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Applied Physiology, Nutrition, and Metabolism
Physiologie appliquée, nutrition et métabolisme

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Journal:	*Applied Physiology, Nutrition, and Metabolism*
Manuscript ID:	apnm-2014-0274.R1
Manuscript Type:	Article
Date Submitted by the Author:	n/a
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Keyword:	muscle damage < muscle

Regenerated *soleus* muscle shows reduced creatine kinase efflux after contractile activity *in vitro*

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Abstract

Regenerated skeletal muscles show less muscle damage after strenuous muscle exercise. The aim of the studies was to investigate if the regeneration is associated with reduced muscle creatine kinase (CK) efflux immediately after the exercise. Cryolesion was applied to the *soleus* muscle (SOL) of 3 month old C57BL/6J male mice. Then total CK efflux was assessed *in vitro* in the regenerated (REG) muscles without exercise or after 100 eccentric contractions. The same measurements were performed in the control (CON) muscles which were not exposed to cryolesion. REG muscles generated weaker ($P < 0.05$) twitches, but stronger ($P < 0.05$) 150-Hz and 300-Hz tetani with prolonged ($P < 0.01$) contraction times compared to the control muscles. There was no difference between REG and CON muscles in the total CK efflux without exercise, but only CON muscles showed an increase ($P < 0.001$) in the CK efflux after the exercise. Our results suggest that muscle regeneration is associated with modulation of contractile properties and improvement in muscle resistance to damage after eccentric exercise.

Keywords: cryolesion, primary damage, muscle damage, repeated bout effect, eccentric exercise, lengthening contractions, mice.

Introduction

Efflux of creatine kinase (CK) from skeletal muscles is a popular marker of muscle damage after exercise and disease (Brancaccio et al. 2007). It is often assessed by measuring plasma CK activity. It has been known for some time that eccentric exercise is associated with a particular large increase in plasma CK compared to other types of contractile activity (Newham et al. 1986). Plasma CK and other indicators of muscle damage show ameliorated response to a repeated bout of exercise even if it is performed several weeks after the first exercise bout (Clarkson et al. 1992). The mechanisms underlying this repeated bout effect remain unclear.

Eccentric contractions can induce disruption of myofibrils and thus cause a prolonged impairment in muscle force generating capacity (McHugh 2003). The reasons for CK efflux from skeletal muscles are controversial since muscle exercise might promote an increase in permeability of muscle fibers which is not necessarily associated with damage to muscle fibers (Yu et al. 2013). It is believed that inflammatory cell infiltration of skeletal muscles can also promote secondary muscle damage after exercise and thus contribute to muscle CK efflux after exercise (McHugh 2003; Tidball 2011). It is important to examine effects of primary muscle damage during exercise and secondary damage after exercise separately in order to clarify the mechanisms of repeated bout effect. Reduction in primary muscle damage would reflect increased resistance of muscle structures to disruption during exercise while modulation of the inflammatory responses would determine the secondary damage. Indeed, it is unclear if the increase in plasma CK activity after exercise is due to the primary or secondary muscle damage. There is often no or only a minor increase in plasma CK activity immediately after exercise and plasma CK peaks 1-3 days after the exercise coinciding with the peak in muscle soreness

(Armstrong 1984; Fredsted et al. 2008). Isolated skeletal muscles *in vitro* are well suited for studying primary muscle damage since measurements of muscle CK efflux can be performed immediately after exercise with limited contribution of the secondary muscle damage. Mouse *soleus* muscle (SOL) provides a good model for such studies since it contains approximately equal proportions of type I and type II fibres type (Kilikevicius et al. 2013; Denies et al. 2014) and thus resembles human quadriceps muscle, which is often used in human studies (Staron et al. 2000).

