"Effect of creatine supplementation on skeletal muscle of mdx mice."

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Abstract
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EFFECT OF CREATINE SUPPLEMENTATION ON SKELETAL MUSCLE OF mdx MICE

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Recent evidence suggests that oral creatine supplementation is an effective intervention to increase maximal voluntary contraction and resistance to fatigue, and also to prevent joint contractures in patients with Duchenne muscular dystrophy (DMD).14 DMD is the most frequently inherited human myopathy; its etiology is a mutation of a gene on the locus Xp21 coding for dystrophin. The mdx mice share the same genetic alteration and are often used as an animal model for DMD.10 The body mass and muscle mass of mdx mice are larger than those of C57/Bl10 control animals.1 Nevertheless, their muscles develop 20% less tetanic tension than normal mice.19 The fast-twitch extensor digitorum longus (EDL) muscles of mdx mice show a dramatic fall in isometric tension when submitted to repeated forced lengthening contractions in vitro.17,20 Histological analysis indicates that this higher susceptibility to damage is associated with membrane disruption, supporting the idea of a reinforcement role for dystrophin.17 Moreover, mdx muscles exhibit focal areas of degeneration, necrosis, and regeneration of centrally nucleated fibers.1 The calcium homeostasis of mdx muscles is disturbed, leading to a twofold increase of the total muscle calcium content.2

Even if muscle creatine concentration is significantly increased after creatine supplementation in DMD patients,8,14 the mechanisms explaining any positive effects are not understood. Since dystrophic patients never perform high-intensity exercises, it is unlikely that a high level of phosphocreatine by itself influences muscle performance. A lower cytosolic Ca²⁺ concentration has been found in mdx myotubes grown in the presence of 20 mM creatine and an enhancement of formation and survival of myotubes was concomitantly observed.21 Moreover, a strong reduction of the first wave of muscle necrosis observed 4 weeks after birth was also reported in mdx mice fed with a creatine-enriched diet.18

In this study, we hypothesized that creatine supplementation in mdx mice tends to normalize the phenotype of the animals and increases tetanic tension developed by isolated EDL muscles, but does not affect the other criteria of dystrophinopathy (e.g., low muscle resistance to a forced lengthening protocol and elevated total calcium content) as there is no reason to believe that creatine corrects
the defect in cell membrane integrity that characterizes the disease.

**MATERIALS AND METHODS**

**Animals.** Male dystrophin-deficient (mdx, *n* = 15) mice and their genetically identical controls (C57/Bl10, *n* = 18), aged between 100 and 110 days and weighing 20–30 g, were kept on a constant light-to-dark cycle (12:12) and given access to powdered chow and drinking water ad libitum. This protocol was approved by the Ethics Committee for Animal Experimentation of the Université Catholique de Louvain.

**Creatine Supplementation.** Creatine was given for 30 days in both food (47 g kg⁻¹) and water (14 g L⁻¹). The average creatine intake was about 13.7 g kg⁻¹ body mass per day. The animals were divided into four groups: C57 control (*n* = 7); C57 creatine (*n* = 11); mdx control (*n* = 6); and mdx creatine (*n* = 9).

**Muscle Contractile Properties.** Mice were anesthetized by an intraperitoneal injection of a mixture of ketamine (100 mg kg⁻¹) and xylazine (10 mg kg⁻¹). After the dissection, the EDL muscles were mounted horizontally between a force transducer and a motor in an organ bath containing Krebs solution consisting of (mM): NaCl, 118; NaHCO₃, 25, KCl, 5; KH₂PO₄, 1.0; CaCl₂, 2.5; MgSO₄, 1.0; glucose, 5, which was continuously gassed with 95% O₂ and 5% CO₂ and maintained at 20°C.

The muscle was adjusted at the reference length, *L₀*, defined as the length at which the muscle develops maximal isometric force during a 300-ms 125-Hz tetanus. The muscle was stimulated by passing current (condenser discharges) between two platinum electrodes fixed along the two sides of the chamber.

**Tetanic Force Measurements.** The following parameters were calculated from isometric force records: maximum isometric force; maximum rate of force rise at the beginning of the tetanus (in milli-newtons per millisecond), calculated as the slope of the tangent to the initial fast rising phase of the record); and the half-relaxation time (T50%; the time necessary to obtain a 50% decrease in force after the last stimulus of the tetanus). Maximal tetanic tension was defined as the ratio of maximal isometric force to cross-sectional area of the muscle. Tension was calculated as follows: tension = force (newtons) × length (millimeters) × muscle density (1.06 mg mm⁻³) × cos of pennation angle (0.48) × muscle mass⁻¹ (milligrams).

