Aus der Klinik und Poliklinik für Orthopädie, Physikalische Medizin und Rehabilitation

Klinikum der Ludwig-Maximilians-Universität München

Vorstand: Prof. Dr. med. Dipl.-Ing. Volkmar Jansson

A novel hydrostatic pressure bioreactor for cartilage regeneration

Dissertation
zum Erwerb des Doktorgrades der Medizin
an der Medizinischen Fakultät der
Ludwig-Maximilians-Universität zu München

vorgelegt von

Ge Zhu

aus

Xinjiang

Jahr 2016

Mit Genehmigung der Medizinischen Fakultät der Universität München

Berichterstatter:	Prof. Dr. med. Peter Müller
Mitberichterstatter:	Priv. Doz. Dr. Denitsa Docheva Prof. Dr. Marcus Schmitt-Sody
Mitbetreuung durch den promovierten Mitarbeiter:	Dr. biol. hum. Oliver Betz
Dekan:	Prof. Dr. med. dent. Reinhard Hickel
Tag der mündlichen Prüfung:	06.10.2016

LMU LUDWIG-MAXIMILIANS-UNIVERSITÄT MÜNCHEN

Dean's Office Medical Faculty

Cumulative Dissertation

Confirmation pursuant to § 4a Paras. 3 and 5 Doctoral Degree Regulations for Dr. med., Dr. med. dent. and Dr. rer. boil. hum.
and
pursuant to § 7 Para. 4 Doctoral Degree Regulations for Dr. rer. nat. at the Medical Faculty
Please note: for each published article, a separate "Cumulative Dissertation" form has to be submitted!

Ge Zhu

Name of doctoral candidate

A CLOSED LOOP PERFUSION BIOREACTOR FOR DYNAMIC HYDROSTATIC PRESSURE LOADING AND CARTILAGE TISSUE ENGINEERING

Title of publication

Journal of Mechanics in Medicine and Biology Vol. 16, No. 2 (2015)

Journal (Name, issue, year, etc.)

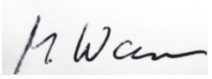
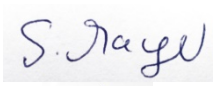
I hereby confirm that none of the articles submitted for this doctoral degree have been the subject of another (current or completed) dissertation.

Signature of doctoral candidate

By signing, the following **co-authors** confirm that:

- the extent of their contributions (content-related and volume) in the publications submitted,
- their agreement to the submission of the publications, and
- the article in question is not the subject of another (current or completed) dissertation.

Name of co-author	Extent of contribution (content-related and volume)	Signature of co-author

1. CHRISTIAN SCHRÖDER	Development and engineering design of the whole bioreactor, electronics, programming, supervising project related staff, Writing Manuscript;	
2. ANDREAS HÖLZER	Idea, funding, project coordination, supervising project related staff writing and reviewing manuscript	
3. MATTHIAS WOICZINSKI	FE and CFD Analysis, reviewing manuscript	
4.OLIVER B. BETZ	reviewing manuscript	
5. HELENA GRAF	Construction and Calculation of the dimensions of the bioreactor- chamber.	
6. SUSANNE MAYER-WAGNER	reviewing manuscript	
7. PETER E. MÜLLER	reviewing manuscript	

LMU LUDWIG-MAXIMILIANS-UNIVERSITÄT MÜNCHEN

Confirmation pursuant to § 4a Paras. 3 and 5 Doctoral Degree Regulations for Dr. med., Dr. med. dent. and Dr. rer. boil. hum.
and
pursuant to § 7 Para. 4 Doctoral Degree Regulations for Dr. rer. nat. at the Medical Faculty
Please note: for each published article, a separate "Cumulative Dissertation" form has to be submitted!

Ge Zhu

Name of doctoral candidate

Comparing effects of perfusion and hydrostatic pressure on gene profiles of human chondrocyte

Title of publication

Journal of Biotechnology 210 (2015) 59–65

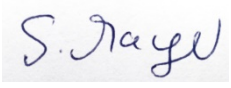
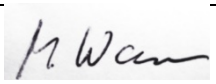
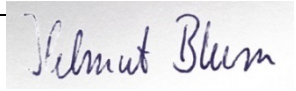
Journal (Name, issue, year, etc.)

I hereby confirm that none of the articles submitted for this doctoral degree have been the subject of another (current or completed) dissertation.

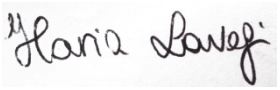
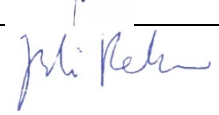
Signature of doctoral candidate

By signing, the following **co-authors** confirm that:

- the extent of their contributions (content-related and volume) in the publications submitted,
- their agreement to the submission of the publications, and
- the article in question is not the subject of another (current or completed) dissertation.

Name of co-author	Extent of contribution (content-related and volume)	Signature of co-author
1. Susanne Mayer-Wagner	Concept and design of the experiment, data analyzing, manuscript writing	
2. Christian Schröder	Development and engineering design of the whole bioreactor, electronics, programming, reviewing manuscript	
3. Matthias Woiczinski	FE and CFD Analysis, reviewing manuscript	
4. Helmut Blum	RNA-sequencing, reviewing manuscript	

Cumulative dissertation Date: 24.02.2016

5. Ilaria Lavagi	data analyzing of RNA-sequencing	
6. Stefan Krebs	RNA-sequencing, reviewing manuscript	
7. Julia I. Redeker	Perpetration of cells, PCR data analyzing, reviewing manuscript	
8. Andreas Hölzer	Idea and design of the bioreactor	
9. Volkmar Jansson	reviewing manuscript	
10. Oliver Betz	Funding, reviewing manuscript	
11. Peter E. Müller	reviewing manuscript	

Publication list

Publication 1

A closed loop perfusion bioreactor for dynamic hydrostatic pressure loading and cartilage tissue engineering

Christian Schröder, Andreas Hölzer, Ge Zhu, Matthias Woiczinski, Oliver B. Betz, Susanne Mayer-Wagner, Peter E. Müller

Journal of Mechanics in Medicine and Biology, Vol. 16, No. 2 (2015) 1650025 (16 pages)

Publication 2

Comparing effects of perfusion and hydrostatic pressure on gene profiles of human chondrocyte

Ge Zhu, Susanne Mayer-Wagner, Christian Schröder, Matthias Woiczinski, Helmut Blum, Ilaria Lavagi, Stefan Krebs, Julia I. Redeker, Andreas Hölzer, Volkmar Jansson, Oliver Betz, Peter E. Müller

Journal of Biotechnology 210 (2015) 59–65

Contents

Introduction

Construct and property of cartilage

Hyaline cartilage is the most widespread cartilage in the human body. In diarthroidal joints hyaline cartilage covers the contact zones of two interlocked bones and is called articular cartilage.[1] Articular cartilage is a unique type of well-characterized connective tissue. It has the lowest cellular volume of any tissue in the human body. In humans, chondrocytes contribute only about 1% of the tissue volume. The remaining 99% is made up of a complex ECM. The chondrocytes themselves synthesize all necessary ECM components. The ECM is composed of collagen and Proteoglycans. [1]

Collagen is the primary constituent of the ECM of articular cartilage. 90-95% of the collagen is collagen II which is often used as a marker for chondrogenic differentiation. The collagen II fibrils are responsible for the tensile strength of the tissue.[2] Aggrecan is the most common proteoglycan in articular cartilage, and it is responsible for the compressive strength of the tissue.[1] Thus, the expressions of GAGs and Aggrecan are also used as specific markers for chondrogenic differentiation.

Repair of cartilage injury

Articular cartilage is avascular, aneural, and alymphatic tissue. Therefore, the intrinsic ability of articular cartilage to repair itself is limited. Due to the poor ability of articular cartilage to heal itself, and the limited clinical treatment options, tissue engineering may provide the most promising approach to articular cartilage regeneration. The matrix assisted autologous chondrocytes transplantation (MACI), which follows the tissue engineering approach, is the current state of the art treatment option.[3]

For this treatment, cartilage cells are taken from the patient, expanded in vitro and then seeded onto a scaffold (the matrix). This scaffold will then be implanted into the cartilage defect, replacing the damaged cartilage respectively the lost cartilage tissue.

Unfortunately a major obstacle in applying this method is the limited number of native cartilage cells, the so called chondrocytes, from the patient. This fact makes it necessary to expand the limited number of cartilage cells. A principal of cartilage tissue engineering is the application of mechanical stimulation to simulate in vivo condition and enhance chondrocyte metabolic activity and ECM production.[2]It is well studied that HP is the most important mechanical loading in cartilage regeneration. HP provides a robust method for chondrocyte stimulation, as it can be applied to chondrocytes in monolayer [4] , 3-D constructs [5], as well as explants [6].

Hydrostatic pressure bioreactors

Bioreactors are widely used for cartilage tissue engineering. In general, there are two methods of applying HP, and they both showed advantages and disadvantages. In the first method, HP is applied by compressing a gas phase that transmits load through the medium to the cells. Those systems use a strong pressure chamber, therefore, could provide high pressure of up to 50 MPs.[7, 8] However, this method may alter the gas concentration within the culture medium. Alternatively, a less complicated approach involves applying HP by compressing only the fluid phase, which limits any changes in gas solubility within the chamber.[9, 10] This method generally involves connecting a fluid-filled chamber by hose to a piston attached directly to a hydraulic press, controlled by a computer. On the contrary to the first method, this type of bioreactor often uses a relatively fragile construct that can only resist low pressure. Both types of bioreactors also include temperature control, generally by placing the chamber in a water bath. Finally, either type of bioreactor may be altered to allow for semicontinuous medium perfusion. This alternation can avoid frequent medium changes, and reduce the risk of contamination. However, such additions will make the bioreactor more complex. While a separate perfusion system is inevitable for method two, it is possible to generate various

pressure protocols by controlling two electromagnetic valves of the in-and outflow of medium. However, due to the restrict relationship of flow rate, volume of the chamber and frequency, bioreactors of the second type still needs to add a separate perfusion system and can only apply to 3-D constructs. Thus, it is necessary to establish a novel bioreactor for HP loading that not only simultaneously permits medium perfusion but also has a less complex design.

Hydrostatic pressure and perfusion

HP has been extensively used for increasing the metabolic activity of chondrocytes in tissue engineering studies. The effects of HP on chondrocytes cultured in monolayer, 3-D constructs, as well as cartilage explants. In studies involving HP application, there are large amount of variations of the magnitude, frequency, and duration of HP. It was shown, that a window of effective loads and frequencies exist between 0.1 and 15 MPa and 0.05 and 1 Hz, respectively that produce positive results when culturing chondrocytes.[11, 12] The duration varies from 4 days to 8 weeks. With appropriate parameters, HP appears to be a promising method of stimulating chondrogenic differentiations. However, it should be noted that chondrocytes in monolayer respond differently to HP than 3-D constructs because of the absence of abundant ECM.[12] Additionally, despite the large number of studies involving hydrostatic pressure, most of the studies used animal cells. Animal cells have been shown to have a different response to HP stimulation than human chondrocytes.[13]

Perfusion supplies nutrition and removes waste from the culture. Is also affects the chondrogenic activity.[14, 15] However, the effects of perfusion can be highly dependent on the medium flow-rate and the type of object. Numerous bioreactor systems involving HP also include a perfusion system. Nevertheless, all the studies neglected the effect of perfusion in addition to HP. Thus, the question remains what role did perfusion and HP play respectively in stimulating chondrogenic differentiation?

Objective of the study

Motivated by the problems mentioned above, it is hypothesized that 1) a novel bioreactor for dynamic hydrostatic pressure loading that simultaneously permits medium perfusion with a simple construct can be established for chondrocyte cultures; and 2) both HPP and P can effect differentiation and metabolism of human chondrocytes, whereas, the effect of HP and P addresses different aspects. To test the hypotheses, two studies were carried out and demonstrated in publication 1 and publication 2 respectively.

Publication 1 described the mechanical design and properties of the bioreactor. It consists of a medium reservoir, a pump that pressurizes the medium to create pressure, a pressure reservoir, an inlet valve, the test chamber including a temperature sensor and a pressure sensor, an outlet valve and a medium reservoir which are all connected by flexible tubes. The medium reservoir provides a hole covered with a filter for gas exchange. The test chamber provided space for one well-plate, with could meet requirements of various studies. The bioreactor was controlled via a purpose-built real-time LabVIEW software program which controls the pump, the valves and the pressure sensor self-sustaining. Frequency, pressure and the duration in hours per day can be adjusted by the user to set up the pressure mode of the bioreactor. The pressure could be achieved up to 270 kPa, and frequency up to 0.1 Hz which is within the range of 0.1~15MPa and 0.05~1Hz of benefit chondrogenic differentiation. Additionally, a cell test with human articular chondrocytes was performed. Chondrocytes were put in the bioreactor for four days with pressure of 100 kPa and a frequency of 0.01 Hz for 24 h/day before WST-1 assay to measure the proliferation of the cells. The results showed that cell viability was not decreased by the bioreactor. In this study a novel bioreactor for simultaneous dynamic hydrostatic pressure and perfusion was successfully introduced. A purpose-built program was developed to control the pump and valves, **which** reduced the complexity of the construct. This study showed the bioreactor has extensive prospects in cartilage tissue

engineering studies.

In this study, as a co-author, I helped to modify the bioreactor and the software and optimized the parameters of the loading. I performed the cell viability test and provided the results. Finally, I took part in writing the manuscript.

