Relationship between Force and Histological Changes in Mouse Anterior Crural Muscles Following Eccentric Exercise

Mike Newton[(1)], Kazunori Nosaka[(1, 2)], and Paul Sacco[(1)]

(1) School of Biomedical and Sports Science, Edith Cowan University, Western Australia, Australia, and (2) Exercise and Sports Science, Department of Environmental Science, Yokohama City University, Yokohama, Japan

Abstract

The purpose of this study was to determine the relationship between mononuclear cell number and maximal isometric force (P_o) following active lengthening exercise. The tibialis anterior muscles (TA) of mice were actively lengthened 120 times from a dorsiflexed to plantarflexed position in 300 ms while stimulating the peroneal nerve (100 Hz, 310 ms). P_o at 150 Hz was measured immediately before and after,10 min, 3 and 10 days after exercise and the total number of mononuclear cells per field of view (MN) for the TA muscles were counted. P_o declined to 55% of the baseline value both immediately and 10 min after exercise, declined further to 25% at 3 days, and was still significantly reduced at 10 days following exercise. TA muscles at 3 and 10 days post-exercise showed marked increases in MN, however, the only significant relationship between P_o and MN was at 3 days following exercise ($r = -0.64$). The results of this study do not support the use of P_o following active lengthening exercise to either predict the extent of subsequent injury, or estimate the concurrent degree of histological damage.

Key words: eccentric exercise, E-C coupling failure, maximal isometric force, mononuclear cells, muscle damage.

Basic Appl Myol 10 (5): 225-229, 2000

Novel or unaccustomed exercise has been shown to produce temporary and repairable damage to muscle [1]. This phenomenon has been termed exercise-induced muscle damage (EIMD). Exercise involving a predominance of high force eccentric contractions (active lengthening) has been found to produce the greatest magnitude of muscle damage in both human [10] and other animal [9] models. A temporary decline in voluntary muscular force and power production [7], impairment of fine motor control [11], swelling [6], reduction in range of motion [3], and muscle soreness [13] are all associated with damage resulting from eccentric exercise and have obvious implications for athletic performance [14].

Researchers have employed a number of measures as indicators of EIMD. These include abnormally high plasma levels of intramuscular proteins, strength loss, and altered muscle contractile properties [4]. In order to quantify the degree of damage to myofibres, in humans, it is necessary to extract tissue samples via either needle or open biopsies. Although this process is useful for experimentation purposes, the procedure is invasive, usually requires the services of a trained medical practitioner, and may not be indicative of damage to the whole muscle. Non-invasive approaches to this problem may be to use strength loss following eccentric exercise as an estimator or predictor of muscle fibre "damage" [15].

It seems reasonable to assume that a decrement in maximal isometric force (P_o) is associated with loss of contractile proteins due to mononuclear cell phagocytosis. In rodents, it has been shown that following novel maximal eccentric contractions mononuclear cell invasion of the exercised muscle peaks at 3 days [9]. By 10 days post exercise, lower numbers of monocytes are present around the regenerating myofibres [8]. If indeed, mononuclear cell phagocytosis is a major determinant of loss in muscle force generation capacity, there should be a relationship between mononuclear cell number and P_o following eccentric exercise. Therefore, the purpose of this study was to determine the relationship between the number of mononuclear cells present and P_o of tibial anterior muscle at selected time points following active lengthening exercise.

