Executive Summary

Optimization of the dehydration process of the prefixed bovine pericardium

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1. Introduction

As of today, in case of valvular heart disease (VHD), a phenomenon only projected to increase in the coming decades, valve replacement remains the standard of care with over 300,000 implants performed worldwide per year and a mortality rate below 2%. [1]
When discussing valve choice during patient consultation, younger and active patients prefer biological valves over the mechanical ones because of the better hemodynamic performances and no need for long-term anticoagulant therapy. However, one of the main criticisms of this type of valve is the durability of its biological tissue, which tends to calcify leading to failure due to structural valve deterioration (SVD). For this reason, in view of the higher life expectancy, ESC/EACTS guidelines suggest the use of mechanical valves only for younger patients (<60 years old), which are less prone to this phenomenon. However, the mechanical alternative carries significant limitations and a substantial change in lifestyle.
With the aim of overcoming the limits of both types of prostheses, focusing on the biological valves structure, a novel bovine pericardium treatment, comparable to the Resilia tissue treatment developed by Edwards Lifesciences, is presented. The Resilia tissue, a unique example available on the market, is bovine pericardium treated with a proprietary tissue integrity preservation technology designed to eliminate free aldehyde groups, a known cause of calcification, resulting from the fixation and sterilization process in glutaraldehyde (GA) of the tissue. [2, 3] In addition to better anticalcification properties and greater durability, valves with Resilia tissue can be dry-stored, which is easier to manage than liquid storage, and do not require an intraoperative rinsing procedure.
This work is developed in the industrial setting of Corcym, a global leader in the production of cardiac valve prostheses.
The objective is to identify a treatment that effectively dehydrates (drying process) the prefixed bovine pericardium in order to optimize the production process. The properties of the dehydrated pericardium must guarantee the same performances as the ones of the wet pericardium, currently used in the manufacture of valve bioprostheses. Defining such treatment is a complex process: multiple aspects, such as materials, tools, times, temperatures, etc., must be combined together correctly. Improper calibration of any of these can result in a dry tissue instead of a dehydrated one, thus not usable. Moreover, it should be noted that bovine pericardium is a biological material and, as such, exhibits high intrinsic variability.
There are multiple reasons to work with dehydrated tissue related to both the improvement of the properties of the valve (e.g. Resilia tissue performances) and the optimization of the industrial production process. In particular, this work aims to achieve the latter because the dehydrated bovine pericardium: i) does not dry out when exposed to air and therefore a dry process does not require the management of all the

liquids associated with a "wet" work cycle, *ii*) does not expire, allowing for the creation of a bank of pre-treated material from which to draw for valve manufacturing, *iii*) does not require the complicated and expensive sterilization in aldehyde-based sterilizing liquid, and *iv*) can be dry-stored, simplifying storage and shipping.

2. Materials and methods

All the necessary equipment and materials for the tests have been provided by Corcym R&D labs in Via Crescentino sn, 13040 Saluggia (VC), Italy (September 2022 to March 2023). This study consists of 3 experimental phases, each phase built upon the findings of the previous one: a preliminary phase, where process parameters are defined; a factorial analysis, where the treatment that best dehydrates the pericardium is identified; and a verification phase, where it is ensured that the identified treatment does not alter the properties of the pericardium over time, up to 3 months. Although each phase followed a completely different procedure due to its distinct objectives, the starting point for all phases is the bovine pericardium treated with a GA solution at room temperature for 3 to 4 hours and then subjected to the current Corcym's FREE treatment. [2]
The desired dehydration occurs through a proprietary solution based on glycerol. Glycerol is a compound that has a strong hygroscopic effect, allowing it to remove and replace most of the water molecules that are governed by weak intermolecular forces (e.g. van der Waals forces) present in the tissue. Additionally, the drying mixture retains in the pericardium patch a sufficient degree of residual humidity that prevents irreversible dehydration, even when exposed to air for an extended period of time.

