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SHORT COMMUNICATION

Force loss induced by inhibiting cross-bridge cycling is mitigated in eccentric contraction

Atsuki Fukutani[1,*], Shiho Kunimatsu[2] and Tadao Isaka[1]

ABSTRACT

We examined whether the force loss induced by 2,3-butanedione monoxime affects isometric and eccentric forces differently. Single skinned muscle fibers were activated at an average sarcomere length of 2.4 μm and then stretched to 3.0 μm. This trial was performed with and without 2,3-butanedione monoxime to calculate the magnitude of force loss attained at several time points: pre-stretch phase at 2.4 μm, eccentric phase, end of eccentric contraction, and post-stretch phase at 3.0 μm. The magnitude of force loss was significantly larger in the pre-stretch phase than at the other time points. Further, the mitigated force loss in the eccentric contraction was more prominent in the long condition than in the short condition. We suggest that the eccentric force is relatively preserved compared with the reference isometric force (pre-stretch) when cross-bridge cycling is inhibited, possibly because of the contribution of the elastic force produced by titin.

KEY WORDS: Soleus, Skinned fiber, Titin, Residual force enhancement

INTRODUCTION

It is widely accepted that muscle force is produced by cross-bridge cycling (Huxley, 1957). Thus, when cross-bridge cycling is inhibited in certain situations, the muscle force decreases. However, it has recently been suggested that in addition to the cross-bridges, titin, a protein responsible for passive elasticity of muscle fibers, also contributes to muscle force (Linke, 2018; Freundt and Linke, 2019; Eckels et al., 2019). The current proposed mechanism is that once muscles are actively stretched, the elastic force produced by titin (hereafter, titin force) at a given sarcomere length during or after stretch increases, which contributes to the increased active muscle force (Nishikawa, 2016; Hessel and Nishikawa, 2017; Herzog, 2018; Fukutani and Herzog, 2019). The current proposed mechanisms for enhancing titin force are the increase in stiffness by Ca^{2+} binding (Linke, 2018; Freundt and Linke, 2019) and titin–actin interaction (Schappacher-Tilp et al., 2015; Nishikawa, 2016; Herzog, 2018; Dutta et al., 2018; Fukutani and Herzog, 2019). Based on these mechanisms, the increased titin force is considered as one of the mechanisms for residual force enhancement, i.e. the increase in the isometric force attained after an eccentric contraction compared to that attained in a purely isometric contraction (Nishikawa, 2016; Herzog, 2018; Fukutani and Herzog, 2019).

[1]Faculty of Sport and Health Science, Ritsumeikan University, 1-1-1 Noji-higashi, Kusatsu, Shiga 525-8577, Japan. [2]College of Sport and Health Science, Ritsumeikan University, 1-1-1 Noji-higashi, Kusatsu, Shiga 525-8577, Japan.

*Author for correspondence (afr15171@fc.ritsumei.ac.jp)

A.F., 0000-0002-8572-7940

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Because this elastic force comes from a passive structure, titin, one can speculate that the magnitude of this titin force does not change under conditions where cross-bridge cycling is inhibited. Regarding this aspect, we recently examined whether the magnitude of residual force enhancement induced by an active stretch differed between normal and reduced force conditions using the cross-bridge inhibitor 2,3-butanedione monoxime (BDM), which inhibits the power stroke and/or reduces the proportion of actin-bound myosin heads (Higuchi and Takemori, 1989; Herrmann et al., 1992; Iwamoto, 2018), reducing the Ca^{2+} concentration or reducing the pH. We found that the magnitude of residual force enhancement was preserved even under these reduced force conditions (Fukutani and Herzog, 2018). Similarly, several studies using BDM reported that the magnitude of residual force enhancement was preserved (Rassier and Herzog, 2005a,b). If we assume that residual force enhancement is mainly caused by the titin force (Nishikawa, 2016; Herzog, 2018; Fukutani and Herzog, 2019), this result implies that the titin force is preserved even under reduced force conditions.