Muscle incubation with damaging agents leads to injury and subsequent regeneration of rat skeletal muscles (Jackson et al. 1987). In another study the regenerated *extensor digitorum longus* muscle of rats showed reduced ultrastructural damage compared to the control muscle when examined 3 days after plyometric exercise (Devor and Faulkner 1999). It is, however, unclear if this apparent resistance to muscle damage was caused by modulation of primary or secondary mechanisms of muscle damage. The aim of our study was to test the hypothesis that muscle regeneration after cryolesion is associated with a reduction in muscle CK efflux immediately after exercise and could be attributed to reduction in the primary muscle damage. We compared CK efflux from the regenerated and control SOL *in vitro* without any prior exercise and immediately after repeated eccentric contractions.

Methods

Animals and experiments; All procedures involving mice were approved by the Lithuanian Republic Alimentary and Veterinary Public Office (Nr. 0223). As in our previous studies, C57BL/6J mice were housed in the standard cages without exercise equipment, one to three mice

per cage at a temperature of 22-24º C and 40-60 % humidity (Ratkevicius et al. 2010; Kilikevicius et al. 2013) with the normal 12/12-h light/dark cycle. Animals were fed a standard chow diet (76.9% kcal from carbohydrate, 16.9 % kcal from protein, 6.2 % kcal from fat; Kedainiu grudai, Kedainiai, Lithuania) and received tap water ad libitum. As described below (see "Muscle contractile properties and CK efflux"), we assessed CK efflux from SOL muscle at rest without any prior exercise (n = 6) and after 100 eccentric contractions (n = 8) in 12 week old male mice. These muscles are referred to as control (CON) muscles. We have also performed the same measurements at rest (n = 9) and after exercise (n = 9) in SOL after regeneration and refer to these muscles as regenerated (REG). In a separate series of experiments, we have also assessed effects of two-hour *in vitro* incubation on peak isometric force of CON muscles (n = 4) using the same procedures described for the assessment of muscle CK efflux.

Muscle regeneration; Muscle regeneration was induced by cryolesion as described elsewhere (Irintchev et al. 2002). Briefly, at age of 2 months male mice (n = 18) were anesthetized by intraperitoneal injection of the anesthetics: ketamine (120 mg/kg; Richter Pharma AG, Wels, Austria) and xylazine (14 mg/kg; Eurovet Animal Health B.V., Bladel, Netherlands). The hair from the leg was removed using electric shaver. SOL was exposed by making the incision through the overlaying skin and connective tissue, and retracting the adjacent gastrocnemius muscle. Muscle cryolesion was induced by touching the middle portion of SOL with flat end of a copper rod (3x0.7 mm) precooled in liquid nitrogen and maintaining its position for 5 s. After 2 min when the muscles had thawed, the skin incision was closed with polyamide threads (4–0 Ethilon; Ethicon, Norderstedt, Germany) and mice were placed on 37^0C temperature plate for several hours to avoid hypothermia. After 29 days (at age of ~3 months) we assessed contractile properties and CK efflux.

Muscle contractile properties and CK efflux; Mice were euthanized by cervical dislocation and weighted immediately afterwards (Kern, ABS 80-4, Balingen, Germany). Sutures were attached to the proximal and distal tendons of SOL from left leg. The muscle was then excised and fixed between two platinum plate electrodes in 50 ml Radnotti tissue bath filled with Tyrode solution (121 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, 1.8 mM $CaCl_2$, 0.4 mM NaH_2PO_4, 0.1 mM EDTA, 24 mM $NaHCO_3$, 5.5 mM glucose, pH adjusted to 7.4) bubbled with 95 % O2 and 5 % CO2. The distal tendon of the muscle was attached to a hook and the proximal end was tied directly to the lever of muscle test system (1200A-LR Muscle Test System, Aurora Scientific Inc., Aurora, Canada). The muscle was then left to equilibrate in the solution for 7 min. Afterwards muscle length was increased in steps every 2 min and the muscle was stimulated at 150 Hz for 3 s. This procedure was continued until no further increase in muscle force was seen with the increase in muscle length. Thereafter the muscle was photographed with the length scale in the background to assess muscle length with a precision of 0.5 mm. The subsequent force measurements were performed at this optimal muscle length. Firstly, single twitch was evoked. Then force frequency relationship was determined by stimulating muscle at 30, 50, 75, 150, 300 Hz for 3 s with 2 min intervals in between the stimuli trains. Afterwards, SOL was subjected to repeated eccentric contractions every 10 s or the control experiment without exercise. For the eccentric exercise, SOL was stimulated at 150 Hz stimulation for 700 ms. Over the last 200 ms of this stimulation 3.5 mm ramp stretch was performed followed by 200 ms gradual return of the muscle to the initial length without any stimulation. In the control experiment, SOL was left at rest for ~ 20 min. After the exercise or the control experiment, SOL was placed in 2 ml of Tyrode solution for 2 hours. Afterwards, muscles were dried and weighed. 250 µl of Tyrode solution was taken to assess CK activity with biochemical analyzer (Spotchem™ EZ SP-4430,