2.1. Preliminary phase

A preliminary activity was carried out to roughly range the time required for glycerol to dehydrate the prefixed bovine pericardium. At room temperature, groups of 20 samples (20 x 20 mm) were exposed to the drying solution for 30 seconds, 30 minutes, 2 hours, and 16 hours, respectively. The residual humidity was then evaluated to assess the degree of tissue dehydration, using the weight difference of each sample measured with a precision analytical balance before and after being placed in an environmental chamber at 100-102°C for 16-18 hours, as per AOAC Official Method 950.46 - Moisture in Meat. [4]
However, since this method cannot precisely differentiate the percentage of weight lost in the chamber due to water and the weight lost due to glycerol, it only serves as an indicator of potential trends in sample behavior under different treatment conditions, considering the large number of samples available. The confirmation of such trends was done using the more precise yet expensive Karl Fischer (KF) titration, a known chemical analysis used to determine specifically the water content. This method, performed by the company labs on 4 samples for each exposure time, is based on the oxidation of sulfur dioxide by iodine in a solution of anhydrous methanol. In principle, the hygroscopic nature of methanol enables the extraction of water from the sample. The sample is weighed before analysis, and the water content is calculated based on the quantity of titrant consumed. Specifically, 1 ml of titrant is capable of titrating 5 mg of water.
It is important to note that while both the oven-drying method and the KF method serve the same purpose, they provide completely different information about the samples: the former allows for the calculation of weight loss, while the latter detects the exact amount of water content.

2.2. Factorial analysis

During the factorial analysis, the optimal treatment for dehydrating the pericardium was determined by performing a Design of Experiment (DoE) factorial analysis, according to Table 1. DoE is a statistical technique that aims to optimize reactions and processes by defining the relationships between sets of variables.

Design of Experiments factorial analysis	
Inputs	**Outputs**
Time (5 to 115 min)	T_s [°C]
Temperature (7 to 30 °C)	UTS [MPa]
Rolling (Y/N)	E [MPa]

Table 1: DoE factorial analysis summary table.

The DoE approach examines the effects of 3 treatment conditions (input variables), which are exposure time, glycerol temperature, and mechanical post-treatment, on each of the differ-

ent properties of the pericardium post-treatment (output variables), including shrinkage temperature (T_s) and stiffness of the dehydrated tissue. The T_s is the temperature at which the pericardium sample starts to contract due to the denaturation of collagen fibers. The Shrinkage Test involves heating a pericardium strip sample in a physiological solution bath at a specific temperature ramp rate. The sample is connected to a system capable of detecting displacement and the temperature at which the contraction begins.
The tissue stiffness was evaluated through two features of the stress-strain curve, defined for each dogbone shaped sample of pericardium during the uniaxial tensile test performed in accordance with ASTM D1708 using a strain rate of 0.1 mm/sec and a load cell of 1 kN: the Ultimate Tensile Strength (UTS = F_{max}/A_0, where A_0 is the sample cross-section and F_{max} is the maximum force), which is the maximum stress value reached, and the elastic modulus after the flex point (E), which is the linear tract of the high stiffness part of the curve.
The mechanical post-treatment refers to a quick drying process used to eliminate the excess of glycerol present on the patch after extraction from the drying mixture. This process involves a steel roller that mimics an automated industrial application, allowing the evaluation of potential damages.
To give statistical significance to the results, each possible combination was repeated 5 times (the maximum number of replicates suggested by the statistical software Minitab) for all 10 possible conditions, each condition was then tested for all 3 outputs. Some useful examples of the conditions are: 5 samples immersed in glycerol at 7 °C for 5 minutes, with and without post-treatment, or 5 immersed at 30 °C for 115 minutes, with and without post-treatment, or 5 immersed at room temperature for 60 minutes, with and without post-treatment, etc. All 150 samples were laser-cut from pericardium patches. Once the treated samples are tested, Minitab calculates the relationships between variables and identifies which factor or combination of factors is responsible for the obtained results.
Moreover, for each of the 10 identified conditions, residual humidity was checked in 3 tissue samples using the KF method to determine whether the dehydrating treatment was successful or not.