If these concepts are correct, we can also speculate that the influence of cross-bridge cycling inhibition on muscle force differs depending on the type of contraction, i.e. isometric, concentric or eccentric. Because the enhanced titin force induced by active stretch affects the eccentric force attained during an active stretch and the isometric force attained after an active stretch, but does not affect the isometric force before an active stretch, the magnitude of force loss caused by inhibiting cross-bridge cycling might be different among contractions. Specifically, the magnitude of force loss caused by inhibiting cross-bridge cycling would be mitigated in eccentric contraction or isometric contraction after an active stretch because a part of force derived from titin (not sensitive to the inhibition of cross-bridge cycling) is preserved. In contrast, because the force exclusively comes from cross-bridge cycling in the reference isometric contraction, this reference isometric force is simply decreased by inhibiting cross-bridge cycling. Therefore, the purpose of this study was to compare the magnitude of force loss during reference isometric contraction, eccentric contraction and isometric contraction after an active stretch. We hypothesized that the magnitude of force loss is smaller in the eccentric than in the reference isometric contraction because of the preserved titin force. To strengthen this hypothesis, we also compared the magnitude of the above force losses in the short and long sarcomere length conditions. If the titin force mitigates the force loss, this effect should be prominent in the longer sarcomere length condition because the influence of elastic force (i.e. contribution of the titin force) becomes larger.

MATERIALS AND METHODS

Muscle samples and experimental setup

We purchased isolated rabbit muscle tissues (harvested from New Zealand white rabbits) from the SHIMIZU Laboratory Supplies. These processes were conducted according to the Guidelines for

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Proper Conduct of Animal Experiments (1 June 2006) and approved by the Japanese Society for Laboratory Animal Resources (17-026). Strips of the soleus muscle were harvested and tied to wooden sticks to preserve the *in situ* sarcomere length. The strips were then placed in a 50% rigor, 50% glycerol solution with protease inhibitors (one tablet per 50 ml) (cOmplete™, Roche Sigma-Aldrich, USA) to chemically disrupt the muscle membrane. Subsequently, the strips were stored in a freezer at −20°C for at least 2 weeks. On the day of the experiments, a single fiber was isolated using fine forceps under a dissecting microscope (SM-1TSW2-L6W-M, AmScope, USA). The isolated fiber was then transferred to a custom-made experimental chamber containing a relaxing solution with protease inhibitors (one tablet per 100 ml) (cOmplete™, Roche Sigma-Aldrich). One end of the fiber was attached to a force transducer (model 403B, Aurora Scientific, Ontario, Canada), and the other was attached to a length controller (model 315D, Aurora Scientific) using a cellulose membrane dissolved by acetone. The sarcomere length was measured using an He-Ne laser-based diffraction system (HNLS008L-JP, THORLABS, Japan), and the fiber length (1.9±0.7 mm) and diameter (74.8±11.2 µm) were measured using a stereomicroscope (SM-8TW2-144S, AmScope, USA). Experiments were performed at room temperature (24.9±0.4°C). A previous study reported that in the case of chemically skinned fibers, more than 90% of passive force was derived from titin (Irving et al., 2011).

Experimental procedures and measurements

In this study, we performed the normal and BDM trials to calculate the magnitude of force loss caused by inhibiting cross-bridge cycling. In experiment 1 (N=15), the BDM trial was conducted first, followed by the normal trial (Fig. 1, top panel). The fiber length was set at an average sarcomere length of 2.4 µm. The fibers were activated at this length isometrically and then actively stretched to an average sarcomere length of 3.0 µm in 2 s (Fig. 1). After the end of the active stretch, the isometric contraction was continued for 15 s. The same protocol was employed for the normal and BDM trials, except for the activating solution (normal activating solution or BDM-containing activating solution). In experiment 2, a similar experiment was conducted in a shorter operating region (Fig. 1, middle panel). Specifically, the end of the fiber attached to the motor was moved by 25% of fiber length to shorten the fiber. After that, the fiber was isometrically activated at that length, and then