Methods

Experimental animals

Thirty-six female mice (C57 BL / 10 strain) aged 18-22 weeks were obtained from an Animal Resource Centre and were housed in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes. Food and water were available ad libitum. All animal care and use procedures met the guidelines set by the Animal Ethics Committee of Edith Cowan University and permission to conduct the study was obtained from this committee prior to commencement. Animals were anaesthetised with 25% pentobarbitone sodium (0.03 ml per gram body weight) injected into the peritoneal cavity and the right knee was clamped to restrict movement of the lower limb. An incision was made laterally and slightly below the level of the patella, the peroneal nerve exposed and a stainless steel hook electrode positioned around the nerve. The animal's foot was taped to a footplate containing a force transducer that was directly attached to the axle of a Vexta stepping motor (Oriental Motor Co T.T.D.). The foot was positioned so that rotation of the axle caused it to move through approximately 100° from a dorsiflexed to plantarflexed position in 300 ms. The contraction velocity and range of motion of the stepping motor were controlled via an Oregon Micro Systems Inc MH10 Microstep Drive (10 μ steps/step) which received its instructions from a batch program containing stepper motor commands run through a digital to analogue board housed in an IBM 286 personal computer.

During active lengthening the peroneal nerve was stimulated using a Digitimer DS-7 electrical stimulator (Digitimer Ltd) at a frequency of 100 Hz for 310 ms with 100 μs square wave pulses. Voltage was fixed at 200 V and the optimal amperage of 0.3 A, as determined in previous pilot work, by increasing from zero until maximum force production of the dorsiflexor muscles was recorded at the foot plate in the absence of any noticeable co-contraction of the plantarflexor muscles or drop in P_o. Stimulation commenced 10 ms prior to and throughout muscle lengthening, the return phase being passive. The duration of the entire cycle, from a fully flexed starting position to a fully flexed final position was five seconds. Animals were randomly assigned to an active lengthening or passive lengthening group. The lengthening phase was either active or passive (i.e., stimulated or non-stimulated) and was repeated every 5 seconds for five sets of 24 repetitions (total of 120 repetitions). Each set was separated by a one-minute interval, during which P_o was recorded as the peak force generated during a one second stimulation of the anterior crural muscles at 150 Hz. Following the lengthening protocol the electrode was removed, the incision area was sutured and cleaned, and the animals allowed to recover. The passive group was treated in an identical fashion as the active lengthening group, except that no stimulation was applied to the peroneal nerve during lengthening.

Muscle force (P_o)

Force output was determined via a force transducer mounted into a footplate that was attached to the axle of a stepping motor. Analogue signals from the force transducer were amplified and sent for processing to an analogue to digital converter, located in an IBM 486DX-33 personal computer. Converted digital signals were captured by a software program (Waveview – 1993, Eagle Appliances Pty LTD) at 1000 data points per second (1000 Hz) and displayed visually in a format which allowed measurement of the captured forces. The foot was positioned at a fixed angle of 90 degrees to the foreleg and the anterior crural muscles were stimulated via the peroneal nerve. Previous pilot work examining force-angle relationships at 10-degree intervals from 70 to 100 degrees revealed that a fixed foot angle of 90 degrees to the foreleg consistently produced optimal force. The P_o at 150 Hz was collected immediately prior to exercise, immediately following the 120^{th} lengthening movement, 10 minutes following the final lengthening movement, and 3 and 10 days after exercise. Data from preliminary studies showed that no further increases in P_o were obtained when stimulation frequencies above 150 Hz were employed.

Histological preparation and analysis

Mice were terminated with a lethal intraperitoneal dose of pentobarbital sodium. Lengthened and contralateral TA muscles were carefully dissected out and a portion was separated from the belly of the muscles, mounted adjacent to and in contact with each other on a corkboard with the base of the muscles surrounded by Optimum Cutting Temperature (OCT) fluid, and frozen in isopentane cooled in liquid nitrogen. The muscle blocks were oriented in such a way that the long axis of the muscle fibres lay perpendicular to the base of the cork board allowing transverse sections to be separated in a cryostat.

Eight μm transverse sections were separated from all muscle blocks in a Tissue Tek cryostat at minus 20°C, fixed in 10% formalin and stained with haematoxylin and eosin. The stained tissue samples were viewed via microscopy under x40 magnification, and histological data were gathered on every second field of each muscle section. Ten fields were counted for all treatment and contralateral muscle sections using a Leitz Wetzlar 42 point graticule. The total number of mononuclear cells per field of view was counted and the average of the ten fields was calculated for each muscle.

