Dietary NaCl supplementation prevents muscle necrosis in a mouse model of Duchenne muscular dystrophy

Mizuko Yoshida,1 Akira Yonetani,2 Toshihiro Shirasaki,2 and Keiji Wada1

1Department of Degenerative Neurological Disease, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo, Japan; 2Hitachi High Technologies, Ibaraki, Japan

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Yoshida, Mizuko, Akira Yonetani, Toshihiro Shirasaki, and Keiji Wada. Dietary NaCl supplementation prevents muscle necrosis in a mouse model of Duchenne muscular dystrophy. Am J Physiol Regul Integr Comp Physiol 290: R449–R455, 2006. First published September 22, 2005; doi:10.1152/ajpregu.00684.2004.—The mdx mouse is an animal model for Duchenne muscular dystrophy. Mdx mice fed a 12% NaCl diet from birth up to 20 days of age (mdx-Na mice) had an ~50% reduction in serum creatine kinase (CK) activity compared with mdx mice fed a standard diet. Most notably, necrotic fibers in tibialis anterior (TA) muscle of mdx-Na mice were reduced by 99% and were similar in control mice. These mdx mice displayed significantly elevated blood Ca2+ and Na+ levels, while the total calcium content of their TA muscle was reduced to the level of control mice. In addition, mdx-Na mice had elevated zinc and magnesium contents in their TA muscle. These results suggest that elevated serum Na+ leads to Ca2+ extrusion from muscle via the Na+/Ca2+ exchanger causing a decrease in intracellular Ca2+ levels and an increase in blood Ca2+ levels. Extracellular Ca2+ and, in addition, Zn2+ and Mg2+ might also contribute to the stabilization of the cell membrane. Other possibilities explaining the surprisingly efficacious beneficial effect of dietary sodium exist and are discussed.

MATERIALS AND METHODS

Duchenne muscular dystrophy (DMD), a severe X-linked recessive muscle-wasting disease, is a health problem with an incidence of 2–3 per 10,000 males in the world population (15). The disease is caused by a defect in the gene encoding dystrophin, a protein located on the inner surface of the plasma membrane (44). The exact function of dystrophin is unknown, and the prospects for successful treatment of DMD remain uncertain, although numerous studies of gene therapy for DMD remain uncertain, although numerous studies of gene therapy for DMD (21, 22, 42, 43) and of pharmacological treatment (8, 17, 23, 33, 37, 40, 46) have been published.

In DMD patients and in mdx mice, an animal model of DMD, the disease is characterized by necrosis of muscle fibers that causes increased serum levels of the muscle enzymes creatine kinase (CK) and pyruvate kinase (32, 48).

Dystrophin-deficient muscle membranes allow excess Ca2+ influx, causing calcium accumulation in necrotic muscle fibers (6, 11). The total calcium content of skeletal muscle fibers in DMD patients (3, 4, 24, 29) and mdx mice (19, 30, 34) is higher than that found in normal muscle fibers. The causes leading to excess Ca2+ influx into dystrophin-deficient muscle fibers are still unclear, although the following mechanisms have been proposed: 1) because dystrophin is thought to have a mechanical function, dystrophin-deficient muscle fiber membranes are predisposed to rupture (2, 10); 2) Ca2+ leak channels or stretch-activated channels open more easily in dystrophic myotubes, leading to poor Ca2+ regulation (18); and 3) the function of L-type Ca2+ channels is abnormal (12, 47).

We found earlier that serum CK activity in mdx females more than 60 days of age is less than 50% of that observed in males in agreement with published observations (38). Also, we previously found that mdx mice given saline from ages 7 to 24 days exhibit significantly reduced serum CK activity by day 25. These interesting findings led us to ask the following questions: 1) Does saline decrease serum CK activity and, presumably, prevent muscle necrosis in mdx mice? 2) Why is serum CK activity in mdx females lower in males? The answers to these questions may lead to effective therapies for halting the progression of DMD.

