Cartilage Tissue Engineering:

Controlled Release of Growth Factors.

Effects of GDF-5, Sexual Steroid Hormons and Oxygen

Dissertation to obtain the Degree of Doctor of Natural Sciences

(Dr. rer. nat.)

from the Faculty of Chemistry and Pharmacy

University of Regensburg

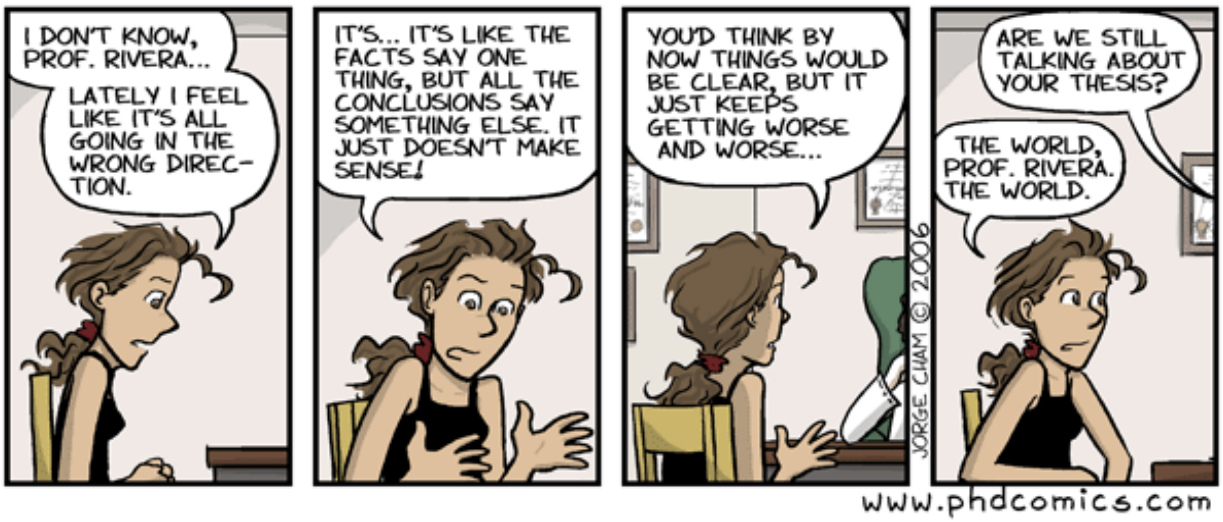
Presented by

Bernhard Appel

from München

September 2006

To my family and Maria



"Piled Higher and Deeper" by Jorge Cham

www.phdcomics.com

This work was carried out from August 2001 until Oktober 2005 at the Department of Pharmaceutical Technology of the University of Regensburg.

The thesis was prepared under supervision of Prof. Dr. Achim Göpferich.

Submission of the PhD. application :		21.08.2006
Date of examination :		20.09.2006
Examination board :	Chairman:	Prof. Dr. S. Elz
	1. Expert:	Prof. Dr. A. Göpferich
	2. Expert:	PD Dr. Peter Angele
	3. Examiner:	Prof. Dr. J. Heilmann

Table of Contents

Chapter 1

Introduction

Tissue engineering

On the quest for tissue and organ replacement, the usage of donated organs represents a first successful step, but is connected with strong side effects, e.g. the lifelong medical suppression of the immune system to prevent rejection of the allograft donor material. To overcome these disadvantages of allografts, a major target in transplantation medicine represents the utilization of autograft tissue material, harvested directly from the patient as primary or stem cells and prepared by tissue engineering (TE) methods for the desired application [1]. The methods of tissue engineering are based on simulation of the regular tissue growth and trauma healing processes utilizing artifical cell carrier systems, which can act as some kind of artifical extracellular matrix, morphogens like growth factors, which have the potential to retain cellular phenotype, increase growth rate and increase differentiation to a specific cell phenotype, and pre-cultivation under specific in vitro conditions and subsequent re-implantation.

Since the establishment of the field in the 1990 the number of cultivated tissue types, applicable biomaterials, morphogens and engineering techniques have increased rapidly, as well as the possible field of application in vivo and in vitro.

Articular cartilage biology

Articular cartilage consists of a small number of chondrocytes embedded in an extracellular matrix (ECM). Primary function of chondrocytes is to synthesize and maintain this ECM that is able to withstand physical deformation and facilitates tissue function [2]. The chondrocytes, which compose only 5% of wet weight of articular cartilage, furthermore orchestrate a balance between ECM synthesis and breakdown and, thus, regulate the normal tissue metabolism. Articular cartilage provides outstanding mechanical properties, as it is durable against countless numbers of repetitive strains. This durability results from the special composition of ECM consisting of collagen fibers, proteoglycans and embedded water, the latter of which accounts for approximately three-quarter of wet weight. The cross-linked and interconnected collagen fibrils consist mainly of collagen type II and some minor fractions of collagen type IX and XI. The proteoglycans are dominated by aggrecan, a highly glycosylated protein. Aggrecan consists mainly of glycosaminoglycan chains, which contain chondroitin sulfate and keratin sulfate. The aggrecans show high affinity to hyaluronic acid, a long polysaccharide chain molecule. The forming of these aggrecan-hyaluronic acid aggregates is

stabilized by link proteins. Further minor proteoglycans can be found in cartilage, namely decorin, biglycan and fibromodulin, which help to stabilize the matrix [3].

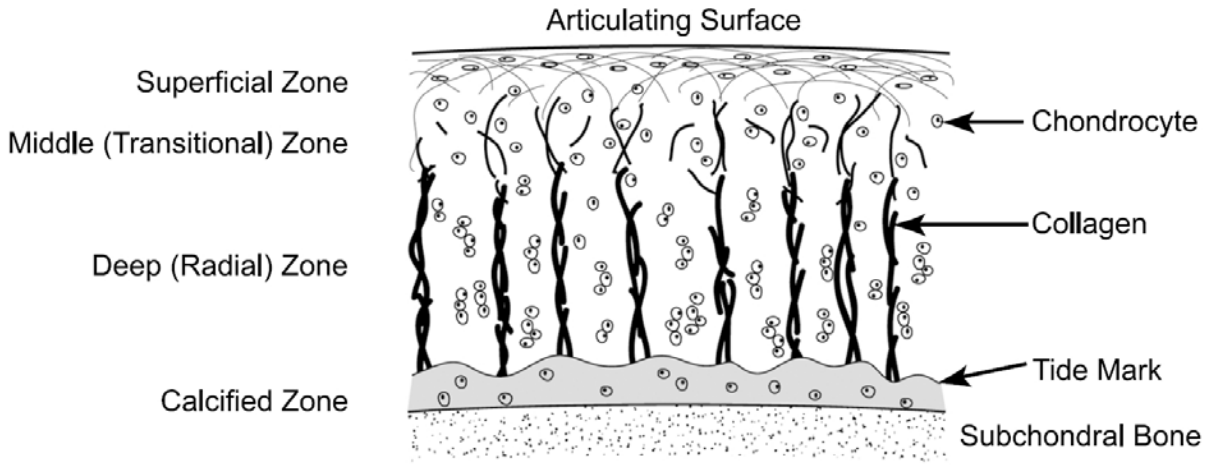


Fig. 1: Articular cartilage structure. Schematic representation of adult articular cartilage organization of matrix and cells into zones [4].

Articular chondrocytes form a 2 to 5 mm thick, gel-like, avascular white composite matrix tissue. This tissue is divided in three different zones, each with different morphology and arrangement of type II collagen fibers. The superficial zone (Fig. 1) has more flattened chondrocytes and collagen fibrils aligned parallel to the surface. Subsequently the transitional zone which is of the largest thickness shows rounded cells and transversely to the surface orientated collagen fibers. In the deep zone cells and collagen fibers are arranged perpendicular to the cartilage surface [3].

The supply of articular cartilage with nutrients and further metabolite exchange relies on diffusion from the articular surface, because the tissue is not vascularised. So the cell metabolism is adapted to a low oxygen tension (between 7-10% at the surface to below 1% in the deep layers). As a result the energy generation comes from glycolysis and so the chondrocytes do not contain abundant mitochondria [2]. Even so, the cells can be remarkably active synthetically. The adaption to low oxygen tensions seems to be regulated by expression levels of several factors, e.g. hypoxia inducible factor (HIF) [5] and TGF-β [6].

Articular cartilage in focus of TE

The biology and anatomy of articular cartilage is characterized by a low cell density, avascularity and high extracellular matrix content. It is a highly specialized tissue with only limited capacity for self repair. Articular cartilage undergoes degradation processes in response to e.g. metabolic, genetic and mechanical disorders as well as mechanical trauma [3] and this is often connected with constant deep pain and restriction in motion of the patients. A generally progressive loss and degeneration of cartilage is known as osteoarthritis (OA) and in most severe cases lead to joint deformities and subluxations [7, 8, 8]. The prevalence of OA increases with age, as more than one third of people over 45 years report joint symptoms such as intermitted aching up to permanent pain or occasional joint stiffness [8]. Of people over 65 years more than 75% have OA [7]. But even young people suffer from secondary osteoarthritis in consequence of e.g. joint trauma from accidents. Therefore, the wide prevalence of articular cartilage diseases makes therapeutic treatments eligible which not only relieve pain but lead to a healing of the morbid cartilage tissue. Some first commercial products for the tissue engineering of cartilage have reached the market (Carticel ®, Hyalograft C ®) yielding promising results in repair of punctual defects of the articular cartilage surface. First studies reported from acceptable long-term results monitored by magnetic resonance imaging (MRI) observation with a subjective improvement rate of about 81% [9, 10].

But most basic questions and problems for tissue engineering of cartilage still remain unanswered or unsolved. They need to be answered to produce a tissue that fully regenerates the natural zonal organization of articular cartilage, which is still not achieved even after several months in vivo [11]. The main points are the polymer material used for scaffolding, the type of cells used for TE (differentiated or progenitor), the use of suitable growth factors to influence cellular development and an improvement in integration of the newly formed tissue implants into the existing natural cartilage tissue. The usage of growth factors or other morphogens can be improved by controlled release devices [12, 13], which might also function as cell carrier matrix, e.g. smart polymeric scaffolds loaded with growth factors, or as a pure release device within the synovial joint [14].

A further problem can be seen in the limitation in the number of harvestable cells to form autologous TE implants. So an approach to overcome this problem consists in the prior proliferation of harvested cells in a 2-D culture, which commonly results in cell dedifferentiation rendering the cells less suitable for tissue engineering purposes [15].
In general, the limited capacity of self repair paired with the reduced nutrient consumption makes cartilage an interesting tissue material for tissue engineering [16, 17]. Furthermore, as a non-vascularized tissue consisting of only one cell type, articular cartilage is likely a major candidate to be successfully regenerated by tissue engineering methods.´

Goals of Thesis

In this thesis three major problems in the field of tissue engineering of cartilage were focused on in an established bovine cartilage culture [18]. Firstly, the suitability of controlled release devices for long-term delivery of protein growth factors was determined. Secondly, morphogens were investigated with regard to their ability either to enable the redifferentiation of expanded chondrocytes and the concurrent generation of relevant cartilaginous constructs or to improve extracellular matrix composition of constructs made from primary chondrocytes. As a third aspect, the influence of differential oxygen partial pressure on the development of tissue engineered cartilage was investigated.

Drug delivery systems in tissue engineering

The method of delivery of morphogenetic effectors like growth factors to the cells draws increasing attention in the field of tissue engineering [12], as the emulation of natural growth factor production could lead to improved developing or regenerating tissues [19]. However, the administration and utilization of protein growth factors is limited by their short half-lives at in vivo application [20]. The release of protein growth factors from controlled release devices is further limited by a loss of bioactivity during production of the device. This loss of activity results from denaturation and deactivation of protein during the formulation process with a polymer matrix [12]. This makes protein carriers desirable, which could ensure a sustained release and at the same time retain the biological activity of the growth factors [21]. Within our research group, recently a lipid-based controlled release matrix system was

developed in which insulin was incorporated as model growth factor [22]. These lipid matrices were investigated with regard to the release of bioactive insulin in our chondrocyte TE cell culture (**chapter 3**).

Furthermore, smart cell carriers are desirable, which combine the function of an artificial extracellular matrix ensuring cell attachment and the release of adequate growth factor concentrations at the site of action over an extended period of time. Recently, in our group the manufacturing of macroporous scaffolds using the newly developed lipid-templating technique has been demonstrated [23, 24]. These macroporous scaffolds were also loaded with insulin and tested in our chondrocyte TE cell culture with regard to the development of cartilaginous constructs (**chapter 4**).

Morphogens for cartilage tissue engineering

The second aim of this thesis was to investigate the effects of morphogens on the development of TE cartilage constructs. Growth and differentiation factor-5 (GDF-5) is known as a prominent growth factor in the embryonic precartilaginous mesenchymal condensation and in digit and limb formation [25-28, 28]. The influence of GDF-5 alone or in combination with insulin on the development of TE cartilage constructs either made from primary or from expanded chondrocyte was investigated in this thesis (**chapter 5**).

As articular cartilage is known as a steroid hormone-sensitive tissue, intensive research has been previously done on the role of estrogen and androgens and other steroids on the growth plate cartilage and longitudinal bone growth [29-31]. In cartilage engineering, only little is known about the potential of steroid hormones. Therefore, the effects of testosterone, estrogen and dehydroepiandrosterone on the development of cartilage TE constructs were investigated (**chapter 6**).

Oxygen conditions in tissue engineering of cartilage

Despite the fact that in articular cartilage an oxygen partial pressure of 10% and lower is observed, most cell culture experiments including cartilage engineering approaches are conducted using an oxygen concentration of 21%. As a third aspect of this thesis, the effects

of differential oxygen partial pressure (5% and 21% O_2) on the development of cartilage TE constructs were investigated. These experiments were combined with the effects of morphogens like insulin and GDF-5 (see chapter 5) and sexual steroid hormones (see chapter 6) (**chapter 7**).

Chapter 2

Materials and Methods

Materials and Methods

Lipid matrices production (chapter 3)

For the aseptic production of insulin-loaded matrices, Dynasan 116 (glycerol tripalmitate), a kind gift from Sasol (Witten, Germany), was sterilized for 2 hours at 160°C. Subsequently crystallization of the lipid in the stable beta-orientation was achieved by tempering the molten lipid for 3 days at 55°C. Lipid modification was investigated by scanning calorimetry using a 2920 differential scanning calorimeter (TA Instruments, Alzenau, Germany) at a heating rate of 5 K/min. The lipid was powdered in a mortar and sieved through a sieve with a pore size of 106 µm under aseptic conditions. For the preparation of the desired insulin/lipid powder mixtures, 58.84mg human insulin (Sanofi-Aventis, Frankfurt/Main, Germany) were dissolved in 300 µl 0.01N HCl (Carl Roth, Karlsruhe, Germany) and 11 ml double-distilled water were added. The solution was filtered through a 0.22 µm filter (Corning, Schiphol-Rijk, Netherlands) and the concentration of insulin was determined by HPLC (see insulin measurements). Calculated amounts of the insulin solutions required for the desired insulin/lipid ratios were added to mortars filled with sterile Dynasan 116® (glycerol tripalmitate). The mixtures were freeze-dried in a desiccator, cooled with dry ice, and evacuated using a RV5 two-stage pump (Edwards, Crawley, Sussex, UK) for 24 hours under aseptic conditions. The dried powder mixtures were manually homogenized in a mortar. For the manufacture of lipid matrix cylinders, a set of 2 mm diameter cylindrical punches and a die were machined from hardened steel and V4A steel, respectively. Cylindrical matrix discs (2 mm height, 2 mm diameter) were obtained by manual compression of accurately weighed amounts of the insulin-loaded lipid powder in the die, applying a force of approximately 250 N for 10 s. Cylinders with an average weight of 6.3±0.60 mg were used for the experiments. The matrices were visualized by scanning electron microscopy (SEM). For this procedure, samples were mounted on aluminum stubs with conductive carbon tape and coated with gold-palladium. All micrographs were obtained at 10kV on a DSM 950 (Zeiss, Oberkochen, Germany).

Solid lipid templated scaffolding (chapter 4)

The scaffolds (chapter 4) were prepared by a recently developed anhydrous solid lipid templating technique using solid lipid microparticles as porogens as previously described [23, 24]. Solid lipid templating combines the principles of phase separation and porogen leaching to generate spongy scaffolds [24]. In brief, Resomer RG756 (PLGA), synthesized from 75% lactic acid (LA) and 25% glycolic acid (GA), kindly provided by Boehringer Ingelheim (Ingelheim, Germany) was dissolved in ethylacetate. If applicable an appropriate amount of crystalline bovine insulin (Sanofi-Aventis, Frankfurt, Germany) was suspended in the RG756 solution. Solid lipid microparticles were further suspended under ice cooling with the polymer solution. The homogeneous dispersion was transferred into Teflon molds (1.9 cm x 1.9 cm x 1.2 cm) with a cylindrical cavity of 0.8 cm in diameter. After a pre-extraction treatment step in *n*-hexane at 0°C, the molds were submerged in warm *n*-hexane to induce solvent extraction followed by the precipitation of the polymer and extraction of the lipid porogen. The resulting porous, cylindrical polymer constructs were allowed to cool in cold (0°C) *n*-hexane and were removed from the molds. After drying under vacuum for 48 h, the constructs were cut into 2 mm slices, which were then designated as scaffolds.

Insulin measurements (chapter 3 and 4)

The drug loading of the matrices (chapter 3) and the scaffolds (chapter 4) was determined by HPLC. Matrices/scaffolds were dissolved in 600 µl chloroform (Merck, Darmstadt, Germany) each and insulin was then extracted with 600 µl 0.01N HCl (Carl Roth, Karlsruhe, Germany). After 10 s of mixing on a vortex mixer (Genie-2, Scientific Industries, NY; USA), phase separation was achieved by letting the mixture settle for 10 minutes. This procedure was repeated two times and followed by centrifugation at 1600 g (5415R, Eppendorf, Hamburg, Germany).

The insulin content of the aqueous phase was analyzed by HPLC analysis, using an HPLC system with a degasser (Knauer, Berlin, Germany), LC-10AT pump, FCV-10AT$_{vp}$ gradient mixer, SIL-10Ad$_{vp}$ autosampler, CTO-6a oven, SPD-10AV UV-Detector, RF-551 fluorescence detector and SCL-10A$_{vp}$ controller (all from Shimadzu, Duisburg, Germany).

100 µl of the insulin solutions were analyzed at 37°C using a C18-reversed phase precolumn (LC318, 4,6mmx20mm, Supelco, Bellefonte, USA) combined with an analytical C-18 reversed phase column (Supelcosil, LC318, 4.6 mm x 250 mm; Supelco, Bellefonte, USA) and a linear gradient method (mobile phase A: 90% H_2O, 10% acetonitrile, 0.1% TFA; mobile phase B: 90% acetonitrile, 10% H_2O and 0.1% TFA) with a flow rate of 1 ml/min. A linear gradient from 20% to 36% B over 24 min was applied. The chromatograms were recorded at wavelengths of 210 nm and 274 nm (UV-detection) and fluorescence detection was carried out at 274 nm excitation and 308 nm emission.

Cell culture media from the four-weeks trial (chapter 3) were collected at the times of media change every two to three days and frozen at -80°C. The insulin content was determined by enzyme-linked immunoassay (ELISA) in appropriate dilutions. The absorption was measured at 450 nm on a plate reader (Shimadzu, Duisburg, Germany). Possible adsorption of insulin on the surfaces of the well-plates was minimized by the use of FBS in the releasing cell culture media [32]. Previous studies employing two similar culture systems indicated that when exogenous insulin was applied at the time of media change and remaining insulin was assessed at the next medium change after 2-3 days, approximately 75-100% of the originally applied insulin were measured by ELISA [33].

Chondrocyte isolation

Knee joints from three-months-old bovine calves were obtained from a local slaughterhouse (EGN Vilshofen, Vilshofen, Germany or SBL Landshut, Landshut, Germany) within 6-12 hours of slaughter. Fresh articular cartilage was gained from the surface of the femoral patellar groove. The cartilage was cut into small pieces and primary chondrocytes were isolated by enzymatic digestion with collagenase type II, obtained from Worthington (CellSystems, St.Katharinen, Germany). The digest was filtered through a 149 µm filter (Spectrum, Rancho Dominguez, CA, USA)., centrifuged at 1200 rpm for 5 minutes, and washed three times with PBS (Gibco-Invitrogen, Karlsruhe, Germany) [16]. Isolated cells were resuspended in culture medium (DMEM) containing 4.5 g/l glucose, 584 mg/l glutamine, 10% FBS, 50 U/ml penicillin, 50 µg/ml streptomycin, 10 mM HEPES, 0.1 mM non-essential amino acids, 0.4 mM proline (all from Gibco-Invitrogen, Karlsruhe, Germany), and 50 µg/ml ascorbic acid (Sigma-Aldrich, Taufkirchen, Germany). The cell number was

determined by cell counting using a hemocytometer and an inverted phase-contrast microscope. Pipettes, petri dishes, falcon tubes, and well plates were purchased from (Corning, Schiphol-Rijk, Netherlands).

Cultivation under 21% and 5% oxygen partial pressure (chapter 6,7)

Cells were kept under an atmosphere of 21% O_2, 5% CO_2, and 74% N_2 in a CO_2-regulated incubator (Heraeus Instruments, Hanau, Germany) or under 5% O_2, 5% CO_2, and 90% N_2 in also an CO_2-regulated incubator (Heraeus Instruments, Hanau, Germany). For the reduced oxygen atmosphere a ready-made gas mixture was used containing 5% O_2, 6% CO_2, and 90% N_2 (Linde, Pullach, Germany). The slight increase in CO_2 from 5% to 6% partial pressure allowed the CO_2 sensor in the incubator adaptive control of gas concentration.

Chondrocyte expansion (chapter 5-7)

Primary chondrocytes were seeded in 150cm^2 cell culture flasks (7.500 cells/cm^2) (Corning, Schiphol-Rijk, Netherlands) and cultivated in medium described above to confluence over 13 days. The cells (passage 1) were washed three times with PBS and trypsinized with trypsin 0.25% (Gibco-Invitrogen, Karlsruhe, Germany), After centrifugation and resuspension in chondrocyte medium they were counted (see above, 10-fold increase in cell number) and seeded again in 150cm^2 cell culture flasks. After 8 days they were confluent again (passage 2 = expanded chondrocytes, 9-fold increase in cell number), harvested as described above and seeded onto the PGA scaffolds.

In vitro cell culture

Cell seeding (Fig. 2) was performed in self-made spinner flasks (250 ml volume, 6 cm bottom diameter, side arms for gas exchange). Polyglycolic acid (PGA) non-woven meshes (12-14 µm fiber diameter; 96% porosity; 62mg/cm^3 bulk density; initial molecular weight of PGA approx. 70 kDa) [34] were obtained from Albany International (Mansfield, MA, USA) and die-punched into discs 5 mm in diameter and 2 mm thick (scaffolds). Scaffolds were threaded onto needles (10 cm long, 0.5 mm diameter, from Unimed, Lausanne, Switzerland) and held

in place with small pieces of silicone tubing (1 mm long, from Cole Palmer, Niles, IL, USA),. Four needles with two scaffolds apiece were inserted into a silicone stopper (Schuber & Weiss, München, Germany), which was placed into the mouth of a spinner flask containing a magnetic stir bar. The cell suspension with $5*10^6$ isolated chondrocytes per scaffold (i.e., $40*10^6$ cells per flask) in 110 ml of culture medium was filled into the flask. Flasks were placed in an incubator at 37 °C, 21% O_2, 5% CO_2, and 74% N_2 in a CO_2-regulated incubator (Heraeus Instruments, Hanau, Germany) and 95% humidity; stirring with 50 rpm on a magnetic stirrer (Bellco, Vineland, NJ, USA) for two days allowed for cell attachment.

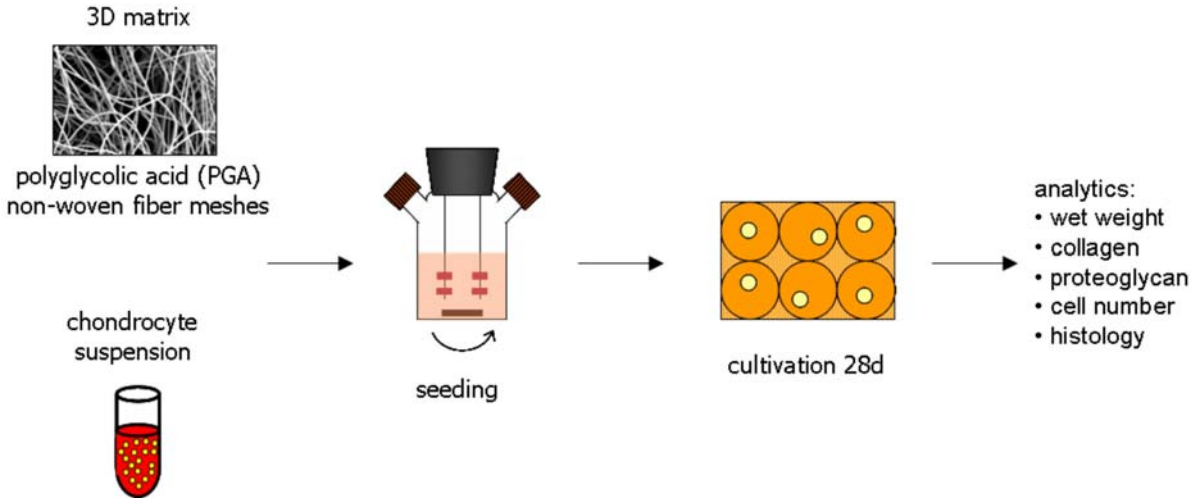


Fig. 2: Experimental tissue engineering set-up including seeding in spinner flask bioreactors and cultivation in well-plates.

Each scaffold was then placed in a 6-well plate (Corning, Schiphol-Rijk, Netherlands) filled with 6 ml of culture medium (same medium as above except for 1% FBS instead of 10%); plates were placed on an orbital shaker at 50 rpm (SO5, Stuart Scientific, Surrey, UK).

For the reduced oxygen culture conditions an additional incubator

After one day, morphogens were added to the wells and from then on with each medium change. In all groups, medium was replaced three times per week.

In vitro cell culture and controlled release of insulin

Cell culture with lipid matrices (chapter 3)

Two independent studies were conducted: In the first study, which was conducted over two weeks and which in the following is designated "two-weeks trial", cell-polymer constructs were cultured in the presence of matrices loaded with 2% (m/m) insulin; groups with unloaded matrices (0%) or no matrices served as controls. For further comparison, a fourth group was included receiving exogenous insulin at 2.5 µg/ml with each medium change (concentration eliciting maximum response based on previous studies [18]). Four individual constructs were cultured per matrices group, two constructs in each of the no-matrices groups. In the second study, conducted over four weeks and designated "four-weeks trial", cell-polymer constructs were cultured in the presence of matrices with three different loading concentrations of insulin (0.2%, 1%, 2% (w/w)) or unloaded matrices (0%). Four individual constructs were cultured per group.

Cell seeding and cell culture on solid lipid templated scaffolds (chapter 4)

The PLGA scaffolds were disinfected in 70% ethanol for 2 hours. Afterwards they were thoroughly rinsed three times in PBS. The treated scaffolds were transferred into bioreactors and pinned onto needles. To reduce effects from potential burst release of insulin from the scaffolds and for improved cell attachment scaffolds were rinsed in the bioreactors with complete culture medium under stirring for 24 hours. Subsequently, medium was changed and a cell suspension containing 5 x 10^6 chondrocytes per scaffold was added and stirred at 80 rpm in a humidified (37 °C / 5% CO_2) incubator for two days to allow for cell attachment to the polymers. Cell-polymer constructs were transferred into 6-well plates (one construct and 6 ml culture medium per well) and cultured for three weeks on an orbital shaker at 50 rpm. Medium was completely exchanged three times per week. Four independent constructs were cultured per group. Three different groups with different concentrations of insulin loading were investigated (designated as I0.1%, I0.7%, and I1.7%) and compared to an unloaded control group (control). In a further control group, unloaded scaffolds received exogenous insulin with each medium change at a concentration of 1.4 mg/ml, based on theoretical calculations of the maximum release rate from the insulin-loaded scaffolds (I1.4)

The concentration of exogenously applied insulin was calculated on the basis of the medium loading rate of 0.69% insulin (w/w) and an average scaffold mass of 11mg. The resulting loading mass of 76µg insulin per mesh was divided by 9 media changes during the culture period. The resulting mass of about 8.4µg insulin released between each media change divided by 6ml of cell culture medium gave a concentration of about 1.4µg/ml insulin that was taken as exogenous insulin control. In this calculation loss of insulin during the procedure of cell seeding in the bioreactor, burst release or incomplete release were not taken into account.

In order to control for a potential insulin burst release, in two further groups single insulin doses, either 10 or 200 µg/ml, were applied after transfer into the 6-well-plates (I10µ and 200µ). After that, these groups received only standard medium without insulin.

Biochemical analysis of the engineered tissues

Analytical assays were performed as previously described [18]. In brief, cell-polymer constructs were weighed (= wet weight, ww) and cut in half. One part was freeze-dried and digested by papain solution (3.2 U/ml in buffer) for 18 h at 60°C. The cell number was determined measuring the DNA content using Hoechst 33258 (Polysciences, Warrington, PA, USA) dye in a fluorometrical assay [35]. The sulfated glycosaminoglycan (GAG) content was determined spectrophotometrically at 525 nm as chondroitin sulfate (ICN, Aurora, Ohio, USA) after reaction with dimethylmethylene blue (Sigma-Aldrich, Taufkirchen, Germany) [36, 37]. Hydroxyproline content was measured spectrophotometrically at 550 nm after acid hydrolysis and reaction with chloramine-T (Merck, Darmstadt, Germany) and p-dimethylaminobenzaldehyde (Merck, Darmstadt, Germany) [38]. The total collagen amount could be calculated using a hydroxyproline to collagen ratio of 1:10 [39].

Histology of the engineered tissues

The constructs were fixed in 2% glutaraldehyde (Merck, Darmstadt, Germany) for 30 minutes and stored in 5% formalin. After fixation, the tissues were embedded in paraffin and sliced into 5 µm sections. The deparafinized sections were subjected to a hematoxylin, fast green and safranin-O staining (Sigma-Aldrich, Taufkirchen, Germany) [40].

Immunohistochemistry of the engineered tissues

The constructs were fixed in a mixture of methanol/PBS and 9% formaldehyde (Sigma-Aldrich, Taufkirchen, Germany). They were incubated in ascending concentrations of sucrose solution up to 40% and frozen in Tissue-Tek (Sigma-Aldrich, Taufkirchen, Germany). Frozen samples were cryosectioned at 10µm in a cryostat (HM550OMP, Microm, Walldorf, Germany). Dried sections were rehydrated in PBS containing 0.1% tween-80 (Sigma-Aldrich, Taufkirchen, Germany) (PBS/tween) and processed with a pepsin (Sigma-Aldrich, Taufkirchen, Germany) digestion. Non-specific antigen binding was blocked by a 5% normal horse serum in PBS. Primary antibody solution was spreaded on the slides and incubated overnight. Antibody solution was dissolved with PBS/tween in a ratio of 1:1000 for collagen type I antibody (col-1) (Sigma-Aldrich, Taufkirchen, Germany) and 1:6 for collagen type II antibody (CIIC1) as previously described [41], for control sections pure PBS containing 0.1% tween-80 was used. Anti-collagen type II antibody (CIICI) developed by Rikard Holmdahl and Kristofer Rubin was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD (University of Iowa, Department of Biological Sciences, Iowa City, USA). After a washing procedure slides were incubated with a secondary biotinylated antibody for 30 minutes and washed with PBS/tween vigorously. Staining procedure was performed following the manufacturer operating procedures using a Vectastain ABC-kit and a DAB-kit for the avidin-biotin-peroxidase complexes formation. Secondary biotinylated antibody (BA-1400) anti-mouse anti-rabbit antibody, Vectastain ABC-kit and DAB staining-kit were from Vector Laboratories (Linaris, Wertheim-Bettingen, Germany).

Reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR analysis was performed as described elsewhere [42]. In brief, native and engineered cartilage blocks were frozen in liquid nitrogen and lyophilized. RNA was extracted using RNA-Bee (Biozol, Eching, Germany) according to the single-step acid-phenol guanidinium method. cDNA synthesis was performed by using Superscript RNase H-reverse transcriptase (Invitrogen, Karlsruhe, Germany) in the presence of oligo-dt primers. Primers were sequenced as detailed in [42]. RT-PCR was conducted using Light Cycler analysis with Light Cycler Kits (Roche, Mannheim, Germany). The level of each target gene was normalised to the reference gene glyceraldehyde phosphate dehydrogenase (GAPDH).

Statistical analysis

Statistical significance was assessed by one-way analysis of variance ANOVA in conjunction with Tukey's studentized range test using SPSS 12 for Windows from SPSS Software (Munich, Germany).