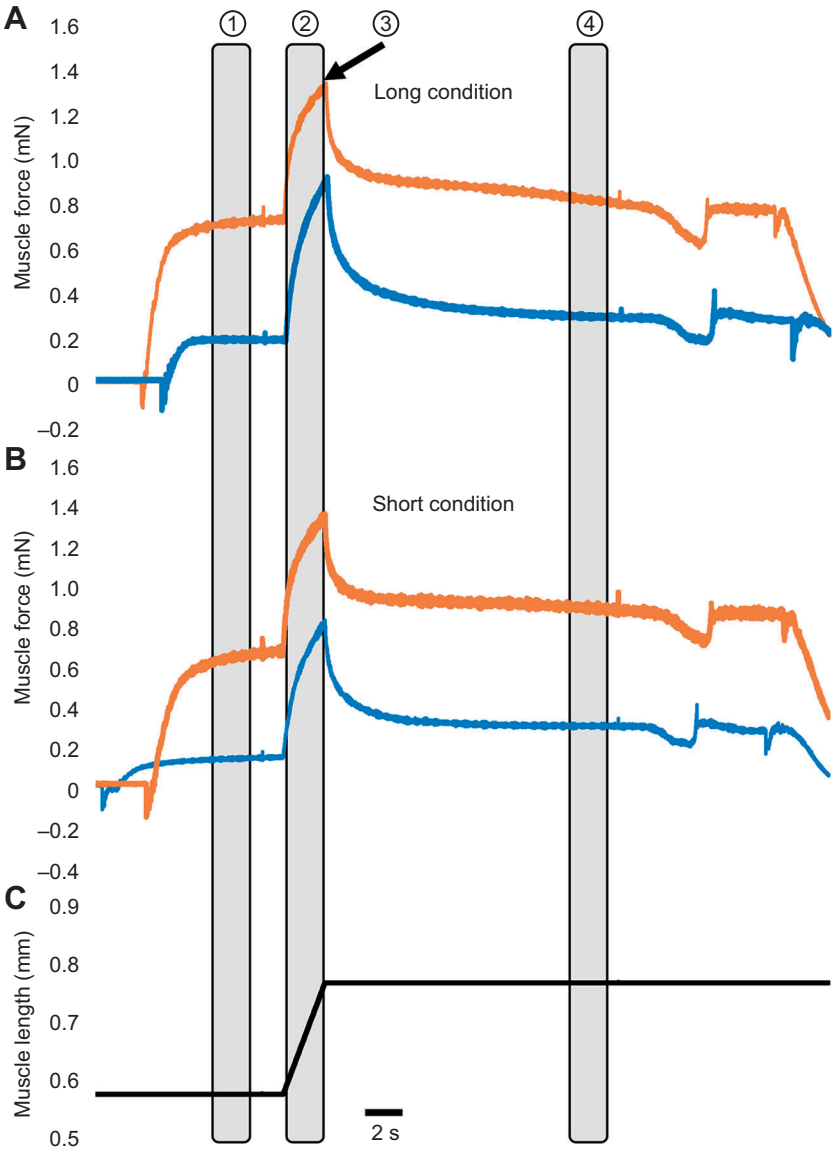

Fig. 1. Force and length response of the normal (orange line) and 2,3-butanedione monoxime (BDM; blue line) trials. (A) Long condition; (B) short condition. The black line indicates the length change, which is identical for all four trials (C). Relative force was calculated at the following four time points: (1) pre-stretch phase, (2) stretching phase, (3) end of stretch and (4) post-stretch phase. The muscle force was evaluated at these four time points to calculate the force loss between normal and BDM trials.

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actively stretched to an average sarcomere length of 2.4 µm in 2 s. After the end of the active stretch, the isometric contraction was continued for 15 s. This condition was defined as the short condition and experiment 1 was defined as the long condition. In the short condition, the fibers were passively shortened by the 25% of fiber length (theoretically, to an average sarcomere length of 1.8 µm). However, all the fibers became slack by this 25% shortening, indicating that the sarcomere length before contractions was not 1.8 µm. Owing to this, when fibers were activated by BDM-containing activating solution (i.e. inhibited weak contractions), the slack was not eliminated (nine out of 15 fibers) because the force was too small to eliminate the slack within the limited contraction duration. Thus, we eliminated nine fibers for the following analyses in experiment 2 (N=6). The force and length responses were recorded at 10 kHz. In this study, 'force' is considered as the total force including active and passive forces unless mentioned.

The magnitude of force loss was expressed as the relative value of the muscle force attained in the BDM trial with respect to the muscle force attained in the normal trial. This relative force was calculated at the following four time points: the isometric phase before the active stretch (pre-stretch), during the stretch (averaged value attained during the stretch, ECC mean), the end of the stretch (ECC peak) and the isometric phase 14 s after the end of the stretch (post-stretch) (Fig. 1). These calculations were conducted under both long (experiment 1) and short (experiment 2) conditions.