We hypothesize that 1) saline may affect the concentration of some ions in the extracellular space (ECS), as well as in muscle fibers differently in mdx and control mice, and 2) these changes may be different between mdx female and male mice.

Duchenne muscular dystrophy
Table 1. Mice serum creatine kinase (CK) activity, blood $K^+$ and $Ca^{2+}$ concentration in male and female mdx and B10 mice from days 7 to 90

<table>
<thead>
<tr>
<th>Age, days</th>
<th>Strain</th>
<th>Sex</th>
<th>CK activity $\times 10^3$, U/l</th>
<th>$K^+$, mM</th>
<th>$Ca^{2+}$, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>mdx</td>
<td>M</td>
<td>0.360±0.122 ($n$=28)$^a$</td>
<td>3.9±0.29 ($n$=31)$^a$</td>
<td>1.51±0.0387 ($n$=31)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F</td>
<td>0.347±0.107 ($n$=29)$^b$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>M+F</td>
<td></td>
<td>3.8±0.30 ($n$=29)</td>
<td>1.54±0.0695 ($n$=29)</td>
</tr>
<tr>
<td></td>
<td>B10</td>
<td>M</td>
<td>0.355±0.160 ($n$=32)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>F</td>
<td>0.397±0.178 ($n$=26)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>mdx</td>
<td>M</td>
<td>7.80±4.58 ($n$=33)$^c$</td>
<td>4.9±0.44 ($n$=28)$^h$</td>
<td>1.31±0.0417 ($n$=28)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F</td>
<td>7.86±4.31 ($n$=33)$^d$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>M+F</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B10</td>
<td>M</td>
<td>0.235±0.0598 ($n$=37)$^e$</td>
<td>3.7±0.30 ($n$=29)</td>
<td>1.30±0.0363 ($n$=29)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F</td>
<td>0.244±0.0442 ($n$=33)$^f$</td>
<td></td>
<td>1.23±0.0431 ($n$=31)$^a$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M+F</td>
<td></td>
<td></td>
<td>1.25±0.0330 ($n$=32)</td>
</tr>
<tr>
<td>60</td>
<td>mdx</td>
<td>M</td>
<td>15.3±13.1 ($n$=32)$^g$</td>
<td>5.0±0.47 ($n$=56)$^b$</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>F</td>
<td>6.73±3.97 ($n$=33)$^h$</td>
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<td></td>
<td>M+F</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>B10</td>
<td>M</td>
<td>0.105±0.0771 ($n$=23)</td>
<td>3.6±0.31 ($n$=60)</td>
<td>1.17±0.0334 ($n$=30)$^a$</td>
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<td></td>
<td></td>
<td>F</td>
<td>0.051±0.0145 ($n=28$)</td>
<td></td>
<td>1.23±0.0344 ($n$=31)$^b$</td>
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<td>M+F</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>mdx</td>
<td>M</td>
<td>16.8±10.1 ($n=33)$^i</td>
<td>5.0±0.61 ($n=58)$^b</td>
<td>1.17±0.0243 ($n=30)$^j</td>
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<td></td>
<td>F</td>
<td>7.91±4.97 ($n=32)$^j</td>
<td></td>
<td>1.20±0.0280 ($n=29)$^k</td>
</tr>
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<td></td>
<td></td>
<td>M+F</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B10</td>
<td>M</td>
<td>0.084±0.0500 ($n=34$)</td>
<td>3.6±0.32 ($n=59)$^k</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>F</td>
<td>0.065±0.0542 ($n=34$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>M+F</td>
<td></td>
<td></td>
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</tbody>
</table>

Values are presented as means ± SD. Mice were fed a standard diet. M, male; F, female; n, number of mice examined. CK activity (serum): a vs. c, b vs. d, e vs. f, g vs. h, i vs. j, P < 0.001; c vs. g, c vs. h, i vs. j, P < 0.004. $K^+$ concentration: a vs. b, b vs. c, P < 0.001; b vs. c, P < 0.001; d vs. e, P < 0.01; f vs. g, P < 0.001. Ca$^{2+}$ concentration: a vs. b, c vs. e, d vs. f, g vs. h, i vs. j, P < 0.001; c vs. g, P < 0.004. $K^+$ concentration: a vs. b, b vs. c, P < 0.001; b vs. c, P < 0.001; d vs. e, P < 0.01; f vs. g, P < 0.001.

