Does Low-Intensity Pulsed Ultrasound Stimulate Maturation of Tissue-Engineered Cartilage?

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Abstract: Traumatic events are a primary cause of local lesions of articular cartilage. Tissue engineered, cartilage-like structures represent an alternative to current treatment methods. The time necessary for tissue maturation and the mechanical quality of the regenerate at implantation are both critical factors for clinical success. Low-intensity pulsed ultrasound has proven to accelerate chondrogenesis in vitro. The goal of this study was to evaluate whether low-intensity pulsed ultrasound is capable of accelerating the process of cartilage maturation and increasing regenerate stability. Hyaline-like cartilage specimens were generated *in vitro* and subcutaneously implanted in the backs of nude mice. Twenty-eight animals received 20 min of low-intensity pulsed ultrasound treatment daily, and 28 animals received a sham treatment. Specimens were explanted after 1, 3, 6, and 12 weeks, mechanically tested with the use of an indentation test, histologically examined, and processed for RT-PCR. The Young's moduli significantly increased from 3 to 12 weeks, and at 6 weeks were comparable to those of native articular cartilage. In histological examination, specimens showed neocartilage formation. There was no significant difference between ultrasound-treated and sham-treated groups. The mechanical stability of the neocartilage specimens increased with treatment time and reached values of native cartilage after 6 weeks *in vivo*. Low-intensity pulsed-ultrasound stimulation showed no stimulatory effect on tissue maturation. In contrast, ultrasound-treated specimens showed a reduced Col 2 expression at 1 week and were significantly less stiff compared to native cartilage at 6 and 12 weeks. An acceleration of the maturation of tissue-engineered neocartilage in a clinical setting by means of low-intensity pulsed ultrasound therefore appears rather unrealistic. © 2003 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 68B: 21–28, 2004

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INTRODUCTION

Traumatic events in sports or vehicle accidents are a main cause of local lesions of articular cartilage.[5,13,18,19] The initial damage produced in a traumatic event may be a disruption of the articular surface. Another possibility is that the underlying bone may be compacted, resulting in a bone bruise and derangement of the subchondral structure.[13,18,19] Injuries or illnesses may lead to a further softening of the cartilage composite structure.[11]

In such instances, the cartilage composite structure may be irreversibly destroyed. Self-repair processes may not be capable of adequately restoring the articular joint surface and integrity. Without further surgical treatment, the cartilage lesions or defects may, in the long term, lead to large-scale degenerative changes and osteoarthritis.[4]

Currently, the damaged tissue is replaced and defects are filled. Material for defect filling may be obtained from an-

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other, nominally unloaded region and transferred to the defect site.[22] However, suitable autologous tissue is only available to a limited extent. Heterologous transplantation is not well accepted due to the risk of infection.[37] Alternatively, engineered cartilage tissue, which may be cultured for orthopedic purposes from nonarticular locations, provides the potential for an ample quantity to fill large defects.[10,30,31] As an alternative to the replacement of defect tissue by cartilage or cartilage-like tissue, such voids may be filled with a substance that matures *in vivo* and may potentially bear full weight in the long term.[3]

If post-traumatic lesions are treated sufficiently early, restoration of the initial joint geometry and integrity may be achieved.[3] The long-term outcomes of the clinical treatment methods currently available are, however, still unknown: Data from double-blind randomized clinical studies are scarce, and results are usually limited to single clinical centers or surgeons.[3]

A crucial factor for the initial as well as long-term performance of these surgical treatment methods are the mechanical properties of the defect fillings.[3,8] A number of methods exist that allow the static[15,36] and dynamic behavior of articular cartilage to be determined.[1,33] Models have been established to describe the mechanical behavior of cartilage.[12,17,23,25] Previous investigations have demonstrated a maturation of neocartilage in an *in vivo* situation within 12 weeks.[8] Furthermore, it was demonstrated that the mechanical stability (stiffness and failure load) of the tissue-engineered regenerate is similar to that of nasal septal cartilage but below that of articular cartilage. Even though specimens showed hyaline-like appearance, both mechanical quality as well as the necessary maturation time appeared to be rather unpractical for clinical applications.[8]

