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Short communication

# Density and hydration of fresh and fixed human skeletal muscle

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**Abstract**

The maximum tetanic tension of skeletal muscle ($P_0$) is often estimated based on calculation of physiological cross-sectional area (PCSA). PCSA depends on muscle volume, pennation angle, and fiber length. Studies documenting PCSA in fixed human muscles usually compute muscle volume by dividing muscle mass by density. These studies use a density value of 1.0597 g/cm$^3$, which was originally based on unfixed rabbit and canine muscle tissue. Due to the dehydration effects of different fixation methods, the variable hydration that occurs when fixed tissue is stored in buffered saline, and the potential for species-specific muscle density, this value may be incorrect and an accurate value for fixed human muscle density is needed. To obtain an accurate density and water content values, 4% formaldehyde-fixed ($n = 54$) and 37% formaldehyde-fixed ($n = 54$) cadaveric human muscle samples were divided into 6 groups (0, 6, 12, 18, 24, or 30 h) for hydration in phosphate buffered saline (PBS). Measurements of volume, water content, and mass were made enabling calculation of muscle density. Additionally, water content was measured in living muscle ($n = 4$) to determine the appropriate hydration time in PBS. Comparisons among groups demonstrated a significant increase in muscle water content and muscle volume over time, reaching living tissue levels after 24 h, but, interestingly, the hydration process did not affect muscle density. These data yield a density value (mean ± SE) of 1.112 ± 0.006 g/cm$^3$ in 4% formaldehyde-fixed muscle and 1.055 ± 0.006 g/cm$^3$ in 37% formaldehyde-fixed muscle. These results indicate that the use of inappropriate hydration times or density values can produce PCSA errors of 5–10%.


*Keywords:* Human muscle; Muscle density; Muscle hydration; Muscle architecture; Modeling

## 1. Introduction

Muscle function is often inferred from its architecture. One key parameter of muscle function is maximum tension ($P_0$) or maximum force producing capacity. Given the difficulty of directly measuring this value in humans, muscle architecture is often used to estimate $P_0$. Specifically, physiological cross-sectional area (PCSA) has been shown to be an excellent predictor of $P_0$ and is calculated using the following equation (Powell et al., 1984; Sacks and Roy, 1982).

$$\mathrm{PCSA(cm^2)} = \frac{\mathrm{Muscle\ mass\ (g)}\cos(\theta)}{\rho(\mathrm{g/cm^3})\,\mathrm{fiber\ length\ (cm)}}, \quad (1)$$

where muscle density ($\rho$) is 1.0597 g/cm$^3$ (Mendez and Keys, 1960) and $\theta$ is fiber pennation angle.

Current biomechanical modeling techniques rely on PCSA to estimate peak muscle force production during a task (Anderson and Pandy, 2003; Buchanan and Shreeve, 1996). However, Brand and colleagues (Brand et al., 1986) demonstrated that muscle force predictions are highly sensitive to changes in PCSA, therefore, it seems apparent that the accuracy of measured values used to compute PCSA is important. Muscle architecture reports typically do not directly measure muscle density (Lieber et al., 1992; Wickiewicz et al., 1983). Instead, most studies use the value 1.0597 g/cm$^3$, which was derived from unfixed rabbit and canine muscle tissue (Mendez and Keys, 1960).

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Given the fact that human muscle architecture is often characterized in formaldehyde-fixed tissue, this previously defined value may be inaccurate for several reasons. First, a species effect may exist so that rabbit or canine muscle density may differ from human muscle density. Second, the method and duration of fixation may cause shrinkage and thus dehydration, which may alter muscle density. Finally, the time in which stored muscle samples hydrate in buffered saline may affect volume and thus density. If these variables affect density either separately or in combination, current estimates of muscle PCSA and thus predictions of muscle force may be inaccurate. Thus, the purpose of this study was to measure muscle density directly as a function of fixation method and hydration time in human skeletal muscle.

## 2. Methods

Muscle samples from three living subjects (four samples), three immersion-fixed cadavers (54 samples) and three perfusion-fixed cadavers (54 samples) were obtained for this investigation. Subject groups were not significantly different in terms of age, however, living subjects were younger on average ($59 \pm 14$ years) compared to immersion-fixed cadaver specimens ($76 \pm 8$ years) and perfusion-fixed cadaver specimens ($79 \pm 9$ years). Causes of death in the cadaveric specimens included respiratory arrest, cardiac arrest, and cancer. All living subjects signed informed consent prior to participation in accordance with Human Research Protection Program at the University of California, San Diego.