Analysis of data

All statistical analyses were carried out using the software program SPSS for Windows release 10. A two-way ANOVA with Bonferroni post-hoc comparisons was used to test for significant differences between the passive and active groups during the course of lengthening (i.e., pre-lengthening to 10 minutes post lengthening) and 3 and 10 days of recovery. The alpha level was set at 0.05 for the ANOVA. One-tailed Pearson product moment correlations were used to correlate the average number of mononuclear cells per field of view with normalized P_o at 10 min, 3 days, and 10 days, respectively. Significance was set at an alpha level of 0.05 for all correlation analysis. Results are displayed as mean ± standard error of the mean (SEM).

Results

Muscle force (Po)

For muscles which underwent active lengthening the normalized P_o declined to 55.3±2.8% of the baseline value immediately following the 120th repetition ($p<0.05$), and showed no evidence of recovery after 10 minutes rest (Figure 1). After 3 days of recovery from active lengthening, P_o further declined to 24.8±10.1% of baseline, and whilst there was some recovery in force by 10 days, it was still significantly ($p<0.05$) reduced from baseline (Figure 1). No significant correlation between the normalized P_o at 10 minutes following exercise and at 3 and 10 days after exercise was evident. There were no significant declines in the passively lengthened muscles at any time during the post-exercise period (Figure 1).

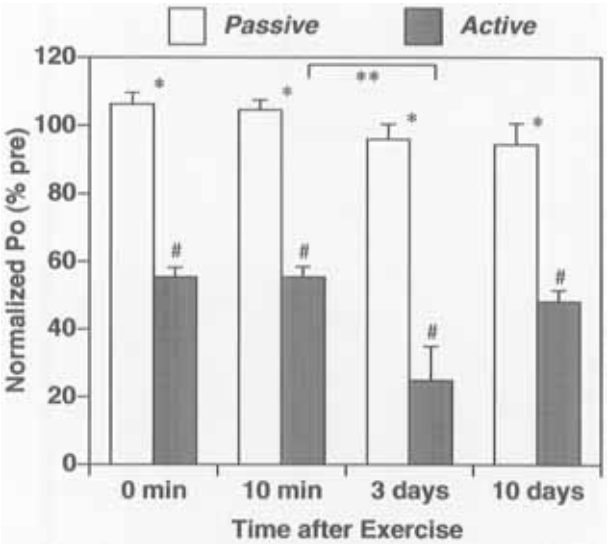

*Figure 1. Changes in normalized Po from the pre-exercise level (100%) immediately (0 min), 10 min, 3 days and 10 days after lengthening exercise for passive and active conditions. Mean and S.E.M. values are shown. # indicates significant ($p<0.05$) difference from base line, and * indicates significant ($p<0.05$) difference between the conditions. ** denotes significant ($p<0.05$) difference between 10 min and 3 days for the active condition.*

Histology

Histological examination of the TA muscles at 3 days post-exercise showed marked mononuclear cell infiltration of the actively lengthened compared to the non-exercised contralateral muscles. Pilot work revealed that the average number of mononuclear cells per field of view of the non-lengthened contralateral muscles did not significantly differ from the passively lengthened muscles, therefore the contralateral muscles were used for histological comparison. This allowed mice to act as their own immunological and age matched controls.

As shown in Figure 2, an average of 0.6±0.1 mononuclear cells per field of view were present in contralateral muscles, whereas, actively lengthened muscles contained an average of 4.8±0.8 mononuclear cells, this difference being significant ($p<0.05$). Mononuclear cell number of the actively lengthened muscles was slightly lower after 10 days (3.9±0.8), but this difference did not prove statistically significant from that of 3 days.