Low-intensity pulsed ultrasound is known to stimulate tissue regeneration.[2] Ultrasound appears to be specifically beneficial by accelerating the inflammatory response during the initial healing phase.[35] In experimental and clinical situations, low-intensity pulsed ultrasound has been successfully proven to stimulate fracture healing.[20,21,28,39] and bone growth.[34] It has been demonstrated that influencing the expression of proteins relevant to cartilage generation (e.g., proteoglycans),[38,39] results in enhanced mechanical stability.[28,38,39] *In vitro*, low-intensity pulsed ultrasound has demonstrated an influence on the expression of cartilage-specific matrix proteins and growth factors in cell cultures from chondrocytes, fibroblasts, and osteoblasts.[7,24,27]

Based on experimental and clinical experience, the hypothesis of the present work was that low-intensity pulsed ultrasound stimulates cartilage formation and maturation and may eventually increase the mechanical quality of tissue-engineered cartilage and thereby help to reduce the *in vitro* maturation period.

The goals of this study were (a) to monitor the process of maturation of neocartilage structures and (b) to stimulate this process by means of low-intensity pulsed ultrasound.

MATERIALS AND METHODS

Scaffold and Tissue Engineering

The methodology of cell isolation, cultivation, and implantation has been previously reported and is only briefly described here.[8,10,31] A local abattoir supplied freshly slaughtered adult bovine forelimbs. Pieces of articular cartilage were surgically removed from the humeral head under sterile conditions. The cartilage specimens were enzymatically digested with 1 mg/ml collagenase P (Boehringer), 0.1 mg/ml Hyaluronidase (Serva, Frankfurt, Germany), and 1.5 mg/ml DNAse (Paesel, Frankfurt, Germany) in RPMI 1640 medium (Seromed, Berlin, Germany) to obtain the cellular contents of the tissue. The cells were passed through a nylon filter (Reichelt Chemie, Heidelberg, Germany) and washed three times in Hanks solution. The vital cells were assessed by hemocytometer counting, with the use of Trypan blue exclusion staining.

Cell amplification was performed in culture flasks (Nunc, Naperville) with modified RPMI 1640 medium (10% FCS, 2% Hepes, 1% penicillin/streptomycin): $4–5 \times 10^6$ cells were plated on 75-cm^2 culture flasks and incubated under standard culture conditions. The medium was renewed every second day.

A commercially available fibrin glue system was used as the first component of the carrier composite (Tissucol Duo S 1, Immuno, Heidelberg, Germany). The fibrin sealant consisted of a fibrinogen component (human plasma protein fraction 80–120 mg; fibrinogen 70-110 mg; factor XIII 10–50 E; plasminogen 0.02–0.08 mg; aprotinin 3000 KIE) and a thrombin compound (human thrombin 500 IE; calcium chloride 2 H_2O 5.88 mg). Thrombin was diluted in a ratio of 1:10 with RPMI 1640 medium. The chondrocytes were suspended in the fibrinogen component in a ratio of 1:1. Lyophilized bioresorbable co-polymer fleeces of vicryl (PGLA, Ethisorb 210, Ethicon, Norderstedt, Germany) served as a second component of the carrier composite The 2-mm-thick fleeces were cut into 10×10-mm slices and soaked with the chondrocyte-fibrinogen suspension.[30]

A native, freshly isolated chondrocyte population, which had been cultivated less than four passages, was used to prepare three-dimensional chondrocyte cultures. The cells were suspended in the fibrinogen solution and transferred into separate wells of a 24-well tissue plate (Becton Dickinson) with a cell density of $30 \times 10^6/cm^3$. Next, chondrocytes were loaded into the polymer constructs by soaking the biomaterial with the chondrocyte-fibrinogen suspension. Adding 50 μl/cm3 thrombin solution polymerized the fibrin. Cell culture was performed under standard conditions with a continuous medium supply and with separate cell perfusion chambers (Minucell, Regensburg, Germany) over a period of 8 days.[31]