Cadaveric specimens were processed in one of two ways. Immersion-fixed specimens were frozen ($-20\,^{\circ}C$) within 24 h of death, thawed to room temperature for 24 h before use, dissected free of skin and superficial fascia, then immersion-fixed in 10% buffered formalin (4% formaldehyde, 3% methyl alcohol, 92% water, 1% buffer) for 48 h. After fixation, intact extensor carpi radialis longus, biceps brachii, and flexor carpi ulnaris muscles were dissected for further study. Perfusion-fixed specimens were fixed with 37% formaldehyde (37% formaldehyde, 15% methyl alcohol, 48% water) within 24 h of death, followed by dissection of vastus lateralis, psoas major and tibialis anterior muscles. These two fixation methods (immersion-fixation and perfusion-fixation) have been previously described in the literature (Lieber et al., 1992 and Wickiewicz et al., 1983) and will be identified as the 4% and 37% group, respectively. In each fixation group, muscles were divided into small samples, evenly separated into 6 groups and placed in 0.2 M phosphate-buffered saline (PBS) for 0, 6, 12, 18, 24, or 30 h for hydration.

Muscle sample volumes were measured directly by scanning each sample using a 1.5 T GE Signa MR imaging system (GE Medical Systems, Milwaukee, WI). Axial 3D fast-spoiled gradient recalled echo (FSPGR) images were obtained using the following parameters; TR: 7.7 ms, TE: 2.2 ms, flip angle: 60°, NEX 2, FOV $12 \times 12\,cm^2$, matrix $256 \times 256$, and slice thickness 1 mm. These parameters yielded a voxel resolution of $0.47 \times 0.47 \times 1\,mm^3$. Muscle volumes were calculated from the MRI data using a semi-automated segmentation and reconstruction routine (Analyze version 6.0, Analyze Direct, Lenexa, KS).

To evaluate the accuracy of this volumizing technique, phantoms were constructed by pipeting 0.5–10 $cm^3$ samples of vegetable oil into centrifuge tubes which were then scanned and processed using the above reconstruction routine. Regression and RSME analyses yielded excellent agreement between the MR derived and the pipeted volumes ($ICC_{2,1} = 0.99$, $P < 0.001$, RMSE $0.07\,cm^3$; Fig. 1).

After imaging, samples were weighed, snap frozen in liquid nitrogen ($-196\,^{\circ}C$), and stored at $-80\,^{\circ}C$. Samples were then lyophilized for 12 h and reweighed. Water content (%) was calculated as the percentage difference between wet and dry weights and density ($g/cm^3$) was calculated by dividing wet muscle mass by muscle volume. For living tissue, muscle biopsies were stored in saline gauze for 1 h before water content was determined, to simulate the procedure used clinically.

Statistical comparisons of water content and density were made with separate two-way analyses of variance (fixation method × hydration time). For water content, post hoc one-way analyses of variance and Tukey tests were used to determine water content differences within and between fixation methods at each hydration time point. This statistical design allowed water content differences between 4% and 37% fixed tissues to be determined as well as comparisons between each tissue and hydration time with living muscle water content to be made. Hydration rates and the correlation between wet-weight and volume (density) were determined using

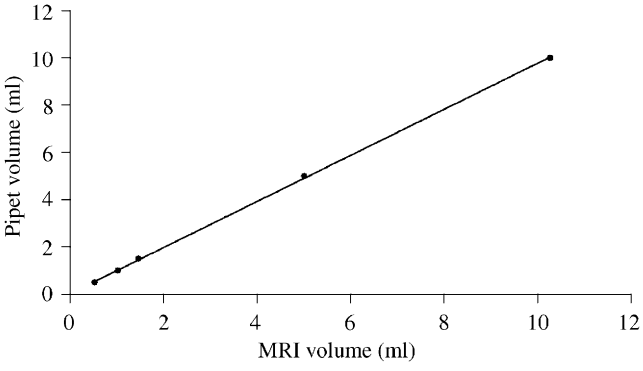


Fig. 1. Scatter plot of phantom volumes. MRI calculated volumes (*x*-axis) and known volumes (*y*-axis). The data demonstrate that the MR measurement method is a valid for measuring volumes over the range of 0.5–10 $cm^3$.

simple linear regression. $P$ values $<0.05$ were determined to be significant and all reported values are mean $\pm$ SE unless otherwise noted.