2.3. Verification phase

After determining the process conditions, a verification step was carried out to ensure that the identified treatment did not have any adverse effects on the properties of the pericardium over time, up to 3 months.
The pericardial patches were treated and stored in heat-sealed aluminum pouches. On the date of use, the pouch was opened, 93 samples were laser-cut from the patches in the required shape, and then rehydrated in Steridet solution, since mechanical tests have to be performed on wet samples. After this, the samples were tested for mechanical properties.
To determine the correct sample size, an internal company document was used. It recommends the use of 31 samples for each output to be evaluated for a two-tailed statistical test with a confidence level of 95% on Class III risk devices (i.e., "serious or critical risk"). The same properties as in the previous phase, namely T_s, UTS, and E, were evaluated. The verification was performed at different aging times: immediately (T0), after 1 month (T1), and after 3 months (T2). All sample groups were compared against a reference sample of pericardium that had not undergone glycerol treatment.
Residual humidity was also evaluated on 3 samples using the KF method, as in the previous phase, to determine whether the treatment was successful.
Furthermore, cutting the patches and assembling the valve after treatment with glycerol requires a dimensional verification process. In order to ensure that the performance of a hypothetical cardiac valve, with bovine pericardium leaflets treated in glycerol, is not altered in vivo, it was necessary to verify that the glycerol absorbed by the tissue did not cause any dimensional changes once rehydrated. To achieve this, 31 circular specimens with a diameter of 30 mm were laser-cut and marked with references (A and B) that define two directions at 90° to each other. The programmed dimensions at the time of cutting must match the dimensions of the specimen after rehydration in Steridet. The dimensions, measured through an optical measur-

ing system, were compared against a dry sample and evaluated, in 15 specimens, after 5 minutes of immersion and after 1 day of immersion.

3. Results and discussions

3.1. Results of the preliminary activities

The analysis of the 4 treatment conditions (groups), namely immersion for 30 seconds, 30 minutes, 2 hours, and 16 hours was conducted. To evaluate the consistency of the results, untreated bovine pericardium was used as reference. Table 2 shows the experimental results, in terms of mean and standard deviation, of the pericardium samples obtained by the oven-drying method. The mean values below indicate the average amount of weight lost after drying in the oven, based on 20 samples analysed per group.

Weight lost - Environmental chamber		
Group	**Mean**	**SD**
Reference	72.15%	2.01%
30 sec	65.54%	3.62%
30 min	69.67%	4.48%
2 h	72.88%	4.21%
16 h	70.05%	4.19%

Table 2: Experimental results obtained by the oven-drying method.

The analysis of variance (ANOVA) resulted in a p-value ($P < .001$) lower than the significance level of the test ($\alpha = 0.05$), indicating a statistical difference between the groups. Since the ANOVA test does not indicates which group in particular differs from the others, a post hoc test has to be performed. The Tukey's HSD test confirmed that the pericardium samples immersed for 30 seconds in glycerol behaved differently from the other groups. No significant difference was found between the 30 minute and 16 hours baths, which is an important finding when optimizing production time.
However, this technique has the major limitation of not being able to distinguish between the weight lost due to water and that lost due to glycerol. For this reason, the large number of oven data points was used to identify trends in group behavior, while the water content measurement precision of the KF titration was used to confirm or refute such trends.
The findings from both techniques were consistent, and the results confirmed that dehydration of the pericardium is proportional to the exposure time to glycerol.

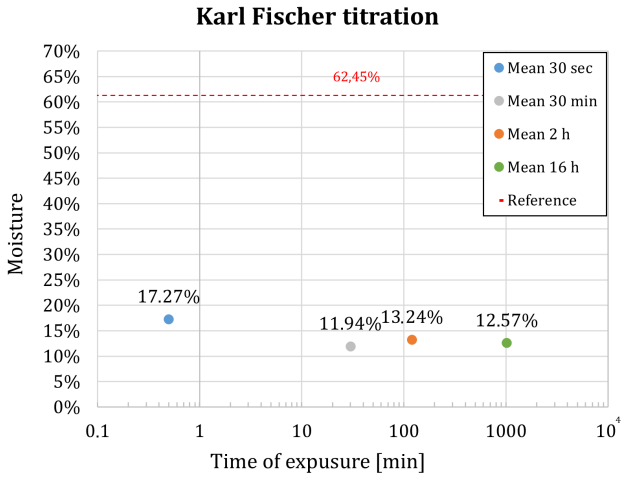

Figure 1: Graph of the experimental results obtained by KF titration.

Figure 1 shows the average amount, out of 4 samples analysed per group, of water content in the tissue after treatment in glycerol. The samples immersed for 30 seconds are more hydrated than those immersed for 30 minutes or longer, and over time, the percentage of residual water tends to plateau around 13%. The high percentage of moisture lost in the first few seconds of the bath is referred to the water present in the superficial layers of the tissue, after which the extraction of water dispersed in the internal layers becomes more gradual. Considering industrial needs, an effective immersion time in glycerol falls between 30 minutes and 2 hours.