Transplantation and *In Vivo* Model

Fifty-six anesthetized female homozygotic athymic nude mice (CD 1 nu/nu, Charles River Wiga, Sulzfeld, Germany; age: 6–8 weeks, weight: 30–35 g) were randomly distributed

into eight groups with seven animals in each. For transplantation, subcutaneous pockets were prepared in the back of all 56 mice. All animal experiments were carried out according to the policies and principles established by the Animal Welfare Act, the NIH Guide for Care and Use of Laboratory Animals, and the national animal welfare guidelines. The experiments were approved by the local legal representative.* The animals were anaesthetized with 5 mg/kg of xylazinhydrochloride 2% (Rompun®, Bayervital, Leverkusen, Germany) and 100 mg/kg ketamine hydrochloride (Ketanest® 50, Parke-Davis, Freiburg, Germany) in a ratio of 1:6. The backs of the mice were disinfected with iodine. A skin incision was performed with a scalpel, followed by subcutaneous pocket preparation. A cartilage implant was inserted and skin closure was performed with a 4-0 monofil nonreabsorbable bond (Ethicon, Norderstedt, Germany).

Ultrasound Treatment

The low-intensity pulsed-ultrasound treatment was performed with the use of a commercially available device (SAFHS-2a, Exogen, Smith & Nephew GmbH, Norderstedt, Germany). Animals were treated for 20 min per day, 7 days a week, with treatment starting on the second postoperative day. The animals were placed in a custom-made device that allowed ultrasound application directly to the skin covering the cartilage specimens. The device was disinfected prior to individual treatments. A drop of ultrasound gel ensured firm transmission of the stimulating energy (high-viscosity coupling gel, Exogen, Smith & Nephew GmbH, Norderstedt, Germany). The treated group of seven animals per time point received the regular ultrasound dosage of 30 mW/cm^2 at a frequency of 1.5 MHz. In the control group, the ultrasound device was disconnected from the power supply but animals received otherwise identical treatment as those in the treated group.

Biomechanical Testing

After 1, 3, 6, and 12 weeks, the mice were sacrificed and the cartilage implant was separated from the surrounding fibrous tissue and transferred for macroscopic and microscopic analysis.

As a standard protocol for mechanical testing, similarly sized (10 × 10 mm, accuracy 0.5 mm) native articular cartilage specimens were obtained from bovine hip and shoulder joints.

For the 3-, 6-, and 12-week specimens, as well as for the native tissue group, biomechanical testing was performed (Figure 2) within 3 h of sacrifice. Throughout the experiment, specimens were kept moist using Ringer solution. Mechanical indentation tests were performed under sterile conditions on a materials-testing machine (Zwick 1455, Ulm, Germany), with the use of a calibrated load cell (accuracy 0.15 N). Testing was carried out in displacement control (accuracy 0.02 mm) with a velocity of 5 mm/min. The diameter of the indenter was selected to be 4 mm.[8] For testing, specimens were placed unconstrained on a rigid metallic surface to allow free lateral deformation, thereby eliminating possible effects of surrounding specimen holders. The Young's modulus was determined with the use of the formula of Hayes et al. for indentation tests.[12] The Poisson's ratio was set to 0.4.[14,16] To correct for friction and large deformation effects, the modified calculation of the Young's modulus according to Zhang et al. (1997) was applied.[40] Prior to testing, the zero position of the indenter was determined by positioning the indenter tip on a rigid metallic surface. At a preload of 1 N, the thickness of the specimens was determined from the distance reading. Statistical analysis was performed on Young's modulus data with the use of ANOVA and Bonferroni correction (SPSS 10.0, SPSS Inc., Chicago, IL).

Histology and RT-PCR

For the 3-, 6-, and 12-week specimens, one half of the specimen was fixed with formalin immediately after biomechanical testing for histological analysis. Cartilage transplants were embedded in paraffin, and 5-μm sections were prepared for a series of histochemical examinations. For morphological measurements, hematoxilin and eosin (HE) staining was performed. Matrix formation was visualized by azan (AZ) staining. Proteoglycan synthesis was analyzed by alcian blue (AB) staining.

For RNA extraction, the remaining half of each specimens from the 1-, 3-, and 6-week groups were placed on dry ice. As an internal control, four completely processed but not implanted fleece implants loaded with cell were also placed on dry ice for RNA extraction.