## 3. Results and discussion

For water content, there was a significant main effect for fixation method ($P<0.001$), hydration time ($P<0.001$), and fixation method by hydration time interaction ($P=0.003$) (Fig. 2). Post hoc testing demonstrated that 4% samples had significantly greater water content than 37% samples at each hydration time point (Fig. 2). The largest difference being before hydration (10%, $P<0.001$) and the smallest difference after 24 h of hydration (5%, $P<0.05$).

Post hoc comparisons also demonstrated water content significantly increased over time in 4% samples ($P<0.001$) and in 37% samples ($P<0.001$). In 4% samples, water content was significantly lower at 0 h compared to every other time point, however, the only significant increase between sequential time points was between 0 and 6 h (78% vs. 79%, respectively). In 37% samples, water content was significantly lower at 0 h compared to every other time point, but significant sequential increases were observed up to 24 h (68% at 0 h and 75% at 24 h). Regression analysis demonstrated that the rate of hydration in PBS was significantly greater over the entire 30 h time period in 37% samples (0.3%/h) compared to 4% samples (0.1%/h).

Water content in living muscle samples was 77% $\pm$ 2%. Post hoc testing demonstrated that 4% samples did not have significantly different water content from living tissue except after 30 h of hydration (81% vs. 77%, respectively). However, 37% samples had significantly lower water content than living tissue until 24 h of hydration (Fig. 2).

In terms of density, 4% samples were significantly more dense than 37% samples ($P<0.001$), but there was no main effect of time and no fixation method $\times$ time interaction (Fig. 3). Given the lack of differences in density between hydration time points, sample volumes were plotted against wet-weights to calculate average muscle density for 4% samples ($1.112\pm0.006$ g/cm$^3$, $r^2=0.973$, Fig. 4) and 37% samples ($1.055\pm0.006$ g/cm$^3$; $r^2=0.990$, Fig. 4).

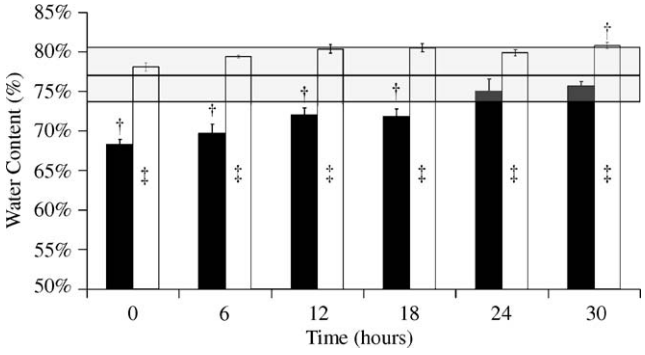


Fig. 2. Muscle hydration vs. time (mean $\pm$ SE) in 4% formaldehyde-fixed tissue (open bars) for 37% formaldehyde-fixed tissue (solid bars) and living tissue (horizontal line with 95% confidence interval shaded). † indicates a significant difference from living tissue. ‡ indicates a significant difference between 37% and 4% fixed tissues.

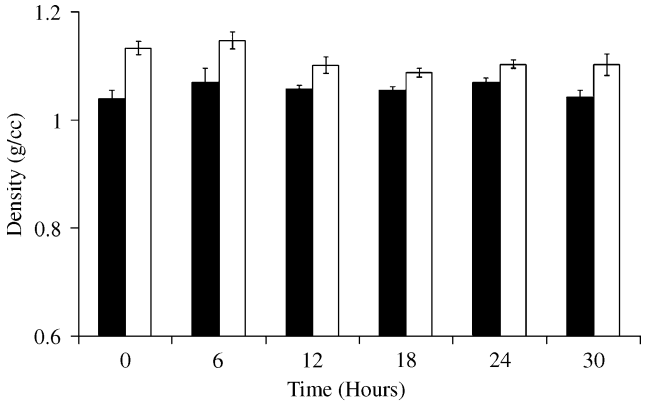


Fig. 3. Muscle density vs. time (mean $\pm$ SE) in 4% formaldehyde-fixed tissue (open bars) and 37% formaldehyde-fixed tissue (solid bars). 4% muscle samples were significantly more dense than 37% muscle samples, but hydration time did not influence density in either group.