Muscle force versus histology

Figure 3a displays the relationship between the average number of mononuclear cells per field of view of actively lengthened muscles at 3 days post exercise and normalized P_o following active lengthening at 10 min and 3 days post exercise. Although there was no relationship between force at 10 minutes and subsequent invasion by mononuclear cells, a significant negative relationship ($r = -0.64$; $p<0.05$) was evident between

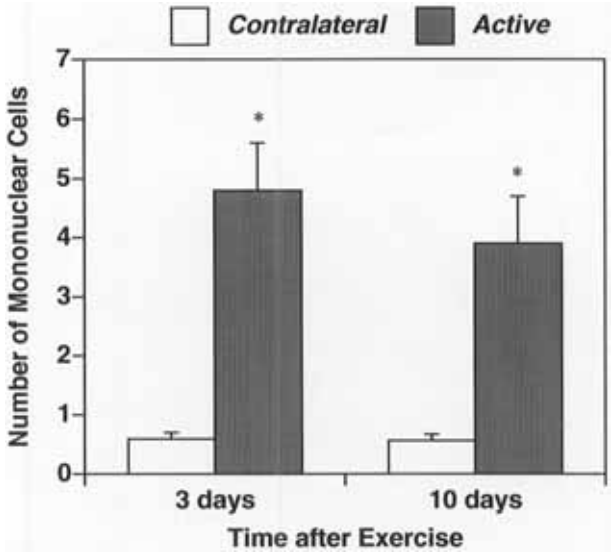

*Figure 2. The average number of mononuclear cells per field of view at 3 and 10 days following exercise for actively lengthened (Active) and contralateral muscles. Mean and S.E.M. values are shown. * indicates significant ($p<0.05$) difference between the conditions.*

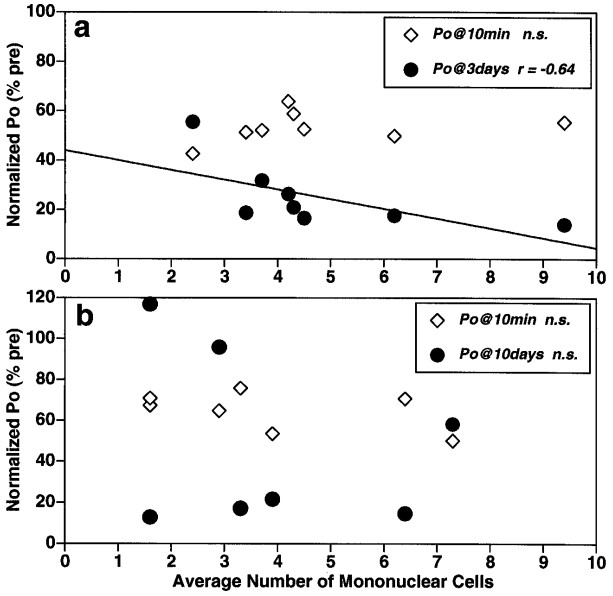

Figure 3. Correlation between the average number of mononuclear per field of view at 3 (a) or 10 (b) days after active lengthening exercise and normalized Po at 10 min post exercise (shown by ◇; Po@10min) or the day when the mononuclear cell number was counted (shown by ●; P@3days for a, Po@10days for b). n.s.: not significantly different.

force and histology at 3 days. At 10 days following active lengthening no significant relationship between force at either time point and mononuclear cell numbers was evident (Figure 3b).

Discussion

The present study demonstrated significant decreases in normalized P_o for actively exercised muscles immediately following 120 lengthening repetitions, the magnitude of which was similar to that previously reported in a similar model of injury [5, 9]. The loss of force immediately post exercise was unlikely the result of metabolic fatigue, since 10 minutes of passive rest resulted in no recovery of P_o. Balnave and Allen [2] and Warren et al. [16] suggested that the main cause of force loss immediately following such exercise is excitation-contraction (E-C) coupling failure, which is consistent with our findings. Ingalls et al [7] injured the anterior crural muscles of mice in vivo with 150 active lengthening contractions, and P_o, caffeine and potassium-induced contracture forces, sarcoplasmic reticulum Ca^{2+} release and uptake rates, and intracellular Ca^{2+} concentration were measured in EDL muscles at varying times post active lengthening. They determined that E-C coupling impairment could account for at least 75% of the reduction in P_o immediately after the injury and at least 57% of the decrement in P_o following 5 days of recovery. In the present study there was no significant correlation between the normalized P_o at 10 minutes following exercise and at 3 and 10 days after exercise. This suggests that the mechanisms contributing to the force decrement in the minutes following eccentric exercise are different to those operating at 3 and 10 days.