Menarini Diagnostics, Winnersh-Wokingham, UK) with soft reagent strips (ARKRAY Factory, Inc., Shiga, Japan).

Statistical analysis; All data analysis was performed using Prism 5.0 software. The two factor analysis of variance (ANOVA) was used to assess effects of regeneration and exercise on muscle contractile properties and muscle CK efflux. The post hoc testing was carried out using t-tests with a Bonferroni correction for multiple comparisons. Person's correlation coefficient was calculated to investigate the association between the variables. All the tests were two-tailed with significance level was set at $P < 0.05$.

Results

The data on body mass, muscle mass, tetanic force, specific force and optimal muscle length of CON and REG muscles are presented in Table 1. There were no differences between CON and REG muscles in these parameters.

Data on force-frequency relationship as well as twitch and tetanus properties are shown in Fig. 1. In comparison to CON muscles, REG muscles generated relatively less ($P < 0.01$) force in single twitches and more force ($P < 0.001$) in tetani at 150 Hz and 300 Hz. Twitch contraction times did not differ between REG and CON muscles, but REG muscles showed shorter ($P < 0.001$) relaxation times than CON muscles. On the other hand, REG muscles had prolonged ($P < 0.01$) contraction time in 150 Hz tetanus compared to CON muscles.

Data on peak isometric and eccentric force during eccentric exercise are shown in Fig. 2. Peak isometric force decreased more than eccentric force during the exercise ($P < 0.001$). REG

158 muscles tended to maintain isometric force better than CON muscles though the difference was
159 not significant. There were no differences between CON and REG muscles in eccentric force.
160
161 A separate set of experiments on control SOL showed that peak isometric force decreased ($P <$
162 0.05) by 6.9 ± 2.8 % when muscles were incubated for 2 hours in Tyrode solution as during the
163 assessment of muscle CK efflux. Data on the total muscle CK efflux without any prior exercise
164 and after 100 repeated contractions are shown in Fig. 3. The CK efflux did not differ between
165 REG and CON muscles when muscles were not subjected to prior exercise. However, the total
166 muscle CK efflux was significantly ($P < 0.001$) larger in the exercised CON compared to
167 exercised REG muscles and the non-exercised muscles. The exercised REG muscles did not
168 differ from the non-exercised muscles. The total muscle CK efflux did not correlate with force
169 loss by the end of exercise in CON muscles, but the there was a positive correlation between
170 these parameters in REG muscles.
171
172 **Discussion**
173 The main aim of the study was to test the hypothesis that muscle regeneration is associated with
174 a reduction in exercise-induced increase in the total muscle CK efflux. Our results show that the
175 regenerated muscles produced weaker single twitches, but stronger tetani with longer contraction
176 times compared to the control muscles. The regenerated muscles did not differ from the control
177 muscles in the total CK efflux without exposure to exercise. However, in contrast to the control
178 muscles, these muscles did not show any increase in the total CK efflux after the repeated
179 eccentric contractions. These results suggest that muscle regeneration is associated with

