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BRIEF COMMUNICATION

Prior Freezing Has Minimal Impact on the Contractile Properties of Permeabilized Human Myocardium

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BACKGROUND: Experiments measuring the contractile properties of human myocardium are important for translational research but complicated by the logistical difficulties of acquiring specimens. Accordingly, many groups perform contractile assays using samples that are acquired from patients at one institution and shipped to another institution for experiments. This necessitates freezing the samples and performing subsequent assays using chemically permeabilized preparations. It is unknown how prior freezing affects the contractile function of these preparations.

METHODS AND RESULTS: To examine the effects of freezing we measured the contractile function of never-frozen and previously frozen myocardial samples. Samples of left ventricular tissue were obtained from 7 patients who were having a ventricular assist device implanted. Half of each sample was chemically permeabilized and used immediately for contractile assays. The other half of the sample was snap frozen in liquid nitrogen and maintained at –180 °C for at least 6 months before being thawed and tested in a second series of experiments. Maximum isometric force measured in pCa 4.5 solution, passive force measured in pCa 9.0 solution, and Hill coefficients were not influenced by prior freezing (P=0.07, P=0.14, and P=0.27 respectively). pCa_{50} in never-frozen samples (6.11±0.04) was statistically greater (P<0.001) than that measured after prior freezing (5.99±0.04) but the magnitude of the effect was only ≈0.1 pCa units.

CONCLUSIONS: We conclude that prior freezing has minimal impact on the contractile properties that can be measured using chemically permeabilized human myocardium.

Key Words: biobanking ■ contractile function ■ human myocardium

Cardiovascular research is a multidisciplinary field that seeks improved therapies for patients. Among the most important techniques are assays of contractile function performed using chemically permeabilized samples. These experiments provide insights into the fundamental mechanics of sarcomeres and the molecular effects of emerging therapies for heart failure such as omecamtiv mecarbil, mavacamten, and danicamtiv. Although animal models can be useful in these experiments, there is always a challenge in generalizing these results to human patients. For this reason, human myocardial samples provide a robust and translationally significant opportunity to study heart disease and potential therapies.

The procedures used to collect myocardial samples for research cannot be allowed to compromise patient care. Accordingly, ventricular myocardium is typically collected only during surgeries in which standard of care involves removal of sections of the heart. Heart transplants and left ventricular assist device implantations serve as the primary opportunities for fresh specimen collection. In comparison to the over 200 000 coronary artery bypass graft procedures that occur annually in the United States, heart transplants and

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left ventricular assist device implantations are relatively rare with ≈3200 and 2000 procedures, respectively, performed annually in United States. This can make it difficult to schedule experiments and plan research projects because it is unknown when the next opportunity to collect fresh myocardium may arise. Many researchers use previously frozen samples to overcome these challenges because the tissue can be acquired at a separate time or place and stored. The collection and freezing of human myocardium also allow for more unique tissue selection as a researcher could collect tissue with a specific pathology, for example, nonischemic heart failure secondary to myocarditis, over the course of many months to years before doing research on the set of collated samples.

Although prior freezing is a common practice, it is not clear what impact it may have on the contractile properties of human myocardium when thawed and used for permeabilized, multicellular preparations. In this study multicellular preparations were chemically permeabilized to disrupt the cell membrane and allow the calcium concentration near the myofilaments to be controlled. This allowed for the measurement of various aspects of contractile function such as force generation and calcium sensitivity, among others. This study compared the contractile properties of preparations of never-frozen human myocardium with previously frozen preparations to investigate the effects of prior freezing.

METHODS

The data that support the findings of this study are available from the corresponding author upon request.

Procurement of Human Samples

Through-wall apical cores of ventricular myocardium were obtained from patients who were receiving a left ventricular assist device at the University of Kentucky. The samples were placed immediately in an insulated cooler with ice and transferred to clinical pathology where one third of the sample was taken for clinical analysis. The remainder was taken to the research laboratory where it was split into 2 equal parts. One portion was placed in a cryogenic vial, dropped into liquid nitrogen, and subsequently transferred to the vapor phase of liquid nitrogen where it was stored for at least 6 months before being used for contractile experiments. The vial containing a frozen sample of myocardium was removed from the storage tank on the day of the experiment and allowed to thaw at room temperature before homogenization. The remaining sample was immediately chemically permeabilized and used for contractile experiments within hours. All samples were kept in an insulated cooler with ice until they were frozen or permeabilized no later than ≈1 hour after being removed from the patient. This method of temporary storage and short time frame minimizes ischemic damage to the tissue.[1] The workflow for preparing these frozen and never-frozen samples is illustrated in Figure 1. The University of Kentucky Institutional Review Board approved all procedures and patients gave informed consent for the use of their specimens (IRB 46103). A detailed description of the procurement system has already been published.[2] The demographics and clinical characteristics of the patients used in this study are reported in Table S1.