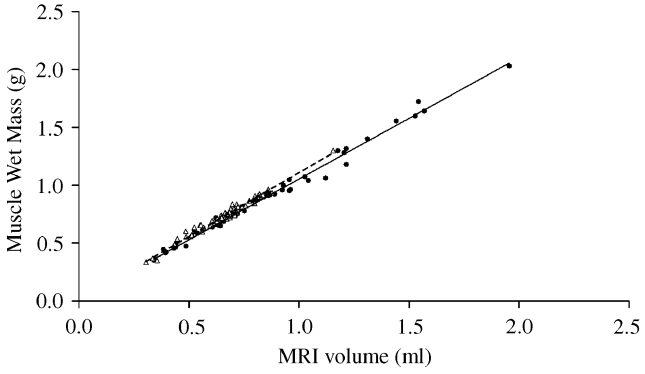


Fig. 4. Scatter plot of muscle wet-weight vs. volume in 4% formaldehyde-fixed muscle (open shapes) and 37% formaldehyde-fixed tissue (closed shapes) regardless of hydration time. The slope of the mass vs. volume regression line (4%-dashed and 37%-solid) represents density.

## 4. Summary

Living muscle water content averaged 77% in this study, which agrees with the 76% value reported by Hargens et al. (1983). When immersed in PBS, fixed muscle-water content increased over time regardless of fixation method. Although 4% formaldehyde-fixed muscle had greater water content than 37% formaldehyde-fixed muscle at each time point, they had similar water content to living muscle after 24 h of hydration. The fact that 37% formaldehyde-fixed muscle had

greater hydration rates than 4% formaldehyde-fixed muscle over the 30 h time period, likely indicates that it was relatively dehydrated initially. This was not surprising as the 37% formaldehyde solution had about 5 times the methyl alcohol content and half the water content as the 4% formaldehyde solution.

Most importantly, water content did not influence muscle density, whereas fixation method did (1.112 g/cm$^3$ in 4% tissue versus 1.055 g/cm$^3$ in 37% tissue). Although the 1.055 g/cm$^3$ value for formaldehyde-fixed tissue was not dramatically different from the value reported by Mendez and Keys (1960), the 1.112 g/cm$^3$ value observed in formalin-fixed tissue was greater by 5% compared to the previously reported value. The fact that a 4% formaldehyde fixative produced significantly more dense tissue than the 37% formaldehyde fixative is likely due to the method of fixative delivery. The 4% muscles were immersed in solution for 48 h, while the 37% muscles were perfusion-fixed. It is possible that the former method allows for greater fixation of the tissue.

There are several methodological limitations to this study. First, the 4% samples were obtained from upper extremity muscles, while the 37% samples were from lower extremity muscles. This limitation was due simply to tissue availability. However, there are no reasons to suspect that muscles from different limbs would have different densities, as there are no reported structural differences between upper and lower extremity muscles. Second, fixed-muscle density was not compared to living muscle density. This was due to the inability to obtain living biopsies large enough to scan for volume. However, estimates of living tissue $P_0$ are based on volume-based PCSA calculations, and therefore, density is not needed. Finally, analysis of the variability in our density measurements, although very small, appears to be related to our volume measurement technique and not true variability in muscle density.

These data address two important methodological considerations in human muscle architecture research, and therefore, biomechanical modeling. First, muscle architecture studies using immersion-fixation in 10% formalin should use a value of 1.112 g/cm$^3$ for muscle density. Those that use the traditional value of 1.0597 g/cm$^3$ will be overestimating PCSA by 5%. Second, fixed-muscle samples should be rehydrated in 0.2 M PBS for at least 24 h. Muscle architecture studies that do not rehydrate muscle, specifically in 37% formaldehyde-fixed muscle, will underestimate muscle mass, and therefore PCSA, by 9%. Estimating maximum muscle force generating capacity ($P_0$) requires numerous assumptions. This study serves to minimize errors in estimating $P_0$ from muscle architecture by generating accurate values of muscle density and water content.

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