Solutions

The rigor solution contained 50 mmol l^{-1} Tris, 100 mmol l^{-1} potassium chloride, 2 mmol l^{-1} magnesium chloride and 1 mmol l^{-1} EGTA, pH 7.0. The relaxing solution contained 170 mmol l^{-1} potassium propionate, 2.5 mmol l^{-1} magnesium acetate, 20 mmol l^{-1} MOPS, 5 mmol l^{-1} K_2EGTA and 2.5 mmol l^{-1} ATP, pH 7.0. One tablet of protease inhibitors was added to 100 ml of the relaxing solution. The washing solution contained 185 mmol l^{-1} potassium propionate, 2.5 mmol l^{-1} magnesium acetate, 20 mmol l^{-1} MOPS and 2.5 mmol l^{-1} ATP, pH 7.0. The normal activating solution contained 170 mmol l^{-1} potassium propionate, 2.5 mmol l^{-1} magnesium acetate, 10 mmol l^{-1} MOPS, 2.5 mmol l^{-1} ATP and free Ca^{2+} buffered with EGTA (CaEGTA and K_2EGTA were mixed to obtain a solution with a pCa value of 4.2), pH 7.0. The BDM-containing activating solution was the same as the normal activating solution, except it included 20 mmol l^{-1} BDM.

Statistical analysis

Descriptive data are presented as means±s.d. For experiment 1 (N=15), the magnitude of force loss was compared among pre-stretch, ECC mean, ECC peak and post-stretch using a one-way repeated-measures ANOVA. If the main effect was found, a subsequent *post hoc* test (Tukey's HSD) was conducted. For experiment 2 (N=6), a paired t-test was conducted to examine whether the magnitude of force loss differed between short and long conditions at each time point (pre-stretch, ECC mean, ECC peak and post-stretch). The level of significance was set at α<0.05.

RESULTS AND DISCUSSION

For experiment 1, a one-way repeated measures ANOVA revealed that the magnitude of force loss was significantly different at the different time points (F=209.02, P<0.001). The subsequent *post hoc* test revealed that the magnitude of force loss was significantly larger in pre-stretch than in the others (relative force: pre-stretch 41.0 ±11.4%, ECC mean 64.1±7.9%, ECC peak 72.6±5.3%, post-stretch 51.3±8.3%; P<0.001–0.008) (Fig. 2). This result indicates that the eccentric and isometric forces after an active stretch are relatively preserved when cross-bridge cycling is inhibited compared with the reference isometric force.

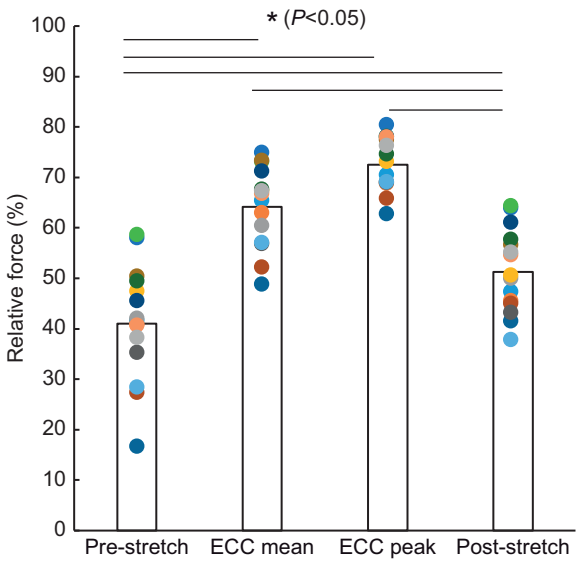

Fig. 2. Magnitude of the relative force (force loss) evaluated at the four time points of the long condition. Individual values are overlaid on the bar graphs. Asterisk indicates a significant difference between time points (P<0.05, N=15). The magnitude of force loss was significantly larger in pre-stretch than in the other time points.

