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Initial study on removing cellular residues from hydrostatic high-pressure treated allogeneic tissue using ultrasound

Abstract: Hydrostatic high-pressure technology (HHD) devitalizes tissue quickly and gently, without negatively affecting the structural properties. HHD-treated tissues must be cleaned from devitalized cells. A partially automated, gentle, reproducible and timesaving rinsing test setup utilizing ultrasound is demonstrated in this study. The test setup is used to clean HHD-treated bone allografts of tissue residues and prevent microbiological contamination. A rinsing procedure is investigated. Residual DNA content determination is utilized to analyze cleaned bone allograft tissue for rinsing procedure evaluation.

Keywords: HHD, allograft, cell residue, decellularization, rinsing device, ultrasound, test setup, bone tissue

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1 Introduction

The regeneration capacity of the human body for large tissue defects is limited. Such large tissue defects can have a multitude of causes such as tumour diseases, trauma, infections, diabetic complications, chronic inflammations or surgical interventions. The missing tissue must be replaced with adequate structures, which adopt its form and functionality. Autograft (endogenous) tissue substitution is immunologically identical to the missing tissue. However, it is associated with extensive surgery and stress for the patient, because of donor site morbidity. Therefore, allogenic (exogenous) tissue substitution (allograft) is becoming increasingly important. However, allografts can lead to an extensive immune response and inflammation [1], which should be prevented.

The hydrostatic high-pressure treatment (HHD) allows a fast and gentle devitalization of allografts of various tissues to reduce the immune response of the allograft recipient after transplantation while keeping the structural properties of the tissue intact [2]. Nevertheless, HHD-treated tissue must be cleaned from devitalized cells (decellularization) via an adequate and gentle rinsing process.

For this purpose, a novel, semi-automatic, gentle, reproducible and timesaving rinsing test setup is demonstrated in this study. Here, the test setup is used for the decellularization of HHD-treated bone allografts. The ultrasound test setup is to be used in a cell lab's laminar flow cabinet.

2 Methods

2.1 Hydrostatic high-pressure treatment (HHD) of bone tissue samples

The HHD setup as shown in [2] is utilized in this study. Therefore, bone cylinders (Ø diameter = 7 mm, Ø length = 10 mm), harvested from the distal epiphysis of femora from tissue donors (Ethics Proposal Number: A2016-0083) were HHD-treated at 300 MPa for 10 min. After the

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HHD-treatment half of the bone tissue samples were decellularized using the rinsing test setup.

2.2 Ultrasound test setup

The rinsing test setup uses ultrasound as mechanic rinsing principle. Therefore, an ultrasound processor (Figure 1) vibrates at 24 kHz. A connected sonotrode transfers the vibration into a fluid such as water or an aqueous solution where alternating high-pressure and low-pressure waves are generated. Since the fluid pressure falls below its vapour pressure gas bubbles are generated. During the subsequent high-pressure wave these bubbles collapse again which causes micro-jets, the so-called cavitation [3]. If cavitation occurs near surfaces it erodes material from the surface. In this study the cavitation caused by ultrasound is supposed to erode cell residue from the allograft.

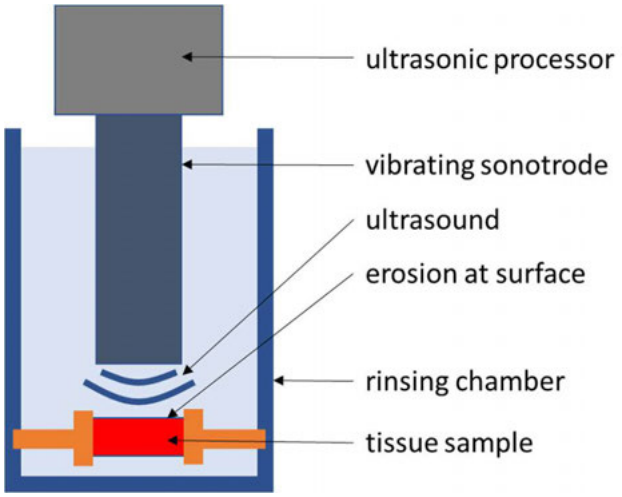

Figure 1: Principle of ultrasonic decellularization

The ultrasound test setup (Figure 2) consists of the Ultrasonic Homogenizer UP200S (Hielscher Group, Germany) and a 3D-printed holder for a CELLSTAR 50 ml polypropylene centrifugation tube (Greiner Bio-One, Austria). The tube can be positioned vertically by a SAW-1080 linear stage (IGUS GmbH, Germany) that is powered by a NEMA23 stepper motor (Nanotec Electronic GmbH & Co. KG, Germany) at 48V and is controlled by a SMCI47-S (Nanotec Electronic GmbH & Co. KG, Germany) motor control. These components are mounted on 20 mm aluminium profiles (MayTec GmbH, Germany).

