The Effects of Cryopreservation on the Biaxial Mechanical Properties of Canine Saphenous Veins

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The experimental and analytical methods presented in the companion paper [1] are used here to study the effects of cryopreservation on the in vitro biaxial random-elastic mechanical properties of canine saphenous veins. The properties of specimens tested in their physiological range of loadings immediately after thawing were not significantly different from their properties before cryopreservation. However, they stiffened significantly in the few hours following thawing. This effect was not observed for aging fresh specimens, nor for cyanide-poisoned specimens, indicating that the static tone of the venous smooth muscle may be affected by cryopreservation and thawing, but the elastin and collagen fibers are most likely unaffected.

Introduction

Cryopreservation is a process by which biological tissues or fluids are frozen at controlled rates. Optimized freezing and thawing protocols, associated with storage, preferably at the temperature of liquid nitrogen (−196°C), ensure that the cells of the tissue remain viable [2]. Cryopreservation considerably extends the possibilities for organ and tissue banking [3].

Although autograft arteries and veins are the conduits of choice for vascular graft surgery, they come in short supply and alternate graft materials, such as synthetic grafts or cryopreserved vein allografts, are often necessary. All the biological and physical properties relevant to the patency of these grafts need to be assessed [4]. This includes the mechanical properties, despite the fact that it is not yet clear which mechanical factors have an impact on graft patency [5].

The biaxial mechanical properties of fresh, let alone cryopreserved, saphenous veins are not yet documented. This is due in part to long-standing difficulties associated with obtaining reliable mechanical property data on highly nonlinear blood vessels. The experimental and analytical methods presented in the companion paper, which address these difficulties, are used here to determine and compare the biaxial mechanical properties of fresh and cryopreserved canine saphenous veins.

Methods and Materials

The hardware and the experimental design have been described in [6] and [7]. For this study, canine saphenous vein specimens, approximately 25 mm in length and 4.5 mm in diameter *in situ,* were systematically tested twice within 8 hours of procurement. Procurement, storage, perfusion and testing of the vessels took place in Hank's Balance Salt Solution (HBSS) supplemented with papaverine (12 mg/l) and Penicillin/Streptomycin (5 ml/l of HBSS solution). This solution was changed before each test sequence.

After mounting, specimens were equilibrated at 37°C for 30 minutes during which time preconditioning, consisting of several inflation and stretching cycles within the physiological range of loading, took place. Tests followed the randomized Central Composite Design (CCD), described in the companion paper [1], with the measured biaxial loads lying in the physiological range. The total plugged-end force F ranged from 4.9–201 mN (0.5–21 g) during longitudinal stretch and the luminal pressure P varied from 133–5453 Pa (1–41 mmHg) for inflation. The order of collection of the data was randomized, resulting in a different sequence for each test. The radius and length of the central region of the specimens were measured before, during and after each test as well as at the reference loaded state, the CCD center, defined by F_c = 103 mN (10.5 g) and P_c = 2793 Pa (12 mmHg).

Data in the companion paper [1] indicated that no significant change occurs in fresh specimens up to 8 hours after dissection. Thus, for any specimen, fresh or thawed, the time interval between consecutive CCD tests was not controlled exactly, but was between 1 and 6 hours. Between tests, specimens were kept at 37°C in the same solution.

Seventeen fresh specimens were tested and subdivided into three groups as outlined in Fig. 1.

Cryopreserved Group. After the initial series of two tests, eight specimens were refrigerated at 3–5°C until the next day when cryopreservation took place. After at least 4 days storage at −196°C, specimens were thawed by a stepwise process, achieving dilution of the cryoprotectant dimethyl sulfoxide [4]. The testing protocol described above was then repeated on the thawed specimens. The first test on thawed specimens took place a average of 2.4 (standard deviation, Std. = 1.1) hours after the completion of the thawing process, and the second followed thawing by an average of 5.4 (Std. = 2.9) hours.

