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Electromagnetical stimulation of human articular chondrocytes by the Algonix device.

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Materials and Methods

Preparation of collagen gel seeded with human chondrocytes

Cartilage samples were harvested from knee joints of 10 patients (2 male, 8 female; mean age 67,8) undergoing total knee replacement. All patients gave their written consent prior to operation.

Samples were collected in DMEM medium containing 10 % fetal calf serum (FCS), 100 U/ml penicilline, 100 μg/ml streptomycine. The cartilage was cut into 1-2 mm^3 pieces, and digested with 1 mg/ml Liberase (Roche Diagnostics, Indianapolis, USA) overnight. The released chondrocytes were centrifuged for 10 min at 1200 rpm and washed subsequently 3 times with 10 ml serum-free DMEM.

Rat tail collagen type-I gel was provided by Ars Arthro (Esslingen, Germany). The collagen type-I was supplied as a 6 % aqueous solution in 0.1 % acetic acid. It remained liquid when stored at 4 °C and gelled when transferred to 37 °C.

$2x10^5$ chondrocytes/ ml gel were centrifuged and resuspended in 1 vol collagen type-I gel mixed with 1 vol 2x DMEM/2 M HEPES (0.93:0.07). 1.5 ml cell-seeded collagen gel was pourn into each well of a 12-well-plate and allowed to gel for 30 min. After gelling, samples were overlaid with DMEM/FCS medium and cultivated under standardized in-vitro conditions (37°C, 5% CO_2, humidified atmosphere) for up to 3 weeks. Every three days, samples were fed with fresh medium.

Electromagnetical stimulation of cell-seeded collagen samples

Electromagnetical stimulation was carried out by use of the Algonix device. Untreated samples of the same patients were included as control. Sterile electrodes were inserted into the cell-seeded collagen type-I gel on opposite sites of the samples. A program specific for muscles surrounding joints and rheumatoid complaints (sweep 48) was applied, stimulation was carried out twice every two days. After up to 3 weeks, samples were processed as described.

Histochemical and immunocytochemical analysis

Samples were fixed overnight in a phosphate-buffered solution of 4 % paraformaldehyde and embedded in paraffin. 5 µm sections were stained with hematoxylin-eosin and safranin O according to standard protocols.

For detection of collagen type-II protein, 5 µm sections were deparaffinized, blocked and incubated with a polyclonal antibody to human collagen type-II (Biotrend, Köln, Germany) diluted 1:50 overnight.

Proliferating cells were detected by indirect immunohistochemistry using the Ki-67 polyclonal antibody (NeoMarkers, Fremont, USA) specific for proliferating cells.

Apoptotic cells were detected by TUNEL staining of fragmented DNA (Dead End Colorimetric Apoptosis Detection System, Promega, Madison, USA) according to the manufacturer's instruction.

Immunohistological staining was visualized using the streptavidin/biotin technique (Vectastain ABC Kit, Vector Laboratories, Burlingame, USA). Diaminobenzidine (DAB Peroxidase Substrate Kit, Vector Laboratories, Burlingame, USA) was used as the developing substrate leading to a brownish color of the immunopositive cells. The specificity of the staining was verified by omission of the primary antibody and the use of matrix samples without enclosed cells, giving the background staining.

All images were captured by a Leica microscope (Leica, Wetzlar, Germany) and prepared using the Discus software by the same manufacturer.

Analysis of mRNA expression

To quantify gene expression of collagen type-II and aggrecan, RNA was isolated with the Oligotex Direct mRNA Kit (Quiagen, Hilden, Germany) according to the manufacturer's instruction. Isolated mRNA was used to create the corresponding cDNA by the SuperScript First Strand Synthesis System (Invitrogen, Carlsbad, USA). Gene expression was quantified by real time PCR on a LightCycler (Roche Diagnostics, Indianapolis, USA). The housekeeping gene β-actin was used as an internal standard. Because of the large variability between the patients, data were presented as relative expression with respect to control samples of the same patients.

Statistical analysis

For statistical evaluation the ANOVA test with repeated measurements was performed.

Results

The collagen type-I gel was easily and homogeneously to mix with the freshly prepared chondrocytes. It could be pourn into samples of different size, was easy to handle with a forceps and remained stable even when cultivated for 3 weeks in-vitro. Electromagnetic stimulation by use of the Algonix device did not lead to a significant improvement of mechanical stability after 3 weeks.

