Effect of energy compound on skeletal muscle strain injury and regeneration in rats

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Abstract
Objective To determine whether the supplement of energy compound could attenuate strain-induced damage to skeletal muscle in rats. Method Experimental animals were injured by a strain injury model. Energy compound groups were given energy compound 10 ml/kg body weight per day, while saline groups were given saline at the same dose. Plasma was centrifuged to LDH, La and CK. Muscles were fixed for histology observation and immunohistochemistry assay. Results The results showed a similar tendency of plasma CK, La and LDH in saline and energy compound groups, while the lower levels was found in the energy-compound group. The histological examination of muscle sections revealed a lower degree of damage in the energy compound group in which the expression levels of desmin and vimentin were higher than in the saline group. Conclusion It is suggested that energy compound supplement may attenuate train-induced muscle damage and facilitate its regeneration.

1. INTRODUCTION

Skeletal muscle injuries often occur in professional and recreational sports or daily activities. Most of them are strain injuries caused by an episode of sudden stretch. Microcirculation disorders and intense inflammation occurred after injury, followed by metabolic disturbance such as abnormal regulation of oxygen free radicals, pH and energy supplement. Peeze reported that soleus muscle inflated at the region of contraction, microcirculation blockage caused by increased pressure derived from swollen muscle fiber may be involved in this\(^1\). However, microcirculation disturbance is not the original cause of muscle damage, it relates to cell injury by inducing metabolic alteration. Rapid depletion of ATP has been reported in intense or consistent contraction. ATP deficiency is common in affecting the stability of muscle fiber.
Energy compound is used as an auxiliary therapy to treat energy metabolism dysfunction caused by cardiovascular and cerebrovascular diseases, myocarditis, renal failure and other diseases. It is reported that supplement of energy compound has a protective effect on ATP activity and ultramicrostructure in skeletal muscle ex vivo. However, its protection for skeletal muscle in vivo has not been fully addressed yet. Therefore, considering that energy metabolism dysfunction and deficiency play an important role in the process of muscle damage, in the present study we investigated the in vitro effects of energy compound on skeletal muscle strain injury and regeneration in rats, in the hope to explore whether the supplement of energy compound could attenuate strain-induced damage to skeletal muscle and facilitate its regeneration.

1.1 Methods

56 adult male Sprague-Dawley rats weighing 250 to 300g were used in this study. All animals were housed in cages and allowed free access to standard rat food and water. After 2 weeks of acclimation, the rats were divided randomly into group. One group (n=8) served as the control group (control group). The other 2 groups were designed separately as saline group (n=24) and energy compound group (n=24).

The experimental procedures were reviewed and approved by the Peking University Health Science Center for Animal Care and Use Committee.

1.1.1 Development of an animal model for skeletal muscle strain injury

The model used was developed in rats according to the method described by Almeskinders and Gilbert. A controlled strain injury of the gastrocnemius muscle was produced on the right hindlimb of saline and energy-compound group while a sham operation was performed on the right hindlimb of control group. The animals were anesthetized with chloral hydrate (400mg/kg) injected intraperitoneally. The right hindlimb was shaved and the distal tendon of the gastrocnemius muscle was exposed and separated from other tendons and tissues through a 0.5 cm incision, which was kept moist by 37 °C normal saline. The rat lay on the back on a specially adapted platform attached to the materials testing machine (Bei Hang University, Peking). A needle was inserted transversely through the distal femur of the right leg and the needle was secured to a frame on the machine. The distal tendon of gastrocnemius was connected to the load cell by a surgical silk and the muscle was pulled at a speed of 6 cm/min under the control of a computer until the horizontal plateau of recorded load-elongation curve was reached. Then the needle and the surgical silk were immediately removed and the gastrocnemius muscle was gently laid back into its bed. Subsequently, the incision was closed after desinfection. Gastrocnemius muscles of the control group were treated by the same procedure but without the strain injury. Each animal was given benzylpenicillin for 3 days to avoid infection and allowed to feed and move freely. In this model of muscle strain injury, experimental injury was limited in degree to the plastic region of muscle deformation. This simulated the clinical situation where most common injuries to the musculotendinous region are partial tears but not complete ruptures.