The lack of correlation between P_o 10 minutes following active lengthening and mononuclear cell numbers 3 days after lengthening exercise (Figure 3a) was unexpected. We proposed that a significant proportion of the P_o decrement immediately, and at 10 minutes following active lengthening exercise would be due to mechanical stress to the sarcolemma and contractile / cytoskeletal proteins, and would be related to the number of mononuclear cells invading these damaged myofibres at 3 days. Mechanical stress to these structures may be responsible for a portion of the P_o decrement in the minutes following eccentric exercise, although the present data suggests that this factor would be minor compared to that attributable to E-C coupling failure. However, if there was a small involvement of mechanical stress at this time, especially to sarcolemmal and other cytoskeletal structures, it may still be effective in initiating secondary injury to susceptible myofibres. Data from the present study, though, do not support the use of P_o 10 minutes following active lengthening to predict the extent of myofibre damage by infiltrating mononuclear cells. It was consistent with the work of Balnave & Allen [2] and Ingalls et al. [7] suggesting that E-C coupling failure plays a significant role in the P_o decrement in the short term following eccentric exercise.

At 3 days post exercise the normalized P_o of actively lengthened muscles were significantly lower than the passive group, and this time point also coincided with the lowest normalized P_o recorded following lengthening (Figure 1). The 3 day time-point was chosen as this was previously shown to be when the greatest force loss occurred following 240 active lengthening repetitions of mouse foot dorsiflexor muscles [12]. We found that the P_o decrement and average number of mononuclear cells were significantly correlated 3 days after active lengthening (Figure 3a). At this time muscle weakness would likely be due primarily to loss of contractile and cytoskeletal proteins caused by mononuclear cell phagocytosis. In contrast, Ingalls et al. [7] suggested that by 5 days following active lengthening exercise loss of contractile proteins may account for only 57% of the P_o decrement, and this could explain why the negative correlation between these variables in the present study was not stronger. Extrapolation of the relationship between force loss and the number of monocytes in Figure 3a would suggest that if there were no mononuclear cells present in the myofibres at 3 days following active lengthening P_o would still be reduced below pre-exercise levels. This data is in agreement with the work of Ingalls et al. [7], described above, and indicates con-

tribution from factors other than mononuclear cell phagocytosis in the P_o decrement as late as 3 days following active lengthening. The lack of a stronger significant relationship between P_o and mononuclear number at 3 days following active lengthening would question the use of P_o at this time point to estimate the extent of histological disruption to the exercised myofibres.

At 10 days following active lengthening both the average number of mononuclear cells per field of view and the P_o decrement were significantly greater than that of the non-actively lengthened muscle (Figures 1 and 2). However, when P_o at 10 minutes, and 10 days following active lengthening were correlated with the average number of mononuclear cells present at 10 days, no significant relationships were apparent (Figure 3b). This was the case even though Ingalls et al. [7] reported that the contribution of decreased contractile proteins could account for nearly all of the P_o decrements from 2 to 4 weeks.

In support of Warren et al. [16] contention, E-C coupling failure has been implicated in the reductions in P_o following active lengthening exercise. As stated above, this cause of P_o decrement may not be associated with damage to myofibrillar structures, or infiltration of immune mononuclear cells due to maintenance of sarcolemmal integrity. In conclusion, it seems that the results of this study do not support the use of P_o following active lengthening exercise to either predict the extent of subsequent injury, or estimate the concurrent degree of histological damage. In addition, whether a relationship exists between E-C coupling failure and subsequent monocyte infiltration following active lengthening remains to be elucidated.

