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Ultrasonic treatment for decellularisation of cartilage and dura mater

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Abstract
Due to limited availability of autografts for tissue reconstruction, allografts have been increasingly used in recent years. The importance of regenerative medicine demands improvements of decellularisation methods to process allogeneic materials. Different chemical, enzymatic, and physical methods intend to remove allogeneic antigens while retaining the complex ultrastructure of the extracellular matrix. The chemical treatment, whereby important molecules such as glycosaminoglycans dissociate from collagenous tissues and chemical residues remain in the tissue, can compromise the biological response of these materials and the successful tissue transplantation. Physical methods such as snap freezing, sonication or direct pressure systems are effective but can also lead to disrupted or fractured extracellular matrix by the treatment. Hence, further research regarding tissue specific intensities, process time and depth of penetration are necessary. In order to create new perspectives for the decellularisation of allografts from supporting and connective tissue, the aim of this preliminary study was to investigate the effects of ultrasonic treatment with different frequencies and intensities in a temperature-controlled ultrasonic bath. The initial results show an antigene reduction by removing cells and DNA from the porcine dura mater and cartilage treated with ultrasound. However, results are limited because of the biological fluctuations and the small sample size. Hence, further investigations have to be carried out to investigate the impact of ultrasound treatment on allograft decellularisation.

Keywords: Ultrasonic treatment, decellularisation, allograft transplants

1. Introduction

Malformations, traumas and cancer of the head and musculoskeletal system cause severe tissue defects, which require reconstruction. Alloplastic (synthetic), autogeneic (from the same individual) and allogeneic (from another individual) tissues are used for surgical reconstruction. All materials have specific limitations: 1) Alloplastic materials suffer from foreign body reactions and extrusion. 2) The availability of autogenic materials is limited and the harvest causes donor side morbidity. 3) Allogeneic materials need expensive processing to eliminate donor antigens to prevent a severe immune reaction after transplantation. Allografts can be devitalised using various methods such as irradiation or high-pressure treatment. After devitalisation, cells, cellular components and genetic material of the donor must be removed from the tissue while preserving the extracellular matrix (ECM). Decellularisation (DC) is achieved by chemical, enzymatic or physical methods. Enzymatic and chemical methods are most frequently used. However, they cause certain disadvantages such as possible toxicity from remaining residues of the DC solution or destruction of the glycosaminoglycans (GAGs) or collagen, which can result to an extensive immune response and inflammation [1]. Regarding current DC methods, a systematic analysis and evaluation of different methods is still required in order to provide more effective DC protocols [2]. Within the research project HOGEMA, various physical DC methods are examined, as these may offer an alternative to the currently used methods. The aim of our preliminary study was to investigate the effects of the application of ultrasonic treatment with different frequencies and intensities in a temperature-controlled ultrasonic bath.

2. Allogeneic transplants and decellularisation

Allogeneic transplants, which are taken either from living and deceased donors, must be cleaned from cellular components without damaging the ECM before they can be used as tissue replacement materials. One of the main issues with using allografts is the immune response to the major histocompatibility complex (MHC). MHC proteins are expressed on the cell surface and induce an immune reaction against foreign tissues/cells. Therefore, it is very important to remove cellular and MHC components completely during decellularisation, while retaining the complex infrastructure of the extracellular matrix [3, 4]. For this, a variety of decellularisation methods, which are characterised by chemical, enzymatic or physical treatment, have become established in regenerative medicine. Depending on the tissue, these DC methods are used individually or combined to fulfil two tasks: a cell lysis and cell removal from the ECM [4].

2.1. Chemical decellularisation

For chemical DC, detergents or surfactants are used to dissolve (solubilisation) membrane-bound proteins, followed by cell lysis, and removal of the cellular components from the tissue. Because of these advantages chemical DC methods are currently widely used in regenerative medicine [1, 5, 6]. Despite this, there are also disadvantages such as the dissociation of important matrix molecules such as GAGs and collagen as well as chemical residues in the tissue that can compromise the biological response of the allograft material. In the case of dense tissue there are limitations to achieving a homogeneous distribution of

the detergent, since the concentration gradient decreases from the surface of the tissue to the centre [1].