Chapter 3

Lipidic implants for controlled release of bioactive insulin: Effects on cartilage engineered in vitro [43]

Appel, B. *, Maschke, A. *, Weiser, B., Sarhan, H., Englert, C., Angele, P., Blunk, T., Göpferich, A.

*equally contributing authors

Published at Int. J. Pharm. 314 (2), 170-178 (2006)

Introduction

The field of tissue engineering [1] aims at the regeneration of mechanical and functional body tissue or organ defects that cannot be sufficiently cured by self-healing processes. One strategy in tissue engineering is to seed and culture cells on 3-D scaffold structures in vitro in order to generate tissue constructs for implantation. Cell proliferation and differentiation as well as the formation of an adequate extracellular matrix (ECM) in an in vitro culture largely depend on the supplementation of growth factors and other morphogens [20]. In addition growth factors can strongly improve the integration of the engineered tissue after implantation. These effects render growth factors an important tool for tissue engineering purposes, however, their efficacy is limited by their short half-lives and their potential toxicity at systemic levels [20]. To overcome these problems the use of protein carriers that ensure a sustained release and at the same time retain the biological activity of the growth factors is desirable [21]. Unfortunately, protein stability is easily compromised during the manufacture, storage, and drug release (Schwendeman et al., 1996). For example, for the well established biocopolymer poly(lactic-co-glycolic acid) (PLGA) it has been demonstrated that degradation products from the release matrix can influence protein stability due to changes in the microclimate of the microspheres during degradation, e.g., higher osmotic pressure or acidic environment [44, 45]. In order to overcome such problems, stabilizing additives were introduced such as $Mg(OH)_2$ [46], $Ca(OH)_2$ [47], and, especially for insulin, zinc salts [48, 48-50]. The latter was additionally used to prolong the release of insulin [51] [32] [52]. As an alternative approach, controlled release systems based on lipids as a matrix material have recently attracted increasing attention, as they avoid detrimental effects of breakdown products of the biomaterial [53-55]. However, the processes used for the production of a lipid matrix often include organic solvents likely resulting in organic-water interfaces, which in turn are known as destabilizing factor for proteins [56]. We recently developed cylindrical matrices based on solid triglycerides, especially designed for the purpose of a long-term release [57]. For the production of these protein-loaded matrices neither emulsions with organic solvents, surfactants nor ultrasonification are needed, which in other systems may lead to a loss of bioactivity of the incorporated proteins [22]. These matrices may not only be of major interest in the field of tissue engineering, but also can be loaded with proteins and

other types of drugs for the local treatment of tissues such as needed in the treatment of brain cancer [57] or neurodegenerative CNS diseases.

Previously, we established a 3-D cartilage engineering culture (Fig. 1) that can be utilized as a test system for sustained-release carriers [18].

Readily available insulin is used as a model protein; insulin was demonstrated to have strong anabolic effects on engineered cartilaginous constructs similar to those of insulin-like growth factor-I (IGF-I). The model provides quantifiable data and responds sensitively to supplemented insulin in a dose-dependent manner over a cultivation period of several weeks [18]. Even if sustained-release carriers are typically applied in an in vivo situation, this 3-D culture offers the opportunity to evaluate newly developed release systems with regard to their effects within a defined tissue engineering setting.

In this study, insulin-loaded triglyceride matrices were manufactured in order to investigate the biological effects of released insulin in the 3-D cartilage engineering culture. The first specific aim was the determination of the release kinetics of matrices with varying amounts of incorporated insulin. Further specific aims were the analysis of the effects of released insulin on the tissue construct weight, cell number, and amounts of ECM components, namely glycosaminoglycans and collagen, within the engineered tissue.

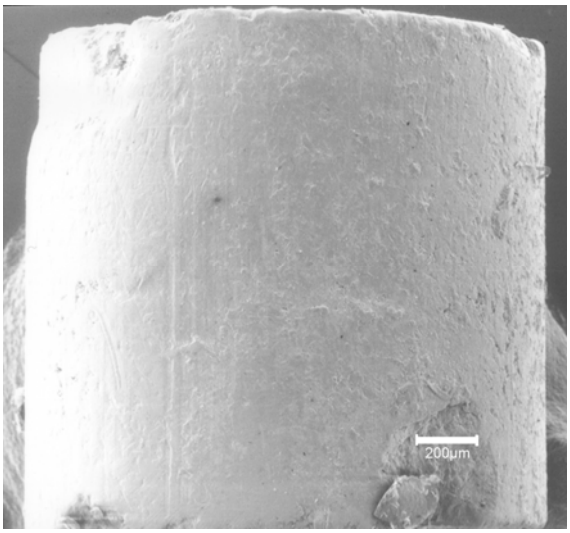
Results

Lipid matrices

The manufactured insulin-loaded matrices had a well-defined cylindrical geometry**.** Examination by scanning electron microscopy revealed a generally smooth surface (Fig. 3A). After sterilization and tempering, differential scanning calorimetry analysis showed only the endothermic peak caused by the melting of the stable β-modification of glycerol tripalmitate (same peak as before treatment) indicating that complete crystallization of the lipid in the stable orientation was achieved (Fig. 3B).

Insulin loadings of the matrices were 0.20% (w/w) ± 0.003, 1.08% ± 0.043, and 2.08% ± 0.114, as determined by HPLC, and correlated well with the intended and designated insulin contents of 0.2%, 1%, and 2%, respectively.

(A)



(B)

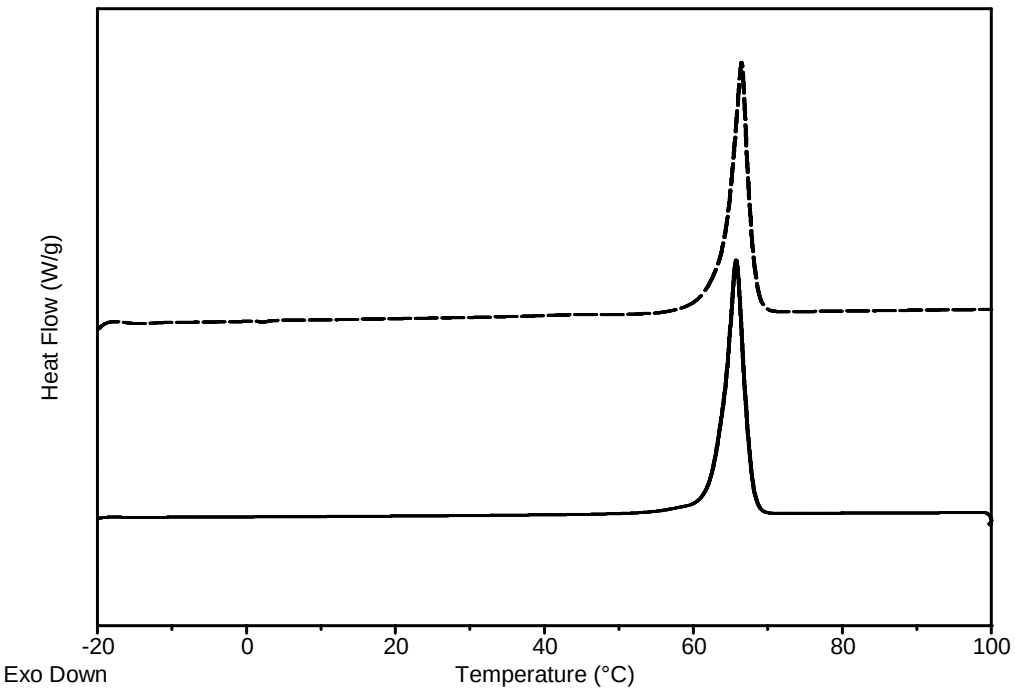


Fig. 3: (A) Scanning electron microscopy image of the manufactured cylindrical matrix, loaded with human insulin. (B) Determination of crystallinity of untreated glycerol tripalmitate (-) and sterilized and tempered glycerol tripalmitate (- -) by differential scanning calorimetry. The thermograms were recorded at a heating rate of 5K/min. Only the endothermic peak for the melting of the stable β-modification of glycerol tripalmitate was detected before and after treatment, indicating that complete crystallization in the stable modification was achieved also after treatment.

Two-weeks trial

In the two-weeks trial, a significant 1.9-fold increase in wet weight was detected for the cell-polymer constructs cultured for two weeks in the presence of the insulin-loaded matrices (2%) (68 mg), as compared to the control group with unloaded matrices (35.5 mg) (Fig. 4).

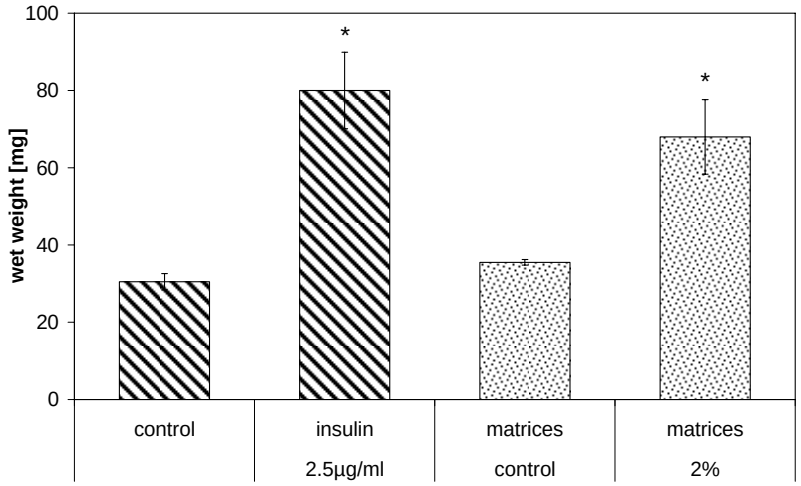


Fig. 4: Effects of unloaded and insulin-loaded lipid matrices on the wet weight of tissue engineered cartilage in the two-weeks trial. Statistically significant differences to the control and matrices control (unloaded matrices) are denoted by * ($p<0.05$). Data represents the average ± SD of four independent measurements for the matrices groups and two independent measurements for the control and insulin 2.5µg/ml groups.

The wet weight of the unloaded matrices group was not significantly different from the control group with no matrices. The constructs receiving exogenous insulin (2.5 µg/ml) showed similar wet weights as the constructs cultured in the presence of the insulin-loaded matrices (Fig. 4).

The insulin-loaded matrices also significantly increased the absolute amounts of the ECM components GAG and collagen in the cell-polymer constructs (2.4-fold and 3.2-fold, respectively) and the GAG and collagen content per cell (1.8-fold and 2.5-fold, respectively), as compared to the control group with unloaded matrices (Table 1). The cell number per wet weight was decreased to 67% (Table 1); a lower cell density is commonly observed in more mature engineered cartilaginous constructs [18]. All values obtained for the constructs grown in the presence of the insulin-loaded matrices were on the same order of magnitude as the values obtained for the constructs receiving exogenous insulin (Table 1).

	control	insulin 2.5µg/ml	matrices control	matrices 2%
GAG [mg]	1.2±0.47	2.7±0.20 *	1.2±0.27	2.9±0.63 *
GAG per cell [pg]	181±35.9	347±54.2 *	200±10.1	368±47.7 *
collagen [mg]	0.39±0.113	1.3±0.24 *	0.36±0.047	1.2±0.34 *
collagen per cell [pg]	63.1±4.09	171±8.1 *	58.0±1.93	144±29.0 *
cell number per wet weight [1/µg]	202±31.4	96.8±10.27 *	174±25.3	117±11.3 *

Tab. 1: Effects of unloaded and insulin-loaded lipid matrices on tissue-engineered cartilage in the two-weeks trial. Data represents the average ± SD of four independent measurements for the matrices groups and two independent measurements for the control and insulin 2.5µg/ml groups. Statistically significant differences to the control and matrices control (unloaded matrices) are denoted by * ($p<0.05$).

For all parameters investigated, no significant differences were detected between the two control groups cultured in the absence of matrices or in the presence of unloaded matrices (Table 1).

The histological analysis of the constructs correlated well with the obtained quantitative biochemical data. All cross-sections of the constructs appeared to be cartilaginous with round chondrocytes in lacunae surrounded by large areas of extracellular matrix deposition. However, the control group constructs cultured without insulin had a smaller, more fractured appearance compared to those of the insulin groups. Constructs from the groups cultured either in the presence of insulin-loaded matrices or exogenous insulin showed a more regular and coherent GAG distribution when stained red with safranin-O (data not shown).

Four-weeks trial

Insulin release

Released insulin was sampled at the times of media change directly from the well plates of the cell culture of the four-weeks trial. Thus, the collected insulin had been released over a period of two to three days. Over the first 3 days, a small burst release was observed for matrices 2% (10.3%), matrices 1% (23.8%), and matrices 0.2% (21.6%). Within the first 12 days, the matrices continuously liberated up to 67% (matrices 2%), 89% (matrices 1%) and 99% (matrices 0.2%), respectively, of the total loaded protein (Fig. 5A). Insulin concentrations at the times of media change within the first 12 days were between 0.4 and 5.7µg/ml for matrices 2% (day 12, day 8), between 0.9 and 3.4 µg/ml for matrices 1% (day 12, day 8), and between 0.01 and 1.0 µg/ml for matrices 0.2% (day12, day 8) (Fig. 5B).

Generally, after day 12, lower amounts of insulin were released. However, at the times of media change the insulin concentrations were still above 0.005 µg/ml in the groups of the matrices 1% (except for day 19, 0.004 µg/ml) and matrices 2% (except for day 24, 0.004 µg/ml) (Fig. 5B). Even on day 29, the last day of this study, insulin concentrations of 0.019 µg/ml and 0.025 µg/ml were observed for matrices 1% and matrices 2%. In contrast, insulin concentrations in the group of the matrices 0.2% were always below 0.002 µg/ml after day 17 (Fig. 4B).

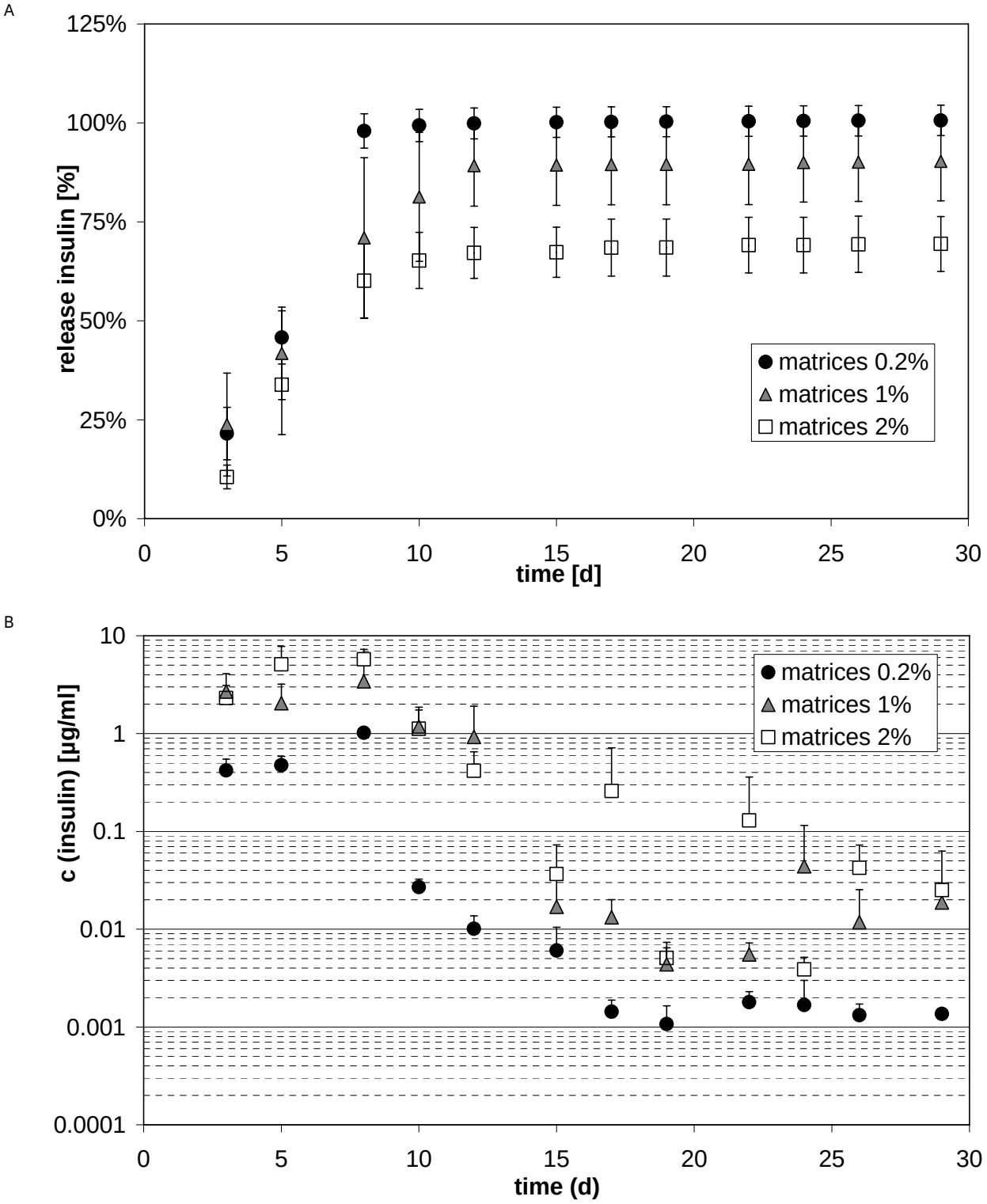


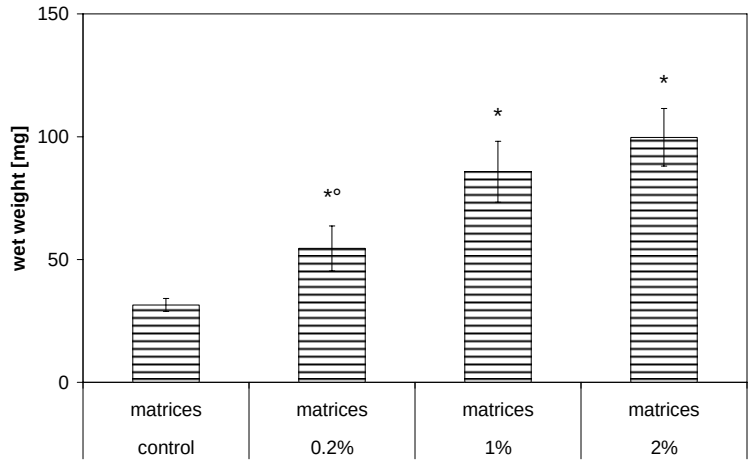
Fig. 5: (A) Cumulative release of insulin from lipid matrices in the four-weeks trial under cell culture conditions determined by ELISA. (B) Insulin concentrations measured in the cell culture medium at the time of media change every second or third day determined by ELISA. Data represents the average ± SD of four independent measurements.

Wet weights and cell number

All cell-polymer constructs cultured in the presence of insulin-loaded matrices exhibited significantly increased wet weights after four weeks of culture (54.5 – 100 mg), as compared to control constructs cultured in the presence of unloaded matrices (31 mg). The dose-dependent increases of the matrices groups were 1.7-fold, 2.7-fold, and 3.2-fold, respectively. Values for the matrices 1% and 2% were significantly higher than those for matrices 0.2% (Fig. 6A).

The cell number per wet weight was reduced in all constructs receiving released insulin, as compared to the constructs receiving no insulin. A dose-dependent trend was observed with lowest cell densities for the highest insulin loading (Fig. 6B).

A



B

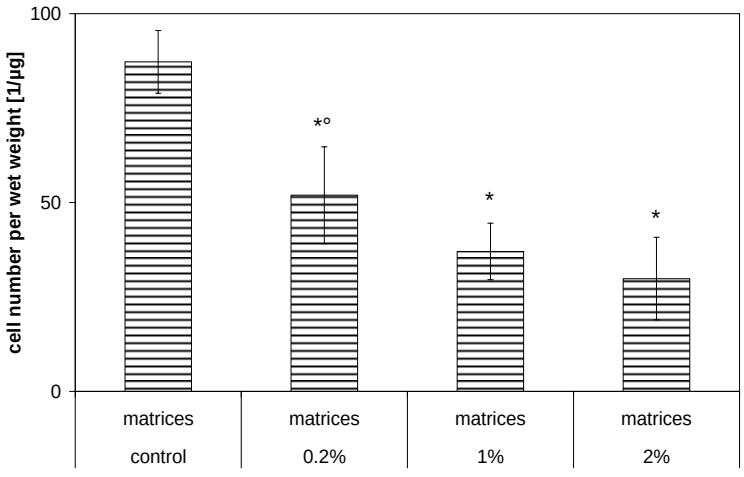


Fig. 6: Effects of insulin-loaded matrices on the wet weight (A), and the cell number per wet weight (B) of engineered cartilage after 28 days in the four-weeks trial. Data represents the average ± SD of four independent measurements. Statistically significant differences to the matrices control (unloaded matrices) are denoted by * , to the matrices 2% group by °($p<0.05$).

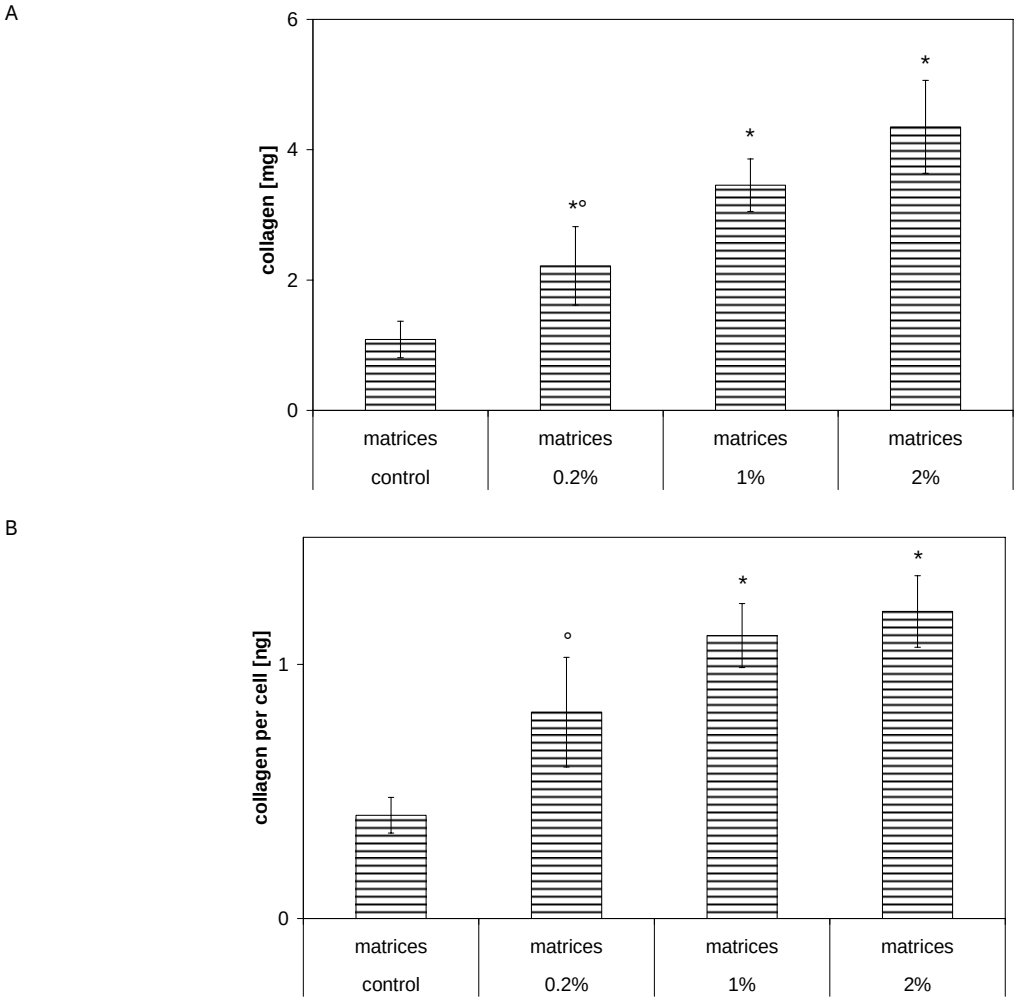


Fig. 7: Effects of insulin on the absolute amount of collagen per construct (A), and on the amount of collagen per cell in engineered constructs (B) in the four-weeks trial. Data represents the average ± SD of four independent measurements. Statistically significant differences to the matrices control (unloaded matrices) are denoted by *, to the matrices 2% group by ° ($p<0.05$).

Collagen and glycosaminoglycans

Insulin released from the lipid matrices dose-dependently increased the absolute amount of collagen within the cell-polymer constructs. Increases were between 4-fold (4.4 mg for matrices 2%) and 2-fold (2.2 mg for matrices 0.2%), as compared to constructs receiving no insulin (1.1 mg) (Fig. 7A). The collagen content per cell was also increased by released insulin; an almost 3-fold increase was observed for matrices 2% (Fig. 7B).

Similar observations were made for the GAG content: Absolute amounts of GAG were increased in the matrices 1% and 2% groups (up to 3.5 mg), as compared to the unloaded matrices group (1.2 mg). The matrices 0.2% only led to a slight, but not significant increase

(Fig. 7A). The same applied to GAG content per cell, which was increased 2.2-fold in the matrices 1% and 2% groups (Fig. 7B). For all parameters, no significant differences could be detected between the matrix 1% and matrix 2% group (Fig. 7, Fig. 8).

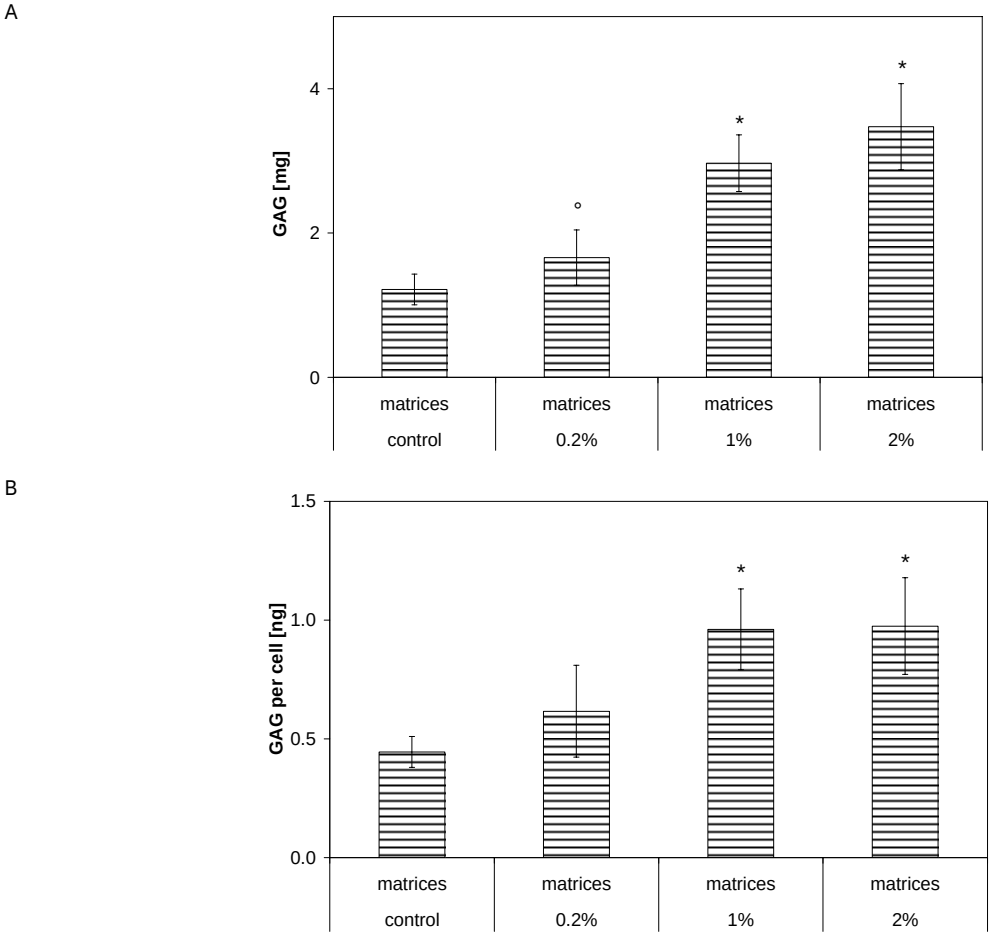


Fig. 8: Effects of insulin on the absolute amount of glycosaminoglycans (GAG) per construct (A), and on the amount of GAG per cell in engineered constructs (B) in the four-weeks trial. Data represents the average ± SD of four independent measurements. Statistically significant differences to the matrices control (unloaded matrices) are denoted by *, to the matrices 2% by ° ($p<0.05$).

Histology

Histological cross-sections showed an increasing construct size with increasing insulin content of the matrices correlating with the construct weights (Fig. 6A, Fig. 9). In all cross-sections, round chondrocytes in lacunae surrounded by large areas stained red with safranin-O for GAG were detected. However, the constructs grown in the presence of matrices 1% and 2% exhibited a more even distribution of GAG, as compared to the constructs of groups with matrices 0.2% and unloaded matrices, which showed an irregular GAG distribution with distinct areas containing no safranin-O stain (Fig. 9).

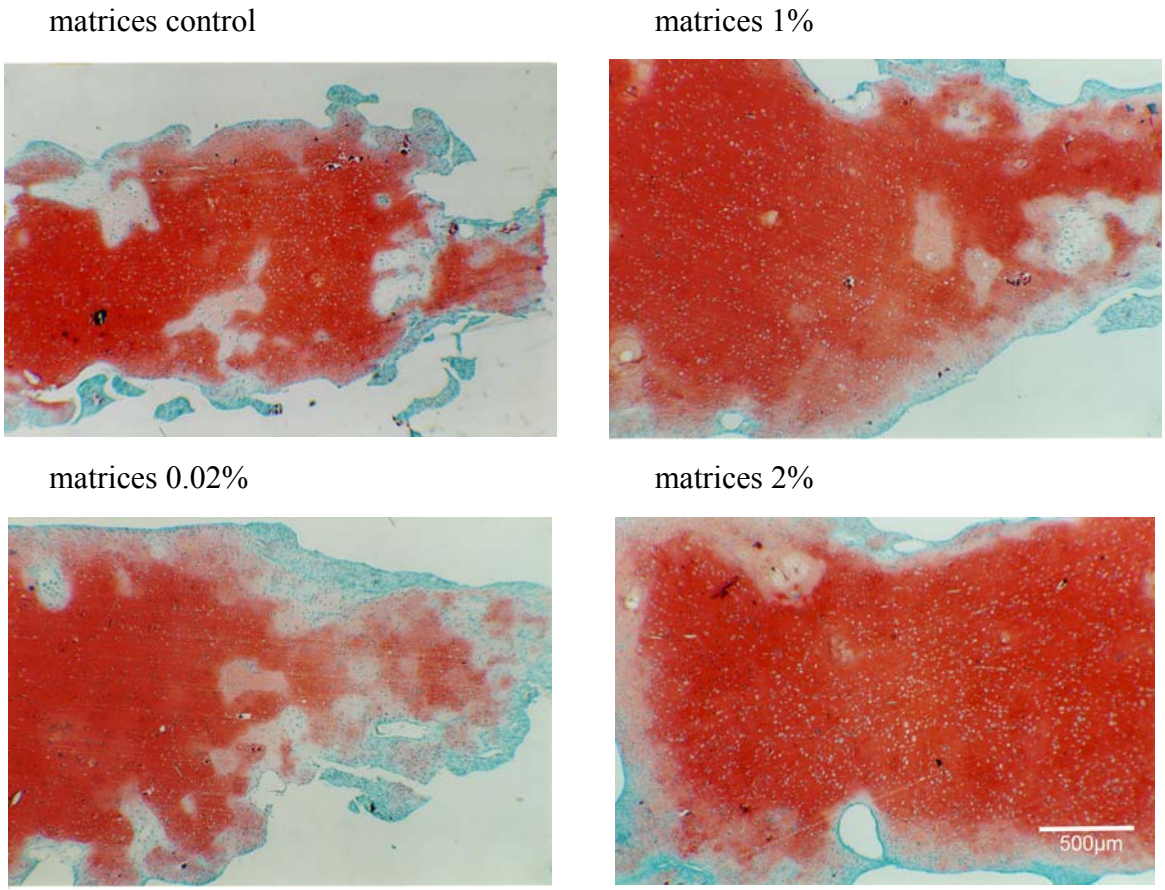


Fig. 9: Glycosaminoglycan (GAG) distributions in cross-sections of tissue-engineered constructs. GAG was stained red with safranin-O (appears dark gray in the black & white print).

