The Effect of Repeated Freeze-Thaw Cycles on Human Muscle Tissue Visualized by Postmortem Computed Tomography (PMCT)

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The aim of this study was to determine whether effects of repetitive freeze-thaw cycles, with various thawing temperatures, on human muscle tissue can be quantified using postmortem computed tomography (PMCT) technology. An additional objective was to determine the preferred thawing temperature for muscle tissue in this study. Human cadaver upper extremities were divided into two different thawing temperature groups and underwent a series of four freeze-thaw cycles in total. Axial CT scans were performed after each cycle. CT attenuation (in Hounsfield units, HU) was measured in four muscles of the upper extremities. HU values changed significantly with the introduction of each subsequent freeze-thaw cycle. Moreover, the changes in HU values were different for each thawing group. There was a significant increase of HU values in both groups between $t_0$ and $t_1$. Unfrozen tissue showed large variation of HU values in all samples. It was possible to distinguish between samples thawed at different thawing temperatures based on their respective HU values. It is advisable to keep the number of freeze-thaw cycles to just one, if the human cadaveric tissue is to be used for educational purposes. The preferred thawing temperature in this study is 2°C. Clin. Anat. 30:799–804, 2017.

Key words: freezing; cadaver; humans; muscles; temperature; tomography; X-ray computed

INTRODUCTION

Freezing of fresh human material, also referred to as fresh-freezing, is a frequently employed preservation method of human bodies donated to science. This method entails that the cadaver be frozen in total or be dismembered with body parts being separately packaged, frozen and stored immediately upon arrival at the respective institute. Despite the disadvantages associated to fresh-freezing cadavers, such as the need of freezing facilities, rapid decomposition of the samples once thawed and risk of infection, when compared to other, more complex preservation techniques, fresh-freezing is still commonly preferred (Hayashi et al., 2016). This is mainly because fresh-frozen cadavers retain a realistic appearance as well as native flexibility once thawed, particularly in comparison to cadavers which have been preserved by other means such as embalming (Eisma and Wilkinson, 2014; Hayashi et al., 2016). In support of this, studies on muscle tissue, trabecular bone and tendons have indicated that fresh-freezing has no (Panjabi

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Received 22 April 2017; Revised 27 April 2017; Accepted 27 April 2017

Published online 19 June 2017 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/ca.22917

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et al., 1985; Woo et al., 1986; Linde and Sørensen, 1993) or only marginal effects (Hirpara et al., 2008; Lee et al., 2009; Lee and Jasiuk, 2014) on the biomechanical properties of the respective material. Fresh-frozen human cadaver material is, *inter alia*, used for the teaching of basic human anatomy, as well as for the development and training of novel surgical techniques and devices (Anderson, 2006; Reed et al., 2009; Eisma and Wilkinson, 2014; Hayashi et al., 2016). However, to efficiently make use of the donated bodies in multiple sessions, materials may often be thawed and refrozen several times. Although fresh-freezing has shown to be an acceptable preservation method, the effects of repeated freeze-thaw cycles have proven to significantly impair the integrity of the tissue under test (Lewis et al., 2008; Huang et al., 2011). From these studies, the overall conclusion can be drawn that onetime freezing of human tissue is virtually harmless concerning the integrity of the respective tissue. Repeated freeze-thawing, however, leads to a decline in integrity with increasing number of freeze-thaw cycles.

The purpose of the present study is to determine whether effects of repeated freeze-thaw cycles at various thawing temperatures on human muscle material can be quantified using postmortem computed tomography (PMCT) technology. This is of importance from an educational point of view, since human cadaveric material ought to be unaltered with respect to physical properties as well as appearance if it is meant to be used for dissection-based teaching and training.

