment (d), where the empty lacunae of DECM

were still visible. TEM illustrates the differences between untreated zones of DECM (b, e) and the CO<sub>2</sub> (c) and Er:YAG (f) laser-modified areas. In untreated DECM (b, e), loosely arranged collagen fibrils with the typical crossbanding were visible. In contrast, the matrix was compressed and coarse in the laser-treated regions, while crossbanding was absent

non-ablative laser treatment of cartilage leads to physical and chemical alterations of the ECM which contribute to tissue regeneration. Furthermore, he pointed out that the effectiveness of the irradiation depends on the correct choice of laser parameters [1]. In the current study, histology confirmed that human nasal chondrocytes proliferated on the DECM scaffolds after ablative laser treatment. Immunohistochemistry demonstrated the *de novo* synthesis of cartilage-specific aggrecan and collagen type II by the chondrocytes. We substantiated the hypothesis that the irradiation process did not have a cytotoxic effect on chondrocytes. Although we cannot prove that laser treatment stimulated cell proliferation, we have strong evidence from the cytotoxicity assay that it did not inhibit cell proliferation.

In this study, laser irradiation caused a compacted matrix adjacent to the borders of the produced channels most likely as a result of thermal damage (Fig. 3). These changes were much more pronounced after the use of the CO<sub>2</sub> laser. It is a well-known fact that the Er:YAG laser creates a reduced thermal tissue damage in contrast to other ablative lasers such as the CO<sub>2</sub> laser [35, 36]. The extent of thermal tissue damage due to laser application depends on the absorption by the tissue and on the irradiation parameters of the laser. The absorption of the Er:YAG laser in water-containing tissues is much higher than that of the CO<sub>2</sub> laser because of the different absorption coefficients (12,800 vs. 800 cm<sup>-1</sup>) [37]. Furthermore, the pulsed operation mode (pulse duration < 1 ms) of the Er:YAG laser leads to thermomechanical ablation, which is much more efficient and with minor thermal effects compared to the tissue evaporation by the CO<sub>2</sub> laser in continuous or modulated operation mode (pulse duration > 30 ms) [36, 38, 39]. This is in accordance with our SEM and histological and immunohistochemical results, as the surfaces treated with the Er:YAG laser were not as dense as surfaces treated with the CO<sub>2</sub> laser. The denaturation zone in our experiments was about 80–100 µm thick after applying the CO<sub>2</sub> laser while it was hardly visible and could not be measured after using the Er:YAG laser.

Already in the 1980s, Whipple et al. showed that CO<sub>2</sub> laser energy causes thermal injury of human fibrocartilage [40]. Electron microscopy revealed changes in the organization and microstructure of collagen fibers as well as alteration in cell structure with loss of cell organelles and damage of endoplasmic reticulum architecture [40]. Similar changes in collagen structure became obvious in the DECM scaffolds in the current study. On the other hand, another experimental study on ear cartilage of rabbits showed that 8 W CO<sub>2</sub> laser evaporation stimulates the regeneration of cartilage [41] as postulated by Sobol for the non-ablative lasers [1]. Furthermore, the structure of the treated tissue determines the extent of thermal alterations [36]. Walsh compared the effect of Er:YAG laser irradiation on different tissues and found destruction of collagen fibers but not of elastic fibers

