

New
Methods

Impact on Mechanical Properties of 10 versus 20 Minute Treatment of Human Pericardium with Glutaraldehyde in OZAKI Procedure

Luca Koechlin, MD,^{1,*} Giuseppe Isu, PhD,^{1,2,*} Vladislav Borisov, MD,^{1,2} Diana Robles Diaz,^{1,2} Friedrich S. Eckstein, MD,^{1,2} Anna Marsano, PhD,^{1,2} and Oliver Reuthebuch, MD¹

Purpose: The aim of this study was to analyze the effects of 10-minute (standard term) versus 20-minute treatment with glutaraldehyde (GA) on mechanical stability and physical strength of human pericardium in the setting of the OZAKI procedure.

Methods: Leftover pericardium (6 patients) was bisected directly after the operation, and one-half was further fixed for 10 additional minutes. Uniaxial tensile tests were performed and ultimate tensile strength (UTS), ultimate tensile strain (uts), and collagen elastic modulus were evaluated.

Results: Both treatments resulted in similar values of uniaxial stretching-generated elongations at rupture (10 minutes $25 \pm 7\%$ vs. 20 minutes: $22 \pm 5\%$; $p = 0.05$), UTS (5.16 ± 2 MPa vs. 6.54 ± 3 MPa; $p = 0.59$), and collagen fiber stiffness (elastic modulus: 31.80 ± 15.05 MPa vs. 37.35 ± 15.78 MPa; $p = 0.25$).

Conclusion: Prolongation of the fixation time of autologous pericardium has no significant effect on its mechanical stability; thus, extending the intraoperative treatment cannot be recommended.

Keywords: pericardium, OZAKI, mechanical stability

Introduction

Aortic valve replacement using autologous pericardium, the so called “OZAKI” technique (also referred to as “aortic valve neocuspidization” or “aortic valve reconstruction”), has gained popularity in recent years.^{1,2} First

published in 2011 by Ozaki et al.,¹ the technique uses autologous glutaraldehyde (GA)-treated pericardium to rebuild the aortic valve.

Ozaki and colleagues suggest a 10 minute treatment of the autologous pericardium with 0.6% GA solution. The treated pericardium is then rinsed three times for 6 minutes each in a physiological saline solution.¹

However, protocols for GA-fixation treatments of animal-originated pericardium, largely employed in medical applications, typically describe fixation durations of multiple hours up to even weeks.^{3,4} Yet, the OZAKI technique requires a fast, timely well-coordinated fixation since it is implemented intraoperatively. We consider 20 minutes of intraoperative treatment as the utmost extension to restrict cross-clamp time. Thus, we aimed to compare the mechanical properties of pericardium treated according to Ozaki’s original procedure (10 minutes, 0.6% GA) with pericardium treated for 20 minutes (0.6% GA), hypothesizing extended mechanical endurance in causal dependency on prolonged treatment. To the best of

¹Department of Cardiac Surgery, University Hospital Basel, Basel, Switzerland

²Department of Biomedicine, University of Basel, Basel, Switzerland

Received: May 7, 2020; Accepted: September 22, 2020

Corresponding author: Oliver Reuthebuch, MD, Department of Cardiac Surgery, University Hospital Basel, Spitalstrasse 21, CH-4031 Basel, Switzerland

Email: oliver.reuthebuch@usb.ch

*These authors contributed equally to this manuscript.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives International License.

©2021 The Editorial Committee of *Annals of Thoracic and Cardiovascular Surgery*

our knowledge, this is the first study analyzing the impact of 10-minute versus 20-minute treatment time in human pericardium in the setting of the OZAKI procedure.

Methods

Ethical approval

The local ethical committee (EKNZ BASEC Req-2017-01334) approved the study protocol, which is in accordance with the principles of the Declaration of Helsinki. Informed consent was obtained from all the patients. The trial was registered at ClinicalTrials.gov (ID NCT03667235).

Materials

Patients were included between September 2017 and July 2018 in our institution. After OZAKI valve replacement, leftover and one-time treated pericardium of six patients (four males and two females) was bisected longitudinally (cranial to caudal orientation). One half of the tissue was treated for additional 10 minutes (thus, 20 minutes in total) with 0.6% GA, and then rinsed again three times for 6 minutes in saline according to the original protocol.¹⁾