Two subgroups of specimens were defined. In order to investigate the effect of short-term post-thaw refrigeration, specimen H4 was tested four times before cryopreservation (instead of two times) and refrigerated for four hours before a series of four post-thaw CCD tests. Also, the effect of long-term (a few days) refrigeration after thawing was investigated using five specimens tested on subsequent days after thawing.

Cold-Stored Control Group. Four specimens were refrigerated and tested on subsequent days to document aging effects. Using the protocol described above, specimens were refrigerated between test sequences on different days.

KCN-Poisoned Control Group. Five specimens were poisoned after fresh testing by perfusion with potassium cyanide (200 mg/l KCN) for 2 hours at 37°C, tested again, cryopreserved the next day and tested after thawing.

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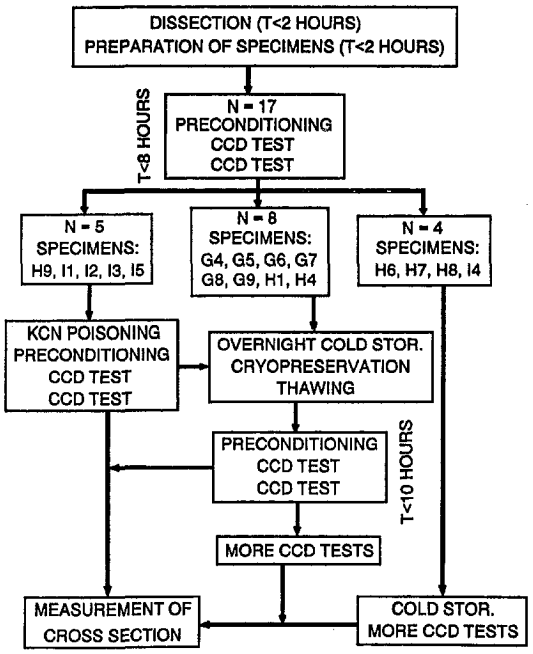


Fig. 1 Summary of the experimental protocol showing the division of the specimens into the various groups

Data Analysis

Following the notation used in the companion paper [1], the model used to reduce the data expresses the complementary energy per unit length of a vessel segment as a function $W'_C(F', P')$ of the two variables F' and P' [6, 7]. The function $W'_C(F', P')$ is assumed to be a linear combination of three basic functions g_k, the principal component functions, chosen as fourth-order polynomials, as described in the companion paper [1]:

$$W'_C(F', P') = F_R \sum_{k=1}^{3} d_k g_k(F', P') \tag{1}$$

where $F_R = 98$ mN (10 g) is the radius of the CCD, d_k are the fitted parameters and

$$F' = \frac{F - F_C}{F_R} \tag{2}$$

$$P' = \frac{P - P_C}{P_R} \tag{3}$$

$$g_k(F', P') = \sum_{j=1}^{10} a_j(F', P') m_{jk} \tag{4}$$

In Eq. (4), the $a_j(F', P')$ are the monomials F'^2, $F'P'$, P'^2, F'^3, F'^2P', $F'P'^2$, P'^3, F'^4, F'^3P', $F'^2P'^2$. The functions g_k are assumed to be common to all specimens in any state (fresh, cryopreserved, aged or . . .), and are obtained from a reference database of CCD data sets from 16 different specimens by the Principal Component Analysis-based method exposed in the companion paper [1]. This reference CCD database included data from the first fresh test on 14 of the 17 specimens referenced in this paper, the exceptions being I3, I4, and I5.

All CCD data sets (fresh, cryopreserved or poisoned) were fitted to the three regressors, values of the functions at the design points, resulting in three material parameters d_i per test. Changes in mechanical properties were assessed by Analysis of Variance [8] on the values of the material parameters determined using linear regression with indicator variables. Two-sided p-values were computed with Bonferroni's correction for multiple comparisons [8, 9].