It has been widely confirmed that the isometric force attained after an active stretch is increased compared with the isometric force attained in a reference isometric contraction. This phenomenon is called residual force enhancement (Abbott and Aubert, 1952; Edman et al., 1982; Herzog et al., 2006) and is considered to be derived from titin (Nishikawa, 2016; Herzog, 2018; Fukutani and Herzog, 2019). This idea is partially supported by our recent study, which showed that the magnitude of residual force enhancement was preserved even by inhibiting cross-bridge cycling (Fukutani and Herzog, 2018), suggesting the contribution of a non-cross-bridge component, possibly titin, to residual force enhancement and eccentric muscle force. Because the titin force originates from its elasticity, it can be preserved when cross-bridge cycling is inhibited. Here, we discuss the possible explanation of our finding based on the two different forces, suggested by Pinniger et al. (2006), that is, cross-bridge force and non-cross-bridge force (primarily caused by titin force). Based on this, the force attained before the active stretch (pre-stretch) was composed of (1) cross-bridge force and (2) non-enhanced titin force, i.e. titin force produced without active stretch, whereas that attained after the active stretch (post-stretch) was composed of (1) cross-bridge force and (2) enhanced titin force, i.e. titin force produced with active stretch. When cross-bridge cycling was inhibited by BDM, component 1 decreased while component 2 was preserved. Because the relative contribution of component 2 to the total force was larger in the post-stretch than in the pre-stretch owing to the enhanced titin force, the magnitude of force loss should become smaller in the post-stretch, which was experimentally confirmed in this study. A similar concept can be applied to the force attained during the active stretch (ECC mean) and the end of the active stretch (ECC peak) because the effect of enhanced titin force exists not only after an active stretch but also during an active stretch (Schachar et al., 2004; Bullimore et al., 2007). Although we

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could not confirm whether residual force enhancement was successfully induced in this study because we did not perform a reference isometric contraction, it is likely that residual force enhancement was successfully induced because the stretching protocol was identical to that used in our previous study, which confirmed the existence of residual force enhancement (Fukutani and Herzog, 2019).

This speculation was supported by the results of experiment 2. In this experiment, we conducted similar trials with experiment 1 in the shorter operating region (from 1.8 to 2.4 µm). If the titin force explains the smaller force loss in the ECC mean, ECC peak and post-stretch than in the pre-stretch, this effect should be smaller in the shorter operating region. This is because the magnitude of elastic force is dependent on the length, which is smaller in short conditions. Therefore, the effect of titin force should be smaller in the short length condition. As a result, a paired *t*-test revealed that, compared with the long condition, the magnitude of force loss was significantly larger in the short condition (relative force: pre-stretch 38.7±10.6%, ECC mean 57.6±8.1%, ECC peak 64.5±6.3%, post-stretch 45.8±9.9%; P=0.002 for the ECC mean, P<0.001 for the ECC peak and P=0.012 for the post-stretch), except for the pre-stretch (P=0.057) (Fig. 3), which support our hypothesis. Taken together, it is reasonable to assume that decreased force loss in the ECC mean, ECC peak and post-stretch should be caused by titin force.

In addition to the isometric force after an active stretch (post-stretch in this study), the eccentric force during stretch (ECC mean and ECC peak in this study) is considered to be affected by titin force. This is based on the result that the force attained during and at the end of stretch with the same stretching velocity and at the same muscle length was larger when the active stretch was initiated from the shorter length, i.e. the magnitude of stretch was larger (Schachar et al., 2004). Because the effect of residual force enhancement is known to be large when the stretching magnitude is large (Edman et al., 1978), a reasonable interpretation is that the larger force in the longer stretch condition is derived from residual force enhancement, i.e. titin force (cross-bridge force should be the same in that study because the force–velocity and force–length conditions were identical). Thus, it is reasonable to assume that enhanced titin force exists not only isometric contractions after an active stretch but also during active stretches. The present study provides support for the growing body of evidence that titin contributes to the active muscle force in eccentric contractions and isometric contractions after an active stretch and suggests the influence of enhanced titin force on the magnitude of force loss induced by inhibiting cross-bridge cycling.