RNA analysis was performed for the 1-, 3-, and 6-week specimens with the use of RT-PCR techniques and GAPDH as a housekeeping gene (Table I). In addition, a RT-PCR analysis was performed for the 1-week specimens and the loaded implant specimens with β-Actin used as a housekeeping gene: The specimens were homogenized, the total mRNA was isolated and converted into cDNA. Bovine-specific PCR primers were designed with sequence analysis software (DNA Star) and obtained from Invitrogen (Karlsruhe, Germany). The sequences of the primers used for the RT-PCR are given in Table I. Equal volumes of PCR products were loaded on a 1% agarose gel. The quantification of transcripts (intensity calculation) was done with the use of densitometry analysis software (Scion Image, Frederick, MD). PCR expressions were reported as relative intensities of GAPDH and β-Actin expressions. Statistical analysis was performed on relative intensity data with the use of ANOVA and Bonferroni correction (SPSS 10.0, SPSS Inc., Chicago, IL).

RESULTS

At explantation, the tissue-engineered specimens appeared whitish in color, not unlike normal hyaline cartilage (Figure

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TABLE I. The Sequences of the Primers Used for the RT-PCR. GAPDH and β-Actin Served as Housekeeping Genes

Primers	f/r	X-mer	5′→3′	Annealing Temperature (°C)
GAPDH	for	25	cgtccccactcccaacgtgtctgtt	56.9
	rev	26	gcatcgaaggtagaagagtgagtatc	
Coll1/a2	for	22	agtcaagaactggtacagaaat	52.2
	rev	17	gggccaatgttcaatct	
Coll2/a1	for	21	caacccggccagcgtccccaa	61.9
	rev	27	ttcgtgcagccatccttcagaacggta	
TGF-β1	for	17	ggtgcccgaacctgtgt	56.9
	rev	22	ccgttaatgtccacttgaagcg	
β-Actin	for	24	cccaaggccaaccgtgagaagatg	61.5
	rev	24	aagtccagggccacgtagcagagc	

1). No infection in any of the mice was observed at any point in the experiment. After 6 and 12 weeks, implants appeared elastic and of solid consistency. At sacrifice, the implants were attached to the subcutaneous pocket, due to a superficial infiltration of adjacent tissue.

In biomechanical testing, the 6- and 12-week implants showed superior mechanical properties compared to those at 3 weeks ($p < 0.001$; Figure 2). This increase in Young's modulus was independent of the ultrasound treatment, however.

The mechanical stiffness of the pure polymer fleece was below the sensitivity of the biomechanical test setup. During maturation, the neocartilage specimens showed an increase in their mechanical stiffness. No statistical difference, however, was found in the material properties between the 6- and 12-week tissue-engineered specimens and the native articular cartilage specimens.

There was no statistically significant difference between the ultrasound-treated and the control groups at any time point ($p > 0.05$). However, the ultrasound-treated groups at 6 and 12 weeks proved to have a lower Young's modulus than the native articular cartilage specimens, whereas the sham-treated group was not significantly different.

With any staining method used, there was no visible difference between sham-treated and ultrasound-treated specimens. Tissue-engineered specimens showed neocartilage formation during macroscopic and histological examination with the use of hematoxilin and eosin staining. The neocartilage showed typical histological features of normal cartilage with a chondron-like cell-matrix formation. At 6 and 12 weeks, round-shaped chondrocytes were homogeneously embedded within the cartilage matrix. Azan staining demonstrated only slight differences in matrix formation after 12 weeks *in vivo* compared with specimens after 6 weeks *in vivo* (Figure 3). At 12 weeks, the pericellular matrix appeared more compact than at 6 weeks. Thin layers of fibrous tissue covered the cartilage transplant. Because of the nonspecific process of wound healing, it is difficult to conclude to what extent the infiltrating fibrous tissue led to the slight resorption of engineered cartilage at the margins of the implants at both time periods. Alcian blue staining demonstrated homogeneous purple staining, indicating the presence of sulphate glycosaminoglycans such as chondroitin sulphate. At the 12-week time point, no pieces of the polymeric fleece were detectable within the tissue-engineered specimens.