Results

Cold-Stored Control Group (Fig. 2). No significant trend for any of the parameters d_i is observed for these specimens ($p > 50$ percent for all d_i). The reference loaded radius of the specimens did not change either ($p > 50$ percent) [6].

Cryopreserved Group (Figs. 3, 4, 5). The parameter d_1 decreases slightly, indicating stiffening of the specimens (difference in the means, $\Delta d_1 = -0.03$), between the first and second

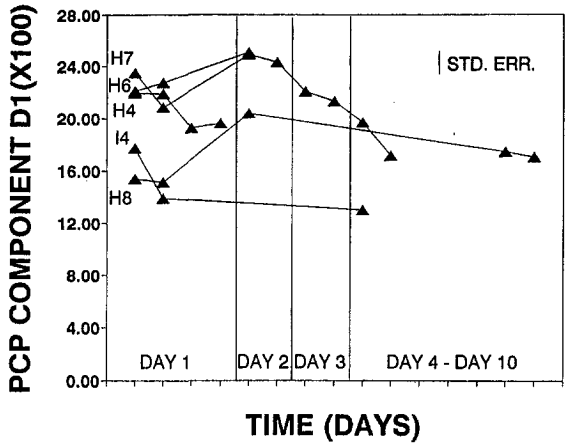
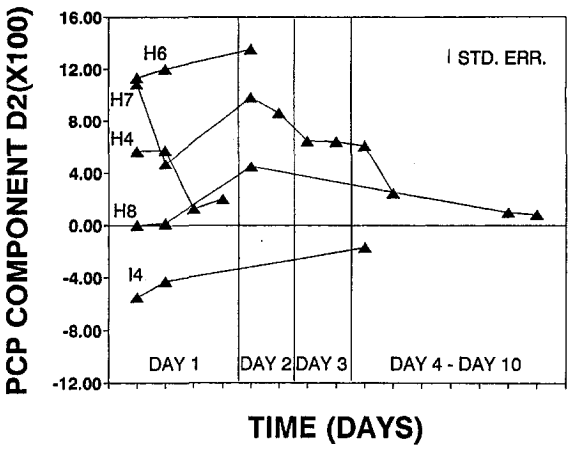


Fig. 2(*a*)



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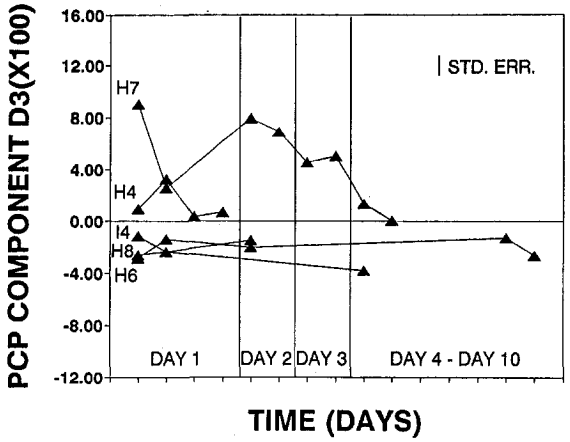

Fig. 2(*c*)

Fig. 2 The effects of aging of the fresh specimens on the coefficients d_i of the principal component polynomials g_i: (*a*) d_1, (*b*) d_2, (c) d_3

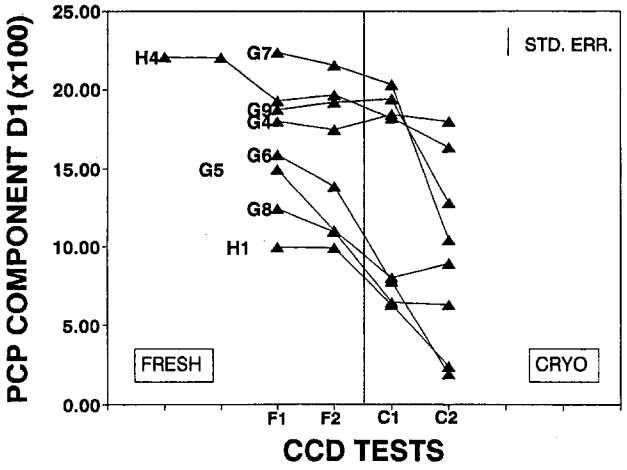

Fig. 3(*a*)