**Stretch Resistance.** After 5-minute recovery, the forced lengthening protocol was initiated. The EDL muscle was stimulated for 300 ms. The length remained constant for the first 150 ms (isometric contraction); then, the muscle was forcibly stretched by 7.7% of *L₀*, at a constant velocity of 1 fiber *L₀* per second. Muscle length was returned to *L₀* 2 s after the last stimulus. This procedure was repeated six times with a time interval of 3 min between successive contractions. The force drop was expressed as the percentage decrease of maximal isometric force between the first and the last contraction of the series. At the end of the study the muscle was blotted, weighed, and stored at −80°C for later analyses.

**Muscle Calcium Content.** Isolated gastrocnemius muscles were rinsed for 15 min in a Ca²⁺-free Krebs solution containing 2 mM ethylene-glycol tetraacetic acid (EGTA) to remove extracellular calcium. They were then freeze-dried and dissolved in 0.5 ml of 10N HCl. The concentration of calcium was measured by atomic absorption flame photometry, after adequate dilution in the presence of 1% LaCl₃. Results are expressed in micromoles of calcium per gram dry weight.

**Histology.** Tibialis anterior muscles were frozen in isopentane cooled with liquid nitrogen. Cryostat 7-µm-thick transverse sections were prepared and stained with hematoxylin and eosin; they were used for morphometric analysis (total muscle section surface, and total cell count in the section; mean cell surface was obtained by dividing the section surface by the number of cells in the section). Centrally nucleated fibers were counted in separated Gomori-stained sections.
Table 1. Effect of creatine supplementation on total creatine contents, muscle contractile properties, and muscle histology in C57 and mdx mice under control (−) and supplemented (+) conditions.

<table>
<thead>
<tr>
<th></th>
<th>C57 − (7)</th>
<th>C57 + (11)</th>
<th>mdx − (6)</th>
<th>mdx + (9)</th>
<th>Race</th>
<th>Treatment</th>
<th>Race* treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total creatine (mM)</td>
<td>34.2 ± 3.4</td>
<td>33.0 ± 1.2</td>
<td>31.1 ± 4.1</td>
<td>34.8 ± 3.5</td>
<td>NS</td>
<td>NS</td>
<td>P = 0.04</td>
</tr>
<tr>
<td>Phosphocreatine (mM)</td>
<td>18.8 ± 3.7</td>
<td>17.7 ± 2.4</td>
<td>19.1 ± 1.6</td>
<td>22 ± 4.3</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Rate of force rise (mN ms⁻¹)</td>
<td>12.5 ± 3.2</td>
<td>11.4 ± 1.1</td>
<td>12.8 ± 3.3</td>
<td>10.7 ± 2.6</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>T50% (ms)</td>
<td>41.6 ± 3.6</td>
<td>50.5 ± 0.7</td>
<td>43.4 ± 2.7</td>
<td>50.5 ± 1.7</td>
<td>NS</td>
<td>P &lt; 0.001</td>
<td>NS</td>
</tr>
<tr>
<td>Force drop (%)</td>
<td>7 ± 1</td>
<td>6 ± 1</td>
<td>72 ± 3</td>
<td>63 ± 7</td>
<td>P &lt; 0.001</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Mean fiber surface (μm²)</td>
<td>2476 ± 238</td>
<td>2609 ± 227</td>
<td>4270 ± 261</td>
<td>3414 ± 206</td>
<td>P &lt; 0.001</td>
<td>NS</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Centrally nucleated fibers (% of fibers)</td>
<td>0.1 ± 0.6</td>
<td>0.1 ± 0.6</td>
<td>94.7 ± 0.7</td>
<td>95.1 ± 0.5</td>
<td>P &lt; 0.001</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS, not significant; T50%, time necessary to obtain 50% decrease in force after last stimulus.

Statistical Analysis. All comparisons were made using a two-way analysis of variance (ANOVA; race × treatment). Values are expressed as mean ± SD.

RESULTS

Phosphocreatine (PCr) and Total Creatine (TCr) Contents. In control conditions, TCr was lower in the EDL of mdx than C57 mice (P = 0.02). In the normal mice, creatine supplementation did not modify EDL TCr, but in mdx mice creatine feeding increased EDL TCr by 12% (Table 1). Thus, after the period of creatine supplementation, the creatine content in mdx EDL muscles was similar to that observed in C57 EDL muscles (Table 1). The ratio PCr/TCr remained similar in all conditions.