Acknowledgments

The authors would like to acknowledge Mrs Frances Lloyd for her assistance with the histological preparation, and Dr Colin James for his contribution during the early stages of the study.

Address correspondence to:

Mike Newton, School of Biomedical and Sports Science, Edith Cowan University, 100 Joondalup Drive, Joondalup, WA 6027, AUSTRALIA, tel. 61 8 9400 5171, fax 61 8 9400 5717, Email m.newton@ecu.edu.au.

References

[1] Armstrong RB, Warren GL, Warren JA: Mechanisms of exercise-induced muscle fiber injury. *Sports Med* 1991; 12: 184-207.

[2] Balnave CD, and Allen DG: Intracellular calcium and force in single mouse muscle fibres following repeated contractions with stretch. *J Physiol* 1995; 488: 25-36.

[3] Clarkson PM, Nosaka K, Braun B: Muscle function after exercise-induced muscle damage and rapid adaptation. *Med Sci Sports Exer* 1992; 24:512-520.

[4] Ebbeling CB, Clarkson PM: Exercise-induced muscle damage and adaptation. *Sports Med* 1989; 7: 207-234.

[5] Faulkner JA, Jones DA, Round JM: Injury to skeletal muscles of mice by forced lengthening during contractions. *Quart J Exper Physiol* 1989; 74: 661-670.

[6] Howell JN, Chelboun G, Conaster R: Muscle stiffness, strength loss, swelling and soreness following exercise-induced injury in humans. *J Physiol* 1993; 464: 183-196.

[7] Ingalls CP, Warren GL, Williams JH, Ward CW, Armstrong, RB: E-C coupling failure in mouse EDL muscle after in vivo eccentric contractions. *J Appl Physiol* 1998; 85: 58-67.

[8] Lowe DA, Warren GL, Ingalls CP, Boorstein DB, Armstrong RB: Muscle function and protein metabolism after initiation of eccentric contraction-induced injury. *J Appl Physiol* 1995; 79: 1260-1270.

[9] McCully KK, Faulkner JA: Injury to skeletal muscle fibers of mice following lengthening contractions. *J Appl Physiol* 1985; 59: 119-126.

[10] Newham DJ, Jones DA, Clarkson PM: Repeated high-force eccentric exercise: effects on muscle pain and damage. *J Appl Physiol* 1987; 63: 1381-1386.

[11] Pearce AJ, Sacco P, Byrnes ML, Thickbroom GW, Mastaglia FL: The effects of eccentric exercise on neuromuscular function of the biceps brachii. *Aus J Sci Med* 1998; 4: 236-244.

[12] Sacco P, Jones DA, Dick JRT, Vrbova G: Contractile properties and susceptibility to exercise-induced damage of normal and mdx mouse tibialis anterior muscle. *Clin Sci* 1992; 82: 227-236.

[13] Smith LL: Acute inflammation: the underlying mechanism in delayed onset muscle soreness. *Med Sci Sports Exer* 1991; 23: 542-551.

[14] Smith LL, Keating MN, Holbert D, Spratt DJ, McCammon MR, Smith SS, Israel RG: The effects of athletic massage on delayed onset muscle soreness, creatine kinase, and neutrophil count: a preliminary report. *J Sports Phys Ther* 1994; 19: 93-99.

[15] Warren GL, Lowe DA, Armstrong RB: Measurement tools used in the study of eccentric contraction-induced injury. *Sports Med* 1999; 27: 43-59.

[16] Warren GL, Lowe DA, Hayes DA, Karwoski CJ, Prior BM, Armstrong RB: Excitation failure in eccentric contraction-induced injury of mouse soleus muscle. *J Physiol* 1993; 468: 487-499.