In experiment 2, fibers were shortened by changing the position of motor. As a result, fibers became shorter than the optimal length and finally became slack. Activation was started from this slack state. As such, fibers were shortened in the early phase of

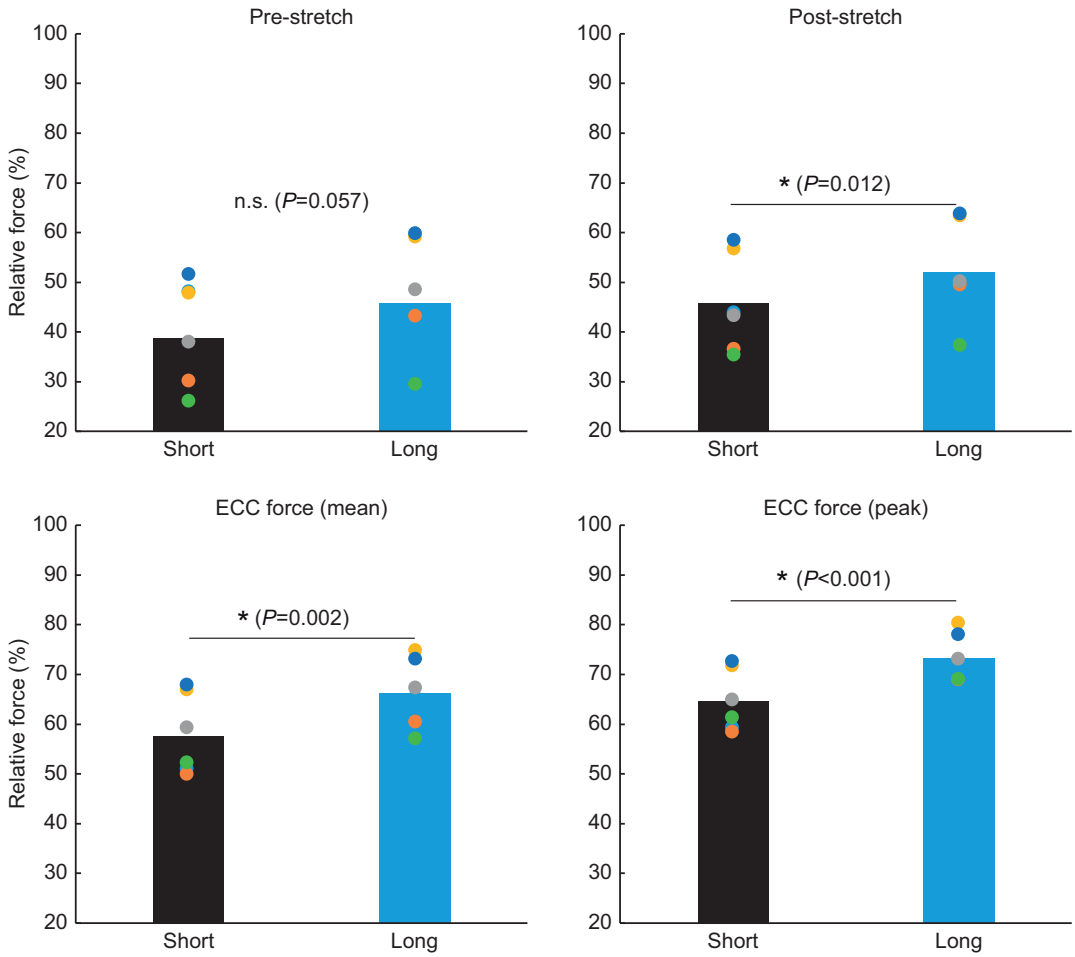

Fig. 3. Comparison of the magnitude of the relative force (force loss) evaluated at the four time points between short (black bar) and long (blue bar) conditions. Individual values are overlaid on the bar graphs. Asterisk indicates a significant difference between short and long conditions (P<0.05, N=6).

contractions. This shortening might induce force depression (Abbott and Aubert, 1952), which affects our main finding. However, even if this force depression exists, its influence on our result should be very small because the magnitude of force depression is strongly related to the magnitude of mechanical work attained during shortening (Herzog and Leonard, 1997), and the force produced in our early phase of contraction (shortening phase for eliminating the slack) was very low (virtually zero).

In conclusion, the eccentric and isometric forces after an active stretch are less susceptible to cross-bridge cycling inhibition than the reference isometric force. This phenomenon can be explained by the elastic force produced by titin.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: A.F., T.I.; Methodology: A.F., S.K.; Validation: A.F., S.K.; Formal analysis: A.F., S.K.; Investigation: A.F., S.K.; Resources: A.F., S.K.; Data curation: A.F., S.K.; Writing - original draft: A.F.; Writing - review & editing: A.F., S.K., T.I.; Visualization: A.F.; Supervision: T.I.; Project administration: T.I.; Funding acquisition: A.F., T.I.

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