4°C (Tomy Seiko, Japan). Serum CK activity was determined with a Cica liquid CK test (Kanto Chemical, Japan) using Synchron CX7 (Beckman Coulter, Fullerton, CA).

Measurement of $K^+$, $Ca^{2+}$, and $Na^+$ concentrations in blood. Blood was collected into 1-ml syringes without anticoagulants by heart puncture under ether anesthesia. The blood was placed immediately in an i-STAT cartridge (EC6+) (i-STAT, Princeton, NJ). The measurement of $Ca^{2+}$, $Na^+$, and $K^+$ concentrations was performed within 2.5 min after drawing blood.

Measurement of total potassium, calcium, sodium, magnesium, and zinc contents in muscle and food. The muscle was excised under deep ether anesthesia. TA muscle or food was placed in Teflon tubes and digested with 0.5 ml of ultrapure HNO$_3$ (Kanto Chemical) at 100°C for 2 h. The samples were diluted to 10 ml with ultrapure water. Potassium, calcium, sodium, magnesium, and zinc contents were determined using inductively coupled plasma emission spectrometry (P-4010, Hitachi High-Technologies, Tokyo, Japan).

Morphological analyses of TA muscle tissue. TA muscles of mice at day 20 were excised and frozen immediately in isopentane cooled in liquid nitrogen. Cryostat transverse sections were stained by Carrazzi’s hematoxylin and eosin Y (1% solution; Muto Pure Chemical, Tokyo, Japan). Necrotic areas of muscle fibers were identified as described (6, 16) and analyzed with a Color Image Analyzer (SP-500 Olympus Optical, Tokyo, Japan). Necrotic areas correspond to the whole TA muscle area minus the white color area and normal (peripherally nucleated) fibers.

Statistics. Data are expressed as means ± SE or ± SD. The data were analyzed using the two-tailed Student’s t-test.

RESULTS

Critical starting point of muscle fiber degeneration and differences between adult males and females. At the start of the study of the effect of NaCl on the degeneration of mdx muscle fibers, we decided to inhibit mdx muscle degeneration before the onset of muscle fiber necrosis. As shown in Table 1, the serum CK activity and blood $K^+$ concentration of mdx mice at day 7 were similar to those of control B10 mice. Average serum CK activity and blood $K^+$ concentration in mdx mice began to increase only after day 7, and the $K^+$ concentration at day 8 (day 8: $4.2 \pm 0.1$ mM, $P < 0.02$) and CK activity at day 10 (day 10: 499 ± 28 U/l, $P < 0.001$) were significantly different from those at day 7. Serum CK activity and blood $K^+$ concentration also were significantly different for mdx mice between days 7 and 20 ($P < 0.001$; Table 1). The results shown in Table 1 show that the serum CK activity and $K^+$ concentration in blood of mdx mice began to increase after day 7 compared with those of B10 mice, suggesting that the mdx cell membrane started to rupture after day 7, causing the CK and the $K^+$ to leak from muscle fibers to ECS. On the basis of these results, we started NaCl administration before day 7.

Blood $Ca^{2+}$ concentration of both mdx and B10 female mice at day 90 was significantly higher than that of males (Table 1). Although the differences of $Ca^{2+}$ concentration between female and male mdx and B10 mice were small, the differences may be advantageous for the discussion, since blood $Ca^{2+}$ concentration is strictly controlled (36). Serum CK activity of female mdx mice was about half of males’ serum CK activity, as described in the introduction (Table 1). The values for serum CK activity in adult mdx mice are consistent with those reported by Suh et al. (38) and Takagi et al. (40).