1.1.2 Experimental protocol

In the energy-compound group, the animals were given energy-compound 10ml/ kg body weight per day by intraperitoneal injection since the day of injury. The saline group received an equal volume of normal saline as the energy-compound group. Eight randomly selected animals in the energy-compound and saline group were sacrificed 3, 7 or
14 days after the injury, while the control group was sacrificed 3 days after the sham operation. Blood obtained from the descending aorta of rats were centrifuged immediately and the plasma was frozen at -80℃ until analysis. Plasma acid concentration and lactic dehydrogenase were measured to analyze the status of muscle energy supply. Creatine kinase (CK) activity in the plasma was determined as indicators of muscle injury. The right gastrocnemius muscles of each group were removed and fixed on day 3, 7 and 14 for histology examination or immunohistochemistry assay.

1.1.3 Chemical analysis

Plasma La, CK, LDH levels were analyzed using a detecting kit obtained from Nanjing Jiancheng Bio-engineering Institute (Nanjing, PR.China)

1.1.4 Immunohistochemistry assay

After anesthesia and perfusion of phosphatebuffered 4 % paraformaldehyde through the aorta, the medial head of right gastrocnemius muscles was removed and immersed in freshly prepared buffered formalin. The fixed muscle samples were embedded routinely in paraffin and cut into 6 /im sections. The expression of desmin and vimentin were monitored by immunohistochemistry using specific antibodies. The first antibodies used to detect desmin and vimentin were rabbit antibodies (Sigma Immunochemicals, St. Louis, MO) at a dilution of 1/100 overnight. Following several rinses in phosphate-buffered serum saline (PBS), a goat anti-rabbit was used at a dilution of 1/200 for 2h. The visualization of the immunohistochemistry was done by microscopy.

The amounts of desmin and vimentin immunoreactivity were computerized as index of regeneration. To measure the area of expression/field under microscope, 33-36 random fields (equal size area 331647.57 µm²) were selected for each group. Images were rendered to monochrome, the features extracted, and the absolute area of positive staining collected in each image field. We collected the positive area and the mean optical density of each selected field (LEICA Q550CM, LEICA Germany), and the product of their multiplication, called integral optical density, was used to indicate the expression level of each protein.

1.1.5 Data analysis

All data were expressed as mean ± SD. Statistical analysis was performed by SPSS (Version 11.0) statistical software, and comparisons were made using one-way ANOVA. P values less than 0.05 were considered to be significant.

2. RESULTS

Table 1 shows the values of plasma CK, La and LDH of each group on days 3, 7 and 14 after injury.

<table>
<thead>
<tr>
<th></th>
<th>Control (n=8)</th>
<th>Number of days after the injury</th>
<th>Saline</th>
<th>Energy</th>
<th>Saline</th>
<th>Energy</th>
<th>Saline</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK (U/L)</td>
<td>85.78±5.61</td>
<td></td>
<td>433.36±31.53*</td>
<td>373.19±34.97*</td>
<td>207.19±32.95*</td>
<td>177.08±32.34*</td>
<td>91.35±9.15*</td>
</tr>
<tr>
<td>La (mmol/L)</td>
<td>2.63±0.38</td>
<td></td>
<td>4.42±0.71*</td>
<td>3.41±0.66*</td>
<td>3.62±0.67*</td>
<td>2.87±0.62*</td>
<td>3.15±0.60*</td>
</tr>
</tbody>
</table>
2.1 Plasma CK
On day 3, plasma CK activity increased significantly in saline group and energy-compound group compared to the control group. On day 7, plasma CK tends to decrease in these two groups but still higher than the control group. On day 14, plasma CK activity decreased to the basal level. Lower plasma CK activity was found in the energy-compound group than in the saline group on day 3 and day 7.

2.2 Plasma La
In the saline group, plasma La increased significantly on day 3 and began to fall on day 7 but still higher than the control group. And no significant difference was found on day 14. In energy-compound group, there was a significant higher level of plasma La on day 3, while no difference was shown on day 7 and day 14 compared to the control group. Plasma La was lower in the energy-compound group than in the saline group at these three-time points.

Plasma LDH
On day 3, plasma LDH increased significantly in saline group and energy-compound group compared to the control group. On day 7, plasma LDH tends to decrease in these two groups but was still higher than the control group. On day 14, plasma LDH activity decreased to the basal level. Lower plasma CK activity was found in the energy-compound group than in the saline group on day 3 and day 7.

2.3 Histological evaluation
On day 3 after injury, histological observation showed the following changes in the saline group: muscle fiber rupture, intense inflammation with limited muscle fiber necrosis, infiltration of leukocytes and hemorrhage. Muscle regeneration was also shown in the sections but most of myotubes were small with little cytoplasm. In the energy-compound group, inflammation was present with minor fiber necrosis and swelling. Muscle regeneration was found active with obvious phenomenon of fiber fusion.

