Evaluation of ultrasound application for the decellularization of small caliber vessels

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Abstract—**Decellularized matrices for tissue engineering seem to be an attractive material for providing biological vascular grafts for patients with advanced peripheral arterial disease who require bypass surgery, but do not have suitable autologous small-caliber vessels (<6 mm diameter). Currently, a variety of decellularization (DC) techniques have been proposed such as physical, chemical, and/or enzymatic methods; however, identifying an optimal protocol resulting in preservation of favorable physiochemical properties of the vascular scaffold is still elusive.**

The goal of the proposed study was to examine the capacity of sonication to completely decellularize small-diameter blood vessels when applied alone, to test the effect of waves' parameters on the processing quality and matrix microarchitecture preservation, and to evaluate the possibility to reduce the time required for cell removal when ultrasound is used in combination with non-ionic detergents.

Contradictory to other DC protocols reported previously, we were not able to record completely or even partially cell removal in all studied groups. Interestingly, the combination of conventional chemicals, as Triton X-100, with physical method did not result in improving the DC efficiency and did not offer tissue permeabilization and easier chemicals access towards deeper tissue layers. In addition, when high sonication power was applied, significant destruction of the vessel matrix was determined. In summary, the use of sonication had no beneficial effect on DC in this study.

Keywords—**decellularization, small caliber vascular grafts, ultrasound, detergent, histological assessment.**

I. Introduction

Increased interest for tissue engineering and regenerative medicine is determined by the scarcity of available organs and the gap between demand and supply [1].

Decellularization (DC) techniques seem to be a promising method for the preparation of natural scaffolds for regenerative medicine, because the retrieved matrices maintain biochemical and biomechanical properties, are biodegradable, biocompatible, stimulate cell migration and proliferation, and do not stimulate immune response, if allogeneic material is used [2, 3].

Taking the existing advantages of acellular materials into consideration, they represent an attractive option for patients with advanced end-stage cardiovascular disease, as peripheral arterial disease or coronary artery disease, who require bypass surgery, but do not have suitable autologous small-caliber vessel (<6 mm diameter) [1, 4, 5].

DC can be realized by a variety of methods. The majority of the publications in the field refer to application of chemical and biological agents or combined approaches; physical methods being assessed inadequately so far [4, 5, 6, 7].

Till now, the contributions of physical methods in DC, such as rapid freeze-thaw (thermal shock), perfusion, supercritical carbon dioxide, immersion and agitation, hydrostatic pressure and sonication [2] when used alone or in combination with chemical and biological approaches are insufficiently evaluated.

For instance, so far, sonication treatment has been used for DC of tissues such as larynx [8, 9], trachea [10], kidney [11, 12, 13], meniscal tissue [14, 15, 16, 17], aorta [3, 18, 19, 20, 21], skeletal muscle [22], or osteochondral tissue [23]. It is assumed that sonication may have the potential to decrease the time required for complete tissue decellularization by inducing cell membrane perforation and enhancing detergent (Det) molecules uptake. This effect is determined by its ability to reversible loosen the junctions sealing the cells together [21, 24, 25].

The effects induced from the ultrasonic waves, including homogenization, dispersing, deagglomeration, emulsification, altering cell membrane integrity, debris removal, disintegration, loosening cells junctions, and sonochemical effects, are caused by cavitation. Cavitation phenomenon consists in the creation of vapor cavities (micro bubbles) in a fluid determined by altering high-pressure and low-pressure cycles, with rates depending on the frequency (the number of bubbles increasing with increasing sonication power). During the low-pressure cycle (the rarefying phase of sound wave), these bubbles grow. During this event the gas inside the bubble reaches very high temperature [26].

When the bubbles cannot longer absorb energy, they collapse violently during a high-pressure cycle (the compression phase), inducing significant local temperature, pressure increase, and generating a violent shock wave through the medium. In this way the sonic energy is transformed into mechanical energy responsible for the majority of biological effects caused by the ultrasound [14, 27, 28, 29].

The sonication process can be carried out by different types of ultrasonic systems, as probe-type ultrasonic homogenizer (direct sonication) or an ultrasonic bath (indirect sonication).

However, the cavitation process generated in an ultrasonic bath seems to be uncontrollable due to by the uneven distribution of the ultrasonic irradiation field in the ultrasonic tanks; thus, the repeatability and scalability of the process is very poor. Alternatively, a probe-type device offers full control over the sonication parameters ensuring reproducibility of the experimental results [12, 23].

This study was carried out to evaluate the capacity of sonication treatment to complete cell removal from small-diameter blood vessels when used alone or in combination with a non-ionic Det, as Triton X-100.

II. Materials and Methods

A. Decellularization system

Porcine vessels were collected from German Landrace pigs (3-5 months old) in the animal facility of the Medizinische Hochschule Hannover (MHH). Vessels were cleaned from fat and adjacent tissue using forceps and scissors. Vessels were stored at -80ºC.

DC of the vascular tissue has been done by physical (sonication and osmotic shock) and combined (sonication with Det) methods.