**MATERIALS AND METHODS**

**Study Design**

All specimens used for this study were human bodies donated to the department of Anatomy, Embryology and Physiology (AEF) of the Academic Medical Center (AMC) in Amsterdam, The Netherlands, for research purposes between February and April 2013. Written informed consent for the use of body material for research purposes was given prior to death by means of the standard procedure of the body donation program. Upon arrival at our institute in 2013, the bodies were subject to an initial total body PMCT scan. This scan served as the *t*₀ measurement in the consecutive analysis. After scanning, the upper extremities were amputated proximally to the humerus, placed in plastic bags, sealed and stored in a morgue refrigeration cell at a temperature of 2°C until further usage. Two plastic bands were attached to each extremity to establish a point of orientation for subsequent CT scans. The bands were removed from the plastic bags and three iButton temperature loggers (Maxim Integrated, San Jose, USA) were placed on the skin of one sample in each thawing group to monitor the gradual temperature change during the 48- and 24-hr thawing process, respectively. Additionally, a probe thermometer was used to measure the internal temperature. After leaving the upper extremities to thaw at the respective temperatures, axial CT scans were performed on all upper extremities. Subsequently, the samples were rebagged, refrozen and stored at −20°C for five and six days, respectively. Therefore, one freeze-thaw cycle consisted of a 48-hr or 24-hr thawing period and a five or six day freezing period. This cycle was repeated four times in total (Fig. 1).

**PMCT Imaging**

The initial total body PMCT scan was performed on a Siemens Sensation 64 (Siemens, Erlangen, Germany). For total body PMCT scans, the cadavers were placed in a supine position with arms crossed above the body or placed next to the body and scanned in craniocaudal direction. The subsequent axial CT scans of the upper extremities were performed using a Philips Brilliance 64 (Philips Healthcare, Best, The Netherlands). All scanners were routinely calibrated according to clinical protocol. The upper extremities were placed in a pronated position and scanned from distal to proximal. All scans were performed using the following scan parameters: 3 mm slice thickness; 3 mm increments; 120 kV tube voltage; 400 mAs tube current; B30f (soft tissue filter) convolution kernel. Two plastic bands were attached to each extremity prior to the first thawing process. The bands were placed two centimeters proximal to the wrist and two centimeters distal to the elbow in each sample. This was done to establish a point of orientation for subsequent CT value measurements in the CT images of the respective samples.

**Image Viewing and CT Value Measurement**

CT attenuation value measurements were performed using an image viewing software (AGFA, Impax 6.5, Mortsel, Belgium). Measurements were taken in the mid sections of four muscles of the upper extremities: *M. pronator quadratus* (M1), *M. flexor digitorum profundus* (M2), *M. brachioradialis* (M3), and *M. flexor carpi ulnaris* (M4) (Fig. 2). CT attenuation values were measured in Hounsfield units (HU) by setting a circular region of interest (ROI) in the mid sections of the respective muscles. The ROI was
set as large as possible, to capture the average HU value for that particular muscle. All measurements were performed by two independent investigators.

Statistical Analysis

The systematic variation between the individual specimens used in this study was not of interest, but rather, hampered further analysis. Therefore, this variation was removed using factor correction as described in (Ruijter et al., 2006). Assuming a normal distribution of the data, an initial four-way analysis of variance (ANOVA) was performed with two repeated factors (investigator and time points) and two fixed factors (analyzed muscle and thawing temperature). Because no significant inter-observer differences were found between the measurements taken by the two investigators (\( P = 0.055 \)) in the four-way ANOVA, data obtained by the two investigators was combined and averaged for further three-way analysis. This was done with one repeated factor (time points) and two fixed factors (analyzed muscle and thawing temperature) to determine which factors contributed significantly to the observed differences. A \( P \) value of \(< 0.05\) was considered statistically significant. Data analysis was performed using SPSS (IBM, SPSS Statistics 22.0, Armonk, USA). Factor correction was performed with a software program specifically designed for this purpose and available from the website of the Heart Failure Research Center (HFRC) of the AMC (HFRC, 2015).

In three cases the upper extremities were not captured on the initial total body PMCT scan and therefore it was therefore not possible to perform CT value measurements for time point \( t_0 \) in these samples. The missing \( t_0 \) CT values were imputed by SPSS using a model based on the total distribution of HU values in groups, muscles and time points. To this end, five possible substituted CT values per missing sample were calculated and the mean of the five values was taken to be the best approximation for the missing value.