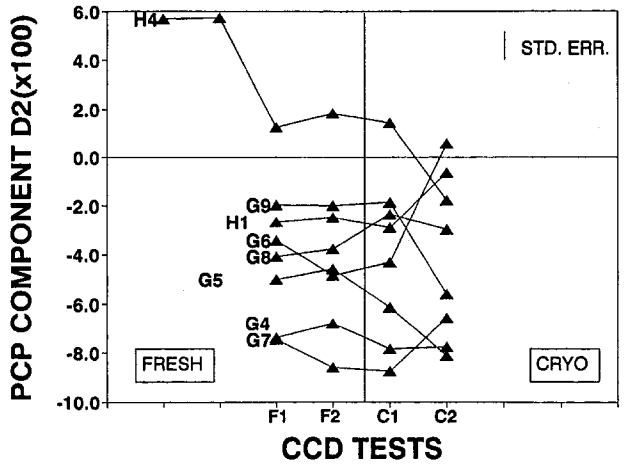

Fig. 3(*b*)

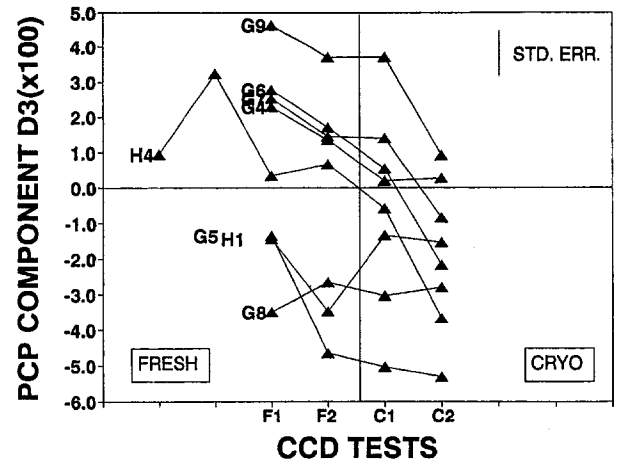

Fig. 3(*c*)

Fig. 3 Evolution of the principal component polynomial coefficients d_i for cryopreserved specimens (first day) (*a*) d_1, (*b*) d_2, (*c*) d_3. Axis labels F1 and F2 refer to two tests conducted within 6 hours of disection. Axis labels C1 and C2 refer to tests conducted 2.4 hrs (avg.) and 5.4 hrs (avg.), respectively, post-thaw. Specimen H4, which was refrigerated before testing (see text), is included for comparison.

CCD test after thawing (Fig. 3(*a*)). The largest decrease (44 percent) is observed for specimen G6. The difference between the last fresh test and the first cryopreserved test is borderline significant ($t = -2.9$, $5 < p < 6$ percent) but becomes statistically significant when the second cryopreserved test is compared to fresh results ($\Delta d_1 = -0.06$, $t = 6.4$, $p < 1$ percent, $n = 8$).