Five strip samples of $25 \times 5 \text{ mm}^2$ were cut in random orientations to average the high tissue anisotropy of leftover pericardium biopsies harvested in each patient. The samples were cut as shown in **Fig. 1A**, and characterized with regard to processing time (i.e., GA-10 = 10 min GA, GA-20 = 20 min GA), size, and thickness (to evaluate intra- and inter-sample thickness inhomogeneities) by means of an in-house image-based quantification software (Matlab 2017b, Mathworks). Pictures of each sample ($3456 \times 2304 \text{ px}$ resolution) were taken with a high-resolution CMOS camera (Canon EOS 700D, Tokyo) mounting a macro objective (objective 20 \times), with a resulting digital resolution of up to 20 $\mu\text{m}/\text{px}$. We captured frontal pictures for the measurement of length and width, and we took two lateral views of the construct (one for each side) for the extraction of the thickness profiles. We then combined the obtained values of width and length of each sample with the thickness profiles to calculate the minimum resistant cross-section, which represents the section of minimum thickness for each sample. Thus, uniaxial tensile tests were performed using a standard test machine (**Fig. 1B**) (MTS Synergy, Lüdenschied, Germany), applying a pre-load of 0.01 N and an elongation ramp of 2.5%/s.⁵⁾ Stress–strain curves were elaborated, and ultimate tensile strength (UTS),

ultimate tensile strain (uts), and collagen elastic modulus were calculated as engineering stress at the minimum resistant cross-section (**Fig. 1B**). When not specified in the text, unpaired t-test with Welch's correction was performed to compare the two experimental groups.

Immunofluorescence staining and tissue clarification

Small randomly oriented pieces of the original biopsies of the two experimental groups (size approximately $1 \times 1 \text{ cm}$) were cut with a scalpel. Each sample was blocked with goat serum blocking solution for 48 hours at room temperature (94.75% tris-hydroxymethyl aminomethane + NaCL, 5% goat serum, 0.25% Triton x-100), then stained for type I collagen for 24 hours at room temperature (1:200, primary antibody NB-600-450, Novus Biologicals, Zug, Switzerland), and finally incubated with mouse IgG1 A546-red for additional 24 hours at room temperature. Thereafter, the samples were kept in the dark and clarified in cubic clarification solution until completely transparent⁶⁾ (approximately 2–3 days under gentle shaking). Images of the samples were acquired using a confocal microscope (LSM 710, Zeiss, Oberkochen, Germany).

Results

Leftover pericardium from six patients (median [interquartile range] age 61 [51,70] years, 4 males [67%]) was analyzed. Baseline characteristics are presented in **Table 1**. Pericardium samples excised from random locations (as shown in **Fig. 1A**) exhibited thickness variations ranging from 0.4 to 1 mm (**Fig. 1A**). The thickness varied mainly between the samples; thickness variations of the same range within one sample were rare. Mean (SD) thickness of the samples was 0.72 (0.19) mm in the GA-10 group and 0.64 (0.17) mm in the GA-20 group showing that the different GA treatment time did not produce significant variations of the thickness properties of the construct ($p = 0.12$).

Uniaxial stretching generated mean (SD) elongations at rupture of 25% (7) in the GA-10 group and 22% (5) in the GA-20 group ($p = 0.05$), corresponding to mean (SD) UTS values of 5.16 (2) MPa in the GA-10 group and 6.54 (3) MPa in the GA-20 group ($p = 0.59$). The high standard deviation values resulted from the high anisotropy of the tissue, which was averaged by randomizing sample orientation. Gender difference had an impact on the heterogeneity of the UTS values. Samples from women exhibited 1.5 and 1.9 times lower UTS values than samples from men, respectively (men: 6.17 MPa in