180 modulation of contractile properties and increased resistance to loss of muscle proteins during
181 exercise.
182
183 Myotoxin (bupivacaine) injection has been often applied to induce muscle injury with
184 subsequent regeneration (Devor and Faulkner 1999). Muscle cryolesion induces a similar
185 phenomenon (Irintchev et al. 2002; Pereira et al. 2014). There were no differences in either body
186 mass or muscle mass between the mice exposed to muscle cryolesion and the control mice in our
187 study. Peak tetanic force, specific force and optimal length were also similar in the regenerated
188 and control muscles. Thus mice and treated muscles recovered fully within 29 days after the
189 cryolesion. However, the regenerated muscles showed slow rate of force generation in tetani.
190 Indeed, muscle regeneration is associated with a shift towards slower muscle fiber types and
191 myosin isoforms (Whalen et al. 1990; Irintchev et al. 2002). These changes might contribute to
192 the lower rate of tetanic force generation and a tendency towards better force maintenance in
193 repeated contractions. A robust contractile response of regenerated muscle suggests a full
194 recovery from the damage caused by the intervention. This would be consistent with earlier
195 findings showing that the number of fibres do not decline in the muscle after the cryolesion
196 (Irintchev et al. 2002). However, it is unclear what mechanisms are responsible for the
197 improvement in force output at high frequencies of electrical stimulation coupled with small
198 amplitude and fast relaxation of single twitches. This might be due to alteration in intracellular
199 calcium handling, but changes in muscle fibre force summation are also possible. It appears that
200 the total number of muscle fibres tends to increase in the regenerated muscles after the
201 cryolesion (Irintchev et al. 2002). However, it is unclear if all these fibres can contribute equally
202 to force output at different frequencies of electrical stimulation. Assessment of glycogen

depletion patterns in muscle fibres after electrical stimulation might be a useful experimental strategy to resolve this uncertainty in future studies.

We used a precooled copper rod to induce muscle cryolesion. This procedure followed by subsequent muscle regeneration might have resulted in altered permeability of sarcolemma. However, our results are inconsistent with such scenario. The total CK efflux at rest, when muscles were not subjected to exercise, did not differ between the regenerated and control muscles. The magnitude of this CK efflux was also similar to the previously reported for rat soleus muscle in vitro (Jackson et al. 1987). Thus it is unlikely that there were significant differences in muscle membrane permeability to CK molecules between the control and regenerated muscles.

We assessed the total CK efflux during the two-hour muscle incubation in Tyrode solution. Consistent with previous studies (Plant et al. 2001), peak force generating capacity showed only a small decline during the two-hour muscle incubation which suggests that there was no major disruption of muscle contractile apparatus. We did observe a tendency for an increase in muscle mass after this procedure (unpublished observation). This might be a reflection of an increase in muscle water content (Sjøgaard et al, 1985), and it is likely that muscle CK efflux is partially associated with the osmotic stress generated by muscle incubation in Tyrode buffer. However, skeletal muscles are well adapted to withstand such stresses as there is an increase in the muscle's extracellular and intracellular water content after exercise of submaximal and maximal intensity, respectively (Sjøgaard et al, 1985). Our results suggest, however, that muscle regeneration is not associated with increased muscle resistance to CK efflux at rest under the influence of mild osmotic stress.

There were significant differences between the control and regenerated muscles after the exercise. The regenerated muscles showed no increase in CK efflux whereas the control muscles showed a substantial, 2.8-fold, increase. These differences between the regenerated and control muscles could not be explained by the variation in the mechanical stresses experienced by muscles since the regenerated muscles produced more force than control muscles during the exercise. Thus, regenerated muscles showed a true increase in resistance to exercise-induced muscle CK efflux. Interestingly, CK efflux from the regenerated muscles correlated with force loss during the exercise, but there was no such correlation for the control muscles (see Fig. 3). This suggests that there was a qualitative difference between the muscles. It is likely that a disruption of muscle structure is needed to cause a significant increase in CK efflux from the regenerated muscles while the control muscles show an increase in the muscle CK efflux after exercise even without damage to the contractile machinery. Indeed, skeletal muscles often show a significant CK loss even when there are no clear signs of the ultrastructural damage (Yu et al. 2013).

