Effect of freeze-drying on the mechanical, physical and morphological properties of glutaraldehyde-treated bovine pericardium: evaluation of freeze-dried treated bovine pericardium properties

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ABSTRACT

Purpose: Biomaterials have been widely used in the field of regenerative medicine. Bovine pericardium tissue has been successfully used as a bioprosthetic material in manufacturing heart valves, but studies concerning the tissue are ongoing in order to improve its storage, preservation and transportation. This article provides an overview of the characteristics of bovine pericardium tissue chemically treated after the freeze-drying process. These characteristics are essential to evaluate the changes or damage to the tissue during the process.

Methods: The mechanical properties of the tissue were analyzed by three different methods due to its anisotropic characteristics. The physical properties were analyzed by a colorimetric method, while the morphological properties were evaluated by scanning electron microscopy (SEM).

Results: The freeze-dried bovine pericardium showed no significant change in its mechanical properties. There was no significant change in the elasticity of the tissue (p>0.05) and no color change. In addition, SEM analysis showed that the freeze-dried samples did not suffer structural collapse.

Conclusions: It was concluded that glutaraldehyde-treated bovine pericardium tissue showed no significant change in its properties after the freeze-drying process.

Key words: Bovine pericardium, Glutaraldehyde, Freeze-drying

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INTRODUCTION

Bovine pericardium is a tissue widely used as a biomaterial in the manufacture of heart valves (1, 2). It is usually treated with glutaraldehyde, which reduces thrombogenicity and antigenicity, modifying its properties. Treatment with glutaraldehyde alters the structure of the tissue due to cross-linking between the amino groups of collagen present in the tissue and the aldehyde groups of glutaraldehyde (3).

The mechanical properties of bovine pericardium are one of the characteristics that determine the functioning of heart valves. These properties are provided by the orientation of their collagen fibers. The tissue has an anisotropic characteristic which means that the cutting direction of the sample might change the final result of the analysis (4, 5). The measurement of the color of the tissue is also important because a change in this parameter could demonstrate that the structure of the product was not preserved. Scanning electron microscopy (SEM) is an observation method of the structure of a product. It may be used to characterize structural collapse in freeze-dried products, as well.

Currently, heart valves are preserved in formaldehyde, which requires numerous procedures before use. When subjected to the freeze-drying process, these valves, if properly packaged, are preserved, dried at room temperature for a long time, and easily stored, transported and manipulated.

Freeze-drying is a sensitive drying method. It is a technique where freezable water is removed from a frozen material first by sublimation, called primary drying, followed by desorption of the unfrozen water, under reduced pressure, called secondary drying. Freeze-drying has a num-
number of advantages when compared to other drying methods. The material remains frozen until it is completely dry, so it eliminates shrinkage, while physical and chemical changes are inhibited. In addition, the loss of biological activity is minimized; and, furthermore, due to a porous texture, the lyophilized product is readily reconstituted, regaining its original size and shape when immersed in water (6-9).

This study evaluated the effect of freeze-drying on mechanical, physical and morphological properties of bovine pericardium tissue submitted to reticulation by glutaraldehyde. Three different methods were applied in order to verify the mechanical behavior of the tissue under the different type of efforts. It aimed at offering an alternative in the preservation of heart valves.

MATERIALS AND METHODS

The bovine pericardium tissue was obtained from a slaughterhouse where it was cleaned to remove fat and washed in a saline solution (0.9% NaCl). The tissue was treated with a 0.2% solution of glutaraldehyde in 0.2 M phosphate buffer (pH 7.4) at 4 °C for 2 hr. Before each analysis the samples were washed in a saline solution (0.9% NaCl) under constant stirring for 15 min repeated three times.

Freeze-drying of bovine pericardium

The freeze-drying of the product was performed by an FTS Systems freeze-dryer, model TDS-00209-A, microprocessor-controlled tray dryer (Dura-Stop, Dura-Dry-MP). Samples were placed on Petri dishes and submitted to freezing (2 °C/min) on trays until product temperature reached -50 °C. A thermal treatment was applied by heating the frozen samples to -20 °C and the temperature was maintained for 1 hr at this fixed temperature. Finally, the samples were cooled to -50 °C and the samples were freeze-dried. Primary drying was conducted at a shelf temperature of -5 °C and a pressure of 160 mTorr. For secondary drying the shelf temperature was increased to 25 °C at the same pressure. The residual moisture of the samples was 9.67 ± 2.71%. The dried samples were rehydrated in a saline solution (0.9% NaCl) at a temperature of 25 °C.

Tensile tests

The tensile tests were performed on a Stable Microsystem TA-XT2 texturometer in accordance with the ASTM D638 rule. The tests were done with 50 samples, cut randomly in different directions. The thickness of the samples was determined in three places using a digital micrometer (Mitutoyo). Tests were conducted applying an extension rate of 0.2 mm/s. The equipment registered the load (F) vs. displacement (D) data until the sample broke. The data were converted to stress vs. strain. Then, the tensile strength to break (F/Ar, where Ar is the rectangular cross-sectional area of the sample) and the elongation to break (D/Lo, where Lo is the original length of the sample) were determined. The Young’s Modulus of elasticity is given by the angular coefficient of the linear region of the stress-strain curve.