Compared to the cell-loaded implants prior to implantation, the Col 1 RNA expression was significantly reduced in the 1-week specimens, independent of the ultrasound treatment (Figure 4). In addition, there was a slight but significant reduction in Col 1 and Col 2 RNA expression in the ultrasound-treated group compared to the sham-treated group. The TGF-β1 RNA expression was similar between the loaded but unimplanted specimens and the 1-week sham and ultrasound-treated ones. At the later time points of 3 and 6 weeks *in vivo*, no differences in RNA expression could be detected either between the two time points or the two treatment options.

DISCUSSION

The goal of this study was to determine if low-intensity pulsed-ultrasound treatment is capable of accelerating the process of maturation in tissue-engineered neocartilage structures. This goal was approached by using a well-established experimental animal model.[8,9,31] and *in vivo* stimulation of tissue-engineered cartilage structures in the backs of nude mice.

The macroscopic examination, as well as the histological staining, indicated no differences between the sham- and ultrasound-treated groups. In each case, the tissue-engineered cartilage specimens showed a hyaline-like nature after 6 and 12 weeks. Cells appeared round in shape and homogeneously distributed within a compact pericellular cartilage matrix. During the course of healing, the increasing homogeneity of the pericellular matrix staining indicated further development of a more compact pericellular matrix. Due to the remodeling nature of wound healing, signs of resorption with infiltration of fibrous tissue were observed at the periphery areas after 6 and 12 weeks. After 12 weeks, none of the polymeric fleece was histologically detectable.

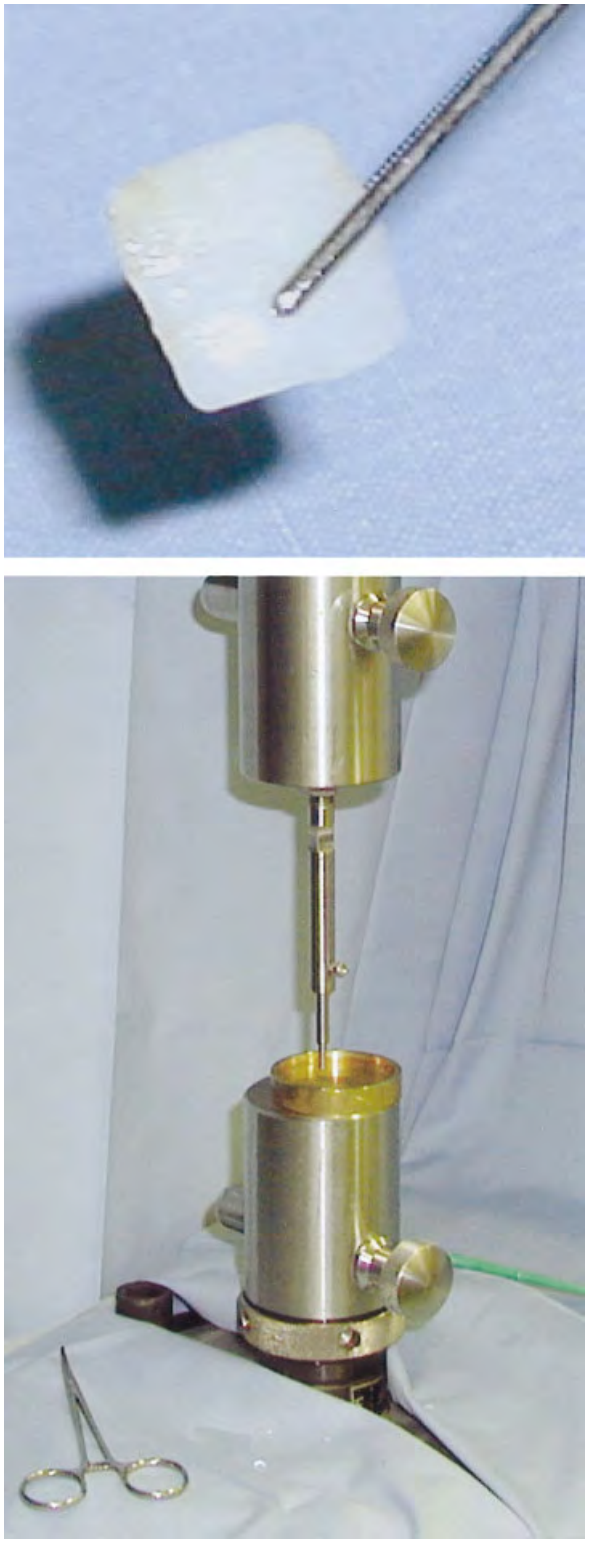

Figure 1. Specimen after 6 weeks appeared whitish in color and not unlike normal hyaline cartilage (top). Specimens were subjected to unconfined compression testing under sterile conditions to determine the Young's modulus (bottom).[Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