Fig.4: Real-time PCR analysis of aggrecan gene expression. Data are presented as relative aggrecan gene expression of stimulated samples in comparison to untreated samples of the same patients.

In summary, the presented data show a positive effect of electromagnetical stimulation of hyaline chondrocytes with the Algonix device under in-vitro conditions. When replacing the in-vitro cultivation by in-vivo conditions, the observed positive effects might even be stronger.

In all samples cells remained viable, as TUNEL staining of apoptotic cells revealed. Proliferation was low but constant; in all samples only few proliferating cells could be detected.

Morphological phenotype in both control samples and samples treated with the Algonix device was prevailed to a large extent, although some cells showed signs of a partial morphological dedifferentiation (Fig.1). Chondrocytes in hyaline cartilage show a roundish phenotype, while chondrocytes cultivated in monolayer culture express a tendency to dedifferentiate morphologically and biochemically to become fibroblast-like cells.

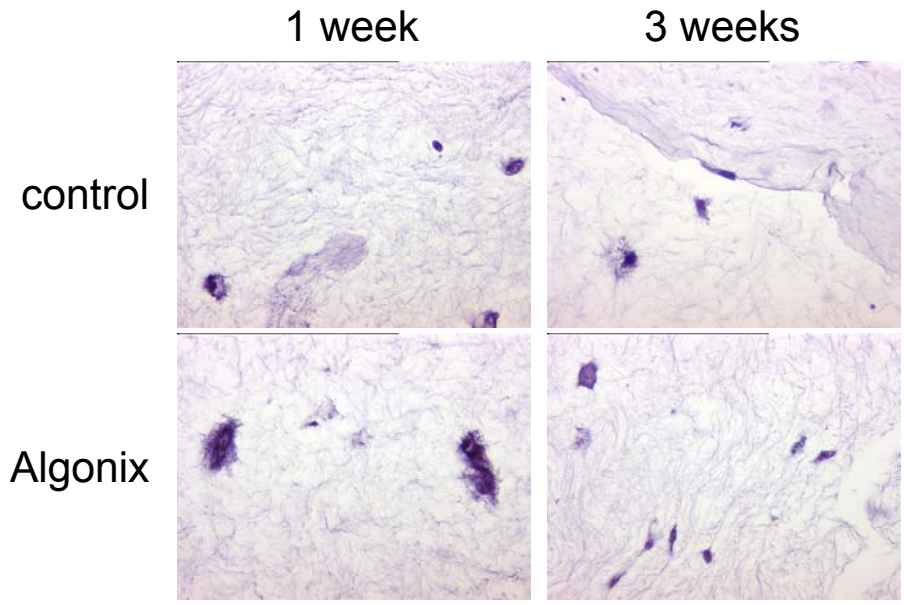


Fig.1: Hematoxylin-Eosin staining of untreated collagen gel samples and cell-seeded collagen gel samples stimulated with the Algonix device. Objective x63.

Collagen type-II protein was produced in both control samples and Algonix-treated samples and stored mainly pericellularly. The building of a territorial matrix characteristic for hyaline cartilage could not be observed under the given in-vitro conditions (Fig.2). The amount of collagen type-II protein as detected by

semiquantitative immunocytological staining did not show any striking differences between control and Algonix-treated samples.