Muscle Mass. As seen in Figure 1, EDL muscles of mdx control mice were heavier than those of C57 control mice (P < 0.001). In mdx mice, the mean muscle mass was lower in animals fed with creatine than in control animals (P < 0.01), reaching values similar to those observed in EDL muscle of C57 mice.

Maximal Tetanic Tension. Figure 1 shows that EDL muscles of C57 mice developed more tetanic tension than mdx muscles (P = 0.003), and that creatine supplementation slightly increased this tension to the same extent in both groups, even if this improvement did not reach significance (P = 0.08). In Table 1, it can be seen that the slope of force rise and half-relaxation time was similar in control C57 and mdx mice, but after creatine supplementation half-relaxation time was greater in EDL muscles of both C57 and mdx mice. The rate of force rise was unaffected.
Effect of Stretch. As previously reported, dystrophin-deficient muscles were particularly susceptible to forced lengthening applied during maximal muscle activation: in mdx mice, the force drop was ten times larger than in C57 animals ($P < 0.001$; Table 1). Creatine supplementation had no protective effect (treatment nonsignificant).

Total Calcium Content. Total Ca\(^{2+}\) content of gastrocnemius muscles was three times larger in mdx than in C57 mice ($P < 0.001$; Fig. 1). Large differences in Ca\(^{2+}\) content values were observed among mdx mice. Creatine supplementation significantly increased the total muscle Ca\(^{2+}\) contents in mdx mice, but not in C57 mice (race \times treatment effect: $P = 0.001$).

Centrally Nucleated Fibers. As shown in Table 1, the number of centrally nucleated fibers was dramatically increased in tibialis anterior muscle of mdx mice, compared with C57 mice, and creatine supplementation did not reduce this difference.

Mean Fiber Area. The mean fiber area in mdx mice was larger ($P < 0.001$) than in C57 animals; it was reduced after creatine supplementation ($P < 0.05$, Table 1). No such effect was observed in control C57 mice.

DISCUSSION

The results of the present study clearly demonstrate that EDL muscles of mdx mice have a lower TCr than C57 mice. After creatine supplementation the levels of TCr were similar in both strains (Table 1): TCr increased by 12% in muscles of mdx mice and remained unchanged in C57 animals. A lack of TCr increase in EDL muscle of C57 mice has been reported previously. Recently, Brault et al. showed that, after creatine depletion by guanidino-propionic acid (GPA), the rate of creatine uptake was related to fiber type and independent of the level of creatine reached. In the same study, the authors did not show any effect of creatine feeding (1%) on creatine concentration of various muscles (soleus, red gastrocnemius, and white gastrocnemius). However, using a larger dose of creatine (5%), Eijnde showed that the magnitude of TCr increase after creatine supplementation was dependent on the initial TCr level in muscle. Thus, it seems that the lower the initial TCr concentration, the larger the increase will be. Our results suggest that a maximal level of TCr was already reached in the EDL muscle of control C57 mice. The dose of Cr used in this study may appear very large compared to the dose currently given in human studies (about 20 g/day). Preliminary experiments and data from the literature have indicated that such a high dose was required to increase significantly the TCr content in EDL muscles of mdx mice. The animals did not show any side effects during the supplementation period and the food intake was similar in both groups.

The muscle mass of EDL is known to be about 20% larger in young mdx compared to C57 mice. We found similar results. In humans, clinical manifestations of dystrophin deficiency ranged from mild calf muscle hypertrophy with cramps to the classic progressive degenerative hypertrophic myopathy of Duchenne. A common feature of clinical signs of dystrophin deficiency in humans and animal models is the presence of muscle fiber hypertrophy. In mdx mice, injection in tibialis anterior muscle of a vector encoding murine dystrophin leads to an improvement of all studied features of the pathology in treated muscles, including a reduction of muscle hypertrophy. Consequently, a reduction of muscle mass in dystrophinopathy may be taken as a sign of improvement in muscle function. A 16% decrease in EDL muscle mass was observed in mdx mice treated with creatine, giving rise to values similar to those in C57 mice (Fig. 1). A similar finding was observed in gastrocnemius and tibialis anterior muscles, for which the mass and the mean fiber cross-sectional area were reduced in muscles of mdx mice after creatine supplementation. This muscle mass reduction was not due to a loss of water, because the ratio of dry to frozen weight was not modified after treatment. The small-diameter fibers in mdx mice show less necrosis, again suggesting that a reduction in muscle mass is associated with an inhibitory effect on disease progression. However, a higher length/surface ratio does not by itself prevent dystrophy, as creatine-supplementated mdx mice showed the same susceptibility to eccentric contractions (see below). Whether this decrease in muscle surface area is predominant in type 1 or type 2 fibers is unknown.