Female TA muscle zinc contents of mdx and B10 mice were significantly higher than those in males of mdx and B10 mice. Muscle Zn content of mdx males was markedly higher than that of B10 males (Table 2).
Effect of NaCl ingestion on serum CK activity and on morphology of mdx mice. We preliminarily examined whether a diet containing 1, 3, and 5% Na weight/weight (wt/wt) fed to mothers reduced serum CK activity of the offspring. Although 1 and 3% Na was insufficient, the diets containing 5% Na (In the following expression, a diet with 12% NaCl was used.) effectively reduced serum CK of mdx mice at day 20 (Fig. 1). Therefore, these conditions were used in this study. The effect of NaCl ingestion on serum CK activity of mdx on day 20 is shown in Fig. 1. The activity of this marker enzyme of muscle fiber degeneration was reduced to about 60% of that of mdx mice fed control food.

TA muscle fibers of mdx mice showed variations in fiber size, shape, and the incidence of large, dark fibers, and necrotic fibers were increased (Fig. 2, mdx). In contrast, the TA muscle fibers of mice whose mothers ate a diet with elevated NaCl were nearly normal in appearance (Fig. 2, mdx-Na). The area of necrosis in TA muscle sections from mdx-Na mice was drastically lowered (0.46% of total area, \( n/H11005 \ 12 \) compared with mdx mice (46%, \( n/H11005 \ 17 \) (\( P < 0.001 \)). In addition, sections from the mdx-Na mice showed a nearly normal muscle pattern.

Effect of NaCl ingestion on blood K\(^{+}\), Ca\(^{2+}\), and Na\(^{+}\) concentrations. The effect of NaCl ingestion (via mothers’ milk) on blood K\(^{+}\) and Ca\(^{2+}\) concentration of mdx-Na and control mice is shown in Fig. 3. Although NaCl had no effect on blood K\(^{+}\) concentration in control mice, this ion was 11% lower in mdx-Na than in mdx mice (\( P < 0.001 \); Fig. 3A).

Blood Ca\(^{2+}\) and Na\(^{+}\) levels in mdx-Na and control mice were significantly elevated compared with those on the control diet (Ca\(^{2+}\); Fig. 3B, Na\(^{+}\); data not shown).

Effect of NaCl ingestion on calcium, sodium, potassium, magnesium, and zinc content in TA muscle. The calcium and sodium content of TA muscles from mdx mice were markedly higher than those from B10 mice (Fig. 4, Ca and Na). NaCl administration significantly increased B10-Na muscle calcium (\( P < 0.02 \)) and Na contents (\( P < 0.001 \)). However, muscle calcium and sodium contents of mdx-Na mice were dramatically lower than those of mdx mice (both \( P < 0.001 \)) and close to the levels in B10-Na mice.

Muscle potassium and magnesium contents in mdx mice were significantly lower compared with those on the control diet (Fig. 4, K and Mg). In mdx-Na mice, muscle potassium and magnesium contents were noticeably higher as a result of NaCl supplementation (both \( P < 0.001 \)).

The zinc content of muscle from mdx mice was significantly lower than that of the B10 controls (both \( P < 0.001 \); Fig. 4, Zn). NaCl administration markedly elevated the muscle zinc content of both mdx-Na and B10-Na mice (both \( P < 0.001 \)). Muscle Zn content of mdx-Na mice was close to that of B10 mice, but the difference was significant (\( P < 0.01 \)).