[36]. He also postulated that the shortening of the pulse duration of the CO<sub>2</sub> laser under the thermal relaxation time of the tissue will lead to less thermal tissue damage [42]. So one can conclude that the variation of the adjustments of the used laser will strongly influence the damage of the tissue and therefore could probably improve the migration of cells into the tissue. However, as the DECM scaffolds predominantly consist of collagen fibers tissue damage cannot be totally inhibited when irradiated by ablative lasers such as the CO<sub>2</sub> and Er:YAG laser. TEM likewise revealed that in both types of lasers used there is a denaturation of the collagenous network with loss of crossbanding. The concurrent condensation of the DECM surface areas inhibited chondrocytes to migrate into the scaffolds. This effect was clearly more pronounced when the CO<sub>2</sub> laser was applied, as it lasted for at least 42 days *in vitro*; after Er:YAG laser irradiation, several chondrocytes migrated through the denatured area on day 42 after seeding. Meister et al. examined the effects of Er:YAG laser irradiation on porcine knee joint cartilage with special respect to the thermal damage and tissue necrosis. They demonstrated a significant increase of the ablated volume and the cut depth with increasing pulse duration and decreasing distance to the cartilage surface but found no loss of proteoglycan or collagen type II or alterations on cell levels, such as increased apoptosis or altered cell morphology [18]. A study on decellularized fibrocartilaginous temporomandibular joint discs even demonstrated that CO<sub>2</sub> laser micropatterning might lead to a uniform cell spread within the scaffold in contrast to the matrices which were not laser treated. The increased permeability of the scaffolds after laser irradiation supported cell migration and remodeling [19]. The rapid distribution of the cells is contradictory to our findings but is most likely caused by the different composition of the hyaline and fibrous cartilage and may be influenced by the modification of the laser adjustments.

In fact, the denaturation of the collagen fibers due to the CO<sub>2</sub> laser as well as the Er:YAG laser application leads to a consolidation of the matrix along the laser channels, thus hindering the chondrocytes to migrate into the scaffold and finally revealing the current limitation of the applied method. In spite of successful surface enlargement, the expected increase of cellular migration and the subsequent increment in stability of the collagen matrix could not be accomplished.

Some limitations of this study have to be mentioned explicitly: first of all the number of scaffolds used for the different experiments was rather limited, namely 28 scaffolds for each laser type and an equal number of unlasered scaffolds; furthermore, the detection of the *de novo* synthesis of collagen type II, aggrecan, and glycosaminoglycans by histological and immunohistochemical stainings could have been quantified by real-time PCR to clarify slight differences between the laser treated and untreated scaffolds. Also, different adjustments of



the laser parameters might have influenced the thickness of the denaturation zone; still these parameters were not included in the current study.

To impede the thermal damage of the collagen structure, other laser types like those in the infrared spectrum with known minimal attendant destruction could be an option to alter the surface of the scaffolds and to accelerate cellular migration into the matrix at the same time. The femtosecond and excimer laser are two examples which are both employed in corneal refractive surgery [43]. A preclinical study on articular cartilage ablation by a femtosecond laser demonstrated the possibility of creating extremely precise channels through the cartilage without noticeable thermal damage [44]. Similar results were obtained by the application of an excimer laser on different components of porcine temporomandibular joints [45]. Thus, infrared lasers could be a promising alternative for modifying the surface of DECM scaffolds.

## Conclusion

Thermal denaturation, which has been demonstrated to be a typical effect of ablative laser irradiation [35], is also obvious in DECM scaffolds from porcine nasal septum. Changes in the superficial structural composition of DECM are much more intense after CO<sub>2</sub> laser application than after Er:YAG laser treatment. Alterations of the collagenous fibers are predominantly visualized as compacted amorphous area around the laser-treated cavities, which inhibit migration of chondrocytes into the scaffold. Although there are no relevant cytotoxic effects along with an increase in surface area after laser application, modifications of laser settings are inevitable. Due to its physical characteristics, the Er:YAG laser obviously caused a thinner denaturation zone than the CO<sub>2</sub> laser. Especially modifications of the Er:YAG laser settings such as optimization of the pulse duration or of the beam parameters like spatial beam profile (top-hat) and depth of focus are expected to minimize damage to the collagen network and might therefore improve the migration of the cells into the DECM scaffolds. Also, alternative ablative techniques with minimal tissue damage such as femtosecond or excimer laser might be used instead. In future, successful modification of the scaffolds' surface which shortens the time cells need to migrate into the scaffolds and which at the same time prevents loss of stability by resorption and remodeling processes would be a major step to a prosperous use of the DECM matrices in vivo.

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**Compliance with ethical standards** The study has been approved by the University of Ulm Ethical Committee (Ethic application number 152/08) and has therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

**Conflict of interest** The authors declare that they have no conflict of interest.

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