Publication 2 addresses hypothesis 2) by comparing effects of HPP and P for differentiation and dedifferentiation of human chondrocytes. In order to approach clinical applications, human chondrocytes are used in this study. The cells were cultured in 37^{o}C, 5% CO^{2} until passage 2 before divided in to 3 groups: HPP, P and C. For P and HPP, perfusion was applied with a medium flow rate of 2 ml/min for 20 h/day for 4 days. HPP, hydrostatic pressure of 0.1 MPa for 2 h (loading), followed by2 h rest (off-loading), was applied each day. Cells of group C were kept in static cultures. The bioreactor system introduced in publication 1 was utilized in this study. The chondrocytes were evaluated with real-time PCR for COL2A1, COL1A1, ACAN and MMP 13. More importantly, a full gene expression profile was displayed via RNA-sequencing. This study compared for the first time the effects of HP and P. Also, it is the first whole gene expression profile of human chondrocytes after either stimulation that has been published. Under those circumstances, not only the well-known cartilage genes like COL2A1, ACAN and SOX9 but also genes which have not been commonly tested in bioreactors such as CYTL1 and GDF5 were examined. In addition, the state of art RNA-sequencing provided changes of gene expression influenced by HPP and P. Thereby, the study provides new insight into how HP and P enhance cartilage differentiation and inhibit catabolic effects.

As a first author, I designed and performed the experiment except for the RNA-sequencing while Dr. Susanne Mayer-Wagner introduced the RNA-sequencing technique, which was an important part of this study. Apart from this, Dr. Susanne Mayer-Wagner provided a lot of advises though out the whole study. Last but not least, both authors wrote the manuscript together. In this case, both authors contributed equally to this study. Hence, a shared-authorship came to be.

References

1.Schulz, R.M. and A. Bader, Cartilage tissue engineering and bioreactor systems for the cultivation and stimulation of chondrocytes. Eur Biophys J, 2007. 36(4-5): p. 539-68.

2.Kessler, M.W. and D.A. Grande, Tissue engineering and cartilage. Organogenesis, 2008. 4(1): p. 28-32.

3.Harris, J.D., et al., Autologous chondrocyte implantation: a systematic review. J Bone Joint Surg Am, 2010. 92(12): p. 2220-33.

4.Smith, R.L., et al., Effects of intermittent hydrostatic pressure and BMP-2 on osteoarthritic human chondrocyte metabolism in vitro. J Orthop Res, 2011. 29(3): p. 361-8.

5.Correia, C., et al., Dynamic culturing of cartilage tissue: the significance of hydrostatic pressure. Tissue Eng Part A, 2012. 18(19-20): p. 1979-91.

6.Tarng, Y.W., B.F. Huang, and F.C. Su, A novel recirculating flow-perfusion bioreactor for periosteal chondrogenesis. Int Orthop, 2012. 36(4): p. 863-8.

7.Kunitomo, T., et al., Influence of extracellular matrix on the expression of inflammatory cytokines, proteases, and apoptosis-related genes induced by hydrostatic pressure in three-dimensionally cultured chondrocytes. J Orthop Sci, 2009. 14(6): p. 776-83.

8.Smith, R.L., et al., Time-dependent effects of intermittent hydrostatic pressure on articular chondrocyte type II collagen and aggrecan mRNA expression. J Rehabil Res Dev, 2000. 37(2): p. 153-61.

9.Mizuno, S., Novel cell culture model using pure hydrostatic pressure and a semipermeable membrane pouch. Cell Transplant, 2011. 20(5): p. 767-74.

10. Carver, S.E. and C.A. Heath, Semi-continuous perfusion system for delivering intermittent physiological pressure to regenerating cartilage. Tissue Eng, 1999. 5(1): p. 1-11.

11. Darling, E.M. and K.A. Athanasiou, Articular cartilage bioreactors and bioprocesses. Tissue Eng, 2003. 9(1): p. 9-26.

12. Elder, B.D. and K.A. Athanasiou, Hydrostatic pressure in articular cartilage tissue engineering: from chondrocytes to tissue regeneration. Tissue Eng Part B Rev, 2009. 15(1): p. 43-53.

13. Grogan, S.P., et al., Effects of perfusion and dynamic loading on human neocartilage formation in alginate hydrogels. Tissue Eng Part A, 2012. 18(17-18): p. 1784-92.

14. Yu, L., et al., Tubular perfusion system for chondrocyte culture and superficial zone protein expression. J Biomed Mater Res A, 2014.

15. Forsey, R.W., et al., Perfusion bioreactor studies of chondrocyte growth in alginate-chitosan capsules. Biotechnol Appl Biochem, 2012. 59(2): p. 142-52.

Abbreviations

ACAN	aggrecan
COL1A1	collagen type 1 α1 chain
COL2A1	collagen type 2 α1 chain
CYTL1	cytokine like 1
ECM	extracellular matrix
GAGs	glycosaminoglycans
GDF5	growth differentiation factor 5
HP	hydrostatic pressure
HPP	hydrostatic pressure and perfusion
MACI	matrix assisted autologous chondrocytes transplantation
MMP 13	matrix-metalloproteinase 13
P	perfusion
RNA	ribonucleic acid
SOX9	SRY (sex determining region Y)-box 9

Publication 1

Journal of Mechanics in Medicine and Biology
Vol. 16, No. 2 (2015) 1650025 (16 pages)

DOI: 10.1142/S0219519416500251

World Scientific
www.worldscientific.com

A CLOSED LOOP PERFUSION BIOREACTOR FOR DYNAMIC HYDROSTATIC PRESSURE LOADING AND CARTILAGE TISSUE ENGINEERING

CHRISTIAN SCHRÖDER*, ANDREAS HÖLZER†, GE ZHU‡, MATTHIAS WOICZINSKI§ and OLIVER B. BETZ¶

Department of Orthopaedic Surgery
University Hospital of Munich (LMU)
Physical Medicine and Rehabilitation
Marchioninistrasse 15, Campus Grosshadern
Munich D-81377, Germany
**christian.schroeder@med.uni-muenchen.de*
†*andreas.hoelzer@med.uni-muenchen.de*
‡*ge.zhu@med.uni-muenchen.de*
§*matthias.woiczinski@med.uni-muenchen.de*
¶*oliver.betz@med.uni-muenchen.de*

HELENA GRAF

Department of Medical Engineering
RWTH Aachen University
Aachen, Germany
h.graf@ceramtec.de

SUSANNE MAYER-WAGNER‖ and PETER E. MÜLLER**

Department of Orthopaedic Surgery
University Hospital of Munich (LMU)
Physical Medicine and Rehabilitation
Marchioninistrasse 15, Campus Grosshadern
Munich D-81377, Germany
‖*susanne.mayer@med.uni-muenchen.de*
***peter.mueller@med.uni-muenchen.de*

Received 9 August 2013
Revised 5 December 2014
Accepted 7 April 2015
Published

In the present study, a novel bioreactor for dynamic hydrostatic pressure loading that simultaneously permits medium perfusion was established. This bioreactor enables continuous cultivation without manual attendance. Additional emphasis was placed on a simple bioreactor design which was achieved by pressurizing the medium directly and by applying pressure loading and perfusion through the same piping. Straight forward pressure control and at the same time maintaining sterility were achieved by using a peristaltic pump including inlet and outlet magnetic pinch valves connected with a real-time control. Cell tests using chondrocytes

were performed and similar cell proliferation rates in the bioreactor and in the incubator were found. We conclude that the novel bioreactor introduced here, has the potential to be easily applied for cartilage tissue engineering on a larger scale.

Keywords: Bioreactor; hydrostatic pressure; cartilage engineering; perfusion; chondrocytes.

1. Introduction

Articular cartilage injuries are a common disease of human joints; a promising new treatment option is tissue engineering of functional articular cartilage. The autologous chondrocyte implantation (ACI) which follows the tissue engineering approach is already successfully established in the clinical practice of the repair of articular cartilage lesions in the knee joint. In the latest generation, the so-called matrix-assisted ACI, chondrocytes are isolated from healthy cartilage of the patient, cultured and seeded in a scaffold matrix *in vitro* and implanted in the defect.[1] Clinical results of ACI show a significant improvement in clinical outcomes from baseline scores.[2–5] However, complications such as graft hypertrophy are commonly reported after ACI[1,2] and fibrocartilage mainly develops instead of hyaline cartilage.[6–8] The generation of constructs with more hyaline cartilage-like properties *in vitro* could improve the outcome of ACI and enable its application for larger cartilage defects.

It is well established that the differentiation, proliferation and expression of cells are, among other parameters, influenced by the mechanical loading.[9] Articular cartilage is subjected to continuous dynamic mechanical compressive pressures *in vivo*. Due to the biphasic nature of cartilage, the interstitial fluid is pressurized during loading and initially almost completely supports the applied load. Thus, an almost pure hydrostatic pressure acts on the solid components.[9,10] Exposing dynamic hydrostatic pressure *in vitro* had beneficial effects on chondrocytes in terms of protein production and gene expression in literature studies.[9]

For the application of hydrostatic pressure two types of bioreactors were used in the literature. In the first type a fluid is compressed by a piston driven by a hydraulic or pneumatic pump[3,11–13] or a hydraulic testing machine.[14–19] Due to sterility issues two separate fluid systems were used. The fluid is compressed by the piston and the fluid medium with the specimen that is surrounded but sealed from the first fluid system. In the second type of bioreactor gas compressed by a pump[20–23] or from a compressed gas cylinder[24,25] is injected to the medium that surrounds the specimen. Problems that arise with a compressed gas are changed gas concentrations and a possible generation of gas bubbles. Both bioreactor types are complex since the pressure is not directly applied to the medium. Furthermore, only one bioreactor from the literature worked with medium perfusion,[11] but pressure loading and perfusion were implemented through separate pipings in this bioreactor, i.e., an additional perfusion system including a pump, pipes, valves and connections were necessary making the bioreactor even more complex.

The present study will address this issue by establishing a novel bioreactor which enables continuous operation without manual intervention to investigate the effect

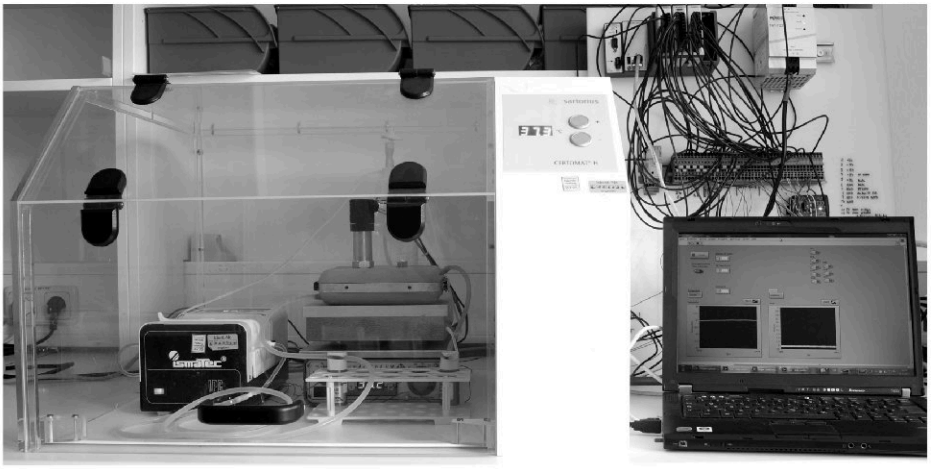
of a continuous dynamic application of hydrostatic pressure on a long-term basis in the future. The main innovations of the novel bioreactor are the direct pressurizing of the culture medium using a pump and the application of pressure loading and medium perfusion through the same piping. In this way, only one pipe and fluid system is necessary. Perfusion occurred automatically due to periodic pressurizing/ depressurizing of the medium.

Therefore, the aim of the present study was to develop a novel bioreactor for dynamic hydrostatic pressure loading that simultaneously permits medium perfusion (1) and an easy as well as self-sustaining valve, pressure and temperature control for cell cultivation (2). First result of human chondrocyte cultures will be presented to show the applicability of the novel bioreactor (3).

2. Materials and Methods

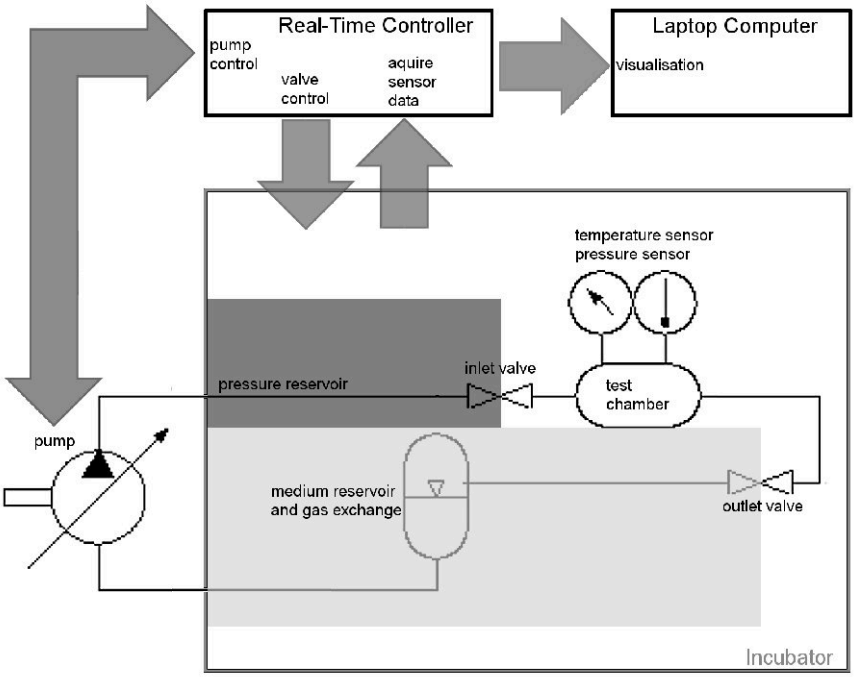
2.1. *Setting of the bioreactor*

The assembly scheme of the bioreactor is depicted in Fig. 1, the bioreactor consists of a medium reservoir, a pump (Ismatec, Wertheim, Germany) that pressurizes the medium to pump pressure, a pressure reservoir, an inlet valve (PN100P2NO12, Bio-Chem Valve, Berlin Germany), the test chamber including a temperature sensor (Pt1000, Heraeus Sensor Technology GmbH, Kleinostheim, Germany) and a pressure sensor (K-Line, Kistler Instrumente AG, Winterthur, Switzerland), an outlet valve (PN100P2NO12, Bio-Chem Valve, Berlin Germany) and a medium reservoir which are all connected by a flexible tube (10025-05S, Bio-Chem Valve, Berlin Germany).