2.2. Enzymatic decellularisation

The enzymatic decellularisation for example with trypsin, causes the peptide bonds on the carbon side of arginine and lysine to split, nucleases and exonucleases catalyse the hydrolysis in the interior or terminal bonds of the ribonucleotide or deoxyribonucleotide chains, which leads to the degradation of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) [5]. Mostly enzymatic methods were combined with other DC methods, to obtain effective removal of DNA without causing significant damages to the ECM. Many studies have shown that cellular components have been removed, but prolonged exposure can destroy the ECM structure. Furthermore, residues of the enzymatic solutions are difficult to wash out and can cause immune responses [6].

2.3. Physical decellularisation

An alternative, as it leads to a homogeneous distribution of the forces within the tissue and therefore depends less on the penetration efficiency of the decellularisation solutions, physical DC methods were also applied in regenerative medicine. These methods use snap freezing, direct pressure, sonication and agitation to disrupt the cell membrane and cells, whereby cell contents and residues are released into the ECM and have to flushed out by further washing processes. By using snap freezing cells are killed and cell membranes are destroyed by intracellular ice crystals, which in turn causes cell lysis. This technique, which is often used to decellularise tendons, ligaments and nerve tissue requires a subsequent wash cycle to remove the cell debris. Confident temperature monitoring is needed to prevent thermal ECM damage [7]. Cell lysis by direct pressure is limited to tissues without dense ECM [8]. Mechanical agitation can be performed with a magnetic stir plate, orbital shaker or low profile roller but there is a lack of studies on cycle times, intensity, and optimal size. The same applies to ultrasonic treatment, which is sometimes presented in the literature as a capable DC method in combination with other methods. During the process, tissues or organs are sonicated with a sonotrode or an ultrasonic bath, whereby cavitation and its effects are responsible for decellularisation. When ultrasound acts on liquid media, strong alternating stress creates small cavitation bubbles. Under the influence of the external pressure of the medium, the unstable bubbles implode after brief growth with high pressure and temperature peaks, creating high shear forces at the boundary layer [9]. An effective ultrasonic cleaning process depends on parameters such as intensity, temperature, viscosity and the dissolved gas in the liquid as well as the position of the sample in the ultrasonic field [10, 11]. Azhim et al. [11] and Syazwani et al. [12] reported that under optimal fluid conditions, low frequency sonication treatment in 2 % SDS without saline can thus produce decellularised blood vessels for tissue engineering. Ingram et al. [13] reported a decellularised porcine patella tendon scaffold treated with ultrasound (P = 360 W, pulse time of t_p = 1 s for a total of t_t = 1 min) using 0.1 % SDS. This leads to a mechanical easing of collagen structures within the scaffold, which in turn fosters the seeding of new cells. Another study by Starnecker et al. [2] showed that the combination of shaking, whirling and sonication (P = 120 W, f = 45 kHz) treatment followed by ten washing cycles in phosphate buffered saline (PBS) as an effective DC method with achieved penetration depths of x_d = 800 µm. An ultrasonic treatment in combination with detergent has a DC effect as it supports the penetration of the decellularisation material into the cartilage. This improves the speed of the decellularisation process while it has no significant defect on the structure of the tissue by soft treatment [14]. Chemical and enzymatic DC methods have their limitation in terms of potential toxicity caused by the presence of residual decellularising agents due to the chemical concentration gradient and the destruction of ECM proteins, which also leads to a significant ECM change. Regarding to ultrasonic treatment as a decellularisation method, there is a lack of studies on intensity, temperature, frequencies and treatment times as well as different tissues [6], although the method shows potential for DC. A few selected current studies on ultrasonic decellularisation are summarised in table 1.