Discussion

Growth factors, cytokines and morphogens are powerful protein molecules that, if adequately released from carrier systems, potentially play a key role in many tissue engineering applications. Unfortunately, in controlled release studies in general, processing during the manufacture of the carriers, storage, and the release itself often strongly compromise protein stability [58]. Therefore, in the development of new release systems it is paramount to determine not only the release kinetics by standard assays such as ELISA, but also to determine the bioactivity of the released protein in a relevant environment. In this study, recently developed cylindrical matrices were investigated with regard to the biological effects of released protein. The lipid devices require neither stabilizing additives within the matrix nor possibly detrimental organic-water interfaces during the production process. Insulin served as model protein and its effects were determined in a recently established cartilage engineering test culture [18].

Cylindrical lipid matrices were produced from glycerol tripalmitate by manual compression with an insulin load of 0.2%, 1, or 2%. As a possible change in lipid modification could have an impact on release characteristics, the lipid matrices were subjected to differential scanning calorimetry analysis. It was demonstrated that no detrimental effects occurred during processing; the glycerol tripalmitate showed crystallization exclusively in the stable β-modification before and after treatment.

In the two-weeks trial, distinct beneficial effects on tissue construct development were observed elicited by released insulin. Increases in construct wet weight and amounts of ECM components, as compared to controls receiving no insulin, were on the same order of magnitude as for constructs in the group receiving exogenous insulin at a concentration of 2.5 µg/ml (Fig. 4, Tab. 1); this concentration was previously shown to elicit the maximum response in the same culture system [18]. Furthermore, the constructs grown in the presence of the empty control matrices were not significantly different from the control constructs cultured in the absence of any matrices only in basal medium, indicating that the lipid matrices themselves had no effects on tissue construct development (Fig. 4, Tab. 1).

Therefore, in the four-weeks trial the control group cultured only in basal medium was omitted; the insulin-loaded matrices were compared to unloaded controls.

For all three different insulin loadings, an insulin burst release of only 10-24% was detected within the first three days (Fig. 5A). In preliminary experiments, we could show that high concentrations of exogenously applied insulin (up to 50 µg/ml) only during the first days of culture, i.e. simulating a burst release at concentrations by far exceeding the concentrations reached in the presented study, resulted in distinctly inferior constructs compared to a continuous supply of insulin at concentrations below 0.01 µg/ml over 21 days (data not shown). Within the presented study, in general, higher amounts (>0.4 µg/ml) of insulin were released during the first twelve days from the matrices with higher loadings and during the first eight days from the matrices with the lowest loading. Although the released amounts dropped afterwards, for the matrices 1% and 2% still concentrations above 0.005 µg/ml of insulin were achieved (Fig. 5B). In preliminary experiments, 0.005 µg/ml was the minimum insulin concentration eliciting a biological response in the 3-D cartilage engineering culture (data not shown). In the group cultured in the presence of the matrix with the lowest loading, insulin concentrations were always distinctly below this concentration after day 17 (Fig. 5B).
The measured concentrations of the released insulin were well reflected by the effects on the quality of the engineered cartilage constructs. The culture in the presence of the lipid matrices 0.2% resulted in significant increases of the wet weight and collagen content of the constructs, furthermore in a beneficial decrease of the cell number per wet weight, as compared to the unloaded matrices controls (Fig. 6, Fig. 7). This indicated that even the comparably low concentrations of insulin released from these matrices were sufficient to elicit relevant biological effects.

Even stronger effects were observed for the matrices 1% and 2%: Large improvements in wet weight, cell number per weight, collagen and GAG amounts and also differences in the histological appearance were detected. Even the amounts of the ECM components per cell were distinctly increased (Fig. 6- Fig. 8). The effects on wet weight and amounts of ECM components were significantly larger than those of the matrices 0.2%, which was in agreement with the release data. Only a small trend in favor of the matrices with the highest loading (2%) was observed, but no significant differences could be detected between

constructs grown in the presence of matrices 1% and 2%, indicating that the minor differences in release data were also of minor relevance for the development of the constructs.
In the presented study, the cartilage engineering culture was successfully employed to prove the bioactivity of the released insulin. Up to now, the only methods to directly investigate the efficacy of an insulin releasing device over a period of weeks involved in vivo assays such as the determination of the blood glucose level in rats over a period of 14 days [50]. Here, it was demonstrated that the cartilage engineering in vitro assay facilitates the testing of controlled release devices with regard to their biological efficacy in a complex system without the need for the expense of laboratory animals.

In conclusion, in this study we demonstrated the sustained release of bioactive insulin from cylindrical lipid matrices. The released insulin elicited strong dose-dependent effects on tissue engineered cartilage. The lipid matrices, preserving bioactivity of incorporated and released proteins, are suggested as a suitable carrier system for growth factors and morphogens in regenerative medicine.

Chapter 4

Insulin-loaded macroporous poly(lactic-co-glycolic acid) scaffolds evaluated in a 3-D cartilage engineering culture

Introduction

In the field of tissue engineering [1] new strategies and techniques have been developed for the growth of functional tissues in-vitro or the enhanced repair of damaged tissues in-vivo. Recent tissue engineering approaches utilize biocompatible, biodegradable polymeric cell carriers (scaffolds) as an artificial extracellular matrix (ECM) in combination with morphogenetic effectors like growth factors [12]. The success of those tissue engineering approaches often depends on the suitability of the scaffold and the method of growth factor delivery to the cells within the regenerating tissue. In particular the emulation of natural growth factor production in developing or regenerating tissues became of major interest for tissue engineering applications [19].

However, the administration and utilization of proteinic growth factors is limited by their short half-lives and their potential toxicity at systemic levels [20]. This makes protein carriers desirable, which could ensure a sustained release and at the same time retain the biological activity of the growth factors [21]. The adaption of drug delivery systems for tissue engineering application led to the development of promising controlled release devices, which guaranteed adequate growth factor concentrations at the site of action over an extended period of time. They were developed by incorporation of the growth factor in an appropriate carrier, which made it possible to apply this combination of growth factors and their carriers at the site of action [13]. Recently, in tissue engineering applications also the cell carrier system has been utilized as release device, e.g., providing growth factors embedded in a hydrophilic gel [59], which enabled accelerated diffusion of the growth factor, or manufactured in a double emulsion process [19]. However, it has to be kept in mind that in many manufacturing processes of protein release devices the protein stability may be compromised, e.g., by elevated temperatures or at organic-water interfaces, which lead to protein aggregation and changes in protein structure, in turn resulting in a loss in bioactivity [56, 58]. Therefore, in the development of new protein release devices the evaluation with regard to bioactivity in a relevant application-oriented setting is a sine qua non.

In tissue engineering applications, also solid macroporous scaffolds have been used as a release device[60-67]. In general, such scaffolds can be produced by various techniques, such as salt leaching [68, 69], fibrous fabric processing [70], gas foaming [71], thermally induced

phase separation [72]. Recently, in our group the manufacturing of macroporous scaffolds using the newly developed lipid-templating technique has been demonstrated [23, 24].
In this study, the suitability of the latter scaffolds as protein release system was investigated. Insulin served as model protein and was incorporated into the scaffolds during manufacture. The scaffolds were evaluated within an established insulin-sensitive cartilage engineering culture in vitro [18, 43].

Results

Insulin loading

The insulin content of the scaffolds was determined by a two phase extraction and phase separation method followed by HPLC analysis as described in chapter 2. Originally, insulin contents of 0.1% - 2.5% were aimed at. Loading rate analysis revealed actual insulin contents of 0.09%, 0.69%, and 1.71% (w/w); the scaffolds were designated I0.1%, I0.7%, and I1.7% (Tab. 2).

group	insulin content (w/w)	SD
scaffold I 0.1%	0.09%	0.005%
scaffold I 0.7%	0.69%	0.003%
scaffold I 1.7%	1.71%	0.064%

Tab. 2: Insulin content of the insulin-loaded scaffolds determined by two-phase extraction and HPLC quantification. Each group was determined in triplicate.

Construct wet weight, size, and cell number

The constructs generated with the insulin-loaded scaffolds dose-dependently increased in wet weight after three weeks of culture (Fig. 12A); the weights of the constructs with the highest insulin loading (I1.7%) increased 1.2-fold, compared to the control (I1.7%: 105mg vs. control: 87mg). In contrast, insulin applied in a high single dose (“burst”) did not result in increased weights, as compared to the control (Fig. 12A). For comparison, insulin exogenously applied over the whole cultivation period (I1.4) led to a 1.5-fold weight increase.

The weight measurements were well reflected by the size of the contructs (Fig. 11). With regard to cell content, no significant differences were measured between all groups.

Glycosaminoglycans

The insulin-loaded scaffolds resulted in a loading dose-dependent increase in the mass of glycosaminoglycans (GAG) (Fig. 12D). The I0.7% and I1.7% groups showed significant increases (1.4-fold for I0.7%, 1.7-fold for I1.7%) compared to the control (I0.7%: 2.4mg; I1.7%: 2.9mg vs. control: 1.7mg). The scaffolds of the groups with one high single insulin application ("burst"), I10µ and I200µ, performed significantly weaker compared to the scaffolds with highest insulin loading (I10µ:1.3mg; I200µ: 1.6mg vs. I1.7%: 2.0mg). Highest increase in GAG was observed after exogenous application of insulin over the whole culture period (I1.4), resulting in a 2.5-fold increase of GAG, as compared to the control.
Similar differences between groups were also detected for GAG content expressed per cell and per wet weight, respectively (Fig. 12E, F). Also dose-dependent increases were observed for the differently loaded scaffolds, with 1.5-fold (Fig. 12E) and 1.4-fold (Fig. 12F) increase of the scaffolds with the highest loading compared to the control. Again, values of the "burst" groups (I10µ, I200µ) were considerably lower.

Collagens

Insulin-releasing scaffolds resulted in no significant increase in generated mass of collagen (Fig. 12G), compared to the control. A significant difference could be found between both insulin-loaded scaffolds I0.7%/I1.7% and the external burst group I10µ (I0.7%: 2.5mg; I1.7%: 2.7mg vs. I10µ: 2.0mg). The continuous external insulin application, I1.4, led to 1.4-fold increase in collagen mass (I1.4: 4.3mg vs. control: 2.7mg). The collagen per cell (Fig. 12E) and collagen per wet weight (Fig. 12F) fractions of all groups showed no significant differences to the control.

Macroscopizc appearance and histology

In the histological sections (Fig. 10), GAG distributions were represented by red areas of the safranin-O staining. The insulin-loaded scaffolds resulted in a loading dose-dependent increase in the area and strength of safranin-O staining (Fig. 10: Safranin-O staining of cross-section of scaffolds after three weeks of cultivation, after formalin fixation and paraffin embedding.); the insulin-loaded scaffolds I0.7% and I1.7% clearly increased GAG area and

staining, as compared to the control, well reflecting the GAG quantification (compare Fig. 12D-F). The burst release groups I10µ and I200µ showed distinctly less staining for GAG, as compared to the insulin-loaded scaffolds I0.7% and I1.7%, and similar staining as the control group (Fig. 3). The strongest staining was observed for the group receiving exogenous insulin over the whole cultivation period (I1.4) (Fig. 10), again well reflecting the quantification data (Fig. 12D-F).

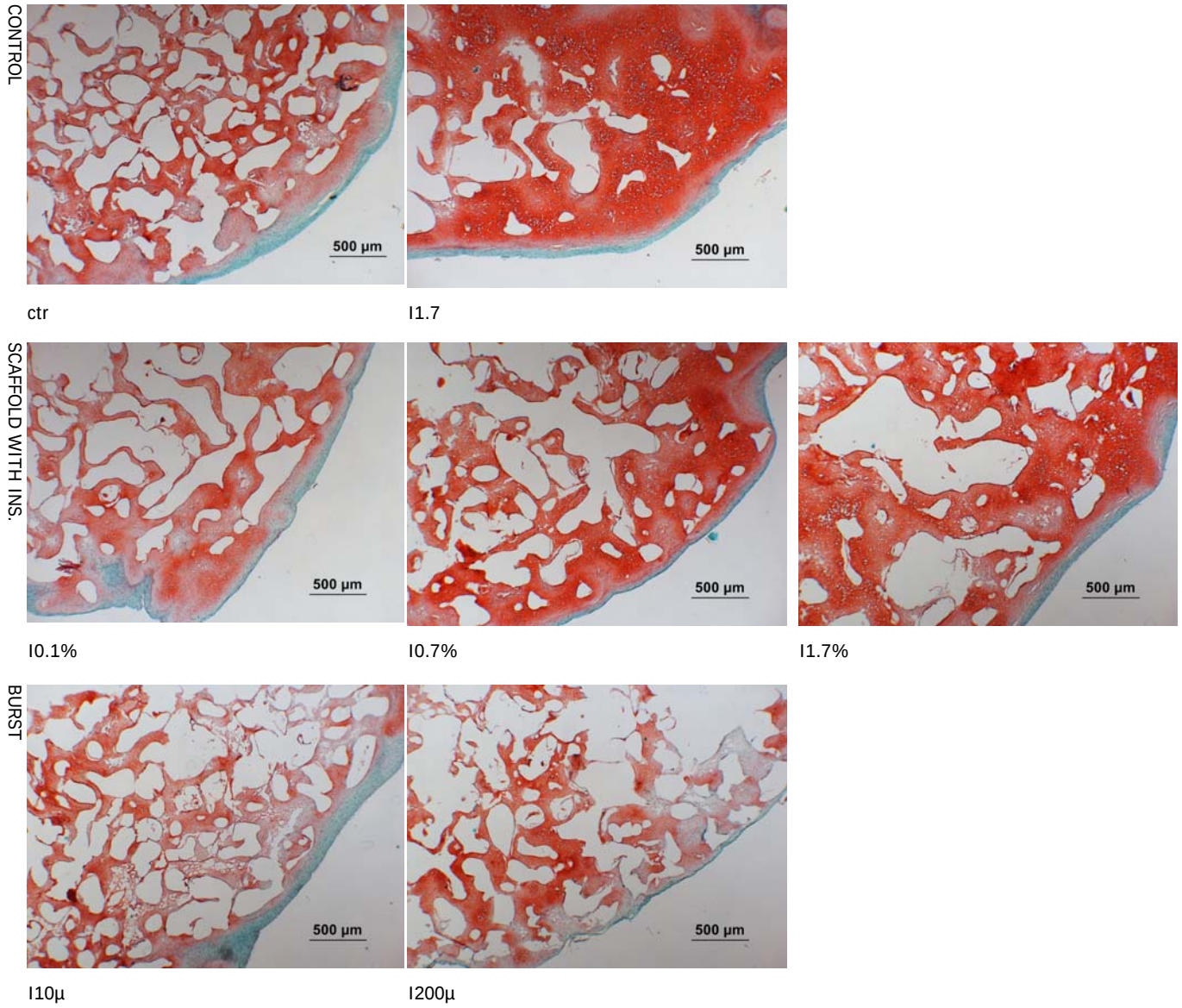


Fig. 10: Safranin-O staining of cross-section of scaffolds after three weeks of cultivation, after formalin fixation and paraffin embedding.

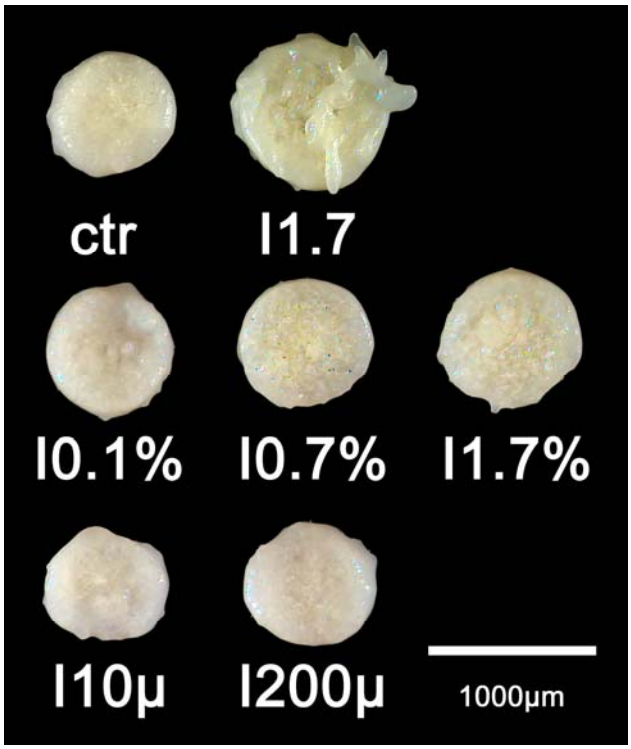


Fig. 11: Stereooptic photo of the cultivated scaffolds after 21 days of cultivation

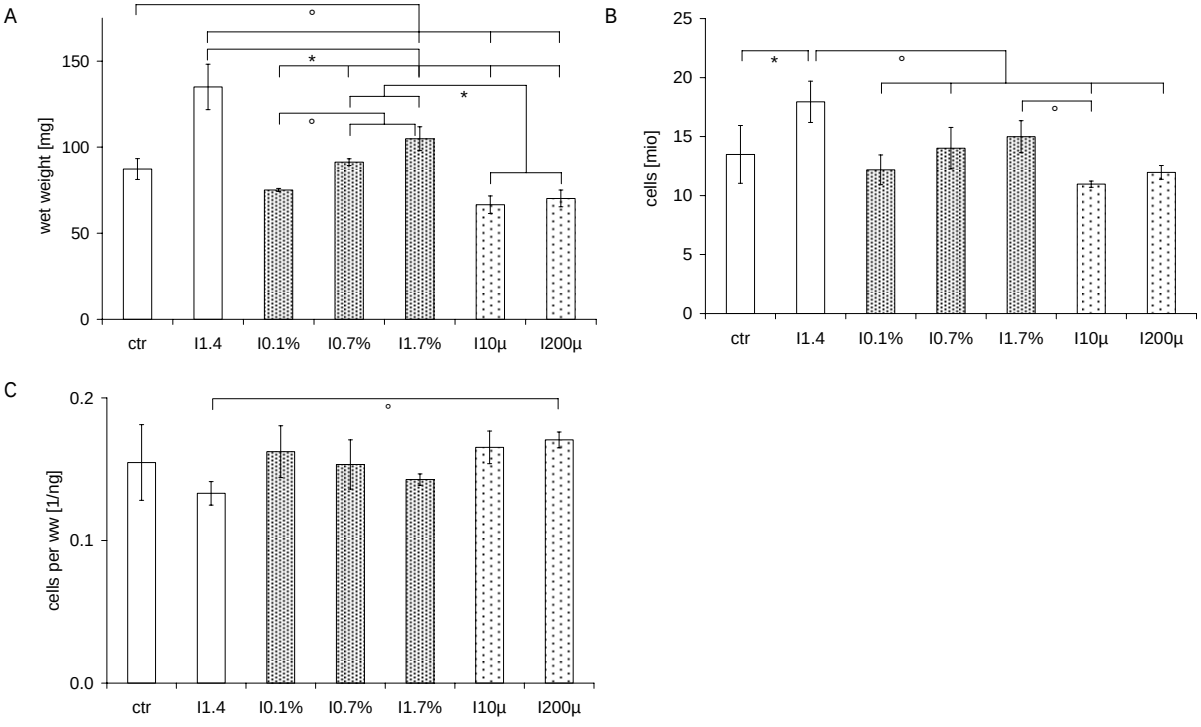


Fig. 12.: Data from cell culture experiments after three weeks of cultivation Data represents the average ± SD of four independent cartilaginous constructs. Statistically significant differences between the groups are denoted by symbol (°) ($p<0.05$) or asterisk (*) ($p<0.01$).

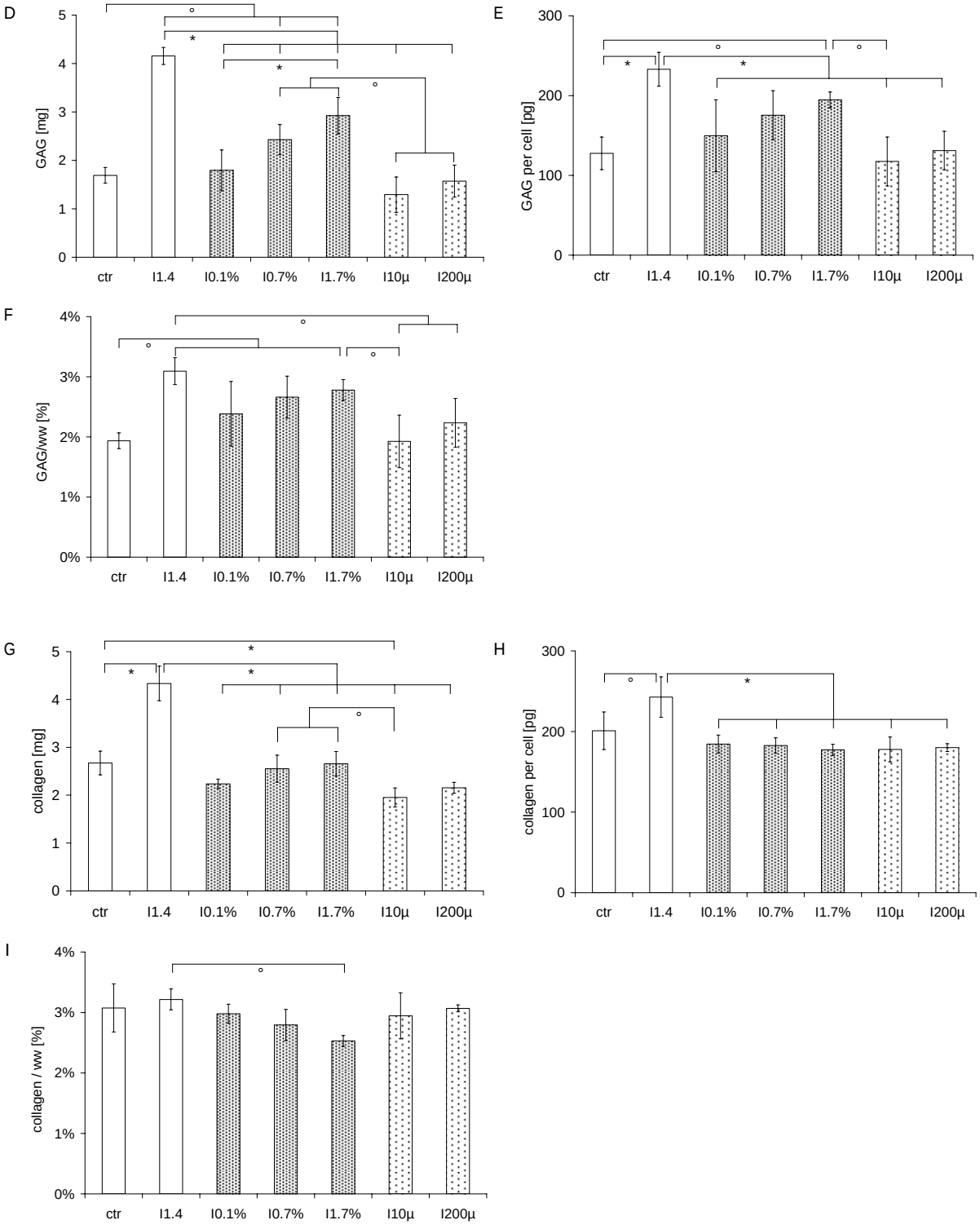


Fig. 12.: Data from cell culture experiments after three weeks of cultivation Data represents the average ± SD of four independent cartilaginous constructs. Statistically significant differences between the groups are denoted by symbol (°) ($p<0.05$) or asterisk (*) ($p<0.01$).

Discussion

In this chapter the suitability of recently developed macroporous PLGA scaffolds (Hacker, Biomaterials 2003) as a controlled release device was investigated in a 3-D cartilage engineering culture. The interconnectivity and pore size of 100-300µm, adjusted by the size of the chosen lipid microparticles, which were used as porogens, has been proven to be suitable for cell attachment and homogenous cell distribution in previous studies [24]. However, when used as release device for proteins, manufacturing processes of the scaffolds, storage and the release itself may compromise protein stability [58]. Thus, besides the determination of the release kinetics by standard assays such as HPLC or enzyme-linked immunosorbent assay, it is also paramount to check the bioactivity of the released protein in a relevant environment [43]. Here, insulin was used as a model protein, which we have previously demonstrated to be suitable to evaluate controlled release devices in the same insulin-sensitive 3-D cartilage engineering culture that was also employed in the presented study [18, 43].

The produced scaffolds were loaded with bovine insulin in concentrations of 0.1%, 0.7%, and 1.7% (Tab. 2). Prior to cell seeding, insufficiently embedded insulin crystals were washed out over 24h to prevent burst release effects. Additionally, in two control groups, single doses of exogenous insulin (10 and 200 µg/ml, respectively) were applied at the beginning of the culture period in order to simulate a potential burst release.

Insulin-loaded scaffolds resulted in dose-dependent increases of wet weight, size, and GAG content of the generated cartilaginous constructs after three weeks of culture (Fig. 12A, D-F; Fig. 10). Especially the scaffolds with the highest loading resulted in significant increases of weight, size, and GAG content, as compared to control constructs receiving either no insulin or receiving a single exogenous insulin dose at the beginning of the culture period. The latter comparison implied that the effects of the insulin-releasing scaffolds could not be attributed to a burst, but rather a prolonged release. For all groups, differences in GAG content quantified by DMMB assay (Fig. 12D-F) were confirmed by histological analysis (Fig. 10).
With regard to collagen content, all insulin-loaded scaffolds did not improve the constructs over control. Only insulin exogenously applied over the whole cultivation period resulted in

increased collagen content, it also led to the highest values for all other parameters measured. As the exogenously applied insulin concentration of 1.4 µg/ml was well above the concentration that was theoretically achievable with the scaffolds with the highest insulin loading, these results were to be expected. The observation that the insulin-loaded scaffolds increased construct weight and GAG content, but not collagen content may be attributed to low insulin release in the later period of the culture: In previous studies with several harvesting time points it was shown that, in contrast to early increases in GAG content, collagen increase mostly occurs at later stages, e.g., beginning from the third week of cultivation [18]. To validate this suggestion it becomes mandatory to analyze the released insulin in further studies.

In literature recent approaches manufacturing macroporous scaffolds for controlled release of growth factors, mostly dealt with protein loaded microspheres which were entrapped within the polymeric scaffold (PLA/PLGA) during scaffolding process [67, 73-76]. This double wrapping of growth factors may lead to very low loading rates within the resulting scaffold. Another approach utilized biphasic emulsion technique to embed proteinic growth factors into PLA/PLGA scaffolds during scaffold molding [77]. The emulsion processes often include organic solvents likely to result in organic-water interfaces, which in turn are known as destabilizing factor for proteins [56]. Supercritical carbon dioxide ($scCO_2$) was used in single-phase gas foaming scaffolding process utilizing polymer material (PLA/PLGA) and protein [60, 61, 64, 78]. For improved pore forming NaCl crystals were embedded within gas foamed scaffolds. The following aqueous leaching process might lead to a loss of proteins. Other approaches dealt with protein loading techniques for the prefabricated scaffolds. One group used polymeric protein containing films, which coated a prefabricated scaffold with a protein-containing polymeric emulsion on top [67, 73]. Another group loaded macroporous polymeric scaffolds with protein containing hydrogels [79]. These basic approaches seem to have promise, but need further research. Also different scaffold materials as e.g. chitosan were utilized, but they lack a FDA approval [62, 63, 80].

In conclusion, in this study bovine insulin released from macroporous scaffolds improved cartilaginous constructs, specifically weight and GAG content. The observed effects were not caused by a single burst release, as the burst release controls did not increase weight and GAG content. Further analytics need to be performed to get a closer understanding of the release profile and the amounts of released insulin. However, already from this data it can be concluded that the embedded insulin was released over an extended period of time under retention of its bioactivity.

Chapter 5

Synergistic effects of growth and development factor-5 (GDF-5) and insulin on primary and expanded chondrocytes in a 3-D environment

Introduction

Articular cartilage shows only limited capacity for self repair. Large defects often develop as a point of origin of osteoarthritis in the synovial joint. So the treatment of cartilage defects still remains a challenge. Tissue engineering applications represent an important therapeutic option, especially for larger defects in the joint. The limited source of autologous harvestable cells represent one major limiting factor for engineering of cartilage substitutes. An approach to overcome this problem consists in the prior proliferation of harvested cells in a 2-D culture. Afterwards the expanded cells are seeded on a 3-D cell carrier and further cultivated. However, proliferation in 2-D culture commonly results in cell dedifferentiation rendering the cells less suitable for tissue engineering purposes [15]. One approach to improve engineered cartilage constructs is the application of growth factors, either during cell expansion or during tissue construct development. The aim of this study was to elucidate the potential of the protein growth and differentiation factor-5 (GDF-5) to improve tissue construct development either made from primary or from expanded chondrocytes.

GDF-5 is a member of the bone morphogenetic protein (BMP) subfamily; BMPs in turn are members of the TGF-β superfamily and were originally identified as inducers of bone and cartilage formation in vivo. Homodimeric BMPs induce ligand-dependent type I and II receptor heterodimerization of transmembranic serine-threonine kinases [81, 82]. GDF-5, also known as cartilage-derived morphogenetic protein 1 (CDMP1), is a known partner in digit and limb formation [25-27] and is also found in the stage of precartilaginous mesenchymal condensation and throughout the cartilaginous cores of the developing long bones of bovine embryos [28]. It has also been postulated to be involved in the maintenance of normal cartilage and regenerative response in diseased tissue as it was found in the superficial layer of normal cartilage and throughout osteoarthritic cartilage [83].

Within this study, the effects on development of engineered cartilage of either GDF-5 alone or in combination with bovine insulin were investigated. Insulin has previously been demonstrated to have strong anabolic effects on engineered cartilaginous constructs from primary cells similar to those of insulin-like growth factor-I (IGF-I) [18]. Specifically, in the experiments presented here, engineered cartilage constructs were assessed with regard to their histological appearance and extracellular matrix (ECM) composition, i.e., glycosaminoglycan and collagen content. The study demonstrates the synergistic effects of GDF-5 and insulin to improve cartilaginous cell-polymer constructs made from either primary or expanded (passage 2) chondrocytes.

Results

Primary chondrocyte culture

Wet weight and cell number

Within 21 days of culture, GDF-5 at 0.1 µg/ml and 0.5µg/ml increased construct wet weight 2.3-fold and 5.3-fold, respectively, as compared to control; no effect was observed at 0.01 µg/ml (Fig. 13A). Insulin at the routinely used concentration of 2.5 µg/ml increased the wet weight 3-fold. Strikingly, in combination with insulin, even the low concentrations of GDF-5 led to a strong increase in wet weight (5.7-fold for GDF-5 at 0.01 µg/ml and 7.5-fold for GDF-5 at 0.1 µg/ml) (Fig. 13A).

The cell number per wet weight decreased by the supplementation with growth factors (Fig. 13B). GDF-5 applied alone resulted in a slight dose-dependent decrease to 73 % and 67 % for GDF-5 at 0.1µg/ml and 0.5µg/ml, respectively. The addition of insulin, either alone or in combination with GDF-5 decreased the relative cell number to 60-53 %.

Glycosaminoglycan and collagen content

The glycosaminoglycan (GAG) content in the cartilaginous constructs from primary cells was strongly increased by the addition of supplemented proteins (Fig. 13C). The absolute amount of GAG was increased dose-dependently up to 9.9-fold in the presence of GFD-5 alone and 5-fold in the presence of insulin alone; the combination of GFD-5 and insulin yielded an increase up to 12-fold compared to the control (Fig. 13C). For the GAG content per cell highest values were obtained for insulin, GDF-5 alone at the highest concentration, and the combinations (up to 3.1-fold increase) (Fig. 13D). The differential GAG content in the scaffolds was also confirmed histologically by safranin-O staining (data not shown), in which all all growth factor recieving groups showed a strong red staining for GAG.

The addition of proteins affected the amount of total collagen in a similar fashion as observed for GAG. Again, a strong dose-dependent increase was observed for GDF-5 and highest values were obtained for the combination of GDF-5 and insulin (5.2-fold increase) (Fig. 13E). The collagen content per cell was increased for all insulin-receiving groups; GDF-5 alone had no significant effect (Fig. 13F). Immunohistochemical analysis demonstrated a homogeneous anti-collagen II staining throughout the whole cross-section of all scaffolds, only at the edges all scaffolds showed a positive anti-collagen I staining (data not shown).