(a)

Fig. 1. (a) Picture of the complete bioreactor system; (b) Assembly scheme of the bioreactor with high (dark grey) and ambient (light grey) pressure areas. The pressure in the test chamber depends on the state of the inlet and outlet valves.



(b)

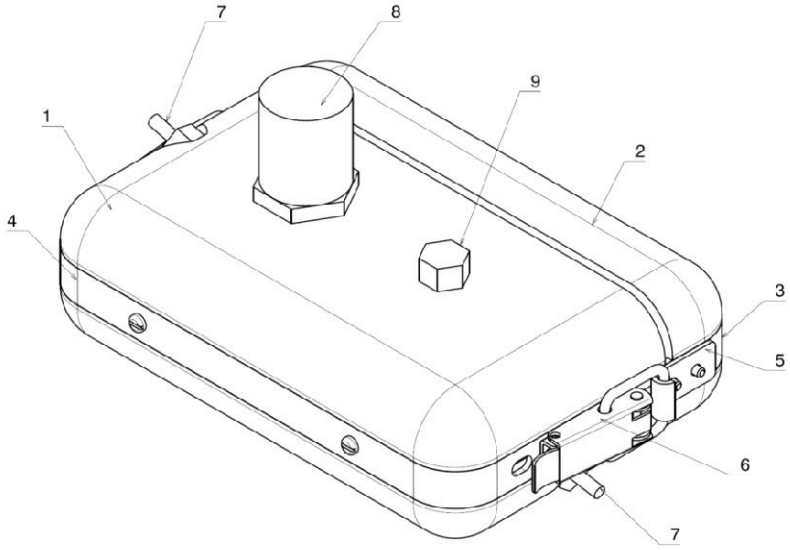
Fig. 1. (*Continued*)

For the medium reservoir a laboratory glass bottle (Schott, Mainz Germany) with two hose connections and a hole covered with an air filter was used. In this way, the medium reservoir had contact with the ambient atmosphere through the filter to allow gas and pressure exchange. Additionally, easy replacement of the medium was allowed by simply changing the bottle with culture medium.

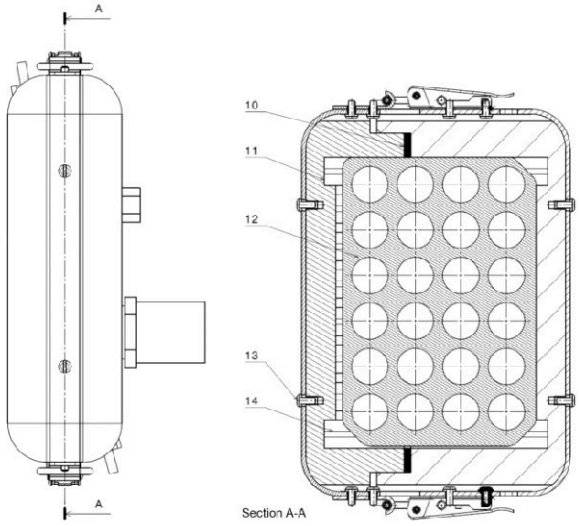
As a pressure reservoir an 1.8 m long flexible tube was applied; this could store pressure well due to its elastic walls (effective stiffness: 1593.0 ± 75.4 kPa). The pressure reservoir was necessary, since the power of the pump was limited. Without the reservoir it would take a long time to build-up the pump pressure inside the test chamber. The pressure in the test chamber was controlled by one inlet and one outlet valve.

The test chamber provided space for one well-plate and had hose connections for medium inflow and outflow, for the pressure sensor and for the temperature sensor (Fig. 2). The test chamber was custom-made of laser sintered polyether ether ketone (PEEK) with an ethylene propylene diene monomer (EPDM) gasket. The assembled test chamber contained a volume of 217 ml and has an effective stiffness against inflation of 9654 ± 808 kPa.

Due to sterility reasons and to reduce contamination a roller pump and magnetic pinch valves were used which had no contact to the medium. All parts which were in



(a)



(b) (c)

Fig. 2. CAD drawing of the test chamber (a) isometric projection including (1) carrier body, (2) closure head, (3,4) fixing brackets, (5) clamp, (6) fastener, (7) hose connections, (8) pressure and (9) temperature transducer; (b) frontal projection with definition of the section A–A; (c) Section A-A including (10) gasket, (11) flute for fluid circulation, (12) wellplate, (13) fixation screws, (14) flute for fluid circulation.

contact with the medium were sterilized before use in an autoclave (e.g., medium reservoir and test chamber) or were sterile products for single-use.

The test chamber was placed on a precision heat plate (Präzitherm, Störktronik; Stuttgart, Germany) which kept the test chamber at 37°C. Additionally the whole assembly was placed in a thermostat-controlled incubator (Certomat H, Sartorius; Göttingen, Germany) during the cell tests. In this way, the whole cultivation medium was set to 37°C (Accuracy 1.0 K).

The pressure and temperature sensors, the pinch valves and the pump were connected to a real-time controller (CRio-9076, National Instruments, Austin, Texas, USA) via analog input, digital output and analog output modules.

The bioreactor was controlled via a purpose-built real-time LabVIEW (Version 2011, National Instruments, Austin, Texas, USA) software program which control the pump, the valves and the pressure sensor self-sustaining. Frequency, pressure and the duration in hours per day can be adjusted by the user to set up the pressure mode of the bioreactor. All acquired data were saved in an ASCII-File with 10 Hz.

2.2. *Finite element model*

2.2.1. *Static analysis*

A finite element (FE)-model of the test chamber was developed based on the computer-aided-design files (Ansys 14.0, Inc., Canonsburg, Pennsylvania, USA) to investigate process safety under pressure mode as well as to identify potential leakage points between the gasket and the camber parts. Sufficient high mesh accuracy (quadratic tetrahedral elements) of the FE-model was ensured by convergence studies of the meshes. The final mesh for the bioreactor had a maximum element size of 1.5 mm for the gasket and 4 mm for the pressure chamber and consisted in total of 88,943 elements. Material properties for the model were adapted from the datasheet of the manufacturer and are shown in Table 1.

The contact between cover plate and main pressure chamber was assumed to be frictionless and the contact conditions for the gasket and the pressure chamber was frictional with a friction coefficient of 0.7. Pressure and temperature sensor were fixed with a joint and the clamps were simulated with a linear longitudinal spring with a stiffness of 1000 N/mm. In order to simulate a closed clamp the springs were preloaded with 650 N. The pressure inside the chamber of 200 kPa was applied normal to each face and the test chamber was fixed to avoid rigid body motion.

Table 1. The material properties of the test chamber.

	PEEK (Chamber parts)	EPDM (Gaskat)
Young's modulus	4250 MPa	5 MPa
Density	1.30 g/cm^3	1.08 g/cm^3
Poisson's ratio	0.40	0.49
Critical strength	90 MPa (ASTM D638)	12 MPa (DIN 53504)
Critical strain	2,8% (ASTM D638)	350% (DIN 53504)

2.2.2. *Computational fluid dynamics*

A FE model of the test chamber was developed based on the computer-aided-design files (Ansys 14.0 CFX, Inc., Canonsburg, Pennsylvania, USA). The improved mesh for the bioreactor had a maximum element size of 1.0 mm and for the fluid the maximum element size was 0.5 mm.

To simplify the simulation the cell culture medium was replaced by water with a reference pressure of 101325 Pa (ambient pressure). The fluid turbulence option was set to "k-epsilon", which solves two additional transport equations for turbulence generation (k) and for turbulence dissipation (epsilon). This option was used for turbulences of small eddies.

A mass flow rate of 3.3e-5 kg/s, which equates to 2 mL/min, was defined as inlet boundary condition while the inlet turbulence option was adjusted to intensity and length scale with fractional intensity (FI) of 0.117 and eddy length scale (ELS) of 0.28 mm. The outlet boundary condition was set to a mass flow rate of 3.3 e-5 kg/s, to guarantee perfusion and to enable us to make a statement about pressure and velocity distribution of the fluid in the test chamber.

2.3. *Cell test*

Hyaline cartilage was collected from the knee joint of one human donor (male, 17 years) within 12 h of death. Chondrocytes were isolated from hyaline cartilage by digestion with collagenase. The cells were cultured in Dulbecco's modified Eagle's medium/Ham's F12 (1:1, Biochrom AG, Berlin, Germany) supplemented with 10% fetal calf serum (PAA Laboratories GmbH, Pasching, Austria), 1% MEM amino acids (50x, Biochrom AG), 1% l-glutamine (200 mM, Biochrom AG), 25 μg/mL ascorbic acid (Sigma-Aldrich Co., St. Louis, USA), 50 IU/mL penicillin/streptomycin (Biochrom AG) and 0.25 μg/mL amphotericin B (Biochrom AG) at 37°C. After three passages the cells were trypsinized and seeded on a 96-well plate at a cell density of 20,000 cells/cm^2 in the same medium as described above.

One group was left for four days (96 h) in the incubator and one group was exposed to dynamic hydrostatic pressure loading and perfusion using the novel bioreactor for four days with pressure amplitude of 100 kPa and a frequency of 0.01 Hz for 24 h/day. The same culture medium as described above was used in the bioreactor. The optical density which corresponds to the viable cell density was measured using the WST-1 assay initially and after four days.

3. Results

3.1. *Function of the bioreactor*

Before starting the regular operation both valves were opened in order to fill the entire bioreactor system with medium from the medium reservoir, no pressure was generated. If both valves are open, the bioreactor works like a perfusion chamber

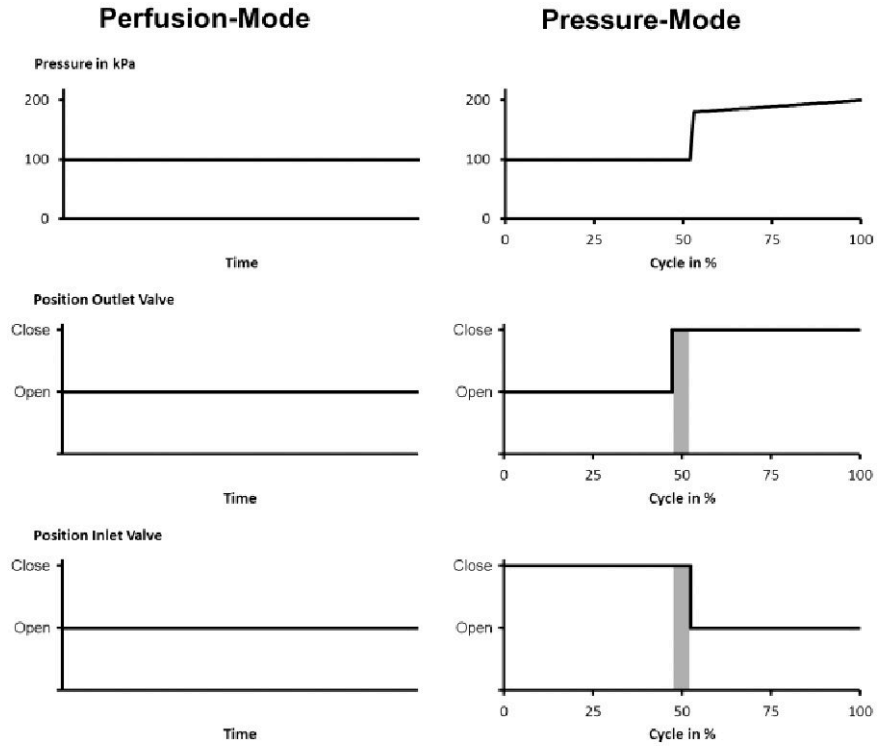


Fig. 3. Position of the valve and the resulted pressure in the bioreactor. Both valves are open during perfusion mode and ambient pressure remains in the bioreactor. Perfusion occurs by a constant fluid flow (2 mL/min) in the bioreactor. During pressure mode the inlet valve is closed and pressure is stored in the flexible tube. The outlet valve closed, afterwards inlet valve were open and the pressure were transferred to the reactor. There is an overlap of 500 ms (grey area) to secure that the outlet valve is closed before the inlet valve is open.

and the medium can circulate (constant volume flow: 2 mL/min) with ambient pressure (Fig. 3(a)).

During the pressure mode, one of the valves was always closed and, thus, the medium in the pressure reservoir remained pressurized under pump pressure. In detail, it can be distinguished between pressure mode and nonpressure mode. In pressure mode the inlet valve is open and the outlet valve is closed, i.e., the test chamber has access to the pressure reservoir only (Fig. 1) so that the pressure in the test chamber approaches the (high) pump pressure. In nonpressure mode the inlet valve is closed and the outlet valve is open, i.e., the test chamber has access to the medium reservoir under atmospheric pressure (Fig. 1) and the pressure in the test chamber approaches the (low) ambient pressure. In this way the chamber pressure could be easily changed between pump pressure and ambient pressure by switching

the valves (Fig. 3(b)). The pump pressure could be adjusted by changing the rotational frequency of the roller pump.

As mentioned above, one of the two valves was always closed during pressure mode. However, due to the tube deformation while switching the valves and, in addition, due to the very small but existing compressibility of the fluid medium, a low perfusion occurred during pressurizing.

Closed loop operation as shown in Fig. 1 was used in the present study, which means that the same medium remained in the system. It should be mentioned that the bioreactor can of course also operate in batch operation, i.e., the medium that flows out from the test chamber is not fed into the medium reservoir again.

3.2. *FE-model*

3.2.1. *Static analysis*

For the pressure chamber the maximum equivalent stress (von Mises stress) was 10.5 MPa and the maximum principle strain was 0.25% localized inside the test chamber (Fig. 4(a)). A maximum principle strain of 6.2% and a maximum equivalent stress (von Mises Stress) of 0.38 MPa was calculated for the gasket made of EPDM. The FE-model showed a homogeneous pressure distribution between the gaskets the other chamber parts (Fig. 4(b)). Therefore, potential leakage between these parts can be excluded.