In this study, we did not observe any decrease in the number of centrally nucleated fibers (Table 1). This contrasts with the observations of Passaquin and coworkers, who reported less muscle damage (a 60% reduction of EDL necrosis/regeneration at 28 days) in supplemented mdx pups. This difference is probably due to the age of the animals. In our study, the mice were 3 months old (i.e., after the first wave of degeneration had occurred) and they showed 95% of centrally nucleated fibers at the end of the study, suggesting that the necrosis was too advanced.
to permit any decrease in number of centrally nucleated fibers. The use of adult mdx mice prevented bias in our results. Indeed, during the period of intensive necrosis/regeneration, necrotic parts of the muscle would have temporarily produced no contraction, whereas interstitial inflammatory and regenerating cells could have been influenced by creatine, as suggested by other investigators. However, because early diagnosis and treatment are not the rule in dystrophinopathies, it is critical to know whether a treatment initiated after the disease process is established can still improve muscle function.

Compared with control mice, EDL muscles of mdx mice produced 20% less isometric tension. The values reported herein fall within the same range. The increase of tetanic tension observed in both strains after Cr supplementation was also in the same range (9.2%) as the increase previously reported in healthy humans and in DMD patients. These results are thus in agreement with the observed ergogenic effect of Cr supplementation.

A surprising result of this study is the positive effect observed on the tension of C57 EDL muscles despite an unchanged TCr level. This finding suggests that intracellular Cr level by itself is probably not the only signal involved in the effects observed after Cr supplementation. An effect of aging is unlikely as we tested mice aged from 100–110 days at the start of the study, that is, during a period of relative stability.

Creatine supplementation was recently reported to reduce relaxation time during intermittent maximal isometric muscle contractions in human subjects, but the same investigators did not find an equivalent effect on isolated mouse EDL muscle. On the contrary, our observations indicate that creatine supplementation increased relaxation time in C57 as well as mdx mice, but did not affect the rate of force rise. Thus, after creatine supplementation, tetani are more fused during contractions. The measurements of all mechanical properties of muscle contraction were highly reproducible. Therefore, the likelihood of a type 2 error in such results is low. Wallimann et al. showed that creatine increases the availability of PCr to an active form of the sarcoplasmic reticulum (SR)-bound creatine kinase (CK), which increases the local supply of the SR-Ca++ pump with adenosine triphosphatase and, consequently, the efficiency of Ca++ sequestration. Our apparently contradictory results could be reconciled if a larger amount of Ca++ is released by the SR after creatine supplementation. In this case, the initial rate of rise of force should increase, but such an effect was not observed. Therefore, this point remains to be clarified.

Elevated total calcium content in muscles is found consistently in both DMD patients and mdx mice. In our study, calcium measurements were made on the entire gastrocnemius, implying that Ca++ content was detected in a mixture of fast-twitch and intermediate muscle types. Creatine supplementation increased total Ca++ contents of gastrocnemius muscles in mdx mice. In this respect, creatine supplementation did not improve the dystrophic phenotype of mdx mice, but probably did not aggravate it either, as the plasma CK values were not increased (data not shown). We did not identify the compartment(s) in which Ca++ accumulated. In another report, Pulido et al. showed that Cr supplementation decreases the rise in cytosolic Ca++ induced by either high extracellular Ca++ concentrations or hypoosmotic stress, and suggested that creatine lowers cytosolic Ca++ by stimulating SR-Ca++ adenosine triphosphatase. These results suggest that the higher cell Ca++ contents observed in our mdx mice after creatine supplementation are localized in the sarcoplasmic reticulum. However, the experiments by Pulido et al. were carried out on mdx myotubes, and such results cannot be directly transposed to living animals.

EDL muscles of mdx mice showed a dramatic fall in isometric force when submitted to repeated forced lengthening contractions in vitro. The findings of this study confirm the effect: the force drop was ten times larger in mdx than C57 mice. However, creatine supplementation did not prevent the loss of force (Table 1).

In conclusion, creatine does not mitigate the disease process, although it may represent a positive intervention to improve muscle function.

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REFERENCES

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AQ1: No “Walliman et al.” entry in References. Please advise.

AQ2: Note change from “correct” to “mitigate.” Please advise.

AQT1: Please indicate/explain asterisk in footer