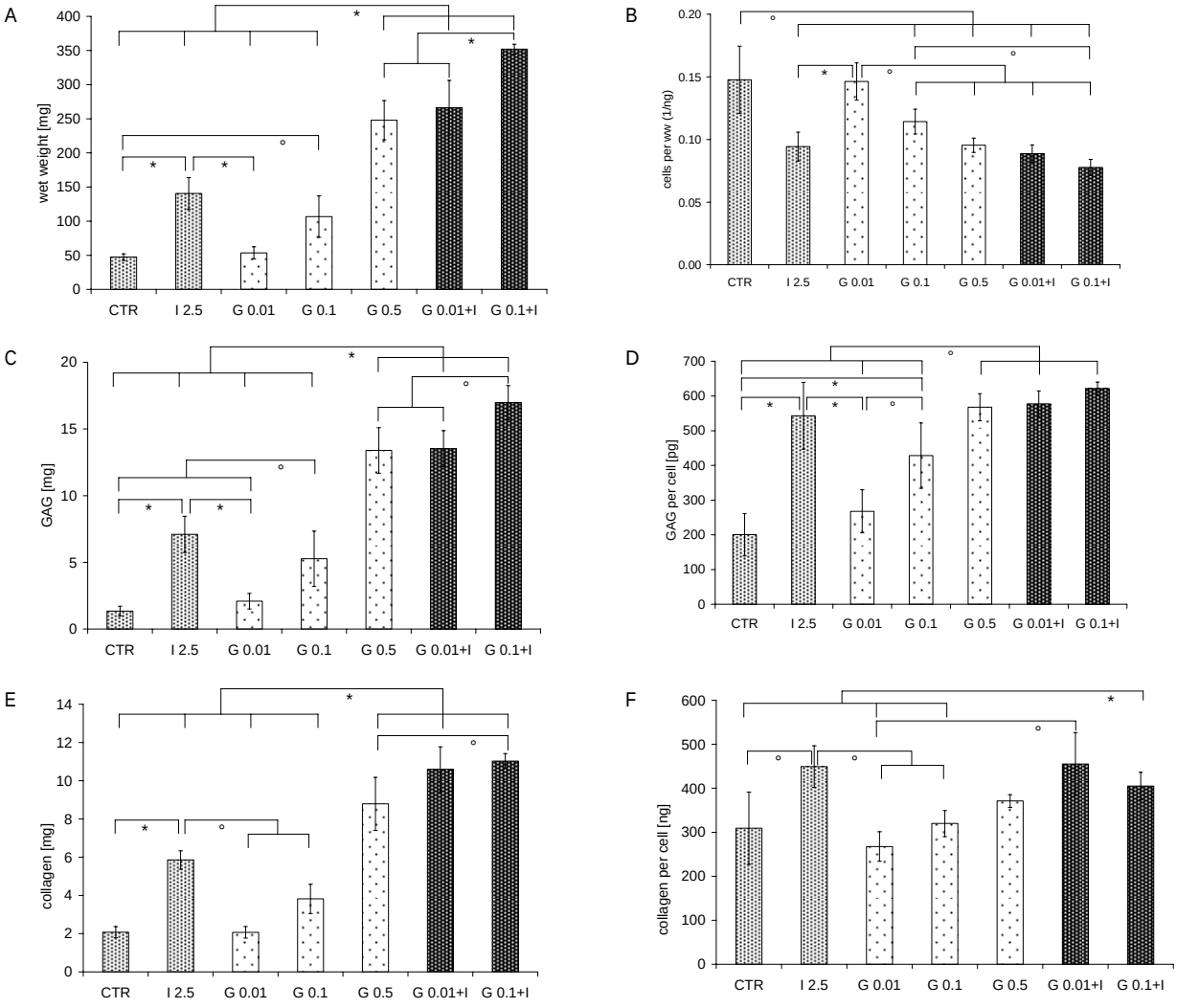


Fig. 13: Wet weight (A), cell number (B), GAG content (C-D), and collagen content (E-F) of cartilaginous constructs generated with primary chondrocytes after three weeks of culture. CTR: control; I2.5: insulin at 2.5µg/ml; G: GDF-5 at 0.0.1, 0.1, or 0.5 µg/ml; G + I: GDF-5 at 0.01 or 0.1 µg/ml combined with insulin at 2.5 µg/ml. Data represents the average ± SD of four independent constructs. Statistically significant differences between the groups are denoted by symbol (°) ($p<0.05$) or asterisk (*) ($p<0.01$).

Passage 2 chondrocyte culture

Macroscopic appearance, wet weight, and cell number

Employing passage 2 chondrocytes, after a culture period of 21 days constructs of the control group were comparably small (approx. half of the diameter of the original scaffold (0.5 mm)) (Fig. 14) and had only about 16% of wet weight of primary chondrocyte control constructs (Fig. 15A, compare Fig. 13A). Whereas insulin increased the wet weight by 2.2-fold, GDF-5 alone led to no significant increase in wet weight. However, in combination with insulin, GDF-5 increased the wet weight by 3.2-fold (GDF-5 at 0.01 µg/ml) and 4.8-fold (GDF-5 at 0.1 µg/ml), respectively (Fig. 15A). The constructs of the latter group were the only ones that maintained the original scaffold size (Fig. 14).

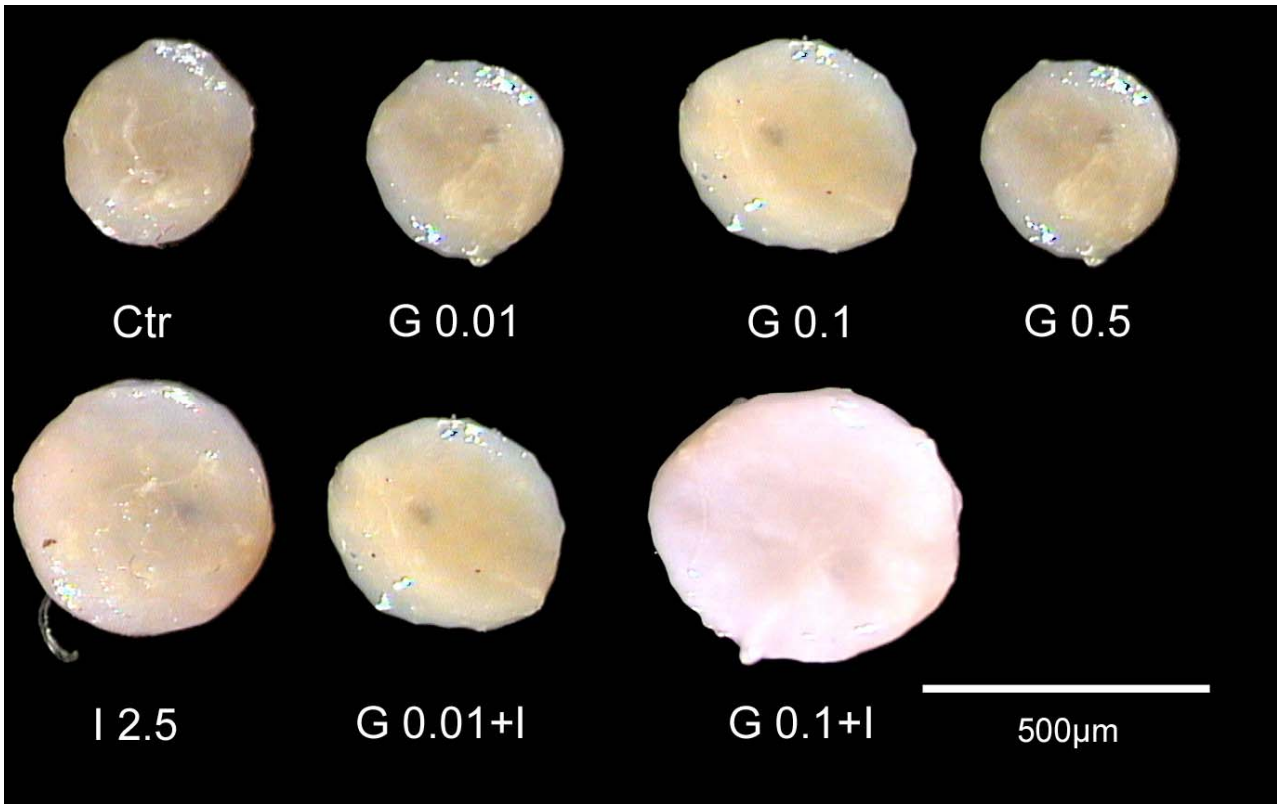


Fig. 14: Macroscopic appearance of cell-polymer constructs generated with passage 2 chondrocytes after three weeks of culture. .CTR: control; I2.5: insulin at 2.5µg/ml; G: GDF-5 at 0.0.1, 0.1, or 0.5 µg/ml; G + I: GDF-5 at 0.01 or 0.1 µg/ml combined with insulin at 2.5 µg/ml.

The cell number per wet weight was not significantly affected by GDF-5 alone. It was decreased for all insulin receiving groups, as compared to the control, with lowest values for the combinations of insulin and GDF-5 (decrease down to 32%) (Fig. 15B).

Glycosaminoglycan and collagen content

The administration of a single protein did not cause a significant difference in GAG content. However, both combinations of GDF-5 and insulin resulted in significant increases in absolute GAG amounts (up to 5.7-fold) and GAG per cell (up to 3.7-fold) (Fig. 15C, D).

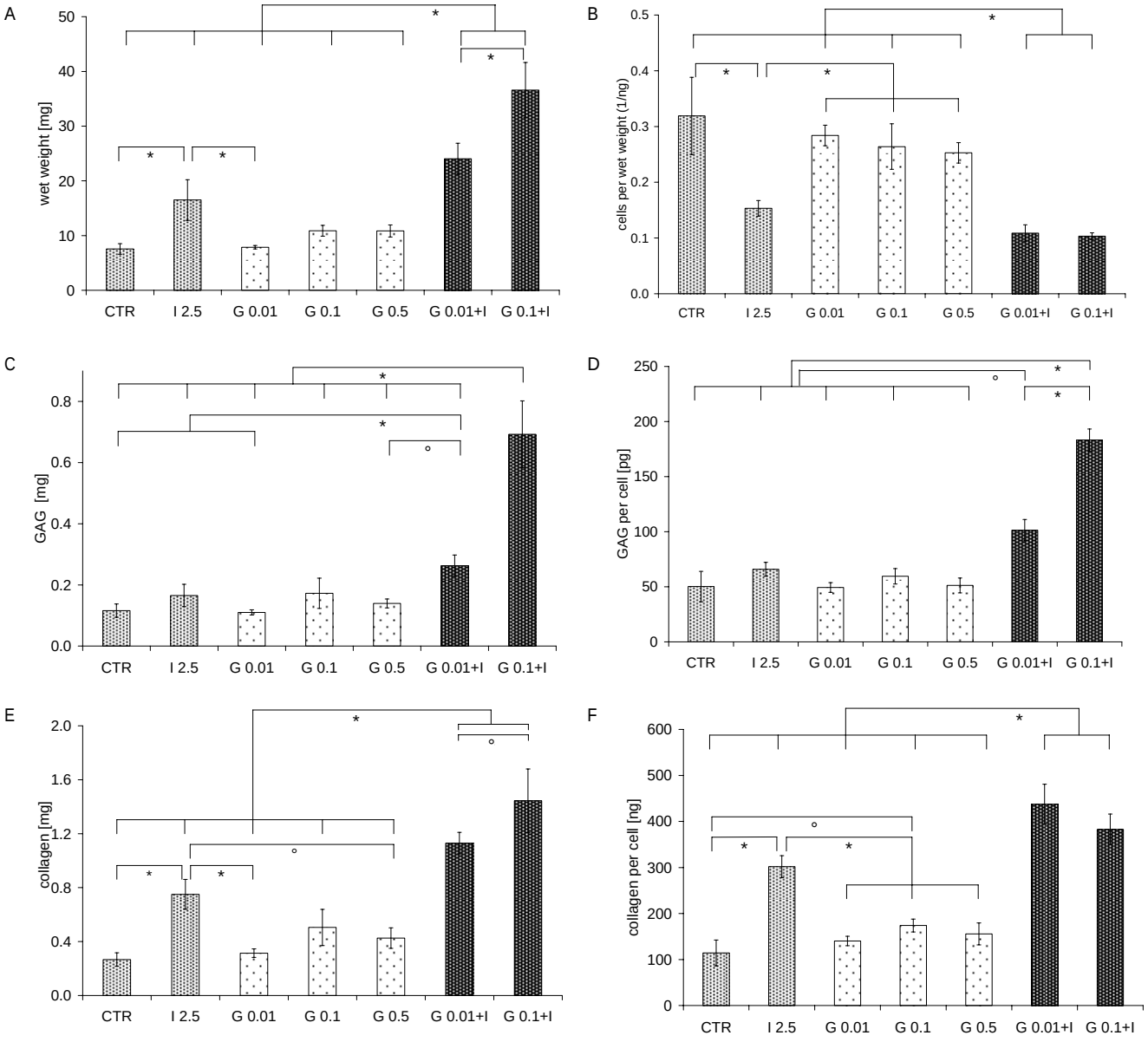
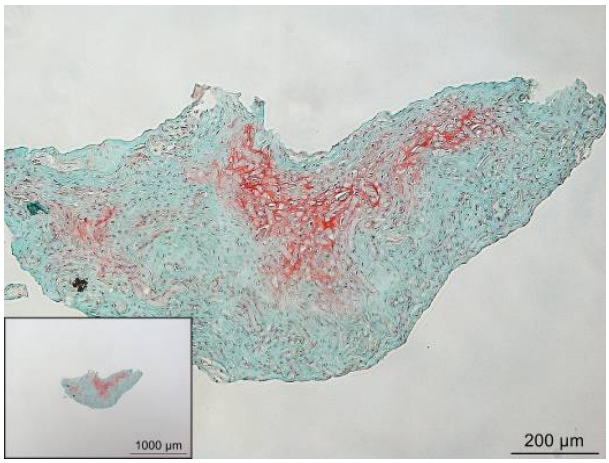


Fig. 15: Wet weight (A), cell number (B), GAG content (C-D), and collagen content (E-F) of cell-polymer constructs generated with passage 2 chondrocytes after three weeks of culture. CTR: control; I2.5: insulin at 2.5µg/ml; G: GDF-5 at 0.0.1, 0.1, or 0.5 µg/ml; G + I: GDF-5 at 0.01 or 0.1 µg/ml combined with insulin at 2.5 µg/ml. Data represents the average ± SD of four independent constructs. .Statistically significant differences between the groups are denoted by symbol (°) ($p<0.05$) or asterisk (*) ($p<0.01$).

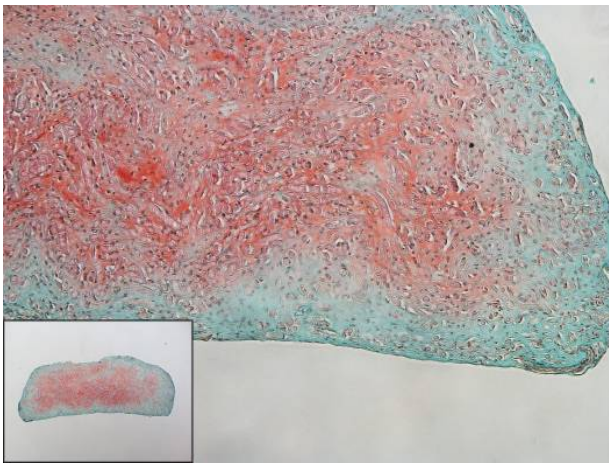
The collagen content was also not significantly affected by GDF-5 alone. Insulin alone increased the collagen content, but highest values were again obtained for the combinations of GDF-5 and insulin, with increases in absolute amount of collagen up to 5.3-fold and in collagen per cell up to 3.8-fold (Fig. 15E, F).

Histology

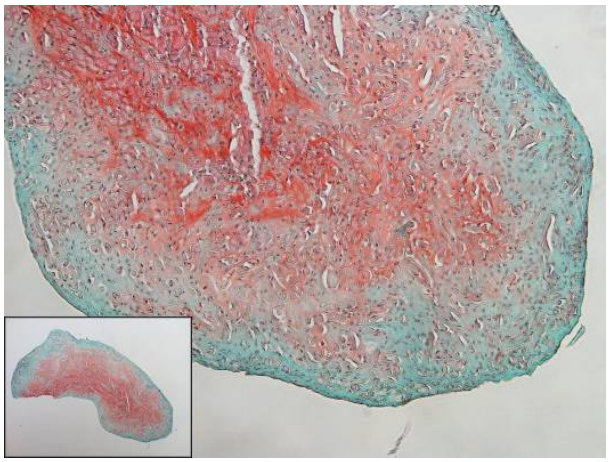
The control group exhibited small constructs with large areas of cells with a fibroblastic phenotype (stained blue) and a small core stained red for GAG with safranin-O (Fig. 16). The addition of insulin resulted in larger constructs, but still with large blue stained areas containing fibroblastic cells and a core with only weak GAG staining. Supplementation of GDF-5 alone resulted in an enhanced staining for GAG in the inner region of the construct, as compared to the control and insulin group; the blue stained areas were reduced to the outer regions of the scaffolds. The combination of GDF-5 and insulin resulted in scaffolds with an even stronger staining for GAG. In the combination receiving GDF-5 at 0.1 µg/ml, GAG was distributed throughout the constructs; small blue stained areas with fibroblastic cells could only be found on the edge of the constructs (Fig. 16).



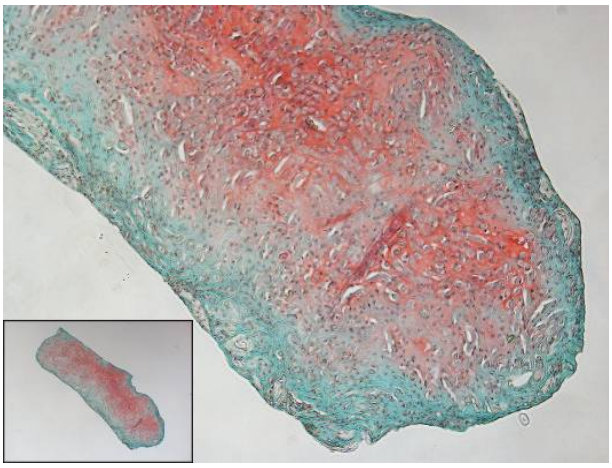
ctr



G 0.01

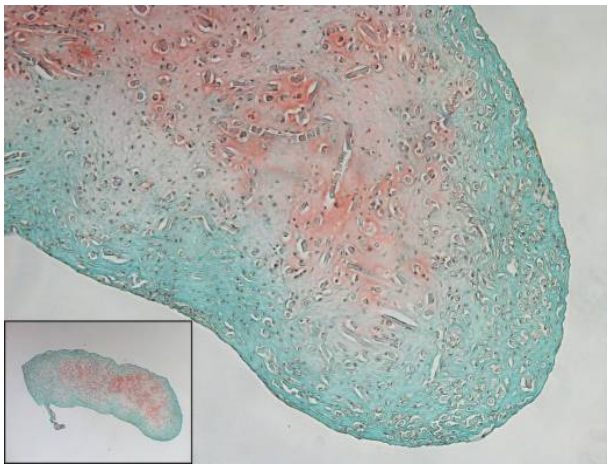


G 0.1

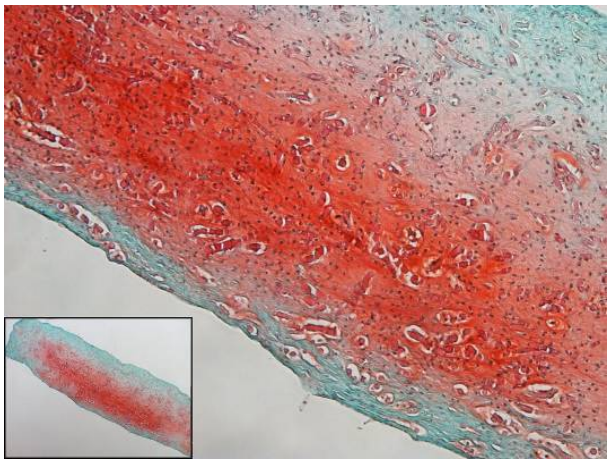


G 0.5

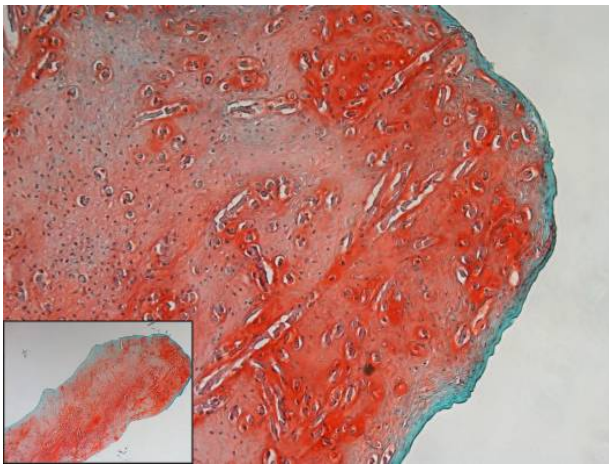
Fig. 16: Glycosaminoglycan (GAG) distribution in cross-sections of cell-polymer constructs generated with passage 2 chondrocytes. GAG was stained red with safranin-O (appears dark gray in black & white print). . CTR: control; I2.5: insulin at 2.5µg/ml; G: GDF-5 at 0.0.1, 0.1, or 0.5 g/ml; G + I: GDF-5 at 0.01 or 0.1 µg/ml combined with insulin at 2.5 µg/ml.



I 2.5



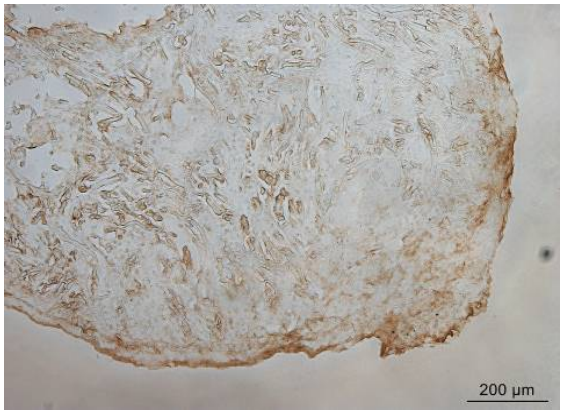
G 0.01+I



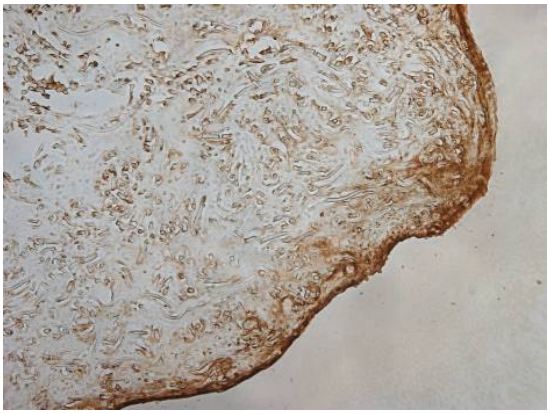
G 0.1+I

Fig. 16: Glycosaminoglycan (GAG) distribution in cross-sections of cell-polymer constructs generated with passage 2 chondrocytes. GAG was stained red with safranin-O (appears dark gray in black & white print). . CTR: control; I2.5: insulin at 2.5µg/ml; G: GDF-5 at 0.0.1, 0.1, or 0.5 g/ml; G + I: GDF-5 at 0.01 or 0.1 µg/ml combined with insulin at 2.5 µg/ml.

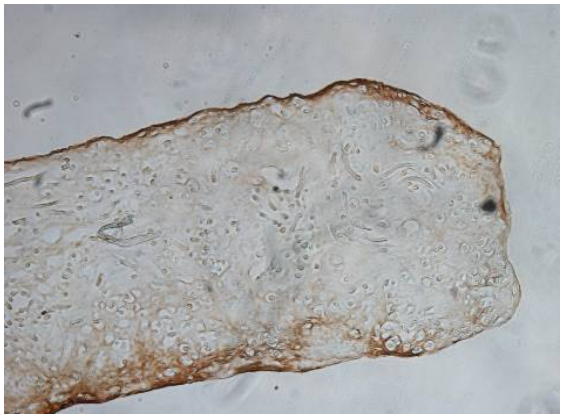
A



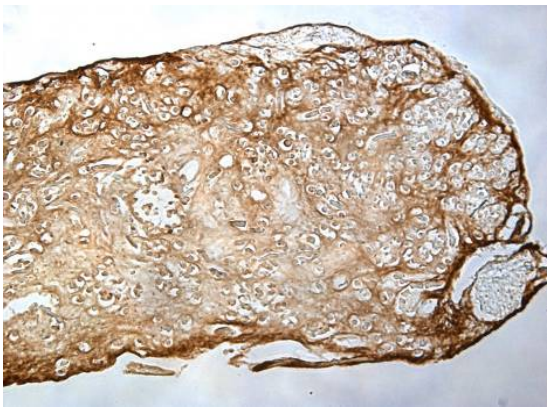
ctr



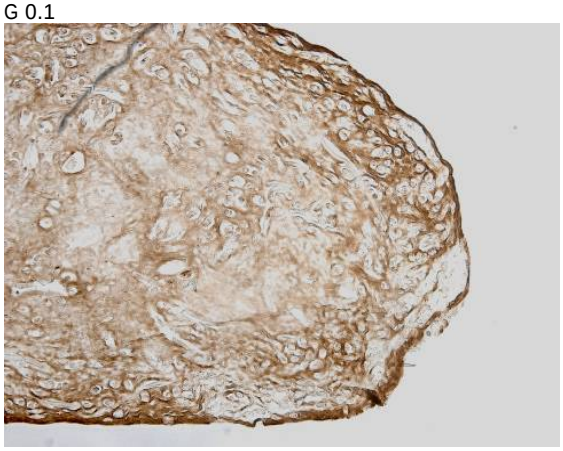
G 0.01



G 0.1

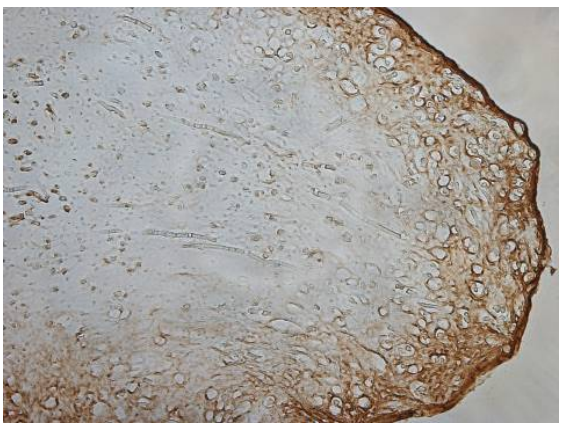


G 0.5



I 2.5

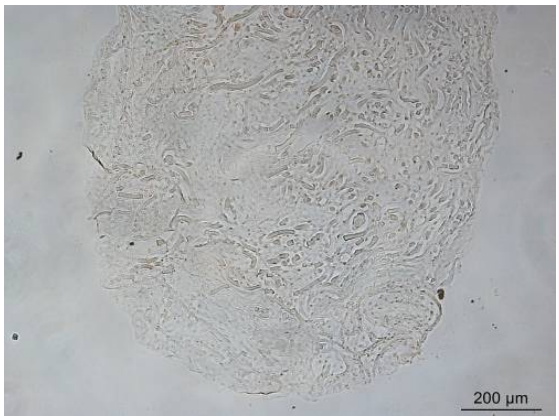
G 0.1+I



G 0.01+I

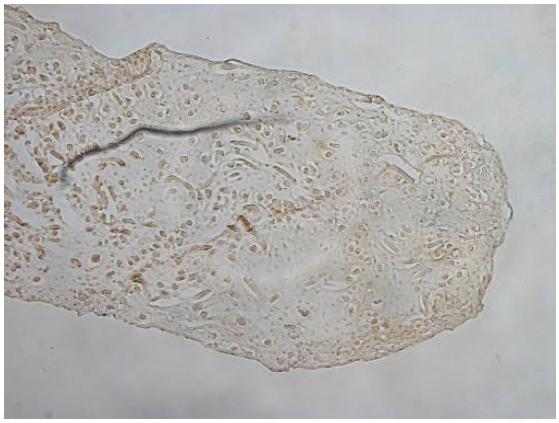
Fig. 17: Collagen type I (A) and collagen type II (B) distributions in cross-sections of cell-polymer constructs generated with passage 2 chondrocytes. Collagen type I and II were immunohistochemically labelled with anti-collagen type I and anti-collagen type II antibody, respectively, and stained brown with DAB (appears dark gray in the black & white print). CTR: control; I2.5: insulin at 2.5µg/ml; G: GDF-5 at 0.0.1, 0.1, or 0.5 µg/ml; G + I: GDF-5 at 0.01 or 0.1 µg/ml combined with insulin at 2.5 µg/ml.

B

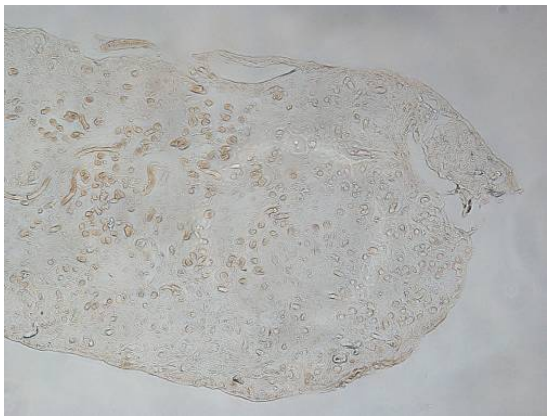


ctr

G 0.01

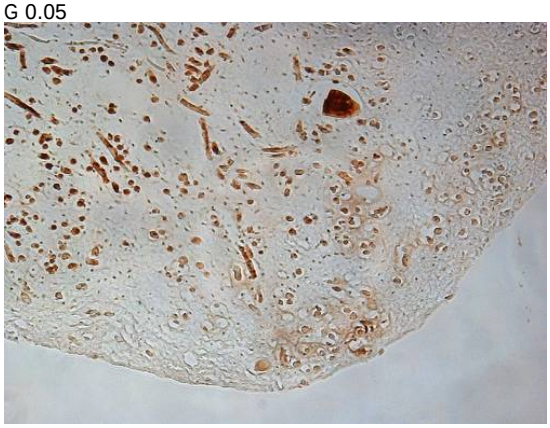


G 0.1

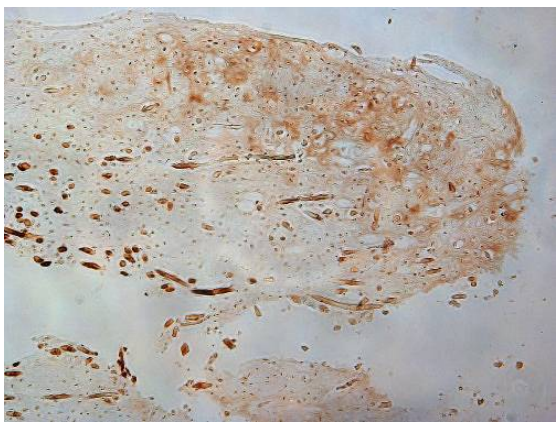


G 0.05

I 2.5



G 0.01+I



G 0.1+I

Fig. 17: Collagen type I (A) and collagen type II (B) distributions in cross-sections of cell-polymer constructs generated with passage 2 chondrocytes. Collagen type I and II were immunohistochemically labelled with anti-collagen type I and anti-collagen type II antibody, respectively, and stained brown with DAB (appears dark gray in the black & white print). CTR: control; I2.5: insulin at 2.5µg/ml; G: GDF-5 at 0.0.1, 0.1, or 0.5 µg/ml; G + I: GDF-5 at 0.01 or 0.1 µg/ml combined with insulin at 2.5 µg/ml.

Immunohistochemical staining

Staining for collagen type I was detected throughout the control constructs; insulin alone led to even more intensive staining (Fig. 17A). GDF-5 alone elicited a biphasic response: At low concentration of 0.01 µg/ml, a collagen type I staining similar as in the control constructs was observed; at 0.1 µg/ml GDF-5, a reduction of collagen type I in large areas of the core of the construct was observed; in contrast, at 0.5 µg/ml GDF-5, the strongest staining for collagen type I of all groups was detected. Strikingly, the combination of GDF-5 and insulin led to a distinct reduction of collagen type I staining; especially in the combination GDF-5/insulin 0.1/2.5 µg/ml, there was no collagen I detectable throughout the construct except for a thin area at the edge (Fig. 17A). Decreased collagen I expression for GDF-5 at 0.1 µg/ml and for the GDF-5/insulin combinations, and increased expression for GDF-5 at 0.5 µg/ml were confirmed by RT-PCR analysis (data not shown).

Collagen type II (Fig. 17B) could not be detected in control constructs and in constructs receiving either insulin or GDF-5 alone. However, the combination GDF-5/insulin at 0.01/2.5 µg/ml showed collagen type II staining in small areas, and at 0.1/2.5 µg/ml the combination resulted in well-distributed staining in large areas of the constructs. As compared to the control group, an increased collagen II expression was detected using RT-PCR for all groups except for GDF-5 at 0.5 µg/ml, for which a decrease was observed (data not shown).

Discussion

The use of in vitro expanded chondrocytes is still one of the major obstacles in the generation of engineered cartilaginous constructs. In order to improve constructs made from passaged chondrocytes, different approaches have employed various growth factors either during the expansion phase or during construct development [84-88]. To the best of our knowledge, this study for the first time investigated the effects of growth and development factor-5 during in vitro construct culture, either alone or in combination with insulin, on the quality of cartilaginous constructs made from primary or expanded chondrocytes. With primary chondrocytes, GDF-5 elicited similar improving responses as compared to insulin, which has been previously established as beneficial in cartilage engineering [18, 41, 43]. However, with passage 2 chondrocytes the use of neither insulin nor GDF-5 alone led to convincing cartilaginous constructs. In contrast, the combination of GDF-5 and insulin strongly improved the macroscopic and histological appearance as well as extracellular matrix composition of the engineered cartilage.