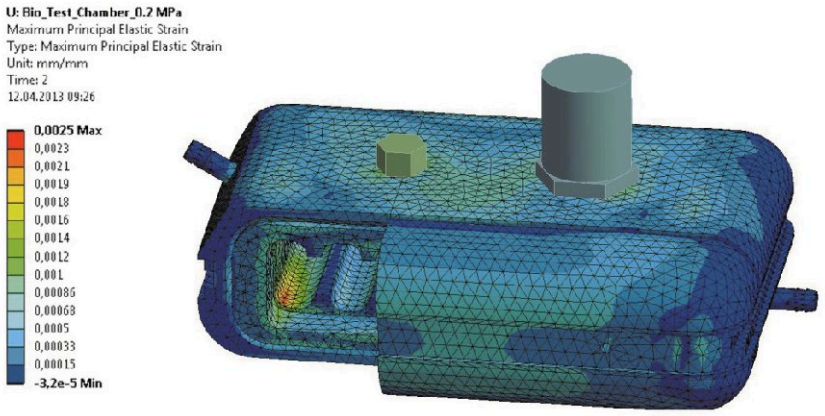
Both, the critical strain and stress of PEEK and EPDM (see Table 1) are approximately Nine times higher than the calculated values for the pressurized test chamber.

3.2.2. *Computational fluid dynamics of the perfusion mode*

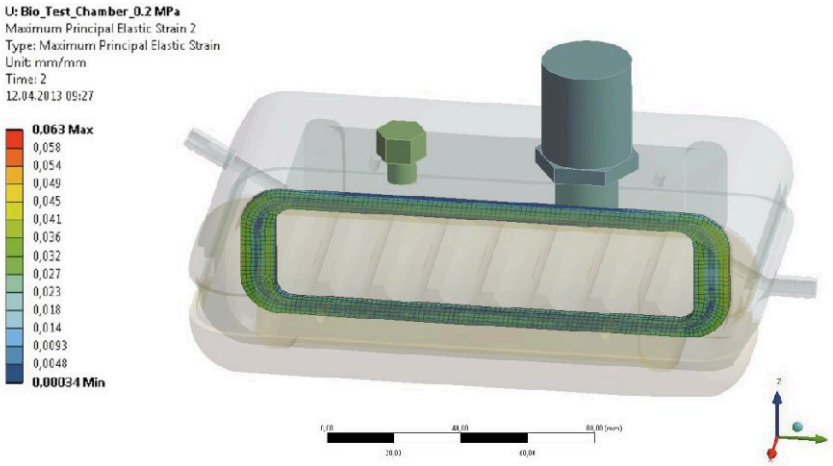
The velocity of the inlet was defined to 2.6e-3 m/s. This value decreased to 4.0e-5 m/s due to the larger cross-section area of the upper sector of the bioreactor. The restriction of the outlet caused an increase of velocity (Fig. 5). To exclude shear effects on the cell layer the velocity in the wells were determined. The mean velocity in the wells was $0.54 \pm 0.05\,\mu$m/s. The local distribution of the velocity in a single well varies from $0.35 \pm 0.04\,\mu$m/s at the boundary area to $0.70 \pm 0.06\,\mu$m/s in the center of the well. Respecting the flow direction a decrease of $0.10\,\mu$m/s was calculated between the wells in front and in the back of the plate.

3.3. *Pressure*

The maximum pump overpressure that could be achieved was approximately 270 kPa. The maximum pump overpressure decreased with increasing frequency at periodic loading, e.g., at 0.1 Hz the maximum pump overpressure was approximately 100 kPa. Figure 6 show the test chamber pressure over time for four cycles of periodic loading with the pressure amplitude of 100 kPa and frequencies of 0.1 Hz,



(a)



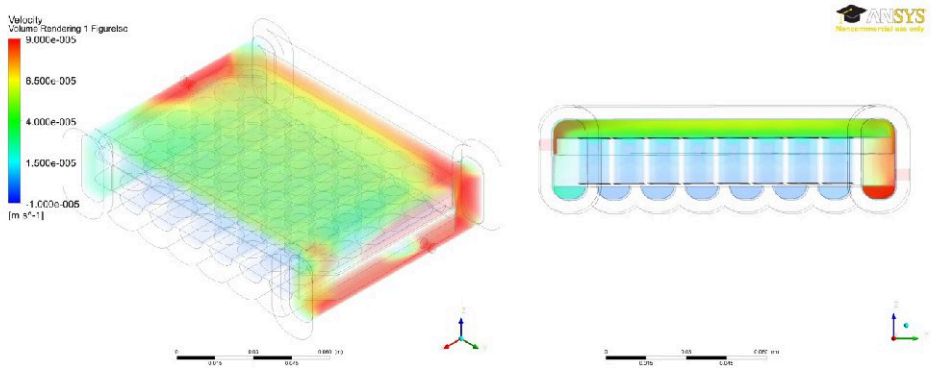
(b)

Fig. 4. (Color online) Maximum principal strains of the test chamber (a) and the gasket (b) under loading conditions.

0.01 Hz, and 0.001 Hz, respectively. The used roller pump created periodical flow interruptions. Therefore, the linear increase of the pressure was transient. Also pressure relaxation appears at all frequencies after opening the outlet valve. However, this relaxation subsides after approximately 2 s for all measured frequencies.

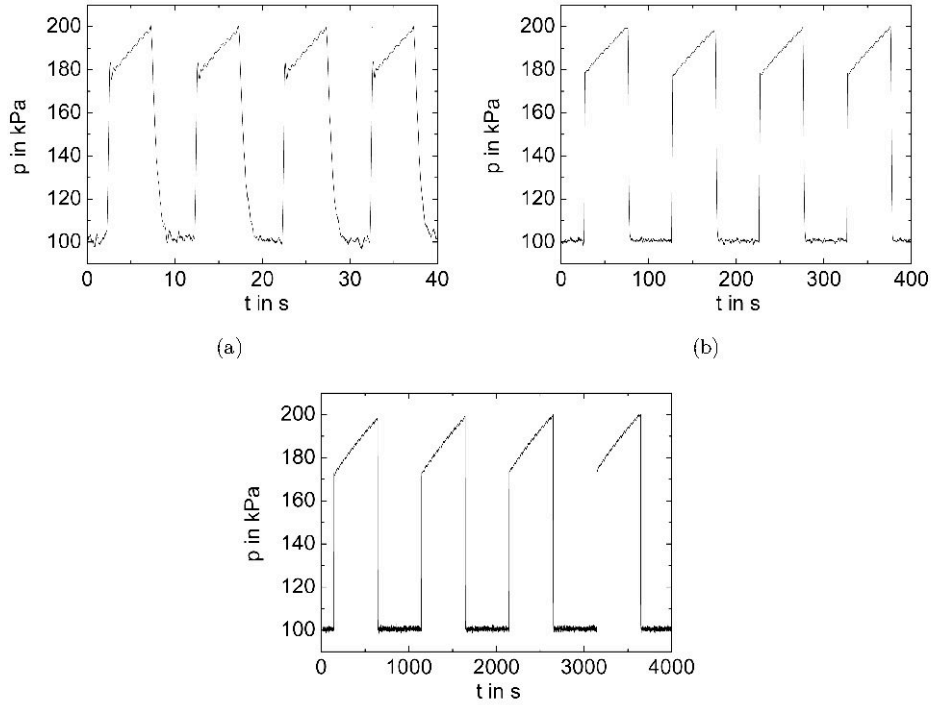
3.4. *Cell test*

The optical densities using the WST-1 assay of the different groups studied are displayed as box plots in Fig. 7. The densities were statistically compared using the independent two-sample t-test. The densities of the incubator group and bioreactor group were not significantly different from each other after four days ($p = 0.49$). For



(a) (b)

Fig. 5. (Color online) Velocity distribution of the test chamber during perfusion mode. (a) 3D View (b) View perpendicular to the flow direction.



(a) (b)

(c)

Fig. 6. Test chamber pressure over time for periodic loading with the pressure amplitude of 100 kPa and frequencies of (a) 0.1 Hz, (b) 0.01 Hz and (c) 0.001 Hz.

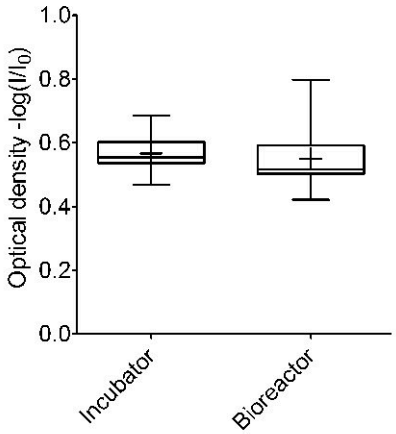


Fig. 7. Comparison of the optical density using WST-1 assay of the medium without cells, cells after four days in the incubator and bioreactor.

comparison, the optical cell density of the medium without cells is significantly smaller ($p < 0.001$) than the densities of both the incubator and bioreactor group after four days, i.e., that there are still viable cells in the incubator and the bioreactor after four days.

4. Discussion

A novel bioreactor for simultaneous dynamic hydrostatic loading and medium perfusion was successfully established.

Before manufacturing, a FE-model of the test chamber ensured suitable component dimensions for pressure loading. Leakage between gasket and chamber parts could be excluded while circumferential pressure loading was found.

Due to the fact, that continuous perfusion tests can be performed without manual intervention and contaminations during medium, change cannot occur. An easy pressure control was achieved by using a pressure reservoir, one inlet and one outlet valve. This enables the user to switch between pump pressure and ambient pressure quick and easy. In contrast to other bioreactors with two different fluid[15,16] or gas[22,26] systems, only a single closed loop fluid system was used to improve manageability and sterility. A real-time architecture including self-written software and hardware were archive a self-sustaining and failsafe long term control of the bioreactor.

However, in the present study the pressure over time was solely no square wave; e.g., in the example runs plotted in (Fig. 7) the pressure still increased linearly from about 180 kPa to 200 kPa at pressure mode. The transient behavior of this rising is an artefact of the roller pump. In addition, the maximum pump pressure decreased with increasing frequency. Both issues mentioned above indicate an unadjusted pressure reservoir for the pump power used. Furthermore, the

maximum pump pressure was restricted to approximately 270 kPa due to the limited power of the pump.

As been reviewed, loads between 0.1 and 15 MPa and frequencies between 0.05 and 1 Hz produce positive results when applied on chondrocytes.[27] Several studies[25,28] harnessed the lower end in the range mentioned above to mimic loads during continuous passive motion (CPM). In clinical practice, the use of CPM has been demonstrated to enhance cartilage healing.[29] The average loads on cartilage during CPM were estimated to be between 0.1 and 0.2 MPa, which are within the limit of our system. Therefore, the novel bioreactor in the present study shows a good potential in application for cartilage tissue engineering.

Initial cell tests using chondroyctes showed good cell viability in the bioreactor up to four days, similar to the viability of cells kept in the incubator for the same time period. Different results about the proliferation of cartilage cells subjected to dynamic pressure exist in the literature; mostly, the proliferation was reduced[20,21,25] or similar[11,19] compared to incubated control cells. Our results are in agreement to the latter references. Reasons for the different results mentioned could be, amongst other things, different culturing procedures, bioreactor types or loading parameters.

The present study includes only a basic cell-biological test because the main objective was to describe the novel bioreactor from a technical point of view. The bioreactor is capable of individual investigation of different pressure magnitudes, frequencies and test durations and such cell studies are under way. In addition, nonsymmetric loading schedules, which had been shown to have an influence on proliferation and collagen production,[25] can be readily applied using the novel bioreactor as well.

It should be mentioned that the medium perfusion of the novel bioreactor is approximately 1 mL per cycle at the pressure amplitude of 100 kPa, which is very low compared to the total test chamber volume of approximately 220 mL.

Compression is the superficial loading case in a cartilage tissue and provides i.e., the maintenance of the cells.[9,10] However, shear stresses exist in the cartilage tissue, due to the movement and friction of the joint *in vivo*. Tribological investigations measure the friction coefficients of cartilage/cartilage pairings show high variations in the range of 0.0013–0.34.[30] Therefore, the amplitude of shear force acting on a cartilage layer and their apportionment between extracellular matrix and cells are still unknown and therefore not included in the bioreactor yet. Perfusion occurs with a constant flow rate of 2 mL/min which results in a negligible small velocity in the wells of the plate ($< 1\,\mu$m/s). Thus, the shear stress that arises due to perfusion can be neglected and the fluid stress inside the test chamber is almost pure hydrostatic pressure.

Another limitation is that the pressure distribution in the chamber cannot be measured *in vitro*. Due to the hydrodynamic resistance, a slightly decrease (approximately 30 Pa) of the acting pressure in flow direction over the whole test chamber can occur. It was tried to minimized that limitation by using only the wells near the pressure sensor for the presented cell test.

A monolayer culture was used in the present cell test in accord with the majority of studies from the literature, which means that the hydrostatic fluid pressure acts mainly from the inlet side only. However, three-dimensional (3D) cultures such as pellet cultures, alginate or agarose beads,[14,24] membrane-based cultures[31] or with a scaffold can all be studied using the novel bioreactor.

In conclusion, in the present study a novel bioreactor for simultaneous dynamic hydrostatic pressure loading and perfusion was successfully introduced and its mechanical design and properties were described. The cell viability in the bioreactor was assured in a cell experiment. The novel bioreactor design enables the investigation of different pressure amplitudes, frequencies and schedules under continuous conditions. Thus, it has the potential to improve the quality of tissue engineered cartilage under additionally providing a mechanical stimulus.

Acknowledgments

The authors would like to thank Tatjana Müller for creating and solving the CFD-model and Florian Hammerschmid for the first cell culture experiments. Furthermore, we want to thank Dr. Yan Chevalier for his help in improving the language of this manuscript.

Conflict of Interest Statement

None.