The biomechanical testing exposed no significant advantage of the ultrasound stimulation compared to the sham-treated group. Independent of the ultrasound treatment, tissue maturation was observed as an increase in Young's modulus between the 3-week and the 6- and 12-week specimens. This is especially remarkable because the polymeric fleece, which determined the stability at implantation, had a notably lower mechanical stiffness compared to the values measured at 3, 6, and 12 weeks.[8] After 3 weeks, the tissue-engineered but sham-treated specimens showed comparable values of Young's modulus to those of native articular cartilage.

In contrast to the sham-treatment group, the ultrasound-treated group showed a statistically reduced Young's modulus at 6 as well as at 12 weeks compared to those of the native tissue. Even though the mechanical quality increased with treatment time, the values of native cartilage tissue were not met by the ultrasound-treated specimens. This was a clear difference from the findings in the sham-treated group, which, at 3 weeks, had already reached the mechanical quality of the native tissue.

In the RT-PCR analysis, no significant advantage of the ultrasound stimulation was detectable at the 3- and 6-week time points: The expression of Col 1, Col 2, and TGF-β1 were comparable between the ultrasound- and sham-treated groups. No difference in expressions were visible between the 3- and 6-week time points, even though the specimens seemed to undergo a considerable maturation process, as demonstrated by the biomechanical and histological findings.

A significant reduction in Col 1 and Col 2 expression in the 1-week ultrasound-treated group compared to the sham-treated group was detectable, however. TGF-β1 expression tended to lower with ultrasound treatment, but this difference was not statistically significant. The reduced expression of Col 2 and to a lesser degree of TGF-β1, illustrates no stimulating but rather an inhibiting effect of the ultrasound treatment in the analyzed tissue-engineered specimens.

In contrast to the preoperative situation, the Col 1 expression was significantly reduced after 1 week of implantation.

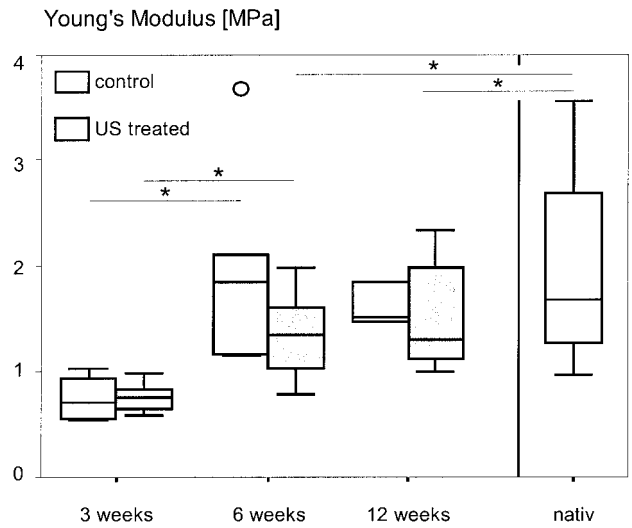

Figure 2. Young's modulus of sham-treated and US-treated groups at 3, 6, and 12 weeks in comparison to native articular cartilage specimens (*$p < 0.001$).