From previous reports in the literature, GDF-5 is well known for its role in early chondrogenesis. Apparently GDF-5 plays an essential role in the embryonic chondrogenesis and limb formation, however the function of GDF-5 in adult cartilage is still to be elucidated [82, 89]. Nevertheless, GDF-5 remains an interesting candidate molecule for cartilage engineering applications, as it was found to be expressed in adult bovine and human cartilage in both normal and osteoarthritic tissue [83]. In a rabbit cartilage defect model (trochlear groove in adult rabbits), the application of GDF-5 adsorbed on collagen-I sponges improved the histological appearance of the repair tissue at 4 and 8 weeks after surgery, but after 24 weeks no beneficial effect of the growth factor was detectable, as compared to the control [90]. Furthermore, the use of GDF-5 has been suggested in the healing of Achilles tendon defects [91, 92].

Within this study, GDF-5 has been evaluated with regard to its ability to improve engineered cartilage constructs made from bovine juvenile chondrocytes in vitro. On primary chondrocytes, GDF-5 elicited strong dose-dependent anabolic effects which were reflected by total wet weight and the extracellular matrix content of the treated scaffolds, i.e., both GAG

and collagen content (distinct effects already at 0.1 µg/ml GDF-5 and up to 9.9-fold increase in mass of GAG (Fig. 13C) and up to 4.2-fold increase in mass of collagen (Fig. 13E) for GDF-5 at 0.5µg/ml). Previously, in a cartilage defect model system with trypsin-treated bovine cartilage, at a concentration of GDF-5 of 0.1 µg/ml also an increase in proteoglycan synthesis was detected by increased ^{35}S-sulfate incorporation at day 5 [83]. Intervertebral disk cells isolated from GDF-5 deficient mice and embedded in alginate beads responded to GDF-5 treatment in concentrations up to 0.1 µg/ml with upregulated expression of type II collagen and aggrecan genes in a dose-dependent manner [93].

In the present study, strikingly, when combining GDF-5 at concentrations of 0.01 and 0.1 µg/ml with insulin (2.5 µg/ml), wet weight as well as GAG and collagen amounts were strongly increased in a synergistic fashion as compared to either factor alone at the same concentration. This was especially remarkable for GDF-5 at 0.01 µg/ml, as at this concentration GDF-5 alone resulted in no significant increase, neither for total weight nor for the ECM fractions; however, combined with insulin, GDF-5 at this concentration increased the weight and ECM fractions compared to those achieved with insulin alone for both 1.9-fold (Fig. 13A,C,E). The ECM content expressed per cell was not affected in the constructs receiving the combinations as compared to those receiving insulin alone (Fig. 13,F), with similar cell numbers per weight for the combinations and insulin alone (Fig. 13B). Thus, combining GDF-5 with insulin led to distinctly larger constructs than insulin alone, with similar relative cell numbers and cells that were equally active in producing ECM.

Constructs generated with passage 2 cells, in general, were a lot smaller and lighter than their primary cell counterparts; histological analysis of control constructs revealed a dedifferentiated phenotype with a fibroblastic appearance, correlating staining for collagen type I throughout the construct, and no staining for the cartilage-specific collagen type II (Fig. 16, Fig. 17).

GDF-5 alone did not significantly affect the wet weight and the amounts of GAG and total collagen (Fig. 15A, C, E). However, GDF-5 elicited a biphasic response with regard to collagen type I expression: Whereas staining for collagen type I was distinctly reduced in large parts of the construct at 0.1 µg/ml, it was increased at 0.5 µg/ml, as compared to control

constructs (Fig. 17). The reason for this biphasic response remains to be elucidated in future studies. Previously, it has been reported that GDF-5 supplementation in concentration of 500ng/ml led to a down-regulation of collagen type I expression in human mesenchymal stem cells [94]. Also, GDF-5 stimulated the ECM response of fetal rat calvarial cells towards the chondrogenic phenotype [95].

In our study, for passage 2 cells also striking advantageous effects were observed when combining GDF-5 with insulin, which were even more pronounced than for primary chondrocytes. Insulin alone increased the weight and the collagen fraction (Fig. 15A,E), however, no collagen type II staining was detected and collagen type I staining was clearly increased, as compared to control (Fig. 17). The combination of GDF-5 (at 0.01 and 0.1 µg/ml) and insulin (2.5 µg/ml) yielded increases of weight and GAG and collagen amounts which were distinctly higher than those of insulin alone (Fig. 15A,C,E). Moreover, in the constructs receiving the combinations, also the GAG and collagen contents expressed per cell were distinctly increased, as compared to control and insulin-receiving constructs, which again was especially remarkable as GDF-5 alone resulted in no significant increases at all (Fig. 15D,F). Even more important, the combination GFD-5/insulin led to a clear shift in collagen subtype expression towards that of a more cartilaginous phenotype: Employing GDF-5 at 0.1 µg/ml yielded a staining for collagen II throughout the construct and a reduction of collagen I staining to only a thin layer at the edge of the construct. Taken together, whereas neither factor alone was able to overcome the dedifferentiation resulting from chondrocyte expansion, the combination of GDF-5 and insulin led to cartilaginous constructs with more actively ECM producing cells and a distinctly improved collagen subtype content.

In conclusion, in the presented study, GDF-5 was demonstrated to be a useful protein in cartilage engineering in vitro. Especially in combination with insulin, GDF-5 appears to enable the redifferentiation of expanded chondrocytes and the concurrent generation of relevant cartilaginous constructs. As an alternative approach, a recent study has used growth factor supplementation during chondrocyte expansion in order to improve redifferentiation in a 3-D environment [85]. In analogy to this approach, it may be also worthwhile investigating the effects of GDF-5 on the quality of cartilaginous constructs when applied during chondrocyte expansion. With regard to the synergistic effects of GFD-5 and insulin observed

in the present study, further research is required to elucidate the mechanism on the molecular level. The fact that GDF-5 was demonstrated to decisively modulate the response to another cartilage-effectice protein contributes to the emerging picture of the role GDF-5 apparently plays in chondrogenesis and cartilage physiology.

Chapter 6

Effects of steroid hormones on cartilage engineering in vitro

Introduction

Cartilage tissues have been proven to be steroid hormone-sensitive. Articular cartilage was found to be an estrogen-sensitive tissue [96]. Further evidence on the influence of steroid hormones on the development of cartilage came from menopausal women, where supplementation of estrogen resulted in an improved cartilage matrix, higher mobility and reduced pain within the synovial joint [97-99]. In rat cartilage, receptors specific for 17-β-estradiol and testosterone could be found [100] and estrogen receptors were further specified for human articular cartilage. Two different estrogen receptor (ER) in human chondrocytes could be detected, ER-α and ER-β [101] .

Estrogens and androgens in peripheral cells are connected with each other in complex pathways under inclusion of androgen precursors, such as dehydroepiandrosterone (DHEA). DHEA, a 19-carbon steroid hormone, is a member of the adrenal androgen family, released from the adrenal gland. From the findings of its decreasing concentration with age in plasma, it is known as an "antidote from aging" [102]. DHEA was found to reduce IL-1β-induced syntheses of catabolic enzymes, such as MMP-1 and -3, and to up-regulate inhibitors of MMPs, such as TIMP-1 gene expressions [103]. It was also found to be capable of suppressing interleukin 6 (IL-6) secretion in man in vitro [104]. Other studies demonstrated the suppressing influence of DHEA and testosterone on the secretion of IL-1β, IL-6 and tumor necrosis factor (TNF) [104, 105]. DHEA as one of the major androgen precursors can be enzymatically converted to testosterone by consecutive reaction with 17β-hydroxysteroid dehydrogenase and 3β-hydroxysteroid dehydrogenase (alternatively in reverse order) [106]. A conversion to estrogens is possible for testosterone by aromatase to 17-β-estradiol, and for DHEA by 3β-hydroxysteroid dehydrogenase via androstenedione, which is in turn converted by aromatase to estrone. Estrone and 17-β-estradiol can be converted into each other by 17β-hydroxysteroid dehydrogenase [106].

In this study, preliminary experiments were conducted in order to investigate the effects of steroid hormones on tissue engineering of cartilage in our bovine cell culture model. Hormones were tested with regard to their effects on primary and expanded chondrocytes. For primary chondrocytes, hormones were additionally evaluated under different oxygen partial pressures (21% and 5%).

Results

First study – 2 and 4 weeks primary culture

In the first study with primary chondrocytes and two harvesting points of time after two and four weeks, the scaffolds generally increased in wet weight and especially collagen content after four weeks of cultivation, compared to the results from the two weeks groups (Fig. 18).

Wet weight

For control constructs, a 1.4-fold increase in wet weight (Fig. 18A) was found after 4 weeks as compared to after two weeks (48.3mg vs. 68.7mg for control groups after 2 and 4 weeks). Similar increases were observed for the testosterone 10^{-7}mol/l (T1) and the DHEA 10^{-7}mol/l (D1) groups; all other groups showed lower increases. Within the two weeks groups no differences could be found.

Within the 2 and 4 weeks groups, no significant differences in wet weight could be found.

Glycosaminoglycans

Between the 2 and 4 weeks groups no definite trends were obvious and detectable. Only for the controls a distinct 1.6-fold increase (Fig. 18D) in the mass of GAG (1.6mg vs. 2.5mg GAG for 2 vs. 4 weeks group) and a 1.7-fold increase for the GAG per cell fraction (Fig. 18E) (152pg vs. 264pg for 2 vs. 4 weeks group) could be detected, but no difference in the GAG per wet weight fraction (Fig. 18F) (3.4% vs. 3.6% for 2 vs. 4 weeks group).

Within the two weeks groups, a significant increase in GAG per wet weight fraction for estrogen 10^{-9}mol/l (E-09) (3.4% vs. 5.0% for control vs. estrogen 10^{-9}) (Fig. 18F) was demonstrated. Further a tendency to increased GAG amount for estrogen 10^{-10}mol/l (E-10) and 10^{-9}mol/l (E-09) groups were detectable (1.6mg (control) vs. 2.6mg (estrogen both 10^{-10}mol/l (E-10) and 10^{-9}mol/l (E-09)) (Fig. 18D). Increased GAG per cell fraction for estrogen 10^{-9}mol/l (E-09) (151.7pg vs. 261.3pg for control vs. estrogen 10^{-9}mol/l (E-09)) (Fig. 18E). The four weeks groups showed no significant differences in the GAG content.

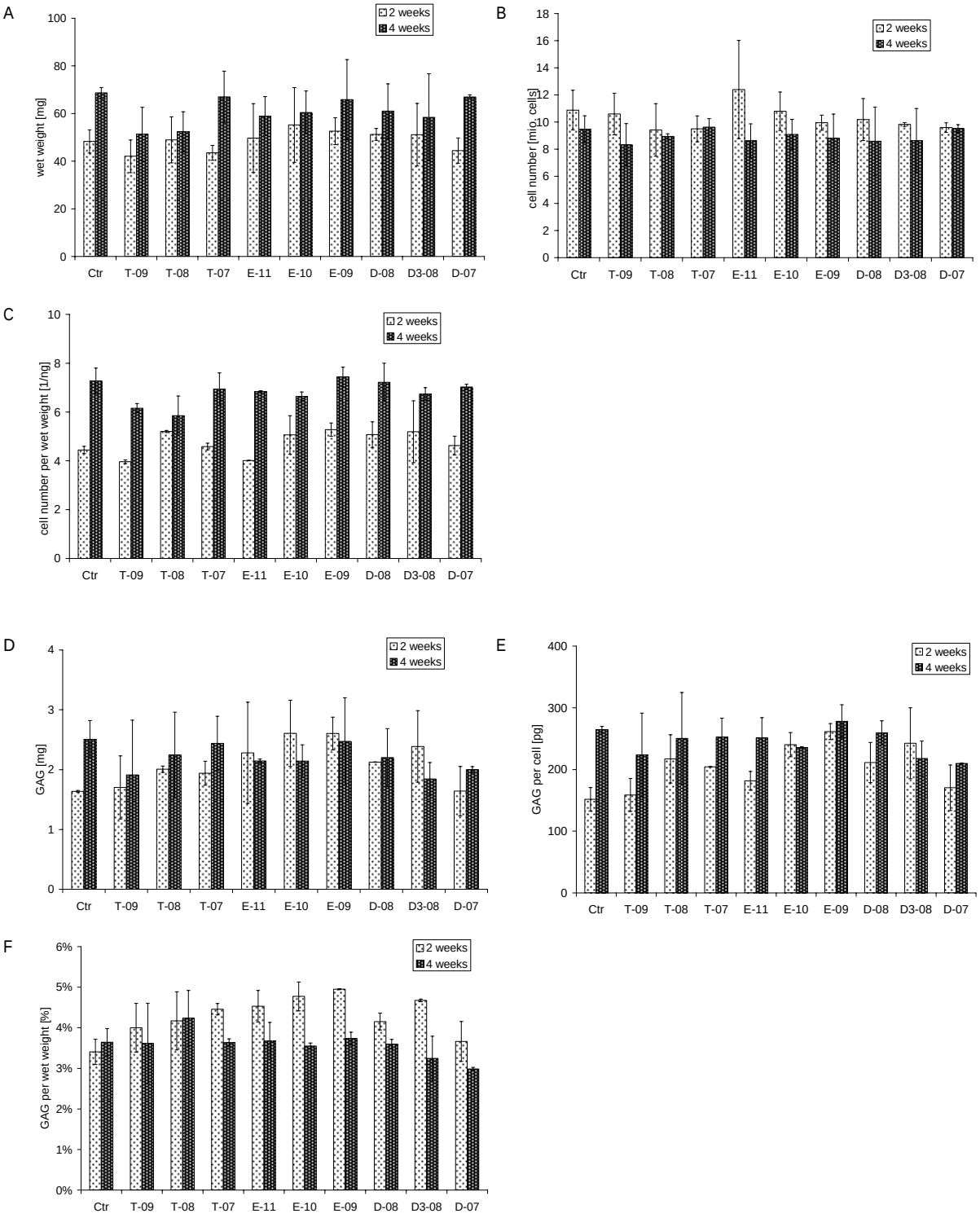


Fig. 18: Data from primary chondrocyte constructs after 2 and 4 weeks of cultivation. All experiments were performed with two independent constructs per group. Group abbreviations: testosterone (T): T-09: 10-9mol/l, T-08: 10-8mol/l, T-07: 10-7mol/l; 17β-estradiol (E): E-11: 10-11mol/l, E-10: 10-10mol/l, E-09: 10-9mol/l; dehydroepiandrosterone (DHEA) (D): D-08: 10-8mol/l, D3-08: 3·10-8mol/l, D-07: 10-7mol/l.

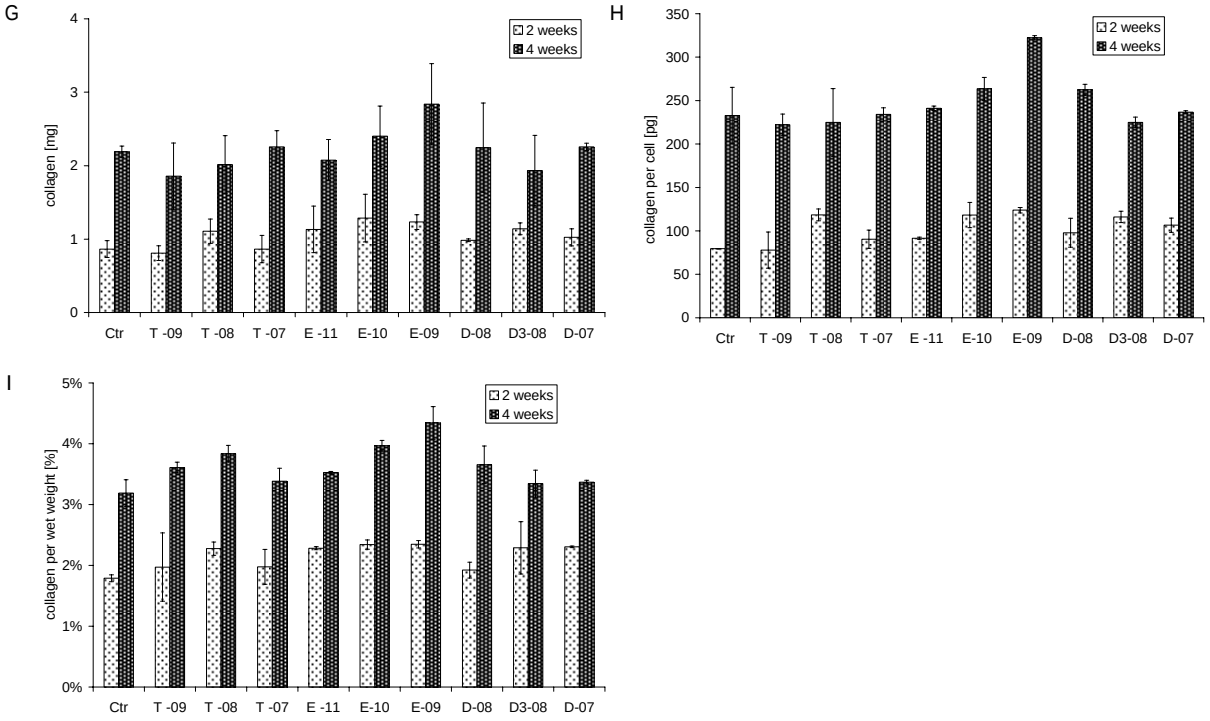


Fig. 18: Data from primary chondrocyte constructs after 2 and 4 weeks of cultivation. All experiments were performed with two independent constructs per group. Group abbreviations: testosterone (T): T-09: 10^{-9}mol/l, T-08: 10^{-8}mol/l, T-07: 10^{-7}mol/l; 17β-estradiol (E): E-11: 10^{-11}mol/l, E-10: 10^{-10}mol/l, E-09: 10^{-9}mol/l; dehydroepiandrosterone (DHEA) (D): D-08: 10^{-8}mol/l, D3-08: $3 \cdot 10^{-8}$mol/l, D-07: 10^{-7}mol/l.

Collagens

The four weeks groups resulted in tremendously higher collagen mass, collagen per cell and collagen per wet weight fraction, as compared to the two weeks groups.

For the two weeks groups no significant increase in collagen mass or collagen fraction could be found. A tendency to increased collagen weight could be found for testosterone 10^{-8}mol/l (T-08) (0.86mg vs. 1.11mg for control vs. testosterone 10^{-9}mol/l) with a 1.3-fold increase and estrogen 10^{-9}mol/l (E-09) (0.86mg vs. 1.23mg for control vs. estrogen 10^{-9}mol/l) with a 1.4-fold increase. The same groups showed increase in the GAG per cell (79.4pg for control, 118.4pg for testosterone 10^{-9}mol/l, 123.8pg for estrogen 10^{-9}mol/l) and in the GAG per wet weight fractions (1.8% for control, 2.3% for testosterone 10^{-9}mol/l, 2.3% for estrogen 10^{-9}mol/l).

Within the four weeks groups, significant increases in collagen per wet weight for estrogen 10^{-10}mol/l (E-10) and 10^{-9}mol/l (E-09) groups (3.2% vs. 4.0%/4.3% for control vs. estrogen

0.1/1) (Fig. 18I) were detected. Further tendencies of an increase in collagen per cell fraction for estrogen 10^{-9}mol/l (E-09) (233pg vs. 322pg for control vs. estrogen 1) (Fig. 18H) were observed.

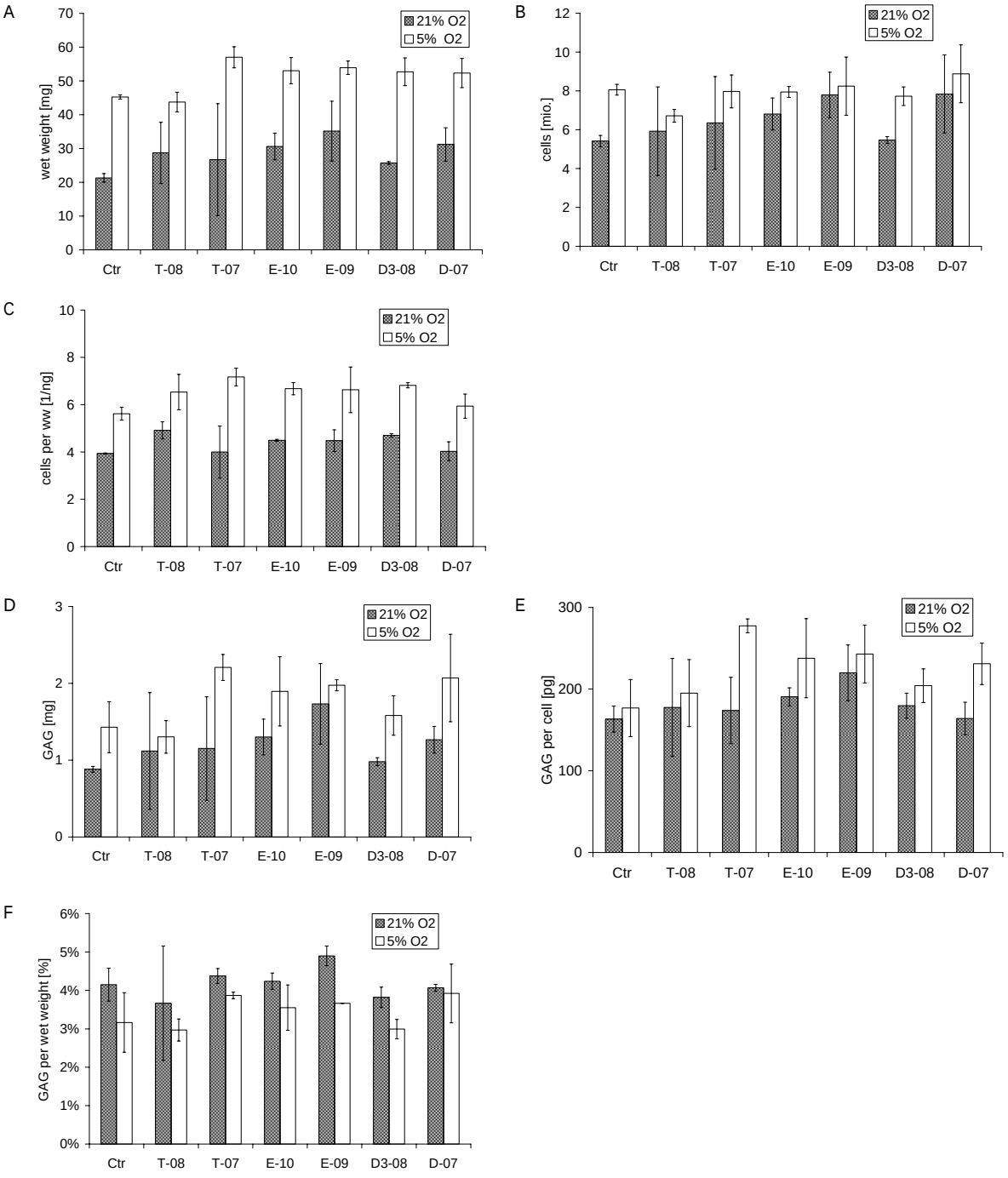


Fig. 19: Data from primary chondrocyte constructs after 3 weeks of cultivation under regular (21% O2) and low (5% O2) oxygen partial pressure. All experiments were performed with two independent constructs per group. Group abbreviations: testosterone (T): T-09: 10-9mol/l, T-08: 10-8mol/l, T-07: 10-7mol/l; 17β-estradiol (E): E-11: 10-11mol/l, E-10: 10-10mol/l, E-09: 10-9mol/l; dehydroepiandrosterone (DHEA) (D): D-08: 10-8mol/l, D3-08: 3·10-8mol/l, D-07: 10-7mol/l.

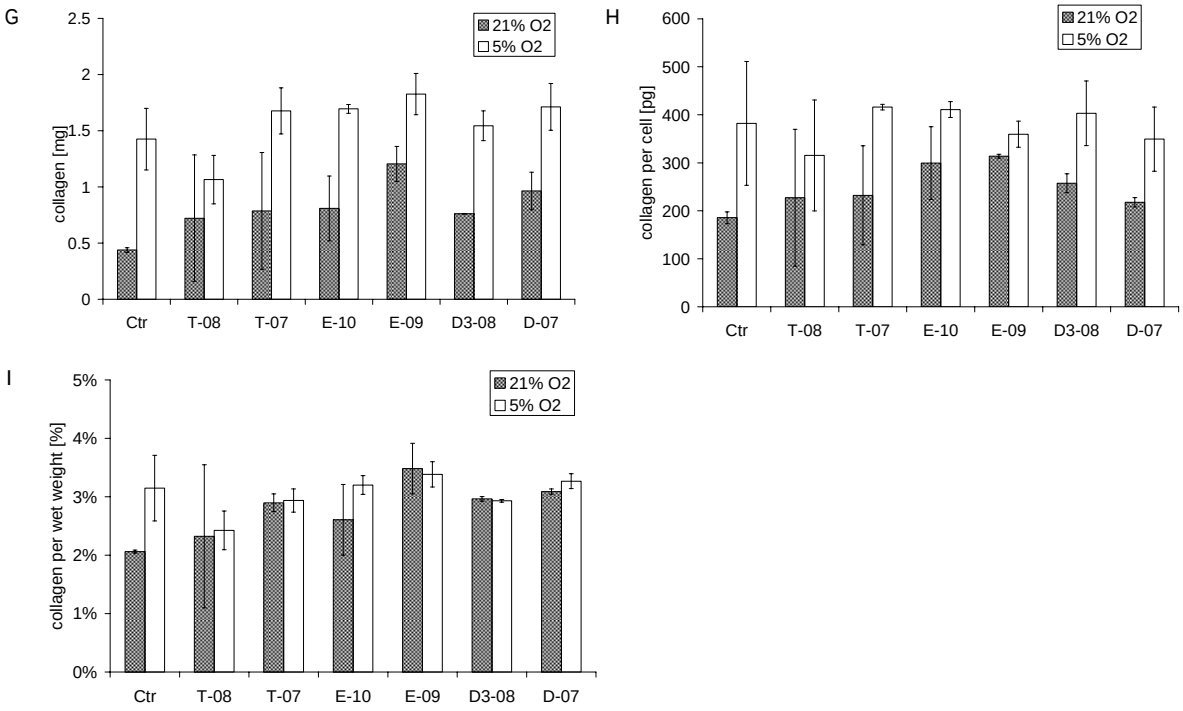


Fig. 19: Data from primary chondrocyte constructs after 3 weeks of cultivation under regular (21% O_2) and low (5% O_2) oxygen partial pressure. All experiments were performed with two independent constructs per group. Group abbreviations: testosterone (T): T-09: 10^{-9}mol/l, T-08: 10^{-8}mol/l, T-07: 10^{-7}mol/l; 17β-estradiol (E): E-11: 10^{-11}mol/l, E-10: 10^{-10}mol/l, E-09: 10^{-9}mol/l; dehydroepiandrosterone (DHEA) (D): D-08: 10^{-8}mol/l, D3-08: $3 \cdot 10^{-8}$mol/l, D-07: 10^{-7}mol/l.

Second study – differential oxygen partial pressure (three weeks)

Wet weight

As a general tendency, low oxygen conditions resulted in increased wet weight (Fig. 19A) for all groups. At 21% oxygen, constructs of the estrogen 10^{-9}mol/l (E-09) group increased in wet weight 1.7-fold, compared to the control (21.3mg vs. 35.2mg for control vs. estrogen 10^{-9}mol/l). At 5% oxygen, a 1.2-fold increase of wet weight were detected for testosterone 10^{-7}mol/l (T-07), estrogen 10^{-10}mol/l and 10^{-9}mol/l (E-10, E-09) as well as for DHEA $3 \cdot 10^{-8}$mol/l (D3-08) and 10^{-7}mol/l (D-07).

Glycosaminoglycans

At 21% oxygen, constructs of the estrogen 10^{-9}mol/l (E-09) group increased 2-fold in mass of GAG (Fig. 19D), compared to the control (0.9mg vs. 1.7mg for control vs. estrogen 10^{-9}mol/l). At 5% oxygen, testosterone 10^{-7}mol/l (T-07) increased the mass of GAG 1.5-fold, estrogen 10^{-9}mol/l (E-09) 1.4-fold, and DHEA 10^{-7}mol/l (D-07) 1.4-fold (control: 1.4mg vs. T-07: 2.2mg, E-09: 2.0mg, D-07: 2.1mg). Same trends were also detectable for the GAG per cell fraction (Fig. 19E): Under high oxygen partial pressure, constructs of the estrogen 10^{-9}mol/l (E-09) group increased 1.3-fold (163pg vs. 220pg for control vs. E-09, 21% O_2). Under low oxygen pressure, the testosterone 10^{-7}mol/l (T-07) group increased in GAG per cell 1.6-fold, estrogen 10^{-9}mol/l (E-09) increased 1.4-fold and DHEA 10^{-7}mol/l (D-07) 1.3-fold (control: 177pg vs. T-07: 277pg, E-09: 243pg and D-07: 231pg, 5% O_2).

Collagens

Under high oxygen partial pressure, mass of collagen was especially increased for estrogen 10^{-9}mol/l (E-09) (3-fold, 0.4mg vs. 1.2mg for control vs. E-09) (Fig. 19G). Similar results were observed for the collagen per cell (Fig. 19H) and the collagen per weight fraction (Fig. 2I), for which estrogen 10^{-9}mol/l (E1) yielded the largest increases (1.7-fold and 1.7-fold**)**, as compared to the control. .

All low oxygen groups increased mass of collagen compared to the related high oxygen group. Within low oxygen groups, a 1.3-fold increase in mass of collagen (Fig. 19G) could be detected for estrogen 10^{-9}mol/l (E1) and a 1.3-fold decrease for testosterone 10^{-8}mol/l (T0.1), both compared to the control (control: 1.4mg vs. estrogen 1: 1.8mg and testosterone 0.1: 1.1mg).

Third study - Expanded chondrocytes (three weeks)

Wet weight

The addition of steroid hormones had no influence on the resulting wet weight (Fig. 20A) of the constructs after a cultivation period of 3 weeks.

Glycosaminoglycans

A tendency to decreasing mass of GAG (Fig. 20D), decreasing GAG per cell (Fig. 20E) and GAG per wet weight fraction (Fig. 20F) could be found for all steroid hormone-receiving constructs.

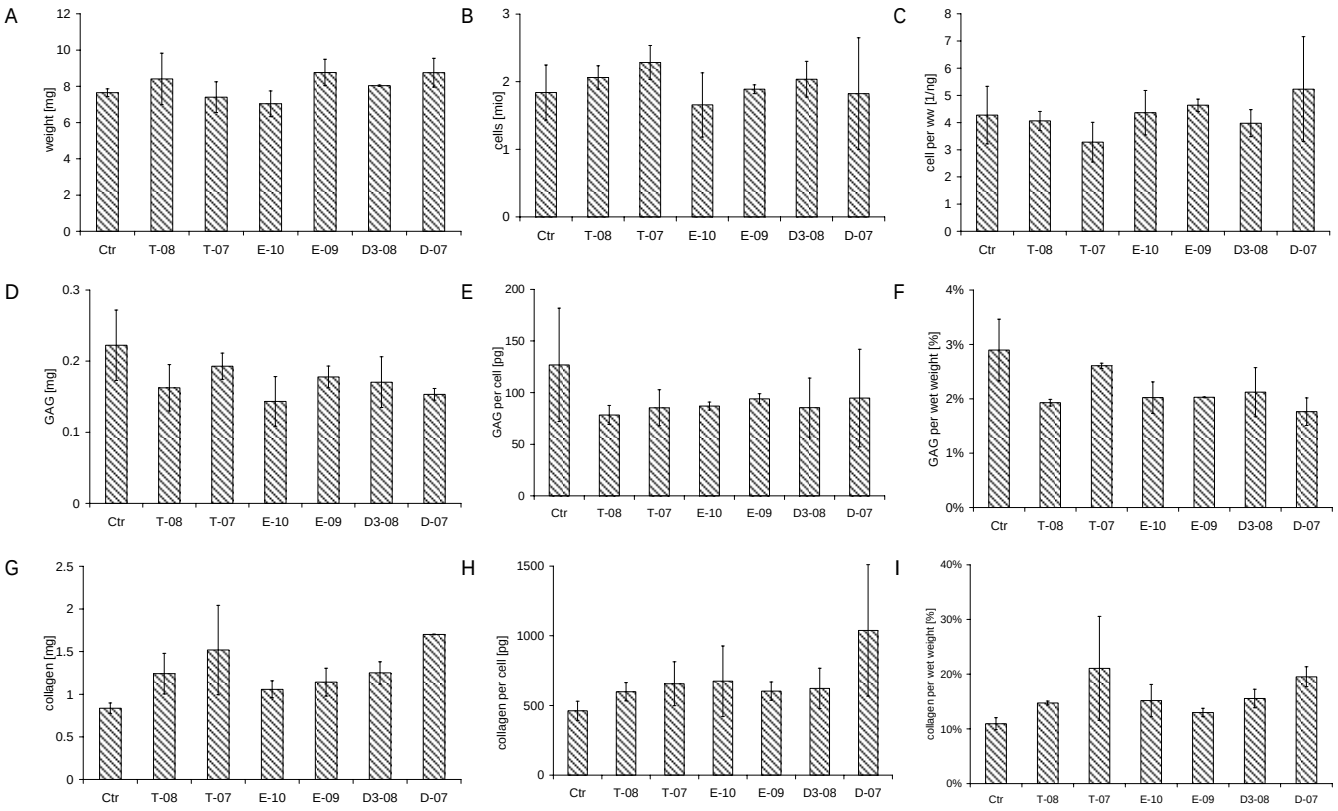


Fig. 20: Data from expanded chondrocytes constructs after 3 weeks of cultivation with 21% oxygen partial pressure. All experiments were performed with two independent constructs per group. Group abbreviations: testosterone (T): T-09: 10^{-9}mol/l, T-08: 10^{-8}mol/l, T-07: 10^{-7}mol/l; 17β-estradiol (E): E-11: 10^{-11}mol/l, E-10: 10^{-10}mol/l, E-09: 10^{-9}mol/l; dehydroepiandrosterone (DHEA) (D): D-08: 10^{-8}mol/l, D3-08: $3 \cdot 10^{-8}$mol/l, D-07: 10^{-7}mol/l.