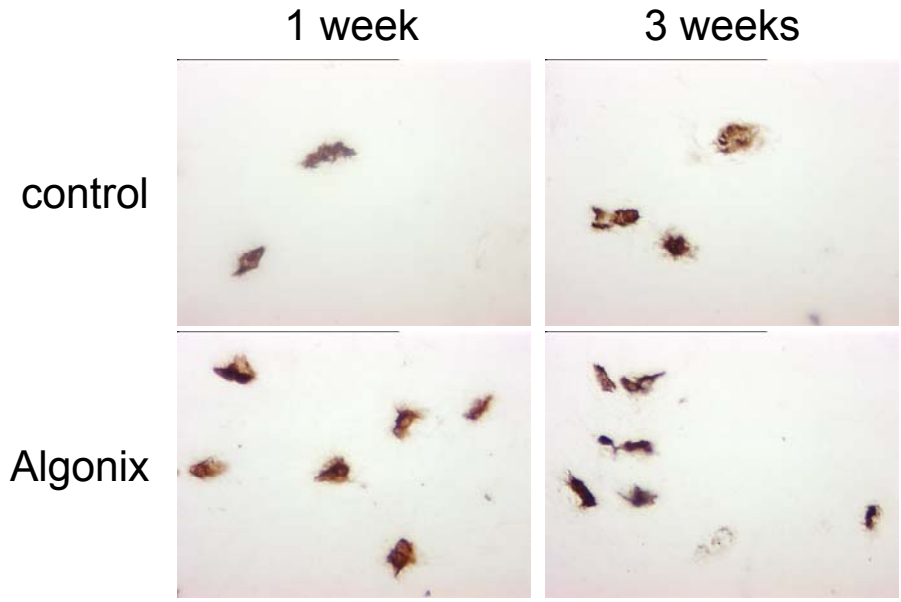


Fig.2: Immunohistological staining of collagen type-II protein. Cell-seeded collagen type-I gel samples were either left untreated or stimulated with the Algonix device. Objective x63.

In contrast to the detection of collagen type-II protein, the determination of col-II gene expression showed significant differences between control and Algonix-treated samples. When looking at the relative expression level of Algonix-treated samples in comparison to control samples, electromagnetic stimulation led to a significant increase of gene expression (Fig.3). Even after 3 weeks of in-vitro cultivation, expression of the col-II gene, which is characteristic for chondrocytes of hyaline cartilage, was about 1.8 times elevated.