It is often argued that exercise training can lead to an increase muscle collagen content which might affect mechanical properties and thus improves muscle resistance to exercise-induced muscle damage (McHugh 2003; Mackey et al. 2004). Indeed, eccentric exercise training can lead to an increase in dynamic and passive stiffness of skeletal muscles (Reich et al. 2000). However, we did not observe any difference between the regenerate and control muscles in forces generated during the eccentric phase of the contractions when the controlled stretching of the muscles was imposed. These findings speak against muscle stiffness being of importance for exercise-induced muscle CK efflux in the regenerated muscles. A shift in muscle fiber

249 composition towards slower contraction muscle fibers and myosin isoforms could be of greater
250 importance. Slow twitch muscle fibers show less damage than fast twitch fibers after exercise
251 (Chapman et al. 2013).
252
253 Our findings agree with previous studies on rat muscles showing less structural damage in
254 regenerated muscles 3 days after plyometric exercise (Devor and Faulkner, 1999). Our results
255 suggest that reduced primary muscle damage is likely to be a major factor in regeneration-
256 induced resistance to muscle damage. It appears that stimulation of muscle regeneration might be
257 a useful strategy in increasing resistance to exercise-induced muscle damage. Leucine
258 supplementation increased the gain in myofiber size during regeneration though its effects on
259 muscle resistance to exercise-induced damage are less clear (Pereira et al. 2014).
260
261 In summary, our results show that muscle regeneration is associated with modulation of
262 contractile properties and increased resistance to the primary muscle damage during exercise, but
263 it is not protecting against muscle CK efflux at rest when mild osmotic stresses are applied.
264

Acknowledgements

We are very grateful to Petras Jeneckas and Audrius Capskas for technical assistance. The study was supported by a grant (No. MIP-067/2012) from the Research Council of Lithuania.

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335

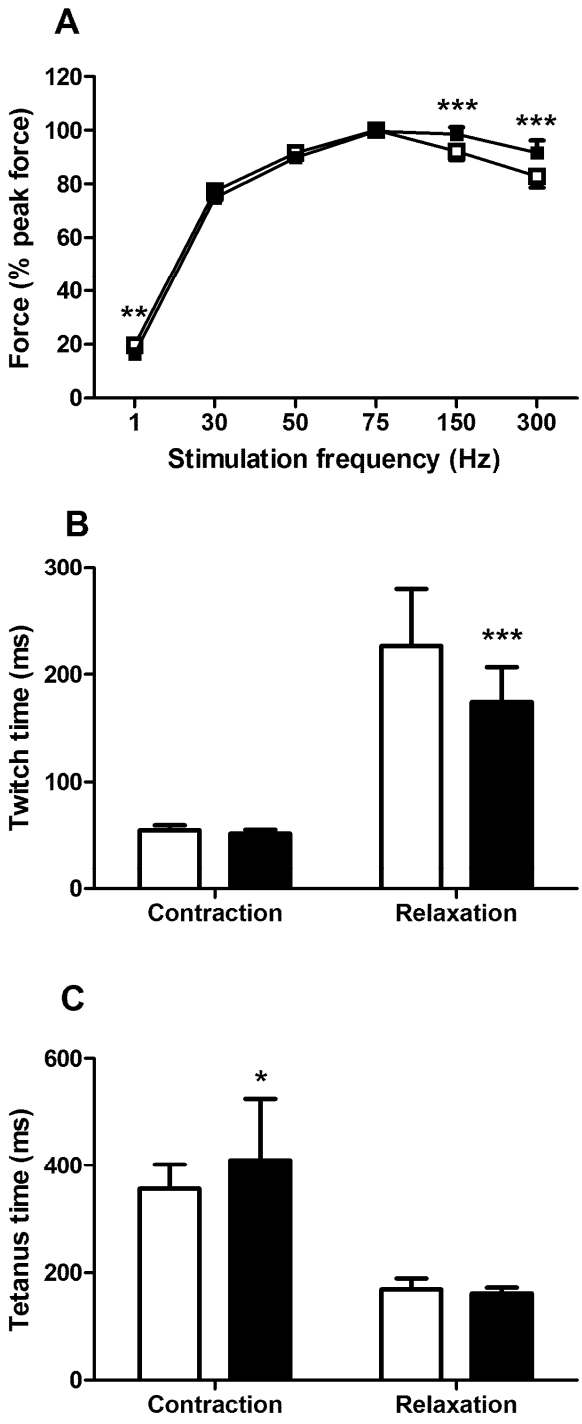
336 **Table 1.** Body mass as well as *soleus* (SOL) muscle mass, peak tetanic force, specific force and
337 optimal muscle length in mice with the control (CON) and regenerated (REG) muscles. Values
338 are means ± S.D.