Table 2. Total zinc content in the tibialis anterior muscle of mdx and B10 male and female mice on day 60

<table>
<thead>
<tr>
<th>Sex</th>
<th>mdx</th>
<th>B10</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>13.7 ± 0.216 (( n/H11005 \ 30 ))</td>
<td>12.2 ± 0.216 (( n/H11005 \ 30 ))</td>
</tr>
<tr>
<td>F</td>
<td>14.8 ± 0.272 (( n/H11005 \ 20 ))</td>
<td>15.7 ± 0.339 (( n/H11005 \ 30 ))</td>
</tr>
</tbody>
</table>

Values are means ± SE. Zinc content is given in mg/kg wet weight. Mice were fed a standard diet. a vs. b, \( P < 0.005 \); c vs. d, a vs. c, \( P < 0.001 \); b vs. d, \( P < 0.04 \).
and vertical lines are SE.

and B10-Na (n = 28) were fed a diet containing 12% NaCl. a vs. b, P < 0.001. B: Ca2+ concentration; mdx (n = 27), mdx-Na (n = 28), B10 (n = 30), and B10-Na (n = 31), a vs. b, c vs. d, P < 0.001. Bars are mean, and vertical lines are SE.

DISCUSSION

We found earlier that mdx mice given saline by injection exhibited significantly reduced serum CK activity. We have now investigated whether oral supplementation of NaCl reduced serum CK activity and inhibited mdx muscle degeneration if given before the onset of muscle fiber necrosis.

Results in Table 1 show that the serum CK activity and the K+ concentration in the blood of mdx mice began to increase after day 7 compared with those of B10 mice, suggesting that the mdx cell membrane started to rupture after day 7, causing the CK and the K+ to leak from muscle fibers into ECS. On the basis of these results, we started NaCl administration before day 7. NaCl-supplement administration significantly reduced muscle fiber degeneration of mdx mice at day 20 (Figs. 1 and 2). NaCl administration also reduced blood K+ concentration (Fig. 3A) and muscle sodium content (Fig. 4, Na), and increased potassium and magnesium in muscle fibers in mdx-Na mice (Fig. 4, K and Mg). The muscle calcium content of mdx-Na mice decreased drastically compared with that of mdx mice (Fig. 4, Ca) and close to the levels in B10-Na mice. The 99% decrease in the area of necrotic fibers in mdx-Na mice shows that NaCl supplementation almost completely inhibits mdx TA muscle fiber necrosis (Fig. 2). In mdx-Na and B10-Na mice, blood Na+ and Ca2+ concentrations (Ca2+; Fig. 3B) and muscle zinc content (Fig. 4, Zn) increased on day 20 relative to the control. It is unknown why NaCl administration increased both zinc content in muscle fibers and blood Ca2+ concentration concurrently.

These results may have important implications for the inhibition of muscle necrosis, as Ca2+, Na+ and Zn2+ play critical physiological roles. We expect that the process of muscle degeneration in DMD or mdx mice causes excess Ca2+ to accumulate gradually in fibers after reaching a critical point in time, because their muscle fibers appear to function normally until around age 7 days (Table 1). When muscle fibers, with high levels of accumulated calcium, receive stimulation, the fibers subsequently undergo extreme hypercontraction at the critical point (after age 7 days) and may lead to membrane rupture (47). At this moment, K+ may leak into the ECS and might produce the contraction of other muscle fibers around the ruptured fibers [potassium-induced contracture (27)].

We believe that group necrosis of muscle fibers following the efflux of K+ may be one of the causes of necrosis of muscle fibers, in addition to the “theories on grouped necrosis” described by Gorospe et al. (20). The TA muscles of mdx-Na mice did not show group necrosis except in one case. Aside from the Ca2+-stabilizing effect, reduced serum CK activity, blood K+ concentration, and the group necrosis fibers of mdx-Na mice may have been caused by a quick, excessive K+ excretion with excessive Na+ excretion in urine, as the urinary potassium excretion rate depends on urinary volume (35). The mice that ingested the Na supplement drank excessive water and evacuated a large amount of urine, although we do not have data on the volume of their urine. Atrial natriuretic peptide (ANP) in the blood of the mice that ingested the Na supplement might have increased, since the high-salt diet induces an increase in ANP plasma levels (28). ANP has been also known to block sarcolemmal L-type Ca2+ channel activity and the Ca2+ release from the sarcoplasmic reticulum (SR) (25). The L-type Ca2+ channels and the Ca2+ release from the SR in mdx-Na muscle fibers may have been inhibited by the ANP, which prevents calcium accumulation in mdx-Na muscle fibers.