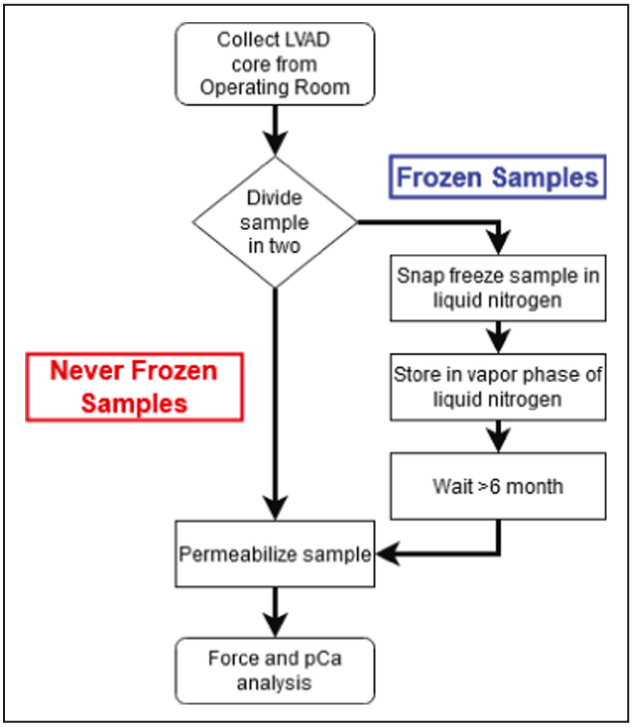

Figure 1. Procedure for paired fresh and frozen experiments. LVAD indicates left ventricular assist device; and pCa, a measure of calcium concentration where $pCa=-\log_{10}[Ca^{2+}]$

Preparations

Multicellular preparations were obtained by mechanical homogenization as described[3] and then chemically permeabilized (30 minutes, 1% v/v Triton detergent). Myocardial preparations from 7 patients were attached between a force transducer (resonant frequency, 600 Hz; Model 403, Aurora Scientific, Aurora, Ontario, Canada) and a motor (step time 0.6 ms; model 312B, Aurora Scientific) as previously described.[4,5] Each preparation was stretched to a sarcomere length of 2.26±0.01 μm (range: 2.24–2.28 μm) in pCa 9.0 solution (where $pCa=-\log_{10}[Ca^{2+}]$). All measurements were performed at 37 °C using SLControl software.[6]

Respective dimensions of the never-frozen and frozen preparations were as follows: cross-sectional area: 5.00±1.59×10^{-8} m^2, 5.26±1.84×10^{-8} m^2, P=0.651; preparation length: 998±197 μm, 740±116 μm, P<0.001. Multicellular preparations often vary in both length and width. By scaling the length changes imposed during the experiments to each preparation's length and normalizing force to cross sectional area, we can account for this variation and compare force generation between preparations of different sizes. Plots comparing the preparation dimensions can be found in Figure S1.

Preparations were placed into each calcium solution, allowed to reach a steady state force (≈3 seconds), subjected to a series of length changes (≈2 second), and then returned to a relaxing solution. The preparations were therefore activated for only ≈5 seconds per calcium solution for a total of ≈60 seconds of activation. Preparation rundown was assessed as the percent difference in maximal force per cross-sectional area measured at the beginning and end of each experiment session (respective never-frozen and frozen preparation rundown: 13.4±7.4%, 8.6±7.8%, P=0.091). This level of rundown is similar to previously published data in human myocardium at 37 °C.[7]