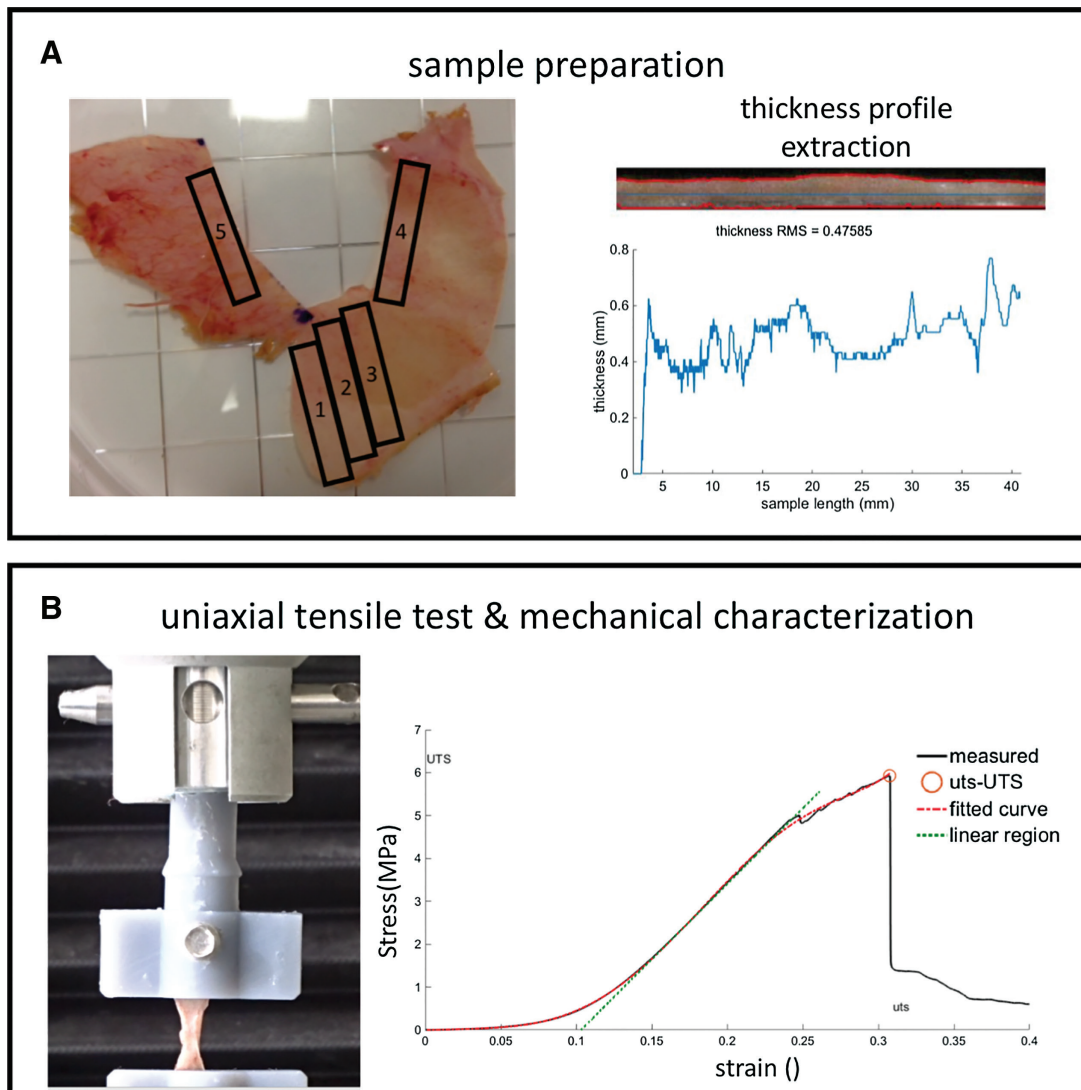


Fig. 1 Representative photos of the five strip samples cut with random orientation to average the high tissue anisotropy from leftover pericardium (**A**). Representative images of the sample's cross-section, and a plot representing the extracted thickness profile (**A**). Example of the uniaxial tensile test and of the mechanical characterization (**B**). UTS: ultimate tensile strength; uts: ultimate tensile strain.

Table 1 Patient characteristics

Age in years, mean (m) (IQR)	61 (51, 70)
Male Gender, n (%)	4 (67)
Body mass index in kg/m ² , m (IQR)	25 (21, 28)
Diabetes mellitus, n (%)	0 (0)
Dyslipidemia, n (%)	4 (67)
Coronary artery disease	1 (17)
Renal insufficiency	2 (33)

IQR: interquartile range

GA-10 vs. 8.62 MPa in GA-20; women: 4.22 MPa in GA-10 vs. 4.62 MPa in GA-20; Mann-Whitney test: $p = 0.0005$ for GA-10 and $p = 0.007$ for GA-20). The

elastic modulus, representing the stiffness properties of the collagen fibers, showed similar mean (SD) values in both groups: 31.80 (15.05) MPa in the GA-10 group and 37.35 (15.78) MPa in the GA-20 group ($p = 0.25$).

Tissue clarification and collagen staining

The different fixation time did not result in any observable difference at the microscopic level, as appreciable in the collagen structures depicted in **Fig. 2A** and **2B**. Similar anisotropic structures were present in both constructs, exhibiting no sign of compaction of the fibers related to enhance cross-linking (**Fig. 2A** and **2B**).