	Body mass (g)	*Soleus* mass (mg)	Tetanic force (mN)	Specific force (N/g muscle)	Optimal muscle length (mm)
CON	24.6 ± 1.9	10.0 ± 1.4	166.2 ± 19.4	18.7 ± 2.3	16.2 ± 1.6
REG	23.8 ± 1.2	9.1 ± 0.7	175.6 ± 23.2	19.2 ± 1.7	15.8 ± 0.8

339

340

341 **Figure 1.**



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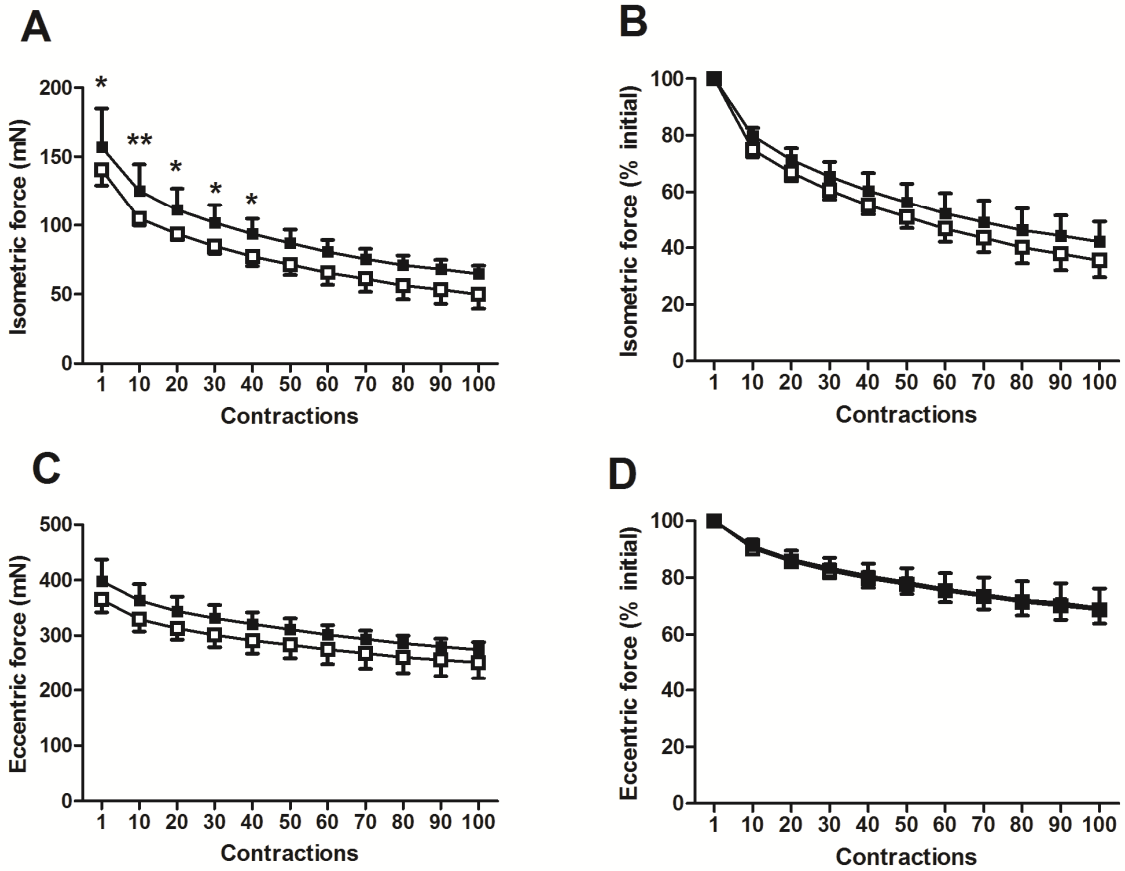
344 **Figure 2.**