References

1. Vasiliadis HS, Wasiak J, Salanti G, Autologous chondrocyte implantation for the treatment of cartilage lesions of the knee: A systematic review of randomized studies, *Knee Surg Sports Traumatol Arthrosc* **18**(12):1645 1655, 2010.
2. Harris JD, Siston RA, Pan X, Flanigan DC, Autologous chondrocyte implantation: A systematic review, *J Bone Joint Surg Am* **92**(12):2220 2233, 2010.
3. Parkkinen JJ, Ikonen J, Lammi MJ, Laakkonen J, Tammi M, Helminen HJ, Effects of cyclic hydrostatic pressure on proteoglycan synthesis in cultured chondrocytes and articular cartilage explants, *Arch Biochem Biophys* **300**(1):458 465, 1993.
4. Niethammer TR, Pietschmann MF, Horng A, Rossbach BP, Ficklscherer A, Jansson V, Müller PE, Graft hypertrophy of matrix-based autologous chondrocyte implantation: A two-year follow-up study of NOVOCART 3D implantation in the knee, *Knee Surg Sports Traumatol Arthrosc* **22**(6):1329 1336, 2014.
5. Kon E, Verdonk P, Condello V, Delcogliano M, Dhollander A, Filardo G, Pignotti E, Marcacci M, Matrix-assisted autologous chondrocyte transplantation for the repair of cartilage defects of the knee: Systematic clinical data review and study quality analysis, *Am J Sports Med* **37**(1):156S 166S, 2009.
6. Horas U, Pelinkovic D, Herr G, Aigner T, Schnettler R, Autologous chondrocyte implantation and osteochondral cylinder transplantation in cartilage repair of the knee joint. A prospective, comparative trial, *J Bone Joint Surg Am* **85-A**(2):185 192, 2003.

24. Heyland J, Wiegandt K, Goepfert C, Nagel-Heyer S, Ilinich E, Schumacher U, Portner R, Redifferentiation of chondrocytes and cartilage formation under intermittent hydrostatic pressure, *Biotechnol Lett* **28**(20):1641 1648, 2006.
25. Hansen U, Schunke M, Domm C, Ioannidis N, Hassenpflug J, Gehrke T, Kurz B, Combination of reduced oxygen tension and intermittent hydrostatic pressure: A useful tool in articular cartilage tissue engineering, *J Biomech* **34**(7):941 949, 2001.
26. Tarng YW, Huang BF, Su FC, A novel recirculating flow-perfusion bioreactor for periosteal chondrogenesis, *Int Orthop* **36**(4):863 868, 2012.
27. Darling EM, Athanasiou KA, Articular cartilage bioreactors and bioprocesses, *Tissue Eng* **9**(1):9 26, 2003.
28. Luo ZJ, Seedhom BB, Light and low-frequency pulsatile hydrostatic pressure enhances extracellular matrix formation by bone marrow mesenchymal cells: An *in-vitro* study with special reference to cartilage repair, *Proc Inst Mech Eng H* **221**(5):499 507, 2007.
29. Reinold MM, Wilk KE, Macrina LC, Dugas JR, Cain EL, Current concepts in the rehabilitation following articular cartilage repair procedures in the knee, *J Orthop Sports Phys Ther* **36**(10):774 794, 2006.
30. Schwarz ML, Schneider-Wald B, Krase A, Richter W, Reisig G, Kreinest M, Heute S, Pott PP, Brade J, Schutte A, [Tribological assessment of articular cartilage. A system for the analysis of the friction coefficient of cartilage, regenerates and tissue engineering constructs; initial results], *Orthopade* **41**(10):827 836, 2012.
31. Mayer-Wagner S, Schiergens TS, Sievers B, Redeker JI, Schmitt B, Buettner A, Jansson V, Muller PE, Scaffold-free 3D cellulose acetate membrane-based cultures form large cartilaginous constructs, *J Tissue Eng Regen Med* **5**(2):151 155, 2011.

Publication 2

Journal of Biotechnology 210 (2015) 59–65

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Comparing effects of perfusion and hydrostatic pressure on gene profiles of human chondrocyte.

Ge Zhu[a,1], Susanne Mayer-Wagner[a,*,1], Christian Schröder[a], Matthias Woiczinski[a], Helmut Blum[b], Ilaria Lavagi[b], Stefan Krebs[b], Julia I. Redeker[a], Andreas Hölzer[a], Volkmar Jansson[a], Oliver Betz[a], Peter E. Müller[a]

[a] Department of Orthopaedic Surgery, Physical Medicine and Rehabilitation, Campus Großhadern, Ludwig-Maximilians-University, Marchioninistr. 15, 81377 Munich, Germany
[b] LAFUGA Genomics, Gene Center Munich, Ludwig-Maximilians-University, Feodor-Lynen-Str. 25, 81377 Munich, Germany

ARTICLE INFO

Article history:
Received 28 May 2015
Received in revised form 17 June 2015
Accepted 22 June 2015
Available online 29 June 2015

Keywords:
Perfusion
Hydrostatic pressure
Bioreactor
Sequencing
Human chondrocytes

ABSTRACT

Hydrostatic pressure and perfusion have been shown to regulate the chondrogenic potential of articular chondrocytes. In order to compare the effects of hydrostatic pressure plus perfusion (HPP) and perfusion (P) we investigated the complete gene expression profiles of human chondrocytes under HPP and P. A simplified bioreactor was constructed to apply loading (0.1 MPa for 2 h) and perfusion (2 ml) through the same piping by pressurizing the medium directly. High-density monolayer cultures of human chondrocytes were exposed to HPP or P for 4 days. Controls (C) were maintained in static cultures. Gene expression was evaluated by sequencing (RNAseq) and quantitative real-time PCR analysis. Both treatments changed gene expression levels of human chondrocytes significantly. Specifically, HPP and P increased COL2A1 expression and decreased COL1A1 and MMP-13 expression. Despite of these similarities, RNAseq revealed a list of cartilage genes including ACAN, ITGA10 and TNC, which were differentially expressed by HPP and P. Of these candidates, adhesion related molecules were found to be upregulated in HPP. Both HPP and P treatment had beneficial effects on chondrocyte differentiation and decreased catabolic enzyme expression. The study provides new insight into how hydrostatic pressure and perfusion enhance cartilage differentiation and inhibit catabolic effects.

1. Introduction

In cell-based therapies for cartilage regeneration, a large number of differentiated chondrocytes are required to repair cartilage defects. However, chondrocytes undergo dedifferentiation and lose their characteristic phenotype when expanded in monolayer culture (von der Mark et al., 1977). Biomechanical stimulation is widely used to stimulate chondrogenic differentiation (Madeira et al., 2015). Various forms of bioreactors have been implemented to apply mechanical stimuli to chondrogenic cells (Darling and Athanasiou, 2003; Responte et al., 2012). Hydrostatic pressure is among the most important forces used in chondrocyte culturing. Hydrostatic pressure on bovine chondrocytes results in an increased collagen and glycosaminoglycan content (Benjamin et al., 2009) and an increased COL2A1 expression in human osteoarthritic (OA) chondrocytes (Smith et al., 2011). Hydrostatic pressure combined with medium perfusion at a very low rate upregulates the expression of COL2 and COL1 and maintained the expression of aggrecan in bovine chondrocytes on collagen gels (Mizuno and Ogawa, 2011). There have also been studies using perfusion without hydrostatic pressure, which showed decreased ACAN and collagen type 2 expression of bovine chondrocytes in a three-dimensional tubular perfusion system after 7 days and re-increased at day 14 (Yu et al., 2014). The question remains whether it is necessary to invest so much effort in applying hydrostatic pressure, or could perfusion by itself, be sufficient enough to improve chondrocyte differentiation? Due to different application methods, cell sources and other parameters it is problematic to compare the outcomes of various bioreactor systems. To obtain an overview of processes regulated by hydrostatic pressure and perfusion in bioreactors, it is important to leave behind restrictions of relative gene expression levels. Microarray analyses of chondrocytes under dynamic expression revealed mechanosensitive genes in mouse chondrocytes (Bougault et al., 2012). To our knowledge there has been no approach to compare perfusion versus perfusion combined with

* Corresponding author. Fax: +49 89 44007 4863.
E-mail address: susanne.mayer@med.uni-muenchen.de (S. Mayer-Wagner).
[1] Both authors contributed equally.

http://dx.doi.org/10.1016/j.jbiotec.2015.06.409
0168-1656/

hydrostatic pressure using gene profiles. Another problem regarding bioreactor studies is the use of animal cells or human OA chondrocytes. Animal cells have been shown to be less sensitive to biomechanical influences and might therefore not reflect clinical findings (Grogan et al., 2012; Tran et al., 2011). Human OA chondrocytes do not reflect clinical conditions, as healthy human chondrocytes are used in the repair of cartilage defects. In order to approach clinical applications, healthy human chondrocytes are required to fully examine the effects of hydrostatic pressure and perfusion.The aim of the present study was to compare effects of HPP and P on the complete gene profile screen of human chondrocytes.

2. Materials and methods

2.1. Cell culture

Human articular cartilage was obtained through triple arthrodesis (16 years, male). The study was approved by the responsible Ludwig-Maximilians-University medical center ethics committee. Chondrocytes were isolated by pronase (Roche Diagnostics GmbH, Mannheim, Germany) and collagenase (Sigma–Aldrich Co., St. Louis, USA). Isolated cells were cultured in a humidified atmosphere at 37 °C, 5% CO_2 until passage 2. Culture medium consisted of Dulbecco's Modified Eagle Medium (DMEM)/F12 (1:1, Biochrom AG, Berlin, Germany), 10% fetal bovine serum (FBS) (Biochrom AG, Berlin, Germany), 1% MEM amino acids (Biochrom AG, Berlin, Germany), 25 μg/ml ascorbic acid (Sigma–Aldrich Co., St. Louis, USA), 50 IU/ml Penicillin/Streptomycin (Biochrom AG, Berlin, Germany) and 0.25 μg/ml Amphotericin B (Biochrom AG, Berlin, Germany). The cells were plated 24 h prior to stimulation at a density of 10^6 cells/cm^2 in 6-well plates.

2.2. Bioreactor and stimulations

A simplified bioreactor (Fig. 1) described earlier (Schröder et al., 2015), was used to culture human chondrocytes in high-density monolayer cultures. In brief, loading and perfusion were applied through the same piping by pressurizing the medium directly. Parallel chambers were used for different stimulations: Perfusion (P) ($n = 6$), hydrostatic pressure plus perfusion (HPP) ($n = 6$) and control (C) ($n = 6$), where cells were kept in static cultures. For P and HPP, perfusion was applied with a medium flow rate of 2 ml/min for 20 h/day for 4 days. The perfusion flow rate of 2 ml/min was chosen in accordance with cell viability tests (data not shown). In HPP, hydrostatic pressure of 0.1 MPa for 2 h (loading), followed by 2 h rest (off-loading), was applied each day. During this 4 h period perfusion was stopped in P simultaneously.

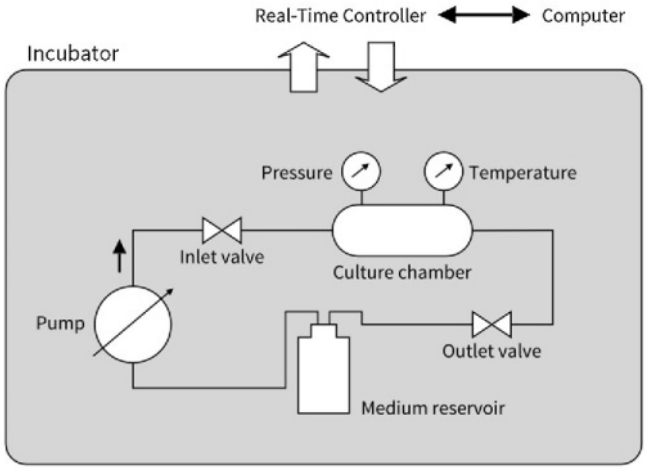


Fig. 1. Schematic setup of the bioreactor system including culture chamber, peristaltic pump and medium reservoir in an incubator.

2.3. Finite element method analysis

To analyze fluid flow of the medium and interaction with cells a computer fluid dynamics (CFD) simulation of the test chamber was developed (Ansys 14.0, Inc., Canonsburg, Pennsylvania, USA). In the numerical simulation, the final mesh for the bioreactor had a maximum element size of 1 mm and the fluid had a maximum mesh size of 0.5 mm. The medium was given the characteristic properties of water. Fluid turbulences were taken into account with the k-epsilon option which is used for small eddies. The inlet and outlet mass flow rates were set to 3.3 e-5 kg/s, matching a 2 ml/min volume flow there. All results were analyzed at end of the transient simulation which occurred after 3 s.

2.4. RNA isolation

After 4 days of culture, the total RNA was isolated using Qiazol™ Lysis Reagent (Qiagen, Hilden, Germany) according to manufacturer's instructions. Briefly, 1 ml of Qiazol™ Lysis Reagent was added in each well of 6-well plates. RNA was extracted with 0.2 ml of chloroform (Sigma–Aldrich Co., St. Louis, USA), precipitated with 0.5 ml isopropanol (Sigma–Aldrich Co., St. Louis, USA) and washed with 75% ethanol. Isolated total RNA was checked for purity (Nanodrop ND-1000, ThermoFisher, Waltham, USA) and integrity (Bioanalyzer 2100, Agilent, Santa Clara, USA). Total RNA with A260/A280 > 1.8 and integrity > 9 was used for analysis.

2.5. Generation of RNAseq libraries

An amount of 100 ng total RNA was first treated with double-strand specific DNAse (Fermentas Inc., Hanover. MD, United States) to remove any traces of genomic DNA. After heat-inactivation of the DNAse, cDNA was synthesized and converted to Illumina-compatible sequencing libraries with the Encore complete RNAseq kit from NuGen (NuGen, San Carlos, USA). Briefly, first strand cDNA was generated by selective priming, second strand cDNA was synthesized using dUTP and the generated double stranded cDNA was fragmented using a Covaris M220 sonicator (50W peak incident power, 20% duty factor, 200 cycles per burst, 160 s treatment time). Then cDNA was end-repaired, ligated to Illumina-Adapters and the second-strand was selectively removed. After 18 cycles of PCR the final library was quantified on an Bioanalyser (Bioanalyzer 2100, Agilent, Santa Clara, USA) and diluted to 10 nM prior to pooling and sequencing. The pooled libraries were sequenced on two lanes of a rapid flowcell in 100 bp single end mode on a HiSeq1500 instrument (Illumina, San Diego USA).