On day 7 after the injury, there were still manifestations of inflammatory cells infiltration and endocytosis in the saline group, but not as serious as on day 3. In the energy-compound group, inflammation was present in local area, and the regenerated myotubes were rich in cytoplasm with scattered nucleus.

On day 14 after the injury, there was limited inflammation in the saline group, and obvious fibrosis was present in the zone of necrosis. In the energy-compound group, infiltration of inflammatory cells was hard to see, most of the regenerated fiber developed well with obvious striations. And scar tissue was shown in the injured zone, but the area was smaller than that in the saline group.

There were obvious ultrastructural changes in muscle specimens from the saline rats at study days 7, characterized by collapse and disarray of sarcomeres, fiber vacuolar degeneration and occasional disorganization of Z-disc. Swollen mitochondria with disorganized cristae, mitochondrial membrane segmentation were also observed, as well as lightly accumulated chromatin and actively regenerated myoblasts. In the energy-compound group, disorganization of sarcomeres
was also present with occasional vacuolar degeneration. Most mitochondria appear normal. Thick cristae is seen in some enlarged mitochondria with many electron-dense vesicles. The regenated muscle fibers are rich in cytoplasm.

Effect of energy-compound on desmin and vimentin expression

Table 2 shows integral optical density of desmin and vimentin stained sections in each group.

Table 2. Integral optical density (IOD \( \times 10^3 \)) of desmin, vimentin -stained sections in each group (mean±SD ).

<table>
<thead>
<tr>
<th>Number of days after the injury</th>
<th>Control</th>
<th>Saline</th>
<th>Energy</th>
<th>Saline</th>
<th>Energy</th>
<th>Saline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>desmin</td>
<td>10.31±4.89</td>
<td>6.35±2.16*</td>
<td>373.19±34.97*(^\Delta)</td>
<td>207.19±32.95*</td>
<td>177.08±32.34*(^\Delta)</td>
</tr>
<tr>
<td></td>
<td>vimentin</td>
<td>1.23±0.56</td>
<td>7.76±4.00*</td>
<td>3.41±0.66*(^\Delta)</td>
<td>3.62±0.67*</td>
<td>2.87±0.62(^\Delta)</td>
</tr>
</tbody>
</table>

\(^*\) Statistically significant differences vs. control group (\(P<0.05\));
\(^\Delta\) Statistically significant differences vs. saline group at the same time points (\(P<0.05\)).

In sections from the control group, desmin immunostaining was positive with a normal stained pattern while vimentin was negative.

After injury, the desmin expression in saline group was notably decreased on day 3 but enhanced significantly on day 7, then decreased again on day 14 but still higher than the control group. The desmin expression in energy- compound group was significantly higher than that in the saline group at these three time points.

After injury, the vimentin expression in saline group was notably increased on day 3, and then decreased from day 7 to day 14, but still higher than that in the control group. In the energy- compound group, the vimentin expression was significantly increased on day 3 and day 7 compared to the saline group. On day 14, the vimentin expression decreased to the basal level with no significance in comparison with the saline group.

3. Discussion

90 % creatine kinase (CK) resides in skeletal muscle, located in the cytoplasma. It is a key enzyme involved in regulating energy metabolism in tissues with fluctuating energy requirements such as muscle and brain. CK catalyses the reversible transfer of the phosphoryl group from phosphocreatine to ADP to regenerate ATP\(^7\). Elevations of plasma CK are characteristic responses to strenuous exercise and are often used as indicators of muscle damage. Many investigators suggest that this enzyme is a basic diagnostic indicator useful in early detection of changes caused by pathological change of the muscle\(^8-10\). In our study, we observed a major elevation of CK level after injury. This suggests that our strain injury model had caused pathological damage to the gastrocnemius muscle. The production of ATP was affected by the emission of CK from the cytoplasma, which caused the dysfunction of energy supply to the energy. We found that plasma CK was lower in the energy- compound group than in the saline group. This indicated that the status of energy metabolism was
improved after the supplement of energy materials. This helps to reduce the damage caused by the free radical and other factors.