Contractile Function Measurements

Once positioned between the force transducer and motor, preparations were activated briefly in a saturated Ca^{2+} solution with a pCa of 4.5. Maximum isometric force was defined as the steady-state force while fully saturated with calcium at pCa 4.5. A preactivation solution with reduced calcium buffering was not used in these experiments. Additional trials measured force in solutions with lower Ca^{2+} concentrations ranging from pCa 9.0 to 4.8. Tension pCa curves were generated by fitting these data to a form of the Hill equation:

$$F = F_{pas} + F_{act}\frac{\left[Ca^{2+}\right]^{n_H}}{\left[Ca^{2+}\right]^{n_H} + \left[Ca^{2+}_{50}\right]^{n_H}}$$

where F_{pas} is passive force, F_{act} is maximum active force, n_H is the Hill coefficient, and Ca^{2+}_{50} is the concentration of Ca^{2+} in solution, which generates half-maximum active force. The rate of tension recovery, k_{tr}, was assessed by rapidly (≈1 ms) shortening the muscle by 20%, holding it at the short length for 20 ms, and then stepping the muscle back to its original length. k_{tr} was determined by fitting a function of the form $y=A\cdot(1-B\cdot\exp(-k_{tr}\cdot t))$ to the force recovery trace where A and B are constants.[8]

Statistical Analysis

Hill-curves $\left(F = F_{pas} + F_{act}\left(\left[Ca^{2+}\right]^{n_H}/\left(\left[Ca^{2+}\right]^{n_H} + \left[Ca^{2+}_{50}\right]^{n_H}\right)\right)\right)$ were fit to experimental data from each preparation using MATLAB (Mathworks, Natick, MA) to calculate 4 physiological parameters: passive force (F_{pas}), active force (F_{act}), Hill coefficient (n_H), and Ca^{2+}_{50}. Physiological parameters and fiber dimensions were analyzed in SAS version 9.4 (SAS Institute, Inc., Cary, NC) using linear mixed models to examine the effects of prior freezing. Each statistical model included freezing status as a fixed effect, a random effect for subject, and the appropriate physiological parameter or fiber dimension as the response variable. This approach accounts for repeated measures with different preparations from the same heart and takes advantage of the paired study design. P<0.05 was considered significant. Data are reported as mean±SEM, unless otherwise specified.

RESULTS

Figure 2 shows pCa-tension curves of both never-frozen and frozen preparations and a comparison of the 4 parameters obtained from the curve fits. Force generated at pCa 9.0 and pCa 4.5 were unaffected by prior freezing (P=0.135, P=0.071, respectively). Similarly, there were no observed changes in the Hill coefficient (n_H) of preparations after freezing (P=0.267). There was an increase in the pCa_{50} of preparations after freezing (P<0.001). Figure 3 shows representative force records from a never-frozen preparation. The k_{tr} values of both experimental groups increase similarly with increasing isometric force. Additionally, statistical analysis shows the k_{tr} values of both experimental groups measured in maximally activating solution of pCa 4.5 are unchanged by prior freezing (P=0.977).

DISCUSSION

Because of the scarcity and sporadic availability of fresh human myocardium for use in research, many researchers have chosen to use samples that have been frozen before use. However, there has been little research on the potential effects of freezing on myocardium. Previous work has anecdotally shown myofibrillar disorganization in previously frozen permeabilized single cell fragments but did not compare contractile function.[10] To our knowledge this is the first study to examine how prior freezing of human myocardium affects the contractile properties of chemically permeabilized myocardial preparations.

In this study, analysis of paired never-frozen and frozen samples shows that prior freezing has minimal effect on the force generation of isolated human