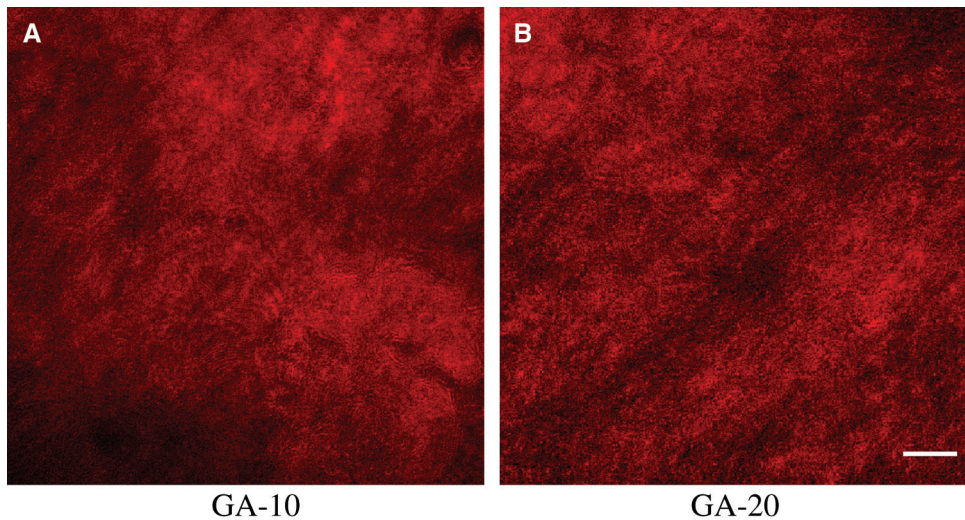


Fig. 2 Representative images of immunofluorescence staining for type I collagen of pericardium biopsies treated for 10 (A) or 20 (B) minutes in GA. Scale bar = 50 μ m. GA: glutaraldehyde

Discussion

The aim of this study was to assess whether prolongation of the relatively short time of 10 minutes, that is typically applied during the OZAKI procedure to treat autologous human pericardium in 0.6% GA modifies the stability of the treated tissue. We could show that extending the treating time to 20 minutes had no significant effect on the mechanical properties of the tissue, as expressed in comparable resulting values of UTS and elastic modulus ($p = 0.59$ and $p = 0.25$). Yamashita and colleagues,⁵⁾ who compared fixated pericardium (0.6% GA for 10 minutes) with fixated native calcified and non-calcified leaflets, proved the same orientation-dependent variability, UTS and elastic modulus values within the equal range of our analysis.

The confocal microscope qualitative observation of the collagen structures exhibited similar anisotropic patterns in both groups, with the same level of fiber compaction. This suggests that doubling the fixation time has no effect on the degree of fixation (e.g., on the collagen crosslinking); it results in similar mechanical properties. Indeed, Lee and colleagues⁷⁾ showed that tensile strength was not affected by GA fixation time (0.6% GA, 10 vs. 20 min) using bovine pericardium, although they revealed higher elongation at break with the longest 0.6% GA treatment.

This is the first study conducted to compare different fixation periods of human pericardium. The number of possible tests and readouts was restricted by the limited available biopsy material, which additionally had to be split into the two different experimental groups.

Conclusion

In conclusion, we disclaimed the hypothesis that doubling the GA fixation time of human pericardium results in ameliorated mechanical properties. Our results showed no significant differences in mechanical stability of pericardium treated for 20 minutes and pericardium treated for 10 minutes, as suggested by Ozaki. Thus, we recommend to further proceed with 10-minute fixation time in clinical cases.

Disclosure Statement

There are no disclosures to declare.

References

- 1) Ozaki S, Kawase I, Yamashita H, et al. Aortic valve reconstruction using self-developed aortic valve plasty system in aortic valve disease. *Interact Cardiovasc Thorac Surg* 2011; **12**: 550–3.
- 2) Reuthebuch O, Koechlin L, Schurr U, et al. Aortic valve replacement using autologous pericardium: single centre experience with the Ozaki technique. *Swiss Med Wkly* 2018; **148**: w14591.
- 3) Pasquino E, Pascale S, Andreon M, et al. Bovine pericardium for heart valve bioprostheses: in vitro and in vivo characterization of new chemical treatments. *J Mater Sci Mater Med* 1994; **5**: 850–4.
- 4) Hülsmann J, Grün K, El Amouri S, et al. Transplantation material bovine pericardium: biomechanical and immunogenic characteristics after decellularization vs. glutaraldehyde-fixing. *Xenotransplantation* 2012; **19**: 286–97.

- 5) Yamashita H, Ozaki S, Iwasaki K, et al. Tensile strength of human pericardium treated with glutaraldehyde. *Ann Thorac Cardiovasc Surg* 2012; **18**: 434–7.
- 6) Susaki EA, Tainaka K, Perrin D, et al. Advanced CU-BIC protocols for whole-brain and whole-body clearing and imaging. *Nat Protoc* 2015; **10**: 1709–27.
- 7) Lee C, Lim HG, Lee CH, et al. Effects of glutaraldehyde concentration and fixation time on material characteristics and calcification of bovine pericardium: implications for the optimal method of fixation of autologous pericardium used for cardiovascular surgery. *Interact Cardiovasc Thorac Surg* 2017; **24**: 402–6.