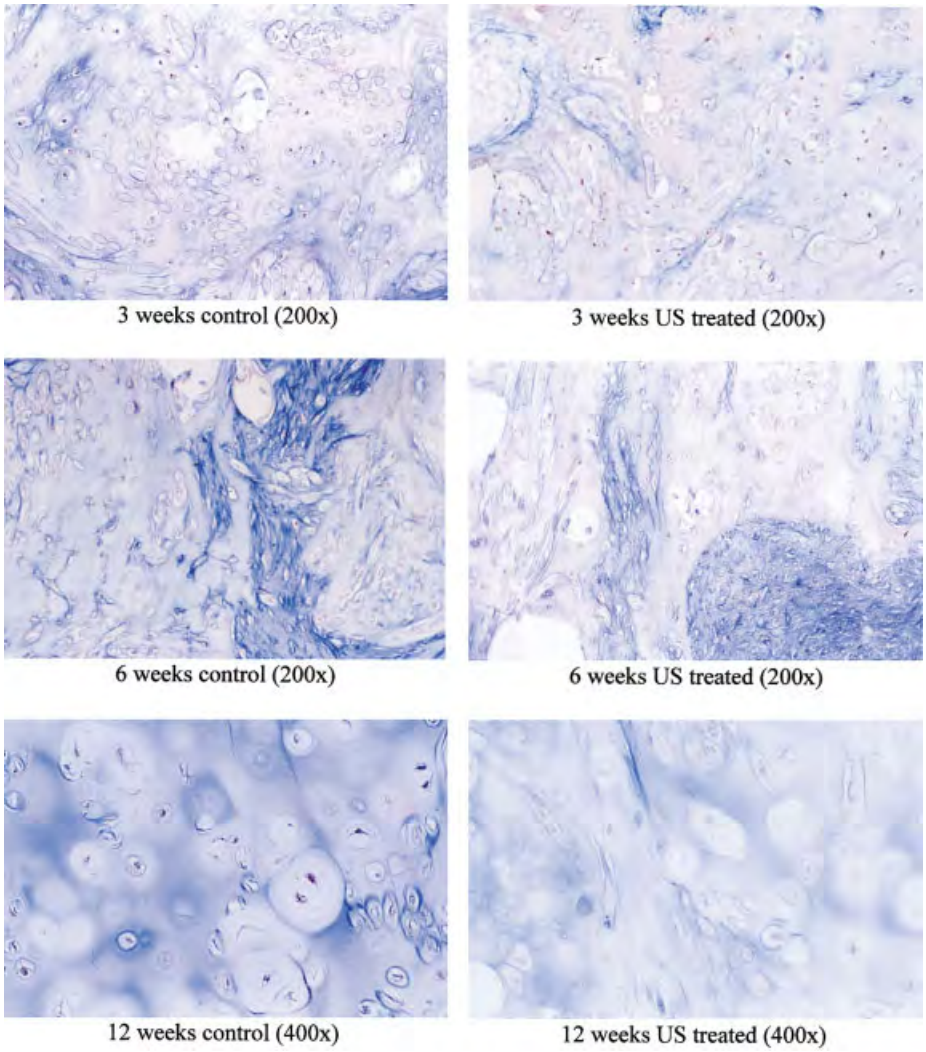

Figure 3. Azan staining of sham-treated and US-treated specimens at 3, 6, and 12 weeks. Azan stains the collagen matrix and thereby allows the process of tissue maturation to be judged.

This finding is in agreement with earlier findings,[6,32] demonstrating increased expression of Col 1 during cell amplification and a reduction after implantation in a three-dimensional fleece structures. This finding illustrates the sensitivity of the selected RT-PCR approach. Unfortunately, an expected increase in Col 2 expression during the tissue maturation as documented by the biomechanical and histological findings could not be detected. To further elucidate the underlying mechanisms of tissue maturation in respect to mRNA expression, a protein analysis by means of Western-blot techniques may be indicated. However, because no positive effect of the low-intensity pulsed-ultrasound treatment was detectable in biomechanical, histological, or RT-PCR analysis, a further analysis of the underlying mechanisms of tissue maturation appeared not to be effective.

Low-intensity pulsed ultrasound is known to stimulate tissue regeneration.[2] In vitro, low-intensity pulsed ultrasound has been demonstrated to influence the expression of cartilage-specific matrix proteins and growth factors in cell cultures from chondrocytes, fibroblasts, and osteoblasts.[7,24,27] Some of these studies have used an identical applicator to the one in the presented study. In contrast to all *in vitro* cell-culture experiments, the current study analyzed chondrocyte maturation in a tissue-engineering environment, including a three-dimensional construction of the tissue, a polymeric fleece, as well as an *in vivo* animal model. All of these factors may have compromised the positive effect reported from the *in vitro* cell-culture experiments. It appears that the positive effects found during *in vitro* cell-culture experiments appeared not to be reproducible in the selected *in vivo* analysis of tissue maturation.