RESULTS

The HU values per muscle, time point and thawing temperature group are shown in Figure 3. The three-way ANOVA of this data showed that time and time-temperature interaction had a statistically significant influence on the HU values (both \( P < 0.001 \)); no statistically significant time-muscle (\( P = 0.258 \)) or time-muscle-temperature (\( P = 0.975 \)) interactions were found. This indicated that the time effect was dependent on the thawing temperature, but that the time effects were similar for each thawing group. This phenomenon is underpinned by the gap between the HU values of the two thawing groups (Fig. 3). The thawing temperature had a significant influence on the change of HU values during the freeze-thaw cycles (\( P < 0.001 \)). Within each group, the HU values significantly changed with the introduction of each subsequent freeze-thaw cycle (\( P < 0.001 \)). In the 19°C thawing group, an increase of HU values could be seen throughout the experiment, most distinctly the increase from \( t_0 \) to \( t_1 \). After \( t_1 \), the HU values remained relatively constant. In the 2°C setting, HU values first increased between \( t_0 \) and \( t_1 \) and showed a slight decline at \( t_3 \) (Fig. 3). A significant muscle effect (\( P = 0.001 \)) as well as a significant temperature-muscle interaction (\( P = 0.015 \)) were found, indicating that the HU values differed significantly in each muscle and that these effects depended on the thawing temperature. Averaged values from both investigators are shown in Table 1 and Figure 3 together with the standard error of the mean (SEM).

DISCUSSION

HU values significantly changed with the introduction of each subsequent freeze-thaw cycle. The changes that occurred to HU values also differed between the applied thawing temperature (2°C/19°C) resulting in the statistically significant thawing temperature effect that was measured. Thus temperature
had a critical effect on the change of HU values in the respective muscles in this study.

It was possible to differentiate between samples thawed at 2°C and 19°C based on their respective HU values, because the HU values behaved differently in each thawing group. The changes that occurred to HU values in the course of repeated freeze-thaw cycles can be explained by cellular and subcellular processes that take place in tissue when exposed to freezing and thawing (Schäfer and Kaufmann, 1999). Autolysis and putrefaction, the two sub-processes of decomposition, are inhibited by low temperatures and freezing (Levy et al., 2010). These processes, which are “reactivated” when frozen tissue reverts to higher temperatures, lead to an irreversible disruption of cell-to-cell connections in tissue (van der Made et al., 2013). With increasing number of freeze-thaw cycles, the number of disrupted cells, which release intracellular components into the extracellular area, increases as well. Due to this, the size of the extracellular area increases (Schäfer and Kaufmann, 1999). This hypothesis might explain underlying cellular processes that account for the increase of HU values in the 19°C thawing group. In the 2°C group, this effect was less marked, as tissue in this group was still partially internally frozen after 48 hr of thawing. The processes of thawing and decomposition were, therefore, less pronounced. Additionally, this study delivered results, which were difficult to assess objectively. With increasing number of freeze-thaw cycles, a gradual change of color of the muscle tissue on the amputation surface of the upper arm was observed: from red to greyish and dark brown. The odor accordingly intensified with increasing number of freeze-thaw cycles, suggestive of beginning decomposition. Furthermore, the firmness of the samples decreased. These findings were more pronounced, but not restricted to the samples in the 19°C thawing group. The distinct increase in all HU values in both groups from t₀ to t₁ is not likely to be due to the use of different CT scanners at these two time points. The CT scanners, which were employed in this study are used predominantly for diagnostic purposes and are therefore routinely calibrated. Moreover, scanning parameters did not differ between the scans made at t₀ and those made at t₁. The distinct increase in HU values from t₀ to t₁ may have been due to the extended freezing period of 2.9 years. However, studies have shown that the extracellular area of frozen tissue does not increase when tissue is frozen for longer than 72 h at −12°C or lower (Schäfer and Kaufmann, 1999). From this it follows that it is not possible to discriminate between freshly frozen tissue or tissue...
that has been frozen for extended periods such as was the case in this study. Alternatively, the observed increase in HU values may be due to dormant decomposition processes that take place even at −20°C, albeit at a comparatively slow rate. Published research indicated a decrease of 40 to 60 HU when comparing HU values of muscle tissue in unfrozen and fully frozen conditions (Pech et al., 1987). This may explain the systematically lower HU values in the 2°C thawing group, as the deep muscles (M. pronator quadratus and M. flexor digitorum profundus) may still have been partially frozen at the time of scanning. Superficial muscles (M. brachioradialis and M. flexor carpi ulnaris), however, were fully thawed at the time of scanning. A high variation of HU values in unfrozen tissue coincides with inconsistent HU values reported in literature for skeletal muscle tissue (Aubrey et al., 2014). Variation in muscle attenuation is suggested to be due to physiological as well as pathological variation. As a result of this variation, it was difficult to determine the integrity of muscle tissue with the use of HU values. As indicated by others, a defined range of attenuation values for muscle tissue would benefit a comparison between studies (Aubrey et al., 2014). Moreover, a deviation range for muscle attenuation values would enable an assessment of integrity of the tissue. Any significant deviation from a defined range could be interpreted as an increase in disruption of cell-to-cell connections and this in turn as a decrease of muscle integrity. The increase in HU values between \( t_0 \) and \( t_1 \) was the least in the 2°C group. Thus, if HU values are taken as a measure of integrity of muscle tissue, it can be concluded, that the 2°C thawing temperature is preferable over the 19°C thawing temperature when trying to keep tissue integrity at a high level. In other words, to keep HU values at a comparable level as those measured in fresh muscle tissue it is advisable to thaw frozen muscle tissue at 2°C, albeit for longer periods, to allow the tissue to become completely thawed.