Indentation tests

The indentation tests were performed on a Stable Microsystem TA-XT2 texturometer. The device consists of a spherical probe 5 mm in diameter and a film holder. The samples were put in the holder between the two plates. The probe was driven through the sample at a speed of 0.2 mm/s. The tests were done with 50 samples of 3 cm². The equipment determined the load required for indentation (F1), the displacement of the probe from the point of contact to the point of indentation (D1) and the area under the curve related to energy to indentation. Then, the indentation strength (F1/Acs, where Acs is the cross-sectional area of the film located in the cylindrical opening of the film holder) and the elongation to indentation ([((R² + D₁²)⁰.⁵ - R)/R]*100, where R is the radius of the film exposed in the cylindrical hole of the film holder) were calculated.

Dynamic mechanical analysis

The analysis was conducted in a dynamic mechanical analysis (DMA) 242 Cell equipment (NETZSCH). Samples were submitted to compression at frequencies of 0.1, 0.5, 1, 5 and 10 Hz at room temperature. The tests were done with 50 samples of 1 cm². Only the storage modulus (E’) of the samples was determined since this parameter characterizes the mechanical behavior of the tissue.

Colorimetric methods

The analysis was conducted in a Hunter Lab spectrophotometer, model UltraScanTMXE. The reflectance of the samples was analyzed before freeze-drying and after rehydration following the CIELAB system (10, 11).

Scanning electron microscopy

The cross-section and surfaces of freeze-dried samples were observed with a scanning electron microscope JSM-7401F (JEOL Ltda) at an accelerating voltage of 2.0 kV. Samples submitted to natural evaporation in an oven at 30 °C and 30% relative humidity for 24 hr were also analyzed to permit comparison with the structure of lyophilized samples. The samples were coated with gold.
Evaluation of freeze-dried treated bovine pericardium properties

Statistical analysis

Data are expressed as mean values ± standard deviation. The differences in data average were compared by the t-test. The significance of the difference was determined at a 95% confidence limit.

RESULTS AND DISCUSSION

Color is a parameter usually measured to verify alterations in products due to processing. The results of colorimetric measurement are represented by L*, a* and b* values, where L* represents the lightness of the color, red for positive values of a*, green for negative values of a*, yellow for positive values of b* and blue for negative values of b* (10, 11). The results revealed that freeze-drying had no significant effect on the color of the samples (Tab. I).

The tensile test is a common testing procedure to characterize materials mechanically (12-15). It measures the elasticity of a material when submitted to a tensile load. Table II presents the mean values of data obtained in the tensile tests. The tensile strength describes the maximum resistance of the sample before break. The elongation rate at break represents the increase in the original length of the sample due to the applied load; and the Young’s Modulus is a measure of the elasticity of the product, given by the slope of the linear region of the stress-strain curve. The data showed that the effect of freeze-drying on the glutaraldehyde-treated bovine pericardium (GBP) was insignificant (p>0.05). Hafeez et al (14) also reported no effect of freeze-drying on tensile properties of untreated bovine pericardium.

The high standard of deviation is due to the anisotropic characteristic of the bovine pericardium (16). Because of this characteristic, the tissue was submitted to different types of stretch.

The indentation test evaluates the toughness of the material. It measures its resistance to indentation. Unlike the tensile test in which the sample is submitted to a deformation (Δl1) in its original length (l) until break (Fig. 1A), in the indentation test the sample is submitted to another kind of deformation (Δl2) in its original radius (r), the radius of the film exposed in the cylindrical hole of the film holder, until indentation (Fig. 1B). The indentation strength, elongation to indentation, energy to indentation and elasticity to indentation were determined (Tab. III). In this case, the elasticity was also calculated as the slope of the stress-strain curve before film break but was called the film holder, until indentation (Fig. 1B). The indentation strength, elongation to indentation, energy to indentation and elasticity to indentation were determined (Tab. III). In this case, the elasticity was also calculated as the slope of the stress-strain curve before film break but was called

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Fig. 1 - Schematic of the sample deformation under tensile test (A) and indentation test (B).
change in their mechanical, physical and morphological properties after the freeze-drying process, encouraging freeze-drying as a preservation method of the tissue.

Based on the data in Tables II and III, the tissue presented higher strength, elongation and elasticity to indentation than to break. No significant difference was observed from the data for fresh GBP and freeze-dried GBP ($p>0.05$).

The DMA exposes the sample to a sinusoidal deformation. Figure 2 shows the storage modulus ($E'$) of fresh and freeze-dried samples as a function of frequency. This parameter is related to the amount of stored energy during the cycle, indicating its elastic behavior.

The $E'$ values were the same for fresh and freeze-dried samples ($p>0.05$). Examination of the mechanical data from different tests showed that they complemented each other, demonstrating the maintenance of the elastic property of glutaraldehyde-treated bovine pericardium after the freeze-drying process.

GBP samples were submitted to natural evaporation to cause structural collapse, and their structure was compared to freeze-dried samples by SEM, as shown in Figures 3 and 4.

A tangle of fibers was observed on the surfaces of the evaporated samples (Figs. 3C, D) when compared to freeze-dried samples (Fig. 3A, B). In Figure 4 a structure with loss of porosity is clearly visible in the evaporated samples (Fig. 4B) when compared to the freeze-dried samples (Fig. 4A).

It was found that GBP retained its structure after the freeze-drying process. Borgognoni et al (9) also demonstrated the maintenance of the untreated bovine pericardium structure by Raman spectroscopy after the freeze-drying process.

It was concluded that bovine pericardium reticulated in glutaraldehyde demonstrated no significant
Evaluation of freeze-dried treated bovine pericardium properties

Conflict of interest: The authors certify that there is no conflict of interest with any organization regarding the material discussed in the manuscript.

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REFERENCES