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Normalization of force to muscle cross-sectional area: A helpful attempt to reduce data scattering in contractility studies?

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There have been several attempts to reduce data scattering in contractility studies. When using superfused muscle preparations for contractility experiments, there is always the question how to compensate for variabilities in muscle thickness. A simple but rather imperfect approach is the normalization of force to cross-sectional area. However, this attempt does not consider the possibility of hypoxic cores in thick muscles, which probably do not really contribute to force development. Han et al. [1] recently published a new computer model to investigate the relevance of oxygen distribution for force generation when intact muscles are superfused, as frequently used in physiological experiments. According to the mathematical model from Han et al. [1] the relationship between radius of the muscle tissue (R) and the fraction of whole cross-section of muscle supplied with oxygen (θ) is:

$$R = \sqrt{\frac{4 \times K \times P}{(m_b + m_a) \times (\theta + (1-\theta) \times \ln(1-\theta))}} \quad (1)$$

where P is pressure relative to atmospheric pressure, K is Krogh's constant (product of solubility and diffusion constant of oxygen in the muscle tissue), m_b and m_a are basal and active oxygen consumption rates in the muscle, and $\theta = 1 - (r'/R)^2$ and r' is the radius at which $p = 0$. The numerical values are $P = 1$, $K = 1.3098*10^{-9}$ mol*m^{-1}*Pa^{-1}*s^{-1}, and $m_b + m_a = 0.1904$ mol*m^{-3}*s^{-1}. The cross-section of muscle supplied with oxygen is calculated further as:

$$A_{oxy} = \theta \times \pi \times R^2 \quad (2)$$

Based on the relationship between radius of the muscle tissue (R) and the fraction of whole cross-section of muscle supplied with oxygen (θ) defined by Han et al. [1], the cross-section of muscle supplied with oxygen was calculated as: $A_{oxy} = \theta * \pi * R^2$.

In the past, normalization of force to cross-sectional area gained much popularity but has severe limitations. First report about normalization to cross-sectional area originates from Kelly [2]. They used a larger number (n = 70) of rat left ventricle papillary muscles with a radius between 0.25 and 1.2 mm. Muscle thickness was measured under pre-tension. Raw data for force were not presented, but force normalized to cross-sectional area correlated negatively to cross-sectional area. Koch-Weser [3] studied the relationship between muscle cross-sectional area and force in very detail. They used rather thin papillary muscles from cat right ventricles. Muscle cross-sectional area was between 0.2 and 1.5 mm^2 (= radius between 0.12 and 0.35 mm). Force normalized to cross-sectional area was constant up to an area of 1 mm^2 (radius of 0.55 mm) when paced at slow rate (18.8 bpm) but declined already above an area >0.6 mm^2 (radius 0.44 mm) when paced ten times faster. From this finding, the authors concluded that the critical thickness depends on the actual work of the muscle. It remains

unclear from that study what the critical radius at 1 Hz could be. However, based on interpolation, we would expect a value between 0.44 and 0.55 mm. Findings of Koch-Weser were replicated in cat right ventricular papillary muscles by Fisher & Lee 1967 [4]. They found a constant relationship between cross-sectional area and force (n=4). This finding is at contrast to several other publications where researchers failed to detect any stable plateau for normalized force values even at very small radius. Bing et al. [5] had used rat left ventricular trabeculae (smallest radius 0.4 mm) and rat left ventricular papillary muscles (smallest radius 0.35 mm). Only Raman [6] could demonstrate a stable plateau for force normalized to cross-sectional area in a population of very thin free running right ventricular trabeculae prepared from mouse heart. However, it should be noted that the critical radius was found to be 0.06 mm, much lower than reported in the above-mentioned studies [5 3].

Trying to overcome the limitation of normalization to total muscle area, we retrospectively analysed whether normalization of force of contraction to oxygenated area rather than normalization to total area reduces data scattering in isolated heart muscle preparations.

We therefore analysed 856 human right atrial tissues from 124 patients paced at 1 Hz and 37 °C. Isometric force of contraction was normalized to cross-sectional area or the area of muscles oxygenated as predicted from the computer model by Han et al.

Muscle thickness has only a minor effect on basal force in atrial trabeculae from human right atrial appendages

Cross-sectional area as well as force of contraction showed huge variability (Figure 1A). In order to investigate whether variability in force is related to differences in muscle thickness, we plotted individual force data against respective muscle thickness, expressed as cross-

sectional area (Figure 1A). A simple linear regression curve was fitted to the data points. There was a weak positive correlation between cross-sectional area and basal force. However, the slope was rather low and the coefficient of determination R^2 accounted to only 0.01. Both results argue against a simple relationship between muscle cross-sectional area and force.

Even if the relationship between force and cross-sectional area is not perfect, normalization could reduce data scatter. Therefore, we have calculated coefficients of variation for raw data and data normalized for total cross-sectional area. The coefficients accounted to 0.818 and 1.079. Variability was statistically significant higher ($p<0.001$) when data were normalized to cross-sectional area than raw data (two-sided p-values for differences between coefficients of variation where estimated using bootstrapping).