For sample processing a direct sonication method (direct sonicator UP200S Hielscher, Germany and Sonotrode S1 for samples from 0.1 to 5 mL) was used. Taking into consideration that collapsing bubbles may produce significant thermal loads emitted in the surrounding liquid [8], the experiments were performed in a cold room (+4ºC). In addition, the samples were placed in an ice bath in order to prevent tissue over-heating.

Physical treatment: For decellularization, the vessels (1 cm long segment, internal diameter 4 mm) were flushed with PBS and submerged in 2.0 mL Eppendorf tubes containing 1.5 mL hypotonic lysis buffer (0.3% NaCl in distilled water). The samples were exposed to sonication with a frequency of 24kHz, 200 watt, control mode "1" (permanent acoustic irradiation). Two different amplitude values and two different exposure times for DC were applied: 20% *vs* 100% and 3 hours *vs* 12 hours, respectively.

Combined method: The samples from this group were placed in a 2.0 mL Eppendorf tubes containing 1.5 mL 1% Triton X-100 and exposed to sonication with a frequency of 24kHz, 200 watts, amplitude 20%, control mode "1" for 48 hours. Distance of the sample to the tip of the ultrasound probe was set at 1 cm. As control, samples were treated with the same solution under continuous rotation (50 rpm speed, Biometra WT 17).

B. Histological analysis

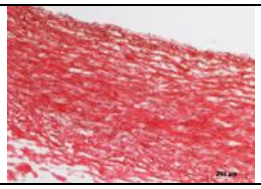
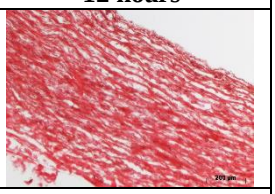
The retrieved matrices were evaluated qualitatively for remaining cellular components and DNA through H&E and DAPI staining, respectively. Native porcine vessels were used as controls.

H&E staining. Frozen samples were cut into 7 µm sections. Sections were transferred on slides and stored overnight at -80ºC. Samples were fixed in acetone at -20ºC for 8 minutes. The fixed slides were placed in a staining rack and moved through hemotoxylin (8 minutes), eosin (20 seconds), and a series of alcoholic solutions (namely, 5 minutes in 95% ethanol and 5 minutes in 100% ethanol). Finally, after clearing the samples in xylene (10 minutes), they were covered with cover slips using corbit balsam and left to dry overnight.

DAPI staining. Sections from snap frozen samples were used for DAPI staining. The sections were washed in PBS and fixed in 4% paraformaldehyde for 20 minutes in a wet chamber. Sections were washed for 15 minutes with PBS, and incubated for 15 minutes with DAPI (0.33 µg/mL in PBS). After three washing steps in PBS, 5 minutes each, the samples were covered with cover slips using Shandon Immu-Mount fluorescent mounting medium. The stained sections were then analyzed with a fluorescence microscope.

III. Results

The pictures of the histological stainings of samples treated by sonication without supplementary Det exposure for 3 and 12 hours-treatments are presented in Figure 1.

		3 hours	12 hours
H & E	**20%**		

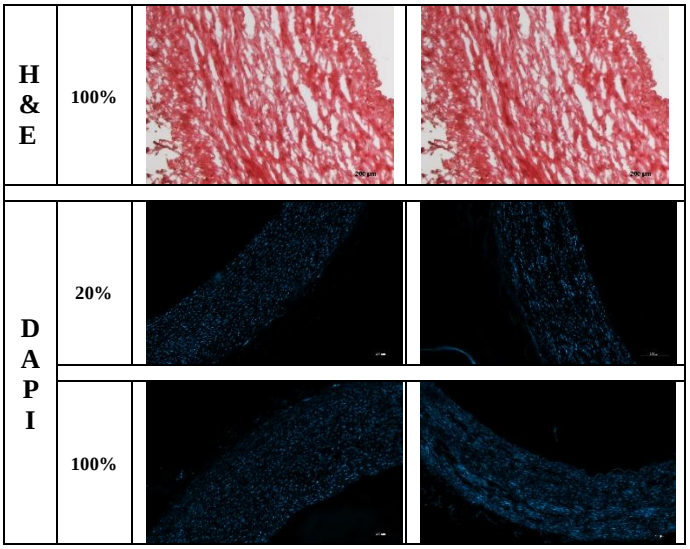
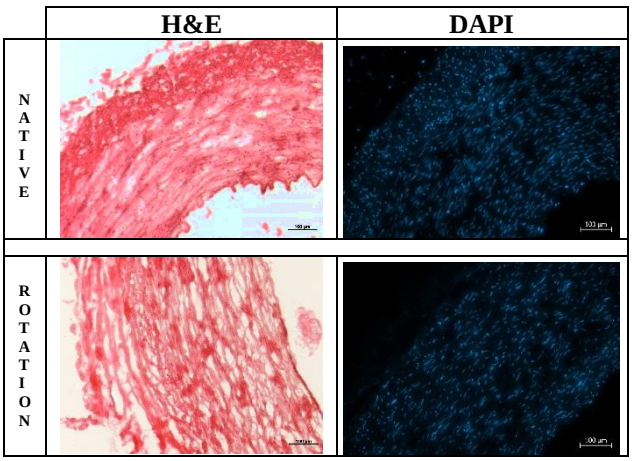


Fig. 1 Ultrasound application in carotid artery decellularization (histological assessment)

Interestingly, H&E and DAPI staining revealed the presence of huge amounts of intact cells under all investigated conditions demonstrating that sonication was not able to remove cells efficiently from the tissue even with high amplitude setting and prolonged exposure. In addition, H&E staining analysis showed that the tissue structures were significantly affected by high amplitude waves (100%).