Table 1. Summary of some currently used decellularisation techniques, - negative effect, + positive effect.

Physical DC	Tissue	Outcome
Direct sonication and ultraviolet light	small intestines	- + increase in interfibril spaces - disrupted ECM structure [15]
Sonication (P = 360 W) and detergens (SDS 0.1 %)	porcine patella tendons	+ no reduction in collagen and GAG content + good decellularisation [13]
Shaking, whirling, direct sonication (P = 120 W, f = 45 kHz) and detergents	aortic walls	+ nuclei were not detectable - heavy ultrasonic cavitation could damage the ECM fiber network [2]
Freeze-drying, washing in PBS and direct sonication (P = 180 W, f = 37 kHz)	larynx	+ good cellular removel + complete decellularisation by combination of DC methods - structural damage to the scaffold [16]
Direct sonication (P = 200 W, f = 24 kHz) in PBS for t = 2 min	bone	+ DNA content decrease + direct sonication is suitable for DZ [17]
Direct sonication (P = 15 W, f = 40 kHz) in SDS (0.1 %) for t_t = 10 h	meniscus	+ sonication treatment did not affect ECM properties + complete nuclei removal + preservation of collagen and GAG structure [18]
Direct sonication (P = 80 W, f = 12 kHz and f = 24 kHz), ulltrasonic bath (P = 170 W, f = 42 kHz) and detergens (SDS, 0.1 % and 1 %)	articular cartilage	+ ultrasonic bath (t_t = 5 h) significantly decreased the cell nucleus residue + GAG and collagen were maintained in ECM structure + good penetration of the DC material into the cartilage + speed of DC increased - disruped the ECM [15]
Direct sonication (P = 15 W and P = 30 W, f = 20 kHz)	aorta	+ low frequency sonication is capable to completely DC + effectivity of DC incease by positioning at an optimal ultrasonic pressure fields + increase cellular removal - heavy treatment can dis-rupting cell membranes [11]
Direct sonication (P = 15 W, f = 20 kHz) in 2 % SDS without saline for 3 h ≤ t_t ≤ 24 h	aorta	+ complete decellularisation after 24 h + complete removal of DNA + complete decellularised tissue treatment - slightly ECM disruption [12]

However, it must be noted that intensive physical treatment can destroy the tissue and the ECM, which is counterproductive with regard to successful tissue integration [2, 11, 16].
Further, ultrasonic treatment causes increasing interfibril spaces and slight damage to the ECM, so that cells and cell components can be flushed out, whereby the depth of the treatment

depends on the treatment time and intensity [2, 11-15, 18]. A study with a comparison between the direct sonication and an ultrasonic bath showed that the ultrasonic bath is smoother for the tissue while it has less damage on collagen and GAGs [15].

3. Methods

3.1 Ultrasonic set-up and characterisation

For the reasons mentioned above, the application of a temperature-controlled ultrasonic bath as DC method is examined in this research work in order to evaluate the single effect of ultrasonic treatment. Frequencies in range of $28 \leq f \leq 40$ kHz with low powers of $60 \leq P \leq 120$ W were used for gentle treatment of the tissue. Furthermore, different process times (t_t =10 min, t_t = 40 min, t_t = 60 min) were selected in order to analyse the influence of treatment time on the DC process, whereby each sample was turned after half of the time. For the experimental investigations a commercially available bowl (length = 90.5 mm, width = 90.5 mm, height = 40 mm) were attached with different transducers (P = 60 W, f = 28 kHz and f = 40 kHz; P = 100 W, f = 28 kHz and f = 40 kHz, P = 120 W with f = 40 kHz), as shown in Fig. 1.