Despite the limitation that only two thawing temperatures were used, the current study represents a first step toward quantifying effects of freeze-thaw cycles on human muscle tissue by means of PMCT technology. More research is needed to verify the results and to determine if the findings hold for other thawing- and freezing temperatures. This is of importance from an educational point of view, since the most striking advantage of using fresh frozen human specimens for dissection-based teaching and training purposes is the retention of its native, lifelike appearance and physical properties. Repeated freeze-thaw cycles, however, result in an alteration of the HU values of the respective tissue and tissue is therefore no longer representative of fresh cadaveric material. Human cadaveric material should, if possible, not be submitted to multiple freeze-thaw cycles if it is to be used for educational and training purposes, since decomposition processes alter the native properties of the tissue. In this study we were able to show that this effect can be measured using HU values and PMCT technology. The results of this study show that 2°C is preferred over 19°C as thawing temperature as the HU values remain more stable and similar to HU values of fresh tissue.

ACKNOWLEDGMENTS

First and foremost, the authors wish to thank the persons who donated their bodies to the department of Anatomy, Embryology and Physiology of the Academic Medical Center, without which this study would not have been possible. The authors would also like to thank M. Lida van der Merwe for her assistance in this project as well as Mara Clerkx, Inge Dijkman and Erik Lichtenberg for their technical support.

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### TABLE 1. Averaged HU Value Measurements Per Thawing Group

|       | 2°C          | 19°C         |
|-------|--------------|--------------|
|       | M1           | M2           | M3           | M4           | M1           | M2           | M3           | M4           |
| \( t_0 \) | 44.8 ± 4.3   | 37.3 ± 3.3   | 50.5 ± 3.4   | 49.6 ± 2.8   | 40.3 ± 4.1   | 43.8 ± 1.7   | 50.6 ± 2.9   | 48.2 ± 3.4   |
| \( t_1 \) | 61.0 ± 4.0   | 50.7 ± 1.8   | 56.0 ± 3.6   | 57.2 ± 2.8   | 62.0 ± 2.3   | 63.6 ± 1.6   | 67.7 ± 2.1   | 69.5 ± 1.8   |
| \( t_2 \) | 55.5 ± 3.2   | 46.8 ± 1.4   | 51.4 ± 3.7   | 55.0 ± 2.8   | 64.2 ± 1.7   | 63.0 ± 1.7   | 65.5 ± 2.3   | 69.7 ± 2.4   |
| \( t_3 \) | 53.0 ± 3.5   | 38.8 ± 1.7   | 44.8 ± 3.2   | 49.0 ± 1.5   | 65.4 ± 0.9   | 62.8 ± 2.1   | 65.6 ± 1.6   | 71.6 ± 1.3   |
| \( t_4 \) | 54.0 ± 3.2   | 42.1 ± 1.9   | 51.7 ± 4.7   | 53.5 ± 3.3   | 61.9 ± 2.5   | 65.5 ± 2.3   | 65.8 ± 1.4   | 69.7 ± 3.3   |

Averaged HU value measurements from both investigators and standard error of the mean (SEM) per time point (\( t_0 \)–\( t_4 \)) per thawing Group (2°C/19°C) in M. pronator quadratus (M1); M. flexor digitorum profundus (M2); M. brachioradialis (M3); M. flexor carpi ulnaris (M4).
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