Results from combined treatment with Det and ultrasound are shown in Figure 2. Native carotid artery was used as control. H&E and DAPI staining demonstrated the pres ence of intact cells and nuclei in treated samples (Triton X-100 in combination with sonication or rotation) to the same extent as in the control. Nuclei within the vascular wall remained intact in treated tissue, suggesting that the reagent was not able to solubilize the membranes and to induce cell removal even with sonication.



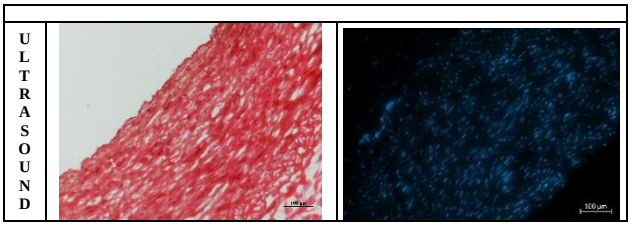


Fig. 2 Combined approach in carotid artery decellularization (histological assessment)

IV. Discussion

Azhim A *et al* (2011) demonstrated for the first time that using low frequency (20kHz), high power (15 or 30 Watts) ultrasound in combination with strong ionic Det (2% sodium dodecyl sulfate (SDS) complete DC of aortic tissue can be obtained. The results were compared to a typical DC process (immersion or shaking with the same chemical solution). A novel DC system consisting of a commercially available ultrasonic horn and a roller pump was used by the group. They stated that adding sonication significantly improved DC depth with cells being completely removed from the scaffold, which did not happen in immersion treatment [20].

In a second study, the recellularization potential of such DC matrix with vascular smooth muscle cells (VSMCs) was evaluated. In this case, DC was carried out at 36±1ºC temperature in continuous oscillation with ultrasonic power of approximately 500 mVrms and ultrasonic frequency of 170 kHz for 10 hours in 2% SDS solution. The study confirmed successful VSMCs adherence and its infiltration into the tissues [21]. Further, the same working group reported in 2019 the development of a closed sonication DC system used together with 0.1% or 2% SDS for preparing acellular aortic scaffolds [3].

Also other reports demonstrated the efficiency of ultrasound application in combination with other chemicals in larynx (probe-type ultrasonic homogenizer Sonifier 250, output control setting 6, 6% DMSO and 1% Triton X-100) [9], meniscus (closed sonication system, 40 kHz, 0.1% SDS) [16, 17], and kidney (bath-type sonicator, sonicator power 0 W/ 60 W/ 120 W, 0.25% / 0.625% / 1% SDS, Triton X-100 OR 150 W/ 200 W/ 250 W, frequency 20 kHz, perfusion 10 mL/min, 1% SDS) DC [11, 12, 13].

Only the report byTchoukalova YD *et al* (2018) did not confirm the efficiency of sonication combined with Det (4% sodium deoxycholate, SDC) in washout of cells from the cartilaginous tissue [10].

In the current study, we were not able to obtain DC by treating porcine carotides with ultrasonic waves even in combination with a Det; however, using Det and rotation together was not resulting in DC as well. As Triton X-100 is

considered a weak Det this might explain the inefficiency of the treatment compared to other studies. To investigate if sonication may offer permeabilization of the matrix and easier access of the chemicals to the deep layers, we suggest the application of stronger detergents such as SDS or SDC.

In addition, the tissue structure seemed to be significantly affected by high amplitude waves (Fig. 1). The negative impact of long exposure to high sonicator settings can be explained by prolonged subjection to the cavitation effect and microbubbles action that can puncture and destroy the matrix. The same results were reported previously [11, 14, 18, 19, 22, 30].

V. Conclusions

No study thus far has provided results for small-diameter blood vessel decellularization, as porcine carotid artery, when using sonication alone or combination of chemical immersion and ultrasound. Nevertheless, using ultrasound in DC did not appear to be an efficient strategy to remove cells from the tissue. The same results were obtained when using ultrasonic waves in combination with a non-ionic detergent.

In addition, the lack of DNA reduction when applying Triton X-100 suggests the necessity of using sonication in combination with stronger chemicals, such as sodium dodecyl sulfate or sodium deoxycholate, for small caliber vessel DC.

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Conflict of Interest

No competing interests were disclosed.

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