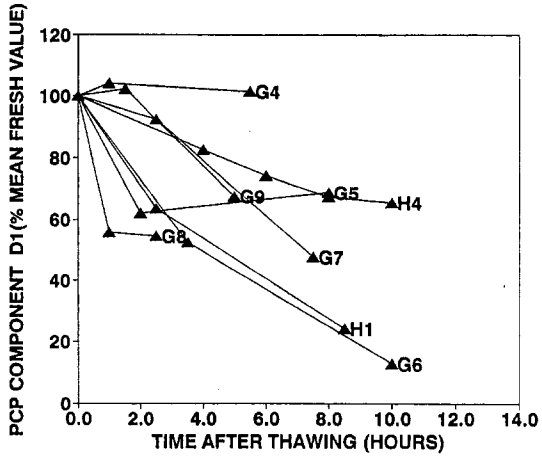

Fig. 4 Effects of cryopreservation on d_1 with increasing post-thaw time

No such changes are observed for the second component d_2 (Fig. 3(*b*), $\Delta d_2 = -0.006$, $t = -0.78$ for both tests, $p > 20$ percent), nor for the reference radius ($p > 40$ percent). The evolution of d_3 (Fig. 3(*c*)) parallels that of d_1: a borderline significant decrease ($\Delta d_3 = -0.01$, $t = -1.8$, $10 < p < 16$ percent) at first, confirmed by the second cryopreserved test ($\Delta d_3 = -0.04$, $p < 1$ percent).

Figure 4 shows the evolution of d_1, normalized by its average fresh value, with time after thawing. An approximate exponential fit shows that d_1 decreases by about 10 percent of its fresh value per hour. Additional data on later days was collected on 5 of these 8 specimens (Fig. 5). Although no meaningful statistics can be drawn from such a small subgroup, the trend seems to level off at about 25 percent of the fresh d_1 value 2 days after thawing (G7 = 22, H1 = 23 percent, G6 = 27 percent). There is no consistent pattern to the further evolution of d_2 and d_3 and the reference radius (data not shown, $p > 10$ percent [6]).

Specimen H4, subjected to refrigeration between thawing and testing, showed a much less dramatic decrease in the parameter d_1 (Fig. 3(*a*)).

KCN-Poisoned Control Group (Fig. 6). The coefficient d_1 decreased significantly after poisoning by $\Delta d_1 = -0.05$ ($t = -6.3$, $p < 2.5$ percent) on the average, but no further changes are observed after cryopreservation and thawing ($t = -2.8$, p

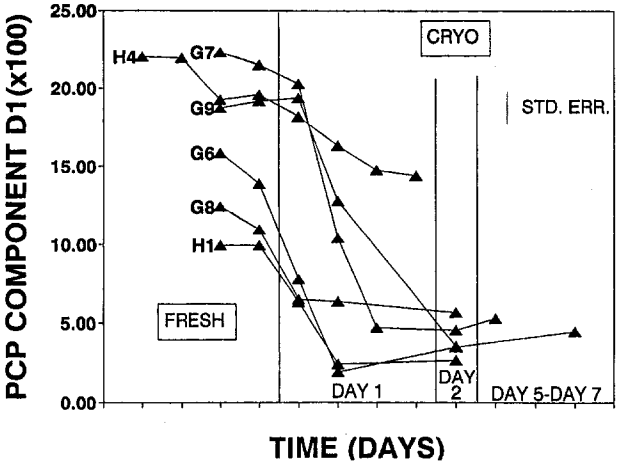

Fig. 5 Effects of cryopreservation and subsequent aging on d_1 values. Specimen H4, which was refridgerated prior to testing (see text), is included for comparison.

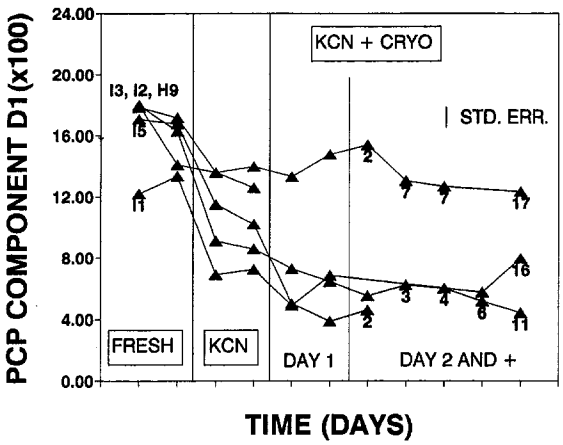

Fig. 6 Effects of cyanide poisoning and subsequent cryopreservation on d_1 values

$>$ 10 percent). The reference radius and components d_2 and d_3 show no significant changes at either stage (data not shown, p $>$ 20 percent [6]).