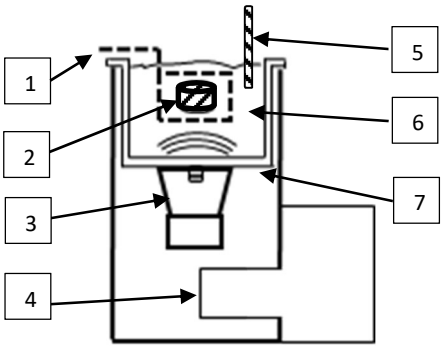

Figure 1. Ultrasonic bath system consisting of sample holder (1), tissue (2), transducer (3), cooling system (4), temperature sensor (5), PBS (6) and bowl (7).

A generator (Oursultrasonic, Hesentec, ShenZhen, China) was used to supply the ultrasonic signal, with which the frequency in the range of 28 kHz ≤ f ≤ 40 kHz as well as the treatment and cycle time could be set. For the cooling of the bowl and transducer, a cooling system based on Peltier elements was added. A minicomputer Raspberry PI was used to control the system. All tissues were held in a jig made of polylactide (PLA), fabricated by fused deposition modelling. For each examination, the bowl was filled with 250 ml of PBS with a temperature of T = 6 °C. Additionally, to ensure a temperature below T = 40 °C, the PBS was exchanged after t_t = 20 min during one hour treatments despite the cooling.

To characterise the ultrasonic field at the beginning of the investigation, the area of the most intense cavitation was identified by the aluminium foil test and sono-chemiluminescence. During cavitation, high forces and temperatures generated during a bladder collapse create free hydroxyl radicals. This radical reacts with other chemically dissolved substances in the medium [19]. A typical reagent used for sonochemiluminescence visualisation is luminol ($C_8H_7N_3O_2$). A luminol solution 0.01 mol/l with 0.25 mol/l sodium hydroxide (NaOH) and a camera with a long exposure time (Canon EOS 5D Mark II, Canon Deutschland GmbH) were used for the investigation.

3.2 Tissue sample preparation for ultrasonic treatment

The porcine thyroid cartilage and dura mater sample were donated by a local abattoir. After rinsing the tissues with sterile PBS (Sigma-Aldrich, St. Louis, USA) the samples were punched out with a diameter of d_c = 10 mm (cartilage) or d_d = 22 mm (dura mater). Before storage in PBS at T = 6 °C until ultrasonic treatment, the perichondrium of the thyroid cartilage was removed and the sample was cut to a height of h_c = 1.5 mm.

3.3 Determination of DNA content

To determine the efficiency of the decellularisation method, the DNA content of the tissue samples were measured. For this, the cartilage and dura mater were cut into small pieces and stored at T = -20 °C after ultrasonic treatment. Then, small-cut cartilage and dura mater specimens were freeze-dried for at least t = 7 h followed by weight measurements. Afterwards, the tissues were pre-digested with 3 ml collagenase A (0.46 U/ml; Roche Diagnostics GmbH, Rotkreuz, Switzerland) and additional 0.5 ml dispase (3 U/ml; Roche Diagnostics GmbH, Rotkreuz, Switzerland) for dura mater overnight at T = 37 °C in a Thermo-Shaker. Furthermore, each sample was incubated with 480 µl water, 480 µl solid tissue buffer and 40 µl proteinase K for at least t = 3 h at T = 55 °C in a Thermo-Shaker. The DNA isolation was done by using the "Quick-DNA Midiprep Plus Kit" (Zymo Research, Irvine, USA) and was performed according to the user manual. After the DNA was eluted, the contents were determined by absorptiometry at λ = 260 and λ = 280 nm (Tecan infinite® F200Pro, Maennedorf, Switzerland).

4. Results and discussion

Firstly, an aluminium foil test and sonochemiluminescence were carried out to characterise the ultrasonic field in order to identify the optimal position with the greatest cavitation effect. The results of the aluminium test are shown in Fig. 2 (left), with the foil being most damaged precisely in the middle at a distance of x = 30 mm. This was also confirmed by an experiment with sonochemiluminescence, see Fig. 2 (right).