3.2. Results of the factorial analysis

The statistical analysis results suggested that none of the input factors considered have a significant effect, with a 95% confidence interval, on the tensile mechanical properties, namely UTS and E. It should be noted, however, that the non-significance of the results may be due to the chosen operating range, and if different and more extreme parameters were used, significant differences may have been observed. Nonetheless, among the non-significant results, it was found that the mechanical post-treatment had the greatest impact on UTS.
In contrast, the T_s of dehydrated pericardium

was found to be significantly influenced by the immersion time in glycerol, particularly with prolonged immersion (115 minutes), resulting in a higher average T_s (Figure 2).

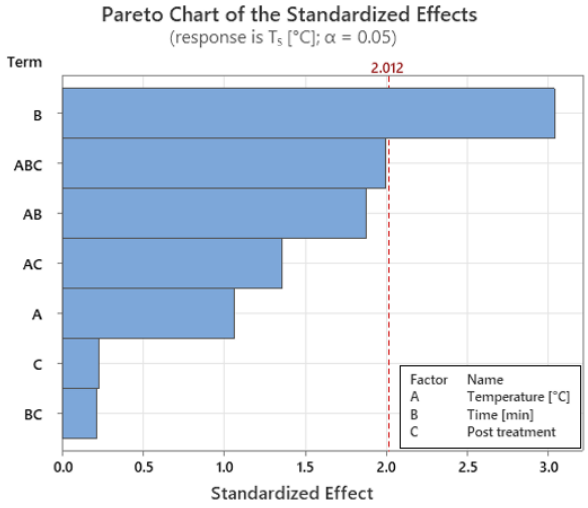

Figure 2: Pareto Chart of Standardized Effects, with $\alpha = 0.05$, used to evaluate the effects of factors on T_s.

As mentioned before, T_s is the temperature at which the pericardium begins to contract, following an increase in temperature. Thus, proteins that act as bridges between collagen macromolecules denature, and the molecular structure loses stability. An increase in T_s occurs when the virgin bovine pericardium is fixed in GA, and the number of cross-links increases, mechanically stabilizing the tissue. Therefore, this result was unexpected: glycerol does not have a cross-linking function, yet the beginning of contraction is somehow delayed. However, the observed T_s variation remained within the acceptable range defined by Corcym for untreated patches, indicating that this parameter is sensitive to other secondary factors in addition to the degree of cross-linking.
The residual moisture in the tissue was found to be significantly affected by both time and temperature. Furthermore, it was noted that immersion in a solution at 7 °C for 5 minutes was a failure: glycerol treatment is not able to prevent dehydrated samples from drying out once exposed to air. In fact, despite acceptable residual moisture values (20.63%), all samples in this group, when analysed by company labs, were found to be dry to the touch and sight. This phenomenon is due to the lack of protective action of glycerol, which did not migrate into the tissue due to the short immersion time and slowed down by the low temperature of the solution. These results demonstrate that a treatment of only a few seconds, such as the one shown in Figure 1, despite extracting large amounts of water, is not sufficient to ensure glycerol shielding and effective tissue dehydration. The highest degree of dehydration was achieved by immersing samples in a room-temperature glycerol solution for 60 minutes, with moisture values close to the plateau identified in the previous section (14.63%).
In conclusion, through the results presented above, it was possible to identify the treatment that best dehydrates the pericardium: a 2 hour immersion in a room-temperature glycerol solution. Finally, it was determined that the post-mechanical treatment with the steel roller had no influence on the properties of the tissue.

3.3. Results at different aging time

The verification activity, carried out on a statistically significant number of samples, of the identified process conditions, namely immersion for 2 hours in room-temperature glycerol solution, showed that effective dehydration of the prefixed bovine pericardium was achieved without altering its properties over time.
The glycerolized pericardium was evaluated for 3 time intervals (T0, T1, and T2), in terms of T_s, UTS, and E and compared to untreated pericardium. A MANOVA test was performed resulting in a Wilks' Lambda of 0.74 and a p-value ($P < .001$) lower than the significance level of the test ($\alpha = 0.05$). The results suggest that the treated samples, for all intervals considered, maintain the same characteristics in terms of UTS and E as the untreated one. For non-aged samples (T0), a higher average T_s and statistically different behavior were observed. This result was not confirmed in the subsequent intervals, and therefore, it could be due to the high sensitivity of the test.
It is concluded that, based on these results, up to 3 months from the treatment, the tissue's performances are not affected by glycerol. Therefore, operating with dehydrated bovine pericardium could represent an advantage in terms of optimizing the production process.