Discussion

Advantages of the Method. We present here the first use of Principal Component Analysis in quantitative biomechanical modeling and in application to a clinically relevant problem: the first fully biaxial study of fresh and cryopreserved canine saphenous veins. First, the advantages of the method over classical stress-strain modeling are discussed, with respect to its numerical stability and its independence of thickness measurements or changes. The clinical relevance of the study is then discussed.

Biomechanical studies of biaxially tested tissues accompanied with classical nonlinear regression techniques are fraught with numerical instability, making comparative studies difficult. In contrast, the companion paper [1] demonstrated that the three parameters arising from the model can be repeatably measured from test to test ("test-to-test" standard error $<$ 0.02) and are clearly different from specimen to specimen ("specimen-to-specimen" standard deviation $>$ 0.03). Not only can these coefficients be assumed to be representative of three different material properties, but they are minimal in number and ordered by decreasing importance, most of the data (85 percent of the total variance in the case of the fresh database) being captured by d_1, which is always positive. The two remaining terms provide small corrections on either side of the general pattern established by d_1. The predominance of the first component conveniently reduces the discussion of the mechanical properties to that of the first coefficient.

Another major numerical advantage is that the parameters are quite easy to calculate using a simple dot product, the simplest form of linear regression, once the preliminary work of determining the regressors, the principal component functions, from the reference database is completed.

An additional advantage of this description of biaxial blood vessel mechanics, based on the measured total force F and luminal pressure P, instead of stresses and strains, is that thickness is not needed to compute the coefficients of the complementary energy $W'_C(F', P')$. This is also the case for thin-wall models which are formulated in terms of resultants and hence the reference value for thickness does not appear. In contrast, absolute values for the reference and deformed thickness are needed in thick-wall, large-deformation models. The reference thickness of a given specimen must also be assumed to be unchanged by a given treatment. This hypothesis is not necessary within our formulation. For the sake of completeness, the thickness of the specimens was measured after all tests were finished. No correlations were found between thickness and the material properties d_i's [6].

We conclude that, for practical applications, such as controlled comparative studies like this one, the first material parameter d_1 has the properties required of a compliance index characterizing the biaxial properties of blood vessels: repeatability, ease of measurement and computation, and independence from thickness.

Effects of Cold Storage and Cryopreservation. The cold storage control group indicates that the mechanical properties of fresh canine saphenous vein are not affected consistently by cold storage and repeated testing over a few days. However, this variation, on the order of 0.2 for d_1, is not all accounted for by the test-to-test variation level of the parameters (test-to-test standard error $<$ 0.02). Day-to-day variation is, in all likelihood, due to residual smooth muscle tone variation, despite the presence of the smooth muscle relaxant papaverine in the testing and storage solution. Our success in keeping specimens free from spasm, a usually fast, irreversible and therefore often damaging contraction of the muscle, confirms the beneficial role of papaverine for vein grafts ([10], [11]). However, the data seem to indicate that smooth muscle activity is not completely inhibited by papaverine.

Specimens that have not been kept in papaverine-supplemented solutions usually spasm either during dissection or mechanical testing. For instance, a specimen not referenced here [6] spasmed between fresh tests. Results showed that d_1 decreased from 0.118 to 60 percent of its original value and the reference diameter decreased by 50 percent.

When cryopreserved veins are tested for the first time after thawing, they are either similar to or stiffer than fresh. By the time of the second test, most specimens have started stiffening. The time course of the stiffening strongly suggests that specimens usually have the same elasticity just after thawing as they had before cryopreservation, and start stiffening shortly thereafter, sometimes before the first test takes place. Such a trend was not noticed with aging fresh specimens. The effect of cryopreservation on the mechanical properties of saphenous veins is thus distinct from that of cold storage, aging or spasm.