2.6. RNAseq data analysis

Raw sequence reads were demultiplexed and mapped to the human genome (hg19 release) using Tophat 2.0 (Kim et al., 2013). Read counts for each gene were obtained by the python script HTseq count (Anders et al., 2015). Read counts were then normalized by r-log transformation with the program DESeq2 (Love et al., 2014). Differentially expressed genes were identified by pairwise comparison with DESeq2 using an fdr threshold of 5%. A list of cartilage relevant genes was obtained from www.genecards.org using "cartilage" as keyword including all genes with a relevance score of >0.5. The scoring was calculated by a Lucene defined algorithm. From the lists of significant genes that overlapped with the list of relevant cartilage genes was plotted as a heatmap. In order to visualize the difference of the expression between C, P and HPP, the data

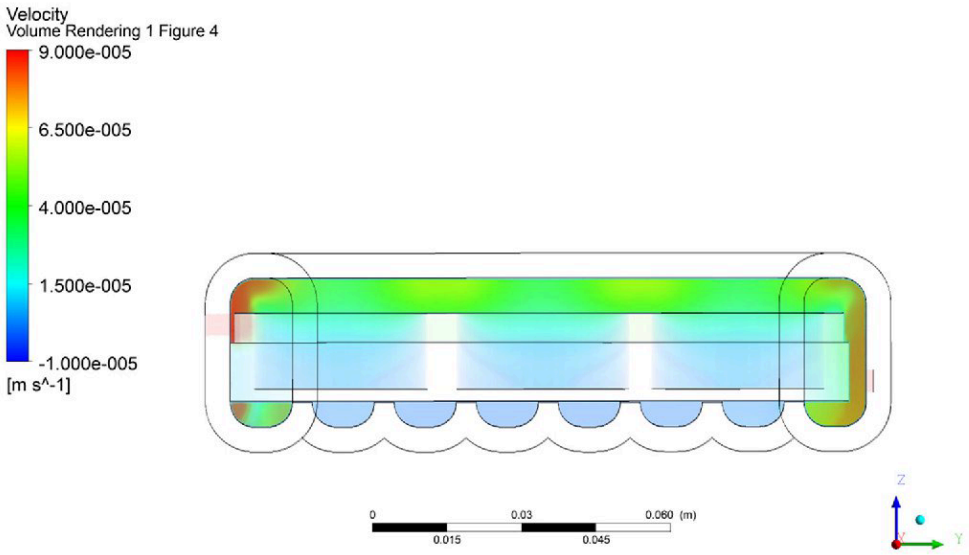


Fig. 2. Finite element method analysis illustrating even velocity within the bottom of the wells.

were centered by subtracting the mean of the log2 Fold Change of all samples for each gene from the original log2 Fold Change value. The centered data of all samples were then plotted into two distinct heat maps: any comparison (Fig. 3A) and P vs HPP (Fig. 3B). The rows of the heatmaps (genes) were ordered by foldchange, and the columns (C, P and HPP) were arranged by hierarchical clustering.

2.7. Real time PCR

1 μg of total RNA of each sample was reverse-transcribed using QuantiTect® Reverse Transcription Kit (Qiagen, Hilden, Germany) which included a DNase treatment according to the manufacturer's protocols. Relative quantitative real-time PCR was performed using LightCycler® 96 System (Roche, Mannheim, Germany) with 5 μl of FastStart Essential DNA Green Master Mix (Roche Diagnostics GmbH, Mannheim, Germany) and 2.5 μl of cDNA resulting in a final volume of 10 μl. Amplification parameters were identical for each primer pair (10 min at 95 °C preincubation, 10 s denaturation at 95 °C was followed by 10 s annealing at 60/65 °C and 15 s extension at 72 °C for 40 cycles). Reactions were performed in triplicates.

Messenger RNA expression levels of Glycerinaldehyd-3-phosphat-Dehydrogenasef (GAPDH) (Vandesompele et al., 2002), collagen type II α_1 chain (COL2A1) (Varas et al., 2007); collagen type 1 α_1 chain (COL1A1) (Neidlinger-Wilke et al., 2005), aggre-

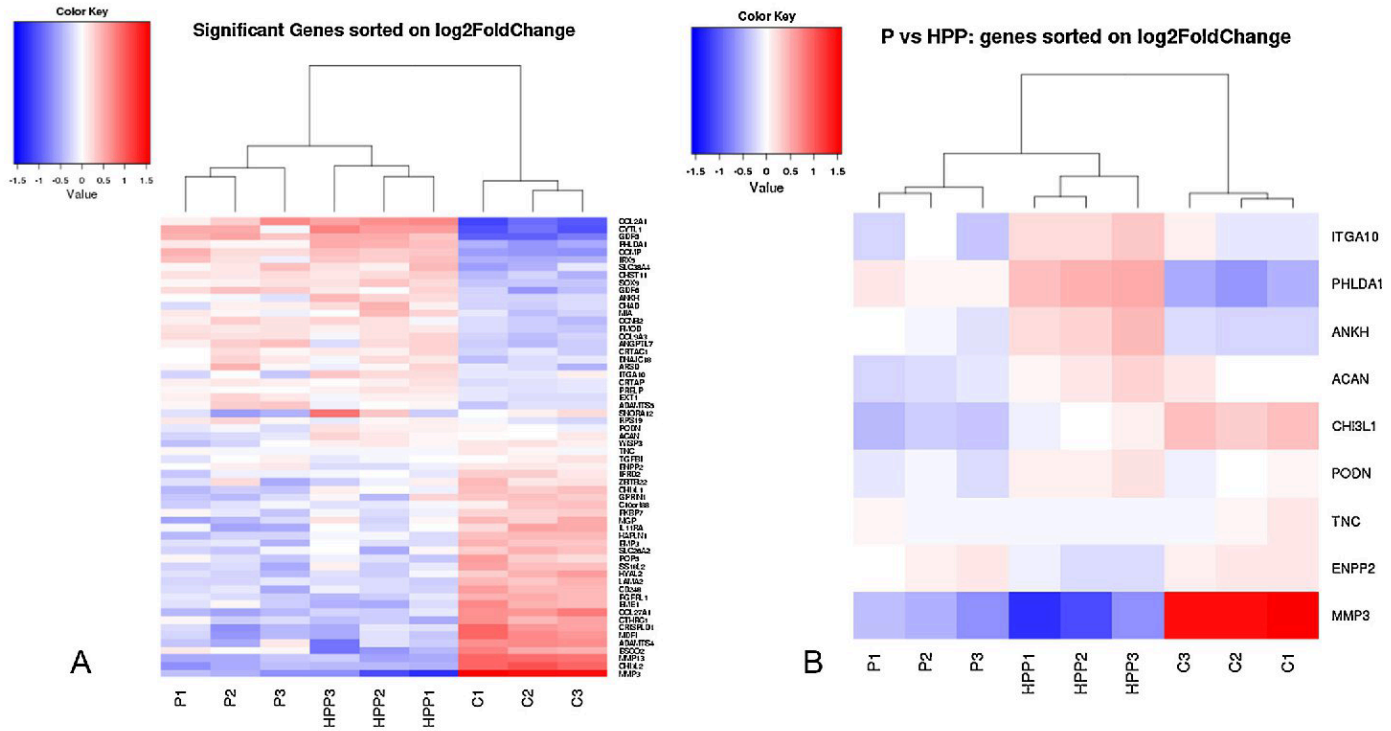


Fig. 3. Heatmap visualization of selected RNAseq results. Each heatmap shows differentially expressed genes (DEG) significant for one pairwise test. Only genes that are functionally annotated with the term 'cartilage' are shown. Heatmap colors indicate log2 distance from the mean expression level of each gene. Rows are ordered by increasing log2 foldchange and columns are ordered by hierarchical clustering. (A) DEG for any comparison. (B) Comparison HPP vs P.

Table 1
Gene sequences of primers.

Gene	Annealing temperature	Sequence
GAPDH forward	60	TGCACCACCAACTGCTTAGC
GAPDH reverse	60	GGCATGGACTGTGGTCATGAG
COL2A1 forward	65	GTTATCGAGTACCGGTCACAGAAG
COL2A1 reverse	65	AGTACTTGGGTCCTTTGGGTTTG
ACAN forward	65	CAGCACCAGCATCCCAGA
ACAN reverse	65	CAGCAGTTGATTCTGATTCACG
COL1A1 forward	65	TGACCTCAAGATGTGCCACT
COL1A1 reverse	65	ACCAGACATGCCTCTTGTCC
MMP-13 forward	60	GACTTCACGATGGCATTGCTG
MMP-13 reverse	60	GCATCAACCTGCTGAGGATGC

can (ACAN) (Varas et al., 2007) and matrix-metalloproteinase 13 (MMP-13) (Hong et al., 2009) were tested. Mean relative quantification values were calculated by the ΔDCt method using GAPDH as an endogenous control. Sequences of primers are shown in Table 1. Each primer pair was tested and only 90–100% efficient primer pairs were used for the analysis.

2.8. Statistical analysis

At least 2 independent sets of experiments for each condition were performed in triplicate. Data were pooled and expressed as mean ± standard error of the mean (SEM). Results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test with GraphPad Prism 5 (GraphPad Software, Inc. USA). *P*-values < 0.05 was considered to be statistically significant.

3. Results

3.1. Bioreactor

Perfusion within the bioreactor was monitored in order to exclude inhomogeneous perfusion conditions caused by a non-laminar flow. Finite element method (FEM) analysis was performed to analyze velocity within wells. FEM showed that, velocities of the fluid within the bottom of all wells were nearly identical ranging between 5 and 6.5 μm/s (Fig. 2) and that no velocity gradient existed. At the inlet and outlet of the bioreactor the velocity of the fluid was higher due to the smaller diameter.

3.2. Sequencing

3.2.1. Count of up/down- regulated genes

Sequencing analysis comparing gene expression patterns of C, P and HPP provided multiple significant changes ($p < 0.05$) in gene expression according to the log2 Fold Change. The number of up-regulated and down-regulated genes when comparing C vs P, C vs HPP or P vs HPP is shown in Table 2. The two treatments showed large effects on gene expression relative to the control. The two stimulations also differed in up-and downregulated genes to a certain degree (Table 2).

Table 2
Number of up/down-regulated genes.

Treatments	Up-regulated genes	Down-regulated genes	Total number of genes
C vs. HPP	2573	2468	5039
C vs. P	2169	2135	4304
P vs. HPP	152	95	247

3.2.2. Heat maps and hierarchical cluster analysis

The clustering showed separation into three groups, consistent with the three different treatments and a high similarity between the replicates. Both treatments showed a large variation to the control group (Fig. 3A), but a relatively high similarity between the treatments (P and HPP) (Fig. 3B).

Cartilage relevant genes, which changed more than 2-fold ($p < 0.05$) between HPP and C and between P and C are displayed in Table 3. Sox9 was upregulated by HPP (1.5-fold) and *P* (1.4-fold).

The fold changes of genes ($p < 0.05$) comparing P and HPP displayed an upregulation of cartilage specific integrin alpha 10 ITGA10 (1.4-fold), pleckstrin homology-like domain A1 (PHLDA1) expression (1.3-fold), ANKH inorganic pyrophosphate transport regulator (ANKH) expression (1.3-fold), ACAN expression (1.3-fold), Chitinase 3-Like 1 (CHI3L1) expression (1.3-fold), podocan (PODN) expression (1.2-fold), Tenascin C (TNC) expression (1.2-fold) and a downregulation for ectonucleotide pyrophosphatase/phosphodiesterase 2 (ENPP2) expression (1.2-fold) and matrix metalloproteinase 3 (MMP 3) expression (1.5-fold).

The following link has been created to allow review of the record GSE69206 while it remains in private status: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=cdunucumhfirhyv&acc=GSE69206

3.3. Real time PCR

The HPP cultures expressed a significantly higher (3.1-fold; $p < 0.05$) COL2A1 mRNA level compared to C. For P treatment cultures showed an upregulation (2.4-fold) of COL2A1 mRNA expression compared to C, which was not significant according to Tukey's test. No significant difference of COL2A1 expression was found when comparing P and HPP.

For ACAN there was no significant difference observed between HPP and C. There was a significant lower ACAN expression level (decreased 2.4-fold) observed in cultures from P compared with C.

Table 3
Up- and down-regulated genes from gene card "cartilage" in HPP and P cultures compared to C.