The centrifugation tube holder is 3D-printed from titanium alloy on an ARCAM A1 Selective electron beam melting machine (Arcam AB, Sweden). The thread of the original screw cap of the centrifugation tube has been integrated into the centrifugation tube holder design. It enables quick and easy mounting and switching of single-use centrifugation tubes in a reproducible, fix position. After printing, the centrifugation tube holder has been plasma polished (Plasotec, Germany) to reduce the roughness of the surface and increase the functionality of the thread for an optimal surface cleaning and smooth mounting of the tube.

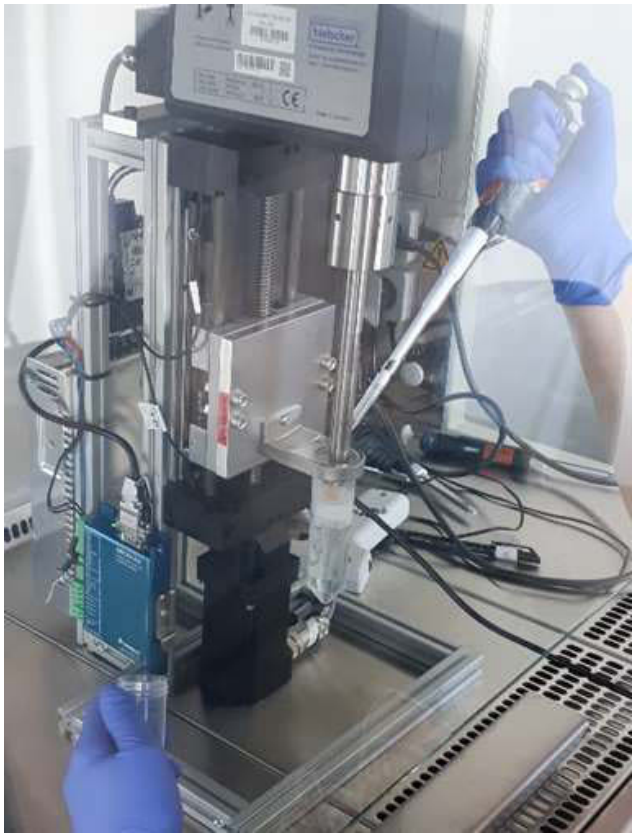

Figure 2: Ultrasound test setup with the ultrasonic processor and the 14 mm sonotrode reaching into the 50 ml centrifugation tube that is mounted at the centrifugation tube holder

2.3 Rinsing procedure parameters

The sonotrode UP200S S14 (Hielscher Group, Germany) with a diameter of 14 mm has been used to decellularize the tissue. To perform a rinsing procedure, the sonotrode is dipped into the PBS filled centrifugation tube via automated positioning (see section 2.2). It is positioned 10 mm above the bone tissue sample in the tube. An amplitude of 50% and a cycle 0.5 has been set. Rinsing procedure was performed for $t = 2$ min at a temperature of $T = 21$ °C.

Since the bone tissue, a 7 mm cylinder, is not fixed at the bottom of the centrifugation tube it turns continuously during the ultrasonic treatment of 2 min so that all sides of the sample are treated equally.

2.4 Determination of residual DNA content

To evaluate the efficiency of the rinsing procedure, the residual DNA content of the bone tissue samples was determined. For that, the bone tissue samples were stored at T = -20 °C for at least 12 h. Afterwards, the initial weight of the bone tissue samples has been determined and they were freeze-dried to an average weight loss of 60 %. To granulate the samples, they were treated with mortar and pistol under the influence of liquid nitrogen. After that, the granules were incubated with a mixture of water, solid tissue buffer and proteinase K (provided by the Quick-DNA™ Midiprep Plus Kit, Zymo Research, Irvine, CA, USA) for 3 hours at 55 °C. Afterwards, DNA was isolated as per protocol.