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347 **Figure 3.**

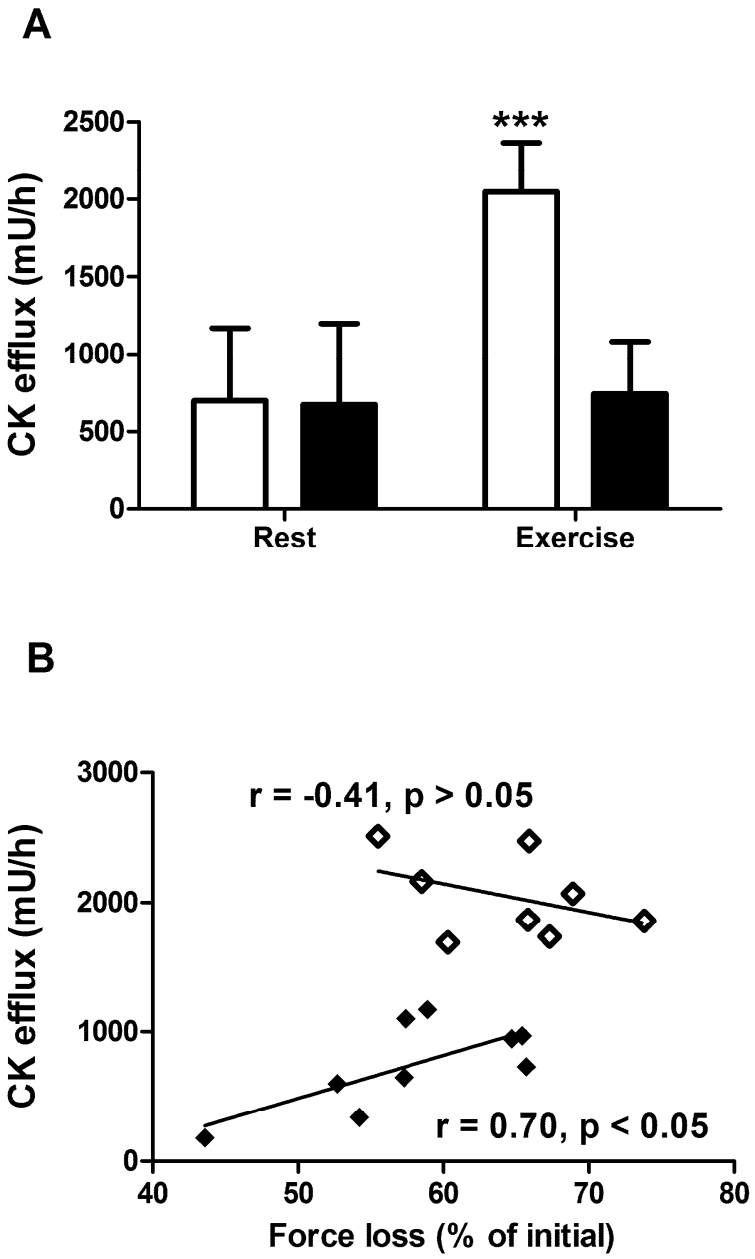

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Figure captions

Figure 1. Force-frequency relationship (A), twitch speed (B) and 150-Hz tetanus speed (C) in control (CONT, white symbols) and regenerated (REG, black symbols) *soleus* muscles. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Values are means and S.D.

Figure 2. Peak isometric and eccentric force for the control (CONT, white symbols) and regenerated (REG, black symbols) muscles during 100 contractions repeated every 10 s. * $P < 0.05$; ** $P < 0.01$. Values are means and S.D.

Figure 3. A) Muscle CK efflux for the control (CON, white bars) and regenerated (REG, black bars) muscles at rest without prior exercise and after 100 eccentric contractions; B) Scatter plot of muscle CK efflux versus isometric force loss for CON muscles (white symbols) and REG muscles (black symbols). Values of Pearson product-moment correlation coefficient are also indicated. *** $P < 0.001$. Values are means and S.D.