Gene symbol	Gene name	Fold change	
		HPP vs. C	P vs. C
COL2A1	Collagen type II α 1	4.066239	3.424199
CYTL1	Cytokine-like 1	3.631163	2.91454
GDF5	Growth differentiation factor 5	2.936348	3.092956
COMP	Cartilage oligomeric matrix protein	2.064972	2.028494
MMP3	Matrix metallopeptidase 3	−5.36686	−4.72598
CHI3L2	Chitinase 3-like 2	−3.06323	−3.13046
MMP13	Matrix metallopeptidase 13 (collagenase 3)	−2.92568	−2.905
ADAMTS4	ADAM metallopeptidase with thrombospondin type 1 motif 4	−3.16154	−2.68689
MDFI	MyoD family inhibitor	−2.73354	−2.7729
CRISPLD1	Cysteine-rich secretory protein LCCL domain containing 1	−2.38318	−2.51458
COL27A1	Collagen type XXVII alpha 1	−2.26605	−2.49131
EME1	Essential meiotic structure-specific endonuclease 1	−2.32827	−1.9803

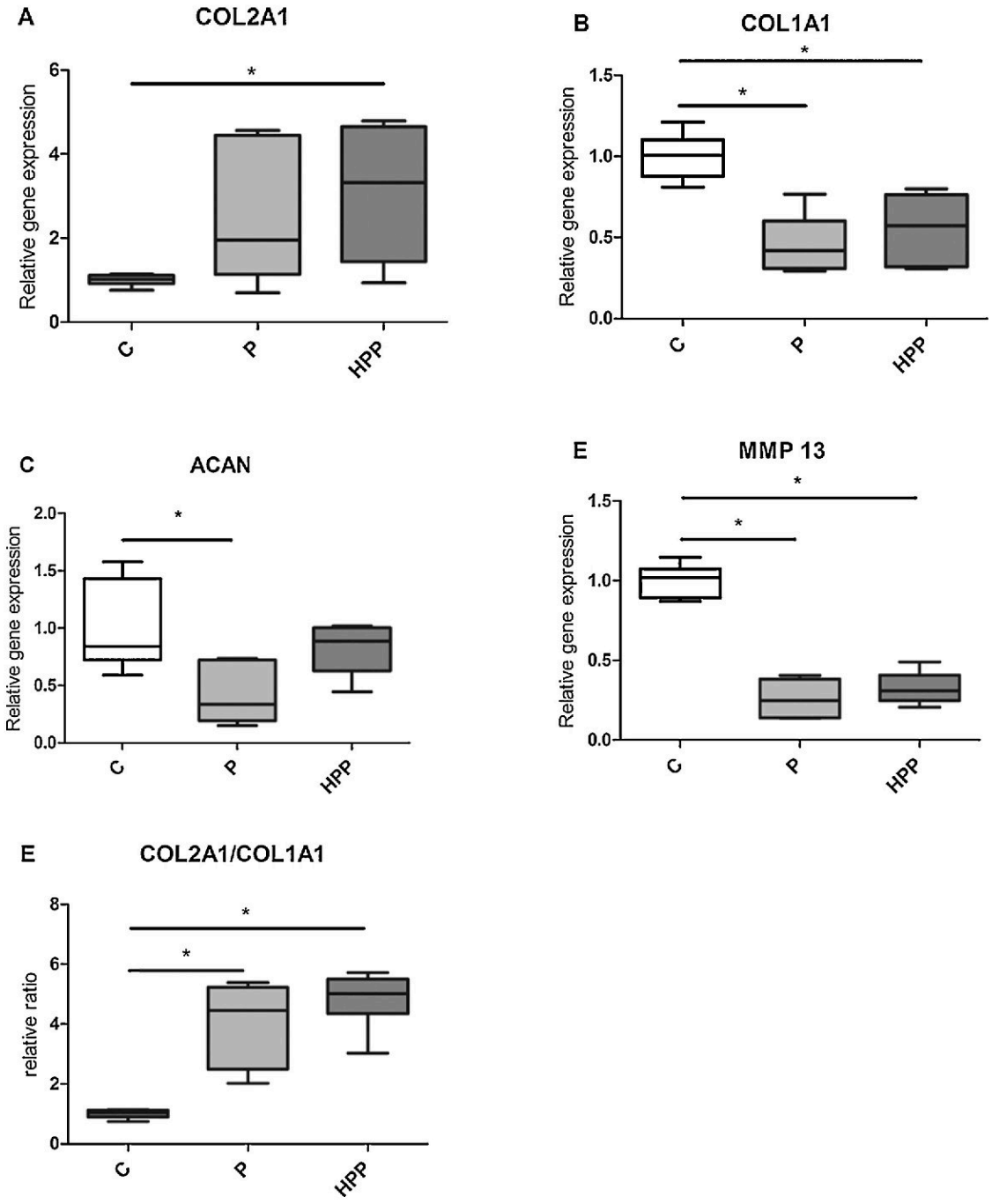


Fig. 4. Gene expression levels of (A) COL2A1, (B) COL1A1, (C) ACAN, (D) MMP 13 and (E) COL2A1/COL1A1 ratio expressed by chondrocytes cultured for 4 days in P and HPP conditions relative to controls.

COL1A1 mRNA expression levels were significantly lower for HPP (1.8-fold) and P (2.2-fold) compared with C. The COL2A1/COL1A1 quotient was significantly higher for HPP (4.8-fold) and P (4.0-fold) conditions compared with C. MMP 13 expression levels of P and HPP decreased by 3.9-fold and 3.1-fold respectively compared with C (Fig. 4).

4. Discussion

A simplified bioreactor system was constructed, which examined the effect of HPP and P versus control simultaneously. Both treatments changed the gene expression levels of human chondrocytes significantly. Sequencing illustrated similarities between the two treatments. Specifically, HPP and P increased COL2A1 expression and decreased COL1A1 expression according to sequencing and RT-PCR results. The COL2A1/COL1A1 quotient was significantly higher for HPP and P compared with C.

A full gene expression profile of human chondrocytes undergoing HPP and P was displayed. Well known cartilage genes like COMP and SOX9 were upregulated by HPP and P respectively. Furthermore, genes which have not been commonly tested in bioreactor studies were described in addition. CYTL1, which is required for the maintenance of cartilage homoeostasis (Jeon et al., 2011), was upregulated by HPP and P. GDF5, which is able to raise the expres-

sion of COL2A1, SOX9 and ACAN (Murphy et al., 2015) was also elevated by HPP and P. The sequencing results give a first insight into how both stimulations enhance cartilage differentiation.

Both treatments were also able to inhibit catabolic effects. HPP and P decreased the expression of MMP-3, MMP-13 and ADAMTS4, which play an important role in ACAN degradation and development of osteoarthritis (Song et al., 2007). In regards to the literature, changes of hydrostatic pressure have been shown to increase the expression of catabolic molecules in bovine chondrocytes on collagen gels, whereas an off-loading hydrostatic pressure reduced catabolic mRNA (Mizuno and Ogawa, 2011). The simplified bioreactor system using a static hydrostatic pressure set-up with off-loading and a high-density monolayer culture reached beneficial effects in both anabolic and catabolic processes for HPP and P. It may be assumed that the loading regime used in combination with the high cell density was combating the destructive processes in chondrocytes.

Despite of the similarities demonstrated in the clustering analysis and mRNA expression, HPP showed more beneficial effects than P regarding chondrocyte differentiation. HPP treatment maintained ACAN expression while P decreased it. When comparing HPP and P there was a significant difference in ITGA10 (Camper et al., 1998), which has been shown to interact with collagen type 2 (Durr et al., 1993) and mediates chondrocyte adhesiveness (Mitani et al., 2009). HPP treated cultures also showed a significantly higher expression of TNC, an adhesion-modulatory extracellular matrix molecule (Huang et al., 2001). This might further indicate that hydrostatic pressure promotes adhesive forces (Kim et al., 2008) also in human chondrocytes. Enhanced adhesion might be a reason why HPP resulted in stronger effects on the chondrogenic development than P.

In conclusion our results demonstrated that both HPP and P benefit cartilaginous cells but hydrostatic pressure was needed in addition to perfusion to evoke the full beneficial effects on chondrocytes. As the effects gained by adding hydrostatic pressure were not significantly superior to effects obtained by pure perfusion, the parameters of hydrostatic pressure might require further optimisation. Our findings furthermore demonstrated that sequencing can be a useful tool in exploring interrelationships in bioreactor studies. The simplified bioreactor could be a financially rewarding alternative to conventional bioreactor systems and be suitable for clinical use in expanding chondrocytes for cartilage repair.

Conflict of interest

The authors indicate no potential conflict of interests.

Author contributions

Study conception and design: G Z, S M-W, C S, M W, H B, I L, SK, JI R, A H, V J, O B, PE M.

Acquisition of data: G Z, S M-W, H B, I L, SK, JI R.

Analysis and interpretation of data: G Z, S M-W, H B, I L, SK, JI R, PE M.

Drafting of manuscript: G Z, S M-W, C S, M W, H B, I L, SK, JI R, A H, V J, O B, PE M.

Critical revision: GZ, S M-W, F H, H B, S K, JI R, BM H, V J, OB, PE M.

All authors have read and approved the final submitted manuscript.

Acknowledgements

We thank Bärbel Schmitt and Maryna Bondarava for excellent technical support and advice, Lars Schliack for sophisticated graphical assistance, Alexander Crispin (IBE Munich) for statistical advice and Christopher Johnson for revising the manuscript. This work was presented by Ge Zhu in partial fulfillment of the requirements for an MD degree, Ludwig-Maximilians-University Medical School, Munich, Germany. This work was supported by the China Scholarship Council (CSC).

References

Anders, S., Pyl, P.T., Huber, W., 2015. HTSeq–a Python framework to work with high-throughput sequencing data. Bioinformatics 31, 166–169.

Benjamin, D., Elder, P.D., Kyriacos, A., Athanasiou, P.E., 2009. Effects of temporal hydrostatic pressure on tissue-engineered bovine articular cartilage constructs. Tissue Eng.: Part A 15.

Bougault, C., Aubert-Foucher, E., Paumier, A., Perrier-Groult, E., Huot, L., Hot, D., Duterque-Coquillaud, M., Mallein-Gerin, F., 2012. Dynamic compression of chondrocyte-agarose constructs reveals new candidate mechanosensitive genes. PLoS One 7, e36964.

Camper, L., Hellman, U., Lundgren-Akerlund, E., 1998. Isolation, cloning, and sequence analysis of the integrin subunit alpha10, a beta1-associated collagen binding integrin expressed on chondrocytes. J. Biol. Chem. 273, 20383–20389.

Darling, E.M., Athanasiou, K.A., 2003. Articular cartilage bioreactors and bioprocesses. Tissue Eng. 9, 9–26.

Durr, J., Goodman, S., Potocnik, A., von der Mark, H., von der Mark, K., 1993. Localization of beta 1-integrins in human cartilage and their role in chondrocyte adhesion to collagen and fibronectin. Exp. Cell Res. 207, 235–244.

Grogan, S.P., Sovani, S., Pauli, C., Chen, J., Hartmann, A., Colwell, C.W. Jr., Lotz, M.K., D'Lima, D.D., 2012. Effects of perfusion and dynamic loading on human neocartilage formation in alginate hydrogels. Tissue Eng. Part A 18, 1784–1792.

Hong, H., Park, Y.K., Choi, M.S., Ryu, N.H., Song, D.K., Suh, S.I., Nam, K.Y., Park, G.Y., Jang, B.C., 2009. Differential down-regulation of COX-2 and MMP-13 in human skin fibroblasts by glucosamine-hydrochloride. J. Dermatol. Sci. 56, 43–50.

Huang, W., Chiquet-Ehrismann, R., Moyano, J.V., Garcia-Pardo, A., Orend, G., 2001. Interference of tenascin-C with syndecan-4 binding to fibronectin blocks cell adhesion and stimulates tumor cell proliferation. Cancer Res. 61, 8586–8594.

Jeon, J., Oh, H., Lee, G., Ryu, J.H., Rhee, J., Kim, J.H., Chung, K.H., Song, W.K., Chun, C.H., Chun, J.S., 2011. Cytokine-like 1 knock-out mice (Cytl1-/-) show normal cartilage and bone development but exhibit augmented osteoarthritic cartilage destruction. J. Biol. Chem. 286, 27206–27213.

Kim, D., Pertea, G., Trapnell, C., Pimentel, H., Kelley, R., Salzberg, S.L., 2013. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol. 14, R36.

Kim, Y.J., Park, S., Lee, Y.J., Shin, J.W., Kim, D.H., Heo, S.J., Park, K.D., Shin, J.W., 2008. Effects of intermittent hydrostatic pressure on cell adhesive forces and other related parameters under various resting periods. J. Biomed. Mater. Res. Part B, Appl. Biomater. 85, 353–360.

Love, M.I., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 550.

Madeira, C., Santhagunam, A., Salgueiro, J.B., Cabral, J.M., 2015. Advanced cell therapies for articular cartilage regeneration. Trends Biotechnol. 33, 35–42.

Mitani, G., Sato, M., Lee, J.I., Kaneshiro, N., Ishihara, M., Ota, N., Kokubo, M., Sakai, H., Kikuchi, T., Mochida, J., 2009. The properties of bioengineered chondrocyte sheets for cartilage regeneration. BMC Biotechnol. 9, 17.

Mizuno, S., Ogawa, R., 2011. Using changes in hydrostatic and osmotic pressure to manipulate metabolic function in chondrocytes. Am. J. Physiol.-Cell Physiol. 300, C1234–C1245.

Murphy, M.K., Huey, D.J., Hu, J.C., Athanasiou, K.A., 2015. TGF-beta1, GDF-5, and BMP-2 stimulation induces chondrogenesis in expanded human articular chondrocytes and marrow-derived stromal cells. Stem Cells 33, 762–773.

Neidlinger-Wilke, C., Wurtz, K., Liedert, A., Schmidt, C., Borm, W., Ignatius, A., Wilke, H.J., Claes, L., 2005. A three-dimensional collagen matrix as a suitable culture system for the comparison of cyclic strain and hydrostatic pressure effects on intervertebral disc cells. J. Neurosurg. Spine 2, 457–465.

Responte, D.J., Lee, J.K., Hu, J.C., Athanasiou, K.A., 2012. Biomechanics-driven chondrogenesis: from embryo to adult. FASEB J. 26, 3614–3624.

Schröder, C., Hölzer, A., Zhu, G., Woiczinski, M., Betz, O.B., Graf, H., Mayer-Wagner, S., Müller, P.E., 2015. A closed loop perfusion bioreactor for dynamic hydrostatic pressure loading and long term cartilage tissue engineering. J. Mech. Med. Biol. http://www.worldscientific.com/doi/abs/10.1142/S0219519416500251?

Smith, R.L., Lindsey, D.P., Dhulipala, L., Harris, A.H., Goodman, S.B., Maloney, W.J., 2011. Effects of intermittent hydrostatic pressure and BMP-2 on osteoarthritic human chondrocyte metabolism in vitro. J. Orthop. Res. 29, 361–368.