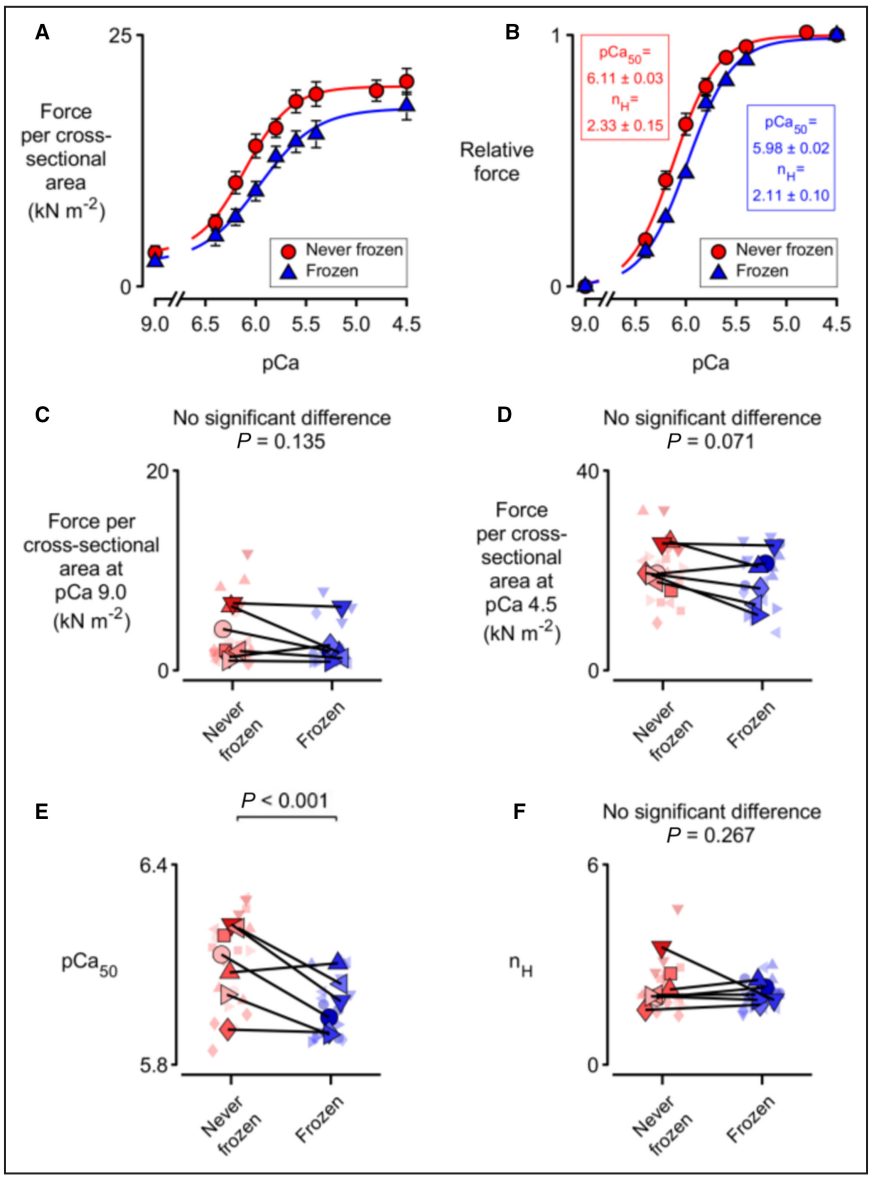

Figure 2. Prior freezing does not alter force generation nor the Hill coefficient but does minimally increase pCa_{50}.
Data from multicellular preparations of never-frozen and frozen myocardium fit to (**A** and **B**) pCa-tension curves. Analysis with linear mixed models shows prior freezing has no significant effect on (**C**) force generation at minimal activation, (**D**) force generation at maximal activation, and (**F**) cooperativity (n_H); however, freezing does result in a slight decrease in (**E**) calcium sensitivity (pCa_{50}). Superplots display each patient as a unique symbol with pale symbols representing individual multicellular preparations and filled symbols representing the mean values for each patient.[9] pCa is a measure of calcium concentration where $pCa=-\log_{10}[Ca^{2+}]$

myocardium. Figure 2C shows no significant difference in the force generated by frozen and never-frozen preparations in pCa 9.0 solutions. Force generated at 9.0 pCa reflects the passive tension generated by the extracellular matrix and elastic proteins such as titin. The lack of observed change in force generated at pCa 9.0 indicates prior freezing has no significant effect on the passive tension of multicellular, chemically permeabilized human myocardial preparations. Likewise, Figure 2D shows no significant difference in the force generated at maximal activation between never-frozen and frozen preparations.