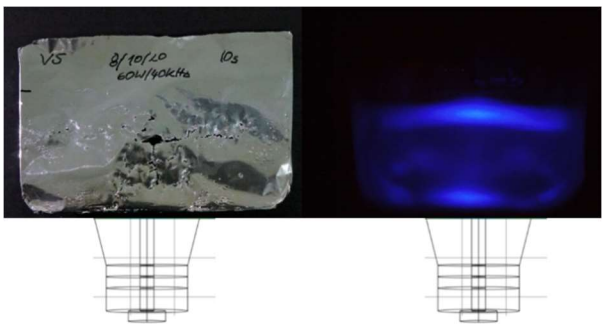

Figure 2. Left: Aluminium foil test with a large hole in the middle and small holes around after a processing with t_t = 10 s, f =40 kHz and P = 60 W.
Right: Sonochemiluminescence image, where the highest forces acting at the brightest point.

Cartilage and dura mater DNA isolation was performed with and without the ultrasonic treatment to evaluate the DC process in relation to the power, frequency and treatment time. Fig. 3 shows the results of the treatment of the cartilage with increasing power from left to right. While increase of power and frequency seemed to decrease DNA content, the impact of time was marginal.

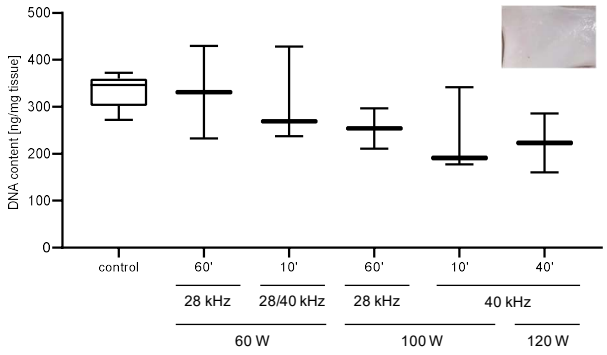

Figure 3. Residual DNA content of porcine thyroid cartilage with control sample (left) and increasing power from left to right (n ≥ 2). Lowest DNA content of DC cartilage of 169.19 ng/mg.

The lowest value of residual DNA content of 169.19 ng/mg for cartilage was achieved with a frequency of f = 40 kHz and an output of P = 120 W. Fig. 4 shows the results of the residual content of DNA for the DC of dura mater, whereby the best result of 30.74 ng/mg could be also achieved with a frequency of f = 40 kHz and an output of P = 120 W. Starnecker et al. [2] and Norzarini et al. [18] also observed in their studies that treatment with higher frequencies is more effective for decellularisation. However, high fluctuations occur due to the small number of samples and the known biological differences.

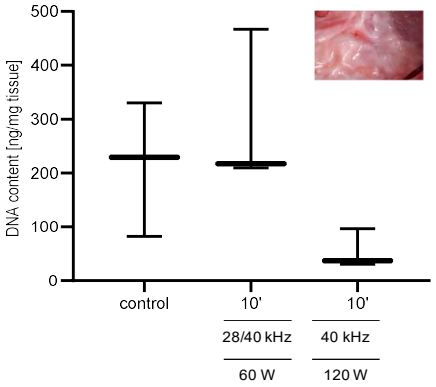

Figure 4. Residual DNA content of porcine dura mater with control sample (left) and increasing power from left to right (n = 3). Lowest DNA content of DC dura mater of 30.74 ng/mg.

5. Conclusion

Our preliminary results show that ultrasound treatment for the decellularisation of allograft transplants may be an interesting alternative to avoid toxic agents. For this purpose, a mini ultrasonic bath with temperature cooling was developed based on the ultrasonic DC methods presented in the literature. The subsequent determination of the DNA content by DNA isolation showed a reduction in the content of both types of tissue. It was observed that at a frequency of f = 40 kHz and the highest power of P = 120 W, the DNA content decreases. In this initial study, a trend towards ultrasound treatment can be seen, even if the results are limited because of the biological fluctuations and the small sample size. Hence, further investigations with a larger number of samples as well as structural and biomechanical ECM characterisation will be carried out.

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