Weak association between muscle cross-sectional area and force is not restricted to human atrial trabeculae: Guinea pig left ventricle papillary muscles

Next, we wanted to evaluate whether our findings may represent a peculiarity of human atrial trabeculae. For that purpose, we used data from guinea-pig right ventricular papillary muscles, published by one of us [7]. The muscles were conical in shape like human atrial trabeculae, but slightly thicker (radius 0.76 ± 0.02 vs.0.62 ± 0.01 mm, n = 64 vs. 856/124, $p<0.0001$ unpaired t-test). The coefficient of variation of raw force data was much lower than in human right atrial trabeculae (0.532 vs. 0.818, $p<0.001$). In contrast to human atrial trabeculae, there was no positive correlation between cross-sectional area and force. As seen before for human right atrial trabeculae, normalization of force data to cross-sectional area increased data scattering (coefficients of variation was 0.956 vs. 0.532, $p<0.001$).

Human right atrial trabeculae and guinea pig left ventricular papillary muscles are always too thick to be fully oxygenated

That theoretical model was used to derive the values for fully oxygenated "active" area as a function of muscle radius for right atrial trabeculae (Figure 1C). While total cross-sectional area increased exponentially, the slope for fully oxygenated area increase was rather low and almost linear. The model predicted incomplete oxygenation for muscles with radius > 0.2 mm. Regression lines for fully oxygenated area and for force generation run in parallel. The same holds true for guinea-pig papillary muscle (Figure 1D).

To test whether normalization to fully oxygenated (active) area could reduce data scatter in right atrial trabeculae, we calculated coefficients of variation for data normalized to active area and compared to coefficients of variation of raw data and those normalized to total cross-sectional area. The coefficient for normalization on active area amounted to 0.832 and was significantly ($p<0.001$) lower than for data normalized simply to total cross-sectional area (. However, normalization to active area did not result in lower data scattering than using raw data ($p = 0.414$). For guinea-pig papillary muscles, the coefficient of variation was 0.665 when normalized to active area and again significantly lower compared to normalization to total cross-sectional area, but significantly higher than for raw data ($p<0.001$).

Inotropic responses in right atrial trabeculae

The fraction of fully oxygenated fibres in a superfused muscle preparation does not only depend on muscle thickness but also on actual oxygen demand [1]. Any positive inotropic intervention that increases oxygen consumption should therefore increase the fraction of the muscle being hypoxic. Obviously, thicker muscles should be at higher risk for hypoxia when

force is increased. In order to evaluate whether muscle thickness has an impact on effect size of a positive inotropic intervention, we have plotted the maximum positive response evoked by high concentrations of Ca^{2+} (8 mM) as a function of muscle radius (Figure 1E). Unexpectedly, there was a weak, but clearly positive correlation between the inotropic effect of high calcium concentrations and muscles radius in right atrial trabeculae. We tested whether a much simpler approach could work better. By simply plotting stimulated (maximal) force vs. basal force, we found a clear positive linear correlation with a rather high correlation coefficient of 0.6 (Figure 1F).

The concept of critical oxygenation and inotropic interventions

Following the initial concept of Koch-Weser [3] but also the more sophisticated computer model proposed by Han et al. [1], limited oxygen supply should be a particularly critical factor when oxygen demand is increased, i.e. under inotropic stimulation. Since we saw a rather flat curve for force vs. area we have chosen the simplest approach and plotted delta force vs. radius. As seen before for basal force there was a very low association between positive inotropic effect and radius. There was no hint that inotropic responses could be limited in thicker muscles. Again, R^2 values were rather low.

In conclusion, force values measured in intact muscle preparations differ widely. There is only a very weak correlation between force and cross-sectional area. Correlation to a predicted oxygenated area of muscles works better than normalization to cross-sectional area. However, cross-sectional area and oxygenated area do not contribute to more than 1 and 2 % of variability in force. Other, not yet identified properties may contribute to variability.

Conflict of Interest:

There are no competing interests in any of the authors.

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Figure legend

Fig. 1)

Relationship between muscle cross-sectional area and force in trabeculae from human right atrial appendages under control conditions (A, B)

Plotted are individual force data, expressed in absolute values (**A**) or normalized to its muscle cross-sectional area (**B**) vs. respective muscle cross-sectional area for right atrial trabeculae (n/n indicate number of trabeculae/number of patients). The red dotted lines indicates linear regression fit curves to the data points.

Calculated fully oxygenated muscle area and force as a function of muscle radius (C, D)

Total muscle area (total) and fully oxygenated muscle area (active) are calculated and given as dotted lines and plotted against the left y axis (for details compare Results). For comparison, measured force data are plotted against the right y-axis. Date are given for trabeculae from human right atrial trabeculae (**C**) and for Guinea pig papillary muscle (**D**).

Positive inotropic responses does not relate to muscle thickness in trabeculae from human right atrial appendages (E, F)

Force increase upon high Ca^{2+} (8 mM) expressed as delta values in trabeculae from human right atrial trabeculae plotted vs. individual muscle radius (**E**). Absolute force in the presence of high Ca^{2+} as a function of basal force (**F**).