It is well documented from *in vivo* animal experiments and clinical investigations that low-intensity pulsed ultrasound has successfully been proven to stimulate fracture healing[20,21,28,39] and bone growth[34] Previous experimental analyses have explained the stimulatory effects of low-intensity pulsed ultrasound by an acceleration of the chondrogenic pathway of callus healing.[26,27,29] However, the current study could not identify an accelerating effect of low-intensity pulsed ultrasound on the chondrogenesis or the maturation of

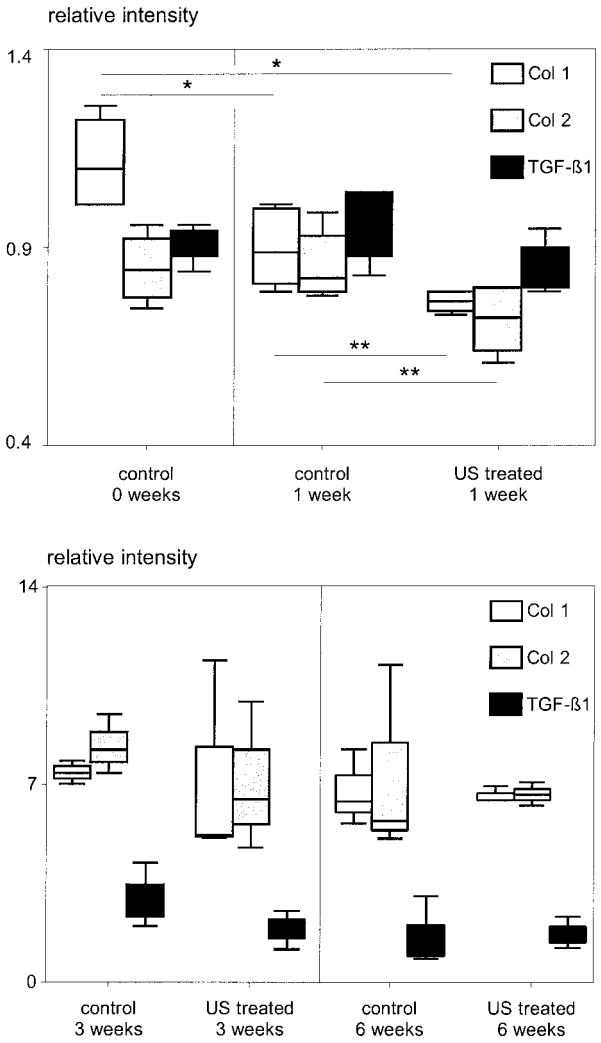

Figure 4. Relative intensity of Col 1, Col 2, and TGF-β in the RT-PCR after 0 and 1 week (top) and 3 and 6 weeks (bottom) *in vivo* (*$p \leq 0.004$, **$p \leq 0.04$).

chondrocytes. The biomechanical and RT-PCR data rather indicate an inhibitory effect of the ultrasound treatment on the maturation of neocartilage specimens. Therefore, direct positive effects of the ultrasound treatment on the general process of chondrogenic differentiation might be questioned. Whether the positive effects seen from low-intensity pulsed ultrasound in fracture healing are directly related to an acceleration of the chondrogenic pathway, however, remains to be seen.

CONCLUSIONS

The current animal experiment demonstrates the maturation process of tissue-engineered cartilage specimens in an *in vivo* environment. The original Young's modulus of native cartilage as well as neocartilage structures was reestablished in the tissue-engineered cartilage specimens after 6–12 weeks.

An additional stimulation by means of low-intensity pulsed ultrasound showed no positive effects with respect to the biomechanical quality, histological outcome, or mRNA expression compared to the sham-treated groups. Low-intensity pulsed ultrasound does not seem to accelerate the maturation process of tissue-engineered cartilage. In the selected animal model, the ultrasound treatment even appeared to have an inhibitory effect on the maturation of tissue-engineered cartilage specimens.

For the clinical situation, an acceleration of the healing of cartilage lesions or the integration and maturation of tissue-engineered cartilage implants by means of low-intensity pulsed-ultrasound treatment appears, therefore, rather unrealistic.

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