The Hill coefficient (n_H) is graphically interpreted as the steepness of the pCa-tension curves, which in Figure 2A and 2B are similar for both experimental groups. Further analysis in Figure 2F shows no

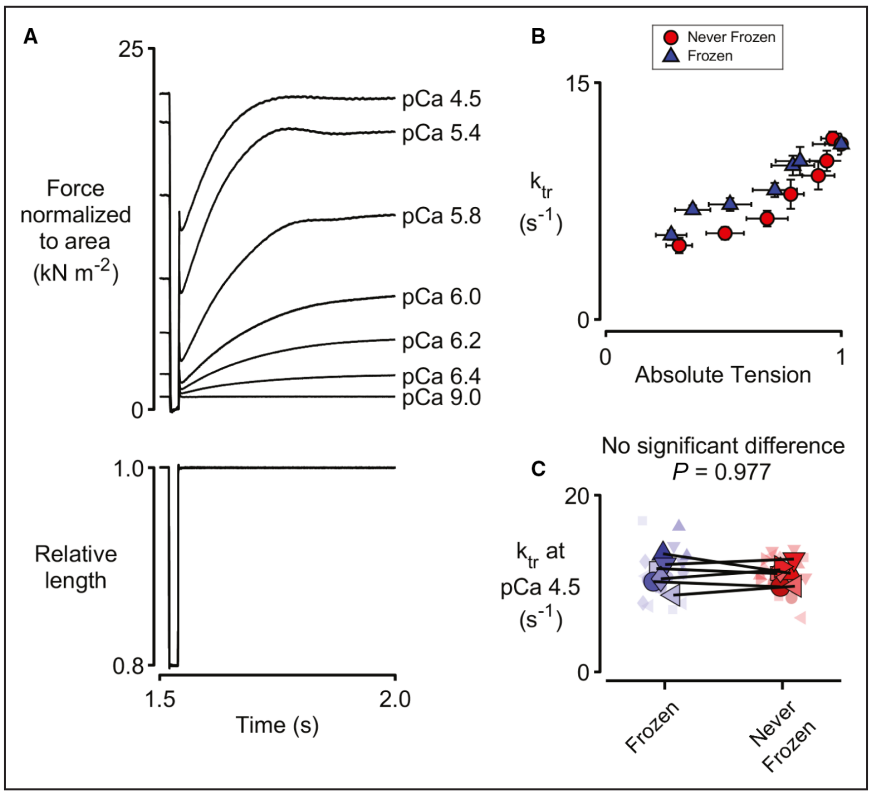

Figure 3. Rate of tension recovery is not affected by prior freezing.
A, Representative traces showing (top) force normalized to cross-sectional area and (bottom) relative length for a preparation during a shortening/restretch protocol (20% muscle length reduction, 20 ms hold). **B**, k_{tr} values plotted against absolute tension. Symbols show mean±SEM for trials in different pCa solutions. **C**, Comparison of k_{tr} values at pCa 4.5 with linear mixed model shows no change across frozen status. Muscles were shortened 20% their initial length for k_{tr} measurements, which accounts for differences in preparation length. Superplots display each patient as a unique symbol with pale symbols representing individual multicellular preparations and filled symbols representing the mean values for each patient.[9] pCa is a measure of calcium concentration where $pCa=-\log_{10}[Ca^{2+}]$

significant difference in cooperativity between frozen and never-frozen preparations. Although the cooperativity and force generation were unchanged with freezing, Figure 2E shows a slight increase in pCa_{50} with prior freezing. Figure 2A and 2B also illustrates this slight increase in pCa_{50} in frozen preparations with the right shifting of the frozen pCa-tension curve. The pCa_{50} of a preparation is the pCa at which the muscle generates half-maximal active force and serves as a measurement of the sensitivity of the contractile system to calcium. The increased pCa_{50} of previously frozen tissue corresponds to a slight decrease in calcium sensitivity. Because the curve is right shifted, the force produced by the previously frozen samples at a given submaximal pCa value was lower than that generated by never-frozen preparations in the same solution.

The k_{tr} values, as displayed in Figure 3C, of both frozen and never-frozen preparations were similar and were not affected by prior freezing. The points in Figure 3B appear horizontally displaced as a result of the decreased calcium sensitivity of the frozen preparations and therefore decreased force at submaximal activation. However, the vertical displacement, representative of a difference in k_{tr}, is not statistically different between the frozen and never-frozen preparations (P=0.095). These k_{tr} values represent the rate at which the preparation is able to redevelop tension after a brief, rapid shortening and provides insight into cross-bridge kinetics of the muscle.