Collagens

Testosterone 10^{-7}mol/l and DHEA 10^{-7}mol/l increased mass of collagen (Fig. 20G) by 1.8-fold and 2-fold, respectively (0.17mg for control, 0.3mg for testosterone 1, 0.34mg for DHEA 10^{-7}mol/l). For the collagen per wet weight fraction (Fig. 20I), the same trends were observed: Collagen per wet weight increased 2-fold for testosterone 10^{-7}mol/l and DHEA 10^{-7}mol/l, both compared to the control (2% for control vs. 4% for both testosterone 10^{-7}mol/l and DHEA 10^{-7}mol/l).

Discussion

In this study, the effects of steroid hormones on tissue engineered cartilage were investigated in preliminary experiments. In contrast to the previous chapters, the effects of steroid hormones on extracellular matrix development and increase of wet weight were generally small and remained distinctly behind the anabolic efficacy of protein factors like insulin and GDF-5. In the first study between the two and four weeks group increases in wet weight (Fig. 18A) and collagen (Fig. 18G-I) were detectable. After 2 weeks, increases in GAG (Fig. 18D-F) were detectable for estrogen in concentrations of 10^{-10}mol/l and 10^{-9}mol/l.

Surprisingly, these increases were not sustained in the 4 weeks constructs, which showed similar or weaker GAG content (Fig. 18D-F). The collagen content of all constructs distinctly increased between 2 and 4 weeks of culture (Fig. 18G-I). Estrogen 10^{-9}mol/l resulted in higher collagen content after 4 weeks, as compared to the control. In the second study, the responses of extracellular matrix development under the high oxygen culture conditions (21% O_2) were comparable to those of the first study after 4 weeks of cultivation. Increases in GAG (Fig. 19D-F) and collagen (Fig. 19G-I) content could be detected for the estrogen 10^{-9}mol/l group.

The response of extracellular matrix development to the steroid hormones changed with low oxygen culture conditions (5% O_2). Increases in GAG (Fig. 19D-F) content in addition to estrogen 10^{-9}mol/l could also be found for lower concentrated estrogen 10^{-10}mol/l, testosterone 10^{-7}mol/l, and DHEA 10^{-7}mol/l. For collagen mass (Fig. 19G), in the low oxygen groups (5% O_2), an increase was detected for estrogen 10^{-9}mol/l, as well as a decrease for testosterone 10^{-8}mol/l.

One side aspect of this chapter dealt with the effects of a combination of insulin and estrogen. In literature interesting effects were described of this combination (insulin and estrogen) on primary bovine cells from adult cows cultivated in monolayer culture under low oxygen (5% O_2) partial pressure.

Besides the insulin dose-dependent increase of proline incorporation, a suppression of anabolic insulin effects on proline incorporation by estradiol administration was reported [107]. These findings could not be confirmed in our cell culture model, as collagen contents (data not shown) of constructs receiving a combination of estrogen and insulin were not significantly different to those constructs receiving solely insulin. The postulated antagonism of estrogen on the anabolic effect of insulin on the proline incorporation and collagen production could not be found in our 3-D cell culture model. The differences of these findings may be attributed to a 100-fold higher estrogen concentration used in our study and/or to the 3-D environment in contrast to the 2-D cell culture. In the third study, the effects of steroid hormones on constructs generated with expanded (passage 2) bovine chondrocytes were investigated. The influence of steroid hormones on wet weight (Fig. 20A) and GAG distribution (Fig. 20D-F) were only marginal. Interesting differences were found for collagen content (Fig. 20G-I): Testosterone 10^{-7}mol/l and DHEA 10^{-7}mol/l supplementation resulted in increased collagen contents, in contrast to the results from primary chondrocytes (which were originally from the same donor calf), for which only estrogen influenced the collagen content of the extracellular matrix.

Articular cartilage acts as a steroid hormone-sensitive tissue. Intensive research was already done for the role of estrogens and androgens and other steroids on growth plate cartilage and longitudinal bone growth, which was recently reviewed [29-31]. For tissue engineered cartilage only little is known about the role of steroid hormones on the development of the constructs. From the results of this study, as of yet no definite recommendation can be made for the application of steroid hormones in vitro cartilage engineering.

Further research needs to be conducted to get a more comprehensive view of the role of steroid hormones in growing engineered tissues. In future studies, attention should be paid to the selected concentrations of hormones, to find optimum concentrations with regard to a

potential biphasic dose response, as it was shown for estrogen elsewhere [108]. Furthermore, also the gender of the donor cells may be taken into consideration [31].

Chapter 7

Effects of different oxygen partial pressures on engineered cartilage generated with primary and expanded chondrocytes

Introduction

Articular cartilage is a blood vessel free tissue. So the supply of the cartilage cells with oxygen and other essential nutrients mostly occurs via diffusion from the synovial fluid or via "forced" convection by mechanical compression of the cartilage matrix. In this chapter we will focus on the effects of oxygen on the development of engineered cartilage tissue. Oxygen is required for cellular survival. It is known for its central role in the mitochondrial respiratory chain and also for its signalling function in regulating cell metabolism and response [109]. Furthermore oxygen has shown to influence the development of cells and tissues, including cartilage formation. However, the specific role of oxygen in these processes remains still unclear and the data reported is controversial [109].

The regular oxygen tension in the synovial fluid is about 7% partial oxygen pressure, thus, it is lower compared to 12% oxygen in the arterial blood and vascularised tissue [110]. In vitro measurements within bovine tissue engineered constructs and native articular bovine cartilage showed a tendency to lower oxygen concentration for the tissue engineered constructs, compared to the native tissue, i.e., on the edge as well as in the centre of the constructs. Also an oxygen gradient was detected within the tissue engineered constructs from the edge to the near anoxic core [111]. The role of oxygen within developing tissues, both in embryonic tissues and engineered tissues remains unclear. It can be postulated, that oxygen plays an important role for those developing tissues, as oxygen was found as a mediator of developmental processes in cartilage formation [109]. Also for tissue engineering purposes further research on the effects of oxygen is mandatory, as it may also influence the redifferentiation of dedifferentiated cells and modulate the effect of growth factors on the targeted cells.

The cell response of bovine chondrocytes to IL-1β was influenced by different oxygen partial pressures [112]. Furthermore, the collagen II deposition from dedifferentiated cartilage cells in alginate was also influenced by oxygen partial pressure [113, 114]. In Tab. 3, studies on cartilage and the influence of oxygen found in literature are summarized. All these data came from 2-D or alginate beads cell culture.

To the best of our knowledge, in this chapter we present the first data on the influence of oxygen partial pressure (21% vs. 5%) on a complex 3-D cartilage tissue engineering culture; this includes the effects on primary and expanded chondrocytes as well as the modulation of the effects of the growth factors insulin and growth and development factor-5 (GDF-5).

	Species	Type of culture, culture periode	Low vs high oxygen tension	Effect on ECM	Reference
Primary	Bovine	AC in alginate	5%/20%	Coll II ↑ Proteoglycan↑	[113]
	Bovine	AC in alginate, 7d	<0.1%/20%	mRNA↓↓ Lactat ↓	[110]
	Bovine	AC in alginate, 7d	<0.1%/20%	mRNA Coll II↓ mRNA TIMP-1↑	[6]
	Bovine	AC monolayers	5%/20%	Coll II ↑ Coll IX ↑ Coll I ↓ Proliferation ↑	[115]
	Bovine	Alginate and collagen I/III membrane cultures with AC	5%/20%	Ratio of protein and mRNA Coll II/Coll I ↑	[114]
	Bovine	Articular cartilage explants 4.5h	0%/21%	Sulfage incorporation ↓	[116] [117]
	Bovine	PGA scaffold, 35d	w/o gas exchange/20%	GAG↓ Collagen↓	[118]
	Bovine	AC in porous polylactic acid constructs	5%/20%	GAG ↑ Collagen +/-	[119]
	Bovine	Articular cartilage explants, 7d	6%/24%	Sulfate incorporation ↓	[120]
	Human	OA AC on collagen I/III membranes	5%/20%	Proline and sulfate incorporation ↑	[121]
Dedifferentiated	Bovine	Cells in alginate	5%/20%	Coll II ↑ Coll IX ↑ Coll I ↓	[113]
	Bovine	AC on collagen I/III membranes	5%/20%	Coll II ↑	[114]
	Bovine	AC in alginate	5%/20%	mRNA Coll II ↑ mRNA aggrecan ↑	[122]
	Human	Nasal cells in pellet culture	5%/20% 1%/20%	GAG ↑ Coll II ↑ GAG ↑↑ Coll II ↑	[123]
	Human	AC in alginate	5%/20%	mRNA Coll II ↑ mRNA aggrecan ↑	[124]

Tab. 3: Data from literature on the influence of low oxygen tension on matrix synthesis of primary and dedifferentiated articular chondrocytes (AC) taken from [125] and completed by the author.

Results

Due to the numerous plots presented in this chapter, only the major differences can be presented and discussed in the Results and in the Discussion section. All the experiments were performed in quadruplicate, i.e., four independent scaffolds were cultured per group.

Wet weight and cell number

Primary chondrocytes

For the primary chondrocytes (Fig. 18A) a tendency to increasing wet weights with reduced oxygen partial pressure was found for the control (47.3/68.5mg : 21%/5% O_2) and all single growth factor groups (insulin 2.5µg/ml: 140.5/204.6mg; GDF-5 0.1µg/ml: 106.7/148.7mg for 21%/5% O_2). The combination of GDF-5 and insulin resulted in a tendency to lower wet weights for 5% oxygen partial pressure (GDF-5/insulin 0.1/2.5µg/ml: 352.0/291.8mg. for 21%/5% O_2). For the cell numbers per wet weight (Fig. 18C) no significant differences between 21%/5% O_2 groups could be found. All insulin-receiving groups had a tendency to decrease cell number per wet weight.

Expanded chondrocytes

The constructs generated with expanded chondrocytes exhibited up to 8-fold lower wet weight (Fig. 22A), than constructs made from primary chondrocytes (up to 43.5mg for passage 2 vs. 352.0mg for primary chondrocytes). Anabolic effects were detected for all insulin-receiving groups. The addition of GDF-5 only resulted in no significant increase in wet weight (control: 7.6/8.6mg, GDF-5 0.1µg/ml: 10.9/10.7mg for 21%/5% O_2). The combination of GDF-5/insulin produced a dose-dependent increase in wet weight (GDF-5/insulin 0.01/2.5µg/ml vs. 0.1/2.5µg/ml: 24.0 vs. 36.6mg for 21% O_2). In all insulin-supplemented groups, 5% oxygen resulted in higher wet weight compared to the high (21%) oxygen partial pressure. The cell number per wet weight (Fig. 22C) decreased for all insulin-receiving groups.

Glycosaminoglycans

Primary chondrocytes

The mass of GAG (Fig. 18D) increased after addition of growth factors. Between the different oxygen groups, the same trends as from wet weight could be found, i.e. the control (1.3/2.6 mg : 21%/5% O_2) and the single growth factor-receiving groups showed higher values for 5% than for 21% oxygen (insulin 2.5µg/ml: 7.1/10.6mg; GDF-5 0.1µg/ml: 5.3/7.5mg for 21%/5% O_2). The combination of GDF-5 and insulin resulted in inverted results, i.e., a tendency to decreased GAG mass for the constructs cultivated under 5% oxygen partial pressure (GDF-5/insulin 0.1/2.5µg/ml: 17.0/13.8mg. for 21%/5% O_2). In the GAG per cell (Fig. 18E) and GAG per wet weight (Fig. 18F) data differences could only be found between the control and the growth factor groups, but not within the different oxygen concentrations.

Expanded chondrocytes

Constructs supplemented solely with GDF-5 produced about equal mass of GAG (Fig. 22D), compared to the control (GDF-5 0.01µg/ml: 0.12mg; GDF-5 0.1µg/ml: 0.11mg; control: 0.11mg; for 5% O_2). All insulin-receiving groups resulted in significant increase of GAG mass (insulin 2.5µg/ml: 0.33mg), the combination of GDF-5/insulin even led to distinctly higher GAG content (GDF-5/insulin 0.01/2.5µg/ml: 0.26mg; GDF-5/insulin 0.1/2.5µg/ml: 0.69mg; for 21% O_2). The lower oxygen concentration of 5% resulted in higher GAG mass in the insulin groups (GDF-5/insulin 0.01/2.5µg/ml: 0.54mg; GDF-5/insulin 0.1/2.5µg/ml: 1.11 mg; for 5% O_2), compared to the respective groups receiving 21% oxygen. The higher GAG content of the insulin-receiving groups became also apparent in the GAG per cell (Fig. 22E) fraction.

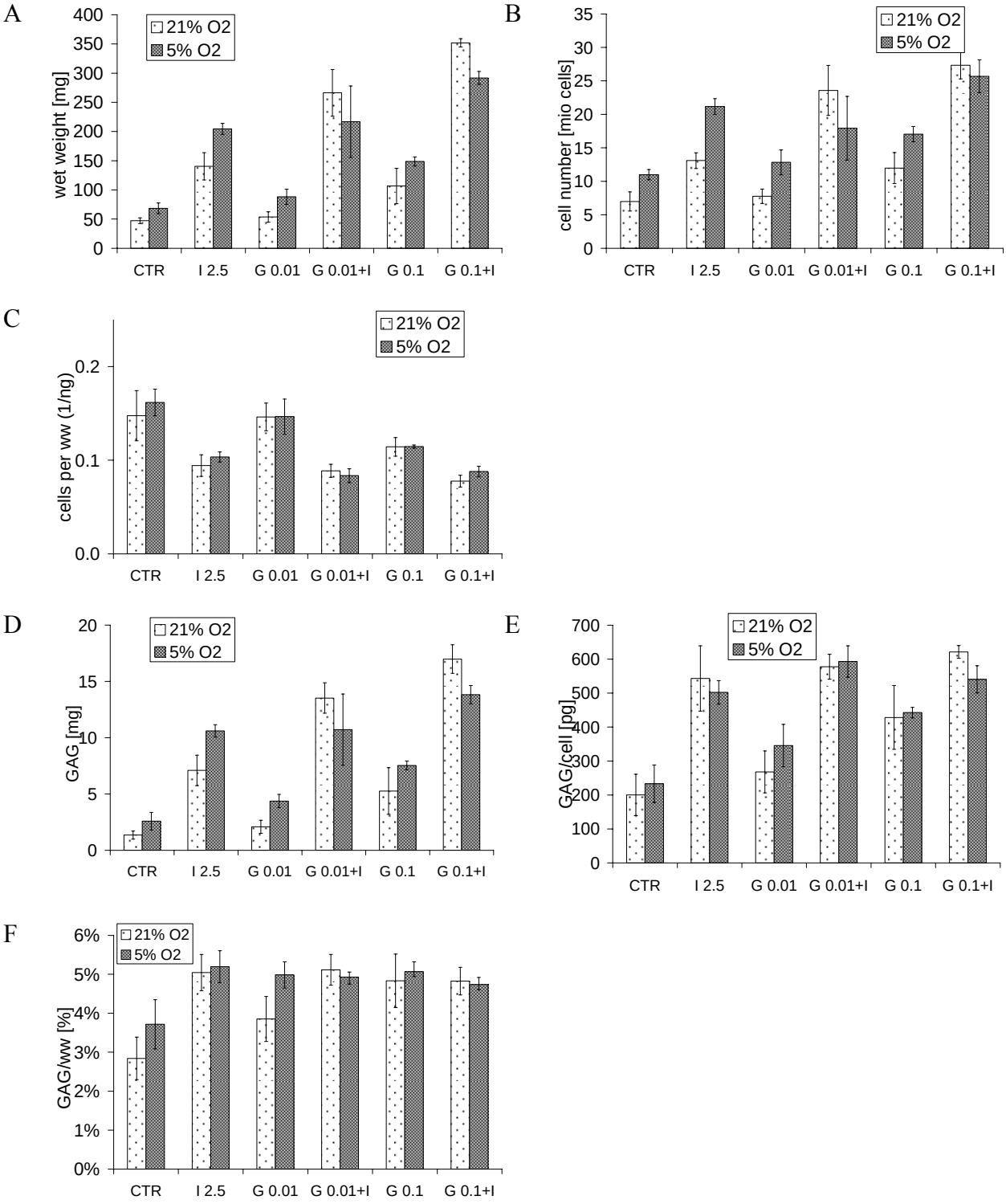


Fig. 21: Data from primary chondrocyte constructs after 3 weeks of cultivation. All experiments were performed with four independent constructs per group. Group abbreviations: (I2.5): insulin 2.5µg/ml; (G0.01): GDF-5 0.01µg/ml; (G0.1): GDF-5 0.1µg/ml; (G0.01+I) or (G0.1+I): combination of insulin 2.5µg/ml and GDF-5 0.01µg/ml or 0.1µg/ml, respectively.

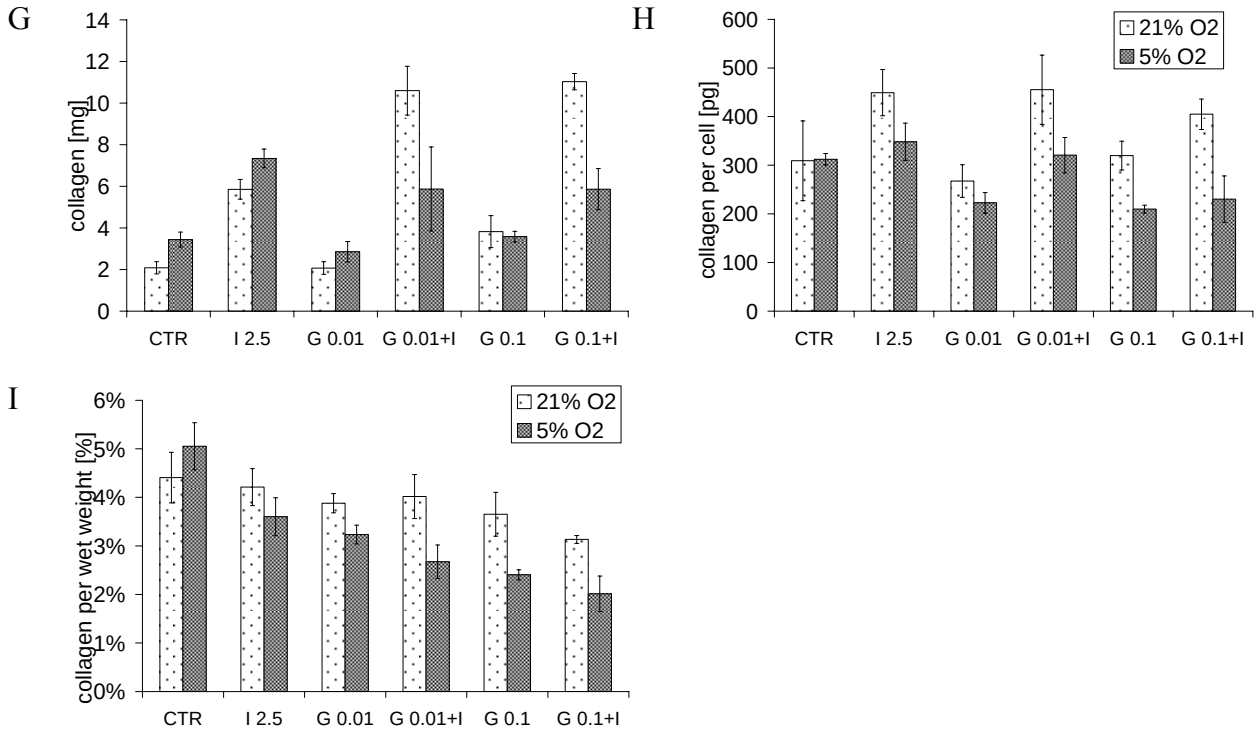


Fig. 21: Data from primary chondrocyte constructs after 3 weeks of cultivation. All experiments were performed with four independent constructs per group. Group abbreviations: (I2.5): insulin 2.5µg/ml; (G0.01): GDF-5 0.01µg/ml; (G0.1): GDF-5 0.1µg/ml; (G0.01+I) or (G0.1+I): combination of insulin 2.5µg/ml and GDF-5 0.01µg/ml or 0.1µg/ml, respectively.

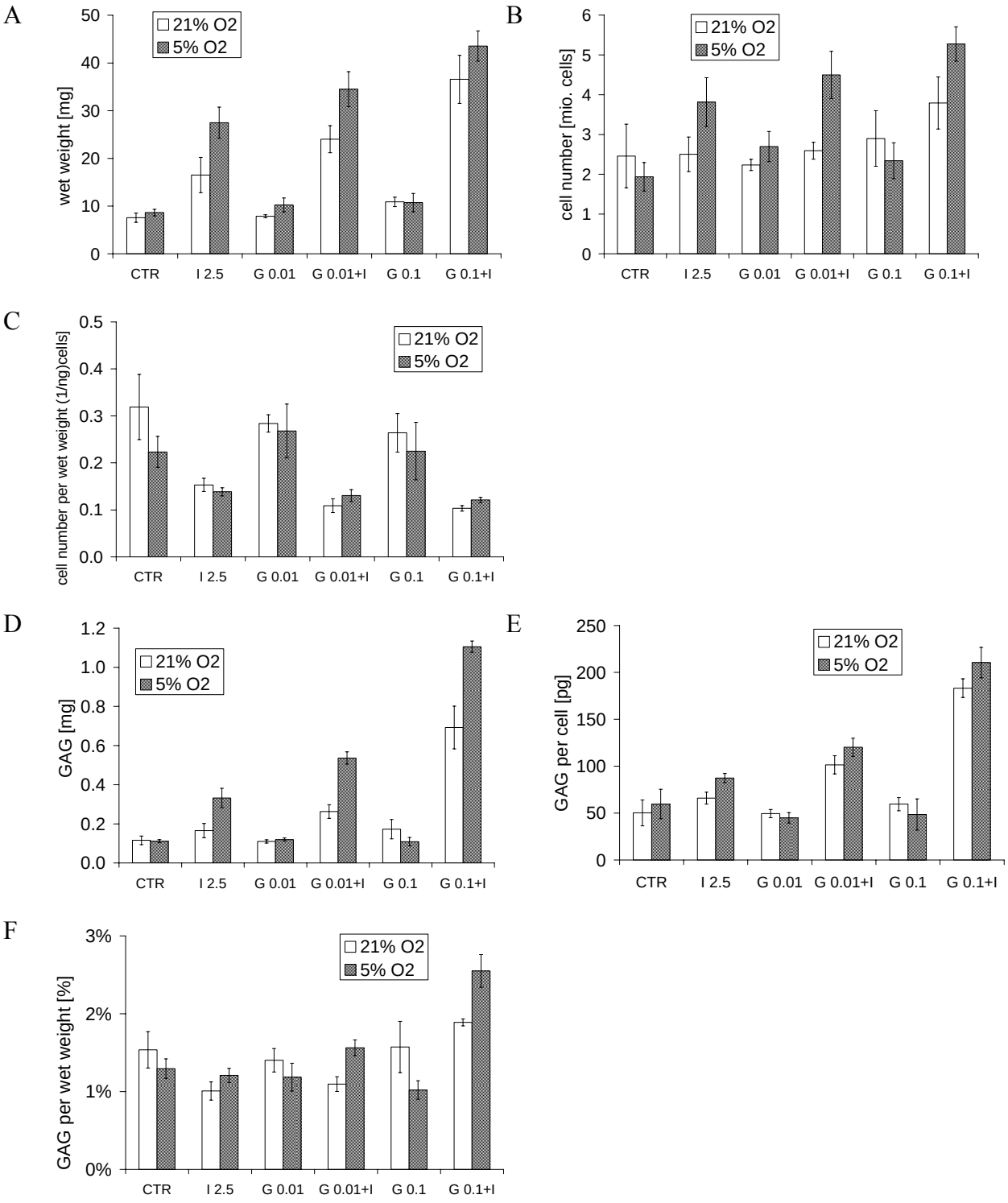


Fig. 22: Data from expanded (passage 2) chondrocyte constructs after 3 weeks of cultivation. All experiments were performed with four independent constructs per group. Group abbreviations: (I2.5): insulin 2.5µg/ml; (G0.01): GDF-5 0.01µg/ml; (G0.1): GDF-5 0.1µg/ml; (G0.01+I) or (G0.1+I): combination of insulin 2.5µg/ml and GDF-5 0.01µg/ml or 0.1µg/ml, respectively.

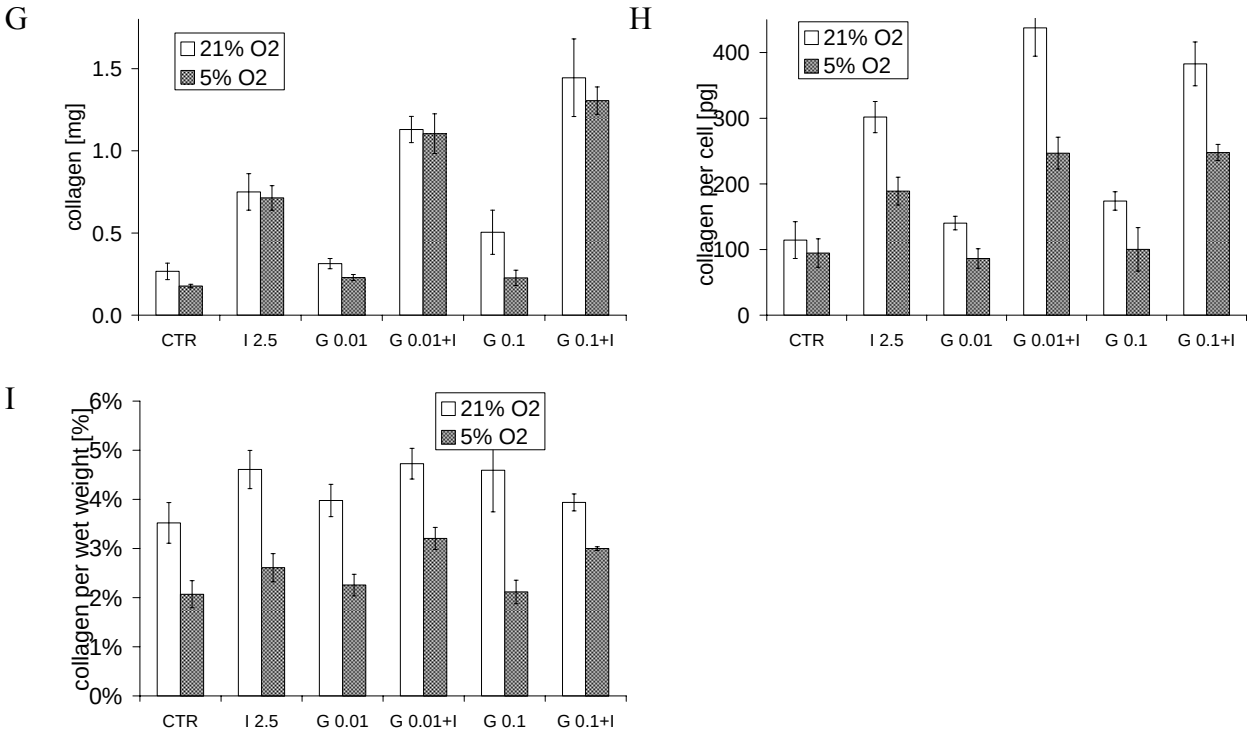


Fig. 22: Data from expanded (passage 2) chondrocyte constructs after 3 weeks of cultivation. All experiments were performed with four independent constructs per group. Group abbreviations: (I2.5): insulin 2.5µg/ml; (G0.01): GDF-5 0.01µg/ml; (G0.1): GDF-5 0.1µg/ml; (G0.01+I) or (G0.1+I): combination of insulin 2.5µg/ml and GDF-5 0.01µg/ml or 0.1µg/ml, respectively.

An especially strong increase could be found for the GDF-5/insulin 0.1/2.5µg/ml combination (for 21% O_2: GAG per cell: 183.2pg; GAG per wet weight: 1.89%); its GAG content increased distinctly compared to all other groups, especially when cultured under 5% oxygen (for 5% O_2: GAG per cell: 210.4pg; GAG per wet weight: 2.55%). The differential oxygen-dependent response with regard to GAG production was also confirmed by preliminary experiments (data not shown).

Collagen

Primary chondrocytes

The amount of produced collagen (Fig. 18G) increased with the application of insulin. Collagen mass ranged from 2.1mg (control, 21% O_2) and 5.8mg (insulin 2.5µg/ml, 21% O_2) to 11.0mg (GDF-5/insulin : 0.1/2.5µg/ml, 21% O_2), which meant an 5.3-fold increase. Under lower oxygen partial pressure (5% O_2) similar or only slightly higher results compared to 21% oxygen could be found for the control group (2.1mg vs. 3.4mg; 21% vs. 5% O_2), insulin 2.5µg/ml (5.8mg vs. 7.3mg; 21% vs. 5% O_2) and the GDF-5 groups (0.01 and 0.1µg/ml). The groups receiving the growth factor combination with GDF-5 and insulin exhibited more obvious differences between the different oxygen conditions: The high oxygen groups resulted in significantly higher collagen production than the low oxygen groups (11.0mg vs. 5.9mg, 21% vs. 5% O_2 and GDF-5/insulin: 0.1/2.5µg/ml). Resulting collagen per cell (Fig. 18H) and collagen per wet weight fraction (Fig. 18I) showed a tendency to lower values for all 5% oxygen groups compared to the respective 21% oxygen group, except for the controls. Further differentiations of the collagen composition were made by immunohistology and can be found below.

Expanded chondrocytes

The supplemented insulin led to increased collagen production in the scaffolds (insulin 2.5µg/ml: 0.75mg for 21% O_2, 0.71mg for 5% O_2). For the groups receiving GDF-5 only, no significant differences in the mass of collagen (Fig. 22G) could be found. The combination of GDF-5/insulin (0.1/2.5µg/ml) led to the highest results in collagen production, as it increased up to 1.44mg and 1.30mg (for 21% and 5% O_2, respectively. In the collagen per cell (Fig. 22H) (e.g., insulin 2.5µg/ml: 301pg vs. 189pg for 21% vs. 5% O_2) and collagen per wet weight (Fig. 22I) fraction (e.g., insulin 2.5µg/ml: 4.6% vs. 2.6% for 21% vs. 5% O_2), all high oxygen groups (21% O_2) resulted in higher collagen content, as compared to the respective low oxygen groups (5% O_2). This finding was also confirmed by preliminary experiments (data not shown).

Histology

Primary chondrocytes

The GAG distribution within the primary chondrocyte constructs (Fig. 23) was further differentiated by safranin-O staining. Among the cross-sections from constructs of the high oxygen culture (Fig. 23A), a more saturated and continuous red staining of GAG could be found for increasing GDF-5 concentrations, which was further intensified by the supplementation of insulin, with a reduced blotchiness in the staining within the core of the scaffold.

The cross-sections from the lower (5%) oxygen culture (Fig. 23B) exhibited a slightly lower saturation of the red staining and a tendency to a more scattered staining in the core of the scaffolds.

Expanded chondrocytes

The safranin-O staining of the expanded (passage 2) chondrocyte constructs (Fig. 24) showed smaller constructs with weaker staining, as compared to the constructs made from primary cells. By supplementation of growth factors strengthening of the staining was achieved. Especially the combination of GDF-5/insulin led to a saturated staining throughout the scaffold. For the combination with the high GDF-5 concentration (GDF-5/insulin 0.1/2.5µg/ml) differences between 21% oxygen (Fig. 24A) and 5% oxygen (Fig. 24B) could be detected. The cross-sections from constructs cultured under 5% oxygen showed a more continuous and intense staining and lower blotchiness within the scaffold.