Song, R.H., Tortorella, M.D., Malfait, A.M., Alston, J.T., Yang, Z., Arner, E.C., Griggs, D.W., 2007. Aggrecan degradation in human articular cartilage explants is mediated by both ADAMTS-4 and ADAMTS-5. Arthritis Rheum. 56, 575–585.

Tran, S.C., Cooley, A.J., Elder, S.H., 2011. Effect of a mechanical stimulation bioreactor on tissue engineered, scaffold-free cartilage. Biotechnol. Bioeng. 108, 1421–1429.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR

data by geometric averaging of multiple internal control genes. Genome Biol. 3.

Varas, L., Ohlsson, L.B., Honeth, G., Olsson, A., Bengtsson, T., Wiberg, C., Bockermann, R., Jarnum, S., Richter, J., Pennington, D., Johnstone, B., Lundgren-Akerlund, E., Kjellman, C., 2007. Alpha 10 integrin expression is up-regulated on fibroblast growth factor-2-treated mesenchymal stem cells with improved chondrogenic differentiation potential. Stem Cells Dev. 16, 965–978.

von der Mark, K., Gauss, V., von der Mark, H., Muller, P., 1977. Relationship between cell shape and type of collagen synthesised as chondrocytes lose their cartilage phenotype in culture. Nature 267, 531–532.

Yu, L., Ferlin, K.M., Nguyen, B.N., Fisher, J.P., 2014. Tubular perfusion system for chondrocyte culture and superficial zone protein expression. J. Biomed. Mater. Res. Part A.

Summary

Articular cartilage has poor self-repairing capacity. Hence tissue engineering is regarded as a promising approach for cartilage regeneration. Hydrostatic pressure (HP) and/or perfusion (P) have been widely used in tissue engineering studies to increase the chondrogenic activity of chondrocytes. The objective of this study was to establish a hydrostatic pressure bioreactor which permits perfusion and in a second step, to investigate the effect of HP and P on human chondrocytes.

There were various types of bioreactors used in previous studies, although only a few included a perfusion system. The absence of a perfusion system causes frequent manual intervention but bioreactors with perfusion systems were considered to be too complex. Therefore, a simple design for a bioreactor which allows applying HP and P was necessary for further studies. For HP studies involving a perfusion system, the effect of perfusion has been neglected. When applied simultaneously the individual effect of HP and P was still unknown. In this study, a novel bioreactor was established. With this system, HPP and P were applied on human chondrocytes respectively. Complete gene profile was generated by RNA-sequencing.

The bioreactor was validated by finite element model, computational fluid dynamics and cell proliferation test. The results showed that the bioreactor could provide dynamic HP of 0.1 Mpa, 0.001-0.1 Hz by controlling the valves and pump by a computer. Furthermore, the bioreactor and stimuli did not affect proliferation of the chondrocytes. HPP and P were applied on human chondrocytes utilizing the bioreactor system mentioned above. A complete gene profile was generated by RNA-sequencing. Highly relevant biomarkers such as COL2A1, COL1A1, ACAN and MMP 13 were examined by real-time PCR. Both treatments showed a large variation to the control group, but a relatively high similarity between the treatments (P and HPP). HPP and P increased COL2A1 expression and decreased COL1A1, Both treatments were also able to inhibit catabolic effects. HPP and P decreased the expression of MMP-3, MMP-13 and ADAMTS4. HPP treatment maintained ACAN expression while P decreased it. When compared with P, HPP also increased expression of ITGA10 and TNC which are involved chondrocyte adhesiveness.

Taken together, both HPP and P benefit cartilaginous cells but hydrostatic pressure was needed in addition to perfusion to evoke the full beneficial effects on chondrocytes. As the effects gained by adding hydrostatic pressure were not significantly superior to effects obtained by pure perfusion, the parameters of hydrostatic pressure might require further optimization. Our findings furthermore demonstrated that sequencing can be a useful tool

in exploring interrelationships in bioreactor studies. The simplified bioreactor could be a financially rewarding alternative to conventional bioreactor systems and be suitable for clinical use in expanding chondrocytes for cartilage repair without causing dedifferentiation of the expanded cartilage cells. This would resolve a mayor problem of current autologous chondrocyte transplantation (ACT) treatments.

Zusammenfassung

Gelenkknorpel besitzt ein ungenügendes Selbstheilungsvermögen. Daher werden Methoden des Tissue Engineerings als vielversprechender Heilungsansatz gesehen. Dabei wird hydrostatischer Druck (HP) und/oder Perfusion (P) häufig in Tissue Engineering Studien eingesetzt, um die chondrogene Aktivität von Chondrozyten zu erhöhen.

Das Ziel dieser Studie war es einen neuartigen Hydrostatikdruck-Bioreaktor zu etablieren, der zusätzlich die Perfusion der Zellen zulässt um in einem zweiten Schritt den Effekt von sowohl hydrostatischem Druck als auch der Perfusion bzw. deren Kombination zu untersuchen.

Es wurden verschiedenste Bioreaktoren in bisherigen Studien eingesetzt, allerdings nur eine kleine Anzahl besitzen zusätzlich eine Perfusionssystem. Das Fehlen eines Perfusionssystems erfordert häufiges, manuelles eingreifen. Dies ist aufwändig und erhöht die Gefahr von Kontaminierung und Anwendungsfehlern. Jedoch wurden Bioreaktoren mit Perfusionssystemen als zu Komplex angesehen. Daher war es notwendig, einen einfach designten Bioreaktor zu entwickeln, welcher sowohl die Belastung mit hydrostatischem Druck als auch einen Perfusion zulässt. Außerdem wurde bisher in Studien mit hydrostatischem Druck und einem Perfusionssystem der Einfluss der Perfusion vernachlässigt. Daher gab es keine Angaben zum Einfluss der Perfusion. In den hier beschriebenen Studien wurde aus diesen Gründen ein neuartiger Bioreaktor konstruiert. Mit diesem System wurde dann der Einfluss von HP bzw. P auf Knorpelzellen untersucht und unter anderem umfangreiches Genprofil der so stimulierten Knorpelzellen mit Hilfe von RNA-Sequenzing erstellt. Zuerst wurde der Bioreaktor wurde über ein Finite Elemente Model und eine numerische Stömungsberechnung charakterisiert sowie ein Proliferationstest (WST-1 – Test) durchgeführt. Die Ergebnisse zeigen, dass der Bioreaktor mit einem dynamischen hydrostatischen Druck von 0,1 Mpa bei einer Frequenz von 0,001-0,1 Hz betrieben werden kann, wobei die Ventile und die Pumpe von einem Computer gesteuert werden. Dies hatte auserdem keinen negativen Einfluss auf die Proliferation der im Bioreaktor kultivierten Chondrozyten. HPP und P wurden in einem zweiten Experiment auf humanen Knorpelzellen appliziert. Danach wurde ein umfangreiches Genprofil mittels RNA-Sequencing erstellt. Hochrelevante Biomarker wie Collagen2A1, Collagen1A1, Agrecan, und MMP 13 wurden mit Hilfe von real-time PCR untersucht. Beide Stimuli zeigten große Unterschiede zur Kontroll-Gruppe aber keine großen Unterschiede zwischen den Stimuli P und HPP. Beide Stimuli hatten eine Erhöhung der COL2A1 und eine Erniedrigung der COL 1A1 Expression zur Folge.

Damit unterdrückten beide Stimuli einen katabolen Effekt. HPP und P verminderten weiterhin die Expression von MMP-3, MMP-13 und ADAMTS4. HPP wirkte erhaltend auf die ACAN Expression während nach Perfusion allein diese vermindert war. Im Vergleich zu P war bei HPP die Expression von ITGA10 und TNC, beides beteiligt an der Chondrozyten Adhäsion, erhöht.

Zusammengefasst hat sowohl HPP als auch P einen positiven Effekt auf den Erhalt der chondrogenen Eigenschaften von kultivierten Chondrozyten wobei der Effekt der Kombination von hydrostatischem Druck und Perfusion einen zusätzlichen Vorteil gegenüber der Perfusion zeigte. Allerdings konnte in dieser Studie mit den untersuchten Parametern keine signifikanten Unterschiede gezeigt werden. Daher sollte der Parameter Hydrostatischer Druck in nachfolgenden Untersuchungen weiter optimiert werden. Unsere Ergebnisse zeigen weiterhin, dass das Erstellen eines Genprofils mittels RNA-Sequencing ein nützliches Instrument bei der Untersuchung der Zusammenhänge der Parameter bei Untersuchen von Bioreaktor-Studien sein kann. Außerdem zeigt diese Studie, dass ein Bioreaktor mit vereinfachtem Design eine attraktive Alternative gegenüber konventionellen Bioreaktoren oder Kultivierungsmethoden sein kann, um als klinische Methode zur Expansion von Chondrocyten zur autologen Chondrozyten Transplantation (ACT) eingesetzt zu werden, ohne dass die expandierten Zellen Ihre chondrogenen Eigenschaften verlieren. Dies würde ein signifikantes Problem der gegenwärtig angewandten ACT lösen.

Acknowledgement

I would like to thank Prof. Dr. med. Peter E. Müller for giving me this precious opportunity to work in the Department of orthopedics, Klinikum Großhadern as a doctoral student. My thanks also go to Dr. Oliver Betz, who is always supportive of me throughout my time in Munich. He provides me lots of help and advices both in my academic and daily life. I cannot make it without his supervision on my thesis and publications. Additionally, I would like to thank Dr. Susanne Mayer-Wagner, who is very encouraging and caring. She offered me lots of interesting work experiences. I will not have my publication without her supports.

I am especially thankful for all of the support and help I have received form current and previous members of the lab for biomechanics. I especially would like to thank Miss Bärbel Schmitt, who taught me all the basic experiment techniques and is always available to help. I would like to thank Dr. Maryna Bondarava for her help in my cell culture. I appreciate her rigorous attitude of science. My additional thanks go to Miss Julia Redeker, who help me with RNA extraction and RT-PCR. I would like to thank Dr. Christian Schröder and Mr. Matthias Woiczinski for their works on the bioreactor system. I'm grateful to Mr. Michael Kraxenberger for IT support and useful software.

Finally, I would like to thank my family for their unending support and love. I would like to thank my girlfriend, Dr. Shu Zhou, for her support and company.

My work in LMU was supported by scholarship China Scholarship council.

Curriculum vitae

Ge Zhu
Birth Date:01/11/1986 Nationality：Chinese
Telephone：13476012021 027-67813585
E-mail：ZHU.ge861101@gmail.com
Address：Department of orthopedic 5, Zhongnan Hospital of Wuhan University, 430071 Wuhan, CHINA

Research Interest

- Biomechanics
- Joint surgery(Arthroplasty and Arthroscopy)

Language Skills

- Chinese: mother tongue
- English: competent command(IELTS:6.5)

Education

9/2012-date **Ludwig-Maximilians-University of Munich**

09/2005-6/2012 **Medical College of Wuhan University Wuhan, CHINA**
Degree: Bachelor & Master (combined Bachelor's/ Master's Degree Program)
Subject: Clinical Medicine, Orthopedic.
Expected date of Graduation: 30/06/2012

09/2006-05/2007 **Central China Normal University Wuhan, CHINA**
Second Degree: Bachelor's Degree of Psychology

Practice Experience

10/2010-6/2012 **Department of orthopedic（Joint Surgery）, Hospital of Wuhan University, CHINA**
Extern Specialty medical practice, in-patient department and OPD.

09/2009-09/2010 **Hospital of Wuhan University, CHINA**
Extern medical practice in 18 departments, including neurology, imageology, Emergency, Internal Medicine, Surgery.

07/2006 **Province of Yunnan, CHINA**
Summer vacation social practice in group: Investigation of conditions of country doctor in poor area of southwest China.

Research Experience

04/2010-date **Department of orthopedic 5, Hospital of Wuhan University, CHINA**
Treatment of recurrent patellar dislocation by lateral release and medial reefing with arthroscopy；
Symptomatic intraarticular ganglia of the cruciate ligaments of the knee.
Articles expected to be published.

skills

- **Academic** Solid basic knowledge of musculoskeletal system anatomy and disease, certain clinic experience, especially in Arthroplasty and Arthroscopy. Epidemiology, Statistics. preclinical medicine knowledge and basic experimental skills in Pathology, Biochemistry, Molecular Biology ect.
- **Practical** Basic surgery technique of Trauma, Arthroscopy and Total Knee/Hip Replacement. Common experimental technique(Biochemistry, Physiology, Molecular Biology, Mechanics)
- **Computer literacy** Proficient in Microsoft Office; familiar with SPSS.
- **Hobby** Badminton, Basketball.

Awards

Excellent academic scholarship 3rd class of Wuhan University,2005-2006 and 2010-2011 academic year.
Title of Outstanding student cadres OF SPORTS, 2005-2006 and 2006-2007 academic year

Publications

Zhu G, Mayer-Wagner S, Schröder C, et al. Comparing effects of perfusion and hydrostatic pressure on gene profiles of human chondrocyte. **J Biotechnol.** 2015 Sep 20;210:59-65 **(IF 2.87)**
Schröder C, HÖLZER A, **Zhu G**, et al. A CLOSED LOOP PERFUSION BIOREACTORFOR DYNAMIC HYDROSTATIC PRESSURE LOADINGAND CARTILAGE TISSUE ENGINEERING. **Journal of Mechanics in Medicine and Biology.** 2015, Vol. 16, No. 2 **(IF 0.8)**

Eidesstattliche Versicherung und Erklärung

Zhu, Ge

Name, Vorname

Ich erkläre hiermit an Eides statt,
dass ich die vorliegende Dissertation mit dem Thema

„A novel hydrostatic pressure bioreactor for cartilage regeneration."

selbständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und
alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als
solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle
einzeln nachgewiesen habe.

Ich erkläre des Weiteren, dass die hier vorgelegte Dissertation nicht in gleicher oder in
ähnlicher Form bei einer anderen Stelle zur Erlangung eines akademischen Grades
eingereicht wurde.

Ort, Datum
Munich, 19.4.2016

Unterschrift Doktorandin/Doktorand