Control experiments on KCN-poisoned vessels demonstrated that the stiffening does not happen in specimens whose smooth muscle components have been rendered inactive implying that smooth muscle is responsible for post-thaw stiffening. However, the elastic components of the wall (elastin and collagen fibers) are relatively unaffected by cryopreservation and should be equally able to withstand loads as well as before cryopreservation. Cryopreservation therefore increases the passive elastic properties of vascular smooth muscle, probably through gradual depressing of the ability of the smooth muscle to change dimensions passively in response to purely mechanical loads, a phenomenon possibly related to the onset of rigor. On the other hand, aldehyde tanning, another source of material for vascular or valvular prostheses, eliminates smooth muscle viability, promotes cross-linking of collagen and results in a very stiff material. Aldehyde-fixed material, however, is prone to degeneration [12]. In contrast, cryopreserved veins are able to synthesize collagen [13] and are thus expected to demonstrate a lesser tendency to aneurysm, as clinical studies tend to show.

These observations confirm previous uniaxial studies of saphenous veins. In isometric smooth muscle tests, Brockbank et al. [3], [13] found that cryopreserved vein rings could only contract to 60 percent of the force elicited in the fresh state by vasoactive reagents. Showalter et al. [14] showed that the compliance of cryopreserved saphenous veins at arterial pressures was half that of fresh veins, but did not identify the process nor the time course of the stiffening. Furthermore, their study did not distinguish the effects of cryopreservation from those of arterial pressures on the venous tissue. The present investiga-

tion, limited to venous pressures, was able to single out the effects of cryopreservation alone.

It is remarkable that none of the groups of specimens studied demonstrated any significant change in the reference diameter. Hence, the stiffening of cryopreserved specimens is not related to spasm nor is it related to microbial decay. Both fresh or cryopreserved specimens kept in antibiotic-free solution suffer a dramatic loss of strength of the vessel after a few days [6]. Finally, results for specimen H4 (Fig. 3), which was kept refrigerated for three hours before the second series of tests, indicate that refrigeration may be beneficial in slowing the stiffening process. Therefore, pre-cryopreservation refrigeration in antibiotic-laced storage media seems to have little effect on the strength of vascular tissue. However, under these conditions, cellular viability decreases over time.

This study provides several elements relevant to vascular grafting of cryopreserved saphenous veins. First, cryopreserved veins should be grafted as soon as possible after thawing and should be refrigerated if implantation is delayed. Showalter's data [14] indicate that post-thaw evolution of vein mechanics might not be interrupted by renewed exposure to blood flow in vivo. However, a better-controlled study and the development of a storage solution for vein grafts, able to preserve the properties of the vascular smooth muscle, are needed.

The reference radius was not affected by any of the different means of storage investigated [6]. A vein graft stored in a solution with papaverine will, after either refrigeration or a cycle of cryopreservation/thawing, have the same radius. The vascular surgeon will therefore manipulate a graft with the same diameter as the original vessel. Accurate diameter matching for a bypass to a given site in the arterial tree is thus possible. Finally, assuming that the ability of the surgeon to manipulate the tissue is related to mechanical properties, this study shows that, at least in the canine model, the handling of cryopreserved vein grafts is similar to that of fresh vein grafts.

Conclusion

The practical relevance of innovative approaches to biaxial tissue mechanics has been demonstrated. Experimental design and modern data-processing methods yielded reliable numerical results without sacrificing the rigor and mechanical formalism favored by biomechanicists.

Canine saphenous veins were shown to be affected, in a mechanical sense, by cryopreservation in ways that differ from those of spasm and cold storage. Cryopreservation effects the smooth muscle of the vessels resulting in a gradual loss of biaxial compliance which seems to be slowed by short-term refrigeration.

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