This study used myocardium from patients with heart failure. We have not performed similar experiments with nonfailing myocardium obtained from organ donors. On balance, we think that prior freezing induces gross structural changes in myocardial samples and is likely to affect failing and nonfailing preparations in a similar way. Owing to the limited availability of human myocardial samples this study was restricted to tissue from 7 patients. A study with more patients would have had greater statistical power and might have detected additional statistical differences. Some

of these effects might have been too small to be physiologically important. The experimental approach balanced the practicalities of sample collection, statistical design, and clinical relevance.

We are not aware of equivalent experiments testing animal myocardium (perhaps because these samples are more readily available) but again predict that prior freezing will induce similar effects to those measured in our work.

CONCLUSIONS

Biobanking and freezing of human myocardial samples has become a common practice and vital to conduct translational research on the contractile properties of human myocardium. This study shows that most of the contractile properties of multicellular, chemically permeabilized human myocardial preparations are not affected by prior freezing. The conclusions of this study can be compared only with studies with similar freeze/thaw protocols. The only impact of freezing on the contractile function of human myocardium observed here was a small decrease in calcium sensitivity. These results support the validity and continued use of previously frozen human myocardium in research.

ARTICLE INFORMATION

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Disclosures

None.

Supplemental Material

Table S1
Figure S1

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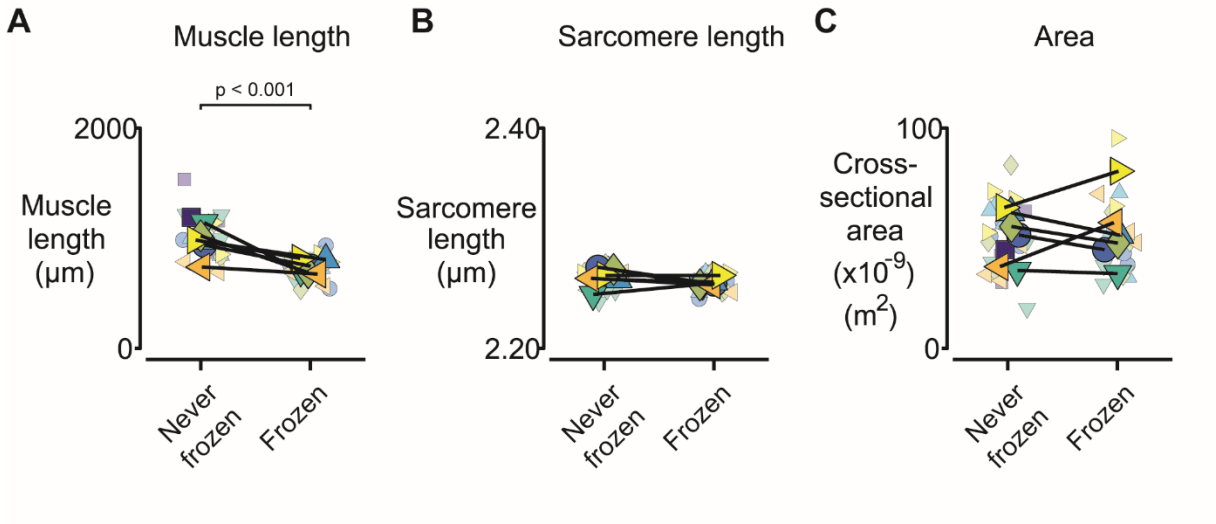
SUPPLEMENTAL MATERIAL

Table S1: Patient Characteristics

Data presented as either a mean ± standard deviation or n

	Cohort (n=7)
Age (years)	45.2±14.5
Male	6
White	3
Black	4
Ejection fraction (%)	19.9±3.7
Diabetes	4
Number of patients on medication	
B-blockers	4
ARBs	1
ACEs	1
Statins	3
Aldosterone antagonist	3
Inotrope	7
Digitalis	0
Vasopressor	3
Aspirin	2
Vasodilators	2

Figure S1: Comparison of frozen and never frozen multicellular preparation dimensions



The dimension of the multicellular preparations used in this study analyzed by linear mixed models. A) The overall length of previously frozen preparations was greater than that of never frozen preparations. This is not expected to have affected the experimental results since all preparations were held at a fixed sarcomere length of 2.26 ± 0.01 µm (range: 2.24 to 2.28 µm) and the k_{tr} measurements were obtained using a stretch/re-stretch perturbation that was normalized to 20% of the preparation length. B) Sarcomere length and C) cross-sectional area were not significantly different between the groups.