Mouse skeletal muscle fibers possess a Na+/Ca2+ exchange mechanism (1). High Na+ levels in the ECS serve to force efflux of Ca2+ from fibers via the Ca2+/Na+ exchanger and inhibit Ca2+ release from the SR (13). The average blood Na+ concentration in mdx-Na and B10-Na mice was significantly elevated. Thus the Na+/Ca2+ exchanger of mdx-Na muscle fibers may have prevented calcium accumulation in mdx-Na muscle fibers and might contribute to increase Ca2+ concentration in blood (and ECS).

Lijnen and Petrov (26) demonstrated reduction of the total calcium content of erythrocytes and the intraplatelet Ca2+ concentration by calcium supplementation. The mean value of serum Ca2+ concentration and the plasma total calcium content of the treated calcium group was higher than in the placebo group, although there are no significant differences between the calcium and placebo groups. They also showed a reduction in the plasma concentrations of intact parathormone and 1,25-dihydroxyvitamin D3 that raise calcium uptake in cells (7, 9). Increased Ca2+ concentration in the blood of mdx-Na might have been produced to reduce the activity of parathormone and
1,25-dihydroxyvitamin D3 and may have prevented calcium accumulation in muscle fibers of mdx mice.

Activation of Ca^{2+}-ATPase of erythrocytes by calcium supplementation (49) and high activity of Ca^{2+}-ATPase in dystrophic muscle sarcolemma (14, 39) were demonstrated. High Ca^{2+} concentration in the blood of mdx-Na mice may have induced Ca^{2+}-ATPase to higher activity for the prevention of Ca^{2+} accumulation in muscle fibers and might have protected them from calcium accumulation in muscle fibers of mdx mice. From the above discussions, it is conceivable that higher Ca^{2+} in the blood (or in ECS) of female mdx mice reduced the calcium accumulation in their muscle fibers, inhibited necrosis of muscle fibers, and stabilized membrane effects of Ca^{2+}.

The zinc content of TA muscle of mdx-Na or female mdx mice was higher than that of muscle of mdx mice fed a control diet or of male mdx mice. Zinc may be important to inhibit muscle necrosis because Zn^{2+} stabilizes cell membranes (5) and blocks L-type Ca^{2+} channels (45). One of the reasons why serum CK activity in mdx females is lower than in males may be higher zinc content of muscle than that of muscle of males. Muscle zinc content of mdx males was markedly higher than that of B10 males (Table 2). These results suggest the importance of zinc in mdx mice surviving as long as control B10 mice. Mdx males seem to require higher muscle zinc content to survive as long as B10 mice. Zn^{2+} also plays a role in protein synthesis and may contribute to the regeneration of muscle fibers. Therefore, Zn^{2+} may be important for muscle regeneration in mdx mice. The assumption is supported by the results of Tameyasu et al. (41), which suggest that the administration of a zinc compound ameliorates muscle function in the mdx
mouse. The standard feed used in this study and in the Laboratory Animal Facility of the National Institute of Neuroscience, NCNP, contains approximately two times the calcium, six times the potassium, six times the magnesium, seven times the sodium, and six times the zinc as the estimated minimal mineral requirements for mice (31). Hence, the mice in this study and at our institute may have ingested necessary calcium, sodium, and zinc in their diet to survive as long as B10 mice. Our results suggest that blood Ca\(^{2+}\) concentration, muscle zinc content, and potassium excretion are important for the inhibition of muscle fiber necrosis in mdx mice.

In future studies, we will further investigate the cause-and-effect relationship of NaCl supplementation to identify effective therapies for reducing the rate of muscle degeneration and improving the quality of life of DMD patients.

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