The DNA content was measured with a Tecan-Reader Infinite®200 Pro (Maennedorf, Switzerland) with an absorption of 260 nm and a reference of 280 nm. To evaluate rinsing procedure, three different bone tissue treatments will be compared via DNA content determination (n = 3 for each):

(1) untreated bone tissue samples (control)
(2) bone tissue samples treated with ultrasound
(3) bone tissue samples treated with HDD and ultrasound

3 Results

The ultrasound test setup was utilized successfully (Figure 3). Design-features as tube positioning via linear stage enable a reproducible tissue sample treatment and an easy and fast usage. Moreover, the ultrasound test setup is designed to be adequately and easily cleaned.

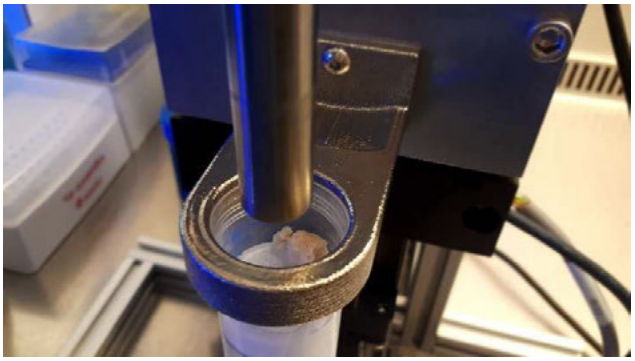

Figure 3: Centrifugation tube holder with integrated thread and a mounted 50 ml centrifugation tube with a decellularized bone sample after ultrasound treatment.

For rinsing performance analysis, the residual DNA content for treated bone tissue samples (n=3) (ultrasound and HHD + ultrasound) and the control sample (untreated bone tissue) is shown in Figure 4.

The median of the control sample is at about 73 µg DNA per g tissue while the median of the ultrasound samples is at about 61 µg DNA per g tissue and the median of the HHD + ultrasound samples is at about 57 µg DNA per g tissue. The residual DNA content is reduced about 16% from the control sample to the ultrasound treated tissue and 21 % from the control sample to the HHD + ultrasound treated tissue. Although both treatments decrease the residual DNA content significantly, they do not decrease it below 50 µg DNA per g tissue, as it is desired and described in the literature [4,5].

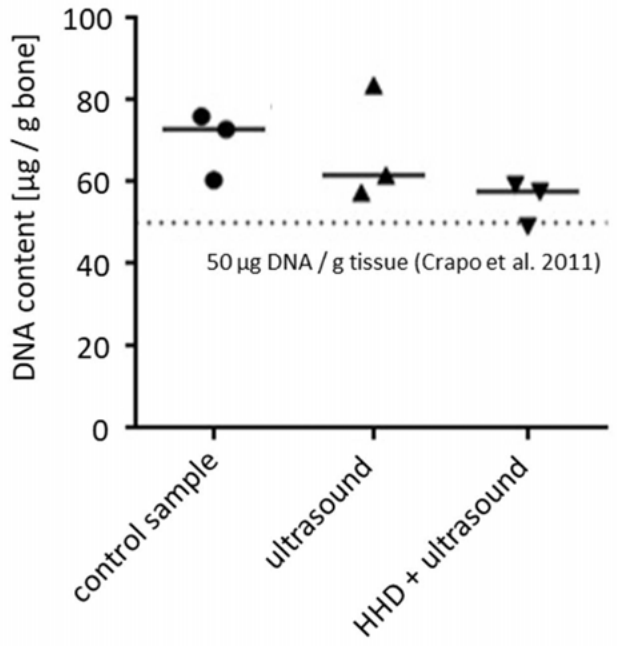

Figure 4: Residual DNA content for control sample (left), ultrasound treated tissue (middle) and HHD + ultrasound treated tissue (right). While there is a significant decrease in DNA content of about 16 % for ultrasound and 21 % for HHD + ultrasound the goal of 50 µg DNA / g tissue is not reached yet.

4 Conclusion

The ultrasound-based test setup enables a rinsing procedure for the decellularization of bone allografts. As result of the rinsing-treatment, there was a reduction of residual DNA content for HHD-treated bone tissue as well as bone tissue without HHD-treatment before the rinsing procedure. While the decrease in residual DNA content is significant, it is planned to be decreased even more.

In further studies, aiming for more efficiency of decellularization, it is planned to increase the amplitude of the ultrasound to the maximum of 100%, increase the cycle from 0,5 to 1 and increase the duration of the experiment from 2 min up to 5 min.

Author Statement

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