3.4. Results of the dimensional verification

The distance between the laser-etched references was measured on the 31 circular samples. The dry reference sample had a distance of 29,58 mm for reference A and 29,51 mm for reference B. After 5 minutes of rehydration in Steridet, the average distance changed to 29.64 mm (-0.21%) and 29.56 mm (-0.17%) for references A and B, respectively. After 1 day of immersion, the average distance changed to 29.57 mm (+0.04%) and 29.56 mm (+0.13%) for references A and B, respectively. Data show a normal distribution and no difference between groups was detected by the ANOVA test. It can be concluded that the effect of rehydration in Steridet on sample dimensions is negligible.

4. Conclusions and future developments

The aim of this work was to identify a treatment capable of effectively dehydrating prefixed bovine pericardium while maintaining the same properties and performance as wet pericardium, in order to optimize the production process of biological heart valve prostheses. To this end, an experiment was designed comprising of three phases: a preliminary phase to define process parameters, a factorial analysis to identify the treatment that best dehydrates the pericardium, and a verification phase to ensure that the identified treatment does not alter the properties of the pericardium over time.

This study demonstrates that the performances of the tissue remain unaffected by glycerol treatment (2 hours immersion in a room-temperature glycerol solution) up to 3 months and the effect of rehydration in Steridet over dimensional changes is on average negligible.

Therefore, operating with dehydrated bovine pericardium could represent an advantage in terms of optimizing the production process. As the achieved results are encouraging, it would be interesting to overcome the limitations of this work and further investigate the following future developments.

1. Studying failure modes. Creating and studying intentionally flawed batches of samples can be a useful method for preventing potential process errors. With these samples, investigation methods could be developed to identify patches that were not adequately treated or stored and avoid them becoming valves.

2. Improving mechanical characterization. Bovine pericardium is mechanically anisotropic, as the tissue's response to the load in terms of material strength and stiffness is regulated by multidirectional bundles of collagen fibers. However, since bovine pericardium is industrially treated as an isotropic material, as per ISO 5840, which does not identify the preferred direction of fibers, this work evaluates the biomechanical properties of dehydrated tissue only through uniaxial tensile tests. Bulge tests capable of estimating the degree of anisotropy of the material or alternatively biaxial tests could allow for more refined mechanical characterization. Furthermore, to refine the overall evaluation, not just the mechanical one, of post-treated pericardium samples in glycerol, additional outputs of interest could be evaluated, such as histological assessment of the tissue.

3. Extending aging times. A higher level of reliability regarding the results obtained by aging samples up to 3 months could be achieved by further extending aging times up to 1 year in thermosealed aluminum pouches.

4. Increasing statistical power. Increasing the sample size means increasing the value of the obtained results, but also the costs. In particular, the low number of samples dedicated to KF titration in the 3 phases is one of the major limitations of this experiment. Furthermore, an interesting future development could be to identify an alternative and more efficient methodology.

References

[1] Shahbudin H. Rahimtoola. Choice of Prosthetic Heart Valve in Adults: An Update. *Journal of the American College of Cardiology*, 55(22):2413–2426, 2010.

[2] Bart Meuris, Herbert De Praetere, Marina Strasly, Piero Trabucco, John C. Lai, Peter Verbrugghe, and Paul Herijgers. A novel tissue treatment to reduce mineralization of bovine pericardial heart valves. *Journal of Thoracic and Cardiovascular Surgery*, 156:197–206, 7 2018.

[3] Joseph E. Bavaria, Bartley Griffith, David A. Heimansohn, Jacek Rozanski,

Douglas R. Johnston, Krzysztof Bartus, Leonard N. Girardi, Thomas Beaver, Hiroo Takayama, Mubashir A. Mumtaz, Todd K. Rosengart, Vaughn Starnes, Tomasz A. Timek, Percy Boateng, William Ryan, Lorraine D. Cornwell, Eugene H. Blackstone, Michael A. Borger, Philippe Pibarot, Vinod H. Thourani, Lars G. Svensson, and John D. Puskas. Five-year Outcomes of the COMMENCE Trial Investigating Aortic Valve Replacement with RESILIA Tissue. *The Annals of Thoracic Surgery*, 2022.

[4] AOAC Official Method 950.46, Moisture in Meat, 1950.