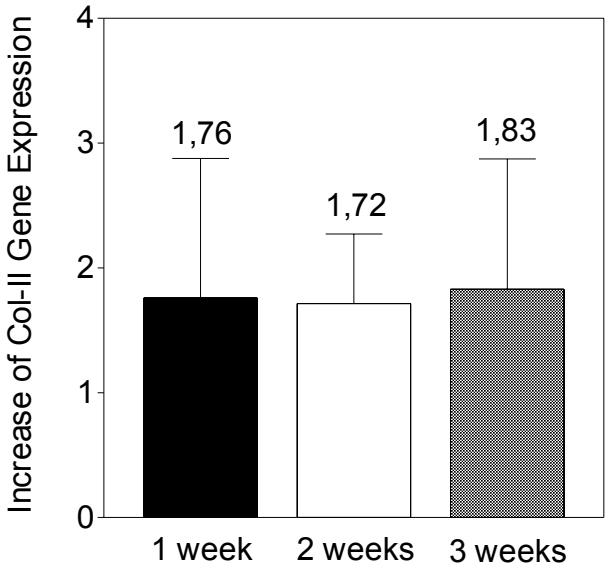
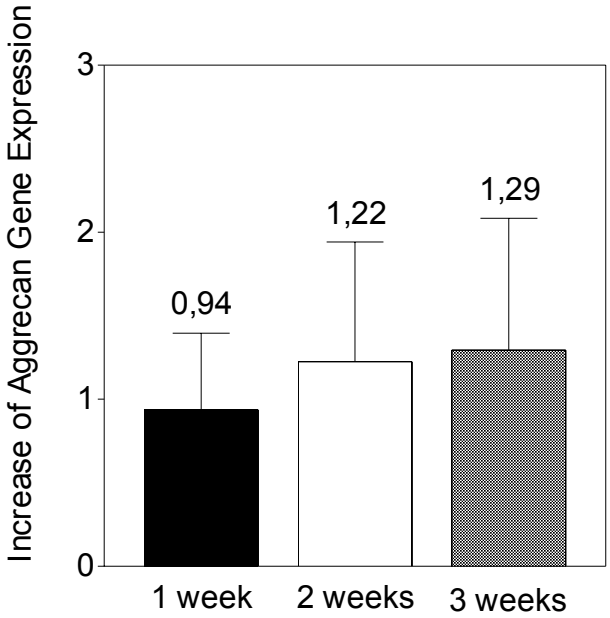
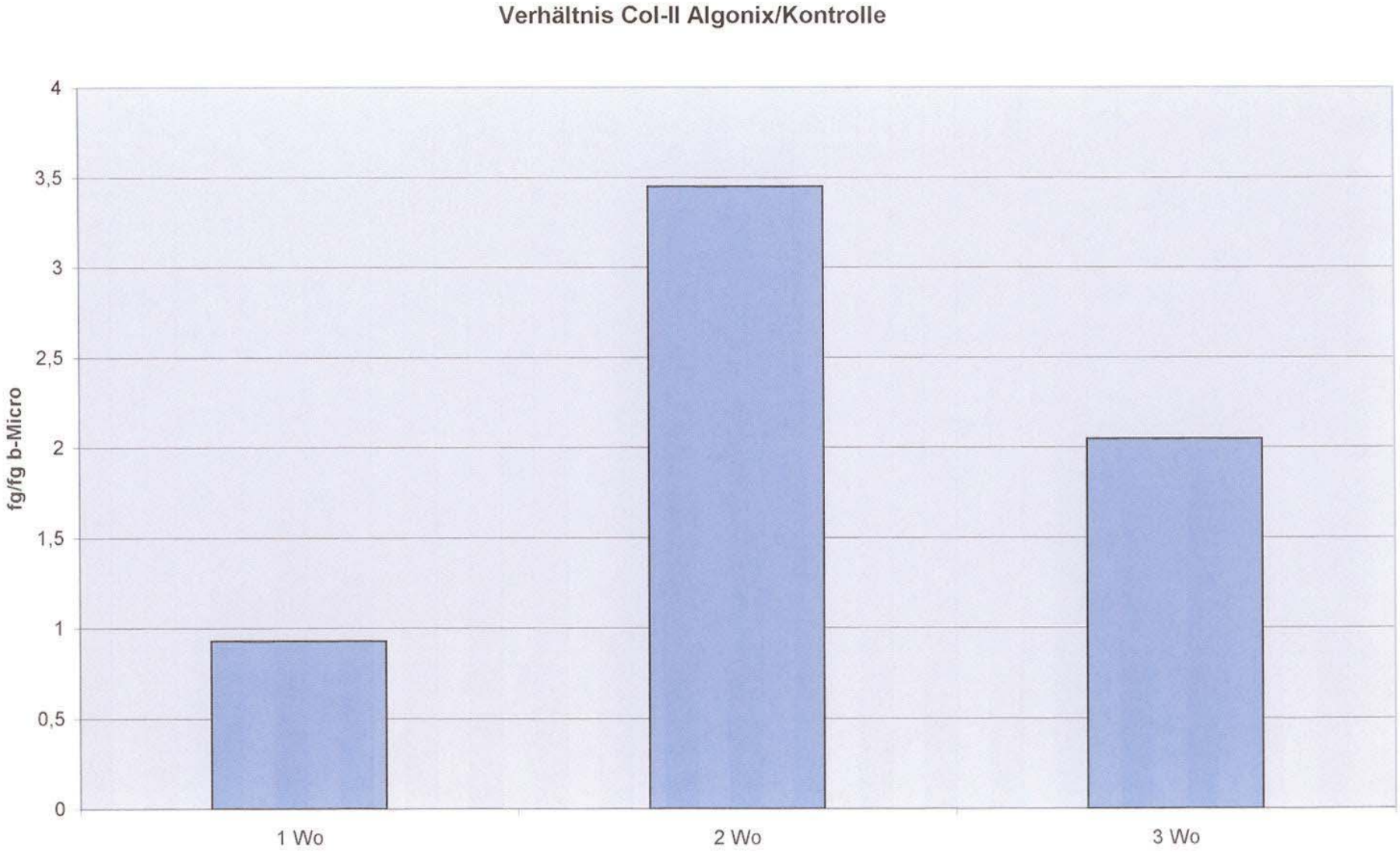


Fig.3: Real-time PCR analysis of col-II gene expression. Data are presented as relative col-II expression of stimulated samples in comparison to untreated samples of the same patients.

Real-time PCR examination of the aggrecan gene, another gene characteristic for chondrocytes in hyaline cartilage, confirmed the data obtained for collagen-II. Expression of the aggrecan gene was elevated after electromagnetical stimulation, and gene expression remained strong even after 3 weeks of cultivation (Fig.4).




Verhältnis Col-II Algonix/Kontrolle
fg/fg b-Micro
4
3,5
3
2,5
2
1,5
1
0,5
0
1 Wo
2 Wo
3 Wo

Conclusion

We demonstrated that millicurrent therapy directly stimulates cell metabolism of human articular chondrocytes in human explants in a selective way. Therefore, millicurrent treatment may support regeneration of affected cartilage and may be a helpful tool to complement conventional therapy.

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Authors' contributions
KG carried out the stimulation of the samples, the histological examinations and prepared the draft of the manuscript. BSR performed the statistical analysis. JS evaluated the millicurrent treatment protocol. US, SA and RMR participated in the study design and helped to draft the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests. The Arthro Nova Clinic was not involved in the funding of this study.

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