Lactic acid is the important intermediate product of energy supply system. It’s an end product of the process of glycolysis and ground substance of aerobic metabolism. Lactic acid is another form of energy reserve, can easily transfer between the tissues to provide energy. Lactic acid generated in skeletal muscle is cleared through the following mechanism: 1) to be oxidized to carbon dioxide and water; 2) to construct glucose and glycogen; 3) to be shifted to fatty acid and alanine. In the skeletal muscle, the process of production is accompanied with the metabolism, and the content of plasma lactic acid depends on the balance of its production and elimination. In this experiment, we observed a significant increase of plasma lactic acid after the injury, this indicates that the balance was broken by the strain, the produced lactic acid can not be removed in time, so the content of lactic acid was raised in the plasma. The accumulated lactic acid resulted in the elevated pH, and aggravated the metabolic disorder. In the energy compound group, the plasma lactic acid was reduced. This indicated that the process of metabolism was improved, the accumulated metabolite can be removed immediately.

LDH is present in many tissues and localized in the cytoplasm. Pyruvic acid generated in the process of glycolysis can be converted to lactic acid by LDH. Therefore, LDH is used as an indicator to evaluate the anaerobic capacity in skeletal muscle, liver, kidney and heart. In this experiment, plasma LDH was significantly increased as CK after muscle damage, suggesting the similar mechanism may be involved as CK. Cellular swelling occurred after muscle injury, the microcirculation disturbance caused the accumulation of poisonous matters, which resulted in the increased permeability of cell membrane. Plasma enzymes increased as a result of leakage from muscle cells. After supplement of energy compound, the plasma LDH decreased significantly in compared to the saline group. This indicates that the energy compound can ameliorate the status of metabolism and lessen the damage of membranous structure.

Based on the results of histology and immunochemistry, we can see that administration of energy compound after injury may promote the rehabilitation of cell function and structure. The mechanism may be in relation to the change of local energy metabolism. The results we observed in this experiment may be the mutual effect of these compositions.

ATP in this energy compound is the primary material to supply energy in maintaining the normal metabolism. In physiological condition, the membrane potential and the contents of K⁺, Na⁺, Ca²⁺ and Mg²⁺ were modulated actively by two important enzymes, Ca²⁺-ATPase and Na⁺-K⁺-ATP, which got energy from ATP. After injury, the supply of ATP was influenced by the microcirculation disturbance. Then the reduced enzyme activity caused the entrance of water and Na⁺ into cytoplasm, cellular swelling and structure damage occurred. In addition, mitochondrial dysfunction can also affect the process of ATP generation.

Coenzyme Q₁₀, a lipid-soluble molecule found in all cellular membranes and serum, is involved in multiple pathways essential for growth and homeostasis. CoQ₁₀ is an essential component of the mitochondrial respiratory chain. CoQ₁₀ plays a vital role in ATP production. CoQ₁₀ also has membrane-stabilizing properties and acts as an antioxidant in both mitochondrial and lipid membranes. Several reports have found that CoQ₁₀ protects creatine kinase and other key proteins from oxidative inactivation during reperfusion, a functional crucial in preserving energy metabolism. Therefore, CoQ₁₀ in this energy compound can ameliorate the mitochondrial function and protect the membrane from the damage of free radicals.
Coenzyme A plays a huge role in intermediary metabolism, in which cells synthesise, break down or use nutrient molecules for energy production, growth, etc. It can still promote the utilization of CoQ10. In this experiment, CoA administration helps to facilitate the process of substance metabolism and energy production, and to lessen the metabolic disturbance.

Cytochrome C (Cyt-C) is an important part of Complex III electron transfer chain, and is encoded by nuclear gene. Its active form resides in mitochondrial inner membrane, and combined with Cyt-C oxidase. Cyt-C is used as a respiratory activating enzyme in clinical medicine to treat hypoxia. Cyt-C in this energy compound may assist the maintainance of normal function of mitochondrial respiratory chain.

Vit B₆ is the derivative of pyridine, acts as a co-enzyme in transamination, decarboxylation and racemization. So Vit B₆ is considered to be associated with energy metabolism. It has been reported that Vit B₆ plays an active part in promoting amino acid metabolism and recovery of peripheral nerve. In other paper, administration of Vit B₆ can enhance the utilization of selenium, increase GSH-Px activity in erythrocyte, skeletal muscle, myocardium and testis, and decrease lipid peroxides levels. Therefore, VitB₆ can lessen the damage to cellular structure and promote the regenerative process by reducing the lipid peroxides levels.

In conclusion, the supplement of energy compound after strain-induced injury may reduce the degree of damage in the skeletal muscle and facilitate its healing. It is suggested that energy compound may serve as an auxiliary drug in the treatment of strain-induced injury to skeletal muscles.

References