As detected by the anti-collagen type II immunostaining (Fig. 25), the groups receiving the combination GDF-5/insulin were the only ones to produce collagen type II in the extracellular matrix, as an important marker for articular cartilage. Differences between the high oxygen (Fig. 25A) and the low oxygen (Fig. 25B) groups could not be found.

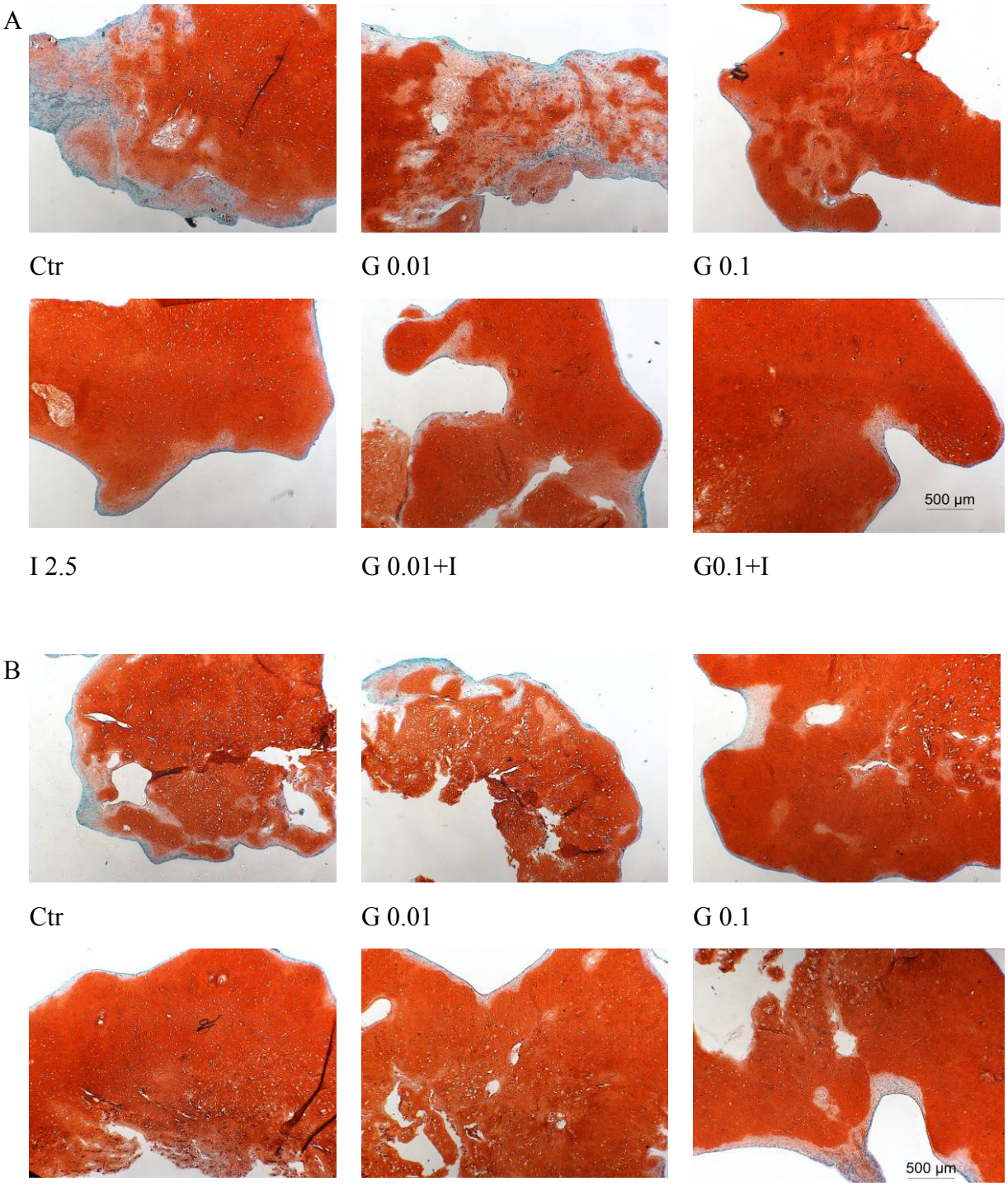


Fig. 23: Safranin-O staining of primary chondrocyte constructs after 3 weeks of cultivation. Cross-sections in (A) were from constructs cultivated under 21% oxygen, in (B) from constructs cultivated under 5% oxygen. Group abbreviations: (I2.5): insulin 2.5µg/ml; (G0.01): GDF-5 0.01µg/ml; (G0.1): GDF-5 0.1µg/ml; (G0.01+I) or (G0.1+I): combination of insulin 2.5µg/ml and GDF-5 0.01µg/ml or 0.1µg/ml, respectively.

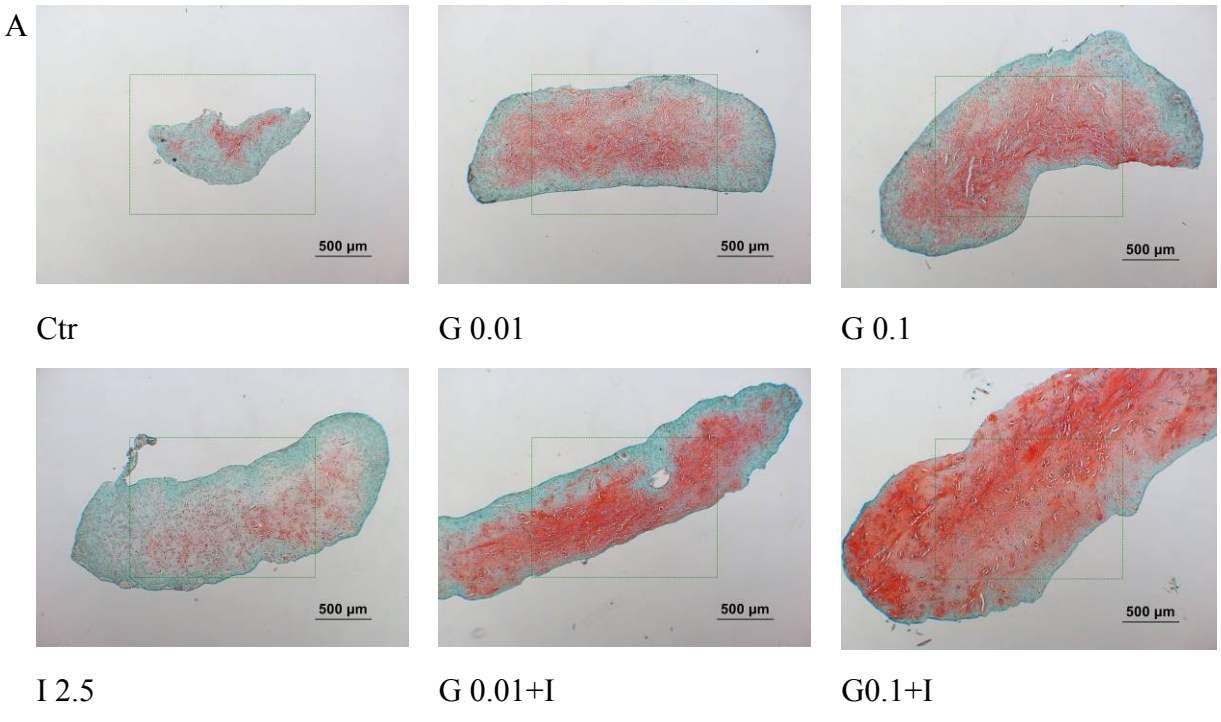
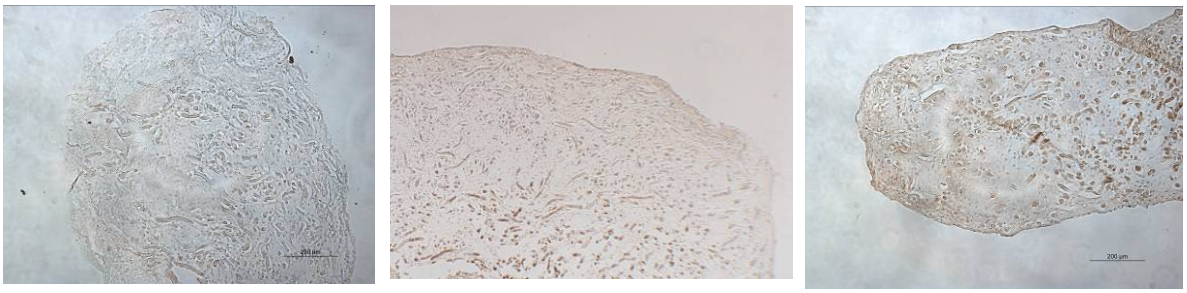
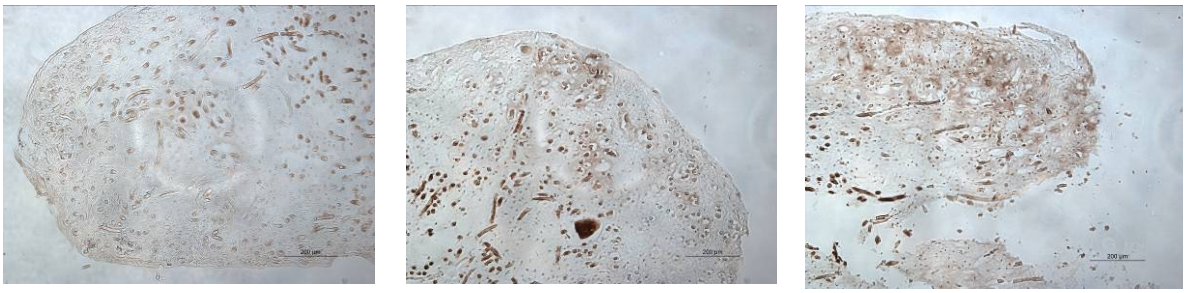


Fig. 24: Safranin-O staining of expanded (passage 2) chondrocyte constructs after 3 weeks of cultivation. Cross-sections in (A) were from constructs cultivated under 21% oxygen, in (B) from constructs cultivated under 5% oxygen. Group abbreviations: (I2.5): insulin 2.5µg/ml; (G0.01): GDF-5 0.01µg/ml; (G0.1): GDF-5 0.1µg/ml; (G0.01+I) or (G0.1+I): combination of insulin 2.5µg/ml and GDF-5 0.01µg/ml or 0.1µg/ml, respectively.

A



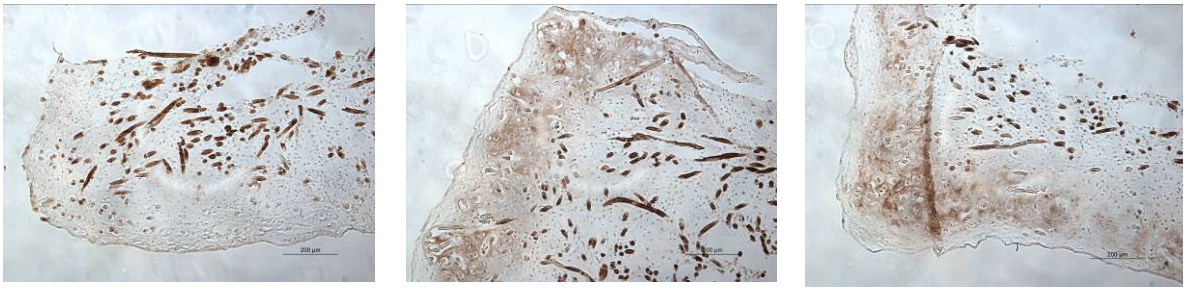
Ctr G 0.01 G 0.1



I 2.5 G 0.01+I G0.1+I

B

Ctr G 0.01 G 0.1



I 2.5 G 0.01+I G0.1+I

Fig. 25: Anti-collagen type II immunohistochemical staining of passage 2 chondrocyte constructs after 3 weeks of cultivation. Cross-sections in (A) were from constructs cultivated under 21% oxygen partial pressure, in (B) from constructs cultivated under 5% oxygen partial pressure. Group abbreviations: (I2.5): insulin 2.5µg/ml; (G0.01): GDF-5 0.01µg/ml; (G0.1): GDF-5 0.1µg/ml; (G0.01+I) or (G0.1+I): combination of insulin 2.5µg/ml and GDF-5 0.01µg/ml or 0.1µg/ml, respectively.

Discussion

In the primary chondrocyte culture, constructs in the control group and the groups supplemented with a single growth factor showed increased wet weight under low oxygen (5%) partial pressure, as compared to 21% oxygen (1.4-fold increase for control, insulin 2.5µg/ml and GDF-5 0.1µg/ml, 1.6-fold increase for GDF-5 0.01µg/ml), but not a change in extracellular matrix composition. The constructs supplemented with the growth factor combination GDF-5/insulin gave contrasting results, as the wet weight decreased for the lower oxygen culture conditions (1.2-fold decrease in wet weight for both GDF-5/insulin combinations, as compared to 21% oxygen); also the collagen fractions were reduced under 5% oxygen, as compared to 21%. In histological analysis, under 5% oxygen the core of the GDF-5/insulin (0.1/2.5 µg/ml) constructs were found to be stained in a more scattered fashion as under 21% oxygen. The weaker extracellular matrix production under 5% oxygen might be an effect of the unfavorable nutrition situation in the core of the scaffold.

Our findings are in agreement with the measurements of the oxygen pressure from Kellner et al. They measured the oxygen pressure in the center of the chondrocyte constructs, resulting in a oxygen pressure close to zero, and a strong oxygen gradient within the scaffold [111]. The relevance of these findings for natural cartilage were further discussed in a modeling analysis [126]. So it can be postulated, that the oxygen supply at 5% oxygen in this study may not be sufficient to support the metabolically highly active cartilage tissue or, as a second point, the increased size and volume of the scaffolds act as a diffusional barrier. In a cartilage pellet culture cells could cope with oxygen pressure below 0.1% for a minimum of 7 days [110]. After further culture for 21 days the low oxygen groups showed no significantly lower cell numbers, compared to their related high oxygen group. In the study presented here, also the cell fraction (cell number per weight) did not change with different oxygen partial pressure in any group. Thus, consistent with the report by Grimshaw and Mason [110], at low oxygen partial pressure the chondrocytes within the core of the scaffolds in our study might have reduced their metabolic activity resulting in the observed reduced ECM production.

For the expanded chondrocytes, the low oxygen tension caused partially contrasting results compared to those for primary chondrocytes. At low oxygen partial pressure, all insulin-receiving groups increased in wet weight (1.7-fold increase for insulin 2.5µg/ml, 1.4-fold for

GDF-5/insulin 0.01/2.5µg/ml) and cell number (1.5-fold increase for insulin 2.5µg/ml, 1.7-fold for GDF-5/insulin 0.01/2.5µg/ml), as compared to culture of the respective groups under high oxygen. For the insulin-receiving constructs, also an increase in the GAG mass became detectable under 5% oxygen, as compared to 21% oxygen (1.2-fold increase for insulin 2.5µg/ml, 2-fold for GDF-5/insulin 0.01/2.5µg/ml); a similar trend was observed for the GAG fractions per cell and per weight, respectively, suggesting a modulation of the tissue structure. In contrast, for almost all groups, low oxygen partial pressure led to decreasing collagen fractions per cell and weight, respectively, as compared to culture under high oxygen partial pressure. Especially in the collagen per cell (1.2-fold decrease for control, 1.6-fold for insulin 2.5µg/ml, 1.8-fold for GDF-5/insulin 0.01/2.5µg/ml) and collagen per wet weight fraction (1.7-fold decrease for control, 1.8-fold for insulin 2.5µg/ml, 1.5-fold for GDF-5/insulin 0.01/2.5µg/ml) this decrease became evident. With regard to the type of expressed collagen, expression of type II collagen did not seem to depend on the oxygen partial pressure, but on the usage of a suitable growth factor. Under both, 5% and 21% oxygen, the groups receiving the combination GDF-5/insulin were the only ones to distinctly express collagen type II.

This result differs from literature, where dedifferentiated bovine chondrocytes in alginate beads deposited new collagen type II protein, but only when they were kept under 5% oxygen partial pressure, not under 21% oxygen [113, 114]. Similar results were found for dedifferentiated human chondrocytes embedded in alginate beads, which showed, under 5% oxygen partial pressure, increased expression of collagen II, Aggrecan and sox9, compared to the 20% oxygen groups [124]. The differences to the results of our study might predominantly be an effect of the different scales in cell construct size, as our scaffolds range from 4-7mm in diameter and are 2mm in height (for constructs from dedifferentiated chondrocytes); thus, they might be hard to compare with scaffolds cultivated in alginate beads with a diameter below 1mm. In our cell culture, more complex aspects of diffusion and nutrition appear to influence the development of the larger constructs..

In general, the contrasting results of the conducted studies to date including the one presented here indicate that further research needs to be done with regard to the role of oxygen partial pressure within tissue-engineered constructs. Elucidating the influence of differential oxygen concentrations, also in distinct oxygen gradients within the scaffold [111], on cell spreading, cell density and extracellular matrix deposition within the scaffold, might lead to a tissue-engineered cartilage material that possesses a layer composition similar or equal to natural cartilage. In parallel, further research in understanding ATP synthesis of the chondrocytes need to be done to explain the negative Pasteur effect and the source of non-oxygen related energy generation, as described elsewhere [116, 117]. Also we need a better understanding of the cellular response of chondrocytes to low oxygen, e.g., it has been suggested that it might be mediated by hypoxia inducible factor (HIF-1) [125].

In our cell culture model, oxygen has a potential as a supporting tool which modulates the response to an applied growth factor. In general, it might be useful to improve cell culture conditions in 2-D or micro 3-D cell culture, as proven in alginate beads, and, in the future, might contribute to the engineering of small cartilage allografts. However, as of yet, specific standard oxygen conditions for cartilage engineering cannot be defined, as the tissue response, e.g., the extracellular matrix deposition has been too controversial in different studies.

Chapter 8

Summary and Conclusions

Summary and Conclusions

Articular cartilage is likely a major candidate to be successfully regenerated by tissue engineering methods; first commercial products have already reached the market. However, to fully regenerate articular cartilage tissue, further optimization appears mandatory with regard to the application of morphogens, the maintenance of chondrocyte phenotype by applied morphogens and improved culture conditions, and the composition of engineered cartilage constructs.

Drug delivery systems in tissue engineering

In this thesis, it was demonstrated that cartilage-effective proteins such as insulin can be released from cylindrical lipid matrices, preserving the bioactivity of incorporated and released proteins (chapter 3). In addition, it was demonstrated that the employed cartilage engineering in vitro assay facilitates the testing of controlled release devices with regard to their biological efficacy in a complex 3-D system without the need for the expense of laboratory animals (chapter 3).

As a second controlled release device the suitability of solid lipid templated macroporous scaffolds as protein release system and at the same time as artificial cell carrier was proven (chapter 4). The embedded insulin was released from those smart scaffolds over an extended period of time under retention of its bioactivity facilitating the generation of cartilaginous constructs.

Morphogens for cartilage tissue engineering

In the area of morphogens, experiments with GDF-5 alone and in combination with insulin on expanded chondrocytes resulted in a clear shift of the cells towards the cartilaginous phenotype (chapter 5). Especially in combination with insulin, GDF-5 appeared to enable the redifferentiation of expanded chondrocytes and the concurrent generation of relevant cartilaginous constructs exhibiting substantial fractions of the extracellular matrix components glycosaminoglycans and collagen. With regard to the synergistic effects of GDF-5 and insulin observed, further research is required to elucidate the mechanism on the molecular level. The fact that GDF-5 was demonstrated to decisively modulate the response to another cartilage-

effective protein contributes to the emerging picture of the role GDF-5 apparently plays in chondrogenesis and cartilage physiology.

Further experiments were conducted investigating the effects of sexual steroid hormones on tissue engineered cartilage (chapter 6). In contrast to the previous chapters, the effects of testosterone, estrogen and dehydroepiandrosterone on extracellular matrix development and increase of wet weight of cartilaginous constructs were generally small and remained distinctly behind the anabolic efficacy of protein factors like insulin and GDF-5. From these preliminary experiments, no definite recommendation could be made for the application of steroid hormones in in-vitro cartilage engineering. Thus, further research needs to be conducted to get a more comprehensive view of the potential of steroid hormones in growing engineered tissues.

Oxygen conditions in tissue engineering of cartilage

Despite the oxygen partial pressure of 10% and lower in native articular cartilage, the majority of in vitro investigations including cartilage engineering approaches are conducted using an oxygen concentration of 21%. As a third aspect of this thesis, the effects of differential oxygen partial pressure (5% and 21% O_2) on cartilaginous constructs were investigated (chapter 7). Taking together both the controversial results from many studies in the literature and the results obtained here, still specific standard oxygen conditions for cartilage engineering cannot be defined. Nevertheless, in our cell culture model it could be demonstrated that oxygen can be utilized as a supporting tool which modulates the response to an applied growth factor.

Chapter 9

References

[1] Langer R and Vacanti J P, 'Tissue engineering', *Science* **260** (5110), 920-926 (1993).

[2] Archer C W and Francis-West P, 'The chondrocyte', *Int J Biochem Cell Biol* **35** (4), 401-404 (2003).

[3] Ulrich-Vinther M, Maloney M D, Schwarz E M, Rosier R, and O'Keefe R J, 'Articular cartilage biology', *Am Acad Orthop Surg* **11** (6), 421-430 (1999).

[4] Leipzig N D and Athanasiou K A, 'Cartilage Regeneration ', Marcel Dekker, Inc., 283-291 (2004).

[5] Rajpurohit R, Koch C J, Tao Z, Teixeira C M, and Shapiro I M, 'Adaptation of chondrocytes to low oxygen tension: relationship between hypoxia and cellular metabolism', *J Cell Physiol* **168** (2), 424-432 (1996).

[6] Grimshaw M J and Mason R M, 'Modulation of bovine articular chondrocyte gene expression in vitro by oxygen tension', *Osteoarthritis And Cartilage / OARS, Osteoarthritis Research Society* **9** (4), 357-364 (2001).

[7] Buckwalter J A and Martin J A, 'Osteoarthritis', *Adv Drug Delivery Rev* **58** (2), 150-167 (2006).

[8] Buckwalter J A and Mankin H J, 'Instructional Course Lectures, The American Academy of Orthopaedic Surgeons - Articular Cartilage. Part II: Degeneration and Osteoarthrosis, Repair, Regeneration, and Transplantation*{{dagger}}', *J Bone Joint Surg Am* **79** (4), 612-632 (1997).

[9] Henderson I J P, Tuy B, Connell D, Oakes B, and Hettwer W H, 'Prospective clinical study of autologous chondrocyte implantation and correlation with MRI at three and 12 months', *The Journal Of Bone And Joint Surgery British Volume* **85** (7), 1060-1066 (2003).

[10] Marlovits S, Singer P, Zeller P, Mandl I, Haller J, and Trattnig S, 'Magnetic resonance observation of cartilage repair tissue (MOCART) for the evaluation of autologous chondrocyte transplantation: Determination of interobserver variability and correlation to clinical outcome after 2 years', *European Journal of Radiology* **57** (1), 16-23 (2006).

[11] Temenoff J S and Mikos A G, 'Review: tissue engineering for regeneration of articular cartilage', *Biomaterials* **21** (5), 431-440 (2000).

[12] Tabata Y, 'The importance of drug delivery systems in tissue engineering', *Pharmaceutical Science & Technology Today* **3** (3), 80-89 (2000).

[13] Tabata Y, 'Significance of release technology in tissue engineering', *Drug Discovery Today* **10** (23-24), 1639-1646 (2005).

[14] Gerwin N, Hops C, and Lucke A, 'Intraarticular drug delivery in osteoarthritis', *Adv Drug Delivery Rev* **58** (2), 226-242 (2006).

[15] Elima K and Vuorio E, 'Expression of mRNAs for collagens and other matrix components in dedifferentiating and redifferentiating human chondrocytes in culture', *FEBS Letters* **258** (2), 195-198 (1989).

[16] Freed L E, Marquis J C, Nohria A, Emmanual J, Mikos A G, and Langer R, 'Neocartilage formation in vitro and in vivo using cells cultured on synthetic biodegradable polymers', *J Biomed Mater Res* **27** (1), 11-23 (1993).

[17] Freed L E and Vunjak-Novakovic G, 'Tissue Engineering of Cartilage',(120), CRC Press, 1788-1806 (1996).

[18] Kellner K, Schulz M B, Goepferich A, and Blunk T, 'Insulin in tissue engineering of cartilage: A potential model system for growth factor application', *J Drug Targeting* **9** (6), 439-448 (2001).

[19] Whitaker M J, Quirk R A, Howdle S M, and Shakesheff K M, 'Growth factor release from tissue engineering scaffolds', *The Journal Of Pharmacy And Pharmacology* **53** (11), 1427-1437 (2001).

[20] Babensee J E, McIntire L V, and Mikos A G, 'Growth factor delivery for tissue engineering', *Pharm Res* **17** (5), 497-504 (2000).

[21] Tabata Y, 'Tissue regeneration based on growth factor release', *Tissue Eng* **9** (Supplement 1), S5-S15 (2003).

[22] Maschke A, Lucke A, Vogelhuber W, Fischbach C, Appel B, Blunk T, and Goepferich A, 'Lipids: An Alternative Material for Protein and Peptide Release',(13), American Chemical Society, 176-196 (2004).

[23] Hacker M, Tessmar J, Neubauer M, Blaimer A, Blunk T, Goepferich A, and Schulz M B, 'Towards biomimetic scaffolds: anhydrous scaffold fabrication from biodegradable amine-reactive diblock copolymers', *Biomaterials* **24** (24), 4459-4473 (2003).

[24] Hacker M, Ringhofer M, Neubauer M, Vogel T, Appel B, Blunk B T, Goepferich A, and Schulz M, 'Solid Lipid Templating: A Versatile Lab-scale Fabrication Technique for Macroporous Tissue Engineering Scaffolds',(6), University of Regensburg, Germany, 167-191 (2004).

[25] Storm E E, Huynh T V, Copeland N G, Jenkins N A, Kingsley D M, and Lee S J, 'Limb alterations in brachypodism mice due to mutations in a new member of the TGF beta-superfamily', *Nature* **368** (6472), 639-643 (1994).

[26] Tsumaki N, Nakase T, Miyaji T, Kakiuchi M, Kimura T, Ochi T, and Yoshikawa H, 'Bone morphogenetic protein signals are required for cartilage formation and differently regulate joint development during skeletogenesis', *J Bone Miner Res* **17** (5), 898-906 (2002).

[27] Francis-West P H, Abdelfattah A, Chen P, Allen C, Parish J, Ladher R, Allen S, MacPherson S, Luyten F P, and Archer et a, 'Mechanisms of GDF-5 action during skeletal development', *Development (Cambridge, England)* **126** (6), 1305-1315 (1999).

[28] Chang S C, Hoang B, Thomas J T, Vukicevic S, Luyten F P, Ryba N J, Kozak C A, Reddi A H, and Moos M e a, 'Cartilage-derived morphogenetic proteins. New members of the transforming growth factor-beta superfamily predominantly expressed in long bones during human embryonic development', *The Journal Of Biological Chemistry* **269** (45), 28227-28234 (1994).

[29] Nilsson O, Marino R, De Luca F, Phillip M, and Baron J, 'Endocrine regulation of the growth plate', *Hormone Research* **64** (4), 157-165 (2005).

[30] Sävendahl L, 'Hormonal Regulation of Growth Plate Cartilage', *Hormone Research* **64** (Suppl. 2), 94-97 (2005).

[31] Kinney R C, Schwartz Z, Week K, Lotz M K, and Boyan B D, 'Human articular chondrocytes exhibit sexual dimorphism in their responses to 17beta-estradiol', *Osteoarthritis And Cartilage / OARS, Osteoarthritis Research Society* **13** (4), 330-337 (2005).

[32] Brange J and Langkjaer L, 'Insulin Structure and Stability', **1**(11), Plenum Press, 312-350 (1993).

[33] Weiser B, 'Effekte von Insulin im Tissue Engineering von Knorpel:
Etablierung eines kontinuierlichen Applikationssystems
im Vergleich zur herkömmlichen Dosierung', Fachhochschule Weihenstephan, University of Applied Sciences, Dept. of Biotechnology and Bioinformatics (2002).

[34] Freed L E, Vunjak-Novakovic G, Biron R J, Eagles D B, Lesnoy D C, Barlow S K, and Langer R, 'Biodegradable polymer scaffolds for tissue engineering', *Bio/Technology (Nature Publishing Company)* **12** (7), 689-693 (1994).

[35] Kim Y J, Sah R L, Doong J Y, and Grodzinsky A J, 'Fluorometric assay of DNA in cartilage explants using Hoechst 33258', *Anal Biochem* **174** (1), 168-176 (1988).

[36] Farndale R W, Buttle D J, and Barrett A J, 'Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue', *Biochim Biophys Acta* **883** (2), 173-177 (1986).

[37] Taylor K B and Jeffree G M, 'A new basic metachromatic dye, I:9-dimethyl methylene blue', *Histochem J* **1** (3), 199-204 (1969).

[38] Woessner J, 'The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid', *Arch Biochem Biophys* **93** (2), 440-447 (1961).

[39] Hollander A P, Heathfield T F, Webber C, Iwata Y, Bourne R, Rorabeck C, and Poole A R, 'Increased damage to type II collagen in osteoarthritic articular cartilage detected by a new immunoassay', *J Clin Invest* **93** (4), 1722-1732 (1994).

[40] Martin I, Obradovic B, Freed L E, and Vunjak-Novakovic G, 'Method for quantitative analysis of glycosaminoglycan distribution in cultured natural and engineered cartilage', *Ann Biomed Eng* **27** (5), 656-662 (1999).

[41] Kellner K, Tessmar J, Milz S, Angele P, Nerlich M, Schulz M B, Blunk T, and Goepferich A, 'PEGylation Does Not Impair Insulin Efficacy in Three-Dimensional Cartilage Culture: An Investigation toward Biomimetic Polymers', *Tissue Eng* **10** (3-4), 429-440 (2004).

[42] Angele P, Schumann D, Angele M, Kinner B, Englert C, Hente R, Fuchtmeier B, Nerlich M, Neumann C, and Kujat et a, 'Cyclic, mechanical compression enhances chondrogenesis of mesenchymal progenitor cells in tissue engineering scaffolds', *Biorheology* **41** (3-4), 335-346 (2004).

[43] Appel B, Maschke A, Weiser B, Sarhan H, Englert C, Angele P, Blunk T, and Gopferich A, 'Lipidic implants for controlled release of bioactive insulin: Effects on cartilage engineered in vitro', *Int J Pharm* **314** (2), 170-178 (2006).

[44] Lucke A, Fustella E, Tessmar J, Gazzaniga A, and Goepferich A, 'The effect of poly(ethylene glycol)-poly(D,L-lactic acid) diblock copolymers on peptide acylation', *J Controlled Release* **80** (1-3), 157-168 (2002).

[45] Lucke A and Goepferich A, 'Acylation of peptides by lactic acid solutions', *Eur J Pharm Biopharm* **55** (1), 27-33 (2003).

[46] Zhu G, Mallery S R, and Schwendeman S P, 'Stabilization of proteins encapsulated in injectable poly (lactide- co-glycolide)', *Nat Biotechnol* **18** (1), 52-57 (2000).

[47] Zhu G and Schwendeman S P, 'Stabilization of proteins encapsulated in cylindrical poly(lactide-co-glycolide) implants: mechanism of stabilization by basic additives', *Pharm Res* **17** (3), 351-357 (2000).

[48] Kim Y J, Choi S, Koh J J, Lee M, Ko K S, and Kim S W, 'Controlled release of insulin from injectable biodegradable triblock copolymer', *Pharm Res* **18** (4), 548-550 (2001).

[49] Surendrakumar K, Martyn G P, Hodgers E C M, Jansen M, and Blair J A, 'Sustained release of insulin from sodium hyaluronate based dry powder formulations after pulmonary delivery to beagle dogs', *J Controlled Release* **91** (3), 385-394 (2003).

[50] Choi S and Kim S W, 'Controlled release of insulin from injectable biodegradable triblock copolymer depot in ZDF rats', *Pharm Res* **20** (12), 2008-2010 (2003).

[51] Brange J and Langkjaer L, 'Chemical stability of insulin. 3. Influence of excipients, formulation, and pH', *Acta Pharm Nord* **4** (3), 149-158 (1992).

[52] Cai L, Okumu F W, Cleland J L, Beresini M, Hogue D, Lin Z, and Filvaroff E H, 'A slow release formulation of insulin as a treatment for osteoarthritis', *Osteoarthritis Cartilage* **10** (9), 692-706 (2002).

[53] Dellamary L, Smith D J, Bloom A, Bot S, Guo G R, Deshmuk H, Costello M, and Bot A, 'Rational design of solid aerosols for immunoglobulin delivery by modulation of aerodynamic and release characteristics', *J Controlled Release* **95** (3), 489-500 (2004).

[54] Prego C, Garcia M, Torres D, and Alonso M J, 'Transmucosal macromolecular drug delivery', *J Controlled Release* **101** (1-3), 151-162 (2005).

[55] Thomas T T, Kohane D S, Wang A, and Langer R, 'Microparticulate formulations for the controlled release of interleukin-2', *J Pharm Sci* **93** (5), 1100-1109 (2004).

[56] Fu K, Klibanov A M, and Langer R, 'Protein stability in controlled-release systems', *Nat Biotechnol* **18** (1), 24-25 (2000).

[57] Vogelhuber W, Magni E, Gazzaniga A, and Goepferich A, 'Monolithic glyceryl trimyristate matrices for parenteral drug release applications', *Eur J Pharm Biopharm* **55** (1), 133-138 (2003).

[58] Schwendeman S P, Cardamome M, Klibanov A M, Langer R, and Brandon M, 'Stability of Proteins and Their Delivery from Biodegradable Polymer Microspheres',(1), Dekker, 1-49 (1996).

[59] Isogai N, Morotomi T, Hayakawa S, Munakata H, Tabata Y, Ikada Y, and Kamiishi H, 'Combined chondrocyte-copolymer implantation with slow release of basic fibroblast growth factor for tissue engineering an auricular cartilage construct', *Journal Of Biomedical Materials Research Part A* **74** (3), 408-418 (2005).

[60] Howdle S M, Watson M S, Whitaker M J, Davies M C, Shakesheff K M, Popov V K, Mandel F S, and Don Wang J, 'Supercritical fluid mixing: preparation of thermally sensitive polymer composites containing bioactive materials', *Chem Commun* (1), 109-110 (2001).

[61] Sheridan M H, Shea L D, Peters M C, and Mooney D J, 'Bioabsorbable polymer scaffolds for tissue engineering capable of sustained growth factor delivery', *Journal Of Controlled Release: Official Journal Of The Controlled Release Society* **64** (1-3), 91-102 (2000).

[62] Lee J E, Kim K E, Kwon I C, Ahn H J, Lee S H, Cho H, Kim H J, Seong S C, and Lee M C, 'Effects of the controlled-released TGF-beta 1 from chitosan microspheres on chondrocytes cultured in a collagen/chitosan/glycosaminoglycan scaffold', *Biomaterials* **25** (18), 4163-4173 (2004).

[63] Lee J E, Kim S E, Kwon I C, Ahn H J, Cho H, Lee S H, Kim H J, Seong S C, and Lee M C, 'Effects of a chitosan scaffold containing TGF-beta1 encapsulated chitosan microspheres on in vitro chondrocyte culture', *Artificial Organs* **28** (9), 829-839 (2004).

[64] Murphy W L, Peters M C, Kohn D H, and Mooney D J, 'Sustained release of vascular endothelial growth factor from mineralized poly(lactide-co-glycolide) scaffolds for tissue engineering', *Biomaterials* **21** (24), 2521-2527 (2000).

[65] Salvay D M and Shea L D, 'Inductive tissue engineering with protein and DNA-releasing scaffolds', *Mol Biosyst* **2** (1), 36-48 (2006).

[66] Huang Y C, Kaigler D, Rice K G, Krebsbach P H, and Mooney D J, 'Combined angiogenic and osteogenic factor delivery enhances bone marrow stromal cell-driven bone regeneration', *Journal Of Bone And Mineral Research: The Official Journal Of The American Society For Bone And Mineral Research* **20** (5), 848-857 (2005).

[67] Sohier J, Vlugt T J H, Cabrol N, Van Blitterswijk C, de Groot K, and Bezemer J M, 'Dual release of proteins from porous polymeric scaffolds', *Journal Of Controlled Release: Official Journal Of The Controlled Release Society* **111** (1-2), 95-106 (2006).

[68] Mikos A G, Thorsen A J, Czerwonka L A, Bao Y, Langer R, Winslow D N, and Vacanti J P, 'Preparation and characterization of poly(-lactic acid) foams', *Polymer* **35** (5), 1068-1077 (1994).

[69] Mikos A G, Sarakinos G, Leite S M, Vacanti J P, and Langer R, 'Laminated three-dimensional biodegradable foams for use in tissue engineering', *Biomaterials* **14** (5), 323-330 (1993).

[70] Freed L E, Vunjak N G, Biron R J, Eagles D B, Lesnoy D C, Barlow S K, and Langer R, 'Biodegradable polymer scaffolds for tissue engineering', *Biotechnology (N Y)* **12** (7), 689-693 (1994).

[71] Mooney D J, Baldwin D F, Suh N P, Vacanti J P, and Langer R, 'Novel approach to fabricate porous sponges of poly(,-lactic-co-glycolic acid) without the use of organic solvents', *Biomaterials* **17** (14), 1417-1422 (1996).

[72] Nam Y S and Park T G, 'Biodegradable polymeric microcellular foams by modified thermally induced phase separation method', *Biomaterials* **20** (19), 1783-1790 (1999).

[73] Sohier J, Haan R E, de Groot K, and Bezemer J M, 'A novel method to obtain protein release from porous polymer scaffolds: emulsion coating', *J Controlled Release* **87** (1-3), 57-68 (2003).

[74] Hu Y, Hollinger J O, and Marra K G, 'Controlled release from coated polymer microparticles embedded in tissue-engineered scaffolds', *J Drug Targeting* **9** (6), 431-438 (2001).

[75] Meese T M, Hu Y, Nowak R W, and Marra K G, 'Surface studies of coated polymer microspheres and protein release from tissue-engineered scaffolds', *Journal Of Biomaterials Science Polymer Edition* **13** (2), 141-151 (2002).

[76] Nof M and Shea L D, 'Drug-releasing scaffolds fabricated from drug-loaded microspheres', *Journal Of Biomedical Materials Research* **59** (2), 349-356 (2002).

[77] Whang K, Goldstick T K, and Healy K E, 'A biodegradable polymer scaffold for delivery of osteotropic factors', *Biomaterials* **21** (24), 2545-2551 (2000).

[78] Hile D D, Amirpour M L, Akgerman A, and Pishko M V, 'Active growth factor delivery from poly(D,L-lactide-co-glycolide) foams prepared in supercritical CO(2)', *Journal Of Controlled Release: Official Journal Of The Controlled Release Society* **66** (2-3), 177-185 (2000).

[79] Holland T A, Tabata Y, and Mikos A G, 'Dual growth factor delivery from degradable oligo(poly(ethylene glycol) fumarate) hydrogel scaffolds for cartilage tissue engineering', *J Controlled Release* **101** (1-3 SPEC. ISS.), 111-125 (2005).

[80] Kim S E, Park J H, Cho Y W, Chung H, Jeong S Y, Lee E B, and Kwon I C, 'Porous chitosan scaffold containing microspheres loaded with transforming growth factor-beta1: implications for cartilage tissue engineering', *Journal Of Controlled Release: Official Journal Of The Controlled Release Society* **91** (3), 365-374 (2003).

[81] Yamashita H, ten Dijke P, Franzen P, Miyazono K, and Heldin C H, 'Formation of hetero-oligomeric complexes of type I and type II receptors for transforming growth factor-beta', *The Journal Of Biological Chemistry* **269** (31), 20172-20178 (1994).

[82] Lories R J U and Luyten F P, 'Bone Morphogenetic Protein signaling in joint homeostasis and disease', *Cytokine & Growth Factor Reviews* **16** (3), 287-298 (2005).

[83] Erlacher L, Ng C K, Ullrich R, Krieger S, and Luyten F P, 'Presence of cartilage-derived morphogenetic proteins in articular cartilage and enhancement of matrix replacement in vitro', *Arthritis And Rheumatism* **41** (2), 263-273 (1998).

[84] Martin I, Suetterlin R, Baschong W, Heberer M, Vunjak-Novakovic G, and Freed L E, 'Enhanced cartilage tissue engineering by sequential exposure of chondrocytes to FGF-2 during 2D expansion and BMP-2 during 3D cultivation', *J Cell Biochem* **83** (1), 121-128 (2001).

[85] Jakob M, Demarteau O, Schafer D, Hintermann B, Dick W, Heberer M, and Martin I, 'Specific growth factors during the expansion and redifferentiation of adult human articular chondrocytes enhance chondrogenesis and cartilaginous tissue formation in vitro', *J Cell Biochem* **81** (2), 368-377 (2001).

[86] Kino-oka M, Maeda Y, Ota Y, Yashiki S, Sugawara K, Yamamoto T, and Taya M, 'Process design of chondrocyte cultures with monolayer growth for cell expansion and subsequent three-dimensional growth for production of cultured cartilage', *Journal of Bioscience and Bioengineering* **100** (1), 67-76 (2005).

[87] Mandl E W, Jahr H, Koevoet J L M, van Leeuwen J P T M, Weinans H, Verhaar J A N, and Van Osch G J V M, 'Fibroblast growth factor-2 in serum-free medium is a potent mitogen and reduces dedifferentiation of human ear chondrocytes in monolayer culture', *Matrix Biology* **23** (4), 231-241 (2004).

[88] Martin I, Vunjak-Novakovic G, Yang J, Langer R, and Freed L E, 'Mammalian chondrocytes expanded in the presence of fibroblast growth factor 2 maintain the ability to differentiate and regenerate three-dimensional cartilaginous tissue', *Experimental Cell Research* **253** (2), 681-688 (1999).

[89] Hotten G C, Matsumoto T, Kimura M, Bechtold R F, Kron R, Ohara T, Tanaka H, Satoh Y, Okazaki M, and Shirai et a, 'Recombinant human growth/differentiation factor 5 stimulates

mesenchyme aggregation and chondrogenesis responsible for the skeletal development of limbs', *Growth Factors* **13** (1-2), 65-74 (1996).

[90] Simank H G, Sergi C, Jung M, Adolf S, Eckhardt C, Ehemann V, Ries R, Lill C, and Richter W, 'Effects of local application of growth and differentiation factor-5 (GDF-5) in a full-thickness cartilage defect model', *Growth Factors (Chur, Switzerland)* **22** (1), 35-43 (2004).

[91] Rickert M, Jung M, Adiyaman M, Richter W, and Simank H G, 'A growth and differentiation factor-5 (GDF-5)-coated suture stimulates tendon healing in an Achilles tendon model in rats', *Growth Factors (Chur, Switzerland)* **19** (2), 115-126 (2001).

[92] Chhabra A, Tsou D, Clark R T, Gaschen V, Hunziker E B, and Mikic B, 'GDF-5 deficiency in mice delays Achilles tendon healing', *Journal Of Orthopaedic Research: Official Publication Of The Orthopaedic Research Society* **21** (5), 826-835 (2003).

[93] Li X, Leo B M, Beck G, Balian G, and Anderson G D, 'Collagen and proteoglycan abnormalities in the GDF-5-deficient mice and molecular changes when treating disk cells with recombinant growth factor', *Spine* **29** (20), 2229-2234 (2004).

[94] Koch H, Jadlowiec J A, Fu F H, Nonn J, Merk H R, Hollinger J O, and Campbell P G, 'The effect of growth/differentiation factor-5 (GDF-5) on genotype and phenotype in human adult mesenchymal stem cells', *Zeitschrift Fur Orthopadie Und Ihre Grenzgebiete* **142** (2), 248-253 (2004).

[95] Heidaran M A, Daverman R, Thompson A, Ng C K, Pohl J, Poser J W, and Spiro R C, 'Extracellular Matrix Modulation of rhGDF-5-Induced Cellular Differentiation', *e-biomed: The Journal of Regenerative Medicine* **1** (9), 121-135 (2000).

[96] Rosner I A, Manni A, Malemud C J, Boja B, and Moskowitz R W, 'Estradiol receptors in articular chondrocytes', *Biochem Biophys Res Commun* **106** (4), 1378-1382 (1982).

[97] Spector T D and Campion G D, 'Generalised osteoarthritis: a hormonally mediated disease', *Annals Of The Rheumatic Diseases* **48** (6), 523-527 (1989).

[98] Wluka A E, Cicuttini F M, and Spector T D, 'Menopause, oestrogens and arthritis', *Maturitas* **35** (3), 183-199 (2000).

[99] Nevitt M C, Cummings S R, Lane N E, Hochberg M C, Scott J C, Pressman A R, Genant H K, and Cauley J A, 'Association of estrogen replacement therapy with the risk of osteoarthritis of the hip in elderly white women. Study of Osteoporotic Fractures Research Group', *Archives Of Internal Medicine* **156** (18), 2073-2080 (1996).

[100] Nasatzky E, Schwartz Z, Soskolne W A, Brooks B P, Dean D D, Boyan B D, and Ornoy A, 'Evidence for receptors specific for 17 beta-estradiol and testosterone in chondrocyte cultures', *Connective Tissue Research* **30** (4), 277-294 (1994).

[101] Ushiyama T, Ueyama H, Inoue K, Ohkubo I, and Hukuda S, 'Expression of genes for estrogen receptors alpha and beta in human articular chondrocytes', *Osteoarthritis And Cartilage / OARS, Osteoarthritis Research Society* **7** (6), 560-566 (1999).

[102] Jo H, Park J S, Kim E M, Jung M Y, Lee S H, Seong S C, Park S C, Kim H J, and Lee M C, 'The in vitro effects of dehydroepiandrosterone on human osteoarthritic chondrocytes', *Osteoarthritis And Cartilage / OARS, Osteoarthritis Research Society* **11** (8), 585-594 (2003).

[103] Jo H, Park J S, Kim E M, Jung M Y, Lee S H, Seong S C, Park S C, Kim H J, and Lee M C, 'The in vitro effects of dehydroepiandrosterone on human osteoarthritic chondrocytes', *Osteoarthritis Cartilage* **11** (8), 585-594 (2003).

[104] Straub R H, 'Serum dehydroepiandrosterone (DHEA) and DHEA sulfate are negatively correlated with serum interleukin-6 (IL-6), and DHEA inhibits IL-6 secretion from mononuclear cells in man in vitro: possible link between endocrinosenescence and immunosenescence', *Journal of Clinical Endocrinology and Metabolism* **83** (6), 2012-2017 (1998).

[105] Danenberg H D, Alpert G, Lustig S, and Ben Nathan D, 'Dehydroepiandrosterone protects mice from endotoxin toxicity and reduces tumor necrosis factor production', *Antimicrobial Agents And Chemotherapy* **36** (10), 2275-2279 (1992).

[106] Schmidt M, Weidler C, Naumann H, Anders S, Scholmerich J, and Straub R H, 'Androgen conversion in osteoarthritis and rheumatoid arthritis synoviocytes--androstenedione and testosterone inhibit estrogen formation and favor production of more potent 5alpha-reduced androgens', *Arthritis Research & Therapy* **7** (5), R938-R948 (2005).

[107] Claassen H, Cellarius C, Scholz-Ahrens K E, Schrezenmeir J, Gluer C C, Schunke M, and Kurz B, 'Extracellular matrix changes in knee joint cartilage following bone-active drug treatment', *Cell And Tissue Research* **324** (2), 279-289 (2006).

[108] Calabrese E J, 'Estrogen and related compounds: biphasic dose responses', *Critical Reviews In Toxicology* **31** (4-5), 503-515 (2001).

[109] Malda J, Martens D E, Tramper J, van Blitterswijk C A, and Riesle J, 'Cartilage tissue engineering: controversy in the effect of oxygen', *Critical Reviews In Biotechnology* **23** (3), 175-194 (2003).

[110] Grimshaw M J and Mason R M, 'Bovine articular chondrocyte function in vitro depends upon oxygen tension', *Osteoarthritis And Cartilage / OARS, Osteoarthritis Research Society* **8** (5), 386-392 (2000).

[111] Kellner K, Liebsch G, Klimant I, Wolfbeis O S, Blunk T, Schulz M B, and Goepferich A, 'Determination of oxygen gradients in engineered tissue using a fluorescent sensor', *Biotechnology And Bioengineering* **80** (1), 73-83 (2002).

[112] Mathy-Hartert M, Burton S, Deby-Dupont G, Devel P, Reginster J Y, and Henrotin Y, 'Influence of oxygen tension on nitric oxide and prostaglandin E2 synthesis by bovine chondrocytes', *Osteoarthritis Cartilage* **13** (1), 74-79 (2005).

[113] Domm C, Schunke M, Christesen K, and Kurz B, 'Redifferentiation of dedifferentiated bovine articular chondrocytes in alginate culture under low oxygen tension', *Osteoarthritis Cartilage* **10** (1), 13-22 (2002).

[114] Kurz B, Domm C, Jin M, Sellckau R, and Schunke M, 'Tissue Engineering of Articular Cartilage under the Influence of Collagen I/III Membranes and Low Oxygen Tension', *Tissue Eng* **10** (7), 1277-1286 (2004).

[115] Hansen U, Schunke M, Domm C, Ioannidis N, Hassenpflug J, Gehrke T, and Kurz B, 'Combination of reduced oxygen tension and intermittent hydrostatic pressure: a useful tool in articular cartilage tissue engineering', *Journal Of Biomechanics* **34** (7), 941-949 (2001).

[116] Lee R B and Urban J P, 'Evidence for a negative Pasteur effect in articular cartilage', *The Biochemical Journal* **321 (Pt 1)** 95-102 (1997).

[117] Lee R B and Urban J P G, 'Functional replacement of oxygen by other oxidants in articular cartilage', *Arthritis And Rheumatism* **46** (12), 3190-3200 (2002).

[118] Obradovic B, Carrier R L, Vunjak-Novakovic G, and Freed L E, 'Gas exchange is essential for bioreactor cultivation of tissue engineered cartilage', *Biotechnology And Bioengineering* **63** (2), 197-205 (1999).

[119] Saini S and Wick T M, 'Effect of low oxygen tension on tissue-engineered cartilage construct development in the concentric cylinder bioreactor', *Tissue Eng* **10** (5-6), 825-832 (2004).

[120] Ysart G E and Mason R M, 'Responses of articular cartilage explant cultures to different oxygen tensions', *Biochim Biophys Acta* **1221** (1), 15-20 (1994).

[121] Scherer K, Schunke M, Sellckau R, Hassenpflug J, and Kurz B, 'The influence of oxygen and hydrostatic pressure on articular chondrocytes and adherent bone marrow cells in vitro', *Biorheology* **41** (3-4), 323-333 (2004).

[122] Murphy C L and Sambanis A, 'Effect of oxygen tension and alginate encapsulation on restoration of the differentiated phenotype of passaged chondrocytes', *Tissue Eng* **7** (6), 791-803 (2001).

[123] Malda J, van Blitterswijk C A, van Geffen M, Martens D E, Tramper J, and Riesle J, 'Low oxygen tension stimulates the redifferentiation of dedifferentiated adult human nasal chondrocytes', *Osteoarthritis Cartilage* **12** (4), 306-313 (2004).

[124] Murphy C L and Polak J M, 'Control of Human Articular Chondrocyte Differentiation by Reduced Oxygen Tension', *J Cell Physiol* **199** (3), 451-459 (2004).

[125] Henrotin Y, Kurz B, and Aigner T, 'Oxygen and reactive oxygen species in cartilage degradation: friends or foes?', *Osteoarthritis And Cartilage / OARS, Osteoarthritis Research Society* **13** (8), 643-654 (2005).

[126] Zhou S, Cui Z, and Urban J P G, 'Factors influencing the oxygen concentration gradient from the synovial surface of articular cartilage to the cartilage-bone interface: a modeling study', *Arthritis And Rheumatism* **50** (12), 3915-3924 (2004).

Appendix

Abbrevations

ANOVA	analysis of variance
BMP	bone morphogenetic protein
DAB	diaminobenzidine
DMEM	Dulbecco's Modified Eagles Medium
DNA	desoxyribonucleinic acid
ECM	extracellular matrix
ELISA	enzyme-linked immunoassay
FBS	fetale bovine serum
GAG	glycosaminoglycans
GDF	growth and differentiation factor
HEPES	N-2-hydroxyethylpiperazine N'-2-ethansulfonic acid
HIF	hypoxia inducible factor
HPLC	high performance liquid chromatography
IGF	insulin like growth factor
MMP	matrixmetalloproteinase
MRI	magnetic resonance imaging
MW	molecular weight
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PGA	poly(glycolic acid)
PLA	poly(lactic acid)
PLGA	poly(lactic-co-glycolic acid)
SD	standard deviation
SEM	scanning electron microscopy
TE	tissue engineering
TGF	transforming growth factor
UV	ultraviolett light
Ww	wet weight

Curriculum vitae

Name	Bernhard Appel
Geburtsdatum	15.03.1974
Geburtsort	München
Staatsangehörigkeit	deutsch
Familienstand	Ledig

Schulbildung

09/1980 - 07/1984	Grundschule Haar
09/1984 - 07/1990	Naturwissenschaftliches Gymnasium Haar
09/1990 - 07/1994	Naturwissenschaftliches Gymnasium München

Hochschulbildung

11/1994 - 04/2000	Studium der Pharmazie, LMU München
06/2001	Approbation als Apotheker

Berufliche Tätigkeit

05/2000 - 10/2000	Praktikum Klinge Pharma GmbH, München
11/2000 - 04/2001	Praktikum Jagdfeld Apotheke, Haar
08/2001 – 10/2005	Promotion am Lehrstuhl für Pharm. Technologie der Universität Regensburg unter Anleitung von Prof. A. Göpferich
11/2005 -12/2005	Ratisbona-Apotheke, Regensburg, Tätigkeit als Apotheker

List of Publications

T. Blunk, A.L. Sieminski, B. Appel, C. Croft, D.L. Courter, J.J. Chieh, A. Goepferich, J.S. Khurana: Bone morphogenetic protein 9: a potent modulator of cartilage development in vitro.
Growth Factors 21 (2), 71-77 (2003).

A. Maschke, A. Lucke, W. Vogelhuber, C. Fischbach, B. Appel, T. Blunk, A. Goepferich: Lipids: An alternative material for protein and peptide release. In: ACS Symposium Series 879. Ed. Sönke Svenson, 176-196 (2004).

B. Appel, A. Maschke, B. Weiser, H. Sarhan, C. Englert, P. Angele, T. Blunk, A. Goepferich: Lipidic implants for controlled release of bioactive insulin: Effects on cartilage engineered in vitro.
Int.J.Pharm. 314 (2), 170-178 (2006).

A. Maschke, N Cali, B. Appel, Josef Kiermaier, T. Blunk, A. Goepferich: Micronization of Insulin by High Pressure Homogenization.
Pharm.Res. in press (2006).

M. Hacker, M. Ringhofer, B. Appel, M. Neubauer, T. Vogel, T. Blunk, A. Göpferich, M. B. Schulz: Solid Lipid Templating: A Versatile Lab-scale Fabrication Technique for Macroporous Tissue Engineering Scaffolds.
submitted to: Biomaterials (2006)

Conference Abstracts:

B. Appel, A. Maschke, C. Guse, H. Sarhan, K. Kellner, T. Blunk, A. Goepferich: 3-D cartilage engineering culture as a test system for growth factor application devices.
GZG Jahrestagung, Regensburg (2002).

B. Appel, A. Maschke, C. Guse, H. Sarhan, K. Kellner, T. Blunk, A. Goepferich: Lipid matrices release bioactive insulin in a 3-D cartilage engineering culture.
CRS Annual Meeting, Wien (2002).

H. Sarhan, B. Appel, D. Eyrich, A. Göpferich, T. Blunk: Effect of interleukin-4 on engineered cartilage in 3D cell culture.
International Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, Nuremberg, Germany (2004).

H. Sarhan, B. Appel, D. Eyrich, A. Göpferich, T. Blunk: Effects of interleukin-4 on glycosaminoglycan content and matrix metalloproteinase expression in engineered cartilage.
Joint Meeting of the Tissue Engineering Society International (TESI) and the European Tissue Enginee, Lausanne, Switzerland (2004).

A. Maschke, N. Cali, B. Appel, T. Blunk, A. Goepferich: Micronization of insulin by high pressure homogenization in non-aqueous environment.
AAPS Annual Meeting, Baltimore (2004).

B. Appel, D. Eyrich, H. Sarhan, A. Goepferich, T. Blunk: Growth and differentiation factor-5 improves in vitro engineered cartilage.
ETES Annual Meeting, München (2005).

Acknowlegdements:

Mein herzlicher Dank gilt allen Kollegen und Mitarbeitern, die mit mir die Zeit der Promotion durchlebt und am Gelingen dieser Arbeit beigetragen haben:

Herrn **Prof. Göpferich** möchte ich ganz herzlich danken, sowohl für die Promotionsmöglichkeit an seinem Lehrstuhl, als auch für viele gute Gespräche bei einer guten Tasse Espresso. Der Lehrstuhl war und ist dank hervorragender Ausstattung, innovativer Forschungsausrichtung und motivierten Mitarbeitern der denkbar beste Ort für meine Promotion.

Mein besonderer Dank gilt auch **Dr. Torsten Blunk**, dem Betreuer der Arbeit. Seine guten Ideen und Anregungen sowie viel Geduld haben diese Arbeit erst möglich gemacht. Seine Beharrlichkeit und stetiger Optimismus haben mir sehr bei der Erstellung dieser Arbeit geholfen.

Großer Dank gilt auch meinen Labor-Lebens- und Arbeitsgemeinschaften, zuerst mit **Hans-Jürgen Untch**, später dann für lange Zeit mit **Allison Dennis** und zu guter letzt **Wolfgang Hild**. Unsere Laborgemeinschaften machten die Forschung erst lebenswert.

Dr. Hatem Sarhan (Agus) und **Dr. Daniela Eyrich** danke ich herzlich für die gemeinsame Arbeit am Kalbsknorpel, Cell-Sitting, gemeinsame Analytik und viele sehr gute Gespräche über Gott/Allah und die Welt an der Flow-Box.

Allison Dennis danke ich besonders für Ihre Freundschaft und natürlich auch für Ihre Hilfe in Übersetzungsfragen.

Dr. Michael Hacker gilt großer Dank für die hervorragende Zusammenarbeit bei der Computerbetreuung am Lehrstuhl, viele gemeinsame Nachtschichten und natürlich für die Herstellung der Insulin-beladenen PLGA Scaffolds.

Dr. Sigrid Drotleff und **Dr. Uta Lungwitz** danke ich sehr für Ihre Freundschaft und Ihre Bereitschaft, immer für meine Probleme ein offenes Ohr gehabt zu haben. Auch der neuen Besatzung vom Nachbarlabor **Annina Seitz**, **Conny Rose** und **Anna Hezinger** danke ich sehr dafür, immer vorbeischauen zu dürfen und Ansprache zu finden. Sigrid, Annina und Conny danke ich auch für gemeinsame Trainingseinheiten im Unisport.

Ferdinand Brandl danke ich besonders für viele gute Gespräche neben der Wissenschaft und für seine Freundschaft.

Dr. Jörg Tessmar danke ich für sein offenes Ohr, seine immer guten Ratschläge und einfach, weil es ihn gibt.

Meinen Kolleginnen **Andrea Schüssele**, **Stephanie Könnings**, **Barbara Weiser** und natürlich **Beaker** danke ich für Ihre Kameradschaft und die sehr schöne gemeinsame Zeit.

Axel Ehmer und **Ferdinand Brandl** danke ich für die gute Zeit im bitsandbytes-Team.

Den Motivationsbeauftragten (**Christian Becker**, **Florian Sommer**, **Robert Knerr**), sowie auch meinen früheren Kollegen **Dr. Christoph Koelwel** und **Dr. Markus Neubauer** danke ich ganz herzlich für hervorragende Stunden voller Heiterkeit und Spass am Lehrstuhl und auch ausserhalb. **Dr. Christoph Koelwel** danke ich für die Freundschaft und auch für eine sehr gute Zeit in seiner Apotheke, für die ich Ihm auch weiterhin viel Erfolg wünsche.

Herzlicher Dank gebührt auch dem Lehrstuhlsekretariat mit **Frau Lydia Frommer** und **Frau Liane Öttl**. Ihre stetige und zuverlässige Organisation macht so vieles am Lehrstuhl erst möglich.

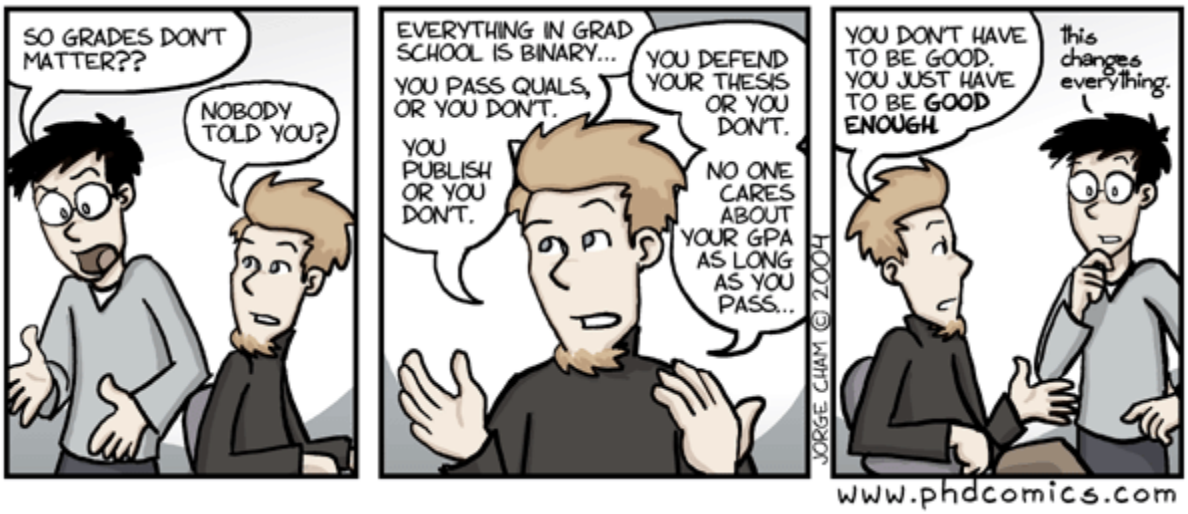
Dr. Angelika Maschke gilt mein Dank für die Herstellung der Insulin-beladenen Lipid Matrices und Ihrer Geduld beim Insulin Bioassay.

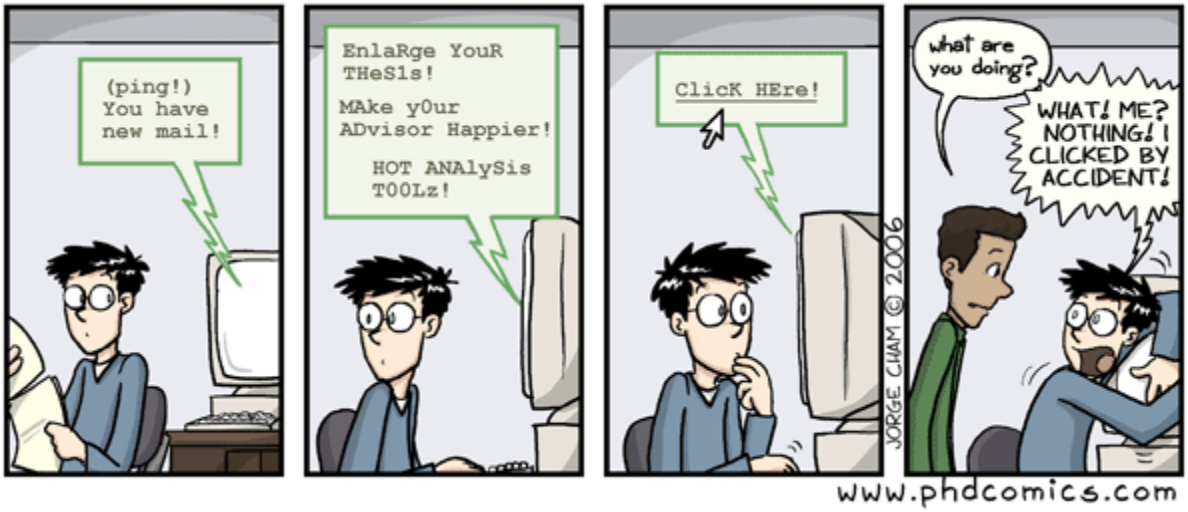
Dr. Peter Angele, Abteilung für Unfallchirurgie am Universitätsklinikum Regensburg, und seinem Team, insbesondere **Tom Böttner**, danke ich für die hervorragende Kooperation innerhalb der Hightech Offensive Bayerns.

Frau Thienel und Ihrem Team der Pathologie des Universitätsklinikums Regensburg danke ich für immer zuverlässige Anfertigung der Paraffinschnitte.

Dem Rechenzentrum der Universität, insbesondere **Herrn Österreicher** und **Herrn Werling** danke ich für die gute Zusammenarbeit bei der Computerbetreuung.

Specials thanks belong to **Jorge Cham**. His wonderful comics helped me not to lose my sense of humour during the PhD work. "Piled Higher and Deeper" (PhD) is the comic strip about life (or the lack there of) in academia. Here are my personal favourites:





"Piled Higher and Deeper" by Jorge Cham. http://www.phdcomics.com